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# HMW gDNA purification and ONT ultra-long-read data generation

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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. 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 1-2 Gb of ultra-long-read ONT data with an N50 ~150 kbp on one MinION flow cell.

MATERIALS

| NAME | CATALOG # | VENDOR |
|---|---|---|
| UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) | 15593031 | Thermo Fisher Scientific |
| Tris-EDTA, pH 8.0 | AM9849 | Ambion |
| Proteinase K | 19131 | Qiagen |
| RNAse A | 19101 | Qiagen |
| 200 Proof Ethanol pure | E7023 | Sigma Aldrich |
| Disposable Inoculating Loops and Needles, Flexible Loop; Volume:10µ; Color: Yellow; Individual wrapped | 22363600 | Thermo Fisher |
| Ammonium Acetate (5 M), RNase-free | AM9070G | Thermo Fisher |
| Lysis buffer [10 mM Tris-Cl (pH 8.0) 0.1 M EDTA (pH 8.0) 0.5% w/v SDS] | | |
| Phase Lock Gel Light | 2302820 | Quantabio |
| Rapid Sequencing Kit | SQK-RAD004 | Oxford Nanopore Technologies |
| FRA Buffer | | Oxford Nanopore Technologies |
| RAP buffer | | Oxford Nanopore Technologies |

## Cell collection and lysis

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

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 glass Erlenmeyer flask.
- For 1 mL of cell suspension, use a 50-mL flask; for 2 mL of cell suspension, use a 125-mL 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.

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 once per hour.

11 Cool the solution to RT.

## Phenol-chloroform extraction

12 Add light phase-lock gel to 4 15-mL polypropylene tubes.
- Phase-lock gels typically come in 2-mL tubes. Transfer the gel by cutting the lid off 3 2-mL phase-lock tubes, placing them upside down into the 15 mL tube, and spinning it at 4000 rpm for 1 min each.

13 Split the viscous lysate into the 2 15-mL Falcon tubes prepared with phase-lock gel.
- This is easiest using a 10 mL serological pipette at low speed.

14 Add an equal volume of ultra-pure phenol:chloroform:isoamyl alcohol (~5-6 mLs) to each Falcon tube containing lysate.

15 Gently mix the two phases by slowly turning the tube end-over-end for 20 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.

16 Spin in a centrifuge at 4500 rpm for 10 mins.

17 Pour the aqueous phases into two new 15-mL Falcon tubes containing phase-lock gel.

18 Add an equal volume of ultra-pure phenol:chloroform:isoamyl alcohol (~5-6 mLs) to each Falcon tube containing lysate.

19 Gently mix the two phases by slowly turning the tube end-over-end for 20 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 Spin in a centrifuge at 4500 rpm for 10 mins.

21 Combine the aqueous phases from the two tubes by slowly pouring them into a new 50-mL Falcon tube.

## Ethanol precipitation

22 Add 0.4 volume of 5 M ammonium acetate to the purified DNA, and gently swirl to mix (usually takes several minutes).
- It is very important to mix gently here. The gentler you mix, the longer your DNA will be.

23 Add 2 volumes of ethanol at RT and gently swirl to mix (usually takes ~30 mins).
- Swirl gently, sometimes rocking back and forth slowly to get the DNA in solution with the salt and ethanol.

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

25 The DNA forms a precipitate. Remove the precipitate in one piece from the ethanolic solution with a disposable inoculating needle shaped into a U.
- If the DNA precipitate becomes fragmented, abandon the needle and collect the precipitate by centrifugation 4500 rpm for 5 mins at RT.

26 Wash the DNA precipitate 2x with 70% ethanol, and collect the DNA by centrifuging at max speed (~15000 rpm) for 15 secs.

27 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 (~30 mins to 1 hr).
- Do not allow the pellet of DNA to dry completely. Desiccated DNA is very difficult to dissolve.

28 Add 125-250 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

29 Equilibrate an ONT MinION/GridION flow cell to room temperature.

30 Pre-set a thermocycler to the following program:
a. 30°C for 1.5 min
b. 80°C for 1 min
c. 4°C forever

31 Pre-warm the thermocycler by starting the program and then immediately pressing “Pause”.

32 Add the following reagents to a PCR tube on ice (in order).
Note: Use a wide-bore pipette tip for the gDNA to avoid shearing.

10 uL HMW gDNA (2-3 ug)
5 uL $dH_2O$
3 uL FRA Buffer

Total = 18 uL

33 Pipette the gDNA mixture up and down slowly >20 times on ice with a wide-bore pipette tip. Do not worry about shearing the DNA. Try to avoid creating bubbles.

34 In a lo-bind tube on ice, dilute FRA 1:12 in FRA buffer on ice
- FRA buffer composition is proprietary, but ONT will send you free FRA buffer if you ask them.
- This dilution can be modified depending on the needs of the sequencing experiment. In our hands, increased FRA concentration gives shorter reads but greater throughput.

35 Add 1.5 µl FRA (diluted) to the gDNA mixture on ice.

36 Pipette the gDNA mixture up and down slowly >20 times with a wide-bore pipette tip while keeping the tube on ice. Try to avoid creating bubbles.

37 Place the tube in the thermocycler and let the program continue.

38 After the thermocycler is finished, move the tube to ice, and bury it in the ice to cool it down from all sides.

39 In a lo-bind tube on ice, dilute RAP 1:12 in RAP buffer.
- RAP buffer composition is proprietary, but ONT will send you free RAP buffer if you ask them.

40 Add 0.5 µl RAP (diluted) to the gDNA mixture on ice .

41 Pipette the gDNA mixture up and down slowly >20 times with a wide-bore pipette tip while keeping the tube on ice. Try to avoid creating bubbles.

42 Incubate the gDNA mixture at room temperature for 2 hours.
- This incubation time can be reduced to 30 min, but we obtain greater yield with an extended incubation.

## Flow cell priming and loading

43 Place the flow cell on a MinION/GridION and run platform QC.

44 Remove the flow cell from the GridION and place in its holder to let it cool down for at least 10 mins.

45 Add 30 µl FLT to the tube of FB. Invert to mix and spin down.

46 Using a P1000 set to ~800 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.

47 Load 800 µl FLB + FLT via the inlet port slowly. Wait 5 minutes.

48 Meanwhile, thoroughly mix SQB by inverting.

49 Add 20 µl $dH_2O$ and 34 µl SQB to the gDNA mixture.

50 Pipette the gDNA mixture up and down slowly >20 times. Try to avoid creating bubbles.

51 Lift the cover off the SpotON port. Load 200 µl FLB + FLT via the inlet port slowly using a P1000 pipette. Try to dispense at a speed where a bead of liquid becomes visible over the SpotON port which then gets siphoned back in.

52 Using a P200 set to 75 µl with a wide-bore tip, pipette the diluted library onto the SpotON port as it gets siphoned in.

53 Close the SpotON sample port cover, and close the priming port.

54 Let incubate for 10 min before placing on the MinION/GridION and starting the run.