Protocols for Wet Lab Anaerobic Work

**Innoculating New Media**

Before Adding the Cells

1. Test for pressure in each tube of media by flame sterilizing all the tubes and then pushing an unattached needle into each one, making sure to hear the hiss of depressurizing.
2. Switch on Nitrogen for sparging, switch it to the gassing canula, and turn on the Bunsen burner
3. Assemble a small syringe (1 mL) for adding Na2S and an additional small syringe for each tube being inoculated
4. Flame sterilize the canula, then sparge the syringe ~10 times. Finish with a full syringe of nitrogen to replace Na2S you’ll be removing
5. Quickly flame sterilize the Na2S, insert the syringe, and push out all the nitrogen.
6. Invert the Na2S bottle and remove 0.1 mL for each tube being inoculated.
7. Pull out the syringe/needle, then flame sterilize each tube of media, insert the syringe, and inject 0.1 mL. Do this until each tube has Na2S

Adding the Cells

1. Flame sterilize the canula, then sparge the syringe ~10 times. Finish with the syringe full of nitrogen (plunger extended) to replace the cells you’ll be taking out
2. Quickly flame sterilize the tube with cells, insert the syringe, and push out all the nitrogen.
3. Invert the tube with the syringe, pull back the syringe to gather cells and push up to clear bubbles. Pull back a second time to take 0.1 mL of cells and hold the syringe plunger in place with the side of the finger. Take out the syringe
4. While holding the syringe, quickly flame sterilize the tube with medium. Insert the syringe, inject the cells, yell “BAMM!”, then remove and dispose of the syringe.

Adding the Nutrients

1. Grab a sterile gassing needle and place it in the test tube holder.
2. Switch from gassing canula to gassing manifold, turn off the Bunsen burner, and turn off the nitrogen. Switch on the H2/CO2 mix so that it’s coming out of the manifold
3. Remove the cap from the gassing needle and insert the manifold needle into the gassing needle top until it’s all the way in.
4. Sterilize the top of the cell + medium tube with ethanol, then quickly blow it off with the gassing needle apparatus and insert the needle into the tube.
5. Hold the gassing needle in the media tube until the pressure equilibrates, then remove the needle apparatus and put it back in its tube.
6. Remove the manifold needle from the gassing needle and place it back in its rubber tube. Place the cap back on the gassing needle and place it in the “Used” tube rack.

**Preparing the Chemostat for Autoclave**

1. Decide where each component goes (condenser, temperature probe, inoculation port), keeping in mind where the gas and liquid inlets are. Try to minimize clutter and crossing lines
2. Unscrew the inoculation port, remove the rubber stopper, and clean the port. Grab a new rubber stopper that matches the old one, then cut the new one to the correct size. Put it back into the port and screw it in.
3. Place the components in, with a gasket in each piece to make sure it’s airtight. Tighten each until it’s finger-tight.
4. Take out the apparatus and put the gasket on the chemostat. Rotate it outward as you push out, so that as it tries to rotate outward, it doesn’t roll back in. Replace the top of the chemostat.
5. Remove the liquid delivery apparatus and use a 20 mL syringe to push water through each part of the assembly, collecting the refuse in a container. Set apparatus aside
6. Make a full reservoir of media (9 L) following the posted recipe
7. With the help of another person, transfer 1 L of media to the chemostat. First, pour 1 L into a graduated cylinder, then have the other person lift off the top of the chemostat as you pour the contents of the graduated cylinder into the chemostat. Replace the top of the chemostat.
8. Place the reservoir on the plate next to the chemostat and place its top w/tubing into it
9. Add the nuts to the top of the chemostat and tighten until just catching
10. Temporarily set the liquid delivery apparatus back on its perch to assist in measuring tube sizes.
11. Add new tubing for the liquid inlet and outlet, but not for the sampling outlet. Use existing tubes as guides for measuring new tubes. Add clamps to all three liquid lines. Remember that inlet is the smallest, outlet is middle, and sampling is the biggest.
12. Measure new tubing from the chemostat gas inlet (smallest tube) to gas inlet filter and from the media reservoir gas inlet (shorter tube) to the reservoir gas inlet filter. Unscrew each of the gas inlet filters and attach them to their new measured tubing. Add clamps to each of them
13. Remove the metal inlet piece from the outlet liquid pump and add it to the new outlet tubing.
14. Put tin foil on the outlets of ALL the tubing (all three liquid tubes, gas inlet tube, both reservoir tubes), plus the top of the condenser (after putting in the inner coil of the condenser).
15. Put the red gasket inside the condenser top and attach it to the clear plastic tube of the refuse flask. Tinfoil both the flask opening and the condenser top.
16. Check every tubing connection to ensure it has a clamp, is tin-foiled, the clamp is tight, and the tubing is going to the correct place. Do this for the reservoir and the chemostat
17. Check every fitting to make sure the chemostat nuts are just catching and the other fittings are finger tight. Check to make sure the rubber top of the reservoir isn’t in tight.
18. Create the 500 mL solution of divalent cations in a 1 L bottle. Make sure to mix it thoroughly between components
19. Reserve 1/9 (55 mL) of the divalent cations and put it in a serum bottle to add to the chemostat. Put the cap back on the divalent cations, tightening a bit but keeping it loose. Cover both the divalent cation vessels with foil.
20. Assemble chemostat, reservoir, liquid delivery apparatus, refuse flask, and both divalent cation solutions in the autoclave bins and place on the cart. The chemostat can stand alone, but the reservoir and large cations should be in one bin with the liquid delivery apparatus in a second bin with the refuse flask and small cations in a basket in that bin.
21. Sign up on the near signup sheet for autoclave time. Take the cart down and open the autoclave. With hot gloves on, remove the rack and set aside. Reset the controls
22. Slide in the bin with the reservoir and push to the back, making sure the rubber isn’t touching the top. Slide in the bin with the liquid delivery apparatus and be sure that the rubber isn’t touching the sides. Lastly, set in the chemostat. Close the autoclave and lock it securely.
23. Hit the “Change Values” button until you get to the settings for cycle 4. Change the time to 90 minutes, then hit “Change Values” again. Hit the “4” button twice to start the cycle. Set a timer for 2 hours
24. After the timer goes off, take the cart back down to the autoclave to get the bins. Check that the display is at 0 PSIG, then carefully open the autoclave and place the cart by the opening. Put on the hot gloves and take out the chemostat and each bin very carefully. Put them on the cart as you take them out, being careful not to get burnt.

**Harvesting Chemostat Cells**

1. In preparation, leave a 1-L collection bottle with a syringe-friendly in the anaerobic hood the day before harvesting