VERSION 1
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Extraction and ONT MinLibrary Preparation of uHMW gDNA V.1

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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 large 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-109 gDNA Ligation Sequencing protocol
- Zymo® DNA Clean & Concentrator™ Magbead Kit protocol (best-testing phase only)

PROTOCOL CITATION

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BEFORE STARTING

- 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.
- For best results, allow AMPure XP beads (stored at 4°C) to come to RT prior to use.

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

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 1) to each tube containing tissue and pestle

6 Continue using the pestle to grind the tissue within the master mix until fully 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

2h 50m

8 Incubate sample in dry bath at 55 °C for 02:30:00 or 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

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 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 supernatant to move forward for Part 2.

2h 20m

Part 2: Ultra-HWM gDNA purification | Zymo Quick-DNA HWM MagBea

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 each time before beads are added to a sample

13 To ensure DNA binds to beads, mix on a rotator mixer at a low speed for 40 min at room temperature. Spin down briefly before proceeding with the next step

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 20 min. Spin down briefly before proceeding with the next step

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 1.5 mL microcentrifuge tube

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 1.5 mL microcentrifuge tube

28 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

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

20m

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

> 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 DNA 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

2h

31 Incubate in dry bath at 37 °C for 02:00:00 with occasional gentle flicking and spinning down throughout incubation

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 DNA) to a new 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

1h

Part 3: DNA repair and end-prep | Zymo Clean & Concentrator, ONT L

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 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 49 µL gDNA, add 196 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 each time before beads are added to a sample

20m

44 Mix samples on rotating mixer at a low speed at Room temperature for 00:20:00

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

10m

48 Air dry the beads for 00:10:00

> 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

49 Add 50 µL Zymo DNA Elution Buffer **Zymo Research Catalog #D3004-4-10**

10m

50 Manually agitate samples by gently flicking (and occasionally spinning down) for 00:10:00

> Note
>
> This volume is too small to be able to a the rotator mixer effectively at this step. Manually agitation is necessary

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

52 Remove and retain the 50 µL of eluate (containing repaired & end-prepped DNA) to a new clean 1.5 mL microcentrifuge tube

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

1h

Part 4: Adaptor ligation and clean up | ONT Ligation Sequencing & NE

54 Set dry bath to 37 °C

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

56 Spin down ONT Adaptor Mix (AMX) **Oxford Nanopore Technologies** and Quick T4 DNA Ligase **New England Biolabs Catalog #E7180S** and place on ice

57 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

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

59 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

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

25 µL	ONT Ligation Buffer (LNB) **Oxford Nanopore Technologies**
10 µL	Quick T4 DNA Ligase **New England Biolabs Catalog #E7180S**
5 µL	ONT Adaptor Mix (AMX) **Oxford Nanopore Technologies**

60.1 Keep master mix on ice after mixing

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

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

61.1 Keep titrated wash mix on ice after vortexing

62 Pipette 40 µL of master mix (prepared in Part 4 Step 60) 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

15m

63 Incubate the reaction 00:15:00 at Room temperature

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

64 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 each time before beads 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

65 Mix on a rotator mixer at a low speed for 01:00:00 at Room temperature

1h

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

67 Wash the beads by adding 250 µL 1:3 SFB:LFB titrated wash mix (prepared in Part 4 Step 61). 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

68 Wash the beads by adding 250 µL 1:3 SFB:LFB titrated wash mix (prepared in Part 4 Step 61). 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

2m 30s

69 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

Note

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

70 Remove the tube from the magnetic stand and resuspend the pellet in 15 µL

Elution Buffer (EB) **Oxford Nanopore Technologies**

71 Briefly spin down and incubate in dry bath at 37 °C for 02:00:00 with occasional gentle flicking and spinning down throughout incubation 2h

Note

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

72 Incubate on the bench top at Room temperature overnight

73 1.After overnight incubation, pellet the beads on a magnet until the eluate is clear and colorless (at least 00:01:00) 1m

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

75 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