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Protocol status: Working
 We use this protocol in our group, and it is working.

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 33033

🌐 HMW gDNA purification and ONT ultra-long-read data generation V.1

👤 In 1 collection

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High molecular weight DNA extraction from all kingdoms
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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.

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

MATERIALS

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

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

⊗ Proteinase K **Qiagen Catalog #19131**

⊗ RNase A **Qiagen Catalog #19101**

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

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

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

⊗ Lysis buffer [10 mM Tris-Cl (pH 8.0) 0.1 M EDTA (pH 8.0) 0.5% w/v SDS] **Contributed by users**

⊗ Phase Lock Gel Light **Quantabio Catalog #2302820**

⊗ Rapid Sequencing Kit **Oxford Nanopore Technologies Catalog #SQK-RAD004**

⊗ FRA Buffer **Oxford Nanopore Technologies**

⊗ RAP buffer **Oxford Nanopore Technologies**

Cell collection and lysis

- 1 Freeze down $2-7 \times 10^7$ 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 5×10^7 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.

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

21 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 µL 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 dH₂O
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 dH₂O 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.

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- 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.