bio-protocol

Published: Jun 05, 2022

A Modified Fluctuation Assay with a *CAN1* Reporter in Yeast

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Abstract

Understanding the generation of mutations is fundamental to understanding evolution and genetic disease; however, the rarity of such events makes experimentally identifying them difficult. Mutation accumulation (MA) methods have been widely used. MA lines require serial bottlenecks to fix *de novo* mutations, followed by whole-genome sequencing. While powerful, this method is not suitable for exploring mutation variation among different genotypes due to its poor scalability with cost and labor. Alternatively, fluctuation assays estimate mutation rate in microorganisms by utilizing a reporter gene, in which Loss-of-function (LOF) mutations can be selected for using drugs toxic to cells containing the WT allele. Traditional fluctuation assays can estimate mutation rates but not their base change compositions. Here, we describe a new protocol that adapts traditional fluctuation assay using *CAN1* reporter gene in *Saccharomyces cerevisiae*, followed by pooled sequencing methods, to identify both the rate and spectra of mutations in different strain backgrounds.

Keywords: Mutation rate, Mutation spectrum, Fluctuation assay, *CAN1*, *Saccharomyces cerevisiae*

This protocol was validated in: eLife (2021), DOI: 10.7554/eLife.68285

Background

Mutation accumulation assays (Lynch *et al.*, 2008, Zhu *et al.*, 2014, Sharp *et al.*, 2018), though useful in providing a genome-wide estimate of the mutation rate, are usually cost- and labor-intensive, and do not scale up to dozens of genotypes. This protocol utilizes traditional fluctuation assays by accumulating LOF mutations in the *CAN1* reporter gene, with the addition to pool and sequence mutants. This yields not only mutation rate estimates (Lang *et al.*, 2008) but also mutation spectrum estimates for each strain assayed, enabling comparison across different genotypes. This protocol has been used by Jiang *et al.* (2021), which explored mutation rates and spectra of 16 haploid natural isolates of *Saccharomyces cerevisiae*. Jiang *et al.* (2021) found a 10-fold mutation rate difference among the isolates and different mutation spectra in two mosaic beer strains, which show an excessive C>A mutations, demonstrating the usefulness of this protocol.

Materials and Reagents

Consumables:

1. Omni plates (Nunc OmniTray with Lid, catalog number: 242811)
2. Costar Round-bottom 96-well plates (Costar, catalog number: 3788)
3. Costar Flat-bottom 96-well plates (Costar, catalog number: 3370)
4. 50 mL Conical tube (VWR Scientific, catalog number: 89039-656)
5. 50 mL Reservoirs (VWR Scientific, catalog number: 89003-382)
6. Breath-easy sealing membrane (Sigma-Aldrich, catalog number: Z380059)
7. Transparent Sealing Films (VWR, catalog number: 60941-078)
8. 15 mL Conical tube (Thermo Fisher Scientific, catalog number: AM12500)
9. Locking-lid microcentrifuge tube (Fisher Scientific, catalog number: 02-681-291)
10. Illumina Nextera XT DNA Library Prep Kit (Illumina, catalog number: FC-131-1024, or FC-131-1096)
11. Ampure beads (Beckman Coulter, catalog number: A63880)
12. 6% TBE gel (Invitrogen, catalog number: EC62655BOX or EC6265BOX)
13. Zymo PCR clean up kit (Zymo, catalog number: D4004)
14. Phusion Hifi polymerase (NEB, catalog number: M0530L)
15. 1.7 mL microcentrifuge tubes (VWR, catalog number: 87003-294)
16. 2 mL locking lid microcentrifuge tubes (Thermal Fisher Scientific, catalog number: 3458PK)
17. Toothpicks and/or 10 µL tips (USA Scientific, catalog number: 1120-3710)
18. 200 µL pipette tips (USA Scientific, catalog number: 1120-8710)
19. Glass culture tubes with cap
20. 0.2 mL 8-well PCR Strip tubes (Genesee Scientific, catalog number: 22-161A) and caps (Genesee Scientific, catalog number: 22-170R)
21. 100 mm regular Petri dish (SSE, catalog number: PMP35-01)
22. Omni plates (VWR, catalog number: 62409-600)

Media/plates needed:

Ingredients:

1. Yeast Extract (Difco, catalog number: 212750)
2. Peptone (Difco, catalog number: 211677)
3. Agar (BD Bacto, catalog number: 214030)
4. Dextrose (Fisher Scientific, catalog number: D16-3)
5. Yeast Nitrogen Base (Difco, catalog number: 233520)
6. Ammonium Sulfate (Spectrum, catalog number: AM185)
7. L-canavanine (Sigma-Aldrich, catalog number: C9758-5G)
8. SC Amino acid mix (see Recipes)
9. SC-Arg dropout mix (see Recipes)

10. SC-Arg-Ser dropout mix (see Recipes)

Media/Plates:
1. SC-Arg media (see Recipes)
2. SC-Arg-Ser+Can media (see Recipes)
3. SC-Arg-Ser+Can in Omni plates (see Recipes)
4. YPD plates (see Recipes)

Equipment

1. Misonix Sonicator
2. BioTek Synergy H1 Plate reader
3. Qubit (Invitrogen)
4. Thermo Scientific Sorvall ST 8/8R Centrifuge (for 96-well plates)
5. 12-channel 200 µL multi-channel pipette
6. Tabletop Microcentrifuge
7. Bio-Rad C1000 Thermal Cycler (Bio-Rad, 1851148)
8. Thermo Scientific mechanical convention incubator 30M (at 30°C)
9. Invitrogen XCell SureLock Mini-Cell (for TBE gel)
10. Thermo Scientific MaxQ 2000 open-air shaker (VWR, 47742-750)
11. Elmeco Drum-X tissue Culture Rotator (Elmeco Engineering, L-85)
12. Thermo Scientific Owl Easycast B1, B1A, B2 gel box systems
13. Bio-Rad Powerpac Basic Power Supply (Bio-Rad, 1645050)

Procedure

A. [Step 1: Fluctuation assay]

This section of fluctuation assay protocol is adapted from Lang *et al.* (2018). With modification to plate onto Omni canavanine plates using multi-channel instead of regular round plates. Sterile technique is needed for all live yeast cell-related procedures (Step 1 and Step 2).
Before you start: Count and prepare the Omni plates needed for this round of the experiment.
Omni plates: SC-Arg-Ser+Can, dry for at least 2 days in the incubator at 30°C before use [If the plates are freshly made (within a month), dry for at least 3 days, ideally for 4 days].

Day 1
1. Streak out strains with pipette tips onto YPD plates. Place in the incubator at 30°C for approximately 2 days.

Day 3
2. Inoculate a single colony of each strain in ~3 mL of SC-Arg media in a sterile culture tube with a cap allowing for aeration in the afternoon. Place in the incubator at 30°C overnight on a roller drum.

Day 4
Note: One 96-well plate (round bottom) will be used for one estimate of mutation rate of the inoculated strain. Test up to six plates in one batch. If doing multiple batches (do not exceed four), test each batch approximately 1 h apart.
3. Dilute 4 µL from overnight culture into 40 mL of SC-Arg media (1:10,000) in a 50 mL conical tube. Vortex to mix well. Pour entire mixture into a sterile reservoir. Transfer 50 µL into each well of the 96-well round-

bottom plate using a 200 µL multi-channel pipette. Seal each plate with a Breath-easy membrane. Leave the plates in the incubator at 30°C with shaking (~150 RPM).

Day 6 (48 h from setting up 96-well plate on day 4)

4. Use the sonicator to separate cells (put one plate at a time in the sonicating water bath).
   Protocol: Amplitude: 10. Time 1min 30s. 1s ON, 1s OFF.
5. Pulse centrifuge for 30 s (top speed reach to 4,500 RPM) of the plates.
6. Leave two rows (24 wells) to pool and estimate overall cell numbers (use the same rows for different strains for consistency). Use the remaining six rows to estimate mutation events. Resuspend the cells and transfer the entire culture from each well using multi-channel from the remaining six rows (72 wells) onto previously dried SC-Arg-Ser+Can Omni plates. Take care not to let the individual droplets on the plates run together. If it happens, these replicates cannot be used to estimate mutation rates.
   Note: For each strain, the desired dilution should be previously tested when plating onto the canavanine plates. If plating 50 µL of culture results in large background on canavanine plates, test diluting with sterile H_2O in 1:1, 1:2, or 1:3, 1:4 ratios, then transfer to ensure plating initial culture entirely onto the canavanine plates (e.g., 1:1 dilution with H_2O will end up plating two rows per culture instead of one). The 1:1 dilution works for most strains tested. The dilution that results in low background growth on canavanine plates should be used for that strain.
7. Let the Omni plates dry at room temperature. Then place in the incubator at 30°C for 48 h.
8. Estimating overall cell numbers:
   Mix the two rows of cells into a 1.5 mL microcentrifuge tube. Then dilute them into ~1:20,000 with sterile ddH_2O (usually try dilutions from 1:10,000, 1:20,000, and 1:40,000. The 1:20,000 dilution works the best for most cases), with at least two replicates per dilution. This amount should be adjusted based on the saturation density of your strain with initial test runs by plating dilutions on YPD. Plate 100 µL of the dilution on YPD plates. Incubate at 30°C and count cells after ~2 days. Optimal dilution will result in 100–200 cells per plate to count. Then estimate the total number of cells per 50 µL culture from the cell counts. If the cell count of the 100 µL from the 1:x dilution is y, the estimated actual cell count in the non-diluted culture is x*y/2, averaged from replicates.

Day 8 (48 h from day 6, after placing Omni plates in the incubator at 30°C)

Mutants should grow as colonies on the canavanine plates (Figure 1). Only strains with at least one or two mutants from the 72 independent cultures grown are used. Plates can be placed at 4°C for storage (up to 2–3 weeks) before next steps.

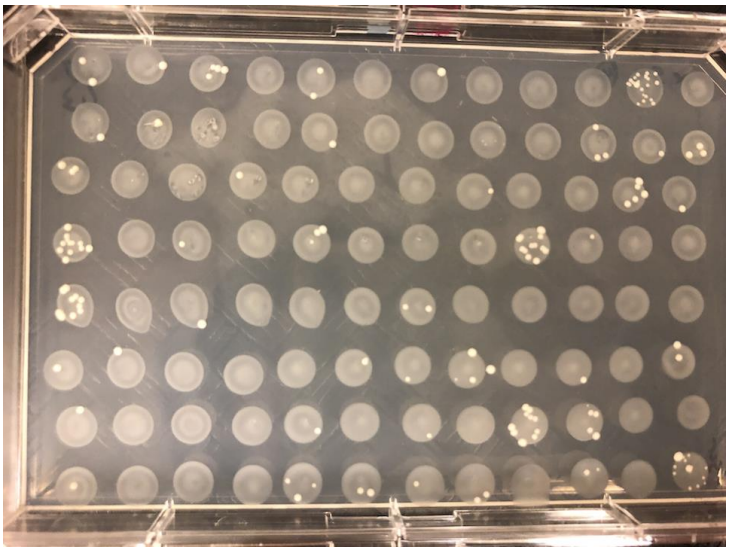

Figure 1. Example of mutants grown on Omni plates.

Here, eight rows of mutants were plated. When used for estimating mutation rates, only six rows are plated, and the other two rows are mixed to estimate total cell numbers.

Then estimate mutation rates using "rsalvador" R package (Zheng, 2017). Script to estimate mutation rate in Jiang *et al.* (2021) can be found: https://github.com/harrispopgen/elife_CAN1_paper/tree/main/fluc.

B. [Step 2: Pooling mutants]

Day 1

1. Start in the afternoon ~3 p.m., depending on how many plates to set up. Incubate for ~43 h.
2. Add 200 µL of SC-Arg-Ser+Can selective media to the 96-well flat bottom plate (easy for plate reader to read OD). Use a 10 µL sterile pipette tip or toothpick and pick at most one independent mutant colony from each replicate for which there are independent pickable CanR colonies. Place each mutant colony into a well on the fresh 96-well plate containing SC-Arg-Ser+Can. Use the VWR transparent film for sealing after finishing picking for the whole plate. Leave at least 1–2 empty wells on the plate to calibrate the plate reader. This process takes approximately 30 min per plate. Leave the inoculated 96-well plates in the incubator at 30°C with shaking for ~43 h.
   Note: Optionally, place one plate in a plate reader to determine if all cultures grow to saturation during the incubation.

Day 3

Morning (~9 a.m.)

3. For each plate of every strain, resuspend by pipetting up and down with a multi-channel pipette. Put on a new transparent seal. Low speed centrifuge if required, but avoid as it makes resuspension difficult. Measure the OD_{600} of each well using a plate reader, making sure the empty wells yield a close-to-zero number. The saturated culture will yield an OD_{600} close to 1 and should be similar among cultures of the same plate but can be different among different strains.
4. The pooling is done by combining independent saturated cultures of mutants from the same strain into a mix, to ensure each mutant is roughly the same and relatively high frequency (~0.03) in the pool so that they can be detected by Illumina Sequencing. Exclude cultures with below average OD_{600} as the determination of mutational frequency assumes a close to equal number of cells in the pool. Determine which mutants to pool together based on the total number of mutants collected by the strain, up to 35–40 mutants from the same strain per pool. Record the number of mutants per pool.
5. Pool mutants for each strain one at a time. Transfer 150 µL of saturated culture from each well with the multi-channel pipette into a reservoir. Then transfer all the liquid to a 15 mL conical tube. Vortex to mix well, and then evenly distribute into three 2 mL locking-lid microcentrifuge tubes.
6. Spin down for 3 min to pellet the cells (stored at -20°C).

C. [Step 3: Preparation for sequencing of *CAN1*]

1. Extract genomic DNA for each pool, which usually gives a yield of ~200–500 ng genomic DNA (use 70 ng to set up each 25 µL PCR reaction if possible). This assay was developed with the Hoffman Winston protocol (Hoffman and Winston, 1987), but any genomic extraction protocol will work. For most pools, extract one tube of genomic DNA from cell pellets. Randomly selected some pools to perform two independent extractions as replicates and test consistency in the mutation calling pipeline. Keep the third tube at -20°C in case of DNA extraction failure.
2. Use PCR to amplify the *CAN1* locus from the genomic DNA. Set up three 25 µL reactions for each mutant pool. See below for the PCR protocol [use primers for *CAN1* from Lang and Murray (2008), forward: ATAGTAAGCTCATTGATCCC, reverse: TCTTCAGACTTCTTAACTCC). Use 15 cycles.

   PCR protocol (with Phusion Hifi polymerase):

- 98°C: 3 min
  - 98°C: 10 s
  - 59°C: 18 s
  - 72°C: 2 min
  - (Go to step 2, with a total of 15 cycles)
- 72°C: 5 min
- 12°C: forever

3. Run 10 µL of the 25 µL on the gel; if a band is present, proceed with the other two reactions for PCR cleanup with the Zymo kit.
4. Measure DNA concentration using Qubit (High Sensitivity kit).

D. [Step 4: Library preparation for sequencing of *CAN1*]

1. Use Illumina Nextera XT DNA Library Prep Kit. Follow the protocol with small modifications.
   a. Use starting PCR product at 0.18 ng/µL (5 µL).
   b. For the tagmentation step, use 7 min for most samples; 5 min produces an average library size of ~800 bp–1 kb, and 7min produces the appropriate library size of ~500–600 bp.
   c. Use 1:1 ratio of sample: Ampure beads during clean up.
   d. Elute in 47.5 µL Resuspension Buffer (RSB) and transfer 45 µL.
2. Use Qubit (High Sensitivity kit) to quantify the concentration of each library. Run TBE gel in Invitrogen XCell SureLock Mini-Cell to estimate rough library size (Figure 2). Convert concentration in X ng/µL to Y nM with library size K bp, using the formula Y = X/(660*K)*1000000 (according to the Illumina Handbook). Mix library together with equal mole. Send for sequencing [spike-in each pool to get 0.25 M reads from a 150-bp paired-end Illumina sequencing reads, resulting in an estimated coverage of 44,000 for each pool (*CAN1* size of 1.7kb): $0.25*10^6*300/(1.7*10^3) \sim 44{,}000$].

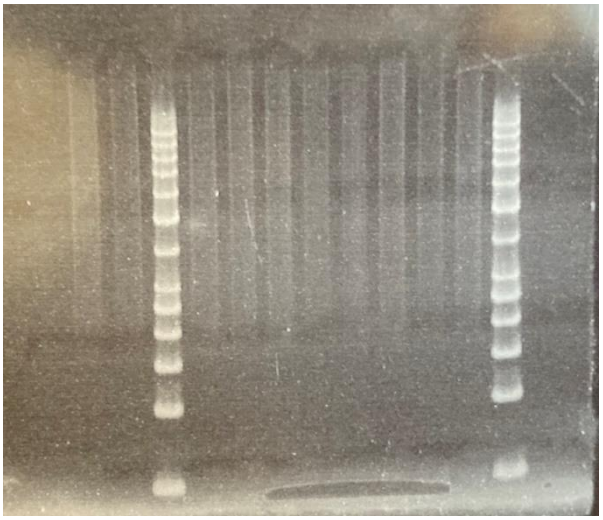

Figure 2. Example of a TBE gel obtained.
Lanes 3 and 12 are ladders, and the rest are libraries. The estimated library size is taken as the mid-point of the spread.

3. Analyze reads using scripts found in: https://github.com/harrispopgen/elife_CAN1_paper/tree/main/CAN1_mut_sequencing.

Recipes

1. SC Amino acid mix (for 1 L)

0.019 g Adenine
0.019 g Arginine
0.096 g Aspartic Acid
0.096 g Glutamic Acid
0.019 g Histidine
0.077 g Isoleucine
0.077 g Leucine
0.058 g Lysine
0.019 g Methionine
0.048 g Phenylalanine
0.384 g Serine
0.192 g Threonine
0.077 g Tryptophan
0.058 g Tyrosine
0.019 g Uracil
0.144 g Valine
(1.402 g total)

2. SC-Arg dropout mix

0.019 g Adenine
0.096 g Aspartic Acid
0.096 g Glutamic Acid
0.019 g Histidine
0.077 g Isoleucine
0.077 g Leucine
0.058 g Lysine
0.019 g Methionine
0.048 g Phenylalanine
0.384 g Serine
0.192 g Threonine
0.077 g Tryptophan
0.058 g Tyrosine
0.019 g Uracil
0.144 g Valine

3. SC-Arg-Ser dropout mix

0.019 g Adenine
0.096 g Aspartic Acid
0.096 g Glutamic Acid
0.019 g Histidine
0.077 g Isoleucine
0.077 g Leucine
0.058 g Lysine
0.019 g Methionine
0.048 g Phenylalanine
0.192 g Threonine
0.077 g Tryptophan
0.058 g Tyrosine

0.019 g Uracil
0.144 g Valine

4. **SC-Arg media (1 L)**
   1.7 g Yeast Nitrogen Base
   5 g Ammonium Sulfate
   20 g Dextrose
   1.383 g SC-Arg dropout mix

5. **SC-Arg-Ser+Can media (1 L)**
   1.7 g Yeast Nitrogen Base
   5 g Ammonium Sulfate
   20 g Dextrose
   0.999 g SC-Arg-Ser dropout mix
   10 mL 100× Canavanine media (60 mg/mL)

6. **SC-Arg-Ser+Can plates**
   1.7 g Yeast Nitrogen Base
   5 g Ammonium Sulfate
   20 g Dextrose
   0.999 g SC-Arg-Ser dropout mix
   10 mL 100× Canavanine media (60 mg/mL)
   17 g Agar

7. **YPD plates (1 L)**
   10 g Yeast Extract
   20 g Peptone
   17 g Agar
   20 g Dextrose

Acknowledgments

Pengyao Jiang was supported by a Burroughs Wellcome Fund Career Award at the Scientific Interface awarded to Kelley Harris. Anja R. Ollodart was supported by the National Human Genome Research Institute of the NIH under award T32 HG00035. The research of Maitreya J. Dunham was supported by NIH/NIGMS award P41 GM103533 and a Faculty Scholar grant from the Howard Hughes Medical Institute. The fluctuation assay part of the protocol is adapted from Lang (2018). The protocol is the experimental procedure of the publication Jiang *et al.* (2021), eLife.

Competing interests

There are no conflicts of interest or competing interests.

References

Hoffman, C. S. and Winston, F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene* 57(2-3): 267-272.

Jiang, P., Ollodart, A. R., Sudhesh, V., Herr, A. J., Dunham, M. J. and Harris, K. (2021). A modified fluctuation assay reveals a natural mutator phenotype that drives mutation spectrum variation within *Saccharomyces cerevisiae*. *Elife* 10: e68285.

Lang, G. I. (2018). Measuring Mutation Rates Using the Luria-Delbruck Fluctuation Assay. *Methods Mol Biol* 1672: 21-31.

Lang, G. I. and Murray, A. W. (2008). Estimating the per-base-pair mutation rate in the yeast *Saccharomyces cerevisiae*. *Genetics* 178(1): 67-82.

Lynch, M., Sung, W., Morris, K., Coffey, N., Landry, C. R., Dopman, E. B., Dickinson, W. J., Okamoto, K., Kulkarni, S., Hartl, D. L., *et al*. (2008). A genome-wide view of the spectrum of spontaneous mutations in yeast. *Proc Natl Acad Sci U S A* 105(27): 9272-9277.

Sharp, N. P., Sandell, L., James, C. G. and Otto, S. P. (2018). The genome-wide rate and spectrum of spontaneous mutations differ between haploid and diploid yeast. *Proc Natl Acad Sci U S A* 115(22): E5046-E5055.

Zheng, Q. (2017). rSalvador: An R Package for the Fluctuation Experiment. *G3 (Bethesda)* 7(12): 3849-3856.

Zhu, Y. O., Siegal, M. L., Hall, D. W. and Petrov, D. A. (2014). Precise estimates of mutation rate and spectrum in yeast. *Proc Natl Acad Sci U S A* 111(22): E2310-2318.