Methods S1. Detailed Replicon-seq protocol, related to STAR Methods.

Replicon-seq protocol

Reagents and solutions needed:

- 10mg/mL of α-factor (dissolved in 100% methanol) custom synthesis from GenScript.
- BrdU stock solution: make a 10mg/mL stock solution by freshly dissolving BrdU (Acros organics CAS # 59-14-3) in water
- 10mM EDTA/1mM EGTA solution
- Zentner buffer A: 15mM of Tris-HCl pH 7.5, 80mM KCl, 0.1mM EGTA
    - Supplemented Zentner buffer A: per 5mL of Zentner buffer A add ½ Pierce mini-protease inhibitor EGTA-free (#A32955), 0.5mM Spermidine and 0.2mM Spermine
- Digitonin (Milipore #300410): make a 2% stock solution by dissolving digitonin in water boiled to 96ºC cool on bench until use.
- 200mM $CaCl_2$
- 2X stop solution: 400mM NaCl, 20mM EDTA and 8mM EGTA.
- Sorbitol solution: 1M sorbitol, 1mM EGTA, 10mM β-mercaptoethanol, and 50mM Tris-HCl pH 7.5
- Zymolyase solution: dissolve 5mg of 100T Zymolyase (nacalai tesque #07665-55) into 250uL of sorbitol solution
- RNase Buffer: 4mM EGTA, 50mM NaCl, 50mM Tris-HCl pH= 8.5, 10mM DTT
- RNase cocktail (Invitrogen #AM2288)
- 20mg/mL of Proteinase K (GoldBio, Cat #P-480-1)
- Chelex 100 resin (Biorad Cat #142-1253)
- Phenol-Chloroform-Isopropanol (24:25:1)
- Agencourt AMPure XP beads (Beckman coulter # A63882)
- SQK-LSK109 or SQK-LSK110 kit Nanopore technology library preparation kit
- MinION FLO-MIN106 flow cell R9
- NEBNext companion module (#E7180S)

Cell growth and arrest

1) Grow 500mL of yeast culture in YPD at 25ºC until OD=0.35.

2) Add 250uL of the 10mg/mL stock of α-factor to the culture (final concentration of alpha factor to the culture is 5ug/mL), incubate at 25ºC for 1 hour.

3) Add another 250uL of 10mg/mL of α-factor and incubate at 25ºC for 1hour.

4) Add 50uL of 10mg/mL of α-factor and incubate at 25ºC for 15 minutes.

5) Check under a light microscope that most of the cells are in G1 phase.

6) Add 20mL of the 10mg/mL BrdU stock solution (final concentration 400ug/mL), incubate at 25ºC for 30 minutes.

7) Pellet cell at 4,500rpm (3,400g) for 2min, wash pellet 3 times with 200mL of 25ºC pre-warmed YPD.

8) Release cells into 500mL of 25ºC prewarmed YPD containing 400ug/mL of BrdU.

9) Collect 100mL of cells at different times (20, 30 and 40 minutes for WT cells) by centrifugation – the sample to sequence will be determined by flow cytometry.

10) Pellet cells at 4,000 rpm (2,700 x g) at 4ºC for 1 minute.

11) Rinse pellet with 25mL of ice-cold EGTA/EDTA solution, spin at 4,000 rpm (2,700 x g) for 1 minute.

12) Flash freeze pellet in liquid nitrogen and store at -80ºC until further processing.

MNase-induced cleavage and DNA extraction

13) Thaw cell pellet on ice and resuspend with 1mL of EDTA/EGTA solution for about 10 minutes.

14) Transfer into 1.5mL Eppendorf tubes.

15) Spin 30 seconds at 10,000 x g.

16) Resuspend cells into 1mL of Supplemented Zentner buffer A.

17) Spin 30 seconds at 10,000 x g.

18) Wash cells twice more with 1mL of Supplemented Zentner buffer A, (pellet cells for 30 seconds at 10,000 x g).

19) Resuspend cells into 570uL of Supplemented Zentner buffer A

20) Incubate 1 min at 30ºC in a heat block.

21) Add 30uL of 2% digitonin solution.

22) Incubate 5 minutes at 30ºC in a heat block.

23) Transfer tube into an ice-water solution for 2 minutes.

24) Add 6uL of the 200mM $CaCl_2$ solution and incubate on ice/water slurry for 10 seconds.

25) Stop reaction by adding 610uL of 2X Stop buffer.

26) Incubate on ice for 2 minutes.

27) Spin at 10,000 x g for 20 seconds and remove supernatant.

28) Wash cells once with 1mL of Zentner buffer A (spin at 10,000 x g for 30 seconds).

29) Wash twice with 1mL of Sorbitol solution (spin at 10,000 x g for 30 seconds between each wash)

30) Resuspend cells into 250uL of Sorbitol solution.

31) Add 250uL of Zymolyase solution, mix by pipetting and incubate at 30ºC for 3 minutes, invert several times to allow mixing.

32) Check under light microscope that cell wall is digested (spheroplasts should burst with addition of 2% SDS), leave longer if necessary.

33) Spin spheroplasts at 10,000 x g for 1 minute.

34) Remove supernatant and wash pellet twice with 1mL of Sorbitol solution (spin at 10,000 x g for 30 seconds between each wash).

35) Wash spheroplasts once with 1mL of RNase buffer.

36) Resuspend cells into 500uL of RNase buffer and add 10uL of the RNase cocktail from Invitrogen

37) Incubate at 37ºC for 2 hours in a heat block.

38) Add 10uL of 20mg/mL of Proteinase K, 50mg of chelex 100 resin and 50uL of 20% SDS solution.

39) Mix by inversion and incubate at 55ºC for 1 hour

40) Prepare 2 phase lock tubes by adding vacuum grease (Dow Corning high vacuum silicone grease) into the cap of a 1.5mL Eppendorf tube, closing the tube and spinning for 2min at 10,000g. Add 560uL of Phenol-Chloroform-Isopropanol (PCI) solution.

41) Transfer the cell solution into the first phase lock tube containing the PCI mixture.

42) Invert slowly to mix, do not vortex and be very gentle to not break the DNA.

43) Spin at 10,000 x g for 5 minutes.

44) Repeat PCI extraction with the second phase lock tube by pouring aqueous phase on top. Mix by inversion and spin as previous steps.

45) Pour aqueous phase into a new 1.5mL Eppendorf tube (you should recover about 500uL of DNA solution).

46) Add 1/10 volume of 3M Sodium Acetate pH 5.2.

47) Add 900uL of 100% ethanol.

48) Mix tube by inversion, very slowly to not break the DNA molecule.

49) Incubate on ice for 5 minutes.

50) Spin at 10,000 x g for 20 minutes at 4ºC.

51) Wash DNA pellet twice with 70% ethanol do not dislodge pellet, do not vortex and do not pipette, spin 5 minutes at 10,000 x g.

52) Dry pellet on the bench for 15 minutes.

53) Add 40uL of ultra-pure DNA/RNase free water (it is important to not pipette nor try to resuspend the pellet, flick the tube a little by hand, do it gently and only flick two or three times).

54) Leave the DNA to resuspend overnight at 4ºC.

Nanopore library preparation and sequencing

Troubleshoot DNA before library preparation

55) Measure DNA concentration by qubit and check for purity with nanodrop. If purity is too low the library and sequencing will not be successful.

56) Run 1uL of the DNA onto a 0.8% agarose gel - the DNA should be high molecular weight. If you see some RNA, you will need to add more RNase and re-precipitate the DNA using the steps listed above.

Library preparation

57) Thaw all the reagent on ice from SQK-LSK109 or SQK-LSK110 kit from Nanopore technology. Ligation protocol was adapted from Genomic DNA by ligation SQK-LSK109 protocol version 14 August 2019.

58) In a thin wall PCR tube add the following (1uL of DNA DS, 3.5uL of NEBNext FFPE DNA repair buffer, 3.5uL of Ultra II End-prep reaction buffer, 2uL of NEBNext FFPE DNA repair Mix, 3uL of Ultra II End-prep enzyme Mix)

59) Mix the reagent by pipetting.

60) Add 1.2-1.8ug of DNA, into a PCR tube containing the end repair reagent, complete with water to final volume 60uL.

61) Mix gently by flicking and use a p1000 pipette to mix twice very gently.

62) Incubate in a PCR machine or heat block set at 20ºC for 10 minutes, switch to 65ºC for 5 minutes and cool on ice or 4ºC.

63) Transfer into 1.5mL Eppendorf tube.

64) Add 60uL of Ampure beads, mix by gently flicking the tube with your finger and incubate 5 minutes at room temperature.

65) Place the tube on a magnet wait 2 minutes, until the beads stick to the magnet and wash twice with 500uL of 70% ethanol (freshly prepared)

66) Remove ethanol by pipette leaving the tube on magnet; dry the bead on magnet for about 1 minute. Do not over-dry pellet.

67) Elute DNA from the beads by adding 61uL of ultra-pure RNase/DNase free water for 10 minutes to 30 minutes at 37ºC.

68) Place beads on magnet, wait 2 minutes and transfer 60uL the elution buffer containing DNA into a new tube (if the DNA is not fully eluted from the beads, most of the beads will move away from magnet when you try to remove supernatant).

69) Add to the 60uL of eluted DNA, 4uL of adaptor Mix (AMX), 25uL of the ligation buffer (LNB), and 10uL of the NEBNext Quick T4 DNA ligase (from the NEBNext companion module).

70) Mix gently by pipetting 2 or 3 times with a p1000.

71) Leave on the bench for 30 minutes.

72) Add 40uL of Ampure beads, mix gently by flicking the tube and incubate for 5 minutes on the bench.

73) Place tube on magnet, remove supernatant and wash twice with 250uL of long fragment buffer (LFB).

74) Dry pellet on magnet for 1 minute.

75) Remove tube from magnet.

76) Elute DNA with 15uL of EB at 37ºC for 30 minutes or until the beads do not come off the magnet when pipetting the eluted DNA.

77) Place tube on magnet and transfer eluted DNA into a new tube.

78) Take 1uL of DNA (make a 1:10 dilution, and measure concentration on qubit).

79) Take about 750ng to 1ug of DNA for sequencing.

Sequencing using MinION FLO-MIN106 flow cell R9

80) Take out the flow cell 5 to 10 minutes prior sequencing and warm up at room temperature; check flowcell pore quality.

81) Into a new tube mix 1mL of Flush buffer (FB) and 27uL of Flush tether (FLT), prepare flowcell by adding 800uL of the mix into the flow cell chamber, wait 5 minutes and add the other 200uL (follow the guidelines from Nanopore for preparing flowcell).

82) Into a new Eppendorf tube mix 25.5uL of loading beads (LB), 37.5uL of sequencing buffer (SQB), add 750ng to 1ug of DNA (store the rest of the DNA at -20C to reload flow cell or re-sequence), and complete with water for final volume to 75uL.

83) Load the mix from step 82 to the flow cell and sequence.

Sequencing run options used

84) Sequencing run options used: Select for SQK-LSK109 or SQK-LSK 110kit, 72h run, 180mV, and no base-calling

Check the run every 24h to see how the sequencing is performing, if pore activity is below 30%, pause the run, wash flow cells as described in Nanopore “flowcell wash protocol”. Then reload the rest of the library by repeating step 82 and 83. Do not forget to reprime your flow cell (step 81) before loading the library. Sequence until exhaustion of pores.

Data analysis

Basic data processing was performed using Guppy, Minimap2 and DNAscent V2. Note: reads were mapped to a custom genome (w303.fa) generated by processing WT Nanopore sequencing reads with Canu. Standard genome builds can be used, but alignment gaps will often be apparent at transposable elements.

1) Converting Fast5 files into FastQ files using guppy (Oxford nanopore technology).

```
guppy_basecaller -i fast5 -s guppy --cpu_threads_per_caller 200 --num_callers 1 -c dna_r9.5_450bps.cfg -x auto

cat guppy/*.fastq > reads.fastq
```

2) Mapping reads to the yeast genome using long read settings

```
minimap2 -ax map-ont w303.fa reads.fastq > alignment.sam

samtools view -Sb -o alignment.bam alignment.sam

samtools sort -@8 alignment.bam -o alignment.sorted.bam
```

samtools index alignment.sorted.bam

3) Call BrdU reads using DNAscent V2

DNAscent index -f fast5 -s guppy/sequencing_summary.txt

DNAscent detect -b alignment.sorted.bam -r w303.fa -i index.dnascent -o output.detect -t 25 --GPU 0 -l 250

DNAscent regions -d output.detect -o output.regions

4) Select reads with BrdU count ≥ 0.5

Output.regions reports the BrdU content of each read, broken into 250 bp segments. The fraction BrdU for each read is calculated by dividing the number of BrdU positive segments, by the total number of segments. Most analysis is performed with a cutoff of 0.5 – we generally avoided lower thresholds to increase stringency of our analysis.