Microscopic Cell Culture Incubator
Preliminary Report

BME 400 Design
October 12 2022

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Abstract

The team was tasked with creating and testing a cell culture incubator that will 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 is integrated into the incubator making it bulky and inconvenient to disassemble, or the incubator is expensive. The team is going to design a cost-effective cell culture incubator that will be portable and small enough to fit on the inverted microscope stage, allowing the user to view live cells inside the incubator. The incubator will include 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 will be conducted to find the optimal working environment for the incubator in order to ensure cellular viability and visibility.

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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 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 describes 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 are embryonic stem cells [1].

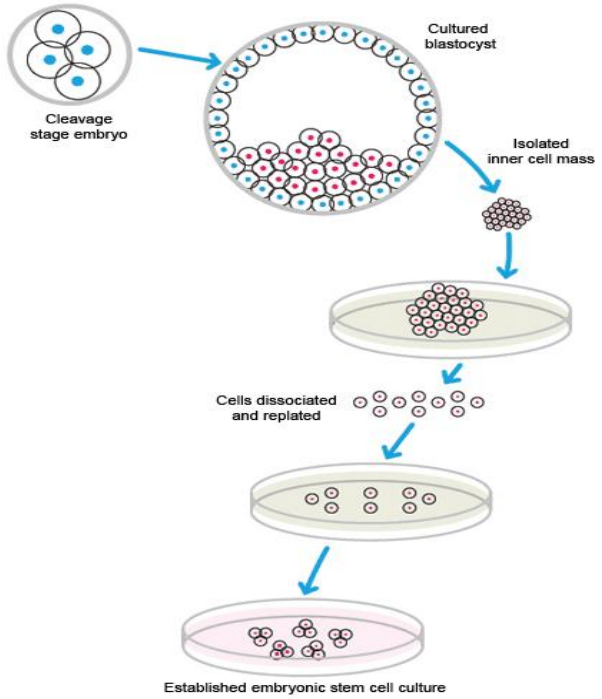

Figure 1: Isolation of Embryonic Stem Cell Lines[7]

Incubators used in cell cultures have to maintain a very 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. 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 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-COVID19 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 is 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 is 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 is 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 is defined as a consistent temperature throughout each section of the chamber. The incubator must fit on an inverted microscope stand (roughly 310 x 300 x 45mm) without interfering with the microscope's optics and functionality. The device must also be able to hold a standard well plate (127.55 x 85.4 x 22.5mm) without disrupting the integrity of the plate of cultures in the plate. The top and the bottom of the incubator must be transparent in order for imaging through the chamber. The aim for this project is to be able to make a device that is low-cost, easily assembled/disassembled, sterilized, and can be easily moved and stored between uses. The market for this product is teaching labs, but if more successful, it could be marketed towards other laboratories and pharmaceutical companies. For more information, see the Full PDS in Appendix A.

Successes of Spring 2022

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 consisted of continuing members, Maya Tanna, Sam Bardwell, Katie Day, Drew Hardwick, and Bella Raykowski. The team was able to create a 195x245x36.83mm incubation chamber using black acrylic[1]. The incubation 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

[1] See Appendix B for more details

chamber to be connected. 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 ⅝ inch holes for copper tubing that was 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 ⅝ inch and ⅛ inch holes for the NDIR CO_2 sensor and thermistor.

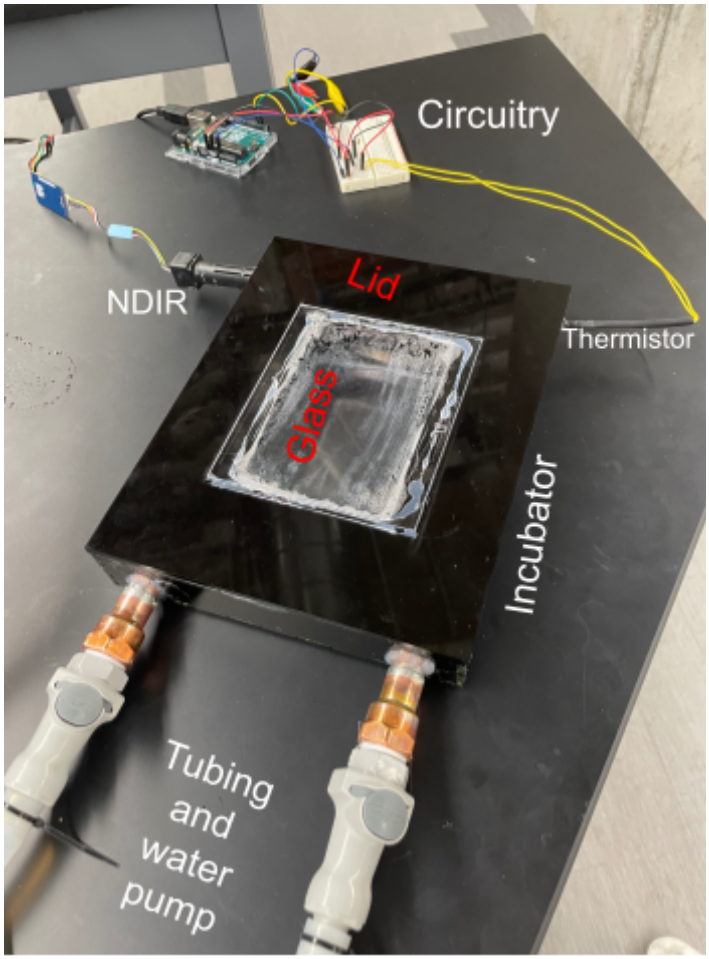

Figure 3: Incubator Prototype Exterior

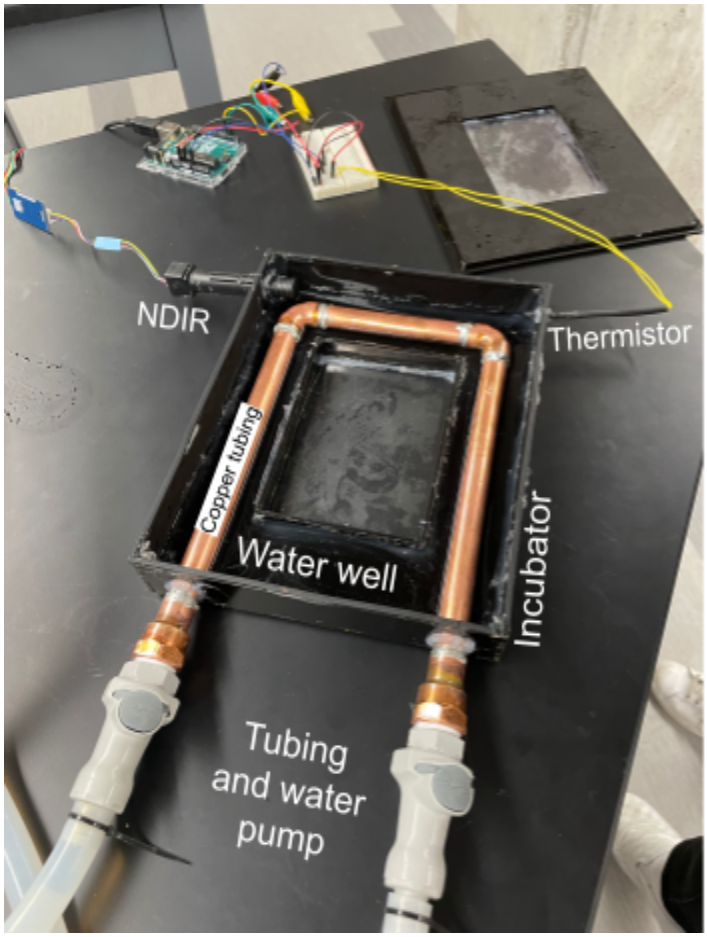

Figure 4: Incubator Prototype Interior

[2] See Appendix A

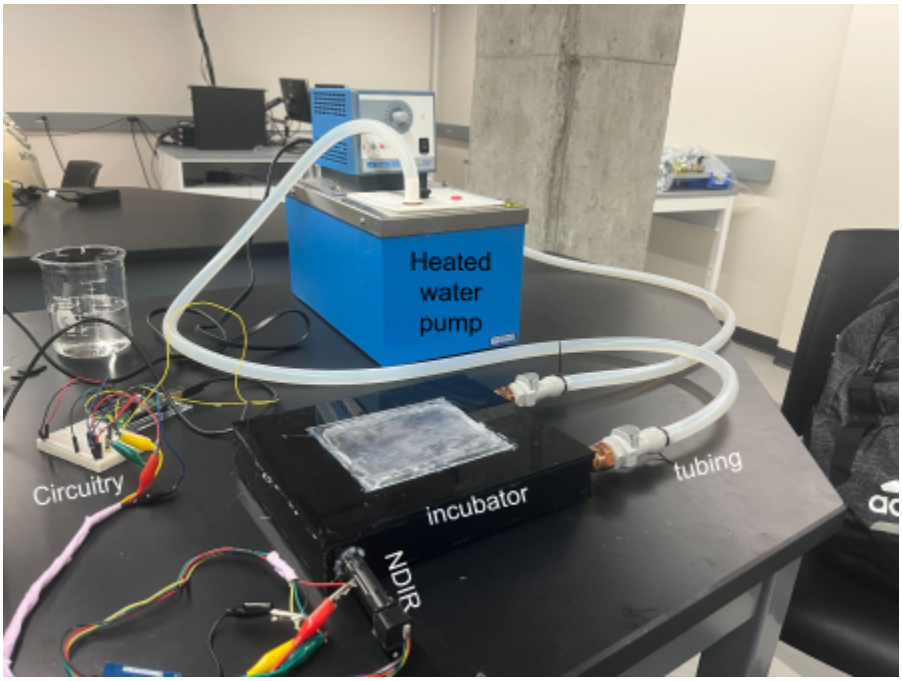

Figure 5: Whole Incubator Set Up

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 is within the standards outlined in the PDS and met the design requirements[5].

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 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 is 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.

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

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.

III. Preliminary Designs

Design #1: Solenoid Valve

The solenoid valve CO_2 input regulator (Figure 6) consists of a normally closed electric solenoid. There will be a 12V power supply that when partnered with a transistor, will excite the solenoid to open when instructed. The solenoid valve will remain open until the correct amount of gas is inserted into the incubator and then will be deactivated, and closed. This design requires adaptors for the CO_2 tubing in order to control air flow because the threads on the solenoid are in European units. Male thread to barbed tubing adaptors are available online. Some benefits to this design are little fabrication, electrical control, and minimal leakage because it is an industrially made part. The solenoid is purchased online and the only fabrication is the circuit building and coding. The electrical control is 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 are 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 will also need to be purchased to allow the team to excite the solenoid.

Figure 6: Solenoid Valve [10]

Design #2: Threaded Pin Valve

The threaded pin valve design (Figure 7) consists of a DC motor being hooked up to a threaded pin, with the typical body design of a pin valve. This valve will be normally closed, and when it needs to be excited, the DC motor will twist and “unscrew” to allow air to pass through

the airway. When enough time has passed to allow air to flow through the tubing, the DC motor will reverse directions and screw in to close the valve. At the end of the threaded pin is a rubber nose that will help provide a nice seal to limit CO_2 leakage. Some benefits to this design are 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 does not have many moving parts so the safety risk of someone getting hurt is minimal. A couple of downfalls to the design would be the risk of leakage. It would be a homemade valve and having precise fabrication is difficult to obtain. The threaded screw design is thought to have a slower response time as well which would lead to less accurate CO_2 input for the incubator.

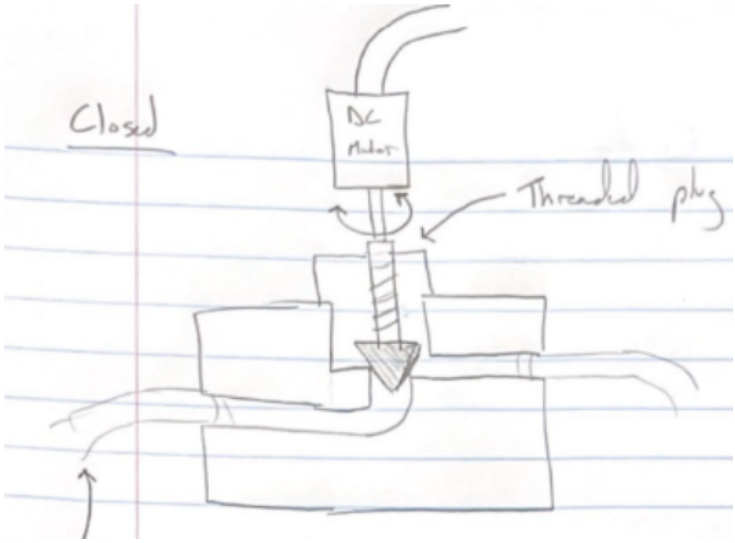


Figure 7: Drawing of the threaded pin valve design

Design #3: Spring Pin Valve

The spring pin valve design (Figure 8) consists of a DC motor being attached to a pin with either a wire or string. When the DC motor is activated, it will wrap the wire or string around the motor shaft, lifting the pin up. As the pin is being lifted up, an attached spring will compress. When the DC motor stops rotating, the pin blocking the airway will have moved, allowing the CO2 gas to pass through. When the correct amount of CO2 has passed, the DC motor will reverse rotation directions, loosening the string, and allowing 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 include 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 is that it is 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 are the complexity of the design and the risk of leakage. This design has multiple little parts and mechanisms that may be hard to fabricate, and this would also lead to not finely machined parts and more CO_2 leakage.

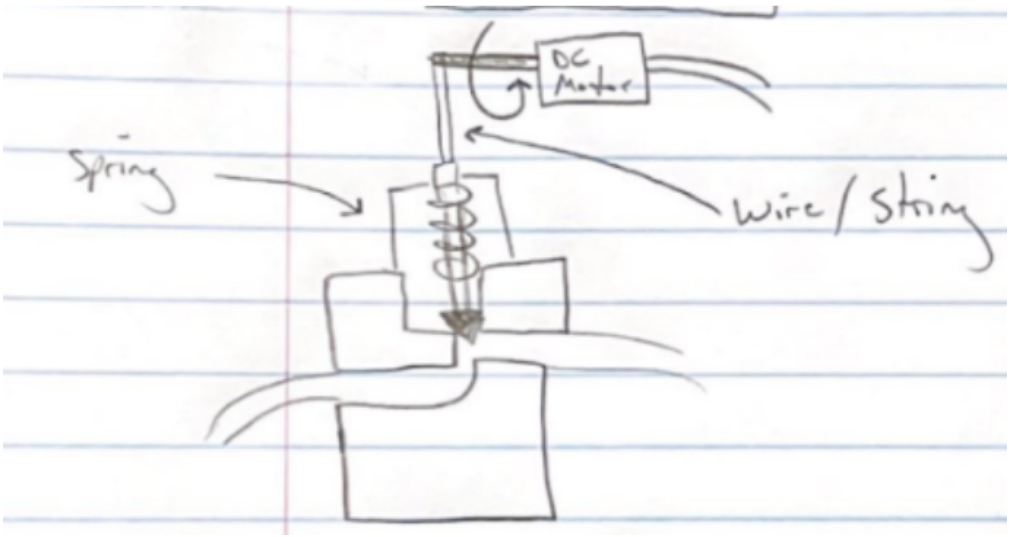
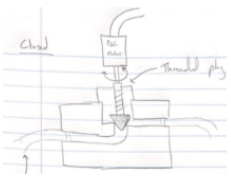
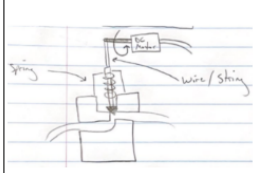


Figure 8: Drawing of the spring pin valve design

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	Live 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 requests 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 has a budget of $100, although the client has 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 will 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 will be left overnight in the teaching lab and must continue to pump CO_2 into the incubator in order to keep the cells alive [11].

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 will 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 needs to adhere to FDA and OSHA standards and regulations [12][13]. Due to the use of tissue cells, the incubator must abide by Biohazard Safety Level 2 and ISO Class 5 air quality standards [14][15].

Proposed Final Design

The team decided to move forward with Design #1, the solenoid valve. Since the valve is industrially made and bought from a third party, the risk of leakage and fabrication difficulties were minimized. Although solenoids can become expensive, cheap products have been found online and will be tested. The solenoid valve will provide the best CO_2 input regulation because it will have a fast response time, based on code, to electrically open and close the solenoid pin. Having these precise response times increase the likelihood of having the correct amount of gas being inserted into the incubator, leading to healthier cells. Some alterations will have to be 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 part outweigh the costs of the solenoids and is 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. This semester, the materials needed include an Arduino-compatible solenoid valve, a diode, and a TIP120 transistor in order to increase the voltage from 5 volts to upwards of 9-12 volts. The solenoid valve is a normally closed valve that will be 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 (70MPa) than PLA, is cheaper, and the black allows for more insulation and protection from light [16]. 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, the black acrylic will be used to laser print a lid that has holes for homogeneity testing. Holes will be filled with a rubber stopper and the stopper will be removed for a thermistor to be inserted into the incubator. This is for the purpose of determining the temperature and humidity of different areas of the incubator.

Methods

In order to determine the performance of the prototyped incubator the team plans to employ CO_2 control methods, Homogeneity testing, and Cell Viability testing.

CO_2 Control

The current fabrication process for the solenoid valve design will require the purchase of adaptors to attach the solenoid valve to the CO_2 tank tubing. G1/4" male thread to 4.5-5.5mm diameter barb adaptors were purchased to allow this connection[17]. The male threads will screw into each side of the purchased solenoid valve and the barb ends will allow a tight connection to the 4.7 mm ID (Inner Diameter) of the CO_2 tank tubing. One side of the solenoid will be connected to the tank via a flexible plastic tube, and the other side will attach to similar tubing being inserted into the incubator. The solenoid valve will be using a transistor circuit, Arduino Microcontroller, and a 12V power supply in order to be excited and opened, allowing the gas to

pass through.The solenoid valve will be coded to open the valve when the percent CO_2 is below 5%. The solenoid valve will then be tested according to the CO_2 Control Testing Protocol in Appendix C, to determine its initial accuracy and reliability. The solenoid valve code will then be implemented into the Arduino Mastercode, which contains a feedback loop for temperature, humidity, and the NDIR CO_2 sensor in order to maintain the internal conditions required for the cells in the incubator[8]. The valve will then be tested to determine its accuracy and reliability with the whole incubation system according to the CO_2 Control Testing Protocol in Appendix C.

Homogeneity

Homogeneity testing will be conducted by laser cutting a second incubator lid with ten precut ½ inch holes surrounding the glass to allow for different areas for the sensors to be inserted. This will provide 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 will be purchased in order to surround the sensors and to provide a tight seal when the sensors are being inserted into the precut lid holes.

Cell-Viability

In order to test the full functionality of the incubator, the team will conduct a cell viability test in order to quantify cellular proliferation which is an indicator of healthy, viable cells. This is done to determine if the prototype can maintain cell viability compared to the standard industry incubator. The control will be the T25 culture flask that is cultured in the standard incubator in order to provide a baseline on appropriate cell proliferation over the course of a 5 days. Another T25 culture flask will be cultured inside the prototype at the same time as the control over the course of 5 days. The images of the cells will be taken using the Zeiss microscope in the teaching lab. Then using ImageJ, the team will be able to quantify the percent area covered by the cells each day over the course of the test. Plotting the time vs percent area coverage of the control group will generate a baseline in which the prototype can be tested against. Tests will be considered successful if there is no significant difference between the percent area coverage over time between the control and the prototype. For more information see Cell-Viability Testing Protocol in Appendix C.

Final Prototype

Final prototype has not been fabricated yet.

Testing

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

[8] For more information see Testing

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 will complete homogeneity testing to ensure that the incubator is reading a uniform distribution of each of the internal environment components (temperature, humidity, and CO_2) of the system. The goal is for each of the conditions to maintain its homeostatic values throughout the incubator: 37°C, 5% CO_2, and >95% humidity. This testing will consist of 3D printing a lid with holes as seen in Figure 9. The thermistor, which reads temperature and humidity, and NDIR CO_2 sensor will be placed in each of the holes evenly spaced in the lid below. The team will record the values for each internal component and then calculate and report the precision result (mean ∓ average deviation). In order to validate the system, all of the values for each component should fall within this precise range to ensure uniformity and accurate internal conditions within the device.

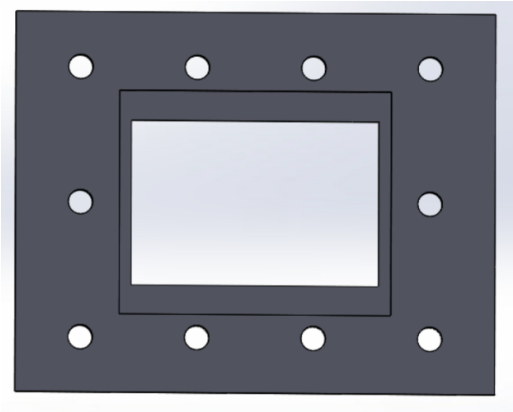


Figure 9: Testing lid with holes throughout to use for sensor placement during homogeneity testing.

CO_2 Testing

The NDIR CO_2 sensor was previously tested for accurate results under testing protocols in Appendix C. For more information please refer to Final Report Spring 2022. The ability of the solenoid valve to regulate the flow of CO_2 will be tested by setting up the incubator for normal use and recording the NDIR sensor values over the course of an hour. If the solenoid valve is able to regulate the correct CO_2 concentration, then it will be tested over the course of 6 hours. If the solenoid valve is able to keep the concentration of CO_2 in the incubator to 5% ± 1%, then the test is passed. See CO_2 Sensor and Feedback System Test Protocol for more information.

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 previous testing.

Currently, anti-fogging methods are being tested to ensure that there is clear visibility through the glass and condensation is prevented within the system as the team had some optical issues last semester. The team recently purchased a silicone-based lens cleaning solution and has been completing testing to determine how much of the solution must be applied to the incubator in order to maintain visibility of the cells for a one-hour period, since that period will be 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.

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 will commence 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 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 Testing

Currently, live cell testing is being conducted on the standard industry incubator in order to create the baseline in which the prototype will be tested against. Dr. Puccinelli provided the team with MC323E1 cells to test in the incubators as well as culture media. 125k cells are seeded into a T25 flask and are allowed to settle for 2 hours before imaging on Day 0. From then on, roughly every 24 hours the cell's media is changed and they are imaged using the Zeiss microscope in the teaching lab. The images are loaded into ImageJ, which calculates the %Area coverage. This is repeated everyday for 5 days in order to determine cell viability during the course of which the prototype will be used. Further live-cell testing once the incubator and CO_2 has been fabricated will follow the Cell Viability Test Protocol outlined in Methods. For more information on the live cell testing protocol, please refer to Appendix C.

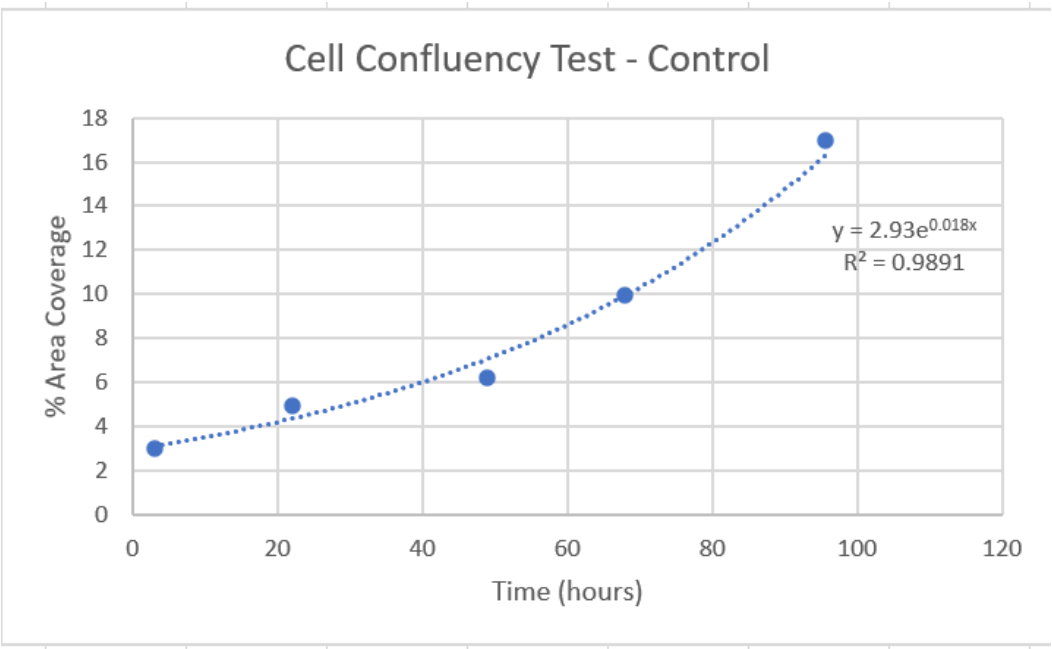


Figure 10: Results from cell confluency test conducted using the standard incubator. This is the baseline in which the prototype will be tested against.

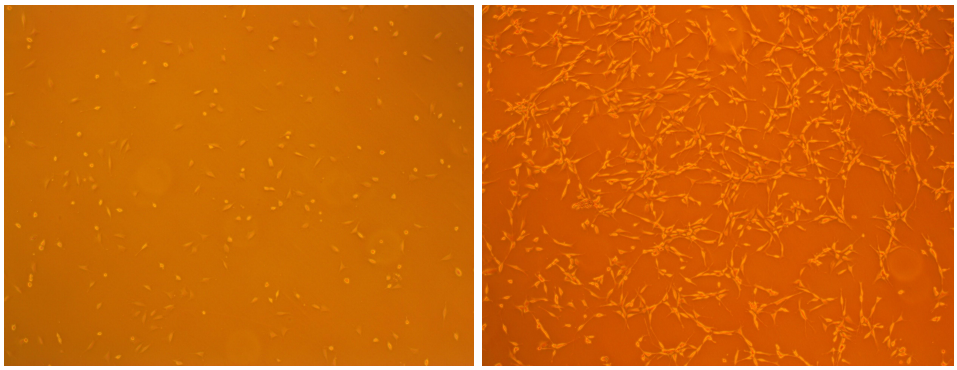


Figure #11a: Image taken by the Zeiss microscope of the control cells on day 0.
Figure #11b: Image taken by the Zeiss microscope of the control cells on day 4.

VI. Results (Future Work for Now)

Now that a final design has been proposed, the prototyping and testing stages of the project can begin. The group plans to break into three teams Fabrication and CO_2, Arduino Coding, and Live-Cell Testing which will each work independently to streamline the design process. The fabrication group will purchase the necessary materials, determine how to control CO_2 input using the solenoid valve, and fabricate a lid for homogeneity testing. The Arduino coding group will begin writing and testing their code for the sensors. The live-cell testing group will start plans for setting up a live-cell culture control group, before moving forward with testing cultures in the fabricated incubator.

VII. Discussion

Discussion will be written once results have been collected.

VIII. Conclusion

The client is in search 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 a placed 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 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 begin the prototyping and purchasing stages of the design process, before moving onto the testing phase.

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X. 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.

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 water tight, and have a transparent appearance.

k. ***Aesthetics, Appearance, and Finish:*** The client does not have a preference in 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. 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 where it 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, 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

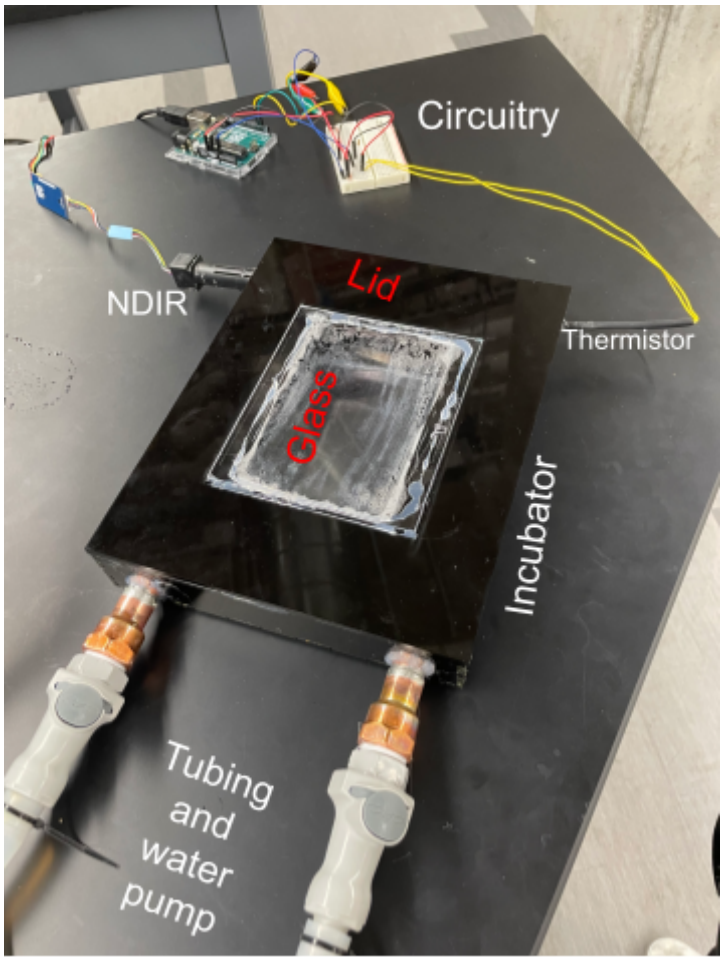


Figure 1: Incubator Prototype Exterior

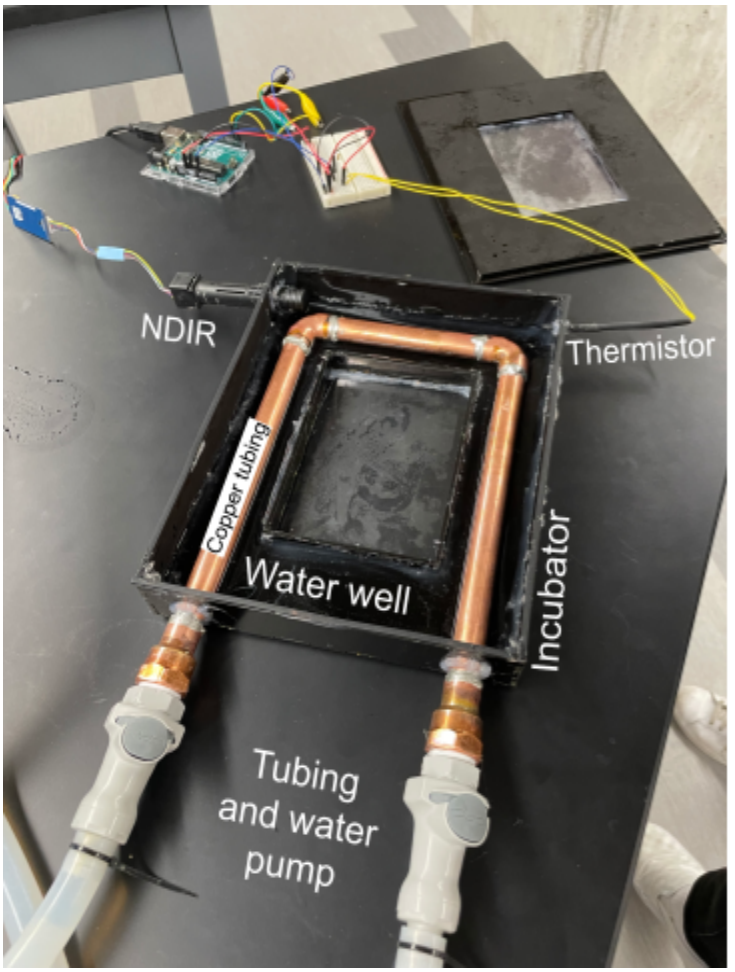


Figure 2: Incubator Prototype Interior

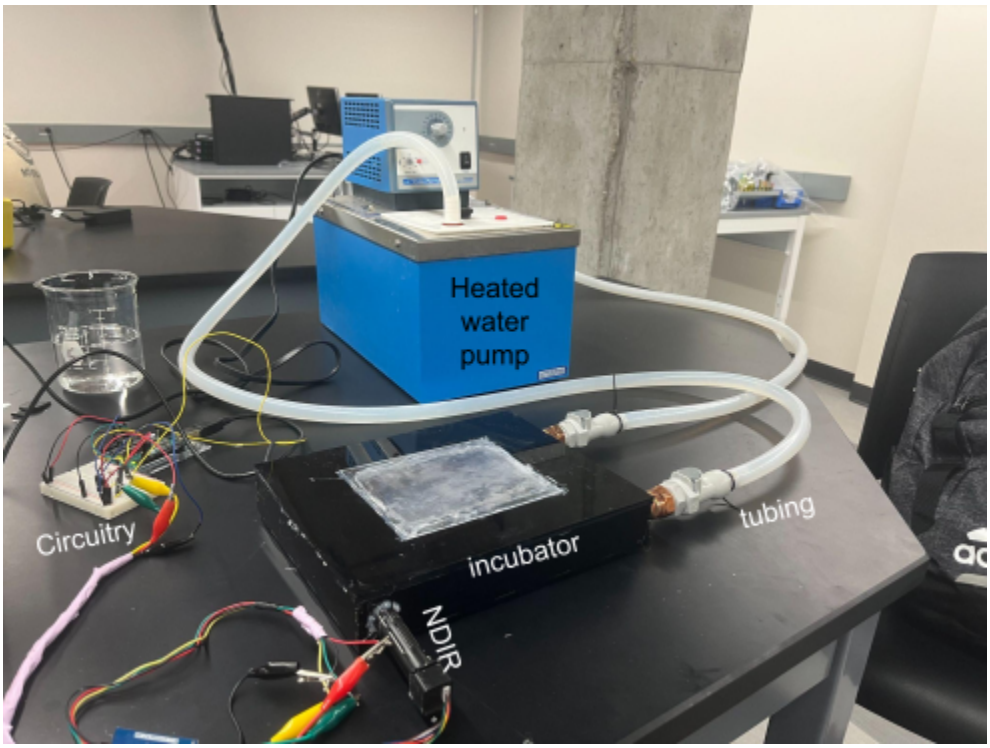
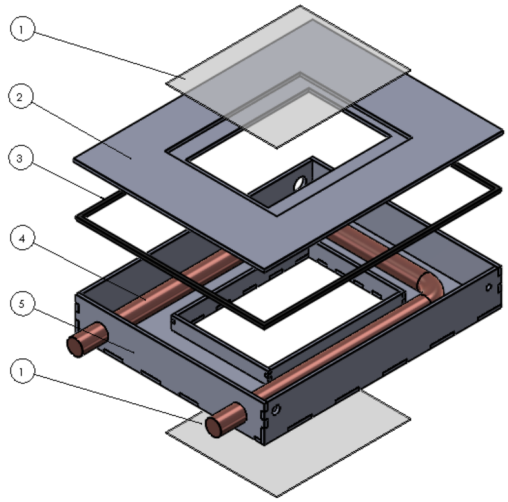


Figure 3: Whole Incubator Set Up

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

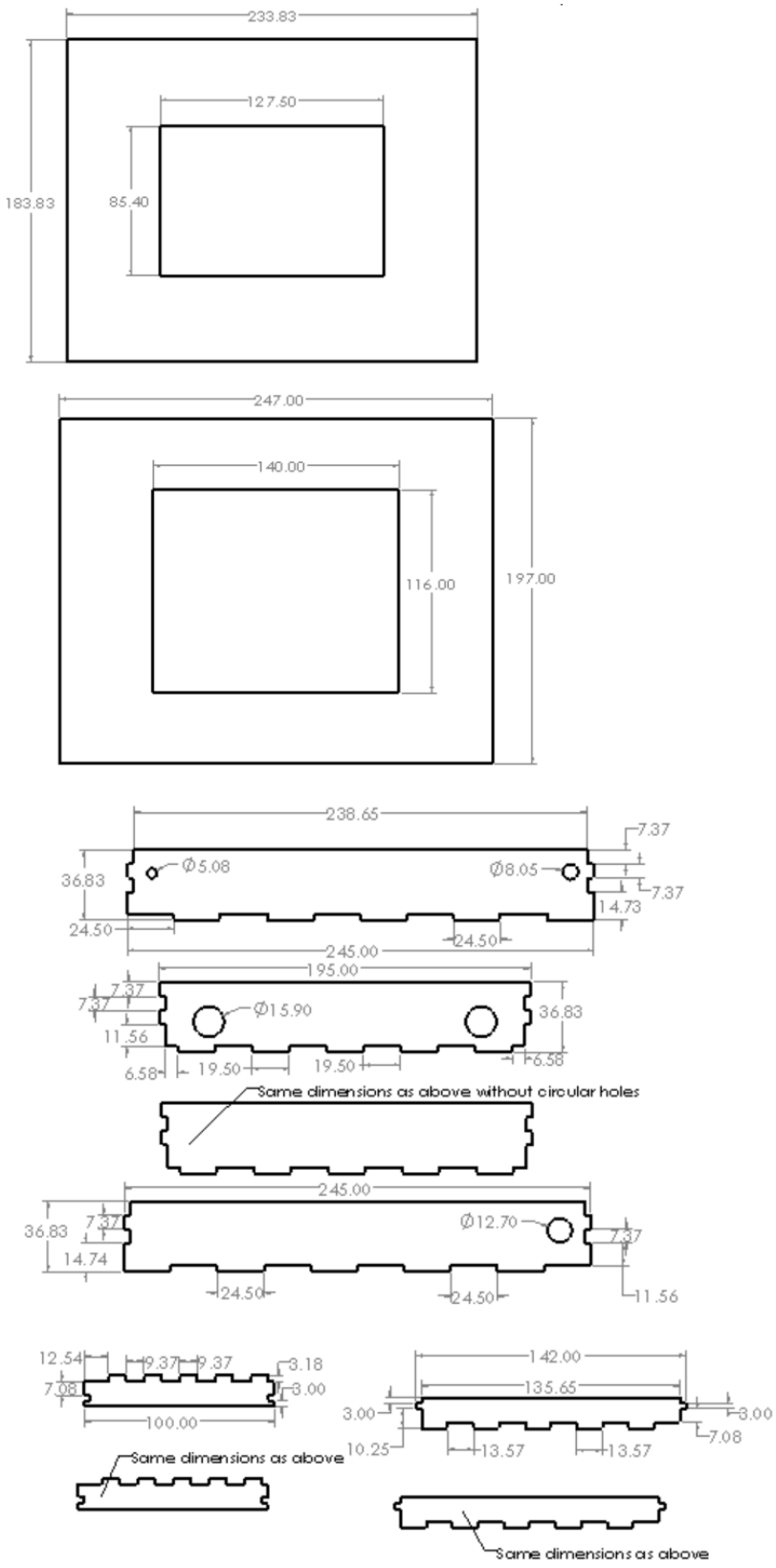
b) DO NOT open until data acquisition is complete and sample isn't required anymore (will compromise internal environment otherwise)

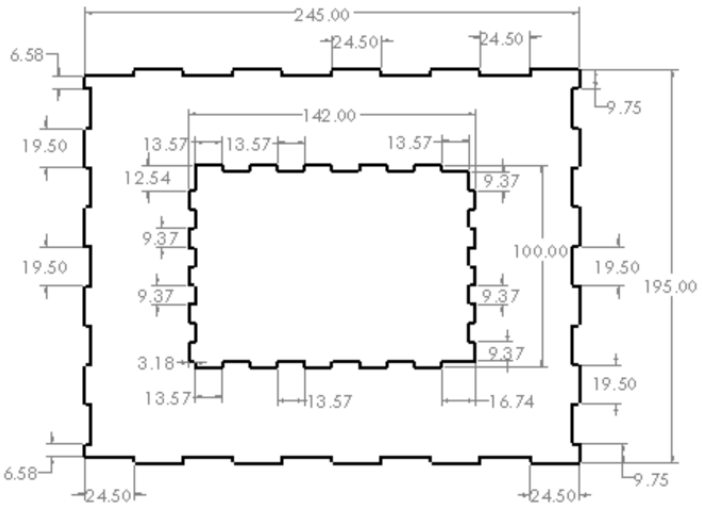
Data Acquisition

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
183.83
85.40
247.00
140.00
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
142.00
135.65
3.00
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

Circuit Diagram and Code

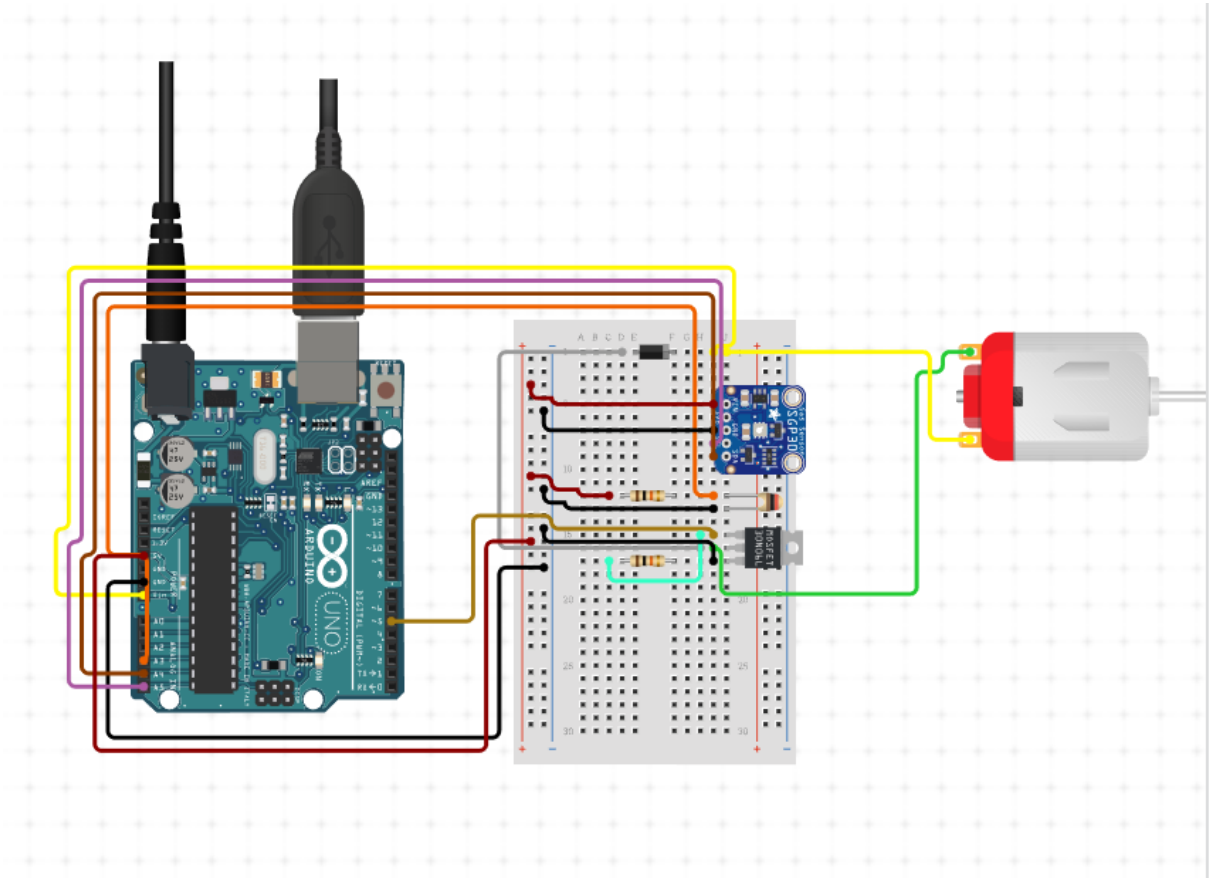


Figure 5: Complete Incubator Circuit Design

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 Clock wise 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[9] with the following graphs showing the results based on the sensor's initial reading ability in the lab incubator in ECB Room 1002.

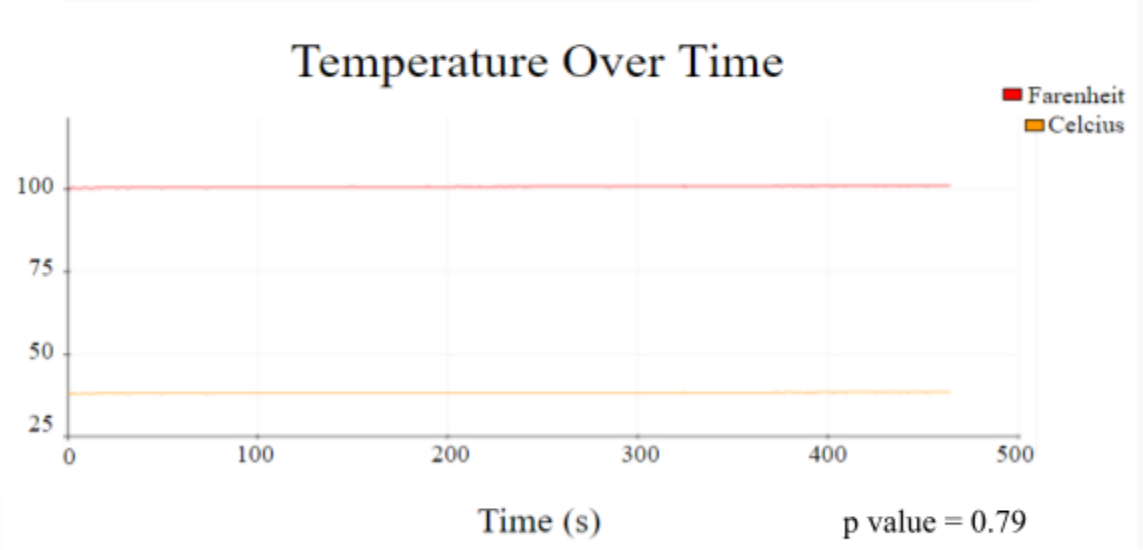


Figure 6: Thermistor Temperature over 10 minute Interval in Lab Incubator

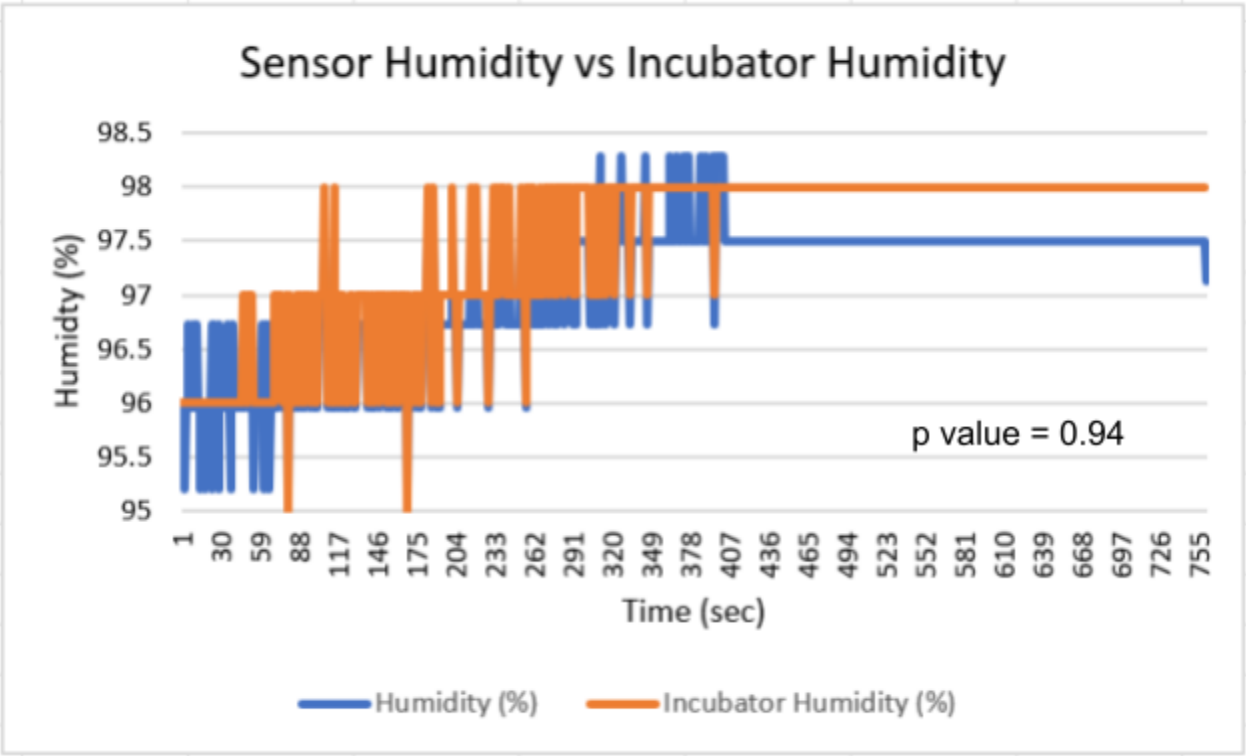


Figure 7: Graph of Humidity Readings in Incubator Over 10 min Time Interval

[9] 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.

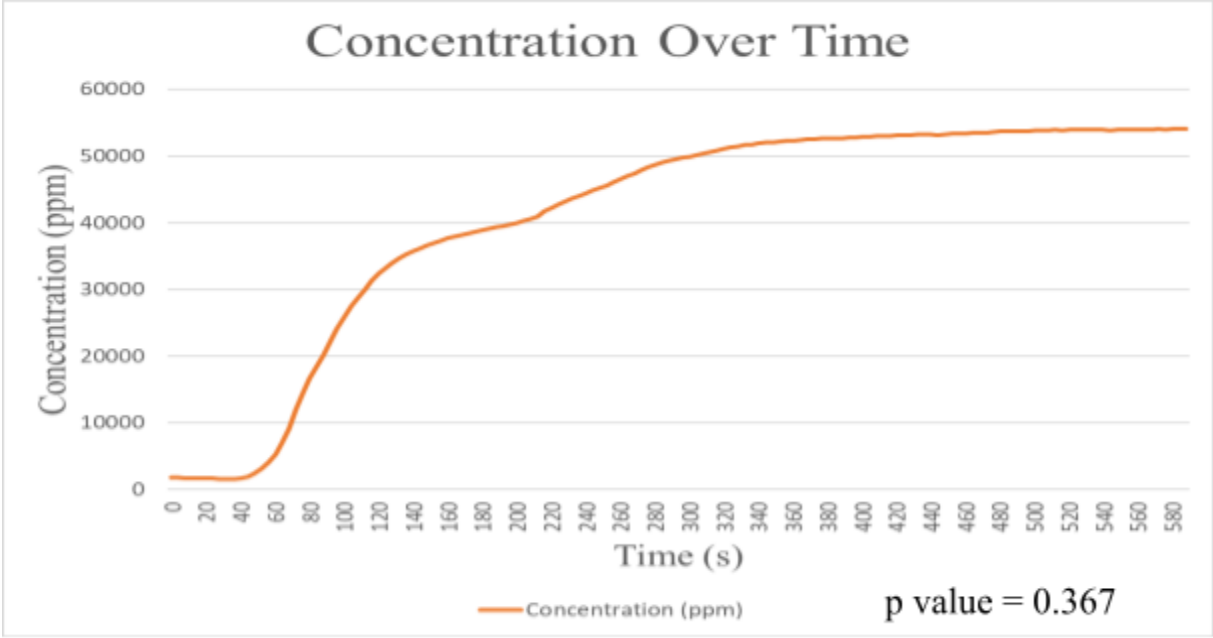


Figure 8: Concentration of CO_2 in Lab Incubator Over approximately 10 minutes

Temperature and Humidity Data in Incubator over 10 minute intervals.

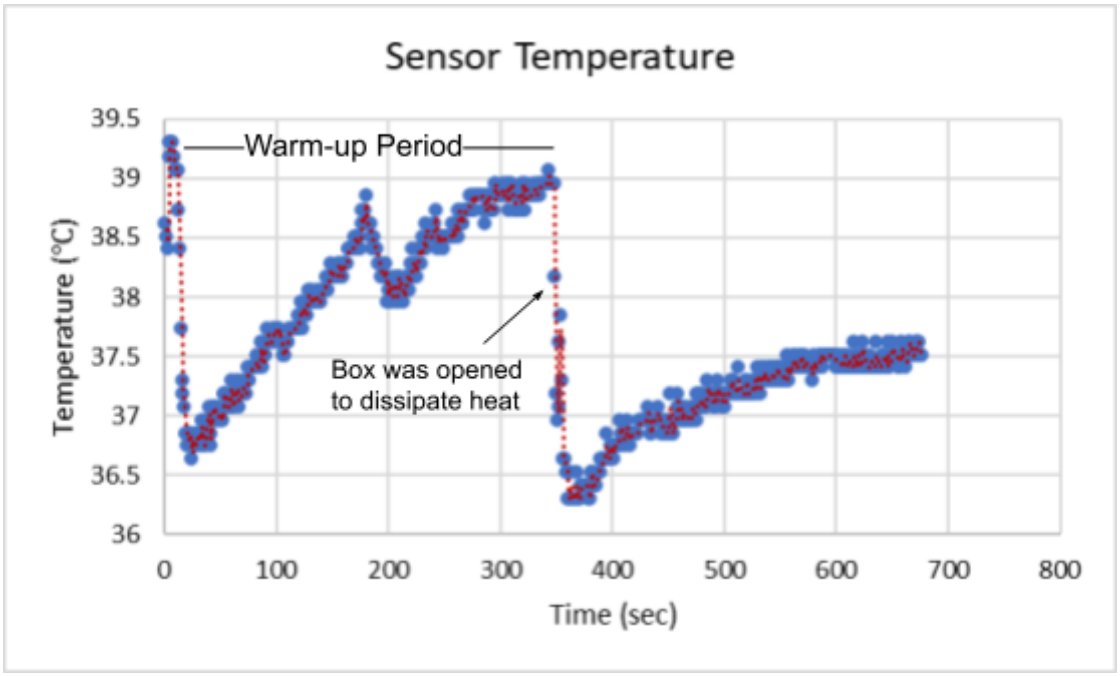


Figure 9: Sensor Temperature in Incubator over 10 minutes

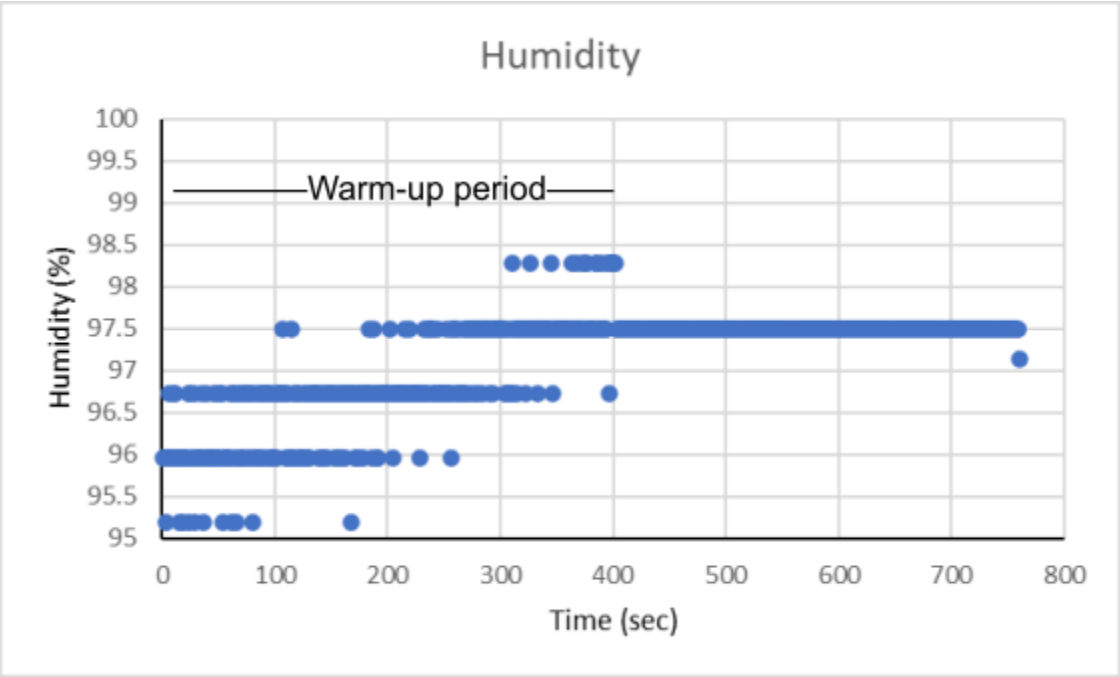


Figure 10: Sensor Humidity in the Incubator over 10 minutes

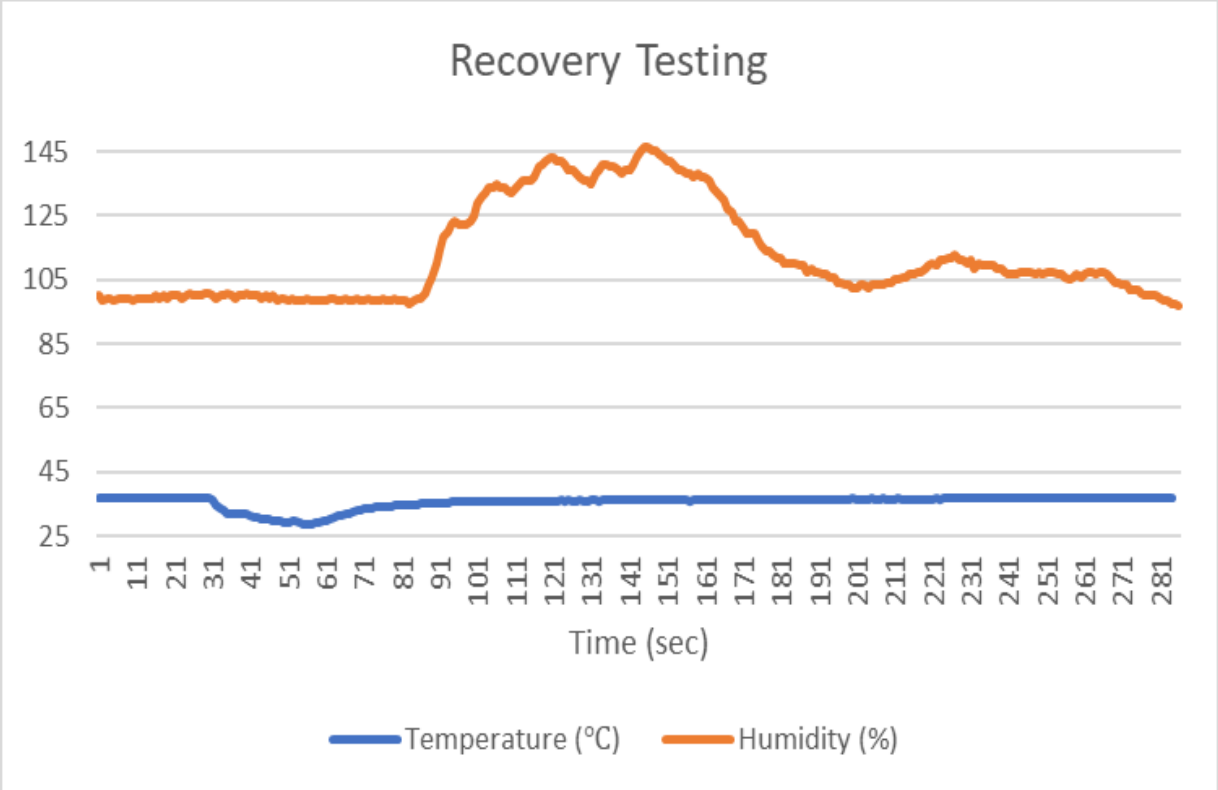


Figure ##: Temperature and Humidity Recovery Testing Data

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/Validation	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	☐ Verified		

	use. Set up a digital thermometer within the system.	Comments:		
4	Set up the Arduino sensor and incorporate the breadboard circuits.	☐ Verified 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/Validation	Pass/Fail	Initials of Tester
1	Test the precision of the sensor by ensuring its values increase and decrease with 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 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.	☐ Verified 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/Validation	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 necessary range, continue the protocol and this step is verified. If this did not occur, stop protocol and record what happened in the comments.	☐ Verified 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 High Transparent Lexan Polycarbonate sheets to determine which best matches the optical properties of well plates. Well Plates have a gloss percentage of 75-90, a haze percentage of 11, and a transparency percentage of 85-90 [16]. The team has researched that the transparency percentage of polycarbonate is 88-89 and the haze is 1%[17]. The team will determine through live-cell imaging, either by fluorescent microscopy or bright field microscopy depending on the client's cell cultures, whether 88% transparency is acceptable.

Steps	Protocol	Verification/Validation	Pass/Fail	Initials of Tester
1	Have one team member complete steps 1-2. Prepare the microscope for use. Place resolution test paper between the 2 sheets of High Transparent Lexan Polycarbonate, and place onto the microscope stage.	☐ Verified Comments:		
2	Adjust the optical components of the microscope to best clarity based on personal judgment. Ensure the resolution test paper is centered under the microscope lens. Take an image of what is observed under the microscope.	☐ Verified Comments:		
3	Repeat steps 1-2 without the polycarbonate sheets, but still including the resolution test paper.	☐ Verified Comments:		
4	Have 3 team members, other than the one who completed steps 1-3, complete this step. The team members will rank the two images on a scale of 1-10 based on focus quality. The image with the higher focus quality will then be determined. Record this image in	☐ Verified Comments:		

	the comments.			

Steps	Protocol	Verification/Validation	Pass/Fail	Initials of Tester
1	Prepare the microscope for use. Get internal conditions of the incubator to those needed for live-cells.	☐ Verified Comments:		
2	Place mammalian cells provided by the client in the incubator. Place the incubator onto the microscope stage.	☐ Verified Comments:		
3	Adjust the optical components of the microscope to best clarity based on personal judgment. Take an image of what is observed under the microscope.	☐ Verified Comments:		
4	Repeat steps 1-3 without the polycarbonate sheets, but still including the cells.	☐ Verified Comments:		
5	Using ImageJ, record the clarity of the images using the microscope focus quality plugin. The images will be divided into regions and assigned a color based on their focus level. Compare these images and their similarity.	☐ 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/Validation**	**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 will be employing image J in order to quantify the percentage of area covered by the cells over time in order to quantify the cell proliferation. This will allow the team to compare cell proliferation in the standard incubator compared to the prototype. The images of the cells will be taken using the Nikon scope in the teaching lab. The control will be the T25 flask that is cultured in the standard incubator in order to provide a baseline on appropriate cell death over the course of a week. Another T25 flask will be cultured inside the prototype at the same time as the control over the course of a week. A section of the flask will be marked to ensure that the same section is imaged each day. Using the Nikon scope in the teaching lab, an image will be taken of the predetermined section, then the image will be loaded into image J. The team will be 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/Validation**	**Pass/Fail**	**Initials of Tester**
1	Day 0: Mark section of the flask with Sharpie and image that section. Analyze in ImageJ and verified if the percent coverage is similar between the industry incubator and the team's device. (verifies that the cell's can be kept alive for 1 week at a time)	☐ Verified Comments:		
2	Day 1: image section. Analyze in image J and verified if the percent coverage is similar between the industry incubator and the team's device. (verifies that the cell's can be kept alive for 1 week at a time)	☐ Verified Comments:		
3	Day 2: image section. Analyze in image J and verified if the percent coverage is similar between the industry incubator and the team's device. (verifies that the cell's can	☐ Verified Comments:		

	be kept alive for 1 week at a time)			
4	Day 3: image section. Analyze in image J and verified if the percent coverage is similar between the industry incubator and the team's device. (verifies that the cells can be kept alive for 1 week at a time)	☐ Verified Comments:		
5	Day 4: image section. Analyze in image J and verified if the percent coverage is similar between the industry incubator and the team's device. (verifies that the cells can be kept alive for 1 week at a time)	☐ Verified Comments:		
6	Day 5: image section. Analyze in image J and verified if the percent coverage is similar between the industry incubator and the team's device. (verifies that the cells can be kept alive for 1 week at a time)	☐ Verified Comments:		
7	Day 6: image section. Analyze in image J and verified if the percent coverage is similar between the industry incubator and the team's device. (verifies that the cells can be kept alive for 1 week at a time)	☐ Verified Comments:		

ImageJ Percent Area Coverage Procedure

Introduction

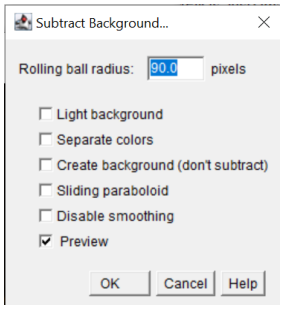
Name of Tester:
Dates of Test Performance:
Site of Test Performance:

Explanation:

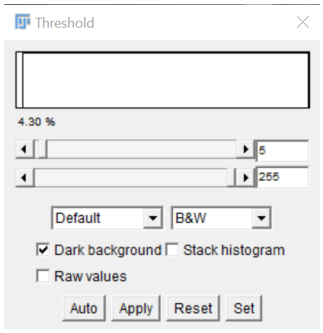
The team will be using ImageJ to analyze the images taken of the cells using the Zeiss microscope. ImageJ will calculate the percentage of surface area covered by the cells and from this, a trend line can be made that tracks the cell proliferation over the course of 5 days.

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/Validation**	**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/Validation**	**Pass/Fail**	**Tester Initials**
1	Apply 2 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:		