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FindingNemo Library 3: KrazyStarFish (KSF)

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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.

We named this protocol **KrazyStarFish (KSF)**. It offers a different approach to UL library prep, by using filter paper shaped as a starfish at the DNA precipitation/clean-up step. It can consistently produced N50 > 100 kb with the right transposase to DNA ratio.

This protocol was developed by John Tyson at UBC, Vancouver.

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PROTOCOL CITATION

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

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PARENT PROTOCOLS

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GUIDELINES

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Please follow on Twitter for latest updates and results:
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MATERIALS TEXT

Chemicals/Compounds

- ☒ Tris-HCl pH 8.0 **Thermo Scientific Catalog #J22638-AE**
- ☒ Ethanol Absolute **Honeywell Catalog #32221-2.5L**
- ☒ Isopropanol Absolute **Fisher Scientific Catalog #P/7500/15**
- ☒ 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

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

Disposables

- ☒ DNA LoBind Tubes, 1.5 mL **Eppendorf Catalog #0030108051**

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DNA LoBind 2.0ml PCR Clean Eppendorf

- Tubes **Eppendorf Catalog #0030 108.078**

8-strip PCR Tubes with

- Caps **Axygen Catalog #14-222-251**

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

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.

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- Avoid unnecessary heating and freezing; isolated DNA should be stable for storage in the fridge for months

Pre Library Prep

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.

Transposase Reaction

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.

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

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.

5 Make 100 µl aliquots into PCR tubes and treat at 30^{O}C for 1 min, 80^{O}C for 1 min and then cool to room temperature.

30 °C **00:01:00**

80 °C **00:01:00**

Room temperature **cool-off**

DNA Precipitation

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

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

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

9 Add 142 µl Isopropanol to the tube.

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

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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.

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

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

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

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

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

DNA Elution

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.

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

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

Adapter Ligation

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

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

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

Room temperature **00:30:00**

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.

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