Protocol

Chemostat Culture for Yeast Experimental Evolution

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Experimental evolution is one approach used to address a broad range of questions related to evolution and adaptation to strong selection pressures. Experimental evolution of diverse microbial and viral systems has routinely been used to study new traits and behaviors and also to dissect mechanisms of rapid evolution. This protocol describes the practical aspects of experimental evolution with yeast grown in chemostats, including the setup of the experiment and sampling methods as well as best laboratory and record-keeping practices.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

Reagents

Defined minimal medium appropriate for the experiment

For examples, see Protocol: ***Assembly of a Mini-Chemostat Array*** *(Miller et al. 2015).*

Ethanol (95%)
Glycerol (20% and 50%; sterile)
Yeast strain of interest

Equipment

Agar plates (appropriate for chosen strain)
Chemostat array

Assemble the apparatus as described in Miller et al. (2013) and Protocol: ***Assembly of a Mini-Chemostat Array*** *(Miller et al. 2015).*

Cryo deep-freeze labels
Cryogenic vials
Culture tubes
Cytometer (BD Accuri C6)
Glass beads, 4 mm (sterile; for plating yeast cells)
Glass cylinder
Kimwipes

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Multiwell plates (96-well)
Sonicator
Spectrophotometer
Tubes, conical (20- and 50-mL)

METHOD

Throughout the experiment, all important parameters and observations should be recorded systematically. For an example of a data collection scheme, see Table 1.

Setting Up the Experiment and Inoculation

Day 1

1. Streak single colonies on an appropriate agar plate and grow at 30°C.

   This is suitable for most Saccharomyces cerevisiae *strains. Optimize the growth temperature for the strain of interest.*

Day 2

2. Assemble the ministats and prepare the medium for the selective conditions desired (see Protocol: **Assembly of a Mini-Chemostat Array** [Miller et al. 2015]). For each evolution culture, inoculate a single colony into 2.5 mL of appropriate medium and let each culture grow to saturation overnight.

   See Troubleshooting.

Day 3

3. Turn the pump on to fill the ministat culture tubes, and then turn it off when the volume reaches the 20-mL mark. Set the heat blocks to the appropriate temperature.
4. Sterilize the tops of the corks with 95% ethanol.
5. Inoculate each chemostat vessel with 0.1 mL from one individual overnight culture using a syringe.

   Do not inoculate multiple vessels from the same overnight culture as this could result in shared mutation content from variants that emerged during the initial outgrowth.

6. Keep a stock of each inoculum by freezing 1 mL of the overnight culture mixed with 0.5 mL 50% glycerol in a cryogenic vial at −80°C.
7. Grow the chemostat cultures to saturation for 30 h before starting the media flow.

Setting Up the Culture Volume and Starting the Continuous Culture

8. Turn the media pump on to a dilution rate of 0.17 vol/h, which corresponds to between 5.75 and 6.5 rpm on the Watson–Marlow pump suggested as part of the ministat setup in Protocol: **Assembly of a Mini-Chemostat Array** (Miller et al. 2015).

TABLE 1. An example of a data collection scheme

Sample	Day	Time	Action	Time elapsed (h)	V_{eff} (mL)	Dilution rate (vol/h)	Generations	Density (cells/µL)	Notes
S1			Inoculation						
S101	1	12 pm	Pump ON 6.25		0				
S102	1	12 pm	Pump ON 6.25		0				
S101	2	9:17 am		21.28	73	0.171	5.3	2000	
S102	2	9:17 am		21.28	72	0.169	5.2	2223	

9. Turn off the air, and adjust the media volume to 20 mL by moving the sampling needle up or down. Turn the air on when done. Wait until the medium starts to exit through the effluent line.
10. Once all the cultures are at 20 mL, empty the effluent bottles. Record the time (this will be time 0), and take samples if desired.

Sampling the Chemostats

The chemostats are ideally sampled every day in a consistent manner and the process may include making a glycerol stock, measuring cell density, sampling for RNA and DNA, etc. The process can take between 30 min (short sampling) and several hours (long sampling). For sampling from overnight effluent collection bottles for DNA extraction, see Steps 19–22. For long-term evolution experiments, samplings can alternate between short and long protocols. Set up all the required materials before you start sampling the culture. Use printer-friendly cryo deep-freeze labels to label the required tubes.

11. Check the whole setup (needle, carboys, and effluent corks) for leaks, contamination, or flocculated cultures.

    See Troubleshooting.

12. Note the time, and start collecting fresh effluent by transferring the sampling corks into labeled 20-mL sterile sampling tubes. Store these tubes on ice while sampling.
13. While the tubes are filling, measure and record the effluent volume (V_{eff}) that has collected in the effluent bottles.
14. Calculate the dilution rate using the time elapsed and the total V_{eff}.
15. Adjust the pump or individual pump cartridges as needed to reach a dilution rate of 0.17 (±0.01) vol/h.
16. Wash the effluent bottles, and replace the sampling corks on the bottles. Note the time.
17. To store frozen aliquots, pipette 1 mL of the fresh sample into 0.5 mL of sterile 50% glycerol in a labeled cryogenic vial. Invert a few times to mix, and store the tubes at −80°C.
18. Measure and record cell density using your favorite method.

    ### *Spectrophotometer*

    i. Dilute the sample to reach a linear range.
    ii. Read the optical density at 600 nm using the spectrophotometer and a matched blank.

    ### *Cytometer*

    i. Vortex the fresh sample and pipette 20 µL into 80 µL of water in a 96-well plate.
    ii. Seal the plate and sonicate to separate the cells.
    iii. Use the C6 cytometer to count the number of cells and record the data.

    ### *Plating Cells for Viable Counts or Drug Resistance*

    i. Vortex a fresh, sonicated sample.
    ii. Make serial dilutions of the culture.
    iii. Plate 250 µL of a 10^{-4} dilution using sterile glass beads onto a labeled agar plate.

    Note that a different dilution may be required depending on your culture density.

    iv. Incubate the plates at the appropriate temperature for 2 d before counting colonies.
    v. Record the data.

Sampling from Overnight Effluent Collection Bottles for DNA Extraction

19. Save 50 mL of the overnight effluent from the effluent bottle. Ideally, sterilize the effluent bottle and effluent tubing before collection begins.

20. Measure and record the remaining volume to calculate total V_{eff}.
21. Centrifuge the samples at 1.5*g* for 3 min at room temperature.
22. Remove the supernatants and freeze the cell pellets immediately at −20°C for later DNA extraction.

TROUBLESHOOTING

Problem (Step 2): The medium to start the overnight culture for the evolution experiment is not available.

Solution: Overnight cultures can be started in YPD or another nutrient-rich medium and cells can be washed with water before inoculating.

Problem (Step 11): There is colonization of the needle or media line.

Solution: Replace the tubing and the needle with autoclaved pieces, and record the information in your database.

Problem (Step 11): Cells begin to flocculate or clump as the culture evolves.

Solution: Increase sonication time or power until cells are dispersed.

DISCUSSION

Evolution in chemostats can operate for hundreds of generations, but these experiments require daily attention and troubleshooting. The possibility of contamination is the biggest concern and unfortunately this is one common reason for stopping experiments. Observation under a microscope for bacterial and fungal contamination of the fresh sample can be performed every 50 generations. Also, careful attention should be given to sterile technique throughout the entire procedure. Another common reason to stop the experiment is the appearance of clumping and wall growth. These are most frequently seen when using wild strains and certain mutants, but also frequently evolve from laboratory strains given enough time. These traits are also correlated with increased colonization of the media port, which should be monitored daily.

Beyond these issues there is no limit, and depending on your experiment you can run the chemostats for hundreds of generations. With experiments of this scale, you should be aware that a lot of samples are going to be collected and that they will require a lot of space to be stored. Organization is key to track the information and the samples. Data can be recorded using different methods, and samples need to be stored in a logical setup using unique identifiers such as:

F1 9/25/13: Fermenter vessel 1, sample collected on 25 September 2013

S10203: Sulfur, experiment 1, vessel 02, day 03

We have not discussed the experimental design in this protocol, but it is obviously of key importance. Strains, media, population size, experiment length, and other important parameters must be carefully considered before embarking on an experiment. Also, proper controls must be performed to ensure the selective condition is as designed. More discussion of these issues can be found in Dunham (2010).

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