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## Cultivation of Green Microalgae in Bubble Column Photobioreactors and an Assay for Neutral Lipids

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September 14, 2018

Dear Dr. Troyer,

We are pleased to accept your invitation to submit a manuscript to JoVE regarding our methods for culturing microalgae in photobioreactors. Enclosed in this submission is the manuscript entitled, "Cultivation of green microalgae in airlift photobioreactors and assay for neutral lipids."

Our photobioreactor approach offers more consistent results than standard flask cultivation but without the high cost of commercially-available photobioreactors. This system is flexible and can be adapted to the cultivation of a wide range of microalgae. We have also included our methods for quantifying growth and neutral lipid content of the algae produced in these photobioreactors. These two metrics are of great interest to the very large community of researchers studying algal biofuels. Although the neutral lipid assay has been published previously, we have received numerous inquiries from researchers requesting our step-by-step protocol for its execution. We have therefore included that with this submission.

To summarize, this protocol includes the following major components:

1. Setup of the photobioreactor system
2. Cultivation of algae in the photobioreactors
3. Harvesting of algae and determination of growth rates
4. Extraction of total lipids
5. Assay for neutral lipids

We believe this article and the associated video will be meaningful contributions to the scientific community. We hope it meets your expectations.

Sincerely,  
Brendan Higgins  
Assistant Professor  
Biosystems Engineering  
Auburn University  
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**TITLE:**

Cultivation of Green Microalgae in Bubble Column Photobioreactors and an Assay for Neutral Lipids

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**KEYWORDS:**

Microalgae; photobioreactor; cultivation; lipid extraction; neutral lipids; biofuel

**SUMMARY:**

Here, we present a protocol to construct lab-scale bubble column photobioreactors and use them to culture microalgae. It also provides a method for the determination of the culture growth rate and neutral lipid content.

**ABSTRACT:**

There is significant interest in the study of microalgae for engineering applications such as the production of biofuels, high value products, and for the treatment of wastes. As most new research efforts begin at laboratory scale, there is a need for cost-effective methods for culturing microalgae in a reproducible manner. Here, we communicate an effective approach to culture microalgae in laboratory-scale photobioreactors, and to measure the growth and neutral lipid content of that algae. Instructions are also included on how to set up the photobioreactor system. Although the example organisms are species of *Chlorella* and *Auxenochlorella*, this system can be adapted to cultivate a wide range of microalgae, including co-cultures of algae with non-algae species. Stock cultures are first grown in bottles to produce inoculum for the photobioreactor system. Algae inoculum is concentrated and transferred to photobioreactors for cultivation in batch mode. Samples are collected daily for the optical density readings. At the end of the batch culture, cells are harvested by centrifuge, washed, and freeze dried to obtain a final dry weight concentration. The final dry weight concentration is used to create a correlation between the optical density and the dry weight concentration. A modified Folch method is subsequently used to extract total lipids from the freeze-dried biomass and the extract is assayed for its neutral lipid content using a microplate assay. This assay has been published previously but protocol steps were included here to highlight critical steps in the procedure where errors frequently occur. The bioreactor system described here fills a niche between simple flask cultivation and fully-controlled commercial bioreactors. Even with only 3-4 biological replicates per treatment, our

approach to culturing algae leads to tight standard deviations in the growth and lipid assays.

## **INTRODUCTION:**

The application of microalgae in engineering and biotechnology has attracted great interest in recent years. Microalgae are being studied for use in wastewater treatment<sup>1-4</sup>, biofuel production<sup>5-8</sup>, and the production of nutraceuticals and other high value products<sup>9,10</sup>. Algae are also being genetically modified at greater rates in an effort to improve their fitness for specific engineering applications<sup>11,12</sup>. Consequently, there is great interest in experimentation with industrially relevant organisms in controlled settings. The purpose of this method is to communicate an effective approach to culture microalgae in a controlled laboratory environment, and to measure the growth and neutral lipid content of that algae. Improving growth rates and neutral lipid content of microalgae have been identified as two key bottlenecks toward commercialization of algal biofuels<sup>13</sup>.

A wide range of approaches have been used to culture algae for experimental purposes. In general, these approaches can be divided between large-scale outdoor cultivation and small-scale indoor cultivation. Outdoor cultivation in photobioreactors and open ponds is appropriate for experimentation aimed at scaling up processes that have already been proven at laboratory scale (*e.g.*, to test scale-up of a new high-lipid strain of algae)<sup>14</sup>. However, indoor small-scale cultivation is appropriate when developing new or improved algae strains or performing experiments aimed at understanding biological mechanisms. In these latter cases, a high-degree of experimental control is required to tease out subtle changes in biological behavior. To that end, axenic cultures are often required in order to minimize the complex biotic factors associated with other organisms (*e.g.* bacteria, other algae) that inevitably grow in large-scale outdoor systems. Even when studying interactions among algae and other organisms, we have found that use of highly-controlled experimental conditions is helpful when examining molecular exchange among organisms<sup>15-17</sup>.

Within the category of small-scale indoor algae cultivation, a range of approaches have been used. Perhaps the most common approach is to grow algae in Erlenmeyer flasks on a shaker table beneath a light bank<sup>18,19</sup>. Exchange of oxygen and CO<sub>2</sub> takes place by passive diffusion through a foam plug in the top of the flask. Some researchers have improved this set-up through active aeration of the flasks<sup>20</sup>. Another approach is to cultivate algae in bottles, mixed by stir bar and active aeration. Despite their simplicity, we have found that the use of flasks and bottles often leads to inconsistent results among biological replicates. Presumably this is due to position effects – different positions receive different amounts of light, which also affect internal reactor temperatures. Daily rotation of reactors to new positions can help but does not alleviate the problem because certain stages of algae growth (*e.g.*, early exponential) are more sensitive to positional effects than others (*e.g.*, log phase).

On the opposite side of the spectrum of technological sophistication are fully-controlled commercial photobioreactors. These systems continuously monitor and adjust conditions in the reactor to optimize algae growth. They have programmable lighting, real-time temperature control, and pH control. Unfortunately, they are expensive and typically cost several thousand

dollars per reactor. Most scientific and engineering journals require biological replication of results, necessitating the purchase of multiple bioreactors. We present here a bubble column reactor system that bridges the divide between the simple (flask) and sophisticated (fully-controlled bioreactor) approaches for lab-scale algae cultivation. Bubble columns use rising gas bubbles to facilitate gas exchange and mix the reactor. This approach provides some degree of control over the lighting and temperature but does so in a way that is cost-effective. Moreover, we have found this system to yield highly consistent results among biological replicates, reducing the required number of biological replicates needed in order to obtain statistically significant results when compared to the flask or bottle approach. We have also used this system to successfully cultivate mixtures of algae and bacteria<sup>21</sup>. In addition to algae cultivation, we outline a procedure for measuring the neutral lipid content in the cultured algae. The latter method has been published elsewhere<sup>22</sup>, but we include the procedure here to provide step-by-step instructions for how to employ it successfully.

## PROTOCOL:

### 1. Setup of Bubble Column Photobioreactors

1.1) Construct a set of vented lids from the plastic lids that came with the 1 L glass bottles and hybridization tubes (see **Figure 1** for schematic and photos). Construct lids for the humidifier, mixing trap, each air lift photobioreactor, and each bottle reactor.

1.1.1) Drill ¼" holes in the lid: 2 holes are needed for bioreactor and humidifier lids; 3 holes are needed for the mixing trap.

1.1.2) Slip a ¼" O-ring over the threads of a 1/8" panel mount Luer fitting, and slide this into the ¼" hole drilled in the lid (**Figure 1A**).

1.1.3) Slip a second ¼" O-ring over the threads so that the lid is sandwiched between the two O-rings. Slip a locknut onto the threads and tighten to fix the panel mount Luer in place.

1.1.4) Snap lock rings onto the exposed male Luer projecting from the lid. Repeat steps 1.1.2-1.1.4 for each hole in the lid.

1.1.5) For lids that will be used on bubble column and bottle reactors, attach 1/8" female Luer to barb fittings to 1.5" pieces of 1/8" ID PVC tubing. Attach these to each of the exposed male Luer fittings on the lid.

1.1.6) Connect a check valve (pointing away from the lid) to the free end of one of the 1/8" pieces.

NOTE: This will serve as the exhaust port for the bioreactor.

1.1.7) Connect a male Luer to barb fitting to the second piece of 1/8" tubing projecting from the lid. Click the rotating lock ring into place and fasten a 0.2 µm air filter to this.

NOTE: This will serve as the inlet port for the reactor.

[Place **Figure 1** here]

1.2) Assemble the air delivery system (see **Figure 2A and 2B** for a schematic and photo).

1.2.1) Attach 1/8" NPT thread to barb fittings to the inlets and outlets on the back of each rotameter.

NOTE: 200-2500 cm<sup>3</sup>/min rotameters are for air pressure modulation upstream of the humidifier, 100-1000 cm<sup>3</sup>/min rotameters are for bottle reactors, 50-500 cm<sup>3</sup>/min rotameters are for air lift bioreactors, and 5-50 cm<sup>3</sup>/min rotameters are for CO<sub>2</sub> flow regulation. It is recommended to mount rotameters to a fixed surface (e.g. plastic sheet) so they do not fall over during operation.

1.2.2) Shut off the compressed air source, then connect 1/4" ID flexible PVC tubing to the compressed air source with a hose clamp. Step down the hose diameter to 1/8" ID PVC flexible tubing using a 1/4" female to barb fitting and a 1/8" male to barb fitting.

1.2.3) Connect the free end of the 1/8" ID tubing to the inlet of a 200-2500 cm<sup>3</sup>/min rotameter.

NOTE: The outlet of this rotameter will feed the humidifier bottle via 1/8" ID tubing.

1.2.4) Connect the 1/8" tubing to an inlet to a vented lid (use a female Luer to barb fitting to make the connection). Then connect a second piece of 1/8" tubing to the inside of the panel mount fitting.

NOTE: This piece will hang down into the humidifier and bubble air through the water.

1.2.5) Attach 1/8" female Luer to barb fittings to each end of a piece of 1/8" ID tubing and use this piece to connect the outlet of the humidifier to the inlet of the mixing trap.

1.2.6) In the same manner as 1.2.5, connect the outlet of the CO<sub>2</sub> regulator to a second port on the mixing trap.

1.2.7) Construct a manifold using 1/8" tubing and 1/8" multiport barb (see **Figure 2C**) to feed air into the rotameter banks.

NOTE: These rotameters will be used to supply the bioreactors. Avoid constructing more than 6 rotameters in series. Instead, use parallel banks of rotameters to expand the system. Ensure that the total flow demand for all reactors is less than 2500 cm<sup>3</sup>/min (or else a larger rotameter will be needed upstream of the humidifier).

[Place **Figure 2** here]

1.2.8) Connect the outlet (3<sup>rd</sup> port) of the mixing trap to the newly constructed rotameter banks using 1/8" tubing and a 1/8" female to barb Luer.

1.2.9) Connect sufficiently long 1/8" tubing to the outlets of each rotameter in the rotameter bank to supply air to the bioreactors. Label the ends of the tubing as well as the rotameters in the bank.

1.2.10) Apply water-proof silicone around all ports on the humidifier and mixing trap lids to ensure they are air tight.

1.3) Set up fish tanks, stir plates, and lights (**Figure 3**).

CAUTION: This system requires a large number of outlets and sufficient circuit capacity to support all components. Avoid stringing together multiple power strips and extension cords in a daisy-chain fashion because this is an electrical hazard. Use of GFI type outlets and power strips is highly encouraged due to the presence of water in the system.

1.3.1) Arrange the low-profile magnetic stirrers on a level surface that is strong enough to hold the weight of water-filled fish tanks.

1.3.2) Place small wooden or plastic blocks (that are slightly taller than the stir plates) around the perimeter of the stir plates to support the weight of the fish tanks.

CAUTION: Avoid placing the fish tanks directly on the stir plates as the weight will crush them.

1.3.3) Place the fish tanks over the stir plates and support blocks and fill the tanks with water.

1.3.4) Cut a piece of a stiff plastic sheet to fit on top of the fish tank as a lid. Cut holes in this cover to slide the hybridization tubes in and out. Also cut a hole for the fish tank heater.

1.3.5) Arrange the fluorescent light banks next to the fish tank to provide horizontal illumination of the bioreactors. Plug the light bank into a light timer to set a day/night cycle.

[Place **Figure 3** here]

## 2. Preparation of Microalgae Inoculum

2.1) Obtain microalgae inoculum from a cryo-preserved, plated, or liquid culture.

NOTE: It is recommended that cryopreserved organisms be plated prior to the use as inoculum to ensure that cells are viable and that the resulting culture is axenic. Agar medium (*e.g.*, ATCC #5 sporulating agar)<sup>21</sup> is a rich medium that works well for reviving species of *Chlorella* and *Auxenochlorella* from cryo-storage.

2.2) Prepare 2.4 L of mineral medium that is appropriate for the particular microalgae species.

NOTE: Examples include N8 medium<sup>23</sup> for species of *Chlorella*, N8-NH<sub>4</sub> medium<sup>21</sup> for species of *Auxenochlorella*. Use of a medium appropriate for the algae strain is one of the most important steps toward ensuring robust algae growth.

2.3) Aliquot 2.4 L of mineral medium equally into three 1 L glass bottles, add stir bars to each bottle and assemble the vented lids (**Figure 1**) for each bottle. Double check that the aeration tube is on the inlet side and each bottle has a stir bar in it.

2.4) Autoclave the stock bottles using a liquid sterilization cycle (121 °C) for 30 min. Autoclave 100 mL of deionized water (dH<sub>2</sub>O) and some 1.5 mL tubes at the same time, which will later be used for plating. Allow the medium to cool overnight. Alternatively, cool the reactor to room temperature and then aerate for 2 h prior to inoculation.

2.5) In a biosafety cabinet (BSC), inoculate microalgae from a plate or axenic liquid culture into the stock bottles. Use sterile technique to maintain axenic cultures in the following steps.

2.5.1) Add 20 mL of autoclaved dH<sub>2</sub>O to a sterile 50 mL centrifuge tube. Use a sterile 10 µL disposable loop to pick several single colonies from the plate from step 2.1. Dip the loop into the 50 mL tube and wash the algae cells into the 20 mL of autoclaved dH<sub>2</sub>O. Shake the 50 mL tube to make a homogenous microalgae solution.

2.5.2) Pipette 6 mL of microalgae solution into each stock bottle with a 10 mL sterile serological pipette. Swirl the bottle to mix the microalgae evenly into the medium.

2.6) Use a 2 mL sterile serological pipette to draw 1 mL samples from each stock bottle and transfer into sterile 1.5 mL tubes.

NOTE: Micropipettes are not recommended for this step due to the risk of contamination. Tighten the vented lids on stock bottles.

2.7) Place the stock bottles on stir plates (~150 rpm) and adjust air flow rate, CO<sub>2</sub>, and lighting levels as appropriate for the species. Rotate the stock bottle position every day.

2.8) Dilute the 1 mL samples obtained during step 2.6 (100-fold dilution in sterile water usually works well) and spread plate onto a rich agar medium.

NOTE: These plates can be used to check for instances of contamination as well as serve as a source of future algae inoculum for further experiments.

2.9) Take samples from the bottles (in the BSC) every two days to check the microalgae growth. Place samples in a 96-well microplate in triplicate (200 µL) and measure optical density (OD) at



550 nm and 680 nm every two days until OD reaches 0.2-0.3 (which typically requires 5-7 days).

2.10) Stop the incubation and place the stock bottles on a bench for 24-48 h to allow the algae cells to settle by gravity.

NOTE: The settled cells will be used next to inoculate the bubble column photobioreactors. If a more rapid cell collection is desired, cells may be centrifuged at no more than 1,000 x g to collect cells.

### 3. Cultivation of Microalgae in Bubble Column Photobioreactors

3.1) The day before bioreactor inoculation, prepare appropriate media and transfer 200 mL (or desired volume) to the bubble column photobioreactor tubes (hybridization tubes). Autoclave tubes with media and vented lids in place.

NOTE: If using wastewater as a growth medium, autoclave the empty bioreactors and add sterile-filtered wastewater (if axenic culture is desired).

3.2) Concentrate the settled microalgae stock by removing the supernatant using a vacuum pump. Leave less than 100 mL of medium in each bottle but avoid removing settled algae.

NOTE: Conduct this procedure inside a BSC and follow sterile technique. A simple vacuum apparatus can be constructed using either a vacuum flask or bottle. Fit a sterile serological pipette onto the end of the tubing.

3.3) Suspend and transfer the algae slurry to sterile 50 mL centrifuge tubes. Centrifuge at 1,000 x g for 5 min to further concentrate algae.

3.4) In the BSC, remove enough supernatant to achieve a total volume of ~80 mL of algae concentrates for 12 photobioreactors. Avoid vacuuming out the pellet. Transfer the algae concentrate to a sterile container (or the used algae stock bottle).

3.5) Add 6 mL of algae slurry into each photobioreactor with a sterile 10 mL serological pipette.

3.6) Sterile filter (0.2 µm syringe or vacuum filter) and add appropriate amounts of any other compounds (e.g. vitamin stocks) which cannot be autoclaved.

3.7) Swirl bioreactors to mix algae into the medium.

3.8) Draw a 2 mL sample from each bioreactor using a serological pipette and transfer to a 2 mL tube. Collect a 2 mL sample (in a BSC) every 24 h to monitor culture progress. Check the sample for pH using test strips and adjust the reactor as needed with either 3 M NaOH or 3 M HCl.

3.9) Tighten the bioreactor lids and place all bioreactors into the fish tank water bath. Adjust the

aeration, CO<sub>2</sub>, and lighting to the appropriate level for the species. Rotate the bioreactor position each day after sampling (Step 3.8).

3.10). Apply 200 µL of each culture sample in triplicate to wells of a 96 well microplate. Measure optical density (OD) at 550 nm and 680 nm.

3.10.1) On the last day of the culture period, measure OD under different dilution factors (*e.g.*, a 1x, 2x, 4x, 8x, 16x and 32x) to establish a correlation between the OD and the actual dry weight after harvest (step 4).

3.11) Centrifuge the 2 mL sample tube at 12,000 x g for 5 min.

3.12) Filter the supernatant through 0.2 µm non-sterile syringe filter and store the supernatant (and pellet if needed) at no higher than -20 °C for long term storage and later analyses of changes in media composition.

#### **4. Harvest and Freeze Drying of Microalgal Biomass**

4.1) Measure a fixed volume of algae culture from each bioreactor with a graduated cylinder (*e.g.*, 160 mL from a bioreactor that originally contained 200 mL of medium) and transfer into centrifuge bottles. Rinse the graduated cylinder with dH<sub>2</sub>O in between each measurement.

4.2) Centrifuge at 4,696 x g for 5 min. Discard the supernatant by carefully vacuuming it out.

4.3) Transfer the pellets to labeled 50 mL tubes. Rinse the centrifuge bottles with dH<sub>2</sub>O, and transfer contents to the 50 mL tubes. Ensure the total tube volume does not exceed 45 mL.

4.4) Wash the algae pellets with dH<sub>2</sub>O to remove salts.

4.4.1) Centrifuge the 50 mL tubes at 4,696 x g for 5 min and discard the supernatant.

4.4.2) Add 40 mL dH<sub>2</sub>O to each 50 mL tube; vortex to mix. Centrifuge again at 4,696 x g for 5 min and discard supernatant.

4.4.3) Repeat step 4.4.2 again.

4.5) Label and weigh empty 15 mL centrifuge tubes on a 4-decimal balance (label both the lid and tube and weigh them together). Weigh one tube per algae culture. Weigh each 15 mL tube twice to minimize error.

4.6) After the last wash, discard the supernatant, and add 7.5 mL of dH<sub>2</sub>O to each 50 mL tube. Vortex and transfer the algae slurries into the pre-weighed 15 mL tubes. Rinse the 50 mL tubes with additional dH<sub>2</sub>O and transfer liquid to the 15 mL tubes. Avoid exceeding 12 mL of total volume in the 15 mL tubes.

4.7) Centrifuge the 15 mL tubes at 4,696 x g for 5 min and decant the supernatant. Freeze the tubes with pellets at -80 °C for at least 30 min in preparation for freeze-drying.

4.8) Freeze dry overnight or until dried.

4.9) Weigh and record the freeze dried 15 mL tubes with algae.

## 5. Lipid Extraction using a Modified Folch method<sup>24</sup>

5.1) Weigh out 20 mg of freeze-dried algal biomass into a 2 mL screw cap polypropylene tube (check manufacturer label to ensure product is suitable for bead extractions).

5.2) Add 1.5 mL of Folch solvent (2:1 chloroform/methanol) to each 2 mL tube (which contains 20 mg of freeze-dried algae). Pour ~0.5 mL zirconia/silica beads (0.5 mm) into each tube until liquid level in tube reaches 2 mL.

CAUTION: Handle chloroform and methanol in a fume hood and avoid breathing fumes or skin contact.

5.3) Homogenize the algae samples in a bead mill for 20 s at a speed of 6.5 m/s. Transfer tubes to ice for 30 s to chill samples. Repeat five more times to fully extract lipids.

5.4) Filter the homogenate through a 5 mL syringe containing a stainless-steel wire mesh disk (#60 mesh) to strain out the beads, collecting filtrate in a 15 mL tube.

5.5) Wash the beads with 1.5 mL of Folch solvent, pushing liquid through with the syringe as necessary. Repeat this wash two more times and collect all filtrate in the 15 mL tube, yielding a final volume of approximately 6 mL.

5.6) Add 1.2 mL of 0.9% (w/v) NaCl solution to the Folch extract in the 15 mL tube and vortex to mix well.

NOTE: If necessary, more Folch solvent can be used to wash beads (use 0.2x the total wash volume of 0.9% NaCl solution to induce phase separation).

5.7) Centrifuge the 15 mL tubes at 6,000 x g for 5 min. Record the bottom chloroform (green) phase volume to the nearest 0.1 mL using lines on the side of the 15 mL tube. Transfer the bottom phase to a glass vial (with the lid) using a glass Pasteur pipette.

5.8) Store the lipid at -20 °C or (-80 °C if there are plans to use this extract for fatty acid analysis).

## 6. Neutral Lipid Assay using a Microplate Method (adapted from Higgins *et al.* 2014<sup>22</sup>)

6.1) Prepare stock solutions. Prepare 10 mL 1 mg/mL vegetable oil standard in chloroform and store at -20 °C.

NOTE: Any vegetable oil may be used in this assay because it is not sensitive to the types of fatty acids. Prepare 10 mL of 200 µg/mL Nile Red solution in dimethyl sulfoxide (DMSO) and store in the dark at room temperature.

6.2) Pre-heat dry microplate block to 55 °C in a fume hood. While this is heating, dilute the lipid extracts and vegetable oil standard 3-fold with methanol.

NOTE: This dilution can be altered based on the lipid content of the algae, but this level works well for most *Chlorella*.

6.3) For each diluted sample, add 80 µL to a 96 well polypropylene microplate in quadruplicate.

CAUTION: Use of polystyrene plasticware is not recommended for use with organic solvents.

6.4) For the solvent blank, apply 80 µL of 2:1 methanol/chloroform in quadruplicate. For standards, add 10, 30, 60, 90, and 120 µL of the diluted vegetable oil standard in quadruplicate.

6.5) Place the microplate in a dry block heater at 55 °C for 20-30 min until all solvent has evaporated. While the solvent evaporates, prepare the working Nile Red solution (need 200 µL of 1 µg/mL solution per plate well). As an example, twelve samples and a full set of standards requires 16 mL of 1.0 µg/mL solution; prepare by dissolving 80 µL of the 200 µg/mL stock (in DMSO) into 16 mL dH<sub>2</sub>O.

6.6) Remove the microplate from the heating block and let cool to room temperature. Add 30 µL of isopropyl alcohol to each well and mix by pipetting up and down. Ensure all pipette channels are mixing the solution and resuspending the lipids, yielding a homogeneous green liquid.

6.7) Add 200 µL of Nile Red solution (1 µg/mL) to each well, pipette up/down 10 times to mix. Incubate the plate for 5 min at room temperature. While waiting, prepare a 50% bleach solution by mixing bleach (6% hypochlorite) with dH<sub>2</sub>O. 20 µL per well are needed. Preparation of 3 mL of 50% bleach is sufficient for 12 samples and a full set of standards.

6.8) Add 20 µL of bleach solution to each microplate well and pipette up and down 5 times to mix well. Incubate 30 min at room temperature.

6.9) After 30 min, read fluorescence in the samples every 5-10 min at 530 nm excitation/575 nm emission with auto cutoff set to 570 nm until the signal from algae samples stabilizes. Typically, 60 min of total incubation is sufficient.

6.10) Create a calibration curve for the vegetable oil standards (in the range of 0-40 ng oil/well).

NOTE: A linear fit works well for low (<30 ng/well) oil concentrations and a polynomial fit may be used if the standard exceeds 30 ng/well. Use this correlation to quantify the neutral lipid in the sample wells.

#### REPRESENTATIVE RESULTS:

This procedure yields a time course of algal optical density data at OD 550 nm (**Figure 4A**). The optical density and dry weight concentration data can be correlated (**Figure 4B**). This is accomplished by first calculating the final dry weight algae concentration after the freeze-drying step. Next, the optical density of the culture serial dilution (performed on the last day of sampling) and the actual dry weight concentrations can be correlated. For low cell concentrations, a linear correlation can be used whereas for higher cell concentrations, a second order polynomial correlation may be used. It is recommended to create a separate correlation for each culture condition. Finally, the correlation can be applied to the time course optical density data to obtain a dry weight growth curve (**Figure 4C**). In this example experiment, *Auxenochlorella protothecoides* (UTEX 2341<sup>25</sup>) was cultured under four conditions: axenic control cultures grown on fresh N8-NH<sub>4</sub> medium<sup>21</sup>, in co-culture with the bacteria *Azospirillum brasilense*, in medium supplemented with 50 mg/L of indole-3-acetic acid, and on spent medium from *A. brasilense*. *A. brasilense* is known to produce indole-3-acetic acid, a plant growth promoting hormone, that also promotes growth in some microalgae. However, at 50 mg/L, the IAA treatment completely inhibited *A. protothecoides* growth. Consequently, optical density data was available, but the quantity of algae was insufficient to obtain an accurate dry weight concentration. In this case, the correlation for the control culture may be applied or optical density data may be reported directly. Because OD data was collected at both 550 nm and 680 nm absorbance, either dataset may be used for the correlation between OD and dry weight. Typically, OD 550 is used because it almost completely excludes the absorbance of chlorophyll<sup>26</sup>, thus suppressing bias from changes in chlorophyll content. In contrast, OD 680 includes the absorbance of chlorophyll and high ratios of OD 680/550 indicate high chlorophyll content in the algae. **Figure 4D** shows a set of twelve algae cultures growing in the bubble column photobioreactors. Even with only three biological replicates per treatment, tight standard deviations were achieved, allowing for high sensitivity to differences among treatments.

[Place **Figure 4** here]

Neutral lipid data is shown for two example experiments in **Figure 5**. This assay has been shown to correlate well with neutral lipid content, particularly triacylglycerol (TAG) content. This can be seen by comparing the neutral lipid assay (**Figure 5A**) to a corresponding thin layer chromatography plate (**Figure 5B**). The same trend can be seen in the second experiment (**Figure 5C and 5D**). In all of these experiments, canola oil was used as a standard and resulted in a linear correlation ( $R^2$  of 0.98 for the first experiment and 0.99 for the second) between fluorescence and canola oil mass in the well. Note that if all samples have low lipid content, then the highest point or two on the standard may be dropped. This correlation can be used to calculate the quantity of neutral lipid ( $\mu\text{g}$ ) in each of the algae sample wells. The oil mass can then be converted to a concentration in the lipid extract applied to the microplate well. This value is multiplied by the dilution factor used (*e.g.*, 3x) to obtain the neutral lipid content of the original Folch extracts.

This concentration is then multiplied by the volume of the extract (should be close to 4 mL) and then divided by the total mass of algae biomass used for lipid extraction (should be close to 20 mg). The result is the neutral lipid content of the microalgae.

[Place **Figure 5** here]

Also, it is good practice to calculate the coefficient of variation (standard deviation divided by the mean) of the raw fluorescence readings across all technical replicates in the neutral lipid assay. Assuming technical replicates were carried out in the microplate in quadruplicate as noted in the procedure, the coefficient of variation typically should not exceed 10%. High coefficients of variation are usually the result of poor mixing (particularly during the addition of isopropyl alcohol) and potentially inaccurate use of the multichannel pipette.

#### FIGURE AND TABLE LEGENDS:

**Figure 1. Schematic and photos for constructing bioreactors.** (A) Schematic for construction of the bioreactor lids (B) photo of the assembled bioreactor lid, and (C) photo of the assembled lid used for the humidifier. Note that the humidifier fittings should be coated in water-proof silicone to ensure an airtight seal with the lid.

**Figure 2. Schematic and photos for assembling bubble column system.** (A) Schematic of the aeration system (B) photo of the humidifier, mixing trap, and rotameter bank, and (C) photo of the manifolds used to connect the rotameter banks together.

**Figure 3. System schematic for the bottle bioreactors (left) and the bubble column photobioreactors (right).** This figure has been modified from Higgins *et al.*<sup>17</sup>.

**Figure 4. Algae growth results in bubble column photobioreactors.** (A) The optical density (550 nm) growth curve of *Auxenochlorella protothecoides* (UTEX 2341) shows cultures entering late logarithmic growth at 120 hours. Control cultures were grown on fresh N8-NH<sub>4</sub> medium, treatment 1 is co-cultures of *A. protothecoides* and *Azospirillum brasilense* grown on fresh N8-NH<sub>4</sub> medium, treatment 2 is axenic *A. protothecoides* grown on N8-NH<sub>4</sub> medium supplemented with 50 mg/L indole-3-acetic acid (IAA), and treatment 3 is axenic *A. protothecoides* grown on spent medium from *A. brasilense*. The spent medium was prepared by culturing *A. brasilense* for 96 hours on N8-NH<sub>4</sub> medium supplemented with 2 g/L malic acid. Cells were removed, and the medium was re-supplemented with ammonium to restore its initial level, pH was adjusted, and the medium was sterile filtered (0.2 µm). Note that the 50 mg/L IAA treatment completely inhibited algae growth. (B) Correlation curves between OD 550 nm and final dry weight concentration using a second order polynomial fit. No correlation is shown for treatment 2 because no algae could be harvested at the end of the culture period. (C) Application of the polynomial correlation to the optical density data yields a growth curve with dry weight concentration on the y-axis. The control culture correlation was applied to the OD data for treatment 2. Error bars are standard deviations based on 3 biological replicates. (D) Photo of the bubble column photobioreactors shortly after culture inoculation.

**Figure 5. Neutral lipid data obtained from cultures of *Chlorella sorokiniana* (UTEX 2714).** (A) Neutral lipid content (% dry weight) for algae in experiment 1 in which algae were grown for 120 hours. The control culture was axenic and cultured on fresh N8 medium<sup>23</sup>, treatment 1 was a co-culture of *C. sorokiniana* and *A. brasilense* on fresh N8 medium, treatment 2 was fresh N8 medium supplemented with 50 mg/L IAA, and treatment 3 was spent medium from *A. brasilense*. The spent medium was prepared by culturing *A. brasilense* for 96 hours in N8 medium supplemented with 2 g/L malic acid, removing cells, supplementing lost nitrate, adjusting pH, and sterile filtering the medium (0.2  $\mu$ m). Unlike *A. protothecoides*, 50 mg/L of IAA did not inhibit *C. sorokiniana* growth. (B) TLC plate image for experiment 1 showing relative TAG abundance. (C) Neutral lipid content (% dry weight) for algae in experiment 2 which had the same treatments as experiment 1 but the cells were harvested after 72 hours of growth. (D) TLC plate image for experiment 2 showing relative TAG abundance. Error bars are standard deviations based on 3 biological replicates.

#### DISCUSSION:

The most important consideration when culturing algae is an understanding of the specific needs of the organism or group of organisms. The algae cultivation system described here can be used to culture a wide range of algae but the specific abiotic factors (temperature, media, pH, light intensity, CO<sub>2</sub> level, aeration rate) need to be adjusted to the needs of the organism. Note the parameters described here were used for the cultivation of *Chlorella* and *Auxenochlorella*. These organisms are of industrial interest because they are tolerant to high nutrient, light, and temperature levels<sup>27</sup>. However, light levels can be reduced through removal of fluorescent bulbs and the day/night cycle can be adjusted to represent seasonality. Likewise, the water heaters can be turned up or down to control the water bath temperature on the system. While the system described here does not employ such a feature, it is possible to chill the fish tank water baths below room temperature through a low-cost cooling system. Place a bucket of water in a small refrigerator and use a variable speed pump to pump water from the cold-water bucket to the fish tank and back. The faster the pumping rate, the colder the fish tank reservoir will become.

Although the algae cultivation system generally provides consistent results, there are a few important caveats that should be considered. The first is water siphoning that occurs in the event that the aeration system experiences a sudden loss of pressure (e.g., a blown fitting or an air compressor failure). Pressure that is in the humidifiers will push humidifier water backward through the tubing, including through the rotameter. Installing an upstream trap or backflow preventer can help. Note that siphoning will not impact the bioreactors themselves because they operate at atmospheric pressure. Routine inspection of the piping and fittings can help alleviate system failures. Another important consideration is to routinely inspect and replace air filters and check valves on the bioreactors. Be sure to follow the manufacturer's recommendation for the number of autoclave cycles allowable. This is particularly important if maintenance of axenic cultures is critical to the experiment. Finally, it is recommended to purchase or construct an autoclavable rack for safe transport of the bioreactors between the water baths, autoclave, and biosafety cabinet. A stainless-steel wire rack can serve this purpose.

The bioreactor system described here has several limitations. A key limitation is the need to handle the reactors in a biosafety cabinet using sterile technique. The reactors lack an anti-siphoning sampling port, requiring the user to move the reactor to a biosafety cabinet to sample without contaminating the culture. The reactors also require manual pH reading and adjustment which introduces potential for human error.

The bioreactor system described here fills a niche between simple flask cultivation and fully-controlled bioreactors. The bioreactor system was developed in response to a need for more consistent results than were achievable using bottles. The data shows that this system generates consistent growth results when operated appropriately. Note that aerated bottles were employed in the procedure for cultivation of algae stock, but this was done only to produce inoculum for the experiment. This is acceptable because stock bottles were pooled for inoculation and hence variability among reactors is not an issue.

As many algae researchers are interested in monitoring both growth and neutral lipid content, we have included our approach to measuring both of these parameters here. Use of optical density to measure growth is standard practice and is unparalleled in its simplicity. However, correlations between dry weight and optical density change over time and depend on culture conditions. It is recommended to produce a correlation equation for each experimental treatment within every experimental batch. This is possible in the proposed procedure because freeze dried algae will be obtained after every culture experiment. A critical assumption of the optical density approach is that the correlation between optical density and dry weight holds constant over the course of batch growth. As long as deviations from this assumption are small, the result will be reasonably accurate. The relative accuracy can be assessed by comparing the calculated algae dry weight concentration at time zero. Assuming cultures were well mixed, and pipetting was accurate, all of the cultures should have the same initial inoculation density. The optical density approach can also be challenged when background absorbance of the medium is high (*i.e.*, when working with certain wastewaters). Subtraction of the medium absorbance (prior to inoculation) from each OD reading can help with this issue.

Extraction of lipids from the dry algae follows the well-established Folch approach<sup>21,24</sup>; however, there are important considerations. Different algae species have different cell walls with varying degrees of toughness. The zirconia/silica beads used here are sharp and are designed to pierce strong, polysaccharide cell walls. A softer bead type (*e.g.*, glass) or fewer bead disruption cycles may be used on algae with weaker cell walls. However, a rule of thumb is that the resulting cell pellet after extraction should be free of pigment, indicating that all chlorophyll was extracted. One of the most common sources of failure during the lipid extraction step occurs due to improper freeze drying. If the sample melts in the freeze dryer before it is fully dry, the result will be a very hard, dark, waxy pellet. This is the result of cells that lysed under the vacuum conditions of the freeze dryer. The pellet may be weighed to obtain a dry weight, but it cannot be used for lipid extraction as the waxy particles do not break down in the Folch solvent. To ensure that freeze drying always yields soft, powdery samples, it is essential to freeze all samples to -80 °C and promptly transfer them to the freeze dryer. Moreover, using parafilm (with a hole poked in it) rather than loosened tube lids will ensure that moisture can be continuously removed from



the sample before it thaws.

The neutral lipid assay described in this procedure has been published previously and includes a discussion of alternative lipid assays<sup>22</sup>. However, some important improvements have been made to that procedure since publication. Most notably, the Nile red solution concentration was increased from 0.5 µg/mL to 1 µg/mL. The effect of this change was higher signal intensity, improved repeatability, and an elimination of signal decline over time during the incubation period. The results show that the assay compares well to results from qualitative thin layer chromatography. This assay was developed and validated using various species of *Chlorella* and *Auxenochlorella* so its applicability to species of significantly different composition has not been determined. All green pigment should be completely removed from the assay during the bleach incubation, leading to samples that are clear or very pale yellow. Also note that lipid extracts that are degraded (as indicated by a change from green to brown color) typically fail to deliver accurate results in this assay. It is thus imperative to store lipid samples at no higher than -20 °C in the dark.

The methods presented here for culturing algae, measuring growth, and quantifying neutral lipids are useful for a variety of engineering applications of algae, but are particularly suitable for research on biofuel production. These methods are also being used to study algal growth inhibition on wastewaters<sup>28</sup> as well as impacts of organism interaction on the growth and composition of microalgae.

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#### DISCLOSURES:

The authors have nothing to disclose.

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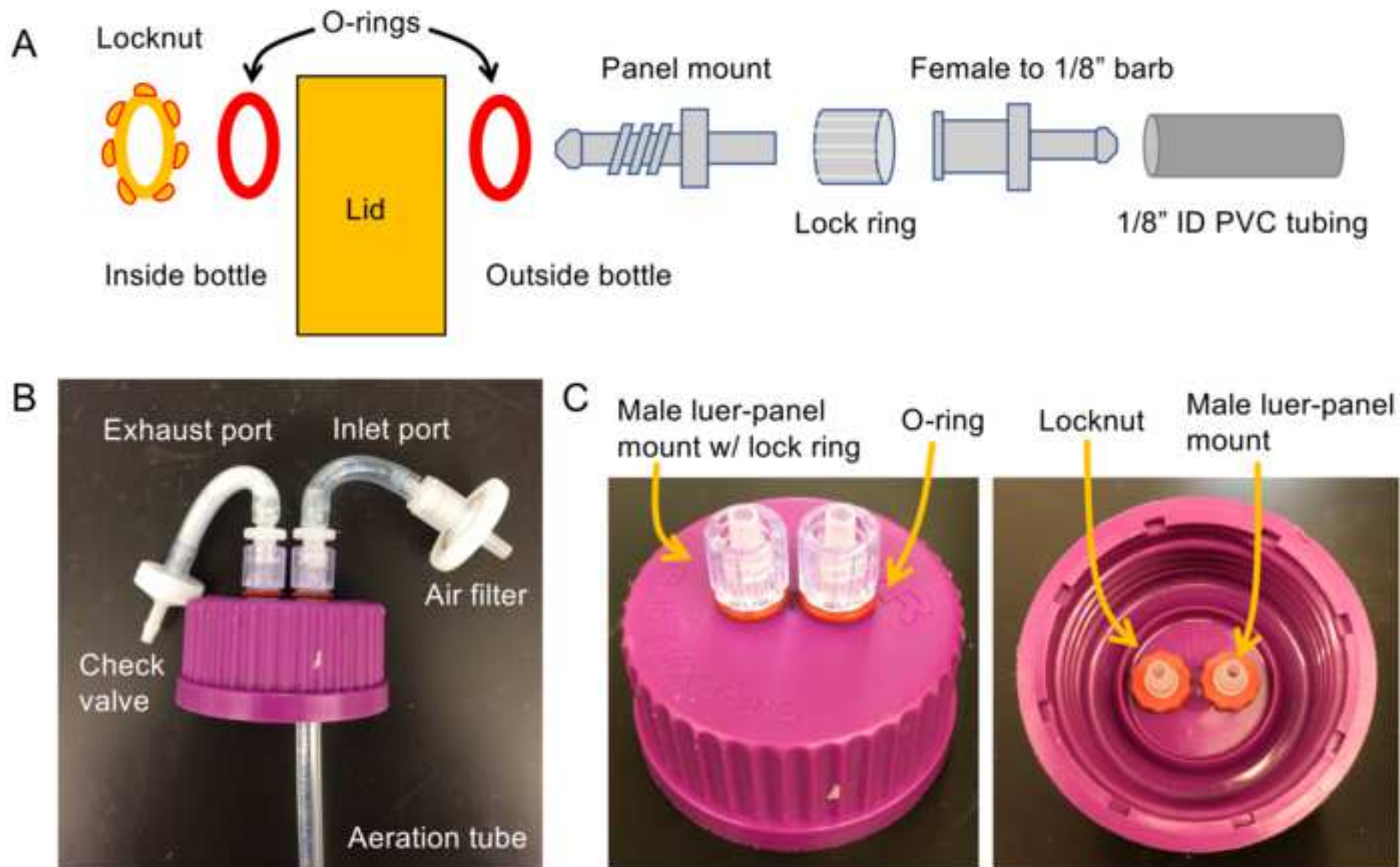
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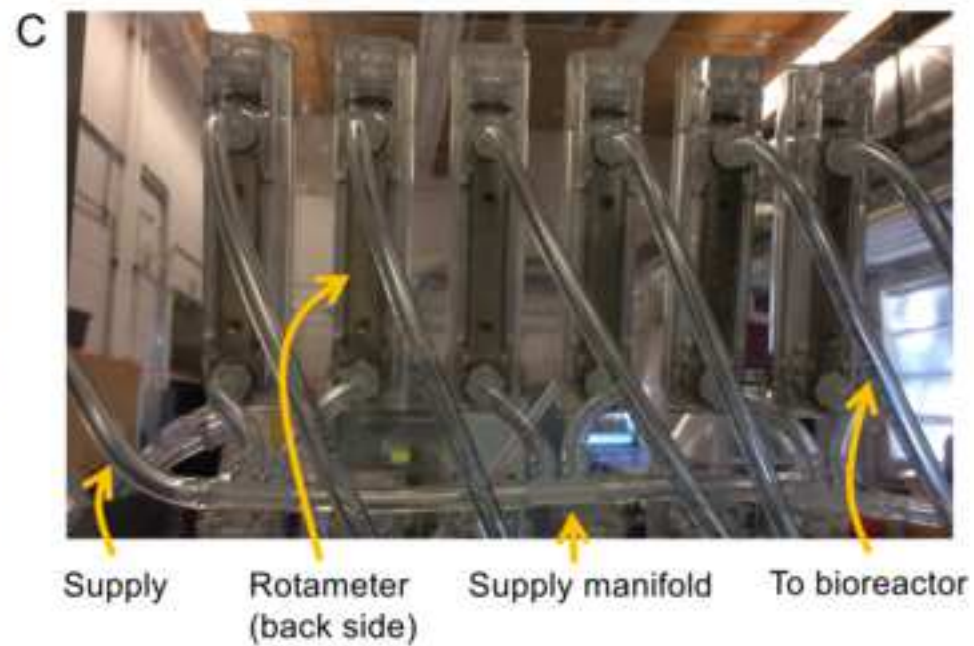
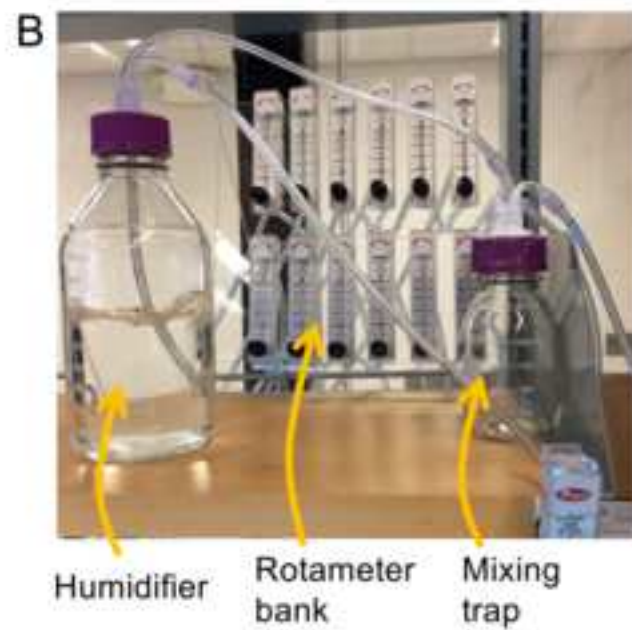
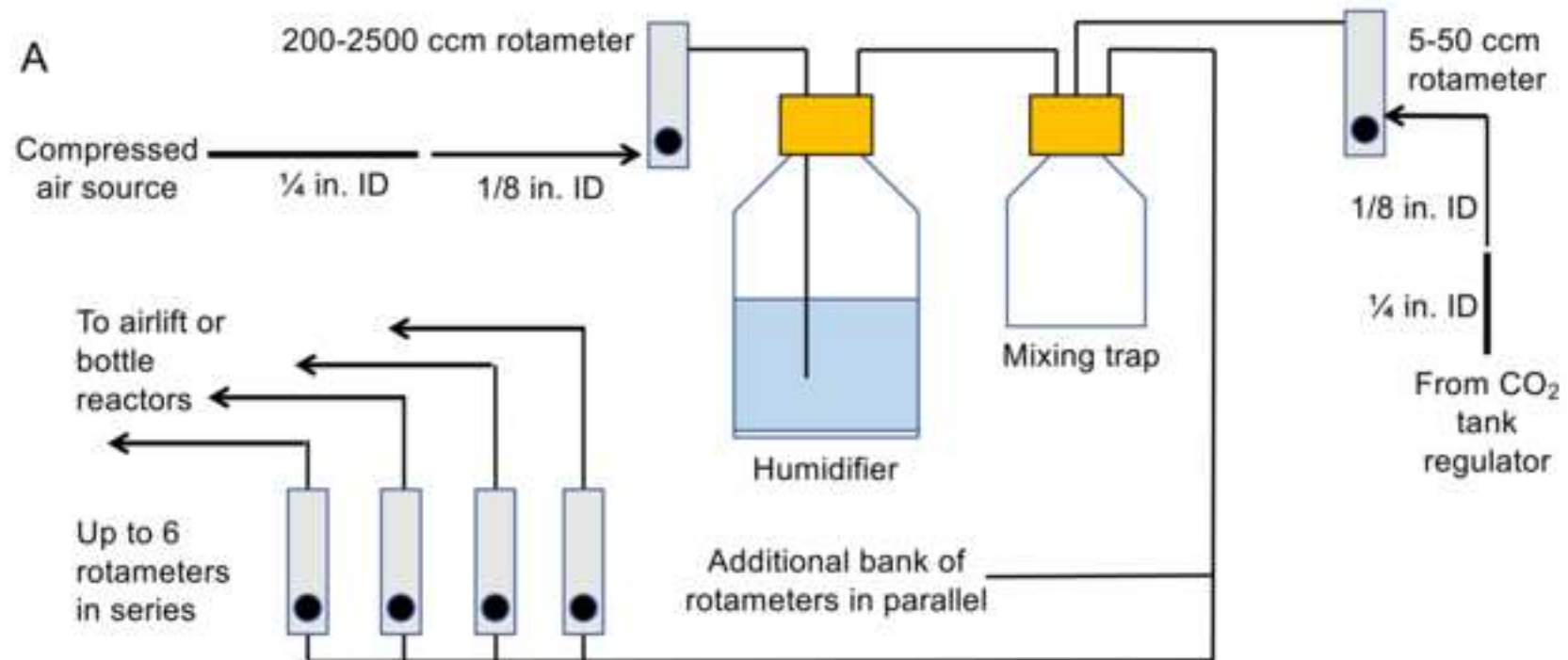
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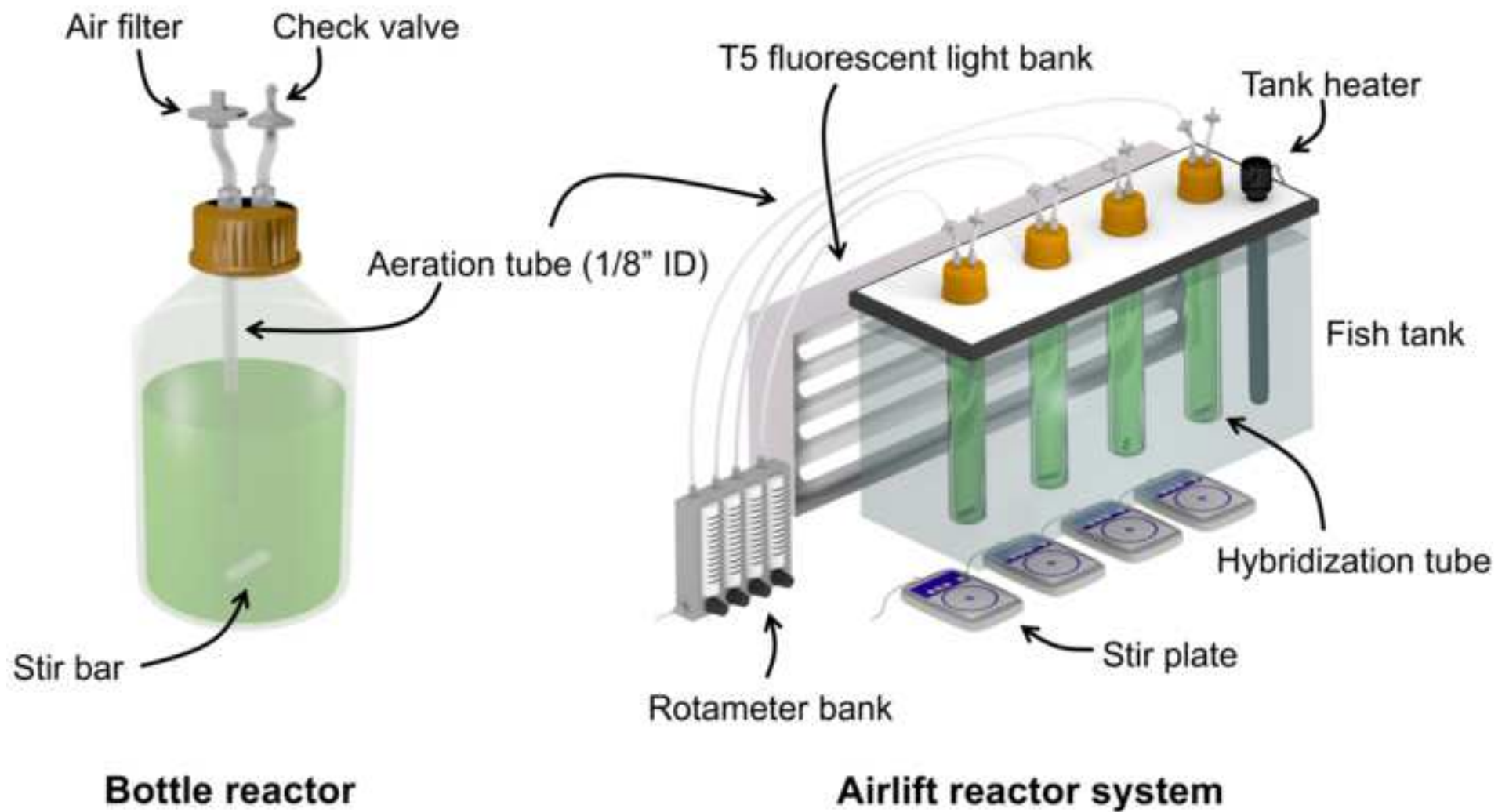
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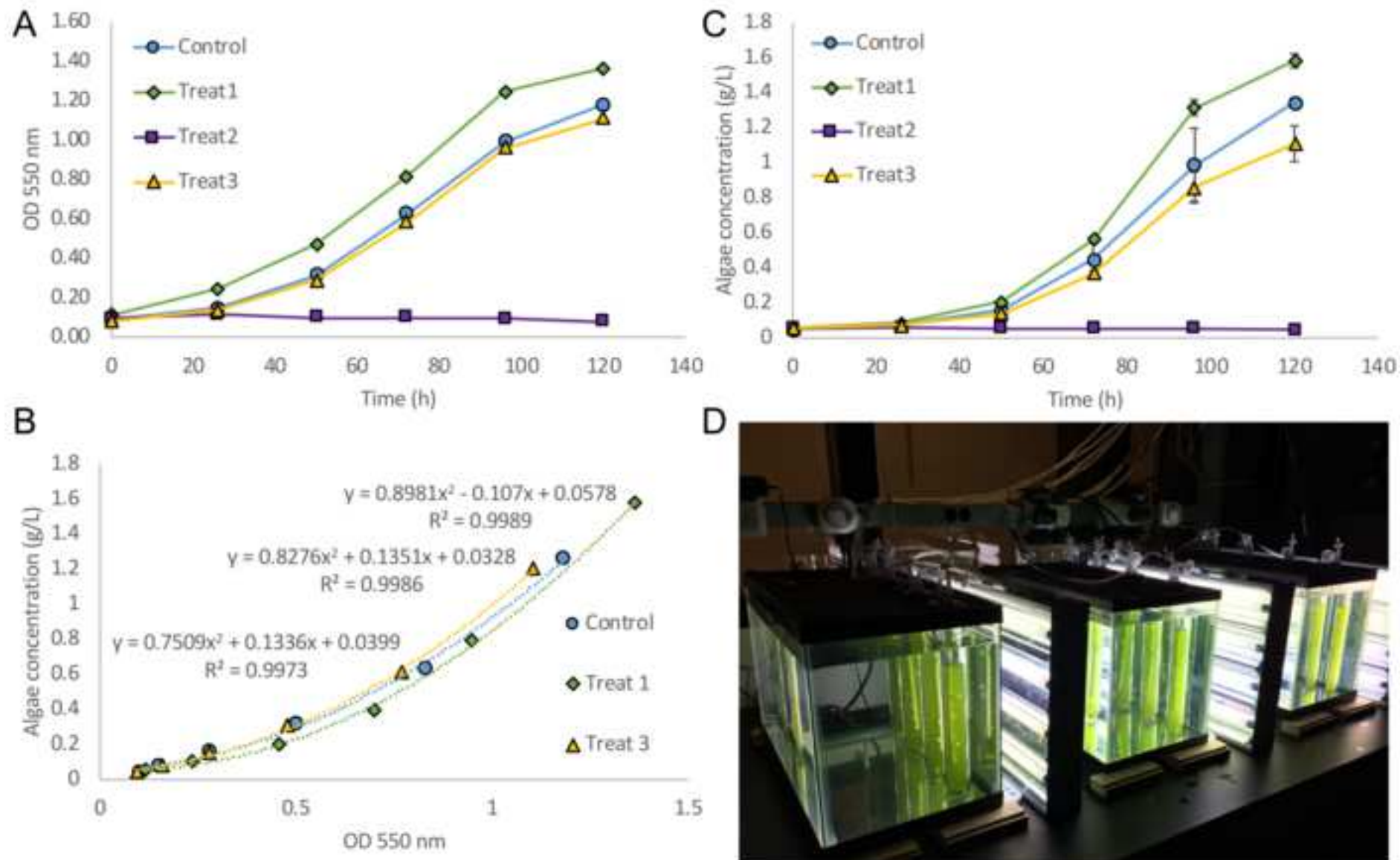
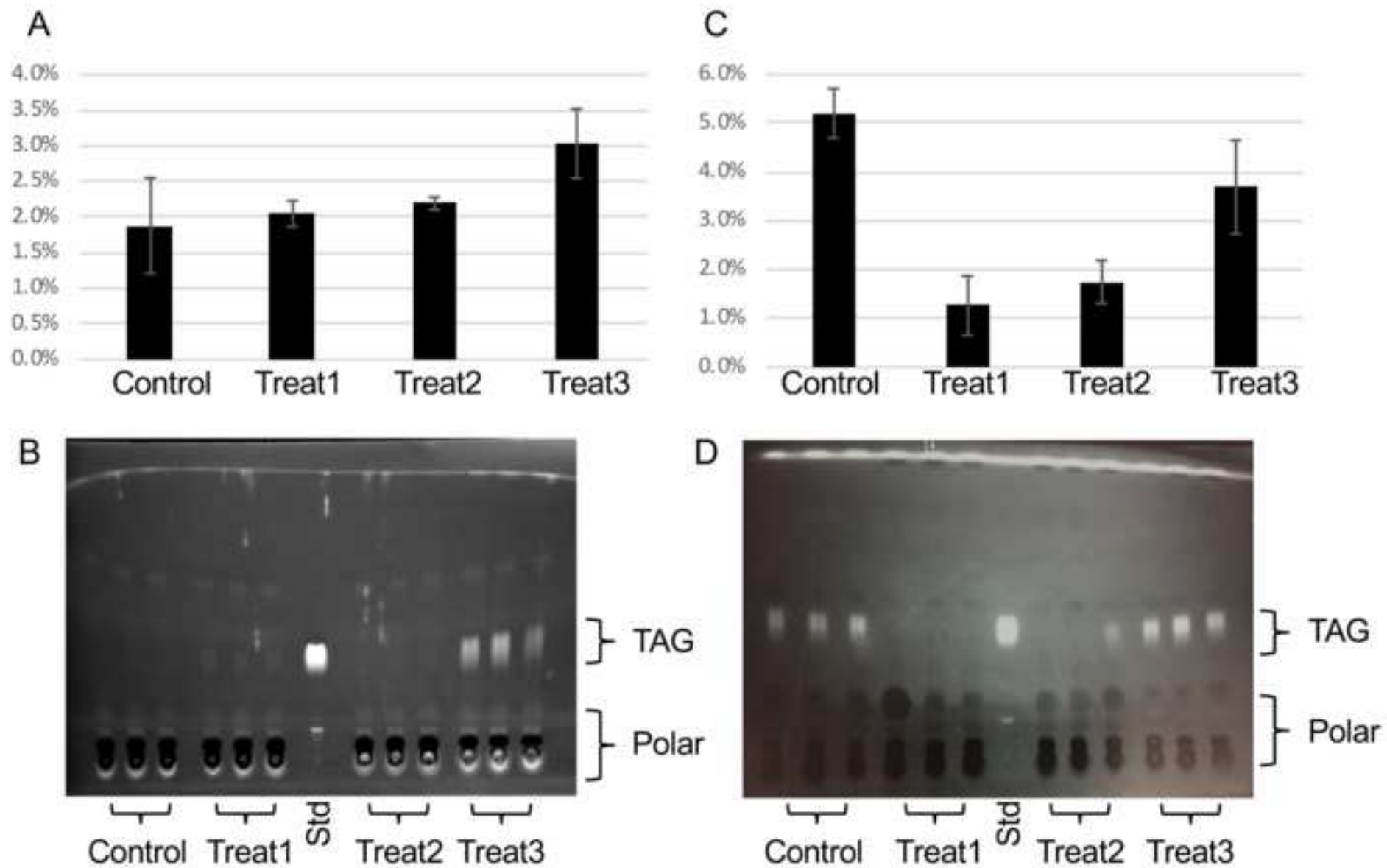


Figure 5

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Name of Material/ Equipment	Company	Catalog Number
<b>Supplies for airlift photobioreactor setup</b>		
1 L Pyrex bottles	Corning	16157-191
1/2" hose clamp	Home Depot	UC953A
1/4" female luer to barb	Nordson biomedical	Nordson FTLL360-6005
1/4" ID, 3/8" OD autoclaveable PVC tube	Thermo-Nalgene	63013-244
1/4" in O-rings	Grainger	1REC5
1/8" Female luer to barb	Nordson biomedical	FTLL230-6005
1/8" ID, 1/4" OD autoclaveable PVC tube	Thermo-Nalgene	63013-608
1/8" male spinning luer to barb	Nordson biomedical	MLRL013-6005
1/8" multiport barb	Nordson biomedical	4PLL230-6005
1/8" NPT to barb	Nordson biomedical	18230-6005
1/8" panel mount luer	Nordson biomedical	Nordson MLRLB230-6005
10 gallon fish tank	Walmart	802262
100-1000 ccm flow meter	Dwyer	RMA-13-SSV
2 ft fluorescent light bank	Agrobrite	FLT24 T5
200-2500 ccm flow meter	Dwyer	RMA-14-SSV
250 mL Pyrex bottles	Corning	16157-136
50-500 ccm flow meter	Dwyer	RMA-12-SSV
5-50 ccm flow meter	Dwyer	RMA-151-SSV
Air filters 0.2 µm	Whatman/ Fisher	09-745-1A
Check valves	VWR	89094-714
Corning lids for pyrex bottles	VWR	89000-233
Female luer endcap	Nordson biomedical	Nordson FTLLP-6005
Hybridization tubes	Corning	32645-030
Light timer	Walmart	556393626
Locknuts	Nordson biomedical	Nordson LNS-3
Low profile magnetic stirrer	VWR	10153-690
Male luer endcap	Nordson biomedical	Nordson LP4-6005
Spinning luer lock ring	Nordson biomedical	Nordson FSLLR-6005
Stir bars - long	VWR	58949-040
Stir bars - medium	VWR	58949-034
<b>Supplies and reagents for culturing algae</b>		
0.2 µm filters	VWR	28145-491

1 mL syringes	Air-tite	89215-216
1.5 mL tubes	VWR	87003-294
10 mL Serological pipettes	Greiner Bio-One	82050-482
100 mm plates	VWR	25384-342
15 mL tubes	Greiner Bio-One	82050-276
2 mL Serological pipette tips	Greiner Bio-One	82051-584
2 mL tubes	VWR	87003-298
50 mL tubes	Greiner Bio-One	82050-348
96 well microplate	Greiner Bio-One	89089-578
Inoculating loops	VWR	80094-478
Liquid carbon dioxide tank and regulator	Airgas	CD-50

**Supplies and reagents for lipid extraction and neutral lipid assay**

2 mL bead tubes	VWR	10158-556
96 well microplates	Greiner Bio-One	82050-774
Bleach	Walmart	550646751
Chloroform	BDH	BDH1109-4LG
Dimethyl sulfoxide	BDH	BDH1115-1LP
Isopropyl alcohol	BDH	BDH1133-1LP
Methanol	BDH	BDH20864.400
Nile red	VWR	TCN0659-5G
Pasteur pipette tips	VWR	14673-010
Sodium chloride	BDH	BDH9286-500G
Vegetable oil	Walmart	9276383
Zirconia/ silica beads (0.5 mm diameter)	Biospec products	11079105z

**Equipment**

Analytical balance	Mettler-Toledo	XS205DU
Bead homogenizer	Omni	19-040E
Benchtop micro centrifuge	Thermo	Heraeus Fresco 21 with 24x2
Dry block heater	VWR	75838-282
Freeze dryer	Labconco	7670520
Large benchtop centrifuge	Thermo	Heraeus Megafuge 16R Tissue

Microplate reader  
Vortex mixer

Molecular Devices    SpectraMax M2  
VWR                      10153-838

### Comments/Description

For bottle reactors, humidifiers  
or equivalent

1/4" ID, PP

50'

#010 Medium Hard Silicone O-Ring, 0.239" I.D., 0.379" O.D.

250'

1/8" multiport barb

1/8" 200 series barb

1/8", PP

Can hold up to 8 bioreactors depending on layout

For bottle reactors

For air regulation upstream of humidifier

For gas mixing after humidifier

For hybridization tube reactors

For CO<sub>2</sub> flow rate control

Polyvent, 28 mm, 0.2 µm, PTFE, 50 pack

10 GL45 lids

Female stable PP

35x300 mm, pack of 2

1/4", red nylon

Low profile magnetic stirrer

Male plug PP

38.1 mm, for bottle reactors

25 mm, for hybridization tubes

13 mm, PTFE, for filtering spent media from daily culture sampling

For filtering spent media from daily culture sampling

Sterile (or equivalent)

Sterile (or equivalent)

100x15 mm stackable petri dishes, sterile

Sterile (or equivalent), polypropylene

Sterile (or equivalent)

Sterile (or equivalent)

Sterile (or equivalent), polypropylene

Polystyrene with lid, flat bottom

Sterile (or equivalent)

Polypropylene tube w/ lid

Polypropylene, flat bottom

Only use regular bleach, not cleaning bleach

Any vegetable oil should work as long as it is fresh

Capable of at least 4 decimal accuracy

Including rotor capable of handling 1.5 and 2 mL tubes

Including dry block for a microplate

2.5L freeze drying system

Including rotors capable of handling 400 mL bottles, 50 mL tubes, and 15 mL tubes

Capable of reading absorbance and fluorescence



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