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

Sep 08, 2021

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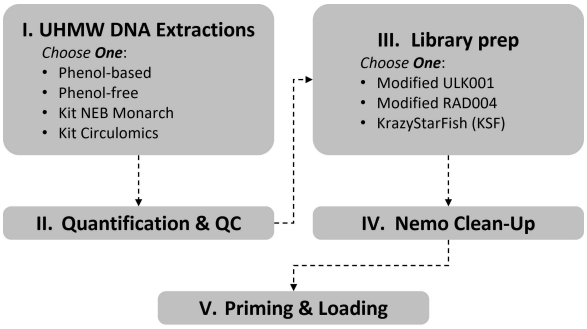
ABSTRACT

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 and library loading

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.



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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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GUIDELINES

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

Results

Below are some results from human genomic DNA runs on MinION flowcells, to give an idea of the range of output produced by certain number of input cells:

A	B	C	D	E	F	G
Input Cell	**Input DNA (µg)**	**FRA (µl)**	**Load DNA (µg)**	**N50 (kb)**	**Yield (Gb)**	**Passed Max Read (bp)**
6 millions	40	6	20	142.9	19.5	1,692,224
3 million	20	3	20	139.5	12.2	1,611,534
1 million	5	1	2	116.7	4.0	1,145,380

MATERIALS TEXT

Chemicals/Compounds

- 5M Ammonium Acetate **Sigma – Aldrich Catalog #A-7330**
- Hexamminecobalt(III) Chloride **Alfa Aesar Catalog #A15470**
- Tris-HCl pH 8.0 **Thermo Scientific Catalog #J22638-AE**
- Ethanol
- Absolute **Honeywell Catalog #32221-2.5L**

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Chloroform:Isoamyl Alcohol [24:1] **Sigma –**

- **Aldrich Catalog #25666**

Jurkat Genomic DNA **Thermo**

- **Fisher Catalog #SD1111**

40% Polyethylene Glycol MW 8000 **Sigma –**

- **Aldrich Catalog #P1458**

Buffer-saturated Phenol **Sigma –**

- **Aldrich Catalog #77607**

Isopropanol Absolute **Fisher**

- **Scientific Catalog #P/7500/15**

1X Phosphate Buffer Saline **Fisher**

- **Scientific Catalog #15453819**

Proteinase K,

- 2mL **Qiagen Catalog #19131**

RNase

- A **Qiagen Catalog #19101**

DNase I (RNase-free) - 1,000 units **New England**

- **Biolabs Catalog #M0303S**

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**
- Calcium Chloride **Contributed by users**

Magnesium Chloride **Fisher**

- **Scientific Catalog #AC223210010**
- Potassium Chloride **Contributed by users**

Triton X-100 **Sigma**

- **Aldrich Catalog #T8787-50ML**

Glycerol **Bio Basic**

- **Inc. Catalog #GB0232.SIZE.500ml**

HEPES **Fisher**

- **Scientific Catalog #BP310**

Made-up Buffers

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

- 100 mM NaCl
- 10 mM Tris-HCl, pH 8.0

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- 25 mM EDTA, pH 8.0
- **TLB-SDS** = 0.5% (w/v) SDS ***or***
- **TLB-CTAB** = 2% (w/v) CTAB

PEGW Buffer

- 10% PEG-8000
- 0.5M NaCl

4x MuA Buffer

- 100 mM Tris-HCl pH 8.0
- 40 mM $MgCl_2$
- 440 mM NaCl
- 0.2% TritonX-100
- 40% Glycerol

Nuclease Flush Buffer (NFB)

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

Kits

Nanobind CBB Big DNA Kit **Circulomics Catalog #NB-900-001-01** (optional)

Monarch HMW DNA Extraction Kit for Cells & Blood **New England Biolabs Catalog #T3050S** (optional)

This kit is necessary for the 1-day protocol

Ultra-Long DNA Sequencing Kit (SQK-ULK001) **Oxford Nanopore Technologies Catalog #SQK-ULK001**

Or use the older SQK-RAD004 rapid kit, complemented with 4x MuA buffer

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

Circulomics Nanobind UL Library Prep Kit **Circulomics Catalog #NB-900-601-01** (optional)

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)

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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**
- ☒ 8-strip PCR Tubes with Caps **Axygen Catalog #14-222-251**
- ☒ 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 Biolabs Catalog #T3004L**

OR

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

or use any 1.5 ml centrifuge tube as collection tube

☒ Whatman Filter Paper No.3 **Contributed by users Catalog #SKU1153211**

KrazyStarFish (KSF) disk:
6 mm star-shaped (or round-shaped) disk punched out of Whatman #3 cellulose filter paper

- 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 A DNA extraction protocol that yields clean and homogeneous UHMW DNA is essential for good UL sequencing output. The choice of protocol should be based on achieving these parameters.

We provide alternatives to achieve this by following kit-based or kit-free extraction protocols as follows:

1. **Kit-free, phenol-based**: a scaled-down version of Josh Quick's protocol (dx.doi.org/10.17504/protocols.io.mrxc57n) with additional glass bead step for DNA precipitation.
2. **Kit-free, phenol-free**: 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 as the DNA precipitation matrix.
3. **Circulomics Nanobind CBB Big DNA Kit**: initially the recommended extraction protocol for UL sequencing using SQK-ULK001.
4. **New England Biolabs (NEB) Monarch HMW DNA Extraction Kit for Cells & Blood**: a quick, tweakable extraction protocol fitting for a one-day library prep

We optimized the above protocols in human cell lines.

To start DNA extraction, choose **one** protocol from below:

1.1

Citation: Inswasti Cahyani, John Tyson, Nadine Holmes, Josh Quick, Nicholas Loman, Matthew Loose (09/08/2021). FindingNemo: A Toolkit of CoHex- and Glass Bead-based Protocols for Ultra-Long Sequencing on ONT Platforms. https://dx.doi.org/10.17504/protocols.io.bxwrppd6

FindingNemo Extraction 1: Phenol-based Method
by Inswasti Cahyani
PREVIEW RUN

1.1.1 This protocol is a scaled-down and modified version of the "**Ultra-long read sequencing protocol for RAD004 V.3**" by Josh Quick (https://dx.doi.org/10.17504/protocols.io.mrxc57n).

Elution volume is adjusted for downstream application of preparing ultra-long DNA library following the new ONT protocol (SQK-ULK001).

1.1.2 Pellet 5 million cells in a 2 ml tube by centrifuging at 500 x g for 5 min at 4°C. 5m
500 x g, 4°C, 00:05:00

1.1.3 Wash with 500 µl cold PBS and centrifuge at 500 x g for 3 min at 4°C. Discard supernatant. 3m
500 x g, 4°C, 00:03:00

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

Thorough resuspension is important for next lysis step.

1.1.5 Add 1 ml **TLB-SDS** and 100 µg RNase A (1 µl) and vortex at full speed for 5 seconds.
2000 rpm, 00:00:05

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

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

1.1.8 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)**

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

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

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1.11 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

1.12 Centrifuge at 4500 rpm for 10 minutes. 10m

4500 rpm, Room temperature , 00:10:00

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

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

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

1.16 Add 1 ml chloroform-isoamyl alcohol.
Repeat step 11-12 and continue to 17.

1.17 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 steps.

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

1.19 Add 3 clean glass beads.

1.20 Add 3 ml absolute ethanol.

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

Citation: Inswasti Cahyani, John Tyson, Nadine Holmes, Josh Quick, Nicholas Loman, Matthew Loose (09/08/2021). FindingNemo: A Toolkit of CoHex- and Glass Bead-based Protocols for Ultra-Long Sequencing on ONT Platforms. https://dx.doi.org/10.17504/protocols.io.bxwrppd6

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.

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

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

1.24 Repeat step 23.

1.25 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.

1.1.26 ⚠

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.)

1.27 30m
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.
37 °C **00:30:00 mix per 10 min**

1.28 1m
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.
12000 rpm, Room temperature , 00:01:00

1.29 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.

Citation: Inswasti Cahyani, John Tyson, Nadine Holmes, Josh Quick, Nicholas Loman, Matthew Loose (09/08/2021). FindingNemo: A Toolkit of CoHex- and Glass Bead-based Protocols for Ultra-Long Sequencing on ONT Platforms. https://dx.doi.org/10.17504/protocols.io.bxwrppd6

Homogeneous DNA sample will ensure consistent output length and yield.

1.30 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.

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

1.2


FindingNemo Extraction 2: Phenol-free Method
by Inswasti Cahyani
PREVIEW RUN

1.2.1 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.

1.2.2 Pellet 3 million cells in 1.5 ml tube by centrifuging at 1000 x g for 1 min at 4°C. 1m
1000 x g, 4°C, 00:01:00

1.2.3 Wash with PBS (make sure all media and serum are rinsed off), spin at 1000 x g for 1 min at 4°C. 1m
1000 x g, 4°C, 00:01:00

1.2.4 Add 149 µl of TLB-SDS **or** TLB-CTAB which has been added with 100 µg RNaseA (1 µl) and vortex at full speed for 3 seconds.

Make master mix of the TLB and RNaseA if handling more than one sample.

1.2.5 Incubate at 37°C for 10 minutes. 10m
37 °C **00:10:00**

Add 140 µl TLB and 200 µg Proteinase K (10 µl). Mix by slow inversion 5 times or with a P1000 wide-bore pipette tip.

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1.2.6

1.2.7 Incubate at 55°C for 20 minutes. 20m

55 °C **00:20:00**

Shaking in a thermomixer at 300-700 rpm can help lysis and homogenization, especially when more than 3 million cells are used.

1.2.8 Add 75 µl of 5M Ammonium acetate, mix well with wide-bore P1000 pipette tip.

1.2.9 Add 3 clean glass beads to the cell lysate.

2.10 Add 275 µl of Isopropanol

2.11 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 4-5 seconds.

2.12 Remove and discard liquid by pipetting.

2.13 Wash bound DNA with 1 ml of 70% ethanol, invert tube 3 times, remove and discard ethanol.

2.14 Repeat step 13 once with 500 µl. Discard the ethanol, taking care not to disturb the DNA precipitate.

2.15 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.

Citation: Inswasti Cahyani, John Tyson, Nadine Holmes, Josh Quick, Nicholas Loman, Matthew Loose (09/08/2021). FindingNemo: A Toolkit of CoHex- and Glass Bead-based Protocols for Ultra-Long Sequencing on ONT Platforms. https://dx.doi.org/10.17504/protocols.io.bxwrppd6

1.2.16 ⚠

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.)

2.17 30m
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.
37 °C **00:30:00 mix per 10 min**

2.18 1m
Insert the bead retainer from step 15 into a clean 2 ml DNA low-bind tube.
Pour the beads from step 17 and centrifuge at 12,000 x g for 1 minute.
12000 rpm, Room temperature , 00:01:00

2.19 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.

2.20 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

1.3 ⊛


FindingNemo Extraction 3: NEB Monarch Kit
by Inswasti Cahyani
PREVIEW RUN

1.3.1 This protocol is using **Monarch® HMW DNA Extraction Kit for Cells & Blood**.
Monarch HMW DNA Extraction Kit for Cells & Blood **New England Biolabs Catalog #T3050S**

1.3.2 We have obtained optimal 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.

> 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.

Citation: Inswasti Cahyani, John Tyson, Nadine Holmes, Josh Quick, Nicholas Loman, Matthew Loose (09/08/2021). FindingNemo: A Toolkit of CoHex- and Glass Bead-based Protocols for Ultra-Long Sequencing on ONT Platforms. https://dx.doi.org/10.17504/protocols.io.bxwrppd6

1.3.3 To complete DNA extraction from cell to library prep and sequencing in one day, start early!

> 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.

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

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

3h

1.3.5 Incubate the eluted DNA at 37^{o}C for about 2-3 hours with regular pipette mixing.

37 °C **03:00:00 max (2-3 hours)**

> 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.

1.3.6 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.

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

4 °C for storage

1.4

FindingNemo Extraction 4: Circulomics Kit
by Inswasti Cahyani
PREVIEW RUN

1.4.1 This protocol is using the **Nanobind CBB Big DNA Kit** (see Materials).

Citation: Inswasti Cahyani, John Tyson, Nadine Holmes, Josh Quick, Nicholas Loman, Matthew Loose (09/08/2021). FindingNemo: A Toolkit of CoHex- and Glass Bead-based Protocols for Ultra-Long Sequencing on ONT Platforms. https://dx.doi.org/10.17504/protocols.io.bxwrppd6

1.4.2 Follow the manufacturer's instructions as described here.

1.4.3 After overnight incubation at room temperature, quantify the DNA as per **"UHMW DNA QC"** and check homogeneity by calculating %CV values.

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

4 °C for storage

UHMW DNA QC

2 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.

- 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.

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

2400 rpm, 00:01:00 vortex max speed

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

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**

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5

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

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.

6

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

7 The UL library protocols we have tested are based on ONT's rapid kits, *i.e.,* RAD004 and ULK001, a transposase based method.

We offer three options for library prep protocols with the following features:

1. **Modified ULK001**: consistently produces N50 > 100 kb from good input quality UHMW DNA and is *our recommended route* for best output. The transposase reaction is performed in a large volume of up to 1 ml.
2. **Modified RAD004**: also consistently produces N50 > 100 kb from good input quality of UHMW DNA. This can be used when the ligation kit ULK001 is not accessible/available. The transposase reaction is again done in a large volume of up to 1 ml. Considering all the steps in the protocol, the Modified ULK001 kit is more cost effective.
3. **KrazyStarFish (KSF; RAD004-based)**: can consistently produce N50 > 100 kb with the right transposase to DNA molecule ratio. In addition, it uses filter paper as a matrix for DNA precipitation/clean-up.

Choose **one** of the protocols we tested below:

7.1

FindingNemo Library 1: Modified ULK001
by Inswasti Cahyani

PREVIEW RUN

7.1.1 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)

Citation: Inswasti Cahyani, John Tyson, Nadine Holmes, Josh Quick, Nicholas Loman, Matthew Loose (09/08/2021). FindingNemo: A Toolkit of CoHex- and Glass Bead-based Protocols for Ultra-Long Sequencing on ONT Platforms. https://dx.doi.org/10.17504/protocols.io.bxwrppd6

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)

7.1.2 ⚠

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.

- 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				

7.1.3 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	

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

7.1.5 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.

Citation: Inswasti Cahyani, John Tyson, Nadine Holmes, Josh Quick, Nicholas Loman, Matthew Loose (09/08/2021). FindingNemo: A Toolkit of CoHex- and Glass Bead-based Protocols for Ultra-Long Sequencing on ONT Platforms. https://dx.doi.org/10.17504/protocols.io.bxwrppd6

On ice

7.1.6 ⚠ 25m

Incubate the reaction as follows:

23 °C 00:10:00

70 °C 00:05:00

Room temperature 00:10:00 at least

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.

7.1.7 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	

- 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.

7.1.8 Incubate for 30 minutes at 23^{o}C. 30m

23 °C 00:30:00

7.2 ⊛

FindingNemo Library 2: Modified RAD004
by Inswasti Cahyani
PREVIEW RUN

Citation: Inswasti Cahyani, John Tyson, Nadine Holmes, Josh Quick, Nicholas Loman, Matthew Loose (09/08/2021). FindingNemo: A Toolkit of CoHex- and Glass Bead-based Protocols for Ultra-Long Sequencing on ONT Platforms. https://dx.doi.org/10.17504/protocols.io.bxwrppd6

7.2.1 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).
We base this protocol on SQK-RAD004 but follow the 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)

7.2.2 The following table lists the replaced reagents between ULK001 and RAD004.

A	B	C
Reagent	**ULK001**	**RAD004 (modified)**
Dilution buffer	FDB	MuA buffer (see Materials)
Transposase	FRA*	FRA^
Sequencing adapter	RAP-F	RAP

(*) FRA enzyme in ULK001 kit
(^) FRA enzyme in RAD004 kit

7.2.3 ⚠

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.

- DNA concentration can still range from 20-50 ng/µl to have optimum transposase 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 molecules 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				

7.2.4 In a 1.5 ml tube, dilute the corresponding amount of transposase (FRA) with MuA buffer to a total volume of 250 µl (or

Citation: Inswasti Cahyani, John Tyson, Nadine Holmes, Josh Quick, Nicholas Loman, Matthew Loose (09/08/2021). FindingNemo: A Toolkit of CoHex- and Glass Bead-based Protocols for Ultra-Long Sequencing on ONT Platforms. https://dx.doi.org/10.17504/protocols.io.bxwrppd6

125 µl if doing half-reaction). More details in the table below.

A Cell No. (million)	B Approx. DNA amount (µg)	C FRA (µl)	D 4X MuA Buffer (µl)	E 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	

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

7.2.6 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

7.2.7 ⚠

Incubate the reaction as follows:

23 °C **00:10:00**

70 °C **00:05:00**

Room temperature **00:10:00 at least**

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.

7.2.8 Add the corresponding volume of sequencing adapter (RAP) as in the table below.

A Cell No. (million)	B Approx. DNA amount (µg)	C FRA (µl)	D RAP (µl)	E 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	

Citation: Inswasti Cahyani, John Tyson, Nadine Holmes, Josh Quick, Nicholas Loman, Matthew Loose (09/08/2021). FindingNemo: A Toolkit of CoHex- and Glass Bead-based Protocols for Ultra-Long Sequencing on ONT Platforms. https://dx.doi.org/10.17504/protocols.io.bxwrppd6

- 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.

7.2.9 Incubate for 30 minutes at 23°C.

23 °C **00:30:00**

7.3

FindingNemo Library 3: KrazyStarFish (KSF)
by Inswasti Cahyani
PREVIEW RUN

7.3.1 This library prep is based on the rapid kit (**SQK-RAD004**) and using a home-made **MuA buffer** (see Materials). Input DNA is based on its concentration/amount without requiring prior knowledge on input cell number.

7.3.2 In a 1.5 ml tube (labelled as DNA), gently mix 75 µl DNA (~100 ng/µl) with 25 µl 4x MuA buffer.

Standard input DNA is 7.5 µg and can be for three loadings on MinION.

7.3.3 In another 1.5 ml tube (labelled as MuA), mix 25 µl 4x MuA buffer with 74 µl water and 0.1-1 µl FRA (depending on N50 targeted).

We consistently obtained N50 >= 100 kb using a ratio of:
0.6 µl FRA per 10 µg human genome DNA

7.3.4 Add/mix the 100 µl content of the MuA tube into the DNA containing tube, pipetting slowly with a wide-bore P200 tip and moving the tip constantly.

7.3.5 Make 100 µl aliquots into PCR tubes and treat at 30°C for 1 min, 80°C for 1 min and then cool to room temperature.

30 °C **00:01:00**

80 °C **00:01:00**

Room temperature **cool-off**

7.3.6 Pool reactions into a single tube and remove ~190 µl into a fresh 1.5 ml tube.

Citation: Inswasti Cahyani, John Tyson, Nadine Holmes, Josh Quick, Nicholas Loman, Matthew Loose (09/08/2021). FindingNemo: A Toolkit of CoHex- and Glass Bead-based Protocols for Ultra-Long Sequencing on ONT Platforms. https://dx.doi.org/10.17504/protocols.io.bxwrppd6

7.3.7 Add 12 µl of 5 M NaCl and mix gently (by flicking or pipetting with a wide-bore P200 tip).

7.3.8 Add a “kRAZYsTARFISH” filter (see Materials) to the tube so it is submerged.

7.3.9 Add 142 µl Isopropanol to the tube.

3.10 Mix contents gently by inversion 20-30 times, allowing UHMW DNA to collect and condense onto the filter.

3.11 Pulse-spin the tube and remove the liquid by passing pipette tip past the filter.

If the filter sticks to the side of the tip during this process gently place it back onto the side of the tube.

3.12 Wash the filter by addition of 500 µl 70% ethanol and gently invert the tube a few times.

3.13 Pulse-spin the tube and remove the liquid leaving the filter behind in the tube.

3.14 Repeat the 70% ethanol wash (step 12) once.

3.15 Pulse-spin the tube and remove any residual liquid leaving the filter behind and allow to air dry for a couple of minutes.

3.16 Transfer the filter to a 2 ml tube by tipping.

3.17 Add 125 µl of EB buffer, covering the filter, and allow tagged DNA to resuspend for 20-30 mins at 37^{O}C with occasional gentle mixing (flicking or pipetting with a wide-bore P200 tip).

37 °C **00:30:00 max**

We have found efficiency of resuspension to be very good from the cellulose filter, but some gains may be had if library is left longer when using larger amounts of UHMW DNA as input.

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3.18 Quantify as per section "**UHMW DNA QC**".

3.19 Store sample at 4^{o}C until ready to proceed to adapter (RAP) addition followed by sequencing.
This will be sufficient for about 3 sequencing runs/reloads.

4 °C for storage

3.20 Remove 37.0 µl of tagged DNA library into a fresh 1.5 ml tube.

3.21 Add 37.5 µl SQB buffer, mix gently (by flicking or pipetting with a wide-bore P200 tip).

3.22 Add 0.5 µl RAP, mix gently and incubate at room temperature for 30 minutes.

Room temperature **00:30:00**

3.23 Continue to Section "Flowcell Priming & Library Loading", **or** an optional "**Nemo**" clean-up step.

Adding the Nemo clean-up step will remove most free adapters. In our experience testing this, the yield/occupancy was only slightly improved as this parameter depends more on the optimum transposase reaction. Using the Nemo clean-up may improve non-optimal transposase-cut library.

NEMO Library Clean-up

8 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.

- For DNA amounts less than 5 µg, 2 glass beads can be used (see table at step 19). 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.

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

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

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

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

- 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.

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

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

13 Wash the glass beads by gently adding 1 ml of PEGW buffer and gently invert 2-3 times. Incubate for 3 minutes at room temperature. 3m

Room temperature **00:03:00**

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

15 Repeat step 13 with 500 µl of the PEGW buffer.

16 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.

17 Insert a bead retainer to a collection tube.

18 Pour the beads from step 16 into the bead retainer and pulse-pin for 1 second in a mini centrifuge (or the shortest time possible) to remove residual wash buffer. Keep the bead retainer.

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.

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19 ⚠

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 (3-mm diameter)**	**Elution Buffer Volume (µl)**
>30-40	3	225
>20-30	3	180
>5-20	3	120
>2-5	2	90
1-2	1	50

- 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.

20 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**

21 Insert the bead retainer from step 18 into a clean 1.5 ml tube. Pour the beads from step 20 into the bead retainer and centrifuge at 12,000 x g for one minute. 1m

12000 rpm, Room temperature , 00:01:00

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

Room temperature **00:30:00 at least**

23 Now - you have found Nemo!

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

4 °C for storage

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

Citation: Inswasti Cahyani, John Tyson, Nadine Holmes, Josh Quick, Nicholas Loman, Matthew Loose (09/08/2021). FindingNemo: A Toolkit of CoHex- and Glass Bead-based Protocols for Ultra-Long Sequencing on ONT Platforms. https://dx.doi.org/10.17504/protocols.io.bxwrppd6

Flowcell Priming & Library Loading

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

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

26 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**

27 Load the library per SQK-ULK001 protocol and let it tether for another 30 minutes before starting the run. 30m
00:30:00 tethering

28 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

29 

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.

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

30.1 Set a P1000 pipette to 200 µl

30.2 Insert the tip into the priming portone

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

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

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

Citation: Inswasti Cahyani, John Tyson, Nadine Holmes, Josh Quick, Nicholas Loman, Matthew Loose (09/08/2021). FindingNemo: A Toolkit of CoHex- and Glass Bead-based Protocols for Ultra-Long Sequencing on ONT Platforms. https://dx.doi.org/10.17504/protocols.io.bxwrppd6

01:00:00 at least

33 Reprime the flow cell as in step 24.

34 Reload the library as in step 26-28.

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