nature portfolio

https://doi.org/10.1038/s41596-022-00793-5

Supplementary information

Genome-wide measurement of DNA replication fork directionality and quantification of DNA replication initiation and termination with Okazaki fragment sequencing

In the format provided by the authors and unedited

Supplementary protocol 1.

CRITICAL The supplementary protocol describes cell culture, EdU labeling, spheroplast preparation, and DNA isolation in yeast *S. cerevisiae*. After preparation of genomic DNA proceed directly to the main protocol step 22 (Size-fractionation of denatured genomic DNA on neutral sucrose gradients).

Materials

Biological materials

Yeast strain

CRITICAL Yeast strain is genetically modified to allow EdU uptake and is auxotrophic for thymidine. The growth mediums should be supplied with 100 µM thymidine.

- *S. cerevisiae* strain *MCM869 (MATa ade2-1 trp1-1 can1-100 leu2-3, his3_ 11,15 URA3::GPD_TK7x AuR1c::ADH_ hENT1 bar1Δ::LEU2 cdc21Δ::kanMX*^{100}*) (A kind gift from Dr. Arach Goldar)*.

Reagents

Cell culture reagents

- YPD Broth (Amresco, Cat. No. J903-500G)
- Thymidine (Sigma-Aldrich, Cat. No. T1895-5G)
- MilliQ water

Common reagents

CRITICAL Note that the reagents shared with the main protocol were not included.

- Glycerol (Sigma-Aldrich, Cat. No. G5516-1L)
- MOPS (Sigma-Aldrich, Cat. No. M1254-100g)
- $MgCl_2$, 1 M (Sigma-Aldrich, Cat. No. M1028)
- Potassium acetate (Sigma-Aldrich, Cat. No P1190)
- Spermine (Sigma-Aldrich, Cat. No. S3256) **CRITICAL** Store at 4 °C. Prepare 1 M stock solution and store at -20 °C.
- Spermidine (Sigma-Aldrich, Cat. No. S0266) **CRITICAL** Prepare 2 M stock solution and store the aliquots at -20 °C.
- Autoclaved MilliQ water **CRITICAL** Chill at 4 °C.

Equipment

CRITICAL Note that equipment shared with the main protocol were not included.

- Culture tubes (sterile; Dutscher, Cat. No. 039220)
- Inoculating loops (sterile, VWR, Cat. No. 80094-476)
- Incubator shaker (Eppendorf, Cat. No. Innova S44i)
- Allegra 64R High-Speed Centrifuge (Beckman, 367588) with fixed angle rotor JLA-9.1000 (Beckman, Cat. No. 366754)
- 1-L centrifuge bottles (Beckman, Cat. No. A98813)
- 0.5-L Erlenmeyer flasks (clean and autoclaved, Fisher Scientific, Cat. No. FB500500)
- 4-L Erlenmeyer flasks (clean and autoclaved, Fisher Scientific, Cat. No. FB5004000)
- Glass beads (0.5 mm diameter, thoroughly cleaned; Dutscher, 079053)
- 0.22 µm filter (Foxx Life Science, Cat. No. 76018-836)

Reagent set-up

Cell culture

- **100 mM thymidine solution**

Dissolve 96.89 mg of thymidine into 4 mL ddH_2O and filter with 0.22 µm syringe filter to prepare 100 mM stock solution. Aliquot and store at -20 °C for up to 1 year. Thaw and mix well before use.

- **YPD medium**

Dissolve 50 g of YPD broth powder in 1000 mL of MilliQ water and autoclave. Store at 4 °C for up to 2 months. Prewarm to 30 °C and add 1 mL 100 mM thymidine to the final concentration of 100 µM for yeast culture.

Common reagents

- **Nuclear isolation buffer**

Prepare as outlined below and adjust pH to 7.2. Filter with 0.22 µm filter, aliquot into 50-mL Falcon tubes, and store at -20 °C for up to 1 year. Thaw and place on ice before use.

Reagent	Final concentration	Stock	for 500 mL
Glycerol	34 % (vol/vol)	100 %	170 mL
MOPS	100 mM	powder	10.46 g
Potassium acetate	250 mM	powder	12.27 g
$MgCl_2$	4 mM	1 M	2 mL
Spermidine	1 mM	2 M	250 µL
Spermine	0.3 mM	1 M spermine	150 µL
ddH_2O			Up to 500 mL

Procedure

Yeast culturing, EdU labelling and harvesting ● **TIMING** 2-3 days of cell culture, 2 hours of labelling and harvesting.

1. In a laminar flow hood, prepare 3 culture tubes, each with 3 mL of YPD medium supplemented with 100 µM thymidine. Using an inoculating loop, transfer 2-3 colonies from YPD-thymidine agar plate into each tube to inoculate the yeast culture. Incubate the tubes in an incubator shaker at 30 °C 200 rpm overnight.
2. Transfer each of the 3 mL of overnight preculture in 4-L Erlenmeyer flasks containing 2 L of YPD medium supplemented with 100 µM thymidine and grow in an incubator shaker at 30 °C, 200 rpm until the culture reaches an optical density (OD) of 0.6-0.8.

CRITICAL Ensure to harvest at least 1×10^{11} cells in exponential growth phase.

3. Transfer the culture into 1-L centrifuge bottles, spin at 4000 g at RT for 10 min, discard the supernatant.
4. Resuspend all collected cell pellets in 400 mL of YPD medium without thymidine, pre-warmed to 30 °C and split into two aliquots of 200 mL in two 500-mL Erlenmeyer flasks.

CRITICAL It is easier to resuspend the cells first in a smaller volume of the medium before adding the rest of the medium.

5. Add 200 µL of 100 mM EdU into each of the Erlenmeyer flasks while gently shaking the cell suspension, and rapidly place in an incubator shaker at 28 °C, 200 rpm for exactly 2 min.

CRITICAL Set a 2 min timer before adding the EdU. Note that the final concentration of EdU is 100 µM for yeast.

6. Chill the flasks immediately by immerging and agitating in an ice-cold water bath. Add 1 mL of 0.5 M EDTA and mix. Store the flasks in the ice-cold water bath until all flasks are treated.

CRITICAL Respect the exact labelling time and immediately cool the flasks to quickly terminate the labelling.

7. Transfer cells to 50-mL Falcon tubes and centrifuge for 10 min at 4 °C, 4000 × g. Discard the supernatant.
8. Resuspend each pellet in 40 ml of ice-cold ddH2O. Centrifuge at 4000 g for 10 min at 4 °C. Discard the supernatant. Instantly proceed to spheroplast isolation.

Isolation of yeast spheroplasts • **Timing** 2 hours

CRITICAL This protocol is modified from Joel Huberman, https://joelhuberman.net/HubermanLabArchives/StandardYeastDNA.html).

9. Resuspend thoroughly the pellet of 1 × 10^{11} cells in 50 mL of ice-cold NIB buffer (final concentration of 1.5~2 × 10^{9} cells per mL).

CRITICAL Thaw the NIB in advance and keep on ice.

10. Split the yeast suspension into 4 equal aliquots of 12.5 mL in 50-mL Falcon tubes.
11. Add an equal volume of glass beads (0.45~0.52 mm at diameter) to each tube. Keep the tubes on ice.

CRITICAL For the efficient spheroplast release the total volume of cell-bead suspension should not exceed 26 mL per 50-mL tube.

12. In a cold room, vortex the tubes vigorously for 30 s at the highest speed. Chill the tubes in ice-cold water bath for ≥ 30 s.

CRITICAL The cell suspension should be adequately cooled down in between the agitations.

13. Repeat step 12 for 10 times more.

14. Control the spheroplast release by measuring the ratio of intact cells to cell wall ghosts by phase contrast microscopy. More than 90% of cell wall ghosts should be observed.

CRITICAL Alternatively the spheroplast release can be controlled by Trypan blue staining. For this mix an aliquot of nuclei suspension with an equal volume of Trypan blue and count the ratio of blue/ white cells (≥ 90% is expected).

15. Collect the suspension with a 10-mL pipette into a new 50-mL tube and keep on ice. Avoid taking the beads.
16. Wash the beads by adding 20 mL of ice-cold NIB and inverting the tubes.
17. Collect the NIB with a 10-mL pipette and combine with the suspension from step 15.
18. Repeat steps 16-17.

CRITICAL Keep the tubes on ice until all tubes are processed. The beads can be reused for the next aliquot of the same sample.

19. Spin the suspension at 4 000 g at 4 °C for 10 min. Discard the supernatant.
20. Proceed immediately to extraction of genomic DNA

PAUSE POINT. Cell pellets can be snap-frozen in liquid nitrogen and stored at -80 °C for up to one year.

Extraction of genomic DNA • **Timing** 2 hours with overnight incubation

21. Resuspend spheroplast pellets gently in 50 ml of Lysis buffer to a final concentration of ~1.5-2 × 10^9 of cells per mL. Distribute 10 mL aliquots of cell suspension to 50-mL tubes. Place the tubes on a rack at room temperature.

CRITICAL STEP Achieve single cell suspension- to ensure homogeneous cell lysis and optimal DNA extraction quality.

22. Follow step 4-13 in the main protocol for phenol-chloroform extraction of genomic DNA.
23. Transfer the upper aqueous phase containing genomic DNA into 50-mL tubes, each with 10 mL.
24. Add 2 mL of 7.5 M ammonium acetate into each tube and mix gently.
25. Add in each tube 25 mL of absolute ethanol, tightly close the caps and gently invert the tubes until all DNA precipitates.
26. Centrifuge the tubes for 20 min at 4000 at 4 °C with a swinging rotor, carefully remove the supernatant.

CRITICAL The pellets may appear brownish.

27. Carefully combine all pellets into one 50-mL Falcon tube with 20 mL of 75 % (vol / vol) ethanol and spin in a swinging rotor at 4000 g, 4 °C for 5 min. Discard the supernatant without disturbing the pellet.
28. Add 30 mL of 75 % ethanol and spin in a swinging rotor at 4000 g, 4 °C for 5 min. Discard the supernatant without disturbing the pellet.
29. Repeat step 28 one more time.
30. Remove any residual ethanol with a 1-mL tip. Keep the cap opened and air dry for 5 min.
31. Add 6 mL of TE to the DNA pellet. Leave the Falcon tube opened for 30 min at 37 °C in a dry oven to allow evaporation of residual ethanol. Close the cap.
32. Proceed to step 22 of the main protocol (Size-fractionation of denatured genomic DNA on neutral sucrose gradients)

CRITICAL Do not pipette or vortex the DNA solution.

PAUSING POINT Leave the DNA solution at 4 °C for at least 3-7 days until complete dissolution. The DNA can be stored for up to 2 weeks at 4 °C.

References:

99 Ma, E., Hyrien, O. & Goldar, A. Do replication forks control late origin firing in Saccharomyces cerevisiae? *Nucleic acids res.* **40**, 2010-2019 (2011).