Microscopic Cell Culture Incubator
Final Report

BME 301 Design
May 4th 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 criteria, 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 of the incubator. The incubator will include a heated water pump and CO_2 pump in order to reach the clients criteria. Transparency, heating, and insulation testing will be conducted on various materials to find the optimal combination for the incubator.

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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].

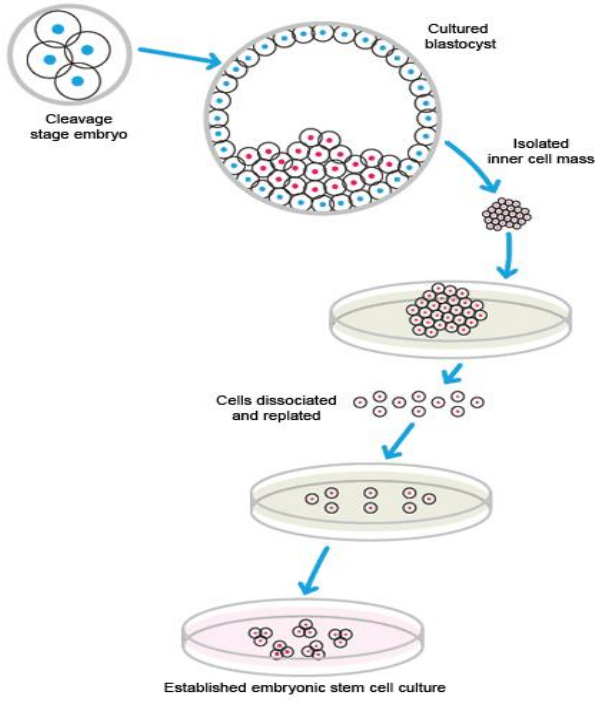


Figure 1: Isolation of Embryonic Stem Cell Lines[7]

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 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℃ ± 0.5°C, 5% ± 0.5% 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 Fall 2021

This project was worked on previously by many BME 200/300/400 students, however last semester, Fall 2021, brought a great deal of success to the project. The team, consisting of continuing members Maya Tanna, Sam Bardwell, and Katie Day and others, was able to create an incubation chamber out of PLA plastic with working temperature and humidity sensors. The incubation chamber was 195 x 245 x 40 mm with a vinyl tubing, inner diameter of ¼ inch and outer diameter of ⅜ inch, wrapped around the interior of the box. The vinyl tubing was connected to nylon barbed vacuum connectors , ⅜ x ⅜ inch, which was then hooked up to a heated water pump. The interior also contained a small water bed, roughly 1 liter in volume, that

in theory would be heated via thermal conductivity of the vinyl tubing induced by the flowing heated water from the pump. However, the results of last semester proved that polyvinyl tubing did not have the right thermal conductivity to heat the water bed higher than 20℃. The temperature and CO_2 sensors were coded and tested, both of which proved that the code ran smoothly and was able to accurately measure the internal environment of the incubator. Last semester, incorporation of CO_2 into the chamber was not possible, however it is of the utmost importance this semester. Appendix B contains more relevant information on the previous semesters work. The goal for this semester is to fabricate a box that does not leak, maintain a temperature inside the box of 37℃, and create an effective CO_2 input.

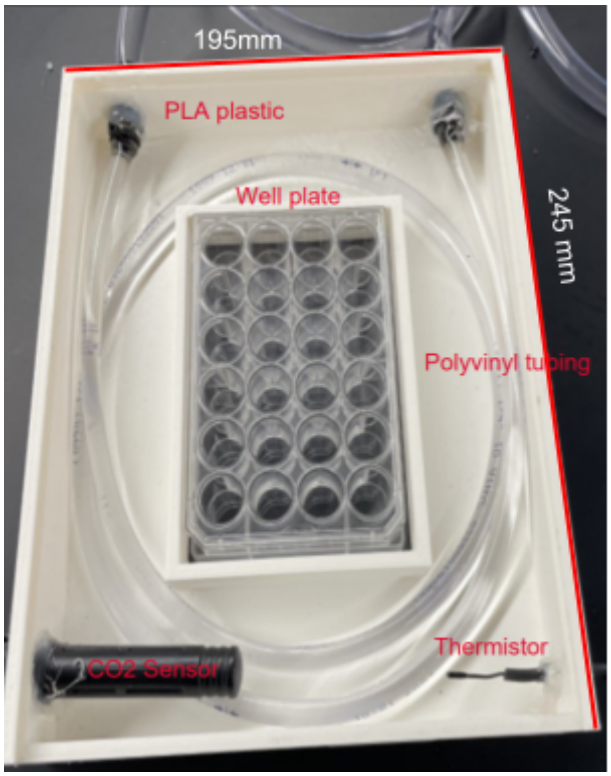


Figure 3: Fall 2021 microscopic cell culture incubator with polyvinyl tubing and sensors without a lid.

III. Preliminary Designs

Design #1 Hinge Top Acrylic Incubator

The hinge top acrylic incubator (Figure 4) consists of a 245x195x40mm black acrylic box with two feet of copper tubing circling the inner box once in order to provide heat via conduction. Copper tubing will be used due to its high thermal conductivity. The hinge top incubator received its name because the lid of the incubator will be placed on a rubber lining on top of the main box. When the lid is placed on top, hinges around the box will hook onto the lid and then be clamped down to compress the lid providing a tight seal for the internal environment. The mechanism will be similar to a hinge and latch on a common tackle box used in fishing. The black acrylic will be designed in SOLIDWORKS with the ability to be laser cut to increase precision, decrease cost, and to expedite the fabrication process. Because of the latched lid and a possible addition of a rubber lining, the ability to maintain an accurate internal environment was scored high. A couple downfalls to this design is with the addition of the latches. There will be more risk of fracturing the acrylic tabs that the latches will hook onto and the latches will increase the amount of fabrication steps, as well as the cost.

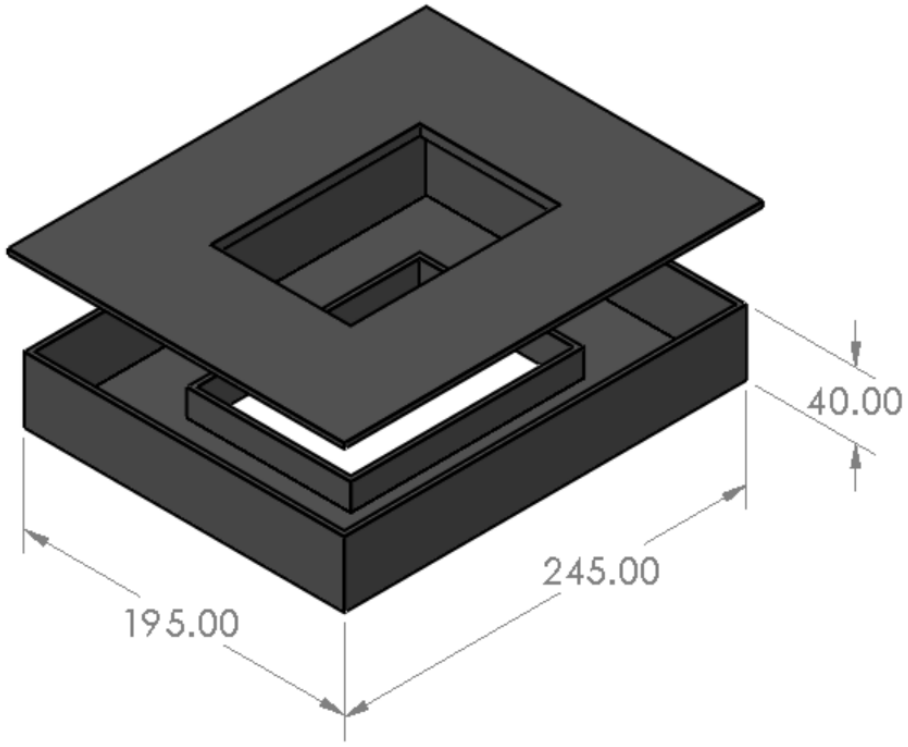


Figure 4: Solidworks Image of Preliminary Design #1 (all dimensions in mm)

Design #2 Slide Top Acrylic Incubator

The slide top acrylic incubator (Figure 5) will be made of laser cut, black acrylic, similar to Design #1. The physical design will be comparable to the previous semester work (Appendix B) with a change in material and fabrication process. The slide top acrylic incubator lid will be able to slide into and out of a slit carved into the inside of the main box. This will allow for easy access into the well plate of the incubator, without disrupting the entire internal environment. This design will include the two feet of copper tubing circling the inner box once in order to provide the 37°C temperature values via conduction. One downfall to this design is the slide top provides more creases and areas for potential loss of the internal environment. If the slide top slit isn't a perfect fit, there will not be a perfect seal between the cover and box causing fogging of the glass impacting the optical clarity. A rubber gasket could be added to have a tight seal between the slide top, but fear of difficulty fabricating and functioning resulted in the possibility of a lower internal environment score.

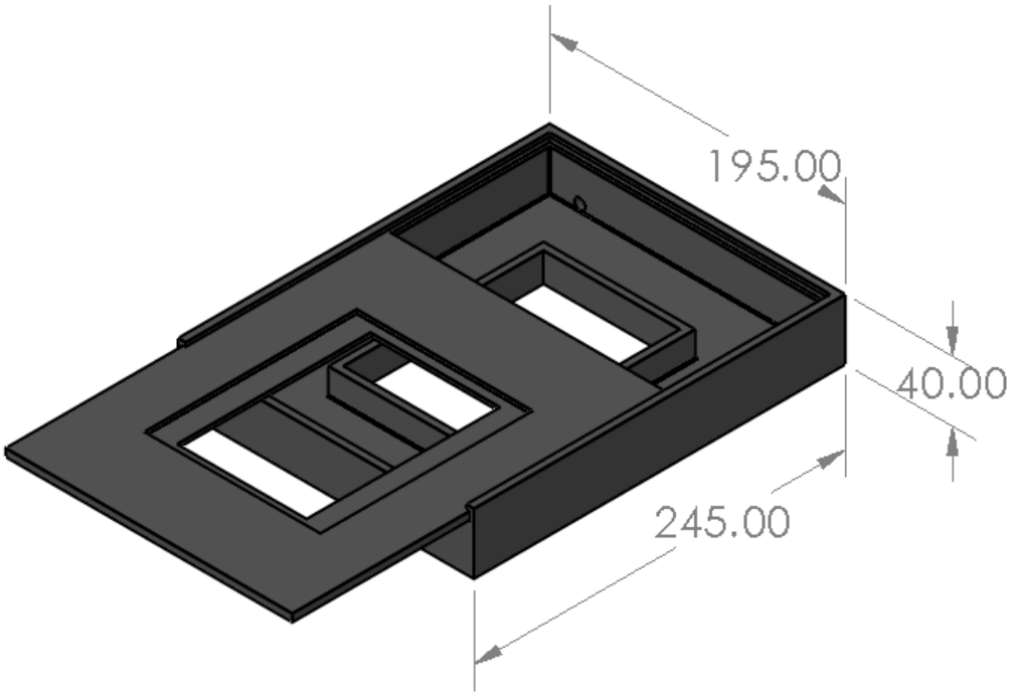


Figure 5: Solidworks Image of Preliminary Design #2 (all dimensions in mm)

Design #3 3D Printed Incubator

The 3D printed incubator (Figure 6) will be made with the same SOLIDWORKS drawings as the previous semester. The box will be made of white PLA plastic with an inner coating of flex seal, insulation spray, liquid concrete, or caulk. The box will have the slide top concept to allow easy access to the inside of the incubator. The inner box will be wrapped with the same two feet of copper tubing as the previous two designs to maximize the heat transfer between the heated water pump and the inner water bed. A couple downfalls to this design is the cost, material properties, and sealant capabilities. 3D printing is much more expensive and with the addition of an extra sealant to prevent the PLA plastic from leaking or cracking, the cost will add up quickly. The PLA is also prone to leakage through microplastics pores which would cause inconsistencies in the internal environment. The one benefit to 3D printing is the ease of fabrication.

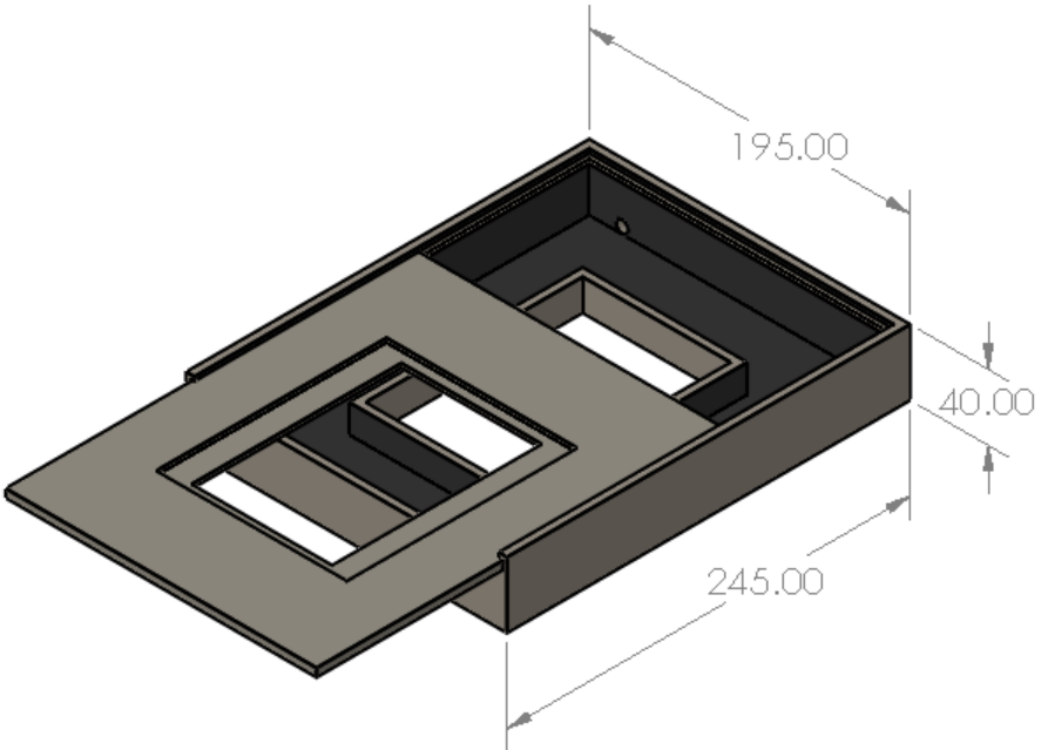
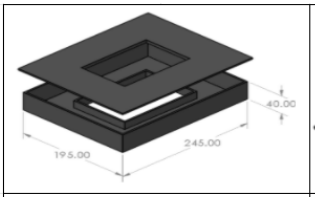
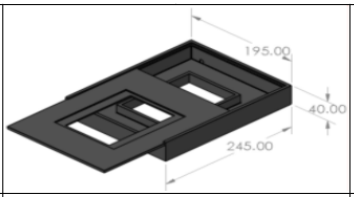
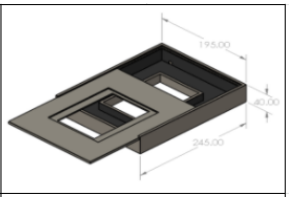


Figure 6: Solidworks Image of Preliminary Design #3 (all dimensions in mm)

IV. Preliminary Design Evaluation

Design Matrix

Table 1: Design Matrix with all methods scored on internal environment maintenance, microscope compatibility, accuracy and reliability, ergonomics, cost, life in service, and safety.

								
			Hinge Top Acryllic Incubator		**Slide Top Acryllic Incubator**		**3D Printed Incubator**	
Rank	Criteria	Weight	Score (5 max)	Weighted Score	Score (5 max)	Weighted Score	Score (5 max)	Weighted Score
1	Internal Environment	25	5	25	4	20	4	20
2	Microscope Compatibility	20	5	20	5	20	5	20
3	Accuracy and Reliability	20	4	16	4	16	3	12
4	Ergonomics	15	5	15	5	15	5	15
5	Cost	10	4	8	4	8	3	6
6	Life in Service	5	5	5	5	5	4	4
7	Safety	5	5	5	5	5	5	5
	Sum	**100**	**Sum**	**94**	**Sum**	**89**	**Sum**	**82**

* All box dimesions are in millimeters

Scoring Criteria

Internal Environment: The internal environment maintenance was weighted the highest due to the client's request that these standards be met as close to industry standards as possible, with some leeway provided the internal environment is viable with live cells. Since live cells are being used in the cell cultures, the incubator must be able to meet 37℃ ± 0.5°C, 5% ± 0.5% CO_2, and 95-100% humidity, in order to survive for the duration of the teaching lab.

Microscope Compatibility: Many currently available incubators are not compatible with inverted microscopes as a result of their size and price. The team needed to design an incubator to fit onto an inverted microscope stand, roughly 310x300x45mm. The team's current designs are much smaller than current incubators. The final product must not interfere with the microscope's optics, allowing for transparency for top and bottom viewing of the cells, along with a maximum thickness of 45mm so that the product does not come in contact with the lens of the scope.

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 accuracy and reliability of the device will be evaluated and monitored using temperature, humidity, and CO_2 sensors connected to the device via an Arduino microcontroller.

Ergonomics: The device must be within a size and weight that the average user can safely handle and move with ease.

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.

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 hinge top acrylic incubator. Since the material was laser cut acrylic at the UW Makerspace, the cost was much lower than 3D printing. This incubator also provided the best internal environment and reduced the majority of leakage throughout the incubator because of the rubber lining addition. With the addition of the inner copper tubing, the heat transfer between the heated water pump water to the water bed was maximized because copper has the second highest thermal conductivity to silver, resulting in the incubator being able to reach the 37℃ temperature as well as the desired humidity of >95%. The incubator was paired with a 100% CO_2 input with sensor readings increasing or decreasing the amount of gas being inserted. Temperature, humidity, and CO_2 sensor coding and circuitry were improved from the previous semester to provide more accurate and precise data readings. A potential constraint with Design #1 is with the addition of latches. Increasing the number of fabrication steps increases the likelihood of design flaws and could even lead to the possibility of fracturing the acrylic walls and tabs. Overall, the first design allows for the most compatibility and improvement of materials, accuracy, and design criteria compared to the other designs.

V. Fabrication/Development Process

Materials

Arduino Materials

The circuitry was made with an Arduino sensing unit for the purpose of measuring temperature, humidity, and CO_2 levels during incubator usage. A DHT22 sensor was previously used in past projects as it accurately and reliably measured both temperature and humidity. However, the downside to this material is that it is not waterproof. The team opted for a thermistor, which measures temperature and is waterproof. The thermistor is also smaller allowing for better implementation into the incubator. In order to make sure that the thermistor can read both temperature and humidity, the team used an equation (see Appendix B) to

determine the relative humidity inside the incubator. The accuracy of this equation was tested against the DHT22 temperature and humidity sensor.

In order to measure CO_2 levels inside the incubator, the team used a MH-Z16 NDIR CO_2 sensor, which has been used in past projects. This sensor was chosen because it is waterproof, has the ability to read temperature which would allow homogeneity of heat throughout the incubator to be checked, and because it was already available for use which would help the team stay under budget. The flow of CO_2 will be monitored via a DC motor with a motor arm attachment that will be controlled by the Arduino microcontroller. The DC motor twists the valve on the CO_2 tank clockwise or counterclockwise to let in more or less CO_2 depending on the NDIR sensor values.

Incubator Materials

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.

Methods

To begin, a prototype of the incubator box was laser cut from high density fiberboard (HDF) and was assembled in order to ensure that the dimensions are accurate and to test if laser cutting the material would assemble properly. Fabrication of the final prototype began with an 18x24x ⅛ inch sheet of black acrylic plastic. The acrylic sheet was placed onto the UW Madison Makerspace laser cutter. The box was laser cut 2-dimensionally using a CAD drawing in SOLIDWORKS and converting the drawing to the laser cutter language. Once the individual pieces were cut out, the team used cement acrylic glue to build the box 3-dimensionally.

Additional materials such as copper tubing, glass, CO_2 input, and sensors were incorporated into the box once it was built. The inner copper tubing was connected to the heated water pump tubing with a threaded metal adaptor. The glass was glued onto its corresponding indents in the bottom of the box and the lid to allow for transparent viewing. A 3D printed motor attachment was connected to a DC motor in order for the 100% CO_2 tank's pressure valve to be controlled from a DC motor. The motor was connected to an Arduino microcontroller that contained code reading values from the NDIR CO_2 sensor, to limit/regulate the amount of CO_2 in the well. The DC motor was needed because the team has decided to use a 100% CO_2 tank in order to meet the budget requirements. Lastly, the sensors were inserted into the same spots as

the previous semester to collect live data on the values of temperature, humidity, and percent CO_2.

The thermal conductivity of copper was assessed along with the heat transfer rate (Q) of copper measured by using equation 1[17].

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

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

Using equation 1, it was determined that if the heated water pump pushes water out at an initial temperature of 50℃, the 1L 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.

The Arduino sensing unit was developed using the materials recommended by the Arduino website in order to build a basic circuit that has both temperature and CO_2 testing. The team used the sample code provided by Arduino with some minor modifications in order to also output the humidity readings.

Final Prototype

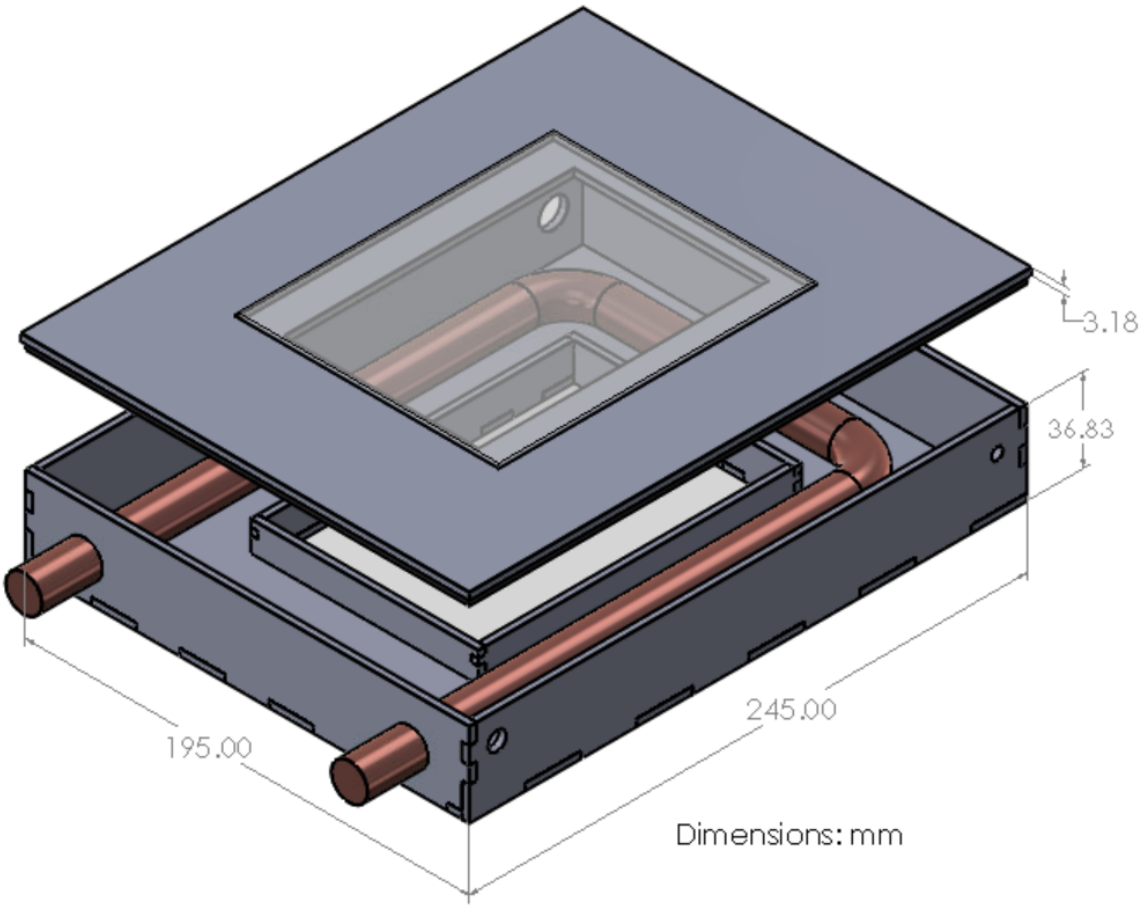


Figure 7: Final SOLIDWORKS drawing of the final design in mm

The final prototype was created using black acrylic. Black acrylic was chosen for its insulation properties, usability with both an inverted microscope and a fluorescent microscope, and its ability to be cheaply fabricated using the UW Makerspace Laser Cutter. An 18x24x ⅛ inch sheet of black acrylic sheet was purchased and cut using the laser cutter and SOLIDWORKS drawing files. The incubation chamber consisted of a top and bottom, with a hole for polycarbonate glass plates, and sides that had filets to prevent leakage in the box and two allow the walls to be connected. The inside of the box had a chamber for the water bath with filets on the side, again to prevent leakage and for joints. The box was glued together using acrylic contact cement glue. The top of the box was lined with rubber so that the lid would be able to fit onto the box. Originally, four latches were going to be cement glued to the sides of the box to provide a compressed seal on the rubber lining, but was later discarded to test the box without latches to produce a lower overall cost. The box also had five holes laser cut into the sides of the box. The front of the box had two ⅝ inch holes for the copper tubing that was

inserted into the water bath space in the box. The copper tubing was curved using copper elbow couplings which allowed the copper to wrap around the interior of the box. The copper couplings were soldered to the copper tubing to prevent any water leakage. There were also ⅝ inch and ⅛ inch holes for both the NDIR CO_2 sensor and the thermistor. Finally a ¼ inch hole was added for the CO_2 polyvinyl input tubing so that CO_2 may enter the box.

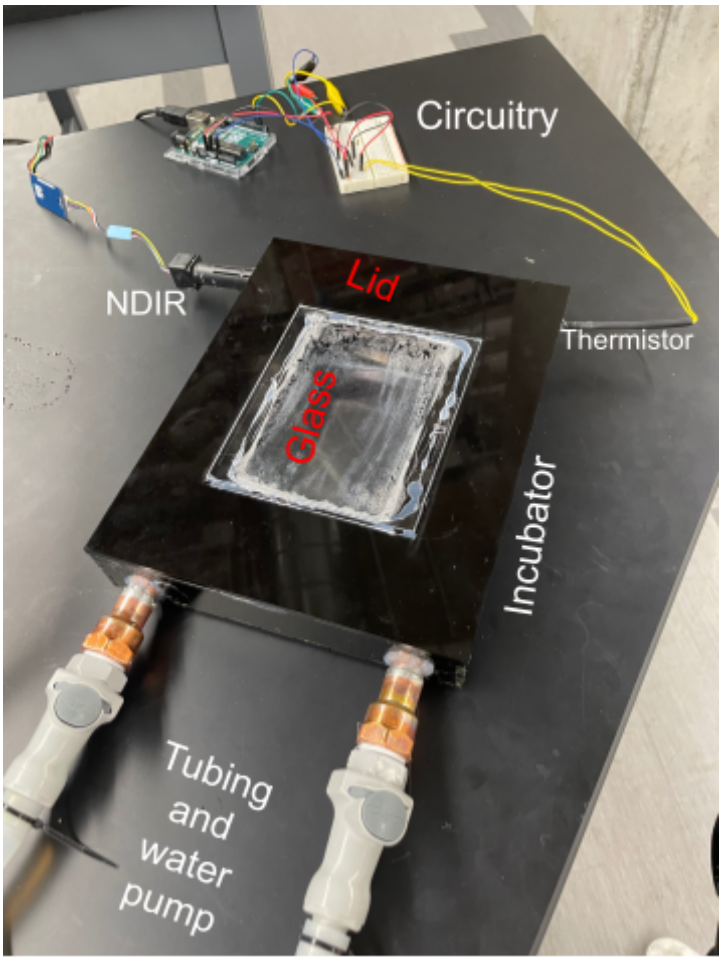


Figure 8: Incubator Prototype Exterior

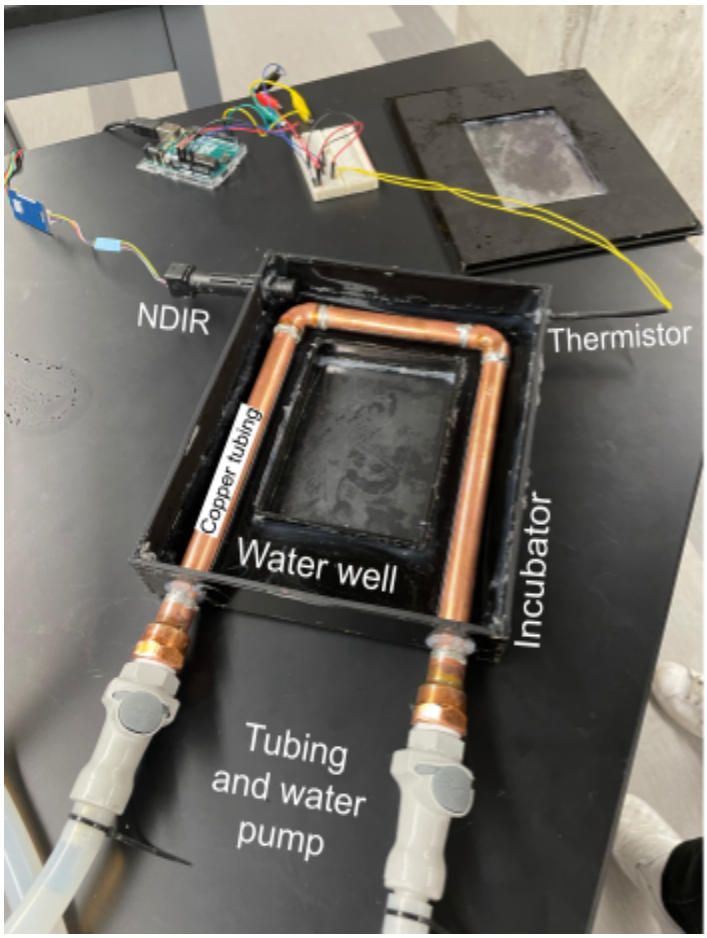


Figure 9: Incubator Prototype Interior

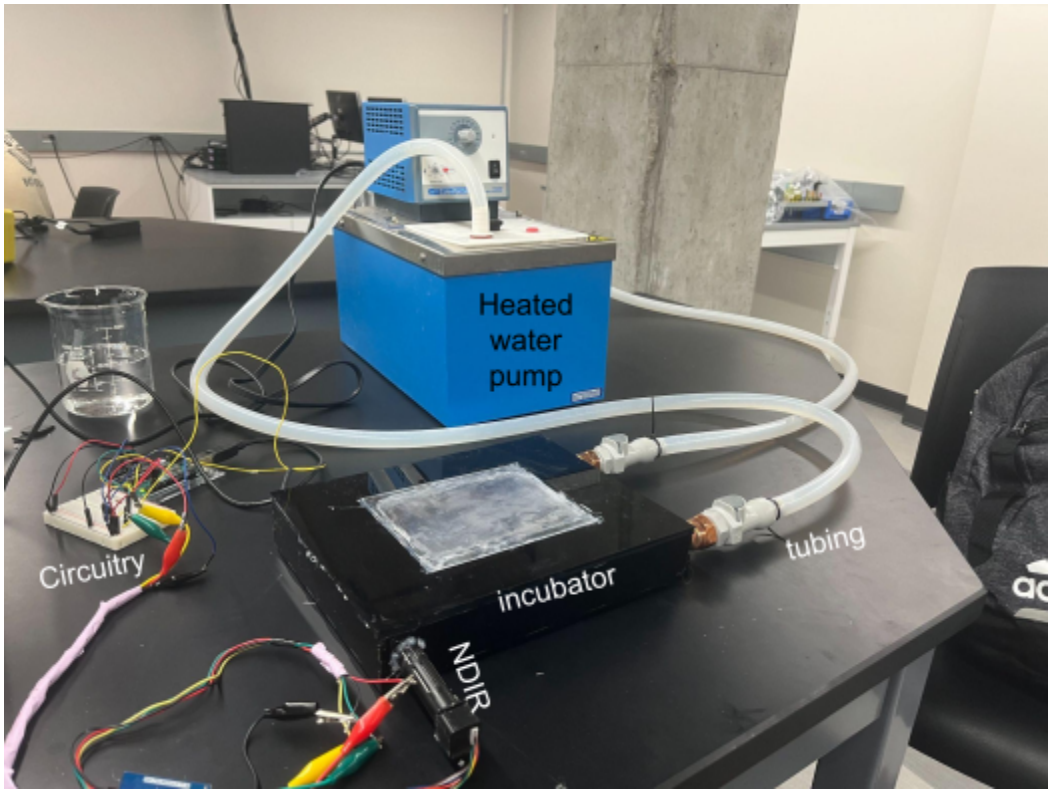


Figure 10: Whole Incubator Set Up

The DC motor was coded to record the percent of CO_2 in the incubator and twist the CO_2 tank valve clockwise or counterclockwise depending on the value[1]. A DC motor attachment was created in order to twist the knob in either direction. This attachment was three-dimensionally modeled in SOLIDWORKS[2] to evenly grip the 100% CO_2 tank regulation valve at four points, evenly spaced, 90° apart. These grip points were modeled in two sets, directly across from each other, spaced to the exact dimensions of the diameter of the regulatory valve, which is circular in shape and has a diameter of 32.62 mm. The grip arms are large and thick with a square cross sectional area of 12.7x12.7 mm to allow for maximum surface area contact with the valve and decrease the chances of the arms cracking or breaking off due to the applied torque. These grip arms were attached to a cylindrical, 101.6 mm shaft that attaches to the DC motor via a small hole drilled into the bottom of the shaft. This valve attachment piece was 3D printed in the UW Madison Makerspace with PLA plastic. The dimensions and SOLIDWORKS image can be seen in Figure 11.

[1] See Appendix F
[2] See Appendix E

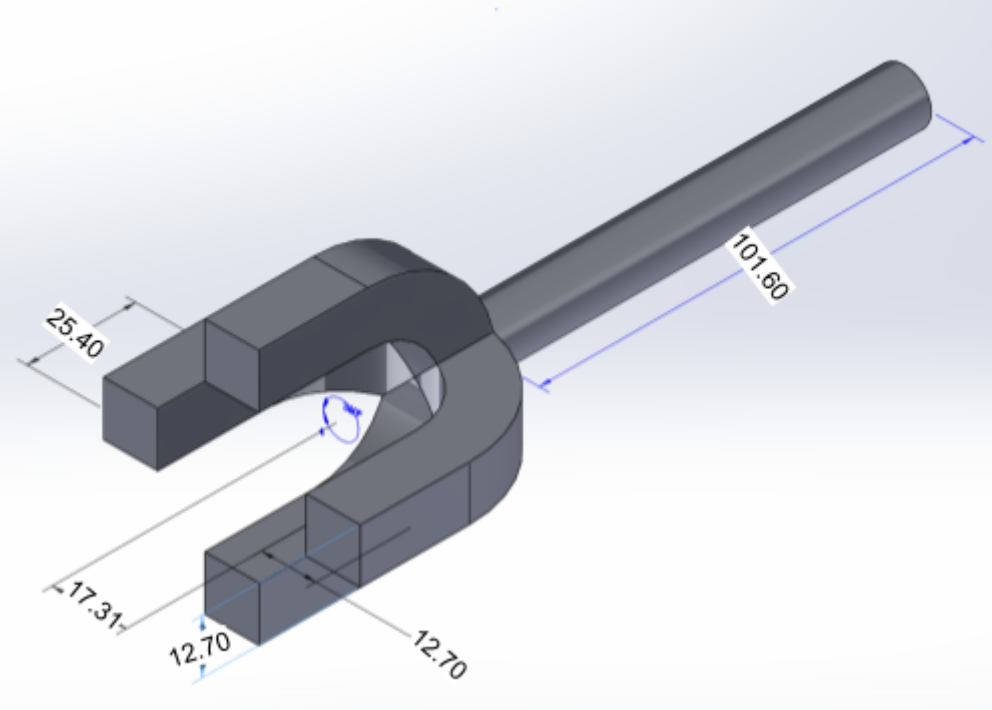


Figure 11: SOLIDWORKS DC Motor Attachment with dimensions shown in mm

Testing

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

Temperature Testing

The ability for the thermistor to accurately record whether the incubator maintains an internal temperature of 37°C ± 0.5°C was evaluated using the Internal Environment - Temperature and Humidity Sensor Testing Protocol[3]. First, the sensor was calibrated using resistance values given by the Arduino website. Once the sensor was calibrated, its precision in a dynamic range was evaluated by first measuring the temperature and humidity of the working environment to gauge if they both worked as expected, and then measured its temperature at extreme high and low temperatures using a hair dryer or heated water cup and freezer.

Next, the accuracy of the thermistor was evaluated by placing it into the lab incubator and ensuring it read the temperature the incubator was set to within an error range of ± 1°C. After placing the sensor in the lab incubator for 10 minutes, the temperature reading was ensured to accurately record the incubator temperature over the entire time interval.

[3] See Appendix C for Testing Protocols

Finally, the temperature sensor was tested within the microscope cell culture incubator itself. The incubator was set up for normal use, and the sensor and a digital thermometer was placed within the incubator before it was sealed. The ability for the incubator to maintain a temperature of 37°C ± 1°C was tested by taking measurements every 10 seconds over a period of 10 minutes and verifying it stayed within the optimal range. Then, the ability for the sensor to accurately measure the temperature within the optimal range was evaluated by taking measurements every 10 seconds over a period of 10 minutes and verifying the thermistor records temperature values of 37°C ± 1°C.

If all these tests were passed, the thermistor and the incubator's ability to maintain the temperature internal conditions was approved. If any of these tests were not verified, then the incubator was reassessed at that point and testing was redone before approval.

CO_2 Testing

The ability for the CO_2 sensor to accurately record whether the incubator maintains an internal environment of 5% ± 0.5% was evaluated using the Internal Environment - CO_2 Sensor and Feedback System Testing Protocol. Once the sensor was calibrated, its precision in a dynamic range was evaluated by ensuring its values increased and decreased with general increase and decrease of CO_2 concentration. The sensor was first tested at standard room conditions to ensure it gave a consistent reading. Then, the sensor was exposed to an increased concentration of CO_2 by having group members breathe on the sensor and the sensor readings were observed to ensure it increased in value. Similarly, the CO_2 supply was cut off and decreased concentration readings from the sensor were verified. If the sensor increased and decreased in CO_2 percentage readings as expected, then its precision in a dynamic range was approved.

Next, the accuracy of the CO_2 sensor was evaluated by placing it into the lab incubator and ensuring it read the concentration the incubator was set to within an error range of ± 0.5%. After placing the sensor in the lab incubator for 10 minutes, the CO_2 sensor reading was ensured to accurately record the incubator temperature over the entire time interval.

Finally, the CO_2 sensor was tested within the microscope cell culture incubator itself. The incubator was set up for normal use, and the sensor and a fyrite were placed within the incubator before it was sealed. The ability for the incubator to maintain a concentration of 5% CO_2 ± 0.5% was tested by taking measurements every 10 seconds over a period of 10 minutes and verifying it stayed within the optimal range. Then, the ability for the sensor to accurately measure the CO_2 concentration within the optimal range was evaluated by taking measurements every 10 seconds over a period of 10 minutes and verifying the sensor recorded concentration values of 5% CO_2 ± 0.5%.

If all these tests were passed, the CO2 sensor and the incubator's ability to maintain the CO_2 internal conditions was approved. If any of these tests were not verified, then the incubator was reassessed at that point and testing was redone before approval.

Optical Testing

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. First, the sheets were evaluated qualitatively. The microscope and its imaging software was prepared for use. Then, one team member placed a prepared slide under a sheet of the High Transparent Lexan Polycarbonate and those two were placed onto the microscope stage. The microscope was then adjusted to the best clarity and an image of what is observed under the microscope was captured. The same procedure was then followed but without the Polycarbonate sheet. To ensure the images quality could be evaluated in a blind and objective fashion, the tester labeled the images and created a key for the naming process. Finally, three team members who were not present for the imaging process assessed the clarity of the two images. Each member chose which image they believed was clearer, or if they looked the same. If the majority saw a difference in clarity between the two images, the test failed and a different transparent material was tested for use. If the majority did not see a difference in clarity between the two images, then the Polycarbonate sheets passed the qualitative test.

In the next testing protocol, the clarity of the Transparent Polycarbonate sheets were evaluated quantitatively. The microscope and its imaging software were prepared for use, and then the same imaging process from before was used to acquire two images of the prepared slide: one with the Polycarbonate sheet and one without. Using ImageJ analysis, the clarity of the images using the microscope focus quality plugin was recorded; the images were divided into gridded squares and each square was assigned a color based on their focus level. The assessments of each image were then compared to evaluate their similarities in clarity. If the majority of the regions in both images were the same, then the Polycarbonate sheets passed the quantitative test and were approved for use in the incubator.

Recovery Testing

The ability of the incubator to return to its internal environment of 37℃, 5% CO_2, and 95-100% humidity after a 30 second opening was evaluated to ensure it returns to these conditions in an efficient manner. The completed incubator was set up for normal use, and the internal conditions were recorded to verify they fall within the correct ranges. Once the ability for the incubator to maintain the internal conditions was confirmed, the data collection from each sensor began. The incubator was then opened for 30 seconds, and it was ensured that each sensor recorded a deviation from the internal conditions. Then, the incubator was closed and a stopwatch was started while conditions were monitored to see if they returned to normal. Once temperature, humidity, and CO_2 individually returned to their respective mark for optimal internal conditions, the time from when the incubator was closed was recorded. If a condition did not return to its range after 5 minutes, this was recorded. If every condition returns to 37℃, 5% CO_2, or 95-100% humidity within 5 minutes after the opening, then the recovery of the incubator was approved. If one of the conditions did not return to its mark, then that condition needed to be

reevaluated and the recovery testing occurred again. This recovery testing ensured that the incubator system can return to optimal homeostatic levels after there is a disruption in the system, validating the effectiveness of the device.

VI. Results

Temperature and Humidity Results

The accuracy and reliability of the thermistor was tested to ensure that the code outputted correct temperature and humidity values, as the internal environment of the incubator is of utmost importance. Following the Temperature and Humidity Sensor Test Protocol[4], the temperature readings for both the thermistor and ECB 1002 Lab Incubator were recorded every 10 seconds for a total of 10 minutes. Next, a two-sample t-Test assuming equal variances was performed to determine the statistical significance between the data obtained. The results showed a p-value of 0.789[5] with a significance value of 0.05, indicating that there is no statistical significance between the thermistor temperature readings and the incubator temperature, proving that the thermistor is working properly.

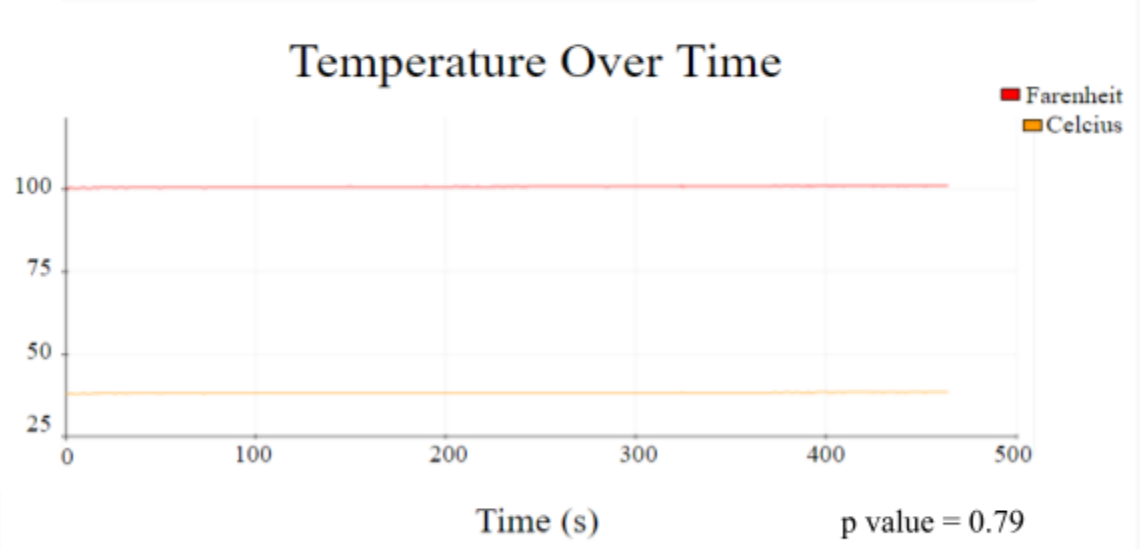


Figure 12: Thermistor Temperature over 10 minute Interval in Lab Incubator

[4] See Appendix C
[5] See Appendix G

The thermistor was coded to calculate humidity, and the accuracy of the formula was tested against a DHT22 temperature and humidity sensor, along with Temperature and Humidity Sensor Test Protocol[6]. Humidity data was collected for twelve and a half minutes using both the thermistor and the DHT22 sensor and a two-sample t-Test assuming equal variances with a significant value of 0.05 was performed to determine the statistical significance between the two collections. The results showed a p-value of 0.943[7], indicating that there was no statistical significance between the two sensors, proving that the humidity formula is working accurately. The Temperature and Humidity Sensor Test Protocol was also passed when the thermistor was placed inside the incubator, validating that the formula provided for the sensor is reliable and accurate.

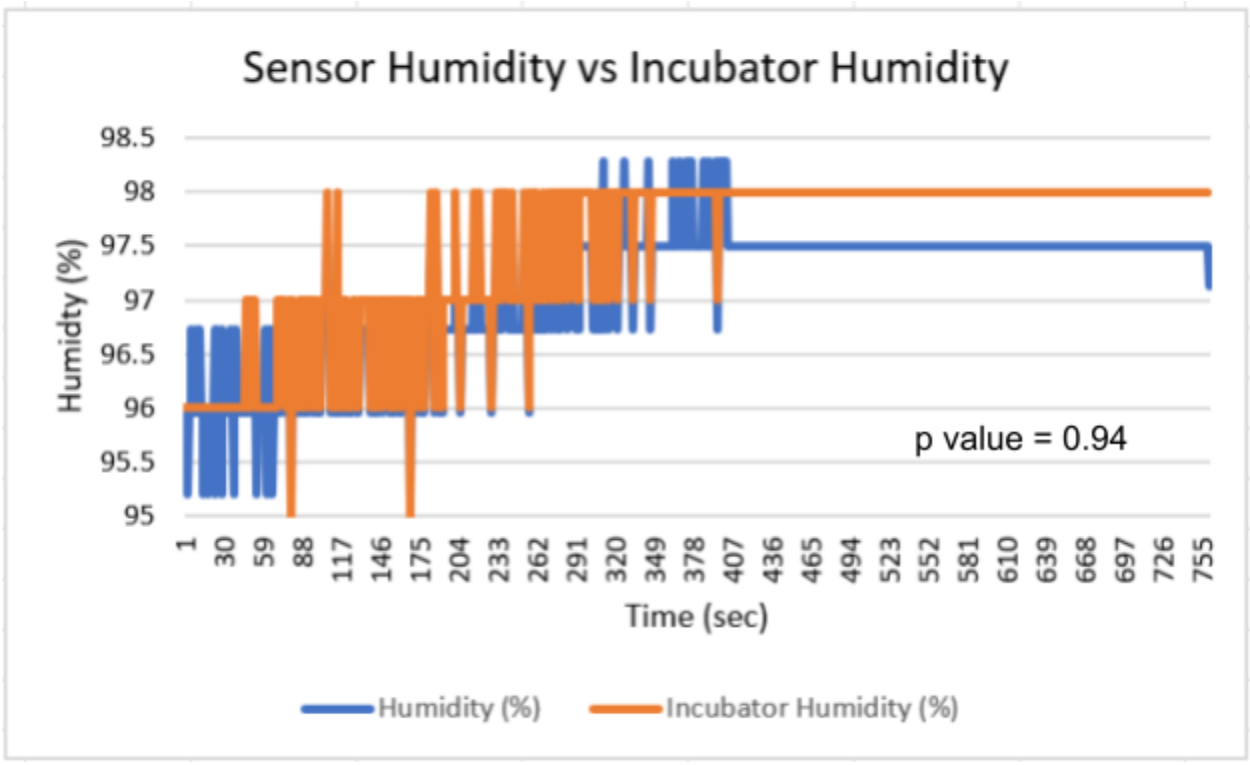


Figure 13: Graph of Humidity Readings in Incubator Over 10 min Time Interval

[6] See Appendix C
[7] See Appendix G

CO_2 Results

The CO_2 sensor was initially tested to ensure accurate sensor readings, before being placed into the fabricated incubator. The CO_2 sensor was placed inside the ECB 1002 Lab incubator and the data was collected for both the sensor and incubator every 10 seconds for a 10 minute interval. A two-sample t-Test assuming equal variances with a significance value of 0.05 was then performed on the resulting values to determine the statistical significance. The results showed a p-value of 0.367[8], indicating that there was no significant difference between the two obtained values. Therefore the CO_2 sensor is working accurately.

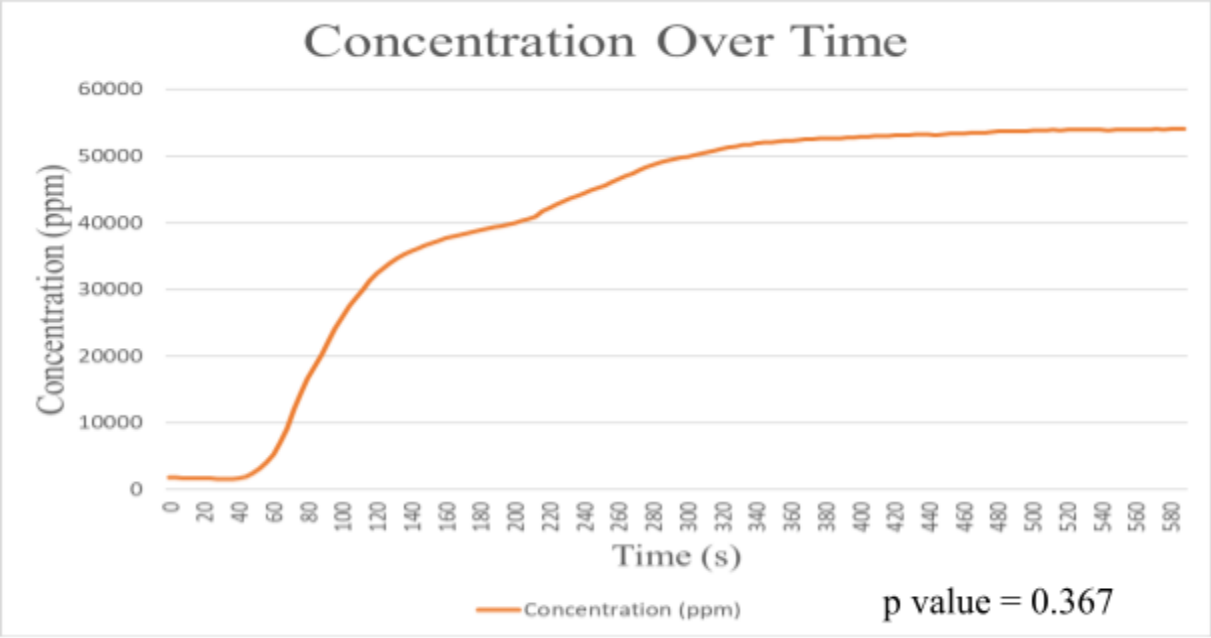


Figure 14: Concentration of CO_2 in Incubator Over approximately 10 minutes

On its own the DC motor was able to spin the DC motor attachment at a high rpm. However, when it was attached to the CO_2 valve for testing the DC motor spun with enough force that the wires broke off of the motor itself. The results show that the DC motor and attachment do not possess the torque needed to turn the valve. See Figure 15 for the DC motor attachment set up and Figure 16 for the broken DC motor.

[8] See Appendix G

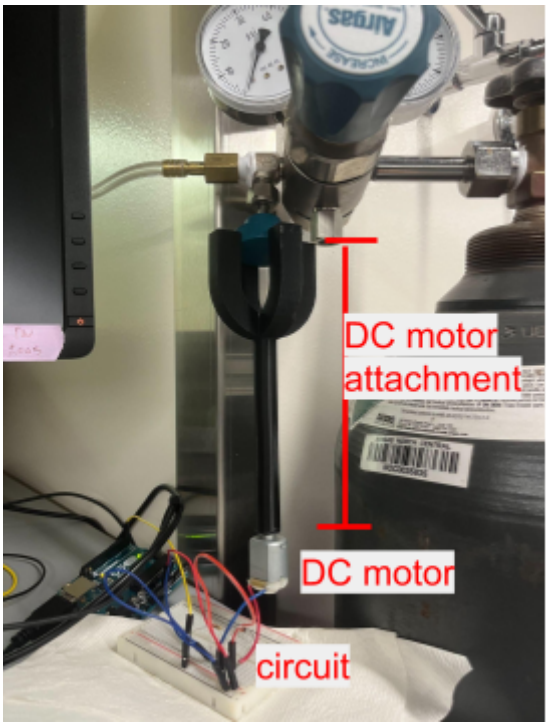


Figure 15: DC Motor and Attachment on CO_2 Tank

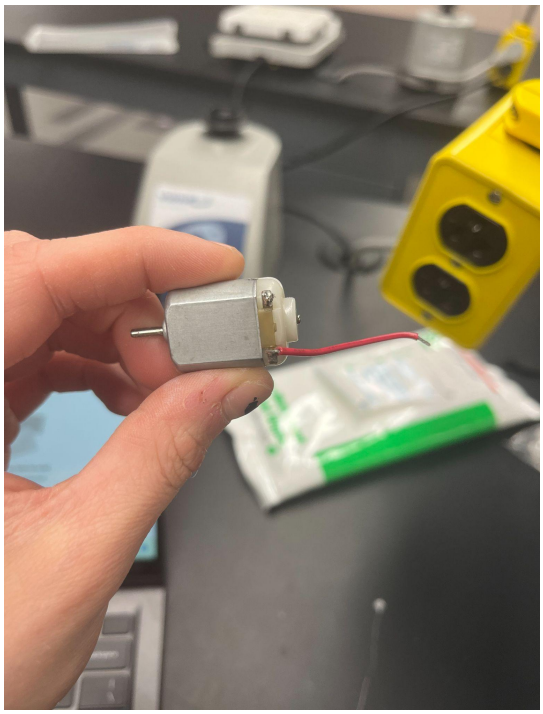


Figure 16: Broken DC Motor

Optical

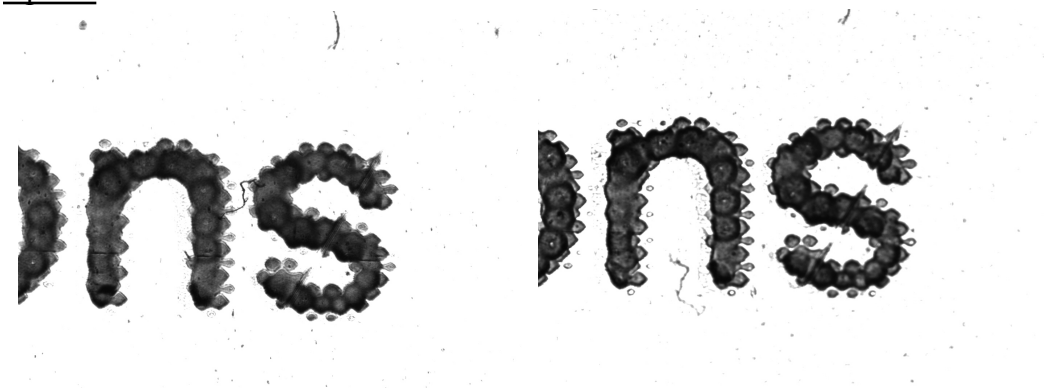


Figure 17: Microscope images with (left) and without polycarbonate sheet (right). The image of the film paper without the polycarbonate sheet has more clarity and a greater focus quality based on qualitative analysis.

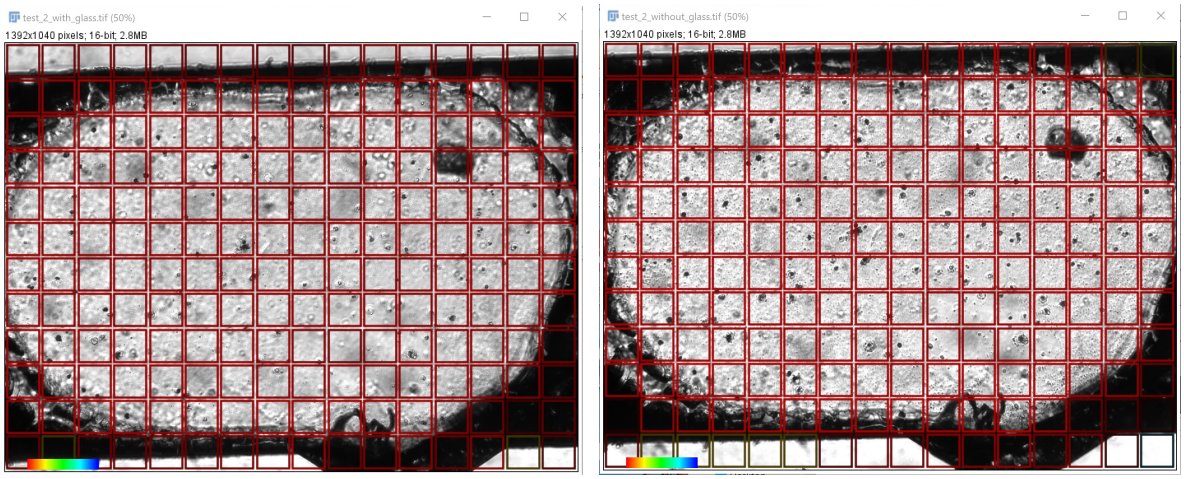


Figure 18: Optical analysis from ImageJ of microscopic cells with glass (left) and without glass (right).

Table 2: Table displaying the number of red (in focus), green (mid focus), and blue (out of focus) squares shown in each image above.

	Microscope Image with Glass	**Microscope Image without Glass**
Red Squares	190	185
Green Squares	2	6
Blue Squares	0	1
Total	192	192

The two optical testing images in Figure 18 above show boxes around the image that outline the clarity and quality of that part of the image. According to the color scale shown at the bottom of both images, the red end of the spectrum indicates that the image is in focus at a specific region, while the blue end of the spectrum indicates that the image is out of focus at a given region. Results from this test show that the image without the glass had a slightly higher, yet very similar focus quality compared to the image with the glass present. Similarly, 100% of randomly selected subjects expressed no difference in clarity between the two optical images. As seen above, the microscope image with glass has slightly more red squares (in focus) and fewer blue squares (out of focus), causing it to have a slightly higher focus quality. However, the two images have very similar values as for each color type as demonstrated in Table 2.

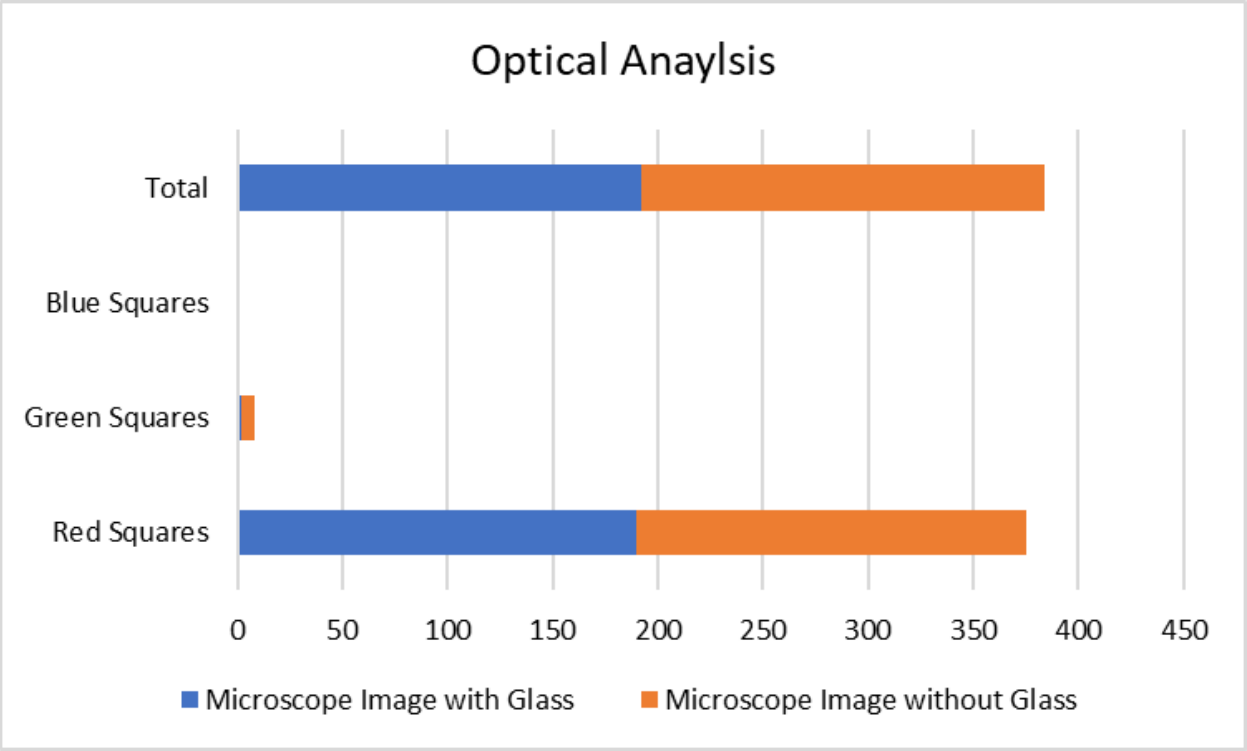


Figure 19: Optical Analysis Stacked Bar Graph of Results

Incubator

The results from testing the incubator's temperature over approximately ten minutes showed an average temperature of 37.6℃. 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℃. This represents the dip in temperature in Figure 9 below. The incubator then had a constant temperature of about 37.6℃ for the remainder of the testing interval. Overall, the results conclude that the temperature inside the incubator is within the standards outlined in the PDS and meets the design requirements. With more tampering of the heated water pump to more precisely get an internal temperature of 37℃ +/- 1℃, the incubator will be able to maintain an appropriate temperature to keep cells viable for up to a week in the teaching lab.

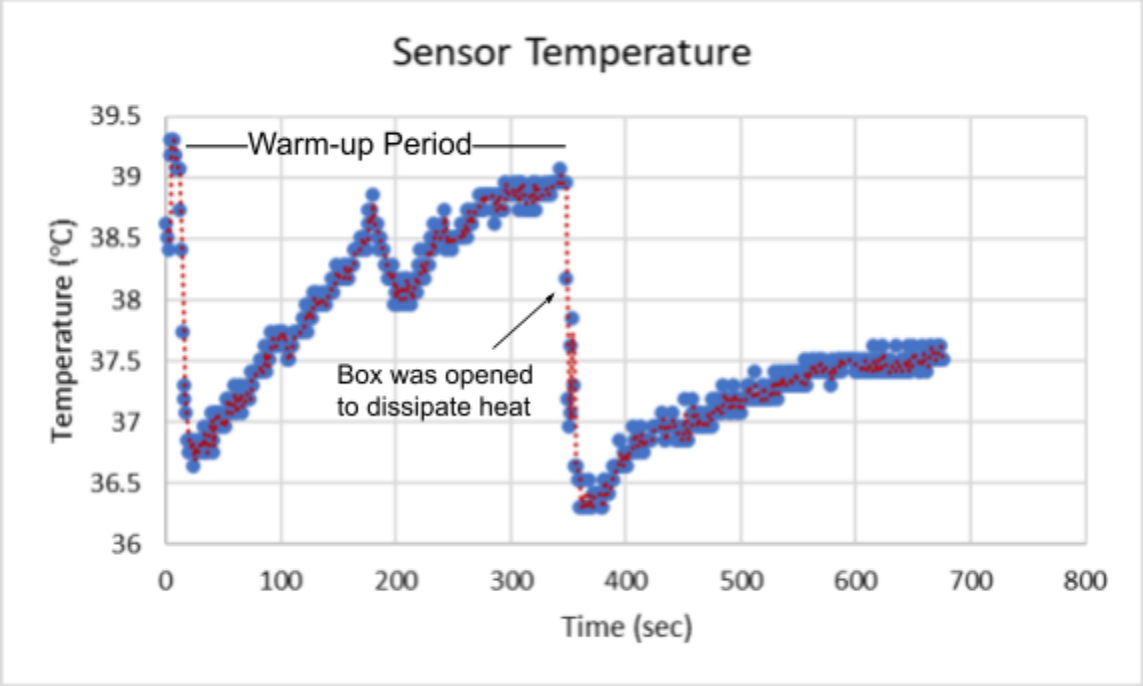


Figure 20: Sensor Temperature in the Incubator over 10 minutes

The results from the incubator's humidity testing initially depicted a decreasing humidity over the ten minute tested time interval. It was concluded that the humidity formula used with the thermistor was inaccurate in some places and the formula was revised by peers to get a more accurate reading. After retesting with the DHT22 sensor to ensure its accuracy, the incubator was set up for a ten minute test trial according to the Temperature and Humidity Testing Protocol[9]. The results proved over a ten minute interval that the humidity was within the range set forth by the design requirements, with an average humidity of 97.1% over the course of testing. Overall, the results show that the incubation design and heated water pump was able to provide an internal humidity environment that will promote cell growth over the course of a week in the teaching lab.

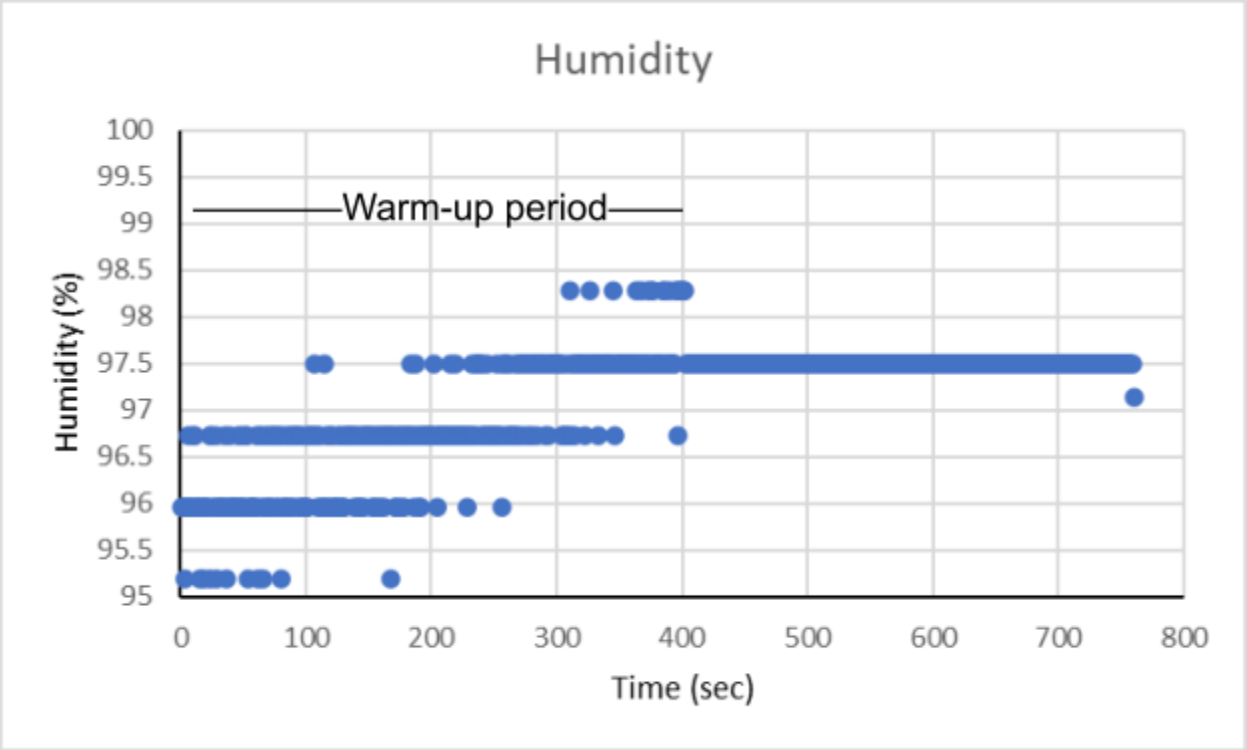


Figure 21: Sensor Humidity Results Over approximately 10 minutes

[9] See Appendix C

Recovery

Recovery testing was completed according to Recovery Test Protocols 1 and 2[10]. The results of Recovery Test 1 showed that after 30 seconds of disruption in the incubation chamber the temperature was able to reach optimal conditions within approximately three minutes. These results are in line with the specifications outlined in the PDS[11].

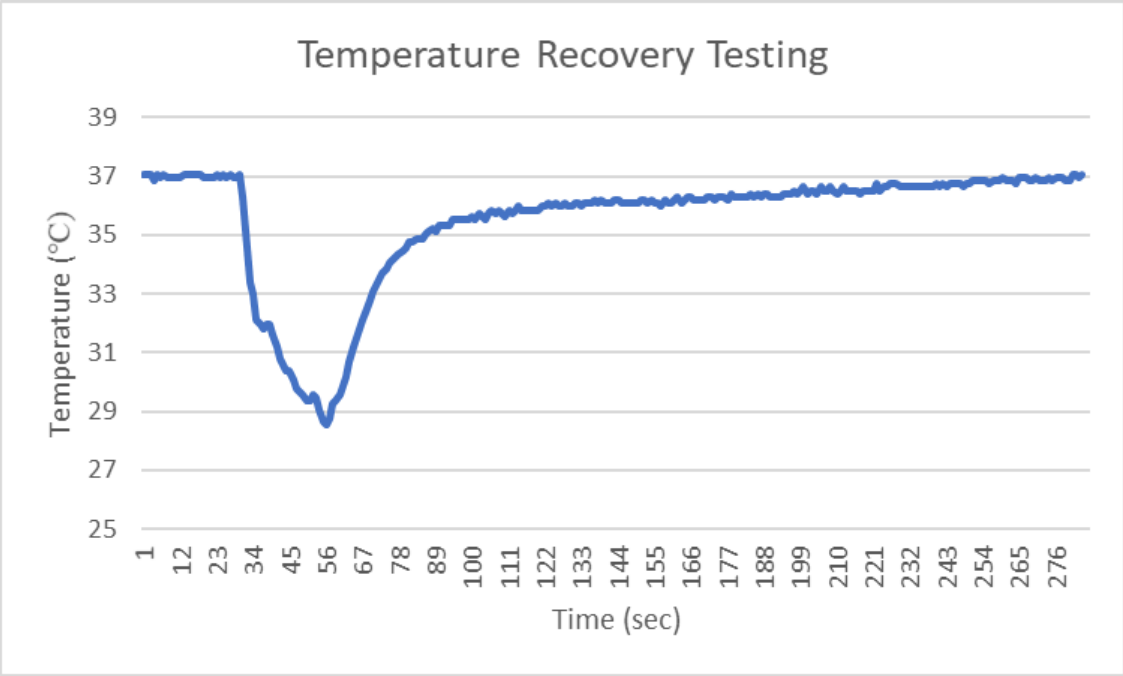


Figure 22: Temperature Recovery Testing

[10] See Appendix C
[11] See Appendix A

The results of Recovery Test 2 showed that after 30 seconds of disruption in the incubation chamber, the humidity was able to reach optimal conditions after 3 minutes and 23 seconds. These results are consistent with the specifications outlined in the PDS[12]. However, humidity values during testing went over 100% which is not theoretically possible. It was concluded that supersaturation, when the air temperature falls below dew point, was responsible for the increased humidity output [22]. Supersaturation values caused the relative humidity formula to respond with humidity values over 100%. The team concluded that although these values are over 100%, the recovery testing was still accurate and showed optimal recovery time.

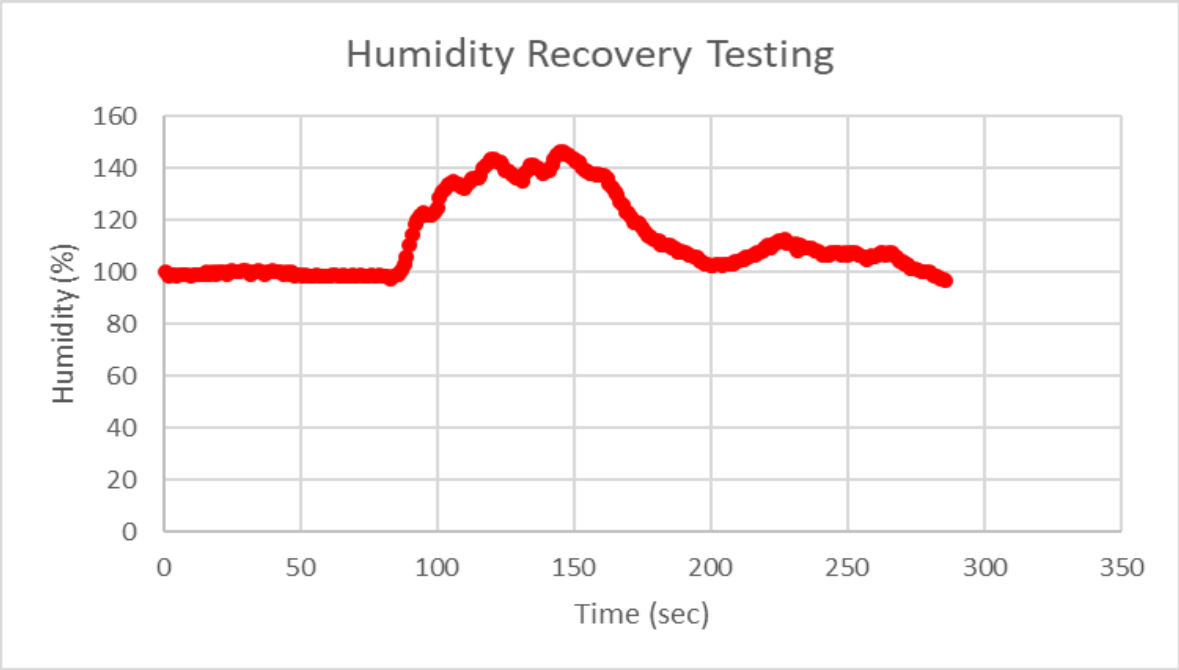


Figure 23: Humidity Recovery Testing

[12] See Appendix A

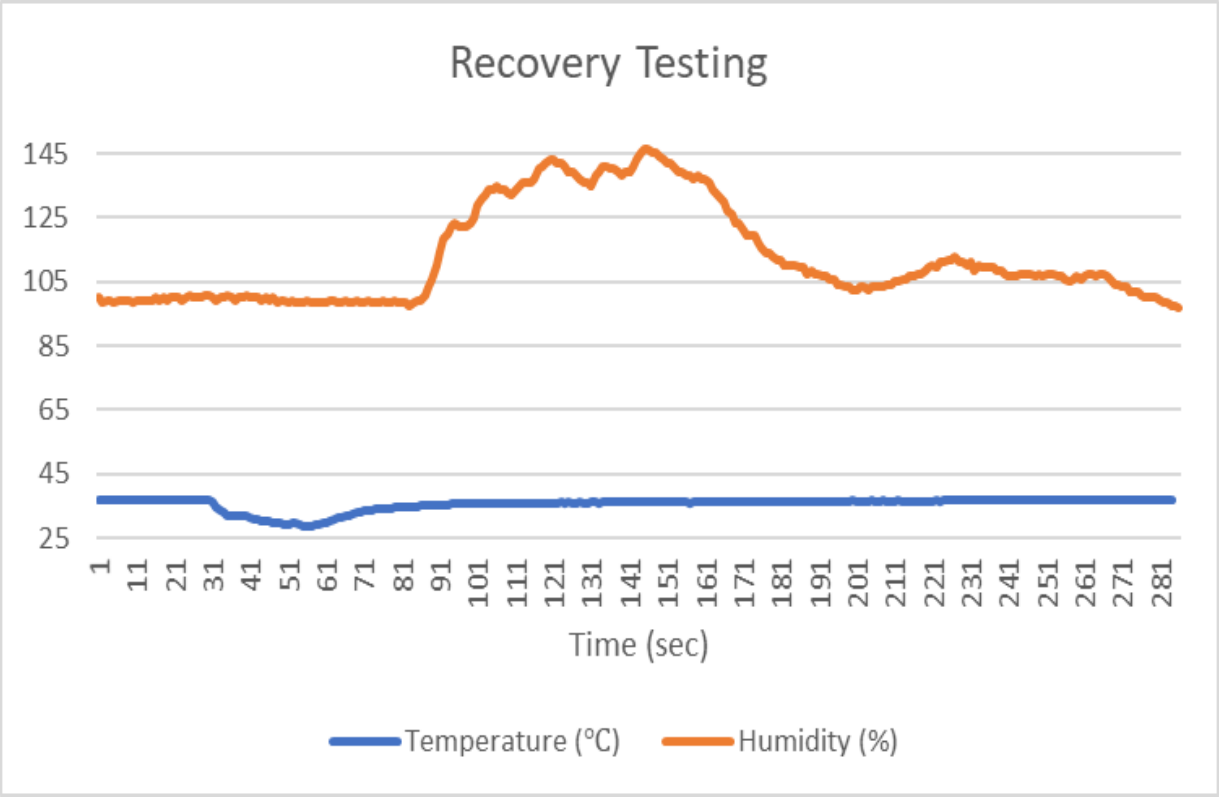


Figure 24: Combined Recovery Testing data

Overall, the recovery testing results show that the incubation chamber is able to withstand short disruptions and will recover completely after approximately 3 minutes and 30 seconds, which is in line with the specifications outlined previously. This allows more ease of use to the product and is optimal for its use in the teaching lab, as the conditions would need to be disrupted for students who are not accustomed to imaging live cells.

VII. Discussion

The results from each initial sensor testing showed that every sensor used was both within the range set forth by the PDS and accurately measured values as compared to a standard incubator. The thermistor was compared to a standard incubator and was not statistically significant with a p value of 0.789. This implies that the code is correct and the circuitry is correctly fabricated. The formula for relative humidity was calculated and tested against both a DHT22 sensor and an incubator. The humidity formula on the thermistor's code was not statistically significant to the DHT22 sensor or lab incubator, both initially and when it was revised, with a p-value of 0.94. This implies that the sensor is working accurately in the incubator. The CO_2 NDIR sensor was tested against the lab incubator and showed a p-value of 0.37 indicating that both the code and circuitry for this sensor are correct.

The optical analysis proved that there was no statistical difference in the optical clarity or microscopic focus quality when images were taken with and without glass ($p > 0.05$). This supported the desired trend, qualitatively and quantitatively, because it demonstrated that the integrity and functionality of the microscope's optics were not compromised, which was a key criterion of the final design.

The incubation testing results proved that temperature and humidity were able to meet the PDS requirements[13]. After testing both variables over the course of a ten minute time period, it was determined that the copper tubing allowed for significant heat transfer. The water pump and copper tubing were able to heat the incubator to a maximum temperature of 39℃, with the average temperature over the testing period being 37.6℃. This implies that the incubator is within the temperature standard of +/- 1℃. Humidity was also tested during the same interval. Humidity had an average value of 97.1% over the course of the ten minute interval which is within the standard of >95% set forth in the PDS. Both temperature and humidity results imply that the incubation chamber designed this semester is capable of growing live cells in the teaching lab.

The results from the DC motor attachment proved that although the DC motor was able to spin the attachment, but the motor didn't have enough torque required to turn the CO_2 valve. This means that the CO_2 input for the incubator was not achieved this semester and must be reworked if the project is to continue. Further considerations should be made to the torque of the valve, types of valves that are within the budget, and the mechanical advantages of different motors in terms of torque.

The changes that need to be made are the regulations for CO_2 input. The DC motor attachment this semester could not withstand the torque needed to turn the valve. In the future, a different valve, such as a solenoid valve, could be considered or a stronger DC motor in order to output CO_2 from the 100% CO_2 tank into the incubator. The overall aesthetic of the box should also be improved in the coming semesters. The box currently has the glass plates held on via hot glue, however that provides a messy finish. Further considerations should be made on more aesthetically pleasing gluing methods, as this will be used in the teaching lab and should look professional. The humidity also has a tendency to occasionally fog up the glass, so considerations should be made as to ways to prevent this in the future using a transparent water repellent or better overall sealant of the internal environment. The electronic circuit should also be soldered using electric solder in order to reduce the amount of set up being used for each electronic test. Further considerations should be made on ways to attach the electronic breadboards onto the incubation chamber in a more aesthetically pleasing way, both to reduce the risk of user error and enhance ease of use. In the future, the team would like to be able to conduct live cell viability and imaging tests in order to determine the accuracy of the design in vitro.

Possible sources of error throughout the project could be found during temperature and humidity testing. The laser cut CO_2 input hole was not covered during temperature and humidity testing which could lead to leakage of the internal environment. The hot glue used to fasten the

[13] See Appendix A

transparent glass and rubber lining had places where the materials were not secure, which could cause leakage of the internal environment as well. The glass plates were also coated with a water-proof repellent during the last week of testing which fully compromised the integrity of the glass. Therefore, while the glass is optically usable, these optics were ruined while trying to reduce the humidity fogging. Additional methods should be used to reduce the amount of fogging without compromising the integrity of the glass.

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 is 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 [20]. 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 [21]. 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 [20]. 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.

VIII. Conclusion

Cell culture incubators must maintain a very stable microenvironment for growth and development of cells. The internal environment must have regulated temperature, humidity, CO_2, and pH levels. Current incubators on the market can be expensive, encompass the whole incubator, and are difficult to use. 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 within the body. The team developed an incubator compatible with an inverted microscope that utilized CO_2 input and a water bath with controllable temperature via a heated water pump which circulated water through copper tubing placed in the well to maintain an environment of 37℃ ± 1°C, 5% ± 0.5% CO_2, and 95-100% humidity. Glass lined both the top and bottom of the

incubator to allow for clear viewing. The top of the box was lined with rubber to provide a tighter seal for the lid to prevent leakage and increase humidity levels. The interior of the water bath was lined with silicone caulk to prevent water and gas from escaping through the cracks where the acrylic pieces were cemented together. Previous semester's incubator yielded lots of leakage due to suspected micropores in the PLA plastic prototype and this semester recorded no water leakage of the internal environment. Temperature, humidity, and recovery testing all produced desired results. The final prototype was able to maintain a constant internal environment of 37.6℃ and >95% humidity on average. The incubator was able to recover, and return to these conditions within four minutes after a disturbance. The team was successful in maintaining the budget requirements with a total production cost of $53.45. However, CO_2 regulation still requires further work to meet design requirements. The DC motor, purchased from the UW-Madison Makerspace, did not provide the necessary torque to turn the 3D printed PLA motor attachment connected to the CO_2 valve. In the future, the use of a solenoid valve or a more powerful DC motor should be used in order to provide successful CO_2 input. Live cell testing can be conducted in the future along with a newly fabricated design to make the project more aesthetically appealing. In the distant future, the team hopes to integrate a homemade heated water pump into the design, rather than the machine from the lab utilized this year. This would allow the team to not only incorporate a closed loop automated temperature control system into the design, but also to potentially market the design as an all-encompassing, compact set which would allow buyers to purchase the entire incubator setup.

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X. Appendix

Appendix A: Product Design Specifications (PDS)

Function: Develop a low cost cell culture incubation chamber with interchangeable culture plates that is compatible with an inverted microscope and capable of live cell imaging.

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 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 ten 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].The bottom and top of the incubator will be transparent. 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 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.
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. 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 Fall 2021

Final Design

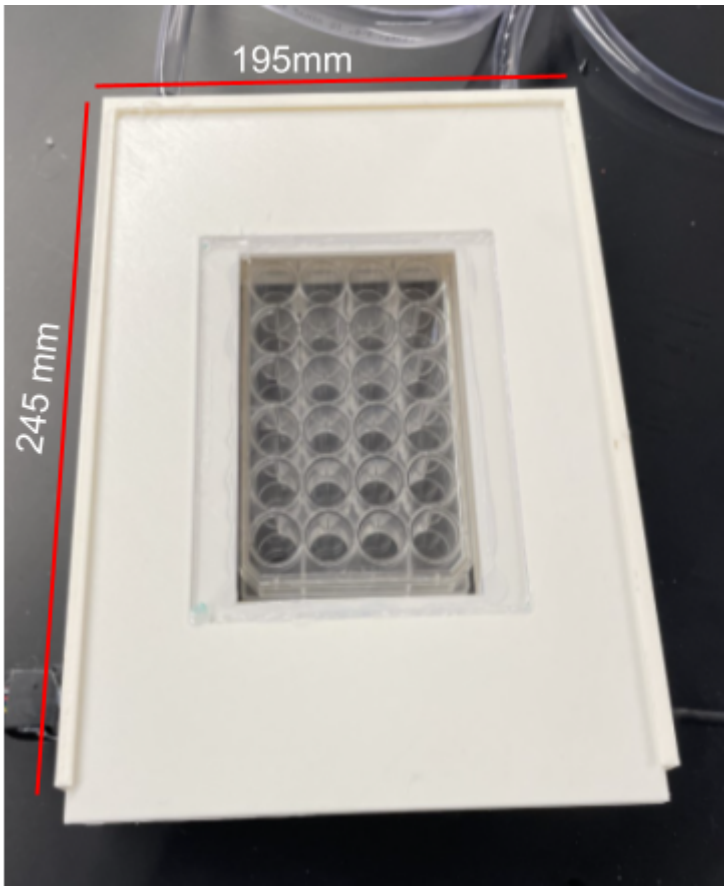


Figure 1: External View of Incubator with a closed lid and visible well plate

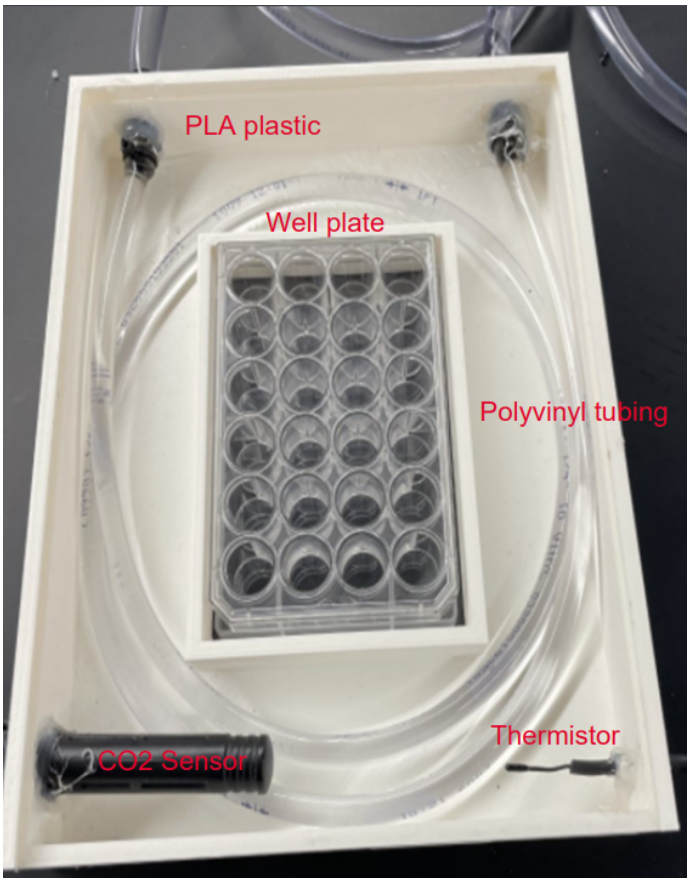


Figure 2: Internal View of Incubator without the lid, highlighting the inner polyvinyl tubing, CO_2 sensor, and thermistor.

SOLIDWORKS CAD Drawing of the Proposed Cell Culture Incubator

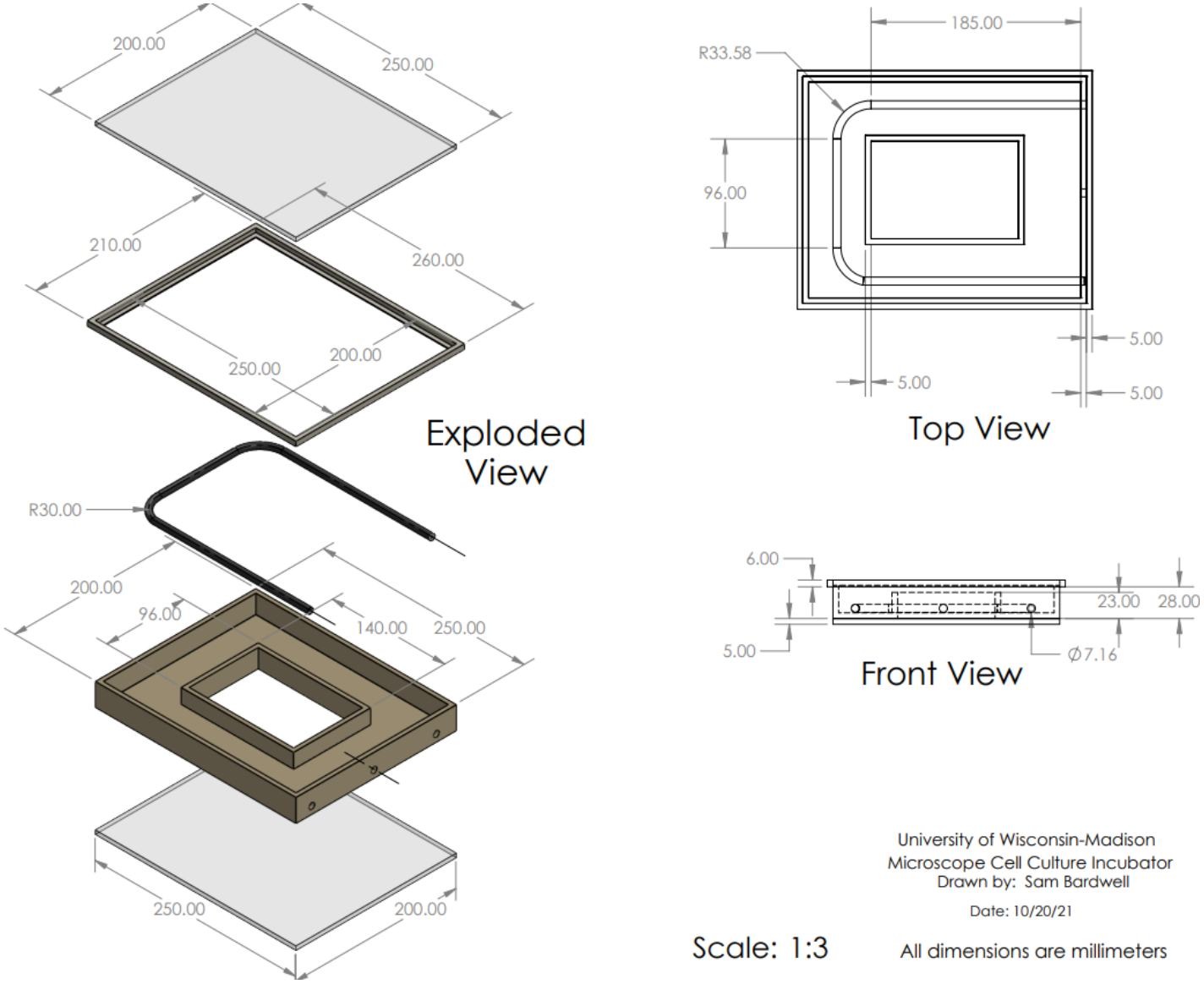
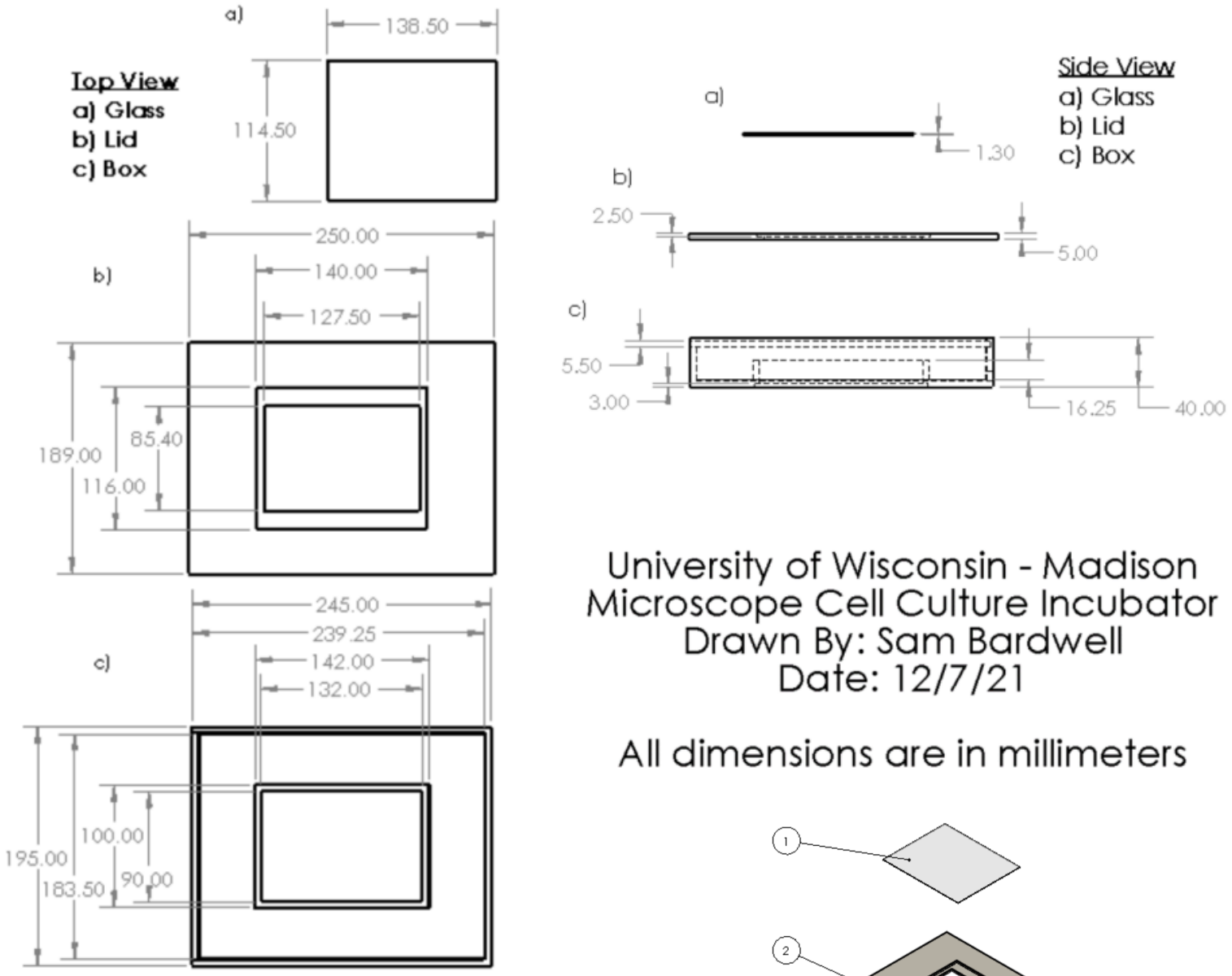


Figure 3: SOLIDWORKS Drawing of Design #2



Item No.	Item Description	Dimensions [mm]	QTY.
1	Glass plates to allow transparent viewing	114.5 x 138.5 x 1.3	2
2	Top removeable crown to allow access to interior	Outside: 189 x 250 x 5 Inside Cut: 85.4 x 127.5 x 2.5	1
3	Well Plate to hold cells	85.4 x 127.5 x 22.5	1
4	Inner heated water pump tubing	Outer Diameter: 12.7 Inner Diameter: 9.525	1
5	Incubator box to maintain a controlled internal environment	Outside: 195 x 245 x 40 Inside Cut: 90 x 132 19	1

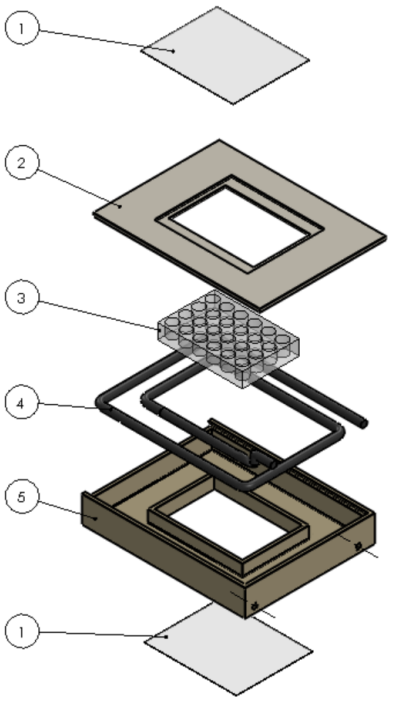


Figure 4: Exploded SOLIDWORKS assembly of the final design along with a table explaining the dimensions and parts

Boot up Process

1) Remove sliding crown from incubator
2) Connect heated water pump tubing to the ribbed cone adaptor on incubator
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
6) Fill incubator with enough DI water to submerge inner tubing
7) Turn on CO_2 tank and gauge to fill the internal environment to 5% CO_2 levels
8) Replace sliding crown back on the incubator
9) Allow time for internal environment to be set to 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) Slide open crown seal 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) Slide crown seal back into place on 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 and inner 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

Thermistor Circuit Diagram and Code

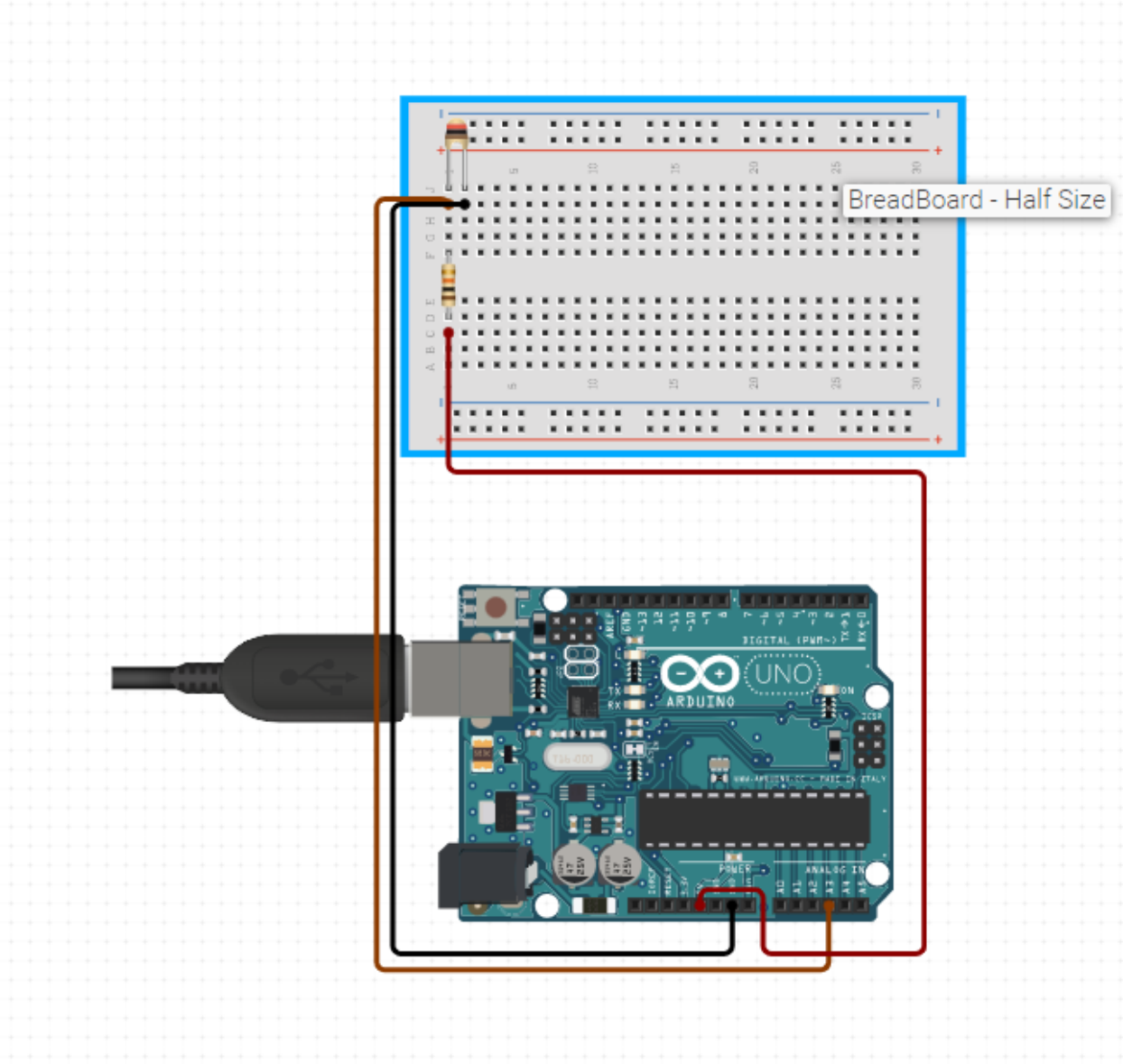


Figure 5: Thermistor Circuit Diagram

Arduino Code

```
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 = 0;
```

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

void loop() {

  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");



  delay(500);
}
```

CO_2 Sensor Code and Circuit Diagram

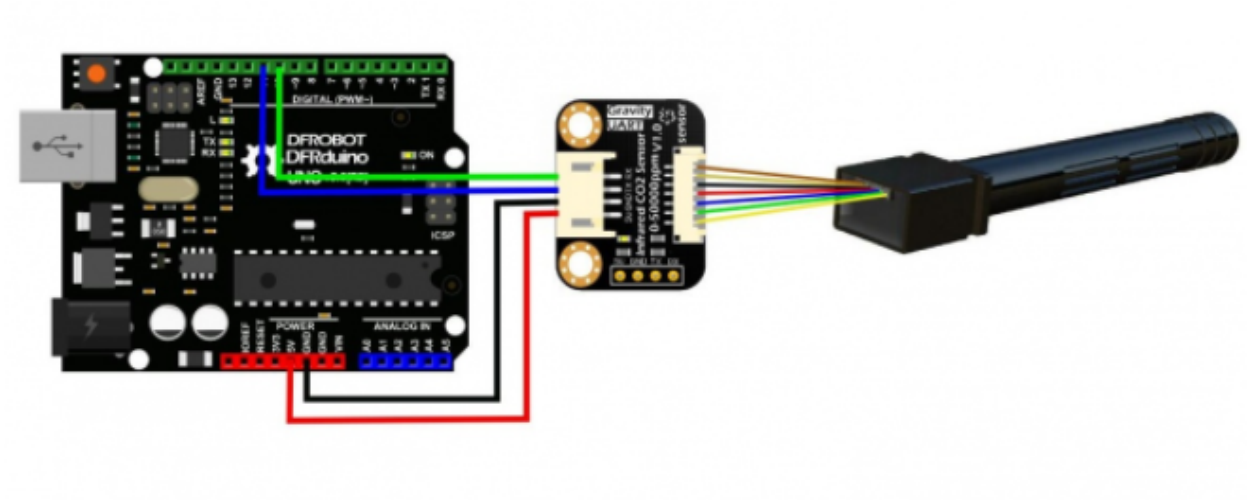


Figure 6: CO_2 Sensor Circuit Diagram [1]

Arduino Code

```
#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;
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() {
  if (mySensor.measure()) {
    Serial.print("CO2 Concentration is ");
    Serial.print(mySensor.ppm);
```

```
    Serial.println("ppm");
    Serial.print("Percent CO2 is ");
    Serial.print((mySensor.ppm/10000));
    Serial.println("%");
  } else {
    Serial.println("Sensor communication error.");
  }
 delay(1000);
  }
```

References

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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 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 extreme high and low temperatures. Afterwards, 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 extreme 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 Comments:		

	verified.			
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 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	☐ Verified Comments:		

	given by the fyrite, the value given by the CO_2 sensor, and the trial number in the 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	☐ Verified Comments:		

	does not occur, stop protocol and record what happened in the 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 smallest element observed without distinct image contrast indicates the approximate resolution limit. Record the group number and	☐ Verified Comments:		

	element number selected by each member in the comments. The team member selecting the resolution limit should assess the image in a blind fashion.			
5	Using the tables and resolution equation provided, calculate the resolution from each team member and the average resolution. Record these numbers in the comments. Higher resolution (lp/mm) is better resolution, and a smaller difference between with the glass and without is better.	☐ Verified 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	☐ Verified Comments:		

	and their similarity.			

Recovery Test Protocol

Introduction

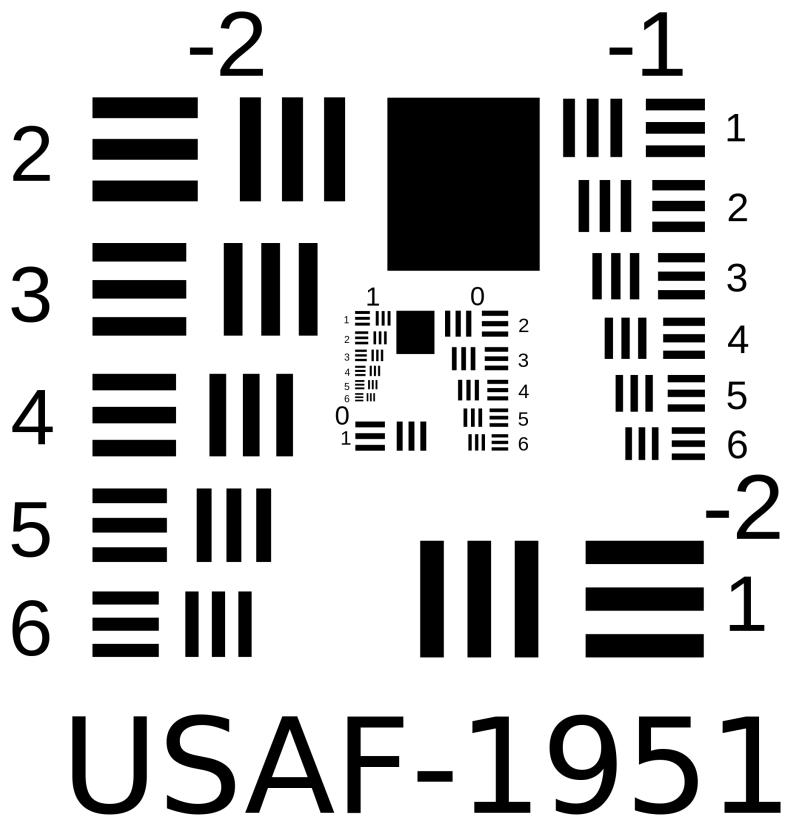
Name of Tester:
Dates of Test Performance:
Site of Test Performance:

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:		

USAF 1951 Resolution Test Chart:



$$\text{Resolution (lp/mm)} = 2^{\text{Group}+(\text{element}-1)/6}$$

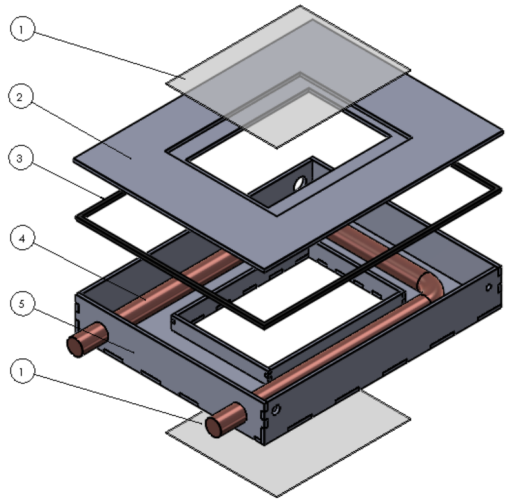
Number of Line Pairs / mm in USAF Resolving Power Test Target 1951

	Group Number											
Element	−2	−1	0	1	2	3	4	5	6	7	8	9
1	0.250	0.500	1.00	2.00	4.00	8.00	16.00	32.0	64.0	128.0	256.0	512.0
2	0.281	0.561	1.12	2.24	4.49	8.98	17.96	35.9	71.8	143.7	287.4	574.7
3	0.315	0.630	1.26	2.52	5.04	10.08	20.16	40.3	80.6	161.3	322.5	645.1
4	0.354	0.707	1.41	2.83	5.66	11.31	22.63	45.3	90.5	181.0	362.0	724.1
5	0.397	0.794	1.59	3.17	6.35	12.70	25.40	50.8	101.6	203.2	406.4	812.7
6	0.445	0.891	1.78	3.56	7.13	14.25	28.51	57.0	114.0	228.1	456.1	912.3

Width of 1 line in micrometers in USAF Resolving Power Test Target 1951

	Group Number											
Element	**−2**	**−1**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	2000.00	1000.00	500.00	250.00	125.00	62.50	31.25	15.63	7.81	3.91	1.95	0.98
2	1781.80	890.90	445.45	222.72	111.36	55.68	27.84	13.92	6.96	3.48	1.74	0.87
3	1587.40	793.70	396.85	198.43	99.21	49.61	24.80	12.40	6.20	3.10	1.55	0.78
4	1414.21	707.11	353.55	176.78	88.39	44.19	22.10	11.05	5.52	2.76	1.38	0.69
5	1259.92	629.96	314.98	157.49	78.75	39.37	19.69	9.84	4.92	2.46	1.23	0.62
6	1122.46	561.23	280.62	140.31	70.15	35.08	17.54	8.77	4.38	2.19	1.10	0.55

Appendix D: Final Design SOLIDWORKS Drawing 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 1: 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

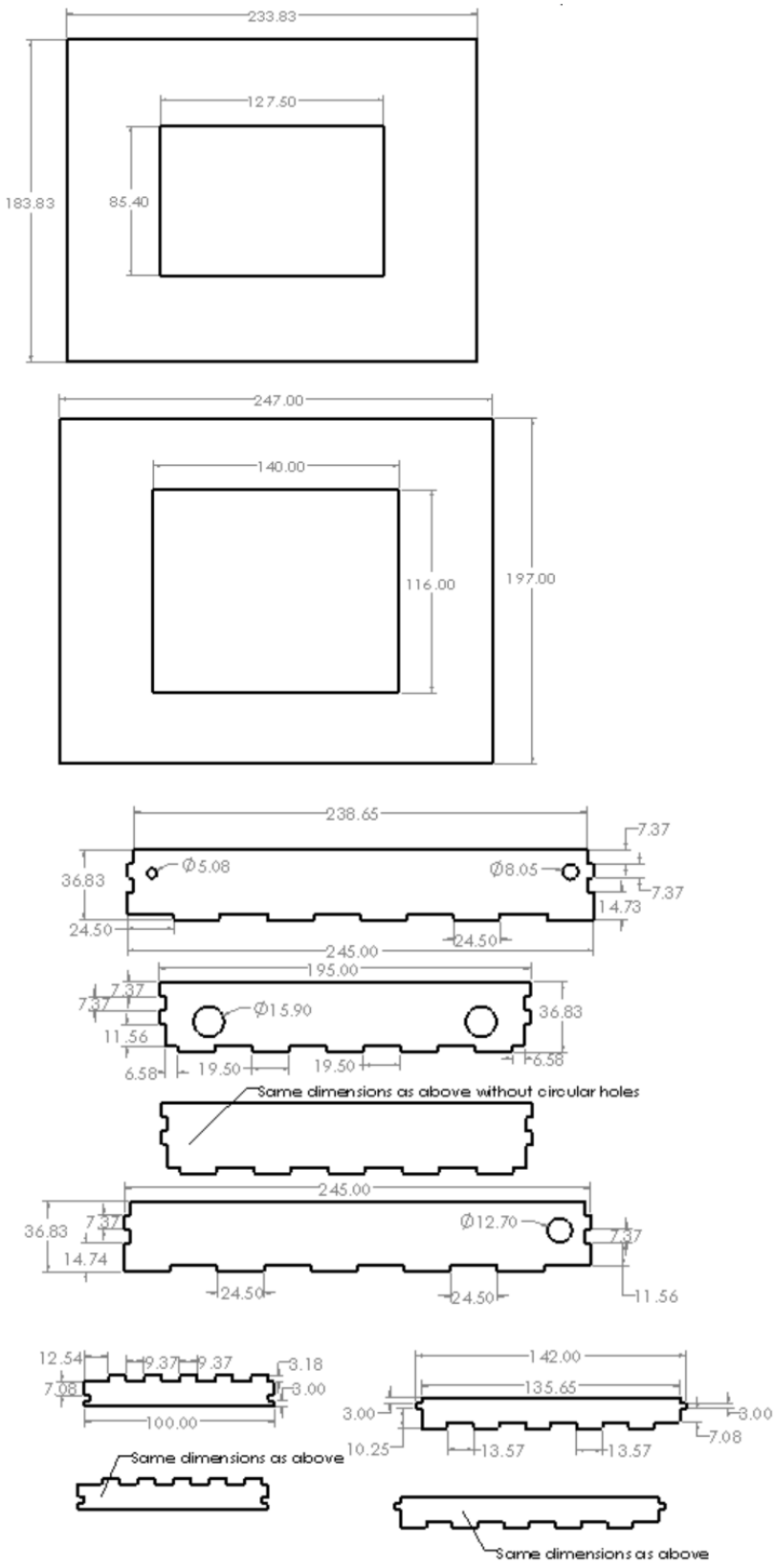
1) Open lid to expose well plate cavity
2) Insert a 138mm x 95mm or smaller well plate into designated cavity
    a) DO NOT use a well plate larger than dimensions given
3) Replace lid back onto incubator
    a) Make sure seal is firmly in place
    b) DO NOT open until data acquisition is complete and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

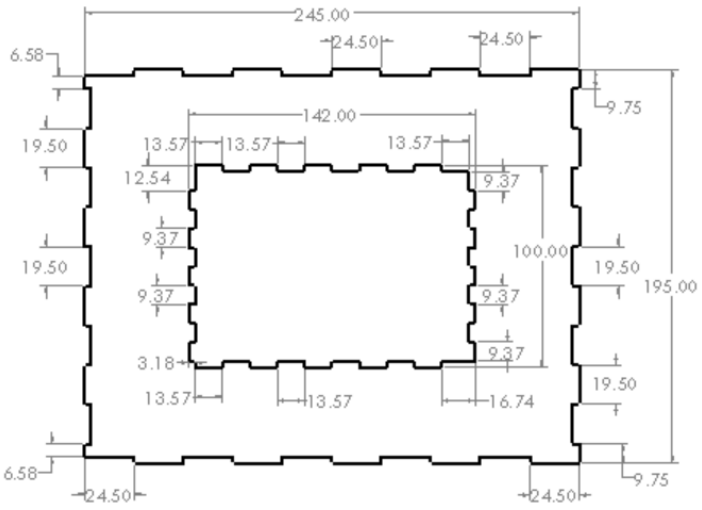
Data Acquisition

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

Appendix E: DC Motor Attachment SOLIDWORKS Drawing

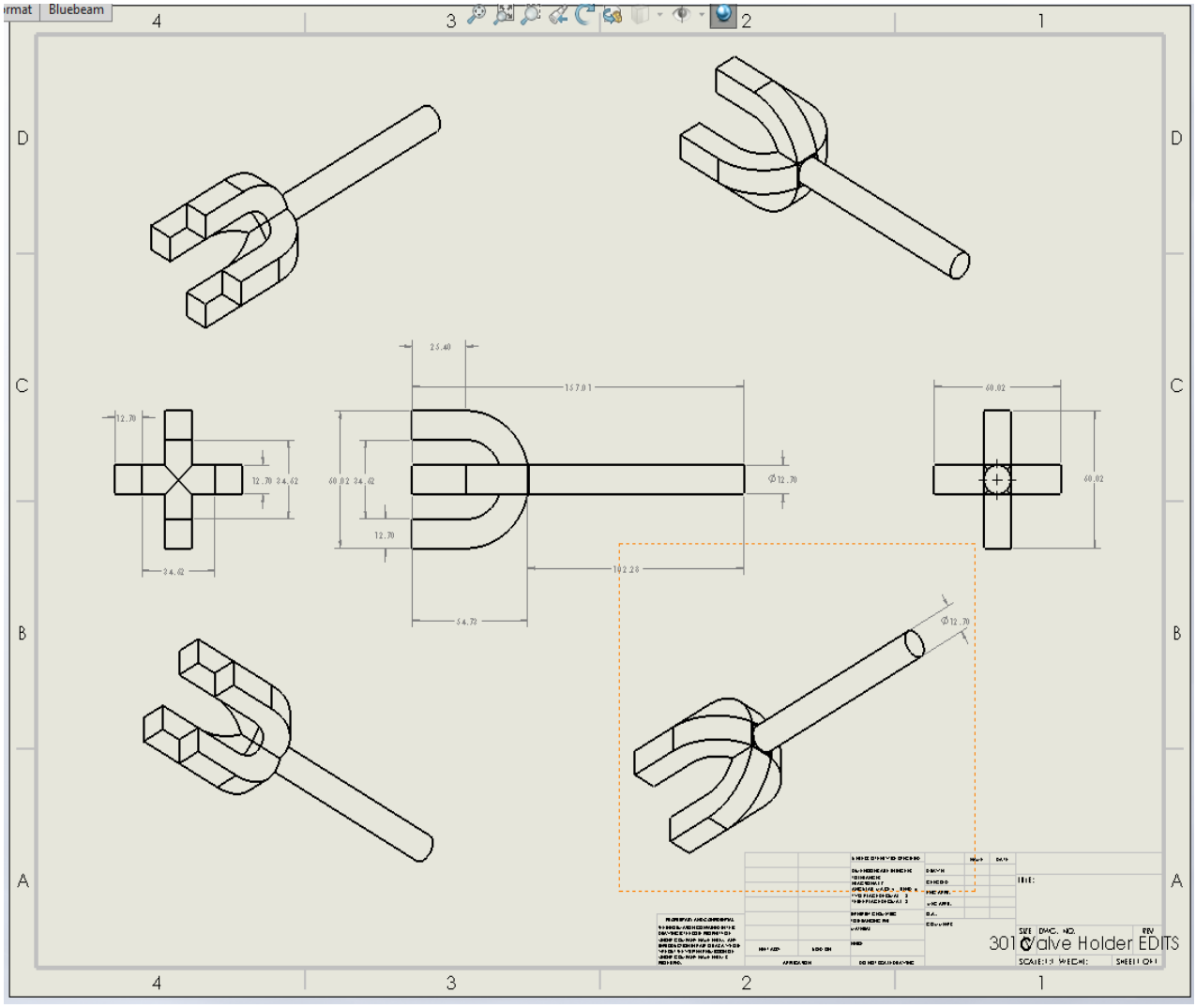


Figure 1: DC Motor attachment/CO_2 Valve Holder SOLIDWORKS drawing (dimensions in mm)

Appendix F: Circuit Diagrams and Final Code

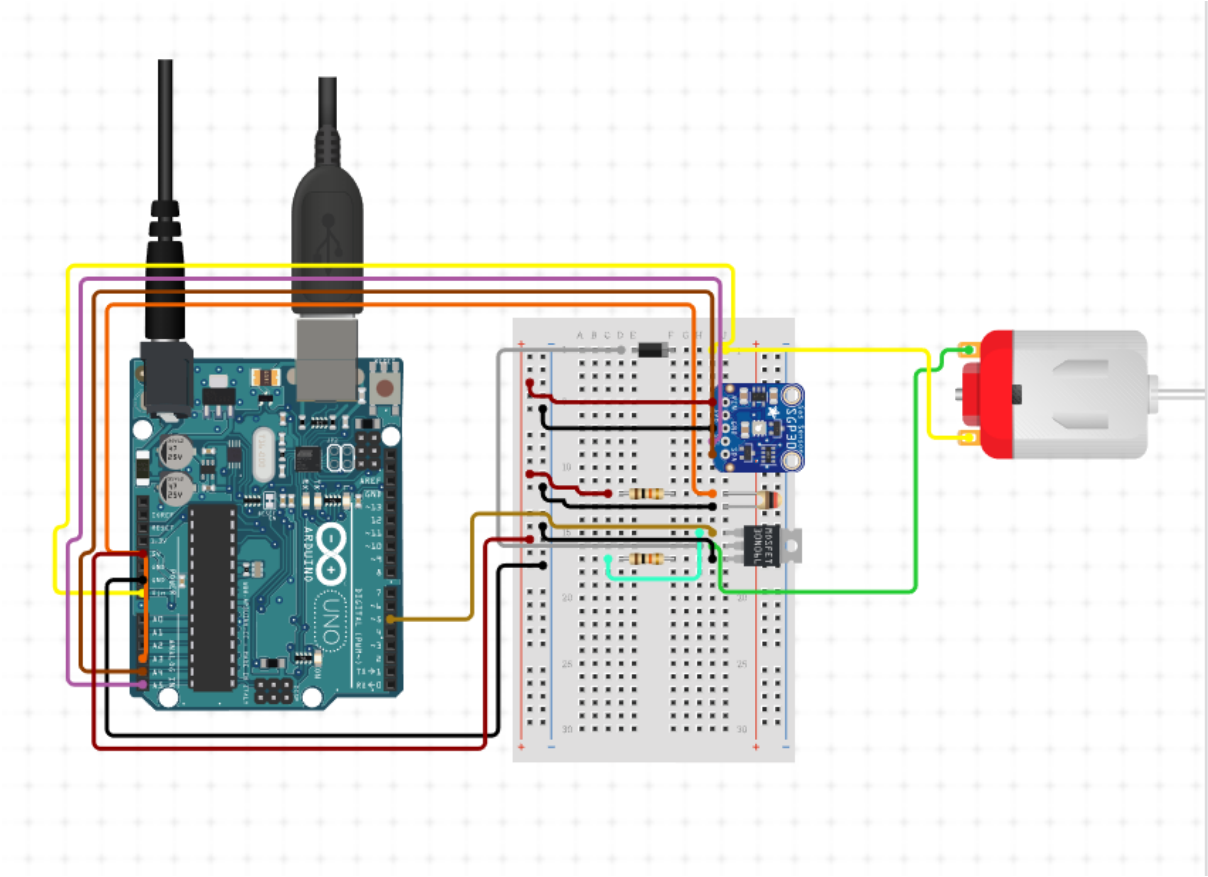


Figure 1: Complete Incubator Circuit Design

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) ;
 }
}
```

Appendix G: Statistical Analysis Data

Temperature Results

Table 1: Temperature Sensor T-Test

Temperature t-Test: Two-Sample Assuming Equal Variances		
	Variable 1	*Variable 2*
Mean	35.6	35.423
Variance	#DIV/0!	0.427998
Observations	1	60
Pooled Variance	0.427998	
Hypothesized Mean Difference	0	
df	59	
t Stat	0.268326	
P(T<=t) one-tail	0.394692	
t Critical one-tail	1.671093	
P(T<=t) two-tail	0.789384	
t Critical two-tail	2.000995	

Humidity Results

Table 2: Humidity Sensor t-Test

Humidity t-Test: Two-Sample Assuming Equal Variances		
	Variable 1	Variable 2
Mean	27.71556	27.73107
Variance	#DIV/0!	0.047972
Observations	1	149
Pooled Variance	0.047972	
Hypothesized Mean Difference	0	
df	148	
t Stat	-0.07062	
P(T<=t) one-tail	0.4719	
t Critical one-tail	1.655215	
P(T<=t) two-tail	0.943799	
t Critical two-tail	1.976122	

CO_2 Results

Table 3: CO_2 Sensor t-Test

CO2 t-Test: Two-Sample Assuming Equal Variances		
	Variable 1	Variable 2
Mean	5.006936	5.402139
Variance	#DIV/0!	0.189928
Observations	1	173
Pooled Variance	0.189928	
Hypothesized Mean Difference	0	
df	172	
t Stat	-0.90422	
P(T<=t) one-tail	0.183572	
t Critical one-tail	1.653761	
P(T<=t) two-tail	0.367144	
t Critical two-tail	1.973852	

Appendix H: Materials and Expenses

Item	Description	Manufacturer	Part Number	Date	QTY	Cost Each	Total	Link
Component 1								
Polycarbonate Transparent Thermal Insulation Sheets	2”x4.25” clear Polycarbonate safety plate for covering cells while viewing	Airgas	RAD64005012	3/9/22	4	$0.53	$2.12	Link
Component 2								
Acrylic Contact Cement	1 oz Clear Contact Cement to mount clasps and assemble acrylic box	Grainger	3EHR7	3/9/22	2	$2.73	$5.46	Link
Component 3								
Buna-N Square Rubber Cord	5ft, ⅛” x ⅛”, 70A, 0℃ - 210℃ square rubber cord to prevent leakage with clasp lid	Grainger	784U15	3/9/22	1	$4.86	$4.86	Link
Component 4								
Hard Wood	36x24x ⅛ Hard wood that was used to fabricate the prototype	UW Makerspace	1	3/21/2022	1	$2.50	$2.50	Link
Component 5								
Hard Wood	18x24x ⅛ Hard wood that was used to fabricate the prototype	UW Makerspace	1	3/21/2022	1	$1.25	$1.25	Link
Component 6								
Barbed Adapter	Barbed x MNPT Adapter, Polyethylene, ⅜ in	Grainger	1	3/29/2022	10	$1.26	$12.63	Link

	barb size, natural used to connect copper tubing to heated water tank							
Component 7								
Black Acrylic	Black Acrylic used to fabricate the incubation chamber 18x24 sheet with ½ inch thickness	UW Makerspace	1	4/11/2022	1	$21.50	$21.50	Link
Component 8								
3D print DC motor attachment	PVA plastic used to fabricate the DC motor attachment for the regulation of CO_2 input into the incubation chamber	UW Makerspace	1	4/11/2022	1	$2.72	$2.72	Link
Component 9								
DC Motor	Actual motor used for CO2 regulation	UW Makerspace	1	4/11/2022	1	$2.00	$2.00	Link
TOTAL:	$53.54							