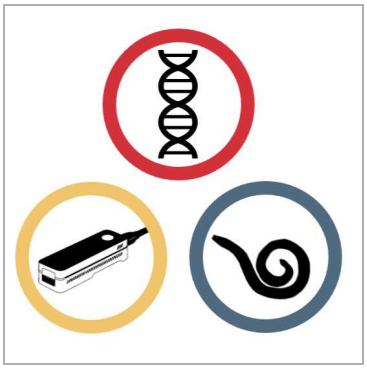

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Extraction and ONT MinION Library Preparation of uHMW gDNA V.5

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ABSTRACT

This custom protocol optimizes extraction, purification, and Oxford Nanopore Technologies (ONT) MinION library preparation for ultra-high molecular weight genomic DNA (uHMW gDNA) from parasitic nematodes. It can be used effectively with both low-input samples (e.g., a single adult hookworm) and high-input samples (e.g., a chunk of tissue from an *Ascaris* sp. adult).

Protocols on which this workflow is based:

- Zymo® Quick-DNA™ Magbead Plus Kit protcol
- Oxford Nanopore Technologies® SQK-LSK-114 gDNA Ligation Sequencing protocol
- Zymo® DNA Clean & ConcentratorTM Magbead Kit

MATERIALS

- Oxford Nanopore Technologies Ligation Sequencing Kit V14 (SQK-LSK-114)
- Zymo Quick-DNA Magbead Plus Kit (D4081)
- Zymo DNA Clean & Concentrator Magbead Kit (D4012)
- NEBNext Companion Module for Oxford Nanopore Technologies Ligation Sequencing (E7180S)
- NEB Monarch Pestle (one per specimen; T3000L)
- AMPure XP strepatavidin beads (A63880)
- Minicentrifuge
- Micropipetters & tips (wide-bore tips STRONGLY recommended for pipetting up gDNA or libraries)
- Dry bath
- Rotator mixer (e.g., PR1MA Roto-Mini Mixer)
- Magnetic separation rack (e.g., MagJET Separation Rack)
- 1.5 mL microcentrifuge tubes (DNase-free, DNA low bind STRONGLY recommended)
- 0.2 mL PCR tubes
- Microcentrifuge (only if processing large [i.e., >2 cm] specimens)

Keywords: extraction, gDNA, MinION, nematode, library preparation, uHMW gDNA, ONT

BEFORE START INSTRUCTIONS

For new kits, add 1,040 µL Zymo Proteinase K Storage Buffer to each tube of Zymo Proteinase K (20 mg) prior to use. The final concentration of Proteinase K is ~20 mg/ml. Store resuspended Proteinase K at -20°C after mixing.

Part 1: Ultra-HWM gDNA extraction | Zymo Quick-DNA HWM..

1 Set dry bath to 55 °C

2 For each sample, add the following to a clean 1.5 mL microcentrifuge tube to create a master mix:

- 95 µL Zymo DNA Elution Buffer **Zymo Research Catalog #D3004-4-1**
- 95 µL Zymo Biofluid & Solid Tissue Buffer **Zymo Research Catalog #D4081-3-25**
- 10 µL Zymo Proteinase K **Zymo Research Catalog #D3001-2-20**

2.1 Vortex the master mix gently to mix, then spin down and keep on ice

3 Using a new pipette tip or sterilized forceps, add one whole worm (or a piece of tissue) directly from tissue preservative to the bottom of a clean 1.5 mL microcentrifuge tube

> Note
>
> Transfer as little tissue preservative liquid as possible to the new tube during this process

4 Use a new NEB Monarch Pestle **NEB Catalog #T3002-1** to grind and crush the tissue in the tube. Keep the pestle in the tube

5 Add 200 µL master mix (prepared in Part 1 Step 2) to each tube containing tissue and pestle

6 Continue using the pestle to grind the tissue within the master mix until homogenized. Remove the pestle, being careful to keep any tissue in the tube by wiping the pestle on the tube edges as it is removed

7 Close the tube and mix by inverting and flicking gently, then spin down briefly to recollect tissue and liquids

8 Incubate sample in dry bath at 55 °C for 02:30:00 to Overnight until tissue solubilizes. During incubation, flick tube every 00:20:00 to agitate tissues, then briefly spin down to recollect liquids and replace tube in dry bath 3h 10m

Note

If a very large amount of input tissue was used: It is likely there will still be visible tissue even after hours of lysis. If so, centrifuge the sample for 00:01:00 at 10000 x g or greater to pellet debris, then pipette all liquids into a new clean 1.5 µL microcentrifuge tube. (The majority of gDNA will be contained in the layer of liquid just above the pellet, so pipette carefully to get as much liquid as possible without disturbing the debris.) Discard the tube contain the pelleted debris and use the retained supernatant for Part 2.

Part 2: Ultra-HWM gDNA purification | Zymo Quick-DNA HW... 2h 20m

9 Set dry bath to 37 °C

10 Add 400 µL Zymo Quick-DNA™ MagBinding Buffer **Zymo Research Catalog #D4077-1-150** to each sample

11 Flick tubes to mix, then spin down briefly to recollect liquids

12 Add 33 µL Zymo MagBinding Beads **Zymo Research Catalog #D4100-2-6** to each sample

> **Note**
>
> MagBinding Beads settle quickly, so ensure beads are kept in suspension while dispensing by vortexing the beads each time before they are added to a sample

13 To ensure DNA binds to beads, mix on a rotator mixer at a low speed for 02:00:00 at Room temperature. Spin down briefly before proceeding with the next step 2h

14 Set sample tubes on a magnetic stand until beads have separated from solution, then remove and discard the supernatant. Remove sample tubes from the magnetic stand.

> **Note**
>
> Some beads may adhere to the sides of the tube. When removing supernatant, aspirate slowly to allow these beads to be pulled to the magnet as the liquid level is lowered.

15 Add 500 µL Zymo Quick-DNA™ MagBinding Buffer **Zymo Research Catalog #D4077-1-150** to each sample

16 Flick to mix initially, then mix on a rotator mixer at a low speed for 00:20:00 at Room temperature. Spin down briefly before proceeding with the next step 20m

17 Set sample tubes on a magnetic stand until beads have separated from the solution, then remove and discard the supernatant. Remove sample tubes from the magnetic stand

> Note
>
> Some beads may adhere to the sides of the tube. When removing supernatant, aspirate slowly to allow these beads to be pulled to the magnet as the liquid level is lowered.

18 Add 500 µL Zymo DNA Pre-Wash Buffer **Zymo Research Catalog #D3004-5-250** to each sample

19 Flick to mix, then spin down briefly

20 Set sample tubes on a magnetic stand until beads have separated from solution, then remove and discard the supernatant. Remove sample tubes from the magnetic stand

> Note
>
> Some beads may adhere to the sides of the tube. When removing supernatant, aspirate slowly to allow these beads to be pulled to the magnet as the liquid level is lowered.

21 Add 900 µL Zymo g-DNA Wash Buffer **Zymo Research Catalog #D3004-2-200** to each sample

22 Flick to mix, then spin down briefly

23 Transfer the entire sample (all liquid and beads) to a new clean 1.5 mL microcentrifuge tube

Note

Transfer to a new tube ensures that any salts that are stuck to the lid of the tube do not get carried over

24 Set samples (now in new tubes) on a magnetic stand until beads have separated from the solution, then remove and discard the supernatant. Remove sample tubes from the magnetic stand

Note

Some beads may adhere to the sides of the tube. When removing supernatant, aspirate slowly to allow these beads to be pulled to the magnet as the liquid level is lowered

25 Add 900 µL Zymo g-DNA Wash Buffer **Zymo Research Catalog #D3004-2-200** to each sample

26 Flick to mix, then spin down briefly

27 Transfer the entire sample (all liquid and beads) to a new clean 1.5 mL microcentrifuge tube

Note

Transfer to a new tube ensures that any salts that are stuck to the lid of the tube do not get carried over

28 Set samples (now in new tubes) on a magnetic stand until beads have separated from the solution, then remove and discard the supernatant. Leave sample tubes on the magnetic stand

Note

Some beads may adhere to the sides of the tube. When removing supernatant, aspirate slowly to allow these beads to be pulled to the magnet as the liquid level is lowered

28.1 Use a P10 pipette to remove any residual liquid from the bottom of the tube

29 Air dry the beads for up to 00:20:00 and proceed to next step once beads are dry, but not over-dry 20m

Note

It may take less time for the beads to dry, so check them often during this process. Beads will change in appearance from glossy black when still wet to a matte black/brown when fully dry. Over drying the beads may result in lower gDNA recovery.

30 Add 50 µL Zymo DNA Elution Buffer **Zymo Research Catalog #D3004-4-50** to each sample and flick gently several times to mix. Spin down briefly

Note

If you plan to Qubit and TapeStation the extraction, it is a good idea to elute in 52 µL (rather than 50 µL) to have 1 µL easily available for each quality control analysis

31 Incubate in dry bath at 37 °C for 02:00:00. During incubation, flick tube every 00:20:00 to agitate tissues, then briefly spin down to recollect liquids and replace tube in dry bath 2h 20m

32 Incubate on bench top at Room temperature overnight.

33 After overnight incubation, set tubes on a magnetic stand until beads have separated from solution, then move the supernatant (now containing eluted gDNA) to a new clean 1.5 mL microcentrifuge tube

Note

The eluted DNA can be used immediately or stored at 4 °C or -20 °C for future use

33.1 Re-suspend beads in 20 µL of Nuclease-free Water **Contributed by users** in case there is no (or not enough) gDNA in final elution

34 Use 1 µL of final elution to quantify extraction via Qubit analysis

35 Use 1 µL of final elution to assess fragment size distribution via TapeStation

Part 3: DNA repair and end-prep | Zymo Clean & Concentrat.. 1h

36 Set dry bath to 65 °C

37 Defrost the needed NEB DNA and End Repair reagents on ice (see Part 3 Step 38)

38 For each sample, add the following to a clean 0.2 mL PCR tube to create a master mix, pipetting 10–20 times between each addition to mix:

3.5 µL

NEBNext® FFPE DNA Repair Buffer **New England Biolabs Catalog #E7180S**

2 µL

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

3.5 µL

NEBNext Ultra II End Prep Reaction Buffer **New England Biolabs Catalog #E7647**

3 µL

NEBNext Ultra II End Prep Enzyme Mix **New England Biolabs Catalog #E7646**

38.1 Keep master mix on ice

39 Add 12 µL of master mix (prepared in Part 3 Step 38) from the PCR tube directly into each 1.5 mL microcentrifuge tube containing extracted & purified uHWM gDNA (from Part 2). Mix all components by gently flicking, and spin tubes down to recollect liquids

40 Incubate samples at Room temperature for 00:10:00 10m

41 Incubate samples in dry bath at 65 °C for 00:10:00 10m

42 Add 4 volumes of Zymo DNA MagBinding Buffer **Zymo Research Catalog #D4012-1-50** to each sample and mix well by flicking and inverting

Note

Example for calculating 4 volumes: If input is 50 µL gDNA, add 200 µL DNA MagBinding Buffer

43 Spin samples down briefly and add 20 µL Zymo MagBinding Beads **Zymo Research Catalog #D4100-5-2**

Note

MagBinding Beads settle quickly, so ensure beads are kept in suspension while dispensing by vortexing beads each time before they are added to a sample

44 Mix samples on rotating mixer at a low speed at Room temperature for 01:30:00 1h 30m

45 Briefly spin down samples and pellet on a magnetic stand (1–2 min) until the supernatant is clear and colorless. With the tubes still on the magnet, pipette off and discard the supernatant

46 Add 500 µL Zymo DNA Wash Buffer **Zymo Research Catalog #D4003-2-24** and then remove from magnetic stand, and mix well by flicking and inverting

47 Briefly spin samples down briefly and transfer to magnetic stand to allow beads to pellet until solution is clear (1–2 min). With the tubes still on the magnet, pipette off and discard the supernatant

48 Add 500 µL Zymo DNA Wash Buffer **Zymo Research Catalog #D4003-2-24** and then remove from magnetic stand, and mix well by flicking and inverting

49 Briefly spin samples down briefly and transfer to magnetic stand to allow beads to pellet until solution is clear (1–2 min). With the tubes still on the magnet, pipette off and discard the supernatant

50 Air dry the beads for 00:10:00 10m

> **Note**
>
> MagBinding Beads utilize a different chemistry than SPRI beads (e.g., AMPure XP beads) so there is not the same risk of over-drying. It is important for optimal elution that the residual buffer is completely removed/evaporated from the beads

51 Add 52 µL Zymo DNA Elution Buffer **Zymo Research Catalog #D3004-4-10**

52 Manually agitate samples for 00:10:00 to 00:20:00 by gently flicking/inverting (and occasionally spinning dow to recollect liquids) 30m

> **Note**
>
> This volume is too small to be able to use most rotator mixers effectively, so manually agitation is necessary

53 Briefly spin samples down and pellet the beads on a magnet until the eluate is clear and colorless (1–2 min)

54 Remove and retain the 52 µL of eluate (containing repaired & end-prepped DNA) to a new clean 1.5 mL microcentrifuge tube

55 Use 1 µL of final elution to quantify via Qubit assay

56 Use 1 µL of final elution to assess fragment size distribution via TapeStation

*

Note

The sequencing adaptors ligated in the next section will affect the validity of TapeStation runs, so assessing the fragment distribution at this stage (i.e., after DNA repair and end preparation) is crucial for being able to estimate the molarity of your final library

Part 4: Adaptor ligation and clean up | ONT Ligation Sequen..

1h

57 Set dry bath to 37 °C

58 Remove AMPure XP Beads **Beckman Coulter Catalog #A63880** from storage at 4 °C and allow them to come to Room temperature

59 Spin down Ligation Adaptor (LA) **Oxford Nanopore Technologies** and Quick T4 DNA Ligase **New England Biolabs Catalog #E7180S** and place on ice

60 Thaw ONT Ligation Buffer (LNB) **Oxford Nanopore Technologies** at Room temperature, spin down, and mix by pipetting. Place on ice immediately after thawing and mixing

61 Thaw Elution Buffer (EB) **Oxford Nanopore Technologies** at Room temperature, vortex to mix, spin down, and place on ice

62 Thaw one tube each of Short Fragment Buffer (SFB) **Oxford Nanopore Technologies** and Long Fragment Buffer (LFB) **Oxford Nanopore Technologies** at Room temperature, vortex to mix, spin down, and place on ice

63 For each sample, add the following, in order, to a new clean 1.5 mL microcentrifuge tube, pipetting 10–20 times between each addition to mix:

- 25 µL Ligation Adaptor (LA) **Oxford Nanopore Technologies**
- 10 µL Quick T4 DNA Ligase **New England Biolabs Catalog #E7180S**
- 5 µL Ligation Adaptor (LA) **Oxford Nanopore Technologies**

63.1 Keep master mix on ice after mixing

64 For each sample, prepare 1:3 SFB:LFB titrated wash mix by adding the following to a new clean 1.5 mL microcentrifuge tube, and then vortex to mix:

- 125 µL Short Fragment Buffer (SFB) **Oxford Nanopore Technologies**
- 375 µL Long Fragment Buffer (LFB) **Oxford Nanopore Technologies**

Note

For samples of sufficiently high input concentration where read length can be prioritized over gDNA retention, you may wish to instead use 1:5 SFB:LFB (i.e., 16.66 µL SBF: 88.34 µL LFB) or untitrated LFB, only

64.1 Keep titrated wash mix on ice after vortexing

65 Pipette 40 µL of master mix (prepared in Part 4 Step 62) directly into entire volume of repaired and end-prepped gDNA from Part 3. Mix all components by gently flicking and spin tube down to recollect liquids

66 Incubate the reaction 00:15:00 at Room temperature 15m

Note

If you have omitted the bead-based purification steps from the second half of Part 3, do not incubate the reaction for longer than 00:10:00

67 Resuspend AMPure XP Beads **Beckman Coulter Catalog #A63880** by vortexing and add 0.4X volume resuspended beads to each sample, then flick to mix

Note

AMPure XP Beads settle quickly, so ensure beads are kept in suspension while dispensing by vortexing beads each time before they are added to a sample

Note

Example for calculating 0.4X volume: If input is 89 µL (after adding master mix), add 35.6 µL AMPure XP Beads

68 Mix on a rotator mixer at a low speed for 01:00:00 at Room temperature 1h

69 Spin down the sample and pellet on a magnetic stand. Keeping the tube on the stand, pipette off and discard the supernatant

70 Wash the beads by adding 250 µL 1:3 SFB:LFB titrated wash mix (prepared in Part 4 Step 63). Flick the beads to resuspend, spin down, then return to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard

71 Wash the beads by adding 250 µL 1:3 SFB:LFB titrated wash mix (prepared in Part 4 Step 63). Flick the beads to resuspend, spin down, then return to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard

72 Spin down the beads and place them back on the magnetic rack. Use a P10 pipette to pipette of any residual liquid and allow beads to air-dry for 00:00:30 to 00:02:00 2m 30s

Note

Do not allow the pellet of beads to dry to the point of cracking! Over-drying beads will result in reduced yields

73 Remove the tube from the magnetic stand and resuspend the beads in 15 µL Elution Buffer (EB) **Oxford Nanopore Technologies**

74 Briefly spin down and incubate in dry bath at 37 °C for 02:00:00. During incubation, flick tube every 00:20:00 to agitate tissues, then briefly spin down to recollect liquids and replace tube in dry bath 2h 20m

Note

For HMW & uHMW gDNA, incubation at 37 °C for longer times can improve the recovery of long fragments

75 Incubate on the bench top at Room temperature overnight

76 After overnight incubation, pellet the beads on a magnet until the eluate is clear and colorless (at least 1 min)

77 Remove and retain the 15 µL of eluate (containing the prepared library) to a new clean 1.5 mL microcentrifuge tube

78 Use 1 µL of final elution to quantify library via Qubit analysis

Note

Note: For same-day or near-future sequencing, store the prepared library on ice or at 4 °C until ready to be loaded onto a flow cell. Otherwise, store libraries at -20 °C