

# Dunham Lab Chemostat Manual

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This comprehensive manual covers the entire process of running a chemostat, including media recipes, chemostat setup, inoculation, data acquisition and storage, daily monitoring, harvests for DNA and RNA, and data analysis. Although it was written with our blown-glass chemostats in mind, many of the procedures are general, and the principles could be applied to other systems. If you have edits, additions, or suggestions for the manual, please email me at [maitreya@uw.edu](mailto:maitreya@uw.edu)

Please feel free to point other people to these instructions. Also, I would appreciate the citation if you use any of this information in a publication or talk.

Visit <http://dunham.gs.washington.edu> for the most recent updates to the manual and for my other protocols on microarrays and yeast genetics. Also available is the old version of the chemostat manual, which covers use of ATR Sixfors Fermenters. That portion of the manual has forked and is now maintained by the Botstein lab.

Thanks to Matt Brauer, my long-time companion in the chemostat lab, for help developing these protocols. Also thanks to Frank Rosenzweig who taught me how to run my first glass-blown chemostats. Many of the protocols were influenced by his chemostat aesthetic. Finally, many members of the Botstein and Dunham labs contributed improvements.

Edits since last version: My new set up at the University of Washington employs blown glass chemostats, and so this manual has been updated to focus on their use and care.

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## **Planning the experiment**

The first thing to do is design your experiment. You need to choose a strain, a limitation, a limiting nutrient concentration, and a dilution rate. Figure out how much media you'll need, and arrange to use the chemostats. Don't try to sign up now, and figure it out later, because you'll end up regretting it. Take the time to read through the manual, and understand the full experiment before you sign up.

When you've read through the manual, arrange to speak with someone in the lab who is practiced with the chemostats, so they can help you. For your first chemostat experiment, plan to run no more than 4 chemostats, and coordinate with someone for setting up, starting the run, and sampling. Then you'll be pretty much on your own until the end of the experiment, when you'll want someone to go over the clean up/ recovery stage.

## **Signup**

Once you've got an experiment planned, sign up on the Chemostat sign-up calendar. That way, other people can plan their own chemostat use. List your name, which chemostats you'll want to use, and for how long. Include time for preparation and clean up in your timeline.

## 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:

Background	Mating type	AKA	DBY number
FY	a/alpha	FY4xFY5	YMD 132
FY	a	FY4	DBY 11069
FY	alpha	FY5	DBY 11070
CEN.PK	a/alpha		DBY 9500
CEN.PK	a		DBY 11092
CEN.PK	alpha		DBY 11093

**Table 1. Yeast strains for the chemostat.**

The FY haploid strains are from Fred Winston. The CEN.PK diploid, DBY9500, is direct from Peter Kotter. The FY diploid and the CEN.PK haploids were derived in my lab by mating and dissection, respectively.

Both strain backgrounds grow well in glucose, phosphate, and sulfur limitation. Oddly, CEN.PK seems to behave better than FY in nitrogen limitation. All S288C derivatives have a Ty element in *HAP1* that decreases its activity. We now also have a *HAP1*<sup>+</sup> derivative of FY from Fred Winston's lab. CEN.PK strains have a mutation in *CYR1*. Also, *LEU2* may not be in the usual location.

You can, of course, use other strains, and we have. The biggest unknown danger of a new strain is its flocculation capacity. Because they can stick to the vessel and sink to the bottom to avoid being diluted out, clumpers are selected for in the chemostat. In addition to complicating cell count data, they also make it very difficult to understand what's going on in terms of selection pressure, clonal selection, etc., so the chemostat run is effectively over once they appear. For CEN.PK and FY, I've gotten clumpers occasionally ~400 generations into the evolutions. Many lab strains carry knock outs of several FLO genes, making the transition to flocculation difficult (although some of the knock out mutations, like the

one in *FLO8* in S288C, are point mutations that may revert). Other strains, such as SK1, frequently flocculate, making them next to useless in the chemostat. When using a new strain, be particularly vigilant about frequently checking the culture with the microscope before and after sonication. If sonication effectively breaks up the clumps, it's probably not a serious enough problem to halt the chemostat, although you should make a note of the phenotype in your log. For short-term cultures, this is not nearly as much of a problem, so you have a wider variety of strains available.

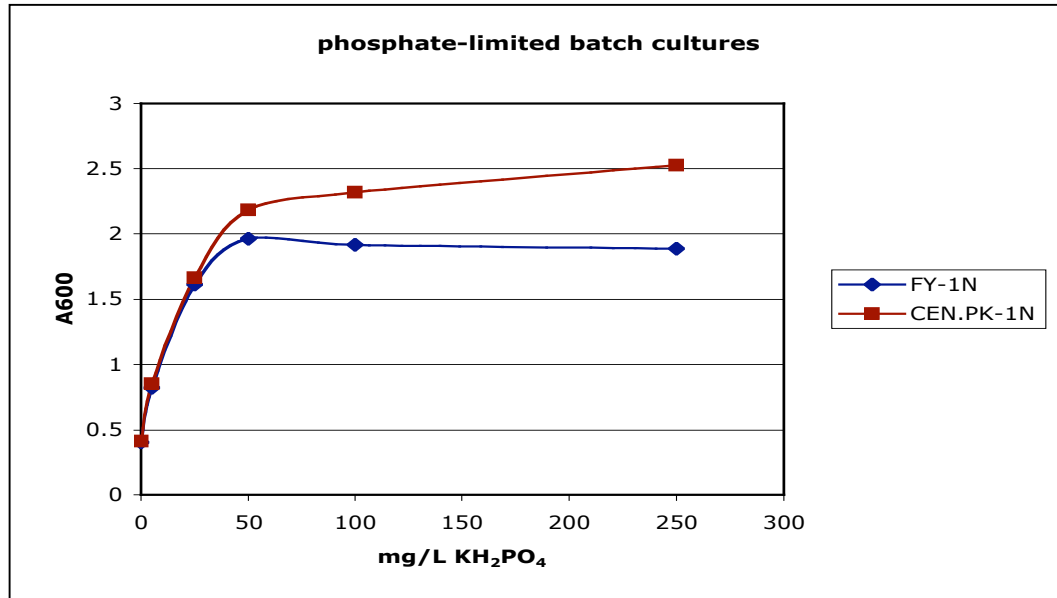
If you do have to use an auxotroph, be very careful with the supplements you add. For example, you can't use adenine sulfate with sulfur limitations. You want to make sure the culture does not become limited for the additive, but you don't want to add so much excess that the culture eats the additive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

## 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. Nitrogen limitation does not work well with FY in my hands, though others have had more success.

If you are not using one of the standard recipe/strain combinations 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. Spin it down and resuspend at a 100X dilution in chemostat media without any limiting nutrient. Aliquot equal volumes into a series of appropriate volume shake flasks that contain different quantities of the limiting nutrient. Be careful that the volumes of limiting nutrient solution are the same in all the flasks so you don't get different dilution factors. You may want to make your media 1.1X and bring them to 1X with the limiting nutrient solution. Let these flasks shake at 30C for a couple of days or until the density stabilizes. You want

them to be completely in stationary phase. Measure the densities. If you graph the concentration of limiting nutrient vs. the final densities of the cultures, you should get a plot with a linear range, a nonlinear range, and a plateau. You want to stay in the linear range. Here's representative data from one of the experiments for phosphate limitation:



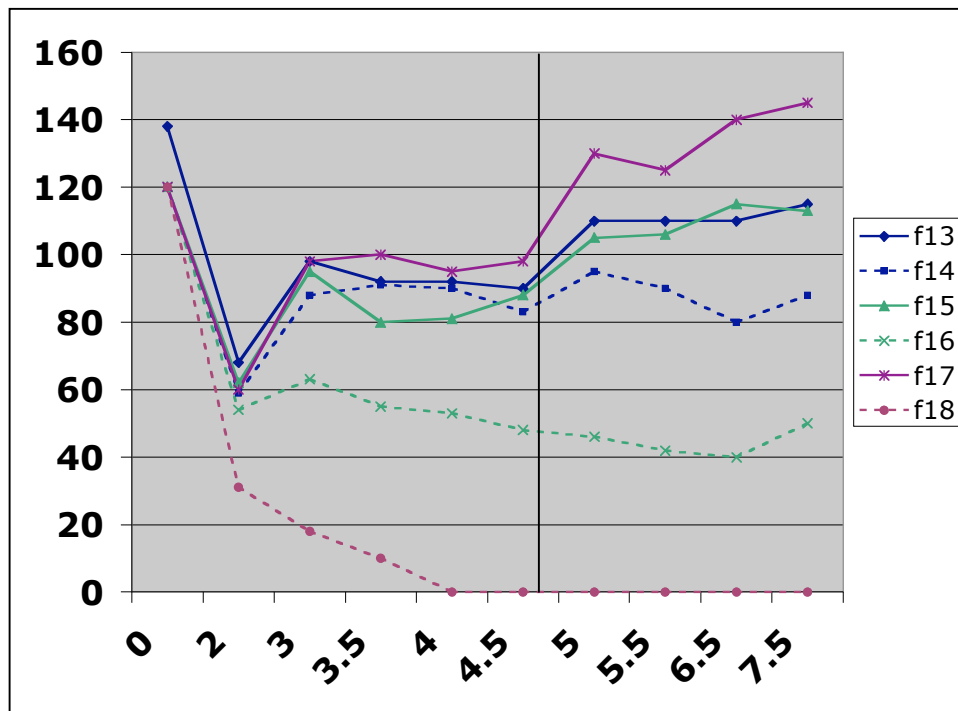
**Figure 1. Testing the limitation in batch.**

Note that the Y-intercept is not zero. That's probably from nutrient the yeast stored from the overnight culture, which was in rich media in this experiment. Take that into account when you decide on a chemostat media concentration that will yield your desired density. We aim to run our evolutions starting from a population size of  $3\text{-}5 \times 10^7$  cells/ml. To measure the final density more exactly, once you have a good idea of what concentration is limiting, grow the first overnight in limiting chemostat media. The cells will use up all the limiting nutrient and the zero will really be zero.

Although the batch results generally match the chemostat quite well, make sure to test the concentration in the chemostat to double check that it's really limiting. As a cautionary tale, we lost over a year of work from failing to properly do this control: a typo in the phosphate limitation media recipe resulted in a low potassium concentration, an error that had no effect on the batch culture results yet profoundly affected the chemostat cultures. To convince yourself that your chemostats are limited by what you think they are, run 2 chemostats to steady state. Once they've hit steady state, in one chemostat, increase the nominal limiting nutrient in the feed media by 50% and watch for an increase in density. In the other, increase the sugar, vitamins and metals by 50%

and see if the density changes. If you are truly limited only for the limiting nutrient, you will see ~50% increase in density in the first one but no change in density in the second one. Density is actually not the greatest indicator since it's really yield you're interested in, but it usually does track pretty well. Klett seems to work the best as a surrogate for yield.

In this example from David Hess, six cultures were grown to steady state with 20 mg/L potassium phosphate. At the indicated point, the feed media was switched to 30 mg/L potassium phosphate. F13, F15, and F17 are all limited by phosphate. F14 and F16 are not. F18 washed out.



**Figure 2. Testing the limitation in the chemostat.**

Also note that not all strains behave exactly the same. In some circumstances, it may be easier to run two separate chemostats, each with a different (supposedly limiting) concentration of the nutrient. Then compare the steady state densities. This experimental design is particularly useful when the strains take a long time to hit steady state and so evolution is a concern.

## Dilution Rate

We most typically use a dilution rate of 0.17 (+/-) 0.01 chemostat

volumes per hour. The Paquin and Adams experiments were all at 0.2 chemostat volumes per hour. You will know if you set the dilution rate too high (i.e., above the maximal growth rate) because your culture will wash out. In glucose limitation, there is a critical dilution rate where the culture switches from respirofermentative growth to primarily fermentative growth. Growth-rate dependent changes have been studied in great detail by the Botstein, Oliver, and Regenberg labs.

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  $\text{hr}^{-1}$ . It is also sometimes called omega.



## Chemostat media

Chemostat media has 4 components that need to be made separately: salts, metals, vitamins, and carbon/sugar.

For each batch of media, you will prepare a carboy, thaw the 1000X vitamins, make 10X salts, and make 10X carbon source. You'll combine these with the pre-made metals and vitamins, and top off to 10L with glass distilled water. It all gets mixed together in a non-sterile "mixing" carboy before it gets filtered into a sterile carboy.

These media recipes come from Julian Adams via Frank Rosenzweig with further modification by me. The glucose limitation recipe is exactly per Adams. I modified the glucose limitation recipe for phosphate, sulfur, and nitrogen limitation. In general, I tried to keep all ions at the same molarity where possible. The Adams version of the phosphate limitation recipe uses the salts at 0.25X to limit the effects of phosphate contamination from the other salts, but I always use 1X salts for everything. You can only get away with this if you use really pure chemicals.

The Adams recipe handed down to me also had a typo in it, which we didn't catch until 2005. The potassium concentration was 10X lower than it should've been. **If you used my phosphate-limited media recipe prior to December 2005, that recipe was wrong!** I'm very sorry about that.

The uracil and leucine limitation recipes use the Adams glucose limitation base plus limiting concentrations worked out by Alok Saldanha.

## **Salts**

Salts can be made as 10X stocks in glass distilled water and kept at room temperature until use. Nonsterile 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. It is crucial that limiting nutrient concentration not vary due to contamination in other salts.

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

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

10X salts for phosphate limitation (1 L)

1 g	calcium chloride·2H <sub>2</sub> O
1 g	sodium chloride
5 g	magnesium sulfate·7H <sub>2</sub> O
50 g	ammonium sulfate
10 g	potassium chloride
100 mg	potassium phosphate monobasic (to 10 mg/L final)

10X salts for sulfur limitation (1 L)

1 g	calcium chloride·2H <sub>2</sub> O
1 g	sodium chloride
4.12 g	magnesium chloride·6H <sub>2</sub> O
40.5 g	ammonium chloride
10 g	potassium phosphate monobasic
30 mg	ammonium sulfate (to 3 mg/L final)

10X salts for nitrogen limitation (1 L)

1 g	calcium chloride·2H <sub>2</sub> O
1 g	sodium chloride
5 g	magnesium sulfate·7H <sub>2</sub> O
10 g	potassium phosphate monobasic
400 mg	ammonium sulfate (to 40 mg/L final)

## Additives

Additives include the carbon source, vitamins, and metals. The following recipes are the standard limitations. Additives should be COMPLETELY dissolved before mixing and filtering.

Additives for sulfur, phosphate, and nitrogen limitations  
(for 10 L media)

50 g	glucose (to 0.5% final)
10 ml	1000X vitamins
10 ml	1000X metals

Glucose (dextrose) limitation additives  
(for 10 L media)

8 g	glucose (to 0.08% final)
10 ml	1000X vitamins
10 ml	1000X metals

Leucine limitation additives  
(for 10 L media)

150 mg	leucine (to 15 mg/L final)
50 g	glucose (to 0.5% final)
10 ml	1000X vitamins
10 ml	1000X metals

Uracil limitation additives  
(for 10 L media)

50 mg	2 mg/ml uracil (to 5 mg/L final)
50 g	glucose (to 0.5% final)
10 ml	1000X vitamins
10 ml	1000X metals

## Metals

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

### 1000X metals

(1 L)

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

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

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

## Vitamins

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

### 1000X Vitamins

(1 L)

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

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

## Setting up a run

It takes 5 days to run a standard short-term chemostat from inoculation to harvest, plus a few days of preparation. A convenient schedule might be:

Thursday	Start 10X salts dissolving ON. Thaw vitamins. Prep carboys and chemostats.
Friday	Autoclave carboys and chemostats. Filter media. Set up and fill chemostats. Start inoculum.
Saturday:	Inoculate.
Sunday:	Start pumps.
Monday:	Measure effluent and check dilution rate.
Tuesday:	Approaching steady state, full sampling.
Wednesday:	At or near steady state, full sampling, ready to harvest and take down chemostats if stabilized.
Thursday and Friday:	At or near steady state, full sampling, ready to harvest and take down chemostats.
Friday:	Finish cleanup of Carboys, etc.

The scheme outlined above is 10-20 generations. Attempts with fewer generations do not reliably hit steady state. However, at more than ~25 generations, you have evolution to worry about. Some strains take longer than others to reach steady state. Do sufficient sampling to convince yourself that the chemostat really is at steady state before you harvest.

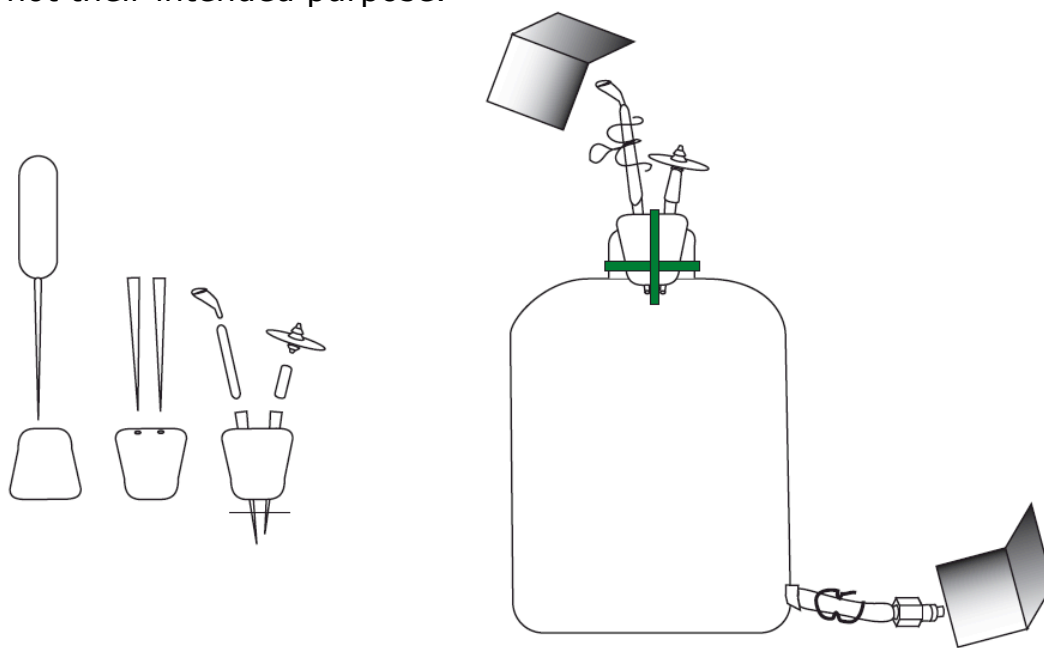
## Mixing and Filtering the media

We use filter-sterilization. You'll autoclave an empty carboy with the appropriate fittings, make up the media in a 'mixing' carboy and then filter it into your sterile carboy as described below.

## Prepping a new Carboy

The media vessel is made up of the 10L glass carboy with a bottom spout, and a Cork Assembly. The bottom spout is fitted with 1/4" tubing, with 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 chemostat. Use of these connectors allows the carboy to be autoclaved separately from the chemostat and its attached media line. 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 filtered.

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 $\mu$ 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 not to injure yourself or the rigid tubing. Whatever you use, it should be monitored for cracks and may occasionally need to be replaced. Then attach a short piece of 1/4" tubing (about 4 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 7 inches), and attach a filter adapter to it. We use Corning filters, and the adapters come with them. They can be autoclaved several times, but should be monitored for cracking, as this is not their intended purpose.



**Figure 3. Setting up the carboy.**

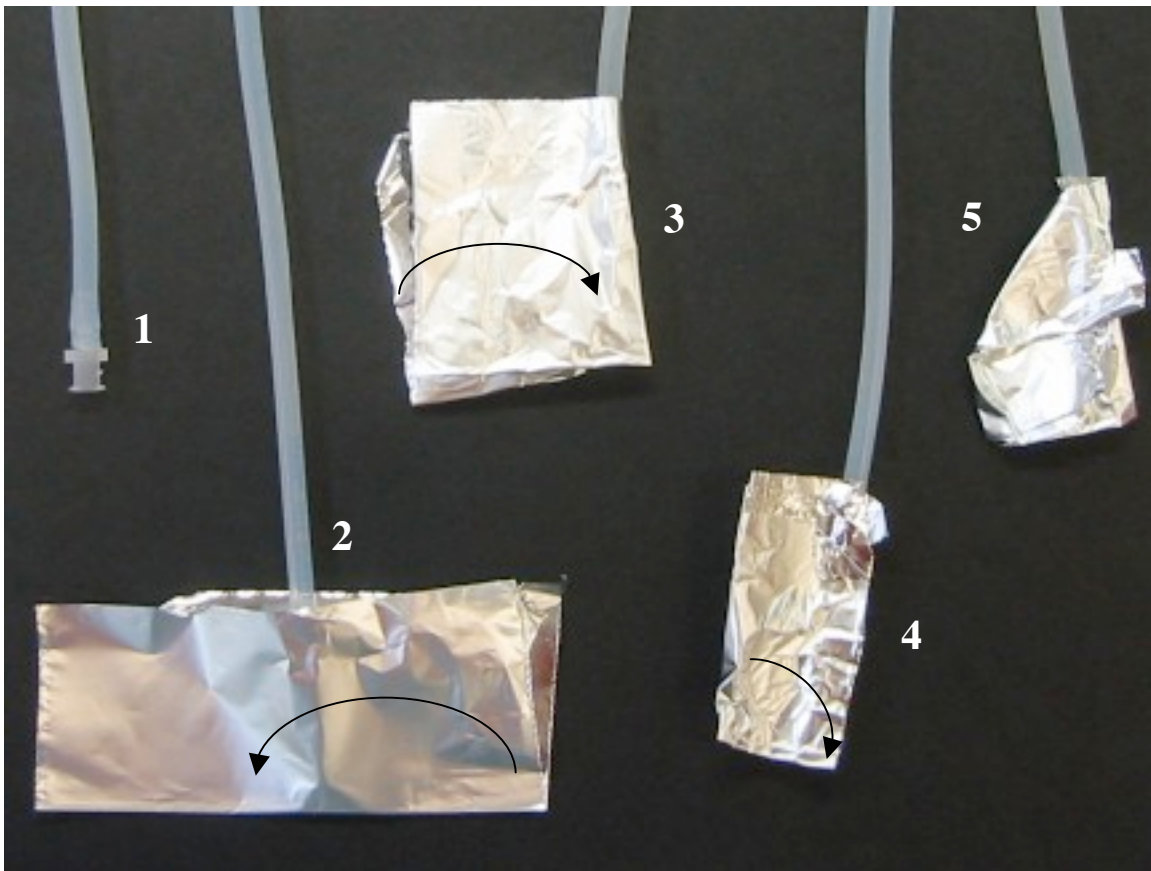


## Foil Origami

Now let's talk about Origami. One nice way to wrap the tubing ends, taught to me by Frank Rosenzweig, is diagrammed below. It is secure, yet can be undone with one hand.

1. Fold a 4 in x 4 in piece of foil in half top-to-bottom and insert the tubing.
2. Fold it in half again left-to-right.
3. Fold the flap back in on itself so the edges meet the tubing.
4. Fold the top corner on the diagonal to lock the end in the packet.
5. If desired, apply autoclave tape to the foil.

When you go to access the connector, you can unfold back to step 2 or 3 with the foil loosely covering the end. It will be protected while you arrange the other piece of tubing.



**Figure 4. Foil Origami**

## **Preparing a Carboy for Media**

Make sure to calculate how much media you'll need, and include additional carboy connectors as needed. Keep in mind that you can NEVER put more than 8 carboys on the carboy shelf at one time.

To prepare the empty carboy for autoclaving, start by attaching a male quick connector to the bottom tube. Then pipet 20ml of water into your clean carboy. This allows for steam sterilization of the interior of the carboy. Then 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. 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.

Autoclave on fluid cycle, for 20 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 20 minutes of sterilization time, because of all the plastic components that we expect to survive multiple sterilization cycles.

## **Fetching Water**

You'll need to fetch enough water for your media, from the 2<sup>nd</sup> floor distiller. Take a cart, and all the water hauling carboys you'll need. You should plan on making no more than 40L per day. We don't have the water hauling vessels to transport much more than that at a time, and if you go back for more later in the same day, you will probably find that the water is too warm to use, since the distiller is replenishing the volume you took. Always use room temperature water to make media. If you do have to get more on the same day, the spigot on the right may have cooler water than the one on the left. Quickly touch the glass to check. Also, the spigot on the right dispenses at twice the rate of the one on the left, so you can fill 2 carboys from the right spigot in the same time it takes the left spigot to fill one. Use the plastic carboys first, and if you have to, use one of the glass ones.

Never use hot water to make your media. If you have to wait a day to allow the water to cool, filter sterilize the 10X sugar. The salts can sit around for a week without being sterilized.

## **Mixing the Media**

Completely dissolve your 10X salts (plus limiting nutrient), and 10X carbon source in separate 2L beakers. Some salts need to dissolve overnight. When these components are dissolved, and adjusted to the proper volume (according to how many carboys you're making ex. 3L of 10X for 3 10L carboys), measure 1 L each of the 10X components in a 1 L glass graduated cylinder, and pour it into a glass 4L graduated cylinder. Then pipet 10ml each, of the 1000X Vitamins (thawed in advance) and the 1000X Metals into the 4 L cylinder. At this point you should have 2 Liters and 20 mls in the 4 L cylinder. Top to 4 L with room temperature glass distilled water, and pour into the mixing carboy. The 4L cylinder can be unwieldy, so use one hand at the top to hold it steady, and the other hand to lift the bottom. You'll have to pour slowly, since the opening of the carboy is relatively small. Refill the cylinder 1.5 times, to reach a total of 10L in the mixing carboy. Turn on the large stirplate to low, so that the large stirbar is not out of control. Stir until thoroughly mixed, at least 5 minutes, and proceed to filtering the media.

## **Filtering the Media**

The media will be filtered into the cooled sterile carboy by manipulating a 1L bottle top filter, attached to a wide mouth 100ml bottle (or a larger one if no 100ml bottles are available). The filter plug will be removed from the usual vacuum attachment with sterile tweezers so that it can instead serve to funnel sterile filtered media into the carboy. The vacuum will be attached to the air vent on the carboy. Filtering 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.

So gather the following:

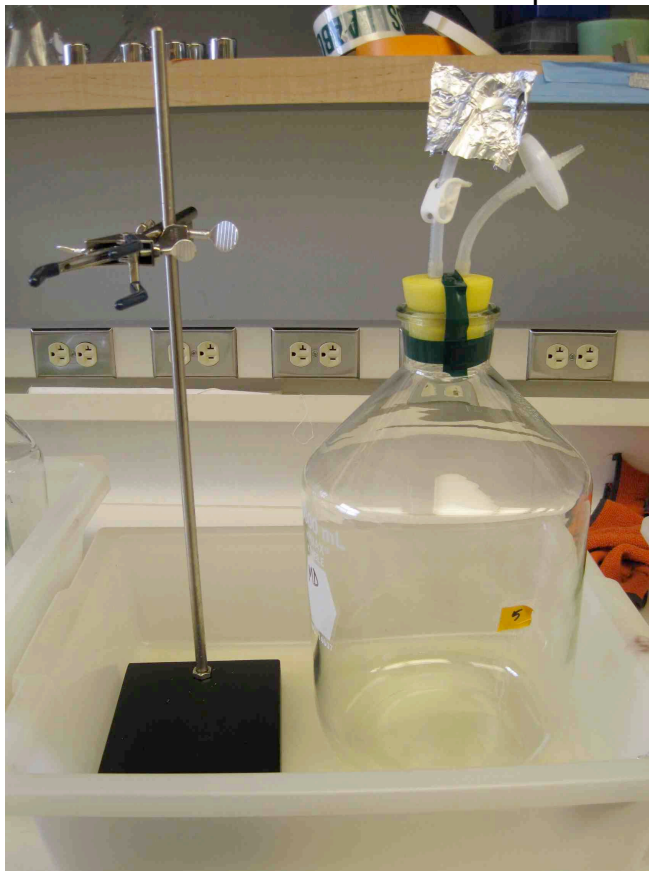
- 1 10 L sterile glass carboy (ambient temperature), labeled and sterilized with plastic piece for filter attachment foiled and clamped off on top, and a male quick disconnect outlet foiled and clamped on the bottom
- 1 10 L non-sterile glass 'mixing' carboy, with a large stirbar, and a

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



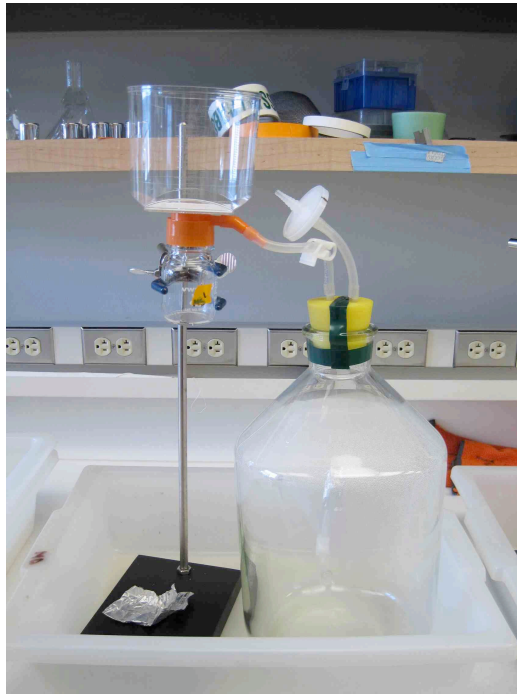
**Figure 5. Getting ready to filter: The Carboy.**

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



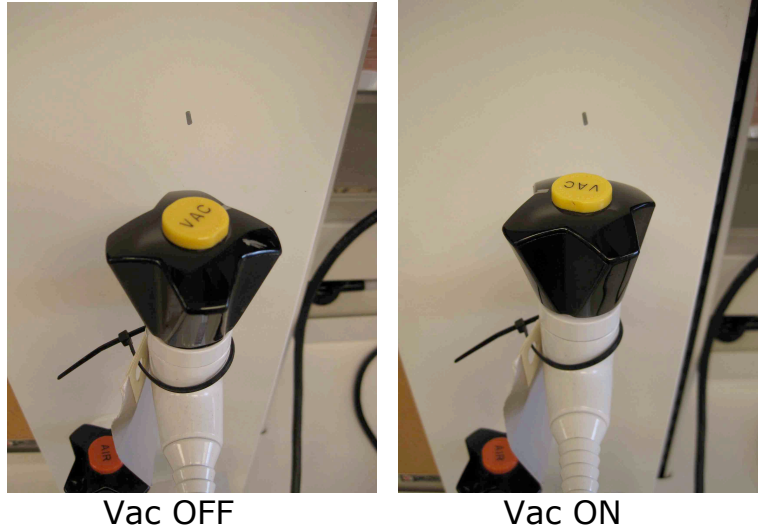
**Figure 6. Setting up the filter.**

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



**Figure 7. Final assembly of filter set up.**

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



**Figure 8. Turn Vacuum on LOW**

12. Turn on the vacuum (Only half a turn!) and unclamp the output hose from the mixing carboy. You can adjust the large clip to constrict the flow if necessary (2-4 clicks seems to work well). The filter should always be covered with media throughout this process.
13. Once the filter cup has started filling, 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 into the carboy. Make sure vacuum lines aren't clamped.
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 (see Disaster Index). Don't turn the vacuum on more than a half turn of the knob!





**Figure 9. During filtration, it's normal for the cork to get *slightly* sucked in.**

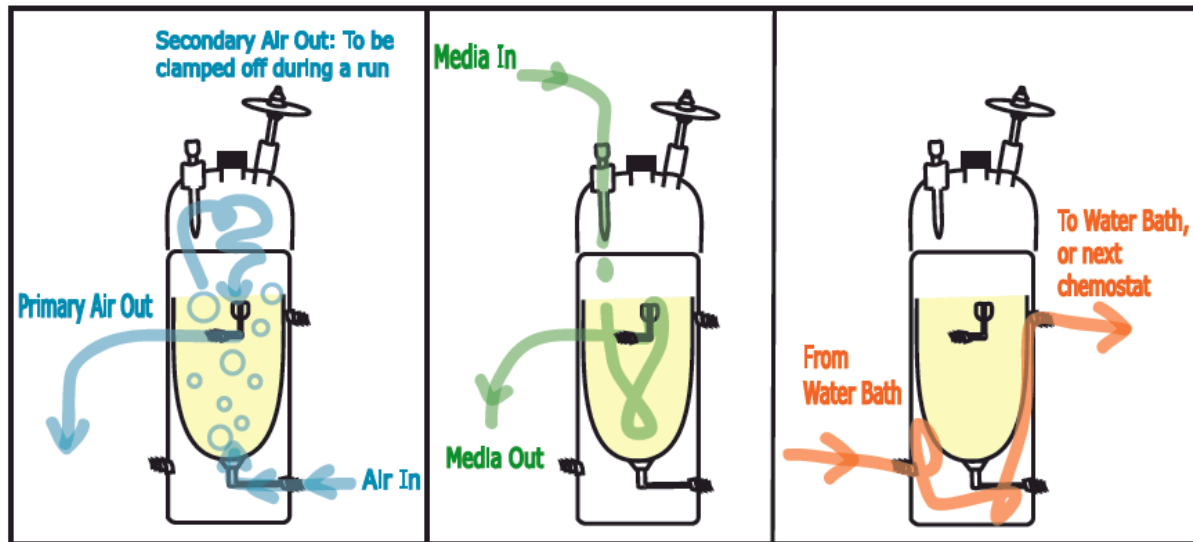
15. When the mixing carboy gets close to the bottom, turn off the stir plate, and tilt the carboy toward the outlet tube until it is drained.
16. When the media is all filtered, unclamp the 100ml bottle, and tilt it toward its outlet, so that media runs into the carboy, and you have some headspace in the bottle.
17. Make sure there is no media in the tube running from the filter to the carboy, and clamp it tightly with a metal clamp. Then turn off the vacuum, and slowly release the vacuum by removing the cork from the trap.
18. Detach the filter and bottle from the carboy's media port. Keeping sterility, cap the bottle. Then toss the filter, and re-cover the media tube with foil.

Move the carboy to the shelf above the chemostats (ask for help if needed). Place the 100mL bottle of media in the 30C incubator, and watch it for a couple of days, to see if any of your carboys may be contaminated. Rinse the mixing carboy, along with any tubing that you used, and the 4L graduated cylinder with DI water, six times each, no soap or bleach, ever.

**NEVER** put more than 8 full carboys on the carboy shelf!



## About the Chemostats



**Figure 10. The three systems of a chemostat.**

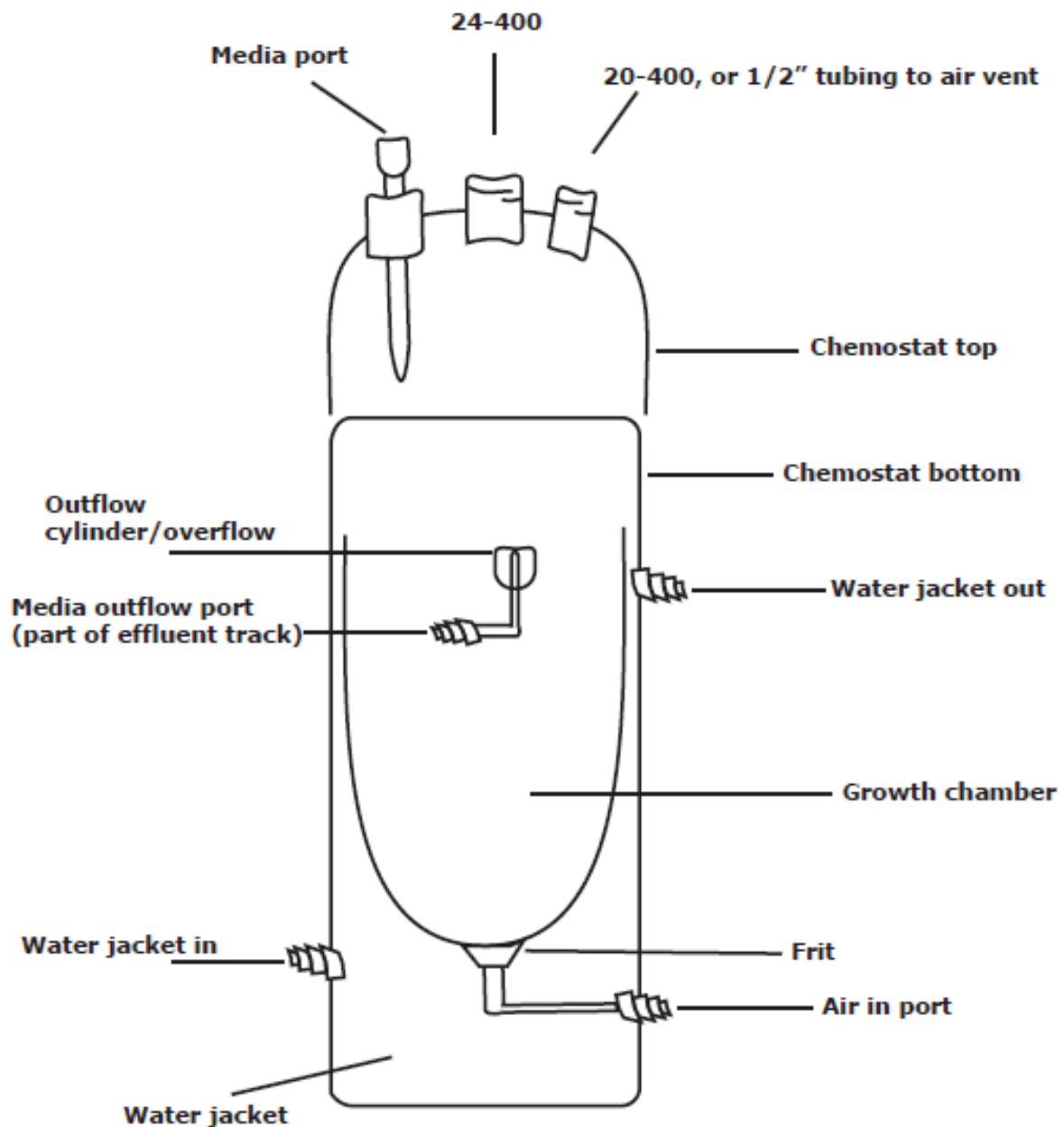
Our chemostats are custom made for us by a scientific glass blower. They require 3 systems functioning together to work properly. They are diagrammed here as Air, Media, and Water jacket, respectively.

Air is pumped from an aquarium pump, through a gas washing bottle, which has a coarse glass frit that disperses the air stream into bubbles within the chemostat. These bubbles not only aerate the media, but they also work to keep cells suspended. Additionally, the positive pressure created by the air keeps the effluent track moving quickly in the correct direction, helping to prevent contamination. The gas washing bottles serve to humidify the air, reducing evaporation. The air filter on the top of the chemostat is clamped off after autoclaving, to direct the air out through the effluent track.

Media drips into the chemostat from tubing that connects a media carboy with the media port on the chemostat. The flow rate is controlled by a peristaltic pump, which massages media through pump tubing that is part of the media line. When the media level reaches the top of the outflow cylinder, it exits by gravity flow, plus the positive pressure created by the air flow pushes the overflow media out through the effluent track.

The temperature of the inner growth chamber is regulated by a water jacket. Water is pumped through the jacket by a circulating waterbath which is set to an appropriate temperature.

**Figure 11. Anatomy of a Chemostat.**



## Setting up the Chemostats

The chemostats should always be handled with great care. They are glass, and have many parts that could easily break off if impacted or handled roughly. Replacing them is costly, in terms of both time and money. Be especially careful of the Pasteur pipet-like media port on the lid of the chemostat. It wants to break off with very little pressure.

## Prepping a new chemostat

When chemostats are brand new, we want to check them out to be sure they are functioning the way they should. Sometimes, there may be a glass seam that isn't completely sealed, and the chemostat might have to be sent back. Here are the tests we usually run for each chemostat, before it can be trusted for an experiment.

1. First, fit the chemostat's air-in and effluent-out ports with tubing (Wet the glass with water first to minimize the pressure you put on the joints). Route the effluent into a beaker, and clamp off the air line. Fill the chemostat with water, until it overflows into the beaker. Watch to be sure the water level is at the top of the outflow cylinder. Check it after 20 minutes or so, to be sure there is no change. Continuing to drain to below the top of the cylinder may indicate a leak where the outflow tube attaches to the side of the chemostat.
2. Take the top of the chemostat, and put caps on the 2 capable ports. Turn it upside down in the sink, and fill it with water. Some water might come through the media port, but you should be able to check for leaks this way.
3. Outfit the chemostat with a media line, and a proper air line (see Setting up a Chemostat for a Run, below). Foil the ends and autoclave it. Fill the chemostat with sterile media, and start the water jacket. Nothing should grow. I usually leave it up for a week before deciding it's really still sterile. If something grows, there is a leak between the water jacket and the inner chamber, or possibly in the chemostat top.

If the chemostat passes these tests, it's ready to be measured and outfitted. With the air bubbling, fill the chemostat with water and put the top on, air vent clamped off, until it finishes overflowing. Then pour the water into a graduated cylinder to measure the working volume. Chemostat volumes vary because they are hand blown. This is ok as long as the volumes (and D's) are similar enough that they can run on the same pump. The following table shows the unaltered working volumes of the chemostats we have. We are working to find a way to alter volumes, so that 4 can run per peristaltic pump.

Chemostat	Volume	Pump#
F1	165	3
F2	170	3
F4	175	3
F5	230	2
F6	230	2
F10	230	2
F11	230	2
F7	235	1
F8	240	1
F9	240	1
F3	195	Too different
F12	265	Too different

**Table 2. Working volumes of unaltered chemostats.**

When setting up a new chemostat, there are a few things to consider. Gravity can work for us, to keep our chemostats from getting contaminated. We employ glass jars to elevate the chemostats, so that media or cells cannot backtrack to the chemostat through the effluent track, resulting in the introduction of bacterial contaminants, or weird subpopulations of yeast into the chemostat. Elevating the chemostats increases the downward angle of the effluent track, reducing this risk. This downward angle should continue from the effluent jar to the 2L flask down below the chemostat table. You should secure this line to the table with tape, eliminating any upward slopes. See photo below for a view of the effluent track.

When fitting the chemostat with tubing, set the chemostat in place on the glass jar and ring stand, and fit the effluent track to an effluent jar to be sure it reaches the jar, but doesn't have a bunch of extra length to introduce weird slopes.



**Figure 12. Downward angle of effluent track prevents contamination from reverse flow.**

## **Clearing the way for aeration**

Aeration is a critical component for the properly functioning chemostat, and a problem with the aeration in a chemostat can end an experiment before it's started. Low aeration effectively increases the volume of the culture in the chemostat, causing an incorrect dilution rate. Vigorous bubbling also ensures that the culture is properly mixed. Once your chemostat is autoclaved, there are only a few things you can do without compromising sterility (See Chemostat Troubleshooting).

At the beginning and end of a run, consider the air line leading up to the chemostat's frit. It should be unobstructed, and the same length and diameter tubing as for the other chemostats. You would be wise to test it before you autoclave it.

## **Cleaning the Bubblers**

The aquarium air pumps emit some kind of oily residue over time, discoloring the tubing, and coating the inside of the gas washing bottle. We replace the water in the bubbler before every run to prevent a buildup of the residue. When you see that the tubing is beginning to be discolored, it's time to replace it. If there's oily residue in the bubbler itself, you'll need to wash out the bubblers.

1. Empty bubblers and over-fill with isopropanol. Allow to sit without bubbling one or two hours, turning air on for a few seconds, 2-4 times during that period.
2. Use a funnel to pour the isopropanol into a jug for reuse or disposal.
3. Check for oily residue, and repeat if necessary.
4. Empty, rinse several times, being sure to flush the entire track. One way to do this is with the water on LOW, connect the bubbler to the faucet, so that it fills up to the top, and spills out the other tube. Leave it flushing for a couple of minutes. Then connect the next one in the same way.
5. After flushing, dump out the water, and blow air through the same track to get the last bit of water out from behind the frit.
6. Over-fill with fresh di water. Allow to bubble overnight
7. Rinse again with di water, then empty and leave air on overnight to dry.
8. Refill with sterile distilled water.

## **Baking the frit**

The aeration in the chemostat should be vigorous, and after your run or after a frit invading strain, you may notice that the bubbles are smaller than they should be. Eventually they get to be tiny like champagne bubbles, and this will undoubtedly affect your experiment. Not only is there low dissolved gas, there is also a change in working volume, making your dilution rate wrong.

To deal with this issue, we excessively clean the frit after each run, and periodically bake the bottom half of the chemostats to incinerate the trapped cells. Remove all tape, then cut the tubing (along the length of the tubing where it is on the glass) to remove it without stressing the glass joints. Arrange to use the drying oven in the autoclave room, and bake them in metal pans without cloth or tape, for 4 hours at 300C.

## Setting up a Chemostat for a Run

Use the metal pans with fabric lining to store, transport, and autoclave the chemostats. Two chemostats fit nicely in each pan. You will quickly notice that there is no good way to lay them down, but you certainly don't want them teetering in an upright stance. The best option is to lay them down with the capped port and the air port down, and with the fragile media port up. However, when autoclaving, you must be sure that the air vent tubing is not kinked.

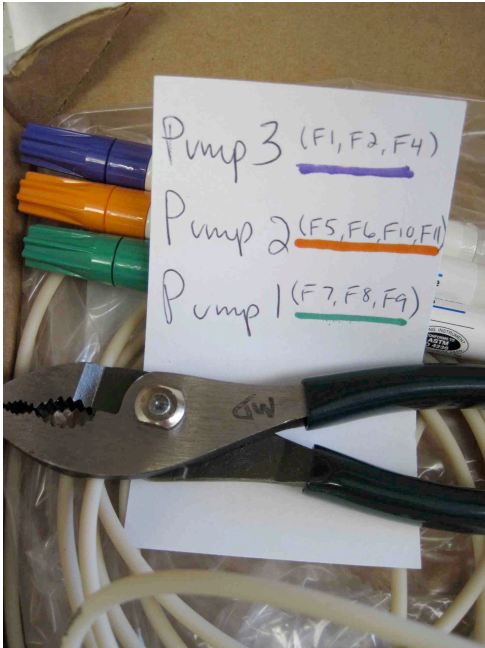
If you're running multiple chemostats, you should consider which ones to use according to which group they fall into based on their volume (see Table 2).

Before assembling the chemostat, check to be sure there is not water under the frit. If there is, hook up an aquarium pump, and turn the chemostat upside down to gently push water from under the frit. Also blow air through the chemostat's media line attached to the chemostat top. Failing to remove this water will result in wet air filters, and reduced air flow.

Put the top onto the chemostat, positioning the air vent over the outflow cylinder. This will be helpful when inoculating, and when adding media, to insure that neither go straight into the effluent.

Decide how you will arrange the media flow tubing network that will connect the carboy(s) to chemostat(s). If you want to run multiple chemostats off one media carboy, you need to make a Y connection out of scrap tubing. Make sure you put the right connectors on all the ends. If you want to run chemostats off multiple media carboys, you will need a more complicated branching connector. Make the connector piece and wrap foil over all the ends. Make a sketch of the media track to be sure you have all the components you'll need (Proper half of quick connector, pump tubing, clamps, etc.). Then, assemble the tubing network according to your set up, and blow air through it to remove any water.

Since pump tubing is reusable for several runs, we color code the tubing in groups according to which pump they were run on, and keep them twist tied together when not in use. If you're not sure about the tubing, ask the person who ran the last chemostats with that tubing, to be sure it is still good. Markers for marking tubing are in the pump tubing box. Use pliers to remove marker caps, and to fit tubing with connectors (they're kind of sharp).



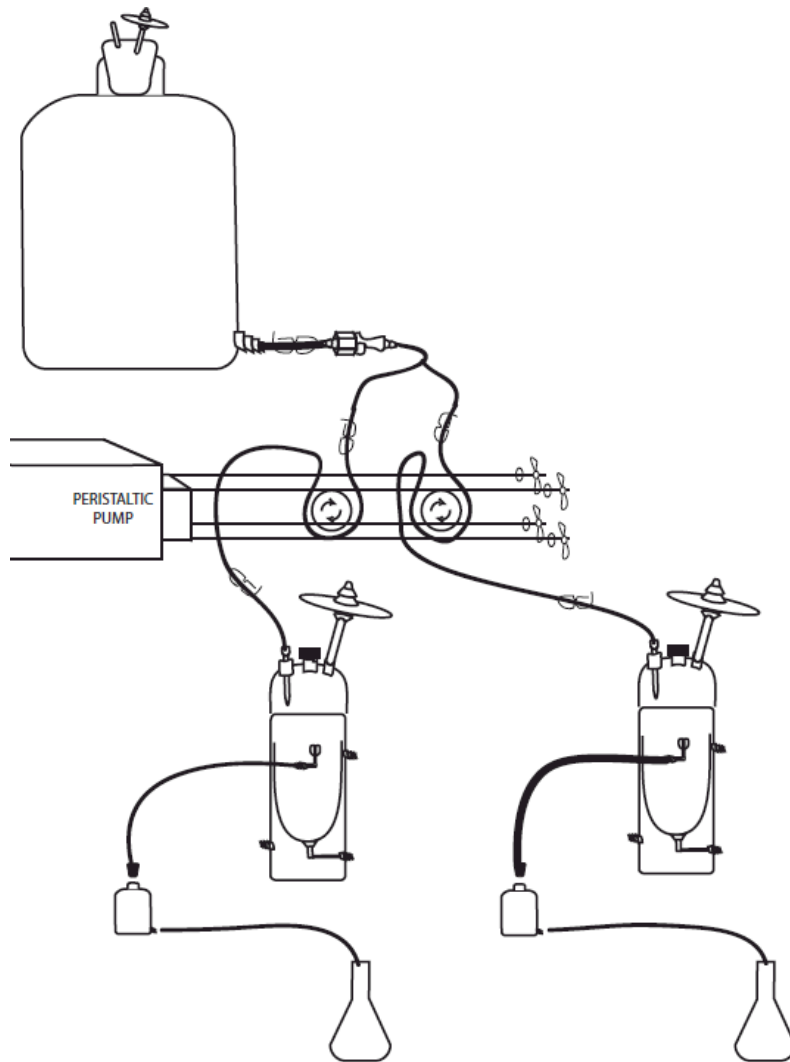
**Figure 13. Set up new pump tubing using pliers and paint pens, or re-use a set of tubing.**

Once the media line is complete and attached to the media port of the chemostat, foil the ends of the media and air tracks, double checking that the air vents and effluent corks are attached to the right ports before autoclaving. See Figure 14 as an example set up for 2 chemostats running off of one carboy.

Autoclave the chemostats and effluent jars on fluid cycle with a 20 minute sterilization time, making sure the air vents are not clamped or kinked, AND that the caps are loose. The cycle will take about an hour total, so plan to go down and get them before someone else does. If a cap has fallen off, or a cork popped out of a carboy, carefully put it back in place immediately to keep it sterile.

While the autoclave is running, check that the bubblers are filled and ready to go. If they aren't full to where the springs attach, dump them out in the sink or in a beaker, and refill with sterile distilled water. While it is difficult to keep them perfectly sterile, we should keep them as sterile as possible. Bubblers can get contaminated.





**Figure 14. Two chemostats running off of one carboy: Media flows from a 10 liter carboy, through tubing to the chemostats. Flow rate is controlled by a peristaltic pump that massages media through pump tubing. Media flows out of the chemostat into an effluent jar. The cork of the effluent jar can easily be transferred to a 50ml conical tube for sampling. The bottom spout of the effluent jar drains into a 2 L flask below, where it collects until sampling time when it is measured (to calculate the dilution rate), and discarded.**

## Filling the chemostats

After they've cooled, set the chemostats up on their pedestals, and secure them with a 3-prong clamp. Don't over-tighten, as the glass is fragile. Ask someone to help you lift the carboys onto the shelf above the chemostats. You want to set the carboy onto the shelf *gently*, which is a challenge because it is quite heavy. If you feel at all unsafe lifting the media yourself, ask someone to help.

Now you're ready to start making sterile connections.

1. Set up the effluent bottles, down in front of the chemostats, and insert the effluent cork in the top. Run the effluent jar tubing down to a sterile 2L flask below. Be sure to keep the foil in place on the flask, to minimize evaporation and contamination. This track serves as the path of least resistance for the media and air flow.
2. Now you can turn the air on. Make sure the 'bubbler' (aka gas washing bottle) is full of water up to where the springs connect, and plug in the air pump. The bubbler should bubble. Connect the output of the bubbler to the sterile inline filter attached to the chemostat.
3. Now for the most critical connection: the media. Loosen the foil on the carboy's bottom port, and on the media line's quick connect end, and then quickly but calmly connect them, being careful to not touch the very ends of the connectors. You might have to use some force to make the connection, and you'll hear a click when they're connected. It's a good idea to practice connecting these ahead of time to get a feel for it. If you should fumble and touch something that should be sterile, use ethanol to sterilize the connector, and keep an eye out for contamination during the run.
4. Now you can fill the chemostat with media. Using gravity instead of the pumps is fastest, and keeps you from having to reset the pumps. Unclamp the tubing, and in a minute, the media will begin dripping into the chemostat. Make sure it is bubbling. Once they have started filling, and you've verified that they are bubbling, you can leave it unmonitored for up to 30 minutes, but don't forget to clamp them off when they start to overflow. The whole carboy will empty onto the floor if you forget about it.
5. While the chemostat is filling, make the water jacket connections to the circulating waterbath. You can daisy-chain up to 6 chemostats together, on one waterbath, with only one degree difference between the first and the last. The image at the top of page 1 shows the daisy chain set up. If the waterbath is set at 30.5C, the coolest chemostat will be 29.5C. Double check all your

connections, and make sure the tubing is all unclamped and unkinked before you turn on the waterbath pump (the button on the front). Stand by with a 2L beaker, so you can add DI water to the reservoir as the chemostat jackets fill. The water level in the reservoir should come up to the divet, about an inch below the top of the reservoir. It takes about 2L to fill the jackets of 4 chemostats.

6. After the chemostat is filled with media and begins to overflow, you need to close the clamps above each chemostat. Then you have 2 options for the timing of the inoculation: you can wait a day to be sure there is no contamination, or if you're in a hurry, you can go ahead and inoculate.

## **Inoculation**

Once the chemostat is filled, you can inoculate. It's best to inoculate the chemostat with fresh overnight culture made from a fresh colony. Plan on 2-3 days to grow colonies streaked from a frozen glycerol stock, plus another day for the overnight culture. You can grow the overnight in YPD or in chemostat media. If you use YPD, you might want to spin down 1 ml of culture and resuspend it in water. Inoculate through the black cap on top of the chemostat. Be quick but careful to minimize risk of contamination. Be careful not to squirt the inoculum into the outflow cylinder, or onto the sides of the growth chamber. After the inoculation, there is a 24 hr waiting period before you turn the pumps on, so that the culture can grow to saturation. Wait to load the pump heads.

## **Starting the pumps**

Your chemostats should be nice and dense before you start the pumps. Usually this takes about 24 hours after inoculation, but might vary in some cases. You might want to top off the chemostat volume via gravity, before loading the pump heads, but be careful not to let the chemostats overflow more than a few drops before clamping the line and loading the pump heads.

## **Setting the Pump Rate**

The best way to be sure that the pump rate is right, is to use the same pump and pump tubing that was used in a previous run, that has shown a correct dilution rate. To help with this, pump tubing is grouped and color-coded according to which pump it has been run on. The pumps can

be difficult to adjust, and you don't want to change the rate during a run, if it is avoidable.

If you have to adjust the pump, you have a couple of options. You can use a 'flow rate tester' before the experiment starts assuming you have a similar piece of pump tubing to what you'll be using in the experiment. Our flow rate tester consists of a water supply with tubing leading through pump tubing, to a 10ml glass pipet. We use a buret clamp on a ring stand, to clamp the pipet in an upright position, and load the pump tubing into a pump head. Measure the amount of time it takes the water to fill 1ml in the glass pipet, and calculate D for the highest and lowest volume chemostat that will be on the pump. Adjust pump if necessary, and repeat until D is between .16 and .18, then time 5 mls to confirm. The other option is to wait until you've loaded all your pump heads and turned the pump on (instructions below), then see how long 5 revolutions of the pump head takes, and adjust as needed to achieve the same rate as in previous runs. Neither method is perfect, and you may still have to adjust the pump rate during the run. If it is corrected early in the experiment, it should be fine. Beware of Pump #1, as tiny adjustments have big effects.

pump	chemostat	Seconds per 5 revolutions	chemostat volume range (ml)
1	F7, F8, F9	27	235 to 240
2	F5, F6, F10, F11	28	230
3	F1, F2, F4	36	165 to 175

**Table 3. Example pump rates for chemostats**

## Loading the Pump Heads

Check the pump head to be sure it has 2 white washers, one on each post, front and back. Replace if necessary. Load the pump tubing into a pump head, and load it onto the pump. If you'll be running multiple pump heads on one pump, BE SURE to include dummy lines in the other pump heads, running water to and from a water reservoir next to the pump. Each head should be moving liquid to simulate the load the pump will be working with during the experiment. Not including these 'dummy lines' will result in an inaccurate flow rate estimate.

While loading more than one pump head per pump, be sure to orient the tubing the same way, since the pump will only be turning in one direction. It can turn in either direction, so either way is fine, but be consistent

from one head to the next.

To load the pump tubing into the pump head, hold the pump head in your left hand, with the male post facing up, and the in/out tubing track toward you. Remove the top half of the pump head, and loosely wrap the pump tubing around the center of the pumphead, entering and exiting at the tubing track. Hold the tubing loosely in place where the tubing track is, with your left thumb. Put your right thumb, (or the special tubing-loading tool) into the gap between the rollers in the pump head, and push the rollers counter-clockwise, one full turn, until the tubing is seated within the pump head. It will stretch out a little, so adjust your left 'holding' thumb so that the tubing is in the tubing track. Then, still keeping the tubing in its track, put the top half of the pump head back on. It should go on clean, with a big click, and no interference from out of place tubing. If you think the tubing might be pinched, turn the center post back and forth a couple of times, to be sure it is moving freely. If it's pinched, start over. It will sever your tubing if not loaded properly.

After all the heads are loaded with tubing, check to be sure all the pump's posts are screwed in all the way. Sometimes they are loose from the last run. Then, load the pump head onto the posts coming off the front of the pump, along with any dummies, with the tubing coming off the top of the pump heads. For each additional pump head, be careful not to disturb the previously loaded heads, as the tubing could come loose and have to be reloaded. Using a tool to turn the center post (the tubing-loading tool, a screwdriver, or scissors work), make sure each head fits together tightly, with no gaps. Secure with a washer and wingnut, not too tight. If you are loading 4 heads onto one pump, you'll also need to include a support foot, after the pump heads, but before the washer.

Check the orientation of the tubing (be sure that the 'from carboy' side of the tubing is the same for all heads on the pump. Determine the direction you want the pump to go, and place a tape arrow indicating which way to flip the switch.

## **Starting the Pump**

Once all the heads are properly loaded, they are acting as the ultimate flow control for the media, so you should open all clamps in the media line.

Now, you can flip the switch in the appropriate direction, and media will start to flow. If you didn't top off by gravity, it will take some time to fill any air gaps that are in the line. Avoid disaster by monitoring the pumps

for the first half hour after you turn them on (see Disaster Index in the Appendix if something does happen).

If you are just checking the flow rate, come back in 30 minutes to see if the pipet is filling with water yet. If it is, grab a timer, a calculator, and a notebook. Start the timer when the meniscus of the water is at one of the mL graduations on the pipet. Make sure you are eye level. When the level has raised by 1mL, calculate the D for the lowest and highest volume chemostats that will be on that pump. If the range of D's isn't between 0.16 and 0.18, adjust the pump and try again. Only very small adjustments are needed, and Pump#1 is especially touchy. Bring your patience for this one. Once you get a good 1mL measurement, confirm it by timing a 3-5ml measurement. After adjusting the pump, cover the knob with a white cap, to insure that it doesn't get bumped.

If this is the actual experiment, you need to stick around to see that effluent is actively draining to the 2L flask below. When you are sure that it is, empty the flask, and note the time. You will use this timepoint to calculate the flow rate.

Below is an example of 'dummy lines'. They run water in and out of a flask to simulate the load of another chemostat line.



**Figure 15. Dummy lines simulate active chemostat lines, so you don't have to adjust the pump.**

## Sampling the Chemostats

The chemostats ideally should be sampled every day, particularly when collecting sweep data. Twice a day is not overdoing it for competition experiments. Try to be as consistent as possible about your technique, and write down anything that you change.

A daily sampling regimen includes measuring effluent volume, OD, Klett reading, Coulter count, Coulter mean cell volume, looking at the culture under the microscope, making a glycerol stock, viable cell counts on YPD and minimal plates (and possibly on selective plates), and possibly sampling for RNA and DNA. 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 1-4 hours.

When you need to change the media, try to do it after sampling to avoid any chance of a perturbation. Note media changes in your records.

To take a sample, place the styrofoam backed conical tube rack next to the outflow jar. Remove the cap from the tube, and place the sampling cork into the tube. Cover this with a loose piece of foil, and place the conical tube cap over the top of the effluent jar. Take care to NEVER elevate the sampling cork! Contaminants could easily be washed into the chemostat. While the tube is filling, pour the effluent into a glass graduated cylinder and write down the volume. You'll use this measurement to calculate the dilution rate later. After 20 minutes, you should have enough culture for typical measurements and stocks.

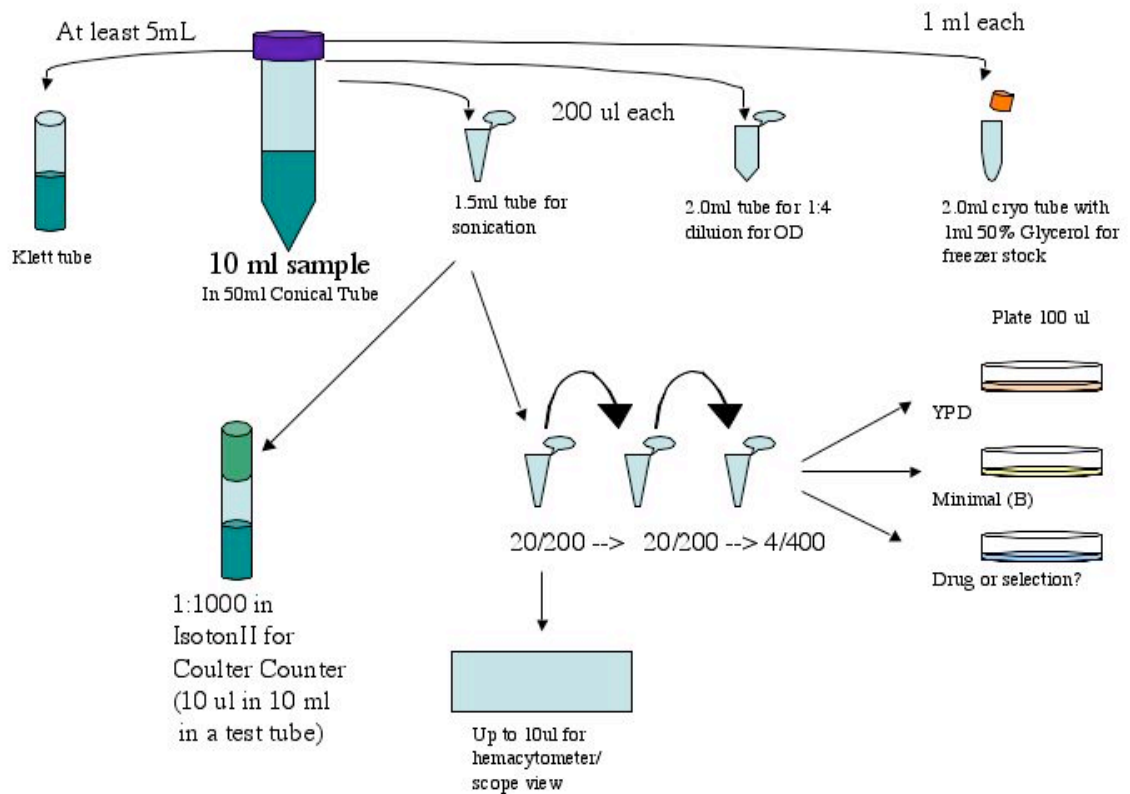


**Figure 16. Taking a sample during a run.**

## Preparing to sample

Before you begin sampling, organize all the required plates and diluents for sampling and prepare to record your data (see below). 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 they're properly calibrated. Run a clean sample of filtered Isoton II through the Coulter Counter to make sure it's clear of excess particles, and refilter it if necessary.

Turn on the Klett at least 30 minutes before using it, and put a water or media blank into place. Check that the pointer is not bouncing off either side, but rather somewhere in the middle.



**Figure 17. Example of typical sampling: Preparing tubes and plates ahead of time.**



## Data collection and record-keeping

It is extremely important that your data be collected in an organized and meaningful way. This will involve a layout sheet, notecards, and an Excel spreadsheet. This system serves to keep both a hard copy and electronic copy in case disaster strikes. The notecards will be added to the lab's card catalog in Maitreya's office, and spreadsheet will be added to the chemostat database on the server.

At the beginning of your experiment, start with a sheet that summarizes your set up. Include Fermenter number, which pump and pump head numbers you used, which carboy went with which fermenter, and so on. The blank forms are kept with the blank notecards.

Date of inoculation: \_\_\_\_\_

Media Pump #3

Media Pump #2

Media Pump #1

--	--	--	--	--	--	--	--	--	--	--	--	--

Notes:

### Figure 18. Experiment Layout Sheet

For the index cards, start with a header card (Figure 19) with all the relevant information about the chemostat, including fermenter number, strain, media composition, limitation, date and time started, method of inoculation, and your initials. Upon completion of the run, you will add the ending date as well.

Fermenter 13 6/4/4 - 6/29/4  
 BC3  
 .089% glucose limitation  
 Inoc w/ 1 ml O/N in H<sub>2</sub>O. 24  
 hr batch  
 Start: 4:05 pm

**Figure 19. Header index card.**

Each day, label all the fields on a new card (Figure 20) before you start sampling. This includes what dilutions should be done for all the measurements, what plates need to be used, and what was written on the frozen sample. Each chemostat gets a new daily card. Fill in the fields as you take the measurements. Be sure to write down any observations and illustrations of the look and smell of the culture on the back of the card. The card then goes into a small 'active chemostats' box. Pull the cards whenever you can fill in another field, such as colony count or dry weight.

F13 6/12/4  
 V<sub>cell</sub> 1325 4:30 pm  
 H<sub>cell</sub> 117  
 Ave. 1/4 .173  
 Coulter count 3.38 × 10<sup>7</sup>  
 1/1000 volume 30.0637  
 Frozen as F13 6/12/4  
 100 μl 10<sup>-4</sup> YPD = 267 c/14  
 min = 302 c/15  
 ~5 ml RNA  
 ~10 ml DNA Look good, oval

**Figure 20. Sampling index card.**

Once the chemostat run is completely finished, fill in the end date on the header card, amend the entry in the chemostat spreadsheet with the end date and any pertinent results (i.e., whether or not you harvested and where you put the tubes) and type all the data into the computer. You

could also input the data periodically while the chemostats are running. Finally, clip all the cards together with a secure paper clip and put them in the "Ready to be Filed" box, so they can be filed in the card catalog. No cards make it into the archives without first getting entered into the computer. The server is automatically backed up, and make sure your computer gets backed up regularly as well. You may also want to burn your data to CD once in a while and store it off-site, or upload it to the cloud.

## **Sample tracking**

The combination of chemostat number (F#) and date gives a unique identifier for sample tracking. Glycerol stocks added to the main strain collection will also have a 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 chemostats, 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 notes (notecard and spreadsheet). There are freezer racks specifically for current chemostat samples.

Please remember that these experiments will be analyzed for years, possibly after you have left the lab, possibly even by other labs. Other people in the future will rely on your notes, records, data, and samples.

## **Contamination issues**

Contamination of the chemostat culture ends the experiment and casts doubt on the previously collected data. Be aware of the sterility of all chemostat accessories at all times. Contamination of the media vessel often manifests as a film of cells on the bottom of the carboy. If the contaminant eats all the limiting nutrient, the first symptom may be that your culture washes out. It's important to look at your culture under a microscope, daily, so that you are aware of any contamination. Contamination of yeast can be difficult or impossible to detect, however. Use as sterile of a technique as possible, at all times.

Rogue yeast from your population can sometimes populate the media line. These yeast can suck all the nutrients out of the media before it gets to the main population. Keep an eye on the media line to be sure you don't have a problem. Below are a couple examples of what an occupied media line can look like.

The first frame shows an unpopulated media line. The second shows a strain that sticks to glass. The third frame shows the most common, and least obvious growth, seen as a ring of cells resting on the top edge of the glass media port. See the Disaster Index for a really bad case.



**Figure 21. Checking for media line occupancy.**

Yeast sub-populations can completely screw up your experiment, and because they can be difficult to detect, you might have no idea of what has gone wrong. Be extremely observant, and careful. Know that the downward sloping effluent tube must always stay downward sloping! Otherwise, yeast that have been experiencing different aeration, temperature, and nutrient levels could be introduced to the main population, and take over the culture. We are developing a couple of ideas to solve this problem.

## **Klett**

The Klett requires about 5-10 ml of culture, and at least 30 minutes to warm up for a good reading. The Klett is nice because it scales very close to linear with dry weight, unlike the other density measures we use. Abuse of the Klett can blow the bulb, which we have to buy from some antique dealer on the web. Read the manual or ask someone if in doubt. Put a water or media blank into place. Switch on the light. Readjust zero with the small knob further back on the machine (aka "light" adjust). Recheck the zero right before you use it.

Mix your leftover undiluted sample by inversion or vortexing, and pour into a spotless klett tube. Let any bubbles clear out and wipe any spilled culture off the tube with a kimwipe. Check that the media only blank is still reading 0. Put the tube in the Klett (with the mark forward) and turn the big knob on the front until the little arrow lines up perfectly with the line on the meter. Record the reading.

Flush the klett tubes with diH<sub>2</sub>O several times, then put them in a rack upside down, and pour more diH<sub>2</sub>O over them. Shake off excess water, and return the rack to the Klett area. Always be careful not to scratch the Klett tubes.

### **Glycerol stock**

If you are freezing aliquots of the culture, pipet 1 ml culture into 1 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 density changes require readjustments. 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. Dilute 0.5 ml culture into 1.5 ml water in a tube. Vortex the tube and pour the contents into a cuvette. 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 on the appropriate notecard. Remember that OD is not comparable across different spectrophotometers. If you use a different instrument, you will need to determine a conversion factor.

### **Sonicator**

Sonication breaks apart cell clumps so cells counts are more accurate. Pipette 0.5 ml culture into a 1.5 ml epi 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. Culture with severe clumping may require more intense 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, but don't touch it to 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 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.

## **Coulter Counter**

The Coulter counter interface is not the most user friendly in the world, and unguided button-pushing can completely screw up the settings. Check the appendix for complete instructions, and ask someone to help you the first time through. Measure a 1:1000 dilution of your samples, then calculate and record the cells/ml. You might also note the cell size, and sketch the curve showing the distribution of sizes (small sizes may indicate a contaminant, and large sizes may indicate flocculation).

## **Plating for viable counts**

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

## Plating for drug resistance

If you are monitoring drug resistances, plate the appropriate volumes of culture on selective plates, or plan to replica later. 250  $\mu$ l 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 transferring them to the 30C incubator.

## Sampling for DNA

If I'm sampling for DNA and RNA, I generally do all of the above first, then go back for the other samples. Taking a sample for RNA probably perturbs the culture a bit, so it should be the last thing you do.

For DNA sampling, I use a modified Hoffman and Winston yeast DNA prep. 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 with a screw-cap lid for future phenol extraction compatibility. Freeze at -80C.

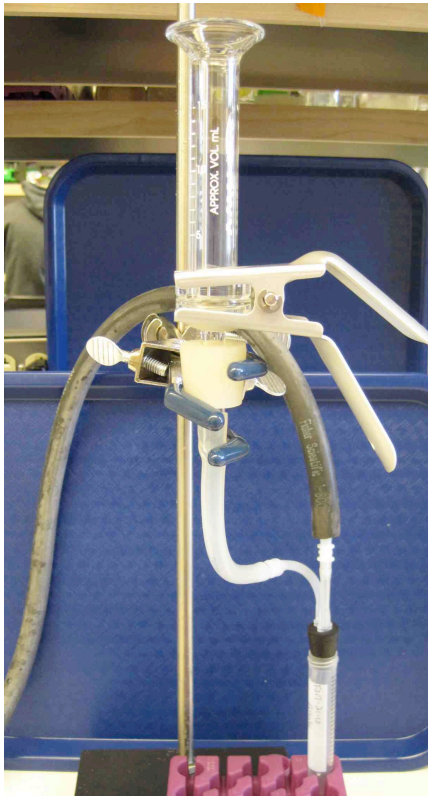
## Sorbitol Solution

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

## Sampling for RNA

A 5 ml sample is adequate to ensure enough RNA for one microarray, and it is such a small fraction of a 200 ml culture that it should not perturb the chemostat much. First, gather the following on a cart: a bucket of liquid nitrogen, 25mm nylon filters, 5ml pipets and pipetboy, tweezers, tongs, the small vacuum apparatus, and one of the vacuum flasks. Label a 15 ml Falcon tube for the filtrate and a 2 ml eppendorf tube with locking

lid for the filter. Using a ring stand, set up the small filter apparatus with the stopper assembly on the cart (see Figure 16), and with a filter in place. Wheel the whole operation into the chemostat room, hook up the apparatus to the house vacuum, and turn it on. Open the 2ml tube, and toss it into the liquid nitrogen, leaving the bucket lid off. Now you're ready to sample.



**Figure 22. Small filtering apparatus.**

Loosen the black cap on the chemostat and turn on the vacuum. Remove the black cap, and pipet 5 ml of culture out of the chemostat, and into the filter assembly. Let it vacuum through. Remove the clamp and glass funnel. Disconnect the vacuum. The order is important to prevent cells from sticking to the glass funnel and to allow all the filtrate to get sucked into the collection tube. Without disturbing the film of cells, remove the filter with tweezers. Roll it over on itself and insert into the 2 ml eppendorf tube. Close the tube and put it in the liquid nitrogen. Cap the filtrate tube and rinse the filter apparatus with DI water. Repeat the procedure for the remaining chemostats. When you've finished collecting all your samples, transfer the frozen tubes to  $-80^{\circ}\text{C}$ , and move the filtrate tubes to  $-20^{\circ}\text{C}$ .



## Cleanup after sampling

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). In particular, make sure you wash out the Coulter counter cups and klett tubes with DI water.

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

## Example of daily sampling

1. Prepare index card. Pre-label a 50 ml falcon tube, a 2ml cryovial, a 2ml epi tube, and 4 1.5 ml epi tubes.
2. Add 0.5ml sterile 50% Glycerol into the cryovial, and 0.6ml water into the 2ml epi. Distribute appropriate amounts of water for serial dilutions in 1.5ml epi tubes
3. Turn the Klett and spec on.
4. Note time, and transfer sampling cork to 50ml Falcon tube, upright in a beaker or rack, covering loosely with foil.
5. While tube is filling (you need 10ml minimum), measure effluent volume from 2L Flask below in a 1000 mL glass graduated cylinder. Record  $V_{\text{eff}}$  on notecard, and calculate D based on  $V_{\text{eff}}$ , and any sample volumes taken that should be added in.
6. Adjust pumps if D is not between 0.16 and 0.18. Make sure effluent is empty and time noted if pump adjustment is made.
7. Remove and cap falcon tube, and replace sampling cork into effluent jar. Note the sample volume taken, so that it can be included in the next D calculation.

8. Vortex sample, then pipet 1ml into cryovial, 0.2 ml into 2 prelabeled epi tubes (one for OD, and one for sonication).
9. Sonicate, then make dilutions for cell counting by hemacytometer and for plating ( $10^{-4}$  dilution) all in water, vortexing extensively at each step.
10. Take remaining undiluted sample along with your OD tubes and cuvettes over to the Klett and Spec area. Vortexing well before each measurement, check the Klett readings of all the samples. Then, check the OD's of the 1/4 dilutions, recording all results on notecards. Be sure to rinse the Klett tubes and cuvettes, so they are clean for next time. Do not leave the Klett on overnight.
11. Plate 100 $\mu$ l of  $10^{-4}$  dilutions on YPD and on minimal media (D and B plates, respectively), using a similar number of 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.
12. Vortex and load 8-10 $\mu$ l of a 1/10 dilution into hemacytometer. Count with a clicker, calculate, and record cells/mL. Alternatively, add 10  $\mu$ l of sonicated sample to 10 mL of Isoton II, for cell count by Coulter Counter.

## Sample Analysis During a Run

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

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

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

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

chemostat volume replacement rate. The spreadsheet will calculate the cell generations elapsed since the last sampling:

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

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

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 Coulter counted cells/ml to get a frequency. The Coulter count is much more well-measured than the viable plate counts, so use it even though it overestimates the viability.

## Media Replacement

It's important to calculate how much media you'll need at the beginning of the experiment. If it runs out, your experiment is jeopardized, so plan ahead. If your carboy is running very low, do the following: When you are down to the last bit of media, put a roll of lab tape under the back of the carboy to pool the remaining media into the exit port. You should be able to get almost all the remaining media this way.

If you have to connect another carboy, hopefully you included an alternate sterile port in your tubing network. If you didn't, you'll be risking contamination when you disconnect the current line, and reconnect it to a fresh carboy. Good luck.

If you are feeding your chemostats off two media vessels connected by a Y connector. You can drain all the media into one carboy by raising one carboy higher than the other. The lower carboy will fill up slowly. Once you've got all you can get out of the elevated carboy, clamp it off and disconnect it. Connect the new carboy, preferably by way of the extra sterile connector you included.

With either technique, watch the supply closely. You don't want to forget about it and run out of media. Once you've almost run out of media, or if you are leaving and the media will run out before you get back, replace the carboy. Any time you alter the media supply, write down the time on your index card. You may also want to take a sample of the media for analysis. See the Filtrate section for advice on nutrient assays.

## Harvest

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

## What is 'Steady State'

The cell density should be pretty stable before you call it steady state. Lab strains usually reach steady state 4-5 days after the pumps are turned on.

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

There are other things that should be taken into consideration when deciding if your chemostat has stabilized. You might give them a little leeway if there is flocculation, since their measurements are likely to be affected. Also, if the flow rate has fluctuated, you can expect flux 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 flux, but much more than 10% and you have to be cautious about your assumptions.

Taking multiple measures of cell density ensures that you aren't misled by an instrument who's having a bad day. That's why we measure Klett, OD600, and cell count, via hemacytometer or Coulter Counter. You will 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 variation could be due to technical artifacts, but it could also be biologically meaningful since the three methods do measure different aspects of cell density. As a rule of thumb, if the numbers are only slightly higher or lower than the previous day's measurements, it has probably reached steady state. If one stays the same, and another

changes significantly, use the 3<sup>rd</sup> measurement as a tiebreaker, and assume that one of the instruments is off. Use your best judgment, or ask an expert.

## **How long should Evolutions go?**

Evolutions can go as long as you want, assuming no one else needs the chemostats and carboys, and you continue to supply media. The goal of your experiment should be the main determining factor. Clumping beyond what can be broken up by sonication is one common endpoint, since these strains may be more difficult to work with and are unrelated to the intended nutrient limitation selection. Colonization of the media feed is another undesirable experiment-ender, which we are trying to reduce in frequency. Contamination is a complete deal-breaker which you cannot recover from, and which may require the entire experiment to be scrapped.

## **Setup for harvesting**

The key to harvesting cells for RNA is to be as fast as possible. Audrey Gasch famously studied the huge number of genes whose expression changes in the face of almost any stress. This stress response sets in within 5 minutes of perturbation and affects almost 1000 genes. Avoid it at all costs.

Because of the time pressure, make sure everything is completely set up before you start sampling. Using a pen that will stand up to liquid nitrogen and the -80, label two 15 ml Falcon tubes, 1 for the RNA prep and 1 for filtrate. Label extra tubes for media samples from each carboy. Get a bucket of liquid nitrogen, the 2 vacuum filter flasks, a pipetboy, and pipets, filters, and the large vac kit (Figure 19). Know where the backup filtering apparatus is in case you break something.

Weigh a 0.45 micron filter for each dry weight measurement. 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.

To set up the filter apparatus, first rinse all parts with DI water. Lubricate the sidearms and the ends of the vacuum tubing with some water and attach them. One will attach directly to the vacuum line, and the other

will connect to the first flask. Put the stopper with the filter support in the top of the second flask. Make sure the metal support is seated in the glass part correctly. The mesh should be level with the glass rim, or slightly below. If it's higher it will leak. Have the clamp and the funnel nearby.



**Figure 23. Large vac kit. All the components should be returned to the pan when you're finished.**

## Harvesting

The active harvesting process should take about 5 minutes. Cleanup and preparation will take longer. First, center a 47mm nylon filter on the filter support. Place the funnel on top and carefully clamp it all together. Start the vacuum. Listen for any whistling noises that may indicate a leak in the seal. If you do get whistling, make sure that the filter is centered and free of wrinkles. Some fraction of the filters have cracks or holes that will interfere with the harvest. Replace the filter with a new one if this seems to be the case.



**Figure 24. Here is the 'Harvest Cart' complete with the large filter apparatus.**

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

Record the time. Remove the top of the chemostat, and set it aside in a safe place. Be aware that if the pump is still running, you will accumulate some media under the top. Pipet 100ml of culture out of the chemostat (being careful not to hit any of the chemostat's internal organs) and into the funnel of the filter apparatus. Watch to make sure it is filtering properly and that no cells are making it into the flask. You can refilter if you have this problem, but try to avoid it.

Once the culture has completely filtered through, remove the clamp and then the funnel. Break the vacuum by removing the cork from the first flask. This order is important to keep cells from sticking to the funnel. Use forceps or a spatula to lift the edge of the filter, avoiding the cells in the center. Carefully roll up or fold the filter. With tongs, dump out the liquid nitrogen in the 15 ml tube, pop the rolled up filter inside, loosely cap it, and dunk it back into the liquid nitrogen. Leave it there until you transfer it to  $-80^{\circ}\text{C}$ . Take the flask and pour filtrate through the sidearm into the room temperature 15 ml tube. Cap and set aside the filtrate. It will be frozen at  $-20^{\circ}\text{C}$ , but you can collect a few more samples first.

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

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

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

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

## **Cleanup**

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

- Allow 20-35 minutes per chemostat for taking them down and washing them.
- All chemostats, reservoir bottles, tubing, and carboys must be flushed with DI water IMMEDIATELY following the end of your run. Otherwise, contaminants will grow in and clog them.



- The 100ml bottles of media you generated while filtering your media need to be emptied, thoroughly rinsed, and autoclaved.
- If you simply cannot take down your chemostats on the same day that you finish, dump the culture, and rinse the chemostat with DI water. If you do this, they can sit for the weekend. If you don't do this, the frit will probably become clogged, and you will have more work.

## **Taking down chemostats**

1. NEVER try to pull tubing off of the glass parts of the chemostat. Use the connectors that are a distance away from the chemostat.
2. Gather the lined metal pans you'll need for the chemostats and for the effluent jars. Also bring 2 plastic 2L beakers to the chemostat room.
3. First, if you won't be disturbing a neighboring chemostat's impending harvest, turn off the pump and clamp off the media line at the chemostat, at the pump, and at the carboy. Then, remove the pump heads, and remove the tubing from the pump heads. If there are other chemostats on the pump that you are not taking down, clamp their lines before removing pump heads, and run a dummy line in the head you're emptying to replace your chemostats load. Then reload the heads, unclamp, and turn the pumps back on.
4. Pull the complete quick connector from the bottom spout of the carboy. Then disconnect the far end of the pump tubing from the line running into the top of the chemostat. Take this tubing to the sink, unclamp it, and flush it with DI water for about 15 seconds. Then hold one end up to let it drain (mostly). Disconnect the male and female parts of the quick connector, and rinse the parts that were to the inside. Put the tubing in a tray.
5. Go back to the chemostat. Holding the media line up, unclamp it so that it drains into the chemostat. Remove the air filter from the top, and put it in a pan. Take the top to the sink, and flush it with DI water at least 5 times, making sure to remove any cells, and flushing the media line and air port for 15 seconds each. Rinse under the black cap, then put the top in its lined metal tray.
6. Get your 2 x 2L plastic beakers. Label 1 'Clean' and fill with DI water from the tap. The other one will be used to collect leftover yeast culture from the chemostats, and water from rinses.
7. With one hand on the chemostat, loosen the clamp which holds it upright on the pedestal.
8. If you won't disturb a chemostat with an impending harvest, turn off the water jacket pump, and clamp off the chemostat's water

- jacket lines. Empty the water jacket into the beaker. This makes the chemostat lighter in weight and easier to pour out (next step). If other chemostats are continuing a run on the same water jacket pump, turn off the pump and clamp the appropriate chemostats off to reroute the water flow. Unclamp and turn back on.
9. Carefully pour the chemostat into the beaker, with the air still connected, and the effluent cork over the beaker, or still in the effluent jar. Using the 'clean' beaker, fill with DI water several times, dumping wash water out in the other beaker. I usually fill it to the top, so the effluent track gets flushed.
  10. After rinsing this way at least 4 times, disconnect the air by detaching the closest inline air filter. Check the water that comes through the frit. It's probably full of cells. To clear the frit, fill the chemostat half full with more DI water, and use a syringe to GENTLY pull it through the line, until the water is clear. It's important to do this slowly, so you don't damage the fragile seams and frit in the air track. Then gently push and pull clean water through several times using the same syringe, to flush it thoroughly. If it gets autoclaved with those cells in there, the cells act as cement to clog the frit.
  11. Once the frit is clean, rinse the chemostat several more times, with more DI water. Remove any crust that may have formed at the top of the chemostat.
  12. Hook it back up to the air line, and turn it upside down to get the water out from under the frit. Also use the air to empty the media line (quick connector to chemostat top), and blot any droplets onto the pan liner. Then put the top onto the chemostat (air vent over outflow cylinder), and place in metal pan with liner. Make sure it has all its air filters, foil the ends, and it's ready to be autoclaved.
  13. Place the chemostat in the metal pan along with its components. Chemostats that share a carboy should also share a pan. Pans go on the shelves above the chemostat bench.
  14. Flush all tubing and effluent bottles with DI water. The effluent bottles go in a separate pan. Rinse the 2L flasks and send them to the dishwasher.
  15. Clean up any spilled culture or media in the chemostat room. Sometimes a spill will spread under the chemostat. Try to soak it all up and wash the bench with diluted Contrad 70 or bleach. Media spills are a haven for contaminants.
  16. Finally, the carboys you used must also be flushed with water (make sure you've taken a sample of the media if you need one). I usually go to the Queitsch Lab sink (not the EtBr one) for carboys, because the sink is bigger. Fill the carboy with a few inches of DI water, and then lift the carboy into a horizontal

position. Swirl the water around the inside of the carboy, rinsing all sides. Do this about 5 times, and be sure to flush the outlet tubing and connectors as well. Please don't leave your carboys sitting for more than a couple of days, and be considerate of the people who need them next. You should be leaving everything clean, like you found it. If the glass is clean, water will not bead up on it.

17. Before you forget, record all details on your notecards and in the accompanying spreadsheet.

## **Taking down all the chemostats**

There are a few things you can do to save time if you're taking all the chemostats down at once.

1. First, turn off the circulating waterbath and clamp the lines in and out on each side of the terminal connector. Disconnect the In and Out lines from the chemostats, and hang them up. Route a piece of tubing from the bottom port of the first chemostat into a large container. Unclamp the bottom port of the first chemostat. Once you unclamp the top port of the last chemostat, they'll all begin to drain into the large container.
2. While they are draining, you can turn off the pumps and clamp the media carboys. Remove all the pump heads and pump tubing. The whole length of tubing from the quick connect to just before the chemostat can be flushed as one big piece.
3. After rinsing the first chemostat top and doing your initial rinsing (still connected to air, filled to top, drained through effluent lines, and dumped 2 times), fill it up a third time, let it drain through the effluent track as before, but then disconnect the air by the closest small filter and turn the chemostat so that you can route that airline into the effluent bottle (where the effluent cork was). As it begins to drip, you can start on the next chemostat's top, and do the same for each chemostat. Pay attention to the rate at which the water drips through the airline. If the dripping is slow (less than several drips per second), use the syringe to pull water through, and leave it to drip. If it's still slow, you may have to work more with the syringe.

## **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 filtrate samples. Next you will

process them. See Appendix A for protocols.

## Chemostat Troubleshooting

Problem	Solution
Cork got sucked into carboy while filtering media.	<ul style="list-style-type: none"> <li>• Hack at the cork with scissors until you can get it all out. Clean up. (See Cork Sucking in Appendix.)</li> <li>• Try lower vacuum next time. One half turn of the knob is high enough.</li> </ul>
Bubbling is not like others	<ul style="list-style-type: none"> <li>• Check for kinked tubing and proper connections.</li> <li>• Clamp the line and replace inline air filters with sterile replacements (foiled in beaker). There may be water/media blocking the line.</li> <li>• Clamp the line and connect the farthest filter to a large syringe. Unclamp while pushing the plunger of the syringe down on the bench, blowing air through both filters and the frit. Reclamp the line before the syringe is empty, and reconnect to the air line.</li> <li>• Clamp the line and Try a different bubbler, or making sure the bubbler top is making good contact with the bubbler bottom.</li> <li>• If air is just not moving through like it should, the frit is probably clogged with cells, and you're out of luck for this run. Bake in a drying oven for 3.5 hours at 300C (be sure to remove all tubing, caps, and tape!)</li> </ul>
Chemostats will not fill by gravity	<ul style="list-style-type: none"> <li>• Double check that the entire media line is not clamped, and that the pump tubing is not loaded into the pump head. Either of these will prevent flow.</li> <li>• If media still won't flow, try loading the pump tubing into a pump head (NOT mounted on the pump drive), and turning the center post a few times in the correct direction to move media toward the chemostat.</li> </ul>

	Once you've done this, remove the tubing from the head, and watch the media flow.
Chemostat fills up, won't eject effluent	<ul style="list-style-type: none"> <li>• Air leak. Make sure effluent track isn't blocked, and clip the air vent.</li> <li>• Check that chemostat top is making good contact with bottom.</li> </ul>
Pump is making a noise	<ul style="list-style-type: none"> <li>• Stop the pump, and clip off the carboy(s) so you can open up the pump head. Make sure the tubing isn't wadding up inside the pump head.</li> <li>• If the tubing is fine, make sure that the posts coming out of the drive are screwed in all the way.</li> <li>• Finally, check to be sure that the small white flat washers are still intact. There should be one on each side of the pump head, for a total of 2. Replace as needed.</li> </ul>
Washout	<ul style="list-style-type: none"> <li>• Dilution rate set too high. Check settings.</li> <li>• Media missing an ingredient.</li> <li>• Media carboy contaminated.</li> <li>• Your strain has problems.</li> </ul>
Carboy is running low	<ul style="list-style-type: none"> <li>• Scoot the carboy back away from the edge, and prop up the back side on a roll of lab tape. This will buy you a small amount of time.</li> <li>• Prepare and connect a new carboy, and write it on the notecard.</li> </ul>
Contamination on viable count plates	<ul style="list-style-type: none"> <li>• Either contamination of the vessel or the water/media used to dilute the cells. Check vessel culture by directly sampling through one of the top ports. Replace water/media.</li> </ul>
Neighboring chemostats correlated	<ul style="list-style-type: none"> <li>• Media inconsistency. Measure limiting nutrient concentration of each carboy of media. Could also be a coincidence.</li> </ul>
Cloudy filtrate during harvest	<ul style="list-style-type: none"> <li>• Faulty filter. Replace and refilter.</li> <li>• Filter not centered on apparatus. Reposition and refilter.</li> </ul>

## **Appendix A: Sample processing**

### **Culture revival**

To revive cells from the glycerol stocks for further study, you should streak them to plates. Be careful about the choice of media, though. Some evolved strains no longer grow well on the rich YPD we generally use for day-to-day growth. You may have to make special low-nutrient plates to ensure good growth. You may want to do a test of different media formulations. Scrape out a nice chunk of the glycerol stock and resuspend it in some media. Count a sample in the Coulter Counter to gauge how much to plate. Assume about a 50% revival. Plate an aliquot to each of your types of plates, let grow up, and count colonies. There are often pretty striking differences. Phosphate- or sulfur-limited strains seem to do the best on YPD (though not always), while some glucose strains like low glucose (0.8%) minimal media better.

No matter what media you choose, watch out for revertant or suppressor colonies that arise on the plates. Always pick an average colony. When growing up culture in liquid media, make only as much cells that you need. This minimizes the number of generations of selection in batch that might allow a revertant/suppressor to take over. Also, don't serial transfer from a culture. Always go back to a fresh colony from the glycerol stock.

If you are selecting clones for further study, clone purify them by two rounds of streaking. Make sure to save a glycerol stock of the clone, labeled in an unambiguous manner (e.g. fermenter number, date, clone number). Be aware that other people may have already isolated clones, so check that your clone number isn't already in use. Clone glycerol stocks should be added to the main strain collection and database.

## **Coulter Counter Instructions**

### **1) Sample preparation:**

Prepare 1:1000 samples ahead of time in test tubes:

- a) make a blank of just Isoton solution, and run that first. If too many particles, refilter it, using a blue bottle top filter.
- b) Pipet 10mL of filtered Isoton Solution and 10 $\mu$ l of sonicated yeast culture into a test tube. Vortex on medium immediately before counting.

### **2) Turn on and Fill:**

- a) Turn on counter (switch on front), stand (switch on top left back), and computer. Open software on computer (Z2 Accucomp).
- b) Allow the machines to warm up for 10 minutes.
- c) Empty WASTE container and fill FILL container with fresh, filtered ISOTON solution (on top of Stand).
- d) Empty and refill the glass with fresh filtered Isoton solution (adjust platform by squeezing release lever under front edge)
- e) Flush line in Stand:
  - i) Turn bottom valve (ALWAYS CLOCKWISE) to 'Fill'
  - ii) Allow Flow for about 10-30 seconds, and then return it to a horizontal position (CLOCKWISE)

### **3) Set parameters:**

- a) With pure Isoton still on the platform, Press 'FULL' button on counter, and wait for it to stop thinking.
- b) Set Manometer select switch to 500 $\mu$ l.
- c) Press 'RESET' on counter. Counter might say, "Current and Gain AutoSet", then "Calculating, Please Wait."

### **4) Measure Sample:**

- a) Pour freshly vortexed blank or sample into a shot glass cuvette.
- b) Lower the platform to remove the cuvette that was already on the platform, and replace it with the new cuvette.
- c) Carefully raise the platform so that the electrode is well submerged in your sample. Be careful not to bend the external electrode!
- d) When light in chamber comes on, and the counter is finished thinking, the system is ready.
- e) Turn top valve to COUNT (clockwise). There will be a delay before you see anything on the screen. You should see little



spikes occurring on the 'monitor.' After about 15 seconds, it will say "Accumulating" at the bottom of the screen.

- f) Counter beeps when count is complete. Make sure the run takes 24-25 seconds. If longer, a clog most likely occurred, and the count should be repeated. With the cuvette in place, and the light on, notice that the upper left gray screen on the stand shows the aperture of the hole. During runs, check here for clogging. If clogging occurs, brush hole with paintbrush until clear.

#### **5) Send data to computer:**

- a) After the count is complete, go to the computer software, and select ACQUIRE from Multisizer.
- b) Press PRINT on counter (to send data to computer).
- c) You will be prompted to name the file and save to your folder.
- d) The number is displayed at the bottom in brackets. This is the number of cells that was in the 500 $\mu$ l of 1:1000 sample. Therefore, multiply this number by 2000 to get cells/ml in your original culture.
- e) Select Analyze -> Statistics to see the other info, including the median cell size, in cubic  $\mu$ L aka femtoliters (fL).
- f) You might want to sketch the curve for your records.

#### **6) To run another sample:**

- a) Press RESET on counter (all unsaved counts will be lost).
- b) Prepare next sample, and position on stand.
- c) With sample in position, light on, and reset complete, move top knob to COUNT.
- d) Monitor aperture, and wait for <BEEP>
- e) Move knob to Reset
- f) Acquire in software, Press PRINT, and Save.
- g) Repeat.

#### **7) To shut down:**

- a) Put the glass with Isoton onto the sample platform, and submerge the electrode.
- b) Set the manometer to 'OUT'
- c) Set the top knob to 'RESET'
- d) Set the bottom knob to 'CLOSE'
- e) Release the vacuum on the waste.
- f) Turn off power switches to stand and counter.

## Troubleshooting the Coulter Counter

Trouble	Shoot
The light won't come on	<ul style="list-style-type: none"> <li>• Double check that you've done everything in the correct order (Ex. Press 'Full' BEFORE setting the manometer)</li> <li>• If the waste container is too full, or if the little gasket on top is not making proper contact, it will not hold the vacuum that pulls the mercury through the tubes, and the light won't come on. Carefully empty the waste, or press down on the knob on top of the waste container for a minute.</li> </ul>
The counter is giving me an error message about the current, and can't Autoset Gain.	<ul style="list-style-type: none"> <li>• Double check that you've done everything in the correct order (Ex. Press 'Full' BEFORE setting the manometer)</li> </ul>
The counter is not resetting	<ul style="list-style-type: none"> <li>• If there is a message bar across the screen, you have to wait until it's gone to press any more buttons. Wait, and try again</li> <li>• If it still isn't working, Press STOP, then RESET.</li> </ul>
The blank is full of stuff	<ul style="list-style-type: none"> <li>• Refilter the Isoton solution.</li> </ul>
The hole keeps getting clogged	<ul style="list-style-type: none"> <li>• Dust is the enemy. Start over with dust free cuvettes and filtered Isoton.</li> </ul>
When I press PRINT, the counter tells me that a device is not connected, or has timed out OR the software can't SAVE because there are too many windows.	<ul style="list-style-type: none"> <li>• Close all the windows within the software, and select Acquire from Multisizer. Then press STOP and then PRINT on the counter.</li> </ul>
After it starts accumulating, it beeps while still counting	<ul style="list-style-type: none"> <li>• Move knob to reset, press STOP then RESET. Start the sample over</li> </ul>

## Testing Filtrates

You may want to measure metabolites and nutrients present in the filtrate. There are a number of commercial kits available from R-Biopharm to measure glucose, ethanol, and many other molecules. Chen, Toribara, and Warner have a great phosphorus assay (see references). It's very easy and accurate, and has a good range. I haven't found a very reliable way to measure sulfur yet, though. You can also measure the pH of the filtrate.

**Protocols for Phosphate, Ethanol, Glucose Assays can be found at <http://dunham.gs.washington.edu>**

## DNA prep

Hoffman-Winston prep modified by Maitreya Dunham and Cheryl Christianson.

Before you start, make the lysis buffer and TE+RNase, and label all your tubes. Process only the number of tubes you can fit in your vortexer at one time (for us that's batches of 12). Use nitrile gloves.

Gently thaw cells and spin to pellet. Decant the supernatant. To resuspended pellet, add:

200 $\mu$ l	lysis buffer (recipe below)
200 $\mu$ l	25:24:1 phenol/chloroform/isoamyl alcohol (kept at 4C).
300 mg	~500 micron acid-washed glass beads (we made a scoop to deliver this amount)

Vortex 8 minutes. Vortexing increases the yield substantially without obviously shortening the DNA on a 1% gel. We use a 'Turbo Mix' attachment. Do not use one of those funny rack vortexers or a large multitube attachment on a normal vortexer. You should make sure your setup actually vortexes the tubes adequately. If you get low yields, this is a key step to check.

Quick spin in a low speed minifuge to get the phenol off the lid.

Add 200  $\mu$ l TE. Invert to mix.

Spin 5 min max speed in a microcentrifuge.

Carefully transfer aqueous (top) layer to a new tube without catching interphase junk.

Add 1 ml room temp 100% ethanol. Invert to mix.

Spin 2 min max speed.

Remove supernatant and add 400  $\mu$ l TE+30  $\mu$ g RNaseA. The pellet may not resuspend easily, but as the incubation proceeds, you can usually get the whole thing to dissolve.

Incubate 30 minutes at 37C. We've lengthened this digestion from the original 5 min to reduce RNA contamination and to make sure the entire pellet gets into solution.

Add 10  $\mu$ l 4 M ammonium acetate and 1 ml room temp 100% ethanol.

Invert to mix.

Spin 2 min max speed.

Remove supernatant completely and dry pellet. We leave the tube inverted on a kimwipe on the bench for about 10 min.

Resuspend in 50  $\mu$ l TE.

Measure DNA concentration using a fluorometer or other DNA-specific method (i.e., NOT the spectrophotometer). Even with the RNase treatment and ammonium acetate precipitation, there's a lot of RNA contamination in these preps. Total yield should be 10-20  $\mu$ g. DNA should restriction digest easily.

### **Lysis buffer for DNA**

We keep a stock of this under the hood in S403, along with everything you need to do this prep. Here's the recipe if you have to make more.

2% Triton X-100  
1% SDS  
100 mM NaCl  
10 mM Tris pH 8  
1 mM EDTA

## RNA prep

Hybrid of an old Joe DeRisi protocol and a standard acid-phenol prep circulating around the Brown/Botstein labs circa 2001

The protocols for the large and small preps are more or less the same with minor volume and centrifuge differences. The phase lock gel makes everything so much easier, but if you aren't using it, add in 1 or 2 extra chloroform extractions to clean things up.

Use RNase free reagents and glass-/plastic-ware throughout! Remember to use glass pipets with chloroform. There is a stock of RNase free solutions in S403.

### Lysis buffer for RNA

(100 ml)

2 ml	0.5 M EDTA
5 ml	10% SDS
1 ml	1 M Tris pH 7.5
92 ml	RNase-free water

## **RNA prep for 5 ml daily samples**

Remove a manageable set of samples from the -80. They should be in locking 2 ml eppendorf tubes.

Before they thaw, add 750 ul lysis buffer. Vortex, trying to get all the cells off the membrane.

Add 750 ul acid phenol (kept in 4C). Vortex.

Incubate 1 hour 65C, vortexing every 20 minutes.

Fish out the filter and discard.

Ice 10 min.

While they are incubating, spin the 2 ml heavy phase lock gel (PLG) tubes for 30 sec full speed in a room temperature microcentrifuge. Set aside.

Spin lysate 5 min.

Transfer the top aqueous layer to the PLG tube.

Add 750 ul chloroform. Invert to mix. Do not vortex!

Spin 5 min.

Pour aqueous layer into a new 15 ml Falcon tube.

Add 75 ul (or 1/10 volume if you lost some) 3 M sodium acetate. Mix.

Add 1.5 ml (or 2 volumes) ethanol. Mix.

Incubate -20C >30 min.

Spin 3000 rpm 10 min in a swinging bucket centrifuge.

Wash pellet 2X with 70% ethanol, with 2 min 3000 rpm spins between washes.

Air dry inverted on the bench 30 min.

Dissolve pellet in 25 ul water at room temperature with frequent flicking.

Measure the undiluted concentration with the fluorometer or nanodrop.

You should get enough for an array.

The Akey Lab has a Bioanalyzer we can use (with our own reagents) to check the quality of the RNA, or you can just run a gel. You will probably have to dilute it ~1/10 if you run it on the Bioanalyzer.

## RNA prep for 50 ml harvest

Remove a manageable set of samples from the -80. They should be in 15 ml Falcon tubes. Be aware for cracked tubes, which will leak phenol. Before they thaw, add 4 ml lysis buffer. Vortex, trying to get all the cells off the membrane.

Add 4 ml acid phenol. Vortex.

Incubate 1 hour 65C, vortexing every 20 minutes.

Fish out the filter and discard.

Ice 10 min.

While they are incubating, spin the 15 ml heavy phase lock gel (PLG) tubes for 2 min 1500g in a room temperature swinging bucket centrifuge. Set aside.

Spin lysate 10 min 3000 rpm.

With a pipet, transfer the top aqueous layer to the PLG tube.

Add 4 ml chloroform. Invert to mix. Do not vortex!

Spin 5 min 1500g.

Add another 4 ml chloroform to the same tube, invert to mix, and spin again.

Pour aqueous layer into a new 15 ml Falcon tube.

Add 400 ul (or 1/10 volume if you lost some) 3 M sodium acetate. Mix.

Add 8 ml (or 2 volumes) ethanol. Mix.

Incubate -20C >30 min or overnight.

Spin 3000 rpm 10 min.

Wash pellet 2X with 70% ethanol, with 2 min 3000 rpm spins between washes.

Air dry inverted on the bench 30 min.

Dissolve pellet in ~250 ul water at room temp, adding more if necessary.

Measure the concentration of a diluted sample with the fluorometer or nanodrop. 1/2 is a good guess, or dilute 1/20 and use the same sample for the Bioanalyzer. You could also run a gel to check the quality.



## **Appendix B: If we had it to do over again**

### **The Disaster Index-Where you don't want to be.**

#### **Cork Sucking**

On a couple of different occasions during media filtration, the cork has been completely sucked into the carboy. We believe this was the result of the vacuum being set too high. When this happens, the media has to be refiltered into a fresh sterile carboy, and the cork can't be removed unless either it or the carboy is destroyed. The cork is much cheaper, and easier to clean up, so it must be hacked to bits. This is a tedious process, but it can be done in less than 10 minutes.



**Figure 25. Hacking at the Cork**

#### **Pump problems**

A lot can go wrong in the period of time right after you turn on the pumps, so you should stick around for about 5 minutes after you turn them on. After the initial 5 minute waiting period, you should check it after 10 more minutes, then after 10 more. If the pumps are still running smoothly, you're good to go.

Why are we paranoid? One rotation student witnessed something reminiscent of arterial spray when his pump tubing popped open. He caught it early, so the damage was minimal. If you forgot to unclamp a media line, it'll pop open and start spraying media all over the place. If you aren't there, there will soon be 10 liters of media on the floor with your name on it. Not to mention the surrounding experiments that might be compromised by this 'arterial spray' type of event.

Another pump problem can occur if the white o-ring gets mangled. This has happened once and was accompanied by a funny noise and white dust. Luckily, it was caught before damage occurred to the equipment.

### **Media line contamination**

Once your media line gets populated, your experiment is probably over. This problem seems to occur most frequently with flocculant strains, like the one shown here.



**Figure 26. Major media line occupation.**

### **Breakage**

So far we've been lucky, and only one chemostat has been broken. With all those fragile glass joints, it's imperative that the chemostats be

handled with great care, and that we never put pressure on the joints.

## Changes to the chemostat design

Our chemostats were made by Reeves Glass, using specifications they already had on file. As with anything, there is always room for improvement, and if we had it to do over again, there are a couple of specifications we would add.

## Consistent chemostat volumes

The distance between the effluent cylinder and the frit should be a fixed distance for reproducible volumes. We have a couple of chemostats whose volumes are too different to run on the same pump (because the volume directly affects the desired pump rate). For us, the consistency of one chemostat's volume to the next is more important than the actual volume, since the pump can be adjusted. Of the chemostats we have, the distance and corresponding volume are defined in the table below, for future reference.

Distance between frit and overflow point at top of effluent cylinder	Working volume of chemostat (with air on)
105mm	175 ml
115mm	195 ml
135mm	230 ml
137mm	240 ml
143mm	265 ml

**Table 4. Distances between frit and overflow.**

## Media line dropper

A perfect solution has yet to be discovered to prevent media line occupation. Here are a couple other things that might work but haven't been completely worked out.

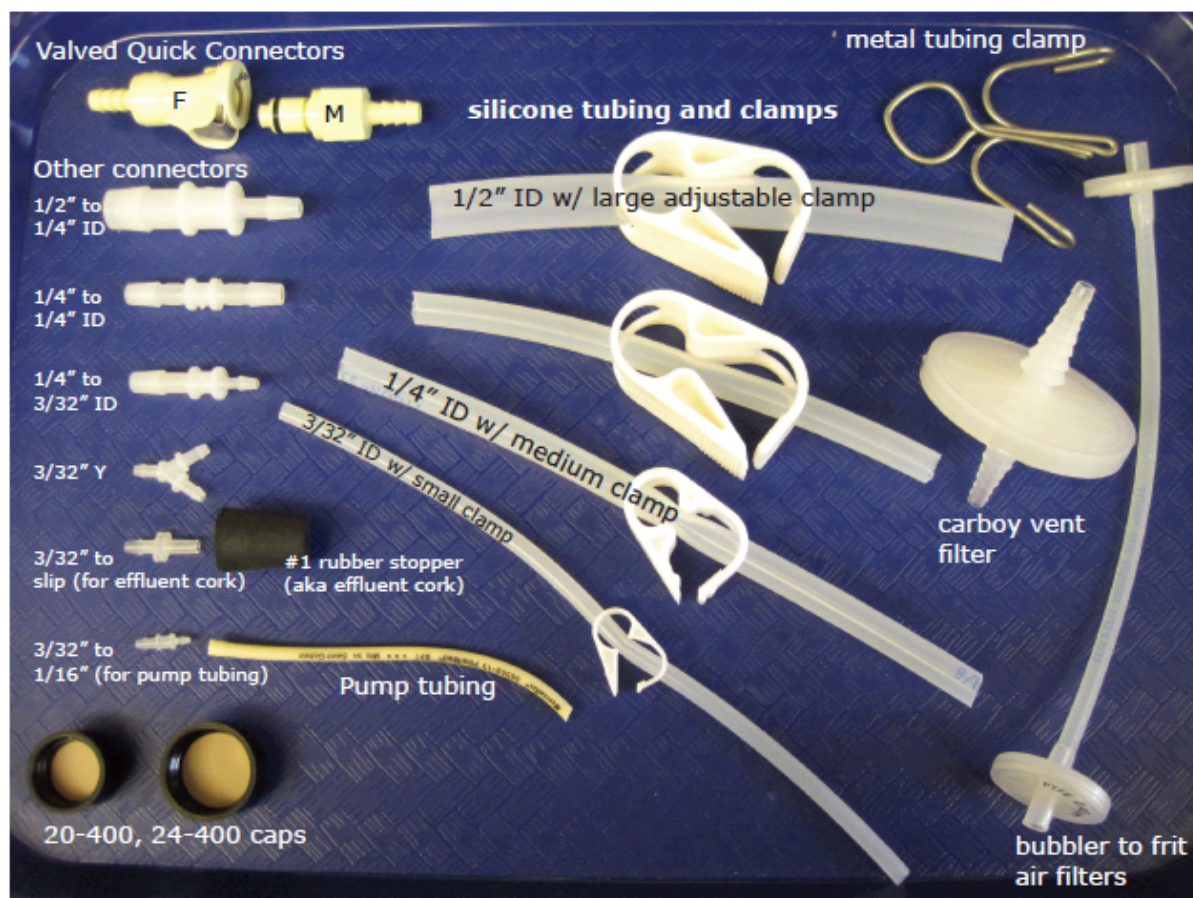
- 1/2" tubing fits over the media dropper surround, giving it more cover from contamination by yeast splashing.
- Establishing an air gap above the media dropper, perhaps by having a 'bulb' inline.

- Heat tape wrapped around the media port, to kill cells that have taken up residence there (without affecting the main population's temperature).
- Instead of the tubing fitting over the media port, fitting a tube or cork snugly inside the port, or in the top of the chemostat itself.

## Appendix C: Parts and Suppliers

### Tubing and Fittings

For reference, here's a guide to the different tubing and connectors and clips we use in setting up the carboys and chemostats. The tubing ID's are 1/2", 1/4", and 3/32". Find more details in the 'Supplies and Suppliers' section.



**Figure 27. Tubing and fittings.**

## Supplies and Suppliers

### VWR

[www.vwrsp.com](http://www.vwrsp.com)

circulating heating/cooling waterbath  
13271-074  
each, \$1,640.32

Corning bottle top filter, 1L, .2um, 45mm  
(for filtering whole carboy of media)  
29442-978  
case of 12, \$105.29

Kontes gas washing bottle, 250 mL, coarse porosity  
KT657750-2523  
each, \$164.29

1/2" x 5/8" silicone tubing (extra large)  
(Useful for fitting onto 20-400 ports on top of chemostat)  
63009-299  
50' coil pack, \$336

1/4" x 3/8" silicone tubing (medium)  
63009-279  
50' coil pack, \$107.41

3/32" x 7/32" silicone tubing (small)  
63009-260  
50' coil pack, \$120

Day Pinchcock (aka metal tubing clamp)  
21730-001  
pack of 10, \$8.24

small tubing clamps  
63022-403  
pack of 100, \$20.20

medium tubing clamps  
63022-405  
pack of 12, \$9.97

large tubing clamps, 12-position  
63022-407  
pack of 6, \$16.36

10 L Reservoir bottle with bottom hose outlet  
(aka carboy)  
89001-530  
case of 1, \$220.44

250 ml Reservoir bottle with bottom hose outlet  
(aka Effluent jar)  
89001-536  
pack of 1, \$33.09

rubber stoppers #1, 1 hole  
(for effluent jars)  
59581-163  
pack, \$18.25

Erlenmeyer flask, 2 L  
89000-370  
pack of 4, \$51.81

20-400 phenolic caps  
KT410121-2000  
case of 24, \$29.71

24-400 phenolic caps  
KT410121-2400  
case of 24, \$31.09

Stainless steel tray, 44x32x6.4 cm  
414004-102  
each, \$37.36

**Fisher**  
[www.fishersci.com](http://www.fishersci.com)

Carboy Venting Filter  
SLFG 050 10  
pack of 10, \$92.70

Nylon Membrane Filters, 0.45um Pore Size; Dia.: 25mm

R04SP02500  
pack of 100, \$79.41

Nylon Membrane Filters, 0.45um Pore Size; Dia.: 47mm  
R04SP04700  
pack of 100, \$108.22

Epoxy ring stand, 6x9 in base  
14-670C  
each, \$54.17

3-prong clamps, 10.5 in  
05-769-8Q  
each, \$37.61

double-buret clamp  
05-779Q  
each, \$42.29

silicone aquarium sealer  
S18180B  
each, \$5.01

Flowmeter #12 standard  
15-078-127  
each, \$113.01

Bench toweling  
(To line metal trays)  
15-235B  
50 yd, \$238.99

**Masterflex/Cole Parmer**  
[www.masterflex.com](http://www.masterflex.com)

Masterflex L/S precision variable speed drive  
EW-07520-50  
each, \$850

Masterflex Standard pump head for L/S 13 tubing  
EW-07013-20  
each, \$112

Masterflex PharMed BPT Tubing, L/S #13  
EW-06508-13  
25", \$67

Mounting hardware for 2 Masterflex L/S pump heads  
EW-07013-05  
each, \$30

Mounting hardware for 4 Masterflex L/S pump heads  
EW-07013-09  
each, \$36.50

Replacement thrust washers for Masterflex® L/S® Standard pump heads,  
EW-07021-04  
pack of 10, \$9.50

Silicone stopper, size 12  
(for carboy)  
EW-06298-22  
each, \$17.50

PTFE filters, 0.45 u, for air filtration  
HV-02915-22  
box of 100, \$143

Hook connector for ring stand  
EW-08041-30  
each, \$9.25

Barbed reducing connector PVDF, 1/4" to 1/8"  
EW-30703-50  
package of 10, \$21.25

Barbed Straight Connector, Kynar, 1/4" ID  
EW-30703-05  
pack of 10, \$23.75

Barbed Y connector, 1/8" ID  
HV-30703-92  
pack of 10, \$20.50

Male luer slip, 1/8" barb  
(for effluent stopper)  
HV-45503-26



25/pack, \$7.25

Barbed reducing connector, PP, 3/32" to 1/16"  
EW-30621-95  
25 pack, \$13.25

Barbed reducing connector, PVDF, 1/2" to 1/4"  
EW-30703-56  
10/pack, \$30

### **Reeves Glass**

200ml Glass Chemostat  
(we've had some trouble in that chemostat volumes vary and the water jacket seams are not always completely sealed)  
RG52086  
each, \$491.90

60/50 teflon sleeves  
RG19445-13  
pack of 3, \$77.10

teflon sleeves, 40/50  
RG19445-09  
pack of 3, \$47.40

### **R-Biopharm AG** [www.r-biopharm.com](http://www.r-biopharm.com)

Ethanol test kit  
10176290035  
\$82

D-Glucose test kit  
10716251035  
\$219

### **Aquarium Guys.com**

Silent Air Pumps

(we've had some trouble with residue from ours. Feel free to shop around)

212422

each, \$24.99

**Amazon.com**

Scotch #35 Electrical Tape, Green

For autoclaving carboy

\$6-8 per roll

Zen Pipe Cleaners Hard Bristle

Amazon.com

\$6.25 for 132

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