

**Fig. 1**

Initial assembly of MBRA. **(a)** Top-view of initial MBRA strip, displaying three entry ports with their assigned uses labeled. **(b)** An image of the male luer adapter w/ a strip of PTFE tubing for use in the waste line. **(c)** Depiction of waste line adapter inside an MBRA chamber. **NOTE**: PTFE tubing should be cut long enough to where the tip of tubing is at water level. **(d)** Assembled MBRA strip with source line, waste line, and sample port labeled.

When using a MiniBioReactor Array (MBRA) strip, there are three separate assemblies that must be done for preparation of a bioreactor experiment: the main system (which includes the MBRA unit), the source system (which includes the media source) and the waste system (which includes the waste line for disposal). The main system can be subdivided into three different components: the MBRA, the media lines for entry of media to the strip’s chambers, and the waste lines for exit of media from the strip’s chambers. Before beginning, there are certain parts that must be prepared (primarily strips of tubing). It is highly recommended that the parts be marked or labeled to help distinguish amongst them.

The ismatec lab tubing will be for use as pump lines, or the lines that will be placed on the peristaltic pumps. There are two sets of ismatec lab tubing, but the tubing should come color coded with one set being red and the other orange. The red tubing will be used for the waste pump line, while the orange tubing will be used for the media pump line. A small female luer barb must be attached to each end of both sets of pump line tubing. This can be a little difficult as the tubing is very narrow, but a good tip is to place the ends of the tubing into a beaker with hot water to allow the tube plastic to become more malleable. It is highly recommended that back-up pump lines be prepared as there can be problems that arise which require replacement of a pump line.

The strips of C-Flex tubing used vary in length depending on the use.

1. For the main system, tubing will be needed with the following lengths: 6.5, 7, 7.5, 8, 8.5, and 9 inches (two strips of each length for one strip). Remember to label the tubing (a good idea would be to write the length of the tube on the side).
2. For the media source system and waste-line systems, the amount of tubing needed is entirely dependent on the set-up for the experiment. Are all six chambers of the strip being used? Is every chamber/strip using the same media source? Based on the answers to these questions, the media source line set-up can be designed. Typically, a fork-like design is used.
3. For the waste exit line, the amount of tubing is entirely dependent on the length of the anaerobic chamber being used. Based on the available port, a plug (e.g. a rubber stopper) can be threaded into the tubing.

Let’s begin with **the main system** (the STL file for the MBRA can be provided upon request).

**Parts and Supplies Required (per strip):**

18 Male Luer Adapter

12 Large Female Luer Barb

6 Medium Female Luer Barb

12 Male Luer Barb

6 Precision Seal Rubber Septa

C-Flex Tubing Strips (two of each of the following: 6.5, 7, 7.5, 8, 8.5, and 9-inch strips)

MiniBioReactor Array Strip

6 Magnetic Stir Bars, 8 x 3 mm

Epoxy, Resin and Hardener

Diba Omnifit Tubing, PTFE

18 Neoprene Rubber Fender Washers

1. The MBRA strip contains six chambers, each with three entry ports located on the roof of the chamber. Each port must have threads produced for the insertion of fittings; this can be done with a ¼-28 hand tap tool (can be found in most hardware stores). After producing threads, wash out trimmings from chambers with water.
2. Insert a magnetic stir bar into each chamber. Also, add a very small amount of water into each chamber. This is important for autoclaving purposes.
3. Prepare waste line adapters for MBRA strip.
   1. The amount of liquid present within the MBRA strip chambers is dictated by the experimenter. Protocol dictates 15 mL; however, PTFE strips can be cut to accommodate a different volume. For 15 mL, cut six 25-mm strips of PTFE tubing.
   2. Prepare the epoxy by carefully mixing the resin and hardener using a pipette tip.
   3. Apply the epoxy on one end of the PTFE tubing strip, then carefully insert the epoxy end into a male luer adapter fitting.
   4. Allow to sit for approximately 5-10 minutes. Tug at PTFE strips once the epoxy has settled to confirm that the strip is secure. Final product should resemble **Fig. 1b**.
4. For all C-flex tubing strips, attach a male luer w/lock ring on one end and a female luer barb (1/8”) on the other end. Screw on male luer adapters to one set of C-tubing strips and the waste line adapters created in the previous step to the second set of C-tubing strips. Position a rubber washer and screw on all strips in their designated position (top holes are for media lines; middle holes are for waste lines). Strips will be placed with the order dependent on the location of the waste exit line/media source line (smallest lines closest to the location of their respective line).
5. Screw on 6 male luer adapters onto female luer barb (3/32”). Position rubber washers onto the inoculation/sampling ports of the strip, and screw on the male luer adapter/female luer barbs. Insert a Precision Seal Rubber Septa on each female luer barb. Fold over the septa until the top is flat. These septa will be used for inoculation of bacteria and sampling.

Now, we’ll start assembling the media bottles and inflow lines used for the **source system**. For one reactor strip, a typical run will start with two media bottles. However, the media intake lines can be modified so only one bottle is used. First, we’ll begin with assembling the bottle caps that will be placed on the media bottles then continue on to the assembly of the media intake lines. **NOTE**: this design is a modified version from the original protocol. Modifications were made to increase flow efficiency and allowing for easier mobility. For the original design instructions, please refer to the original paper.

**Parts and Supplies Required for a 2-Bottle System:**

2 Millex-GV Syringe Filter Unit, 0.22 um

2 Dibafit Adapter, ¼”-28 UNF(M) flat bottom to 3.2 mm ID

2 Kinesis Omnifit “Q” Series Bottle Caps, Two Port

Qosina Male-to-Male Luer Lock Connectors

2 Adapter, nylon, Male Luer to ¼-28 thread

Male Luer Barbs

Female Luer Barbs (1/8”)

2 Mohr Pinchcock Clamps

C-Flex Tubing

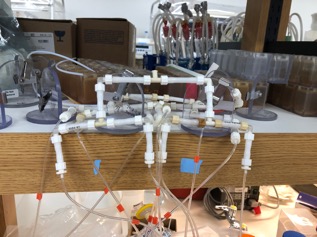
Diba Omnifit PTFE Tubing

Bottle Cap:

**Fig. 2: Bottle w/ bioreactor lid** The inner PTFE tubing must match the length of the bottle to extract as much media possible. The two-port bioreactor lid will have a long strip of C-flex tubing connected to one port for media transport and a shorter second strip connected to the second port. The longer media line has a smaller strip connected to it with a clamp to prevent any media from leaving during the autoclaving process.

1. a) Media port: screw a Dibafit Adapter into one port of the bottle cap. Flip the bottle cap over and you will see two holes corresponding to each port. Attach a strip of PTFE tubing long enough to reach the bottom of the media bottle you will be using in the hole corresponding to the dibafit adapter.
2. Prepare the 16.5/20.5 in. strips (16.5 for one bottle, 20.5 for the other), by attaching a male luer barb on one end of each individual strip. Afterwards, attach the strip end with no barb to the dibafit adapter.
3. Prepare a 2.5. in. strip (one per bottle cap) by attaching one large female luer barb on one end and a male luer barb on the other end. Clamp the tubing and then attach the strip to the 16.5/20.5in. strips. Cover the unattached end of the 2.5in strip with foil.
4. Prepare a single 2.5 in. strip (one per bottle cap) by attaching one large female luer barb on one end and a male luer barb on the other end. Attach the strip to the male luer adapter on the bottle cap. Cover the male luer barb end with foil (after autoclave, a Millipore filter will be attached to this strip).

Media line:



**Fig. 3:** Fork-like design of media line. This design helps promote even flow through media lines and is very easily manipulated to accommodate different set-ups.

Media lines can require some planning depending on experiment set-up. If there are different media types being assigned to certain chambers belonging to the same strip, there will need to be different lines. If there are multiple strips with replicates of the same media, the strips media lines can be combined for use with a singular media source. The set-up is dependent on the experimenter and the amount of space allowed. The steps for an example set-up will be dictated below.

1. For each media line, a total of 10 1-in and 2 1.5-in. strips C-flex tubing strips will need to be cut. At the end of each strip, attach a male luer barb.
2. Connect 1-in. strips to form two fork-like lines. Connect these two forks using 1.5-in. strips and a female luer tee. The length of the strip connecting the media bottle to the media line is dependent on where they will be placed.
3. Screw on the orange pump lines to the media line.

The last system that will be assembled is the most complex system: **the waste system.** In this system, the waste line will be divided into the inner waste tubing and the outer waste tubing. The inner waste tubing will include the tubing that is attached to the waste pump lines; the outer waste tubing includes the exiting waste line and the bottles where the waste will flow into. **NOTE**: The waste design has been modified from the original protocol to increase efficiency in terms of both flow and for compartmentalization. For original design instructions, refer to original MBRA protocol.

The Exit Waste Line is difficult to autoclave and set-up when attached to the fully assembled MBRA system, so it will be kept separate from the rest of the system.

**Parts and Supplies Needed for Exit Waste Line**

C-Flex Tubing (Length of tubing varies on set-up)

1 1L bottle

2 2L bottle

6 Dibafit Adapter, ¼”-28 UNF(M) flat bottom to 3.2 mm ID

3 Kinesis Omnifit “Q” Series Bottle Caps, Two Port

1 Male Luer Adapter

3 Large Female Luer Barb

1 Millex-GV Syringe Filter, 0.22 um

1. If the anaerobic chamber that the experiment will be set-up in contains pass-through ports, such as a butyl rubber stopper, then a 1/4” hole can be drilled through the center to allow the entry of the waste line. After the tubing has been inserted through the rubber stopper, the hole can be resealed with epoxy. After autoclaving, the sealing can be reinforced with an additional layer of epoxy. If there are no pass-through ports, the waste can be collected within the anaerobic chamber and removed daily, or as needed. **NOTE:** The waste must be removed daily if there are gases released as metabolic by-products by the strains being used in experiment.
2. Assemble a bottle by loosely securing a “Q” series two-hole bottle cap to a 1 L bottle. Assign a waste level to the bottle. The waste level is the highest amount of waste you wish the bottle to hold. Cut and attach two PTFE tubing strips, one 2.5in. and one long enough to reach the assigned waste level. Now fill the bottle with enough water so that the 2.5in strip’s end is submerged (for system to remain anaerobic). Attach a Dibafit adapter, ¼”-28 UNF, to both ports of the bottle cap. Attach the waste line assembled in step one to the port corresponding with the 2.5in. strip of PTFE tubing.
3. Assemble waste collection bottles by loosely securing Omnifit “Q” Series two-hole bottle caps to the top of 2 L bottles. Screw on a Dibafit adapter, ¼”-28 UNF, to both ports on the first bottle and to one port on the second bottle. On the second port of the second bottle, attach a male luer adapter. These two bottles will be connected via tubing.
   1. Mark the bottle with two dibafit adapters at the level at which the waste will be kept. Attach a strip of PTFE tubing long enough to reach this level on the hole corresponding to the port which will connect to the second 2 L bottle.
   2. Prepare a 2.5in. strip of C-Flex tubing by attaching a large female luer barb and a male luer barb. Screw on the strip to the male luer adapter on the 2 L bottle. The Millex-GV filter will be attached to this strip to prevent contamination.
4. Connect all the bottles by attaching C-Flex tubing to the dibafit adapters. The tubing strips should all be 8-10 in. in length.

**Parts and Supplies Needed for the Inner Waste Line Tubing:**

Male to Male Luer Lock Connector

Male Luer Barb

Female luer x female luer elbow,

Female Luer Tees

C-Flex Tubing



**Fig. 4:** The set-up displayed in this figure contains 24 outgoing waste lines. This set-up design is recommended to maximize space within the chambers. The lines are made into fork-like arrays of three lines on each side and then stacked on one another.

1. Dependent on the number of chambers having waste flowing out of them, prepare 1-in. strips and attach red-pump tubing to them. These strips are able to be connected using a female luer tee and female luer x female luer elbow connectors.
2. The male to male luer lock connectors are important for compacting the waste line. Maximizing spatial use is very important when working in confined spaces.
3. The last strip to be attached is dependent on the length of the waste exit line. **NOTE:** Make sure that the barb attached to the end of the strip is able to connect to the barb on the end of the waste line. One end must be a large female luer and the other a male luer barb.

Now that all parts have been assembled, connection must be done to fully assemble the MBRA system. Connection between systems will be accomplished via the pump tubing. Connect one end of the pump tubing with their respective systems (orange pump lines to media lines/red to waste lines) and the other end to their corresponding strip of tubing on the reactor strip. Make sure that things do not become too tangled and are very easily handled as when things go into the anaerobic chamber they will become a lot more difficult to maneuver.

**Autoclaving**

* There are three separate items that must be autoclaved: the media bottles w/bottle cap assembly, waste collection system, and the fully assembled reactor strip/media/waste line. **NOTE**: **It is important that all autoclaving be done using the liquid cycle as the tubing is rubber and could possibly melt together.**
* Cover every exposed luer port with foil and assure that all bottles have caps that are semi-loose so that the pressure is able to escape. Likewise, make sure that the fittings screwed onto the MBRA strip are not too tight. **NOTE: If there is no space for the pressure to escape, the MBRA strip will crack.**
* Tubing can be compacted using tape so that it does not become completely entangled. It is advisable to tape together tubing that belongs to the same part (pump tubing being used for the same media line or tubing belonging to one waste line).
* Autoclave all parts on liquid cycle, 20-30 minutes.
* After autoclaving, make sure all fittings are tightened. Tighten all bottle caps and add the Millipore filters to the waste collection and media bottles. Remember to re-apply epoxy to the rubber butyl stopper if a hole was made.
* If there are cracks on your strip, apply a light coating of epoxy onto the cracks for re-sealing. Depending on the damage done to a strip, however, epoxy might not completely re-seal cracks.
* Allow tubing to completely cool. If the pump tubing is clamped too soon, the tubing will be warped permanently closed.
* Be sure to perform sterility checks for all media sources prior to beginning an experiment.

**Set-up**

* For entry into the anaerobic chamber, DO NOT cycle through any of the reactor experiment items. Use the “pass through” method. This method is best done with two people.
  + Fully fill the anaerobic chamber with mixed gas. Open the inner lock door and have one person stand-by to pull in the items while the second person passes the items through the air-lock compartment. Because the chamber is filled with mixed gas, air will be pushed out of the chamber while items are passed through. This will minimize the amount of oxygen introduced into the chamber.
* Always allow all parts of the MBRA set-up that will be placed inside of the chamber to completely reduce (become anaerobic) prior to beginning the experiment. Suggested reduction time is 48-72 hours.
* The strips can be firmly placed onto a multi-position stir plate using clamp holders. The lab currently has an STL file for clamp holders that are available upon request.
* Once everything is in place, connect the media bottles to the media line and the waste-exit line to the waste line. Clamp down all pump lines.
* Turn on both pumps and check that media flows properly and all chambers fill. The design has been modified in an attempt to combat uneven flow, but there is always going to be disparity between chamber flow. The peristaltic pumps being used, however, can be adjusted to individually change the flow rate between pump lines. The clips that hold the pump lines typically have knobs (occlusion knobs) that change how tightly the pump line is held. Always begin the experiment with the knobs turned fully to (+) and turn towards (-) to **increase flow.** It is also suggested to make sure drainage of waste from the chambers is checked in the beginning to avoid issues once the experiment begins.
* Once the chambers have filled, allow the media to sit inside the chamber for at least a day. Sample a small amount of media and perform sterility testing to assure the system is closed.

**Fig. 5:** Fully set-up MBRA array with pump lines all clamped. Inside of the chamber, there is limited space so it is recommended to use twist ties for better organization.

**Sampling and Experiment**

* When sampling, it is very important to maintain sterility to ensure that there is no contamination to the samples present within the chambers. A suggestion would be to cover each septum with a piece of Kim wipe and apply bleach. To accomplish this easily, stuff a cryovial cap with the kim wipe and place over the septum. Each septum should be allowed to soak for about 15 minutes prior to sampling. Sampling should be done with a sterile syringe and needle.

****

**Fig. 6:** Overhead view of cryovial caps on septums. Using these caps helps completely cover the septum and maximize sterility.

* When sampling large quantities, keep in mind that there is a large amount of negative pressure inside the chamber making pulling large volumes very difficult. You can combat this by sampling in increments or loosening one of the fittings and then re-tightening them.
* Constantly monitor waste collection bottles to avoid spill overs. If a puddle of media is noticed during the experiment, use a towel to clean and then place dry towels to localize where the spill is coming from.
* Replacing media bottles is easily accomplished by switching cap over to the new media bottle. It is possible to simply attach a new bottle to the media line but it is important to note that there will be media w/in the lines. The possibility for spillage is much less when simply moving over the bottle cap to a new media bottle. **Be careful not to touch anything with the PTFE tubing when switching the cap over as there is a contamination risk.**

**Post-Experiment Tasks**

* For easier cleaning, connect media bottles containing 10% bleach solution and allow to run until media is empty. It is not important as to how fast the setting is at this point, so both inflow and outflow can be set to max speed. **Do not let MBRA strips sit with bleach inside them for prolonged amounts of time because bleach is corrosive.**
* All c-flex tubing can be re-used but must be flushed out. However, tubing used for media lines should be recycled into waste lines when used again. Pump lines should be thrown away.
* All luer parts can also be re-used. These parts should be placed into a bin and allowed to soak in a SDS/water solution overnight. Afterwards, transfer all luer parts into a bin with only water. Dry and store.
* MBRA strips can also be cleaned and re-used. Allow for strips to soak in a water/sulfur lauryl sulfate solution over-night then shake so that residue will dislodge from the sides of the chambers. Afterwards, flush out MBRA strips with dH2O and allow to dry and store.
* Caps, waste-exit line, and clamp strips can be used again. Clamp strips do not need any cleaning done and can be stored. Caps and waste-exit line only need to be rinsed and flushed with water, allowed to dry, and then can be stored.
* All media waste should be autoclaved and disposed. For bottles used after connecting the bleach bottles, simply dispose of through a sink drain.

**FAQ/Troubleshooting Manual**

* **Q:** There seems to be a flow issue in my experiment, what are some common problems that can cause this?

**A:** There are many issues that can cause this, but the most common issue is flow stoppage. Is the flow issue confined to certain chambers or is there no flow throughout the system?

**Certain Chambers**

1. Confirm that caps on all bottles are securely fastened. If there is any space, no vacuum will be generated for flow.
2. There is a possibility that the bottle cap used for media output is not functional. When this occurs, the only solution is to replace the entire media bottle w/cap.
3. If the tubing is too short, there will be tension created that blocks the flow (**t**hink of a garden hose that has folded). If you find that this is the problem, simply assemble a small strip to attach to the end of the strip that has tension.

**Throughout System**

1. Make sure that **all** fittings are fully tightened, especially the ones attached to the MBRA strip. Peristaltic pumps will be creating a vacuum within the chambers and a loose fitting can cause loss of pressure (thus no flow out of chamber). Pliers can be used to tighten the fittings. This problem can be detected if bubbling is seen around fittings on strip, or leakage around fittings on the tubing.
2. Confirm that the peristaltic pumps have been set to CW/CCW in correspondence to the intended direction of flow.
3. The pump lines are not secured properly. Remove clips and re-position the pump lines. Before removing clips, increase waste pump flow rate to over 20.This is due to the “gravity” flow. The vacuum created within the chambers will suck all the media with no restriction on input. If the waste pump is left on a low output rate, the chamber will overflow and risk contamination.
4. If leakage is detected around a tubing strip or pump line, replace accordingly.

* **Q:** Is there a reason that the flow design is set-up this way?

**A:** The reason for this flow design is to decrease the chances of potential flow blockages, backflow, and address the issue with equal distribution of flow. That is not to say that this design is perfect. Modify accordingly to your experimental design.

* **Q:** Can I still use another design that may require less tubing?

**A:** The answer to this question is variable. From past experiences, higher flow rates require a modified flow system so that tubing is not flooded with media/waste (potentially generating backflow). Flow is also governed by a variety of factors: diameter of tubing being used, flow rate, distribution of flow, viscosity of media/waste, etc. New designs are always welcome if successful!

* **Q:** There are bubbles forming around my fittings and they seem to be tight, how can I stop these bubbles from forming?

**A:** This is due to excess pressure within your chambers. If you have your media pump on and your waste pump off, the pressure inside will accumulate and eventually look for tiny crevices to exit. Simply increase the speed setting on the waste pump for a small amount of time, or turn off the media pump and allow the waste pump to run for a certain amount of time and then turn on media pump again.

* **Q:** The waste-exit line does not seem to flow properly. How can I check that it is functioning properly?

**A:** One way to check if there is proper function is to connect a small luer strip to a syringe with a luer lock connector, attach the syringe/strip to the filter strip located on the last waste collection bottle, and pull to generate suction. If, after a couple pulls, there is no drainage occurring, there is either a problem with the bottle cap sealing or the fittings connecting each bottle. Often, the fix is simply replacing the bottle with another bottle.