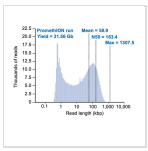

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HMW gDNA purification and ONT ultra-long-read data generation V.3

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This protocol describes the purification of high-molecular-weight genomic DNA from mammalian cells and the generation of ultra-long (N50 >100 kbp) Oxford Nanopore data using the PromethION. It is based on the Sambrook and Russell protocol and Josh Quick's protocol with additional modifications. This protocol improves upon previous protocols developed for ultra-long read sequencing, as it gives longer reads with greater yield. In our hands, we obtain about 30-50 Gb of ultra-long-read ONT data with an N50 ~150 kbp on one PromethION flow cell.

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long-read sequencing, high-molecular-weight, DNA, Oxford Nanopore, ONT, purification, nanopore, sequencing

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Reagents

☒ Tris-EDTA, pH 8.0 **Ambion Catalog #AM9849**

☒ RNAse A **Qiagen Catalog #19101**

☒ Proteinase K **Qiagen Catalog #19131**

☒ UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) **Thermo Fisher Scientific Catalog #15593031**

☒ Ammonium Acetate (5 M), RNase-free **Thermo Fisher Catalog #AM9070G**

☒ 200 Proof Ethanol pure **Sigma Aldrich Catalog #E7023**

☒ Hexamminecobalt(III) Chloride **Alfa Aesar Catalog #A15470**

Kits

☒ Ultra-Long DNA Sequencing Kit (SQK-ULK001) **Oxford Nanopore Technologies Catalog #SQK-ULK001**

Disposables

☒ DNA LoBind Tubes, 1.5 mL **Eppendorf Catalog #0030108051**

☒ DNA LoBind 2.0ml PCR Clean Eppendorf Tubes **Eppendorf Catalog #0030 108.078**

☒ MaXtract high-density tubes (25 x 50 ml) **Qiagen Catalog #129073**

☒ Disposable Inoculating Loops and Needles, Flexible Loop; Volume:10µ; Color: Yellow; Individual wrapped **Thermo Fisher Catalog #22363600**

☒ Glass Beads 3 mm **Scientific Laboratory Supplies Ltd Catalog #DD68501**

☒ Monarch Bead Retainers **New England Biolabs Catalog #T3004L**

OR make a bead retainer by cutting an Eppendorf 1.5-mL tube 2-3 mm from the bottom

Made-up buffers

Note: All buffers should be filter-sterilized with a 0.22 um filter prior to use

Lysis Buffer

- 10 mM Tris-Cl (pH 8.0)
- 0.1 M EDTA (pH 8.0)
- 0.5% w/v SDS

PEGW Buffer

- 10% PEG-8000
- 0.5M NaCl

Cell collection and lysis

1 Freeze down 2-7 x 10^7 cells as a cell pellet, and store at -80°C.

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2 When you are ready to purify the DNA, thaw the cell pellet on ice (usually takes ~30 mins).

3 While the cells are thawing, add RNase A to the lysis buffer at a final concentration of 20 ug/mL. This must be done fresh each time. Keep the lysis buffer + RNase A solution at RT until ready to use.

4 Resuspend thawed cells in ice-cold TE (pH 8.0) at a concentration of 5 x 10^7 cells/mL on ice.

5 Transfer the cell suspension to a 125-mL glass Erlenmeyer flask.
- Make sure that the cells are well-dispersed over the inner surface of the Erlenmeyer flask. This dispersal minimizes the formation of intractable clumps of cells.

6 Quickly add 10 mL of lysis buffer + RNase A for each mL of cell suspension, drop-wise in a circular motion. Swirl to mix.

7 Incubate the cell suspension for 1 hr at 37°C.

8 Add proteinase K to a final concentration of 200 ug/mL in a drop-wise manner.
- For 10 mLs of cell suspension, add 100 uL Proteinase K.

9 Swirl the flask to mix the enzyme gently into the viscous cell lysate.

10 Incubate the lysate in a water bath for 2 hours at 50°C. Swirl the viscous solution twice per hour.

11 Cool the solution to RT.

Phenol-chloroform extraction

12 Add the viscous lysate to a 50-mL conical tube.
- This is easiest using a 10-mL serological pipette at low speed.

13 Add an equal volume of ultra-pure phenol:chloroform:isoamyl alcohol (~10-11 mLs) to the tube containing lysate.

14 Gently mix the two phases by slowly turning the tube end-over-end for 10 mins on a tube mixer. If the two phases have not formed an emulsion at this stage, place the tube on a roller apparatus for 1 hr.

15 Pour the lysate into a MaXtract tube containing a high-density gel.

16 Spin in a centrifuge at 4000 rpm for 10 mins.

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17 Pour the aqueous phase into a new 50-mL conical tube.

18 Add an equal volume of ultra-pure phenol:chloroform:isoamyl alcohol (~10-11 mLs) to the tube containing lysate.

19 Gently mix the two phases by slowly turning the tube end-over-end for 10 mins on a tube mixer. If the two phases have not formed an emulsion at this stage, place the tube on a roller apparatus for 1 hr.

20 Pour the lysate into a MaXtract tube containing a high-density gel.

21 Spin in a centrifuge at 4000 rpm for 10 mins.

22 Pour the aqueous phase into a new 50-mL conical tube.

Ethanol precipitation

23 Add 0.4 volume of 5M ammonium acetate to the purified DNA, and gently swirl to mix (~20 mins).
- It is very important to mix gently here. The gentler you mix, the longer your DNA will be.

24 Add 2 volumes of ethanol at RT and gently swirl to mix (usually takes ~1 hr).
- Swirl gently, sometimes rocking back and forth slowly to get the DNA in solution with the salt and ethanol.

25 Store the precipitating DNA solution overnight at 4°C.
- We have found that storing the DNA overnight results in purer DNA that sequences better and gives more yield.

26 Remove the precipitate in one piece from the ethanolic solution with a disposable inoculating needle shaped into a U and place it into an Eppendorf 2-mL DNA LoBind tube.

27 Wash the DNA precipitate 2x with 1 mL 70% ethanol, and collect the DNA by centrifuging at max speed (~15,000 rpm) for 15 secs.

28 Remove as much of the 70% ethanol as possible. Store the pellet of DNA in an open tube at RT until the last visible traces of ethanol have evaporated (~10 mins).
- Do not allow the pellet of DNA to dry completely. Desiccated DNA is very difficult to dissolve.

29 Add 750-1000 uL EB + 0.02% Triton-X100 and incubate without mixing at 4°C for 2 days to allow the pellet to fully resuspend into a translucent viscous gel.

ONT library preparation with the SQK-ULK001 kit 1h 15m

30 Note: This section follows the SQK-ULK001 protocol published by Oxford Nanopore Technologies (v110_revF_24Mar2021) and is provided here for convenience.

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31 Pre-warm a heat block to 75°C.

32 Thaw the Fragmentation Mix (FRA), FRA Dilution Buffer (FDB) and Rapid Adapter F (RAP F). Spin down briefly and keep on ice.

33 Add the following reagents to a 1.5 mL Eppendorf DNA LoBind tube on ice:

244 uL HMW gDNA
6 uL FRA Buffer

Total = 250 uL

34 Mix the diluted FRA by vortexing.

35 Add 250 uL of diluted FRA to 750 uL of extracted DNA. Stir the reaction with the pipette tip whilst expelling the diluted FRA to ensure an even distribution.

36 Immediately mix the reaction by pipetting 10x with a wide-bore pipette tip.
- Make sure that the solution is well-mixed. The enzyme needs to interact with the DNA in order to tagment it and add an adapter.

37 Incubate the reaction as follows: 25m

1. RT for 5 mins
2. 75°C for 5 mins
3. RT for >10 mins

38 Add 5 uL of RAP F with a regular pipette tip. Use a P1000 wide-bore tip to mix by pipetting. Visually check to ensure the reaction is thoroughly mixed. Inversion can be used to aid mixing.

39 Incubate for 30 mins at RT. 30m

Library clean-up with glass beads 1h

40 Note: This section follows Matt Loose's NEMO bead clean-up protocol (https://www.protocols.io/view/findingnemo-a-toolkit-of-cohex-and-glass-bead-base-bxwrppd6?step=8) and is provided here for convenience. However, please refer to his protocol for additional details and/or alternative procedures.

41 Add 3 glass beads to the DNA solution in a 2-mL Eppendorf DNA Lobind tube.

Note: Glass beads can be washed following an acid-, bleach-, or SDS-wash protocol then sterilized. Sterilization can be by autoclaving or just storing the beads in 70% ethanol.

42 Add an equal volume of 10 mM Hexamminecobalt(III) Chloride (CoHex) into the DNA solution.
-This should be 1000 uL.

43 Rotate the tube with a vertical rotator at 9 rpm for 5-10 minutes.
-If a rotator is not available, invert the tube slowly by hand 30-40 times such that each full cycle takes around 5 seconds.

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44 Invert the tube by hand 3x more to ensure the DNA has precipitated and is tightly bound to the beads.

45 Discard the supernatant. Take care not to disturb the DNA precipitated onto the beads.

46 Wash the glass beads by gently adding 1 mL of PEGW buffer and inverting 2-3 times slowly. Incubate for 3 mins at RT.

47 Discard most of the supernatant, again taking care not to disturb the DNA precipitate.

48 Wash the glass beads by gently adding 500 uL of PEGW buffer and inverting 2-3 times slowly. Incubate for 3 mins at RT.

49 Discard the supernatant, taking care not to disturb the DNA precipitate. It isn't necessary to remove everything; a small volume of liquid can be left behind.

50 Pulse-spin the tube for 1 second and remove the last traces of buffer from under the glass beads with a fine pipette tip.

51 Quickly pour the beads into a new 2-ml LoBind tube and immediately add 225 uL EB Buffer to the beads.
-Do not let the beads with DNA dry out, as it will make DNA homogenization into solution more difficult.
-As an alternative, the elution buffer can be aliquoted into a 2 ml tube prior to this step. The beads can then just be poured into the buffer.

52 Incubate the library at 37°C for 30 min. Gently aspirate and dispense the eluate over the glass beads at regular intervals with a wide-bore P200 tip to aid elution.
-we leave the beads to elute overnight at RT and do not pipette extra.

53 Insert a bead retainer into a clean 1.5 mL LoBind tube. Pour the beads into the bead retainer and centrifuge at 12,000 x g for 1 min.

54 Incubate for at least 30 mins at RT with regular pipette mixing.

55 Store the library at 4°C or proceed to loading it onto a flow cell.

Flow cell priming and loading

56 Let a PromethION flow cell equilibrate to RT for ~10 mins.

57 Place the flow cell onto the PromethION and run platform QC.

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58 Thaw the Sequencing Buffer (SQB), Priming Tether (PT) and one tube of Flush Buffer (FB) at RT. Mix by vortexing and spin down.

59 In a new tube, prepare the DNA library for loading as follows and gently mix with a wide-bore tip:

75 uL SQB
75 uL DNA library

Total = 150 uL

60 Wait 30 mins and gently mix with a wide-bore tip. Visually inspect to ensure the sample is homogenous.

61 Prepare the flow cell Priming Mix in a 1.5 ml Eppendorf tube, and mix by vortexing at RT.

1170 uL FB
30 uL PT

Total = 1200 uL

62 Using a P1000 set to ~400 µl, place the pipette tip in the inlet port of the flow cell and turn the wheel to the right to remove the air and a small amount of storage buffer. This will make the storage buffer flush with the inlet port opening.

63 Load 500 µl Priming Mix (FB + PT) via the inlet port slowly. Wait 5 minutes.

64 Pipette up 150 uL of your DNA library with a P1000 so that it's ready to load. Load another 500 µl Priming Mix (FB + PT) via the inlet port slowly.

65 Immediately load 150 uL of the DNA library into the inlet port. To aid in this process, you can use ther P1000 pipette to draw the DNA into the flow cell by sucking it from the waste port.

66 Ensure there are no gaps between subsequent DNA library loads and avoid introducing air bubbles that can damage the nanopores.

67 Let sit for 10 minutes and then start sequencing.

68 Wash the flow cell and reload a fresh library every 24 hours for a total of three loads per flow cell.

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