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FindingNemo Library 2: Modified RAD004

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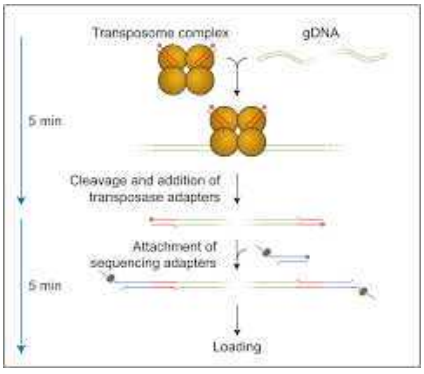
ABSTRACT

This sub-protocol is designed to prepare library from extracted ultra-high molecular weight (UHMW) DNA to obtain ultra-long (UL) reads on Nanopore sequencers. The UL library protocol we tested here is based on ONT's rapid kit, *i.e.,* **SQK-RAD004**, a transposase based adapter ligation kit.

This **Modified RAD004** protocol consistently produced N50 > 100 kb from a good input quality of UHMW DNA, for when ULK001 is not accessible/available. Transposase-based reaction is done in a large volume of up to 1 ml.

The working principle of the RAD004 kit is shown in the diagram below:



source: https://store.nanoporetech.com/uk/rapid-sequencing-kit.html

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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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52892

PARENT PROTOCOLS

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

GUIDELINES

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

MATERIALS TEXT

Chemicals/Compounds

- ☒ Tris-HCl pH 8.0 **Thermo**
- **Scientific Catalog #J22638-AE**
- ☒ Nuclease-free
- Water **Thermofisher Catalog #AM9920**
- ☒ NaCl (5 M) RNase-free **Thermo Fisher**
- **Scientific Catalog #AM9759**
- ☒ Magnesium Chloride **Fisher**
- **Scientific Catalog #AC223210010**
- ☒ Triton X-100 **Sigma**
- **Aldrich Catalog #T8787-50ML**
- ☒ Glycerol **Bio Basic**
- **Inc. Catalog #GB0232.SIZE.500ml**

Made-up Buffer

4x MuA Buffer

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

Kits

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Rapid Sequencing Kit **Oxford Nanopore**

- **Technologies Catalog #SQK-RAD004**

Disposables

DNA LoBind Tubes, 1.5

- mL **Eppendorf Catalog #0030108051**

DNA LoBind 2.0ml PCR Clean Eppendorf

- Tubes **Eppendorf Catalog #0030 108.078**
- 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 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

Library Prep Notes

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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)

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

Transposase Reaction

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				

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

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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)**	**4X MuA Buffer (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	

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

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 ⚠

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.

Adapter Ligation

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

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

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- 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.

9 Incubate for 30 minutes at 23^{O}C.

23 °C **00:30:00**

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