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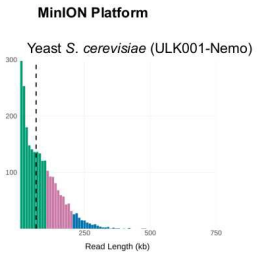
Ultra-Long Sequencing of Yeast Cells (S. cerevisiae) on ONT Sequencers – A Modified FindingNemo Protocol



Forked from ONT Ultra-Long Multiplex Sequencing of Yeast Cells (S. cerevisiae) – A Modified FindingNemo Protocol

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We use this protocol and it's working

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Abstract

This protocol is a bespoke modification of the **FindingNemo** protocol (LongRead Club) to enable ultra-long (UL) sequencing of yeast on Nanopore sequencers.
We have tested and optimised this in *Saccharomyces cerevisae* using the obsolete SQK-ULK001 kit.
It's not tested yet but we think the new ULK114 can be used to replace ULK001, using exact volumes of the corresponding reagents.
Let us know if you have tried it with the new kit!
Our best read N50 so far was ~67 kb (look at profile pic). This is not (yet) ultra-long, but may also be due to the distribution of chromosome sizes which is generally smaller than more complex eukaryotes, e.g. mammals.

Materials

A. Chemicals/compounds

- Sorbitol
- Na_2HPO_4
- 5M Ammonium Acetate Sigma – Aldrich Catalog #A-7330
- Tris-HCl pH 8.0
- 10mM Tris-HCl pH 9.0
- Ethanol Absolute Honeywell Catalog #32221-2.5L
- 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
- NaCl (5 M) RNase-free Thermo Fisher Scientific Catalog #AM9759
- EDTA (0.5 M, pH 8.0, nuclease-free) Thermo Fisher Scientific Catalog #AM9260G
- Cetyltrimethylammonium Bromide (CTAB) MP Biologicals Catalogue #02194004-CF
- Lyticase 5U/ul
- Hexamminecobalt(III) Chloride Alfa Aesar Catalog #A15470
- 40% Polyethylene Glycol MW 8000 Sigma – Aldrich Catalog #P1458
- ONT Ultra long kit #SQK-ULK001

B. Buffers

SpheroBuffer

- 1M Sorbitol
- 100 mM Na_2HPO_4
- 100 mM EDTA pH 8.0

Lysis Buffer

- 2M NaCl
- 20mM Tris-HCl pH 8.0
- 50mM EDTA pH 8.0
- 4% (w/v) CTAB

PEGW Buffer

- 10% PEG-8000
- 0.5M NaCl

C. Disposables

- DNA LoBind Tubes, 1.5 mL Eppendorf Catalog #0030108051
- DNA LoBind Tubes 2.0ml PCR Clean Eppendorf Tubes Eppendorf Catalog #0030 108.078
- 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 (optional), or use 0.5 ml PCR tube cut 2-3 mm from the bottom to make a bead retainer

- Monarch Collection Tubes II - 100 tubes New England Biolabs Catalog #T2018L (optional), or use any 1.5 ml centrifuge tube as a collection tube

Spheroplasting

1 In a 2 ml DNA LoBind tube, pellet ~200 million cells (50 µL circa volume of pellet per reaction).

2 Wash cells in cold **PBS** and centrifuge at 10.000 x g, 00:01:00 1m

3 Remove supernatant.

4 Repeat step 2-3 once more.

5 Resuspend pellet in 480 µL **SpheroBuffer**.

6 Add 20 µL **lyticase** (5U/µl), mix well with a wide-bore pipette tip and incubate at 30 °C 00:30:00 30m. Check spheroplast formation from time to time (if this has not yet been optimised before).

Cell Lysis

7 Add 480 µL **lysis buffer (LB)** and 20 µL **Proteinase K**, carefully mix with wide-bore pipette tip while avoiding any bubbles.

8 Incubate at 56 °C for 00:15:00 whilst shaking at 700 rpm 15m. The solution should clear up when protein digestion takes place.

9 Add 4 µL **RNaseA** and mix with a wide-bore pipette tip while avoiding any bubbles.

10 Incubate at 56 °C for 00:10:00 10m.

11 Add 400 µL **5M Ammonium acetate** and 100 µL **5M NaCl**

12 Mix by vertical rotation (Hula mixer) 9 rpm, Room temperature , 00:02:00

13 Centrifuge at 16.000 x g, Room temperature, 00:10:00 . 10m

14 Transfer the supernatant to a new 2 ml tube, careful not to transfer protein layer/precipitate at the bottom. If lysate is too thick to have layers, move as much clear lysate as possible.

DNA Precipitation

15 Add 3 glass beads (diameter = 3mm) to the cell lysate and 500 µL isopropanol.

> **Note**
>
> If the tube cannot accommodate 500 µl isopropanol, add as much isopropanol as possible (>400 ul). Then after DNA precipitation, take 200-300 µl from the upper part and replace with the same amount of isopropanol and rotate again to further precipitate DNA.

16 Mix on a Hula mixer at 9 rpm, 00:05:00 this can be extended to 10 minutes .

17 Check that the DNA has bound tightly around the beads and let sit for another minute.

18 Remove liquid by pipetting or carefully tipping it over while guarding the beads with the pipette tip.

19 Wash bound DNA with 1 mL of 70% **ethanol**, rotate the tube 2-3 times then discard the wash buffer.

20 Repeat wash once more.

21 Remove ethanol as much as possible, pour beads into a retainer tube on a collection column. Quickly spin (less than 1 second) on a table-top minicentrifuge.

Note

Alternatively, instead of spinning, just absorb excess ethanol with a wipe.

22 Quickly pour beads to a new 2 ml tube previously aliquoted with 100-120 µL **10mM Tris-HCl pH 9.0**

Note

If handling multiple samples, do step 21-22 one sample at a time. Do not let the DNA on the beads to dry as it makes DNA elution and homogenization more difficult.

23 Incubate at 37 °C for at least 00:30:00 with regular pipetting every 10-15 min using a wide-bore tip. 30m

24 Pour the beads into a bead retainer on a new 1.5 ml tube and spin at maximum speed for 00:01:00 . 1m

25 Incubate at Room temperature for a few hours or Overnight (slow rotation may aid elution). 2h

HMW DNA QC

26 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.
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.

27 Measure DNA concentration with Qubit.

Note

(Optional) measure RNA concentration to check that RNase treatment works efficiently. Reading should show only traces amount or <100% of [DNA]. If >100%, DNA concentration needs to be adjusted by this, e.g. halved.

28 For Nanodrop measurement, measure 3 positions from top, middle, and bottom part of the sample. Percent CV can then be calculated from these concentrations to gauge DNA homegeneity.

Note

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

29 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

Tagmentation and Adapter Ligation

30 Take 5 µg DNA and dilute with 10 mM Tris-HCl pH 9.0 to a total volume of 180 µL.
Mix well or rotate and incubate for 10 min. Cool sample on ice.

Note

Final DNA concentration should be between 20-40 ng/µL. Scale-up (or down) final volume if necessary.
DNA sample has to be ice cold when mixing with FRA.

31 In another tube make up FRA dilution (**1.2 µl FRA per microgram of yeast DNA**):

	A	B
		Volume (µL)

	A	B
	FRA	6
	FDB (FRA Dilution Buffer)	55
	Total volume	60

32 Mix the diluted FRA by vortexing. Cool on ice.

33 On ice, add 60 µL of the diluted FRA to the extracted DNA. Stir the reaction with the pipette tip whilst expelling the diluted FRA to ensure an even distribution.

34 Mix by gentle pipetting with a wide-bore pipette tip.

Note

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

35 Incubate the reaction as follows: 20m

22 °C - 00:10:00

70 °C - 00:05:00

22 °C - 00:05:00

36 Add 5 µL of **RAP** to the pooled sample with a regular pipette tip. Use a wide-bore tip to pipette mix. Visually check to ensure the reaction is thoroughly mixed.

37 Incubate for 00:30:00 at 22 °C. 30m

Nemo Clean-up 1h 34m

38 Add 3 glass beads into the tube with the adapted DNA.

39 Add 1:1 volume (240 µL) of **10 mM CoHex**.

40 Rotate tube at 9 rpm, 00:03:00 . Make sure there is no bead stuck at the bottom or at the cap, flick if this is the case.

41 Invert the tube again 2-3 times to ensure all DNA has precipitated and is tightly bound to the beads.

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

43 Wash the glass beads by gently adding 750 µL **PEGW buffer** and gently invert 2-3 times.

44 Incubate for 00:03:00 at room temperature. 3m

45 Aspirate and discard the supernatant, taking care not to disturb the DNA precipitate.

46 Repeat wash once more.

47 Pipette 120 µL of **EB** into a clean DNA LoBind 2 ml tube.

> **Note**
>
> Can also use Tris-HCl pH 9.0

48 Discard the second wash buffer as much as possible.

49 Quick spin the tube and use a 10 µl tip to discard the rest of the buffer at the bottom of the tube (move the beads aside with the tip while doing it). There will be a few microliters buffer left as a dead volume. This will not affect sequencing.

50 Immediately pour the beads into the 2 ml tube with the elution buffer.

51 Incubate the library at 37 °C for 00:30:00. Gently aspirate and dispense the eluate over the glass beads at regular intervals with a wide-bore pipette tip to aid elution. 30m

52 Continue with overnight incubation at room temperature.

53 The next day, insert a bead retainer into a clean 1.5-ml tube.

54 Pour the beads and spin at 10.000 x g, 00:01:00. 1m

55 Incubate for at least 00:30:00 at Room temperature with regular pipette mixing. 30m

56 Quantify the library

57 Load 30-40 µL library with **SQB** in 1:1 volume ratio.

> **Note**
>
> For PromethION flow cell, DNA volume needs to be scaled-up into a total 150 µl. Use EB or Tris-HCl pH 9.0 for this.

58 Incubate for 00:30:00 at Room temperature 30m

59 Load the library on a primed MinION flow cell.