Microscope Incubator for Cell Culture: A Low Cost Alternative

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Abstract

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

1. Introduction

Cell culture is a commonly practiced laboratory method for the use of studying cell biology, replicating disease mechanisms, and investigating drug compounds [1]. Due to the use of live cells during this process, incubators are necessary to keep the cells viable for the duration of the study. Onstage incubators allow for live cell growth because they maintain a highly regulated internal environment of 37 ℃, 5% CO_2, and 95% humidity, without compromising the integrity of the microscope. The COVID-19 pandemic has caused the CO_2 incubator market to increase by 7.69% with an estimated market growth acceleration of 8% over the next decade [2].

Major disadvantages of current commercially available systems are that they tend to be large and bulky enclosing the entirety of the microscope making it difficult to assemble and remove between uses while hindering the use of the microscope in general, and they are often expensive; Fisher Scientific's Enviro-Genie cell incubator is priced at $6,510.68 [3]. This project

will focus on developing a low-cost cell culture incubator that allows for interchangeable culture plates, compatibility with an inverted microscope, easy disinfection, and live cell imaging via maintenance of the internal environment needed for cell growth.

1.1 Cell Cultures in Lab

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

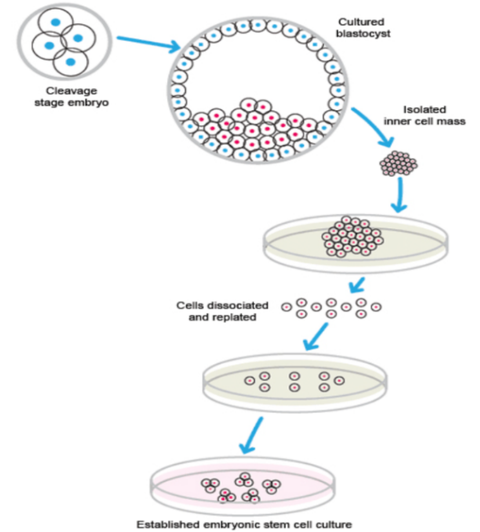

Figure 1: Isolation of Embryonic Stem Cell Lines[7]

Incubators used in cell cultures have to maintain a stable microenvironment and can achieve this via regulated temperature, humidity, CO_2, O_2, and pH levels. Controlling these factors is critical for the viability and growth of the cultured cells, as the incubator is aiming to replicate the cells' environmental conditions in the body (37 ℃ with a pH of 7.2-7.4) [8]. CO_2 is needed as a buffer to help with the pH along with a culture medium. The medium most commonly used is a Basal medium, with occasional serums added (such as fetal bovine serum), which controls the physicochemical properties of the cell cultures' pH and cellular osmotic pressure [1]. Many incubators are therefore larger in size in order to maintain these homeostatic conditions. 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.

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

1.2 Incubator Types

There are two types of commonly used methods to maintain the temperature in industry cell incubators. Many employ the direct heat method which tends to give off heat using electric metal coils that surround the body of the incubator and are programmed to the desired temperature. The other method is the water-jacketed incubators which use a controlled circulating water bath cabinet around the body of the incubator for even heating throughout the entirety of the chamber. Humidity control is achieved most commonly by placing a tray of water at the bottom of the incubator. This method is used in both water-jacketed and direct heat incubators. CO_2 control is achieved through a CO_2 tank that automatically pumps the desired amount of gas into the incubator. Using tubes and a valve connector, the CO_2 tank is able to deliver gas to the inside of both water-jacketed and direct heat incubators. Many incubators also allow for the CO_2 valve to be adjusted when internal conditions are disturbed, such as opening the incubator door to deliver more cell plates, so that the environment is always stable.

1.3 Clinical Significance

There is a significant need for live cells to be cultured via the assistance of an incubator. Pharmaceutical companies often use these methods for drug development and testing as live cell imaging can be used to screen chemicals, cosmetics, and other drug components for their efficacy [8]. Live cell imaging is important because it allows for the observation of internal structures and cellular processes in real time. These observations allow for more insight into the process of a cell, rather than viewing snapshots taken over a period of time. Pharmaceutical companies can also access the drug cytotoxicity in different cell types. Virology and vaccine products benefit from live cell cultures as they can be used to study viruses in order to make new vaccines, such as in the product of the SARS-COVID-19 vaccine [1]. Embryonic stem cells are widely studied for their regeneration properties due to genetic engineering/gene therapy applications of these cell cultures, and the expression of specific genes and the impact they have on other cells can be studied.

The team aimed to create an incubator with an internal environment of 37 ℃ ± 1 °C, 5% ± 1% CO_2, and 95-100% humidity with even heating and humidity across the chamber was both compatible and could operate on an inverted microscope stand (roughly 310 mm × 300 mm × 45 mm) and could fit a standard well plate inside (127.55 mm × 85.4 mm × 22.5 mm).

2. Methods

2.1 CO_2 Control

The CO_2 was measured using a MH-Z16 NDIR sensor, in previous work, and will continue to be used in this experiment. MH-Z16, part number SEN-000030 from Sandbox Electronics, is water/humidity resistant, and able to read the concentration of CO_2 in ppm, which

can then be converted into percentage. A gas permeable RKI Sensor Cover, part number 33-0172RK from RKI, will be utilized to make the MH-Z16 fully water-proof while inside the humid incubator environment. See Appendix E for further sensor and cover specifications.

The sensor is also compatible with an Arduino microcontroller. The input of CO_2 into the incubator was controlled using a relay circuit system and a solenoid valve. The team used a 100% CO_2 tank due to its availability and low-cost. The relay circuit system and solenoid valve allow only 5% of the incubator's internal environment to be CO_2 input at any given time. See Figure 3 for details about the set up.

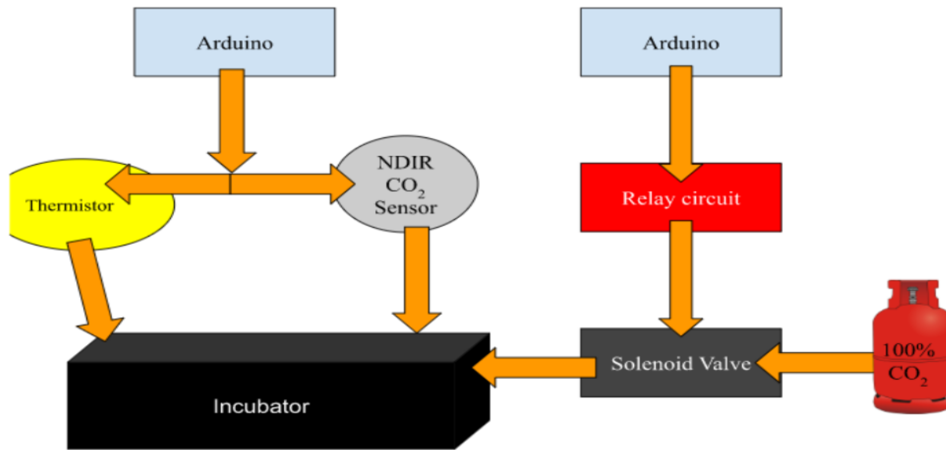

Figure 3: Block Diagram of Circuitry for the microscope incubator

The work this semester will focus on the communication between the NDIR MH-Z16 sensor and the solenoid valve in order to regulate the volume of CO_2 in the incubator. The goal is for the entire incubator interior to maintain a 5% CO_2 environment at all times in order to maintain an internal pH of 7.0-7.4. The pH is important, as it leads to cell proliferation and growth.

2.2 Temperature and Humidity

A thermistor was used to measure the internal temperature of the incubator and was coded to also determine the relative humidity. Relative humidity was calculated using Equation 1 [9].

$$RH = \frac{e}{es}(100) = 100\left(\frac{e^{0.37}}{e^{((17.0625*Tc)/(243.04+Tc))}}\right) \quad (1)$$

RH = relative humidity e = actual vapor pressure e_s = standard vapor pressure Tc = Temperature (℃)

Through previous work, the thermistor was compared against a DHT22 temperature and humidity sensor to ensure its accuracy. It then underwent a Temperature and Humidity Sensor Protocol which is further expanded upon in Appendix C 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 0.406 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. See Figure 4.

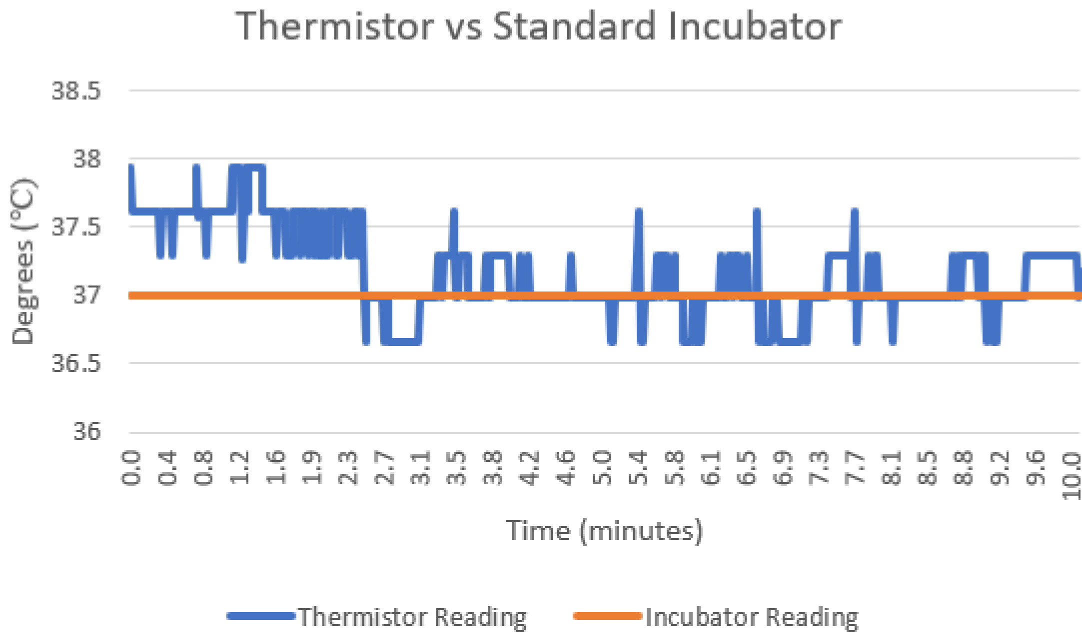

Figure 4: Thermistor Temperature over 10 minute Interval in Lab Incubator

Previously, 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 0.9437 , 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. See Figure 5.

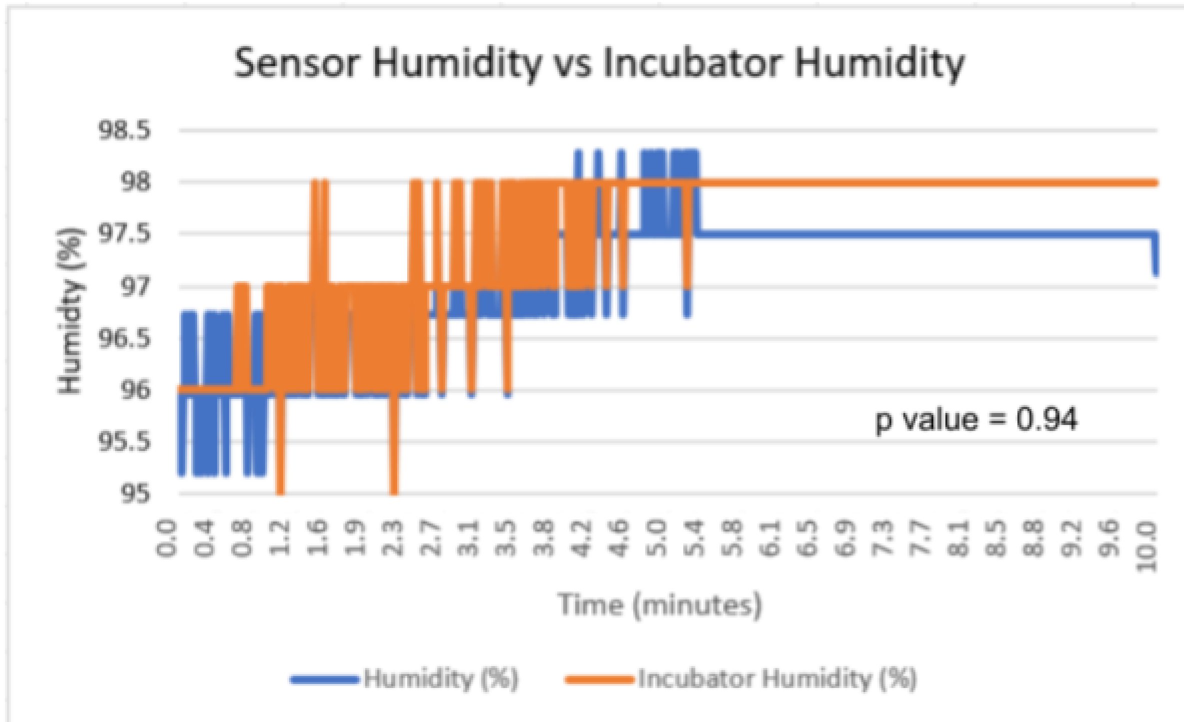

Figure 5: Graph of Humidity Readings in Incubator Over 10 minute Time Interval

These results show that the thermistor was accurate and a viable option for implementation in our incubator.

2.3 Anti-Fog/Anti-Condensation

Previous semester work for anti-condensation included the wiper blade design which was determined to be too mechanically challenging to move forward with. The wiper blade also did not allow the client to perform time lapse imaging due to the need for manual intervention. Nevertheless, the team decided to pursue a mini-fan alternative. Two Coolerguy mini-fans, 25 mm × 25 mm × 10 mm part number 840556070320 , were purchased off of Amazon [10]. These mini fans have a USB power supply cord, which can be connected to a USB wall charger. The fans are small enough to fit inside of the inner box in the incubator and require no fabrication. Once the fans are plugged in, they produce a small amount of air movement, but help prevent the condensation of water droplets onto the glass lid. The removal of the condensation is important for optical clarity and imaging.

Anti-fog and anti-condensation testing was performed by placing two mini-fans in different orientations inside the incubator and then taking images at consistent intervals for approximately an hour. Different orientations of the two mini-fans were tested to determine the best arrangement of each mini fan, because one singular fan did not produce enough air movement. At the 45 minute marker, the incubator lid was removed for 30 seconds and then

placed back on to the incubator box. The lid was open and closed to determine how the mini-fans would respond to an internal environment disruption. In each image, the percentage of condensation buildup was calculated by dividing the total condensation buildup area by the total glass area and multiplying it by 100. The images were analyzed with ImageJ. The team completed the first test by placing the fans on opposite corners of the incubator and the second test by placing the fans on adjacent corners of the incubator, both with the mini fans angled upwards at approximately 45 degrees to maximize the airflow onto the glass lid as seen in Figures 6 and 7.

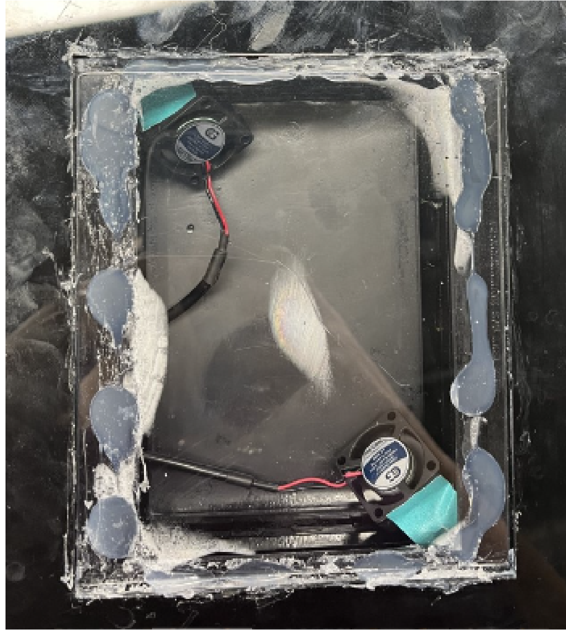

Figure 6: Photo showing the opposite corner dual mini fan setup where the fans are angled up at roughly 45 degrees

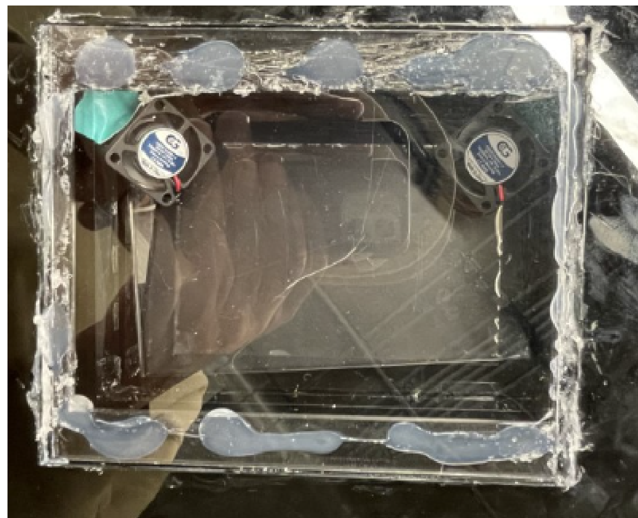

Figure 7: Photo showing the same side dual mini fan set up where the fans are angled up at roughly 45 degrees

2.4 Cell Viability

Cell viability was tested by comparing cell proliferation of mice osteoblast cells in a standard control incubator and in the prototype. Using two T25 flasks each starting with approximately 200,000 cells, one was cultured for 5 days in the standard incubator and the other was cultured for 5 days in the prototype incubator. Cells were passaged using 25% trypsin to remove them from the old flask. Every 24 hours each flask was imaged using the Ziess microscope at 5x magnification. Basal media on the cells was not changed during the duration of the test. Each image was then analyzed using ImageJ to quantify the percent area covered by the cells. The data was compared using a t-test with a p-value of .05 to determine if the data sets were significantly different.

Cell viability baselines are currently being completed to compare the cells conditions in the standard incubator as compared to the team's future result in the microscope incubator.

3. Results and Future Work

Anti-Fog/Anti-Condensation

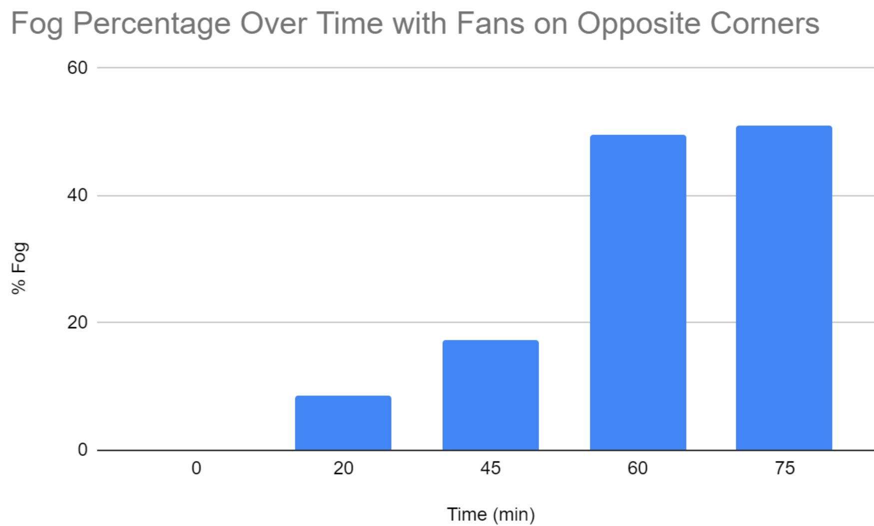

Figure 8: Graph of Fog Percentage Over 75 min Time Interval with Fans on Opposite Corners. At t=45 minutes, the lid was open and closed to test recovery efficiency of the fans.

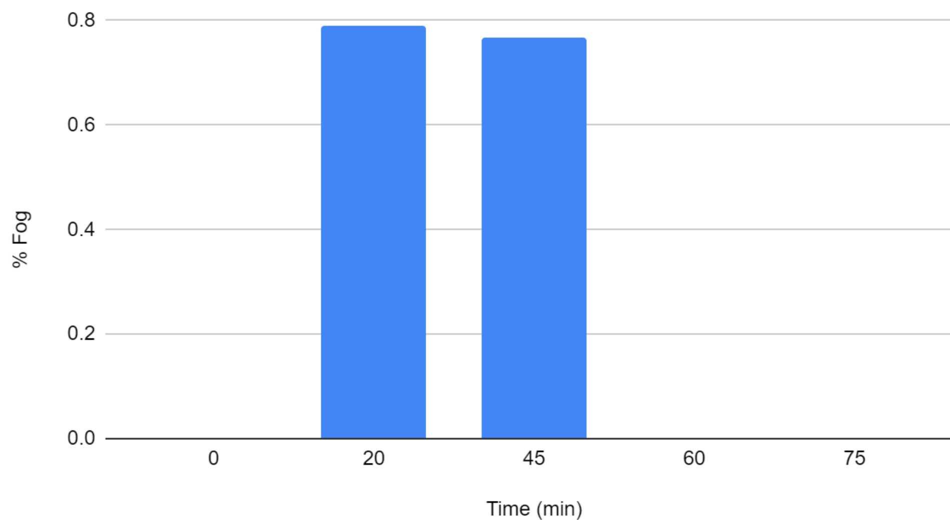

Figure 9: Graph of Fog Percentage Over 45 min Time Interval with Fans on Adjacent Corners. At t=45 minutes, the lid was open and closed to test recovery efficiency of the fans.

Figures 8 and 9 show the results from the anti-fog/anti-condensation testing in the different fan orientations. When the two fans were located on opposite corners of the incubators, there was a much greater percentage of condensation buildup, which is seen in the graph over the 75 minute interval as the fog percentage reaches a maximum of 51%. On the other hand, when the fans were located on adjacent corners inside of the incubator, less than 1% of fog was seen throughout the entire 75 minute interval. Therefore, the team decided to pursue the fabrication of an adjacent side dual mini-fan setup.

Current work for this project has consisted of box fabrication, anti-fog/condensation testing, and preliminary coding. Future work for this team includes obtaining results from cell culture baseline/cell viability testing, CO_2 sensor coding/testing, additional condensation testing, live-cell testing with the prototype, optical testing, homogeneity testing, and recovery testing. Protocols for all of these endeavors can be found in Appendix C.

4. Discussion

The team has not conducted testing on the MH-Z16 CO_2 sensor, nor conducted whole box testing. Further results and their implications will be discussed in the final report.

In terms of anti-fog testing, the results show that when the two fans are located in adjacent corners of the box there is minimal condensation buildup on the glass. The location of the mini-fans was an important factor in preserving optical clarity. The team can conduct optical testing on the viability of this method during whole box-testing and include time lapse imaging if the anti condensation results hold true. The cell baseline is currently being conducted. These

results will eventually be compared to the fabricated prototype. The results will help determine if the prototype is working in accordance with industry standards to keep the cells alive.

The main source of error in the anti-condensation test was exposure to the external environment. In order for the mini fans to be inserted into the incubator, the cords had to be fed underneath the box and up through the center cut out. Since the box rested on top of the cords, the external environment was able to seep in through the bottom, which could have increased or decreased the fans decondensation capabilities. Sources of error in the baseline cell culture testing may include imaging a different area of the flask every day and some dead cells were included in images. The implications of these sources of error are that the number of cells may be slightly different than the true value.

In the future, a protoboard should be fabricated to improve the professional look of the device. The team should also consider the financial implications of using a variety of sensors and devices (mini-fan, display case, etc) that were gifted to the team. The team may need to change the types of materials used in order to fabricate a device that is under $100 in raw material costs, or increase budget to reflect these donations.

Ethical considerations need to be taken into account as this device will be used in a live cell lab. The origin of the cells being studied was of the utmost importance. The client plans to use immortalized pre-osteoblasts isolated from the calvaria of newborn mice. The use of animal cells has caused much ethical controversy over the past half-century. Mice are commonly used in laboratory research as their entire genome has been sequenced and compared to the human genome and they are easily bred and housed [11]. 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 [12]. 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 [11]. 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.

5. Conclusion

The team is underway creating and testing a cell culture incubator that can maintain a specific internal environment while being compatible with an inverted microscope. The internal environment must be 37 °C, greater than 95% humidity, and contain 5% CO_2 in the incubator. Currently, testing and fabrication of the final project are of utmost importance. A new NDIR MHZ-16 CO_2 has been ordered, and upon arrival, the CO_2 system code in Appendix D will be tested and troubleshot. Once the CO_2 regulation is functioning properly, it will be added to the display, and then temperature and CO_2 ppm will be displayed at regular intervals. Upon completion of the CO_2 feedback system and display, Cell Viability testing will be completed, and the entire, cohesive incubator system will be tested for the first time. Upon retrieving cell viability results, the team will make improvements to the current design and complete statistical analysis on the current data. Optical and Anti-Condensation Testing will continue in order to optimize cell imaging and viewing for best results during Cell Viability testing.

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Appendices

Appendix A: Product Design Specifications (PDS)

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

Client requirements:

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

Design requirements:

1. Physical and Operational Characteristics

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

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

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

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

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

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

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

g. ***Ergonomics:*** The device should be portable in that one should be able to carry and store the device easily. Wires should not be hanging freely out of the device, and it should be easy to pick up and put away when needed.
h. ***Size:*** The device must be less than 310x300x45mm in order to fit on the microscope stand without interfering with the optics[1].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. A company called ibidi has developed a stage top incubator compatible with an inverting microscope following all of the temperature, humidity, and CO2 requirements as well as producing anti-condensation glass technology. The incubator is currently on the market for $19,000 [11]. Past BME 200/300 design projects have attempted to build incubators for this client, but none have been completely successful.

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Appendix B: Incubator Fall 2022

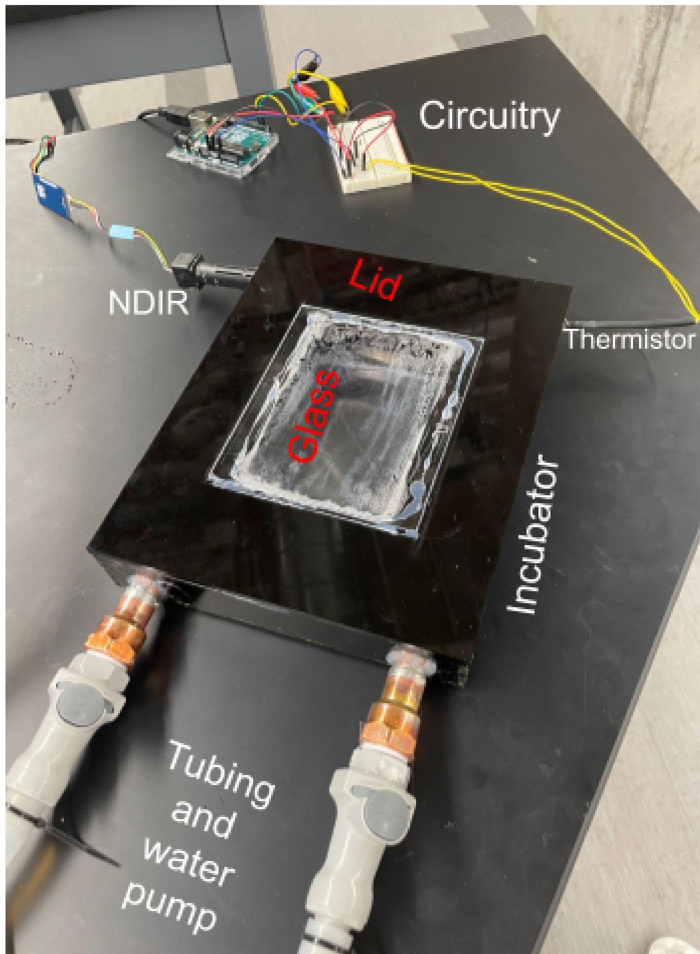


Figure 1: Incubator Prototype Exterior

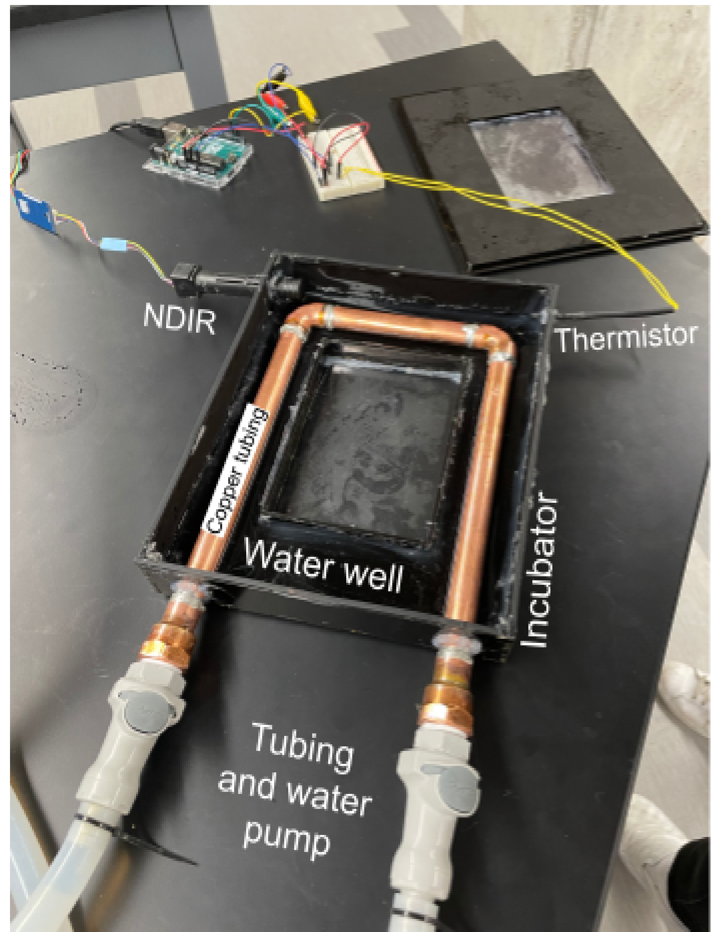


Figure 2: Incubator Prototype Interior

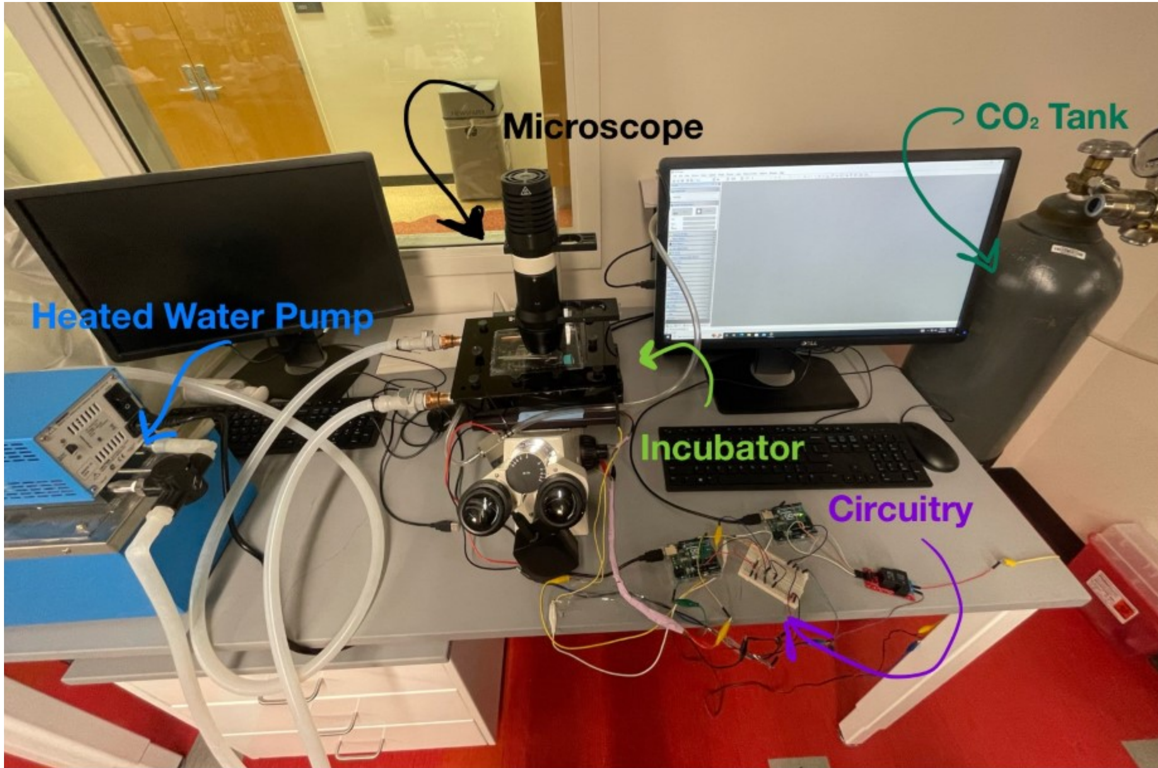
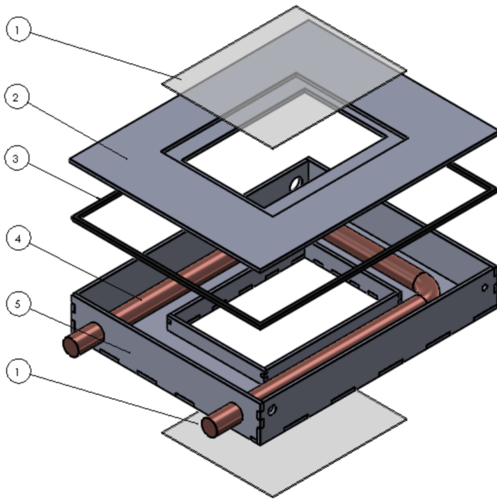
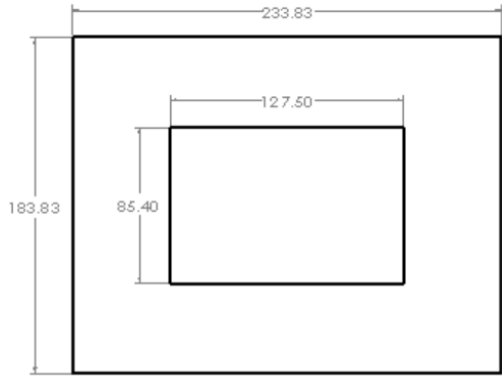


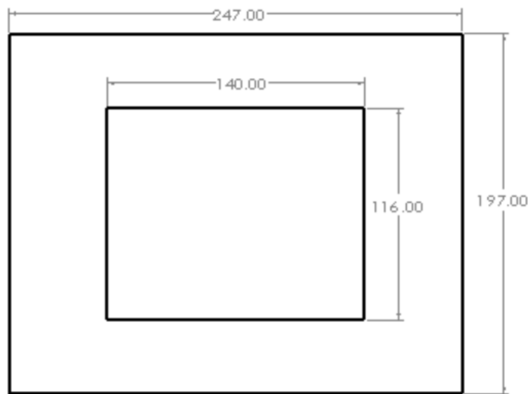
Figure 3: Whole incubator setup



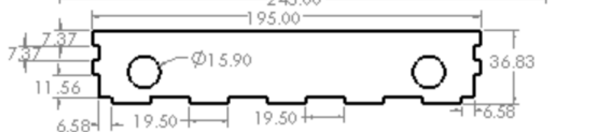
Item No.	Item Description	Dimensions [mm]	QTY.
1	Glass plates to allow transparent viewing	114.5 x 138.5 x 1.3	2
2	Lid of box to enclose the incubator	247 x 197 x 6.35	1
3	Rubber linning to allow tight seal	245 x 195 x 3.175	1
4	Copper tubing to provide heat transfer	Outside Diameter: 15.875 Inside Diamter: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

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


233.83
127.50
183.83
85.40


247.00
140.00
116.00
197.00

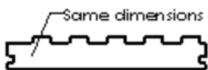
238.65
7.37
Ø5.08
Ø8.05
36.83
7.37
14.73
24.50
24.50
245.00


195.00
7.37
7.37
Ø15.90
36.83
11.56
6.58
19.50
19.50
6.58

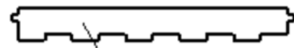
Same dimensions as above without circular holes

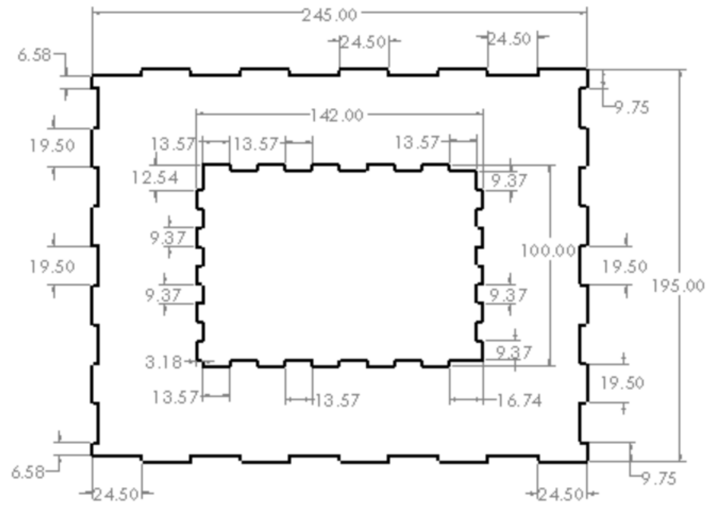
245.00
36.83
7.37
Ø12.70
7.37
14.74
24.50
24.50
11.56

12.54
9.37
9.37
3.18
7.08
3.00
100.00


Same dimensions as above

142.00
135.65
3.00
3.00
10.25
13.57
13.57
7.08


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

Figure 5: Laser Cut designs and dimensions

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**	**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	☐ Verified Comments:		

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

5	Remove the incubator from under the microscope and allow the CO_2 to leave the system so that its value read by the fyrite is nearly the same as room conditions. Repeat steps 5-4 until 5 trials are complete. Record the mean value of difference between the read CO_2 values in the comments.	☐ Verified Comments:		
6	If the CO_2 sensor deviates from the actual CO_2 percentage by ±0.1% or less, then the sensor is verified for use. If not verified, record why in the comments.	☐ Verified Comments:		

Steps	Protocol	Verification	Pass/Fail	Initials of Tester
1	Once the CO_2 sensor is approved for use, set up the incubator for normal use with the CO_2 sensor inside. Seal the incubator.	☐ Verified Comments:		
2	Connect the CO_2 tank to the incubator fixed with a regulator and a solenoid.	☐ Verified Comments:		
3	Verify the sensor is recording values. Then, begin running feedback code in conjunction with the solenoid connected to the CO_2 tank.	☐ Verified Comments:		
4	The solenoid should let CO_2 into the system immediately. Once the CO_2 sensor reads a value within 5% ±0.1% CO_2 the solenoid should stop allowing CO_2 into the incubator. If this occurs, continue protocol and step is verified. If this does not occur, stop protocol and record what happened in the comments.	☐ Verified Comments:		

5	Allow the feedback loop to run for an hour. Record the sensor values read into a graph. Verify that over the hour the CO_2 percentage remained near a level of 5% CO_2 ±0.1%. If the CO_2 remained in this range, continue protocol and step is verified. If this did not occur, stop protocol and record what happened in the comments.	☐ Verified Comments:		
6	Repeat step 5 over the course of 6 hours. If the CO_2 remains in the necessary range, continue the protocol and this step is verified. If this did not occur, stop protocol and record what happened in the comments.	☐ Verified Comments:		

Recovery Test Protocol

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

Explanation:

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

Steps	Protocol	Verification	Pass/Fail	Tester Initials
1	Set up the incubator for normal use. Record internal conditions in the comments and verify that they fall within the correct ranges (37°C, 5% CO_2, and >95% humidity).	☐ Verified Comments:		

2	Open the incubator for 30 seconds. Start stopwatch. Verify that the stopwatch is working.	☐ Verified Comments:		
3	Record internal conditions in the comments at a time of 15 seconds after opening the incubator. Verify that the internal conditions deviate from the normal conditions recorded above.	☐ Verified Comments:		
4	Close the incubator. Verify that the recovery time did not exceed 5 minutes after a 30 second exposure to the external environment. Record the time it took to revert back to optimal conditions in the comments.	☐ Verified Comments:		

Homogeneity Test Protocol

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

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

Steps	**Protocol**	**Verification**	**Pass/Fail**	**Tester Initials**
1	Obtain a lid with the same dimensions as the top of the incubator and ensure that there are holes throughout the frame of the lid.	☐ Verified Comments:		

2	Place probes/sensors for temperature into each hole and record its value.	☐ Verified Comments:		
3	Calculate and report the precision result (mean ∓ average deviation). Verify that all of the values for temperature fall within this precise range (ensures uniformity).	☐ Verified Comments:		
4	Repeat steps 1-3 for the humidity component.	☐ Verified Comments:		
5	Repeat steps 1-3 for the CO_2 component.	☐ Verified Comments:		

Sanitation Protocol

Introduction
Name of tester:
Dates of test performance:
Site of test performance:

Explanation:

This team will be utilizing the prototype to house live cells and therefore needs to follow biosafety level 1 standards. This requires that incubators be sterile in order to reduce contamination and unwanted bacteria growth. Before use the prototype will be placed inside one of the biosafety cabinets in the teaching lab with the inside of the box and lid facing upwards. With a closed sash, the team will turn on the UV light and allow for the box to be sanitized for 15 min. Once complete, the prototype will be removed from the cabinet, sprayed with 70% ethanol, and wiped dry with a kemi wipe. The water bath will consist of DI water in order to further reduce possible contaminants. These steps should be repeated before each use.

Steps	**Protocol**	**Verification**	**Pass/Fail**	**Initials of tester**
1	Raise the sash of the biosafety cabinet and place the prototype	☐ Verified Comments:		

	inside. Ensure the bottom half has the water bath facing the light and the inside of the lid is also facing the light.			
2	Close the sash and turn on the UV light. Leave for 15 minutes.	☐ Verified Comments:		
3	Open the sash and remove the prototype.	☐ Verified Comments:		
4	Spray the entire inside and outside of the prototype with 70% ethanol. Wipe completely dry with a Kemi wipe.	☐ Verified Comments:		
5	Assemble the prototype on the microscope stage, hooking up the water tank, CO_2 tank, and sensors.	☐ Verified Comments:		
6	Fill the water bath inside the prototype with DI water. Close the lid.	☐ Verified Comments:		

Cell Confluency Test Protocol

Introduction

Name of Tester:

Dates of Test Performance:

Site of Test Performance:

Explanation:

The team employed image J to quantify the percentage of area covered by the cells over time in order to quantify the cell proliferation. This allowed the team to compare cell proliferation in the standard incubator compared to the prototype. The images of the cells were taken using the Zeiss scope in the teaching lab. The control was a T25 flask that was cultured in the standard incubator to provide a baseline on appropriate cell death over the course of a week. Another T25 flask was cultured inside the prototype over the course of a week. Using the Zeiss scope, an image was taken every 24 hours, and those images were loaded into image J. The team was able to quantify the percent of cell coverage and track cell confluency over the course of the

week. Tests will be considered successful if there is no significant difference between the confluency between the control and the prototype.

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

Optical Image Analysis Protocol

1. Open ImageJ
2. Insert desired image
    a. Convert type to 16-bit if it is not already
3. Perform Microscope Image Focus Quality plugin
    a. Don’t generate probability image, only do overlay (with border width = 5)
4. Save image as .jpg
5. Edit > Copy to system
6. Put the image into Paint
7. On ImageJ, do Files > New Image (type is RGB)

    a. Check pixels and put in the right numbers (3584 x 2746)
8. Copy image from Paint into ImageJ
9. Image > Color > Split Channels
10. Do Analyze > Measure on all 3 images created (blue, green, and red)
11. Record means into spreadsheet - indicate focus quality

**Red = in focus
**Blue = out of focus

Fan Testing Protocol
1. Open ImageJ
2. Insert desired image
    a. Convert type if needed
3. Create a freehand selection around the well plate area of the incubator
4. Measure the area - this will be normalized to 100%
5. Create another freehand selection encompassing the area that is foggy - do this for all foggy sections
6. Measure the area - these added up will be the foggy areas
7. Calculate the percentage of foggy areas (foggy area / total area)
8. Compare the foggy regions in each of the experimental cases (with each of the varying fan placements)
9. Calculate statistical significance via a chi-squared test - aiming for no statistical significance from the control

Appendix D: Circuitry & Code

D1. Circuitry

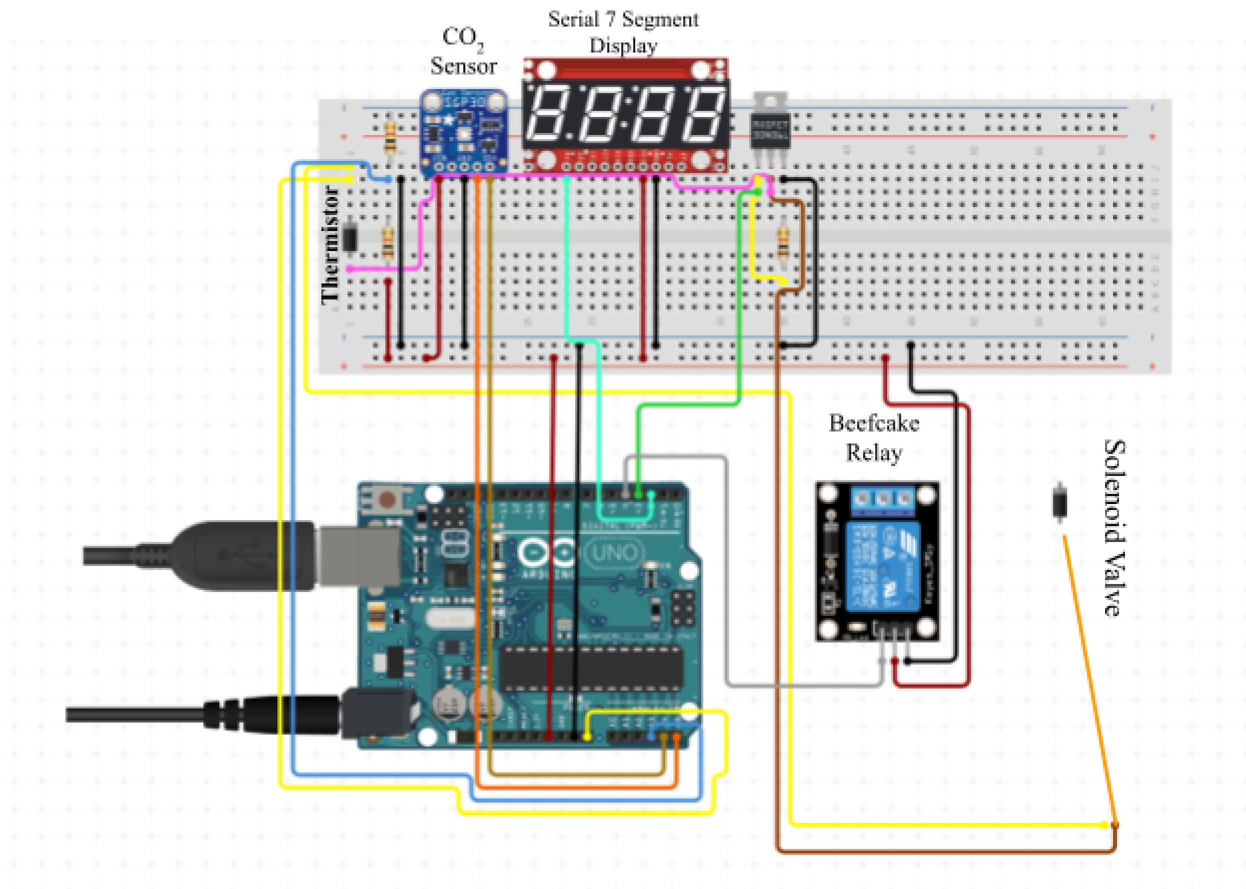


Figure 1: Circuitry

D2. Display Code

```
/* Serial 7-Segment Display Example Code
    SPI Mode Stopwatch
  by: Jim Lindblom
     SparkFun Electronics
  date: November 27, 2012
  license: This code is public domain.

  This example code shows how you could use the Arduino SPI
  library to interface with a Serial 7-Segment Display.
```

```
  There are example functions for setting the display's
  brightness, decimals and clearing the display.

  The SPI.transfer() function is used to send a byte of the
  SPI wires. Notice that each SPI transfer(s) is prefaced by
  writing the SS pin LOW and closed by writing it HIGH.

  Each of the custom functions handle the ssPin writes as well
  as the SPI.transfer()'s.

  There's a custom function used to send a sequence of bytes
  over SPI - s7sSendStringSPI, which can be used somewhat like
  the serial print statements.

  Circuit:
  Arduino -------------- Serial 7-Segment
    5V   --------------------  VCC
    GND  --------------------  GND
     8   --------------------  SS
    11   --------------------  SDI
    13   --------------------  SCK
*/
#include <SPI.h> // Include the Arduino SPI library

// Define the SS pin
//  This is the only pin we can move around to any available
//  digital pin.
const int ssPin = 8;

unsigned int counter = 0;  // This variable will count up to 65k
char tempString[10];  // Will be used with sprintf to create strings

//temp
int sensorPin = A0;    // select the input pin for the potentiometer
int ledPin = 13;      // select the pin for the LED
int sensorValue = 0;  // variable to store the value coming from the sensor
float volt_conversion = 5.0/1023.0;
float ADC_voltage = 0;
float K_temperature = 0;
float C_temp;
```

```
void setup()
{
  // -------- SPI initialization
  pinMode(ssPin, OUTPUT);  // Set the SS pin as an output
  digitalWrite(ssPin, HIGH);  // Set the SS pin HIGH
  SPI.begin();  // Begin SPI hardware
  SPI.setClockDivider(SPI_CLOCK_DIV64);  // Slow down SPI clock
  // --------

  // Clear the display, and then turn on all segments and decimals
  clearDisplaySPI();  // Clears display, resets cursor

  // Custom function to send four bytes via SPI
  //  The SPI.transfer function only allows sending of a single
  //  byte at a time.
  s7sSendStringSPI("DEGC");
  setDecimalsSPI(0b11111);  // Turn on all decimals, colon, apos

  // Flash brightness values at the beginning
  setBrightnessSPI(0);  // Lowest brightness
  delay(1500);
  setBrightnessSPI(255);  // High brightness
  delay(1500);

  // Clear the display before jumping into loop
  clearDisplaySPI();
}

void loop()
{

  // Magical sprintf creates a string for us to send to the s7s.
  //  The %4d option creates a 4-digit integer.
 // read the value from the sensor:
  sensorValue = analogRead(sensorPin);
  ADC_voltage = sensorValue * (volt_conversion);
  K_temperature = (ADC_voltage - 0.205) / 0.0153;
  C_temp = K_temperature - 91;
  counter = C_temp*100;
  sprintf(tempString, "%4d", counter);
```

```
  // This will output the tempString to the S7S
  s7sSendStringSPI(tempString);

  // Print the decimal at the proper spot
  if (counter < 10000)
    setDecimalsSPI(0b00000010);  // Sets digit 3 decimal on
  else
    setDecimalsSPI(0b00000100);

  counter++;  // Increment the counter
  delay(1000);  // This will make the display update at 100Hz.*/
}

// This custom function works somewhat like a serial.print.
//  You can send it an array of chars (string) and it'll print
//  the first 4 characters in the array.
void s7sSendStringSPI(String toSend)
{
  digitalWrite(ssPin, LOW);
  for (int i=0; i<4; i++)
  {
    SPI.transfer(toSend[i]);
  }
  digitalWrite(ssPin, HIGH);
}

// Send the clear display command (0x76)
//  This will clear the display and reset the cursor
void clearDisplaySPI()
{
  digitalWrite(ssPin, LOW);
  SPI.transfer(0x76);  // Clear display command
  digitalWrite(ssPin, HIGH);
}

// Set the displays brightness. Should receive byte with the value
//  to set the brightness to
//  dimmest------------->brightest
//     0--------127--------255
void setBrightnessSPI(byte value)
```

```
{
  digitalWrite(ssPin, LOW);
  SPI.transfer(0x7A);  // Set brightness command byte
  SPI.transfer(value);  // brightness data byte
  digitalWrite(ssPin, HIGH);
}

// Turn on any, none, or all of the decimals.
//  The six lowest bits in the decimals parameter sets a decimal
//  (or colon, or apostrophe) on or off. A 1 indicates on, 0 off.
//  [MSB] (X)(X)(Apos)(Colon)(Digit 4)(Digit 3)(Digit2)(Digit1)
void setDecimalsSPI(byte decimals)
{
  digitalWrite(ssPin, LOW);
  SPI.transfer(0x77);
  SPI.transfer(decimals);
  digitalWrite(ssPin, HIGH);
}
```

D3. CO_2 Control 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);
int relayPin = 13;
float x;
void setup()
{
    Serial.begin(9600);
    pinMode(relayPin, OUTPUT);

    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");
   } else {
      Serial.println("Sensor communication error.");
   }
   x = mySensor.ppm;
   Serial.println(x);
   if( x == 0){
     digitalWrite(relayPin, HIGH);   //switch relay on
     delay(1000);                    //wait 1 second
     digitalWrite(relayPin, LOW);    //switch relay off
     delay(1000);

   }
 // digitalWrite(relayPin, HIGH);   //switch relay on
 // delay(1000);                    //wait 1 second
//  digitalWrite(relayPin, LOW);    //switch relay off
 // delay(1000);

   delay(1000);
}
```

Appendix E: Materials Purchasing

Table 1: Materials Purchasing Table

Expenses

Item	Description	Manufacturer	Part Number	Date	QTY	Cost Each	Total	Link
Component 1								
MH-Z16	NDIR CO_2 Sensor	Sandbox Electronics	SEN-000030	2/7	1	$67.59	$67.59	Link
Component 2								

RKI Waterproof Sensor Cover	CO_2 Waterproof Sleeve	RKI	33-0172RK	2/7	1	$5.37	$5.37	Link
Component 3								
Coolerguys 25mm USB Fan	Miniature fans to decrease condensation on glass	Amazon	840556070320	2/16	2	$6.99	$13.98	Link
TOTAL:	$86.94							