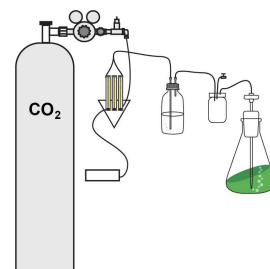


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Assembly and Operation of a Custom Carbon Dioxide and Air Gassing Manifold for Growth of Photoautotrophic Algal Cultures in Illuminated Incubators.



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We use this protocol and it's working

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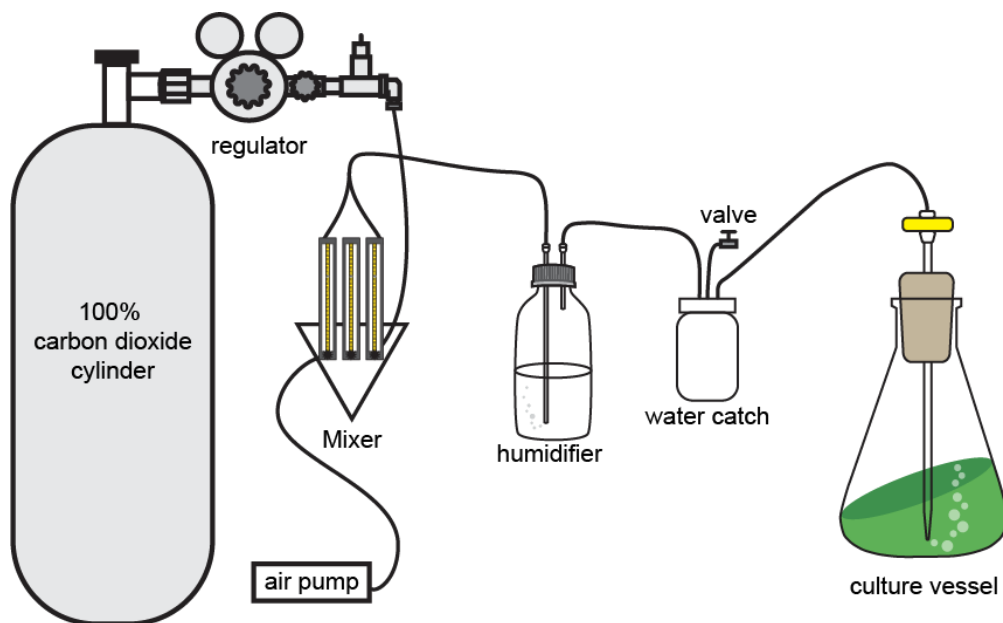
Disclaimer

We would like to thank Usha Lingappa for help with illustration. We appreciate Charles Perrino and Ping Chang for testing the protocol and for feedback.

Abstract

This protocol describes a method for setting up and operating an apparatus to enrich air with 5% CO₂ for growth of algal cultures. The main components of the apparatus consist of an air pump, a CO₂ gas cylinder, a mixer, a humidifier, a water catch, a manifold, air filters, and a gas bubbler. The flowrate of CO₂ can be adjusted to attain a final gas composition of 0% additional CO₂ (only air) up to 10% CO₂ in air. The maximum total flow rate of this system is 1.1 L / min and can steadily feed CO₂ enriched air to 10 culture vessels at a flow rate of up to 100 mL / min for each vessel. This protocol also describes a method for providing a 0.04% CO₂ in 99.96% N₂ pre-mixed gas mixture for anoxic growth of algal cells.

Guidelines



General schematic of gassing apparatus.

Schematics and diagrams are presented at the end of select steps. Please read the entire protocol before attempting to assemble and operate the apparatus.

When tubes are connected via insertion of one tube into another or when tubes are connected to barbs, the inner tube and barb's outer diameter (O.D.) will be 1/32 in. larger than the inner diameter (I.D.) of the outer tube. This is intentional and prevents the connections from coming loose during operation. Gentle force is needed to secure connections. Avoid using metal pliers when connecting fittings because tubes may tear and cause air seepage.

Materials

1. Tetra Whisper aquarium air pump for > 40-gallon aquariums (1.3 L air / min or more).
2. 50 lbs. of 100% high purity CO₂.
3. High-purity tank-mount pressure-regulating valve, CGA 320 female inlet, chrome-plated body, 2 stage, 0-50 psi, 3000 psi max inlet, 316 stainless steel diaphragm. McMaster-Carr Cat. No. 7951A67
4. Medium-pressure aluminum threaded pipe fitting straight connector, 1/4 NPT male, McMaster-Carr Cat. No. 3861T82
5. High-pressure aluminum threaded pipe fitting, tee connector, 1/4 NPT female
6. Fast-acting pressure-relief valve for air and inert gas, 1/4 NPT male
7. Universal-thread push-to-connect tube fittings, short 90-degree adapter, 5/32 in. tube O.D. × 1/4 in. pipe size
8. Universal-thread push-to-connect tube fittings, short 90-degree adapter, 5/32 in. tube O.D. × 1/8 in. pipe size.
9. Firm polyurethane tubing for air and water 3/32 in. I.D. × 5/32 in. O.D. × 50 ft length.
10. Pyrex round medium storage bottle, 1 L capacity, CLS13951I
11. 2-port vent assembly, straight, GL-45, universal, 316L electro polished stainless steel, 6.35 mm / 0.25 in. O.D. hose barbs, complete with gasket and cap, Cat. No. CLS-1429-20
12. Autoclavable clear PVC tubing 3/16 in. I.D. × 5/16 in. O.D. × 1/16 in. wall × 50 ft length. Nalgene® , Cat. No. 8701-9030
13. Autoclavable clear PVC tubing 1/8 in. I.D. × 3/16 in. O.D. × 1/32 in. wall × 50 ft length. Nalgene® , Cat. No. 8701-9020
14. Autoclavable clear PVC tubing 1/8 in. I.D. × 1/4 in. O.D. × 1/16 in. wall × 50 ft length. Nalgene® , Cat. No. 8701-0010
15. 250 mL bottles, style 2104 Nalgene® , Cat. No. B9157-12ea
16. Water catch valve, McMaster-Carr Cat. No. 7033T24
17. Plastic barbed tube fitting for air and water easy-view connector for 5/32 in. tube I.D., McMaster-Carr, Cat. No. 5117K14
18. Autoclavable clear polycarbonate female Luer thread to 200 series barb 5/32 in. I.D. (4.00 mm I.D. tubing) Cole-Parmer® , Cat. No. 50120-51
19. Autoclavable clear polycarbonate male Luer integral lock ring to 200 series barb 5/32 in. I.D. (4.00 mm I.D. tubing) Cole-Parmer® , Cat. No. 50120-30
20. Autoclavable clear polycarbonate male Luer integral lock ring to 200 series barb 3/16 in. I.D. Cole-Parmer® , Cat. No. 50120-32
21. Autoclavable clear polycarbonate Luer fitting, female Luer thread cap, Cole-Parmer® , Cat. No. EW-50120-41
22. Variable-area flowmeter with valve, direct-read, aluminum fitting, 150-mm; 200 mL / min air, Masterflex® Cat. No. MFLX32003-06
23. variable-area flowmeter with valve, direct-read, aluminum fitting, 150-mm; 1.25 LPM air. Masterflex® Cat No. MFLX32033-10
24. Variable-Area Flowmeter with Valve, Direct Reading, PTFE Fittings, 65-mm Scale; 220 std mL/min Carbon Dioxide, Masterflex® Cat. No. MFLX32006-12
25. Flowmeter tripod base for one, two, or three meters. Masterflex® . Cat. No. EW-03226-30
26. Hook and loop cable hangers, 10 pcs. Speedwrap® , Cat No. SWRCHBK
27. PVDF syringe filter, 0.45 µm pore size, Millex, Cat. No. SIhvr33rb
28. Sterile polystyrene disposable serological pipets with magnifier stripe, Fisherbrand™, Cat. No. 13-676-10G.



29. 70% ethanol
30. Foam plugs, 35 – 45 mm openings, Jaece industries Identi-Plugs[®], Cat. No. L800-D
31. Aluminum foil
32. Drill with 11/64" drill bit
33. Sterile hood
34. Autoclave

Safety warnings

- ❗ To prevent injury and possible death, read the safety data sheet (SDS) for compressed CO₂ and the instruction manuals for the regulator and flowmeters. CO₂ may displace oxygen and cause asphyxiation. Make sure that the space has properly engineered and adequate ventilation. Make sure to complete your institution's compressed gas safety training course before attempting this protocol. Verify that each component's rated pressure is above 30 psi and that the pressure relief valve is set to 15 psi. Unrated equipment should be inspected by your institution's safety office (e.g. Office of Environment, Health, and Safety (EHS)). The maximum allowable working pressure (MAWP) of this apparatus is 25 psi and the maximum operating pressure (MOP) is 15 psi. Wear shatterproof ANSI Z87.1+ certified safety goggles.

Assembly

1 Set up the air pump.

1.1. Cut 30 cm of clear PVC tubing (1/8 in. I.D. × 1/4 in. O.D. × 1/16 in. wall) and 10 cm of firm polyurethane tubing (3/32 in. I.D. × 5/32 in. O.D.).

1.2. Firmly insert the polyurethane tube at least 1 cm into the clear PVC tube. The clear 1/8 in. I.D. PVC tubing is flexible and will accommodate the firm 5/32 in. O.D. tube with some force.

1.3. Connect the clear PVC tube to the Tetra Whisper aquarium air pump. The polyurethane tube will be connected to a mixer later.



Aquarium air pump with the lengths of tubing indicated.

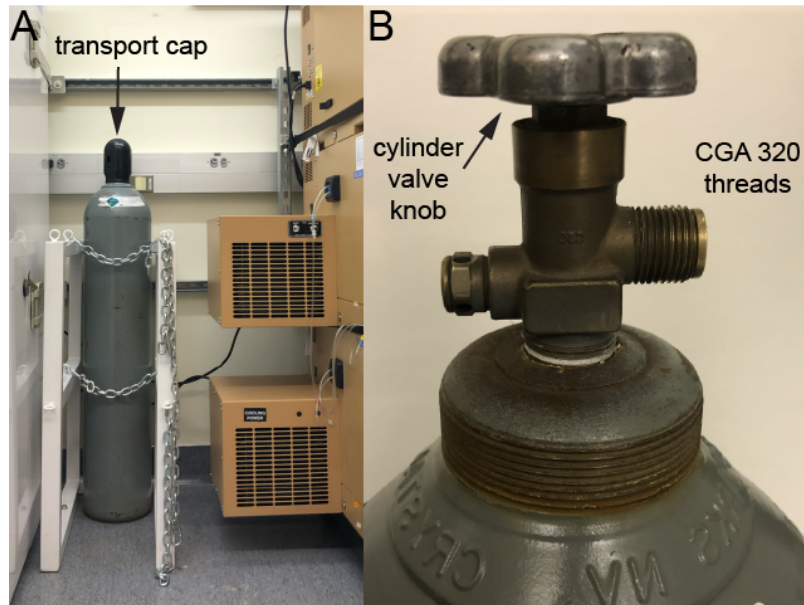
2 Set up the CO₂ cylinder.

2.1. Ensure that the CO₂ cylinder is properly stored in a cylinder rack or chained to the wall near an incubator. Stationary cylinder racks should be anchored to a wall and/or the floor. Chains anchored to the wall or a cylinder rack should restrain the cylinder at 1/3 and 2/3 of its height to prevent toppling. Cylinders should only be transported safely using a specific cylinder hand-truck or cart that is equipped with reinforcing chains. Compressed gas cylinders weighing more than 25 lb. must be transported on an approved cart.

2.2. Remove the cap from the cylinder and any packaging around the valve. Cylinder valves should always be capped unless they are in use.

2.3. Inspect the cylinder valve and threads for signs of damage or corrosion.

2.4. Ensure that the valve on the cylinder is completely closed.



A) CO₂ cylinder restrained at 1/3 and 2/3 of cylinder height and positioned close to an incubator. B) Cylinder valve with a CGA 320 regulator connection.

3 Prepare the regulator, pressure relief valve, and push-to-connect tube fittings.

3.1. Use the correct regulator that is specific to CO₂ gas. Do not change CGA connections. The inlet pressure may fluctuate because CO₂ is not completely a gas nor a liquid in the cylinder; therefore, a two-stage regulator is needed to provide consistent output pressure.

3.2. Remove the 1/4 in. NPT pipe to 1/4 in. O.D. tube Swagelok® Female connector from the output valve of the regulator.

3.3. Install the 1/4 in. NPT male to 1/4 in. NPT male medium-pressure aluminum threaded straight pipe connector to the 1/4 in. NPT female connection on the valve.

3.4. Install the 1/4 in. NPT female high-pressure aluminum threaded pipe fitting tee connector to the 1/4 in. NPT male medium-pressure aluminum threaded pipe straight

connector.

3.5. Install the 1/4 in. NPT male fast-acting pressure-relief valve for air and inert gas at the 90-degree female port of the tee connector.

3.6. Install the universal-thread push-to-connect tube fitting, 90-degree adapter to the 180-degree female port of the 1/4 in. NPT tee connector.

3.7. Connect the firm polyurethane tubing (3/32 in. I.D. × 5/32 in. O.D.) to the universal-thread push-to-connect tube fitting. Cut the polyurethane tubing to a length required to reach the mixer.

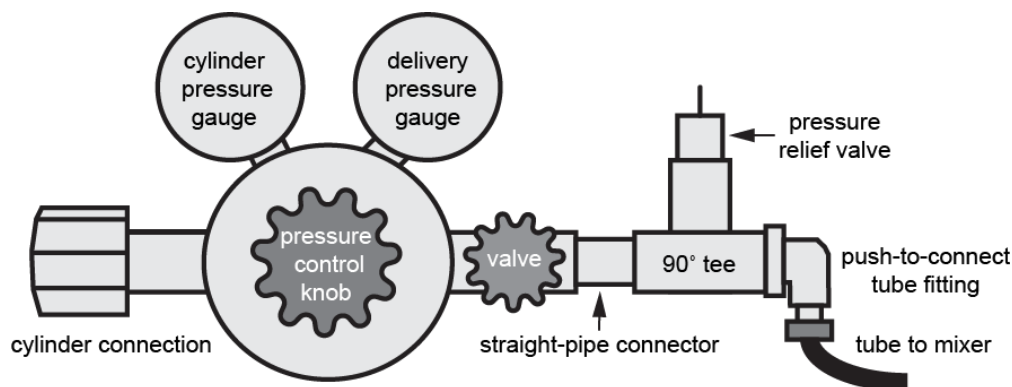


Diagram of regulator

4 Install the regulator to the CO₂ cylinder.

4.1. Be careful not to cross thread the connections. Ensure that the plastic crush washer is in the regulator to cylinder connection. Use a wrench to lightly tighten the CGA connection. Do not over tighten. Position the regulator so that the cylinder is between you and the regulator. Ensure that the control knobs are accessible and that the gauges are readable.

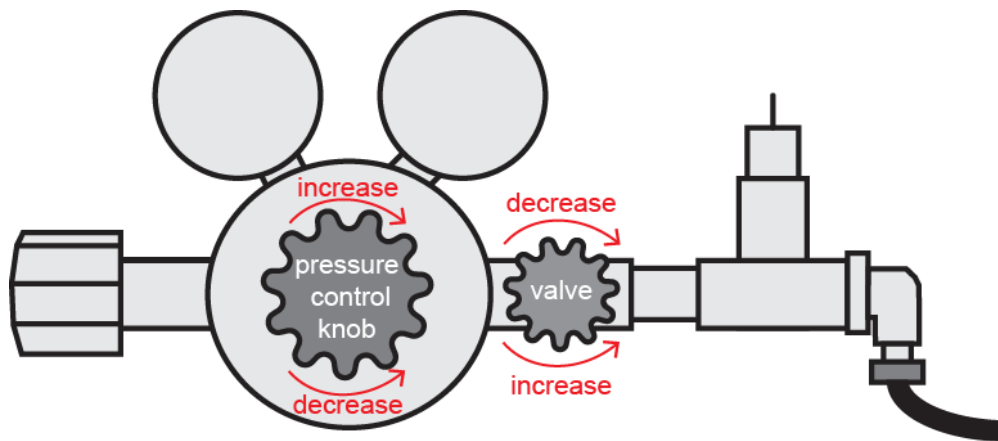
4.3. Do not use Teflon tape to seal the connection between the cylinder and the regulator. Teflon tape may damage the regulator if it enters the regulator.

4.4. Turn the pressure control knob on the regulator to the off position or 0 PSI by turning in the direction indicated on your specific regulator. For most regulators, the directions on the pressure control knob may seem counterintuitive, so please read your regulator's manual carefully. As it closes, turning will become easier.

Safety information

Failure to close the regulator may result in a ruptured diaphragm when the regulator is pressurized. A ruptured diaphragm may cause an uncontrolled release of CO₂ and an over pressurization of the downstream components.

4.5. Close the output valve directly downstream of the regulator.

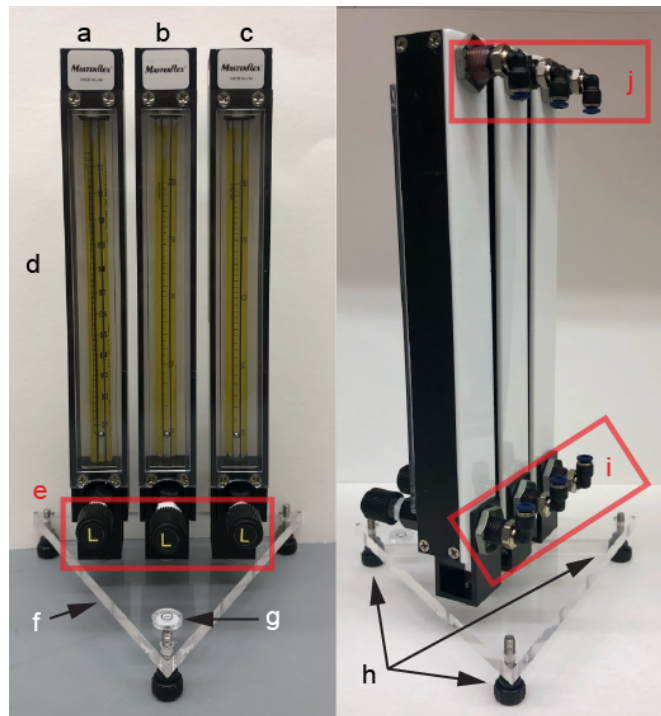


Directions for operating the pressure control knob and valve downstream of the regulator.

- 5
- 5.1. Install the 220 mL / min CO₂ flow meter to the right slot of the triple flow meter tripod base.
- 5.2. Install the 200 mL / min air flow meter in the middle.
- 5.3. Install the 1.25 L / min air flow meter to the left.
- 5.4. Install the universal-thread push-to-connect tube fittings (short 90-degree adapter, 5/32 in. tube O.D. × 1/8 in. pipe size) to each of the six input and outputs of the flow meters.
- 5.5. Turn all flow meter knobs counterclockwise to fully open.
- 5.6. Push the firm polyurethane tube from the aquarium air pump into the push-to-connect tube fitting on the inlet (bottom) of the 1.25 L / min air flow meter.



- 5.7. Push the firm polyurethane tube from the CO₂ regulator into the push-to-connect tube fitting on the inlet (bottom) of the 220 mL / min CO₂ flow meter.
- 5.8. Cut two pieces of 5 cm firm polyurethane tube.
- 5.9. Cut two pieces of 5 cm clear PVC tubing (1/8 in. I.D. × 3/16 in. O.D. × 1/32 in. wall).
- 5.10. Connect the two pieces of 5 cm clear PVC tubing (1/8 in. I.D. × 3/16 in. O.D. × 1/32 in. wall) to the 0-degree and 180-degree ports of a plastic tee barbed tube fitting (5/32 in. tube I.D., McMaster-Carr, Cat. No. 5117K14).
- 5.11. Insert the 5 cm firm polyurethane tube at least 1 cm into each of the PVC tubes (1/8 in. I.D. × 3/16 in. O.D. × 1/32 in. wall).
- 5.12. Connect one of the firm polyurethane tubes to the 220 mL / min CO₂ flow meter and the other polyurethane tube to the 1.25 L / min air flow meter.
- 5.13. Cut a length of clear PVC tubing (1/8 in. I.D. × 3/16 in. O.D. × 1/32 in. wall) required to reach the humidifier.
- 5.14. Install a male Luer lock (Luer lock ring to 200 series Barb 5/32 in. I.D.) to the humidifier end of the PVC tube from the mixer.
- 5.15 Level the tripod base using the bubble level and the adjustable feet beneath the stand.



a) 1.25 L / min air flow meter b) 200 mL / min air flow meter for final airflow measurement
c) 200 mL / min CO₂ flow meter d) Direct read flow tubes e) Flow rate adjustment knobs
f) Flow meter tripod base g) Tripod base bubble level h) Tripod base level adjustment screws i) Inlet universal-thread push-to-connect tube fittings. j) Outlet universal-thread push-to-connect tube fittings. Set up the CO₂ and air mixer.

6 Set up the humidifier. **Perform bold sub-steps in a sterile hood using proper sterile technique.**

Note

A humidifier is needed to saturate the incoming gas with H₂O and hence to prevent the medium from evaporating. Evaporation will inadvertently cause the cell density and concentrations of solutes to rise throughout the experiment.

6.1. Cut and connect 20 cm of clear PVC tubing (3/16 in. I.D. × 5/16 in. O.D. × 1/16 in. wall) to each of two external ports and one internal port. The ports with both internal and external ports are the inlet ports. The port with only an external port is the outlet port.

6.2. Attach a male Luer lock (thread style to 200 series barb, 3/16 in. I.D.) to the output tube.



6.3. Obtain a  1 L Pyrex round medium storage bottle and fill with  800 mL of Milli-Q H₂O.

6.4. Cap the bottle with the 2-port vent assembly cap and the attached tubes. Loosen the cap so that air can escape. The white rubber gasket should sit at the interface of the stainless-steel component and the cap of the assembly, not between the  1 L bottle and the stainless-steel component.

6.5 Wrap the cap, inlet tube, and outlet tube with tin foil.

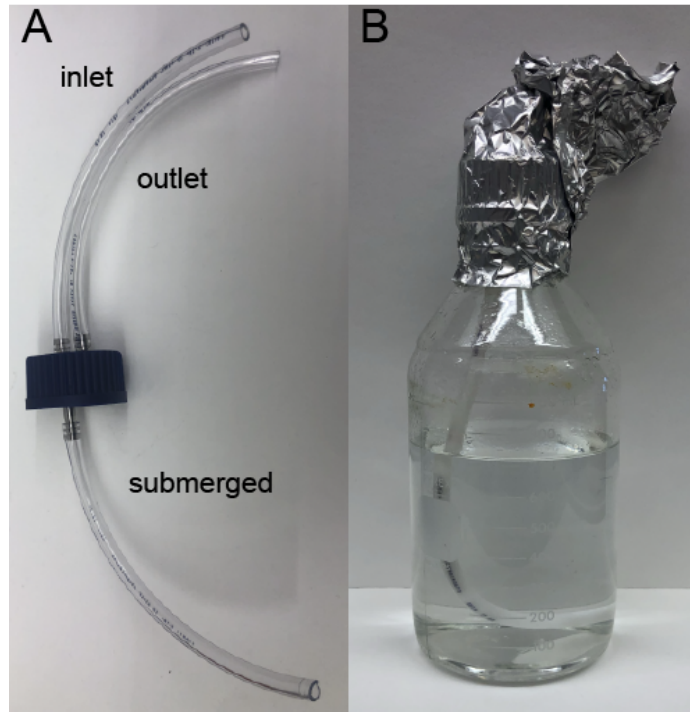
6.6. Sterilize the humidifier assembly using an autoclave. Let the water and bottle cool to room temperature in a sterile hood. Screw the cap on tightly. Unwrap the foil covering the inlet tube.

6.7. Using proper sterile technique, push the output side of a Millex PVDF syringe filter (0.45 µm pore size) into the inlet tube of the humidifier.

6.8. Place the humidifier in a cool place near the incubator. Do not place the humidifier near exhaust vents, or around heat exchangers for the incubators. This will cause the water to warm up in the humidifier and then cool in the downstream tubes, resulting in excessive condensation and potential clogging.

6.9. Cut a length of PVC tubing (1/8 in. I.D. × 3/16 in. O.D. × 1/32 in. wall) required to reach the water catch.

6.10. Connect a male and female Luer lock (Luer lock ring to 200 series Barb 5/32 in. I.D.) to the ends of the tube from step 6.9. Connect the female Luer lock to the male Luer lock on the humidifier.



A) diagram of tubing. B) Humidifier bottle before autoclave sterilization.

7 Assemble the water catch.

Note

A water catch is needed to prevent condensation from clogging PVDF membrane filters.

- 7.1. Drill three 11/64 in. holes into a 250 mL HDPE bottle cap.
- 7.2. Cut three 6 cm long PVC tubes (1/8 in. I.D. × 3/16 in. O.D. × 1/32 in. wall).
- 7.3. Insert the tubes into all three holes with 3 cm on the inside and outside of the cap.
- 7.4. Connect a valve to one tube on the outside of the bottle.
- 7.5. Connect a male Luer lock to another exterior tube.
- 7.6. Connect a female Luer lock to the last exterior tube.
- 7.7. If possible, place the water catch inside the incubator. You may need to install a cable passthrough port through the incubator to connect the external components to the water

catch inside the incubator.

7.8. Connect the male Luer lock from the humidifier (step 6.10) to the female Luer lock on the water catch (step 7.6).

8 Install the apparatus to the incubator.

8.1. Cut nine pieces of 6 cm long PVC tubes (1/8 in. I.D. × 3/16 in. O.D. × 1/32 in. wall).

8.2. Connect each of the 6 cm tubes to the 0-degree and 180-degree ports of the plastic tee barbed tube fitting (5/32 in. tube I.D., McMaster-Carr, Cat. No. 5117K14). Skip the first and last port. Push the barb 1 cm into the tubes.

8.3. Attach the last 6 cm long PVC tube (1/8 in. I.D. × 3/16 in. O.D. × 1/32 in. wall) to the center barbed tee. Install a female Luer lock (Luer lock ring to 200 series Barb 5/32 in. I.D.) to this tube.

8.4. Cut 10 pieces of 65 cm long PVC tubes (1/8 in. I.D. × 3/16 in. O.D. × 1/32 in. wall).

8.5. Install a male Luer lock (Luer lock ring to 200 series Barb 5/32 in. I.D.) on one end of each tube from step 8.4.

8.6. Connect the other end of each of the tubes from step 8.4 to the 90-degree ports of each barbed tee and the remaining 0 and 180-degree ports at the left and right end, respectively. Push the barbed tees 1 cm into each tube firmly.

8.7. Use the adhesive Speedwrap® hook and loop cable hangers to hold the tubing to the top and rear of the incubator. Clean the surface with 70% ethanol before applying the adhesive cable hangers. Make sure that the tubing is suspended and does not get caught on the incubator platform and flask clamps.

8.8. Run a PVC tube (1/8 in. I.D. × 3/16 in. O.D. × 1/32 in. wall) from the middle barb to the water catch. Install a female and male Luer lock (Luer lock ring to 200 series Barb 5/32 in. I.D.) to the upstream and downstream end, respectively. Connect the Luer locks.

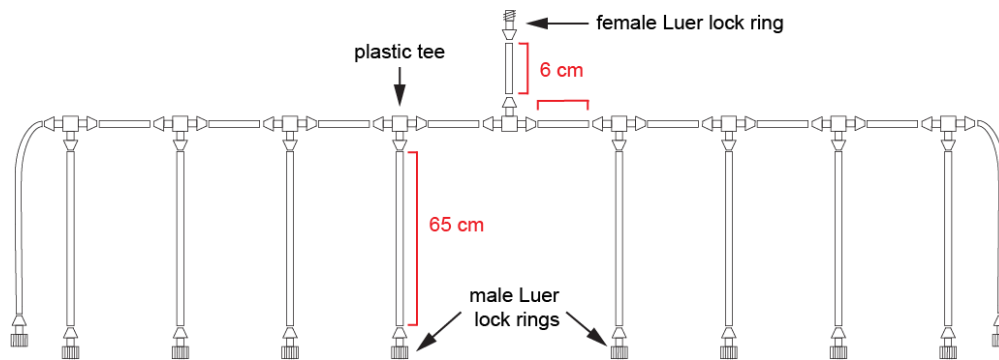


Diagram of manifold.

9 Set up the culture vessel for gas introduction. **Perform bold sub-steps in a sterile hood using proper sterile technique.**

9.1. Use a  1 mL polystyrene serological pipet to cut holes through each foam stopper. Place the foam stopper on a hard surface. Use a spoil board if working on a lab bench to avoid marking the lab bench. Remove the cotton plug from the inside of the pipet. Firmly press the pipettor side of the 1 mL pipet into the center of the foam plug. While maintaining pressure on the pipette, move around in circles to cut a hole. Be careful not to apply too much force or the pipette may snap and cause injury.

9.2. Wrap groups of four foam stoppers with holes in aluminum foil.

9.3. Cut 12 pieces of 2.5 cm long clear PVC tubing (3/16 in. I.D. × 5/16 in. O.D. × 1/16 in. wall).

9.4. Wrap the 2.5 cm long tubes in groups of four with aluminum foil.

9.5. Cut 10 cm wide × 15 cm long aluminum foil. Fold the foil in half to a new shape of 5 cm × 15 cm.

9.6. Autoclave the aluminum foil, foam stoppers, and 2.5 cm long tubes. Let items cool to room temperature in a sterile hood.

9.7. In a sterile hood, tear open a new sterile 1 mL pipet from the pipettor end.

9.8. Hold the aluminum foil to position the foam plug. Do not touch the foam plug. insert the pipettor end of the pipet into the hole in the foam plug.

9.9. Pull the pipet through the foam plug without touching the foam and the dispensing tip of the pipet. Only touch the pipettor side of the pipet.

9.10. Remove the foam plug from the aluminum foil and place it into your culture flask. Make sure that more than 3/4 of the foam is in the flask.

9.11. Wrap the top of the foam plug and 2 cm of top of the flask with the 5 cm x 15 cm aluminum foil from step 9.5.

9.12. Unwrap a PVDF syringe filter, 0.45 µm pore size. Do not touch the output side. Hold on to the syringe side of the PVDF filter.

9.13. Peel the aluminum foil from one of the 2.5 cm long tubes.

9.14. Insert the output side of the syringe filter into one of the 2.5 cm long tubes.

9.15. Insert the pipettor end of the pipette into the other end of the 2.5 cm long tube.

9.16. Bring the culture to the incubator and place the flask into a flask clamp.

9.17. Connect the syringe filter to a male Luer lock.

9.18. Cap all unused tubes from step 8.4 with a female Luer thread cap.

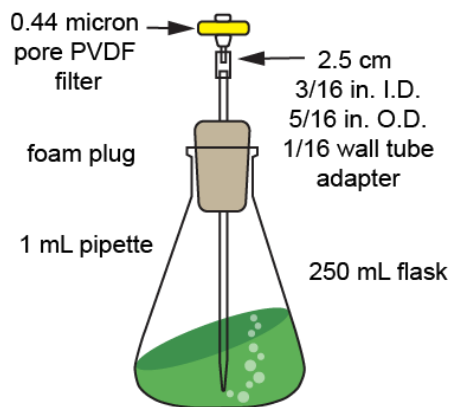


Diagram of culture vessel and gas introduction system.

10 Set up the final flowrate measuring device.

10.1. Connect a 0.45 µm PVDF syringe filter to a 1 mL pipet using a PVC tube adapter (see steps 9.7, 9.12, and 9.14).

10.2. Cut 6 cm of firm polyurethane tubing (3/32 in. I.D. × 5/32 in. O.D.) and enough Clear PVC tubing (1/8 in. I.D. × 3/16 in. O.D. × 1/32 in. wall) to extend from the inside of the

incubator to the flow meter.

10.3. Insert at least 1 cm of the polyurethane tube into the clear PVC tube.

10.4. Connect the polyurethane tube to the inlet of the unused 200 mL / min air flowmeter (center).

10.5. Insert the dispensing end of the pipette into the clear PVC tube.

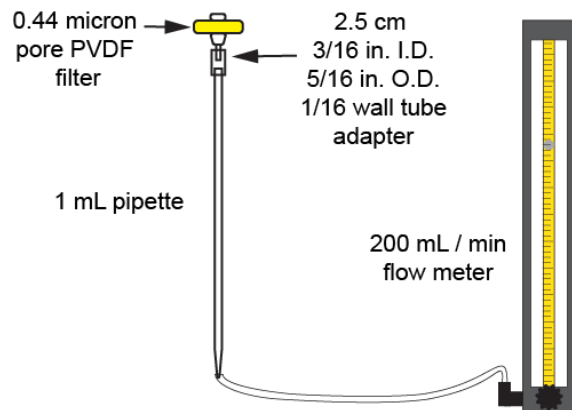


Diagram of final flow rate measuring device.

Operation

11 Verify that all components are properly connected.

11.1. Ensure that the regulator is closed (see step 4.3) and that the valve directly downstream of the regulator is closed. Ensure that all air flow meter valves are shut. Turn the pressure control knob on the regulator counterclockwise and it should turn easily when the regulator is closed.

11.2. Position yourself with the cylinder between you and the regulator.

11.3. Keep your hands off the regulator and look away from the pressure gauges as you open cylinder valve very slowly. Open the cylinder valve slowly to avoid damaging the regulator.

11.4. If no hissing is audible, rotate the cylinder's valve counterclockwise to the completely opened position. Then turn the valve 1/4 of a full rotation clockwise. If hissing

is audible, close the cylinder valve and wait until the inlet high pressure gauge reads 0 PSI. Check the cylinder-regulator connection.

11.5. Inspect the pressure gauges to verify that the outlet or delivery pressure reads 0 PSI. The inlet pressure of a new 50 lbs CO₂ cylinder is typically around 700 PSI.

11.6. Slowly turn the pressure control knob clockwise until 10 PSI is indicated on the delivery pressure gauge.

11.7. Use a soap and water solution to test for leaks around the connections of the cylinder, regulator, and regulator valve. If soap and water cannot be used, make note of the inlet pressure. Close the cylinder valve and wait 5 min to see if the inlet pressure has dropped.

11.8. Label the cylinder with the date, your initials, the gas composition (CO₂), and the inlet pressure.

11.9. Open the downstream regulator valve **very slowly** until fully opened. Turn 1/4 of a turn clockwise.

11.9.1. **Slowly** turn the knob on the CO₂ flowmeter counter clockwise until the float reads 50 mL / min.

11.10. Plug the aquarium pump in and make sure that the air flowmeter reads 1 L / min. As air is supplied, the flow of CO₂ should decrease.

11.11. Use the valves on the flowmeters to fine tune the flowrates of air and CO₂ to 1 L / min and 50 mL / min, respectively.

11.12. Connect the PVDF filter of the final flowrate measuring device from step 10 to one of the male Luer lock outputs from step 8.5. The PVDF syringe filters with a 0.45 µm pore size have a high flow resistance. This resistance is even higher with 0.22 µm pore size filters, so make sure to use only 0.45 µm pore size filters.

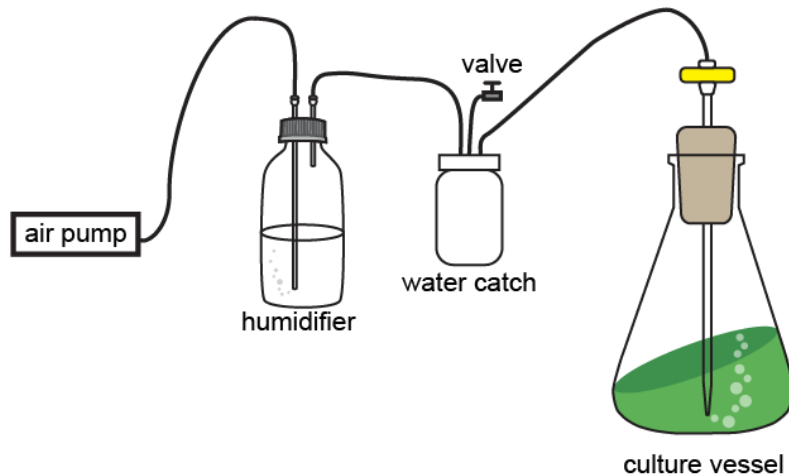
11.13. If the final flowrate is above 60 mL / min, adjust the flowrate of air and CO₂ proportionally using the flowmeter valves. Alternatively, open the valve on the water catch to release some of the air and CO₂ mixture.

11.14. If none of the cultures is bubbling, you may have a leak somewhere in the system. If 1 L / min of air cannot be achieved by the 40 gallon aquarium air pump, you may need

to get a stronger air pump. The Tetra Whisper series air pumps are also available for 60, 100, 150, and 300 gallon aquarium tanks.

Air Only Gassing Procedure

- 12 To supply the cultures with air only, simply connect the aquarium air pump to the humidifier bottle by bypassing the air and CO₂ mixing apparatus. Adjust the flow rates using the valve on the water catch to obtain 50-60 mL / min air / culture vessel.



Simplified diagram for air only gassing of algal cultures.

Anoxic Gassing Manifold Assembly

- 13 The gas manifolds can be adapted to provide a premixed gas mixture of 0.04% CO₂ in 99.96% N₂ for anoxic growth of algal cells. All components are the same except for the gas cylinder, regulator, and flowmeter. The premixed gas cylinder uses a CGA 580 connection so a different regulator dedicated for N₂ gas service is needed.
- 14 Position the new premixed gas cylinder next to the incubator (see step 2).
- 15 The regulator needs to be fitted with the same 1/4 in. NPT connections as the CO₂ regulator (see step 3). You may reuse the components from the CO₂ regulator or choose to purchase a new set of parts (straight connector, tee, pressure relief valve, and push to connect tube fittings).

- 16 Connect the regulator to a single 1.25 L / min air flowmeter. The flowmeter will not be used to measure gas flow; instead it will be used to fine tune the flow rate of the gas mixture.

16.1. Cut a length of firm polyurethane tubing required to reach the flow meter. Connect the polyurethane tube to the push to connect fitting on the regulator and the input (bottom) fitting on the 1.25 L / min air flow meter.

- 17 Connect the flowmeter to the humidifier.

17.1. Cut 5 cm of firm polyurethane tubing and enough PVC tubing (1/8 in. I.D. × 3/16 in. O.D. × 1/32 in. wall) to reach the humidifier.

17.2. Push the firm polyurethane tubing at least 1 cm into the PVC tube (1/8 in. I.D. × 3/16 in. O.D. × 1/32 in. wall). Connect the other end of the firm polyurethane tube to the output (top) push to connect tube fitting on the air flowmeter. Install a male Luer lock (Luer lock ring to 200 series Barb 5/32 in. I.D.) to the other end of the PVC tube.

17.3. Connect the Luer lock to the 0.45 µm pore size syringe filter on the humidifier.

17.4. All components downstream of the air flowmeter are identical to the CO₂ and air manifold components.

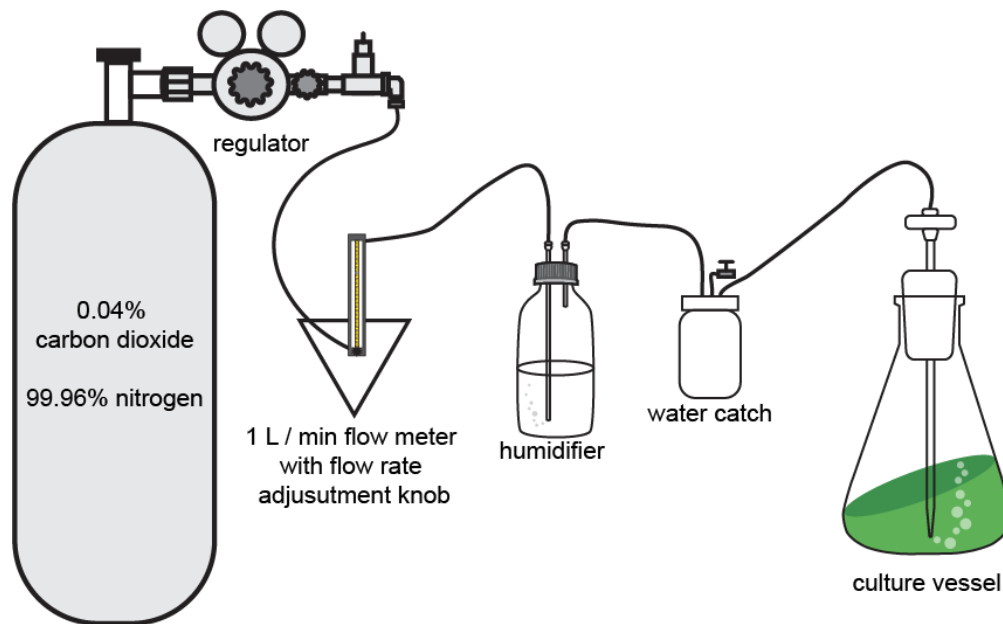


Diagram of anoxic growth gassing apparatus.



Anoxic Gassing Manifold Operation

- 18 Ensure that all components are properly connected and that the all valves (cylinder valve, regulator pressure control valve, downstream regulator valve, air flow meter valve, and water catch valve) are closed.

18.1 Open the cylinder valve **very slowly** and adjust the pressure control knob on the regulator to deliver 10 PSI (see steps 11.1 to 11.8).

18.2. Open the valve on the output of the regulator **very slowly**.

18.3 Slowly open up the valve on the flowmeter until the float reaches a reading of 1 L / min. Use this valve to fine tune the flowrate of the gas mixture.
- 19 Use the final flowrate measuring device from step 10 to read the final flowrate. Adjust the flowmeter valve from the previous step to adjust the flow rate for a final flow rate of 50-60 mL / min / culture vessel.

Shutting Down

- 20 Record the regulator inlet pressure and place a label on the cylinder with the pressure, date, and your initials.

20.1. Close the gas cylinder valve and wait until both the inlet and delivery pressure gauges read 0 PSI.

20.2. Remove the regulator from the cylinder valve very slowly while listening for gas seepage. If a leak is detected, re-tighten the regulator, and make sure that the cylinder valve is completely closed. If the cylinder valve is faulty, keep the regulator tightened and close the valve downstream of the regulator. Call the gas supplier for assistance.

20.3. Close the regulator by turning the pressure control valve counterclockwise.

20.4. Close the valve downstream of the regulator.

20.5. Cap the cylinder and label it as empty if it has a final pressure below 50 PSI. Always try to keep at least 30 PSI left in the cylinders.

20.6. Unplug the aquarium pump.

20.7. Disconnect and drain the humidifier bottle.

20.8. Disconnect and drain the water catch bottle.



20.9. Cap all Luer locks.

Maintenance

1h

21 Wash tubing and connections with soap and water.

1h

21.1. Place all tubing into a bin.

21.2. Use a syringe to flow a soapy water solution through all tubing.

21.3. Soak in soapy water  01:00:00 and cover the bin with plastic wrap.

21.4. Rinse all tubing with Milli-Q H₂O.

21.5. Flow Milli-Q H₂O through all tubing with a clean syringe.

Optional: flow  70 % (v/v) ethanol into the tubes.

21.6. Flow filtered air through the tubing to dry.

Warning: Do not use bleach on polycarbonate, nylon, and polyvinyl chloride (PVC) tubing and fittings.

22 Autoclave sterilize tubes and connections.

22.1. Do not autoclave the aquarium pump, regulator, flowmeters, PVDF filters and  1 mL serological pipets. PVDF filters and serological pipets are single use.

22.2. All polycarbonate tube fittings and PVC tubing described in this protocol can withstand a maximum temperature of  140 °C and can be autoclaved at  120 °C .

23 23.3. Soak used foam plugs in soap and water.

23.3. Rinse thoroughly and squeeze remaining water out of the foam stoppers.

23.3. Air dry and then wrap in aluminum foil and autoclave to re-use.