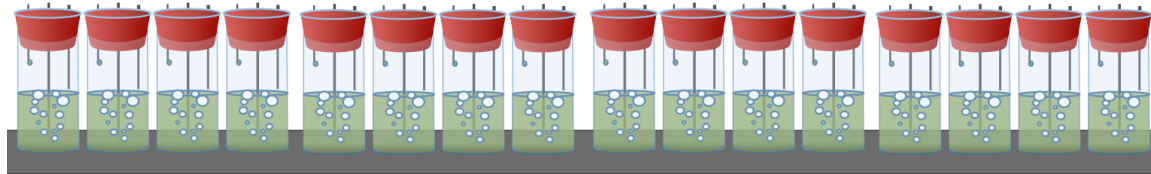


Dunham Lab Ministat Manual

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Presented here is everything you need to construct and run an array of 32 20 ml 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 protocols used in this manual are taken from the ministat training video published in Miller AW, Befort C, Mitchell E, Dunham MJ. *J Vis Exp.* 2013 (72) and in *Budding Yeast: A Laboratory Manual* (ed. Boone C, Andrews B, Fields S, Davis T. 2016 Cold Spring Harbor Laboratory Press). Images 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!

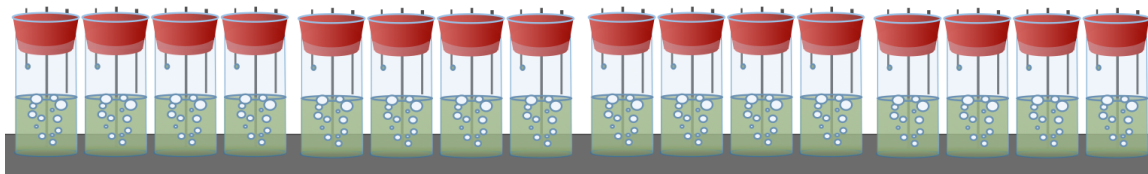


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About the Ministats

Ministats are chemostats with a 20 ml 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 **Figure 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.

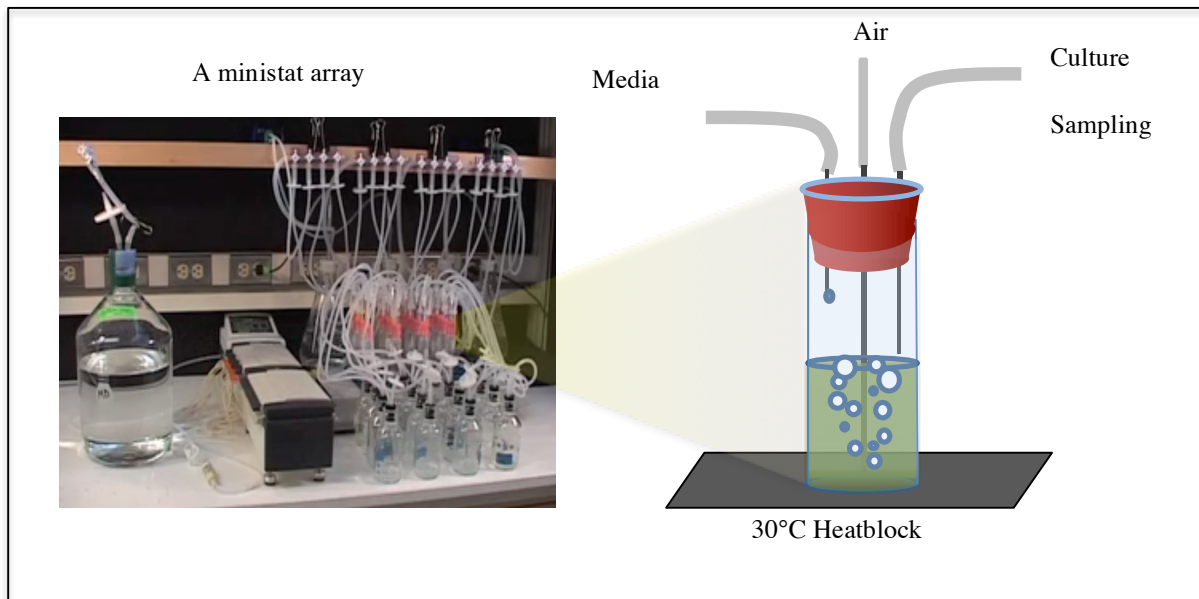


Figure 1: An array of 16 ministats running.

Closeup of a ministat chamber. The ministat chamber is made up of a 50 ml 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

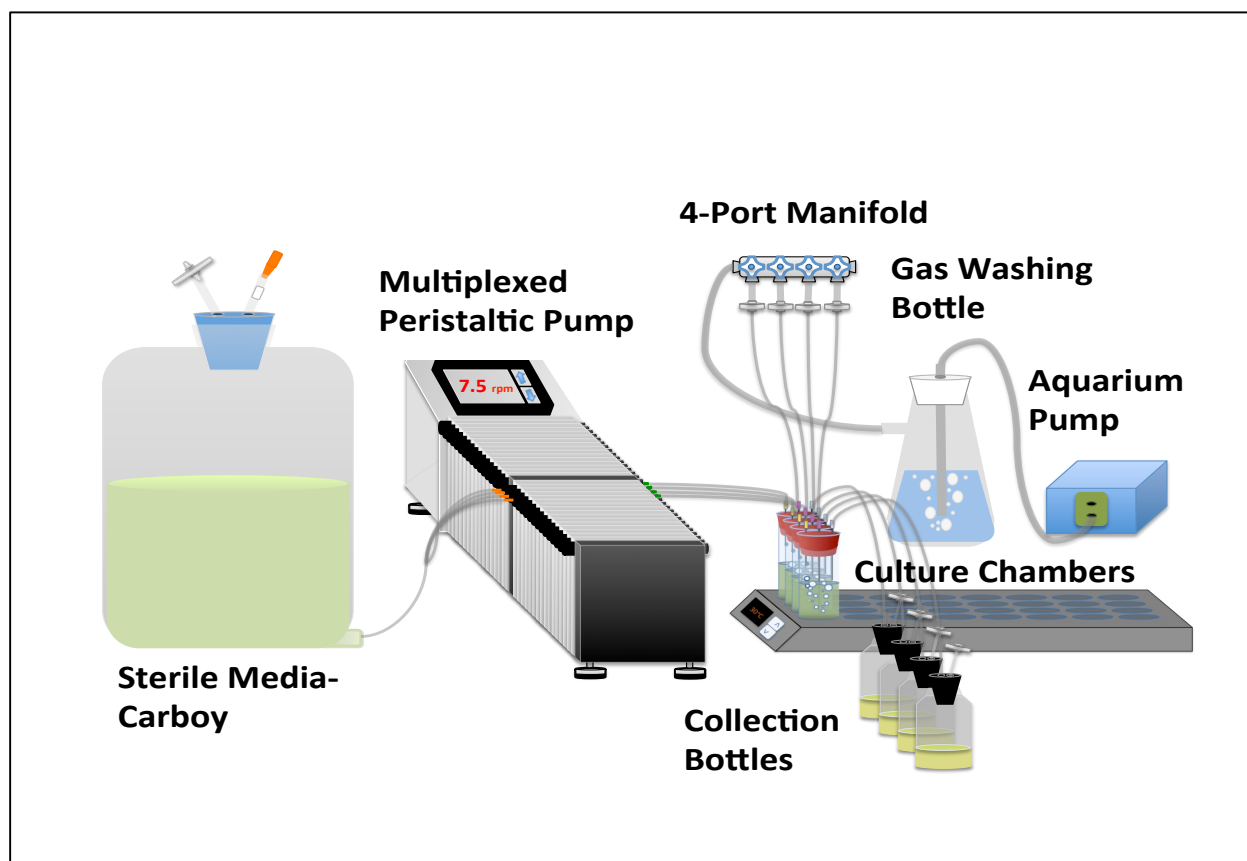


Figure 2: Key components of ministats.

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 $0.45\ \mu\text{m}$ 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 effluent collection chambers. Temperature is regulated by an external heat source. We use a heat-block; however, a water-bath, incubator, or constant temperature room would 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 50 ml capacity allows the experimenter to choose from a variety of culture volumes. We currently use a 20 ml volume, as it allows plenty of headspace to minimize occupation by yeast in the media line, while still offering large enough sample volumes for genetic analysis and reasonable sampling times. However, other vessel geometries, such as squat bottles, may be more appropriate for more clumping-prone organisms such as fission yeast. A stir bar may also be required for such scenarios.

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 ddH₂O 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 20 ml working volume is. 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 21 ml of ddH₂O into each of the tubes and carefully mark the meniscus on the side with a fine tip marker. Remove 1 ml and mark again, and repeat for the final 19 ml mark. Use a glass scorer to make the marks permanent. The ink will wear off. Once the tubes are etched, the vessels can be reused many times without having to remark them.

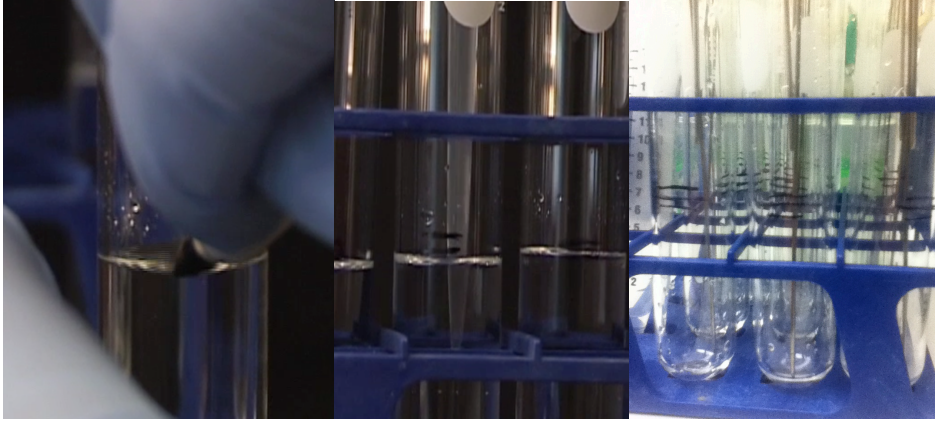


Figure 3: Marking the tubes.

Preparing the cork assemblies for the ministat culture tubes

NOTE: USE OF SAFETY GOGGLES IS ADVISED AT ALL STEPS THAT INVOLVE MANIPULATION OF EXPOSED NEEDLES.

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. 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 in **Fig 4**. The air needle is the longest at 6 in, then the effluent needle is 5 in, and the media needle is 1.5 in. Ideally, they will be evenly spaced and the needles will hang more or less parallel to the glass culture chamber wall. Make sure the media needle isn't pointing directly down the effluent needle so that media isn't immediately removed before mixing with the culture. 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, it's time to connect everything with silicone tubing.



Figure 4: Making the cork assembly.

Assembly of Ministats

Assembling in the block ensures that the lines are of sufficient length to connect the culture chamber to each component: air, effluent, and media. While the blocks we use can hold 36 vessels, we always leave a blank row between each row of vessels, so we can still see what's going on in there. The extra space facilitates handling when you have to get in there for something (troubleshooting, harvest, mixing). Once assembled, the open ends of each line are foiled and the whole array fits into a tub for autoclaving. See section on autoclaving for details.

NOTE: This involves a lot of connectors and tubing. Make straight cuts, not diagonal cuts on the tubing.

Prepping the Air Line

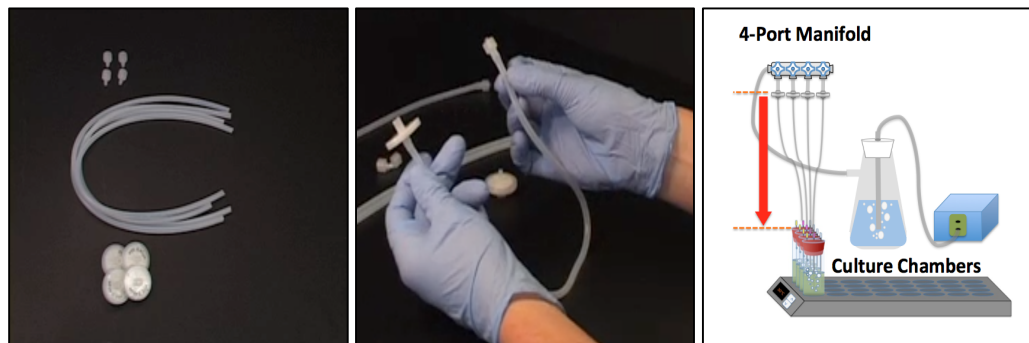


Figure 5: Making the air line.

The air-line is simply a piece of 3/32 in. silicon tubing with a male luer-lock on one end and a 0.45 μm 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 bubbler flask to 4 culture chambers.

The air supply apparatus is located next to the heat block, and does not require sterilization. It is made up of a section of 1/4 in. tubing which connects to the manifold from the sidearm of a 1 L bubbler flask. The one hole stopper in the flask holds a “customized” disposable serological pipet, which directs air from the air pump into a reservoir of sterile water. This step greatly reduces the level of evaporation in the ministats. The bubbler flasks should regularly be emptied, rinsed, and filled with sterile ddH₂O.

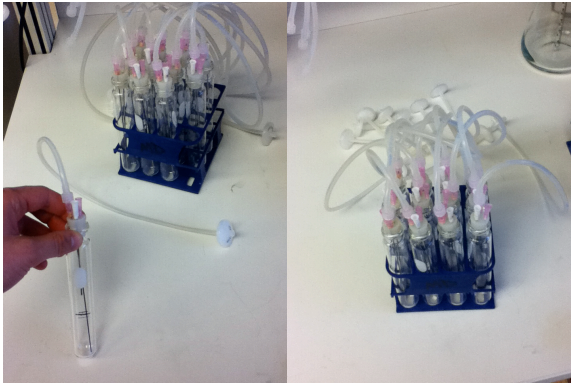


Figure 6: Attaching the air lines to the tops of the culture tubes.

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 foil. The foil will keep the filters from getting wet in the steam autoclave. Do this for all of the culture chambers.



Figure 7: Preparing the air lines for the autoclave.

Prepping the Effluent Line

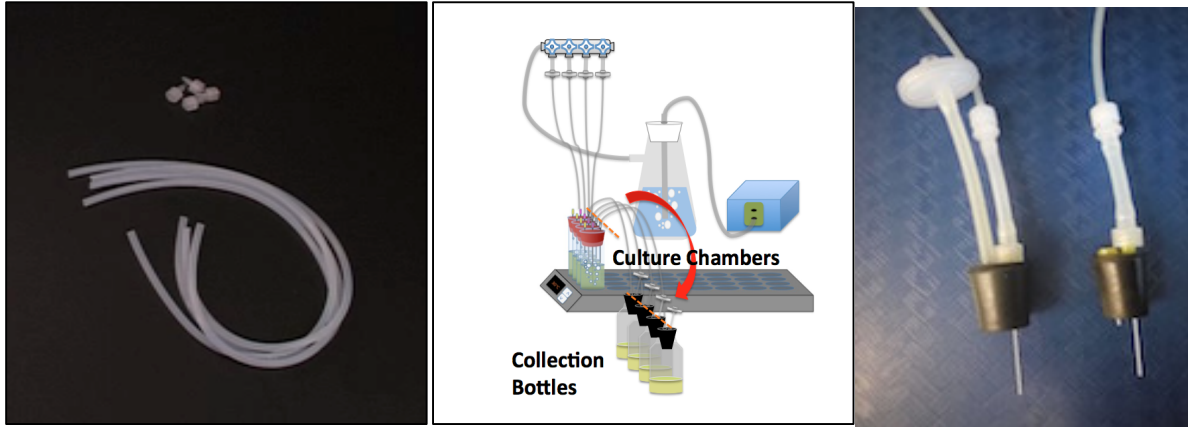


Figure 8: Preparing the effluent lines for the autoclave.

The media effluent and sampling line is a piece of 1/16 in. tubing that has been fitted with one male luer lock which will connect to the effluent needle (the medium length one). The other end of the tube transitions to a short section of 3/32 in. tubing before attaching to the hub of a blunt 1.5 in. needle in the effluent cork. The other hole in the cork is for the efflux of air, and can be set up a couple of ways. Some users, especially during evolution experiments, push a piece of 3/32 in. tubing into the hole, and attach a small air filter. This can sometimes cause backpressure issues. Alternatively, a 0.5 in. blunt needle can be put in through the top. The needle alone is enough to keep fruit flies out, and if the air is constantly blowing out, microbe contamination should be limited. Both options are pictured in **Figure 8**.

The stopper should fit into a 100 ml bottle to be used to catch effluent, but we've noticed some variation in the bottle openings, such that either a #2 or #3 stopper might fit. We've separated the sizes, to keep them as all one size per set up. Cover the separate tub of 100 ml effluent bottles with foil.

As with the air-line, the stoppers should be gathered together, and foiled for autoclaving. Some users place all the stoppers in one big plastic beaker, and foil the top to keep the blunt needles from puncturing the foil.

NOTE: While there are many ways to make the effluent line, make sure you never connect effluent tubing in a way that the upstream is wider bore than downstream, or that it is sloping upward from the chamber.

Prepping the Media Line



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 10 L carboys containing media to the culture chambers. To do this, the media line has a valved female quick-connect attached to $\frac{1}{4}$ in. tubing, an adaptor to adjust the tubing size to $\frac{3}{32}$ in. 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.

NOTE: If you include a Y branch and an additional female quick-connect on your media line, you can use it to combine the contents of the first and second carboys. Look in the Media Replacement section for details.

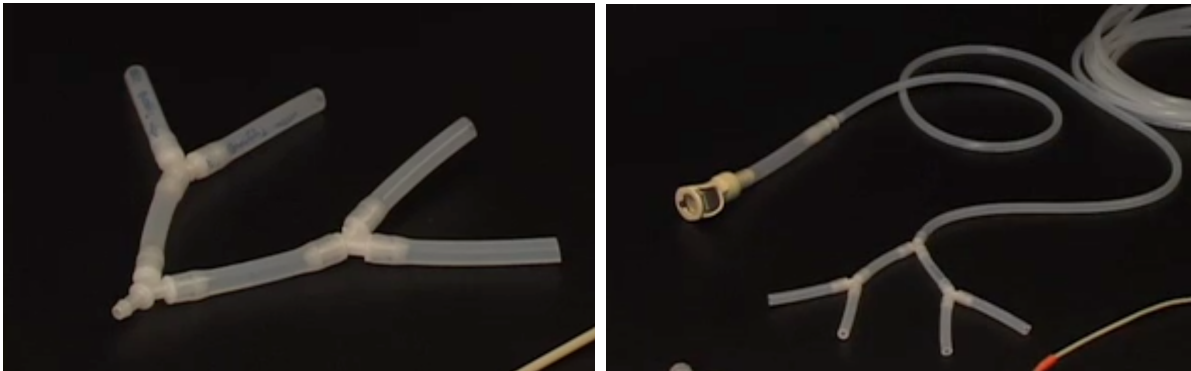


Figure 10: Making branched tubing to divide the media from one source to many ministats.

If one media source is being split to 4 ministats then one would split this 1 media line into 4 using y-connectors and $\frac{3}{32}$ in. 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 in. tubing are actually short blunt needles. Since these needles are not barbed, care needs to be taken to avoid pulling them out during handling. Be careful not to puncture the tubing when you insert the blunt needles. The other end of the pump tubing is connected to another length of 3/32 in. 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.

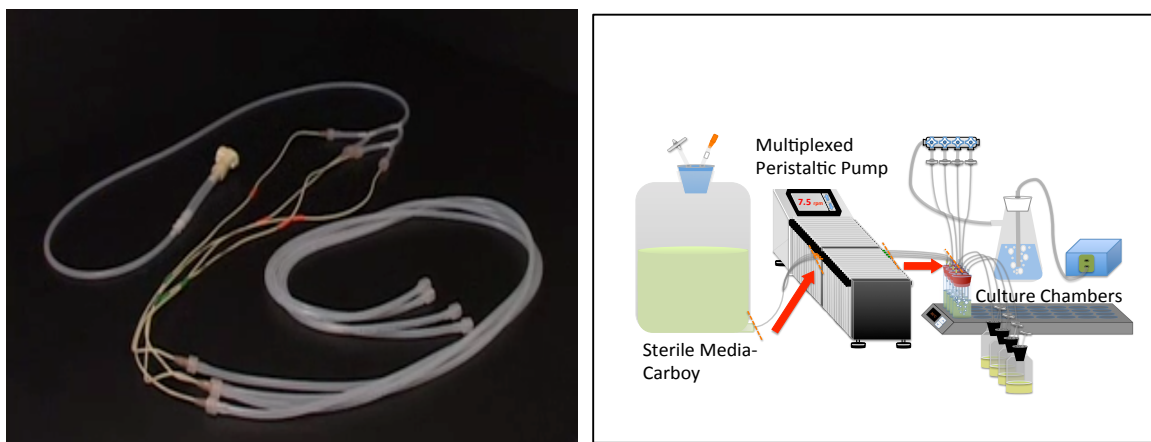


Figure 12: The assembled media tubing.

Assembly of the Carboy

The media vessel is made up of a 5 L or 10 L glass carboy with a bottom spout, and a cork assembly. Described below is the protocol for preparing a 10 L carboy. The procedure is identical to the protocol used for the 5 L carboy with the exception that the 5 L carboy uses a smaller cork, and

gets less water before autoclaving. The bottom spout is fitted with ¼ in. 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 valves in these connectors prevent flow when disconnected from their counterparts. When putting the ¼ in. silicone onto the glass spout, use a small amount of water as lubricant. To remove a silicone tube from glass, cut the tubing off, rather than pulling it. This is to prevent breakage.

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. Find a detailed protocol in the Appendix.

Autoclaving

You will need to autoclave the ministats, carboys, and effluent bottles. You might also want sterile media lines, small air filters, and pump tubing. After outfitting the ministats in the block, transfer them to blue racks. Transfer the racked ministat array with all tubing attached, into one large tub in an organized fashion. Your effluent bottles can be in a separate tub since they are not yet attached. Cover the tub with one big piece of foil to keep them sterile. The carboy may already be sterile, so check around. It's best not to exceed 30 minutes of sterilization time because of all the plastic components that we expect to survive multiple sterilization cycles. Autoclave all components on FLUID cycle for 30 minutes. A dry cycle would melt the plastic components, and possibly break the carboys. Make sure that all containers are vented so that they do not explode or disassemble in the autoclave. See the Appendix for our lab's autoclaving protocol.

NOTE: After autoclaving the ministat array, be sure to double check all needle hub connections, as they tend to loosen up during autoclaving.

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 aka how long the experiment will be running. This depends on whether you will harvest at steady state, compete the strain against another, or allow them to evolve.

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. Our lab, plus Botstein alums Matt Brauer and Alok Saldanha have successfully done this with *leu2* and *ura3* strains, among others. These media formulations are included in the Media Recipes section.

Setting Up the Pump

Once you have received the pump, assemble it according to the manufacturer's instructions. Every pump uses a specific type of tubing, which can be purchased assembled, but to save cost, we assemble our own. See our lab's assembly protocol in the Appendix.

Making Media

You should ideally make media 1-7 days before use in experiments. Monitoring the carboy at room temp, and the bottle of media leftover from the filtering at 30C, could help to identify any possible contamination before you integrate the media into your experiment. The downside of making them a week in advance is that you have to find somewhere to store them. It will also fade in color over time, but that's ok. Media should not be used after two weeks of being made because the

vitamins and other ingredients may degrade over time. It's important to calculate how much media you'll need at the beginning of the experiment.

Special Glassware

None of the glassware we use for chemostat media should be sent to the dishwashers, nor should soap or bleach ever be used. This is not only because they are awkward and likely to be broken, but also because whatever detergent is used by the dishwashing facility may contain phosphate or other chemicals that may be hard or impossible to get rid of. To clean this glassware, rinse glass and tubes thoroughly with tap water, and do a final rinse in the water you will use to make the media. It is wise to start the media making process by rinsing the glassware with this same kind of water.

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 + sterile water. 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.

Water

The source of water you decide to use should be consistent across the board. If you choose distilled water, make sure the water is cooled to near room temperature before you use it. The heat can degrade some of the sensitive vitamins, and using very hot water might affect your final volume. If you share a water source with others, you may want to hoard

what you will need well in advance. We use LDPE carboys for hauling water.

Chemostat Media Recipes

Chemostat media has 4 components that need to be made separately: salts, metals, vitamins, and carbon source (generally dextrose). If your salts will be used immediately, you can include the carbon source in the same concentrate. 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 1000X metals, and top to 10 L with glass distilled or MilliQ water (pick which type of water you will use for all components, and be consistent from batch to batch). The non-sterile media 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 chemicals with very low levels of contamination, specifically of the limiting nutrient. See **Table 1** for examples of purities we are comfortable with.

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. Not all brands make these values available, but currently Fisherbrand and Sigma do provide this information for most of their chemicals. Because of these stringent requirements, it is crucial to keep a substantial supply on hand, as you can't simply borrow from another lab if something runs out. All users must be vigilant about ordering more BEFORE supplies are dwindling.

Table 1: High purity values for media salts reduce contamination of limiting reagent.

Reagent	Contamination
Ammonium Chloride	.002% Sulfate max
Calcium Chloride·2H ₂ O	.005% Ammonium max 0.01% Sulfate max
Dextrose	Less than 0.2% total
Magnesium Chloride·6H ₂ O	.001% Sulfate max
Magnesium Sulfate·7H ₂ O	0.002% Nitrate max 0.002% Ammonium max
Potassium Chloride	0.003% Chlorite and Nitrite max 5 ppm Phosphate max
Potassium Phosphate, Monobasic	0.003% Sulfate max
Sodium Chloride	.001% Chlorate and Nitrate max 1ppm Phosphate max .001% Sulfate max

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

For glucose limitation, you'll add 8 g dextrose for 0.08% final concentration.

1 g	calcium chloride·2H ₂ O
1 g	sodium chloride
5 g	magnesium sulfate·7H ₂ O
10 g	potassium phosphate monobasic
50 g	ammonium sulfate

10X salts for phosphate limitation (1 L)

Separately, mix 50 g dextrose for 0.5% final concentration. Add dextrose to vigorously stirring water.

1 g	calcium chloride·2H ₂ O
1 g	sodium chloride
5 g	magnesium sulfate·7H ₂ O
50 g	ammonium sulfate
10 g	potassium chloride
1 mL frozen aliquot of 100 mg/mL stock	potassium phosphate monobasic (to 10 mg/L final)

10X salts for sulfur limitation (1 L)

Separately, mix 50 g dextrose for 0.5% final concentration. This is full strength sugar. Add dextrose to vigorously stirring water.

1 g	calcium chloride·2H ₂ O
1 g	sodium chloride
4.12 g	magnesium chloride·6H ₂ O
40.5 g	ammonium chloride
10 g	potassium phosphate monobasic
1 mL frozen aliquot of 30 mg/mL stock	ammonium sulfate (to 3 mg/L final)

10X salts for nitrogen limitation (1 L)

Separately, mix 50 g dextrose for 0.5% final concentration. This is full strength sugar. Add dextrose to vigorously stirring water.

1 g	calcium chloride·2H ₂ O
1 g	sodium chloride
5 g	magnesium sulfate·7H ₂ O
10 g	potassium phosphate monobasic
1 mL frozen aliquot of 400 mg/mL stock	ammonium sulfate (to 40 mg/L final)

Metals and Vitamins

The metals and vitamins are made in advance, and require weighing tiny amounts of some reagents. If you don't have a scale that can reliably measure as little as 30 mg, consider making a solution of those reagents instead. Because it is critical that each batch is identical to the next, our practice is that one person makes these. Be careful to avoid losing small amounts of reagent on the weigh dish. Make sure to re-tare the balance each time. High purity of these ingredients is also very important. See our lab's protocol in the Appendix.

About Metals

Metals are made as a 1000X stock that keeps at room temperature for at least a year. While purity of each reagent is important, these are so dilute in the final mix that we are comfortable using ACS grade for these. 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, and the sediment must be thoroughly resuspended before pipetting.

1000X Metals Recipe

(1 L) Everything will not dissolve. You'll bring the final volume to exactly 1 L in a glass graduated cylinder with glass distilled water, and pour into a bottle. Keep it tightly sealed and cover the bottle with foil. Store at room temperature.

Add metal components to a 2 L beaker stirring less than 1 L glass distilled water in the following order:

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

About Vitamins

Vitamins are also made as a 1000X stock. The solution is aliquoted into 50 ml Falcon tubes and stored at -20C. The "working tube" can be stored at 4C for all users. The vitamins will not dissolve completely, so shake before use. Care should be taken to keep the solution well mixed while aliquoting.

1000X Vitamins Recipe

(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 and freeze. To aliquot, with the vitamins stirring vigorously, pipet 40 mL into each 50 ml tube. Don't fill the tubes to the top, or else the lid will split when frozen.

	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 double distilled water. When these components are dissolved, and adjusted to the proper volume (if you're making multiple carboys, e.g., 3 L of 10X for 3 10 L carboys), measure 1 L each of the 10X components in a 1 L glass graduated cylinder, and pour it into a glass 4 L graduated cylinder (The 10X reagents don't have to be 1 L exactly if you're only making 1 carboy). Pipet 10 ml each of the 1000X vitamins (thawed in advance, and resuspended immediately before pipetting) and the 1000X metals (resuspended immediately before pipetting) into the 4 L graduated cylinder. If you measured perfect 10X reagents, you should now have 2 liters and 20 mls in the 4 L cylinder. Top to 4 L with room temperature glass distilled water. Make sure the carboy's downspout tubing is clamped tight and pour into the mixing carboy. The 4 L 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 10 L 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, and proceed to filtering the media.

Filtering the Media

The media will be filtered into the cooled sterile carboy by manipulating a 1 L bottle top filter, attached to a wide mouth 100 ml bottle (or a larger one if no 100 ml 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 10 L 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. Find detailed protocol in the Appendix

STARTING THE RUN

Making an Overnight Culture

Prepare an overnight culture derived from a single colony for each chemostat. Make sure as well that you have a 1 ml glycerol stock derived from 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 – Before You Begin

A Safety Note

There are several electrical components here, in close proximity to liquids. The surge protector should be elevated above the bench surface, where no puddles can form. If your set up has stopped, or there is any other sign of trouble, the strip should be unplugged from the wall before investigating. Also, the two heat blocks should not share an outlet as they will overload the circuit.

The Importance of Flow

The functional design of the ministat system is dependent on the regular flow of media and air, in one direction through the vessel. That flow of air keeps things moving in the right direction, in order to prevent outside contamination from entering into the vessel. If flow is interrupted, great care must be taken to avoid a backpressure event. The ministat always has a level of positive pressure inside, as the flow of air in is what insures the regular effluent of media. Air that goes in must immediately be let out, resulting in a consistent level of pressure inside. When flow is blocked, you risk creating backpressure, and ruining the experiment. Many experiments have ended prematurely due to media line contamination from this kind of incident. While nothing should be able to get through the pump, carboy contamination can occur.

Example: if you were to clamp off the effluent line with the air and media still running, the cork assembly would immediately pop off the ministat. During that seemingly instantaneous event, pressure built up, and pushed in all directions from the vessel until the path of least resistance yielded, thereby releasing the pressure. The most detrimental aspect of this event is that before the cork popped, pressure was being applied to the media line, in the wrong direction. Also, sterility was possibly breached.

Note: No matter which direction the temporary pressure is applied, when it is released, it rebounds in the opposite direction, and contamination can possibly occur.

This is an egregious example, but the concept applies to every aspect of flow, no matter how subtle. If you want to clamp the media line of a vessel, pop out it's cartridge first, so that the pump isn't working against it. Likewise, the carboy must be unclamped before starting the pump. Every line should have only one flow regulator on it, whether it be the pump itself, or a clamp. It should never be both at the same time. Before clamping anything off, be sure to turn off the pump up or downstream of

the clamp. Before starting any pump, make sure there are no clamps on the line. You may still see localized media line contamination, but your carboy (and so all your other vessels on that carboy) is likely to be spared.

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, several at a time.



Figure 13: 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. Many users use long inoculation sticks as a pump tubing guide, running along on the ledge of the cassettes, under where the tubing comes out (perpendicular to the direction of the tubing). When the pump is running, the tubes are moving a bit, and therefore they can get out of their channels, and pinched without something keeping them in line by propping them up. Turn the pump up to maximum speed, and watch to see that all the chambers begin to fill with media. Even at max speed (90 rpm) 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.

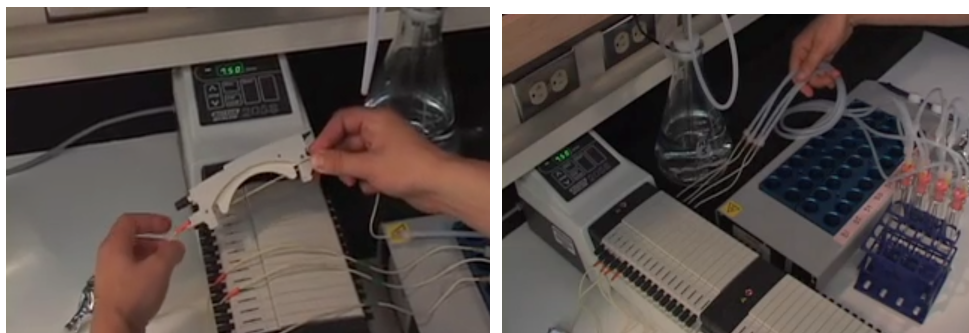


Figure 14: Threading the lines through the peristaltic pump

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. Make sure the switch on the air pump is set to high. 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 or #3 black rubber cork (whichever size fits your set) with two holes and set it in the collection chamber, which is in this case a 100ml bottle.



Figure 15: Effluent vessels with tubing

Inoculating the Chemostats

NOTE: Use appropriate precautions, including safety glasses. Needles and syringes are placed in the sharps container immediately after use. Be sure to start a new sharps container when it is filled to the full line. Proceed with caution!

The ministats are typically inoculated with 1:200 volumes of overnight culture derived from a single colony. With a 20 ml culture volume this would mean that you would inoculate with 100 μ l overnight culture. To do this take a 1 ml syringe with a 5 in. needle (we use 20G or 22G) and fill it completely with your overnight culture. Invert the syringe and tap to cause any air bubbles to go to the top, and depress the plunger until all air is out, spraying into a Kimwipe soaked in 95% ethanol. Next spray the tops of the corks of all ministats you wish to inoculate with 70% ethanol. To inoculate, stab through the cork such that the inoculating needle is in the air away from the 3 needles already in the chamber and making sure not to spray the inoculum onto the wall of the ministat chamber. 4 ministats can be inoculated with a single syringe (assuming the strain is

the same and independence of the inoculating populations is not desired, of course. Always use an inoculum derived from a separate individual colony for each population when initiating evolution experiments.) and then move on to use another syringe/needle. After inoculating make sure the pumps are turned off for 30 hours so that the population can use up all of the available limiting nutrient and grow to saturation. If you cannot turn on the pumps at 30 hours, make sure to refill media in culture vessels at 20-24 hours. Evaporation causes vessels to lose a lot of media volume, and this can result in your strains washing out when you turn the pumps on for your experiment.



Figure 16: Inoculating the ministat

Inoculate using 20G or 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 100 μ l culture.

SAMPLING THE MINISTATS

About Sampling

The chemostats ideally should be sampled every day, particularly when collecting competition or mutation accumulation 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. If you are using a blunt needle instead of the air filter on the effluent cork's air port (described in troubleshooting), you can opt to hang the cork on the side of the 50 ml Falcon tube with the effluent

needle dripping into the tube. The cultures should provide 3.4 mls/hr of culture, so sampling for exactly 2 hours should suffice. While the tube is filling, pour the effluent into a graduated cylinder and write down the date, time, and 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. The following are the different types of sample you might take during a run.



Figure 17: Taking a sample during a run.

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, 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 $\sim 300/\text{plate}$, you should plate less. 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.

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.

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 timecourse, 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.

Sampling for DNA

For DNA sampling, we 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 -80°C . Several members of the lab have also had success freezing cell pellets directly.

Sorbitol Solution Recipe

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

Sampling for RNA

~ 5 ml culture is adequate to yield ~ 50 μg of total RNA. Much less culture is required for many procedures where an amplification step is performed; however, performing RNA preps on tiny quantities of material can be difficult. Therefore, the sampling of RNA from ministats likely marks the end of the run, as removing 5 ml of culture is a significant perturbation. One may choose to risk periodic perturbation in a longer term experiment such as evolution, but removing more than 2 mL at a time is not recommended. Find the RNA harvest protocol in the Appendix.

Data Collection

It is extremely important that your data be collected in an organized and meaningful way so that it can ultimately be organized in 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. Backups are done automatically for files on the server.

At the beginning of your experiment, start the spreadsheet that summarizes your set up. Note the nutrient limitation, and the goal of the experiment. Use a Layout sheet (in Appendix) to organize yourself, and record each ministat's position in the heat block (B#), which strain was inoculated into which location, and the date and time of inoculation. You will add your data to this spreadsheet as you go.

Sample Tracking

The combination of ministat number (B#) and the date should give a unique identifier for sample tracking. Glycerol stocks 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. Use a layout sheet (Find in Appendix) to assign vessel #'s.

Sample Analysis During a Run

Day 1: Dilution Rate

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:

$$D = \text{effluent volume} / (\text{time} * \text{chemostat volume})$$

The dilution rate is in units of hr^{-1} or chemostat volumes per hour. 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.

Since your chemostats will all be running at slightly 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.

Dilution Rate Adjustments

The pump's RPMs essentially translate into approximate flow rates for different numbers of tubes. The vast majority of our experiments have the ideal dilution rate of 0.17 volumes per hour. For our 20 ml cultures this corresponds to 3.4 ml per hour. Dilution rates will be measured daily by measuring the effluent volume collected from the overflow. Calculating the dilution rate once per day, with more volume accumulated is most accurate, as using small volumes yields too much error.

The spreadsheet should calculate the cell generations elapsed since the last sampling:

$$\text{cell generations elapsed} = (\text{time} * D) / \ln 2 = 1.44 * \text{time} * D$$

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

If the dilution rate is high or low across the board, you can bump the pump up or down. If some are high and some are low, you should adjust the occlusion knob of the offending pump cartridge. This knob allows you the option of restricting the flow of one tube at a time, by slightly pinching the tube within the cartridge. Occlusion knobs can vary in their effectiveness, so try swapping cartridges if your adjustment doesn't make a difference. If the pump tubing is highly variable across the set, you'll want to toss it after the run, and use fresh tubing next time. We assemble our own tubing, and the procedure we follow is described in the "Setting up a Run" section.

You'll want to check carefully the next day to be sure that your adjustments were sufficient to get the desired rate.

REACHING YOUR EXPERIMENTAL GOALS

What you do next depends on the goal of the experiment. After a few days, at least 24 hours after the last pump adjustment or other

perturbation, the cultures will reach a steady state, where they have adjusted to the conditions, and are growing on pace with media turnover. For some, this will mark the day of the harvest, and the end of the run. This is also the time when another strain could be introduced for a competition, to see which strain has the higher fitness. This can be done in one week. If you let the cultures grow beyond 25 generations, they will begin to evolve. When we evolve strains, we typically let them go for 200-300 generations, which takes 1-2 months. Special challenges are presented for each type of experiment, and are outlined in the following sections.

'Steady State'

The cell density should be pretty stable before you call it steady state. Lab strains usually reach steady state 2-3 days after the pumps are turned on. Reaching steady state means you can proceed to the next step, which is either to harvest, or to compete the strains.

There are several 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 second day after turning the pumps on they vary less than 5% versus the previous day's measurements, you can proceed. If the numbers are still fluctuating more than that, wait another day, or even half day. If you are competing them, you want the competition to last a couple of days, but be over before 25 generations, so there is time pressure there. You may decide to proceed with more fluctuation than is ideal. If you're up against the 25 generation barrier, you might settle for 10%.

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 if they are much more than 10%, you have to be cautious about your assumptions. You might also give them a little leeway if there is flocculation, since their measurements are likely to be affected.

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 optical density by OD600 and/or klett, 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.

Harvest at Steady State

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.

Setup for harvesting

Final harvest preparation differs depending on what types of downstream measurements you have planned. For example, in addition to regular DNA, microscopy and glycerol samples, you have a unique opportunity to use a lot of culture. You could take 15 ml for RNA, OR you could filter 15 ml for a dry weight measurement. You can't do both because there isn't enough culture volume. Write down final observations about the look and smell of the culture. Does it have chunks? Is there wall growth? 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. Also note whether you have any obvious wall growth. You may want to save a swab of wall growth as a separate glycerol stock. Make sure you physically wipe these with a Kimwipe.

Dry weight

In the past we've had success measuring the dry weight of a certain volume of culture. Our experience is with larger volumes (50 ml on a 47 mm filter). We have not yet explored how well smaller volumes from the ministats work. If you are doing dry weight measurements, weigh a 25 mm 0.45 micron filter for each sample. 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.

You'll follow the same procedure as for RNA described below and in the Appendix, but skip the liquid nitrogen part. Use the weighed filter, and pipet exactly the desired volume of culture into the funnel. 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, 1-2 days. Make sure

the cell side of the filter is not sticking to the foil. After the filter is completely dry, weigh it and subtract off the initial filter weight to get the dry weight per volume of culture.

RNA

If you are sampling for RNA, this should always be the first sample taken. It's important to make sure everything is completely set up before you start. Find a detailed protocol in the Appendix.

You'll use liquid nitrogen to quickly stop the cells. Since RNA expression changes so quickly (the stress response sets in on the order of minutes), you cannot passively sample for RNA. You must instead quickly draw culture directly from the chemostat. Sampling takes about 1 minute from the time the cork is removed to the time the sample is frozen. Reset takes about 3 minutes, so plan on 4 min per sample total.

NOTE: You should use an empty ministat vessel to swap into place of the vessel you're harvesting. This makes things easier in many ways:

- 1) Removing the tube from the crowded block with little headroom makes pipetting culture infinitely more possible.
- 2) It gives the needles a safe place to reside (but handle with care!)
- 3) Turning off the media pump to one, affects others, affecting gene expression before you have a chance to sample them.
- 4) Bumping other stats could alter their volumes, changing their gene expression before you have a chance to sample them.

Once you are finished with all the harvests, you can clean up. Wash out the filter apparatus with DI water, and return all parts to where you found them for next time. Make sure you remove all the RNA harvests from the liquid nitrogen to the -80C. Proceed to the Taking Down an Experiment and Cleanup Section.

Compete at Steady State

If you're doing a competition, the way you set up the block is important. You want competitors adjacent to each other, in the same block to keep it simple while mixing. For example 1-4 would be competitors and 5-8 would be your strain of interest (paired so #1 goes with #5, etc.). The strain you use will need to be easily differentiated from the competitor so that you can measure progress once or twice daily. Our favorite way is to compete our strain of interest against a WT-GFP strain that is fitness neutral in the chosen limitation. We can then use the cytometer to measure what fraction of the population is bright, and whether that

fraction is increasing or decreasing. You could use another neutral marker such as canavanine resistance, but with 32 running at once, counting plates quickly becomes cumbersome.

If we know that a strain has high fitness, we can start the competition with fewer of them, to capture more of the sweep. If you're expecting a quick sweep, you should sample twice daily for the duration of the competition. If we don't expect a speedy takeover, we start with a 50/50 mix, which also allows the competitions to be run in duplicate, since the half volumes can be swapped, and both chambers can continue.

Mixing occurs at steady state, usually after ~2.5 days. Typically, we turn pumps on Monday morning and mix on Wednesday afternoon/evening. Following this timeline, it would end on Friday morning. On mixing day, you'd sample in the morning to be sure you're at steady state, then mix (takes about an hour), and sample again immediately after. Be sure to handle and dispose of all sharps appropriately. Find a detailed protocol for mixing in the Appendix.

It's important to sample immediately after mixing so you have your time zero data point. Usually you have measured the effluent earlier in the day prior to mixing, and so there is only a small amount of effluent in the effluent bottles. Assuming you mixed equal volumes into all the vessels, you can just measure a couple, note the time, and assume that all effluent bottles are at the same volume. Just make sure that however you do it, you account for all volumes taken for the next sampling period.

For competitions, we prefer to use a GFP marked WT strain because of the quick and easy readout from the cytometer. Be sure to use a WT-GFP strain that has been vetted for the limitation you are doing, and of the same background and ploidy as your strain of interest. A set of vetted strains are listed in **Table 2** below. If the one you need is not available, you will need to make/test the strain yourself.

Make sure to dilute samples for the C6 (usually 1:100). Some users have even had luck with refrigerating samples overnight when the C6 was out of order or busy. This isn't ideal but demonstrates the flexibility of this method. Other methods such as plating for a marker can be used. Counting plates from so many vessels is certainly doable, and sometimes necessary, but can be quite labor intensive.

Table 2: GFP strains for competition

Some of the WT-GPP strains in the lab.	Sulfur limitation	Phosphate limitation	Glucose limitation	Additional info
FY <i>MAT_a</i>	YMD1214	Not yet tested	YMD1214	
FY <i>MAT_α</i>	YMD2124	Not yet tested	Not yet tested	
FY 2N GFP het	YMD2196	YMD2196	YMD2196	
CEN.PK <i>MAT_a</i>	YMD1216	YMD1216	Not yet tested	<i>PHO3/5</i> deletion
CEN.PK <i>MAT_α</i>	YMD1217	YMD1217	Not yet tested	<i>PHO3/5</i> WT
CEN.PK 2N GFP het	YMD1689	YMD1689	YMD1689	<i>PHO3/5</i> deletion het
<i>S. uvarum</i> <i>MAT_α</i>	YMD2869	Not yet tested	Not yet tested	

Evolutions

Evolution occurs when you let the cultures grow beyond steady state, and the first changes are observable as early as 25 generations. Because evolutions are more long-term, there are some additional factors to keep in mind.

There is no absolute end point for an evolution. They usually end with a contamination event, if the strain becomes difficult, or when you want your weekends back. The balance between practicalities and experimental goals is generally the deciding factor. Once cells evolve clumping that can no longer be broken up by sonication, we typically pull the plug. Since the experiment typically runs for several weeks (approximately 50 days to get to 300 generations), components of the setup need to be monitored closely and replaced over time to combat media line invasion and flocculation as the strains evolve new traits. One way to slow the emergence of flocculation is to use a *flo1* strain.

While daily checks and effluent emptying are desirable (though could conceivably be skipped given a large enough effluent vessel), daily sampling might not be necessary and could be replaced by weekly or twice weekly sampling, depending on the goals of the experiment. Here is a suggested daily checklist for evolutions. These should be done on the ministats daily to maintain the integrity of the experiment regardless of whether or not you are sampling that day. On sampling days, these

checks should be done in addition to normal sampling. There is additional information in the troubleshooting section.

Media Replacement

It's important to monitor your media as you'll need to keep a steady supply. If you are leaving and there is a chance media might run out before you get back, connect a new carboy. Hopefully you included an alternate sterile port on your media line. If you didn't include a second connector, you'll be risking contamination when you disconnect the current line and reconnect it to a fresh carboy. Spraying the connector with ethanol, and using a quick hand go a long way. The troubleshooting section has some tips for getting the last bit of media out.

If you did include a second carboy connection on your media line, you can combine the contents of the 2 carboys by leaving the first one attached during the transition to the second. Once you connect the second carboy, you can drain all the media into one carboy by raising the 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.

Parts Replacement During a Run

Once an evolution reaches 200 generations, many parts need frequent replacement, particularly media lines. Make sure you have a stock on hand of fresh pre-made, autoclaved media lines (with and without pump tubing) and effluent lines with stoppers. Plan on replacing all of the media lines at some point.

TAKING DOWN AN EXPERIMENT AND CLEAN UP

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 and clog them.

NOTE: ALL MINISTAT COMPONENTS MUST BE COMPLETELY CLEANED DIRECTLY AFTER USE! Never leave media sitting in vessels or tubing.

Decontaminating a Contaminated Carboy

Hopefully your experiment didn't end prematurely due to a yeast, bacteria, or mold that found its way in to the media carboy. If it's in the middle of the run, it usually means your experiment is over. Since we never use soap or bleach in the carboy, we instead must take steps to kill the contaminant by autoclave, and keep the contaminant from spreading through the lab.

Process the carboy as follows:

1. Label it as contaminated, then dump and rinse it with tap water in the sink.
2. Toss all tubing, and the stopper. Don't pull on the tubing, instead use a razor blade to cut along the glass spout so that the tubing easily comes free. Cutting it lengthwise lets you peel it off.
3. Cover the top with foil and leave it at the autoclave bench so it can be sterilized.
4. Now you must BLEACH THE SINK.
5. Once autoclaved, the carboy should be scrubbed with a big carboy brush, located above the sink in S403. Rinse thoroughly. If contamination has been recurring in the lab, foil and autoclave the carboy once more, to be sure no mold spores will be resurrected.

Optional: Using a Water Carboy Prior to Disassembly

This step allows for very excessive flushing of lines with water. It is especially useful if you would like to harvest one day and clean the next. Simply run a carboy of water once harvesting is complete.

1. Harvest or discard your culture from the culture tubes, and empty the effluent bottles.
2. Replace media carboy with a clean (not sterile) carboy filled with ddH₂O. Turn pumps back on and allow water to flush through the system for several hours or overnight.
3. Even if using a water carboy, tubing and culture tubes should still be rinsed after disassembly (see below).

Disassembling the Ministats

1. Get a white rectangular autoclave bin to store parts as you go. Also grab a 1 L plastic beaker to store cork assembly components and enough blue 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 leading to the culture chambers, between the pump and the chambers.
3. Remove all tubing from the ministat culture tube ports, leaving the needles in place. Group each type of tubing separately for cleaning.
4. Place all of the cork assemblies into a 1 L plastic beaker and set this aside in a safe place for cleaning immediately after processing the tubing.
5. Place corks from effluent bottles into another beaker for cleaning.

Cleaning the Ministat Media, Effluent, and Air Lines

1. If your run was an evolution, soak the media lines before proceeding.
2. Make sure “quick connects” are connected so that water will flow through them. They do not flow unless connected.
3. Hook up your media tree directly to a DI water tap. Allow water to flush through lines for ~5 minutes. Add time if there appears to be buildup in the tubing. For best continuous use of lines, disassemble the pump tubing lines where the needles connect, and flush the connectors as well. Any connector area attracts buildup.
4. Use an extra aquarium pump or air line to evacuate as much water as possible from the lines. If needed, a large syringe filled with air can also be used to evacuate lines.
5. Rinse outside of tubes with DI water or ethanol.

6. Hang tubing to dry for next use. Repeat with effluent and air lines
NOTE: Air filters must be removed before this step – do not get air filters wet.

Cleaning the Ministat Culture tubes and effluent bottles.

1. Take the ministat culture chambers and wash them thoroughly with excessive DI water (flush at least 6 times). Store them upside-down in their racks to drain and to keep the dust out.
2. If buildup is present on sides of culture tubes (as with evolutions), rinse with 95% ethanol, then rinse three times with DI water. Wad up 3 Kimwipes and use a set of long tweezers to massage the inside of the tube with the Kimwipes. Re-rinse the tubes with excessive DI water and store upside-down in their racks to drain and for storage.
3. Rinse effluent bottles 3-4 times with DI water.



Figure 18: Using tweezers and 3 folded Kimwipes to clean the culture tubes.

Cleaning the Cork Assemblies.

Take a water squeeze bottle (filled with DI water or ddH₂O) and flush each needle of each cork assembly.

Clean Up the Carboy.

Rinse the carboy and all parts of the cork assembly with excessive DI water 5x. Be sure to remove and rinse 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.

Empty the Bubblers

If these aren't rinsed out regularly, they get scummy. Dump each flask into a beaker, and then swirl some fresh RO water or ddH₂O. Dump that too, and leave the stoppers out if they'll be sitting, so the flasks can dry.

Clean the area

Be sure to clean up any puddles of media under the pump, in the block, and on work surfaces to prevent mold growth in the lab. Also, be sure to put things back where the next user can find them. We have established places to hang drying tubing, beakers for stoppers so that the needles are always pointing down, tubs for used but still good tubing, etc.

RESETTING THE MINISTATS

If you were the last user, then you know that you thoroughly rinsed everything, so you can just reassemble the array, foil as before, and autoclave for reuse. If you weren't the last user, you should be paranoid, and rewash everything before using. As you wash it, inspect everything, and replace parts as needed.

Table 3: When to replace parts

Pump Tubing	<ul style="list-style-type: none">• For an evolution, always make new. Also, it lasts longer if you don't crank up the pump for a water wash.• For competitions, can use 6+ times. You can usually tell from the dilution rate when it's on its way out, and should be disposed of.• Have some sterile media lines and pump tubing on hand for when you need to do a swap mid-run.
Air Filters and Air Line	<ul style="list-style-type: none">• Replace any discolored or wrinkled air filters.• Have a bucket of autoclaved air filters available, individually foil wrapped in case you have to change one mid-run.• We have noticed an oily residue coming from our aquarium pumps, so the sections of tubing closest to them need to be replaced when discolored.
Corks	<ul style="list-style-type: none">• Replace when discolored or if there is any caramelized gunk.• If a needle has been punctured many times, you should replace it.• If a needle needs to be replaced, toss the whole cork and make a new one.
Effluent Tubing	<ul style="list-style-type: none">• Reusable indefinitely if well maintained.
Media Line	<ul style="list-style-type: none">• Reusable until there is residue that cannot easily be removed. If a sticky strain invades and can't be completely removed, toss it.

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>

Appendix A: Parts and Suppliers

Tubing, connectors, and clamps:	Source:	Item:	Price:	Quantity:
1/16" x 1/8" silicone tubing (tiny)	VWR	89068-468	\$41	50' coil pack
3/32" x 7/32" silicone tubing (small) (3/32" tubing uses 1/8" connectors)	VWR	63009-260	\$119	50' coil pack
1/4" x 3/8" silicone tubing (medium)	VWR	63009-279	\$107	50' coil pack
1/2" x 5/8" silicone tubing (extra large)	VWR	63009-299	\$336	50' coil pack
orange green pump tubing, pre-assembled	Watson-Marlow	978.0038.000	\$62	pack of 6
Short Blunt Needle for pump tubing, 24 Gauge x 1/2" Length	Brico Medical Supplies	BN2405	\$90	pack of 1000
Male luer connector to 1/16" barb	Cole Parmer	EW-45513-00	\$28	pack of 25
reducing connector, PP, 3/32" to 1/16"	Cole Parmer	EW-30621-95	\$11	pack of 25
female luer, 1/8" barb, PVDF	Cole Parmer	EW-45512-04	\$24	pack of 25
male luer lock, 1/8" barb	Cole Parmer	HV-45503-04	\$7	pack of 25
barbed Y connector, 1/8" ID	Cole Parmer	HV-30703-92	\$21	pack of 10
male luer slip, 1/8" barb	Cole Parmer	HV-45503-26	\$8	pack of 25
reducing connector, PVDF, 1/4" to 1/8"	Cole Parmer	EW-30703-50	\$21	pack of 10
Male Inline valved connector, Fits tubing: 1/4 in. I.D., Polypropylene	Fisher	05-112-39	\$140	pack of 25
Female Inline valved connector, Fits tubing: 1/4 in. I.D., Polypropylene	Fisher	05-112-37	\$37	pack of 5
large tubing clamps, 12-position	VWR	63022-407	\$16	pack of 6
medium tubing clamps	VWR	63022-405	\$10	pack of 12
small tubing clamps	VWR	63022-403	\$25	pack of 100
Day Pinchcock (metal clamp for tubing)	VWR	21730-001	\$9	pack of 10
Assorted zip ties	VWR	21800-000	\$25	

Optional, if not buying pre-assembled pump tubing				
10 tubes of E6000, 0.18 oz ea	Amazon.com	B007TBYEXM	\$12	10 small tubes
15 m roll marprene tubing .38 mm	Watson-Marlow	905.0003.008	\$197	15m roll
0.187" to 0.062" adhesive lined heat shrink tubing, black (3:1)	Grainger Inc	4RCU9	\$37	pack of 24 x 6" pcs
1/8" Red 2:1 Shrink Ratio Heat Shrink - 10' Section	Amazon.com	B004S2JGN8	\$5	10 foot section
Air Supply:				
Silent Air Pumps	Aquarium Guys.com	212422	\$25	each
PTFE filters, 0.45 µ, for air filtration	Fisher	R04SP02500	\$143	box of 100
Kimax 1 L flask with sidearm	Fisher	10-181F	\$331	pack of 6
#8 silicone stopper, 3/8 in. hole, for babystat bubblers, for vacuum flasks	Fisher	K953715-0801	\$15	each
4 port manifold	Cole Parmer	EW-06464-85	\$9	each
Culture Chamber:				
PYREX® 55 mL Screw Cap Culture Tubes 25x150 mm	Corning Life Sciences	9826-25	\$211	pack of 48
hypodermic needle, 16G, 5 in. length (for effluent line)	Air-Tite Products Co., Inc.			
BD Spinal needles, 18G 6 in. length (for air line)				
hypodermic needle, 20G, 1.5 in. length (for media line)				
Foam Silicone stopper, nonstandard size "2", pink	Cole Parmer	EW-06298-06	\$31	pack of 20
VWR® 8-Well tube Rack	Cole Parmer	82024-452	\$14	each
Effluent				
Blunt Needles for effluent cork, 20 Gauge	Air-Tite Products Co., Inc.	NB20112	\$55	pack of 1000

x 1-1/2" Length				
#3 Rubber Stoppers, Two-Hole	VWR	59582-166	\$25	1 bag
#2 Rubber Stoppers, Two-Hole	VWR	59582-144	\$23	1 bag
100 ml bottles for effluent	VWR	16171-004	\$150	case of 48
Media Carboy:				
10 L Reservoir bottle with bottom hose outlet	VWR	89001-530	\$220	each
Foam Silicone stopper, non-standard size 12	VWR	EW-06298-22	\$18	each
Carboy Venting Filter	Fisher	SLFG 050 10	\$93	pack of 10
Scotch #35 Electrical Tape, Green	Amazon.com	10851-BA-10	\$7	each
Corning bottle top filter, 1 L, 0.2 µm, 45 mm	VWR	29442-978	\$105	case of 12
Carboy with Handles for water hauling, LDPE	VWR	16133-025	\$164.08	each
Media Pump:				
205S/CA16, 16 channel cartridge pump	Watson-Marlow	020.3716.00A	\$5,202	each
16 channel 205CA extension pump head	Watson-Marlow	023.1401.000	\$2,876	each
silicone aquarium sealer	Fisher	S18180B	\$5	each
replacement cassette for 205CA pump	Watson-Marlow	DEA0081A	\$55	each
Heat block:				
6-block dry bath	VWR	12621-120	\$658	each
blocks for drybath, 6 x 25 mm tube	VWR	13259-210	\$64	each
RNA harvest				
small vacuum apparatus	VWR	26316-692	\$84	each
GE Magna* Nylon Membrane Filters, 0.45 µm Pore Size; Dia.: 25 mm	Fisher	R04SP02500	\$108	Pack of 100
Dewar flask, 1 L	VWR	63380-052	\$224	each

Appendix B: Specific Protocols Before the Run

B.1 Assembling Pump Tubing (optional)

We assemble our own pump tubing to cut down costs, but you can buy it preassembled. **See the supplies for each option in Appendix A.**

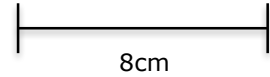


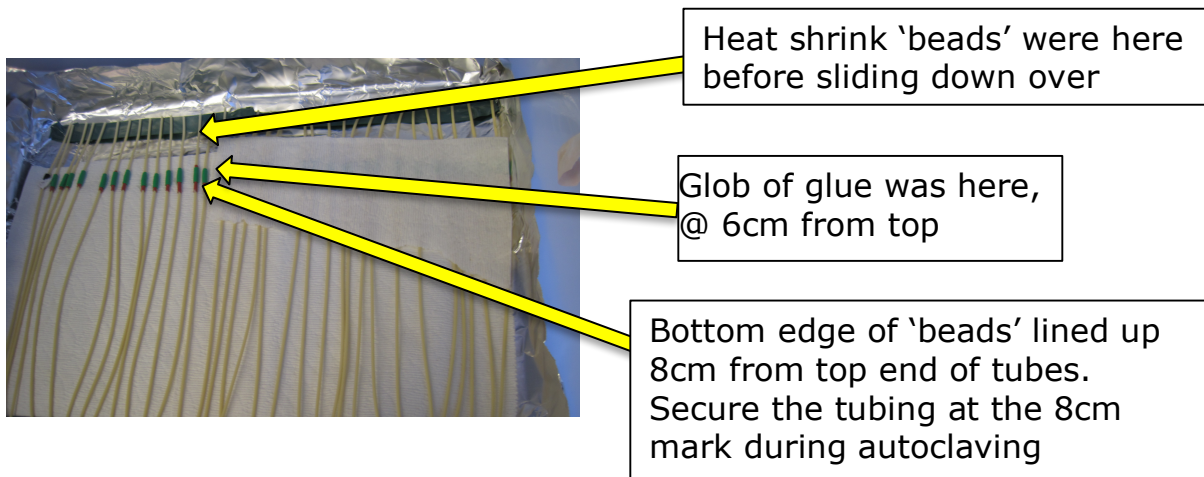
Figure: B.1a: The pump tubing assembly 'kit' and heat shrink 'beads'.



- 1) Get a roll of tubing, and the tubing assembly 'Kit', which includes these instructions, and the items shown at left.
- 2) Cut the roll into 12" lengths, with blunt, not angled cuts. Each roll of tubing (15 m) should make about 49 x 12" lengths (12"=30.5 cm).
- 3) Cut the heat shrink tubing into 'beads' that are @0.5 in. long.
- 4) Line an autoclave bin with foil, and run 2 lengths of green electrical tape, sticky side up, along the long sides of the bin. Put a layer of paper towels in the bottom*.
- 5) Grab a piece of tubing, and put one of each color bead on one of the ends. Secure the tubing to the green tape, keeping the beads gathered at the top. Repeat this step, working from one end of the tub to the

- next. Use small strips of lab tape on top of the green tape as you go, to keep the tubes from coming loose as you manipulate them. Be sure to space the tubes about 1 cm apart, so they don't get all stuck together.
- 6) Use a ruler to measure about 8 cm from the top end of the tubes. This is where you eventually want the bottom edge of the heat shrink beads to rest. You might want to secure this position with a strip of lab tape.
 - 7) Grab a tube of E6000 glue, and apply a glob to the tubing, at about the 6 cm mark. Then use forceps to slide the small red bead over the glob, so that most of the glob goes inside the bead, and stop sliding when the bottom of it is at 8 cm from the top end.
 - 8) Repeat step 6 with the larger, black bead, with the bottom edge not going past the bottom edge of the first bead. It doesn't matter if the distal edge of the beads line up.
 - 9) Using the first tube(s) as a guide, repeat steps 6 and 7 for several tubes at a time, but keep in mind that the glue dries quickly.

Figure: B.1b: Pump tubing overview



- 10) Once all the beads are glued, lay a paper towel on top of the gluey area*, and autoclave using a 20 minute sterilization on the wet cycle.
- 11) If you want to do multiple batches, do the assembly in separate tubs as described, but then nest multiple sets, separated by the bottom aluminum foil layer of each set, and autoclave all in the same tub.
- 12) After autoclaving, allow the tubing to cool completely before removing them. This is because when the glue is hot, it is still sticky, and

letting it cool on paper towels first, allows the paper towel to stick to the glue, making the tubes less likely to stick together in subsequent autoclave runs.

- 13) Allow some time to gently remove the tubing from the bin, and to gently remove any excess paper towel. It doesn't look pretty, but those paper towel bits aren't hurting anything. Tugging on them will compromise the tubing segment, as the glue is stronger than the tubing itself
- 14) Put the batch of tubing in a ziplock with the date, and your initials.

*Surrounding the gluey parts with paper towel is an attempt to capture the excess glue by allowing the fuzz of the paper towel to cover it. There may be a better way.

B.2 Preparing Carboy and Stopper

The media vessel is made up of a 5 L or 10 L glass carboy with a bottom spout, and a cork assembly.

Preparing the Carboy's Cork Assembly

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 1200 μ l 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 the whole stopper will occasionally need to be replaced when damaged, discolored, or too soft. 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, but should be replaced after each run.

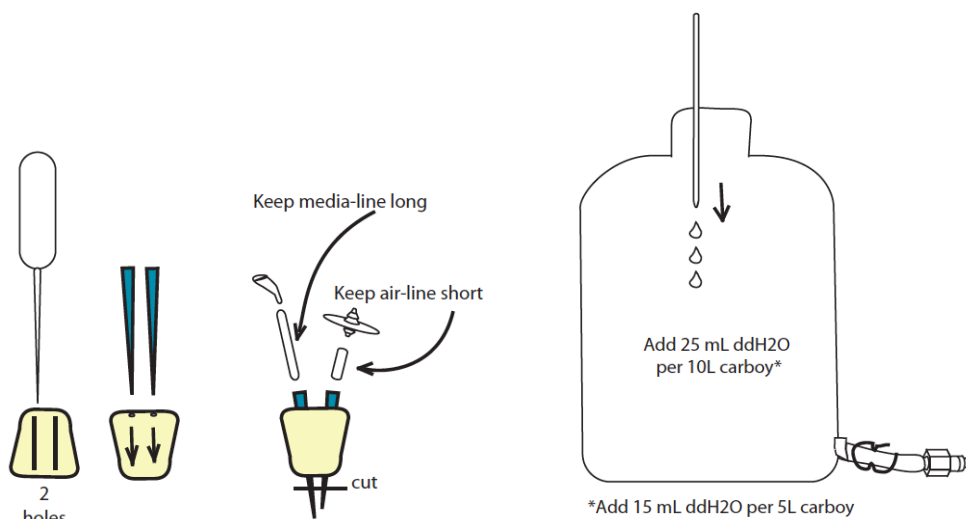


Figure: B.2a: The construction of the carboy stopper and preparation of the uncorked carboy.

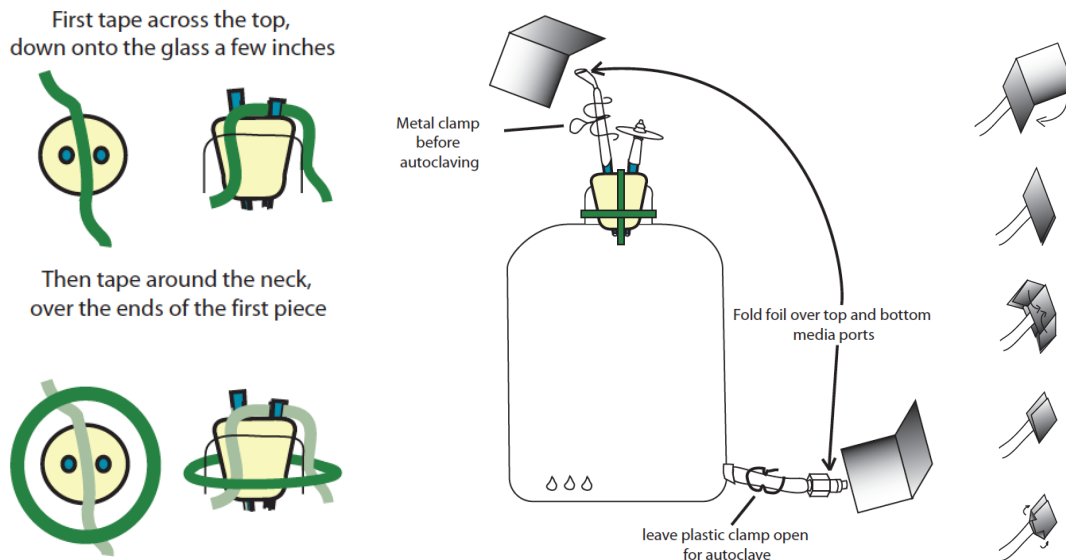


Figure: B.2b: Dressing a carboy for the autoclave.

The Carboy

Make sure to calculate how much media you'll need for your experiment. Since the volume of the carboy is so large, autoclaving on the fluid cycle is required in order to prevent breakage. To assure sterility, we also add a small amount of ddH₂O into the carboy so that it will steam the inside.


1. Rinse carboys and corks 4-6 times with tap water, being sure water is flowing through any tubes (Connect valved connectors so water will flow through them). **Never ever use soap or bleach.**
2. At this point, you can put the carboy on the autoclave bench, and our student helper will take it from here.
3. After the tap water rinses, do a thorough final rinse (inside and outside of the carboy and tubing) with ddH₂O from the sink carboy or diH₂O from the twisty faucet in the Queitsch lab.
4. Rinse the cork assembly the same way, but with the air filters removed.
5. To prepare the empty carboy for autoclaving, start by attaching a male quick connector to the bottom tube. The clamp on that tube should be OPEN.
6. Pipet 25 ml of ddH₂O into your clean 10 L carboy (or 15 ml into your 5 L carboy). This allows for steam sterilization of the interior of the carboy.
7. Insert the cork assembly into the top of the carboy, and give it a firm push in. Use the green electrical tape (Scotch #35), to tape down 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 with the first strip of tape. This should prevent the cork from popping out in the autoclave.

8. Add a large air filter to the short port. This will keep the carboy from exploding in the autoclave.
9. Replace the plastic connector on the media port on the cork.
10. Next, use your favorite foil origami method to cover the media-in port on top, and the media-out port on the bottom. Finally, place a metal clamp on the tubing of the media port.
11. Autoclave on fluid cycle, for 30 minutes, and NEVER clamp off the air vent, as it may cause the carboy to explode in the autoclave. 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 30 minutes of sterilization time, because of all the plastic components that we expect to survive multiple sterilization cycles.

B.3 Autoclaving

1. Make sure you've taken and documented the autoclave safety training.
2. Make a reservation for one or both autoclaves
 - a. Large Autoclave: Can hold 4 large tubs or up to 10 large carboys
 - b. Small Autoclave: for 2 metal pans
 - i. This reservation is for 2 hours, 11:30-1:30

11:00	
11:30	 Dunham S403
12:00	
12:30	
1:00	
1:30	
2:00	
2:30	

Write,
"Dunham, S403"
next to the arrow,
so they can let us
know if there's a
problem.

- ii. If you are 30 minutes late for your reservation, someone else can take it

Chemostats and carboys should be sterilized using the liquid cycle with a 30 minute sterilization time. The total run time will be 1 hour 30 minutes.

3. Prep and gather
 - a. ministats
 - b. effluent bottles
 - c. A sign for the autoclave that says, "Do Not Open!" with your name and phone#
 - d. Plan to be there when the run is complete, with autoclave gloves.
4. When it's done
 - a. Mark the sign up sheet with "fluid cycle" next to your reservation, as described in the training.
 - b. If any tubes have popped off, or foil has come loose, you can fix it quickly after opening the autoclave.
 - c. Allow the setup to cool.

Small Autoclave

The small autoclave can only fit 1 large tub, 2 small tubs, or 2 metal pans.

1. Enter 1949 when prompted for a code.
2. Select and Verify the cycle and settings.
3. After setting cycle parameters, press 9 twice.

4. Press Run.
5. Press enter. Do not enter an operator number; press enter instead.
6. It should start. Set a timer and plan to come back as soon as it's done.

Large Autoclave

Is similar, but with no secret code. Follow the prompts, and refer to the instructions posted above the control panel. Ask for help if unsure.

B.4 Mixing 1000X Vitamins and Metals

- ✓ Allow 2+ hours, make **2 L** of each
- ✓ Make equal batches at the same time. That way, when the vitamins start running low, we know that the metals are too. This protocol describes making 2 L of each.
- ✓ Do the vitamins first. Since they need to be aliquotted, it is more critical that they are incredibly well mixed before and during the aliquot process. Doing them first gives them extra mixing time while you're measuring out the metals.

The weighing process:

1. Use a scale specially designed for tiny amounts. The trick to using this type of scale is to be careful about air and table movement. If there is a lot of activity in the area, wait until everyone goes to lunch, and don't bump the table, or dance around. Close both glass doors for the tare, and for the final weigh.
2. Make sure your hands are completely dry through the weighing process. The tiniest amount of water on the weighboat will throw off your measurements, and some of the reagents are hygroscopic.
3. Get a 2 L plastic beaker, and fill it up to ~1500 ml with ddH₂O. Add a stirbar, and start it spinning vigorously on the weigh bench, near the precision scale. Label it using orange tape for vitamins, and green for metals. This will be the label that ultimately labels the rack of aliquots, or bottle, and should include your initials and the date.
4. Use one weighboat for all the vitamins, and then when the vitamins are all done, use a different one for all the metals. Note any ingredients that there may not be enough of for another batch.
 - a. Select a medium weighboat, and put it on the scale. Close all glass doors, and press the TARE button. It should take less than 10 seconds to zero. If the numbers keep drifting up and down, there is too much activity.
 - b. Add the first ingredient to the boat. Adjust the amount until it's close, then close the door for a final weight. Don't put any of the reagent back in the bottle, put it on a paper towel for disposal instead.
 - i. Sometimes, the vitamin or mineral is too granular to get a perfect weight. If this happens, get as close as possible. (i.e. for particularly difficult ingredients, if it should be 800 mg, allow 795-805, but only if it's impossible to get it perfect!)
 - c. Once the weight is correct, remove the boat from the scale, and tap it over the beaker of stirring water. There will be some

residue on the boat, but that's ok. Return the boat to the scale, and re-tare. Weigh the next ingredients like the first, taring between each one. After the last ingredient, DIP the weighboat into the solution, thereby rinsing all the trace amounts into the final solution (unconventional, but effective). Once rinsed, throw the weighboat into the lab waste, cover the beaker with foil, and move it to a different stirplate in the lab to continue mixing.

- d. Repeat process (a-c) for metals.
5. Get a glass 2 L graduated cylinder, and pour the vitamins in. Add a couple hundred mls of ddH₂O to the mixing beaker, and swirl it around to rinse the sides of any powder. Use this water, and subsequent rinses to bring it up to 2 L. Pour it back into the mixing beaker, and return it to the stirplate.
6. Aliquot the vitamins: this is complicated by the fact that there is quick settling particulate matter in the final solution. Keep them strongly mixed via stirbar during aliquot process.
 - a. To see if it is being mixed enough, use a pipetman and 50 ml pipet to draw 25 ml from the top, and 25 ml from the bottom, and check to see if there is a difference in how cloudy it is. I usually have the 403/microarray stirplate on 8 or 9.
 - b. Remove the caps from a rack of 50 ml conical tubes. Pipet 41 ml into each, then cap and freeze. Make sure the rack is labeled with 1000X Vitamins, your initials, and the date.
 - c. Repeat b.
7. Top up the 1000X Metals using beaker rinses like you did before.
8. Get a funnel, and pour from the cylinder into a 2 L bottle, pausing to resuspend any settling particulates as you go.
9. Wrap the bottle with foil, and label with the green tape from the beaker.
10. Enter the date and # of liters you made in a usage chart, so you can anticipate when stocks might run low again.

B.5 Filtering Media

Be prepared at the beginning and end of the filtering process, to apply or release the clamp and vacuum in the correct order.

Example: if you were to unclamp the media port before you turn on the vacuum, the direction of flow from non-sterile to sterile hasn't been established, so it could go either way, compromising sterility. Even more importantly, at the end when there is significant negative pressure in the carboy, if you were to release the vacuum before clamping the media port, non-sterile air would certainly flow into the carboy through the unrestricted port.

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.
- 100 ml wide mouth bottle (sterile), labeled with its corresponding carboy#, date, and your initials.
- 1 L 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 on the floor in front of the mixing carboy. As you proceed, move quickly but carefully to keep everything sterile.

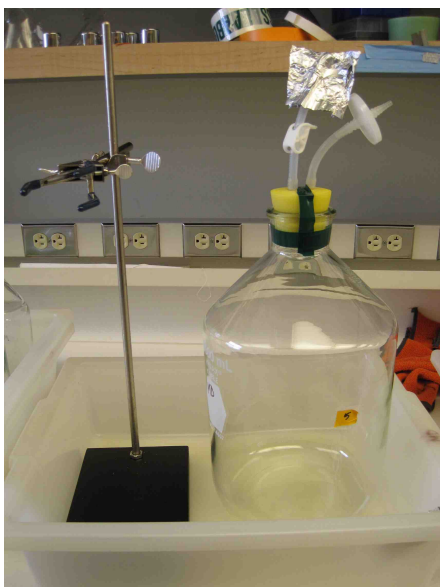


Figure B.5a: Getting ready to filter: the carboy

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



Figure B.5b: Setting up the filter

3. Loosen the cap on the bottle. Open the top end of the bottle-top 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 especially sterile a) where it will screw onto the bottle, and b) on the side port. 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 the side port of the filter.
6. Attach the side port of 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. **DON'T UNCLAMP THE MEDIA PORT** until the vacuum is on.

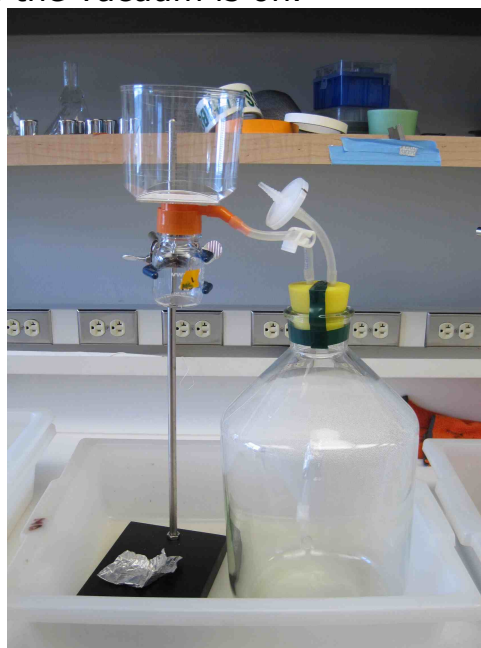


Figure B.5c: Final assembly of filter set up

8. 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 are very tight (filter to bottle, and filter to carboy).
12. Turn on the vacuum (Only half a turn as shown in **Figure B.5e**) and adjust the output hose from the mixing carboy so that media begins to flow into the bottle-top filter. 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, you can finally safely remove the metal clamp on the tube between the filter and the carboy (the media port). The 100 ml bottle will fill first, and then overflow through the side port into the carboy. Make sure vacuum lines aren't kinked.

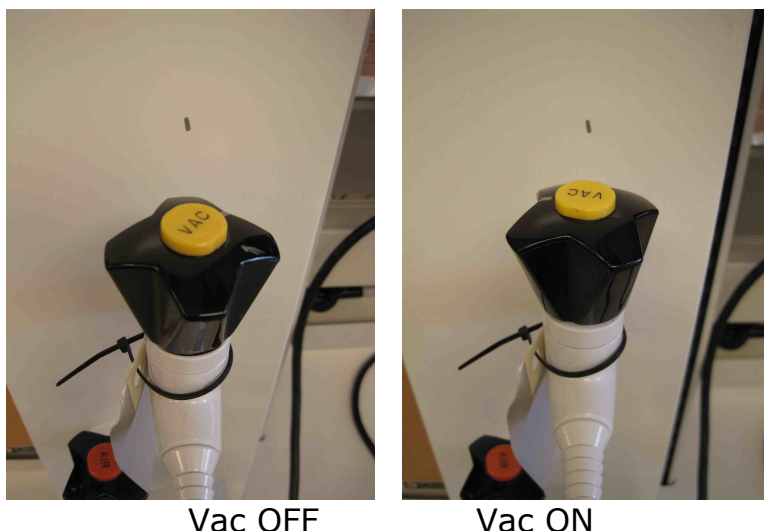


Figure B.5e: Turn vacuum on LOW. If the vacuum is set too high, the media will go too fast, and the cork may get sucked in. If the vacuum is set too low, you risk contamination.

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! 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.
15. When the media is all filtered, unclamp the 100 ml bottle, and tilt it toward its outlet, so that media runs into the carboy, and you have some headspace in the bottle. **DON'T TURN OFF THE VACUUM** until the metal clamp is back in place.

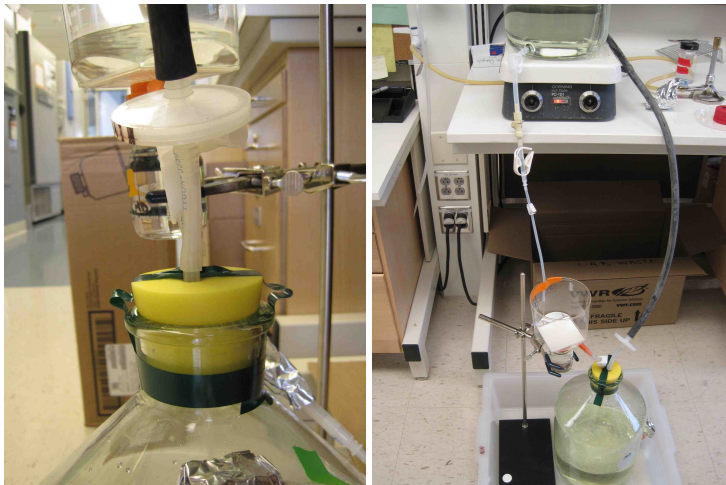







































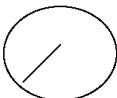


Figure B.5d: During filtration, it's normal for the cork to get *slightly* sucked in

16. 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.
17. Detach the filter and bottle from the carboy's media port. Keeping sterility, cap the bottle. Then toss the filter and re-cover the media tube with foil.
18. Set the carboy on the bench or on a shelf above the setup. Never put more than 8 carboys on the shelf above the setup in the common equipment room.
19. Place the 100 mL bottle of media in the 30C incubator and watch it for a couple of days to see if any of your carboys may be grossly contaminated.
20. Rinse the mixing carboy, any tubing that you used, and the 4 L graduated cylinder with tap water several times each. Then rinse once more with DI water. Do NOT use soap or bleach, ever.

B.5 Ministat Layout and Identifiers Sheet

Date of inoculation_____

Experimental description_____

Appendix C: Specific Protocols During the Run

C.1 During First 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.

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, momentarily turn off the air one manifold at a time 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. You can increase the pump rate to fill the vessels faster if evaporation has occurred and the culture volumes are low.
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 20 ml mark on the tube, and adjust the needle height.
3. Repeat this cycle until you have reached the desired 20 ml culture mark. If you over shoot slightly increase the height of the needle, let the tube refill, and re-do step 2. This process may take several hours to achieve correct volumes for all culture tubes.
4. Once the ideal level has been reached you may begin your first 2-hour sampling on ice as described below.
5. After sampling, it is especially important to calculate the dilution rate on the first day, so you can adjust the pump or cassette if needed. See **Sample Analysis During a Run** for details.

C.2 Typical Daily Sampling

1. For each ministat, pre-label sterile 50 ml glass tubes or 50 ml falcon tubes, a 2 ml cryovial, a 2 ml eppendorf tube, and 4 1.5 ml eppendorf tubes (labeled for serial dilutions as sonicate, 2:20, 2-2:20, 4:400.)
2. Add 0.5 ml sterile 50% glycerol into the cryovial, and 1.5 ml water into the 2 ml eppendorf tube. Distribute appropriate amounts of water for listed serial dilutions in 1.5 ml eppendorfs.
3. Note time, and transfer sampling corks to the 50 ml sampling tube (on ice). Always start in the same place, and move the corks systematically as it takes some time to move them all.
4. While tube is filling (you need 2 ml minimum, although we usually gather ~6.8 mls over the course of 2 hours), measure effluent volume using an appropriate glass graduated cylinder. Record V_{eff} directly into a spreadsheet, then calculate D based on V_{eff} , 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 or other perturbation is made.
6. Cap the sampling tube and put the sampling line back into the 100 ml collection bottle. Note the sample volume taken, so that it can be included in the next D calculation.
7. Vortex sample, then pipet 1 ml into the cryovial and 0.5 ml into 2 prelabeled eppendorf tubes (one 2 ml for OD600 dilution, and one 1.5 ml 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 and changing pipet tips at each step.
9. Take remaining undiluted sample along with 2 ml tubes over to the spectrophotometer area. Vortexing well before each measurement, check the OD of 1/4 dilutions in the 2 ml tubes, 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 100 μ l 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 8-10 μ l of first 2:20 dilution into a hemacytometer. Count with a clicker, calculate, and record cells/mL. Alternatively, use

the second 2:20 dilution (which is 1:100) to count cells with cytometer.

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).

C.3 Example Daily Checklist

Date: _____ Start: _____ End: _____

Steps	Apparatus	Task date:	Who?
1	C6	Turn ON C6	
		Instrument > Cleaning cycle	
2	Ministats	verify no contamination (carboy, media line)	
3		Start fresh effluent collection (tube C1 to C16)	
4	From ON Effluent	Save 50ml from the ON effluent in 50ml conical tubes	
5		Centrifuge tubes 4min at 4000rpm	
6		Add 1ml H2O in effluent tubes and save 100ul for Giang and 1ml for Bonny	
7		Centrifuge, remove H2O, store at -20C	
8		Measure the remaining volume present in the effluent bottle	
9		Wash bottle, dry them quickly replace in the effluent line	
10	From fresh effluent Collection	Save 1ml glycerol stock, store at -80C	
11		Dilute 20ul of the collection in 80ul H2O in V bottom plate	
12		Seal the plate with tape	
13		Sonicate water bath with some ice chips (A=50, 1sec ON, 1sec OFF)	
14		Run the plate in the C6, template > MyTemplate	
15		Save Count, and other information you may need (mean GFP per example)	
16		Edit the spreadsheet (Google doc)	

Ministat	Vol-Effluent
M1	
M2	
M3	
M4	
M5	
M6	
M7	
M8	
M9	
M10	
M11	
M12	
M13	
M14	
M15	
M16	

Ministat	Vol-Effluent
M1	
M2	
M3	
M4	
M5	
M6	
M7	
M8	
M9	
M10	
M11	
M12	
M13	
M14	
M15	
M16	

C.4 Mixing 50/50 for Competition

1. First, spray ethanol on the 2 corks you'll be mixing. A good strategy is to have the competitors close to each other in the same block, such as #1 and #5, the first in each of 2 adjacent rows.
2. Very carefully insert a 5 in. needle with attached 10 ml syringe through the stopper of the strain of interest, and then a separate needle with syringe through the stopper of the competitor. Put the needles in all the way, so that the needles are in the culture.
3. Assuming your ministats are 20 mL volume, draw up 10 mL from each. Often it is useful to take the culture tube out from the block and tilt it, and take culture from the opposite side of the air stream to avoid air in the syringe. Then put the tubes back in the block.
4. Carefully loosen the syringes from the needles, and swap each syringe onto the opposite needle.
5. Make sure the needle is screwed tight to the syringe, before you depress the plunger.
6. Some people leave the needles in the corks until they are all done to mark which have been mixed, but others do not.
7. Proceed through the block until all cultures are mixed.

C.5 Harvesting for RNA

To prepare

- 1) Label your tubes with a pen that will hold up to liquid nitrogen and -80C.
 - a) a 15 ml Falcon tube for each sample's filtrate (if you're keeping it)
 - b) a 2 ml locking eppendorf tube for each sample's filter paper.
- 2) Gather the filter apparatus, filters, vacuum flask, ringstand, tubes with racks, pipets with pipettor, 1 L Dewar flask, tongs, forceps, and DI water bottle. If needed, use a cart as a dedicated workspace.
- 3) Use a ring stand, to set up the small filter apparatus with the stopper assembly as shown.
 - a) Make sure the 3-prong clamp grips the silicone stopper firmly.
 - b) The glass cylinder should be straight up and down, but the 3 prong clamp may appear crooked.
 - c) Fit the 2 hole stopper into a spare 15 ml Falcon tube.
 - d) Hook the vacuum tubing from the vacuum flask to the other stopper port using a ¼" straight connector.
- 4) Fill the 1 L Dewar flask about 1/3 full with a few ladles of liquid nitrogen. Ask someone for help if you haven't been trained in handling liquid nitrogen.
- 5) With the filter screen in place, rinse the apparatus with DI water before using and in between samples to remove any trace of previous samples. This also gives you an opportunity to determine how best to situate the clamp that holds the cylinder in place. You may have to move the clamp slightly away from the glass to ensure a tight fit and no leaks.

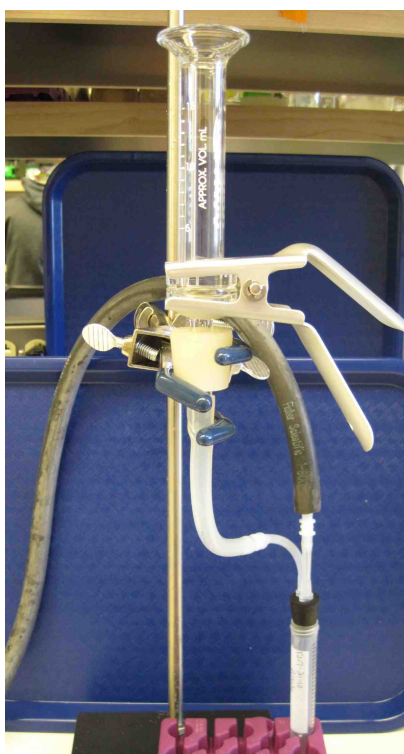


Figure C.5: Small filtering apparatus.

When you're ready to start

- 1) Turn the vacuum on.
- 2) Put the filter apparatus's black stopper into the conical tube for the first sample, and drop the first sample's 2 mL tube into the liquid nitrogen.
- 3) Use forceps to place a fresh 25 mm filter in the screen of the filter apparatus. Check for a tight fit, no whistling. Clamp the cylinder of the apparatus onto the base.
- 4) Being careful of the needles, swap the cork assembly into an empty tube and leave it in the block.
- 5) Quickly remove the culture tube of interest from the block, and pipet 15 mL culture into the cylinder of the filter apparatus, and observe it running through
 - a) If the filtrate is clear, the cells are on the filter
 - b) If it moves slowly, you may want to turn up the vacuum.
- 6) Remove the clamp and glass funnel, leaving the vacuum on.
- 7) Use tongs to retrieve the appropriate 2 mL tube from the liquid nitrogen, dump it out and put it in a tube rack.
- 8) 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 immediately drop it back into the liquid nitrogen. Quickly cap the conical/filtrate tube so that volatiles such as ethanol don't evaporate, and put it on ice. Make sure there is headroom in the tube for the sample to expand upon freezing. It will be frozen at -20C, but you can collect a few more samples first. If you aren't keeping the filtrate, dispose of the liquid and put the tube back in place.
- 9) Rinse the filter apparatus with DI water, and empty the conical tube again if reusing.

With the culture vessel out of the block, take any other samples you want. The vessel you just harvested from can act as the dummy for the next vessel's harvest. Repeat the procedure for the remaining chemostats. You will probably want to pause after every 8 or so samples, so that the liquid nitrogen isn't so full of samples that you can't find the tube you want. Transfer the frozen tubes to a -80C box, and move the filtrate tubes to a rack at -20C, tightly capped.

When all harvests are complete, wash out the filter apparatus with DI water, and return all parts to where you found them for next time. Make sure you remove all the RNA harvests from the liquid nitrogen to the -80C.

C.6 Daily Evolution Checklist

Date and time of effluent dump: _____

CHECKS:

Any unexpectedly high or low effluent levels?	
Any unexpectedly low or high vessel media levels?	
All vessels bubbling?	
Flocculation?	
Air flask contamination?	
Air flask water levels okay?	
Carboy contamination?	
Invasion of cells into effluent lines?	
Invasion of cells into main tubing?	
Invasion of cells into line at port connecting to vessel?	
Development of cell ring at port connecting to vessel?	
Media leak at pump tubing connections?	

REPLACEMENTS MADE:

Air needle:	
Effluent line including stopper:	
Media line (line only):	
Media line + pump tubing:	
Media line + needle:	
Media line + pump tubing + needle:	

At the end of your checks and replacements:

Are your pumps on?

All your media unclamped?

Everything bubbling?

Other Notes:

C.7 Replacing Parts During a Run

Replacing the Media Line

This is the only cure for media line contamination, though replacing pump tubing is usually not necessary, unless it has ruptured.

- 1) Temporarily stop the pump for the line you're replacing.
- 2) Swap out the media line end at the pump tubing connection and clamp off the old media line (metal squeeze clamp) so it doesn't leak
- 3) Quickly swap out the new media line, and connect it to a new sterile media needle.
- 4) Spray the top of the cork with ethanol and insert the new media needle. Remove the old needle safely and dispose in sharps bin.
- 5) When you're swapping in new line, make sure to keep the parts that were foiled as sterile as possible, and take the foil off at the last possible moment.
- 6) Turn the pump back on.
- 7) Rinse the old media line thoroughly using a syringe of RO water to force the water back and forth in the tube. This should dislodge any cells, but if the strain was sticky, the line may need to be tossed.

Replacing Only the Pump Tubing of the Media Line

This is usually only necessary if the pump tubing has ruptured. Replacing one piece of pump tubing in the array may cause that vessel's dilution rate to be higher than the others. Unless the whole set of pump tubing is new, adjust the occlusion knob of that cartridge toward the + to compensate (+ means more occlusion, and so less flow). As the tubing is used, it gets stretched out and less efficient.

- 1) Clamp off the old media line between the pump and the carboy to stop it from leaking.
- 2) Gather the replacement tubing, which is a length of pump tubing with red hub blunt needles in the ends, with the hubs foiled and autoclaved.
- 3) Disconnect the old tube from one hub first, then the other, holding the 2 open ends in one hand. Be careful not to touch the open ends.
- 4) Attach the sterile hubs of the red connectors to the media line on both ends.

Replacing the effluent line

- 1) Do not clamp off the effluent line at the vessel, as this will cause backpressure. You will turn off the air to the vessel for just a moment while you make the replacement.

- 2) Prepare the replacement line, including a fresh luer connector for the needle, with tubing connecting to a new effluent cork. The ends should be foiled and the whole thing autoclaved.
- 3) turn off the air at the manifold and unscrew the luer connector from the top of the effluent needle. Take care not to move the needle up or down, which would change the volume.
- 4) Remove the luer connector from foil and immediately attach to the needle hub on the vessel.
- 4) Unwrap the effluent cork and put it on the effluent bottle.
- 5) For the used line, separate the line and cork and thoroughly wash both with RO water or ddH₂O.

Replacing an air filter

- 1) If you've tried everything but your vessel still isn't bubbling, the small air filter on the manifold may have gotten wet.
- 2) Get a sterile replacement air filter (foiled and autoclaved ahead).
- 3) Examine the foiled filter so you can tell where the smaller outlet is. This is the one you want to keep sterile.
- 4) Remove the top side of the old filter from the manifold, then with the sterile one at the ready, remove the filter from the tubing and quickly plug the small side of the new one into the ministat's air line.

Appendix D: Troubleshooting

General	All bubbling, heat block, or pump has stopped	<ol style="list-style-type: none"> 1) <u>Be careful</u>. Unplug everything from the wall outlet. There may be a DANGEROUS electrical problem due to spilled liquid conducting electricity. 2) If there is no spill in or around the surge protector or other electrical components, check that the heat blocks aren't plugged into the same outlet. They draw the most power, and may overload the circuit if plugged in together, or if you're in the cold room. In the cold room, you might want each one to have a completely dedicated outlet, with pump and bubblers plugged into a third outlet.
	One tube or row of tubes isn't bubbling	<ol style="list-style-type: none"> 1) Make sure all the ports on the stopper(s) are tight 2) Make sure the 4 port manifold is not allowing air to escape 3) Pull air through the effluent line(s) with a syringe as it may be blocked 4) Make sure the air filter isn't wet, and replace it with a sterile one if it is. 5) Replace the air needle with a new, sterile air needle. Spray the surface of the cork with ethanol before inserting the new needle.
	My carboy is contaminated!	<ol style="list-style-type: none"> 1) Assess the damage. 2) What type of contaminant is it and has it obviously spread through the media? 3) If it has spread through the media, the experiment is over regardless of whether it is a competition or evolution. 4) If the contaminant is small and localized, it might be possible to continue: <ol style="list-style-type: none"> a) Clamp off and replace the media immediately. b) Plate 100 μL of the media (continued)

		<p>from the carboy near the contaminant, and 100 μL of media from the carboy far from the contaminant</p> <p>c) If the contaminant is not easily distinguishable from yeast on plating, the experiment is over.</p> <p>d) On next sampling, choose several vessels from the potentially contaminated blocks and plate 100 μL of culture to check for evidence of the contaminant.</p> <p>5) After disconnecting the contaminated carboy, proceed to the protocol for disinfecting a carboy in the Cleanup section.</p>
Making media	Something went wrong during media filtration	If you think it may be contaminated, start over with a fresh filter, bottle, and carboy. If you remove the stopper from your suspect carboy, it will flow freely and you can use it in place of the mixing carboy.
	I hear a hissing sound while the media is filtering	<p>1) non-sterile air is flowing into the media carboy. You may have to re-filter your media into a new sterile carboy.</p> <p>2) There is a loose connection either at the bottle to filter interface, OR at the interface between the filter's side port and filter adapter.</p>
	I think a tiny bit of some other media may have contaminated mine	Toss it and remake. The tiniest amount of glucose rich media can significantly change the makeup of your glucose limited media (also true for each respective limitation).
	My carboy is almost empty! Is there anything I can do to get the last bit of media out while I am making more media?	Lay the carboy on its belly, so that the media pools over the media line, or if it's just a bit low, you can prop up the backside with a roll of tape.

Sampling	My effluent cork keeps popping out or the air filter on it keeps getting wet	Backpressure can cause big problems, so many in our lab use a blunt needle in the air port of the effluent cork, instead of an air filter, to avoid this problem. If air is always flowing through, contamination (including fruit flies) is less likely to get in.
Growth	My cells aren't dense after 30h	<ol style="list-style-type: none"> 1) Double check the strain 2) If it's just one or 2 you can move forward with the ones that are dense, and wait another day. 3) If none of them are grown up, check the temp on the heat block, and revisit your media recipe. If those things look ok, you can re-inoculate them.
	They were doing fine, but then washed out.	<ol style="list-style-type: none"> 1) The media lines can become contaminated. <ol style="list-style-type: none"> a) Check the media port tubing above the vessel, and the bottom portion of hanging loops for yeast accumulation. If you see yeast in the line, proceed to "Contamination of the media line" below. b) Check the carboy for yeast or other contamination. 2) Double check the strain. 3) If none of them are grown up, check the temp on the heat block, and revisit your media recipe. Wait another day to see if they become dense, then proceed if they do. 4) If only a few aren't grown up, proceed with the rest.

(continued on next page)

<p>Growth (continued)</p>	<p>Contamination of the media line</p> <p>NOTE: This commonly occurs, and if caught early, can be removed. Therefore, it is wise to have sterile replacement parts on hand at the beginning of an experiment, especially an evolution.</p>	<ol style="list-style-type: none"> 1) This can be detected through close inspection of the media line, around the needle hub, and in the tubing itself. It is important to investigate the extent of the contamination, to be sure other vessels don't become compromised. <ol style="list-style-type: none"> a) If the media needle's hub is occupied, note these so you can keep close watch. Replace the line if it gets severe. b) Depending on where the yeast is growing, replacing all or part of the media line is the only cure. c) If it is isolated, and adjacent media lines are not also contaminated, replace the entire media line for that vessel, including pump tubing and needle, and keep watch for recurrence. 2) If you have extensive contamination, such that you see yeast near the tops of the media line, or in the carboy itself, the experiment is over. If a yeast from the line made it into adjacent vessels, you would never be able to tell the contaminant from your strain of interest, and all recent data would be suspect.
	<p>Contamination in the bubbler flasks</p>	<p>This usually looks like white ropy scum in the water. The best option is to prepare a new bubbler flask to swap out. However, you may be able to continue instead:</p> <ol style="list-style-type: none"> 1) Remove the stopper from the flask to remove it enough to dump it out into a nearby beaker. 2) Rinse vigorously twice with ddH₂O or MilliQ water, or until there is no visible evidence of the contaminant. 3) Fill with clean water, and reconnect. Monitor for recurrence of the contaminant.

(continued on next page)

Growth (continued)	Contamination of the effluent line	<p>1) If the invasion is minor, a quick squeeze or “massage” of the effluent line around the clump often resolves the problem. Be careful to not pinch off the line, as it will create backpressure.</p> <p>2) If the invasion is major, replace the line including the cork.</p>
	Flocculation/clumping of cells in the ministat vessel	<ul style="list-style-type: none"> • No fix – just let it keep going and manage line invasion by replacing affected parts. • This is most frequently observed on the sides of the vessel near the air needle output. • We observe this 15-35% of the time, typically at 200-300 generations. • Try a <i>flo1</i> strain.
High or low volumes	Unexpectedly low effluent levels	<p>1) Is your effluent line running straight or does it have a kink? If it has a kink, try securing it in better position by twist-tying it to the shelf above the vessel.</p> <p>2) Are there bubbles in your media line? There may be a loose connection.</p> <p>3) Is there a puddle under the pump? Check the cartridge for damaged pump tubing.</p> <p>4) Try replacing the media line including pump tubing.</p>
	Ministat volume is unexpectedly high	<p>1) Check to be sure air is not escaping from the stopper.</p> <p>a) Spray some ethanol around suspected leaky areas and look for bubbles.</p> <p>b) Tighten all hubs.</p> <p>2) Check for a clogged effluent line. Lack of bubbling may also occur, as air and media can’t escape.</p> <p>a) Check for kinks in the line.</p>

		<p>b) Use a syringe to pull media through the effluent line, at the effluent cork.</p> <p>c) Replace the effluent line if necessary.</p>
High or low volumes (continued)	ministat volume is unexpectedly low	<p>This is usually due to an issue with your media line, but can also be an indicator of unchecked contamination in the media line.</p> <p>1) Check to make sure that your media needle isn't running directly into your effluent needle; reposition if necessary.</p> <p>2) Check the pump tubing for that vessel. Pop out the cartridge, and inspect the tubing for wetness/rupture. Replace with sterile pump tubing if ruptured.</p> <p>3) Try replacing the media needle.</p> <p>4) If you have major contamination such that the media line is blocked, you've probably had some backflow, and the experiment is likely over. Check the "contaminated media line" part of this troubleshooting section.</p>