

Experimental Evolution and Resequencing Analysis of Yeast

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Abstract

Experimental evolution of microbes is a powerful tool to study adaptation to strong selection, the mechanism of evolution and the development of new traits. The development of high-throughput sequencing methods has given researchers a new ability to cheaply and easily identify mutations genome wide that are selected during the course of experimental evolution. Here we provide a protocol for conducting experimental evolution of yeast using chemostats, including fitness measurement and whole genome sequencing of evolved clones or populations collected during the experiment. Depending on the number of generations appropriate for the experiment, the number of samples tested and the sequencing platform, this protocol takes from 1 month to several months to be completed, with the possibility of processing several strains or mutants at once.

Key words Yeast, Chemostats, Fitness, Whole genome sequencing, Nextera, MiSeq

1 Introduction

Continuous cultures of microbes have been used since the development of chemostats in 1950 by both Monod [1] and Novick and Szilard [2]. As chemostats allow the culture to grow in a controlled environment at steady state, physiological changes such as transcription, protein and metabolite levels can be accurately observed and compared between strains and conditions [3, 4]. Chemostats also provide an ideal set-up to perform experimental evolution [5]. Microbial experimental evolution studies have demonstrated the rapid accumulation of genetic variation such as point mutations, copy number variations, and genomic rearrangements over time in response to laboratory environments [6–12]. Phenotypic changes in morphology, resistance to drugs, and fitness, for just a few examples, can be observed as early as a few generations [11, 13–15]. The recent advances in sequencing have contributed to the identification of many of the mutations contributing to adaptation. Whole genome sequencing of populations has also contributed to our

understanding of the dynamics of populations undergoing adaptation by following the changes in frequency of mutations over time [10].

In this chapter, we describe an experimental system to perform long term evolution and competitive experiments to measure fitness of yeast using continuous cultures in chemostats. We also describe one method to perform whole-genome sequencing of evolved clones to detect de novo mutations associated with a fitness increase.

2 Materials and Equipment

2.1 *Continuous Culture Using Chemostats*

- Cultures tubes.
- Ethanol 95 %.
- “Ministat” chemostat setup and media as described in ref. [16].
- Kimwipes.
- Syringes: 1 ml.

2.2 *Competition Against GFP Marked Strains*

- 50 ml conical tubes.
- Ethanol 95 %.
- Kimwipes.
- Syringes: 10 ml.

2.3 *FACS Analysis*

- C6 Flow cytometer, CFlow Plus Software (BD Biosciences).
- Locking lid microcentrifuge tubes, 2 ml.

2.4 *Tagmentation*

- 0.5 ml PCR tubes with flat caps.
- Nextera TD buffer and TDE1 enzyme (From Illumina kit).
- Thermocycler.

2.5 *Cleanup of Tagmented DNA*

- 100 % ethanol.
- Eppendorf tubes.
- Nextera RSB buffer (Illumina).
- Zymo DNA clean & Concentrator-5 kit (Zymo Research).

2.6 *PCR Amplification*

- 0.5 ml PCR tubes.
- LightCycler 480 SYBR Green I Master (Roche Applied Science, dilute from 10,000× to 100× in DMSO).
- Nextera NPM and PPC (Illumina kit).
- Nextera P7 and P5 primers buffer (Nextera Index Kit).
- Thermocycler.

2.7 PCR Cleanup

- Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics Inc).
- Eppendorf tubes.
- Ethanol 80 %.
- Magnetic plate: DynaMag-96 Side (Invitrogen/Lifetech Gibco BRL).
- Nextera RSB buffer (From Illumina kit).

2.8 Quantify and Pool Libraries

- 0.5 ml PCR tubes with flat caps.
- Eppendorf tubes.
- Qubit DNA quantification system.

2.9 Library QC

- 5× loading buffer :0.25 % bromophenol blue, 0.5 M DTT, 50 % glycerol, 10 % SDS.
- 6 % TBE gel.
- 50 bp DNA ladder with 6× loading dye.
- SYBR Gold 10,000× concentrate in DMSO.
- 10× TBE: 108 g tris base, 55 g boric acid, 40 ml 0.5 M EDTA (1 l at pH 8.0).
- XCell Sure Lock Mini-Cell CE Mark (Invitrogen Novex Minicell).

2.10 Library Denaturation

- 2 N sodium hydroxide (NaOH).
- Buffer EB :10 mM Tris-HCl pH 8.5.

2.11 Sequencing on the MiSeq

- Ethanol 70 %.
- Illumina MiSeq or HiSeq2000 sequencer.
- Milli-Q water.
- MiSeq reagent kit V2 PE 300 cycles (Illumina).

3 Methods**3.1 Continuous Culture Using Chemostats**

In this protocol, we use the “ministat” chemostat system described in ref. [16] and diagrammed in Fig. 1a. However, the protocol can also easily be modified for use with other chemostat platforms (e.g., [17]).

1. Day 1: Inoculate a single fresh colony of each strain into a separate tube containing 2.5 ml of the media you will use for the chemostat, and let each culture grow to saturation overnight (Fig. 1b) (*see Note 1*). It is important to inoculate each culture with an independent colony in order to avoid shared mutations that might occur during the batch growth phase.

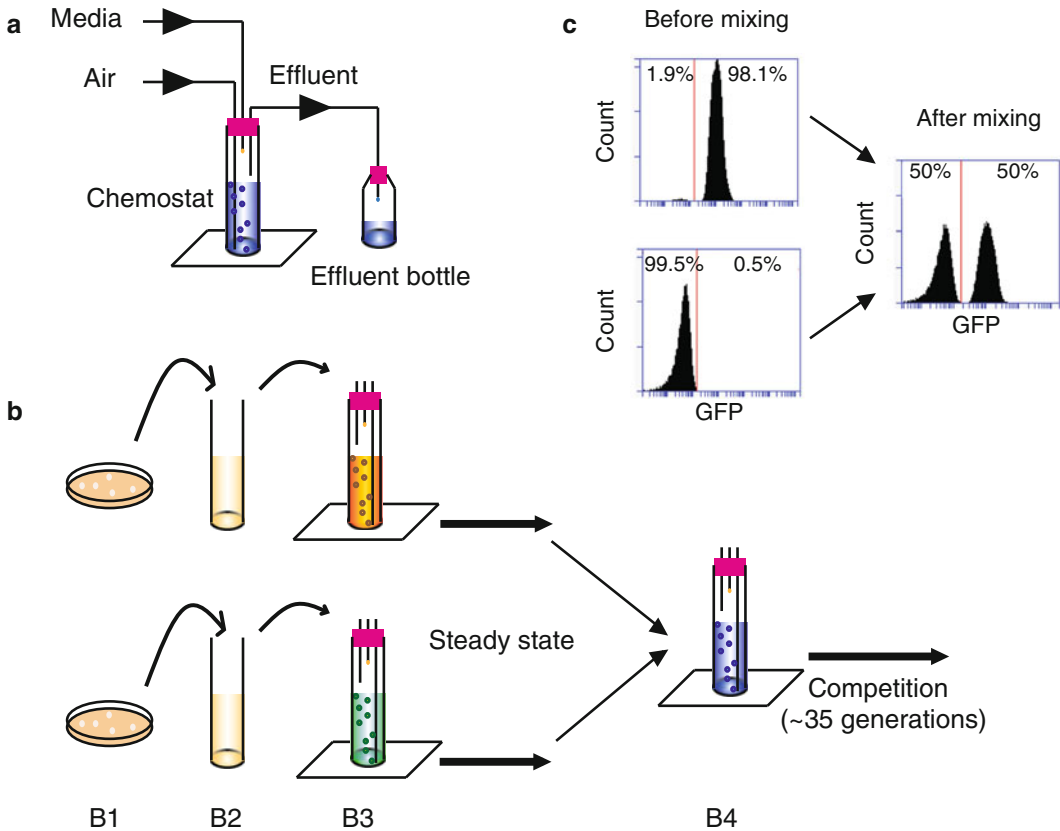


Fig. 1 FACS-based competitive assay. (a) Diagram of a ministat. The ministat chamber is a 50 ml glass tube stopped with a silicon cork (in pink). Air and media are delivered by two independent systems. Culture volume is determined by a third needle connected to an effluent bottle. Positive pressure ejects overflow into the effluent bottle. Samples can be collected passively by switching the effluent bottle with a collection tube. (b) Competitive assay in the chemostats. (b1–b2) Overnight cultures of both the clone and the reference strains from an isolated colony. (b3) Inoculation of the chemostat vessel with 0.1 ml of the overnight cultures. (b4) When the cultures reach steady-state, the two cultures can be mixed together. Samples will be collected twice daily for 35 generations. (c) FACS analysis of the samples. Prior to mixing, each vessel should contain either an unlabeled or a GFP-marked strain. After mixing, the ratio of both populations will be measured

2. Build your array of ministats as described [16] and prepare your media as appropriate for the selective conditions desired (Fig. 1a).
3. Turn the pump on to fill the ministats, and then turn it off when the media reaches the 20 ml mark.
4. Day 2: Sterilize the tops of the corks of the vessels with 95 % ethanol; wipe the cork using a Kimwipe.
5. Inoculate each chemostat vessel with 0.1 ml from one individual overnight culture using a syringe (Fig. 1b).
6. Thirty hours after inoculation, turn the media pump on to a dilution rate of 0.17 vol/h (see Note 2).

7. When the media starts to exit through the effluent line, turn off the air to adjust the culture volume to 20 ml by moving the sampling needle up or down (*see Note 3*). Turn the air on when done.
8. Once all the cultures reach 20 ml, empty the effluent bottles. Record the time and this will be your time 0.
9. Let the culture reach steady state (*see Note 4*).

Experimental evolution can operate for hundreds of generations. With a dilution rate of 0.17 vol/h, 200 generations will be reached in 35 days (*see Note 5*).

3.2 Competition Against GFP Marked Strains

The fitness of a strain of interest is generally measured against a matched reference strain to perform cross comparison. To discriminate between the two strains, the reference strain can be labeled with a fluorescent protein such as *yGFP* or *d-Tomato*, integrated at a neutral locus, such as *HO* [8] or *YEL014C* [17] (*see Note 6*).

1. Sterilize the tops of the chemostats with 95 % ethanol (*see Note 7*).
2. With a 10 ml syringe, collect 10 ml of steady state culture of the culture of interest. Do the same for the vessel containing the reference strain culture (*see Note 8*).
3. Unscrew the syringe from the needle and swap the syringes. Push each needle into the cork of the appropriate destination vessel, eject the culture into the vessels, remove the syringes, and dispose of them in an appropriate container.
4. Note the time of the mixing.
5. Wait 20 min for the volume to stabilize before collecting a sample.
6. Note the time and collect 1 ml passively by transferring the sampling corks into labeled 20 ml sterile sampling tubes.
7. While the tubes are filling, measure and record the effluent volume (V_{eff}) that has collected in the effluent bottles.
8. Use the time elapsed and the V_{eff} to calculate the dilution rate D ($D = V_{eff} / (\text{time elapsed} \times 20)$) and the number of generations elapsed G_e ($G_e = D \times 1.44 \times \text{time elapsed}$) [4].
9. Wash effluent bottles and replace the sampling corks on the bottles.
10. Collect samples for analysis by cytometry for up to 35 generations to follow the relative abundance of the two strains over time. Two samples are generally performed on the day of the mixing, three the following day and one or two the third day to ensure that you will have enough points to fit a normal regression.

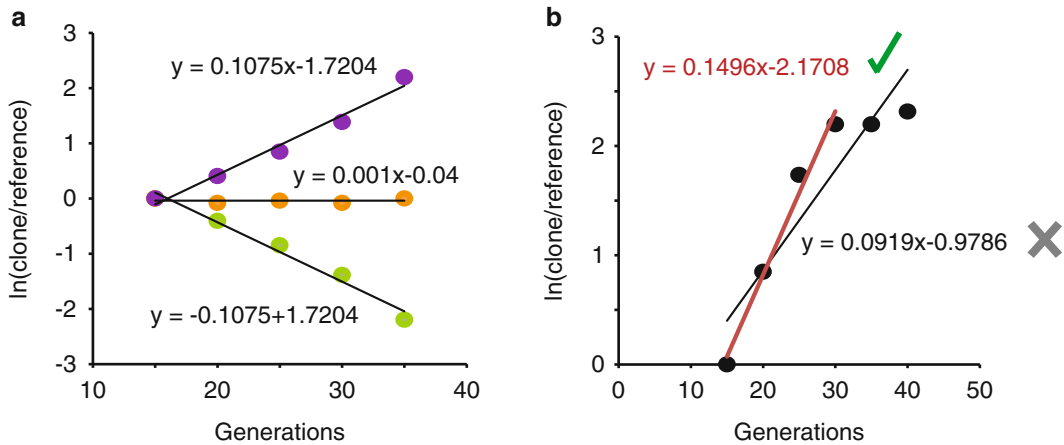


Fig. 2 Relative fitness. **(a)** The relative fitness of a strain is determined by linear regression of the natural log (\ln) of the ratio of the strain over the control strain against the number of generations. A positive slope indicates an increased fitness of the clone compared to the control strain while a negative slope implies a decrease of fitness. A slope around 0 indicates a neutral or near-neutral fitness. **(b)** To accurately measure fitness, exclusion of the later time points might be performed

3.3 FACS Analysis

These instructions are specific for the C6 cytometer from BD Accuri, but could be modified for use with any other appropriate cytometer or FACS machine.

1. Perform a 1:4 dilution of your sample into water. You can either do the dilution in a 2 ml tube or in a 96-well plate.
2. Sonicate your sample to disperse mother and daughter cells and to break up cell clumps.
3. Count 50,000 cells at a medium speed on the cytometer.
4. Create a histogram plot to display the distribution of cell number (Count) and the GFP level (Channel FL1-A) (*see Note 9*).
5. Create vertical marker by clicking the cursor at the point along the x -axis to gate the histogram and separate the two subpopulations. BD CSampler Software will automatically display the percentage of events to the left and the right of the marker (Fig. 1c).
6. Run a cleaning cycle. When the cleaning cycle is finished, you can export your data for further analysis.

3.4 Analysis: Selection Coefficient

1. Per generation selection coefficient is used as a measurement of fitness [4]. The selection coefficient is measured by linear regression of the natural log of the strain abundance ratio against the time in generations (Fig. 2a).
2. Before attempting to fit a linear model to calculate the selection coefficient, you should first determine if your data follow a linear model or if some points are out of the range. This happens generally for the last few time points as one of

the strains may have already reached fixation. Such points should be excluded from further analysis (Fig. 2b).

3.5 Whole Genome Sequencing

1. Whole genome sequencing of clones or populations is often used to determine the genetic changes associated with phenotypic changes such as fitness. Depending on the length of the reads, the capacity of the sequencer and the minimum coverage required to ensure quality results, you might want to sequence more than one library at a time. Using the dual index method you can multiplex up to 96 libraries together. 96 libraries can be processed in less than 3 h using the Nextera kit.
2. As of today, up to 4 libraries of individual clones can be sequenced on one lane of a MiSeq or 24 samples on one lane of a HiSeq for a 30× coverage minimum for each library with ~15 million reads of 150 bp. This level of coverage ensures that SNPs or CNVs can be accurately detected.

3.6 Tagmentation

Nextera technology uses *in vitro* transposition to create whole-genome libraries with a low input requirement (35 ng). This technique is a flexible and simple method capable of fragmenting and tagging DNA in a 5 min reaction, followed by a PCR [18] (Fig. 3a). Genomic DNA can be extracted from overnight cultures or frozen

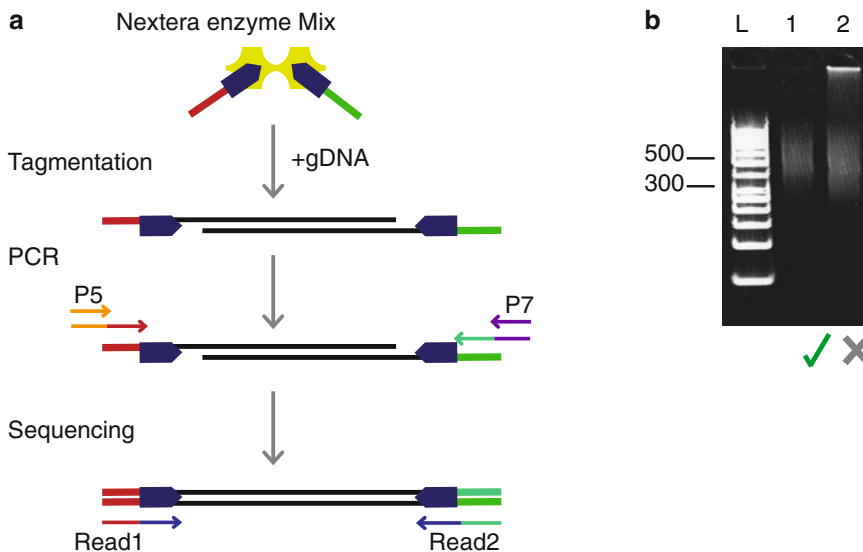


Fig. 3 Nextera library of yeast genomic DNA. **(a)** Generation of libraries by the Nextera method. Genomic DNA is fragmented and tagged with the Nextera Enzyme Mix, followed by a PCR reaction containing four primers to add compatible adaptors (*purple* and *orange*) to the sequencing library. The two sequencing primers correspond to the *red* and *blue* arrows. Figure modified from Adey et al. (2010). **(b)** Lane L: GeneRuler 50 bp DNA ladder. Lane 1: example of a high quality library. The size of the library ranges from 300 bp to less than 1000 bp. Lane 2: example of a low quality library. Large DNA fragments are present. Size selection should be performed on this library to ensure high quality sequencing results

pellets using the Smash-and-Grab method [19]. gDNA should be quantified using a fluorescence-based method such as Qubit.

1. Remove the 2× buffer (TD), the enzyme (TDE1), and genomic DNA from $-20\text{ }^{\circ}\text{C}$ and thaw on ice (*see Note 10*).
2. Ensure that the buffer and the enzyme are mixed by gently inverting the tubes five times, followed by a brief spin in a microcentrifuge.
3. In 0.5 ml PCR tubes mix 35 ng of genomic DNA, 25 μl of 2× buffer (TD), and 5 μl of enzyme TDE1 and bring to 50 μl total volume with ddH₂O (*see Note 11*).
4. Incubate in thermocycler at 55 $^{\circ}\text{C}$ for 8 min.
5. Hold at 4 $^{\circ}\text{C}$.

3.7 Cleanup of Tagmented DNA

1. Thaw the RSB buffer at room temperature.
2. Add 180 μl of Zymo DNA binding buffer to each 50 μl tagmentation reaction.
3. Gently pipet up and down ten times.
4. Load the mixture into a Zymo-Spin Column and centrifuge at full speed ($>10,000\times g$) for 30 s. Discard the flow through.
5. Wash twice with 300 μl of the DNA Wash Buffer.
6. Centrifuge at full speed for an additional 30 s to remove any remaining wash buffer.
7. Place column into a clean Eppendorf tube and pipet 25 μl of RSB onto each column.
8. Incubate at room temperature for 2 min.
9. Centrifuge at full speed for 30 s.
10. Eluted DNA can be stored at $-20\text{ }^{\circ}\text{C}$.

3.8 PCR Amplification

1. Thaw the Nextera PCR Master Mix (NPM) and PCR Primer Cocktail (PPC) from the Nextera kit and the index primers (P7 ($\times 12$) and P5 ($\times 5$)) at room temperature. Plan primer pairs that are compatible for multiplexing and uniquely mark each sample.
2. Set up PCR in individual 200 μl PCR tubes, for easy removal from RT-PCR machine.
3. Mix 5 μl of the chosen index 2 (P7), 5 μl of the chosen index 1 (P5), 1 μl of SYBR green (final concentration $n=0.25\times$), 15 μl of the Nextera PCR master mix (NPM), 5 μl of PCR Primer Cocktail (PPC), and 20 μl of the tagmented DNA.
4. Perform PCR on RT-PCR machine using the following program: 72 $^{\circ}\text{C}$ for 3 min, 98 $^{\circ}\text{C}$ for 30 s, then 5–15 cycles of (98 $^{\circ}\text{C}$ for 10 s, 63 $^{\circ}\text{C}$ for 30 s, 72 $^{\circ}\text{C}$ for 30 s), followed by 3 min at 72 $^{\circ}\text{C}$.

5. Remove the PCR tubes from the thermocycler when the absorbance starts to plateau and spin down using a microcentrifuge.
6. Tubes can be stored at 4 °C for a few days.

3.9 PCR Cleanup

1. Bring the beads to room temperature for 30 min before use and vortex the tube for 30 s.
2. Add 30 µl of the magnetic beads to each PCR reaction.
3. Mix well by gently pipetting up and down ten times.
4. Incubate at room temperature without shaking for 5 min.
5. Place the plate on a magnetic stand for 2 min or until the supernatant has cleared.
6. With the tube still on the stand, use a 20 µl tip to carefully remove the supernatant.
7. With the tubes still on the magnetic stand, wash the beads with freshly prepared 80 % ethanol, twice, as follows:
 - (a) Add 200 µl of freshly prepared 80 % ethanol to each tube. Incubate on magnetic stand for 30 s until the supernatant appears clear.
 - (b) Carefully remove and discard the supernatant.
8. With the tubes still on the magnetic stand, allow the beads to air-dry for 15 min.
9. Remove the tubes from the magnetic stand.
10. Add 32.5 µl of RSB to each tube, making sure to bring the beads into solution.
11. Mix well by pipetting up and down ten times, changing tips for each tube.
12. Incubate at room temperature for 2 min.
13. Place the tubes back on the magnetic stand for 2 min or until supernatant has cleared.
14. Label new tubes.
15. Taking care to not disturb the beads, while keeping the tubes on the magnetic stand, carefully transfer 30 µl of supernatant from each tube on the magnetic stand to a new labeled Eppendorf tube.
16. The libraries can be stored at 4 °C for a few days or at -20 °C indefinitely.

3.10 Quantify and Pool Libraries

We recommend measuring concentration using the Quant-iT dsDNA HS assay kit and a Qubit-iT fluorometer.

1. Set up your 0.5 ml tubes: you will need two tubes for the standards, and one tube per sample (*see Note 12*).

2. Dilute the Quant-iT reagent 1:200 in Quant-iT buffer.
3. Aliquot 190 μl of the diluted reagent in the two standard tubes and 197 μl in the tubes dedicated for the samples.
4. Add 10 μl of the standards in the standards tubes, and 3 μl of your libraries in the tubes dedicated for the samples.
5. Mix the solution by pipetting up and down and incubate the tubes for 2 min in the dark at room temperature.
6. Read tubes in Qubit fluorometer and note the concentration for each sample.
7. Save your individual libraries. Make sure they are clearly labeled.
8. If you plan on multiplexing your libraries, mix together equal ng from each library to make the pooled library.

3.11 Library QC

1. Set up 6 % acrylamide gel in the gel box. Add ~300 ml of 0.5 \times TBE.
2. Mix 5 μl of the library with 5 μl loading buffer.
3. Mix 5 μl ddH₂O with 5 μl loading buffer 5 \times and 0.5 μl 50 bp ladder.
4. Load the ladder and the libraries on the acrylamide gel.
5. Run at 160 V for ~45 min.
6. Incubate gel in ~50 ml of 0.5 \times TBE with 5 μl SYBR Gold on a shaker for 5 min.
7. Take a picture using a gel imaging system.
8. The Nextera method produces libraries with a broad range from 300 to 1000 bp. The ideal size of a Nextera library is around 500 bp (Fig. 3b) (*see Note 13*).

3.12 Sequencing of Your Library on a MiSeq: Sample Sheet

The sample sheet is the set of instructions that the machine uses to know how to sequence your sample.

1. Open the program called “Illumina Experiment Manager”.
2. Select “create sample sheet”.
3. Choose MiSeq, press “next”.
4. Select “FASTQ Only” (*see Note 14*).
5. Fill in the field “Reagent Cartridge Barcode” using the barcode found on the side of the cartridge.
6. Fill in the information about the run—project name, experiment name, investigator name, and a description (*see Note 15*).
7. Fill out the information for your sample(s).
8. Remove the reagent cartridge from the freezer and let it thaw along with the tube of HT1 (hybridization buffer) (*see Note 16*).

3.13 Library Denaturation (Gloves Are Required for This Step)

1. Thaw your pooled library on ice along with your HT1 (*see Note 17*).
2. Prepare a fresh dilution of 0.2 N NaOH.
3. Dilute your library to 2 nM in EB buffer (*see Note 18*).
4. Mix 10 μ l of your library at 2 nM with 10 μ l of NaOH at 0.2 N.
5. Vortex briefly and centrifuge your tube for 1 min.
6. Add 980 μ l of prechilled HT1 buffer to the tube containing the denatured DNA.
7. Dilute the denatured sample to 12 pM (*see Note 18*): 600 μ l of the 20 pM denatured sample, 400 μ l of the prechilled HT1 and 1.2 mM of NaOH.
8. Invert five times to mix the DNA solution.
9. Briefly centrifuge the DNA solution.
10. Place the library on ice until you are ready to load your library onto the MiSeq.

3.14 Load Sample onto Cartridge (Gloves Are Required for This Step)

1. Pierce the foil of the cartridge seal over “Load samples” with a clean pipette tip.
2. Pipette 600 μ l of the sample onto reservoir (*see Note 19*).
3. Proceed directly to the MiSeq.

3.15 Start the Sequencing Run

1. Use plastic forceps and remove the flowcell by the base of the plastic cartridge from the storage buffer.
2. Rinse the flowcell with 2 ml Milli-Q water, making sure to remove the salt.
3. Using care dry the flowcell on a Kimwipe.
4. Clean the flowcell glass with a Kimwipe and 70 % EtOH to remove streaks and fingerprints.
5. Clean the flowcell stage in the flowcell compartment with a Kimwipe.
6. Place the flowcell onto the flowcell stage with the label facing upward.
7. Gently press down. You will hear a click.
8. Follow on-screen instructions (*see Note 20*).
9. Review the run parameters (Experiment name, analysis workflow, read length).
10. The MiSeq will perform a pre-run check.
11. The goal is to reach a cluster density of 750–1200k cluster/ mm^2 .

12. The duration of the sequencing depends on the number of cycles (i.e., read length): ~4 h for 2×36 bp run and up to ~39 h for a 2×250 bp cycle.
13. Always perform a post-run wash just after your run by following the instructions on the screen.
14. After the wash, leave the used flowcell, wash tray and wash bottle in the instrument until your next run.

3.16 Retrieve Your Data

Once the sequencing run is complete, store and back up your data before beginning analysis. The sequencing files (.fastq) will be in the Data/Intensities/BaseCalls/directory of your run folder. If the information about the multiplexing was present in the sample sheet, each file will correspond to an individual sample. The quality of the sequences can be assessed using quality control tools such as FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). The SNPs, INDELS, and CNVs calls can now be generated using various tools such as the SAMtools variants caller [20], mrCaNaVar [21], and SPLITREAD [22].

4 Notes

1. Overnight cultures can be started in YPD or another nutrient rich media and cells can be washed with water before inoculating, though it is preferable to use chemostat media for the overnight culture.
2. A dilution rate of 0.17 vol/h corresponds to between 5.75 and 6.5 rpm on the Watson and Marlow pump.
3. Before use of new chemostat vessels, fill the vessels with 20 ml of clean water and mark the glass to indicate the target volume.
4. The cultures are considered to have reached steady state when the cell count has been stable for 2–3 daily measurements ($\pm 5\%$). Steady state is generally reached 3 days after the pump is turned on and before 25 generations, though some strains may take longer.
5. Evolution experiments can be ended when desired. However, experiments also can be terminated due to the appearance of clumping or wall growth or to contamination of the culture vessels by bacteria or fungi. The set-up as well as the samples must be frequently monitored and recorded.
6. The fitness of the reference strain versus a wild type unmarked strain needs to be tested before its usage to ensure no significant differences were introduced during strain construction.
7. It is good practice to save glycerol stocks and 2 ml of samples for DNA preparation before mixing in case you need to confirm strain genotype or other parameters such as plasmid copy number.

8. The percentage of mixing depends on the fitness estimated. If you do not know, a 50–50 % (vol/vol) mix is a good starting point. If you know your strain is going to be more fit than the reference you can lower its abundance to 20–80 % (vol/vol) to allow for a longer period of time over which informative samples can be collected. The volume of the ministats used in our laboratory is set at 20 ml, so a 50–50 mix will be 10 ml of each culture mixed together.
9. Using the initial pure cultures, set gates on the cytometer to discriminate the two populations (unmarked cells and cells expressing *GFP*). For each mixed sample, record the percentage of cells in each gate (Fig. 1c).
10. The working area and the pipettes should be cleaned with 10 % bleach to avoid cross-contamination.
11. This protocol has been adapted from the Nextera protocol (Illumina). Although 50 ng of genomic DNA is recommended by the official Nextera protocol, we recommend using 35 ng of DNA with 5 μ l of the enzyme or 50 ng of DNA with 3 μ l of the enzyme to ensure a good size selection.
12. The standards are stored at 4 °C and the buffer and the reagent at room temperature. Ensure all reagents are at room temperature before you begin. You will need 200 μ l of working solution for each sample and the two standards.
13. Run each individual library and the pooled library on an acrylamide gel in order to confirm the size and quality of your libraries. The ideal range is around 500 bp. Larger or smaller libraries will not produce high quality reads.
14. This setting means that no on-machine analysis will be performed.
15. If you are not using the Nextera indices, you can at this stage select random sequence. The sample sheet can be edited later.
16. You can thaw the cartridge in a tub of water at room temperature. Be careful not to fill the tub over the line indicated on the side of the cartridge. Thawing in this way will take 1 h, after which you can then store the cartridge at 4 °C.
17. Do not start denaturing your libraries unless your cartridge is thawed.
18. Be sure to use large volumes to reduce pipetting errors. After the dilution you can also double check the concentration of the libraries using the Qubit-iT assay.
19. The Nextera sequencing primers are already loaded in the cartridge.
20. Do not leave the reagent chiller door open for extended periods of time.

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