Microscope Cell Culture Incubator
Final Report

BME 400 Design
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Abstract

The team was tasked with creating and testing a cell culture incubator that can maintain a specific internal environment while being compatible with an inverted microscope. The internal environment must be 37 °C, greater than 95% humidity, and contain 5% CO_2 in the incubator. There are current designs on the market that meet this criterion, but either the inverted microscope was integrated into the incubator making it bulky and inconvenient to disassemble, or the incubator was expensive. The team designed a cost-effective cell culture incubator that would be portable and small enough to fit on the inverted microscope stage, allowing the user to view live cells inside the incubator. The incubator included a heated water pump and CO_2 pump in order to reach the client's criteria. Condensation, CO_2 input regulation, and live cell testing were conducted to find the optimal working environment for the incubator in order to ensure cellular viability and visibility.

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Body of Report

I. Introduction

Cell culture is a commonly practiced laboratory method for the use of studying cell biology, replicating disease mechanisms, and investigating drug compounds [1]. Due to the use of live cells during this process, incubators are necessary to keep the cells viable for the duration of the study. Onstage incubators allow for live cell growth because they maintain a highly regulated internal environment of 37 ℃, 5% CO_2, and 95% humidity, without compromising the integrity of the microscope. The COVID-19 pandemic has caused the CO_2 incubator market to increase by 7.69% with an estimated market growth acceleration of 8% over the next decade [2]. Major disadvantages of current commercially available systems are that they tend to be large and bulky enclosing the entirety of the microscope making it difficult to assemble and remove between uses while hindering the use of the microscope in general, and they are often expensive; Fisher Scientific's Enviro-Genie cell incubator is priced at $6,510.68 [3]. This project will focus on developing a low-cost cell culture incubator that allows for interchangeable culture plates, compatibility with an inverted microscope, easy disinfection, and live cell imaging via maintenance of the internal environment needed for cell growth.

II. Background

Cell Cultures in Lab

Cell cultures are mainly used in the study of cell biology due to their ability to easily manipulate genes, molecular pathways, and culture systems to remove interfering genetic and environmental variables [4]. Cell cultures follow BioSafety Level 2 guidelines[5], which describe the safety procedures for working in a lab that can be associated with human diseases, and any incubators being used in conjunction with cell cultures must follow ISO Class 5 air quality standards [6]. Cell cultures have the ability to work with three different cell types: primary, transformed, and self-renewing cells. Primary cells are directly isolated from human tissue. Transformed cells are those that can be generated naturally with changes to the genetic code, or genetically manipulated. Self-renewing cells are cells that carry the ability to differentiate into a variety of other cell types with long-term maintenance in vitro. An example of self-renewing cells is embryonic stem cells as seen in Figure 1 [1].

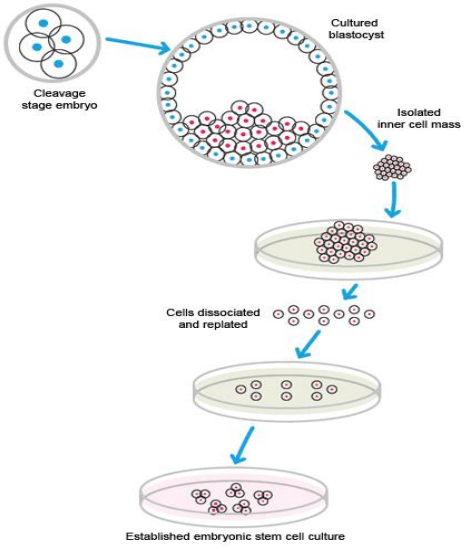


Figure 1: Isolation of Embryonic Stem Cell Lines[7]

Incubators used in cell cultures have to maintain a stable microenvironment and can achieve this via regulated temperature, humidity, CO_2, O_2, and pH levels. Controlling these factors is critical for the viability and growth of the cultured cells, as the incubator is aiming to replicate the cells' environmental conditions in the body (37 ℃ with a pH of 7.2-7.4) [8]. CO_2 is needed as a buffer to help with the pH along with a culture medium. The medium most commonly used is a Basal medium, with occasional serums added (such as fetal bovine serum), which controls the physicochemical properties of the cell cultures' pH and cellular osmotic pressure [1]. Many incubators are therefore larger in size in order to maintain these homeostatic conditions as seen in Figure 2. However, there are some commercially available stage top incubators that are able to adhere to the specifications required to keep cells viable, but they are often more expensive. See Appendix A for more information regarding these competing designs.

Figure 2: Thermo Fisher Heracell VIOS 160i Incubator[9]

Incubator Types

There are two types of commonly used methods to maintain the temperature in industry cell incubators. Many employ the direct heat method which tends to give off heat using electric metal coils that surround the body of the incubator and are programmed to the desired temperature. The other method is the water-jacketed incubators which use a controlled circulating water bath cabinet around the body of the incubator for even heating throughout the entirety of the chamber.

Humidity control is achieved most commonly by placing a tray of water at the bottom of the incubator. This method is used in both water-jacketed and direct heat incubators. CO_2 control is achieved through a CO_2 tank that automatically pumps the desired amount of gas into the incubator. Using tubes and a valve connector, the CO_2 tank is able to deliver gas to the inside of both water-jacketed and direct heat incubators. Many incubators also allow for the CO_2 valve to be adjusted when internal conditions are disturbed, such as opening the incubator door to deliver more cell plates, so that the environment is always stable.

Clinical Significance

There is a significant need for live cells to be cultured via the assistance of an incubator. Pharmaceutical companies often use these methods for drug development and testing as live cell imaging can be used to screen chemicals, cosmetics, and other drug components for their efficacy [8]. Live cell imaging is important because it allows for the observation of internal structures and cellular processes in real time. These observations allow for more insight into the

process of a cell, rather than viewing snapshots taken over a period of time. Pharmaceutical companies can also access the drug cytotoxicity in different cell types. Virology and vaccine products benefit from live cell cultures as they can be used to study viruses in order to make new vaccines, such as in the product of the SARS-COVID-19 vaccine [1]. Embryonic stem cells are widely studied for their regeneration properties due to genetic engineering/gene therapy applications of these cell cultures, and the expression of specific genes and the impact they have on other cells can be studied.

Client

The client for the Microscopic Cell Culture Incubator was Dr. John Puccinelli, an undergraduate advisor, and professor in the Department of Biomedical Engineering at the University of Wisconsin-Madison. The client will be using this product in their teaching lab where students will conduct live cell imaging on tissues for up to one week at a time. The specifics of the experiment are unknown, however, it was believed that this device will be used to teach students how to image cells and watch cellular growth over the course of the week. Having a cell culture incubator that was compatible with an inverted microscope will provide easier teaching and preparation methods for professors. Less time will be spent transferring cells from an incubator to the scope or disassembling a bulky microscope assembly allowing more time to be spent developing the main learning objectives of the course.

Product Design Specifications

The client has asked the team to create an incubation chamber that must be able to maintain an internal environment of 37 ℃ ± 1 °C, 5% ± 1% CO_2, and 95-100% humidity with even heating and humidity across the chamber. Even heating was defined as a consistent temperature throughout each section of the chamber. The incubator must fit on an inverted microscope stand (roughly 310 mm × 300 mm × 45 mm) without interfering with the microscope's optics and functionality. The device must also be able to hold a standard well plate (127.55 mm × 85.4 mm × 22.5 mm) without disrupting the integrity of the cultures in the plate. The top and the bottom of the incubator must be transparent in order for imaging through the chamber. The aim of this project was to be able to make a device that was low-cost, easily assembled/disassembled, sterilized, and can be easily moved and stored between uses. The market for this product was teaching labs, but if more successful, it could be marketed toward other laboratories and pharmaceutical companies. For more information, see the Full PDS in Appendix A.

Previous Work

This project was worked on previously by many BME 200/300/400 students, however last semester, Spring 2022, brought a great deal of success to the project. The team was able to create a 195 mm × 245 mm × 36.83 mm incubation chamber using black acrylic[1]. The incubation

[1] See Appendix B for more details

chamber consisted of a top and bottom, with a hole for polycarbonate glass plates, and sides with filets to prevent leakage in the box and allow for the walls of the chamber to be connected. See Figure 3 for more details. The inside contained a chamber for a water bath with filets on the side, again to prevent leakage and for joints. The box was glued via acrylic contact cement glue and lined with water-resistant caulk to seal the chamber and prevent water leakage. The chamber also had five holes laser cut into the sides of the box. The front had two 15.875 mm holes for copper tubing that were inserted into the water bath space in the box. The copper tubing allowed for heat transfer from a heated water pump into the water bath. This provided temperature and humidity according to the client's specifications[2]. The copper couplings were soldered to the copper tubing to prevent any water leakage. There were also 15.875 mm, 3.175 mm, and 8.05 mm holes for the NDIR CO_2 sensor, thermistor, and CO_2 tubing. See Figure 4 for more information. The entire setup of the incubator can be seen in Figure 5.

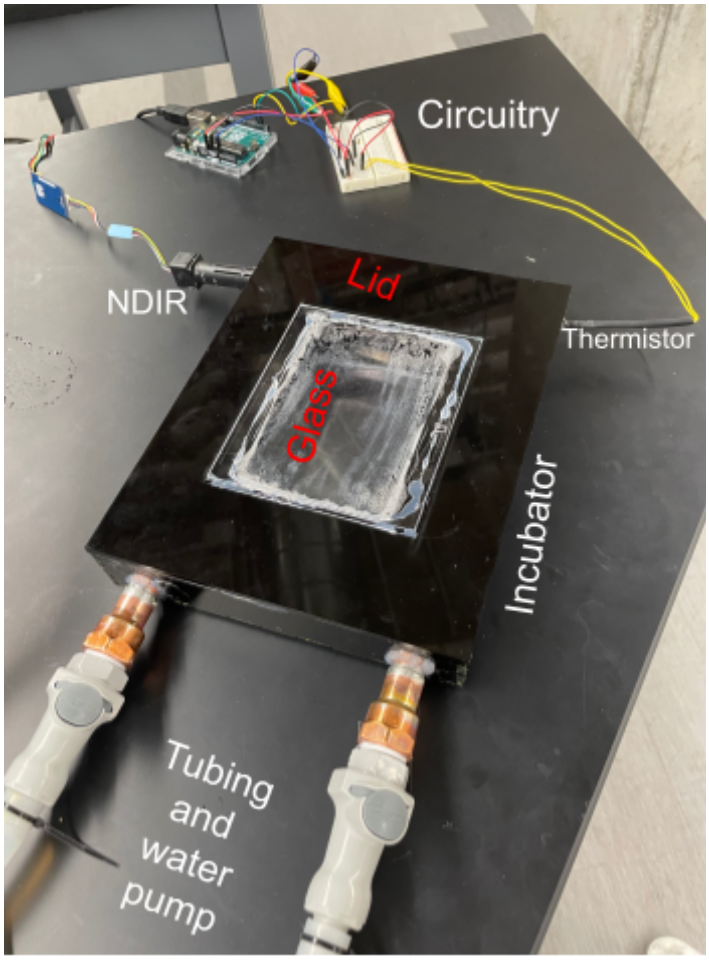


Figure 3: Incubator Prototype Exterior

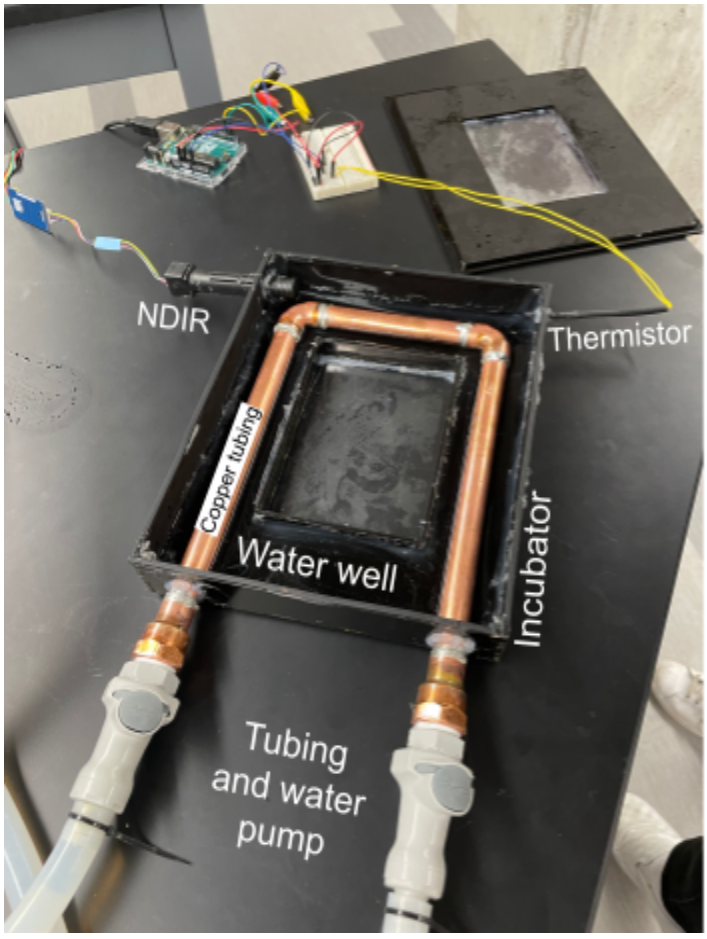


Figure 4: Incubator Prototype Interior

[2] See Appendix A

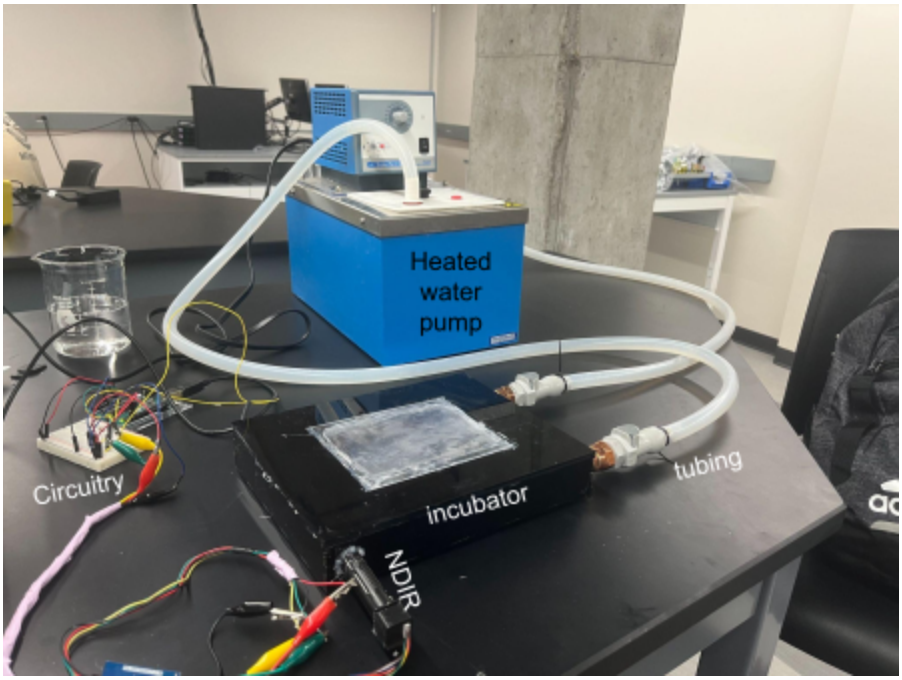


Figure 5: Whole Incubator Set Up

The thermal conductivity of copper was assessed along with the heat transfer rate (Q) of copper measured by using equation 1 [10].

$$Q = mCp\Delta T \quad \text{[kJ]} \ (1)$$

Where m was the mass (kg), Cp was the specific heat capacity of copper (389 J/(kg*K)) and ΔT was the change in temperature (Kelvin).

Using Equation 1, it was determined that if the heated water pump pushes the water out at an initial temperature of 50 ℃, the 1 L water bed should reach the desired temperature of 37 ℃, starting from 20 ℃, within 7.4 minutes. Once the desired temperature was reached, the heated water pump was set to 38 ℃ in order to maintain a 37 ℃ internal temperature and to account for any loss of heat throughout the vinyl tubing of the water pump and acrylic walls of the incubator.

All sensors were tested according to Testing Protocols[3] and standardized results can be found in Appendix B. The incubator underwent whole box testing in regards to temperature and humidity[4]. The incubator was set up for normal use and data collection occurred for approximately ten minutes. The incubator was initially warmed up using a heated water pump, which pumped water at 55 ℃, for approximately 5 minutes, until it was lowered to about 34 ℃. The incubator then had a constant temperature of about 37.6 ℃ for the remainder of the testing interval. The average humidity during this interval was 97.1%. Overall, the results conclude that the temperature and humidity inside the incubator are within the standards outlined in the PDS and met the design requirements[5].

[3] See Appendix C
[4] See Appendix C
[5] See Appendix B for visual representation of data.

The incubator also underwent recovery testing according to Recovery Testing Protocols 1 and 2[6]. The temperature of the incubator was able to return to optimal conditions within approximately three minutes, while the humidity was able to return to optimal conditions after approximately three and a half minutes[7].

Previous data was also collected on the optical transparency of the polycarbonate glass chosen for incubation design. The results shown in Appendix B prove that there was no significant difference between the microscope optics with the glass and without the glass. However, last semester showed that condensation while running the incubator interfered with the optical specifications laid out in the PDS.

Overall, the team found great success in chamber fabrication, temperature, and humidity. The challenges of the current semester are to determine the best way to control the flow of CO_2 from a 100% CO_2 tank and to determine anti-fogging methods to preserve optical clarity.

[6] See Appendix C.
[7] See Appendix B for visual representation of data.

III. Preliminary Designs

Design #1: Solenoid Valve

The solenoid valve CO_2 input regulator (Figure 6) consisted of a normally closed electric solenoid. There would be a 12 V power supply that when partnered with a transistor, would excite the solenoid to open when instructed. The solenoid valve remained open until the correct amount of gas was inserted into the incubator and then would be deactivated, and closed. This design required adaptors for the CO_2 tubing in order to control airflow because the threads on the solenoid are in European units. Male thread to barbed tubing adaptors were available online. Some benefits to this design were little fabrication, electrical control, and minimal leakage because it was an industrially made part. The solenoid was purchased online and the only fabrication was the circuit building and coding. The electrical control was a benefit because the solenoid needs to be opened for specific amounts of time (milliseconds) to have accurate CO_2 input. Some downfalls to solenoid valves were costs and the required high power source. Solenoids range between $25-$400, but cheaper solenoids can be bought with the risk of functionality. A proper power source would also need to be purchased to allow the team to excite the solenoid.

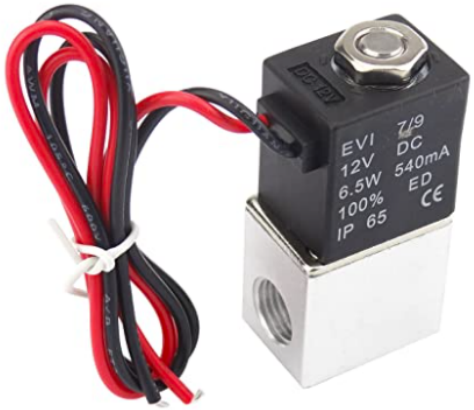


Figure 6: Solenoid Valve [11]

Design #2: Threaded Pin Valve

The threaded pin valve design (Figure 7) consisted of a DC motor being hooked up to a threaded pin, with the typical body design of a pin valve. This valve would be normally closed, and when it needed to be excited, the DC motor would twist and "unscrew" to allow air to pass through the airway. When enough time had passed to allow air to flow through the tubing, the DC motor would reverse directions and screw in to close the valve. At the end of the threaded pin was a rubber nose that would help provide a nice seal to limit CO_2 leakage. Some benefits to this design were the ultimate cost to fabricate the mechanism and the safety risk. The body would be 3D printed and would contain a couple dollar DC motor from the UW Makerspace. This design didn't have many moving parts so the safety risk of someone getting hurt was minimal. A couple of downfalls to the design would be the risk of leakage. It would be a homemade valve and having precise fabrication was difficult to obtain. The threaded screw design was thought to have a slower response time as well which would lead to less accurate CO_2 input for the incubator.

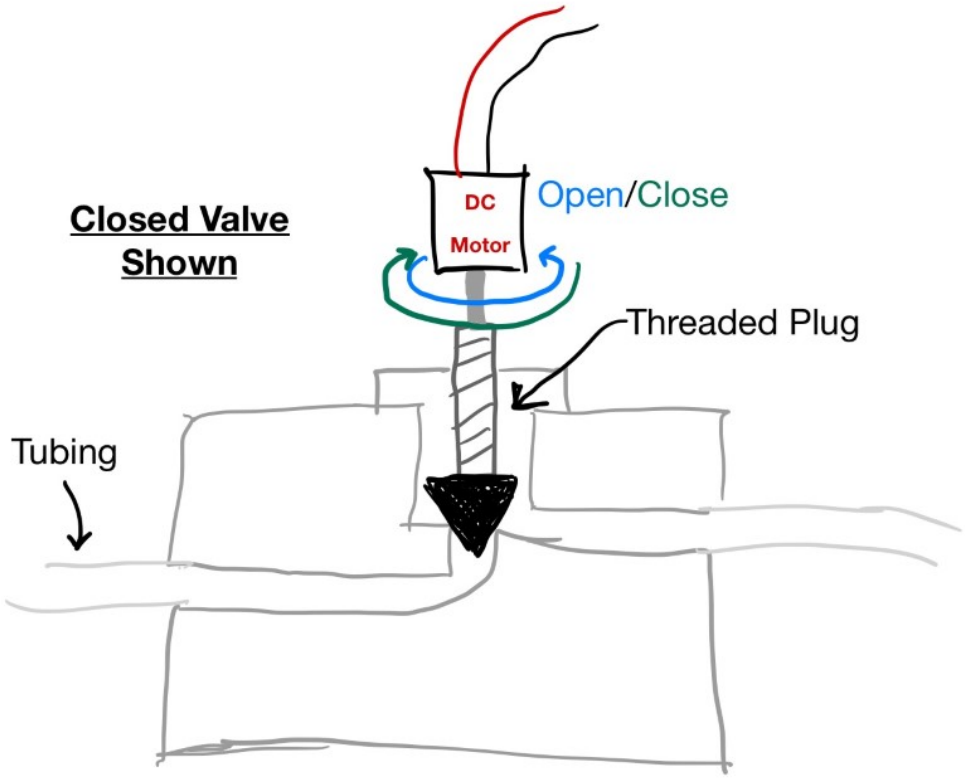


Figure 7: Drawing of the threaded pin valve design

Design #3: Spring Pin Valve

The spring pin valve design (Figure 8) consisted of a DC motor being attached to a pin with either a wire or string. When the DC motor was activated, it would wrap the wire or string around the motor shaft, lifting the pin-up. As the pin was being lifted up, an attached spring would compress. When the DC motor stopped rotating, the pin blocking the airway would have moved, allowing the CO_2 gas to pass through. When the correct amount of CO_2 had passed, the DC motor would reverse rotation directions, loosen the string, and allow the compressed spring to plug the rubber on the end of the pin back into place, ultimately blocking the flow of gas. A couple of benefits to this design included the quick closing response time because CO_2 input accuracy was an important factor in ranking the different valve designs. Another benefit to this design was that it was homemade so the parts can be obtained and made in a lab without the need of outsourcing parts. A couple of downfalls to this design were the complexity of the design and the risk of leakage. This design had multiple little parts and mechanisms that would be hard to fabricate, and this would also lead to not finely machined parts and more CO_2 leakage.

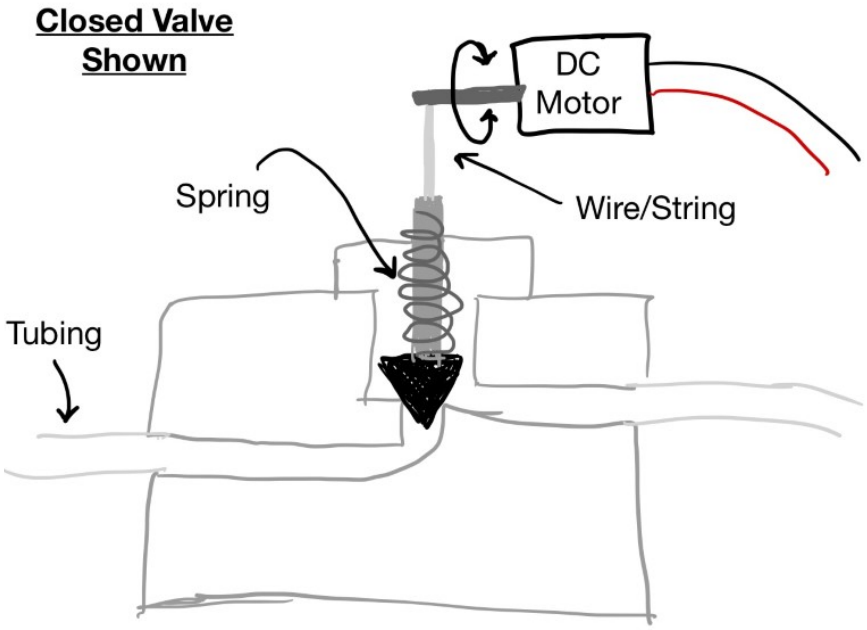
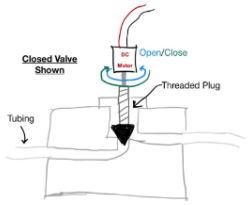
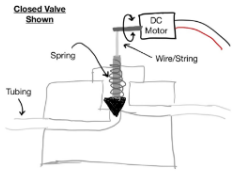


Figure 8: Drawing of the spring pin valve design

IV. Preliminary Design Evaluation

Design Matrix

Table 1: Design Matrix with all methods scored on accuracy and reliability, cost, ease of use, fabrication, life in service, and safety.

			 Solenoid Valve		 Threaded Pin Valve		 Spring Pin Valve	
Rank	Criteria	Weight	Score (5 max)	Weighted Score	Score (5 max)	Weighted Score	Score (5 max)	Weighted Score
1	Accuracy and Reliability	30	5	30	3	18	4	24
2	Cost	20	3	12	4	16	4	16
3	Ease of use	20	5	20	3	12	2	8
4	Fabrication	15	5	15	3	9	2	6
5	Life in Service	10	4	8	1	2	2	4
6	Safety	5	4	4	5	5	5	5
	Sum	**100**	**Sum**	**89**	**Sum**	**62**	**Sum**	**63**

Scoring Criteria

Accuracy and Reliability: Due to the importance of the internal environment for cell growth, the incubator must be able to regulate the conditions within a small margin of error. The client requested that the internal environment of the incubator be as close to industry standards as possible. The CO_2 control system must be able to limit the flow of 100% CO_2 so that the incubator only contains 5% ± 1% CO_2. The control system must also be compatible with an Arduino coding system so that it can be incorporated into the existing feedback loop code.

Cost: The total cost of the product had a budget of $100, although the client had said that more funds may be provided based on the success of the initial prototype. However, the cost of the CO_2 control system should not take up more than 50% of the total budget to save for fabrication and cell testing.

Ease of Use: The flow control system should be relatively easy to use for those unfamiliar with this technology, as it would be used in the teaching lab. The flow system should also be easy to control via Arduino coding. The system also must be able to control itself over time as it would be left overnight in the teaching lab and must continue to pump CO_2 into the incubator in order to keep the cells alive [12].

Fabrication: The flow control system must be easy to fabricate and implement. The fabrication should not be too complex or exceed the cost of materials and testing.

Life in Service: The final product would need to be used for one week out of the semester in the client's teaching lab. The shelf life of this product has a minimum of 10 years.

Safety: The product needed to adhere to FDA and OSHA standards and regulations [13][14]. Due to the use of tissue cells, the incubator must abide by Biohazard Safety Level 2 and ISO Class 5 air quality standards [15][16].

Proposed Final Design

The team decided to move forward with Design #1, the solenoid valve. Since the valve was industrially made and bought from a third party, the risk of leakage and fabrication difficulties were minimized. Although solenoids can be expensive, cheap alternatives were found online and were tested. The solenoid valve provides the best CO_2 input regulation because it has a fast response time, based on code, to electrically open and close the solenoid pin. Having these precise response times increased the likelihood of having the correct amount of gas being inserted into the incubator, leading to healthier cells. Some alterations were made to the solenoid in order to connect the CO_2 tank hose to the solenoid valve and then from the solenoid valve to the incubator. There are current adaptors on the market that the team can acquire to resolve this issue. Overall, the minimal fabrication and industrially fabricated parts outweighed the costs of the solenoids and were the most likely design to allow the team to be successful in having an efficient and accurate CO_2 input regulator.

V. Fabrication/Development Process

Materials

Arduino Materials

Previous materials for electric circuitry include a thermistor and an NDIR CO_2 sensor which have been standardized according to past semester testing results[17]. This semester, the materials needed include an Arduino-compatible solenoid valve and a relay circuit in order to increase the voltage from 5 V to upwards of 9-12 V [18][19]. The solenoid valve was a normally closed valve that was purchased and tested in order to determine its ability to integrate into the Arduino format of previous semesters.

Incubator Materials

Previously, the incubator was equipped with approximately two feet of copper tubing to allow for heat transfer. The copper tubing allowed for sufficient heat to be conducted to the 1L waterbed that sat inside the proposed final design to allow for both optimal temperature and

humidity. The incubator was made using black acrylic from the UW-Makerspace. The acrylic was chosen as an alternative to the PLA plastic used last semester for the prototype. Black acrylic has a larger ultimate tensile strength (70 MPa) than PLA, is cheaper, and the black allows for more insulation and protection from light [20]. Dr. Puccinelli also informed the team that a black acrylic box would be compatible with a fluorescent microscope, as well as an inverting microscope, should the incubator be used in other projects in the future.

This semester, black acrylic was used to laser cut a lid that has ten 12.7 mm holes surrounding the top glass plate for homogeneity testing. Holes were filled with size 00 rubber stoppers which were only removed when a thermistor needed to be inserted into the incubator. This was for the purpose of determining the temperature and humidity of different areas of the incubator.

Methods

In order to determine the performance of the prototype incubator the team employed CO_2 control methods, homogeneity testing, and cell viability testing.

CO_2 Control

The fabrication process for the solenoid valve design required the purchase of adaptors to attach the solenoid valve to the CO_2 tank tubing. G1/4" male thread to 4.5-5.5 mm diameter barb adaptors were purchased to allow this connection [21]. The male threads screwed into each side of the purchased solenoid valve and the barb ends allowed for a tight connection to the 4.7 mm ID (Inner Diameter) of the CO_2 tank tubing. One side of the solenoid was connected to the tank via a flexible plastic tube, and the other side was attached to similar tubing being inserted into the incubator, see Figure 9. The solenoid valve was fabricated using a transistor circuit, Arduino Microcontroller, and a 12 V power supply in order to be excited and opened, allowing the gas to pass through, see Figure 10 and Appendix D for more information. The solenoid valve was initially coded to open when the percent CO_2 was below 5% using a HIGH and LOW digital pin sequence. However, it was discovered that the NDIR CO_2 sensor can only communicate with other electronics using I2C coding. Due to time constraints, it was decided that the solenoid valve would be hard-coded to deliver 5% of CO_2. The flow rate of the solenoid valve was calculated by allowing the solenoid to open for one second into a balloon that was placed into a beaker filled with 550 mL of water. The volumetric displacement of the water was measured which would correspond to the volume of gas outputted in one second from the CO_2 tank. The average flow rate was determined to be 335 mL/s and the time to fill the entire incubator volume with 5% CO_2 was determined to be 0.26 seconds.

The average flow rate determination using water displacement was compared to mathematical analysis using Bernoulli's equation (Equation 2). Where v_1 was the velocity of the gas entering the solenoid ($v_1 = 0$), v_2 was the velocity exiting the solenoid, P_1 was the pressure

entering the solenoid, P_2 was the pressure at the exit of the solenoid, and ρ was the density of CO_2.

$$(v_2^2 - v_1^2) = \frac{2(P_1 - P_2)}{\rho} \tag{2}$$

After plugging values into Bernoulli's equation, using the cross sectional area of the solenoid valve to convert the velocity to volume per second, and performing unit conversions, the expected flow rate came out to be 355.919 mL/s. The expected and actual values had a percent error of 5.87%. One possible source of error that could lead to this difference was the tension from the balloon reducing the volume of the output gas, but overall the mathematical comparison was close. Mathematical calculations can be found in Appendix E.

Due to the value calculated using volume displacement, the solenoid valve was coded to deliver CO_2 once for 0.26 seconds to input 5% CO_2 and then for 30 milliseconds in 1.2 minute intervals to keep the range of CO_2 within specifications. See Appendix for code. The valve was then tested to determine its accuracy and reliability with the whole incubation system according to the CO_2 Control Testing Protocol in Appendix C.

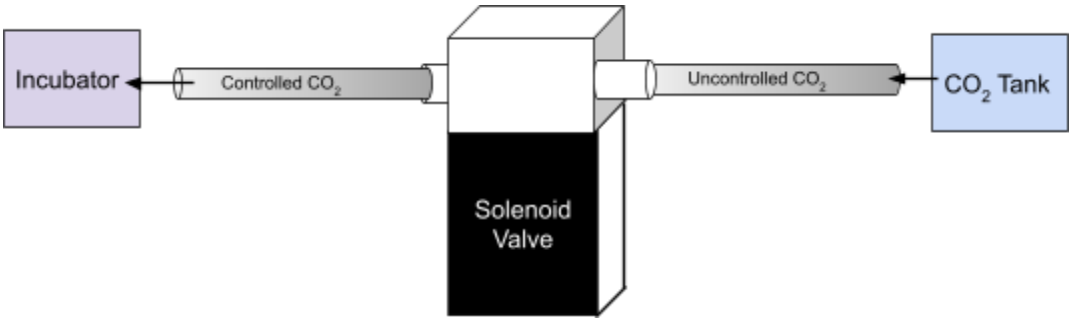


Figure 9: CO_2 Control Block Diagram

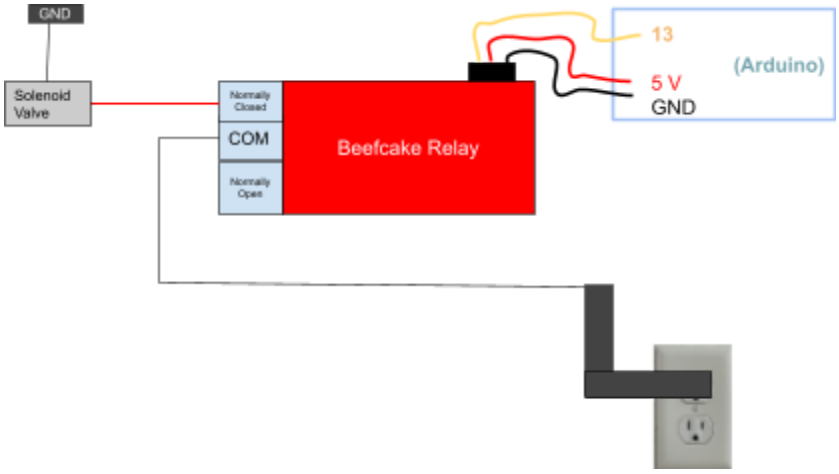


Figure 10: Solenoid Valve Circuit Diagram

Homogeneity

Homogeneity testing was conducted by laser cutting a second incubator lid with ten 12.7 mm holes surrounding the glass to allow different areas for the thermistor sensor to be inserted. This provided the team with data from multiple areas within the incubator to compare discrepancies in temperature, humidity, or CO_2 values and to allow for any corrections. Size 00 rubber stoppers were purchased in order to surround the sensors and provide a tight seal when the sensors were inserted into the precut lid holes, see Appendix F for expenses [22].

Cell-Viability

In order to test the full functionality of the incubator, the team conducted a cell viability test in order to quantify the cellular proliferation over time which was an indicator of healthy, viable cells. This was done to determine if the prototype can maintain cell viability similar to that of a standard industry incubator. One flask of cells would be cultured in the standard incubator and another in the prototype. A comparison of cell growth between the two flasks would allow the team to determine whether the prototype was able to maintain adequate cell viability to be considered functional.

Final Prototype

The final prototype, Figure 13, consisted of the same box design as the previous semester, see Appendix B, with modifications to allow for a wiper blade, Figures 11 & 12, to clear the condensation from the glass plates. The solenoid valve was connected to both the 100% CO_2 tank and the incubator to allow for airflow control, see Methods for more details. These two design changes allowed the team to deliver the necessary amounts of CO_2 to the system and ensure optical clarity.

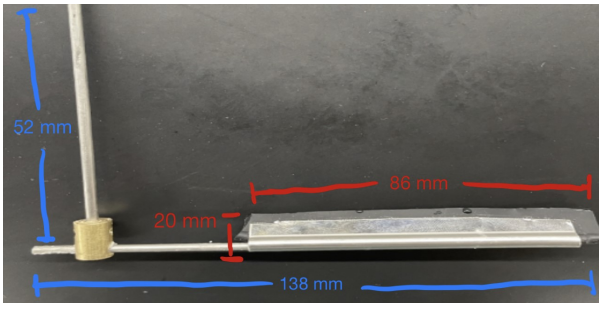


Figure 11: Knob handled wiper blade with dimensions

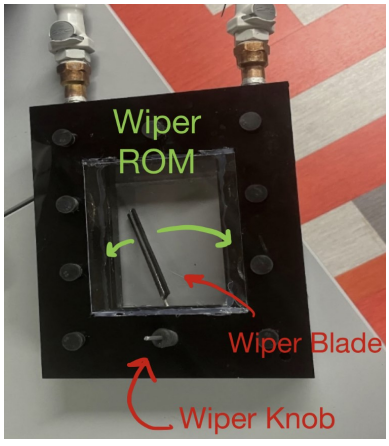


Figure 12: Knob handled wiper blade attached to the incubator with range of motion

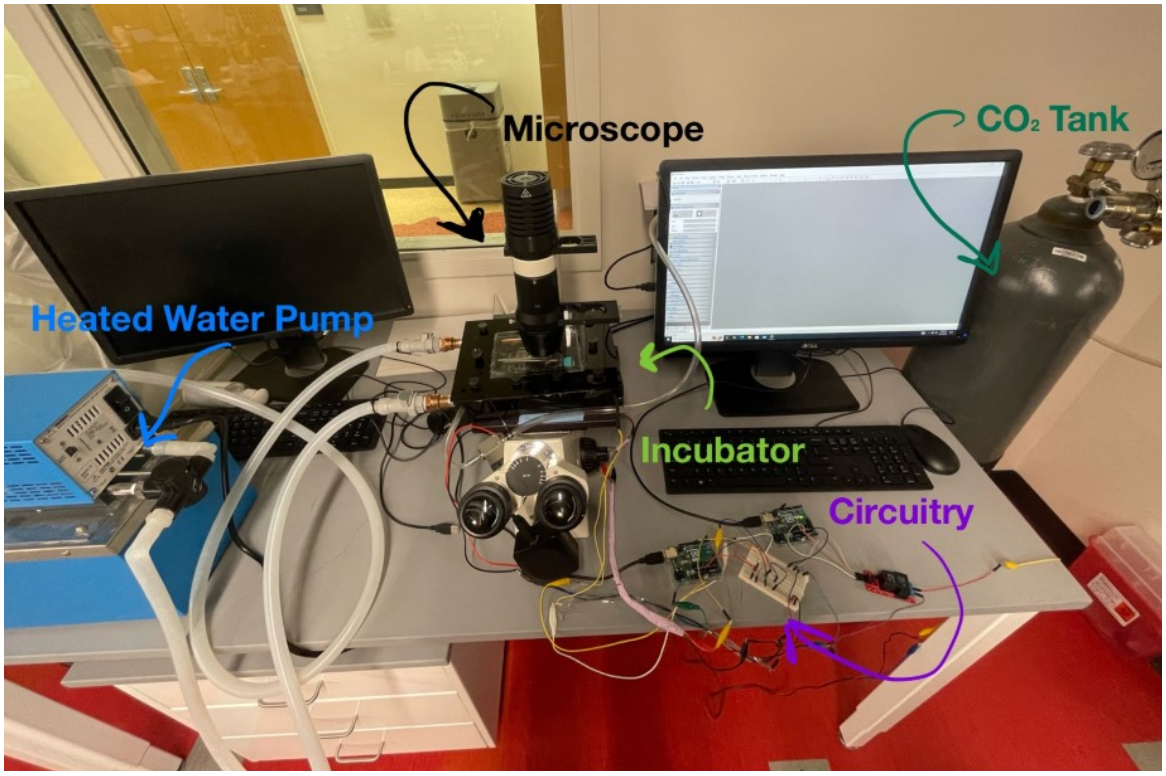


Figure 13: Whole incubator setup

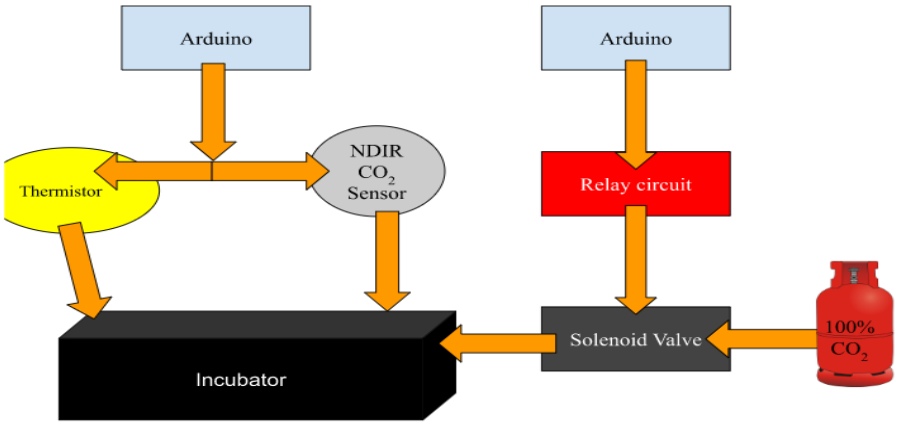


Figure 14: Block Diagram of Circuitry

Testing

The team tested the accuracy of the proposed design in the client's cell culture lab to determine if the internal environment was stable, if cell viability was maintained, and if the microscope optics were not corrupted. (See Appendix C: Testing Protocols for Testing Protocols)

Temperature Testing

The thermistor was previously tested under testing protocols in Appendix C. For more information please refer to Final Report Spring 2022.

Homogeneity Testing

The team completed homogeneity testing to ensure that the incubator was reading a uniform distribution of internal temperature and humidity components of the system. The goal was for each of the conditions to maintain homeostatic values throughout the incubator: 37 °C and > 95% humidity (CO_2 homogeneity was not able to be conducted this semester due to time constraints). This testing consisted of laser cutting an acrylic lid with holes as seen in Figure 14. The thermistor, which reads temperature and humidity, was placed in each of the holes evenly spaced in the lid below. The team recorded the values for each internal component and then calculated and reported the precision result (mean ∓ average deviation). To verify the system, all of the values for each component needed to fall within this precise range to ensure uniformity and accurate internal conditions within the device.

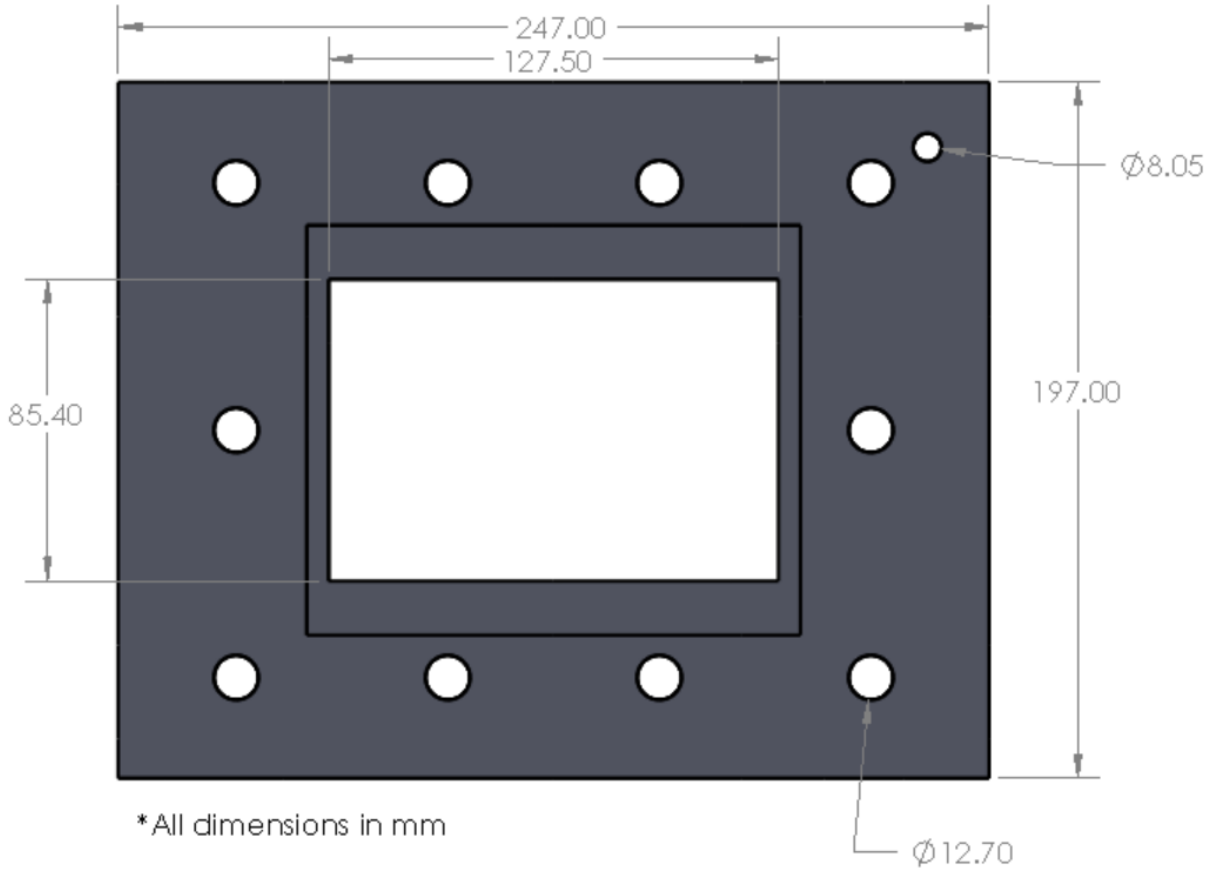


Figure 15: Testing lid with holes to use for sensor placement during homogeneity testing.

CO_2 Testing

The accuracy of the NDIR CO_2 sensor was previously tested for accurate results under testing protocols in Appendix C. For more information refer to Final Report Spring 2022. The ability of the solenoid valve to regulate the flow of CO_2 was tested by running the hard-code according to CO_2 Sensor and Feedback System Test Protocol. The incubator was set up for normal use and the code was implemented for approximately one hour. If the solenoid valve was able to keep the concentration of CO_2 in the incubator to 5% ± 1% then the test passed.

Optical Testing

Previously, the optical clarity of the transparent polycarbonate sheets was evaluated qualitatively and quantitatively to ensure they did not impair the microscope's ability to view the cell culture. Please refer to Appendix C and Final Report Spring 2022 for more information on the previous testing. The team re-conducted the optical clarity tests to ensure that the optics were still maintained. Two control images were taken of a T25 flask with cells inside on the inverted microscope stage and two test images were taken of the same T25 flask inside the prototype incubator on the inverted microscope stage. The images were analyzed using MATLAB to measure the energy of Laplacian. The Laplacian energy of an image is a measurement of intensity changes, therefore a clear image would have a high energy and a blurry image would have a low energy. Please refer to Appendix D for the MATLAB code. The test would be considered successful if the p-value comparing the two image groups was greater than 0.05.

Anti-fogging methods were tested to ensure clear visibility through the glass and condensation was prevented within the system as the team had optical issues last semester. The team purchased a silicone-based anti-fog lens cleaning solution. It was tested to determine how much of the solution must be applied to the glass incubator slides in order to maintain the visibility of the cells for a one-hour period since that period was consistent with the usage of the incubator in the BME teaching lab. For more information on the anti-fog application protocol, please refer to Appendix C. The team tested 8 pumps and 12 pumps of the solution on each glass slide to determine how much solution would be needed. For the control, the team imaged a T25 flask with cells on the inverted microscope stage in multiple places. The images were analyzed using MATLAB to measure the Laplacian energy and take an average for a more accurate representation. After applying 8 sprays to the inside of both glass slides, the same T25 flask was placed inside the prototype system. The heated water bath was operational in order to simulate the temperature and humidity conditions that the prototype would experience. An image of the flask was taken every 10 minutes over the course of 1 hour. The same procedure was applied when the team tested 12 sprays. All images were analyzed in MATLAB to measure the Laplacian energy and plot time vs energy to visualize how the condensation formation impacted the optical properties. Refer to Appendix D for the MATLAB code utilized. The test would be considered successful if no condensation was generated on the glass slides or if the p-value between the control and one or more of the spray levels was greater than 0.05.

Recovery Testing

The ability of the incubator to return to its internal environment of 37 ℃ and 95-100% humidity after a 30-second opening was previously evaluated using the recovery testing protocol outlined in Appendix C. For more information on recovery testing please refer to Final Report Spring 2022. Recovery testing for this semester commenced for CO_2 testing under the same capacity that it was with temperature and humidity last semester. The ability of the incubator and solenoid valve feedback loop was tested by opening the incubator for 30 seconds and recording the time it takes to return to its internal environment of 5% CO_2. See Recovery Test Protocol in Appendix C for more information.

Live Cell Testing

Live cell testing was conducted to ensure that the prototype was able to maintain cellular viability similar to that of a standard incubator. Dr. Puccinelli provided the team with MC323E1 cells to test in the incubators as well as culture media. All images were taken using the Zeiss inverted microscope in the teaching lab. For the control, ~125,000 cells were seeded into a T25 culture flask, were allowed to settle for 2 hours, an image was taken, and the flask was stored in the standard incubator. The cell's media was changed and a new image was taken every 24 hours for 92 hours in order to determine the cellular viability during the course of which the prototype would be used. To test the prototype, ~125,000 cells were seeded into a T25 culture flask, were allowed to settle for 2 hours, an image was taken, and the flask was housed inside the prototype incubator. From then on roughly every 12 hours, the media was changed and a new image was taken. This was repeated for 74 hours due to time constraints.

All images were loaded into ImageJ which was used to calculate the percentage of surface area that the cells covered. Analyzing the percent coverage over time allowed for the quantitative depiction of cell growth and therefore cell viability. The test would be considered successful if the p-value comparing the two groups was greater than 0.05. For more information on the live cell testing protocol, please refer to Appendix C.

VI. Results

Homogeneity Results

The internal environment of the incubator was tested in all locations using the fabricated lid in Figure 15 to ensure uniform conditions within the incubator. The team followed steps 1-4 in the Homogeneity Test Protocol, in which the thermistor was placed within the holes of the fabricated lid and the values for temperature and humidity were recorded for approximately five minutes. The average temperature and humidity values were calculated and used to determine the average conditions in that location. This was repeated for all 10 locations and the well plate area, see Figures 16-18. A two-sample t-test assuming equal variances was performed to determine the statistical significance between the data obtained for each location.

The results showed a p-value of 0.98 with a significance value of 0.05, indicating that there was no statistical significance between the areas in the incubator. Therefore the incubator was homogeneous, uniform, and able to deliver the necessary elements of temperature and humidity within all areas.

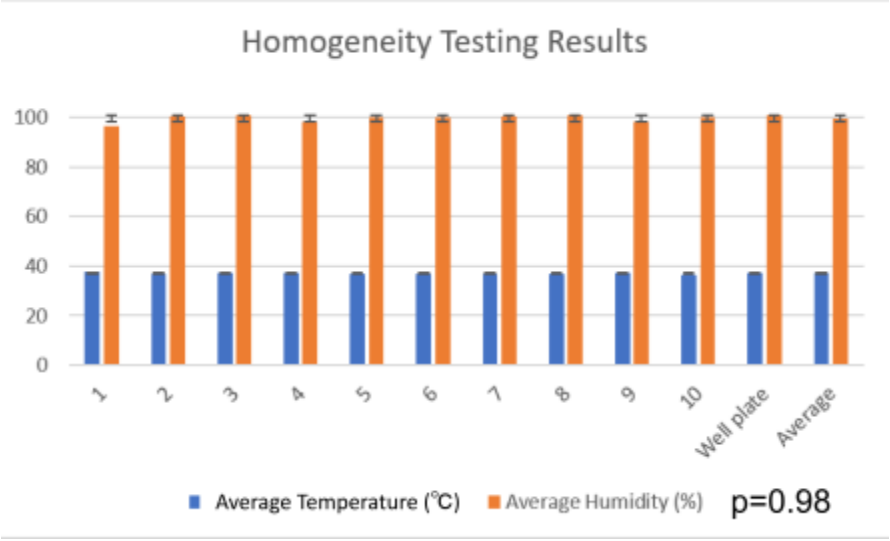


Figure 16: Homogeneity Testing Results

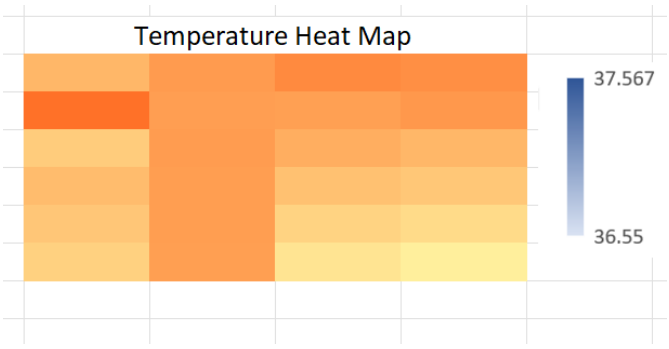


Figure 17: Temperature Heat Map

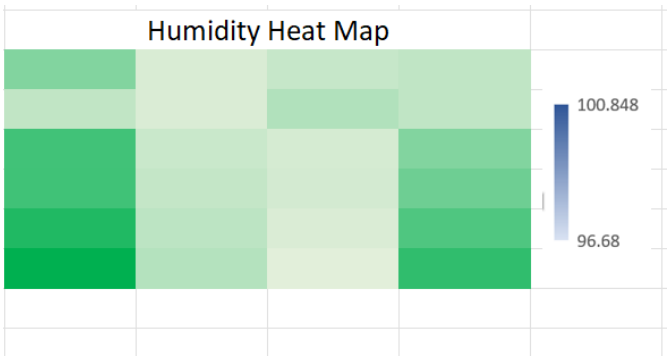


Figure 18: Humidity Heat Map

CO_2 Results

The NDIR CO_2 sensor was calibrated by placing it into the standard incubator and recording the measured percent value for approximately 2.5 hours, following CO_2 Test Protocol (as done in previous semesters). See Figure 19. A t-test was used to determine the statistical significance between the recorded CO_2 and the actual CO_2. The p-value was 0.789 indicating that there was no statistical significance between the values the sensor was outputting and the actual CO_2 in the incubator. The average sensor reading was 5.1%, which matched the output value on the standard incubator of 5.1%. The average standard deviation of the measured CO_2 percentage compared to the actual CO_2 percentage was calculated to be 0.01.

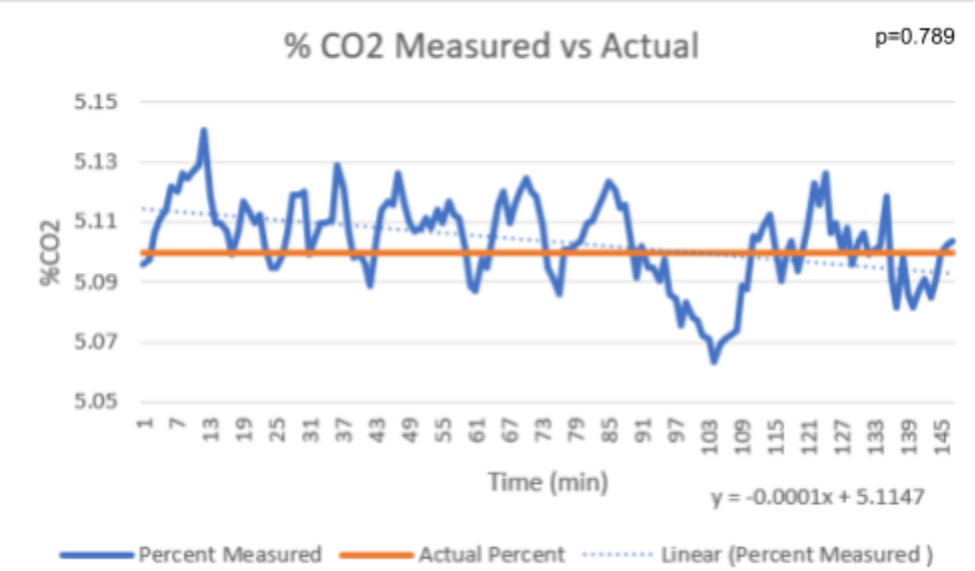


Figure 19: Sensor vs Standard CO_2 Measurement

The incubator was then set up for normal use and the hard-coded solenoid valve was tested for approximately one hour according to the testing protocol, see Appendix D for code. The values were recorded every second for the entire testing period, see Figure 20. The results show a standard deviation of 0.28 and a t-test was performed to obtain a p-value of 0.42. This shows that the results were not statistically significant and therefore can be used in live-cell testing. The hard-coded solenoid valve provided a crude way for the team to allow for 5% CO_2 to be inside the incubator for the entire test period.

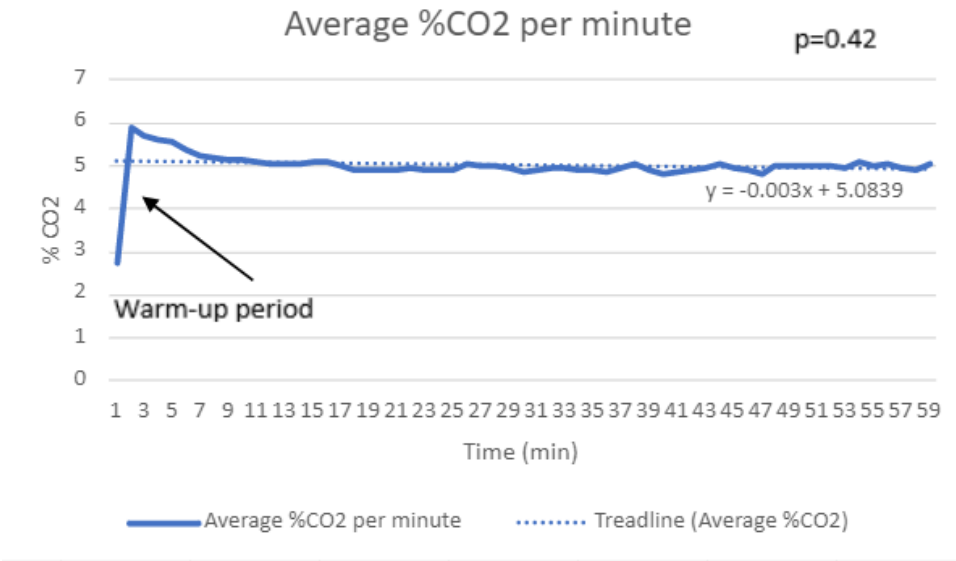


Figure 20: CO_2 Percent over 1-hour

Optical Results

The team conducted optical testing on the glass slides to ensure that they did not interfere with the optics of the microscope. The team followed the Optical Testing Protocol. Data analysis was conducted using MATLAB code in Appendix D. Figures 21a and b were two of the images that were analyzed. Figure 22 shows quantitatively that both control images and both test images had very similar Laplacian energies. A two sample t-test was run on the data and the p-value = 0.8442 therefore there was no statistically significant difference between the two images and the test was successful.

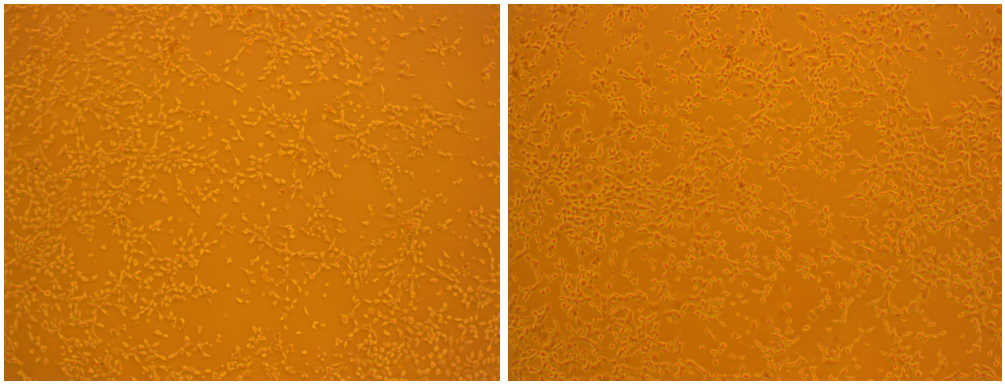


Figure 21a: (left) Control image taken outside of the prototype
Figure 21b: (right) Test image taken inside of the prototype

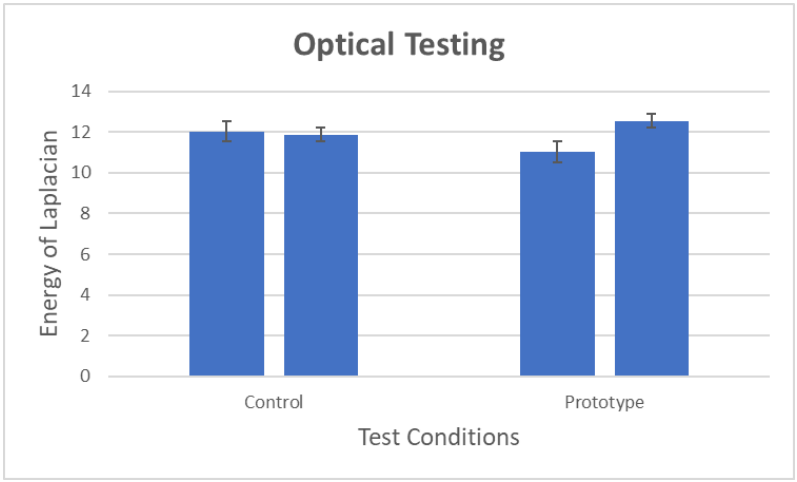


Figure 22: Changes in image intensity with and without the prototype glass slides

Anti-fog Results

The team conducted anti-fog testing to ensure that the system maintained clear visibility and that condensation did not inhibit microscope functionality. The team followed the Anti-Fog Protocol. Data analysis was conducted using MATLAB code in Appendix D. The data showed that in the 8 and 12 spray tests, the Laplacian energy was lower than the control. The images for the experimental groups, therefore, had a lower focus quality than the control group since there

was not as sharp of intensity changes within their images, shown in Figure 23. The lower focus quality of the test images compared to the control images can also be seen by the naked eye in Figure 24a and b. Since quantitatively the Laplacian energy was low and qualitatively the anti-fog spray did nothing to mitigate the condensation formation, the test had been deemed a failure. The team then tested a wiper blade method to remove condensation after its formation.

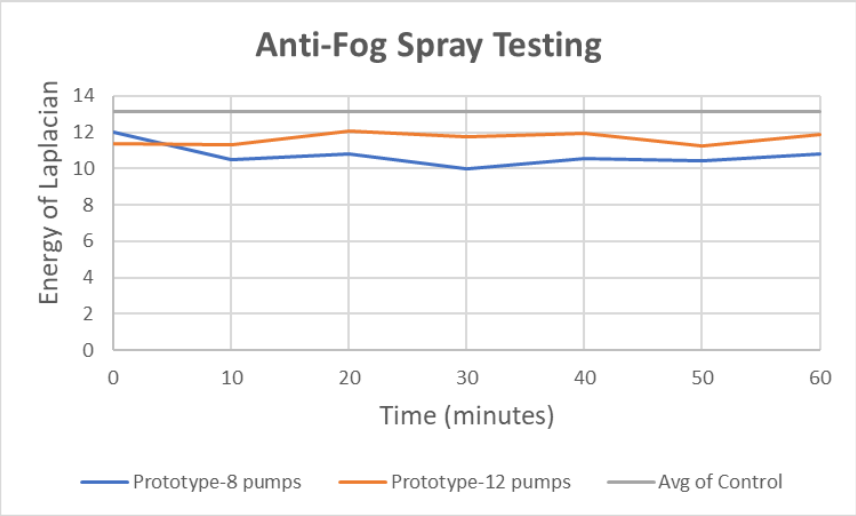


Figure 23: Changes in image intensity with varying levels of anti-fog spray

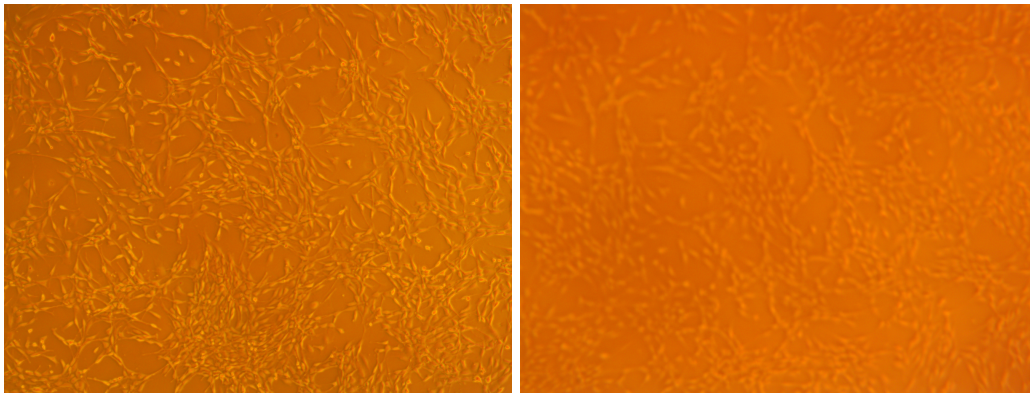


Figure 24a: (left) Control image taken outside of the prototype
Figure 24b: (right) Test image taken at t=50 min after 12 sprays were applied.

Knob Wiper Blade Results

As a result of the anti-fog spray not functioning as needed, the team utilized a fabricated wiper blade to remove condensation. The team qualitatively evaluated the effectiveness of the wiper blade by heating the internal environment to standard incubator temperature and humidity values and assessed wiper usability on the condensation as seen in Figure 25a and b. It was concluded that the wiper blade worked effectively and helped with the mitigation of fog and condensation concerns. Although the wiper blade was effective in removing condensation, the

functionality of the knob wiper blade design was difficult to maneuver and too big to be compatible with the insertion of a well plate so no further testing was done.

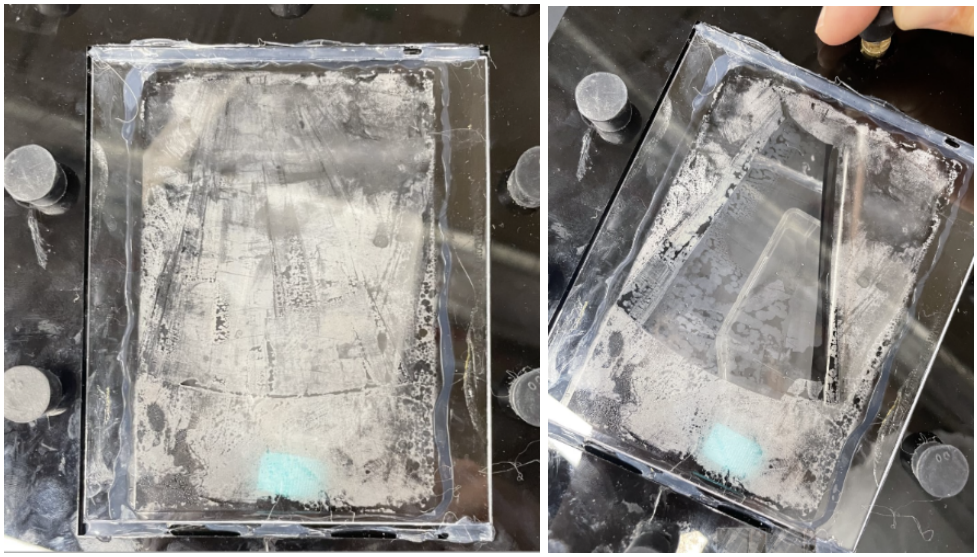


Figure 25a: (left) Incubator glass before a single wiper blade wipe
Figure 25b: (right) Incubator glass after a single wiper blade wipe.

Recovery Results

Recovery testing for CO_2 was not able to be conducted due to having a hard-coded solenoid valve, however, the team would like to use I2C code in the future to test the ability of the incubator and solenoid valve feedback loop. This will be tested by opening the incubator for 30 seconds and recording the time it takes to return to its internal environment of 5% CO_2. See Recovery Test Protocol in Appendix C for more information.

Live-Cell Results

Internal Environment

The internal environment of the cells was measured approximately every 10 minutes during 36-hour live-cell testing to document the cells living conditions. The results show that temperature and humidity allowed for cell growth and maintenance, but CO_2 methods need to be improved. See Figure 26.

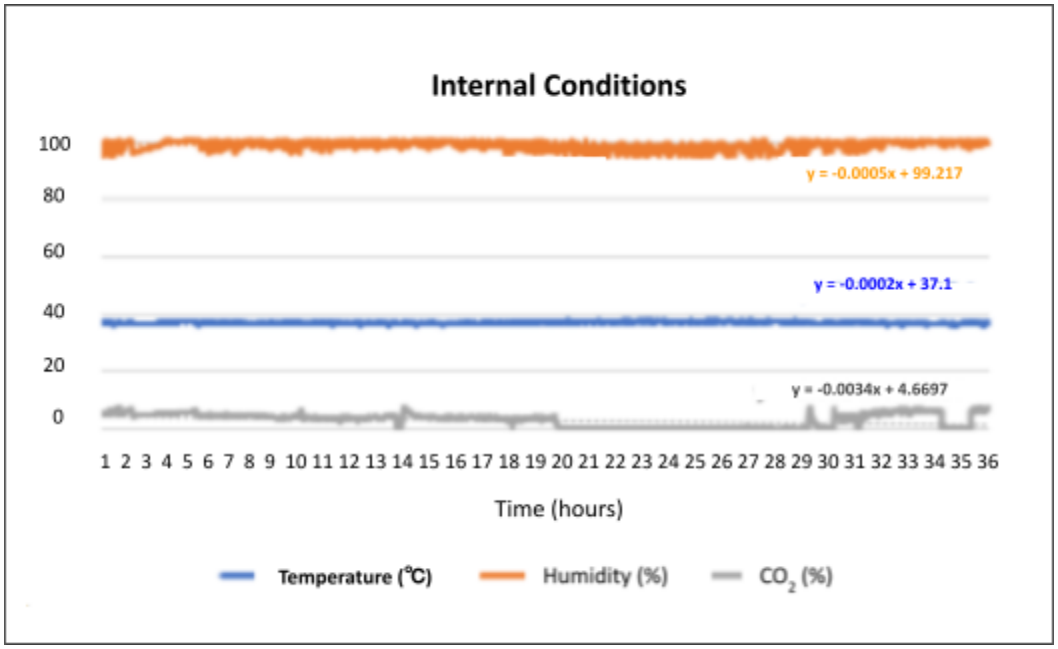


Figure 26: Internal Conditions over 36 hours

Temperature was consistent during the entire 36-hour experiment with an average temperature of 37.18 ℃ with a standard deviation of 0.36 ℃ over the testing period. A t-test was performed to determine the significance between obtained values and the expected value of 37 ℃. The results yielded a p-value of 0.497 showing that the results are not statistically significant and therefore valid. Figure 27 shows the temperature collected approximately every 10 minutes for the entire testing period. Table 2 shows the calculated values listed above.

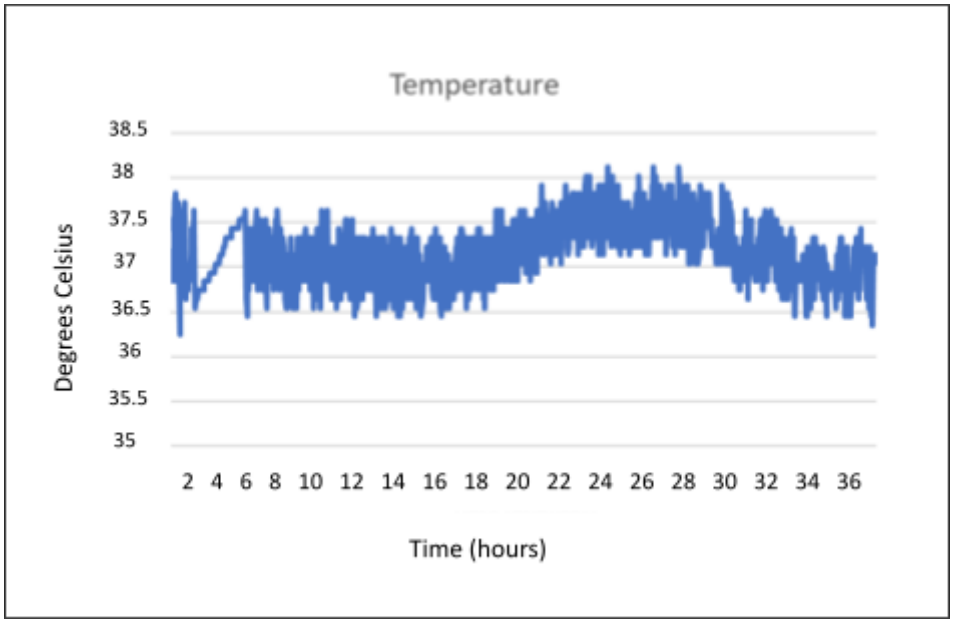


Figure 27: Temperature over 36 hours

The humidity was consistent during the entire 36-hour experiment. The average humidity over the course of the testing period was 98.99% and the standard deviation was 1.006%. A t-test was performed to determine the significance between obtained values and the expected value of approximately 97%. The p-value was calculated to be 0.47 proving that the results are not statistically significant and therefore valid. Figure 28 shows the humidity collected approximately every 10 minutes for the entire testing period. Table 2 shows the calculated values listed above.

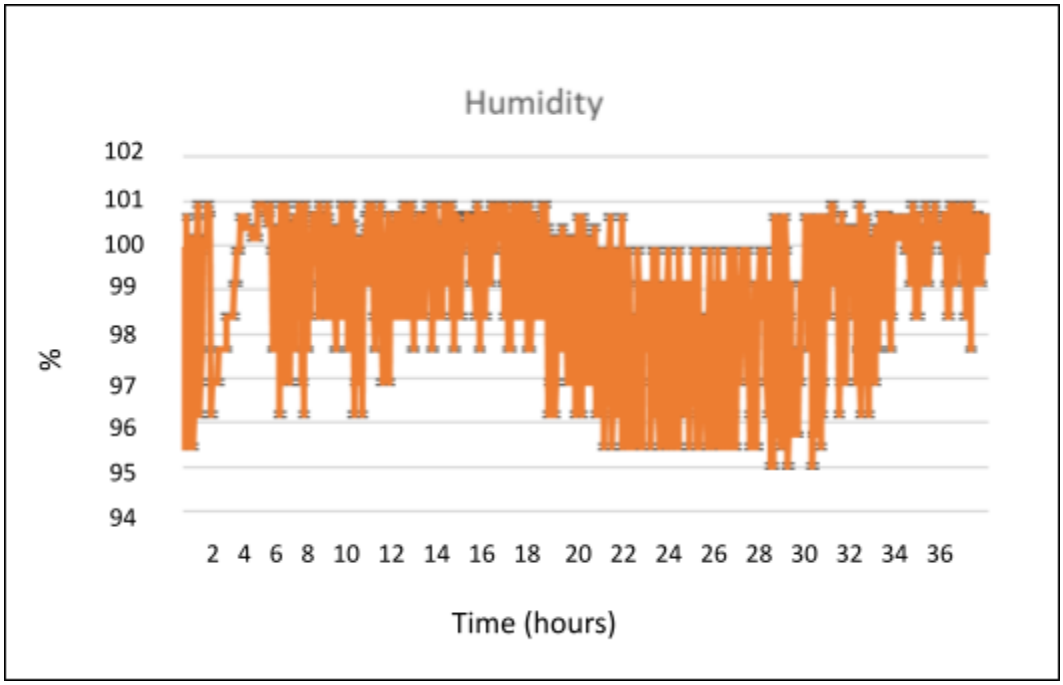


Figure 28: Humidity over 36 hours

CO_2 was extremely inconsistent during Live-Cell testing. Despite the data collected during the hard-coded CO_2 tests, the CO_2 sensor was reading different values than previously predicted. The CO_2 control method is not a viable option for the future as it ranged from values of 0-8% CO_2 over the course of the experiment. The CO_2 tank had a disruption overnight at approximately hour 21 where no CO_2 gas was being inputted into the incubator. The average % CO_2 over the 36-hour experiment was 3.15% with a standard deviation of 2.43%. A t-test was performed to determine the statistical significance of the data compared to the expected value of 5%. The p-value was equal to 1.19×10^{-88} indicating that the results were significantly different than the intended value of 5%. In the future, I2C and protoboard fabrication will be considered as a more permanent solution. Figure 29 shows the CO_2 collected approximately every 10 minutes for the entire testing period. Table 2 shows the calculated values from temperature, humidity, and CO_2 listed above.

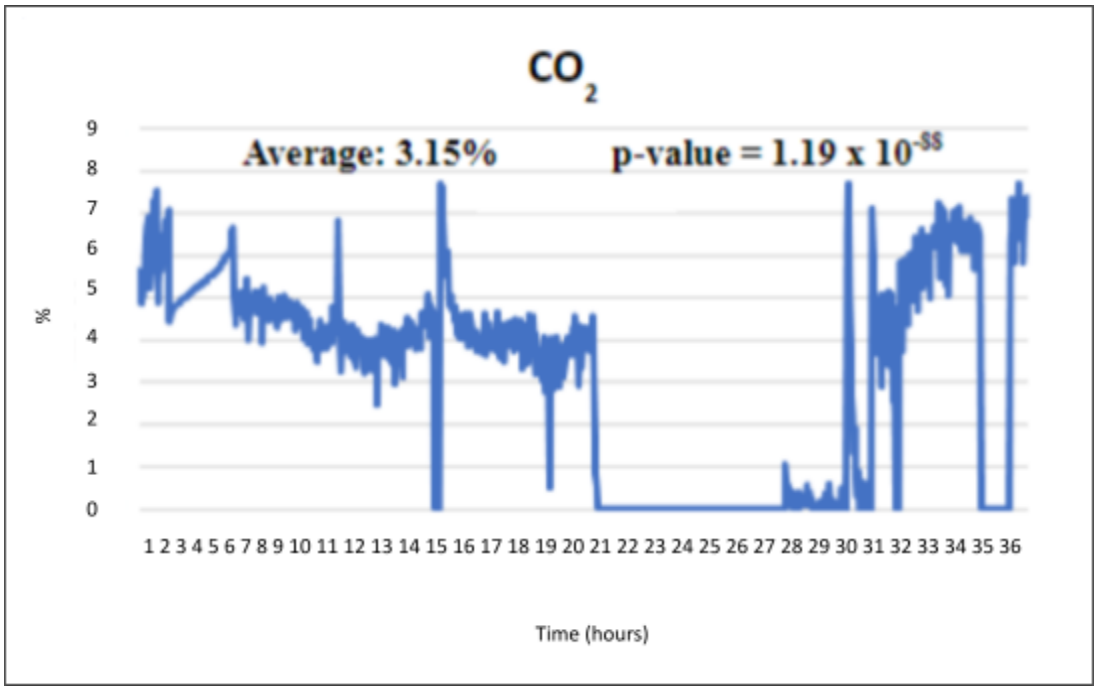


Figure 29: CO_2 over 36 hours

Table 2: Statistical Analysis of Internal Environment

	Temperature	**Humidity**	**CO_2**
Average	37.18	98.99	3.15
STD	0.367	1.006	2.435
p-value	0.497	0.473	$1.19x10^{-88}$

Cell Confluency

The team conducted a cell confluency test to ensure cellular viability was maintained inside the prototype. The team followed the Cell Confluency protocol and data analysis was conducted using the ImageJ percent area procedure. The control group experienced exponential growth over the course of the 100 hours. The test group started to experience exponential growth but at ~22 hours the growth started to trend downwards due to the CO_2 failing. This failure caused the internal environment to become acidic which was conducive to cell growth. This can be seen in Figure 30. Figures 31a and b compare the cell confluency of the control flask at 2 hours and 92 hours in the incubator where large amounts of cell proliferation occurred, indicating healthy cells. Figures 32a and b compare the cell confluency at 1 hour and 74 hours in the prototype incubator, where not very much cell proliferation occurred, indicating no healthy cells. This test was deemed a failure due to the lack of cell proliferation in the prototype meaning the prototype could not maintain healthy cells for one-week periods.

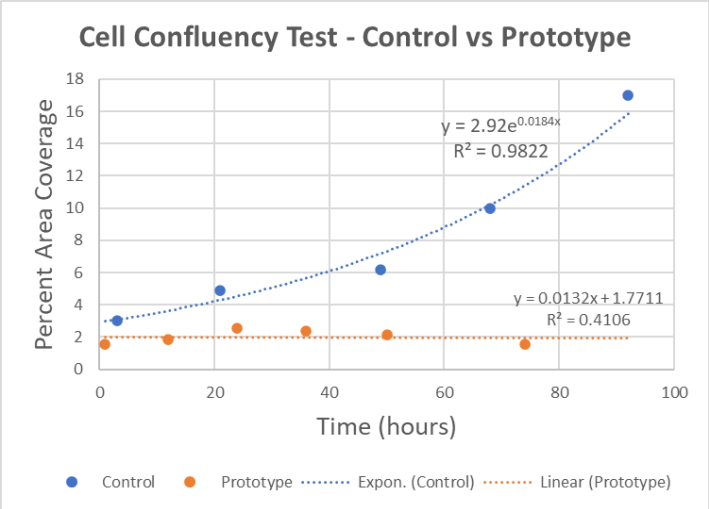


Figure 30: Comparison of cell confluency between the control and test incubator

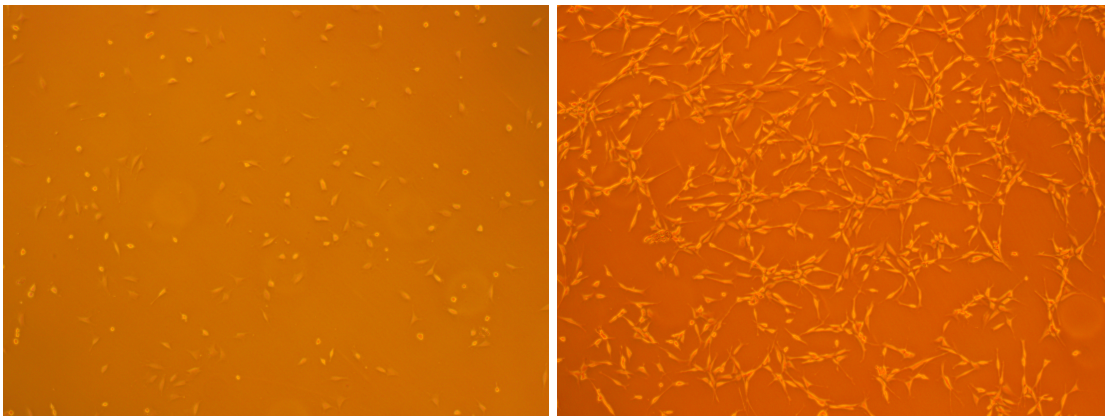


Figure 31a: (left) Control flask at t = 2 hours
Figure 31b: (right) Control flask at t = 92 hours

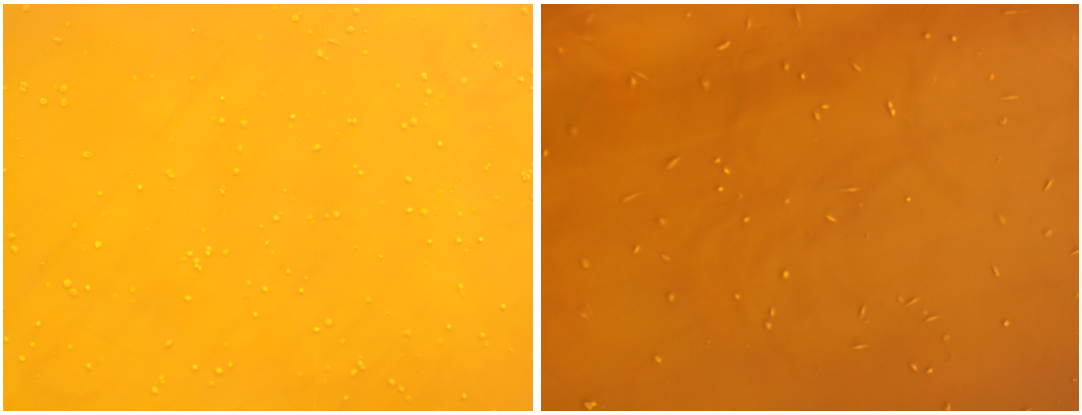


Figure 32a: (left) Prototype flask at t = 1-hour
Figure 32b: (right) Prototype flask at t = 74 hours

VIII. Discussion

Homogeneity testing revealed that there was not a significant difference in temperature or humidity between different locations in the incubator. This allowed each cell to receive the necessary amount of nutrients to grow and proved that one area of cells was not growing faster than the other. Commercially available incubators are able to deliver uniform amounts of temperature and humidity throughout their large incubator devices. The results show that the team's small model incubator was able to perform at industry standard. It can be concluded that the incubator is available for use at a higher academic level in teaching labs due to these results. Optical testing revealed that there was not a significant statistical difference between an image taken of just a culture flask and an image taken in the prototype. This means that the prototype glass covers did not interfere with the optics of the microscope. The NDIR CO_2 sensor was calibrated for accuracy and the results prove that it recorded correctly with an average standard deviation of 0.01. The results showed that the team's data collection was precise and accurate. The hard code used to control CO_2 in the incubator proved to be accurate as the percent CO_2 over the course of the testing period was calculated to be 5% ∓ 0.28%. These results showed that although the hard code will not be a permanent solution, it was able to deliver the necessary amount of CO_2 to the incubator for the testing period. The results compared very similarly with industry-standard incubators that have a specification of 5% ∓ 0.5% on their incubators [23]. The CO_2 code was not a viable option as changes in the internal environment required constant alterations to the amount of CO_2 released. The complexity of the electronics system in general proved the need to create a protoboard in order to condense the system to avoid possible interruptions to the circuitry. The internal environment of temperature and humidity proved to be within the industry standards needed to maintain cell viability.The cells were not able to proliferate and therefore were unhealthy due to the acidic environment they were exposed to. CO_2 levels are the main regulators of pH in cell cultures and because during testing the CO_2 input was interrupted, the environment became very acidic which ultimately stressed the cells. In the future, the team will need to be able to maintain proper CO_2 levels in order to maintain healthy cells.

In the early stages of development, the team planned to use the anti-fog spray in order to reduce the amount of condensation on the glass during the incubation period. However, this was not a viable solution as the spray was not able to properly remove the condensation over long periods of time. This condensation interfered with the optics of the microscope causing the images to be blurry and have low Laplacian energy values.A change was made to incorporate a wiper blade in order to wipe off the condensation during imaging. The team was able to qualitatively assess the functionality of the wiper blade in resolving visibility and the team will finalize and test this design next semester to see if the wiper is able to adequately remove condensation. Originally, the team planned to incorporate the use of digital pins in order to regulate the flow of CO_2 via both the NDIR sensor and the solenoid valve. Upon further evaluation, it was determined that the NDIR sensor only allows for cross-communication using I2C code. Due to the lack of time and knowledge of I2C coding formats, the decision was made

to hard-code the solenoid valve by using the flow rate to determine how much CO_2 would need to be released approximately every minute in order to keep the system at an average concentration of 5% CO_2. In the future, the team plans to research more on I2C coding and implement it next semester in order to have more precise control over the system as a whole. I2C also allows the user to be able to sense any error in the system, avoids having to manually restart after the error is identified, and provides an easier control system. All electronics should be condensed into a protoboard to reduce the amount of human error when the incubator is left unattended.

During live cell testing, a few unforeseen problems arose. First, the hot glue securing the incubator glass to the box melted due to the internal conditions which led the glass to fall off. The glass plates should be secured to the box using heat-resistant and water-resistant acrylic glue to avoid the glass from being disconnected from the incubator during testing and usage in the future. Another issue was the laser cut acrylic lid started to warp after running the incubator for extended periods of time. Acrylic has the tendency to warp when under variable temperature and humidity environments as seen in the incubator. To solve this problem in the future, the team will discuss possible material changes to the lid or implement latches to help reduce the warping. The last unforeseen problem was the growth of mold within the incubator. The internal conditions of the incubator are prime conditions for mold growth when lack of sterilization practices are present. In the future, the team will be more aware of sterilization techniques and disinfection methods to prevent these types of growths in the future.

Possible sources of error throughout the project include leakage, incorrect time intervals, and human error during media changes and live-cell imaging. During homogeneity testing, the laser-cut CO_2 input hole was not covered which could lead to leakage of the internal environment. The hot glue used to fasten the transparent glass and rubber lining had places where the materials were not secure, which could cause leakage of the internal environment as well. The bottom glass was also hot glued together as it was slightly too small for only one sheet to fit over it, which could lead to leakage of heat, humidity, and CO_2 as well. During CO_2 testing, the laser-cut thermistor hole was not covered which could lead to leakage of the internal environment. The CO_2 tests also did not account for the amount of CO_2 that the cells may consume during live cell testing. Due to the hard coding of the solenoid valve in order to control the flow of CO_2, the time interval between the release of air could have been more precise. The intervals were measured twice using a stopwatch but did not account for changes in the system or cellular consumption. The team was trained in cell media changes and imaging techniques, but due to the long period of testing time, different members of the team changed the media and took pictures at 12-hour intervals. This could have caused an error as each team member put in approximately 5 mL of media, which may have varied between each team member. During imaging, each team member also focused on the image before taking a photo. The focus quality between images may have differed depending on the microscope user and their definition of “in-focus”. During live-cell testing, the prolonged temperature and humidity conditions also caused the hot glue holding the bottom glass plate to become loose. There was an error as there

was a considerable amount of leakage of the internal environment until the next morning when team members were able to adjust the heated water pump and CO_2 output to try to combat the significant amount of internal leakage. The live-cell testing was also left unattended where there was a possibility of a lab member accidentally bumping into, turning off, or touching circuitry. This caused the solenoid valve to stop working and the need for rewiring of the solenoid on the second day of testing. There was no input of CO_2 for a considerable amount of time during the night.

Ethical considerations need to be taken into account as this device will be used in a live cell lab. The origin of the cells being studied was of the utmost importance. The client plans to use immortalized pre-osteoblasts isolated from the calvaria of newborn mice. The use of animal cells has caused much ethical controversy over the past half-century. Mice are commonly used in laboratory research as their entire genome has been sequenced and compared to the human genome and they are easily bred and housed [24]. Extra measures must be taken to ensure that the newborn mice are subject to the least amount of harm, distress, and pain in order to conduct an ethical experiment. The Animal Welfare Act, a federal law that outlines the standard of care animals must receive in laboratories, is also a necessary requirement of labs to follow when using mice, and other AWA-approved animals, with the incubator [25]. If in the future, human cells are used, the consent of the subject must be granted before cells are placed in the incubator. Ethical consideration must also be given if the cells are to be manipulated in the future, rather than just watching the growth of the cell. Gene editing has become quite the controversy over the past 20 years, with the ethical considerations of its use in treating cancer, preventing life-threatening diseases in gestation, and its use in what has been termed “designer babies:” the idea that one can alter the DNA in a prenatal cell to fit the desired phenotype or genotype of the parents. Designer babies are currently legal in Sweden, Spain, Belgium, the UK, and the US [24]. Furthermore, ethical considerations must be made when determining how manipulations of the cell will alter not only the DNA but evolution as a whole. The societal implications of prescribed DNA mutations must also be taken into account as the effects of this process can range from the elimination of genetic diseases to the elimination of certain phenotypes altogether.

IX. Conclusion

The client is in need of a microscopic cell culture incubator compatible with an inverted microscope that is lightweight, maintains a stable internal environment, and is cost-effective for the purpose of using it in a teaching lab during the semester. The team has proposed a design that is lightweight, cost-effective, and able to maintain the desired internal environment. The proposed final design will include a copper tube that is wrapped around the inside of the incubator and connected to a heated water pump that will regulate the internal incubator conditions and keep them at their optimal values. The lid to the incubator will be placed on top which will allow for a tighter seal of the internal environment and help prevent leakage. The incubator box will also contain a hole for CO_2 to be pumped in, a CO_2 sensor, and a thermistor

temperature sensor that will in addition be coded to calculate the internal humidity. The CO_2 input will be monitored using a solenoid valve that receives direction from the NDIR sensor via Arduino coding. Moving forward, the team will implement I2C coding, a manual wiper for the removal of condensation build up, and more strict sterilization protocols will be followed. Overall, this is a highly impactful project, as it will be implemented in teaching labs on campus, improving the quality of students' education for future generations to come.

X. References

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XI. Appendix

Appendix A: Product Design Specifications (PDS)

Function: Develop a low-cost cell culture incubation chamber that is compatible with an inverted microscope and capable of live cell imaging over the course of one week.

Client requirements:

- Incubation chamber must be able to maintain an internal environment of 37℃, 5% CO_2, and 95-100% humidity
- Microscope's optics and functionality must not be damaged
- Maintain even heating and humidity across the chamber
- Create a device that stays within a budget of $100
- Ensure that the device can be easily assembled and removed between uses

Design requirements:

1. Physical and Operational Characteristics

a. ***Performance requirements:*** The device must be able to sit on a microscope stand (less than 310 x 300 x 45mm[1]), be transparent on the top and bottom to allow for optical visualization with an inverted microscope, and maintain an internal environment of 37℃, 5% CO_2, and 95-100% humidity. This device should demonstrate no quantitative difference on the microscope when adding glass compared with solely cells, in order to demonstrate full transparency of the top and bottom slides of the system.

b. ***Safety:*** The incubator and the cell culture environment must be in cooperation with BioSafety Level 1 Standards [2]. Any material and electrical or mechanical machinery must be sterilizable and waterproof.

c. ***Accuracy and Reliability:*** The device must be able to maintain a temperature of 37°C ± 1°C throughout the entire internal environment. The humidity must be kept above 95% humidity. CO_2 levels must be 5% ± 1%. The incubator must be able to maintain these conditions constantly for at least two weeks. The device must also be able to reach these conditions after the incubator has been opened and exposed to the external environment within five minutes of interruption.

d. ***Life in Service:*** The device must be able to be used for two weeks, but optimal usage will occur for one week at a time for teaching purposes in the client's tissue lab (including cell viability for one week as well).

e. ***Shelf Life:*** The shelf life of this product should be 10 years.

f. ***Operating Environment:*** The operating environment is a clean room. The incubation chamber must be able to maintain an internal environment of 37°C, 5% CO_2, and 95-100% humidity for at least two weeks, without compromising the integrity of the microscope's optics or functionality. Measures must be taken

to ensure that the temperature is the same in all areas of the chamber with an error of ± 1° C. The box also must be sealed efficiently to ensure that evaporation does not occur.

g. ***Ergonomics:*** The device should be portable in that one should be able to carry and store the device easily. Wires should not be hanging freely out of the device, and it should be easy to pick up and put away when needed.

h. ***Size:*** The device must be less than 310x300x45mm in order to fit on the microscope stand without interfering with the optics[1]. Overall, the product must be compatible with an inverted microscope.

i. ***Weight:*** There are no specific weight requirements. However, minimizing weight would be ideal to promote incubator transportability and usability.

j. ***Materials:*** There are no specific materials that are required for the development of this device. However, it is important to examine different material properties to determine which materials hold heat effectively, are watertight, and have a transparent appearance.

k. ***Aesthetics, Appearance, and Finish:*** The client does not have a preference for color. Well plates are clear, black (to stop contamination), and white (to increase light). Using materials that would block out external light sources would be ideal, but this is not a requirement for the device. The finish should exclude messy elements, such as long wires, and be transparent on both the top and bottom.

2. Production Characteristics:

a. ***Quantity:*** Only one device is necessary to produce, but ideally, it would have the capacity to be produced on a larger scale to be used repeatedly in the teaching labs. The client has also requested a complete guide for others to replicate the work accomplished.

b. ***Target Product Cost:*** The target product cost for this device is $100. It will be financed via UW BME Departmental teaching funds.

3. Miscellaneous

a. ***Standards and Specifications:*** The incubator would need to adhere to the ISO 13485 regulation which outlines requirements for regulatory purposes of Medical Devices [3]. The incubator would also need to follow the FDA's Code of Federal Regulations Title 21, Volume 8 which outlines the requirements for Cell and Tissue Culture products [4].

b. ***Customer:*** The client, Dr. John Puccinelli, is an undergraduate advisor in the Biomedical Engineering Department at the University of Wisconsin - Madison. Dr. Puccinelli is asking for the cell culture incubator in order to amplify the teaching curriculum in his classroom environment. Having an incubator that is

easy to disassemble and compatible with an inverted microscope would result in efficient classroom lessons.

c. ***Patient-related concerns:*** The accuracy of the temperature, humidity, and CO_2 concentration is of utmost concern for the client. Humidity must be 95-100%, otherwise, cells will begin to dry out. Having a set temperature of 37°C will replicate optimal cellular environments. Lastly, the ease of disassembly and disinfecting of the incubator was of concern.

d. ***Competition***: There are currently multiple inverted microscopes and cell culture incubators on the market ranging from $500-$40,000 [4]. Thermo Fisher, NuAire, and New Brunswick all have incubators currently on the market. Thermo Fisher and NuAire are more popular as they have both direct heat and water-jacketed incubators. The most popular Thermo Fisher design is the Heracell VIOS 160i CO2 Incubator with Copper Interior Chambers, which has HEPA filtration for ISO Class 5 air quality and an overnight Steri-Run for total sterilization [5]. Others have also attempted to design low-cost live-cell imaging platforms using 3D-printed and off-the-shelf components. Both okolabs and Elliot Scientific have stage-top microscopic incubators available, both of which use the direct heat method, and have had great success in maintaining a homogeneous environment in terms of temperature and CO2 percentage[6,7]. However, these stage-top incubators are still extremely expensive ranging from $431-$1000, and are only compatible with XY stage inserts[8]. XY stage inserts are roughly 150x150x36mm[9], slightly smaller dimensions than the stage top the team is currently working on. A team of researchers from Australia were able to successfully design a portable low-cost long-term live-cell imaging platform for biomedical research and education for under $1750 [10]. This low-cost incubator also monitored and regulated temperature, CO2, and humidity as per the parameters for successful mammalian cell culture. A company called ibidi has developed a stage top incubator compatible with an inverting microscope following all of the temperature, humidity, and CO2 requirements as well as producing anti-condensation glass technology. The incubator is currently on the market for $19,000 [11]. Past BME 200/300 design projects have attempted to build incubators for this client, but none have been completely successful.

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Appendix B: Incubator Spring 2022

Final Design

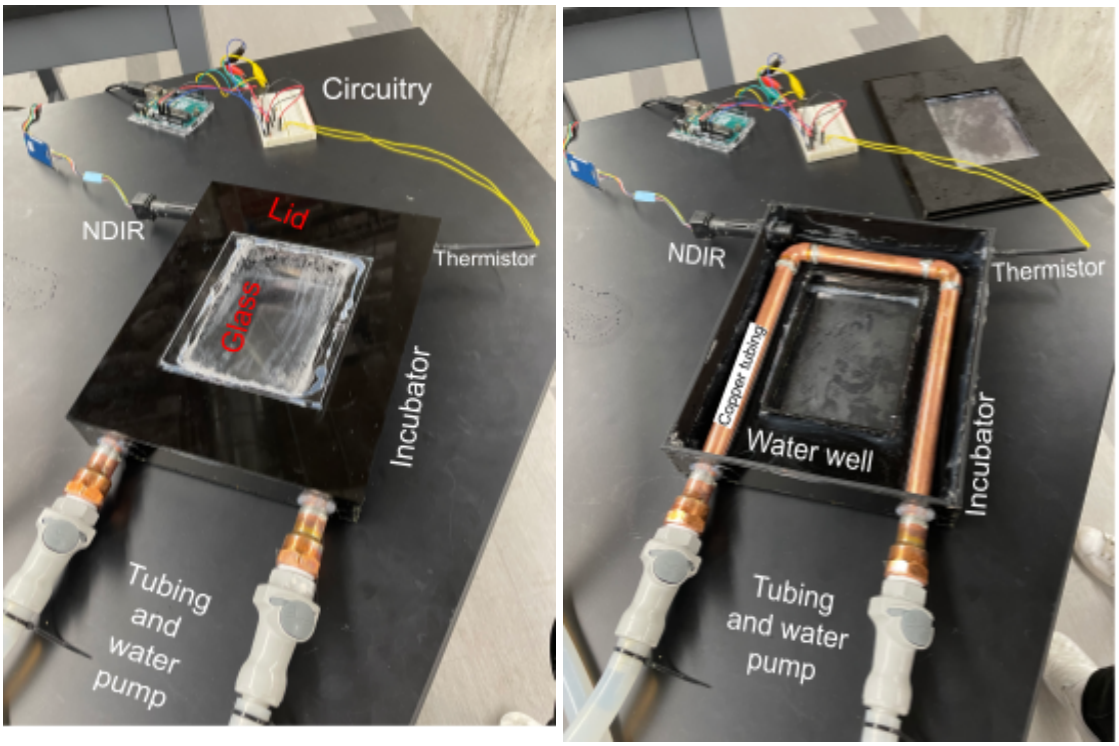


Figure 1: (left) Incubator Prototype Exterior
Figure 2: (right) Incubator Prototype Interior

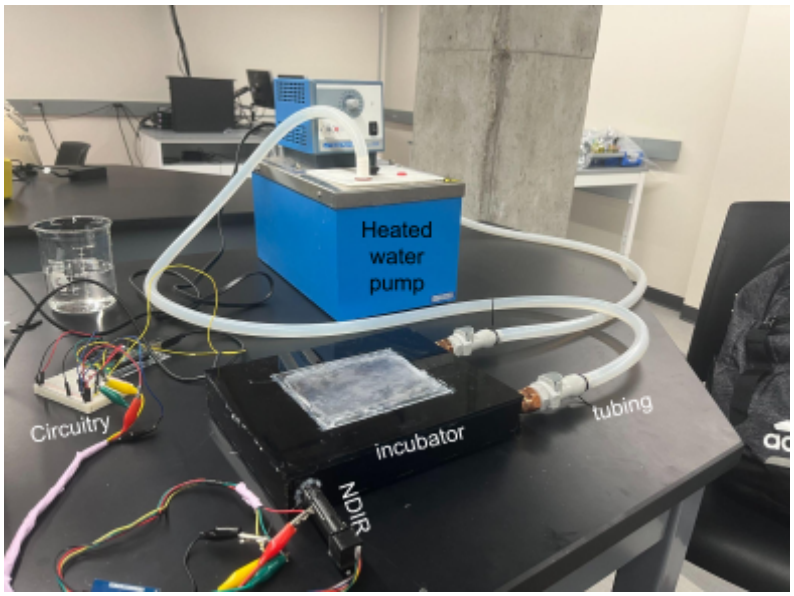
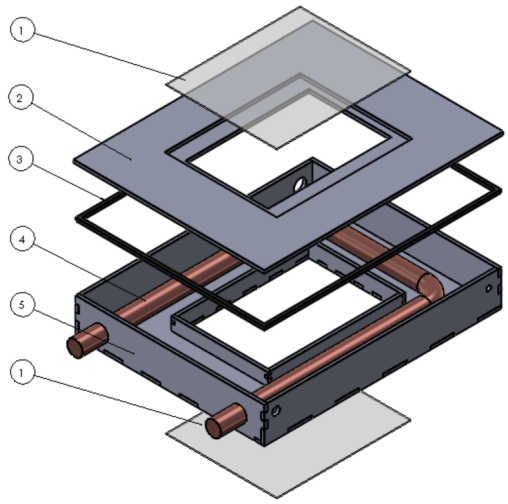


Figure 3: Whole Incubator Set Up

SOLIDWORKS CAD Drawing of the Proposed Cell Culture Incubator and User Manual



Item No.	Item Description	Dimensions [mm]	QTY.
1	Glass plates to allow transparent viewing	114.5 x 138.5 x 1.3	2
2	Lid of box to enclose the incubator	247 x 197 x 6.35	1
3	Rubber linning to allow tight seal	245 x 195 x 3.175	1
4	Copper tubing to provide heat transfer	Outside Diameter: 15.875 Inside Diamter: 12.7 Length: 610	1
5	Black acrylic box to maintain a controlled internal environment	Outside Cut: 245 x 195 x 36.83 Inner Cut: 142 x 100 x 16.25	1

Figure 4: Exploded SOLIDWORKS assembly of the final design along with a table explaining the dimensions and parts

Boot up Process

1) Remove lid from incubator
2) Connect heated water pump tubing to the pipe-tubing adaptor
3) Connect CO_2 tank hosing to incubator
4) Place incubator onto microscope shelf
5) Turn on heated water pump and set water temperature to 37° C
    a) Optional: Start pumping water at a higher temperature at the start to speed up initial heat up process and then lower temperature to 37° C
6) Fill the incubator with enough DI water so the water level is just below the inner square frame, maximizing the amount of water touching the copper piping
7) Turn on CO_2 tank and CO_2 sensor to fill the internal environment to the appropriate 5% CO_2 levels
8) Replace lid back on the incubator
9) Allow time for internal environment to reach 5% CO_2, 37° C, and 95-100% humidity
10) Compare desired inputs to the live sensor readings from the sensors

Inserting Well Plate

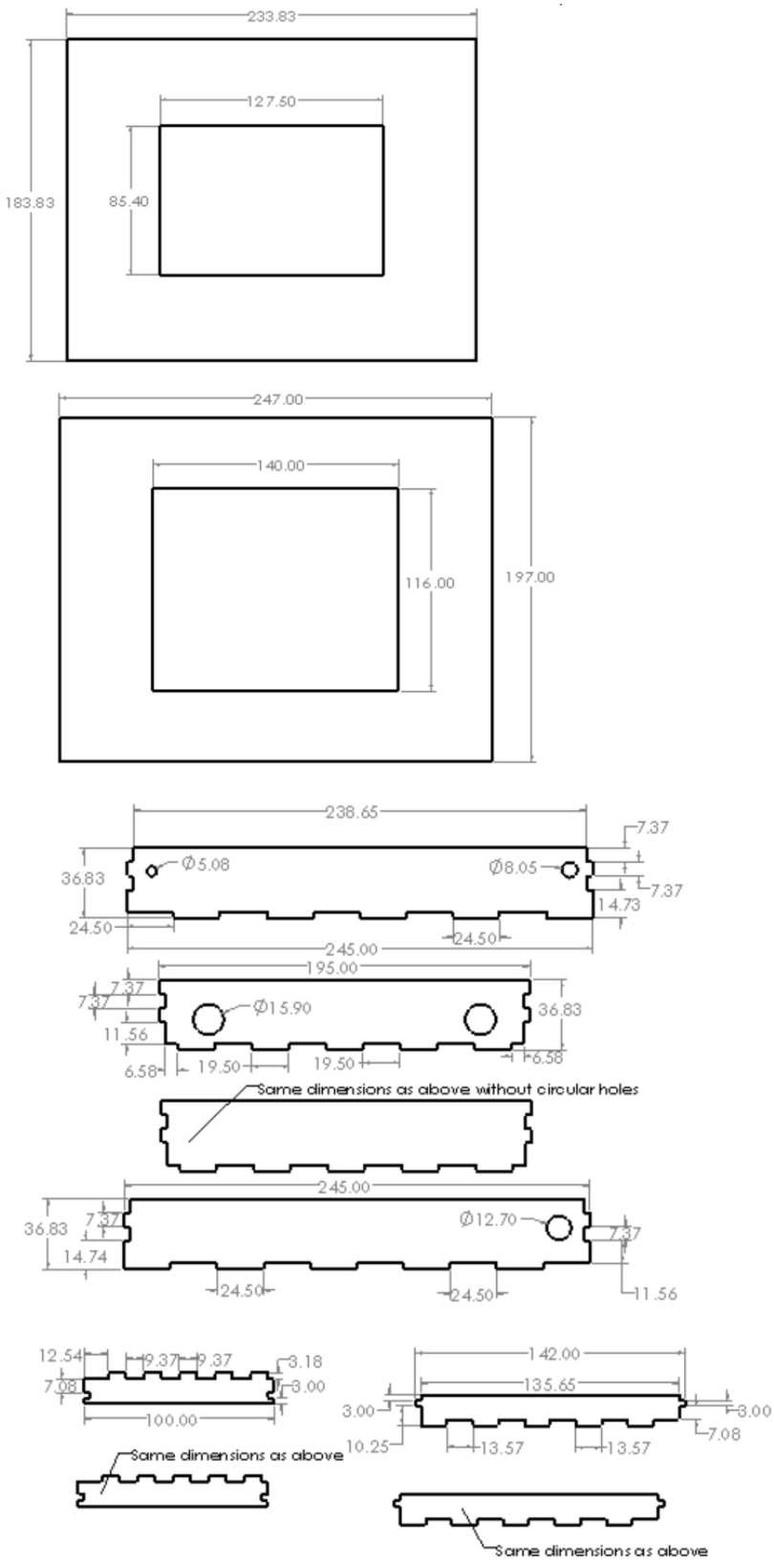
1) Open lid to expose well plate cavity
2) Insert a 138mm x 95mm or smaller well plate into designated cavity
    a) DO NOT use a well plate larger than dimensions given
3) Replace lid back onto incubator
    a) Make sure seal is firmly in place
    b) DO NOT open until data acquisition is complete and sample isn't required anymore (will compromise internal environment otherwise)

Data Acquisition

1) Connect Arduino Microcontroller to a power source
2) Set up sensors to collect internal environment data
3) Upload designated code on Arduino IDE to print live internal environmental data
4) Record any desired values given by data

Cleaning and Disassembly

1) Make sure all power sources are disconnected
2) Empty DI water from inside
3) Remove external tubing from incubator
4) Use ethanol to disinfect the inside of the incubator
    a) DO NOT use an autoclave because of the low melting points of the materials being used


233.83
127.50
85.40
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247.00
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116.00
197.00
238.65
7.37
36.83
Ø5.08
Ø8.05
7.37
14.73
24.50
24.50
245.00
195.00
7.37
7.37
Ø15.90
36.83
11.56
6.58
19.50
19.50
6.58
Same dimensions as above without circular holes
245.00
36.83
7.37
Ø12.70
7.37
14.74
24.50
24.50
11.56
12.54
9.37
9.37
3.18
7.08
3.00
100.00
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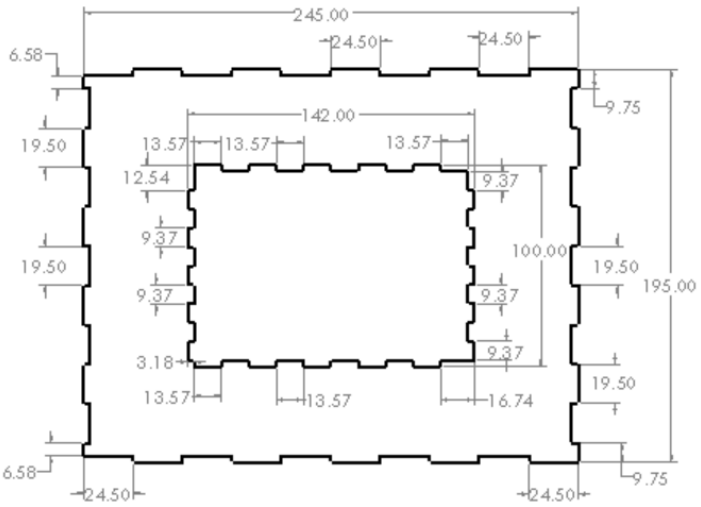
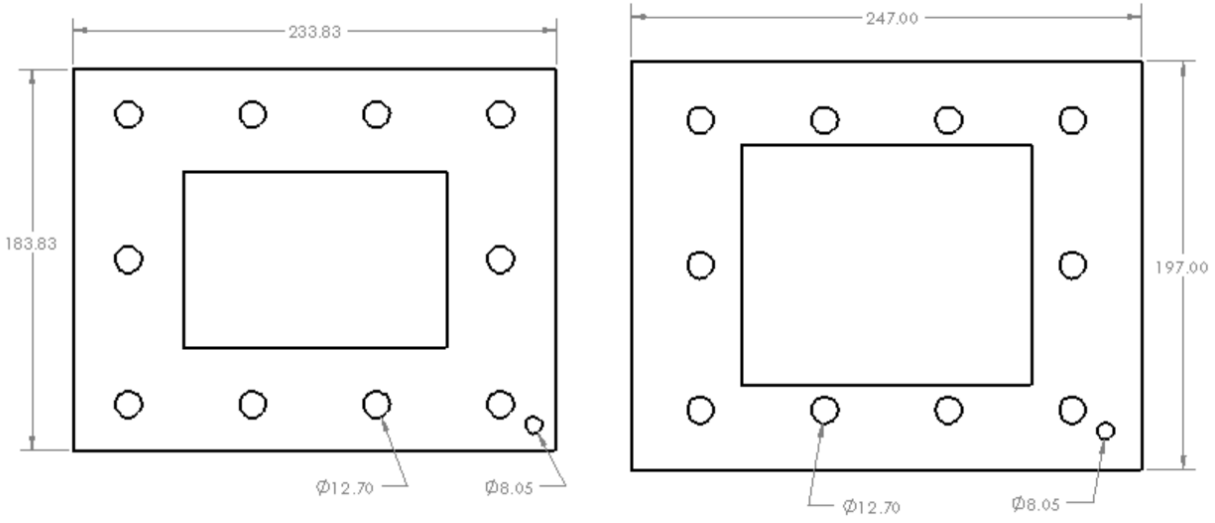
3.00
7.08
10.25
13.57
13.57
Same dimensions as above
Same dimensions as above



University of Wisconsin - Madison
Microscope Cell Culture Incubator
Drawn By: Sam Bardwell
Date: 4/11/2022

All Dimensions in millimeters
*All parts have a thickness of 3.175 mm

Homogeneity Testing Lid



Circuit Diagram and Code

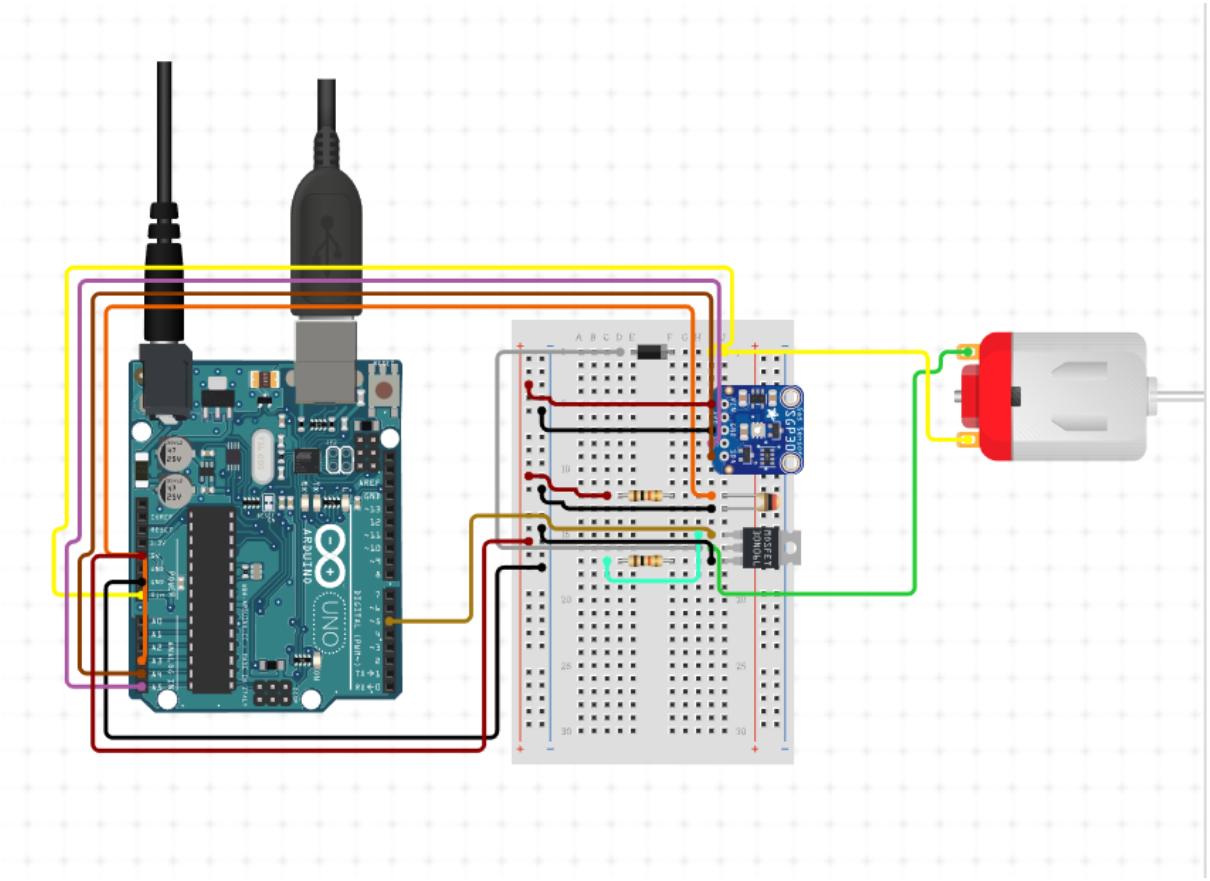


Figure 5: Complete Incubator Circuit Design

Arduino Code

```
//Combined Arduino Code for Temp, Hum, and CO2

//Concentration
#include <SoftwareSerial.h>
#include <NDIR_SoftwareSerial.h>

//Select 2 digital pins as SoftwareSerial's Rx and Tx. For example, Rx=2 Tx=3
NDIR_SoftwareSerial mySensor(2, 3);
double percent = mySensor.ppm/10000;

// temperature variables
int ThermistorPin = 0;
int Vo;
```

```
float R1 = 10000;
float logR2, R2, T, Tc, Tf;
float c1 = 1.009249522e-03, c2 = 2.378405444e-04, c3 = 2.019202697e-07;
float e_s;
float e_d;
float Td = 36.1;

//DC motor variables
const int pwm = 4;
const int in_1 = 8;
const int in_2 = 9 ;
//For providing logic to L298 IC to choose the direction of the DC motor

void setup()
{
   Serial.begin(9600);

   if (mySensor.begin()) {
      Serial.println("Wait 10 seconds for sensor initialization...");
       delay(10000);
   } else {
      Serial.println("ERROR: Failed to connect to the sensor.");
       while(1);
   }
  pinMode(pwm,OUTPUT) ; //we have to set PWM pin as output
  pinMode(in_1,OUTPUT) ; //Logic pins are also set as output
  pinMode(in_2,OUTPUT) ;

}

void loop() {
//  Temperature
  Vo = analogRead(ThermistorPin);
  R2 = R1 * (1023.0 / (float)Vo - 1.0);
  logR2 = log(R2);
  T = (1.0 / (c1 + c2*logR2 + c3*logR2*logR2*logR2));
  Tc = T - 271.15;
  Tf = (Tc * 9.0)/ 5.0 + 32.0;
  float hum =0;
  e_s = 6.11 * pow(10, ((7.5 * Tc)/(237.7 + Tc)));
```

```
 e_d = 6.11 * pow(10, ((7.5 * Td)/(237.7 + Td)));
 hum = (e_d/e_s)*100;
Serial.print("Temperature: ");
Serial.print(Tf);
Serial.print(" F; ");
Serial.print(Tc);
Serial.println(" C");
Serial.print("Relative Humidity: ");
Serial.print(hum);
Serial.println("%");
delay(1000);

//Concentration
  if (mySensor.measure()) {
     Serial.print("CO2 Concentration is ");
     Serial.println(mySensor.ppm);
     Serial.println("ppm");
     Serial.print("CO2 Percentage is ");
     Serial.print((mySensor.ppm/10000));
     Serial.println("%");

   } else {
      Serial.println("Sensor communication error.");
   }
  delay(1000);
//DC Motor
 if (mySensor.ppm < 60000){
  //For Clockwise motion , in_1 = High , in_2 = Low
  digitalWrite(in_1,HIGH) ;
  digitalWrite(in_2,LOW) ;
  analogWrite(pwm,255) ;
  /* setting pwm of the motor to 255 we can change the speed of rotation
  by changing pwm input but we are only using arduino so we are using highest
  value to driver the motor */
 }
 if (mySensor.ppm > 60000){
  //For Anti Clock-wise motion - IN_1 = LOW , IN_2 = HIGH
  digitalWrite(in_1,LOW) ;
  digitalWrite(in_2,HIGH) ;
 }else{
```

```
  //For brake
   digitalWrite(in_1,HIGH) ;
   digitalWrite(in_2,HIGH) ;
  }
}
```

Results from Spring 2022

The thermistor was tested via Temperature and Humidity Sensor Test Protocol[8] with the following graphs showing the results based on the sensor's initial reading ability in the lab incubator in ECB Room 1002.

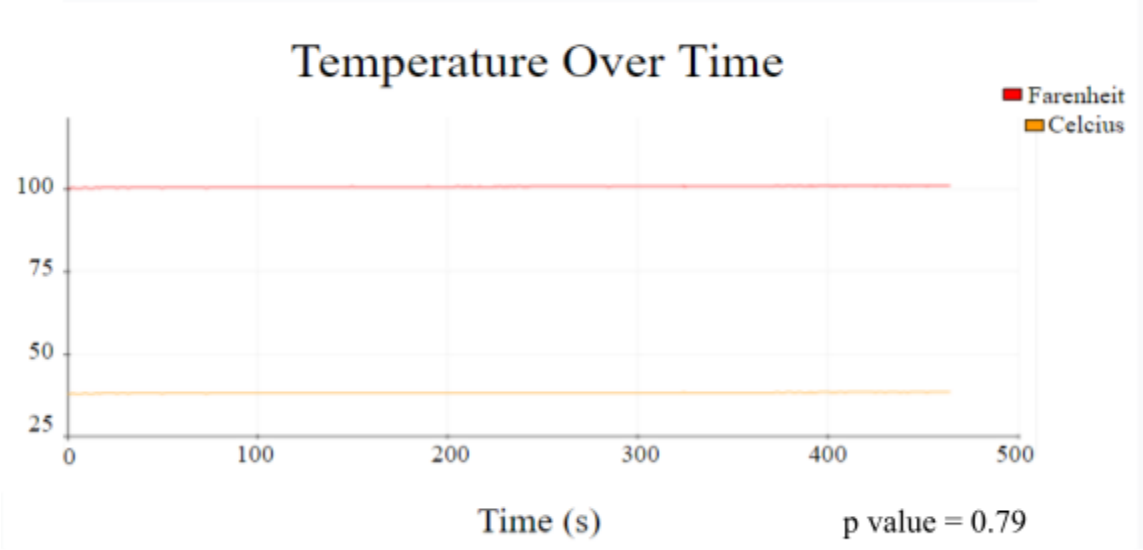


Figure 6: Thermistor Temperature over 10 minute Interval in Lab Incubator

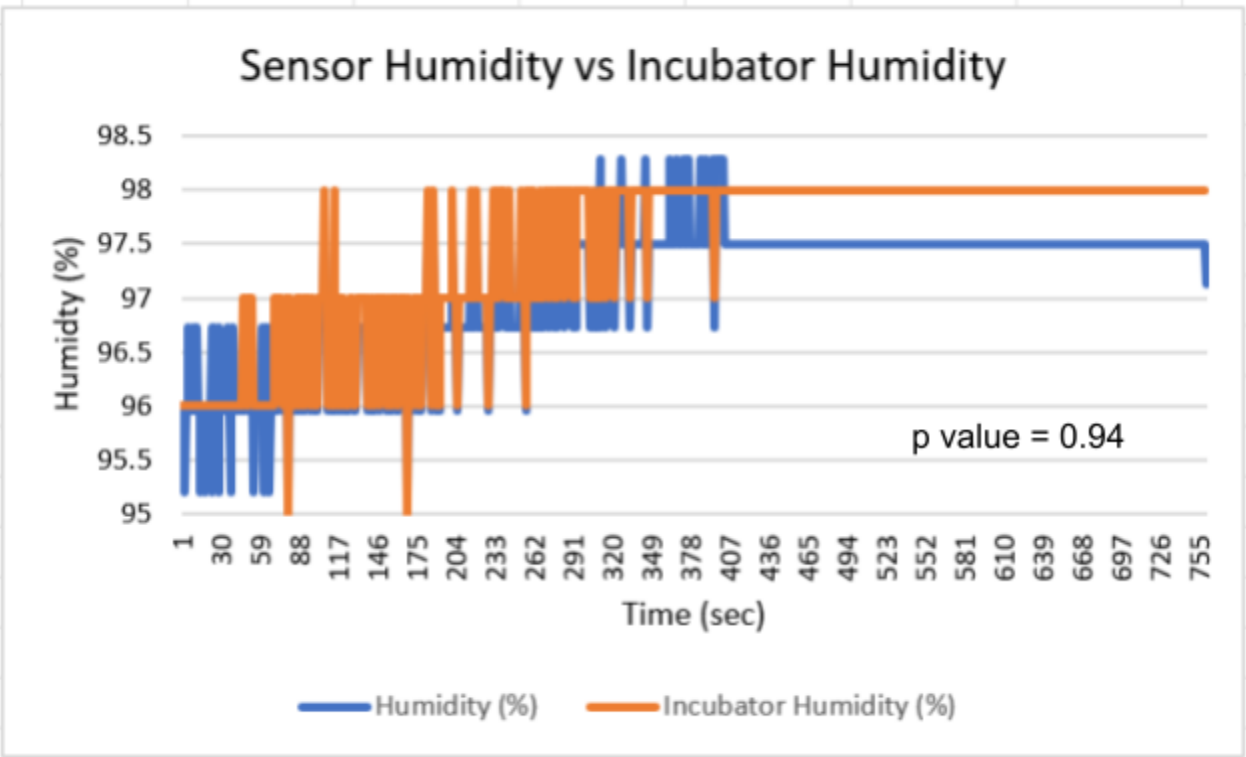


Figure 7: Graph of Humidity Readings in Incubator Over 10 min Time Interval

[8] See Appendix C

The NDIR CO_2 sensor's ability to read the concentration of CO_2 in the ECB 1002 lab incubator was tested.

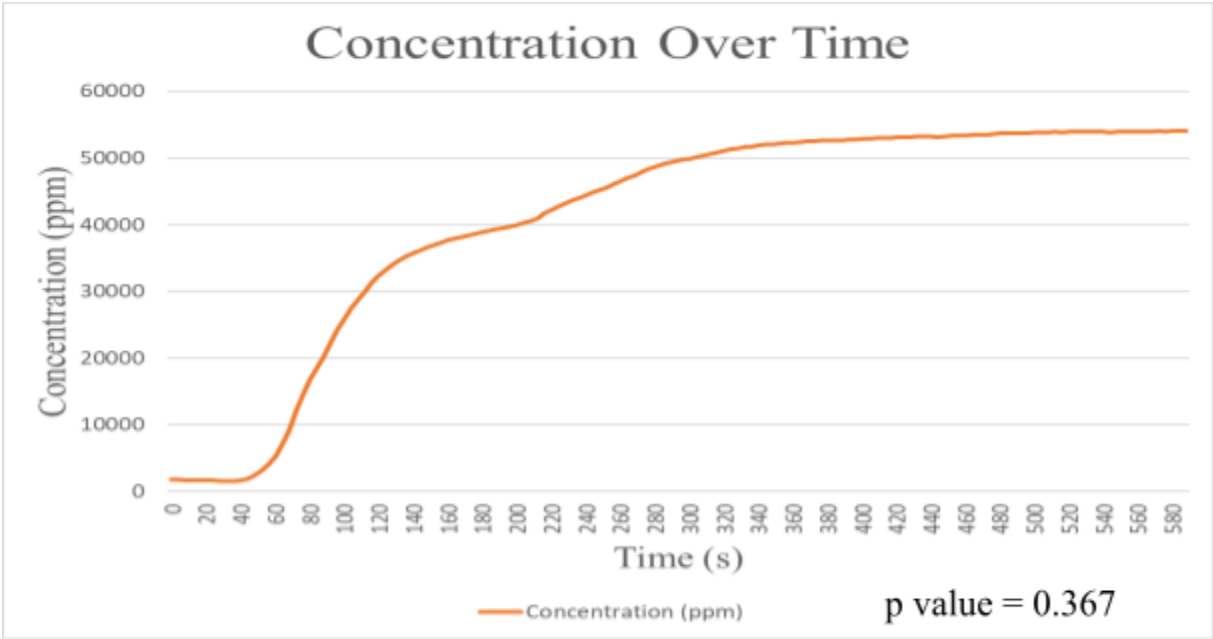


Figure 8: Concentration of CO_2 in Lab Incubator Over approximately 10 minutes

Temperature and Humidity Data in Incubator over 10 minute intervals.

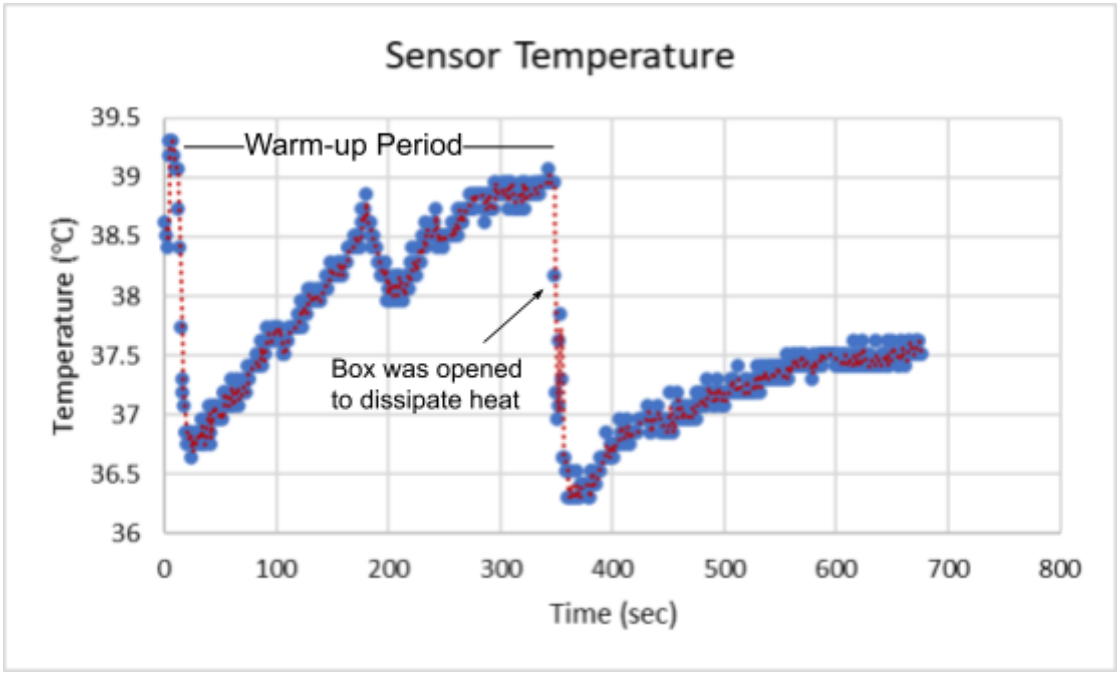


Figure 9: Sensor Temperature in Incubator over 10 minutes

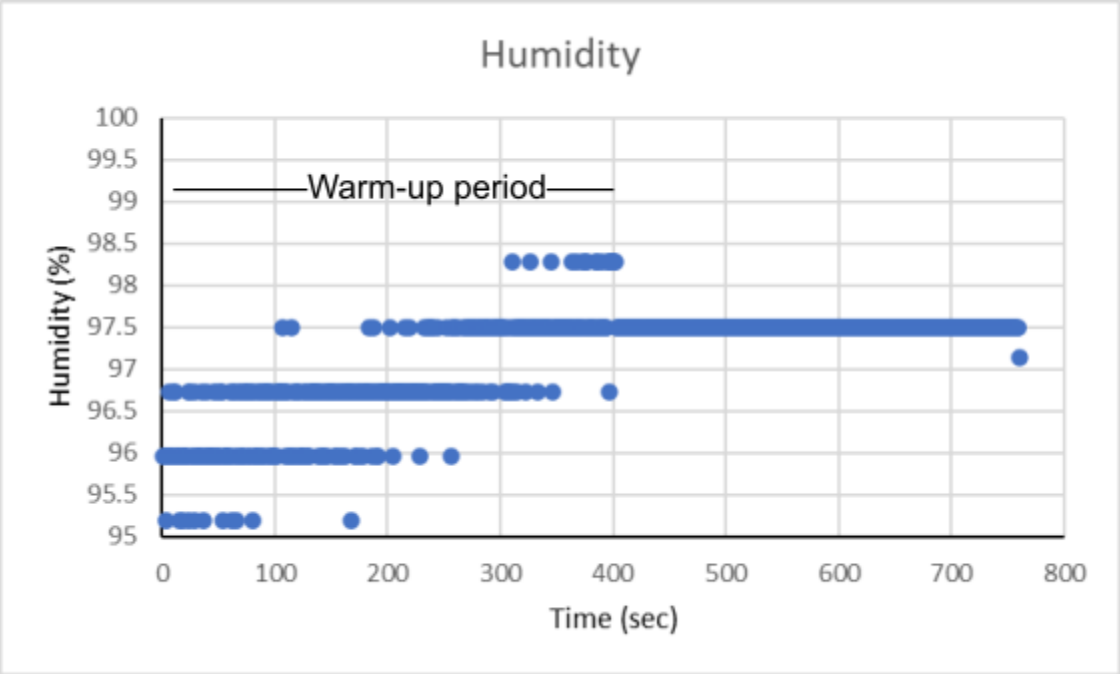


Figure 10: Sensor Humidity in the Incubator over 10 minutes

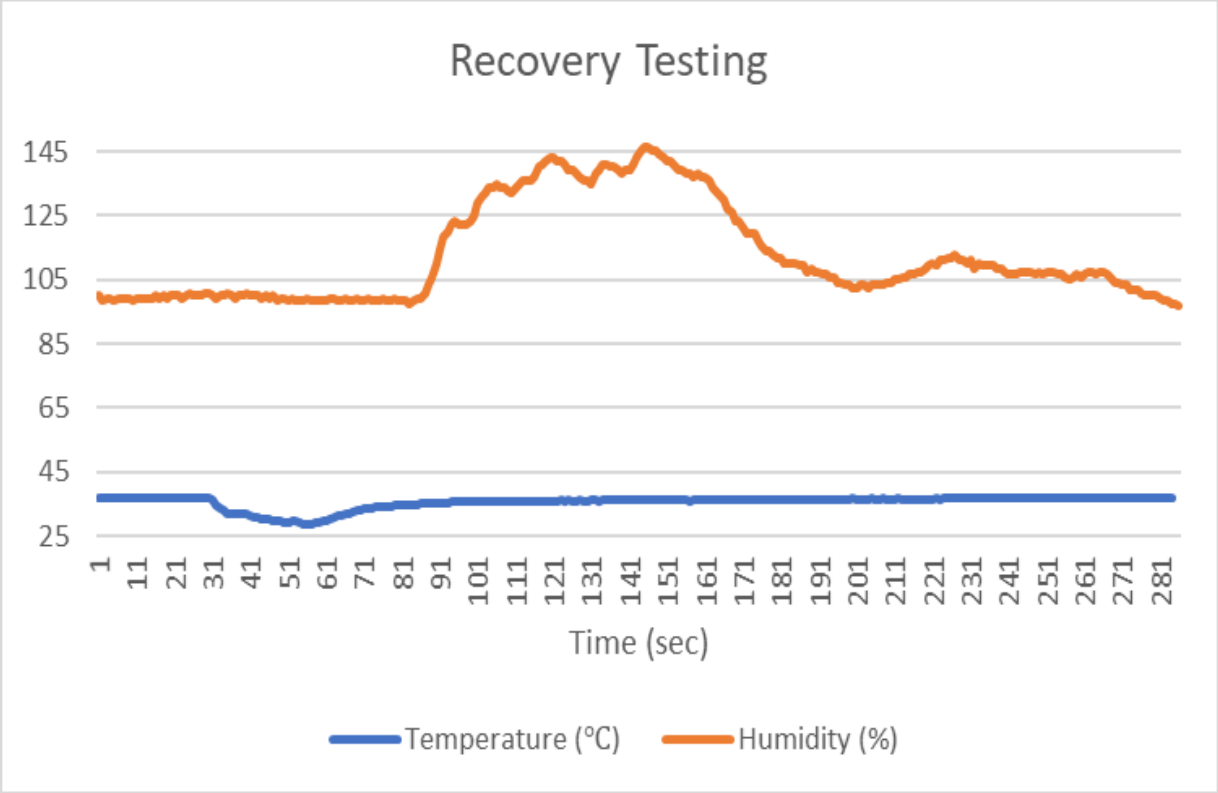


Figure 11: Temperature and Humidity Recovery Testing Data

Appendix C: Testing Protocols

Internal Environment - Temperature and Humidity Sensor Test Protocol

Introduction

Name of Tester:
Dates of Test Performance:
Site of Test Performance:

Explanation:

The team will be employing a sensor inside the incubator in order to measure the internal temperature. The measurements of the humidity and temperature will be obtained by an AOSONG DHT22 Arduino-compatible sensor. The team will test to make sure that the code and the AOSONG are working correctly by calibrating the sensor and then confirming its accuracy at a steady state and precision in a dynamic range using a thermometer. To calibrate the sensor, the team will use resistance values on the Arduino Website. Once the sensor is calibrated, its accuracy will be tested by first measuring the temperature and humidity of the working environment to gauge if they are both working as expected, and then measuring its temperature at extremely high and low temperatures. Afterward, the team will measure the temperature inside the incubator with a thermometer and the sensor. To keep the incubator completely sealed, the thermometer probe and reading display will be inserted into the incubator and read through the glass. The tests will be considered successful if the sensor value is within 2℃ of the thermometer temperature.

Steps	**Protocol**	**Verification**	**Pass/Fail**	**Initials of Tester**
1	Calibrate the sensor using resistance values on Arduino Website.	☐ Verified Comments:		
2	Test the precision of the Arduino microcontroller at extremely high and low temperatures. Heat a cup of water in a microwave for two minutes. Place the sensor in the cup of hot water and ensure the temperature outputs increase the longer it is under heat. Then, place the sensor in the freezer and ensure the temperature outputs decrease the longer it is under there. If the sensor follows these trends, it is verified.	☐ Verified Comments:		
3	Set up the incubator for normal use. Set up a digital thermometer within the system.	☐ Verified Comments:		
4	Set up the Arduino sensor and	☐ Verified		

	incorporated the breadboard circuits.	Comments:		
5	Record the average temperature of the system from the thermometer in the comments, taking measurements every 10 seconds over a period of 10 minutes. Verify that this temperature falls within the optimal range of 37 ℃ ± 2 ℃. **If the thermometer does not seem calibrated correctly, try first measuring the temperature of room temperature water (approximately 25 ℃).	☐ Verified Comments:		
6	Record the average temperature of the system from the Arduino microcontroller in the comments, taking measurements every 10 seconds over a period of 10 minutes. Verify that this temperature falls within ± 2 ℃ of the temperature read by the thermometer.	☐ Verified Comments:		
7	Record the average humidity percentage from the Arduino microcontroller in the comments, taking measurements every 10 seconds over a period of 10 minutes, and verify that this value falls between 95-100%.	☐ Verified Comments:		

Internal Environment - CO_2 Sensor & Feedback System Test Protocol

Introduction

Name of Tester:
Dates of Test Performance:
Site of Test Performance:

Explanation:

The team will be employing sensors inside the incubator in order to measure the internal CO_2. For CO_2, the tank employed in the current lab has a sensor to check the CO_2 levels, but a CO_2 sensor will be placed inside the incubator as well. The measurement of CO_2 recorded by the Arduino sensors should be within 2% of the pressure gauge on the CO_2 tank.

Steps	**Protocol**	**Verification**	**Pass/Fail**	**Initials of Tester**
1	Test the precision of the sensor by ensuring its values increase and decrease with a general increase and decrease of CO_2 concentration. Place the sensor in front of the CO_2 tank dispenser tube. Allow gas to exit the tank at a low flow rate. Ensure the sensor value readings increase as the sensor exposure to CO_2 gas increases. If this occurs, this step is verified.	☐ Verified Comments:		
2	Similarly, once the CO_2 supply from the tank is turned off, ensure the value readings from the sensor decrease. If this occurs, this step is verified.	☐ Verified Comments:		
3	Set up the incubator for normal use. Record the value read by the fyrite at room conditions in the comments.	☐ Verified Comments:		
4	Set up the CO_2 sensor and fyrite within the incubator and seal it. Allow enough CO_2 to enter the incubator that the fyrite reads around 5% CO_2. Record the value given by the fyrite, the value given by the CO_2 sensor, and the trial number in the comments.	☐ Verified Comments:		
5	Remove the incubator from under the microscope and allow the CO_2 to leave the system so that its value read by the fyrite is nearly the same as room	☐ Verified Comments:		

	conditions. Repeat steps 5-4 until 5 trials are complete. Record the mean value of difference between the read CO_2 values in the comments.			
6	If the CO_2 sensor deviates from the actual CO_2 percentage by ±0.1% or less, then the sensor is verified for use. If not verified, record why in the comments.	☐ Verified Comments:		

Steps	**Protocol**	**Verification**	**Pass/Fail**	**Initials of Tester**
1	Once the CO_2 sensor is approved for use, set up the incubator for normal use with the CO_2 sensor inside. Seal the incubator.	☐ Verified Comments:		
2	Connect the CO_2 tank to the incubator fixed with a regulator and a solenoid.	☐ Verified Comments:		
3	Verify the sensor is recording values. Then, begin running feedback code in conjunction with the solenoid connected to the CO_2 tank.	☐ Verified Comments:		
4	The solenoid should let CO_2 into the system immediately. Once the CO_2 sensor reads a value within 5% ±0.1% CO_2 the solenoid should stop allowing CO_2 into the incubator. If this occurs, continue protocol and step is verified. If this does not occur, stop protocol and record what happened in the comments.	☐ Verified Comments:		
5	Allow the feedback loop to run for an hour. Record the sensor values read into a graph. Verify that over the hour the CO_2 percentage remained near a level of 5% CO_2 ±0.1%. If the CO_2 remained in this range, continue protocol and step is verified. If this did not occur, stop protocol and record what happened in the comments.	☐ Verified Comments:		
6	Repeat step 5 over the course of 6 hours. If the CO_2 remains in the	☐ Verified Comments:		

	necessary range, continue the protocol and this step is verified. If this did not occur, stop protocol and record what happened in the comments.			

Optical Testing - Prior to and After Installation

Introduction

Name of Tester:
Dates of Test Performance:
Site of Test Performance:

Explanation:

The team will test the High Transparent Lexan Polycarbonate sheets to determine if they interfere with the optics of the inverted microscope. The control will be an image taken of just the flask with cells inside and the test will be an image taken of the same flask with cells inside of the prototype incubator. Two images will be taken of each conditions and they will be analyzed using MATLAB to determine their Energy of Laplacian. The test is considered a success when the p-value > 0.05 between the control and test images.

Steps	**Protocol**	**Verification**	**Pass/Fail**	**Initials of Tester**
1	Control: place the flask with cells on the inverted microscope stage.	☐ Verified Comments:		
2	Adjust the optical components of the microscope to best clarity based on personal judgment. Ensure the resolution of the cells is as clear as possible. Take an image of what is observed under the microscope.	☐ Verified Comments:		
3	Move the flask so that a new spot of the flask is being imaged.	☐ Verified Comments:		
4	Repeat step 2 in the new location.	☐ Verified Comments:		
5	Test: Repeat steps 1-4 with the same flask placed inside the prototype incubator and place the prototype on the inverted microscope stage.	☐ Verified Comments:		
6	Upload all four images to MATLAB and calculate the Engery of Laplacian.	☐ Verified Comments:		
7	Calculate the average energy for each condition and then run a ttest to determine the p-value between the two conditions.	☐ Verified Comments:		

Recovery Test Protocol

Introduction

Name of Tester:
Dates of Test Performance:
Site of Test Performance:

Explanation:

The team will test the recovery time of the incubator after it has been opened by timing how long it takes for the incubator to return to performance conditions (37°C, 5% CO_2, and >95% humidity). The maximum recovery time should not exceed five minutes after a 30-second exposure to the external environment.

Steps	**Protocol**	**Verification**	**Pass/Fail**	**Tester Initials**
1	Set up the incubator for normal use. Record internal conditions in the comments and verify that they fall within the correct ranges (37°C, 5% CO_2, and >95% humidity).	☐ Verified Comments:		
2	Open the incubator for 30 seconds. Start stopwatch. Verify that the stopwatch is working.	☐ Verified Comments:		
3	Record internal conditions in the comments at a time of 15 seconds after opening the incubator. Verify that the internal conditions deviate from the normal conditions recorded above.	☐ Verified Comments:		
4	Close the incubator. Verify that the recovery time did not exceed 5 minutes after a 30 second exposure to the external environment. Record the time it took to revert back to optimal conditions in the comments.	☐ Verified Comments:		

Cell Confluency Test Protocol

Introduction

Name of Tester:
Dates of Test Performance:
Site of Test Performance:

Explanation:

The team employed image J to quantify the percentage of area covered by the cells over time in order to quantify the cell proliferation. This allowed the team to compare cell proliferation in the standard incubator compared to the prototype. The images of the cells were taken using the Zeiss scope in the teaching lab. The control was a T25 flask that was cultured in the standard incubator to provide a baseline on appropriate cell death over the course of a week. Another T25 flask was cultured inside the prototype over the course of a week. Using the Zeiss scope, an image was taken every 24 hours, and those images were loaded into image J. The team was able to quantify the percent of cell coverage and track cell confluency over the course of the week. Tests will be considered successful if there is no significant difference between the confluency between the control and the prototype.

Steps	**Protocol**	**Verification**	**Pass/Fail**	**Initials of Tester**
1	Day 0: Using the cell flask fron the control incubator, change the cell media, and image a section. Analyze image in ImageJ to determine percent area coverage. Return flask to the control incubator after each imaging.	☐ Verified Comments:		
2	Day 1-6: Repeat step 1 every 24 hours for 6 days for the flask in the control incubator.	☐ Verified Comments:		
3	Day 0: Using the cell flask fron the prototype incubator, change the cell media, and image a section. Analyze image in ImageJ to determine percent area coverage. Return flask to the prototype incubator after each imaging	☐ Verified Comments:		
4	Day 1-6: Repeat step 1 every 24 hours for 6 days for the flask in the prototype incubator.	☐ Verified Comments:		
5	Plot both the control and the test percent area coverage vs time to determine if they are statistically similar.	☐ Verified Comments:		

ImageJ Percent Area Coverage Procedure

Introduction

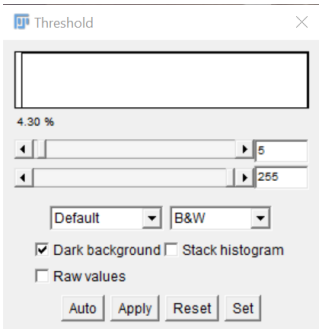
Name of Tester:
Dates of Test Performance:
Site of Test Performance:

Explanation:

The team will be using ImageJ to analyze the images taken of the cells using the Zeiss microscope. ImageJ will calculate the percentage of surface area covered by the cells and from this, a trend line can be made that tracks the cell proliferation over the course of 5 days.

Procedure:

1. Take images of the cells, ideally in the same spot each time
2. Open ImageJ and open the file you just created (file type does not matter)
3. Image → Type → 8-bit; this will turn the image into an 8-bit greyscale
4. Process → Subtract Background; this will remove the background of the image making the cells more visible
    a. Adjust the rolling ball radius until the optimal contrast between the background and cells is achieved (somewhere between 60-150 pixels)
5. Process → Filters → Median; this will reduce noise and sharpen cell selection (a radius between 2 and 5 is usually acceptable)
6. Image → Adjust → Threshold; this will select only the cells
    a. Move the bottom line all the way to the right and adjust the top line until only the cells are white



7. Analyze → Set Measurements → Check Area Fraction; this will calculate the percentage area coverage of a selected image
8. Using the mouse to select the entire image, creating an ROI
9. Analyze → Tool → ROI Manager → Add → Click coordinates → Measure; this will provide you with an output box that has the percent area coverage calculated

Homogeneity Test Protocol

Introduction

Name of Tester:
Dates of Test Performance:
Site of Test Performance:

Explanation:

The team will test the homogeneity of each of the internal conditions throughout the system to ensure that performance conditions are met in a uniform manner. The goal is for each of the conditions to maintain their homeostatic values throughout the incubator: 37°C, 5% CO_2, and >95% humidity.

Steps	**Protocol**	**Verification**	**Pass/Fail**	**Tester Initials**
1	Obtain a lid with the same dimensions as the top of the incubator and ensure that there are holes throughout the frame of the lid.	☐ Verified Comments:		
2	Place probes/sensors for temperature into each hole and record its value.	☐ Verified Comments:		
3	Calculate and report the precision result (mean ∓ average deviation). Verify that all of the values for temperature fall within this precise range (ensures uniformity).	☐ Verified Comments:		
4	Repeat steps 1-3 for the humidity component.	☐ Verified Comments:		
5	Repeat steps 1-3 for the CO_2 component.	☐ Verified Comments:		

Anti Fog Application Test Protocol

Introduction

Name of Tester:
Dates of Test Performance:
Site of Test Performance:

Explanation:

The team will test how often the anti-fog solution must be applied to the system in order to prevent condensation and visibility issues through the glass. The goal is for this anti-fog solution to keep the system's visibility as clear as possible for an extended period of time, but this will be extensively tested through this protocol.

Steps	**Protocol**	**Verification**	**Pass/Fail**	**Tester Initials**
1	Apply 4 pumps of the lens cleaning solution onto a paper towel or wipe and wipe down all glass surfaces within the system.	☐ Verified Comments:		
2	Record the time and date that the solution is administered.	☐ Verified Comments:		
3	Check this system to ensure that there is no condensation or fog on the glass surfaces after 1 hour.	☐ Verified Comments:		
4	Record the time and date that any fog starts to appear on the glass surfaces.	☐ Verified Comments:		
5	Repeat steps 1-4 three times in order to ensure three different trials are conducted.	☐ Verified Comments:		

Appendix D: Circuitry and Code

Solenoid Valve

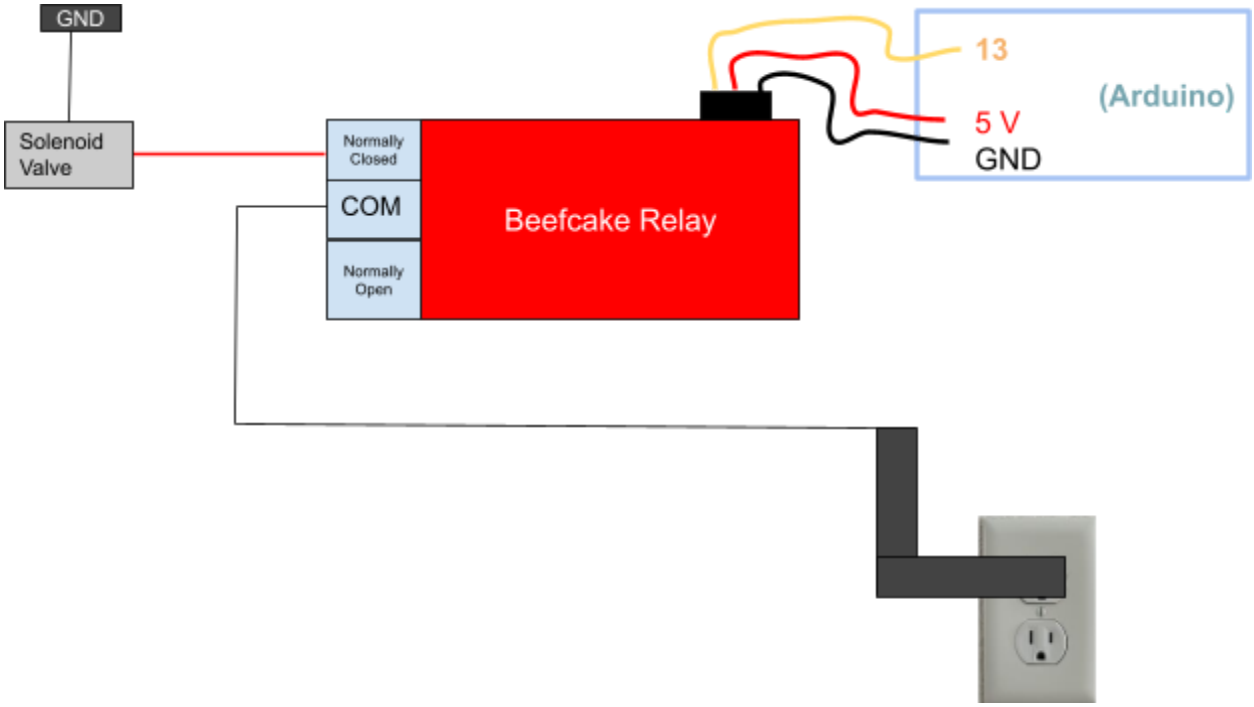
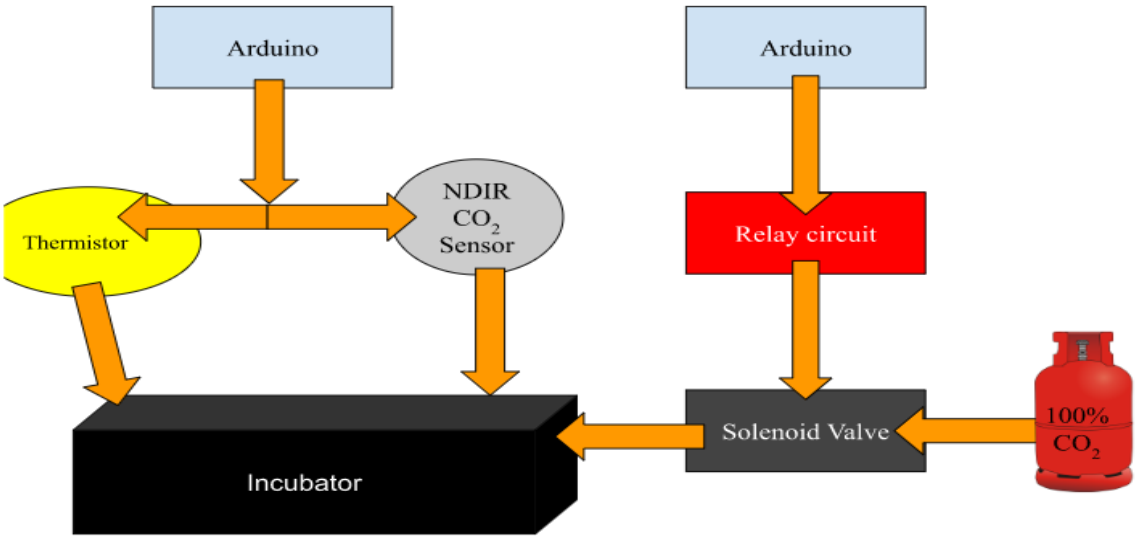


Figure 1: Solenoid Valve Circuitry

Arduino Code

```
#include <SoftwareSerial.h>

int solenoidPin = 4; //Output pin
int relayPin = 13;
int x = 0;

void setup() {
  Serial.begin(9600);
  pinMode(solenoidPin, OUTPUT);    //sets the pin as an output
  pinMode(relayPin, OUTPUT);
}

void loop() {
  int y=x++;
  Serial.println(y);
  if(y <= 1){
    digitalWrite(relayPin, HIGH); //switch relay on
    digitalWrite(solenoidPin, HIGH); //switch solenoid on
```

```
   delay(260);
   digitalWrite(relayPin, LOW); //switch relay off
   digitalWrite(solenoidPin, LOW); //switch solenoid off
   delay(72000);  // wait 1.2 minutes
 }if(y >=2){
    digitalWrite(relayPin, HIGH); //switch relay on
   digitalWrite(solenoidPin, HIGH); //switch solenoid on
   delay(30);
   digitalWrite(relayPin, LOW); //switch relay off
   digitalWrite(solenoidPin, LOW); //switch solenoid off
   delay(72000);  // wait 1.2 minutes
   }

}
```



Arduino Code

Sensors

```
#include <Wire.h>
#include <NDIR_I2C.h>

NDIR_I2C mySensor(0x4D); //Adaptor's I2C address (7-bit, default: 0x4D)

//Temperature
int ThermistorPin = 0;
int Vo;
float R1 = 10000;
float logR2, R2, T, Tc, Tf;
float c1 = 1.009249522e-03, c2 = 2.378405444e-04, c3 = 2.019202697e-07;
```

```
double e_s;

void setup()
{
   Serial.begin(9600);

   if (mySensor.begin()) {
      Serial.println("Wait 10 seconds for sensor initialization...");
      delay(10000);
   } else {
      Serial.println("ERROR: Failed to connect to the sensor.");
      while(1);
   }
}

void loop() {
  //Temperature
  Vo = analogRead(ThermistorPin);
  R2 = R1 * (1023.0 / (float)Vo - 1.0);
  logR2 = log(R2);
  T = (1.0 / (c1 + c2*logR2 + c3*logR2*logR2*logR2));
  Tc = T - 271.15;
  Tf = (Tc * 9.0)/ 5.0 + 32.0;
  float hum =0;
  e_s = 6.11 * pow(10, ((7.5 * Tc)/(237.7 + Tc)));

  hum = pow(10, (((20.85 *e_s) - (9.99*pow(log(e_s), 2))/ ((9.99*log(e_s)) - 7.5))));    //rel
humidity
 Serial.print("Temperature: ");
 //Serial.print(Tf);
 //Serial.print(" F; ");
 Serial.print(Tc);
 Serial.println(" C");
 Serial.print("Relative Humidity: ");
 Serial.print(hum - 42.25);
 Serial.println("%");
 delay(1000);

 //Concentration
   if (mySensor.measure()) {
```

```
      Serial.print("CO2 Concentration is ");
      Serial.print(mySensor.ppm-(1.38*10000));
      Serial.println("ppm");
      Serial.print("CO2 Percent is ");
      Serial.print((mySensor.ppm-(1.38*10000))/10000);
      Serial.println("%");
   } else {
      Serial.println("Sensor communication error.");
   }

   delay(1000);
}
```

Solenoid Valve Hard Code

```
#include <SoftwareSerial.h>

int solenoidPin = 4; //Output pin
int relayPin = 13;
int x = 0;

void setup() {
  Serial.begin(9600);
  pinMode(solenoidPin, OUTPUT);    //sets the pin as an output
  pinMode(relayPin, OUTPUT);
}

void loop() {
  int y=x++;
  Serial.println(y);
  if(y <= 1){
    digitalWrite(relayPin, HIGH); //switch relay on
    digitalWrite(solenoidPin, HIGH); //switch solenoid on
    delay(260);
    digitalWrite(relayPin, LOW); //switch relay off
    digitalWrite(solenoidPin, LOW); //switch solenoid off
    delay(72000);  // wait 1.2 minutes
  }if(y >=2){
     digitalWrite(relayPin, HIGH); //switch relay on
    digitalWrite(solenoidPin, HIGH); //switch solenoid on
    delay(30);
```

```
    digitalWrite(relayPin, LOW); //switch relay off
    digitalWrite(solenoidPin, LOW); //switch solenoid off
    delay(72000);  // wait 1.2 minutes
    }

}
```

Matlab Code for Image Analysis

Optical Test Image Analysis:

```
% Image Upload
c1 = rgb2gray(imread("optical_testing_control_1.bmp","bmp"));
c2 = rgb2gray(imread("optical_testing_control_2.bmp","bmp"));
p1 = rgb2gray(imread("optic_testing_prototype_1.bmp","bmp"));
p2 = rgb2gray(imread("optic_testing_prototype_2.bmp","bmp"));
%case 'LAPE' % Energy of laplacian (Subbarao92a)
LAP = fspecial('laplacian');
energy_c1 = mean2(imfilter(c1, LAP, 'replicate', 'conv').^2)
energy_c1 = 12.0420
energy_c2 = mean2(imfilter(c2, LAP, 'replicate', 'conv').^2)
energy_c2 = 11.8724
energy_p1 = mean2(imfilter(p1, LAP, 'replicate', 'conv').^2)
energy_p1 = 11.0131
energy_p2 = mean2(imfilter(p2, LAP, 'replicate', 'conv').^2)
energy_p2 = 12.5551
% P value
control = [energy_c1, energy_c2];
prototype = [energy_p1, energy_p2];
[h,p] = ttest2(control,prototype)
h = 0
p = 0.8442
```

Anti-fog Test Image Analysis:

```
Trial 1
% Image Upload
t1_c1 = rgb2gray(imread("t1_control_1.bmp","bmp"));
t1_c2 = rgb2gray(imread("t1_control_2.bmp","bmp"));
t1_t0 = rgb2gray(imread("t1_time_0.bmp","bmp"));
t1_t10 = rgb2gray(imread("t1_time_10.bmp","bmp"));
t1_t20 = rgb2gray(imread("t1_time_20.bmp","bmp"));
t1_t30 = rgb2gray(imread("t1_time_30.bmp","bmp"));
t1_t40 = rgb2gray(imread("t1_time_40.bmp","bmp"));
t1_t50 = rgb2gray(imread("t1_time_50.bmp","bmp"));
t1_t60 = rgb2gray(imread("t1_time_60.bmp","bmp"));
%case 'LAPE' % Energy of laplacian (Subbarao92a)
LAP = fspecial('laplacian');
energy_1c1 = mean2(imfilter(t1_c1, LAP, 'replicate', 'conv').^2);
```

```
energy_1c2 = mean2(imfilter(t1_c2, LAP, 'replicate', 'conv').^2);
avg_1c = mean([energy_1c1, energy_1c2])
avg_1c = 14.7851
energy_1t0 = mean2(imfilter(t1_t0, LAP, 'replicate', 'conv').^2)
energy_1t0 = 14.4806
energy_1t10 = mean2(imfilter(t1_t10, LAP, 'replicate', 'conv').^2)
energy_1t10 = 12.1429
energy_1t20 = mean2(imfilter(t1_t20, LAP, 'replicate', 'conv').^2)
energy_1t20 = 10.8411
energy_1t30 = mean2(imfilter(t1_t30, LAP, 'replicate', 'conv').^2)
energy_1t30 = 11.9800
energy_1t40 = mean2(imfilter(t1_t40, LAP, 'replicate', 'conv').^2)
energy_1t40 = 11.8004
energy_1t50 = mean2(imfilter(t1_t50, LAP, 'replicate', 'conv').^2)
energy_1t50 = 13.1720
energy_1t60 = mean2(imfilter(t1_t60, LAP, 'replicate', 'conv').^2)
energy_1t60 = 12.5301
```

Trial 2

```
% Image Upload
t2_c1 = rgb2gray(imread("t2_control 1.bmp","bmp"));
t2_c2 = rgb2gray(imread("t2_control 2.bmp","bmp"));
t2_t0 = rgb2gray(imread("t2_time 0.bmp","bmp"));
t2_t10 = rgb2gray(imread("t2_time 10.bmp","bmp"));
t2_t20 = rgb2gray(imread("t2_time 20.bmp","bmp"));
t2_t30 = rgb2gray(imread("t2_time 30.bmp","bmp"));
t2_t40 = rgb2gray(imread("t2_time 40.bmp","bmp"));
t2_t50 = rgb2gray(imread("t2_time 50.bmp","bmp"));
t2_t60 = rgb2gray(imread("t2_time 60.bmp","bmp"));
%case 'LAPE' % Energy of laplacian (Subbarao92a)
LAP = fspecial('laplacian');
energy_2c1 = mean2(imfilter(t2_c1, LAP, 'replicate', 'conv').^2);
energy_2c2 = mean2(imfilter(t2_c2, LAP, 'replicate', 'conv').^2);
avg_2c = mean([energy_2c1, energy_2c2])
avg_2c = 12.1885
energy_2t0 = mean2(imfilter(t2_t0, LAP, 'replicate', 'conv').^2)
energy_2t0 = 11.9962
energy_2t10 = mean2(imfilter(t2_t10, LAP, 'replicate', 'conv').^2)
energy_2t10 = 10.5021
energy_2t20 = mean2(imfilter(t2_t20, LAP, 'replicate', 'conv').^2)
energy_2t20 = 10.8060
```

```
energy_2t30 = mean2(imfilter(t2_t30, LAP, 'replicate', 'conv').^2)
energy_2t30 = 10.0124
energy_2t40 = mean2(imfilter(t2_t40, LAP, 'replicate', 'conv').^2)
energy_2t40 = 10.5313
energy_2t50 = mean2(imfilter(t2_t50, LAP, 'replicate', 'conv').^2)
energy_2t50 = 10.4311
energy_2t60 = mean2(imfilter(t2_t60, LAP, 'replicate', 'conv').^2)
energy_2t60 = 10.7947
```

Trial 3

```
% Image Upload
t3_c1 = rgb2gray(imread("control 1.bmp","bmp"));
t3_c2 = rgb2gray(imread("control 2.bmp","bmp"));
t3_t0 = rgb2gray(imread("time 0.bmp","bmp"));
t3_t10 = rgb2gray(imread("time 10.bmp","bmp"));
t3_t20 = rgb2gray(imread("time 20.bmp","bmp"));
t3_t30 = rgb2gray(imread("time 30.bmp","bmp"));
t3_t40 = rgb2gray(imread("time 40.bmp","bmp"));
t3_t50 = rgb2gray(imread("time 50.bmp","bmp"));
t3_t60 = rgb2gray(imread("time 60.bmp","bmp"));
%case 'LAPE' % Energy of laplacian (Subbarao92a)
LAP = fspecial('laplacian');
energy_c1 = mean2(imfilter(t3_c1, LAP, 'replicate', 'conv').^2);
energy_c2 = mean2(imfilter(t3_c2, LAP, 'replicate', 'conv').^2);
avg_3c = mean([energy_c1,energy_c2])
avg_3c = 14.1112
energy_3t0 = mean2(imfilter(t3_t0, LAP, 'replicate', 'conv').^2)
energy_3t0 = 11.3502
energy_3t10 = mean2(imfilter(t3_t10, LAP, 'replicate', 'conv').^2)
energy_3t10 = 11.3311
energy_3t20 = mean2(imfilter(t3_t20, LAP, 'replicate', 'conv').^2)
energy_3t20 = 12.0343
energy_3t30 = mean2(imfilter(t3_t30, LAP, 'replicate', 'conv').^2)
energy_3t30 = 11.7195
energy_3t40 = mean2(imfilter(t3_t40, LAP, 'replicate', 'conv').^2)
energy_3t40 = 11.9534
energy_3t50 = mean2(imfilter(t3_t50, LAP, 'replicate', 'conv').^2)
energy_3t50 = 11.2258
energy_3t60 = mean2(imfilter(t3_t60, LAP, 'replicate', 'conv').^2)
energy_3t60 = 11.8699
```

Appendix E: Flow Rate Mathematical Calculations

Bernoulli's Equation

$$\left(v_2^2 - \cancel{v_1^2}^{0}\right) = \frac{2(P_1 - P_2)}{\rho}$$

$$v_2^2 = \frac{2(P_1 - P_2)}{\rho}$$

v = velocity
P = pressure
ρ = density of CO_2
V_2 = v_2 (Exit velocity)
P_1 = 16.5 psi (Entering Pressure)
P_2 = 14.76 psi (Exit pressure)
ρ = 0.1167 lb/ft^3

$P_1 - P_2$ = 1.74 psi

$$1.74\ psi \times \frac{144\ lbf/ft^2}{1\ psi} = 250.56\ lbf/ft^2$$

$$250.56\ lbf/ft^2 \times 32.174\ \frac{(lb \cdot ft/s^2)}{ft^2} = 8061.517\ lb/ft \cdot s^2$$

$$v_2 = \sqrt{\frac{2(8061.517\ lb/ft \cdot s^2)}{0.1167\ lb/ft^3}} = 371.696\ ft/s$$

Cross-Sectional Area of pipe

d = 2 mm

Convert to feet

$A = \pi r^2 = 3.38158e\text{-}5\ ft^2$

$v_2(A) = 371.696\ ft/s\ (3.38158e\text{-}5\ ft^2)$

$= .012569\ ft^3/s$

$$.012569\ ft^3/s \left(\frac{28316.8\ mL/s}{1\ ft^3/s}\right)$$

$= 355.919\ mL/s$

Percent Error

$$\left|\frac{335\ mL/s - 355.919\ mL/s}{355.919\ mL/s}\right| \cdot 100\%$$

$= 5.87\%$ Error

Appendix F: Expense Sheet

Expenses:

Item	Description	Manufacturer	Part Number	Date	QTY	Cost Each	Total	Link
Component 1								
Glass	Polycarbonate Transparent Thermal Insulation Sheets	RADNOR	64005034	9/21/22	4	$1.21	$4.84	Link
Component 2								
Solenoid Valve	¼ inch DC 12V 2 Way NC Electric Solenoid Air Valve	Plum Garden	PL-220101	9/21/22	1	$9.35	$9.35	Link
Component 3								
Anti-Fog Solution	Lens Cleaning Solution: Anti-fog/Anti-Static Silicone	Grainger	4T932	9/21/22	1	$6.58	$6.58	Link
Component 4								
G1/4” Soft Tubing Barbed Adaptor	Barbed Adaptors that screw into Solenoid Valve and attach plastic tubing connected to CO2 tank to valve, to incubator	E-outstanding	N/A	9/29/22	4	$2.40	$9.59	Link
Component 5								
TIP120 Transistor	Transistor needed to power the solenoid valve using an Arduino Circuit.	NTE Electronics, Inc	2368-TIP120-ND	10/3/22	1	$1.00	$1.00	Link
Component 6								

Black Acrylic	Black Acrylic needed to create the homogeneity testing lid. (⅛ x 18 x 24)	UW-Makerspace	N/A	10/17/22	1	$10.75	$10.75	Link
TOTAL:	$42.11							