Dunham Lab Ministat Manual

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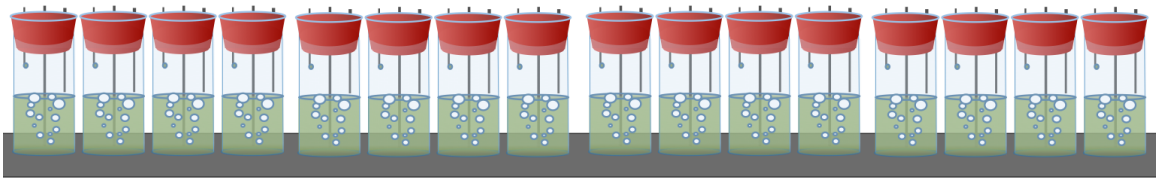
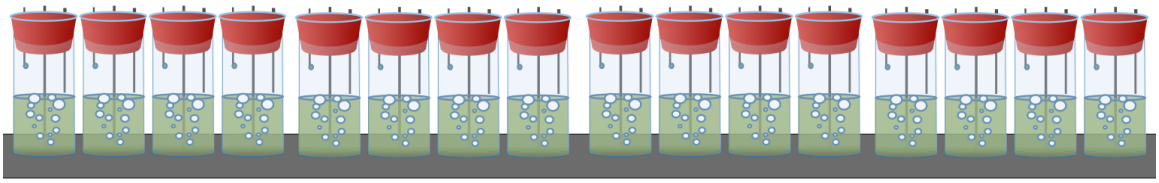


Presented here is everything you need to construct and run an array of 32 20ml chemostats or "ministats." This includes a basic overview of the anatomy of a ministat culture chamber and important components of the array. Additionally this manual provides complete methods used for evolution and competition based experimental studies as well as methods used to characterize physiology in yeast. This includes: how to acquire data, store samples, and harvest DNA/RNA for analysis. Although geared towards assaying competitive fitness and the genomics of experimental evolution in yeast, this guide also describes general elements or methods that can be custom tailored for your own purposes. Many of the images used in this manual are taken from the forthcoming ministat training video (email me for a preprint if interested) and are used with the permission of Corrie Befort. A list of parts and providers is listed in Appendix A.

Visit *http://dunham.gs.washington.edu* for the most recent updates to this and other protocols. Please feel free to point other people to these instructions. Also, we would appreciate the citation if you use any of this information in a publication or talk.

Finally, if you make improvements to any of these methods, please contact me at *maitreya@uw.edu* We would love to hear about them!



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About the Ministats

Ministats are chemostats with a 20ml working volume. This is approximately 1/10th the size normally used by our lab and many others. This volume stands out as being small enough that it can be greatly multiplexed and still fit in a relatively small space, and yet large enough that samples produced will be sufficient for DNA/RNA/protein/metabolite characterization. As depicted below in **Fig. 1**, the chemostat culture chamber is simply a glass tube, fitted with a silicon stopper, which holds 3 needles that deliver air and media as well as continuously sample the culture.

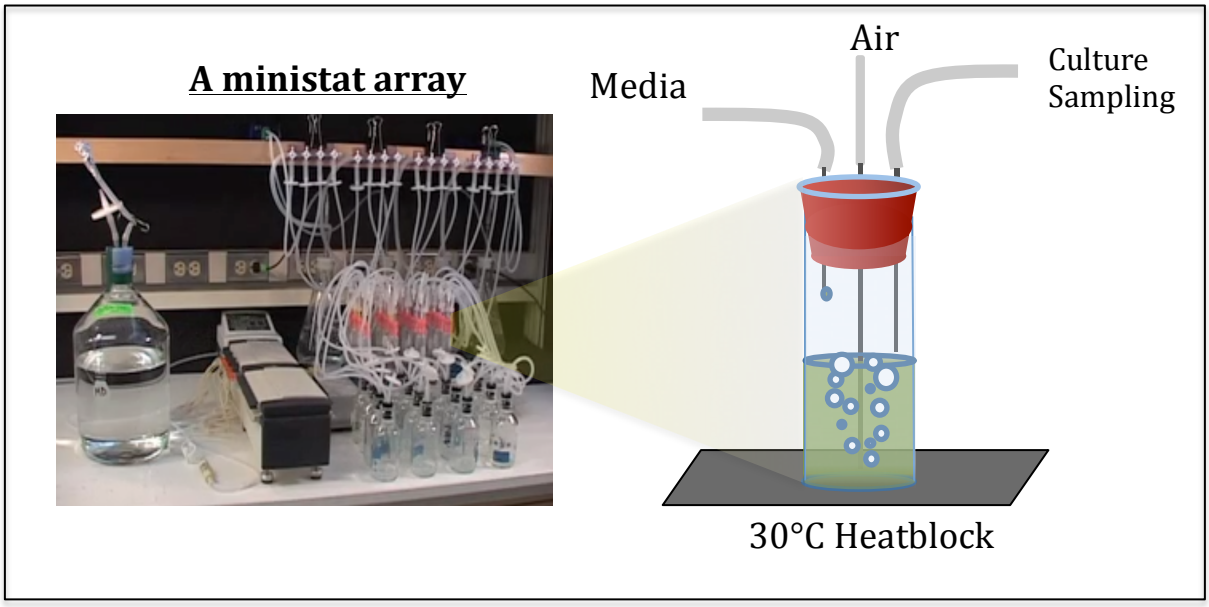


Figure 1: An array of 16 ministats running.

Closeup of a ministat chamber. The ministat chamber is made up of a 50ml screw top glass tube stopped with a silicon cork. Air is delivered to the chamber by way of a long needle, which reaches to the bottom of the tube and delivers robust airflow. Culture volume is determined by a second needle, which removes media and air and samples to a collection chamber for analysis. A third and far shorter needle is used to add media to the chamber. This activity determines the dilution rate and directly controls the rate of cell growth.

Components of the ministat array

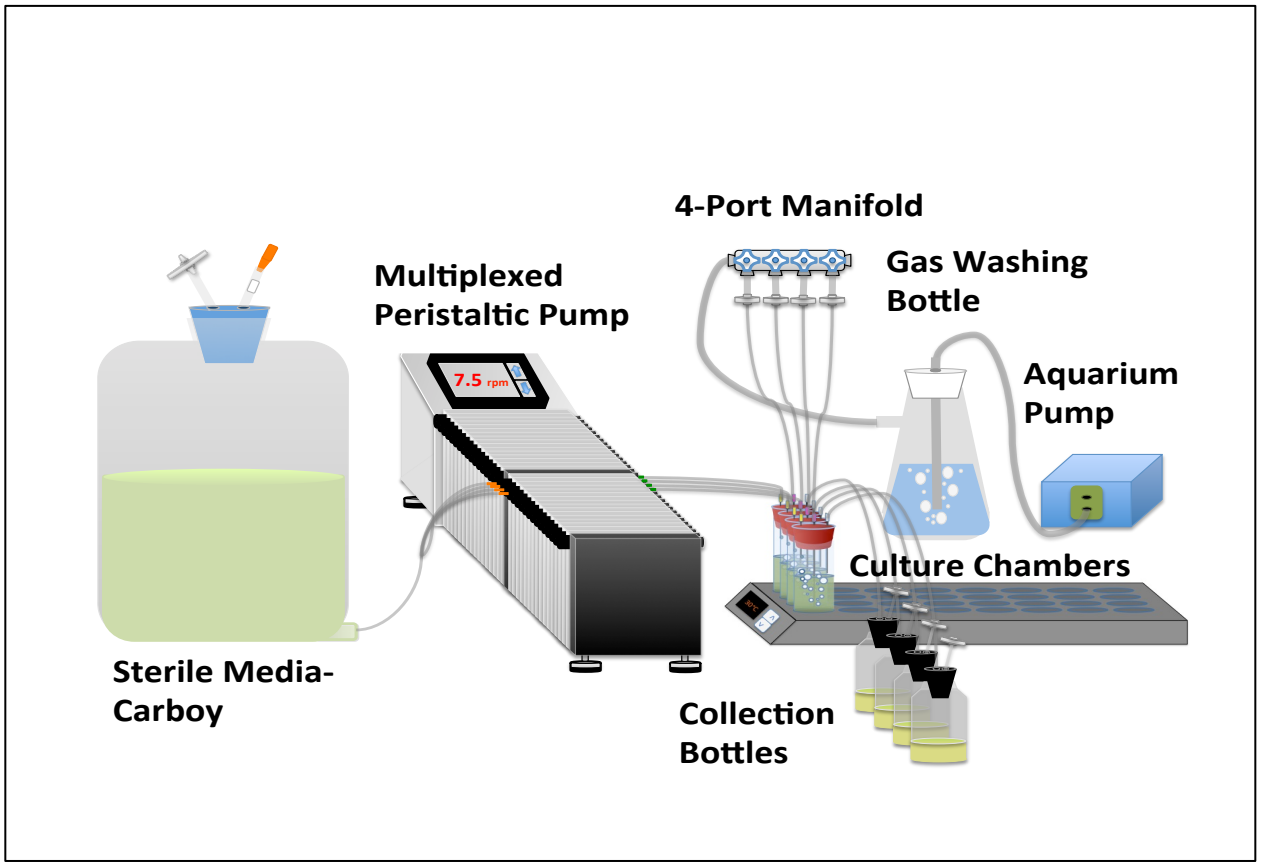


Figure 2: Key components of ministats.

Air is pumped from an aquarium pump, into a gas-washing bottle in order to hydrate the air, which is then routed through a splitter and 0.45um filter before entering the ministat chamber. The gas-washing step humidifies the air, and reduces evaporation in the relatively small volume chemostats. Because the air is routed to the very bottom of the chamber, the bubbling in the ministat not only aerates the media, but it also works to keep cells suspended and from clumping together. Additionally, the positive pressure created by the aeration keeps the effluent track moving quickly in the correct direction, helping to prevent back contamination. **Media** drips into the ministat from tubing that connects a media carboy with the media port on the ministat. The flow rate is controlled by a peristaltic pump, which massages media through pump tubing that is part of the media line. **Effluent** is removed when the media level reaches the height of the culture-sampling needle, and the positive pressure created by the air-flow pushes the media out through the effluent track and into culture collection chambers. **Temperature** is regulated by an external

heat source. We use a heat-block however a water-bath or incubator should work as well. We caution that water-baths may introduce contamination concerns.

BUILDING THE ARRAY

Ordering Parts and equipment

In **Appendix A** is a complete list of all materials, equipment, and ordering info for the 32-plexed ministat array. It will likely take months for all materials to arrive. In our experience the rate-limiting step has been acquisition of the 32-plexed peristaltic pump and autoclavable pump tubing. The carboys we recommend have been slow to order recently as well.

Preparing the parts

Glass culture tubes

We have been using glass tubes as the culture vessel of our chemostat. These come with the advantage that they are relatively inexpensive, and have screw-top capability, which we made use of in earlier designs. The 50ml capacity allows the experimenter to choose from a variety of culture volumes. We currently use a 20ml volume, as it allows plenty of headroom to minimize occupation by yeast in the media line, while still offering large enough sample volumes for genetic analysis and reasonable sampling times.

Cleaning the glass tubes

Thoroughly rinse each of these with ddH2O and 70% ethanol. Next, scrub the interior of the tubes with 3 Kimwipes using forceps. Finally rinse with excess ddH_2O and leave to dry upside-down in a rack. Once dry inspect the tubes and clean again if any residues are apparent.

Marking the glass tubes

The culture tubes we use vary slightly in total volume. Therefore each tube must be marked to know where the 20ml working volume is. I also mark 21 and 19 ml marks as this may be useful in estimating the degree to which volumes deviate from ideal while setting the culture volume. To do this add 21ml of ddH2O into each of the tubes and carefully mark the meniscus on the side with an indelible ink marker or glass scorer.

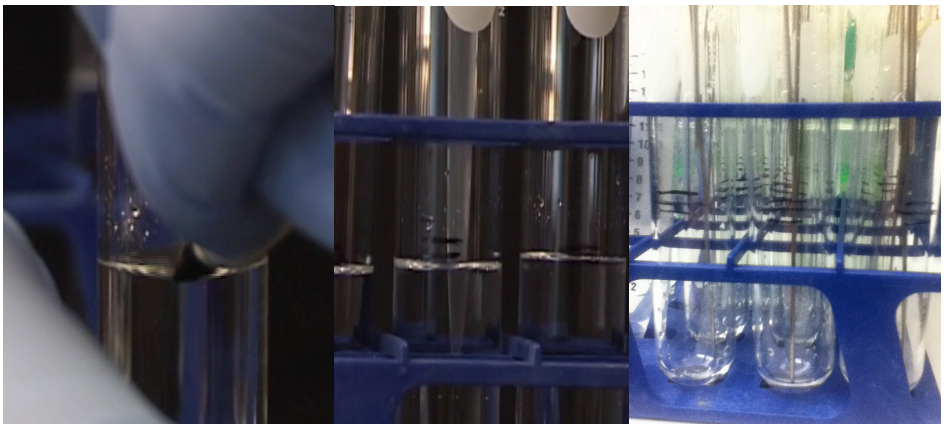


Figure 3: Marking the tubes.

Fill the tubes with 21 ml and remove 1ml increments to mark three lines at 21, 20 and 19ml. These marks identify the ideal working volume and help estimate deviation from the desired volume. Do this for all culture tubes.

Preparing the cork assemblies for the ministat culture tubes

First designate a glass culture chamber for use in punching needles through the silicon corks. This is not a chamber that you'll use for experiments, rather just as a cork stand for placing all 3 needles into the silicon cork. Be sure to take appropriate precautions, especially including wearing protective eyewear. PROCEED WITH EXTREME CAUTION!

One by one place each of the three needle types shown in **Fig 1** into the silicon cork as shown below. Ideally they will be evenly spaced and the needles will hang more or less parallel to the glass culture chamber wall. After the needles are in place, clear any silicon that may have been cored using the spinal-tap needle insert. Once this is done you can move the cork assembly to a new cleaned glass culture tube that will be used as the culture chamber. After you have done this for all the culture chambers you'll need for the experiment you may move on to attaching the silicon tubing responsible for transporting air and media to the culture chamber as well as the tubing used to sample the culture.

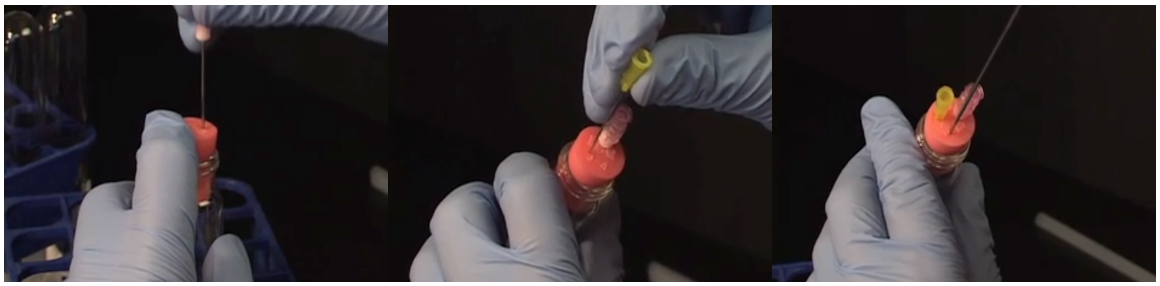


Figure 4: Making the cork assembly.

Prepping the air-line for the autoclave

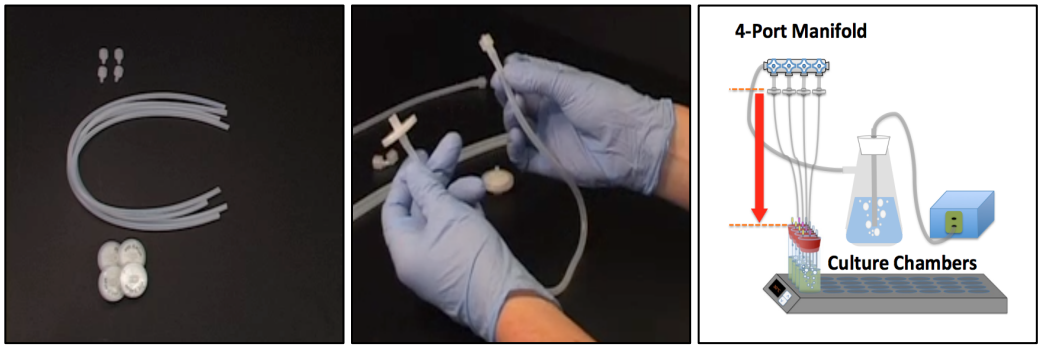


Figure 5: Making the air-line.

The air-line is simply a piece of 3x32in. silicon tubing with a male luer-lock on one end and a 0.45um filter on the other. The tubing should be of sufficient length to connect your culture chamber to the 4-port manifold used to split airflow from one pump to 4 culture chambers.

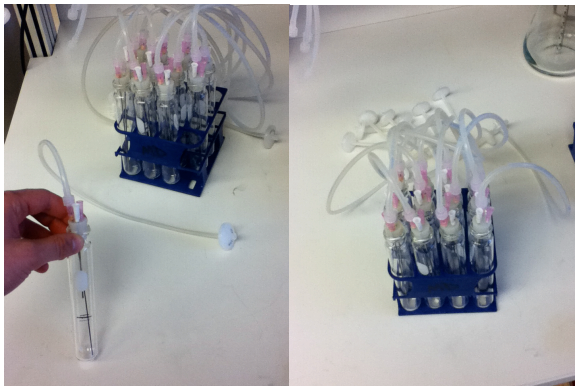


Figure 6: Attaching the air-lines to the tops of the culture tubes.

Once 4 culture chambers have had air-lines with filters attached to the spinal tap needles, group the four filters together and wrap them completely but loosely with tape or cable ties. Do this for all of the culture chambers.

Figure 7: Preparing the air-lines for the autoclave.

Prepping the effluent sampling line for the autoclave

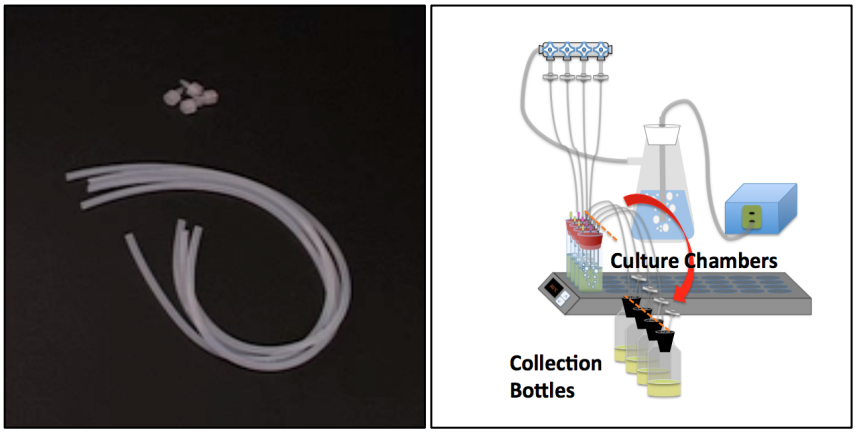


Figure 8: Preparing the effluent lines for the autoclave.

The media effluent and sampling line is a piece of 3x32in tygon tubing that has been fitted with one male luer lock. It must be of sufficient length to connect the culture chamber to the effluent collection chamber. Simply attach the luer-lock end of the tube to the culture chamber's media sampling needle (the medium length one).

As with the air-line, take four of these, bunch the open ends together, and foil them for autoclaving.

Prepping the media-line for the autoclave

Figure 9: Media line parts and assembly of the quick-connect, which attaches to the media carboy.

The media line is used to route media from the sterile 10L carboys containing media to the culture chambers. To do this the media line has a female quick-connect attached to ¼" tubing, an adaptor to adjust the tubing size to 3/32" tubing, a length of tubing sufficient to reach the pump, a short length of pump tubing, and a length of tubing sufficient to reach the culture tube.

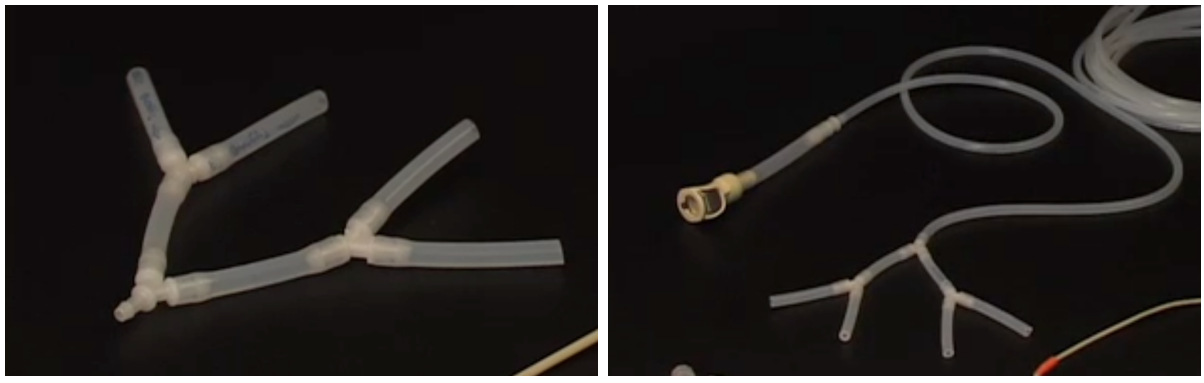


Figure 10: Making branched tubing to divide the media from one source to many ministats.

If one media source is being split to 4 ministats then one would split this 1 media line into 4 using y-connectors and 3/32” tubing. Place the splitter between the carboy’s quick connect and the pump tubing.

Because the pump tubing has such a narrow bore, the connectors used to link them to the 3/32” tubing are actually short blunt needles. Since these needles are not barbed, some care needs to be taken to avoid pulling them out during handing. The other end of the pump tubing is connected to another length of 3/32” tubing which must be long enough to reach the appropriate culture tube in the heating block.

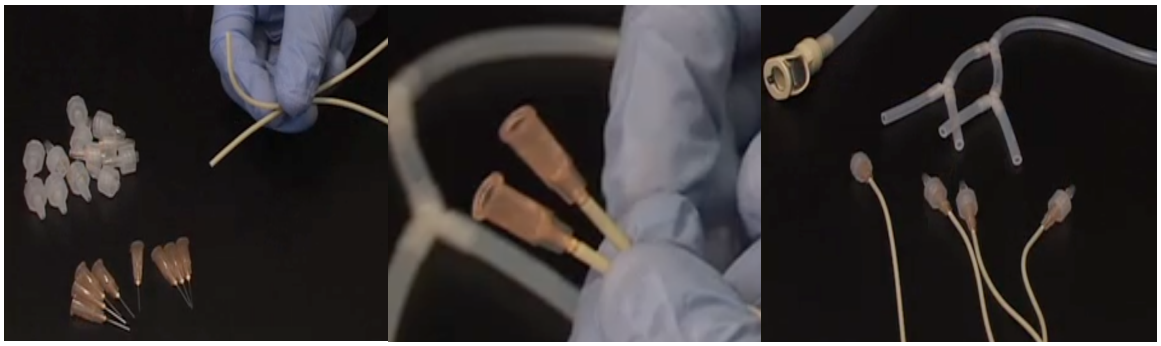


Figure 11: Adding adaptor needles and connecting the pump tubing to the branched tubing.

Once you have this media-line made, attach it to the shortest needle in the ministat’s cork assembly, which will eventually deliver media into the culture tube. Do this for each ministat you’re going to use. Foil the female quick-connect for autoclaving.

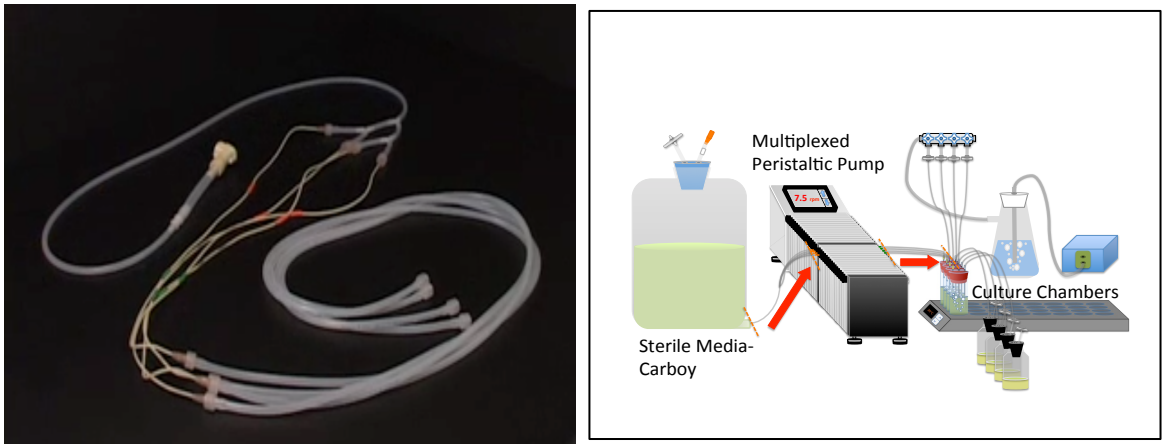


Figure 12: The assembled media tubing.

Make sure this is long enough to reach from the carboy to the pump and from the pump to the culture chambers.

Prepping a new cork for the carboy

The media vessel is made up of a 5L or 10L glass carboy with a bottom spout, and a cork assembly. Described below is the protocol for preparing a 10L carboy which is identical to the protocol used for the 5L carboy with the exception that the 5L carboy uses a smaller cork The bottom spout is fitted with 1/4" tubing, a clamp, and the male part of a 'quick connector,' which will eventually connect to the female part of the quick connector on the media-line running to the ministats. Use of these connectors allows the carboy to be autoclaved separately from the chemostats and their attached media lines. The cork assembly consists of a big silicone stopper with an air filter and a media port outfitted with a connector to fit the filter. This media port will connect to a modified 1 L filter through which the media will be filter-sterilized. Smaller carboys may be used, though we have had some difficulty sourcing appropriately vacuum- and autoclave-safe glassware. We have previously used autoclaving to sterilize chemostat media followed by addition of filter sterilized heat-labile ingredients; however, we found that this method introduces unacceptable variation in volume due to uneven evaporation.

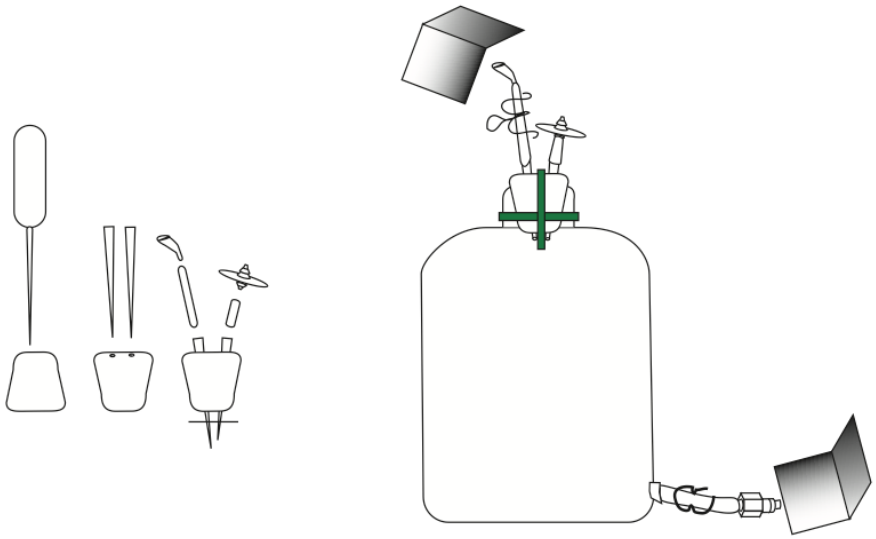


Figure 13: Making the Cork Assembly for a carboy & preparing a carboy for the autoclave.

To make the Cork Assembly, carefully use a cork borer/awl to make a hole from the bottom to the top of the cork. Make 2 of these holes. Then fit a length of rigid tubing (we use a 1200ul pipet tip with the small end clipped off after insertion for unrestricted flow, but you could use metal, or some other autoclavable but not brittle material) into each hole, being careful to not injure yourself or the rigid tubing. Whatever you use, it should be monitored for cracks and may occasionally need to be replaced. Then attach a short piece of 1/4" tubing (about 3 inches) to one of the ports, and attach a carboy vent filter to it. To the other port, attach a longer piece of tubing (about 10 inches) with a clamp on it and attach a filter adapter to it. We use Corning filters, and the adapters come with them. They can be autoclaved several times, but should be monitored for cracking, as this is not their intended purpose.

The Carboy

To prepare the empty carboy for autoclaving, insert the carboy cork assembly into the top of the carboy, and give it a firm push in. Then, use green electrical tape (Scotch #35), to secure the cork. Run one strip from the glass carboy neck on one side, tightly over the cork and in between

the two ports, and onto the other side of the carboy neck. Then run another piece of tape in a ring around the neck, overlapping both ends of the first strip of tape. This should prevent the cork from popping out in the autoclave. Next add 20mls of ddH2O through the top media port, and then clamp it securely with a metal clamp. We have found that this improves sterilization via steam. Next, use your favorite foil origami method to cover the media-in port on top, and the media–out port on the bottom. Make sure to calculate how much media you'll need for your experiment, and include additional carboy connectors as needed to facilitate sterile connection of new carboys.

Autoclaving

Transfer the ministats with attached tubing into a large tub in an organized fashion. Also, don't forget your carboy(s) and effluent bottles. Autoclave all components on fluid cycle for 20 minutes. Make sure that all containers (the ministats and carboy) are vented so that they do not explode or disassemble in the chemostat. Since the volume of air in the carboy is so large, autoclaving on the fluid cycle is required in order to prevent breakage. It's best not to exceed 20 minutes of sterilization time because of all the plastic components that we expect to survive multiple sterilization cycles.

SETTING UP A RUN

The first thing to do is design your experiment. You need to choose your strains, media recipes, and dilution rate. You will also need to figure out how much media you'll need, and construct and sterilize the chemostat array.

Strains

The strains commonly used in the lab are FY, which is an S288C derivative that's been made GAL2+, and CEN.PK, a favorite of the European chemostat community. Using a prototroph is vastly preferred to using an auxotroph. With auxotrophs, you can never really be sure what the cells are using as a source of limiting nutrient. It just complicates matters and makes you less sure of any results. We have prototrophs of FY and CEN.PK, as diploids and as haploids of both mating types, in the strain collection.

If you do have to use an auxotroph, be very careful with the supplements you add. For example, you can't use adenine sulfate with sulfur limitations. You want to make sure the culture does not become limited for the additive, but you don't want to add so much excess that the culture eats the additive instead of the nominal limiting nutrient. See the Limitations section for how to check limiting nutrients. You can also use an auxotroph on purpose and limit with the additive it requires. Matt Brauer and Alok Saldanha have successfully done this with *leu2* strains, and Alok and I have also done *ura3* strains. These media formulations are included in the Media Recipes section.

Setting the pump

Once you have received the pump, assemble it according to the manufacturer's instructions. In our experience it is a good idea to translate RPMs into approximate flow rates for different numbers of tubes. The vast majority of our experiments have a dilution rate of 0.17 volumes per hour. So for our 20ml cultures we desire 3.4ml per hour. Dilution rate will be measured daily by measuring the effluent volume collected from the overflow.

The dilution rate is a simple relation of the effluent volume, length of time (in hours) effluent collected, and chemostat working volume:

D = effluent volume/(time * chemostat volume)

The dilution rate is in units of hr^{-1}. It is also sometimes called omega. We allow a +/- 0.01 vol/hr margin of acceptable dilution rate variation. Deviations beyond this create major gene expression and physiology differences. Please note that pump tubing may grow slack over time and may require you to periodically fine adjust to achieve the desired flow rate.

Making media

You should ideally make media at least one day before use in experiments. Also media should not be used two weeks after being made because the vitamins and other ingredients may degrade over time.

Limitations

The limiting nutrient depends on what your experiment is. Keep in mind that glucose limited cultures seem to be most sensitive to changes in the dilution rate. Lower dilution rates provoke more respiration while

higher dilution rates favor fermentation.

If you are not using one of the standard recipe/strain combinations that are listed in the Chemostat Media section, you should do a preliminary batch culture experiment to figure out the limiting concentration to use. Inoculate an overnight culture in media with a low level of the limiting nutrient. Spin it down and resuspend at a 100X dilution in chemostat media without any limiting nutrient. Aliquot equal volumes into a series of appropriate volume shake flasks that contain different quantities of the limiting nutrient. Be careful that the volumes of limiting nutrient solution are the same in all the flasks so you don't get different dilution factors. You may want to make your media 1.1X and bring them to 1X with the limiting nutrient solution. Let these flasks shake at 30C for a couple of days or until the density stabilizes. You want them to be completely in stationary phase. Measure the densities. If you graph the concentration of limiting nutrient vs. the final densities of the cultures, you should get a plot with a linear range, a nonlinear range, and a plateau. You want to stay in the linear range.

Chemostat media recipes

Chemostat media has 4 components that need to be made separately: salts, metals, vitamins, and carbon source (generally sugar). For each batch of media, you will prepare a carboy, thaw the pre-made 1000X Vitamins, make 10X salts, and make 10X carbon source. You'll combine these with the pre-made metals, and top to 10L with glass distilled water. It all gets mixed together in a clean, non-sterile "mixing" carboy before it gets filter-sterilized into a sterile carboy. These media recipes come from Julian Adams via Frank Rosenzweig with further modification by me. The glucose limitation recipe is exactly per Adams. I modified the glucose limitation recipe for phosphate, sulfur, and nitrogen limitation. In general, I tried to keep all ions at the same molarity where possible. The Adams version of the phosphate limitation recipe uses the salts at 0.25X to limit the effects of phosphate contamination from the other salts, but I always use 1X salts for everything. You can only get away with this if you use really pure chemicals. The uracil and leucine limitation recipes use the Adams glucose limitation base plus limiting concentrations worked out by Alok Saldanha.

Salts

Salts can be made as 10X stocks in glass distilled water and kept at room temperature until use. Non-sterile salts should be used within a couple weeks to avoid contaminant growth. You may be tempted to make

a big carboy of salts, but that experiment has been tried and mysterious floating bits appear eventually. If you want to keep them longer, they can be autoclaved. Make salts using the purest chemicals available/affordable, and try to stick to one brand for replacement purchases. It is crucial that limiting nutrient concentration not vary due to contamination in other salts.

10X salts for carbon, leucine, or uracil limitation (1 L)

1 g	calcium chloride.$2H_2O$
1 g	sodium chloride
5 g	magnesium sulfate.$7H_2O$
10 g	potassium phosphate monobasic
50 g	ammonium sulfate

10X salts for phosphate limitation (1 L)

1 g	calcium chloride.$2H_2O$
1 g	sodium chloride
5 g	magnesium sulfate.$7H_2O$
50 g	ammonium sulfate
10 g	potassium chloride
100 mg	potassium phosphate monobasic (to 10 mg/L final)

10X salts for sulfur limitation (1 L)

1 g	calcium chloride.$2H_2O$
1 g	sodium chloride
4.12 g	magnesium chloride.$6H_2O$
40.5 g	ammonium chloride
10 g	potassium phosphate monobasic
30 mg	ammonium sulfate (to 3 mg/L final)

10X salts for nitrogen limitation (1 L)

1 g	calcium chloride.$2H_2O$
1 g	sodium chloride
5 g	magnesium sulfate.$7H_2O$
10 g	potassium phosphate monobasic
400 mg	ammonium sulfate (to 40 mg/L final)

Metals

Metals are made as a 1000X stock that keeps at room temperature for at least a year. Keep the bottle well wrapped in foil since some of the metals are light sensitive. Make the metals in sterile glass distilled water. Be vigilant about shaking before using since the metals will not totally dissolve.

1000X metals

(1 L) Dissolve chemicals in ~1 L stirring glass distilled water in the following order:

	Metal	Chemical storage
500 mg	boric acid	RT shelf
40 mg	copper sulfate.$5H_2O$	RT shelf
100 mg	potassium iodide	RT, dark, dessicator
200 mg	ferric chloride.$6H_2O$	RT shelf
400 mg	manganese sulfate.H_2O	RT shelf
200 mg	sodium molybdate.$2H_2O$	RT shelf
400 mg	zinc sulfate.$7H_2O$	RT shelf

Bring total volume to 1 L with glass distilled water, and pour into a bottle. Cover the bottle with foil, and store at room temperature.

Vitamins

Vitamins are also made as a 1000X stock. The solution is aliquoted into 50 ml Falcon tubes and stored at -20C. Don't fill the tubes to the top, or else the lid will split when frozen. The "working tube" can be stored at 4C. The vitamins will not dissolve completely, so shake before use. Care should be taken to keep the solution well mixed while aliquoting.

1000X Vitamins

(1 L) Weigh all chemicals and add to a beaker of stirring glass distilled water to dissolve as much as possible. Top off to 1 L, then aliquot about 40 mL per 50 mL tube, and freeze.

	Vitamin	Chemical storage
2 mg	biotin	4C
400 mg	calcium pantothenate	4C
2 mg	folic acid	RT, dark, dessicator
2000 mg	inositol (aka myo-inositol)	RT shelf
400 mg	niacin (aka nicotinic acid)	RT shelf
200 mg	p-aminobenzoic acid	4C
400 mg	pyridoxine HCl	RT, dark, dessicator
200 mg	riboflavin	RT shelf
400 mg	thiamine HCl	RT, dark, dessicator

Mixing the Media

Completely dissolve your 10X salts (plus limiting nutrient), and 10X carbon source in separate 2L beakers. When these components are dissolved, and adjusted to the proper volume (according to how many carboys you're making, e.g., 3L of 10X for 3 10L carboys), measure 1 L each of the 10X components in a 1 L glass graduated cylinder, and pour it into a glass 4L graduated cylinder. Pipet 10ml each of the 1000X vitamins

(thawed in advance) and the 1000X metals into the 4 L graduated cylinder. At this point you should have 2 liters and 20 mls in the 4 L cylinder. Top to 4 L with room temperature glass distilled water, and pour into the mixing carboy. The 4L cylinder can be unwieldy, so use one hand at the top to hold it steady, and the other hand to lift the bottom. You'll have to pour slowly, since the opening of the carboy is relatively small. Refill the cylinder 1.5 times, to reach a total of 10L in the mixing carboy. Turn on the large stirplate to low, so that the large stirbar is not out of control. Stir until thoroughly mixed, about 5 minutes, and proceed to filtering the media.

Filtering the Media

The media will be filtered into the cooled sterile carboy by manipulating a 1L bottle top filter, attached to a wide mouth 100ml bottle (or a larger one if no 100ml bottles are available). The filter plug will be removed from the usual vacuum attachment with sterile tweezers so that it can instead serve to funnel filtered media into the carboy. The vacuum will be attached to the air vent on the carboy. The entire process for making 10L will take ~30 minutes. Although the filters are nominally for only 1 L, this is the most consistent method we've found for sterilizing this volume of media.

Gather the following:

- 1 10 L sterile glass carboy (ambient temperature), labeled and sterilized with plastic piece for filter attachment foiled and clamped off on top, and a male quick disconnect outlet foiled and clamped on the bottom)
- 1 10 L non-sterile glass 'mixing' carboy, with a large stirbar, and a length of tubing sufficient to reach the bottle top filter that will be below it. The tubing should have a large adjustable clamp, to keep the filter cup from overflowing.
- 100ml wide mouth bottle (sterile), labeled with its corresponding carboy#, date, and your initials.
- 1L bottle top filter to fit the bottle (Corning 431174)
- Metal tweezers
- Ethanol for flaming
- Bunsen burner
- Ring stand with 3-prong clamp to hold bottle during filtration
- Large polypropylene tub to catch spills.

Ready, set, filter:

1. Claim an area to work, and set the sterile carboy and ringstand in the large tub. You want the tube with the filter attachment piece closest to the ringstand. Adjust the 3-prong clamp to a couple inches below the filter attachment piece. Set the whole tub aside.

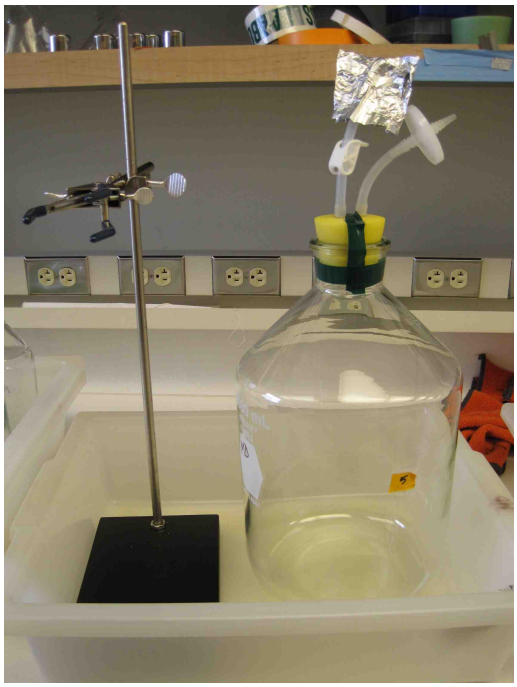


Figure 14: Getting ready to filter: The Carboy.

2. Light the Bunsen burner, and closely position the tweezers, uncapped EtOH, and 100ml bottle. Loosen the origami foil on the top media port of the carboy.

Figure 15: Setting up the filter.

3. Loosen the cap on the bottle. Open the top end of the filter bag, and remove the large sterile filter cover from the package, keeping it sterile. Place it on the bench, sterile side up. Put the sterile bottle cap, sterile side down, on the sterile filter cover.
4. Carefully remove the filter from the package keeping it sterile where it will screw onto the bottle, and where the vacuum usually attaches. Screw it securely onto the bottle.
5. Dip the tweezers in EtOH, shake off excess, and flame them. Use them to pull out the filter plug from where the vacuum usually attaches.
6. Attach the filter to the filter adapter that was autoclaved on the carboy's media port (on the cork assembly).
7. Clamp the bottle into the ring stand, exactly upright, being careful not to tug on the tubes coming from the carboy.

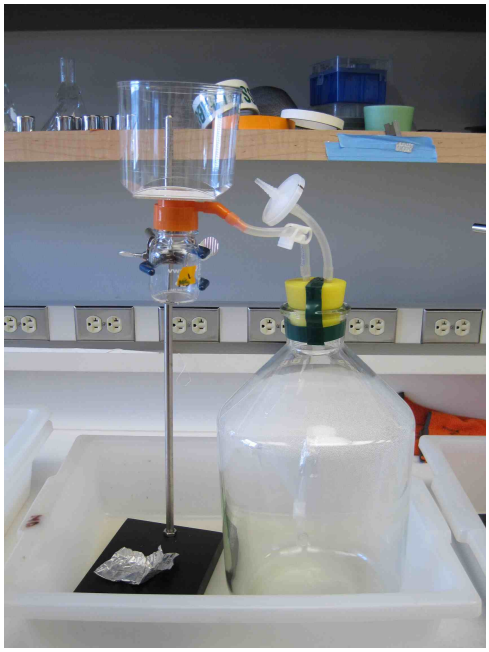
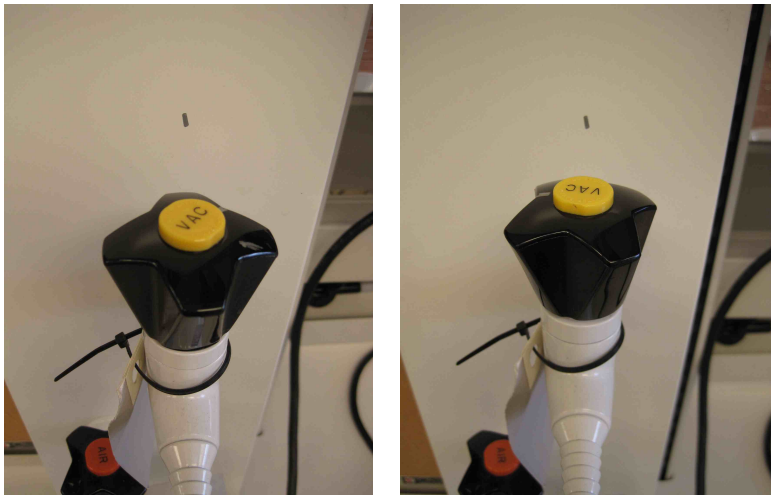


Figure 16: Final assembly of filter set up.

8. Move the tub containing the sterile carboy and ring stand onto the floor below the vacuum.
9. Attach the vacuum hose to the vent filter on the sterile carboy.
10. Route the clamped output tube from the mixing carboy into the top of the filter, and secure it to the top edge with tape.
11. Double check all filter connections (to bottle, to filter, and to carboy).



Vac OFF Vac ON

Figure 17: Turn Vacuum on LOW.

12. Turn on the vacuum (Only half a turn!) and unclamp the output hose from the mixing carboy. You can adjust the large clip to constrict the flow if necessary (2-4 clicks seems to work well). The filter should always be covered with media throughout this process.
13. Once the filter cup has started filling, unclamp the tube between the filter and the carboy (the media port). The 100ml bottle will fill first, and then overflow into the carboy. Make sure vacuum lines aren't clamped. I sometimes wedge another filter adapter in the clamp on the media port to keep it open.
14. Do not walk away! This system is fraught with potential for spillage, so monitor it closely. Stay nearby, and make sure the filter is not going dry or overflowing. Adjust the number of clicks on the large clip to get a good balance. You may notice that the vacuum pulls the cork downward into the carboy. There have been occasions when the vacuum is too high, and the cork gets completely sucked into the carboy. If that happens, you have to autoclave another carboy, refilter your media, and hack the sucked-in-cork to pieces with a pair of scissors. Don't turn the vacuum on more than a half turn of the knob!

Figure 18: During filtration, it's normal for the cork to get *slightly* sucked in.

15. When the mixing carboy gets close to the bottom, turn off the stir plate, and tilt the carboy toward the outlet tube until it is drained.
16. When the media is all filtered, unclamp the 100ml bottle, and tilt it toward its outlet, so that media runs into the carboy, and you have some headspace in the bottle.
17. Make sure there is no media in the tube running from the filter to the carboy, and clamp it tightly. Then turn off the vacuum, and slowly release the vacuum by removing the cork from the trap.
18. Detach the filter and bottle from the carboy's media port. Keeping sterility, cap the bottle. Then toss the filter and re-cover the filling tube with foil.
19. Set the carboy on the bench beside the peristaltic pump or on a shelf above the setup.
20. Place the 100mL bottle of media in the 30C incubator and watch it for a couple of days to see if any of your carboys may be contaminated.
21. Rinse the mixing carboy, any tubing that you used, and the 4L graduated cylinder with DI water, six times each, no soap or bleach, ever.

STARTING THE RUN

Making an overnight culture

Prepare an overnight culture derived from a single colony for each strain that you wish to test. Make sure as well that you have a 1ml glycerol stock of this colony for later use. This overnight culture should ideally be grown in the chemostat media that you will eventually use in your experiment; however, it may be sufficient to grow in YPD or another nutrient rich media, just so long as you spin down and wash with water before inoculating.

Setting up the ministats

Arrange the culture tubes in the heating block

Remove all of the ministats from the racks that were used during autoclaving and place the ministats into the heating block.

Figure 19: Setting each row of four tubes into the heating block.

Clipping the bundled-air and efflux lines is a useful way of keeping things organized while you connect the media lines.

Hooking up the air lines

Take the foil off the filters and hook them up to the 4-way manifold ports used to split air flow. Since the filters act as a barrier against bacteria and other contaminants this does not have to be performed in any specific manner and the filters can be removed and placed back into the manifold as desired. Do not run the air until the pump tubing is locked in place on the pump, since air may bubble back through to the media carboy, creating a risk of back-contamination.

Hooking up the media lines and filling the chambers

The media lines route media from the sterile carboy to the culture chambers by way of a peristaltic pump. When first connecting the media line to the sterile carboy filled with media, simply loosen the foil on the carboy and media line male/female quick connects, quickly remove the foil, and connect the two ends. Next unclip the plastic clip on the base of the carboy and media should begin to fill the individual lines used to provide media to the culture chambers. Massage the tubing to remove air

bubbles. It may help to elevate the carboy. Next set the pump tubing into the cassettes for each ministat, so that you know which tube goes to which ministat and they go in a logical order. Clip in the cassettes, turn the pump up to maximum speed, and watch to see that the chambers begin to fill with media. Even at max speed (90rpm) this still takes about 20 min. Continue with assembling the array; however, turn off the pump once media reaches the efflux needles. NOTE: it is important that you hook up the efflux lines before the media begins being expelled from the culture chamber so do the next step quickly. You have PLENTY of time, but you can't take a break during this portion of assembly. Failure to do this will result in media clogging up the air filters on the efflux lines, which may cause the cork on the culture chamber to come loose and compromise the sterility of your culture chambers.

Figure 20: Threading the lines through the peristaltic pump.

Connecting the effluent lines

The efflux lines connect to the white-topped Air-tite needles. Turn on the air pumps at this point such that air is blowing through the system. This will create positive pressure, which will act to keep contaminants from traveling up the efflux line and into the culture chamber. Place the effluent line into a #2 black rubber cork with two holes and set it in the collection chamber, which is in this case a 100ml bottle. In order to prevent bubbling of the effluent back though the air release vent in the cork, push a 200 ul pipette tip into the bottom of the cork such that it extends from the hole by ~1 cm.

Figure 21: Effluent vessels with tubing.

Inoculating the chemostats

NOTE: USE OF SAFETY GOGGLES IS ADVISED AT ALL STEPS THAT INVOLVE MANIPULATION OF EXPOSED NEEDLES. Needles and syringes are placed in the sharps container immediately after use.

The ministats are typically inoculated with 1:200 volumes of overnight culture derived from a single colony. With a 20ml culture volume this would mean that you would inoculate with 100ul overnight culture. To do this take a 1ml syringe with an extra thin (22Gx5in) needle and fill it completely. Invert the syringe and tap to cause any air bubbles to go to the top. Next depress the plunger until all air is out. I usually spray into a Kimwipe soaked in 95% ethanol. Next spray the tops of the corks of all ministats you wish to inoculate with 95% ethanol. To inoculate, I stab through the cork such that the inoculating needle dangles in the air away from the 3 needles already in the chamber and making sure I'm not spraying the inoculum onto the wall of the ministat chamber. I usually inoculate 4 ministats with a single syringe (assuming the strain is the same and independence of the inoculating populations is not desired, of course) and then move on to use another syringe/needle. After inoculating make sure the pumps are turned off for 30hrs so that the population can use up all of the available limiting nutrient and grow to saturation.

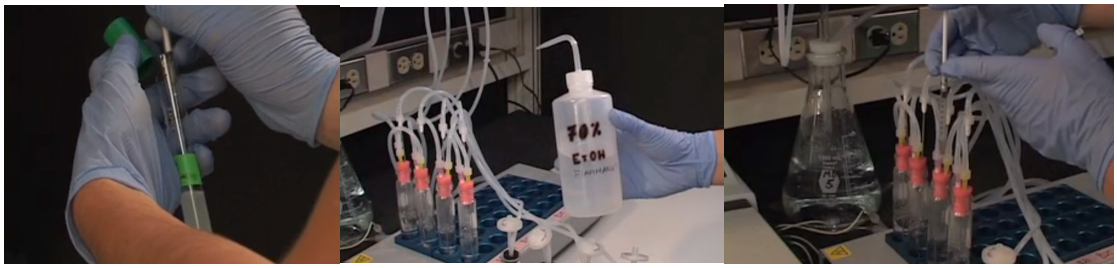


Figure 22: Inoculating the ministat.

Inoculate using 22G needles and an overnight culture grown in the appropriate chemostat media. Spray the tops of the corks liberally with 70% ethanol. Then inoculate the cultures with 100ul culture.

SAMPLING THE MINISTATS

Setting the culture volume

During your first sampling you will set the culture volume. After 30 hours have passed it is time to turn the media pumps back on. First, turn off the air for a moment to check the culture volume.

1. If the media hasn't started to exit through the effluent line, let the media continue filling and move on to the next tube. Check to be sure there is no problem with the media line.
2. With the air off, spray the top of the cork assembly with 70% ethanol. Hold the cork in place with one hand and with the other hand gently adjust the media-sampling needle up or down. Then turn the air back on for several minutes to let the media level stabilize before re-checking. With the air off again, see where the media level is relative to the 20ml mark on the tube, and adjust the needle height.
3. Repeat this cycle until you have reached the desired 20ml culture mark. If you over shoot slightly increase the height of the needle, let the tube refill, and re-do step 2.
4. Once the ideal level has been reached you may begin your first 2-hour sampling on ice as described below.

Sampling regimen

The chemostats ideally should be sampled every day, particularly when collecting sweep data. Try to be as consistent as possible about your technique, and write down anything that you change. A daily sampling regimen might include making a glycerol stock, looking at the culture under the microscope, sampling for RNA and DNA, measuring

effluent volume, optical density, cytometer cell count, viable cell counts on YPD and minimal plates, and sweep marker frequency on selective plates. Depending on the number of chemostats and plates, the whole process, including setup and counting the plates from earlier in the run, takes anywhere from an hour to as much as 3-4 hours. When you need to change the media, try to do it after sampling to avoid any chance of a perturbation. To take a sample, arrange as many clean 50 ml tubes as you have cultures running – place them in racks and pack them in ice. The cultures should provide 3.4 mls/hr of culture and to date I've been doing most of my sampling for exactly 2 hours. While the tube is filling, pour the effluent into a graduated cylinder and write down the volume. You'll use this measurement to calculate the dilution rate later. After 120 minutes, you should have enough culture for typical measurements. A shorter sampling time may be adequate for some purposes.

Figure 23: Taking a sample during a run.

Data collection

It is extremely important that your data be collected in an organized and meaningful way. This will involve a Layout sheet, and an Excel spreadsheet (Dunham Lab version is available upon request). This spreadsheet should be archived in the chemostat database on the server. Furthermore you should backup your data regularly in case disaster strikes.

At the beginning of your experiment, start with a worksheet that summarizes your set up. Include the ministat's position in the heat block (B#), which strain was inoculated into which location, the date and time of inoculation, the nutrient limitation, and the goal of the experiment.

Sample tracking

The combination of ministat number (B#) and date should give a unique identifier for sample tracking. Glycerol stocks added to the main strain collection will also have this unique collection number. Do not institute any sort of shorthand or alternate naming scheme, or if you do, keep track of it in the main chemostat index worksheet. Once you've run a few experiments, you can get awfully confused about what's what. Keep track of where in the freezer you put all the daily samples and the harvests, and record this information in your notebook, spreadsheet, and lab freezer stock. There is a freezer rack specifically for current chemostat/ministat samples.

Sampling

Preparing to sample

Set up all tubes and ingredients necessary before sampling the culture. At a minimum you will want to assay culture density and take a glycerol stock to keep a frozen record of your experiments' progress. You may also wish to label all the required tubes for the density measurements and serial dilutions and fill them with the right amount of diluent. Label all the plates. Turn on all the equipment and check that everything is properly calibrated.

Glycerol stock

If you are freezing aliquots of the culture, pipet 1 ml culture into 0.5 ml 50% sterile glycerol in a clearly labeled cryovial. Invert a few times to mix well and put the sample at -80C. There is a rack for current chemostat samples (Glycerol, RNA, DNA) in the freezer, specifically for these samples.

Spectrophotometer

Dilute the sample appropriately in water or media for the spectrophotometer reading. Use the same dilution for the entire chemostat run unless you leave the linear range. A 1/4 dilution usually allows you to start a little on the low end of the linear range, which is about 0.1-0.5. 0.5 ml culture into 1.5 ml water in a tube works well. Vortex the tube, pour the contents into a cuvette, and tap out any bubbles. Place the cuvette into the spec, so that it is oriented properly

with respect to the light path (arrow indicates direction light comes from). Read the optical density at 600 nm, and record the measurements in your notebook and on the appropriate spreadsheet.

Sonicator

Pour 0.5 ml culture into a 1.5 ml eppendorf tube. Wear safety glasses and ear covers when you use the sonicator. Check the tip occasionally for cracks and other signs of wear. The tip needs to be replaced every once in a while. On our misonix S4000, we use program #1, which consists of 10, 1 second bursts at Amplitude=5, with a 1 second rest in between bursts. This seems to separate cells nicely. As cells become flocculent, they may require more aggressive sonication.

Turn the sonicator on, via the power switch on the back left of the machine. Use the touchscreen to select YES for microtip, and follow the prompts to Run a program. Press 1 to select program #1.

To begin, wipe the tip of the sonicator with a Kimwipe saturated in ethanol. Completely immerse the narrowest part of the tip in the tube of culture, approaching but not touching the bottom of the tube. You'll get to know the usual behavior of the sonicator after using it a few times. If it deviates from the usual behavior, let someone know. Wipe the tip with the ethanol Kimwipe. Keep repeating until you've finished all your samples. Clean the tip thoroughly with ethanol and turn the machine off when you are finished.

Cytometer

Use of the cytometer is beyond the scope of this manual. See someone in the lab for detailed training before use.

Plating for viable counts

Vortex your sonicated sample again. Use it to make appropriate serial dilutions to plate for viable cell counts. Typically, I plate 100 µl of a 10^{-4} dilution, made by 4 dilutions of 100 µl culture into 900 µl water or 2 dilutions of 10 µl culture into 990 µl water. Pay attention to your pipetting technique to ensure accurate dilutions. Pipet 100 µl of the final dilution onto a labeled plate and spread evenly by your favorite method. If your colony counts start getting above ~300/plate, you should plate less. I shoot for 100-300 colonies/plate. See Counting Colonies for more info.

Plating for drug resistance

If you are monitoring drug resistances, plate the appropriate volumes of culture on them as well. 250 µl is about the limit you can comfortably plate without puddles forming. If you need to plate more than this, spin down the volume and resuspend the cell pellet in a smaller volume. It is most accurate if you make 1 tube for each plate and plate all of it, rather than spinning a large sample and trying to resuspend it in exactly the correct volume to split up. Let the plates dry on the bench before inverting them and transferring to the incubator.

Sampling for DNA

If I'm sampling for DNA and RNA, I generally do all of the above first, then go back for the other samples. Taking a sample for RNA probably perturbs the culture a bit, so it should be the last thing you do. For DNA sampling, I use a modified Hoffman and Winston yeast DNA prep (available on the Dunham lab website). Passively collect 10 ml of cells from the effluent tube, spin them down, and resuspend in 0.5 ml of the sorbitol buffer. Transfer to a 1.5 ml eppendorf. Freeze –80C. Several members of the lab have also had success freezing cell pellets directly.

Sorbitol Solution

45 ml	2 M Sorbitol
10 ml	1 M Tris pH 8
20 ml	0.5 M EDTA
25 ml	Water

Sampling for RNA

One home-spotted RNA microarray requires ~50 µg of total RNA. ~5 ml culture is adequate to ensure this yield. Much less culture is required for a typical Agilent array since an amplification step is performed during probe labeling; however, performing RNA preps on tiny quantities of material can be difficult. In general, remove as little culture as possible to get the required yield in a reproducible fashion. The higher the volume and frequency of sampling, the longer and more severe the perturbation to steady state.

First, label a 15 ml Falcon tube for the filtrate and a 2 ml eppendorf tube for the filter. Using a ring stand, set up the small filter apparatus

with the stopper assembly.

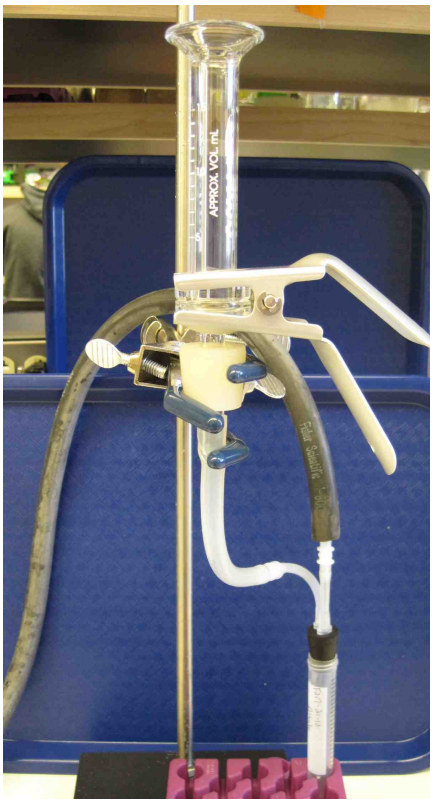


Figure 24: Small filtering apparatus.

The stopper assembly is just 2 connector fittings jammed into the top of a stopper. One of the fittings is connected by a piece of tubing to the filter and the other goes to the vacuum. Fit the stopper into the 15 ml Falcon tube for the filtrate. Clamp down a 25 mm filter in the filter apparatus. Hook the vacuum tubing to the other stopper port. Rinse the apparatus with DI water before using to remove any trace of previous samples. Even a tiny bit of, say, sulfur-limited media contains a huge amount of phosphate relative to the filtrate from a phosphate-limited culture.

You also need a bucket of liquid nitrogen. Since RNA expression changes quickly (the stress response sets in on the order of minutes), you cannot passively sample. You must instead quickly remove culture directly from the chemostat. For the ministats, this can be performed either by removing the cork assembly and pipetting from the tube, or by attaching a syringe to the air port. Remove the appropriate volume of culture and apply to the filter assembly. Let it vacuum through. Remove

the clamp and glass funnel. Disconnect the vacuum. The order is important to prevent cells from sticking to the glass funnel and to allow all the filtrate to get sucked into the collection tube. Without disturbing the film of cells, remove the filter with tweezers. Roll it over on itself and insert into the 2 ml eppendorf tube. Close the tube and put it in the liquid nitrogen. Cap the filtrate tube and rinse the filter apparatus with DI water. Repeat the procedure for the remaining chemostats. When you've finished collecting all your samples, transfer the frozen tubes to –80C, and move the filtrate tubes to –20C.

Cleanup

Make sure to clean up after yourself once you finish sampling. You want to have all the plating and measurements done shortly after you take the sample, so you might leave a bit of a mess in your wake. Clean up while your plates dry. Where it's easy to clean up as you go, do it (i.e., sonicator).

Counting colonies

The different plates will need to grow for different amounts of time. YPD plates need to be left for two days and minimal/YNB plates for 3. Canavanine and 5-FU are good at 4, and alpha-aminoadipate requires 7 days. The most important thing is to **be consistent** about which day you count the colonies. If you deviate, make sure to write it down. Over the course of an evolution, you may see changes in colony size that require changes in the incubation time. Use the touch sensitive colony counter with a pen to quickly count your plates. Sometimes overgrowing the plates can reveal interesting colony morphologies. Record any observations about atypical colony size or morphology.

Typical daily sampling

1. Pre-label pre-autoclaved 50ml glass tubes, a 2ml cryovial, a 2ml eppendorf tube, and 4 1.5 ml eppendorf tubes (sonicate, 2:20, 2-2:20, 4:400.)
2. Add 0.5ml sterile 50% glycerol into the cryovial, and 1.5ml water into the 2ml eppendorf tube. Distribute appropriate amounts of water for serial dilutions in 1.5ml eppendorf tubes (details in #9).
3. Note time, and transfer sampling cork to the 50ml glass sampling tube (on ice).
4. While tube is filling (you need 1.5ml minimum, although we traditionally gather ~6.8 mls over the course of 2 hours), measure effluent volume using an appropriate glass graduated cylinder. Record Veff directly into a spreadsheet, then calculate D based on Veff, and any sample volumes taken that should be added in.
5. Adjust pumps if D is not between 0.16 and 0.18. Make sure effluent is empty and time noted if pump adjustment is made.
6. Cap the sampling tube and put the sampling line back into the 100ml collection bottle. Note the sample volume taken, so that it can be included in the next D calculation.
7. Vortex sample, then pipet 1ml into the cryovial and 0.5 ml into 2 prelabeled eppendorf tubes (one 2ml for OD600 dilution, and one 1.5ml for sonication and subsequent serial dilution).
8. Sonicate, then make dilutions for cell counting by hemacytometer and for plating (10^{-4} dilution = 2:20 x 2:20 x 4:400) all in water, vortexing extensively at each step.
9. Take remaining undiluted sample along with 2ml tubes over to the Spectrophotometer area. Vortexing well before each measurement, check the OD of 1/4 dilutions, recording all results into the same spreadsheet as before. Measure the undiluted sample in the Klett. Be sure to rinse the cuvettes and klett tubes, so they are clean for next time. Do not leave the Klett on overnight.
10. Plate 100ul of 10^{-4} dilutions on YPD and on minimal media (D and B plates, respectively), using a similar number of sterile glass beads to spread the cells on each plate. Count YPD plates after 2 days at 30C, and minimal plates after 3 days at 30C. Use the colony counter. Include any drug or drop out plates depending to your experimental design.
11. Vortex and load 8-10ul of first 2:20 dilution into hemacytometer. Count with a clicker, calculate, and record cells/mL. Alternatively, count cells with cytometer.

Sample Analysis During a Run

Once you've got some sampling data, you'll want to analyze it. At the beginning of a run, it's important to calculate the dilution rate to make sure the pumps are behaving and the settings are correct. The dilution rate is a simple relation of the effluent volume, length of time (in hours) effluent collected, and chemostat volume:

D = effluent volume/(time * chemostat volume)

The dilution rate is in units of chemostat volumes per hour.

Since your chemostats will all be running at different rates, either by experimental variation or by design, generations is often a more useful metric than time for graphing things and talking about run length. The chemostat literature talks about two different types of generations: a culture generation, i.e. one volume replacement of the chemostat; and the cell generation, i.e. the doubling the cells must undergo to keep up with the dilution rate. Since some cells get diluted out before they can divide, the culture as a whole must actually double faster than the chemostat volume replacement rate. The spreadsheet will calculate the cell generations elapsed since the last sampling:

cell generations elapsed = (time * D)/ln2 = 1.44 * time * D

You can cumulatively add up the generations for every sampling point to get a column for making scatter plots.

For measuring drug resistance frequency, add up the total number of colonies on all accurately counted plates and divide by the total volume plated to get resistant cells/ml. Then divide that number by the cytometer-counted cells/ml to get a frequency. The cytometer count is much more well-measured than the viable plate counts, so we use it even though it overestimates the viability.

Media Replacement

It’s important to calculate how much media you’ll need at the beginning of the experiment. If your carboy is running very low, do the following: when you are down to the last bit of media, put a roll of lab tape under the back of the carboy to pool the remaining media by the port. You should be able to get almost all the remaining media this way. If you have to connect another carboy, hopefully you included an alternate sterile port in your tubing network. If you didn’t, you’ll be risking contamination when you disconnect the current line and reconnect

it to a fresh carboy. Good luck.

If you are feeding your chemostats off two media vessels connected by a Y connector, you can drain all the media into one carboy by raising one carboy higher than the other. The lower carboy will fill up slowly. Once you've got all you can get out of the elevated carboy, clamp it off and disconnect it. Connect the new carboy, preferably by way of the extra sterile connector you included. With either technique, watch the supply closely. You don't want to forget about it and run out of media. Once you've almost run out of media, or if you are leaving and the media will run out before you get back, replace the carboy. Any time you alter the media supply, write down the time in your notebook/spreadsheet. You may also want to take a sample of the media for analysis. See the Filtrate section for advice on nutrient assays.

HARVEST

At the end of the chemostat run, you can harvest the cells to make RNA, DNA, media filtrate, and yield measurements. Make sure you've already done all the sampling you want before you harvest.

What is 'Steady State'

The cell density should be pretty stable before you call it steady state. Lab strains usually reach steady state 3-4 days after the pumps are turned on.

There are a few things to consider when deciding if the culture has hit steady state. The first is time. You really don't want them to go more than ~25 generations, because they might start to evolve. If on the third day after turning the pumps on they vary less than 5% versus the previous day's measurements, go ahead and harvest. If the numbers are still fluctuating more than that, wait until the fourth day. If on the fourth day, their cell density varies 10% or less, go ahead and harvest.

There are other things that should be taken into consideration when deciding if your chemostat has stabilized. You might give them a little leeway if there is flocculation, since their measurements are likely to be affected. Also, if the flow rate has fluctuated, you can expect variation in cell density as well. It's not ideal, but if you have to adjust the pump the day before you harvest, it should have stabilized 24 hours after the perturbation. Finally, if you're working with wild rather than lab strains,

you may have more fluctuations, but much more than 10% and you have to be cautious about your assumptions.

Taking multiple measures of cell density insures that you aren't misled by an instrument having a bad day. That's why we take at least 2 measurements to represent culture density. Usually this includes measuring OD600 and cell counting either via hemacytometer, colony forming units, or by using a cytometer. You may find that the instruments don't always completely agree. One instrument may say the density has gone up, while another says it has gone down. This could be noise, or it could be true since the devices measure different aspects of cell density. In general, if the numbers are only slightly higher or lower than the previous day's measurements, the culture has probably reached steady state.

How long should evolutions go?

Evolutions can go as long as you want, assuming no one else needs the ministats, and you have enough media. Practicalities and experimental goals are generally the deciding factor. Once cells evolve clumping that can no longer be broken up by sonication, we typically pull the plug.

Setup for harvesting

Final harvest preparation differs depending on what types of downstream measurements you have planned. If you are sampling for RNA, you have the same concerns as described earlier in the RNA sampling section. It's important to make sure everything is completely set up before you start. If you are filtering a small amount of cells (5-10ml), you may want to use the small filter apparatus, discussed earlier. If you are taking a dry weight measurement, and you are taking most of the ministat volume, you will want to use the large apparatus, described below, but skip the Liquid Nitrogen part. The cells will be dried overnight in a 37C incubator instead.

Using a pen that will stand up to liquid nitrogen and the -80, label two 15 ml Falcon tubes, 1 for RNA preps and 1 for filtrate. Label extra tubes for media samples from each carboy. Get a bucket of liquid nitrogen, the 2 vacuum filter flasks, a pipetboy, and pipets, filters, and the large vacuum filtration kit (Figure 25). Know where the backup filtering apparatus is in case you break something.

If you are doing dry weight measurements, weigh a 0.45 micron filter for each. Write down the weight and fermenter number on the little piece of paper that separates the filters. Make a foil clamshell for each filter and paper pair so you can easily carry them around and keep them separate from the others. We typically have gotten best results for dry weight using 50 ml of culture on a large filter. We have not yet explored how well smaller volumes from the ministats work.

To set up the filter apparatus, first rinse all parts with DI water. Lubricate the side-arms and the ends of the vacuum tubing with some water and attach them. One will attach directly to the vacuum line, and the other will connect to the first flask. Put the stopper with the filter support in the top of the second flask. Make sure the metal support is seated in the glass part correctly. The mesh should be level with the glass rim, or slightly below. If it's higher it will leak. Have the clamp and the funnel nearby.

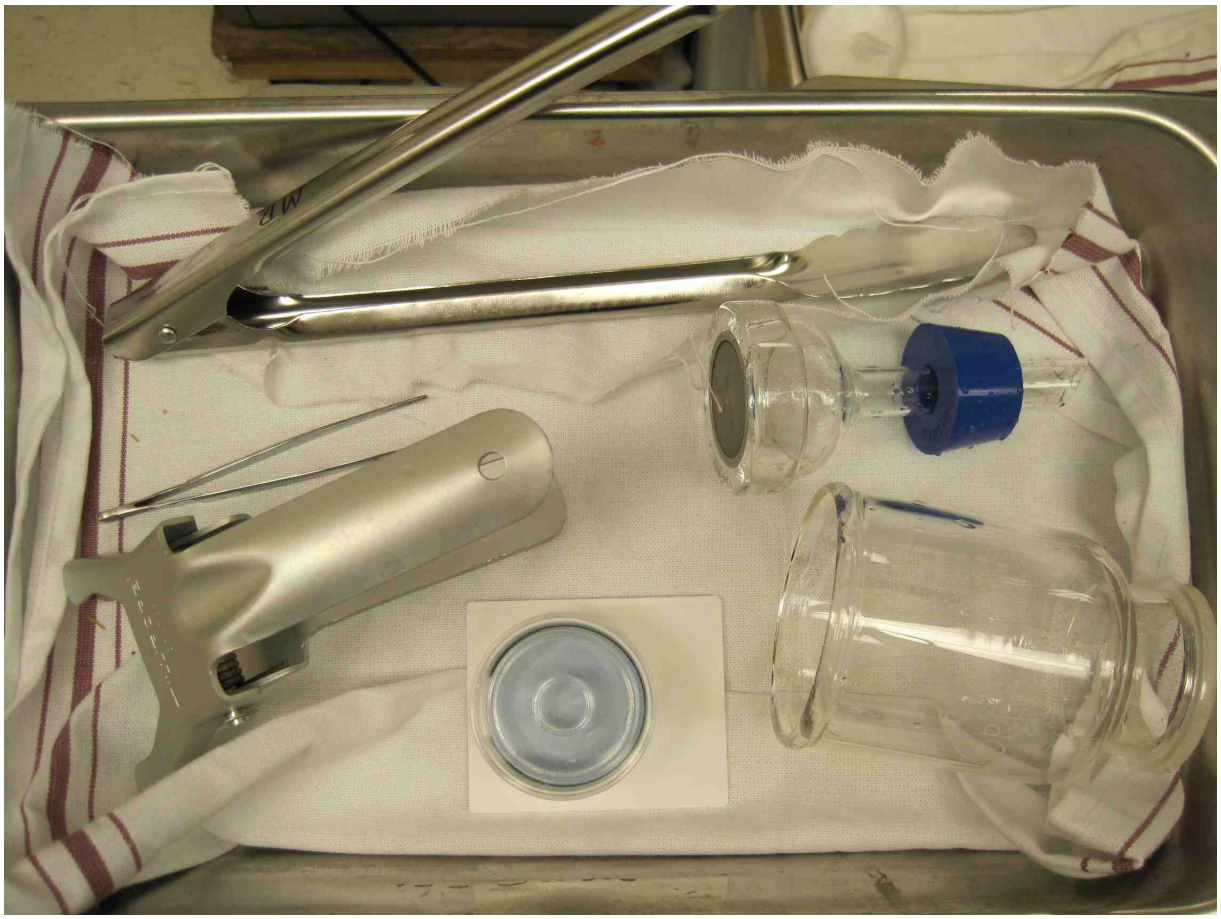


Figure 25: Large Vac kit.

All the components should be returned to the pan when you're finished.

Harvesting

The active harvesting process should take about 5 minutes. Cleanup and preparation will take longer. First, center a 0.45 micron filter on the filter support. Place the funnel on top and carefully clamp it all together. Start the vacuum. Listen for any whistling noises that may indicate a leak in the seal. If you do get whistling, make sure that the filter is centered and free of wrinkles. Some fraction of the filters have cracks or holes that will interfere with the harvest. Replace the filter with a new one if this seems to be the case.

Figure 26: The 'Harvest Cart' complete with the large filter apparatus.

Remove the tops from the 15 ml tube labeled for RNA. Put the open tube in the bucket of liquid nitrogen. Find the correct filter for the dry weight harvest and place it near the filtering apparatus.

Record the time. Being careful of the sharps, remove the cork/needle assembly off the chemostat, and set it aside in a safe place. Pipet the desired volume of culture out of the chemostat, and into the funnel of the filter apparatus. Watch to make sure it is filtering properly and that no cells are making it into the flask. You can refilter if you have this problem, but try to avoid it.

Once the culture has completely filtered through, remove the clamp and then the funnel. Break the vacuum by removing the cork from the first flask. This order is important to keep cells from sticking to the funnel. Use forceps or a spatula to lift the edge of the filter, avoiding the

cells in the center. Carefully roll up or fold the filter. With tongs, Dump out the liquid nitrogen in the 15 ml tube, pop the rolled up filter inside, loosely cap it, and dunk it back into the liquid nitrogen. Leave it there until you transfer it to –80C. Take the flask and pour filtrate through the sidearm into the room temperature 15 ml tube. Cap and set aside the filtrate. It will be frozen at –20C, but you can collect a few more samples first.

After sampling for RNA, I usually put the cork assembly back in the tube until I've sampled for all the experimental endpoints I want. Once you've taken the RNA sample, you may find it easiest to dump the culture into a collection tube on ice for handling

For dry weight, use the weighed yield filter, and pipet exactly the desired volume of culture. Add this to the funnel. Be particularly careful this time to unclamp, remove the funnel, and release the vacuum in that order to make sure you collect all of the cells on the filter. Also, be very careful not to scrape any cells off the filter while handling it. Return the filter to its foil clamshell and let it dry in a 50C oven. Make sure the cell side of the filter is not sticking to the foil. After the filter is completely dry, weight it and subtract off the initial filter weight to get the dry weight per volume of culture.

You can also use some culture for a DNA prep if desired. Spin the cells down and resuspend them in 0.5 ml sorbitol solution (recipe is in the sampling section). Transfer to a 1.5 ml eppendorf tube. Freeze at -80C.

Wash out the filter apparatus with DI water, and return all parts to where you found them for next time. Once you are finished with all the harvests, you can clean up and make final observations about the look and smell of the cultures. Make sure you move all the RNA harvests from the liquid nitrogen to the -80.

Examine the culture as you harvest. Does it have chunks? Is there wall growth? Write down any observations. Smell the culture. Compare it with any other cultures you harvested. The different limitations all have very distinctive smells. Phosphate and sulfur limitations smell very similar, with a fruity, sweet, sharp smell that has some almond or rose in it. Glucose limitations smell awful, like sweatsocks, and even worse when you have other additives. Some other scents that may be present are bready and acrid. Try to be as descriptive as possible and ask others for their opinions. Write down everything. While cleaning the chemostat with DI water, note whether you have any obvious wall growth, and scrub the walls with a wet paper towel or soft bottle-brush.

Taking down an experiment and cleanup.

Don't underestimate the time you'll need to clean up after your experiment. Chemostats are sensitive instruments, and since we never use soap or bleach, extensive flushing with clean water, and minor scrubbing with paper towels is the only way to go, and it takes time.

- Allow a full day for taking the ministats down and washing them if you are running more than just a few.
- All culture chambers, effluent sampling bottles, tubing, and carboys must be flushed with DI water, IMMEDIATELY following the end of your run. Otherwise, contaminants will grow in and clog them.

ALL MINISTAT COMPONENTS MUST BE COMPLETELY CLEANED DIRECTLY AFTER USE!

1. Get a white rectangular autoclave bin to store parts as you go. Also grab a 1L plastic beaker to store cork assembly components and enough purple racks to hold the glass culture tubes as you take them down.
2. Turn off the media and air pumps and use metal clamps to clamp off the media lines, which lead to the culture chambers between the pump and the chamber.
3. Remove all tubing from the ministat culture tube and gather each type of tubing individually for cleaning.
4. Place each of the cork assemblies into a 1L plastic beaker and set this aside in a safe place for cleaning immediately after processing the tubing.

Cleaning the Ministat Culture tubes.

1. Take the ministat culture chambers and wash them thoroughly with excessive ddH2O.
2. Wad up 3 Kimwipes and use a set of long tweezers to massage the inside of the tube with the Kimwipes.
3. Re-rinse the tubes with excessive DI water and let sit upside-down in their racks for later use.

Clean up the carboy.

Rinse the carboy and all parts of the cork assembly with excessive DI water 3x. Be sure to remove the quick connect from the carboy and run water through the spout at the base of the carboy or else water won't properly flush those lines.

Figure 27: Using tweezers and 3 folded Kimwipes to clean the culture tubes.

Sample Processing

That's it. You now have a lot of data and a freezer full of glycerol stocks, cell samples for RNA and DNA, and filtrates. Next you will want to process them. We've posted several useful additional protocols at *http://dunham.gs.washington.edu*