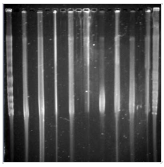

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FindingNemo Extraction 2: Phenol-free Method

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ABSTRACT

This is a sub-protocol designed to extract/isolate ultra-high molecular weight (UHMW) DNA to obtain ultra-long (UL) reads on Nanopore sequencers using a **phenol-free extraction** method.
A DNA extraction protocol that yields clean and homogeneous UHMW DNA is important for a good UL sequencing output. The choice of protocol should be based on achieving these parameters.

Kit-free, phenol-free protocol is a modification of NEB's Monarch HMW DNA Extraction Kit for Cells & Blood, with the option to use SDS or CTAB in the lysis buffer. This protocol also uses glass beads for DNA precipitation matrix.

We tested this sub-protocol in **human cell line**, with input cells of 5 millions but can be varied from 1-5 millions. As a rule of thumb, a million cells will suffice for one load on a MinION.

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PROTOCOL CITATION

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KEYWORDS

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

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GUIDELINES

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

MATERIALS TEXT

Chemicals/Compounds

- 5M Ammonium Acetate **Sigma –**
- **Aldrich Catalog #A-7330**
- Tris-HCl pH 8.0 **Thermo**
- **Scientific Catalog #J22638-AE**
- Ethanol
- Absolute **Honeywell Catalog #32221-2.5L**
- Chloroform:Isoamyl Alcohol [24:1] **Sigma –**
- **Aldrich Catalog #25666**
- Buffer-saturated Phenol **Sigma –**
- **Aldrich Catalog #77607**
- 1X Phosphate Buffer Saline **Fisher**
- **Scientific Catalog #15453819**
- Proteinase K,
- 2mL **Qiagen Catalog #19131**
- RNase
- A **Qiagen Catalog #19101**
- Nuclease-free
- Water **Thermofisher Catalog #AM9920**
- NaCl (5 M) RNase-free **Thermo Fisher**
- **Scientific Catalog #AM9759**
- EDTA (0.5 M, pH 8.0, nuclease-free) **Thermo Fisher**
- **Scientific Catalog #AM9260G**
- 20% Sodium dodecyl sulfate (SDS) **Contributed by users**
- Cetyltrimethylammonium Bromide (CTAB) **MP**
- **Biologicals Catalog #02194004-CF**

Made-up Buffer

Tris Lysis Buffer (TLB-SDS or TLB-CTAB)

- 100 mM NaCl
- 10 mM Tris-HCl, pH 8.0
- 25 mM EDTA, pH 8.0
- **TLB-SDS** = 0.5% (w/v) SDS ***or***

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- **TLB-CTAB** = 2% (w/v) CTAB

Disposables

DNA LoBind Tubes, 1.5

- mL **Eppendorf Catalog #0030108051**

DNA LoBind 2.0ml PCR Clean Eppendorf

- Tubes **Eppendorf Catalog #0030 108.078**

5Prime Phase-lock Gel (Light) 2 mL **VWR**

- **International Catalog #733-2477**

Centrifuge Tubes with CentriStar™ Cap 15

- ml **Corning Catalog #10738771**

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**

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

Monarch Bead Retainers **New England**

OR **Biolabs Catalog #T3004L**

Monarch Collection Tubes II - 100 tubes **New England**

- **Biolabs Catalog #T2018L** (optional)

or use any 1.5 ml centrifuge tube as collection tube

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

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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 STARTING

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

1 This protocol is adapted from Monarch® HMW DNA Extraction Kit for Cells & Blood.

Either SDS (anionic surfactant) or CTAB (cationic surfactant) can be used in the lysis buffer.
Providing alternative surfactants in the lysis buffer may help with different cell systems that require different biochemistry.

Cell Lysis 5m

2 Pellet 5 million cells in a 2 ml tube by centrifuging at 500 x g for 5 min at 4^{O}C. 5m

500 x g, 4°C, 00:05:00

3 Wash with 500 µl cold PBS and centrifuge at 500 x g for 3 min at 4^{O}C. Discard supernatant. 3m

500 x g, 4°C, 00:03:00

4 Resuspend well by pipette mixing in 20 µl cold PBS.

Thorough resuspension is important for next lysis step.

5 Add 1 ml **TLB-SDS** or **TLB-CTAB** and 100 µg RNase A (1 µl) and vortex at full speed for 5 seconds.

2000 rpm, 00:00:05

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6 Incubate at 37°C for 1 hour, mix by inversion every 15 minutes. 1h
37 °C **01:00:00 mix per 15 min**

7 Add 200 µg Proteinase K (10 µl). Mix by slow inversion 5 times.

8 Incubate at 50°C for 2 hours, mix every 30 minutes by slow inversion 3 times. 2h
50 °C **02:00:00 mix per 30 min (3x inversion)**

Phenol Separation

9 Split the lysate into 2 phase-lock gel tubes (ca. 550 ul per tube).

10 Add 550 µl buffer-saturated phenol to each tube containing lysate.

11 Place on a HulaMixer or any vertical rotator at 20 rpm for 10 minutes. If a fine emulsion has not formed after a minute gradually increase the rotation speed. 10m
00:10:00 vertical rotator

12 Centrifuge at 4500 rpm for 10 minutes. 10m
4500 rpm, Room temperature , 00:10:00

13 Transfer the aqueous phase to another phase-lock gel tube by pouring or using a wide-bore P1000 tip.

14 Add 250 µl buffer-saturated phenol and 250 µl chloroform-isoamyl alcohol to each tube.
Repeat step 11-12 and continue to 15.
go to step #11 **go to step #12**

15 Transfer and combine the aqueous phase to a 2 ml tube (sample will be ca. 1 ml).

16 Add 1 ml chloroform-isoamyl alcohol.
Repeat step 11-12 and continue to 17.
go to step #11 **go to step #12**

DNA Precipitation

17 Using a wide-bore P1000 tip, transfer the aqueous phase to a 5 ml conical tube.

Do not bring any liquid from the interface of the chloroform phase, as this will affect DNA purity in the downstream

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Do not bring any liquid from the interface or the chloroform phase, as this will affect DNA purity in the downstream steps.

18 Add 0.4x volume of 5M Ammonium Acetate (ca. 400 µl). Mix by slow inversion of tube.

19 Add 3 clean glass beads.

20 Add 3 ml absolute ethanol.

21 Rotate the tube with a vertical rotator at 9 rpm for 5 minutes. 5m

00:05:00 vertical rotator

If a rotator is not available, hand inversion for 30-40 times can be used. Invert the tube slowly by hand so that a full inversion cycle takes 5-6 seconds.

22 Remove solution, taking care not to disturb bound DNA on the glass beads.

23 Wash bound DNA with 1 ml of 70% ethanol. Invert tube for 2-3 times and discard the ethanol.

24 Repeat step 23.

DNA Elution 31m

25 Insert a bead retainer to a collection tube.
Pour the beads into the bead retainer and spin for 1 s in a mini centrifuge (or the shortest time possible) to remove residual wash buffer. Keep the bead retainer.

26 ⚠

Quickly pour the beads into a new 2 ml low-bind tube and immediately add 250 µl of elution buffer.

Do not let the beads with DNA dry out. (As an alternative, 250 µl of elution buffer can be aliquoted into a 2 ml tube prior to this step.)

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27 Incubate at 37°C for 30 min. Gently aspirate and dispense the eluate over the glass beads at regular intervals with a wide-bore P1000 tip to aid elution. 30m

37 °C **00:30:00 mix per 10 min**

28 Insert the bead retainer from step 25 into a clean 2 ml DNA low-bind tube.
Pour the beads from step 27 and centrifuge at 12,000 x g for 1 minute. 1m

12000 rpm, Room temperature , 00:01:00

29 Add another 510 µl of elution buffer to the eluate from step 28 and mix with a wide-bore P1000 tip.
Leave overnight at room temperature.

Room temperature **Overnight**

- DNA sample will be viscous, but it is important to pipette thoroughly with a wide-bore pipette tip to ensure homogenization.
- Homogeneous DNA sample will ensure consistent output length and yield.

30 Quantify DNA as per "**UHMW DNA QC**" and check homogeneity by calculating %CV values. If the DNA is not sufficiently homegeneous, incubate the DNA for longer.

31 Store at 4°C or continue to **UL Library Preparation** as per "**Modified ULK001**".
If only SQK-RAD004 is available, follow library preparation in "**Modified RAD004**" or "**KrazyStarFish (KSF)**".

4 °C for storage

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