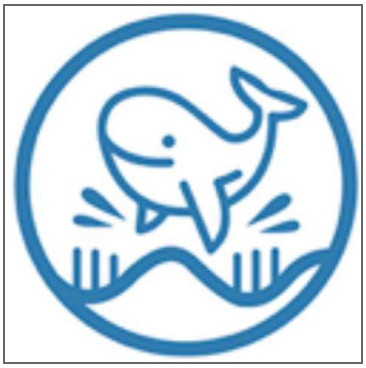

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FindingNemo (v.kit14): A Toolkit for DNA Extraction, Library Preparation and Purification for Ultra Long Nanopore Sequencing

Forked from FindingNemo: A Toolkit of CoHex- and Glass Bead-based Protocols for Ultra-Long Sequencing on ONT Platforms

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

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Keywords: ultra-long sequencing, cohex, glass bead, nanopore, MinION, UHMW DNA, Monarch, Circulomics, phenol, SDS, CTAB, GM12878, Whatman, PromethION, Nanobind, ULK001, RAD004

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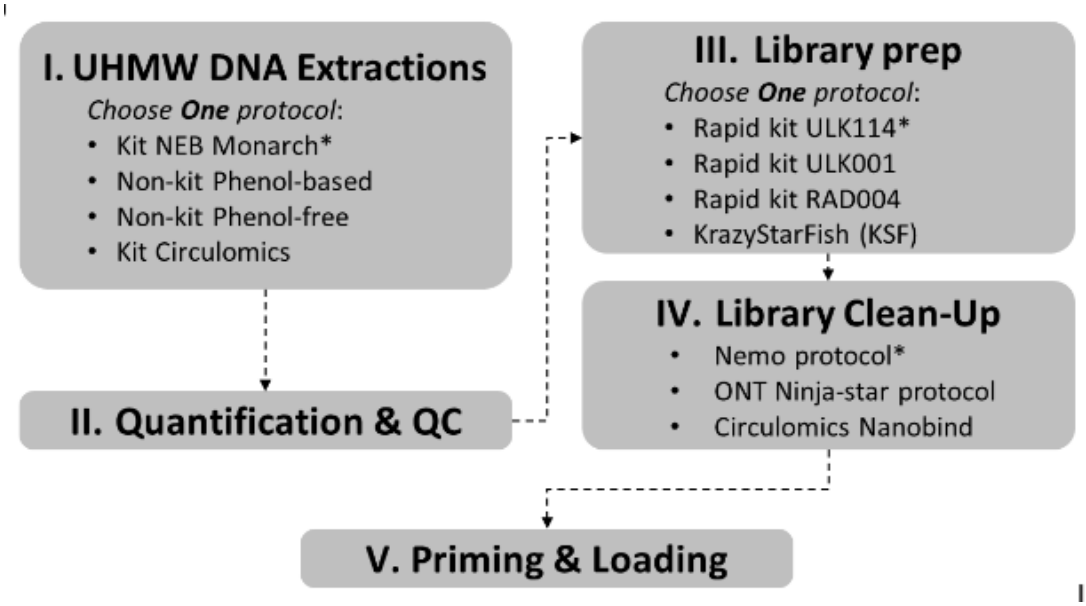
ABSTRACT

This collection of protocols is designed to enable ultra-long (UL) reads on Nanopore sequencers. It is split into five sections dealing with ultra-high molecular weight (UHMW) DNA:

1. Extraction
2. QC
3. Library preparation
4. Nemo clean-up using glass beads and Hexamminecobalt(III) Chloride, aka. CoHex.
5. Flowcell priming, library loading and run

We have tested and optimised the full protocol in **human cell lines**.

Various options are available for each of the steps and we hope that the components here will be useful to the community and provide a long-read toolkit.



Kit 14 version of the protocol is those with asterisks.

GUIDELINES

Acknowledgements

This protocol was developed by Inswasti Cahyani for the Long Read Club with significant contributions from John Tyson and Nadine Holmes, also Josh Quick, and continuous discussion and support of Matt Loose and Nick Loman. We would also like to thank Giron Koetsier (NEB) and Kelvin Liu (Circulomics) for lending their expertise and advance product trials.

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

Results

Below are some results from human genomic DNA runs on PromethION R10.4.1 flowcells after **24 hours** of run, to give an idea of the range of output produced from 6 millions of input cells:

	A	B	C	D	E
	Cell Line	N50 (bp)	Yield (Reads >= 100 kb)	Yield per Pore (Mb)	Average Occupancy
	GM12878	102,692	38.04 Gb (51.26%)	4.87	98.70%
	HG00273	133,881	37.43 Gb (63.36%)	4.76	98.65%

MATERIALS

Chemicals/Compounds

- Hexamminecobalt(III) Chloride **Alfa Aesar Catalog #**A15470
- 10 mM Tris-HCl pH 9.0
- Jurkat Genomic DNA **Thermo Fisher Catalog #**SD1111
- 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
- Nuclease-free Water **Thermofisher Catalog #**AM9920

Made-up Buffers

PEGW Buffer

- 10% PEG-8000
- 0.5M NaCl

Kits

- Monarch HMW DNA Extraction Kit for Cells & Blood **New England Biolabs Catalog #**T3050S
- ONT Ultra-long Kit (#SQK-ULK114)
- Qubit dsDNA BR (Broad Range) assay **Thermo Fisher Scientific Catalog #**Q32850
- ONT Flow Cell Wash Kit **Oxford Nanopore Technologies Catalog #**EXP-WSH004

Disposables

- DNA LoBind Tubes, 1.5 mL **Eppendorf Catalog #**0030108051
- DNA LoBind 2.0ml PCR Clean Eppendorf Tubes **Eppendorf Catalog #**0030 108.078
- DNA LoBind 5.0 ml Tubes **Eppendorf #0030108310**
- Glass Beads 3 mm **Scientific Laboratory Supplies Ltd Catalog #**DD68501
- Wide-bore (or cut off) P1000 and P200 tips
- 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 collection tube

SAFETY WARNINGS

CoHex is a potential carcinogent, handle with care.

BEFORE START INSTRUCTIONS

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.

UHMW DNA Extraction - Monarch Kit Direct Lysis

1 A DNA extraction protocol that yields **clean and homogeneous** UHMW DNA is essential for a good ultra-long (UL) sequencing output. We have routinely used the **New England Biolabs (NEB) Monarch HMW DNA Extraction Kit for Cells & Blood (T3050)** for UL library preparations, with the following modifications.

This protocol has been optimised in human cell lines.

2 For each reaction, use **6 millions** human cells, pellet and wash twice with cold PBS. 1m

7000 x g, 00:01:00

Note

Fresh or frozen cell pellet can be used. Ensure that cells have high viability.
Also, ensure that cell count is accurate as this influence the DNA amount as library input.

3 Heat thermomixer to 56 °C 700 rpm . If available, change thermoblock into one that accommodates 5 ml Eppendorf LoBind tube; the following steps will be done in 5 ml tube. If not available, use 2 ml Eppendorf LoBind tube.

4 Premix 400 µL Nuclei Prep Buffer with 400 µL Nuclei Lysis Buffer and 40 µL Proteinase K from the kit reagents, vortex briefly to mix.

> **Note**
>
> Make a master mix if extracting more than one sample. There is no need to make an excess of volumes.

5 Resuspend cell pellet in 400 µL PBS and move cell suspension into a 5 ml Eppendorf LoBind tube.

6 Add 800 µL Premix Solution to resuspended cells and mix by inverting 10 times.

7 Incubate on thermomixer at 56 °C shaking at 700 rpm for 00:10:00 10m

8 Add 20 µL RNaseA , mix by careful pipetting 5 times using wide-bore P1000 tip, then invert-mix tube 5-10 times.

9 Incubate on thermomixer at 56 °C for 00:10:00 . 10m

Note

Due to the high viscosity of the sample, RNA removal may be incomplete. So, invert the tube from time to time during incubation if necessary.

10 After lysis, add 300 µL Precipitation Enhancer and mix by inverting 10 times.

Note

If using 2 ml tube, split the cell lysate into two tubes, and halve the reagent volumes per tube in the following steps.

11 Using a clean forceps add 3 Capture beads.

Note

In 5 ml tube, the third bead is a 'sacrificed' bead as it will stuck at the bottom of the tube.
If using 2 ml tube, add 2 beads per tube.

12 Add 1.1 mL isopropanol

13 Slowly manually invert until DNA has bound on the beads and no further compacting is observed, up to 25-30 inversions. Alternatively: invert in a vertical rotating mixer 9 rpm, Room temperature , 00:05:00

14 Pre-aliquot 500 µL 10 mM Tris-HCl pH 9.0 into a clean 2 ml tube

Note

Both the Elution Buffer II from the Monarch kit and EEB from the ULK114 kit can be used for elution. We have routinely used Tris-HCl pH 9.0 for elution buffer and found that it homogenises DNA well.

15 Remove supernatant

16 Wash bound DNA with 750 µL Monarch Wash Buffer and carefully invert the tube 2-3 times. The DNA precipitate will contract more tightly around the beads. Remove Wash Buffer.

17 Repeat wash step once more.

18 When most of the wash buffer is removed pour beads in the Monarch Bead retainer sitting in a Monarch Collection tube. Remove residual wash buffer by pulse spin as short as possible.

Note

If DNA seems loose, omit the spin step and just absorb residual wash buffer with a lint-free tissue/Kimwipes. Air dry for 5 seconds.

19 Immediately pour DNA-bound beads into the tube with pre-aliquoted Tris-HCl.

Note

If using 2 ml tube up to this step, combine the 4 beads from the two tubes into one 500 ul elution volume.

20 Incubate on thermomixer at 56 °C for 00:10:00 to elute DNA off the beads. 10m

> **Note**
>
> Optional: shaking at 300 rpm can help homogenizing very viscous UHMW DNA.

21 In the mean time, place the bead retainer back into the collection tube and spin in a minifuge for a few seconds to dry the bead retainer. Remove the collection tube with the wash buffer traces and place the dried bead retainer in a new DNA LoBind 2 ml tube.

22 After elution, pour the beads with the eluted DNA into the bead retainer sitting in a 2 ml tube from the previous step.

23 Centrifuge at 16000 x g, Room temperature, 00:01:00 1m

> **Note**
>
> Check if DNA was completely released from the beads. If after the spin DNA threads are still visible between beads and eluate, centrifuge for 1 additional minute at maximal speed.

24 Add 260 µL 10 mM Tris-HCl pH 9.0 and using a wide bore pipette tip, carefully pipette eluate up and down 5-10 times for homogenization.

> **Note**
>
> If DNA is too viscous, additional 300-400 ul 10 mM Tris-HCl pH 9.0 can be added to dilute and homogenise DNA.

25 Incubate the samples at room temperature Overnight rotating vertically at 9 rpm

UHMW DNA QC

26 Two nucleic acid quantification methods, *i.e.,* fluorometric (Qubit) and spectrophotometric (Nanodrop), can be used in parallel to assess both the quantity and quality 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 (FRA) 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 9 µL DNA is sampled from three different points of the solution: top, middle, and bottom part. Combine in a 2 ml tube.

Note

- Use a cut P10 tip to aspirate DNA sample and if the sample is 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.

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

2400 rpm, 00:01:00 vortex max speed

Note

May use Monarch kit's glass bead (4 mm diameter) or the 3 mm one used in the FindingNemo protocol.

28 Quantify with Qubit kit using the Jurkat genomic DNA as a standard.

Note

If Jurkat genomic DNA is not available, the kit's lambda DNA standard can still be used. Nanodrop measurements will help averaging quantification values.

29 Quantify any RNA carry-over using the Qubit RNA Broad Range kit (optional).

Note

This step is to confirm that RNA content is low to ensure maximum amount of DNA. It should be less than 50% of the DNA concentration measured with Qubit. If RNA content is more than 100% than DNA, DNA quantification needs to be adjusted for library input, i.e. adding more DNA, or repeat RNaseA and glass bead precipitation. RNA quantification step is especially helpful if isolating DNA from a certain cell type for the first time.

30 Next, quantify DNA concentration and purity using Nanodrop (or other spectrophotometric devices), measuring three points in the solution: top, middle and bottom part.

Note

Calculate %CV of the sample. If it is still >=100%, homogenise by more regular pipetting and/or rotation at 9 rpm for few hours up to overnight as before.

31 Whenever 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

32 We are using the transposase-based library preparation kit from ONT, i.e. SQK-ULK114.

33 Dilute or prepare DNA sample extracted from 6 million cells so concentration is between 20-40 ng/µl in a 2 ml tube. Mix to homogenise well and incubate at RT. Then, cool on ice.

Note

Standard DNA volume required per UL library prep is 750 µL . If DNA concentration of this volume is >=50 ng/µl, dilute with 10 mM Tris-HCl pH 9.0 to make a **total** 900 µL volume.

34 Depending on the DNA volume in the previous step, prepare FRA dilution in a separate tube as in the table below:

	A	B	C
	DNA volume	750 ul	900 ul
	FDB (ul)	244	294
	FRA (ul)	6	6
	Final volume	1 ml	1.2 ml

35 Mix the diluted FRA by quick vortexing. While keeping the tube **on ice**, add the diluted FRA to the DNA. Stir the reaction with the pipette tip whilst expelling the diluted FRA to ensure an even distribution. Mix by gentle pipetting with a wide-bore pipette tip 25-30 times.

Note

Make sure sample is homogeneous and cold while mixing with FRA. The enzyme is fast acting and we want to make sure it spreads evenly through the DNA molecules before putting it for incubation at room temperature.

36 Incubate as follow: 22m

Room temperature while rotating 9 rpm for 00:10:00

75 °C for 00:10:00

On ice for 00:02:00

37 Add 5 µL RA to each sample with a regular pipette tip. Use a wide-bore tip to pipette mix. Visually check to ensure the reaction is thoroughly mixed.

38 Incubate at 9 rpm, Room temperature , 00:30:00 vertical rotator

FindingNemo Library Clean-up

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

Add **3** borosilicate clean glass beads (3 mm diameter) into the sample in a 2 ml tube.

Note

- We did not find significant difference when using borosilicate glass beads or the regular ones.
- Glass beads can be washed following an acid-, bleach-, or SDS-wash protocol, rinsed well with nuclease-free water then sterilized. Sterilization can be by autoclaving or just storing the beads in 70% Ethanol solution.

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

Note

When input DNA sample volume is 900 ul, total reaction volume will be 1.2 ml. Add 850-900 µl 10 mM CoHex or until the 2 ml tube is full.

41 Rotate the tube with a vertical rotator at 9 rpm for 3-5 minutes. 3m

9 rpm vertical rotator 00:03:00 5m max

Note

If a rotator is not available, hand inversion for 25-30 repeats can be used. Invert the tube slowly by hand such that each full cycle takes around 5 seconds.

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

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

44 Wash the glass beads by gently adding 1 ml of PEGW buffer and gently invert 2-3 times. 3m
Incubate at Room temperature for 00:03:00

45 Discard most of the wash buffer, again taking care not to disturb the DNA precipitate.

46 Repeat wash with 500 µL PEGW buffer .

47 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, i.e. dead volume.

48 Pulse-spin for 1 second in a mini centrifuge (or the shortest time possible) to collect residual wash buffer at the bottom. Remove last traces of buffer from under the glass beads with a fine pipette tip.

49 Quickly pour the DNA-bound beads into a new 2 ml tube that has been pre-aliquoted with 300 µL 10 mM Tris-HCl pH 9.0 or EB from the ULK114 kit.

Note

Do not let the beads with DNA dry out, as it will make DNA homogenization into solution more difficult.

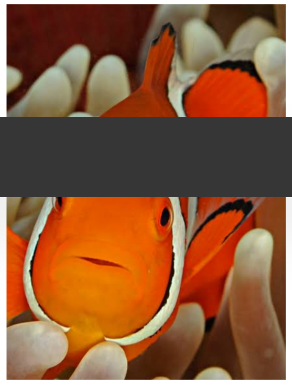
50 Incubate the library at 37 °C 00:30:00 mix per 10 min . Gently aspirate and dispense the eluate over the glass beads at regular intervals with a wide-bore P200 tip to aid elution. 30m

51 Insert a bead retainer into a clean 2 ml tube. Pour the beads from the previous step into the bead retainer and centrifuge at 10000 x g, Room temperature, 00:01:00 1m

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

Room temperature 00:30:00 at least

53 **Now - you have found Nemo!**



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

Flowcell Priming & Library L...

54 Prepare the priming mix as described in the ULK114 protocol using FCF and FTU.

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

Note

When available, check the quality of DNA with Agilent TapeStation genomic DNA protocol. The DIN value will confirm the library quality and the quantity measured can be compared with other methods.

56 Prepare the library mix for loading as follow:

	A	B
	Reagent	Volume (µl)
	Sequencing buffer (SBU)	100
	Loading solution (LSU)	10
	UL library	90
	Total volume	200

The recipe here is for three loadings.

Note

To maximise output, loading can be done four times depending on flow cell quality. The amount of library can be reduced by 10 ul each time, i.e. 90, 80, 70, 60 µl and diluted with 10 mM Tris-HCl pH 9.0 accordingly.

57 Load the library on the flow cell and let tether for at least 30 minutes before starting the run. 30m

00:30:00 tethering

Note

If time allows, it is better to tether for 1 hour.

58 Select the correct UL sequencing script based on the sequencing kit used (with the long winding down setting before every mux scan).

59 How to video from ONT:

YouTube Video: Loading a PromethION Flowcell

Flowcell Nuclease Wash/Flush

1h

60 Flush flowcell after every 20-24 hours or when active pores fall below 1000 (PromethION) using the flow cell wash kit (EXP-WSH004).

> **Note**
>
> Before flushing, ONT provides a protocol to recover library from the flow cell as can be found here. We haven't tested this after the first loads to maintain flowcell health. Principally, any recovered library can undergo another round of Nemo clean-up before reloading.

61 Reprime the flow cell as before.

62 Reload the library as before.