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FindingNemo in OneDay: Ultra-Long ONT Library Preparation from Cell to Flowcell in One Day

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We use this protocol and it's working

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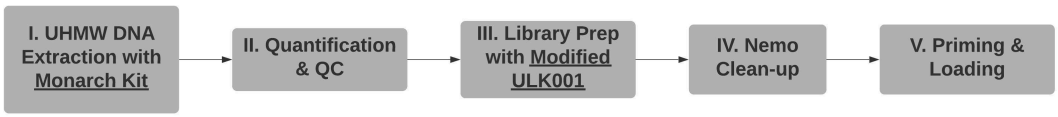
Abstract

This protocol is focussed on the isolation of ultra-high molecular weight (UHMW) DNA, library preparation and clean up ready for sequencing on the Oxford Nanopore Technology (ONT) platforms, all within **one working day**. This protocol is optimised for human cell lines.

Summary of the workflow:



Guidelines

Acknowledgements

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Please follow on Twitter for latest updates and results:
@NininUoN
@mattloose

Sample Results:

- Input cell number: 2 millions (around 13 µg DNA)
- Load amount: ~3.4 µg
- Active channels: 498
- Mean read length: 37,671.4
- Mean read quality: 11.0
- Median read length: 5,994
- Median read quality: 11.8
- Number of reads: 73,641
- Read length N50: 132,133
- Total bases: 2,774,156,337
- Longest read: 1,917,218 (13.6)

Materials

Chemicals/Compounds

- Hexamminecobalt(III) Chloride **Alfa Aesar Catalog #**A15470
- Tris-HCl pH 8.0 **Thermo Scientific Catalog #**J22638-AE
- Jurkat Genomic DNA **Thermo Fisher Catalog #**SD1111
- NaCl (5 M) RNase-free **Thermo Fisher Scientific Catalog #**AM9759
- 40% Polyethylene Glycol MW 8000 **Sigma – Aldrich Catalog #**P1458
- Isopropanol Absolute **Fisher Scientific Catalog #**P/7500/15
- 1X Phosphate Buffer Saline **Fisher Scientific Catalog #**15453819
- DNase I (RNase-free) - 1,000 units **New England Biolabs Catalog #**M0303S
- Nuclease-free Water **Thermofisher Catalog #**AM9920
- Calcium Chloride
- Magnesium Chloride **Fisher Scientific Catalog #**AC223210010
- Potassium Chloride
- HEPES **Fisher Scientific Catalog #**BP310

Made-up Buffers

PEGW Buffer

- 10% PEG-8000
- 0.5M NaCl

Nuclease Flush Buffer (NFB)

- 300mM KCl
- 2mM $CaCl_2$
- 10mM $MgCl_2$
- 15mM HEPES pH 8.0

Kits

- Monarch HMW DNA Extraction Kit for Cells & Blood **New England Biolabs Catalog #**T3050S
- Ultra-Long DNA Sequencing Kit (SQK-ULK001) **Oxford Nanopore Technologies Catalog #**SQK-ULK001
- Qubit dsDNA BR (Broad Range) assay **Thermo Fisher Scientific Catalog #**Q32850
- Qubit RNA BR Assay Kit **Thermo Fisher Scientific Catalog #**Q10211 (optional)

- ONT Flow Cell Wash Kit **Oxford Nanopore Technologies** **Catalog #**EXP-WSH004 (optional) for multiple loadings and storage on one flow cell)

Disposables

- DNA LoBind Tubes, 1.5 mL **Eppendorf** **Catalog #**0030108051
- DNA LoBind 2.0ml PCR Clean Eppendorf Tubes **Eppendorf** **Catalog #**0030 108.078
- Glass Beads 3 mm **Scientific Laboratory Supplies Ltd** **Catalog #**DD68501 **OR**
  Monarch DNA Capture Beads **New England Biolabs** **Catalog #**T3005L
- Thin-wall PCR Tubes 0.5 ml **Fisher Scientific** **Catalog #**12194142

Note

cut tube 2-3 mm from the bottom to make a bead retainer

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

- Monarch Collection Tubes II - 100 tubes **New England Biolabs** **Catalog #**T2018L (optional)

Note

or use any 1.5 ml centrifuge tube as a collection tube

- Wide-bore (or cut off) P1000 and P200 tips

Safety warnings

When handling phenol always wear PPE, keep a solution of 50% (w/v) PEG-400 nearby to treat the burn in the case of accidental splashes.

Before start

Things to observe at all times:

- Excessive and vigorous pipetting and vortexing should be avoided as these may shear the DNA.
- Make up buffers with nuclease-free water to avoid introducing nucleases to solutions.
- Avoid unnecessary heating and freezing; isolated DNA should be stable for storage in the fridge for months.

Monarch UHMW DNA Extraction Kit

1 We have obtained optimal DNA extractions using the NEB Monarch kit. This combines speed with high quality UHMW DNA. Follow the manufacturer's instructions as described **here**, BUT incorporate the following changes as described below.

Note

Our most homogeneous extracted DNA samples were obtained by lysis at 600-700 rpm speed using the Monarch Kit. This is one area that can be optimised depending on the input sample.

2 To complete in one day, start early!

Note

One million human cells are sufficient for a single library load on the MinION. At least two million human cells are required for a single load on the PromethION. Other samples can be scaled according to total amount of DNA recovered. So for a 1 gigabase genome you would require 3 million cells etc.

3 Dilute the eluted DNA with 150 µl of NEB Elution Buffer II.

Note

- This is following the NEB UHMW Monarch kit protocol where DNA is first eluted with 100 µl elution buffer. After this step, sample volume will be 250 µl total.
- Quantification of very viscous UHMW DNA is problematic and will not produce accurate results, hence the dilution.
- Gradual dilution is recommended to achieve a homogeneous final concentration of 50-100 ng/µl.

4 Incubate the eluted DNA at 37°C for 2-3 hours with regular pipette mixing. 3h

37 °C 03:00:00 max (2-3 hours)

Note

During mixing, observe by eye that the viscous DNA 'blob' has been more or less dissolved to the different parts of the tube (*i.e.*, less heterogeneous). This is usually observed after 2 hours of incubation. Otherwise, continue the incubation to 3 hours and proceed to the next step.

5 Quantify the DNA as per "**UHMW DNA QC**" (steps 6-10 below) and check homogeneity by calculating %CV values. If the DNA is not sufficiently homogeneous (*i.e.,* %CV ≥ 100%) incubate the DNA for longer.

UHMW DNA QC

6 Two nucleic acid quantification methods, *i.e.,* fluorometric (Qubit) and spectrophotometric (Nanodrop), can be used in parallel to assess both the quantity and purity of the extracted DNA. The quantification follows the published protocol by **Koetsier and Cantor** with slight modifications as follows.

An accurate measurement of DNA concentration is important as this will determine the optimum ratio of transposase to DNA molecules at the library prep step. Also, the viscous nature of UHMW DNA requires that sample measurement represents all parts of the DNA solution.

A total of 10 µl DNA is sampled from four different locations in the tube:

1. top
2. upper-middle
3. lower-middle
4. bottom

Each sample should be 2.5 µl and combined into a single 2 ml tube.

Note

- Use a cut P10 tip to aspirate the DNA sample and, if too viscous, cut the DNA thread by pushing the tip against the bottom of the tube.
- When available, a positive displacement pipette can also be used to ensure more accurate liquid aspiration.

7 Add a glass bead and pulse vortex at full speed for one minute.

2400 rpm, 00:01:00 vortex max speed

8 Quantify and calculate %CV as described in this paper by **Koetsier and Cantor**.

Note

Typically we take three measurements of 1 µl each on Nanodrop and one measurement of 3 µl using the Qubit DNA BR kit (standardized by the Jurkat genomic DNA).

Qubit dsDNA BR (Broad Range) assay **Thermo Fisher Scientific Catalog #**Q32850

Jurkat Genomic DNA **Thermo Fisher Catalog #**SD1111

9 Next quantify any RNA carry-over using the Qubit RNA Broad Range kit (optional).

Note

This step is to confirm that the RNA content is low to ensure maximum amount of DNA. It should be less than 50% of the DNA concentration measured with Qubit.

10 Wherever possible, the quality of extracted DNA sample should be analysed by method(s) that enable visual inspection of molecule length distribution such as:

- Regular agarose gel electrophoresis
- Pulsed-Field Gel Electrophoresis, *e.g.*, using Pippin Pulse (Sage Science)
- Agilent Bioanalyzer DNA
- Agilent TapeStation DNA

UL Library Prep

11 For library preparation, follow the "**Modified ULK001**" library prep protocol (modified from Oxford Nanopore Technologies SQK-ULK001) as linked below:

Protocol

NAME

FindingNemo Library 1: Modified ULK001

CREATED BY

Inswasti Cahyani

PREVIEW

11.1 Extracted UHMW DNA is often difficult to quantify due to its viscosity. However, accurate measurement of DNA concentration is crucial for calculating optimum ratio of the transposase enzyme to the DNA molecules.
We provide a protocol section for quantifying UHMW DNA in our 'FindingNemo' protocol master file.
Properly quantified DNA can then be processed for this library prep.

Both cell number and DNA concentration/amount are used to calculate the amount of transposase (FRA) and adapter (RAP-F).

We follow the original SQK-ULK001 protocol for the optimum ratio of transposase amount to human genomic DNA:

6 µl FRA to 6 million human cells (or around 40 µg DNA)

For other species, the genome size has to be taken into account and the FRA to DNA ratio optimised, *e.g.,* we had optimised a non-human cell line of **6.2 Gb genome** at:

2.5 µl FRA to 1 million non-human cell (around 12-15 µg DNA)

11.2 In a 2 ml tube, dilute UHMW DNA to a concentration of around 50 ng/µl in a total volume of 750 µl (with water or elution buffer if required).
Mix well with a P1000 wide-bore tip.

Note

- DNA concentration can still range from 20-50 ng/µl to have optimum tagmentation reaction.
- If input DNA amount is less than 20 µg (1-3 million cells used), halve all the reaction volumes, *i.e.,* 375 µl total DNA volume instead of 750 µl as in the table below.
- It is important to have as homogeneous DNA as possible at this step so the transposase can access and cut the DNA solution with an even distribution. It is OK to pipette thoroughly but gently.

	A	B	C	D	E
	Cell No. (million)	**Approx. DNA amount (µg)**	**Total DNA volume (µl)**	**DNA concentration (ng/µl)**	**Total reaction volume (µl)**
	6	>20-40	750	20-50	1000
	5				
	4				
	3	5-20	375		500
	2				
	1				

11.3 In a 1.5 ml tube, dilute the corresponding amount of transposase (FRA) with the dilution buffer (FDB) to a total volume of 250 µl (or 125 µl if doing half-reaction). More details in the table below.

	A	B	C	D	E
	Cell No. (million)	**Approx. DNA amount (µg)**	**FRA (µl)**	**FDB (µl)**	**Total reaction volume (µl)**
	6	>20-40	6	244	1000
	5		5	245	
	4		4	246	
	3	5-20	3	122	500
	2		2	123	
	1		1	124	

11.4 Mix the diluted FRA by vortexing for 2-3 seconds.

11.5 Using a P1000 wide-bore tip, add the diluted FRA to the DNA sample.
Stir the reaction with the pipette tip whilst expelling the diluted FRA to ensure an even distribution.
Mix thoroughly by gentle pipetting.
On ice

11.6 Incubate the reaction as follows:
25m
23 °C 00:10:00
70 °C 00:05:00
Room temperature 00:10:00 at least

Note

It is important that the room temperature at the fragmentation step (first incubation step) does not fall below 20^{o}C to ensure optimum reaction condition. The use of a water bath or heating block is recommended.

11.7 Add the corresponding volume of sequencing adapter (RAP-F) as in the table below.

	A	B	C	D	E
	Cell No. (million)	**Approx. DNA amount (µg)**	**FRA (µl)**	**RAP-F (µl)**	**Total reaction volume (µl)**
	6	>20-40	6	5	1000
	5		5	4.2	
	4		4	3.3	
	3	5-20	3	2.5	500
	2		2	1.7	
	1		1	0.8	

Note

- Use a P1000 wide-bore tip to pipette mix. Visually check to ensure the reaction is thoroughly mixed.
- Tube inversion can be used to aid mixing.

11.8 Incubate for 30 minutes at 23°C. 30m

23 °C 00:30:00

NEMO Library Clean-up

12 This section provides an alcohol-free purification of a nanopore DNA sequencing library from an UL protocol.

For 5-40 µg of input UHMW DNA (corresponding to DNA extracted from 1-6 million human cells), add **3** clean glass beads into the sample in a 2 ml tube.

Note

- For DNA amounts less than 5 µg, 2 glass beads can be used (see table at step 23). Two slightly larger glass beads from New England Biolabs (NEB; see Materials) can also be used per reaction.
- 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.

13 Add a 1:1 volume of 10 mM Hexamminecobalt(III) Chloride (CoHex) into the DNA solution.

Note

When the clean-up follows ONT ultra-long library preparation (SQK-ULK001), volumes can typically range from 500-1000 µl.

14 Rotate the tube with a vertical rotator at 9 rpm for 5-10 minutes. 5m

9 rpm vertical rotator 00:05:00 10m max

Note

- Rotate for 5 minutes if the DNA amount is less than 5 µg and adjust the time when more DNA is used, up to 10 minutes for 40 µg DNA.
- If a rotator is not available, hand inversion for 30-40 repeats can be used. Invert the tube slowly by hand such that each full cycle takes around 5 seconds.

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

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

17 Wash the glass beads by gently adding 1 ml of PEGW buffer and gently invert 3 times. Incubate for 3 minutes at room temperature. 3m

Room temperature 00:03:00

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

19 Repeat step 17 with 500 µl of the PEGW buffer.

20 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.

21 Insert a bead retainer into a collection tube.

22 Pour the beads from step 20 into the bead retainer and pulse-spin for 1 second in a mini centrifuge (or the shortest time possible) to remove any residual wash buffer. Keep the bead retainer.

Note

Omitting Dry-spin Step
When working with large amounts of DNA, this dry-spin step can be omitted to prevent DNA loss from spinning. Follow steps below:

- Remove the supernatant after the second wash as much as possible, pulse-spin the tube for 1 second and remove last traces of buffer from under the glass beads with a fine pipette tip.
- Proceed to the elution step.

23 Quickly pour the beads into a new 2 ml low-bind tube and immediately add the corresponding volume of elution buffer (ONT-EB or 10 mM Tris-HCl pH 8.0) per the table below.

	A	B	C
	DNA Input Amount (µg)	**No. of glass beads**	**Elution Buffer Volume (µl)**

	A	B (3-mm diameter)	C
	>30-40	3	225
	>20-30	3	180
	>5-20	3	120
	>2-5	2	90
	1-2	1	50

Note

- 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.

24 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. 30m

37 °C 00:30:00 mix per 10 min

25 Insert the bead retainer from step 22 into a clean 1.5 ml tube. Pour the beads from step 24 into the bead retainer and centrifuge at 12,000 x g for one minute. 1m

12000 rpm, Room temperature, 00:01:00

26 Incubate for at least 30 minutes at room temperature with regular pipette mixing. 30m

Room temperature 00:30:00 at least

27 Now - you have found Nemo!

Store the library at 4°C or continue loading it to a flowcell.

4 °C for storage

Note

Load at least 1 µg for MinION sequencing (or all of the library for input cells of 1 million).

Flowcell Priming & Library Loading

28 Prime the flow cell as per the MinION or PromethION protocol.
YouTube Video: Priming A MinION Flowcell

29 Quantify 2-3 µl of the library sample using fluorometric method (Qubit DNA BR kit) or alternatively the spectophotometric method (Nanodrop).

30 Mix 38-40 µl library (or at least 1 µg) with the same volume of sequencing buffer (SQB) from the SQK-ULK001 kit or SQK-RAD004, mix and incubate at room temperature for 30 minutes. 30m

Room temperature 00:30:00

31 Load the library per **SQK-ULK001 protocol** and let it tether for another 30 minutes before starting the run. 30m

00:30:00 tethering

32 Select the correct UL sequencing script based on the sequencing kit used (default mux scan should already be set to every 6 hours).

Home-brew Flowcell Wash/Flush (Optional) 1h

33 This section can be used to reload library on the same flowcell.
Add 2 µl DNase I to 398 µl nuclease flush buffer (NFB), vortex to mix.

34 After opening the priming port of the flow cell, check for a small bubble under the cover. Draw back a small volume to remove any bubble:

34.1 Set a P1000 pipette to 200 µl

34.2 Insert the tip into the priming port

34.3 Turn the wheel until the dial shows 220-230 µl, or until a small volume of buffer is seen entering the pipette tip

35 Using a P1000 pipette, load 400 µl of the NFB plus DNase I into the flow cell priming port.

36 Close the flow cell priming port and incubate the flow cell *in situ* for at least 1 hour. 1h

01:00:00 at least

37 Reprime the flow cell as in step 28.

go to step #28

38 Reload the library as in step 30-32..

go to step #30 go to step #31 go to step #32