Extraction and ONT MinION Library Preparation of uHMW gDNA V.4

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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 protocol
- Oxford Nanopore Technologies® SQK-LSK-109 gDNA Ligation Sequencing protocol
- Zymo® DNA Clean & Concentrator™ Magbead Kit

PROTOCOL MATERIALS

Zymo DNA Pre-Wash Buffer Zymo Research Catalog #D3004-5-250

Step 18

Zymo g-DNA Wash Buffer Zymo Research Catalog #D3004-2-200

In 2 steps

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

Step 38

AMPure XP Beads Beckman Coulter Catalog #A63880

In 2 steps

Zymo DNA Elution Buffer Zymo Research Catalog #D3004-4-10

Step 51

NEB Monarch Pestle NEB Catalog #T3002-1

Step 4

Zymo Biofluid & Solid Tissue Buffer Zymo Research Catalog #D4081-3-25

Step 2
PROTOCOL integer ID: 79273

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

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Zymo Proteinase K Zymo
Research Catalog #D3001-2-20  Step 2

Zymo Quick-DNA™ MagBinding Buffer Zymo
Research Catalog #D4077-1-150

In 2 steps

Zymo MagBinding Beads Zymo
Research Catalog #D4100-2-6  Step 12

Nuclease-free Water Contributed by users  Step 33.1

Zymo DNA Wash Buffer Zymo
Research Catalog #D4003-2-24  In 2 steps

Elution Buffer (EB) Oxford Nanopore Technologies  Step 60

Zymo DNA Elution Buffer Zymo
Research Catalog #D3004-4-1  Step 2

Short Fragment Buffer (SFB) Oxford Nanopore Technologies  Step 61

Quick T4 DNA Ligase New England Biolabs Catalog #E7180S  In 2 steps

ONT Ligation Buffer (LNB) Oxford Nanopore Technologies  In 2 steps

Long Fragment Buffer (LFB) Oxford Nanopore Technologies  Step 61

Elution Buffer (EB) Oxford Nanopore Technologies  Step 72

Zymo DNA MagBinding Buffer Zymo
Research Catalog #D4012-1-50  Step 42

NEBNext FFPE DNA Repair Mix - 96 rxns New England Biolabs Catalog #M6630L  Step 38

NEBNext Ultra II End Prep Enzyme Mix New England Biolabs Catalog #E7646  Step 38

Zymo MagBinding Beads Zymo
Research Catalog #D4100-5-2  Step 43

ONT Adaptor Mix (AMX) Oxford Nanopore Technologies  In 2 steps
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 M...

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 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.

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.

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**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.

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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 01:30:00 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
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.

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.

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

Flick to mix, then spin down briefly.

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.

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

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.

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.

32 Incubate on bench top at Room temperature overnight.
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 & Concentrator...**

### 36

Set dry bath to **65 °C**

### 37

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

Keep master mix on ice

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.

Incubate samples at Room temperature for 00:10:00

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

Add 4 volumes of Zymo DNA MagBinding Buffer Zymo Research Catalog #D4012-1-50 to each sample and mix well by flicking and inverting.
Example for calculating 4 volumes: If input is 50 µL gDNA, add 200 µL DNA MagBinding Buffer

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

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.

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

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.

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.

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.

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

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

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

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 51 µL of eluate (containing repaired & end-prepped DNA) to a new clean 1.5 mL microcentrifuge tube.
Part 4: Adaptor ligation and clean up | ONT Ligation Sequenc...  

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

56  Set dry bath to 37 °C

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

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

59  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

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

61  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

62  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 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

62.1 Keep master mix on ice after mixing

63 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 SSB: 88.34 µL LFB) or LFB, only

63.1 Keep titrated wash mix on ice after vortexing

64 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

65 Incubate the reaction 00:15:00 at Room temperature 15m
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**.

**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.

**66**  Resuspend [AMPure XP Beads](https://www.beckmancoulter.com/en-us/ampure-true-nucleic-acid-purification.html?utm_source=Illumina&utm_medium=Search%20Engine&utm_campaign=AMPure%20XP%20Beads%20Catalog%20A63880) by vortexing and add 0.4X volume resuspended beads to each sample, then flick to mix.

**67**  Mix on a rotator mixer at a low speed for **01:00:00** at **Room temperature**.

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

**69**  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.

**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.
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 of beads to dry to the point of cracking! Over-drying beads will result in reduced yields.

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

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.

Note

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

Incubate on the bench top at Room temperature overnight.

After overnight incubation, pellet the beads on a magnet until the eluate is clear and colorless (at least 1 min).
Remove and retain the \(15 \mu L\) of eluate (containing the prepared library) to a new clean 1.5 mL microcentrifuge tube.

Use \(1 \mu L\) of final elution to quantify library via Qubit analysis.

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

Use \(1 \mu L\) of final elution to assess fragment size distribution via TapeStation.