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# An end-to-end workflow to study newly synthesized mRNA following rapid protein depletion in Saccharomyces cerevisiae V.4

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John B. Ridenour[1], Rafal Donczew[1]

[1]Cell Cycle and Cancer Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, USA

Donczew lab

John B. Ridenour
Cell Cycle and Cancer Biology Research Program, Oklahoma Med...



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## Abstract

In this protocol, we describe an end-to-end workflow for rapidly degrading a target protein using the AID system and quantifying newly synthesized mRNA using SLAM-seq in *Saccharomyces cerevisiae*. We describe methods for targeted protein degradation, 4-thiouracil (4tU) incorporation, rapid methanol fixation, RNA purification, RNA alkylation, 3´ mRNA-seq library construction, and data analysis. Although the individual methods described in this protocol are not novel per se, this workflow provides a complete resource for turnkey implementation of these methods, which will benefit others working with *S. cerevisiae*. In addition, this workflow is modular and readily adaptable to other systems, including industrial, pathogenic, or other model fungi, which will benefit the larger research community.

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## Guidelines

Standard laboratory guidelines and practices should be followed when performing this protocol. To ensure accurate size selection, researchers should use careful and consistent pipetting when performing this protocol.

## Materials

**Reagents:**

- Dimethyl sulfoxide, RNase-free (DMSO, ThermoFisher Scientific 327182500)
- 3-indoleacetic acid (IAA, Sigma I3750)
- 4-thiouracil (4tU, Sigma 440736)

Note

4tU is light sensitive and can crosslink upon exposure to light. Avoid exposure to light.

- Dithiothreitol (DTT)
- DNA/RNA Shield (Zymo Research )
- DNase I, RNase-free, and DNA digestion buffer (Zymo Research E1010)
- Iodoacetamide (Sigma I1149)
- Glycogen (20 mg/ml, Roche 10901393001)
- Sera-Mag SpeedBeads, magnetic carboxylate modified particles (Cytiva 65152105050250, ThermoFisher Scientific 09-981-123)

Note

Preparation of speedbeads and related buffers (DNA buffer and DNA binding buffer) is described in:

Protocol

NAME

**A method to prepare Sera-Mag SpeedBeads for purification and size selection of nucleic acids**

CREATED BY

**John B. Ridenour**

PREVIEW

- Maxima H Minus Reverse Transcriptase (200 u/ul) and 5X first-strand buffer (ThermoFisher Scientific EP0752)
- 2X Ex Premier (DNA polymerase) master mix (Takara RR370A)
- dNTP solution set, dATP, dCTP, dGTP, and dDTP (NEB N0446S)
- RNase H (NEB M0297L)
- IRA_UMI_24dTVN oligo (PAGE purification, IDT)
- IFA-isoTSO oligo (standard purification, IDT)
- Working stocks (5 µM ) of i5 (i5-D501 through i5-D508) and i7 (i7-D701 through i7-D512) oligo mixes (standard purification, IDT)

Note

Oligo sequences and methods for oligo preparation are described in (see also Foley et al., 2019, Glenn et al., 2019, and Veeranagouda et al., 2019):

Protocol

NAME

**High-throughput sequencing (HTS) oligos and methods to prepare oligos for HTS applications**

CREATED BY

John B. Ridenour

PREVIEW

- 100% methanol
- 100% ethanol
- Nuclease-free or DEPC-treated water
- MilliQ water

**Solutions:**

- 10 mM Tris-HCl (pH 8.0)
- 0.5 M sodium phosphate buffer (pH 8.0, prepared with nuclease-free or DEPC-treated water)
- 3 M sodium acetate (pH 5.2, prepared with nuclease-free or DEPC-treated water)
- 1 M DTT
- 100 mM DTT
- 20 mM DTT
- 80% ethanol (freshly prepared with nuclease-free or DEPC-treated water)

**Buffers:**

- Speedbead DNA buffer
- Speedbead DNA binding buffer

**Consumables:**

- 50 ml conical tubes (sterile)
- 1.5 ml microcentrifuge tubes (nuclease-free)
- 0.2 ml PCR tubes or strips (low-bind, nuclease-free)
- Quick-RNA Fungal/Bacterial Miniprep kit (Zymo Research R2014)

Note

Other RNA extraction/purification methods can be substituted. Alternative methods should be modified to maintain reducing conditions.

**Equipment:**

- Mini-Beadbeater-24 (Biospec Products)

Note

Other bead beaters or cell homogenizers can be substituted. Processing conditions should be adjusted accordingly.

- Thermomixer (e.g., ThermoMixer F1.5, Eppendorf) or dryblock
- Vortex mixer (e.g., Vortex Genie 2, Scientific Industries)
- Magnetic separation rack for 0.2 ml PCR tubes (e.g., NEBNext Magnetic Separation Rack, NEB)

Note

See Sergi Lab Supplies (https://sergilabsupplies.com) for economical and effective magnetic separation racks.

## Safety warnings

Standard safe laboratory practices and procedures and institution-specific waste management programs should be followed when performing this protocol.

## *Saccharomyces cerevisiae* growth, IAA treatment, 4tU labeling, and rapid fixation

1 Streak appropriate *S. cerevisiae* strain on YPD agar. Incubate culture at 30°C for 2-3 days.

> **Note**
>
> YPD agar and YPD are prepared and supplemented with adenine following standard procedures. Other media can be used. IAA treatment and 4tU treatment are based on Donczew et al., 2020 and Donczew and Hahn, 2021. Sampling and rapid fixation are based on Alexander et al., 2010, Barrass et al., 2015, Aslanzadeh et al., 2018, and Barrass and Beggs, 2019. Samples should be protected from light during Steps 13-21 when possible (e.g., work under red light or low light and keep samples covered with foil).

2 Transfer an individual colony to 5 ml of YPD. Incubate culture at 30°C with shaking at 220 rpm overnight.

3 Dilute overnight culture to an OD600 of 0.2 in 40 ml of YPD. Incubate culture at 30°C with shaking at 220 rpm.

4 When the 40 ml culture reaches an OD600 of 0.3-0.4, cool centrifuge to 4°C and prepare 50 ml conical tubes, 2.0 ml microcentrifuge tubes, and 1.5 ml microcentrifuge tubes.

5 Add 5 ml of 100% methanol to a 50 ml conical tube for each sample to be collected. Keep tubes on dry ice. Three tubes are needed for each 40 ml culture.

6 When the 40 ml culture reaches an OD600 of 0.4-0.5, split the culture into three 10 ml aliquots in 50 ml conical tubes and return them to the incubator.

Each 10 ml culture represents one of three treatments:

| | A | B | C |
|---|---|---|---|
| | Treatment | IAA treatment | 4tU treatment |
| | A | DMSO | 4tU |
| | B | IAA | 4tU |
| | C | DMSO | DMSO |

Note

DMSO is the carrier for both IAA and 4tU. Treatment C is a control treatment and may not be required for all experiments. For example, once background T>C conversion rates have been established for an experimental system Treatment C may not be required.

7 Equilibrate cultures at 30°C for 5 min with shaking at 220 rpm.

8 During equilibration (or just prior to splitting the culture), prepare a 0.2 M IAA solution in DMSO. IAA is freshly prepared. To prepare a 0.2 M IAA solution, add 220 mg of IAA to 6 ml of DMSO and vortex until dissolved. Keep the IAA solution at room temperature covered with foil.

9 **CRITICAL STEP:** Add 50 µl of 0.2 M IAA solution or DMSO to the appropriate 10 ml culture. Vigorously mix the culture and return it to the incubator. The final concentration of IAA is 1 mM.

10 Incubate cultures at 30°C for 25 min with shaking at 220 rpm.

Note

Cultures are generally incubated for 25 min for protein degradation prior to adding 4tU in Step 12. The total time for protein degradation is ~30 min. The time can be adjusted based on the experiment.

11 During IAA treatment, prepare a 2 M 4tU solution in DMSO. 4tU is freshly prepared. To prepare a 2 M 4tU solution, add 260 mg of 4tU to 1 ml of DMSO and vortex until dissolved. Keep the 4tU solution at room temperature covered with foil.

12 **CRITICAL STEP:** Immediately after IAA treatment, add 25 µl of 2 M 4tU solution or DMSO to the appropriate 10 ml culture. 4tU will precipitate when added to the culture; immediately and vigorously mix the culture and return it to the incubator. **It is essential that cultures are uniformly mixed to ensure 4tU is dissolved and homogeneously distributed**. The final concentration of 4tU is 5 mM.

13 Incubate cultures at 30°C for 4 min with shaking at 220 rpm.

Note

Cultures are generally incubated for 4 min for 4tU incorporation. The time can be adjusted based on the experiment.

14 **CRITICAL STEP:** Immediately after 4tU treatment, decant each 10 ml culture directly into 5 ml of 100% methanol in a 50 ml conical tube on dry ice (prepared in Step 5). Loosely cap the slurry and gently mix by swirling to ensure homogenization. Keep the fixed sample on dry ice. The final concentration of methanol is approximately 33%.

Note

**PAUSE POINT:** Samples in methanol can be stored at -80°C. Thaw samples stored at -80°C at room temperature until frozen media has melted (approximately 5 min) before proceeding.

15 Gently mix the fixed sample and transfer 250 µl to a 1.5 ml microcentrifuge tube. Store the 250 µl aliquot at 4°C for cell counting. Estimate cell number using OD600 or count cells using a Neubauer chamber.

Note

The estimated cell number will be used to normalize samples for western blotting. It is generally sufficient to measure the OD600 for one treatment (e.g., Treatment A) for a given strain or replicate; the results for all treatments should be identical.

16 Gently mix the fixed sample and transfer 1.5 ml to a 2.0 ml microcentrifuge tube for western blotting. Centrifuge the sample at max speed for 1 min to pellet cells, discard the supernatant, and freeze cell pellets at -20°C.

Note

Store cell pellets at -20°C (up to 2 weeks) or -80°C (long-term storage). The volume of culture collected is 1 ml. It is helpful to record the OD600 value (Step 16) on the 2.0 ml microcentrifuge tube for future reference. Although specific methods are not described in this protocol, the depletion of target proteins must be validated by western blotting in all experiments.

17 Centrifuge the remaining sample at 3,000 g and 4°C for 10 min to pellet cells.

18 Carefully decant the supernatant and briefly invert the 50 ml conical tube on a paper towel to remove residual supernatant.

19 Adjust samples to a uniform cell number in DNA/RNA Shield. Use the following formula to calculate the appropriate volume of buffer for each sample: volume of buffer = (final OD600 of sample/0.70)*400. Thoroughly resuspend cells by pipetting.

20 Ensure cells are thoroughly resuspended and transfer 400 µl of the cell suspension to a 1.5 ml microcentrifuge tube.

21 **PAUSE POINT:** Flash freeze samples on dry ice or in liquid nitrogen and store at -80°C until proceeding.

## *Schizosaccharomyces pombe* growth, 4tU labeling, and rapid fixation

22 **OPTIONAL STEP:** If experiments require spike-in, prepare 4tU-treated *S. pombe* cells as described in Steps 22-35. Streak appropriate *S. pombe* strain on YE agar. Incubate culture at 30°C for 3-4 days.

> **Note**
>
> YE agar and YE are prepared following standard procedures. Other media can be used.

23 Transfer an individual colony to 5 ml of YE. Incubate culture at 30°C with shaking at 220 rpm overnight.

24 Dilute overnight culture to an OD600 of 0.2 in 250 ml of YE. Incubate culture at 30°C with shaking at 220 rpm.

25 When the 250 ml culture reaches an OD600 of 0.6, cool centrifuge to 4°C and prepare 50 ml conical tubes and 1.5 ml microcentrifuge tubes.

26 Add 125 ml of 100% methanol to a 1 l flask for each culture to be collected. Keep flask on dry ice.

27 When the culture reaches an OD600 of 0.8, prepare a 2 M 4tU solution in DMSO as described in Step 11.

28 **CRITICAL STEP:** When the culture reaches an OD600 of 1.0, add 625 µl of 2 M 4tU solution. 4tU will precipitate when added to the culture; immediately and vigorously mix the culture and return it to the incubator.

29 Incubate the culture at 30°C for 4 min.

> **Note**
>
> Cultures are generally incubated for 4 min for 4tU incorporation. The time can be adjusted based on the experiment.

30 **CRITICAL STEP:** Immediately after 4tU treatment, decant the 250 ml culture directly into 125 ml of 100% methanol in a 1 l flask on dry ice (prepared in Step 26). Gently mix the slurry by swirling to ensure homogenization. Keep the fixed sample on dry ice.

31 Distribute 30 ml aliquots of the culture slurry to 50 ml conical tubes and centrifuge at 3,000 g and 4°C for 10 min to pellet cells.

32 Carefully decant supernatant and briefly invert 50 ml conical tubes on a paper towel to remove residual supernatant.

33 Thoroughly resuspend the cells in each 50 ml conical tube in 540 µl of DNA/RNA Shield by pipetting and pool cell suspensions in a fresh 50 ml conical tube on ice. Samples are adjusted to the equivalent of an OD600 of 7.0.

34 Ensure cells are thoroughly resuspended and distribute 500 µl aliquots of the cell suspension to 1.5 ml microcentrifuge tubes on ice.

35 **PAUSE POINT:** Flash freeze the samples on dry ice or in liquid nitrogen and store at -80°C until proceeding.

## RNA purification and DNase I treatment

36 Supplement the following buffers and reagents with DTT (100 mM DTT working stock):

| | A | B | C | D |
|---|---|---|---|---|
| | Treatment | Reagent vol. per sample | DTT vol. per sample | Final DTT conc. |

| | A | B | C | D |
|---|---|---|---|---|
| | RNA Lysis Buffer | 400 µl | 0.8 µl | 0.2 mM |
| | RNA Wash Buffer | 1.5 ml | 1.5 µl | 0.1 mM |
| | RNA Prep Buffer | 400 µl | 0.4 µl | 0.1 mM |
| | 100% ethanol | 550 µl | 0.55 µl | 0.1 mM |
| | Nuclease-free water | 50 µl | 0.5 µl | 1 mM |

Note

RNA purification and DNase I treatment is performed using a Quick-RNA Fungal/Bacterial Miniprep kit (see Materials). Methods are based on manufacturer's recommendations modified to maintain reducing conditions using DTT essentially as described by Alalam et al., 2022. Prewarm the required volume of nuclease-free water (supplemented with DTT) (see Step 54). Perform all centrifugation steps at 16,000 g and room temperature for 30 s (unless specified). Samples should be protected from light during Steps 36-55 when possible (e.g., work under red light or low light and keep samples covered with foil).

37 Thaw samples stored in DNA/RNA Shield at -80°C at room temperature.

38 Combine cells suspended in DNA/RNA Shield with RNA Lysis Buffer using one of the following options:

38.1 **OPTION A (without spike-in):** Combine 400 µl of RNA Lysis Buffer and 400 µl of cells suspended in DNA/RNA Shield.

38.2 **OPTION B (with spike-in):** Combine 360 µl of RNA Lysis Buffer and 400 µl of cells suspended in DNA/RNA Shield, and combine 500 µl of RNA Lysis Buffer and 500 µl of *S. pombe* cells suspended in DNA/RNA Shield.

Note

See Steps 22-35 for preparation of *S. pombe* cells. *Schizosaccharomyces pombe* cells are combined with *S. cerevisiae* cells in mass-to-mass ratio of 1:19 based on OD600. One aliquot of *S. pombe* cells provides material to spike 24 *S. cerevisiae* samples. If multiple *S. pombe* samples are needed for an experiment, they should be prepared individually, pooled, and thoroughly mixed to ensure the spike-in is consistent across the experiment.

39 Transfer cells suspended in DNA/RNA Shield:RNA Lysis Buffer to a ZR Bashingbead Lysis Tube using one of the following options:

39.1 **OPTION A (without spike-in):** Transfer 800 µl of cells suspended in 1:1 DNA/RNA Shield:RNA Lysis Buffer to a ZR Bashingbead Lysis Tube.

39.2 **OPTION B (with spike-in):** Transfer 760 µl of *S. cerevisiae* cells suspended in DNA/RNA Shield:RNA Lysis Buffer and 40 µl of *S. pombe* cells suspended in DNA/RNA Shield:RNA Lysis Buffer to a ZR Bashingbead Lysis Tube.

40 Process samples in a Mini-Beadbeater-24 at 3800 rpm for 45 s and immediately incubate on ice for 2 min.

41 Repeat Step 40 once.

42 While samples are incubating on ice, prepare the following reaction mixture in a 1.5 ml microcentrifuge tube on ice:

**DNase I reaction mixture**

| | A | B | C |
|---|---|---|---|
| | Reagent | 1X reaction vol. (µl) | Master mix vol. (µl) |
| | DNA Digestion Buffer | 75 | |
| | DNase I, reconstituted (1 u/µl) | 5 | |
| | Total | 80 | |

Note

Do not supplement DNA Digestion Buffer with DTT. DNase I is easily denatured. Do not vortex reaction mixture.

43 Centrifuge samples at 16,000 g for 1 min to pellet debris.

44 **CRITICAL STEP:** Transfer 550 µl of the cleared supernatant to a Zymo-spin IIICG Column in a collection tube and centrifuge at 16,000 g for 30 s. Save the flow through.

45 Add an equal volume (550 µl) of 100% ethanol to the flow through. Thoroughly mix samples by pipetting.

46 Transfer 700 µl of the mixture to a Zymo-spin IICR Column in a collection tube and centrifuge at 16,000 g for 30 s. Discard flow through.

47 Repeat Step 46 with the remaining mixture volume.

48 Add 400 µl of RNA Wash Buffer to the column and centrifuge at 16,000 g for 30 s. Discard flow through.

49 Gently mix DNase I reaction mixture (prepared in Step 42) by inversion and add 80 µl directly to the column matrix. Incubate the reaction at room temperature for 15 min.

50 Add 400 µl of RNA Prep Buffer to the column and centrifuge at 16,000 g for 30 s. Discard flow through.

51 Add 700 µl of RNA Wash Buffer to the column and centrifuge at 16,000 g for 30 s. Discard flow through.

52 Add 400 µl of RNA Wash Buffer to the column and centrifuge at 16,000 g for 1 min to ensure complete removal of buffer.

53 Carefully transfer the column to a labeled nuclease-free 1.5 ml microcentrifuge tube.

54 Add 50 µl of nuclease-free water prewarmed to 50°C directly to the column matrix, incubate at room temperature for 2 min, and centrifuge at 16,000 g for 30 s.

55 **PAUSE POINT:** Store purified RNA at -20°C (up to four weeks) or -80°C until proceeding.

## RNA alkylation and ethanol precipitation

56 Thaw RNA samples on ice. Quantify RNA using a Nanodrop.

Note

RNA alkylation (iodoacetamide treatment) method is based on Herzog et al., 2017 (see also Alalam et al., 2022 and Chappleboim et al., 2022). Alternatively, the SLAMseq Kinetics kit – Anabolic Kinetics module (Lexogen) can be used for alkylation following the manufacturer's recommendations. Iodoacetamide is referred to as IAA in Herzog et al., 2017; IAA refers to 3-indoleacetic acid in this protocol. Samples should be protected from light during Steps 56-62 when possible (e.g., work under red light or low light and keep samples covered with foil).

57 Add 5 µg of total RNA to a 1.5 ml microcentrifuge tube on ice. Adjust the volume of each sample to 20 µl with nuclease-free water.

58 Prepare a 100 mM iodoacetamide solution in DMSO. Iodoacetamide is freshly prepared. To prepare a 100 mM iodoacetamide solution, add 9.25 mg of iodoacetamide to 500 µl of DMSO (1 mg of iodoacetamide to 54 µl of DMSO). Keep the iodoacetamide solution at room temperature covered with foil. Do not store unused iodoacetamide.

59 Prepare the following reaction mixture in a 1.5 ml microcentrifuge tube and mix well by pipetting:

**Alkylation reaction mixture**

| | A | B | C |
|---|---|---|---|
| | Reagent | 1X reaction vol. (µl) | Master mix vol. (µl) |
| | 100 mM iodoacetamide | 5 | |
| | 0.5 M sodium phosphate buffer (pH 8.0) | 5 | |
| | DMSO | 20 | |
| | Total | 30 | |

Note

The sodium phosphate may form aggregates in the reaction mixture. The presence of aggregates will not interfere with the reaction. Prepare an additional 10% volume of master mix to avoid transferring aggregates.

60 Add 30 µl of reaction mixture to the 1.5 ml microcentrifuge tube containing 5 µg of total RNA in 20 µl nuclease-free water (prepared in Step 57). The final concentration of iodoacetamide is 10 mM.

61 Gently mix the reaction and incubate in a thermomixer at 900 rpm and 50°C for 15 min in the dark.

62 Add 1 µl of 1 M DTT to stop the reaction. Briefly vortex to mix. Exposure to light is acceptable following the addition of DTT.

63 Add 1 µl of glycogen (20 mg/ml) and 5 µl of 3 M sodium acetate (pH 5.2) to each sample. Briefly vortex to mix.

64 Add 125 µl of 100% ethanol to each sample. Briefly vortex to mix and incubate at -80°C for 30 min.

65 Centrifuge samples for 30 min at 21,000 g and 4°C to pellet precipitated RNA.

66 Carefully decant the supernatant. Take care not to dislodge pellet.

67 Add 1 ml of 80% ethanol to each sample. Centrifuge samples for 10 min at 21,000 g and 4°C to wash pellet.

68 Carefully decant the supernatant. Take care not to dislodge pellets. Carefully inspect samples and remove remaining ethanol with a 20 µl pipet.

69 Air dry samples for 5 min with lid open. Do not over dry samples.

70 Resuspend RNA in 30 µl of nuclease-free water.

71 **PAUSE POINT:** Store alkylated RNA at -20°C (up to four weeks) or -80°C until proceeding.

## 3´ mRNA sequencing library construction

72 **RNA fragmentation and cDNA synthesis.**

Note

3´ mRNA sequencing library construction is based on Foley et al., 2019 and Veeranagouda et al., 2019. Prewarm required volume of 10 mM Tris-HCl (pH 8.0) (see Steps 73.4 and 74.11).

72.1 Thaw alkylated RNA samples on ice. Quantify RNA using an RNA-specific fluorometry method (i.e., Qubit RNA BR or HS assay kit).

72.2 Add 200 ng of RNA to a 0.2 ml PCR tube or strip on ice. Adjust the volume of each sample to 5 µl with nuclease-free water.

72.3 Prepare the following reaction mixture in a 0.2 ml PCR tube (or 1.5 ml microcentrifuge tube) on ice and mix well by pipetting:

**Fragmentation reaction mixture**

| | A | B | C |
|---|---|---|---|
| | Reagent | 1X reaction vol. (µl) | Master mix vol. (µl) |
| | 5X reverse transcription buffer | 2 | |
| | 20 mM dNTP mix | 0.5 | |
| | 20 µM IRA_UMI_24dTVN oligo | 0.5 | |
| | Total | 3 | |

72.4 Prepare the following reaction mixture in a 0.2 ml PCR tube (or 1.5 ml microcentrifuge tube) on ice and mix well by pipetting:

**Reverse transcription (RT) reaction mixture**

| | A | B | C |
|---|---|---|---|
| | Reagent | 1X reaction vol. (µl) | Master mix vol. (µl) |
| | 20 mM DTT | 1 | |
| | 20 µM IFA-isoTSO oligo | 0.5 | |
| | Reverse transcriptase (200 u/µl) | 0.5 | |
| | Total | 2 | |

72.5 Bring the RNA and fragmentation reaction mixture to room temperature for 2-5 minutes and add 3 µl of the fragmentation reaction mixture to each sample. Mix well by pipetting.

72.6 **CRITICAL STEP:** Incubate samples at 94°C for exactly 4 min in a thermocycler with heated lid to fragment the RNA. Cool samples to 42°C in a thermocycler. Fragmentation time can be optimized to generate libraries of different insert sizes.

72.7 During RNA fragmentation, bring the RT reaction mixture (prepared in Step 72.4) to room temperature for 2-5 minutes.

72.8 Remove samples from the thermocycler, briefly centrifuge, and add 2 µl of the RT reaction mixture to each sample. Mix well by pipetting.

72.9 Briefly centrifuge and return samples to a thermocycler at 42°C.

72.10 Heat samples to 42°C for 60 min followed by 70°C for 10 min in a thermocycler with heated lid. Cool samples to 37°C in a thermocycler. Proceed immediately to RNA removal (Step 73.1).

## 73 RNA removal and single-stranded DNA purification.

73.1 Add 1 µl of RNase H to each sample and mix well by pipetting. Centrifuge briefly at room temperature to collect samples.

73.2 Heat samples to 37°C for 15 min in a thermocycler with heated lid. Proceed directly to speedbead purification.

73.3 Purify the single-stranded DNA (ssDNA). Add 39 µl of DNA buffer and 1 volume (50 µl) of speedbeads to each sample and proceed as described in the general method for speedbead purification (Step 76).

> **Note**
>
> Ensure speedbeads are equilibrated to room temperature and thoroughly mixed. DNA buffer and speedbeads can be mixed prior to use (here and in Step 74.5). If using commercial beads, it may be necessary to supplement beads with 0.05% Tween 20.

73.4 Elute DNA in 21 µl of 10 mM Tris-HCl (pH 8.0) prewarmed to 50°C.

73.5 Transfer 20 µl of the cleared supernatant to a fresh PCR tube.

73.6 **PAUSE POINT:** Store purified DNA at -20°C (up to eight weeks) or -80°C until proceeding.

## 74 Barcoding and enrichment PCR and library purification.

74.1 Transfer 10 µl of purified DNA to a 0.2 ml PCR tube on ice.

74.2 Add 2.5 µl of an appropriate 5 µM i5/i7 primer mix to each sample on ice.

74.3 Add 12.5 µl of 2X Ex Premier master mix to each sample on ice. Mix thoroughly by pipetting.

74.4 Barcode and enrich the library using PCR. Perform initial denaturation at 98°C for 45 s, followed by 12-18 cycles of amplification (98°C for 10 s, 60°C for 15 s, and 68°C for 15 s), and bring the reactions to 20°C.

> Note
>
> Use 12-14 amplification cycles if starting with ≥500 ng of total RNA, 16 amplification cycles if starting with 200 ng of total RNA, or 18 amplification cycles if starting with 100 ng of total RNA.

74.5 Purify the library. Add 25 µl of DNA buffer and 0.8 volumes (40 µl) of speedbeads to the 0.2 ml PCR tube containing the amplified library.

74.6 Mix well by pipetting up and down a minimum of 10 times. Alternatively, vortex samples on setting 4 for 3-5 seconds. If samples require centrifugation after mixing, stop the centrifuge before beads settle.

74.7 Incubate samples at room temperature for 5 min.

74.8 Place samples on magnetic stand for 2-5 min (or until solution is completely clear) to collect beads. Carefully remove and discard cleared supernatant without disturbing the bead pellet.

74.9 Remove samples from the magnetic stand. Add 30 µl of DNA buffer and mix well by pipetting or vortexing. Ensure beads are fully resuspend and incubate samples at room temperature for 2 min.

74.10 Add 0.8 volumes (24 µl) of DNA binding buffer to the 0.2 ml PCR tube containing the library and bead suspension. Proceed as described in the general method for speedbead purification (Step 76).

74.11 Elute the library in 21 µl of 10 mM Tris-HCl (pH 8.0) prewarmed to 50°C.

74.12 Transfer 20 µl of the cleared supernatant to a fresh PCR tube.

74.13 **PAUSE POINT:** Store purified libraries at -20°C (up to four weeks) or -80°C until proceeding.

## 75 Library quality control, quantification, pooling, and sequencing.

75.1 Assess library quality using an Agilent Bioanalyzer or agarose gel electrophoresis.

75.2 Quantify libraries using a DNA-specific fluorometry method (i.e., Qubit dsDNA BR or HS assay kit).

75.3 Pool equimolar concentrations (i.e., 5 nM) of each library. The recommended final concentration of the pool is 5 nM.

> **Note**
>
> See Glenn et al., 2019 for additional information on pooling and a template for calculating molar concentrations and volumes. Methods for library construction that use a template switching oligo (TSO), as described here, generate forward reads that start with three low-diversity bases. It is necessary to increase the diversity of the sequencing pool prior to sequencing on an Illumina instrument. Libraries should be pooled with 10-15% libraries prepared using standard approaches or control libraries (see Veeranagouda et al., 2019 for additional details).

75.4 Sequence the library pool on an Illumina instrument (e.g., NovaSeq 6000). The recommended sequencing depth is 10 million reads per sample.

## General method for speedbead (SPRI) purification

76 Equilibrate speedbeads to room temperature and mix thoroughly.

77 **CRITICAL STEP:** Carefully add the indicated amount of speedbeads to the sample.

78 Mix well by pipetting up and down a minimum of 10 times. Alternatively, vortex samples on setting 4 for 3-5 seconds. If samples require centrifugation after mixing, stop the centrifuge before beads settle.

79 Incubate samples at room temperature for 5 min. Prolonged incubation or incubation at low temperature will increase binding of small nucleic acids (e.g., adapter dimers).

80 Place samples on magnetic stand for 2-5 min (or until solution is completely clear) to collect beads. Keep samples on magnetic stand for Steps 81-85.

81 Carefully remove and discard cleared supernatant without disturbing the bead pellet.

82 Add 200 µl of 80% ethanol (freshly prepared with MilliQ water) and incubate for 30 s. Carefully remove ethanol and discard without disturbing the bead pellet.

83 Repeat Step 82 once. Carefully inspect samples and remove remaining ethanol using a 10 µl pipet.

84 **CRITICAL STEP:** Air dry samples for 2 min with lid open. Do not over-dry samples. Speedbeads (and other SPRI products) will clump and can not be fully resuspended during elution if samples are over-dried.

85 Remove samples from the magnetic stand. Add the indicated volume of appropriate solution for elution and mix well by pipetting or vortexing (see Step 78).

86 Incubate mixture at room temperature for 2-5 min.

87 Place samples on a magnetic stand for 2 min (or until solution is completely clear) to collect beads.

88 Transfer cleared supernatant (volume of solution added for elution less 1 µl) to a fresh PCR tube.

## Data analysis

89 Download and install the following software and dependencies:

- fastp (version 0.23.2)
- BBMAP (version 39.06)
- Python (version 3.10.2)
- SLAM-DUNK (version 0.4.3)
- NextGenMap (version 0.5.5)
- SAMtools (version 1.18)
- VarScan 2 (version 2.4.5)
- R (version 4.1.2-mkl)
- Fastqc (version 0.12.1)
- Multiqc (version 1.21)

**Note**

The most recent versions of the software should be used when possible. The input data for the subsequent steps are demultiplexed, paired-end 150 bp reads. Other data types can be processed without modification (e.g., paired-end 100 bp reads) or with slight modification (e.g., single-end 50 bp reads). The reverse reads (R2) generated from most 3' mRNA-seq methods provide little information beyond barcoding. Unique molecular identifiers (UMIs) in the R2 read are extracted in Step 95. R2 reads are excluded from subsequent steps.

90 Download reference genome and annotation files. The reference genome assembly and annotation for *S. cerevisiae* strain S288C (version R64-3-1, RefSeq Assembly GCF_000146045.2) can be retrieved from the NCBI Datasets repository.

**Note**

Chromosome names should be modified in the reference genome assembly to reflect standard nomenclature (https://www.yeastgenome.org/) and maintain compatibility with BED file described below.

91 Download the reference BED file attached below. This BED file will be used to define counting windows in SLAM-DUNK.

Note

To generate the BED file below, all *S. cerevisiae* open reading frames (ORFs) were extracted from the reference annotation (version R64-3-1) using BEDOPS (version 2.4.3). Libraries constructed as described generate reads that align to gene bodies and 3' untranslated regions (UTRs). All ORFs were extended 250 bp beyond their stop position using SAMtools (version 1.14) and BEDTools (version 2.30.0) to include 3' UTRs. Chromosome names in the BED file were modified to reflect standard nomenclature (https://www.yeastgenome.org/). Reference files (i.e., genome assembly and BED file) must be checked to ensure the same chromosome names are used.

Scerevisiae_R64-3-1_gene3utr.bed

92 **CRITICAL STEP:** Set required variables using the commands below. Directories or files within quotations must be changed appropriately before running.

Command

```
DATA="path_to_zipped_fastq_files"
SAMPLES="file_listing_fastq_files_to_process"
DIRECTORY="path_to_outfiles"
BBMAP_RESOURCES="path_to_bbmap_reference_files"
GENOME_scer="reference_S.cerevisiae_genome_file"
BED_scer="reference_S.cerevisiae_bed_file"
```

Note

As an example, if zipped FASTQ files are stored in a directory named *RDHTSP3* and the path to the directory is */archive/HighThroughputSequencing/*, the command DATA="path_to_zipped_fastq_files" would be changed to DATA="/archive/HighThroughputSequencing/RDHTSP3" before running. An example "SAMPLES" file is provided below.

RDHTSP3_slamworkflow_ids.txt

93 Make subdirectories to store outfiles created in Steps 94-101 using the following commands:

Command

```
mkdir -p $DIRECTORY/logs/fastqc/fastq
mkdir -p $DIRECTORY/logs/multiqc/fastq
mkdir -p $DIRECTORY/fastp
mkdir -p $DIRECTORY/logs/fastp
mkdir -p $DIRECTORY/bbduk
mkdir -p $DIRECTORY/logs/bbduk
mkdir -p $DIRECTORY/logs/fastqc/bbduk
mkdir -p $DIRECTORY/logs/multiqc/bbduk
mkdir -p $DIRECTORY/slamdunk_scer
mkdir -p $DIRECTORY/slamdunk_scer/count_twotcreadcount
mkdir -p $DIRECTORY/slamdunk_scer/alleyoop
mkdir -p $DIRECTORY/logs/multiqc/slamdunk_scer
```

94 Run fastqc to assess raw data quality and multiqc to compile the fastqc output using following the commands:

Command

```
echo "running fastqc on raw data for sample:"
for sample_name in $(cat $SAMPLES | sed "s/_001.fastq.gz//" | uniq)
do
        echo "  ${sample_name}..."

        (fastqc \
                $DATA/${sample_name}_001.fastq.gz \
                --outdir $DIRECTORY/logs/fastqc/fastq \
                --threads 64 \
                --quiet \
                &> /dev/null)

done


echo "running multiqc on raw data..."
multiqc \
        $DIRECTORY/logs/fastqc/fastq \
        --outdir $DIRECTORY/logs/multiqc/fastq \
        --quiet \
        &> /dev/null
```

Note

Here and in subsequent steps, the number of requested threads can be changed based on system capacity.

95 Run fastp to extract unique molecular identifiers (UMIs) using the following commands:

Command

```
echo "processing UMIs for sample:"
for sample_name in $(cat $SAMPLES | sed "s/_R._001.fastq.gz//" | uniq)
do
        echo "  ${sample_name}..."

        (fastp \
                --in1 $DATA/${sample_name}_R1_001.fastq.gz \
                --out1
$DIRECTORY/fastp/${sample_name}_umi_R1_001.fastq.gz \
                --in2 $DATA/${sample_name}_R2_001.fastq.gz \
                --out2
$DIRECTORY/fastp/${sample_name}_umi_R2_001.fastq.gz \
                --disable_adapter_trimming \
                --disable_quality_filtering \
                --disable_length_filtering \
                --umi \
                --umi_loc read2 \
                --umi_len 12 \
                --json
$DIRECTORY/logs/fastp/${sample_name}_umi_fastp.json \
                --html
$DIRECTORY/logs/fastp/${sample_name}_umi_fastp.html \
                2> $DIRECTORY/logs/fastp/${sample_name}_umi_fastp.log)

done
```

Note

UMI information is stored in R1 header. UMI information is not currently used in subsequent steps. In addition to extracting UMIs, fastp produces a quality control report for raw and processed data.

96 Run bbduk from the BBMap package to trim adapter sequences and polyA tails using the following commands:

Command

```
echo "trimming adapters for sample:"
for sample_name in $(ls -1 $DIRECTORY/fastp | sed
"s/_umi_R._001.fastq.gz//" | uniq)
do
        echo "  ${sample_name}..."

        (bbduk.sh \

in=$DIRECTORY/fastp/${sample_name}_umi_R1_001.fastq.gz \

out=$DIRECTORY/bbduk/${sample_name}_trimmed_R1_001.fastq.gz \

ref=$BBMAP_RESOURCES/truseq.fa.gz,$BBMAP_RESOURCES/polyA.fa.gz \
                k=13 \
                ktrim=r \
                mink=5 \
                qtrim=r \
                trimq=10 \
                minlength=20 \
                threads=16 \
                2>
$DIRECTORY/logs/bbduk/${sample_name}_trimmed_bbduk.log)

done
```

97 Run fastqc to assess preprocessed data quality and multiqc to compile the fastqc output using following the commands:

Command

```
echo "running fastqc on preprocessed data..."
fastqc \
        $DIRECTORY/bbduk/*.fastq.gz \
        --outdir $DIRECTORY/logs/fastqc/bbduk \
        --threads 64 \
        --quiet \
        &> /dev/null


echo "running multiqc on preprocessed data..."
multiqc \
        $DIRECTORY/logs/fastqc/bbduk \
        --outdir $DIRECTORY/logs/multiqc/bbduk \
        --quiet \
        &> /dev/null
```

98 Run slamdunk all from the SLAM-DUNK package to align data and quantify total reads and reads that have ≥1 T>C conversions using the following commands:

Command

```
echo "running slamdunk all..."
slamdunk all \
        --reference $GENOME_scer \
        --bed $BED_scer \
        --outputDir $DIRECTORY/slamdunk_scer \
        --trim-5p 5 \
        --topn 100 \
        --multimap \
        --min-coverage 10 \
        --var-fraction 0.8 \
        --max-read-length 151 \
        --min-base-qual 26 \
        --skip-sam \
        --threads 24 \
        $DIRECTORY/bbduk/*.fastq.gz
```

Note

See Neumann et al., 2019 and the SLAM-DUNK documentation (https://t-neumann.github.io/slamdunk/) for detailed information on the package.

99 Rename the *count* directory created by slamdunk all using the following command:

Command

```
mv $DIRECTORY/slamdunk_scer/count
$DIRECTORY/slamdunk_scer/count_onetcreadcount
```

100 Run slamdunk count from the SLAM-DUNK package to quantify reads that have ≥2 T>C conversions using the following commands:

Command

```
echo "running slamdunk count..."
slamdunk count \
        --reference $GENOME_scer \
        --bed $BED_scer \
        --snp-directory $DIRECTORY/slamdunk_scer/snp \
        --outputDir $DIRECTORY/slamdunk_scer/count_twotcreadcount \
        --max-read-length 151 \
        --min-base-qual 26 \
        --conversion-threshold 2 \
        --threads 24 \
        $DIRECTORY/slamdunk_scer/filter/*.bam
```

101 Run the following alleyoop commands from the SLAM-DUNK package to summarize and merge slamdunk output:

Command

```
echo "running alleyoop..."
alleyoop rates \
        --outputDir $DIRECTORY/slamdunk_scer/alleyoop/rates \
        --reference $GENOME_scer \
        --min-basequality 26 \
        --threads 24 \
        $DIRECTORY/slamdunk_scer/filter/*.bam


alleyoop tccontext \
        --outputDir $DIRECTORY/slamdunk_scer/alleyoop/tccontext \
        --reference $GENOME_scer \
        --min-basequality 26 \
        --threads 24 \
        $DIRECTORY/slamdunk_scer/filter/*.bam


alleyoop utrrates \
        --outputDir $DIRECTORY/slamdunk_scer/alleyoop/utrrates \
        --reference $GENOME_scer \
        --bed $BED_scer \
        --multiTCStringency \
        --max-read-length 151 \
        --min-basequality 26 \
        --threads 24 \
        $DIRECTORY/slamdunk_scer/filter/*.bam


alleyoop snpeval \
        --outputDir $DIRECTORY/slamdunk_scer/alleyoop/snpeval \
        --snp-directory $DIRECTORY/slamdunk_scer/snp \
        --reference $GENOME_scer \
        --bed $BED_scer \
        --min-coverage 10 \
        --var-fraction 0.8 \
        --multiTCStringency \
        --max-read-length 151 \
        --min-base-qual 26 \
        --threads 24 \
        $DIRECTORY/slamdunk_scer/filter/*.bam
```

```
alleyoop summary \
        --output
$DIRECTORY/slamdunk_scer/alleyoop/summary_onetcreadcount.tsv \
        --tcountDir $DIRECTORY/slamdunk_scer/count_onetcreadcount \
        $DIRECTORY/slamdunk_scer/filter/*.bam


alleyoop summary \
        --output
$DIRECTORY/slamdunk_scer/alleyoop/summary_twotcreadcount.tsv \
        --tcountDir $DIRECTORY/slamdunk_scer/count_twotcreadcount \
        $DIRECTORY/slamdunk_scer/filter/*.bam


alleyoop merge \
        --output
$DIRECTORY/slamdunk_scer/alleyoop/merge_totalreadcount.tsv \
        --column "ReadCount" \
        $DIRECTORY/slamdunk_scer/count_onetcreadcount/*.tsv


alleyoop merge \
        --output
$DIRECTORY/slamdunk_scer/alleyoop/merge_onetcreadcount.tsv \
        --column "TcReadCount" \
        $DIRECTORY/slamdunk_scer/count_onetcreadcount/*.tsv


alleyoop merge \
        --output
$DIRECTORY/slamdunk_scer/alleyoop/merge_twotcreadcount.tsv \
        --column "TcReadCount" \
        $DIRECTORY/slamdunk_scer/count_twotcreadcount/*.tsv


alleyoop tcperreadpos \
        --outputDir $DIRECTORY/slamdunk_scer/alleyoop/tcperreadpos \
        --reference $GENOME_scer \
        --snp-directory $DIRECTORY/slamdunk_scer/snp \
        --max-read-length 151 \
        --min-basequality 26 \
        --threads 24 \
        $DIRECTORY/slamdunk_scer/filter/*.bam


alleyoop tcperutrpos \
```

```
        --outputDir $DIRECTORY/slamdunk_scer/alleyoop/tcperutrpos \
        --reference $GENOME scer \
```

102 Run multiqc to compile output from the SLAM-DUNK package using the following commands:

Command

```
echo "running multiqc on slamdunk output..."
multiqc\
        $DIRECTORY/slamdunk_scer/alleyoop \
        --outdir $DIRECTORY/logs/multiqc/slamdunk_scer \
        --quiet \
        &> /dev/null
```

Note

See the SLAM-DUNK documentation (https://t-neumann.github.io/slamdunk/) for an example MultiQC report and additional information.

103 Key results from this analysis are the three count tables described below. These files are located in the *alleyoop* directory (i.e., $DIRECTORY/slamdunk_scer/alleyoop).

- merge_totalreadcount.tsv - a table (tab-separated values) providing total read counts for all processed samples
- merge_onetcreadcount.tsv - a table (tab-separated values) providing read counts with ≥1 T>C conversions for all processed samples
- merge_twotcreadcount.tsv - a table (tab-separated values) providing read counts with ≥2 T>C conversions for all processed samples

Note

The *filter* directory (i.e., "$DIRECTORY/slamdunk_scer/filter") contains final mapping files in BAM format that can be useful for visualization and/or further processing. See the SLAM-DUNK documentation (https://t-neumann.github.io/slamdunk/) for additional information on output files.

104 Additional processing, analysis, and plotting can be performed in R (version 4.2.3) (https://www.R-project.org/) and RStudio (version 2023.12.0+369) (http://www.rstudio.com/) based on specific experimental goals.

105 **OPTIONAL STEP:** If experiments used *S. pombe* spike-in cells, set variables and make subdirectories using the commands below. Download the reference *S. pombe* genome assembly. Download the reference *S. pombe* BED file attached below. Repeat Steps 98-103 to generate spike-in count tables.

Command

```
GENOME_spom="reference_S.pombe_genome_file"
BED_spom="reference_S.pombe_bed_file"

mkdir -p $DIRECTORY/slamdunk_spom
mkdir -p $DIRECTORY/slamdunk_spom/count_conversionthreshold
mkdir -p $DIRECTORY/slamdunk_spom/alleyoop
mkdir -p $DIRECTORY/logs/multiqc/slamdunk_spom
```

Note

The reference genome assembly and annotation for *S. pombe* strain 972h- (version ASM294v2, RefSeq Assembly GCF_000002945.1) can be retrieved from the NCBI Datasets repository. The reference *S. pombe* BED file was generated as described in Step 91.

FILE Spombe_ASM294v2_gene3utr.bed

# Protocol references

CITATION

Foley JW, Zhu C, Jolivet P, Zhu SX, Lu P, Meaney MJ, West RB (2019). Gene expression profiling of single cells from archival tissue with laser-capture microdissection and Smart-3SEQ..

LINK

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