Protocol

Assembly of a Mini-Chemostat Array

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Here, we describe instructions for the assembly of an array of miniature (20-mL) chemostats or "ministats" built from relatively inexpensive off-the-shelf parts. In experiments with yeast cultures, we have observed reproducibility in cellular physiology, gene expression patterns, and evolutionary outcomes with different ministats as well as between ministats and commercial large-volume platforms. Growth in continuous culture is a primary means for the characterization of yeast steady-state physiology, competition between strains, and long-term evolution experiments. We hope that these relatively inexpensive and high-throughput devices make the advantages of continuous culture growth more accessible to researchers.

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.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Defined minimal medium appropriate for the experiment
- Glucose-limited chemostat medium <R>
- Nitrogen-limited chemostat medium <R>
- Phosphate-limited chemostat medium <R>
- Sulfate-limited chemostat medium <R>

Select an appropriate nutrient-limited medium, such as one of the above. Alternative sources for each limiting nutrient can be substituted. For example, carbon sources other than glucose can be used in glucose-limited medium. Be sure to confirm that the limiting nutrient is in fact limiting when using any new strains or media formulations. Use high-quality chemicals and water when preparing media. For measuring large volumes, calibrate a graduated cylinder to ensure accurate measurements. Mix the components of the medium in a clean 10-L carboy and then filter them through a 0.2-µm 1-L bottle-top filter into another sterile 10-L carboy (see Steps 27–36).

Ethanol

Equipment

Air filters (0.45-µm; PTFE)
Air pumps (designed for 80-gal aquarium)
Aluminum foil
Autoclave

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Block for dry block heater (6 × 25-mm test tubes per block) (VWR 13259-210)
Bottle (glass; 100-mL; 45-mm-wide mouth; with cap)
Bottle top filter (1 L; 0.2-µm pore size; 45-mm diameter) (Corning 431174)
Carboy (vacuum-safe 10-L reservoir bottle with bottom hose outlet)
Connector (1/8-inch internal diameter; barbed Y)
Connector, female luer (1/8-inch barb)
Connector, male luer lock (1/8-inch barb)
Connector, male luer slip (1/8-inch barb)
Connector, reducing 1/4- to 1/8-inch (PVDF)
Culture tubes (55-mL; outer diameter 25 mm; screw cap; Pyrex)
Day pinchcock (metal clamp for tubing)
Dry block heater (VWR 12621-100)
Electrical tape (Scotch #35; green)
Flask (1-L; with sidearm)
Forceps
Ice pick
Inline valved quick-connectors (male and female; 1/4-inch I.D.; polypropylene)
Manifold, four-port (Cole Parmer EW-06464-85)
Micropipette tips (1-mL)
Needles (16-gauge × 5-inch for effluent port; spinal 18-gauge × 6-inch for air port; 20-gauge × 1.5-inch for media port)
Needles, blunt (20-gauge × 1.5-inch)
Needles, stainless steel (blunt; with polypropylene hub; 25-gauge × 1/2-inch)
Peristaltic pump (16-channel cartridge pump) (205S/CA16 from Watson-Marlow)
Pump head extension (16-channel) (205CA from Watson-Marlow)
Pump tubing (orange/green Marprene from Watson-Marlow)
Ring stand (with 10.5-inch three-prong clamp)
Rubber stopper (#2, with two holes)
Serological pipettes (10 mL; e.g., Stripette)
Silicone tubing, medium (1/4-inch × 3/8-inch)
Silicone tubing, small (3/32-inch × 7/32-inch; uses 1/8-inch connectors)
Stopper, pink foam silicone (Nonstandard size 2) (Cole Parmer EW-06298-06)
Stopper, silicone (no. 8 with 3/8-inch hole)
Stopper, yellow foam silicone (Nonstandard size 12) (Cole Parmer EW-06298-22)
Tube rack (for 25-mm-diameter tubes)
Tubing clamps (large; 12-position)
Vacuum pump
Vent filter (EMD Millipore SLFG 050 10)

METHOD

The ministat array consists of carboys supplying media, a peristaltic pump, an aeration system, a set of culture tubes placed in a heat block, and a set of effluent collection vessels (Fig. 1). Using the instructions below, arrays of up to 32 chemostats can be run off a single 32-plexed peristaltic pump. For further details regarding the design and utilization of these arrays, see Miller et al. (2013).

Assembly of the Culture Chambers and Attached Tubing

1. Clean the glass culture tubes, and rinse them thoroughly with water. Mark the exact location of 21, 20, and 19 mL on each tube (e.g., see Fig. 1) because there is a significant variation in the internal diameter of these tubes.

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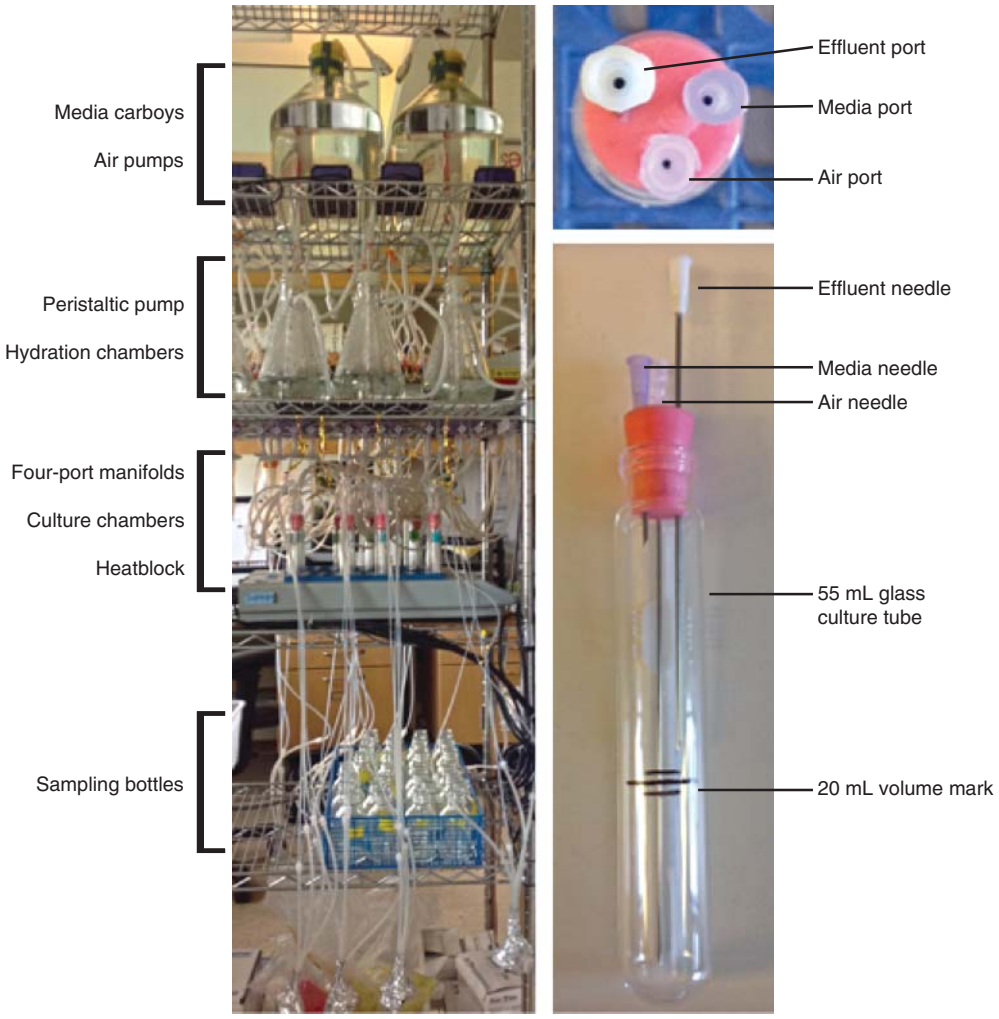

FIGURE 1. Arrangement of a ministat array (*left*) and an individual culture chamber (*right*). An array of 32 ministats and associated parts are shown ready for use in an experiment. The effluent sampling chambers are shown without stoppers, which is acceptable for short-term experiments. Also shown are top and side views of an individual chemostat chamber.

2. Make a cork assembly for each culture tube that comprises a pink size 2 foam silicone stopper containing three needles to deliver air, deliver medium, and remove effluent. Place the needles evenly around the stopper's circumference, and ensure that the needles run roughly parallel to the inside wall of the tube (see Fig. 1).

   Always wear safety goggles when working with exposed needles.

3. Assemble air-line tubing for each chamber by fitting a male luer connector to one end of a piece of small silicone tubing that is of sufficient length to reach from the culture chamber in the heat block to the four-port manifold. Place a 0.45-µm air filter on the other end of this tubing. Attach the luer connector to the longest needle in the cork assembly.
4. Assemble media-line tubing for each chamber by fitting a male luer connector to one end of a piece of small silicone tubing that is long enough to reach from the culture chamber in the heat block to the peristaltic pump. Attach the luer connector to the shortest needle in the cork assembly.
5. Use a female quick-connect to attach a 10-L media carboy with a male quick connect on a piece of medium silicone tubing ~4 inch long. Then, attach a male quick connect, and transition to small silicone tubing of sufficient length to reach from the carboy to the peristaltic pump. Next, use a

series of small Y connectors separated by ~1-inch lengths of small tubing to branch the media line and divide the media flow from one source to up to 32 ministat culture chambers. After the desired number of branches has been put in place, add a male luer connector to the end of each.

6. Gently insert 1/2-inch stainless steel blunt needles into each end of the orange/green Marprene pump tubing. Use this tubing to join the branched end of the media-line tubing with the media-line tubing leading to the culture chamber.
7. Insert a two-hole rubber stopper into each effluent bottle. Fit the underside of the two holes with different length blunt needles. Using a 1/4-inch connector pushed into one hole of the stopper, connect the effluent bottle to the effluent needle on a culture chamber using a sufficiently long piece of small silicone tubing with a male luer connector.

Pre-/Postexperiment Cleanup and Sterilization

Tubing may be reused many times provided it is cleaned immediately after experiments.

8. Place all tubing in separate trays and rinse excessively with ddH_2O. Empty the tubing using an air pump.
9. Clean the cork assemblies with ddH_2O. Wipe the outside of the needles and cork to remove any residual media or cells.
10. Clean the glass tubing with water and ethanol, and remove physical contaminants with forceps and Kimwipes if necessary.
11. Rinse and dry all parts again before use.
12. Immediately before use, reassemble as described in Steps 3–7 and cover all exposed tubing ends and filters with aluminum foil. Wrap the tops of the sampling bottles in foil and autoclave along with the culture chambers in a separate autoclave tray.
13. Fit the assembled array neatly into one or more autoclave trays and autoclave all parts (using the liquid cycle) for 20 min.

Hydrated Aeration of Ministat Chambers

The ministats are aerated with hydrated air delivered from an aquarium pump and bubbled through sterile water.

14. Fill a 1-L sidearm flask with 700 mL of ddH_2O. Remove the cotton filter from a 10-mL serological pipette, and attach a 4-inch piece of medium tubing to the end that previously contained the cotton filter. Carefully work the pipette through a one-hole #8 silicone cork so that it will dip into the water contained in the sidearm flask; shorten the pipette if necessary. Tightly press the cork into the flask.

    One flask will humidify four ministat chambers.

15. Use a 1/4- to 1/8-inch reducing connector to attach a piece of small silicone tubing from the aquarium pump to the medium tubing at the top of each serological pipette.
16. Add medium tubing of sufficient length to the sidearm of the flask so that it reaches the four-port manifolds fastened above the ministat chambers.
17. Place the dials on the four-port manifold so that air is taken in through one of the two side ports and routed through the four main ports and not the other side port.
18. Place 2-inch pieces of medium tubing on each of the four ports to connect to the air-line filters.

Preparation of Sterile Media Carboys

The 10-L carboys used to house media can be assembled, autoclaved, and stored for months before use.

19. Rinse 10-L carboys thoroughly with ddH_2O.

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20. Add two centered holes ~1 in apart in a #12 foam cork using an ice pick.

    Be careful when using the ice pick.

21. Carefully place a 1 mL micropipette tip into each of the two holes such that ~1 inch of the wider end of each pipette tip sticks out of the top of the stopper. Cut the narrow ends off the tips.

    Trimming the narrow ends permits the medium and air to flow more rapidly through the carboy.

22. Place a 4-inch piece of medium silicone tubing onto the wider end of one pipette tip and a 10-inch piece onto the wider end of the other tip.

23. Place the filter adaptor from the bottle-top filter used for media filtration (see Steps 28–30 below) onto the longer piece of tubing. Place a large vent filter onto the other piece of tubing.

24. Insert the cork into the top of the carboy, and give it a firm push. Use green electrical tape to secure the cork. Place at least one piece of tape over the top of the cork (between the two holes) and another around the neck of the carboy to prevent the cork from popping out in the autoclave.

25. Cover the ends of the media-in port (top; with the filter adaptor) and the media-out port (bottom) with foil folded such that it is secure but will be easy to remove.

26. Add 25 mL of water to each carboy and sterilize by autoclaving (using the liquid cycle) for 20 min. Store carboys upright or on their sides.

    They can be stored for months before use.

Filtration of Medium

The medium used in a chemostat is typically a defined minimal medium that is filtered instead of autoclaved to preserve vitamins and metals and to ensure precise nutrient concentrations. Good sterile technique is essential during this procedure.

27. With a flame going nearby, loosen the cap on a sterile 100-mL glass bottle that has threads matched to the bottle-top filter, and carefully screw the bottle-top filter on.

28. Dip forceps in ethanol and shake off any excess. Flame-sterilize the forceps and use them to pull the filter plug from where the adapter and vacuum usually attach.

29. Attach the filter to the filter adapter that was autoclaved on the carboy's media-in port (see Step 23).

30. Clamp the bottle into a ring stand next to the carboy.

31. Attach the vacuum hose to the vent filter on the sterile carboy.

32. Route the clamped outlet tube from the mixing carboy into the top of the filter.

33. Place the sterile carboy in a tray to catch any inadvertent overflow.

34. Turn on the vacuum and unclamp the outlet hose from the mixing carboy. Adjust the large clip to alter the flow of medium out of the mixing carboy.

    The 100-mL bottle will fill first and then overflow into the second carboy. If the vacuum is too strong, the cork can be pulled into the carboy. Filtering should take ~30 min/10 L.

35. Clamp the media line with a metal clamp, and use the same foil that was on the adaptor to cover it once again. Remove the vacuum pump tubing, and turn off the vacuum.

36. Incubate the filter bottle at 30°C to give an early warning of contamination, or clean and autoclave it for future use. Use the filtered medium immediately, or store the carboy for a short period of time before use.

Assembly of Carboy with Chemostats and Collection of Effluent

Assemble the ministat array, culture-sampling bottles, and all nonautoclaved parts on a benchtop or in a metal rack (see Fig. 1).

37. Place the autoclaved culture tubes into the heat blocks.

    We find that placing 16 ministats staggered in a heat block allows for better observation and troubleshooting with the cultures.

38. Place the effluent bottles below or beside the culture chambers. Direct the effluent tubing into each appropriate bottle.

    The culture bottles may be organized in wire racks or plastic tubs to aid in sampling organization.

39. Label each of the culture tubes, effluent lines, and sampling bottles to decrease the likelihood of sampling error.

40. Place filtered sterile media above or next to the ministat array.

RELATED INFORMATION

For descriptions of how to use the ministat array to characterize yeast physiology and perform long-term evolution experiments, see Protocol: **Chemostat Culture for Yeast Physiology** (Kerr and Dunham 2015) and Protocol: **Chemostat Culture for Yeast Experimental Evolution** (Payen and Dunham 2015).

RECIPES

Glucose-Limited Chemostat Medium

Reagent	Quantity (for 10 L)
Calcium chloride dihydrate	1 g
Sodium chloride	1 g
Magnesium sulfate heptahydrate	5 g
Potassium phosphate monobasic	10 g
Ammonium sulfate	50 g
Glucose	8 g
Metals (1000×) <R>	10 mL
Vitamins (1000×) <R>	10 mL

Dissolve the salts in just <1 L of glass-distilled water. In a separate beaker, dissolve the glucose in just <1 L of glass-distilled water. Bring each solution to a final volume of 1 L. Combine the salt and glucose solutions with 8 L of water, the metals, and the vitamins in a mixing carboy to make 10 L of medium. Stir for ~5 min or until thoroughly mixed.

Metals (1000×)

Reagent	Quantity (for 1 L)
Boric acid	500 mg
Copper sulfate pentahydrate	40 mg
Potassium iodide	100 mg
Ferric chloride hexahydrate	200 mg
Manganese sulfate monohydrate	400 mg
Sodium molybdate dihydrate	200 mg
Zinc sulfate heptahydrate	400 mg

Dissolve the reagents in just <1 L of glass-distilled water in the indicated order. Bring the total volume to 1 L, and pour into a foil-wrapped bottle, making sure no residue remains in the original vessel. Store at room temperature. Shake well before using.

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Nitrogen-Limited Chemostat Medium

Reagent	Quantity (for 10 L)
Calcium chloride dihydrate	1 g
Sodium chloride	1 g
Magnesium sulfate heptahydrate	5 g
Potassium phosphate monobasic	10 g
Ammonium sulfate	400 mg
Glucose	50 g
Metals (1000×) <R>	10 mL
Vitamins (1000×) <R>	10 mL

Dissolve the salts in just <1 L of glass-distilled water. In a separate beaker, dissolve the glucose in just <1 L of glass-distilled water. Bring each solution to a final volume of 1 L. Combine the salt and glucose solutions with 8 L of water, the metals, and the vitamins in a mixing carboy to make 10 L of medium. Stir for ~5 min or until thoroughly mixed.

Phosphate-Limited Chemostat Medium

Reagent	Quantity (for 10 L)
Calcium chloride dihydrate	1 g
Sodium chloride	1 g
Magnesium sulfate heptahydrate	5 g
Ammonium sulfate	50 g
Potassium chloride	10 g
Potassium phosphate monobasic	100 mg
Glucose	50 g
Metals (1000×) <R>	10 mL
Vitamins (1000×) <R>	10 mL

Dissolve the salts in just <1 L of glass-distilled water. In a separate beaker, dissolve the glucose in just <1 L of glass-distilled water. Bring each solution to a final volume of 1 L. Combine the salt and glucose solutions with 8 L of water, the metals, and the vitamins in a mixing carboy to make 10 L of medium. Stir for ~5 min or until thoroughly mixed.

Sulfate-Limited Chemostat Medium

Reagent	Quantity (for 10 L)
Calcium chloride dihydrate	1 g
Sodium chloride	1 g
Magnesium chloride hexahydrate	4.12 g
Ammonium chloride	40.5 g
Potassium phosphate monobasic	10 g
Ammonium sulfate	30 mg
Glucose	50 g
Metals (1000×) <R>	10 mL
Vitamins (1000×) <R>	10 mL

Dissolve the salts in just <1 L of glass-distilled water. In a separate beaker, dissolve the glucose in just <1 L of glass-distilled water. Bring each solution to a final volume of 1 L. Combine the salt and glucose solutions with 8 L of water, the metals, and the vitamins in a mixing carboy to make 10 L of medium. Stir for ~5 min or until thoroughly mixed.

Vitamins (1000×)

Reagent	Quantity (for 1 L)
Biotin	2 mg
Calcium pantothenate	400 mg
Folic acid	2 mg
Inositol (*myo*-inositol)	2000 mg
Niacin (nicotinic acid)	400 mg
p-Aminobenzoic acid	200 mg
Pyridoxine hydrochloride	400 mg
Riboflavin	200 mg
Thiamine hydrochloride	400 mg

Mix the reagents in just <1 L of glass-distilled water. (Some of the materials will not completely dissolve.) Bring the total volume to 1 L. Transfer to a beaker and stir. Maintain stirring while transferring 40-mL aliquots into 50-mL conical tubes. Ensure that no residue is left in the original vessel or graduated cylinder. For long-term storage, freeze the aliquots at −20°C. For short-term storage (<1 mo), store the aliquots at 4°C. Shake well before use.

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REFERENCES

Kerr EO, Dunham MJ. 2015. Chemostat culture for yeast physiology. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot089003.

Miller AW, Befort C, Kerr EO, Dunham MJ. 2013. Design and use of multiplexed chemostat arrays. *J Vis Exp* (72): e50262. doi:10.3791/50262.

Payen C, Dunham MJ. 2015. Chemostat culture for yeast experimental evolution. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot089011.

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