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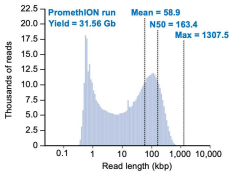
HMW gDNA purification and ONT ultra-long-read data generation V.4



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Protocol status: Working
We use this protocol, and it's working.

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Abstract

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 40-80 Gb of ultra-long-read ONT data with a read N50 of ~110-150 kbp on one PromethION flow cell.

Materials

Reagents

- Tris-HCl, 1M Solution, pH 8.0, Molecular Biology Grade, Ultrapure, Thermo Scientific Chemicals **Thermo Fisher Scientific Catalog #**J22638.AE
- UltraPure 0.5M EDTA pH 8.0 **Invitrogen - Thermo Fisher Catalog #**15575020
- SDS, 10% Solution **Life Technologies Catalog #**AM9822
- 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

Kits

- Ultra-Long DNA Sequencing Kit V14 **Oxford Nanopore Technologies Catalog #**SQK-ULK114

Disposables

- DNA LoBind Tubes, 1.5 mL **Eppendorf Catalog #**0030108051
- DNA LoBind 2.0ml PCR Clean Eppendorf Tubes **Eppendorf Catalog #**0030 108.078
- 15 mL MaXtract High Density tubes **Qiagen Catalog #**129065
- Disposable Inoculating Loops and Needles, Flexible Loop; Volume:10μ; Color: Yellow; Individual wrapped **Thermo Fisher Catalog #**22363600

Equipment

Water bath
Centrifuge
HulaMixer
Heat block
Nanodrop

Made-up buffers

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

Lysis Buffer

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

TE

- 10 mM Tris-HCl (pH 8.0)
- 0.1 mM EDTA (pH 8.0)

Cell collection and lysis

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

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 500 uL ice-cold TE (pH 8.0) 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 5 mL of lysis buffer + RNase A to the cell suspension, drop-wise in a circular motion. Swirl to mix.

7 Cover the flask with a parafilm lid and incubate the cell suspension for 1 hr at 37°C in a water bath.

8 Add proteinase K to a final concentration of 200 ug/mL in a drop-wise manner.
- For 5 mLs of cell suspension, add 50 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 once per hour.

11 Cool the solution to RT.

Phenol-chloroform extraction

12 Add the viscous lysate to a 15-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 (~5-5.5 mLs) to the tube containing lysate.

14 Gently mix the two phases by slowly rotating the tube end-over-end for 10 mins at 18 rpm on a Hulamixer in a fume hood.

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

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

17 Pour the aqueous phase into a new 15-mL conical tube.

18 Add an equal volume of ultra-pure phenol:chloroform:isoamyl alcohol (~5-5.5 mLs) to the tube containing lysate.

19 Gently mix the two phases by slowly rotating the tube end-over-end for 10 mins at 18 rpm on a Hulamixer in a fume hood.

20 Pour the lysate into a 15-mL 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 2 mL of 5M ammonium acetate (0.4 volume of lysate) 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 10 mL of 100% ethanol (2 volume of lysate) 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 Pulse-spin the tube for 1 second and remove the last traces of supernatant.

29 Store the pellet of DNA in an open tube at RT until the last visible traces of ethanol have evaporated (1-2 mins).
- Do not allow the pellet of DNA to dry completely. Desiccated DNA is very difficult to dissolve.

30 Add 760 uL EB and incubate without mixing at RT for 2 days to allow the pellet to fully resuspend into a translucent viscous gel.

ONT library preparation with the SQK-ULK114 kit

1h 45m

31 Pre-warm a heat block to 75°C.

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

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

246 uL HMW gDNA
4 uL FRA Buffer

Total = 250 uL

34 Mix the diluted FRA by pipetting.

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

36 Immediately mix the reaction by slowly pipetting 10x with a wide-bore pipette tip while on ice.

- 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: 35m

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

38 Add 5 uL of RA to the reaction with a regular pipette tip. Use a P1000 wide-bore tip to mix by pipetting 5x. Visually check to ensure the reaction is thoroughly mixed.

39 Incubate for 1 hr at RT. 1h

Library clean-up

40 Thaw PTB and EB at RT. Spin down briefly and keep on ice.

41 Add 500 uL of PTB to the tube with your gDNA.

42 Mix the reaction by rotating end-over-end 10 mins at 3 rpm on a Hulamixer. Check to see if a glassy white mass is visible.

43 Spin in a centrifuge at 1,000g for 1 min.

44 Discard the supernatant, taking care not to disturb the DNA precipitate.

45 Pulse-spin the tube for 1 second and remove the last traces of supernatant.

46 Add 200 uL of EB to the DNA precipitate.

47 Incubate O/N at RT.

48 The next day, gently mix the DNA library by slow pipetting 5-10 times with a P1000 wide-bore tip.

49 Measure the DNA library concentration using a Nanodrop.

50 Keep the DNA library on ice until you're read to load. Alternatively, store the library at 4°C.

Flow cell priming and loading

51 Let a PromethION flow cell equilibrate to RT for ~20 mins. Make sure a light shield is on the flow cell.

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

53 Thaw the Sequencing Buffer (SQB), Loading Solution UL (LSU), Flush Tether UL (FTU), and Flow Cell Flush (FCF) at RT. Mix by inverting and spin down.

54 In a new 2 mL tube, aliquot 100 uL of DNA library using a P1000 wide-bore tip.

55 In a new 1.5 mL tube, add the following and mix thoroughly by pipetting:

100 uL SBU
10 uL of LSU

Total = 110 uL

56 Add 110 uL of buffer mixture to the DNA library.

57 Gently mix the reaction by slowly pipetting 5-10 times with a P1000 wide-bore tip. Visually inspect to ensure the sample is homogenous.

58 Incubate the reaction for 30-60 mins at RT.

59 Prepare the flow cell priming mix in a 1.5 mL Eppendorf tube, and mix by pipetting.

30 uL FTU
1170 uL FCF

Total = 1200 uL

60 Using a P1000 set to ~500 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.

61 Load 500 µl priming mix (FTU + FCF) via the inlet port slowly. Close the cover and wait 5 minutes.

62 Pipette up 200 uL of your DNA library with a P200 with a 200 uL wide-bore tip so that it's ready to load. Load another 500 µl Priming Mix (FTU + FCF) via the inlet port slowly.

63 Immediately load 200 uL of the DNA library into the inlet port. To aid in this process, you can use the P1000 pipette to draw the DNA into the flow cell by sucking it from the waste port.

64 Let the flow cell sit for at least 30 minutes before sequencing.

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

66 Wash the flow cell and reload a fresh library every 24 hours for a total of three or four loads per flow cell. You can recover the DNA from the first two loads, store them at 4°C, and reload them for the third and fourth runs to maximize output.