**Design and Verification of a Sterile Incubator Volume for
Maintaining Post-Deposition Cell Viability for Cell Printing
Processes**

A Thesis

Presented to the Faculty of

California Polytechnic State University—San Luis Obispo

In partial fulfillment of the Requirements for the Degree

Master of Science in Engineering with Specialization in Biomedical Engineering

By

Emily Hakun

June 2008

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TITLE: Design of a Sterile Incubator Volume for Maintaining Post-Deposition
Cell Viability in Cell Printing Processes

AUTHOR: Emily Hakun

DATE SUBMITTED: June 2008

Dr. Lily Laiho

Signature

Dr. Robert Crockett

Signature

Dr. Kristen O'Halloran-Cardinal

Signature

ABSTRACT

Design and Verification of a Sterile Incubator Volume for Maintaining Post-Deposition Cell Viability for Cell Printing Processes

By Emily Hakun

The growing field of tissue engineering requires the design and verification of an environmentally-controlled sterile incubator volume. As this technology advances and the field of cell printing emerges, the need for such a volume increases. This volume shall maintain post-deposition cell viability of printed cells, by maintaining volume sterility and controlling temperature. This becomes more important as more delicate cells are used. Sterility maintenance prevents contamination of the cells, while temperature regulation maintains the optimum temperature for cell viability. Several existing incubator systems are capable of regulating environmental conditions, but none are designed to function with a moving cell deposition head.

The Sterile Incubator Volume System was developed to accommodate cell printing needs. The primary challenge was to create a sterile volume, with environmental conditions suitable for cell growth; it must interface with a moving deposition head. Numerous engineering practices were included in this design process: defining design inputs and outputs, brainstorming, using decision matrices, considering manufacturing constraints, prototyping, and testing.

The final design consists of a portable, self-contained volume capable of maintaining cell viability for at least 4 hours. This environment features feedback-regulated temperature, which is controlled via an external feedback loop by a proportional-integral-derivative (P-I-D) temperature controller. This configuration

optimizes temperature regulation while minimizing the risk of contamination from external elements by placing the heating element external to the sterile volume. The volume is compact (6" x 6" x 2"), with an easily removable snap-fit lid for simple assembly and disassembly in a sterile hood. A latex cover maintains sterility inside the container while allowing adequate movement of the deposition syringe. A septum permits the syringe to penetrate the latex and be removed without compromising the interior sterility of the volume.

The design was verified through a series of tests, including temperature and pH regulation, resterilizability, evaporation, cell viability and systems integration trials. Temperature, pH, resterilizability and evaporation tests yielded quantitative data; while the cell viability and systems integration tests compared cells from the Sterile Incubator Volume System to control cells (from a commercial incubator). These tests verified that the system can maintain cell viability for up to 4 hours; it follows that the allowable cell print time will increase, due to optimized conditions for the cells during deposition and experimentation. These trials found cell viability in the Sterile Incubator Volume System to be comparable to cells from the commercial incubator. This design is simple, autonomous, and can be integrated into most existing tissue engineering and cell culture experiments with minor changes.

The potential for maintaining cell viability could be further enhanced by future developments, including humidity and carbon dioxide regulation, expanding the volume size, and creating additional print-head interface variations to increase the diversity of the printed assemblies. These potential enhancements must consider the design intent and

simplicity. The design of this sterile incubator volume system is an important step in improving tissue engineering technology and the types of tissues that can be engineered.

ACKNOWLEDGEMENTS

Special thanks to the Biomedical and General Engineering Department at Cal Poly for the immense support they have. The academic training, brainstorming, advice, assistance, and funding have made my sterile incubator volume a reality.

I would also like to express my gratitude to my advisors and committee members. Dr. Lily Laiho for countless hours of advice and design brainstorming and analysis; I couldn't have done this without her support and encouragement. Dr. Kristen O'Halloran-Cardinal's expertise in the field of tissue engineering was especially beneficial and her assistance in the lab was instrumental in my testing processes. Dr. Crockett's support has been evident throughout my Cal Poly career; his enthusiasm for this project has been a source of motivation and has helped me to stay focused on the "big picture" of Cal Poly's tissue engineering endeavors.

Other faculty members have also contributed greatly to the success of my thesis project. Thanks to Dave Laiho for taking time out of his busy schedule to assist with heater wiring. Dr. Walsh has provided motivation through his encouragement and enthusiasm throughout my time at Cal Poly.

Many students have also assisted me along the way, by helping in the lab, helping with manufacturing or proofreading my thesis. Special thanks to Brian Wong (my cell culturer), Melanie Jackson, Steffen Hausler, Brian Klofas, Jessica Kiefer, and Derek Benedict.

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LIST OF NOMENCLATURE

BAT	BioAssembly Tool
BioLP	Biological Laser Printing
CICG	Capillary Induced Contact Guidance
CIJ	Continuous Inkjet
DOD	Drop-on-Demand
EPD	Encapsulated Petri Dish
FDA	Food and Drug Administration
ISO	International Standards Organization
LGDW	Laser Guided Direct-Write
LIFT	Laser-Induced Forward Transfer
MAPLE-DW	Matrix Assisted Pulsed Laser Evaporation Direct-Write
RP	Rapid Prototyping
TE	Tissue Engineering

1.0 INTRODUCTION

Tissue engineering has potential to offer effective treatment for thousands of people with organ and tissue disease. Universal tissue engineering goals are ambitious, including aspirations to directly print replacement organs or tissues. If the risk of transplant rejection could be eliminated by utilizing a patient's own tissue, this would reduce transplantation risks and organ wait times. In the United States, 17 people die daily due to a lack of organs for transplant, the need for this technology is evident (Boland 2006).

While there are many methods for tissue engineering, there is much more to consider than just forming cells into the desired architecture. Traditional tissue engineering involves a scaffold seeded with cells, while more recent research has been focused on direct cell printing. Various deposition methods are available, all with strengths and weaknesses. Once the tissue has been engineered, specified environmental conditions must be maintained to ensure that the cells remain viable.

The creation of a sterile incubator volume is aimed at maintaining post-deposition cell viability. The volume's purpose is to ensure that cells are printed into a sterile environment, eliminating many cross-contamination risks of printing in open air or printing in the hood. Cells can be printed in the lab without worrying about airborne residues and other contaminants. This type of technology will also be beneficial in increasing the types of cells that can be printed, by providing optimized environmental conditions, as needed by more fragile cell types.

As the field of tissue engineering expands, the Cal Poly Tissue Engineering Lab is also growing. The lab currently has a functioning inkjet cell printer and is developing a

syringe deposition printer. In addition to depositing cells, the lab would like to print them into precise two-dimensional and three-dimensional patterns with high resolution, create constructs with multiple cell types, and contribute to the field as a whole.

Future iterations of the existing printers will lead to new design features. One goal is to employ multiple deposition heads for printing several types of cells at once and integrating protein and cell printing. When printing conditions are improved, the lab will be capable of printing of larger, more complex structures. The creation of a sterile incubator volume is a significant step towards achieving this goal. These same enhancements should provide the improved printing capabilities to print more sensitive cells, to print more complex structures, to print for longer periods of time, or to print cells without the need for antibiotics.

2.0 BACKGROUND

In 2004, 20 million people in the United States had organ or tissue disease (Bafan 2004). By 2006 there were 17 deaths attributed to these diseases daily (Boland 2006); and as of May 31st 2008 there were over with 98,909 people awaiting transplants (www.optn.org). While there is currently no cure for many forms of organ and tissue disease, new treatment methods are constantly being developed. Organ transplants have traditionally been the primary treatment option; however, limited organ availability and the risk of rejection have led researchers to pursue alternate methods.

There are three types of transplants: xenografts, allografts, and autografts. Each of these may enable patient survival, but none are optimal due to limitations and risks associated with these procedures. Xenografts use animal organs as replacements, which carry the risks of animal viruses being transmitted to the patient and interspecies rejection (Boland 2006). Allografts are transplants from a donor to a recipient within the same species; while interspecies rejection risks have been eliminated, there is still potential for rejection due to incompatibilities between the recipient's major histocompatibility complex and the donated tissue (Boyer 2007). Autografts are transplants where the donor is also the recipient, which reduces the rejection risk. The primary tissue source for autografts is surplus or regenerative tissue, such as skin or vasculature; so, treatment applications are limited. Today's technology and tissue engineering research strive to increase the types of tissue that can be replaced using autograft therapy.

The development and advancement in the field of tissue engineering has proven promising as a renewable and effective therapy for many patients. While there have been many advances in tissue engineering since the term was coined at the 1987 National

Science Foundation meeting, current technology still has its limitations (Miller Smith 2005). Traditional tissue engineering utilizes a scaffold seeded with living cells, to create custom replacement tissue for the patient. Scaffold-based tissue engineering has many practical applications, including grafts of bone, cartilage, skin, and liver tissue (Mironov 2003, Giardino 2006, and Whang 1995). Tissue engineering is a rapidly-advancing field in terms of both scientific research and medical potential. In the late 1990's, tissue loss and organ failure yielded \$400 billion in annual expense in the United States (Niklason 1997, Bafan 2004, Sheridan 1999).

In addition to treatment methods, anatomically accurate organs and tissues are needed for research. Through tissue engineering scientists can develop organs for experiments that would previously have been performed *in vitro*, with animal models, or in formal clinical trials (Boland 2006). For example, new melanoma treatments could be performed on tissue engineered skin; whereas, before they had to be tested *in vivo*, mandating all the regulations associated with a clinical trial (Boland 2006).

There are many methods of tissue engineering which have been explored, including scaffold-based tissue engineering and cell printing. There are several cell printing methods such as inkjet, drop-on-demand, magnetic, lithography, microcontact, capillary induced contact guidance and laser printing. Both scaffold-based tissue engineering and cell printing processes have been utilized for certain applications.

Scaffold-Based Tissue Engineering

Scaffolds were an early endeavor in tissue engineering, and they are still one of the most common methods (Miller Smith 2005). Scaffold-based tissue engineering involves seeding cells onto a pre-made frame (scaffold) to achieve the desired tissue

architecture (see figure 1). The scaffold is made of biocompatible (often bioabsorbable) material. In addition to the scaffold and seeded cells, an environment with conditions suitable for cell growth must be maintained. The environmental conditions, scaffold, and cells must be integrated together properly to achieve the appropriate mechanical, chemical and biological properties for tissue and cell growth (O'Halloran 2007, Bafan 2004, "Tissue Engineered Scaffolds" 2007, Ringeisen 2006). These scaffolds provide structural support and promote cell growth in prescribed three-dimensional patterns; the architecture should enable optimal growth and tissue development within the tissue (Bafan 2004, Ringeisen 2006). After cells have been successfully seeded onto the scaffold, the tissue can be implanted (Bafan 2004, Chen 2005, "Tissue Engineered Scaffolds" 2007).

Figure 1: Cell Seeding on a Scaffold

Tissue engineering scaffolds are constructed of various biomaterials, including: proteins (such as FDA-approved polylactic acids, polyglycolic acids, and collagen), hydrogels, metals, synthetic polymers, and ceramics (Boland 2007, Ringeisen 2006, Niklason 1997, Miller Smith 2005). In many cases these are bioabsorbable—they

dissolve in a biological environment after a specified period of time. In order to successfully integrate cells into these scaffolds, growth factors and proteins are often used to encourage cell growth (Ringeisen 2006). Due to the nature of scaffolds and the need to culture cells (to achieve a large enough cell population) only certain types of cells can be used (Miller Smith 2005). The rigid structure of a scaffold can be utilized to reconstruct both hard and soft tissue (Boland 2007).

While scaffold-based tissue engineering has shown great promise, there are several limitations. Once the scaffold has been created, the cells must be precisely seeded onto it. This process has proven challenging, since the cells tend to adhere in non-uniform, imprecise patterns (Boland 2006, Mironov 2003). Additionally, it is difficult to seed different types of cells in different regions of the scaffold accurately (Boland 2006). If the engineered tissue is not adequately vascularized, the cells will die, since cells must be in close proximity (80-100 microns) to a blood (oxygen) supply in order to survive (Boland 2006; Mironov 2003; Santos 2006; Sheridan 1999).

Scaffolds can be made in several ways, some of which mimic rapid prototyping (RP) technologies. RP is a three-dimensional printing method, which uses a computer model to print a pattern layer-by-layer in order to create a three-dimensional model. General applications for RP include early prototypes, customized parts, and limited quantities of manufactured parts. Today similar technology is being explored with scaffold and cell printing methods, with the hopes that organs can eventually be printed in three-dimensions, in a manner similar to rapid prototyping. These methods include: photolithography, syringe-based gel deposition and solid freeform fabrication (Boland 2007).

Cell Printing

Cell printing research has been intensifying since 1999, when its feasibility was shown (Ringeisen 2006). In direct cell printing various substrates can be used for deposition, leading to a greater variety of cell types that can be utilized. Initial cell printing research focused on the ability to accurately deposit a few cells. The technology has increased to include increasingly complex two-dimensional and three-dimensional constructs (Barron 2004). These methods use deposition devices to directly apply the cells onto a receiving substrate (Barron 2004). While older methods had limited accuracy and reproducibility, today's research involves improving the existing technology to enhance resolution and speed, while creating more complex constructs.

Tissue engineering using cell printing technologies typically involves three steps: pre-processing, processing and post-processing. Pre-processing uses imaging technology to map tissue to create a computer model of the cells to be printed. Processing is the printing of the cells, which frequently utilizes layer-by-layer printing (Mironov 2003). Post-processing involves tissue conditioning and *in vitro* or *in vivo* maintenance to prepare the tissue for implantation (Mironov 2003, Ringeisen, 2006). While technological advances are needed at all stages, the processing phase has proven especially challenging for researchers.

Off-the-shelf Inkjet Heads

Some methods utilize standard "off-the-shelf" inkjet heads, modified to meet the needs of cell printing. In order to prepare a standard inkjet head for cell deposition, the head must be emptied, rinsed (usually with water and ethanol), sterilized (frequently with autoclave) or rinsed thoroughly (with 100% ethanol solution) and filled with cells.

Contamination of cells must be prevented at all stages of printing, and an environment suitable for cell viability must be maintained (Boland 2007). To minimize contamination risks, all components of the printer that will be in close proximity with the deposited cells should be aseptic; any components that will directly contact the cells or the deposited substrate must be sterile.

Modified inkjet printing has been explored in the fields of electronics, microengineering, and biomedical applications, including drug screening, genomics, biosensors and DNA printing (Boland 2006). Inkjet printing is simple and economical as a cell printing method. Additionally, this method is versatile and has the potential to print with multiple cell types simultaneously, comparable to a standard inkjet head printing in different colors (Burg 2003).

There are variations in the deposition method for inkjet printing. Continuous inkjet printing (CIJ) involves a constant stream of fluid, passed through an orifice via electrical or magnetic forces; whereas, Drop-on-Demand (DOD) inkjet printing deposits drops as needed via thermal or piezoelectric forces (Ringeisen 2006, Saunders 2008).

Inkjet printing methods can be either thermal or piezoelectric. In both types, the risk of cross-contamination is minimal, yielding cell viability of 75-90%. Challenges with inkjet methods include the large pixel size (low resolution) and concerns about multipotency and cellular differentiation (Ilkhanizadeh 2007).

Thermal Inkjet Printing

Many common DOD inkjet printers utilize thermal deposition, in which a heated chamber is used to deposit ink. When the ink is heated inside the print-head (temperatures up to 300° C), the pressure increases and bubbles form,

forcing drops of ink out of the print head. Bacterial cells and mammalian cells have been printed using this method (Saunders 2008). Since then, many cell types have been printed with thermal inkjet techniques, including primary motoneurons, primary embryonic hippocampal cells and cortical neurons (Ringeisen 2006).

Researchers have used commercial thermal inkjet heads from HP and Canon. One trial employed quick pulses (10 μ s) to create small temperature increases (4°-10° C) capable of dispensing an 80 pL drop of ink (Boland 2007). The inkjet heads were UV sterilized and cartridges were rinsed with a 100% ethanol solution. The entire printer was placed in the hood, where cells were printed onto a hydrogel receiving substrate, treated, and placed in an incubator. It should also be noted, that thermal inkjet printing has potential to lyse 3% to 10% of cells during the deposition process (Ringeisen 2006, Ilkhanizadeh 2007). This study found that cells can survive the thermal printing process, but called for further research on post-deposition cell integrity, mechanical properties, and the viscosities required for printing (Boland 2006, Ringeisen 2006, Ilkhanizadeh 2007).

Piezoelectric Inkjet Printing

Piezoelectric is another common DOD inkjet method. Application of a voltage changes the physical properties of a piezoelectric material inside the inkjet, causing it to expand. As the element expands, the pressure in the inkjet head increases, and a drop of ink is forced through the nozzle. A greater variety

of media can be used with piezoelectric printing, since temperature-dependent viscosity changes are not a concern.

In one trial, a piezoelectric deposition head (Epson Stylus 700) was used to create bacterial and chemical patterns on a flat surface. Bacterial strands were selected to attempt to control the arrangement of interacting strands, as a measure of the printer's precision and accuracy. Nozzle firing accuracy and large shear forces had an adverse effect on the droplets. Drop volume was measured via direct counting (of beads), weight of the droplet, and fluorescence. 98.5% of bacterial cells and 92% of mammalian cells were found to be viable after piezoelectric deposition (Merrin 2007, Xu 2005).

Other research utilized piezoelectric DOD printing for delivery of human fibroblast cells via a single-jet stationary print head, with a focus on the mechanical and fluid stresses that the cells endure during printing. The amplitude and rise times were varied to determine the effect of these factors on the cells. Changing the amplitude had a minimal effect on cell viability (doubling the amplitude from 40V to 80V yielded a decrease in cell viability from 98% to 94%). Changing the rise time had no noticeable influence on cell viability. More research still needs to be performed on the influence of the inkjet printing stresses and their effect on cell function (Saunders 2008).

Drop on Demand (Scaffold and Cells)

Recent research (Boland 2007) has focused on solid free form fabrication via DOD printing. DOD material deposition is a function of viscosity, deposition radius, and temperature, so all of these elements must be considered in this method (Burg 2003).

This method primarily is used to create scaffolds, by stacking two-dimensional layers on top of each other. This enables the technician to prescribe a specific scaffold architecture (to include: size, shape, geometry, and interconnectivity of the pores) aimed at successful cell seeding (Boland 2007). Once the scaffold has been printed, the cells are seeded onto it using DOD printing methods.

Recent research evaluated the feasibility of simultaneously printing scaffold material and biomaterials or cells (Boland 2007, Burg 2003). A standard HP DeskJet 550 print-head was modified to meet the demands of this method. This research utilized both the two-dimensional printing provided by the printer and a stepper-motor controlled elevator chamber to allow for layer-by-layer printing. Once one layer has been printed, the stage is lowered slightly so that the next layer can be printed on top of the previous layer (Boland 2007, Boland 2006). This method enables accurate placement of cells within a scaffold at a rate of 50,000 cells per minute, but is still restricted by the limitations of scaffold-based tissue engineering (Burg 2003).

Magnetic Printing

Cells have been printed onto arbitrary surfaces using magnetic force and magnetite particles in this form of Magnetic Force Tissue Engineering (MagTE). The target cells are magnetically labeled with tiny (10 nm) magnetite particles which enable the cells to be pulled towards steel plates which position the cells (see figure 2). Both mouse 3T3 fibroblasts and human umbilical vein endothelial cells have been successfully seeded into various patterns with this method (Ino 2007). MagTE is challenged by limited cell adherence and difficulty in producing three-dimensional cell constructs without a scaffold.

Figure 2: MagTE Cell Printing

Lithography

Lithography utilizes chemical processes and hydrophobic interactions to create an image. In cell printing, lithography is used to print patches of cell adhesives which the cells later adhere to. The DNA of the cells must be pre-treated and functionalized prior to printing. Microprinting and soft lithography are the two primary kinds of lithography that are utilized in cell printing (Barron 2004); however, these methods are expensive and yield moderate accuracy (Lenhert 2007).

Microcontact Printing

Microcontact printing is a form of soft lithography that uses an elastomeric polymer to stamp a pattern onto the target surface. The cell solute has an affinity for this pattern and adheres to it (Ilkhanizadeh 2007). While this method provides resolution in micrometers, it also has potential for cell damage and have bee limited to two-dimensional printing (Mrksich & Whitesides 1996). In some research with this method, regions of extra-cellular matrix enabled for cell adhesion whereas, regions without sufficient extra-cellular matrix were non-adhesive and incapable of guiding cell growth patterns of bovine capillary endothelial cells (Chen 1998). In this study cell printing had

to be performed in a clean room due to the contact nature of the printing mechanism and the potential for contamination.

Other researchers created a mold via microcontact printing instead of direct printing and seeded the chick retinal ganglion cells onto this mold (von Philipsborn 2003). A “lift-off” technique was used to create a stamp pattern of proteins. The mold needed to be cleaned after each use, making it less effective than a readily reusable printing method.

Capillary Induced Contact Guidance

Capillary Induced Contact Guidance (CICG) combines lithography and nanoimprinting to create optimized microstructures. In this process, cell adhesion is dependent on membrane elasticity, cytoskeletal tension and dynamic properties. A goal of CICG studies has been to study linear relationships between surface topography and the shape of the cellular interfaces (Lehert 2007, Qijin 2005). For example, surface texture influences morphology, so enhanced knowledge of this topic could improve wound healing, implantology and overall tissue engineering. Grooved polystyrene surfaces were patterned, and a scanning force microscopy was used to examine the surface topographies; various cell types aligned differently with respect to the grooves (Lehert 2007, Qijin 2005).

Biological Laser Printing

Biological laser printing (BioLP) is a laser-based, non-contact method of printing cells and biological materials. Thermal changes, created by lasers focused through an objective lens, move material from the laser absorption layer to the receiving substrate

(see figure 3). Since this method is orifice-free, biological adhesion between the deposition head and the target surface is decreased, minimizing the risk of cross-contamination. The amount of material transferred is a function of the laser spot size, the temperature of the biomaterial and the laser fluorescence (Ringeisen 2004).

Figure 3: BioLP Cell Deposition System

Goals of BioLP include: rapid deposition of biological materials, high resolution printing, reproducibility, sterility of deposition, and the ability to print support materials and other biomaterials in addition to cells and other biological matter. These methods have yielded resolution in micrometers with a deposition rates up to 100 pixels per second. Future goals of BioLP include improved accuracy, increased deposition rate, and creation of heterozygous three-dimensional constructs (Barron 2004, Ringeisen 2004).

Laser Guided Direct Write

Laser-guided direct write uses pressure to deposit cells onto a substrate with a resolution in micrometers. A laser scatters protons via pressure onto a CAD/CAM-created receiving substrate, such as a scaffold (Ringeisen 2006). Matrix Assisted Pulsed Laser Evaporation Direct-Write (MAPLE-DW) is an example of laser-guided direct write that uses the laser's radiation energy to excite and transport material to the target surface

(Chrissey 2003, Ringeisen 2004, Ringeisen 2006). This method is compact, accurate, and gentle on cell membranes; however, it could be further refined to ensure cell viability is not compromised (Ringeisen 2004, Ringeisen 2006).

Cell Viability Maintenance Conditions

In order to maintain cell viability, several conditions suitable for cell life must be maintained in any incubator. Precise temperature, CO₂, and humidity levels are required. The optimal incubator conditions include: temperature at 37° C (98.6° F), 5% CO₂, and a humidity level around 99% (to prevent media evaporation and changes in pH levels) (Boland 2006, Blau 2001, Engelmayr 2003). Additionally, the culture environment must be sterile to prevent contamination which could lead to cell death. Several cell culture systems will be discussed in the next section; these systems strive to meet the following conditions: thermal regulation, CO₂ and humidity maintenance and sterility.

Thermal Regulation

Thermal regulation is important, because even slight temperature deviations can have severe consequences on the cell. Temperatures significantly above 37° C may denature proteins required for cell function, and compromise the integrity of the thermosensitive microtubules, which are crucial for cell division (Cooke 2002, Boyer 2007). This damage delays cell division, and can lead to an increase in apoptosis (cell death), which would be detrimental to the organ being printed (Cooke 2002). Lower temperatures may deter cell growth and division, potentially causing the cells to ball up and die.

CO₂ and Humidity

Both CO₂ and humidity levels have an effect on the viability of the cells, by effecting the media's pH. Insufficient humidity may lead to evaporation of media, which can cause an increase in the media's pH. Likewise, excess CO₂ in the air may alter the media's pH which can have an adverse effect on the cells. Under optimal conditions an incubator environment should be kept at 5% CO₂ (95% air) and the humidity level should be maintained at about 95-99%.

Sterility

The interior of the sterile incubator volume must meet sterility requirements to optimize potential for post-deposition cell viability. Effective sterilization processes reduce bioburden, contamination, and increase the deposited cells' chances for survival. By minimizing airborne and other bioburden, the environment within the sterile volume is more suitable to maintaining viability and encouraging cell growth.

ISO 17664:2004 - Sterilization of Medical Devices defines sterility as a 6 log reduction in bioburden—a one in one million chance of bioburden surviving after sterilization (ISO 17664). Before a device can be sterilized it must be properly cleaned to achieve a 3 log reduction in bioburden (one in one thousand chance of bioburden survival). Cleaning is the removal of contaminants from a device to the extent necessary for further processing or use (ISO 17664). These standards were imposed on medical devices to ensure that devices branded as sterile are suitably decontaminated for use in human medical procedures. This harmonized standard includes a protocol for cleaning and sterilizing medical devices.

Once a device is clean, various sterilization methods can be used, including autoclave, UV/gamma ray, and ethylene oxide sterilization. Autoclaving is a common

steam sterilization method that does not involve harsh chemicals. In order for a device to be autoclavable it must be capable of withstanding temperatures of 250° to 375° F (121°-190° C). Ethylene Oxide (EtO) sterilization does not have the temperature constraints of autoclaving; but it has potential for hazardous residues, which can decrease the efficacy of this sterilization method since contact with these residues may have an adverse effect on the tissue (Leventon 2002). Gamma Ray and UV sterilization are other methods, which can be coupled with EtO sterilization to increase efficacy.

Sterility is necessary for any component that comes into direct contact with living tissue; aseptic standards are applicable for components that do not come into direct contact with the tissue, where contamination is less of a concern. For the sterile incubator volume, the components in direct contact with the cells are the media container (culture dish) and possibly the deposition head (depending on style). The rest of the volume must maintain aseptic conditions. Striving for complete sterility will reduce the risk of contamination, therefore increasing chances for cell viability.

Anything placed in the sterile hood must be thoroughly cleaned. If the sterile incubator volume will be placed in the hood for any reason (e.g. removing the cell culture dish), there must be a way to clean and decontaminate the exterior of the volume. This is achieved by spraying the exterior of the volume thoroughly with 70% ethanol prior to placing it in the hood. Once the volume is in the hood, proper cell culture and hood use procedures shall be followed to ensure the sterility of the cells and to minimize the risk of cross contamination in the hood.

Types of Incubators

There are three main types of sterile incubators for standard cell culture systems: open, closed and box. All of these systems maintain sterility, pH (which is related to humidity and CO₂), and temperature within a specified range. Additionally, none of these systems should interfere with or damage the interfacing devices (e.g. microscope). Interfacing devices, especially electronic or other sensitive devices, must be reasonably protected from the humidity and acidity of the incubator environment (Salierno 2007, Szabo 2007).

In an open incubator system, there is no boundary between the external environment and the cells allowing for external manipulation. However, the culture environment's environmental conditions are dependent on the room's conditions, so slight deviations in room climate could adversely affect the cells. Furthermore, the entire system must be contained in a sterile environment (e.g. the hood) to maintain sterility. If the system is placed in the hood, the entire system must be adequately disinfected to prevent the incubator system from contaminating the hood (Salierno 2007).

A closed incubator system better controls sterility since it is sealed; however, cellular manipulation is more challenging. This type of system can have a regulated environment capable of maintaining cell viability for several days (Salierno 2007). However, since the system is entirely sealed, it can be challenging to reach and manipulate cells inside the system; hence, an entirely closed system is not a feasible option for cell printing. Petri dish heaters are a simple closed-system incubator, but they often yield uneven heating leading to noticeable temperature gradients (Cooke 2002); this often results in evaporation which can make visual inspection of cells very difficult. While more advanced closed systems have been developed, with better temperature

regulations to increase cell viability, this type of system is still not optimal for cell printing.

Traditional objective heaters are another type of closed system; offering a better regulated environment with minimal temperature gradients. Objective heaters were designed specifically for use with inverted microscopes and do not allow for external manipulation of cells within the chamber (Szabo 2007). Because of this, many modifications would be required to modify the devices for external manipulation from a moving deposition print-head.

A third type of heating system is the box incubator system, which encloses the microscope and the volume around it. This enables for some environmental control and some manipulation. However, this system type has an increased risk of contamination over a standard closed system (Salierno 2007). This environment enables for control of temperature, CO₂, and humidity, but limits manipulation (Szabo 2007).

Various examples of incubators will be discussed, including heating method, sterility and their applicability and potential for cell printing. These incubator systems include an autonomous perfusion chamber for long-term culturing and in-situ investigation, encapsulated Petri dish, tissue engineering bioreactors and a bioassembly tool.

Autonomous Perfusion Chamber

Blau's autonomous perfusion chamber is a closed system that allows for electrical and optical investigation of cultured cells, while maintaining cell viability in a controlled environment. This system does maintain the desired environmental conditions; however, it is not intended for use in tissue engineering, as cellular manipulation is limited. This

design is modular, temperature-controlled, transparent, autoclavable, reusable, and internally sterile. While most perfusion chambers are open and only suited for short-term experimentation (Blau 2001), this design allows for longer, climate-controlled trials. The volume's contents are minimized to reduce cross-contamination risks (Blau 2001).

This incubator has its downsides; for example, media pH was not regulated enough which had a negative effect on cell viability. Too much CO₂ permeated through the inlet and outlet tubes, and gas bubbles in these tubes further contributed to CO₂ and pH challenges within the chamber. It was suggested that future iterations of the design consider a simple infusion-type flow mechanism and inert gas to pressure the cell medium supply (Blau 2001).

Encapsulated Petri Dish

The Encapsulated Petri Dish (EPD) system provides a sterile environment compatible with culture dishes and most inverted microscopes. This system is intended for use with tissue cultures and is can be used in mid-term to long-term microscopy experiments, lasting up to 100 hours. A micropipette permanently inserted into the microenvironment enables for some sterile manipulation of the cells; however, these manipulation capabilities are very limited and not suitable for extensive cell printing. The chamber includes regulation of: temperature, CO₂ (via carbogen flow), and humidity (via water flow). Half of the EPD is heated with an aluminum heater to allow for visual inspection of cells on the other half; resulting in temperature gradients. However, this environment is well-suited for maintaining cell viability for a few days (Salierno 2007).

The EPD has many similarities to a potential incubator for printing, in that it is sterile, capable of maintaining cell life and its contents can be manipulated with relative

ease. However, the interface of Salierno's device is stationary; whereas a moving print-head mandates mobility of the culture dish. While this design offers inspiration for the sterile incubator volume design, it is not all-inclusive of the design specifications needed for cell printing (Salierno 2007).

Tissue Engineering Bioreactors

Tissue engineering bioreactors are designed for tissue engineering, with their foci in various aspects of tissue engineering, and are typically placed in a standard humidified incubator (Sodian 2002). Transparent Plexiglas walls and an air-driven respirator pump (to provide O₂) are two important elements of these closed-loop bioreactors. The entire incubator sits inside a sterile hood, is EtO sterilizable, and is easy to assemble. While the bioreactor is capable of maintaining optimal conditions for cell viability, it is not compatible with a moving culture dish.

Bioassembly Tool for Regenerative Medicine

A Bioassembly Tool (BAT) aims to integrate tissue engineering and incubation to increase post-deposition cell viability. The BAT environment is a large cabinet-sized chamber with doors on the front that can be opened to remove the chamber's contents, including engineered tissue (Miller Smith 2005). The cabinet is non-sterile and regulates temperature with three heating sources: a heated stage below the media and two heat lamps above the media. The heaters are controlled by a temperature feedback loop. Ultrasonic and evaporative humidifiers maintain 80% relative humidity, suitable for cell viability. Cell printing has been successfully performed using the BAT; however, this is a very complex system that is beyond the scope of our printer.

Tissue Engineering at Cal Poly

Cal Poly aspires to have its own student created, operated, and maintained cell printers as part of the tissue engineering lab. Two cell printing methods are being developed on campus, and various cell deposition methods may be necessary as tissue engineering and cell printing is researched and enhanced at Cal Poly.

One of the printers under development is a modified inkjet printer. This printer was custom designed with student-created software to dictate the motion of the stages. This printer has substrate deposition rates of up to .3 mL/second and uses mechanically created pressure variations for deposition. Using this deposition method should minimize protein denaturation risks.

The print-head has already been developed and successfully tested for deposition with ink and cells. A preliminary deposition volume (for testing purposes) has been assembled, which is neither climate controlled nor sterile (see figure 4). The volume is made of transparent acrylic, with a hinged door (for retrieval of deposited cells) and a latex top. The interior of the volume may be cleaner than the lab, but the door must be opened frequently, so there is no way to maintain sterile or aseptic conditions inside this volume.

Figure 4: Preliminary Sterile Volume for Inkjet Print-head

A second cell printing option will use a syringe to deposit cells. This method employs mechanical pressure for deposition and is aimed primarily at printing cells (instead of proteins or substrates). Software and mechanical mechanisms for the syringe deposition printer have not yet been devised, but creation of a direct cell printer is a long-term goal.

Cal Poly would like to further develop on-campus cell printing methods. The current inkjet printer is only configured for two-dimensional printing, but the addition of an elevator stage would make three-dimensional printing possible. The inkjet printer has multiple potential deposition volumes, so it should be capable of depositing more than one cell type at a time. Printing organs and further integration of patient imaging and three-dimensional printing technologies are long term goals.

Conclusions

An environmentally controlled sterile incubator volume is needed to preserve cell viability. This sterile incubator volume shall be compatible with, but not limited to, the

inkjet printer and syringe deposition head. This volume shall maintain optimal conditions for cell life throughout the printing process with a focus on temperature regulation. The volume shall also ensure that CO₂ and humidity levels are in a range for maintaining cell viability.

The sterile incubator volume is unique in that it provides an environmentally controlled sterile volume for cell deposition, while allowing for manipulation of the deposited cells. Other incubators are aimed at maintaining cell viability in an enclosed environment, without considering the effects of introducing and removing a foreign component (the deposition print head) into the sterile volume. The volume shall maintain environmental conditions throughout the print process for a specified period of time.

3.0 DESIGN PROCESS

The design process began with the establishment design requirements based on the problem statement. Once these had been established the preliminary design phases could begin, followed by iterative design. Several aspects of the sterile incubator design were considered at each stage in the design process. The design process also adhered to a budget and timeline which were created at the beginning of the design process. With these systems engineering considerations established, the design process can commence.

Goals

The primary objective of the project is: *to design, build and test a volume capable of maintaining cell life for deposited cells*. The volume shall adhere to conditions required for cell life in terms of environment, sterility, and interface with the deposition head. Additionally, the volume must be easy to use, resterilizable, and expenses shall be minimized. These specifications lead to the creation of functional requirements and design goals.

Functional & Non-Functional Requirements

1. The volume must maintain adequate conditions for cell life.
 - a. 37° C (deviation of no more than +/-2° C)
 - b. 5% CO₂, 95% air (to minimize deviations in pH)
 - c. 99% relative humidity (to minimize deviations in pH)
2. The interior of the volume must maintain at least aseptic conditions
3. The volume must be capable of being sterilized

4. The sterile volume must be capable of interfacing with the existing cell deposition print-heads
5. The cost of the volume shall not exceed \$1,000
6. There must be a way to transfer cells from the sterile incubator volume to a commercial incubator
7. There must be a way to ensure that printing is occurring

Desired Design Features

1. Deposited cells should still be viable after 4 hours in the sterile incubator volume*
2. All non-disposable parts should be autoclavable
3. The volume should be resterilizable at least 50 times.
4. The volume should be easy to set-up and use
5. The volume should be compatible with standard cell culture dishes
6. The volume should be compatible with a light microscope

*Based on discussions with Dr. Crockett, this is the maximum foreseeable print time for the printing systems that are under development.

Design Assumptions

1. A modified print head tip is used; this is assumed to be an 18 gage needle or smaller
2. The primary reason for humidity and CO₂ regulation is to maintain pH; if pH can be maintained without meeting these specifications, pH maintenance will be considered acceptable.

3. Cells have a phosphate buffer with a Pk_A of 6.8; therefore they can maintain their pH provided the media pH is within the buffer range (5.8-7.8) (Boyer 2007). pH deviation within the sterile incubator volume shall be kept near this range.

Project Timeline - Proposed and Actual

The following timeline was created with a target date for design, prototyping and verification completion by April 15th, 2008; this allows for nearly 2 months of lead time prior to the final deadline of June 13th, 2008. Some lead time has been built into this schedule, but nearly two months of extra time at the end should accommodate inevitable delays; therefore, proposed and actual deadlines need not align perfectly. Table 1 displays detailed proposed and actual schedules.

Table 1: Proposed and Actual Schedule

Task	Proposed Dates		Actual Dates	
	Start	Complete	Start	Complete
Primary Literature Review	10/1/07	12/31/07	10/1/07	12/28/07
Functional Specifications	10/20/07	12/31/07	10/20/07	12/10/07
Brainstorming & Preliminary Designs	10/20/07	12/31/07	11/1/07	1/15/08
Refined Designs	1/1/08	1/20/08	1/10/08	2/15/08
Order Materials	1/21/08	2/7/08	2/15/08	3/10/08
Prototyping & Design Iterations	1/20/08	2/28/08	2/5/08	4/1/08.
Write Test Protocols	2/1/08	2/28/08	1/26/08	2/20/08
Formal Testing	3/1/08	3/31/08	2/21/08	5/14/08
Rough Draft of Thesis	12/31/07	4/15/08	1/6/08	4/7/08
Advisor Review of Rough Draft	4/15/08	5/15/08	4/28/08	5/19/08
Thesis Defense	5/30/08	6/10/08	6/3/08	

Budget

The projected device budget of \$1,000 includes high-quality monitoring devices, materials, a heater and other expenses. Table 2 displays the anticipated budget and expenses for the sterile incubator volume prototype. This budget is dependent on the quality of the heating, humidifying, and monitoring devices. The accuracy level required

was determined after creation of the budget, so it has potential to vary. Funding for this project is being provided by Cal Poly's Biomedical and General Engineering Department.

The budget does not include lab materials or supplies necessary for culturing cells, since these expenses are related to verification of the design rather than the design itself. These materials were provided by the tissue engineering lab in the Biomedical and General Engineering Department.

Table 2: Projected Budget for Sterile Incubator Volume

Component	Projected Cost
Volume Materials	\$70
Heater	\$100
CO ₂ Monitor/Regulator	\$150
Temperature Controller & Thermocouple	\$250
Connectors/Miscellaneous	\$80
Total	\$650

Preliminary Design

Several preliminary decisions must be made before the sterile incubator volume design can be refined. Sterile volume configuration, heating method, and print-head interface were some of the most important early design decisions.

Volume Size

Determining volume size was important early in the design process. The approximate volume size had to be determined before many other design elements could be determined. This decision involved selecting the approximate size of the volume and determining what to enclose in the volume. There were two options in preliminary volume considerations:

1. **Large Volume:** enclose the entire print-head configuration and both moving stages inside a large sterile volume (approximately 30" x 30" x 10"). This design would enable the entire volume (including the print-head interface) to be constructed of rigid materials. With a large volume, there must be a way to remove and transport the culture dish while maintaining its sterility.
2. **Small Volume:** create a smaller sterile volume that sits on top of the stages (approximately 12" x 12" x 2"), to minimize the risk of contamination. This would require a non-rigid interface to accommodate the moving of the volume with respect to the print-head. The volume itself should be portable; allowing the entire volume to be carried to the hood so the culture dish can be removed.

Figure 5: Volume Size Idea Design Sketches

Table 3: Volume Size Decision Matrix

Parameters (weight)	Large Volume		Small Volume	
	Raw Score	Weighted Score	Raw Score	Weighted Score
Heating/ Heat Maintenance (30%)	2	6	3	9
Least Contamination Risk (25%)	1	2.5	2	5
Resterilizability (20%)	1	2	2	4
Durability (15%)	2	3	3	4.5
Manufacturability (10%)	1	1	3	3
Weighted Total (100%)		14.5		25.5

Parameters are ranked from 1-3, with 3 being the best. Differences in cost are assumed to be negligible, since either volume would be made out of polycarbonate (or similar material), for which either size should be within the budgetary constraints.

Based on the decision matrix the small volume is a better option for the sterile incubator volume, since it is easier to heat and reduces cross-contamination risk. The smaller volume is also easier to sterilize, due to its size. The portability and simplicity of the small volume are also advantageous. The print-head top interface still needs to be determined.

Heating Method (Type)

Once the approximate volume had been determined, heater types could be evaluated. There were two primary types of heaters that were considered:

1. ***Petri Dish Heater:*** readily available, and heat only the Petri dish, which minimizes the energy required. However, this method has potential for non-uniform heating and efficacy concerns have been expressed. A technical sales representative at Bioscience, Inc advised that heating the entire volume would be more effective for this

application. Petri dish heaters are not designed for heating more than a few layers of cells.

2. **Entire-Volume Heater:** this is a method for heating the entire sterile volume, which aims to ensure uniform heating. This may also minimize the external environmental effects.

	OVERVIEW	
	Heat Cell-Containing Vessel	Heat Entire Volume
	<p>$T_{\text{heater}} > T_{\text{cells}}$</p> <ul style="list-style-type: none"> - heater heats cells - rest of volume is unreg.	<p>$T_{\text{heater}} = T_{\text{cells}}$</p> <ul style="list-style-type: none"> - entire volume heated - constant temp throughout
Advantages	<ul style="list-style-type: none"> • Only heats culture dish • "Off-the-shelf" 	<ul style="list-style-type: none"> • Entire volume isothermal • Potential to humidify air • Uniform heating
Disadvantages	<ul style="list-style-type: none"> • Lack of uniformity • Expensive • Sterilization challenges 	<ul style="list-style-type: none"> • Temperature control system must be designed

Figure 6: Heating Method Design Ideas

Table 4: Heating Method (Category) Decision Matrix

Parameters (weight)	Petri-Dish		Entire Volume	
	Raw Score	Weighted Score	Raw Score	Weighted Score
Uniformity of Heating (35%)	1	3.5	2	7
Cost (25%)	1	2.5	2	5
Manufacturability (20%)	2	4	1	2
Resterilizability (20%)	1	2	2	4
Energy required (10%)	2	2	1	1
Weighted Total (100%)		14		19

Parameters are ranked from 1-3, with 3 being the best.

Based on the decision matrix the entire volume shall be heated. Minimizing the size of the volume will help reduce the energy required for the heater.

Print-head Interface

Another early consideration was the print-head interface, which must allow introduction and removal of the print-head from sterile volume, while maintaining sterility of the volume. Several print-head interface options were considered, including:

1. ***Flexible Top:*** an elastic material, capable of stretching to accommodate the movement of the print-head. A concern with this method is the potential for tearing.
2. ***Flexible Top & Septum:*** based on the assumption that a needle can be used, a septum is applicable. The septum maintains sterility inside the volume during needle insertion, deposition, and removal. The septum may also increase the integrity of the flexible top by reducing the shear forces endured by the latex.
3. ***Tent Top:*** a fabric or plastic sheet with additional material to accommodate the motion of the volume. The interface for the tent design would be permanent and would make portability of the volume more challenging (removal of the interface would allow non-sterile air into the volume).

	<p>A</p> <p>print head</p> <p>flexible interface</p>	<p>B</p> <p>septum-less design</p>	<p>C</p> <p>attachment</p> <p>"tent"</p>
Advantages	<ul style="list-style-type: none"> •Simple •Portability of container 	<ul style="list-style-type: none"> •Durable •Maintain Internal Sterility 	<ul style="list-style-type: none"> •Durable •Elasticity not a Concern
Disadvantages	<ul style="list-style-type: none"> •Tearing Potential •Resterilizability •Fatigue 	<ul style="list-style-type: none"> •More complex design •Requires deposition needle 	<ul style="list-style-type: none"> •Potential to sag •More to sterilize and heat

Figure 7: Top Interface Design Ideas

Table 5: Top Print-Head Interface Decision Matrix

Parameters (weight)	Flexible		Septum		Tent	
	Raw Score	Weighted Score	Raw Score	Weighted Score	Raw Score	Weighted Score
Durability (35%)	1	3.5	2	7	2	7
Sterilizability (25%)	2	5	2	5	1	2.5
Design Form (20%)	1	2	3	6	1	2
Manufacturability (15%)	3	4.5	2	3	2	3
Weighted Total (100%)		15		21		14.5

Parameters are ranked from 1-3, with 3 being the best. Durability and

Sterilizability were considered to be the most important, because both are focused on maintaining the internal environmental conditions of the sterile incubator volume.

Design form was weighted slightly more than manufacturability, because all of the designs were considered to be readily manufacturable.

Based on the decision matrix the septum top is the best option since it features superior design form and style. The septum combines the durability of the tent top with the resterilizability aspects of the flexible top.

Morphological Chart

Figure 8 is a chart depicting the preliminary design decisions that lead to the design of the sterile incubator volume. While many refinements were still needed, these early ideas evolved into the final sterile incubator volume design.

Figure 8: Morphological Chart for Preliminary Designs

Preliminary Design Summary

The preliminary design of the sterile incubator volume consists of a small (12" x 12" x 2"), polycarbonate box, heated to 37° C, with a flexible top (see figure 9). The top is an elastic material that accommodates the motion of the print-head, with a septum to ensure that sterility is maintained after the top is punctured and the deposition needle is removed. All these components must integrate into the sterile incubator volume system to achieve the functional and non-functional goals. At this point in the design process, further design iterations and testing are still required to verify these design concepts.

Figure 9: Preliminary Design Sketch

Iterative Design

Once a small, enclosed volume, with a latex-septum top and entire-volume heater was selected, the iterative design process was used to refine the design. This included design discussions aimed at modifying the current design, experimentation with

preliminary prototypes, and refining various design aspects. Exact size, heating method, top interface, container selection, volume sterilization, septum, latex thickness and humidity regulation were design aspects that were refined during this process.

Exact Size

An early design iteration reduced the estimated volume size from 12" x 12" x 2" to 6" x 6" x 2" (see figure 10). This design change was based on to the fact that the initially proposed size was unnecessarily large, since the cells will be deposited into a 3.5" diameter culture dish. With the dish in the center of the volume there would still be ample clearance (at least 1.25") on each side of the dish for water or a heater (if needed). This size reduction minimized the volume to heat, while increasing portability and ease of cleaning.

Figure 10: Original and Refined Size and Volume Comparison

Heating Method (Specific)

Heating methods considered included warm-air fans, mat heaters, and heat lamps. Fans are not optimal since they may yield uneven heating and turbulent air; increasing the

risk of contamination. Heat lamps are difficult to control, which may result in uneven heating. A heating mat was the selected heating method, based on accuracy and uniformity. The heating mat is coupled with a temperature controller to ensure accuracy. The heat mat is placed outside the sterile incubator volume to reduce the risk of cross-contamination.

Silicone rubber heat mats (McMaster-Carr; Los Angeles, CA) and Kapton flexible mat heaters (Omega, Inc.; Stamford, CT) were considered for use with a P-I-D temperature controller and thermocouple (McMaster-Carr; Los Angeles, CA). According to an Omega technical sales representative, the Kapton flexible heater must have constant applied pressure or it will short-circuit. The silicone rubber heat mats can function without constant pressure application. The volume may not have a perfectly flat bottom, so constant pressure on the heater cannot be guaranteed; hence, the heavy duty silicone rubber heat sheet was selected.

In addition to the heat mat, a type J thermocouple and a temperature controller are necessary for temperature feedback. The temperature controller is an Autotuning P-I-D Temperature Controller with relay control output, selected since it can accurately control temperature via integral and derivative parameters. It is a small system (1/16 DIN), meets all requirements, and is cost-effective. A Type 316 SS Type J thermocouple provides feedback to the temperature controller. This thermocouple covers a broad range of temperatures ($0^{\circ}\text{C} - 760^{\circ}\text{C}$) and features a bendable probe capable of taking readings in air or liquid. These features enable for greater potential for design iterations. This three-component heating system will enable for accurate temperature control, including

feedback, and a price that falls within the budgetary constraints of the sterile incubator volume project.

Top Interface

Several concepts were considered for the interface between the latex and the container base. Interface methods included zip-ties or rubber band to secure the latex, a screw on frame, and a magnetic frame capable of holding the latex in place. Primary goals for the interface were ease of use (for in-hood assembly) and sterilizability.

Figure 11: Concept Ideas for Top Interface

The top interface selected is a snap-fit lid to secure the latex. A commercial container will be used due to limited manufacturing capabilities on campus. This will

lock the latex onto the top of the sterile volume. The container should be sterilizable. A 4" x 4" hole will be cut in the lid to create the snap-fit "frame" for the latex.

Container Selection

Many standard snap-fit containers cannot withstand the heats associated with autoclave sterilization, so it was important that the selected container be very durable and temperature resistant. Additionally, the selected container needed to be approximately 6" x 6" x 2".

Two containers were considered, the Rubbermaid Premier 3-cup container and the Snapware Snap'n'Serve 4-cup container. These containers were selected based on size and durability of materials. The Rubbermaid container is square measuring approximately 6" x 6" x 2", while the Snapware container measures 4" x 8.2" x 2". Both containers are made of durable, clear plastic. The lid is easier to seal on the Rubbermaid container, but the Snapware container is acceptable. Both containers must be tested for sterilizability, but the Rubbermaid is the preferred container due to its shape and sealing ease.

Volume Sterilization

Polycarbonate is a durable material that is typically capable of being autoclaved; so this material was considered to be optimal. Both containers under consideration were durable plastic and capable of withstanding high temperatures. Since autoclave is the preferred sterilization method, the ability to withstand these temperatures is considered ideal. Further testing was conducted to ensure that the selected container was, indeed, autoclavable.

Latex Sheet Thickness

Latex sheets of various thicknesses were tested, with the goal of selecting the most durable latex applicable for this application. Thicker latex may be more durable, but thinner latex is easier to stretch; hence a balance must be struck between the two. The initial latex sheets tested were tested were: .006", .0125" and .025" thick (Small Parts, Inc.; Miramar, FL). Based on preliminary tests, the .006" latex was selected; further information on testing can be found in the testing section and in Appendix C.

Septum

The septum allows for insertion and removal of deposition needles, while maintaining sterility inside the volume. Pawling Corporation sent a variety of sample septa, including: natural rubber, butyl rubber, and PTFE, in various sizes. The larger septa were easier to attach to the latex, and also easier to hold in place during needle penetration and removal. Most septa have been validated for needles as large as 18 gage. Prior research has shown cell viability with smaller needle sizes (25 and 33 gage) to extrude collagen, bovine aortic endothelial cells (Miller Smith 2005), so depositing the cells through an 18 gage needle should not be problematic. The septa tested all quickly resealed after insertion and removal of the 18 gage needle.

Primary considerations for the top interface are ease of use (for in-hood assembly), reliability, cost, and ease of manufacturing. The septa shall be attached to the latex outside the hood (prior to sterilization) to minimize the risk of contaminating the hood. Additionally, use of a pre-made snap fit lid ensures

that volume construction is efficient and simple, even when it is performed inside the hood.

Humidity

Maintaining appropriate humidity conditions minimizes media evaporation. Evaporation may result in changes in the media's pH; pH changes may be detrimental to cell viability (Boyer 2007). Since the volume is heated, some evaporation is anticipated; therefore, adequate humidity maintenance aims to prevent excess evaporation. To ensure that appropriate humidity is maintained, several options were considered including a humidifying water bath, a steam or ultrasonic humidifier, humidity packets, and saturated sponges.

Preliminary tests helped to determine that, if anything, only a minimal water supply was needed to maintain adequate humidity to prevent adverse changes in pH; therefore, steam and ultrasonic humidifiers were eliminated. Both of these would lead to an increasingly complex design and increased contamination risks. Sponges were found to assist in pH regulation in a similar way to a water bath; however, evaporation with no humidification source was found to be low. Hence, neither of these options was further developed, and humidification was not considered to be necessary for this iteration of the sterile incubator volume design.

4.0 FINAL DESIGN

The final design was a culmination of several design decisions aimed at optimizing design simplicity and effectiveness. This consists of two pre-made snap-fit containers (the sterile volume and the feedback volume), latex top with septum, silicone rubber heat mat, type J thermocouple, and a P-I-D temperature controller. The container and lid are capable of being autoclaved repeatedly, and the latex top with septum shall be replaced after each use. The septum's integrity will not be compromised during use, but since it is adhered to the latex it makes more sense to simply replace the septum when a new sheet of latex is used. The temperature controller is wired to the power supply, the silicone heat mat, and a thermocouple to accurately measure and regulate the feedback volume temperatures, which correspond to the sterile volume temperatures.

The wiring of the heater and the use of the P-I-D temperature controller enables accurate temperature control. The thermocouple is placed in a feedback volume, both volumes (sterile and feedback) sit on top of the heat mat (see figure 12). The feedback volume is configured in the same way as the sterile incubator volume; both are the same container with culture dishes of media and latex tops. The feedback volume has a thermocouple inserted through a small hole in the lid. This thermocouple tells the temperature controller the temperature of the media in the feedback volume and the controller regulates the heat mat accordingly. It is assumed that the media in the feedback volume and the sterile volume are nearly identical, since both are set up in the same way with only minor differences between the volumes.

Figure 12: Temperature Feedback Setup for Sterile Incubator Volume System

A polycarbonate frame holds both volumes in place on top of the heat mat. This frame was built to allow for simple, accurate placement and removal of both volumes. The frame fits snugly around the volumes, and secures them in place during printing. Once printing is complete and the deposition syringe has been removed, the sterile incubator volume can be lifted out of the frame and returned to the hood for cell post-processing.

The top interface was designed with the primary goal of simplicity, for assembly in the hood. The septum is an important design feature, since it maintains sterility after insertion and removal of the deposition needle. The septum is connected to the latex via Loctite 3186 Medical Device Adhesive (Small Parts, Inc; Miramar, FL). As shown in figure 13, with the septum in place, no foam reinforcement is needed, since the septa helps reinforce the latex.

Figure 13: Latex lid with septa, the deposition syringe is pulled to one edge

The Rubbermaid containers were modified to meet the needs of the sterile incubator volume system. The foot was sanded off of each container to flatten the bottom. The center of each container's lid was removed with a razor blade, leaving an opening approximately 4" x 4" for the latex. One edge of the lip was shaved off each container and lid, so that the containers could be placed closer together on the heat mat. Once these modifications have been completed, the container has been customized for the sterile incubator volume system.

Heater Wiring

Proper configuration and wiring of the heating system is imperative for the sterile incubator volume system. The wiring must properly integrate the three system components and ensure accurate feedback. Wiring is connected directly to the

temperature controller, and wire positions were determined based on the schematics in the temperature controller manual, as depicted in figure 14.

Figure 14: Wiring schematics for the Heating System

Prototypes (Proof-of-Concept)

A functional prototype of the sterile incubator volume was created for proof-of-concept. This prototype was created out of easily-attainable components to maintain design simplicity and keep costs down. Components for this prototype were selected primarily based on their functional capabilities, but cost, availability and ease of use were also considered.

As depicted in the following image (figure 15), the final system consists of a sterile volume (left) and a feedback volume (right) both of which are placed inside a polycarbonate frame, on top of the heat mat. The temperature controller regulates the heat mat's temperature, based on feedback from the thermocouple. The temperature controller indicates the actual thermocouple temperature (AV) on top line and the 37° C set value (SV) on the second line.

Figure 15: Final configuration for Sterile Incubator Volume System

Product Component List and Prices

The following table contains information for the components of the sterile incubator volume, including vendor, name, part number and cost. Whenever possible, components were selected from large vendors to enable for re-ordering as needed.

Table 6: Product Components

Component	Vendor	Name	Part Number	Cost
<i>Sterile Volume</i>				
Snap-fit Container	Rubbermaid	Rubbermaid Premier 3 Cup Container (2)	N/A	\$10.00
Latex	Small Parts, Inc.	Natural Latex Rubber Sheet .006IN thick 24"x15"	SLR-006-B	\$3.00
Septa	Cepure (Pawling Corp.)	20 mm Natural Series - PTFE/ Silicone, 0.125", Loose Septa	CP3200.020.700A	\$0.32
<i>Temperature Controller</i>				
Temperature Controller	McMaster-Carr	Autotuning PID Temperature Controller	38615K13	\$189.46
Thermocouple	McMaster-Carr	Mini-Plug Thermocouple with Bendable Probe	39095K62	\$18.80
Heating Element	McMaster-Carr	Heavy Duty Silicone-Rubber Heat Sheet for Plastic	35285K211	\$80.38
<i>Miscellaneous</i>				
Adhesive	Small Parts, Inc.	Loctite 4541 Medical Device Adhesive	LOC-18690-01	\$21.86
Subtotal				\$323.80
Tax (Subtotal * 7.25%)				\$23.48
Shipping (estimated)				\$25.00
Total				\$372.28

The actual product cost is below the project budget of \$1,000 and the projected cost of \$650. This is largely due to the fact that a cost-effective, pre-made volume was selected, and that CO₂ and humidity regulation were deemed unnecessary, since appropriate pH levels can be maintained within the specified range for up to one hour, based on testing (see Appendix C). The decision to not formally regulate humidity and CO₂ was based on preliminary test results and the desire to maintain design simplicity.

Container Setup

The sterile incubator volume system shall be set up in accordance with the setup protocol (Appendix D). This protocol includes pre-heating the heat mat, temperature adjustment, preparing the sterile incubator volume in the hood, setting up the feedback volume, and placing all components of the system together. This protocol gives directions to set up the system for its intended use.

5.0 TESTING & RESULTS

Each of the functional requirements for the sterile incubator volume shall be verified for its intended use in this design. While some aspects of the design can be verified without formal testing, others must be tested. A summary of testing procedures is, as follows:

Requirement		Testing Method
1a	Temperature	Temperature Testing
1b	CO ₂ levels	Evaporation & pH Test
1c	Relative humidity	Evaporation & pH Test
2	Sterility	Autoclave Test
3	Resterilizable	Autoclave Test
4	Interface (with syringe deposition head)	Viability Test
5	Cost	Cost Chart
6	Cell Transfer	Viability Test
7	Viability	Viability Test

- 1) **Environmental:** temperature, evaporation and pH tests shall be conducted to prove that the volume is achieving and maintaining the appropriate environmental conditions during printing.
- 2) **Sterility:** a protocol for cleaning and sterilizing the volume shall be verified, as much as possible, as part of cell viability tests. Formal sterility verification would require the use of cells without antibiotics. Contamination of these cells would have the potential to contaminate the commercial incubator

causing damage to all the cells inside the incubator; therefore, it is not feasible to perform this form of sterility test in the tissue engineering lab on campus.

- 3) **Resterilizable:** repeated autoclave tests will determine how many times the container can be sterilized prior to failure (defined by cracking, melting, or inability of the container and lid to properly seal). Visual inspection and a drop test shall be performed after each cycle to ensure the device's integrity is uncompromised. Watertight leak tests shall also be performed every 2-4 trials, as defined in the test protocol (Appendix B).
- 4) **Interface:** the interface shall be visually inspected to ensure that the surfaces are tightly mated. Water tight leak tests were used to examine interface integrity in preliminary examinations; since, an inadequate seal could compromise cell viability.
- 5) **Cost:** the cost requirements shall be validated in a chart displaying the costs of all the components for the sterile volume, to include a total cost.
- 6) **Cell Transfer:** the cell transfer is included as part of the cell viability testing, since this test includes the deposition of cells and a check of their viability. This test uses cells in the sterile incubator volume and examines viability after various periods of time.
- 7) **Cell Viability:** the cell viability test examines cell survival after a specified period in the sterile incubator volume.

Preliminary Tests

Preliminary tests examine design feasibility in the early stages. While preliminary tests are not used for verification, successful preliminary test results often

correspond with successful verification tests. Likewise, if a design fails preliminary tests, this may indicate that additional design refinements are necessary.

The preliminary tests, including procedure and results, are summarized in this section. Additional data, including complete test protocols and test reports for all of the preliminary tests can be found in Appendix C.

Temperature - Heat Transfer Testing (Capacity and Maintenance)

Heat transfer testing aimed to ensure that potential containers could withstand the temperatures required to heat or maintain media at 37° C. Heat capacity tests determined how long it took to heat the media at various hot plate temperatures, while maintenance tests examined what temperatures were required to maintain the media temperature.

To heat the media in 10 minutes, both containers required hot plate temperatures of at least 100° C. The Snapware container was more efficient, heating the media from 21° C to 35° C in 10 minutes, while the Rubbermaid heated its media from 20° to 28° C in the same time period. Data from this trial can be found in figure 16 (below).

However, in printing situations, the media would usually still be warm (from being in the incubator prior to printing), so heat maintenance is the more important issue.

Figure 16: Graph of Heat Capacity Test Results

Both containers fared well in heat maintenance trials. For these trials the optimal hot plate temperature was found to be 65° C. With the hot plate set at 65° C, and temperature readings taken every 5 minutes, both containers maintained the media within the specified range (35° C - 39° C). The Rubbermaid container's media ranged from 35.4° C to 37.3° C, while the Snapware container ranged from 36.0° C to 37.8° C. Data

from this trial can be found in figure 17 (below).

Figure 17: Graph of Heat Maintenance Test Results

It was hypothesized that the bases of the container played a large role in determining their heat capacity and maintenance capabilities. The Rubbermaid container has a large lip on its base; while the Snapware container has shorter corner “stands”. To accommodate these differences, the lip was removed from the Rubbermaid base, yielding excellent heat maintenance results in later trials.

CO₂ and Relative Humidity - Evaporation Tests

Preliminary evaporation tests were conducted, since excess evaporation can be detrimental to cell viability. These were performed with the hot plate set an adequate temperature for maintaining the liquid (media or water) at 37° C. The initial and final liquid volumes were measured. The final volume measurement was taken after the

volume and media had been on the hot plate for a specified period of time ranging from 20 minutes to 1 hour. Evaporation losses, by volume, ranged from 0.0% to 5.9%.

Media evaporation can cause changes in pH, which can be detrimental to cell viability. Hour-long tests were performed to calculate how much F-12 media evaporates and to observe pH changes. pH readings were taken using a calibrated Mettler Toledo pH probe. Readings were taken every 10 minutes for one hour, and the media's pH increased from 7.25 to 7.70, since the final pH was still within the cell's phosphate buffer region (5.8-7.8) it is assumed that this change in pH will not adversely affect cell viability.

Another hour long test was performed, this time without taking intermediate readings. This test recorded the temperature, volume, and pH of the media at the beginning and end of the trial to see how leaving the container closed affects the media, in this trial the pH increased from 7.38 to 7.70 which is within the cell's phosphate buffer region. A graph of the changes in pH with respect to time can be found in figure 18. In this trial the temperature increased from 34.7° C to 43.5° C over the course of an hour; while this is beyond the desired temperature range, this should not be an issue once the temperature feedback is introduced in the design. Additionally, a higher temperature would promote more evaporation and changes in pH, so this provides more of a "worst-case" scenario for testing. The initial volume was 40 mL and the final volume was 39.5 mL, a net loss of .5 mL (1.25%).

Figure 18: Graph of Media pH Changes over Time

Sterility Maintenance - Lid Seal Testing

Testing the lid seal integrity was an important preliminary trial, since this test had the potential to verify that the lid could keep the latex in place while maintaining a seal between the lid, latex, and container base. This was performed by removing the center of the lid (4" x 4"), to leave only the "frame" of the lid. Once the frame was prepared, the container was filled approximately 1" of water, and a sheet of latex was used to cover the container. The latex was locked in place with the frame, which is sealed by pressing down firmly on each side; the container was tilted over the sink to check for leaks. There were no leaks in any of the configurations tested (.006" and .012" thick latex with the Snapware container and .006", .012" and .025" thick latex with the Rubbermaid container).

Sterility Maintenance - Latex Tear Testing

Tearing in the latex would expose the cells to non-sterile air and potentially lead to cell death; hence, tear testing was important. Latex sheets of various thicknesses (.006", .012" and .025") were placed on top of the container then locked in place with the snap-fit frame. The frame was pressed down firmly on all sides, to ensure the latex was secured. The latex was punctured with either a push pin (preliminary tests) or an 18 gage needle (final tests). The latex sheets were either unreinforced (latex sheet alone) or reinforced (with craft foam in preliminary tests, with a septum in final tests). The push pin or needle was pulled towards each side of the container at increasing distances ($\frac{1}{4}$, $\frac{1}{2}$, $\frac{3}{4}$ of the way, and all the way) until the latex tore, or the edge of the container had been reached. Latex thicknesses and reinforcement were compared to select the optimal latex thickness.

The three latex thicknesses performed very differently in these tests. The .025" thick latex ripped early in the preliminary tests (push pin pulled halfway to the edge). This latex was also incompatible with the Snapware container, so it was eliminated. The .012" thick latex was durable, relatively difficult to pull, and did not rip during any of the pull tests with the push pin. The .006" thick latex easily stretched to reach all 4 edges of the container. The reinforced latex was the most effective in the preliminary trials (the push pin left the smallest hole, likely due to the foam absorbing some of the shear force). Once the septum was used instead of the foam, it too was able to help disperse the shear force to minimize the needle's hole.

Resterilizability - Container Heat Capacity

Early tests assessed the feasibility of using a polycarbonate container as the sterile incubator volume. These tests involved heating the container in the oven to ensure that autoclave temperatures did not melt the container. These test results were favorable; there was no evident container melting or damage.

Both containers (Rubbermaid and Snapware) were heated in an oven to pre-test for autoclavability. The containers were heated to autoclave temperatures (116° C and 133° C) for a specified period of time (5-15 minutes) then visually inspected for damage. The lid-container seal was tested with a watertight test. Both containers passed the tests with comparable performances.

Resterilizability - Autoclave Tests

The first cycle of the resterilizability trial served as the preliminary autoclave test. Both the lid and the base of the Rubbermaid container were autoclaved with the pouch cycle (steam sterilization at 133° C and 186 kPA for 5 minutes, then a 30 minute drying phase). Once autoclavability was proven, this container proceeded through the entire resterilizability verification for the final testing process. The complete test protocol and report are found in Appendix B, since a separate preliminary test was not required.

Verification Tests

After preliminary testing was completed and the design was refined, verification testing could commence. The purpose of verification testing is to ensure that the design

meets the design requirements as prescribed, and to verify that cell viability can be maintained.

Temperature - Regulation with Feedback System

Validation testing for temperature control was performed with the final sterile incubator volume system configuration. The feedback volume measures the media temperature and sends feedback to the temperature controller, while the sterile volume remains sealed. During feedback tests, all temperature readings were within .2° C of the specified temperature range of 35° C to 39° C. Water temperature tests had a temperature range from 35.0° C to 36.8° C over the 1 hour trial, which is slightly lower than desired. The media temperature ranged from 35.6° C to 39.2° C; however, the reading of 39.2° C was during a period when the thermocouple was not fully submerged in the media. If the thermocouple is not fully submerged, it will take readings in its environment (most likely the air), this air is probably cooler than the media, sending a signal to the temperature controller that the mat needs to heat up. This would lead to an increase in heat mat temperature, which would cause the media temperature to increase (this may have caused the 39.2° C reading). This data is displayed in figure 19 (below).

Figure 19: Temperature Verification Graph

CO₂ and Relative Humidity - pH Changes and Evaporation

When adequate media was in the sterile volume, pH changes were within an acceptable range. The acceptable range was determined to be 5.8 to 7.8, based on the cell phosphate buffer region, with its Pk_A value of 6.8. Adding 15mM of Hepes buffer to the high glucose solution assisted in regulating the pH, which was rising rapidly prior to the addition of the buffer. Once the buffer was added and two trials were conducted using 40 mL of media, the pH levels fell within the desired range for 50 minutes; after one hour the pH levels were slightly above the desired range at 7.85 and 7.88. The results of these pH verification tests can be found in figure 20.

Trials with less media (20 mL instead of 40 mL) experienced a larger spike in pH levels, a pH level of 8.24 after 60 minutes. This may have been due to the lower surface area to volume ratio, yielding more evaporation potential. Minimizing evaporation should help moderate pH and maintain consistent media properties for cell viability; in order to achieve this, the maximum feasible amount of media should be used.

It must be noted that evaporated media caused some condensation on the inside latex sheet and the container sides. This is solid evidence that a substantial amount of evaporation is occurring, which may be contributing to the increasing pH levels. The percent media loss (based on preliminary trials) was relatively negligible (no more than 6%), but this still yields noticeable condensation inside the sterile incubator volume.

Figure 20: pH Verification Test Results

Resterilizability

Autoclave tests were performed with the container to ensure that it could withstand the heat, humidity, and pressure of an autoclave cycle. The Rubbermaid Premier container survived its first autoclave cycle, and went on to perform well through a total of 20 cycles. There was very slight leaking around the corners in the watertight seal test after 8 cycles; however, this was on the order of drops, and was deemed negligible. There was no visible damage to the container or the lids at any point during this testing process.

Cell Transfer and Viability - Testing with Cells

Cell viability was assessed with cells that were contained in the sterile incubator volume for varying periods of time (1.5, 2, 3, and 4 hours). Overall, viability was found to be comparable between the sterile incubator volume and the commercial incubator volume at most time points. Each sterile incubator volume time point was tested twice in order to acquire more data for each time point. A condensed table with photographs from each trial can be found in Appendix A.

1.5 hours

Cell viability was achieved during both 1.5 hour trials, in which cells were poured into the culture dish and the sterile incubator volume was constructed in the hood. The volume was then placed on the heater for 1.5 hours, with a Trypan Blue viability test performed after 30 minutes for one trial. The viability test yielded 100% cell viability for cells in the sterile incubator volume. After 1.5 hours, photos were taken of the cells, which had not yet adhered to the culture dish, but appeared to be alive (the cells were not clumped together or balled up).

After sitting in the sterile incubator volume for 1.5 hours the cells were placed in a standard CO₂ incubator (Shell Labs CO₂ Incubator, Sheldon Mfg.) and photographed at 24, 48 and 80 hours; at all three time points a substantial number of cells were elongated and adhered (viable). Both 1.5 hour trials yielded similar results, with the sterile incubator volume cells appearing similar to the control cells at most time points.

Figure 21: Sterile Incubator Volume Cells from a 1.5 hour trial (after 24 hours in the commercial incubator)

2 and 4 hour trials

The cells were placed in the sterile incubator volume for a period of 2 and 4 hours, then placed in the large incubator and photographed at 24, 48, and 72 hours. The cells in both these trials were non-viable, with the cells from both environments dying in these trials. These cell strains were later deemed non-viable, possibly due to contamination during passing of the strain or non-sterile culture dishes in the incubator. Because of this, these data sets are not being used in the overall analysis. Figures 22 and 23 depict the non-viable, sparse culture dishes from this cell strain, which was not used in later trials.

Figure 22: 4 hour trial - an elongated cell (after 30 hours)

Figure 23: Cells from the 2 hour trial (after 24 hours)

3 Hour Trials

A new strain of cells was cultured and 3 hour viability tests were performed. For both trials a control was placed in the incubator immediately, while the sample was placed in the sterile incubator volume for 3 hours before being placed in the incubator. The first trial yielded minimal success, with cells from the sterile incubator volume being only slightly elongated and adhered; the second trial however yielded great success with the cells from the sterile incubator volume elongated and adhered in a comparable manner to the control cells.

During one of the 1.5 hour trials the cells in the sterile incubator volume elongated better than the cells in the regular incubator, so irregular adherences may be due to something other than the environment. Since the sterile incubator volume produced more viable cells in one situation (1.5 hour trial), and the control yielded more viable cells in the other situation (3 hour trial), this is most likely not related solely to the culture environment; these variations may be due to contamination during cell culturing, inadvertantly pipetting less viable cells into the culture dish, or other factors. Table 7 shows images of cells from the Sterile Incubator Volume and the Control (commercial incubator) at various timepoints. As evidenced in this table, cell elongation and adherence is more evident in the control at 2 hours; however, by 24 hours both samples show a similar proportion of elongated and adhered cells. Differences in elongation and adherence time should be considered for future iterations of the design.

Table 7: Comparison of Sterile Incubator Volume and Control Cells Over Time

Time	Sterile Incubator Volume	Control Incubator
1 Hour
2 Hours
24 Hours

4 Hour Trials

In the 4 hour trials, a batch of cells were placed in the sterile incubator volume, while another batch of cells from the same passage were placed immediately in the

incubator. Cells from the sterile incubator volume and the control were examined hourly during the trial. After 4 hours, the cells from the sterile incubator volume were moved to the main incubator volume and both sets of cells were observed again at 24, 48 and 72 hours.

In both of these trials, cell viability was high. Cells from the sterile incubator volume were elongated and adhered in a similar manner to the control cells after 24 hours. For the first 4 hours, the cells in the cells in the commercial incubator were elongating and adhering sooner than the cells in the sterile incubator volume. Cell sizes, densities, and elongation shapes were all comparable after 24 hours.

Figure 24: Elongated and adhered cells from a 4 hour trial (at 24 hours)

System Integration Trial – 2 hours

The final trial was a 2 hour sterile incubator volume system trial aimed at verifying that all system components were functioning together properly. In this trial a control culture dish of cells was placed in the incubator while the sterile incubator volume was assembled and the deposition syringe was filled with cells in the hood. The sterile incubator volume and deposition syringe were removed from the hood, and the

syringe was used to deposit cells into the culture dish in the sterile incubator volume via a septum. The volume and then placed on the heat mat for 2 hours. The cells from the sterile incubator volume were compared to the cells from the incubator at 24, 48 and 72 hours.

The following figures show sterile incubator volume cells from a systems integration trial at 2 and 24 hours. Figure 25 shows that the cells were starting to elongate and adhere at 2 hours; however, the cells from the control were slightly more elongated and adhered at this point in time (no photo of the control cells is available). Figure 26 shows the sterile incubator volume at 24 hours; at this point, both the control and sterile incubator volume cells were elongated and adhered, with similar cell densities.

Figure 25: Sterile Incubator Volume Cells, System Integration Trial at 2 Hours

Figure 26: Sterile Incubator Volume Cells, System Integration Trial at 24 hours

In the first trial, the cells from the sterile incubator volume were similar in appearance to the control cells. Both sets of cells were elongated and adhered after 24 hours, with only a small percentage (approximately 5%) of cells balled up and non-viable. For this trial the cell density was greater (about twice as much) in the sterile incubator volume than in the control volume.

The second system integration trial yielded similar results, with both the control and sterile incubator volume cells being elongated and adhered within 24 hours. Very few cells were balled up (dead), and cell density between the volumes varied slightly, with the incubator having more cells. Overall cell elongation shapes were similar. After 48 hours, the cells were removed from the incubator and a live-dead Calcein stain was applied; both the control and the sample yielded similar results in this stain, with many living cells, and very few dead cells. This verified that both samples had viable cells in them after 48 hours; it would be optimal to perform this trial at earlier time points in the future.

The results of these trials show that the entire sterile incubator volume system, with deposition syringe cell placement, is capable of maintaining some viable cells. This trial was instrumental in verifying the entire system by proving post-deposition cell viability. System components that were integrated and verified in this trial include temperature control, adequate sterility, pH regulation suitable for maintaining cell viability, and use of the 18 gage deposition needle and septum.

Test Analysis and Discussion

Temperature, pH and sterilizability, and cell viability were verified successfully in this project; however, additional testing could still be performed to determine the

absolute capabilities of the system in terms of maintaining cell viability. Viability maintenance was verified for up to 4 hours, and longer deposition lengths may be possible. At this point, some viable cells can be maintained for up to 4 hours in the sterile incubator volume; further testing could verify if this design is feasible is adequate for maintaining cell viability for future on-campus cell printing endeavors.

The only major difference between cells in the sterile incubator volume and cells in the incubator was adherence time. Within 1.5-2 hours most cells in the incubator had begun to elongate and adhere, while only a small percentage (10-40%) of cells in the sterile incubator volume had begun to adhere. Within a few hours after placing the sterile incubator volume cells in the incubator they were adhered as much as the incubator cells; evaluating ways to make the cells adhere more readily while they are in the sterile incubator volume would be beneficial. It is unknown why this occurred, but it may have been because the cells from the sterile incubator volume were moved more during testing. In a real printing situation cell movement would be minimized, since the researchers would not be examining the cells hourly.

No formal pH or humidity maintenance is being utilized, nor is it deemed necessary. If more delicate cells were to be printed these regulations may be necessary, but the fibroblast cells were viable under non-regulated conditions.

Cells from the sterile incubator volume were generally similar in appearance to the control cells once they had elongated and adhered (usually within 3-24 hours). The sterile incubator volume maintains adequate cell viability to enable some cells to elongate and adhere within 24 hours after deposition, with some adherence occurring as early as 1.5 to 2 hours. After 24 hours (including time in the commercial incubator), both the

sterile incubator volume and the commercial incubator yielded similar cell densities, approximate percentages of cells elongated and adhered, and size of elongated cells, by inspection. There were multiple situations where one of the culture dishes had non-viable cells; however, this cell death was dispersed between both the sterile incubator volume and the commercial incubator, so they are most likely due to a factor other than merely a compromised culture environment.

Adequate preliminary testing ensured that the final testing procedures ran as efficiently as possible. The basic tests (temperature, pH, resterilizability) were performed relatively quickly and easily. But, the cell testing was more challenging, as the variables increase dramatically when dealing with living organisms.

Proposed Future Tests

While viability tests were performed, additional tests are recommended. Due to limited cell availability and time constraints, only basic viability assessments were performed. Several supplemental tests are recommended to verify various aspects of the sterile incubator volume system and to test its capabilities.

Additional testing could be conducted to determine the maximum time cells can remain in the sterile incubator volume without compromising viability. While the design has been verified to maintain some viability for up to 4 hours, there is potential that the current device could maintain viability for a longer period. Additionally, research should be performed to determine how much viability is being maintained after these specified time periods. Since 4 hours is the maximum feasible print time, there is no need to test beyond this time period; however, further validations may be beneficial for future cell printing endeavors.

Longer pH tests could be conducted to evaluate how the pH continues to change over time. Since the pH was increasing relatively rapidly over the hour long trial, additional trials would be recommended to see how the pH continues to change over the full sterile incubator volume incubation period. If the pH continues to rise, it will increase beyond the desired phosphate buffer range, which will mandate that pH control methods be utilized. pH regulation could be achieved in many ways, including use of a buffer, addition of a humidification source (e.g. saturated gauze, humidifier, etc.), or CO₂ regulation.

The tests conducted in this project were based on the use of 3T3 fibroblasts, a robust cell type well-suited for cell printing and other practices which place stresses on the cells that can decrease cell viability. In the future, additional cell types should be used to verify cell viability. Since the current on-campus printer uses 3T3 fibroblasts, it is most important that this cell type be validated. However, in the future the campus will strive to print with new cell types which must be tested in both the printer and the sterile incubator volume.

A sterility test would also be beneficial; however this is not feasible without creating risks for the rest of the tissue in the lab. This would involve depositing cells without antibiotics into the sterile incubator volume, leaving them in the sterile incubator volume for a specified period of time, then placing them in the incubator and checking on them every 24 hours. This would verify that the sterile incubator volume was, indeed, maintaining sterility (because contamination would kill cells without antibiotics); however, if the cells were to become contaminated this could introduce the contaminants to the main incubator. Contamination in the main incubator could compromise the

viability of other students' projects in the incubator; hence, it is not feasible in the current tissue engineering lab.

Alternative sterility and viability testing could be performed using microbiology procedures. It would be optimal to perform cell counts at various points during the incubation process (i.e. at 1, 2, 3, 4, etc. hours) to determine if and when viability begins to be compromised. Utilizing live-dead stains or microbiology approaches to adequately assess cell viability with more frequency would enable for more accurate determination of the limitations and constraints of the sterile incubator volume system.

The cells from the sterile incubator volume could be compared to two different samples, the control (commercial incubator) and a sample placed in the open lab environment. This would enable comparison between optimal cells (commercial incubator), sterile incubator volume cells, and cells exposed to lab conditions (without environmental control), to see if and how much the sterile incubator volume improves viability over printing in the lab. Ultimately, this test would be performed over several time periods and with various cell types.

Finally, an alternate test could assess cells in media without antibiotics. This would show if sterility was adequately maintained to prevent compromises in sterility, which could adversely affect cell viability. The ability to print cells in media without antibiotics could also be beneficial in cases where antibiotic-free media is desired.

6.0 CONCLUSIONS AND NEXT STEPS

The sterile incubator volume is a temperature-controlled environment that maintains conditions suitable for cell viability; verified to maintain some cells for periods of up to 4 hours. This minimizes cross-contamination risks from the external environments, which will be especially beneficial when printing more delicate cells or printing without antibiotics. This method also poses fewer cross-contamination risks than printing in the hood, since mechanical parts are not being introduced into the sterile hood. As the field of tissue engineering expands and Cal Poly's resources grow, the sterile incubator volume design can be enhanced and improved.

Based on background research, most current cell printing practices print either in the open lab or inside a sterile hood; the sterile incubator volume system provides an alternative method. Rather than introducing foreign components to the hood, this system is independent and can be moved freely around the lab. The ability to maintain sterility (rather than printing in the open lab) should enable for printing of more delicate cells, or printing cells without antibiotics.

The following enhancements could be considered: improved humidity regulation, CO₂ gas flow (to maintain precisely 5% CO₂), and other changes to the design. In developing any of these enhancements further, the simplicity of the design must be carefully considered. The current design is very simple, easy-to-use, and self-contained. It is imperative that simplicity of design and ease-of-use are not compromised unnecessarily.

The sterile incubator volume could be enlarged to provide a larger print volume. If there is a need to print a cell volume larger than a culture dish, this may be necessary.

The design features have been verified for the current volume size (6" x 6" x 2"), so the major design features should also work for a larger volume, but enhancements and changes may be required. Primary concerns with an increased volume size include how to maintain heat throughout the volume (multiple heaters or different types of heaters may be necessary), humidity maintenance and CO₂ maintenance. Humidity and CO₂ maintenance were relatively negligible in the current volume, but they should be considered if the volume size is increased substantially.

An interface with the inkjet print-head must be created; which will enable the current print-head to interface with the sterile incubator volume. This interface could be a relatively simple deposition tip that can be attached to the existing print nozzles of the modified inkjet printer. Ultimately, the interface should enable for the printing of multiple cell types. Since an 18 gage needle has been verified with the current design, this is the desired interface; however, it may be possible to verify that other deposition tips are also compatible with the current sterile incubator volume design. Similar interfaces can be created for other means of cell printing, provided each is compatible with both the sterile incubator volume and the deposition system.

The current iteration of the sterile incubator volume system is designed for use with an 18 gage needle deposition tip; this could limit the printing methods that are compatible with the system. For example, the latex barrier could make it difficult for the laser to excite the laser absorption layer in BioLP or LGDW (Barron 2004, Chrisey 2005, Ringeisen 2004, Ringeisen 2006), and MagTE would require that the applicable magnetic forces be compatible with the use of the metal deposition syringe (INO 2007), which could be present challenges.

Once two-dimensional printing is performed, a means of maintaining culture dish placement must be developed. Since the sterile incubator volume is not yet compatible with the inkjet deposition head, this issue has not been fully explored. With a moving sterile incubator volume, the culture dish must be secured to maintain accurate printing. Possible means of connecting the culture dish to the sterile incubator volume include double-sided tape, a culture dish well, magnets, and high-friction material. It is imperative that the connection method be sterilizable, easy to use, and that it does not interfere with heat transfer from the heat mat to the media inside the culture dish.

The sterile incubator volume should assist in achieving Cal Poly's tissue engineering goals of achieving cell viability after long term deposition trials. It should also assist in the universal tissue engineering goals of engineering complex tissues and organs to assist with the organ and tissue disease epidemics.

It is possible that in the future we will be able to print new lungs for patients with lung cancer, new hearts for those with cardiovascular disease, and that printed kidneys will be able to eliminate the need for dialysis in kidney patients. While there is still much to learn before this can be achieved successfully, the field is rapidly advancing and there is good reason to hold great hope for the future of tissue engineering.

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APPENDIX A: CELL VIABILITY TESTING SUMMARY

The following table contains photographs of cells from viability trials. Unless otherwise noted, all photographs were taken 24 hours after deposition. A batch of cells were contained in the sterile incubator volume for the specified time, then they were placed in the Shell Labs (commercial) incubator; the control cells were immediately placed in the Shell Labs incubator.

Trial Description	Sterile Incubator Volume Cells	Control Cells
Trial 1 1.5 hours	<p data-bbox="540 888 870 953">Many cells elongated and adhered, few dead cells</p>	No photo available; these cells looked similar to the sterile incubator volume cells.
Trial 2 1.5 hours	<p data-bbox="488 1333 899 1549">At 1, 1.5 and 24 hours, the cells from the sterile incubator volume and the incubator volume look very similar; well elongated and adhered by 24 hours</p>	<p data-bbox="943 1333 1321 1402">Very few cells elongated and adhered, low cell viability</p>
Trial 3 1.5 hours, printed cells*

	The cells are balled up and dead; this trial used only 3 mL of media (instead of 12)	A few cells are elongated and adhered, but many are dead; more viable than the sterile incubator volume cells
Trial 4 2 hours**	<p>At 1 hour, 2 hours, and 24 hours the cells in both culture dishes looked the same; small and balled up (dead)</p>	<p>Cells are small and balled up (dead)</p>
Trial 5 3 hours	<p>Taken at 48 hours; about 20% of cells are elongated and adhered, many cells seem viable, low cell density</p>	<p>Many cells are elongated and adhered, there are regions of dense cell growth (pictured) and other less dense regions</p>
Trial 6 3 hours	<p>Many cells are elongated and adhered, very few dead floating cells (~5%)</p>	<p>Many cells are elongated and adhered, few dead floating cells (~5%), similar to sterile incubator volume</p>

<p>Trial 7 4 hours</p>	<p>Most cells elongated and adhered; a few round, dead floating cells</p>	<p>Many cells elongated and adhered, several dead cells, similar to sterile incubator volume</p>
<p>Trial 8 4 hours</p>	<p>Somewhat low cell density, many cells elongated and adhered, a few dead (floating) cells</p>	<p>Slightly higher cell density than sterile incubator volume, similar percentage of elongated cells, a few dead cells, very similar to sterile incubator volume</p>
<p>Trial 9 Injection, 2 hours</p>	<p>Many cells elongated and adhered; a few dead (floating) cells (~5%), good cell density</p>	<p>Most cells elongated and adhered; cell density is lower than sterile incubator volume, similar percentages of adhered cells.</p>

<p>Trial 10 Injection, 2 hours</p>	<p>Cells nicely elongated and adhered, good cell density</p>	<p>Cells shape and elongation similar to sterile incubator volume, slightly higher cell density</p>
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*This trial used 3 mL of cells and media instead of 12 mL of cells and media; the cells spent a significant amount of time and were transferred into the culture dish under non-sterile conditions. These reasons may have contributed to the lack of cell viability in the sterile incubator volume sample.

**Cells were later deemed to be from a non-viable strain; there were many issues of cell death with these cell strains, which were disposed of.

APPENDIX B: TEST PROTOCOLS AND REPORTS

This appendix contains test protocols and reports from final verification tests. These were performed using the complete sterile incubator volume system. These tests were conceived early in the design process and modified based on the results of preliminary testing (Appendix C).

Included Final Test Reports:

1. Temperature Verification for Sterile Incubator Volume Test Protocol and Report
2. pH & Humidity Verification for Sterile Incubator Volume Test Protocol and Report
3. Resterilizability Verification for Rubbermaid Containers Test Protocol and Report
4. Cell Viability Test for Sterile Incubator Volume Test Protocol and Report

Temperature Verification for Sterile Incubator Volume Test Protocol and Test Report

DATE: 4/9/08

CONTENTS:

- 1.0 OBJECTIVE**
- 2.0 SCOPE**
- 3.0 BACKGROUND**
- 4.0 MATERIALS AND EQUIPMENT**
- 5.0 PROCEDURE**
- 6.0 EVALUATION CRITERIA**
- 7.0 RESULTS**
- 8.0 DISCUSSION OF RESULTS**
- 9.0 CONCLUSIONS AND RECOMMENDATIONS**

REPORT COORDINATOR: Emily Hakun DATE: 4/9/08
Emily Hakun

1.0 OBJECTIVE

To verify that the specifications for temperature are being met in the Sterile Incubator Volume (SIV); cells must be kept at 37° C to maintain viability.

2.0 SCOPE

This test protocol is aimed at verifying that the SIV, as designed and built (with temperature feedback), is capable of maintaining the specified temperature. If significant modifications are made to the design, new tests shall be performed to ensure efficacy of the SIV.

3.0 BACKGROUND

3.1 Cells must be maintained at 37° C.

3.2 Severe temperature fluctuations can kill cells; changes in temperature can denature proteins crucial for cell life.

4.0 MATERIALS AND EQUIPMENT

4.1 Test Devices

- 4.1.1 Silicone Rubber Heat Mat (McMaster-Carr; Los Angeles, CA)
- 4.1.2 P-I-D Temperature Controller (McMaster-Carr; Los Angeles, CA)
- 4.1.3 Type J Thermocouple (McMaster-Carr; Los Angeles, CA)
- 4.1.4 2 – 3 cup Rubbermaid Premier Containers
- 4.1.5 4 – Sponges (pre cut to fit around Petri dish)
- 4.1.6 2 – Plastic Petri dishes
- 4.1.7 2 – 8” x 8” sheets of .006” thick latex (Small Parts, Inc; Miramar, FL)

4.2 Lab Equipment

- 4.2.1 VWR III hotplate
- 4.2.2 Digital thermometer
- 4.2.3 Sink (water supply)
- 4.2.4 2 – 80 mL beaker
- 4.2.5 2– 200 mL beaker
- 4.2.6 50 mL graduated cylinder

5.0 PROCEDURE

5.1 Preheat hot plate to 65° C.

5.2 Fill each 200 mL beaker with approximately 150 mL of warm water; place the filled beakers on the hot plate.

5.3 Fill each 80 mL beaker with approximately 50 mL of water; place the filled beakers on the hot plate.

5.4 Plug in the temperature controller and check that the set value (SV) is set to 37° C (programmed default). Place the thermocouple on top of the heat mat (so the tip is directly touching the mat).

5.5 Place both Rubbermaid containers on top of the heat mat.

5.6 Place the pre-cut sponges in each container; the circular cavity should be in the center.

- 5.7 Once the water in the large beakers has reached approximately 37° C, use the graduated cylinder to measure and pour 150 mL onto the sponges in each container. The sponges should be well saturated.
- 5.8 Place a Petri dish in each circular sponge cavity.
- 5.9 Once the water in the small beakers is at approximately 37° C, measure 40 mL of water in the graduated cylinder; pour 40 mL water into each Petri dish.
- 5.10 Place the latex and lid onto one of the volumes and insert the thermocouple through one end of the lid (making sure that most of the lid is covered with latex). Check to ensure that the thermocouple tip is submerged in the water. This will provide the feedback to the temperature controller.
- 5.11 Record the temperature of the water in the sterile incubator volume.
- 5.12 Immediately put the latex and lid on the sterile incubator volume and allow it to sit for 10 minutes.
- 5.13 Remove the lid from both volumes (for uniformity) and take the temperature of the water in the Petri dish of the sterile incubator volume.
- 5.14 Repeat steps 5.9-5.10 until 30 minutes has elapsed.

6.0 EVALUATION CRITERIA

- 6.1 Data will be evaluated based on the functional requirements; a deviation greater than 2° degrees C (in either direction) will be considered unacceptable.
- 6.2 While most tests will be performed three times, this test will only be performed twice (once with water and once with media), since future trials should further validate temperature data.

7.0 RESULTS

Trial 1: Water

Time (minutes)	Water Temp. (°C)	Volume (mL)
0	37.0	40 mL
10	35.4	N/A
20	36.0	N/A
30	38.1	N/A
40	35.5	38.5 mL
Net Change	N/A	1.5 mL
% Change	N/A	3.8%

*This trial was continued for an extra 10 minutes due to the fact that all the temperatures were within range. There is no reason to believe that temperatures will leave the specified range (35° C to 39° C) under these conditions.

Trial 2: Media*

Time (minutes)	Media Temp. (°C)	Volume (mL)
0	35.6	19 mL
10	39.2**	N/A
20	35.8	N/A
30	36.3	18 mL
Net Change	N/A	1 mL
% Change	N/A	5.3%

*For this trial media 20 mL of media was used in each Petri dish in place of 40 mL water.

** The alarm light on the temperature controller was on at this point, indicating that the temperature controller was aware that the temperature was too high.

8.0 DISCUSSION OF RESULTS

The changes in temperature were all within the specified range of 35° C to 39° C (or within .2° C). The water temperature range was over 2.7 °C (temperatures from 35.4° C to 38.1° C), while the media range was over 3.6° C (temperatures from 35.6° C – 39.2° C). The larger range for the media trial may have been due to the fact that there was less media, which could have yielded less accurate thermocouple readings.

The only deviation was above the temperature range by .2° C, during a period where the alarm light on the temperature control unit was illuminated. This was most likely due to the temperature controller noting that the initial temperature was lower than the set value (37° C), which caused the heat mat to rapidly increase in temperature to accommodate for the low media temperature. The next reading (trial 2 at 20 minutes) was substantially lowered, indicating that the temperature controller compensated for the alarm signal by cooling down the heat mat.

Overall, the feedback loop for temperature yielded much better results than using the temperature controller to maintain the heater at a set temperature (as in preliminary trials) and kept the temperatures within the specified range.

9.0 CONCLUSIONS AND RECOMMENDATIONS

The feedback loop was successful at maintaining temperatures within the specified range for cell viability. Additional testing will be performed as part of other tests (e.g. cell viability, systems integration).

pH and Humidity Verification for Sterile Incubator Volume Test Protocol and Test Report

DATE: 4/13/08 - 4/15/08

CONTENTS:

- 1.0 OBJECTIVE**
- 2.0 SCOPE**
- 3.0 BACKGROUND**
- 4.0 MATERIALS AND EQUIPMENT**
- 5.0 PROCEDURE**
- 6.0 EVALUATION CRITERIA**
- 7.0 RESULTS**
- 8.0 DISCUSSION OF RESULTS**
- 9.0 CONCLUSIONS AND RECOMMENDATIONS**

REPORT COORDINATOR:

Emily Hakun

DATE: 4/15/08

1.0 OBJECTIVE

To verify that the specifications for pH and humidity are being met in the Sterile Incubator Volume (SIV); all areas containing cells must maintain appropriate pH levels for cell viability.

2.0 SCOPE

This test protocol is aimed at verifying that the SIV, as designed and built, is capable of maintaining the specified pH and humidity levels. If significant modifications are made to the design, new tests shall be performed to ensure efficacy of the SIV.

3.0 BACKGROUND

3.1 Cells must be kept at 5% CO₂ (95% air) to maintain appropriate pH levels.

3.2 Severe fluctuations in CO₂carbon dioxide levels can alter pH and kill cells; changes in pH can denature proteins crucial for cell life.

3.3 The sterile incubator volume must be capable of maintaining pH within a specified range (approximately 5.8-7.8).

4.0 MATERIALS AND EQUIPMENT

4.1 Test Devices

- 4.1.1 Silicone Rubber Heat Mat (McMaster-Carr; Los Angeles, CA)
- 4.1.2 P-I-D Temperature Controller (McMaster-Carr; Los Angeles, CA)
- 4.1.3 Type J Thermocouple (McMaster-Carr; Los Angeles, CA)
- 4.1.4 2 – 3 cup Rubbermaid Premier Containers
- 4.1.5 4 – Sponges (pre cut to fit around Petri dish)
- 4.1.6 2 – Plastic Petri dishes
- 4.1.7 2 – 8” x 8” sheets of .006” thick latex (Small Parts, Inc.; Miramar, FL)

4.2 Lab Equipment

- 4.2.1 VWR III hotplate
- 4.2.2 Digital thermometer
- 4.2.3 Sink (water supply)
- 4.2.4 2 – 80 mL beaker
- 4.2.5 2– 200 mL beaker
- 4.2.6 50 mL graduated cylinder
- 4.2.7 Hyclone High glucose media, with 15mM Hepes buffer
- 4.2.8 Mettler-Toledo pH meter

5.0 PROCEDURE

5.1 Turn on the hot plate and set it at 100° C. Preheat approximately 300 mL

water in the 200 mL beakers (150 mL per beaker) to 37° C. Pour approximately 40 mL media into each of the 80 mL beakers and preheat the media on the hot plate to 37° C.

5.2 Plug in the temperature controller and place the thermocouple directly on the heat mat (to prevent overheating). The temperature controller should be set at 37° C, if it is set at a different value, reset it at 37° C.

- 5.3 Place 2 pre-cut sponges into the containers, leaving the circular cavity in the center. Pour approximately 150 mL of pre-heated water onto the sponges of each container (feedback and sterile volume).
- 5.4 Place a Petri dish in each container's circular cavity and pour 40 mL of the preheated media into the feedback volume's Petri dish. Ensure that the thermocouple tip is fully submerged in the media. Seal the feedback volume by covering the top with latex and pressing down on all sides of the frame to secure it in place.
- 5.5 Measure 40 mL of media in the graduated cylinder. Pour the media into the Petri dish of the sterile volume and take a reading of the media's pH and temperature.
- 5.6 Place a piece of latex over the top of the sterile volume and place the frame in place. Press the frame down on all sides to secure it in place.
- 5.7 After 10 minutes, open the sterile container and record the media's temperature and pH.
- 5.8 Reseal the container by placing the latex on top and pressing down on all sides of the frame to secure it. Place the volume back on the heat mat.
- 5.9 Repeat steps 5.7-5.8 until a total of 1 hour has elapsed. After taking the pH and temperature readings for 60 minutes, use a funnel to pour the media back into the graduated cylinder. Note the final media volume.
- 5.10 This test shall be performed in triplicate to ensure efficacy of the sterile incubator volume.

6.0 EVALUATION CRITERIA

- 6.1 Data will be evaluated based on pH levels (related to CO₂ levels and evaporation); which will be taken to the nearest hundredth (per the pH meter). pH within the range of 5.8-7.8 will be deemed optimal.
- 6.2 The amount of media that has evaporated will also be determined based on initial and final volume measurements. Since some spilling is inevitable, it is likely that the percent media loss will be overestimated, leading to a more conservative estimate. Media volume measurements shall be taken to the nearest .5 mL.

7.0 RESULTS

Trial 1*

Time (minutes)	pH	Volume (mL)
0	7.35	18 mL
10	7.55	N/A
20	7.71	N/A
30	7.82	N/A
40	8.00	N/A
50	8.08	N/A
60	8.24	17 mL
Net Change	N/A	1 mL
% Change	N/A	5.6%

*This trial was conducted with only 20 mL of media, to save media; however, both other trials were conducted with 40 mL of media (per the protocol).

Trial 2

Time (minutes)	pH	Volume (mL)
0	7.42	38 mL
10	7.53	N/A
20	7.60	N/A
30	7.64	N/A
40	7.72	N/A
50	7.78	N/A
60	7.85	37 mL
Net Change	N/A	1 mL
% Change	N/A	2.6%

Trial 3

Time (minutes)	pH	Volume (mL)
0	7.36	36 mL
10	7.48	N/A
20	7.56	N/A
30	7.63	N/A
40	7.73	N/A
50	7.78	N/A
60	7.88	35 mL
Net Change	N/A	1 mL
% Change	N/A	2.8%

8.0 DISCUSSION OF RESULTS

For the first trial (20 mL media), the pH level spiked up to 8.24 within 20 minutes, well beyond the desired maximum pH of 7.8. However, once the volume of media was increased to 40 mL, the pH levels elevated much less dramatically to 7.85 and 7.88 after 1 hour. Both 40 mL trials had pH levels at 7.78 (within the acceptable range) at 50 minutes.

Based on this data it was determined that as much media as possible should be used so that evaporative losses have less of an effect on the media's pH. Since the surface area is the same (roughly 48 square cm), increasing the volume of media will yield a lower surface area to volume ratio and therefore less evaporation.

9.0 CONCLUSIONS AND RECOMMENDATIONS

The buffer seems to have assisted with pH maintenance, and increasing the media volume had an obvious effect on pH regulation. Based on these results, it is feasible to perform cell viability tests; the maximum feasible media volume should be used.

Resterilizability Verification for Rubbermaid Containers Test Protocol & Report

TEST DATES: 3/22/08 – 3/31/08

CONTENTS:

- 1.0 OBJECTIVE**
- 2.0 SCOPE**
- 3.0 BACKGROUND**
- 4.0 MATERIALS AND EQUIPMENT**
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- 6.0 EVALUATION CRITERIA**
- 7.0 RESULTS**
- 8.0 DISCUSSION OF RESULTS**
- 9.0 CONCLUSIONS AND RECOMMENDATIONS**

REPORT COORDINATOR: DATE: 3/31/08
Emily Hakun

1.0 OBJECTIVE

To determine if the Rubbermaid container base and lid are capable of withstanding the autoclave. If they can withstand the autoclave, to determine how many times they can be autoclaved.

2.0 SCOPE

This test protocol is aimed at verifying that the containers can be autoclaved successfully. The container bases and lids will be tested in pouches on the applicable autoclave cycle repeatedly.

3.0 BACKGROUND

- 3.1 Internal sterility is important for the sterile incubator volume since any internal contamination could kill the printed cells.
- 3.2 Autoclave is the preferred sterilization method since it is simple, convenient, and leaves no residues.
- 3.3 It is desired that each component be resterilizable at least 50 times; however, since the container cost was far below the initial budget, 50 cyclic repetitions are not imperative.
- 3.4 There is concern that after repeated autoclave cycles the containers may become brittle; hence drop tests shall be performed.

4.0 MATERIALS AND EQUIPMENT

4.1 Test Devices

4.1.1 Rubbermaid Premier 1.25 Cup Containers*

4.1.2 Rubbermaid Premier 1.25 Cup Container Lid*

*1.25 cup containers were used (instead of the actual 3 cup container) because they fit in the autoclave pouches in the lab. The shape, materials, and thickness of polycarbonate are very similar to the 3 cup container. Larger pouch material was requested (to accommodate the 3 cup containers), but never received. The smaller containers were deemed sufficiently similar for resterilizability testing.

4.2 Lab Equipment

4.2.1 Converters Self-Seal Pouch 7.5" x 13"; Cardinal Health (92713)

4.2.2 Ritter by MD Mark - M9 UltraClave Automatic Sterilizer

4.2.3 Sink or other water source

5.0 PROCEDURE

- 5.1 Place item to be autoclaved (base or lid) in self-seal pouch. Mark the cycle number on the outside of the pouch with a permanent marker.
- 5.2 Seal the pouch by removing the adhesive backing and sticking the adhesive to the plastic side.
- 5.3 Open the autoclave and check to ensure there is adequate water in the autoclave (the water level should be in the green zone). If there is not enough water, add some distilled water in the water tray.
- 5.4 Place sealed pouch in autoclave. Close the autoclave door securely.

- 5.5 Press the pouches button to begin the autoclave cycle. This cycle lasts about 45 minutes.
- 5.6 When the autoclave cycle is complete (as indicated on the autoclave status screen), open the autoclave door and carefully remove the pouch. It may still be hot.
- 5.7 Visually inspect the item in the pouch. If it appears to be in good condition perform a drop test by dropping the pouch from table height (about 3.5 feet) onto the tile floor.
- 5.8 Visually inspect the container or lid inside the pouch, look for cracks or breakage.
Make note of any abnormalities, and take a photograph, if applicable.
- 5.9 If the pouch and container or lid are still in good condition (no brown stains, tears, melting etc.) repeat steps 5.3-5.9, noting the new trial number on the outside of the pouch; otherwise, skip to step 5.10.
- 5.10 Remove the container or lid from the pouch and discard the pouch. Fill the container with approximately .5" of water and put the lid on. Press the lid firmly on all sides to ensure a seal.
- 5.11 Over the sink tip the sealed container full of water to check for leaks. Note any leaks, including severity of leak.
- 5.12 Remove the lid and pour out the water. Repeat the entire process to determine how many times the container and lid can be sterilized.

6.0 EVALUATION CRITERIA

- 6.1 Containers and lids shall be visually inspected for residue/melting; any evidence of either of these shall be noted.
- 6.2 After each drop test, containers and lids shall be visually inspected for cracks, breaks, or other abnormalities; these shall be noted.
- 6.3 The ease of snapping the lid onto the container after autoclaving shall be noted; additionally, it should be noted if the lid appears to have sealed properly.
- 6.4 The results of the waterproof leak test shall be noted, including location of the leak and amount of leaking, if applicable.

7.0 RESULTS

Trial 1: Container Base Autoclave Resterilizability

Completed Cycles	Drop Test	Watertight Test
1	Pass	Pass, no leak
2	Pass	N/A
3	Pass	N/A
4	Pass	Pass, no leak
5	Pass	N/A

6	Pass	N/A
7	Pass	N/A
8	Pass	A few drops at corner
9	Pass	N/A
10	Pass	A few drops at corner
11	Pass	N/A
12	Pass	N/A
13	Pass	N/A
14	Pass	A few drops at corner
15	Pass	N/A
16	Pass	N/A
17	Pass	A few drops at corner
18	Pass	N/A
19	Pass	N/A
20	Pass	A few drops at corner

Trial 2: Container Lid Autoclave Resterilizability

Completed Cycles	Drop Test	Watertight Test
1	Pass	Pass, no leak
2	Pass	n/a
3	Pass	n/a
4	Pass	n/a
5	Pass	Pass
6	Pass	N/a
7	Pass	n/a
8	Pass	A few drops from corner
9	Pass	n/a
10	Pass	A few drops from corner
11	Pass	n/a
12	Pass	n/a
13	Pass	n/a
14	Pass	A few drops from corner
15	Pass	n/a
16	Pass	n/a
17	Pass	n/a
18	Pass	n/a
19	Pass	n/a
20	Pass	A few drops from corner

8.0 DISCUSSION OF RESULTS

Both the container base and the lid are capable of withstanding the autoclave repeatedly. Somewhere between 4 and 8 cycles a slight leak developed with the seal between the base and the lid. This leak was only obvious when the container was held tilted in a position for water to flow out. The leak was very slight, and probably will not pose a real risk for the internal sterility of the container. When

the lid is firmly pressed onto the base there is no significant leaking, even after 20 autoclave cycles.

9.0 CONCLUSIONS AND RECOMMENDATIONS

Additional testing could be performed to determine the exactly when the seal becomes less effective at maintaining sterility, or the sterile incubator volume user can use their best judgment to determine if the integrity of the device has been compromised. As the device is used for printing the number of viable sterilization repetitions shall be noted.

Cell Viability Tests for Sterile Incubator Volume Test Protocol and Test Report

DATE: 4/20/08 – 5/9/08

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- 9.0 CONCLUSIONS AND RECOMMENDATIONS**

REPORT COORDINATOR: Emily Hakun
Emily Hakun

DATE: 5/9/08

1.0 PURPOSE

The purpose of this validation is to ensure that the sterile incubator volume can maintain cell viability (after proper cleaning and cell transfer) and to determine how long cells can remain viable in the sterile incubator volume.

2.0 SCOPE

This protocol specifies the cleaning and cell transfer instructions to be performed when using the sterile incubator volume.

3.0 BACKGROUND

- 3.1. Cleaning is the removal of contaminants from a device to the extent necessary for its further processing or intended use.
- 3.2. In order to be properly sterilized, a device must first be properly cleaned.
- 3.3. A sterile container is needed to optimize chances of cell viability; aseptic conditions are acceptable for the container and lid, but sterile conditions are required for any components of the sterile incubator volume that will come into direct contact with the deposited cells.

4.0 MATERIALS AND EQUIPMENT

4.1. Test Devices

- 4.1.1. Sterile Incubator Volume System
- 4.1.2. Cultured 3T3 fibroblast cells
- 4.1.3. Fibroblast media
- 4.1.4. 2 sterile tissue culture dishes

4.2. Cleaning Equipment

- 4.2.1. Disposable paper towels
- 4.2.2. Cleaning Brushes
- 4.2.3. Alconex
- 4.2.4. 10% Bleach Solution
- 4.2.5. 70% Alcohol Solution
- 4.2.6. Water

4.3. Lab Equipment

- 4.3.1. Personal protective equipment (e.g. gloves, safety glasses)
- 4.3.2. Sterile Hood

5.0 PROCEDURE

5.1. Safety

- 5.1.1. Personnel shall wear protective equipment (safety glasses, gloves, etc.) as necessary when dealing with soiled goods.

5.2. Cleaning

- 5.2.1. Wipe excess soil off the container and lid using absorbent paper towels.
- 5.2.2. Rinse the components in the sink using warm water and Alconex.
- 5.2.3. Use soft-bristled brushes to carefully clean all surfaces of the device, focusing on crevices, corners, and hard-to-clean areas.
- 5.2.4. Rinse the device with water at ambient temperature until there is no visible detergent residue

- 5.2.5. Spray the container, lid and latex generously with the bleach solution. Ensure that all surfaces of each component have been sprayed thoroughly.
- 5.2.6. Allow the bleach solution to remain on all components for 1 minute.
- 5.2.7. Rinse the container thoroughly with water at ambient temperature.
- 5.2.8. Drain excess water from the components; use a paper towel if necessary.
- 5.2.9. Perform a visual inspection for cleanliness of the device
- 5.2.10. If the device still appears to be soiled, repeat the cleaning process, otherwise, continue on.
- 5.2.11. Spray all components (container base, lid, and latex) thoroughly with 70% alcohol and drain the excess alcohol into the sink.
- 5.2.12. Place the components on a clean paper towel and allow excess alcohol to drain off of them.

5.3. Reassembly & Cell Placement

- 5.3.1. The temperature controller shall be turned on and set at 37° C (programmed default).
- 5.3.2. The thermocouple shall be placed directly on the heat mat, and the heat mat shall be allowed to warm up for at least 10 minutes.
- 5.3.3. Pour about 12 mL of fibroblast media into a culture dish and place the culture dish in the middle of the feedback volume.
- 5.3.4. Place the feedback volume directly on the heat mat.
- 5.3.5. The thermocouple shall be threaded through the portal in the feedback volume and the lid shall be placed so that the thermocouple tip is submerged in the media.
- 5.3.6. The latex and lid shall be secured over the feedback volume.
- 5.3.7. Remove cultured cells from the incubator and prepare them using proper protocol.
- 5.3.8. Pour about 2 mL of cells in media into a sterile culture dish; pour 2 mL of cells and media into another sterile culture dish. Add about 10 mL of fibroblast media to each culture dish.
- 5.3.9. Cover one culture dish and place it in the commercial incubator.
- 5.3.10. Place the second culture dish in the center of the sterile incubator volume container.
- 5.3.11. Place the latex (septum-side-up) in the container lid/frame. Place the frame over the container.
- 5.3.12. Press down firmly on each edge of the lid to secure the lid on the container. Gently press down on the latex to ensure that it is in place.
- 5.3.13. Once the device has been reassembled in its entirety, it can be removed from the hood for use.
- 5.3.14. The sterile volume shall be properly closed, removed from the hood and placed directly on the other side heater (next to the feedback volume, directly on top of the heat mat).
- 5.3.15. If Trypan Blue is available; a Trypan Blue test shall be performed after 30 minutes to assess cell viability.
- 5.3.16. After each 1 hour interval, the sterile incubator volume can be carried to the hood and reintroduced to the hood, following proper hood use protocol.

5.4. Cell Transfer & Viability Check

- 5.4.1. The sterile incubator volume can be disassembled in the hood, by carefully removing the latex and lid. The culture dish (containing cells) shall be removed.
- 5.4.2. The culture dish shall be capped with a sterile culture dish lid
- 5.4.3. Cell viability shall be evaluated under the microscope with the capped culture dish every hour during the trial.
- 5.4.4. After each evaluation, the cell culture dish (without lid) shall be re-placed in the sterile incubator volume system and the system shall be reconstructed and placed on the heat mat.
- 5.4.5. Upon completion of the trial, the culture dish shall be placed in the incubator and cell viability shall be visually inspected every 24 hours for a total of 3 days. If cells are deemed non-viable after at least 24 hours the culture dish shall be disposed of to prevent cross-contamination in the incubator.

6.0 EVALUATION CRITERIA

- 6.1. Cells shall be photographed at the following time intervals: post heating, 24 hours, 48 hours, and 72 hours. Cell elongation and adhesion shall be examined.
- 6.2. Cells that have elongated and adhered after 24 hours will be considered viable, while cells that have not will be considered dead.

7.0 RESULTS

Trial 1: 1.5 hours, 4/22/2008

Trypan Blue Data (after 30 minutes):

<i>Time</i>	<i>Live Cells / Square</i>	<i>Dead Cells / Square</i>	<i>Cell Conc. (cells/mL)</i>	<i>Cell Viability</i>
0 minutes	15.4	0	154,000	100%
30 minutes (a)	12.8	0	128,000	100%
30 minutes (b)	2.6	0	26,000	100%

<i>Time (hours)</i>	<i>Picture</i>	<i>Notes</i>
1.5 hours	No photo available	Cells are still clumped, not yet elongated and adhered
24 hours		Many cells are elongated and adhered. Cell density is quite high

48 hours		Some cells are elongated and adhered; many have balled up
72 hours		A few cells are still elongated and adhered; many cells balled up

Trial 2: 1.5 hours, 4/30/2008

<i>Time (hours)</i>	Picture	Notes
1.5 hours		Cells are mostly clumped, some are beginning to elongate; cell density is low.
24 hours		Many cells are elongated and adhered. Cell density is relatively low

48 hours		Most cells are elongated and adhered; cell density is increasing
72 hours		Cells nicely elongated and adhered, cell density is still increasing

Trial 3: 1.5 Hour Trial*

<i>Time (hours)</i>	<i>Picture</i>	<i>Notes</i>
1.5 hours		Cells are already balled up; they do not seem to be adhering. Cell density is fairly high.
24 hours		Most cells balled up, not elongated and adhered, cells are probably dead.
48 hours	Trial Aborted	N/A
72 hours	Trial Aborted	N/A

Trial 4: 2 Hour Trial 4/25/2008

<i>Time (hours)</i>	<i>Picture</i>	<i>Notes</i>
2 hours		Very few cells, not adhered
24 hours		Few cells, not elongated and adhered
48 hours		Cells are balled up and not adhered; the cells from this set of passages were later deemed to be unhealthy cells

Trial 5: 3 hour trial, 5/4/2008

<i>Time (hours)</i>	<i>Picture</i>	<i>Notes</i>
1 hour		Small cell population, not yet adhered; cells look similar to incubator control
2 hours		Small cell population, cells still not elongated and adhered
3 hours		Small cell population; most cells still balled up, not elongated and adhered.
24 hours 48 hours	No Photo Available
	A few cells slightly elongated and adhered, less than optimal cell elongation

72 hours		Many cells balled up, very few cells elongated, few (if any) cells are viable at this point
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Trial 6: 3 hour trial, 5/5/2008

<i>Time (hours)</i>	<i>Picture</i>	<i>Notes</i>
1 hour		Cells still balled up
2 hours		Cells still balled up (photo out of focus)
3 hours		Cells slowly starting to elongate and adhere (blurry photo)

24 hours		Good population of cells elongated and adhered, very similar to the control (regular incubator) volume.
48 hours		Good number of cells elongated and adhered, similar to control volume
72 hours		Cell population is increasing, most cells are elongated and adhered, similar to control volume

Trial 7: 4 hour trial, 5/7/2008

<i>Time (hours)</i>	<i>Picture</i>	<i>Notes</i>
1 hour		Cells still balled up

2 hours		Cells still balled up
4 hours		Cells beginning to elongate and adhere
24 hours		Many cells elongated and adhered, a few dead, floating cells. Similar to sterile incubator volume
48 hours		Cells nicely elongated and adhered, cell density has increased over time, very few dead (balled up) cells.

72 hours		Cell density continues to increase; most cells nicely elongated and adhered, very few dead cells.
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Trial 8: 4 hour trial, 5/9/2008

<i>Time (hours)</i>	<i>Picture</i>	<i>Notes</i>
1 hour		A few cells are beginning to elongate slightly, sterile incubator volume cells very similar to control cells
2 hours		Low cell density, cells beginning to elongate slightly and adhere; slightly more control cells have adhered than sterile incubator volume cells.
4 hours		10-20% of cells elongated and adhered, many cells have not yet elongated and adhered; about 40-50% of the control cells have elongated and adhered.

24 hours		Cells nicely elongated and adhered, overall low cell density, only a few dead (rounded) cells.
48 hours		Cells are elongated and adhered, cell density has increased, only a few dead cells (similar to at 24 hours), sterile incubator volume very similar to control.
72 hours		Cells still viable (elongated and adhered), good cell density, very few dead cells.

8.0 DISCUSSION OF RESULTS

In most cases cell viability in the sterile incubator volume was shown to be comparable to the cells from the control volume (commercial incubator). The only major, consistent difference between the sterile incubator volume and the control cells was that the control cells adhered more readily (most within 1.5 – 4 hours, with full adherence by 24 hours), while the sterile incubator cells took longer (2 hours or more, with full adherence by 24 hours). The reason for the differences in adherence times is unknown, but could be further researched in the future.

There were some situations where either the sterile incubator volume cells or the control cells were non-viable after a period of time. Dead cells were usually found within the first 24 hours, and the sample was disposed of to prevent possible cross-contamination in the incubator. Neglecting situations where the data was eliminated (e.g. Trial 3, which was prepared in a non-sterile environment) there did not seem to

be a correlation between the cell environment (sterile incubator volume or commercial incubator) and potential for cell death.

9.0 CONCLUSIONS AND RECOMMENDATIONS

The results of this trial verified that the sterile incubator volume is adequate for maintaining cell viability for a period of time of 4 hours. The sterile incubator volume may be capable of maintaining cell viability for a longer period of time, which is a potential future test. Additionally, Hepes buffer could be added to the media to regulate pH which may further increase cell viability or the amount of time that cells can be contained in the sterile incubator volume without compromising cell viability. Humidity regulation could also be considered as a potential means of increasing the amount of time that viable cells can remain in the sterile incubator volume. Finally, additional cell types can be tested to determine which cell types are compatible with the sterile incubator volume.

System Integration Cell Viability Tests for Sterile Incubator Volume Test Protocol and Test Report

DATE: 5/12/08 – 5/16/08

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- 9.0 CONCLUSIONS AND RECOMMENDATIONS**

REPORT COORDINATOR: _____ DATE: 5/16/08
Emily Hakun

1. OBJECTIVE

The purpose of this trial is to ensure that the sterile incubator (SIV) volume can maintain cell viability, after proper cleaning and cell transfer, and to determine how long cells can remain in the SIV.

2. SCOPE

This protocol specifies the cleaning and cell transfer procedures to be performed when using the SIV.

3. BACKGROUND

- 3.1. Cleaning is the removal of contaminants from a device to the extent necessary for its further processing or use.
- 3.2. In order to be properly sterilized, a device must first be properly cleaned.
- 3.3. A sterile container is needed to optimize chances of cell viability; aseptic conditions are acceptable for the container and lid, but sterile conditions are required for any components of the sterile incubator volume that will come into direct contact with the cells.

4. MATERIALS AND EQUIPMENT

4.1. Test Devices

- 4.1.1. Sterile Incubator Volume System
- 4.1.2. Cultured 3T3 fibroblast cells
- 4.1.3. Fibroblast media
- 4.1.4. 2 sterile tissue culture dishes
- 4.1.5. 12 mL deposition syringe (sterile)
- 4.1.6. 18 gage needle, 1" in length (sterile)

4.2. Cleaning Equipment

- 4.2.1. Disposable paper towels
- 4.2.2. Cleaning Brushes
- 4.2.3. Alconex
- 4.2.4. 10% Bleach Solution
- 4.2.5. 70% Alcohol Solution
- 4.2.6. Water

4.3. Lab Equipment

- 4.3.1. Personal Protective Equipment (e.g. gloves, safety glasses)
- 4.3.2. Sterile Hood
- 4.3.3. Inverted Light Microscope

5. PROCEDURE

5.1. Safety

- 5.1.1. Personnel shall wear protective equipment (safety glasses, gloves, etc.) as necessary when dealing with soiled goods.

5.2. Cleaning

- 5.2.1. Wipe excess soil off the container and lid using absorbent paper towels.
- 5.2.2. Rinse the components in the sink using warm water and Alconex.

- 5.2.3. Use soft-bristled brushes to carefully clean all surfaces of the components.
 - 5.2.4. Rinse the components with water at ambient temperature until there is no visible detergent residue
 - 5.2.5. Spray the components generously with the bleach solution.
 - 5.2.6. Allow the bleach solution to remain on the components for 1 minute
 - 5.2.7. Rinse the components thoroughly with water at ambient temperature
 - 5.2.8. Drain excess water from the components, use a paper towel, if necessary.
 - 5.2.9. Visually inspect the components for cleanliness.
 - 5.2.10. Spray the clean components generously with the alcohol solution.
 - 5.2.11. Place the components on clean paper towels to allow the alcohol to drain off the components.
 - 5.2.12. If a component still appears to be soiled, repeat the cleaning process.
 - 5.2.13. The temperature controller shall be turned on and set at 37° C (default).
 - 5.2.14. Pour about 12 mL of media (at approximately 37° C) into the culture dish in the feedback volume.
 - 5.2.15. The feedback volume shall be placed directly on one side of the heat mat, with the thermocouple threaded through the portal and the lid sealed (with latex). Ensure that the thermocouple tip is submerged in the media.
 - 5.2.16. Remove cultured cells from the incubator and prepare them for cell deposition using proper protocol.
 - 5.2.17. Pour about 10 mL of media into the culture dish and place it in the center of the sterile volume container.
 - 5.2.18. Place the latex in the frame (septum-side-up) and carefully place the frame over the Rubbermaid container. Seal the latex and lid in place by firmly pressing on each side of the lid. Gently press down on each edge of the latex to make sure it is firmly in place (it should gently resist your pressing).
 - 5.2.19. In the hood fill the syringe with about 2 mL of cells and media.
 - 5.2.20. Remove the sealed sterile incubator volume container and syringe from the hood and place the sterile incubator volume in its place on the heat mat.
 - 5.2.21. Hold the septum between 2 fingers, and carefully insert the needle through the center of the septum.
 - 5.2.22. Gently push down on the syringe to expel the cells into the culture dish inside the sterile incubator volume.
 - 5.2.23. Remove the empty deposition needle from the septum and carefully dispose the syringe and needle in the biohazard bag.
 - 5.2.24. The sterile volume shall be properly closed, removed from the hood and placed directly on the other side heater (next to the feedback volume, directly on top of the heat mat).
- 5.3. Cell Viability Maintenance**
- 5.3.1. Leave the cells in the sterile incubator volume system for 2 hours.
 - 5.3.2. Remove the sterile volume from the system and carry it to the hood.
- 5.4. Cell Transfer & Viability Check**
- 5.4.1. The sterile incubator volume shall be carefully disassembled in the hood, and the tissue culture dish (containing cells) shall be removed
 - 5.4.2. The culture dish shall be closed with a sterile tissue culture dish lid
 - 5.4.3. Cell viability shall be evaluated using the microscope.

5.4.4. The culture dish shall be placed in the incubator and cell viability shall be visually assessed every 24 hours for a total of 3 days. If cells are deemed non-viable between 24 and 72 hours the culture dish shall be disposed of to prevent cross-contamination in the incubator.

6. EVALUATION CRTIERIA

6.1. Cells shall be photographed at the following time intervals: post heating, 24 hours, 48 hours, and 72 hours. Cell elongation and adhesion shall be examined.

6.2. Cells that have elongated and adhered after 24 hours will be considered viable, while cells that have not will be considered dead.

7. RESULTS

Trial 1: 2 hour system integration trial, 5/12/08

<i>Time (hours)</i>	<i>Picture</i>	<i>Notes</i>
2 hours		A few cells beginning to elongate and adhere
24 hours		Many cells are elongated and adhered, about 5% of cells are balled up and floating (dead)
48 hours		Most cells are elongated and adhered, nicely elongated, a few dead cells
72 hours		Increased cell density, most cells elongated and adhered

Trial 2: 2 hour system integration trial, 5/14/08

<i>Time (hours)</i>	<i>Picture</i>	<i>Notes</i>
2 hours		Cells just starting to elongate and adhere
24 hours		Cells elongated and adhered with only a few cells balled up (dead)
48 hours		Cell density has increased, most cells have elongated and adhered, very few dead cells; this portion of the trial was aborted and cells were used for live-dead stain

8. DISCUSSION OF RESULTS

The cells in the sterile incubator volume in both systems integration trials were viable after spending 2 hours in the system. The differences between the cells in the sterile incubator volume and those in the control (incubator) were minimal, considered to be negligible. In both cases cells elongated and adhered within 24 hours.

9. CONCLUSIONS AND RECOMMENDATIONS

Based on the results of this trial, it can be assumed that system integration does not noticeably compromise cell viability. Therefore, it is likely that cells are able to survive substantially longer in system integration conditions. Since the purpose of this test was primarily to verify that the system integration was successful, additional tests are not required. However, future research could compare cell viability levels over longer time periods or differing conditions in the system, to further explore the system's capabilities.

APPENDIX C: PRELIMINARY TEST PROTOCOLS AND REPORTS

This appendix contains test protocols and reports for preliminary tests. These tests were performed to assess feasibility of the sterile incubator volume design during the iterative design process. While these tests cannot be used for verification, poor results from one of these tests would indicate a likely design flaw that should be addressed prior to verification testing. There were four primary types of preliminary tests: container tests, latex test, evaporation tests and pH tests.

Container Tests

- Heating Verification for Potential Containers
- Heating Capacity of Potential Containers
- Heat Maintenance for Potential Containers
- Lid Watertight Seal Testing

Latex Test

- Latex Elasticity and Tearing by Thickness

Evaporation Tests

- Evaporation with Water
- Heater Evaporation with Water

pH Tests

- Evaporation and pH Changes with F-12 Media
- Heater Evaporation and pH Changes with High Glucose Media

Heating Verification for Potential Containers Preliminary Testing Protocol & Report

TEST DATE: 2/22/08

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REPORT COORDINATOR: Emily Hakun DATE: 2/22/08
Emily Hakun

1.0 OBJECTIVE

To determine if the Rubbermaid and Snapware container bases are capable of withstanding the heat of an autoclave (116° C - 133° C).

3.0 SCOPE

This test protocol is aimed at verifying that the containers can withstand autoclave temperatures (116° C - 133° C) since autoclave is the desired sterilization method for the sterile incubator volume. An oven will be used for this preliminary trial.

3.0 BACKGROUND

Standard autoclave temperatures range from 116° C to 133° C. The volume should be autoclavable, otherwise alternate sterilization methods must be explored.

4.0 MATERIALS AND EQUIPMENT

4.1 Test Devices

4.1.1 2—Rubbermaid Premier 3 Cup Containers

4.1.2 2—Snapware Snap N' Serve 4 Cup Containers – 6081OIL1

4.2 Lab Equipment

4.2.1 Oven

4.2.2 Sink or other water source

5.0 PROCEDURE

5.1 Preheat oven to 116° C.

5.2 Place container bases on a metal tray in the oven; the containers should not touch each other. Close the oven and watch the thermometer to make sure desired temperature is maintained.

5.3 After 5 minutes, open the oven door and check on containers; visually inspect for damage and melting.

5.4 If the containers appear to be damaged, carefully remove them from the oven and end the trial. If there is no visible damage, leave containers in oven and close the oven door. Continue heating for an additional 10 minutes.

5.5 Remove containers from oven and close the oven door. Visually inspect the containers looking for melting, debris, and abnormalities.

5.6 Take photographs of each container, noting oven temperature and heating time.

5.7 Place the lid on the container base, note if it seals or not. Remove lid.

5.8 If the lid seals, fill the container with water and put the lid on. Hold the closed container over the sink and try to dump the water out to see if the seal is watertight. Note the results.

5.9 Reset the oven to 133° C and repeat steps 5.1 to 5.8.

6.0 EVALUATION CRITERIA

6.1 Base containers shall be visually inspected for residue/melting; any evidence of these shall be noted.

- 6.2 The ease of snapping on the lid after heating the base shall be noted; additionally, the lid appears to have sealed properly.
- 6.3 The results of the waterproof leak test shall be noted. If there is a leak, note the location and amount of leaking.

7.0 RESULTS

Trial 1: Heat at 116° C for 15 minutes

	Rubbermaid	Snapware
5-Minute Check	Pass (no melting)	Pass (no melting)
Post-Heating Photo (After 15 minutes)
Lid Fit	Yes	Yes
Watertight	Yes	Yes

Trial 2: Heat at 133° C for 15 minutes

	Rubbermaid	Snapware
5-Minute Check	Pass (no melting)	Pass (no melting)
15 Minutes
Lid Fit	Yes	Yes
Watertight	Yes	Yes

8.0 DISCUSSION OF RESULTS

Both the Rubbermaid and the Snapware container bases passed the preliminary oven tests, proving they are capable of withstanding heats of 116° C and 133° C for at least 15 minutes without visible damage or watertight seal damage. Either of these bases should be able to withstand the temperature constraints of an autoclave at up to 133° C.

9.0 CONCLUSIONS AND RECOMMENDATIONS

Since both bases passed the preliminary heat tests, additional testing would be beneficial. This testing should include autoclave testing of the bases and the lids. Since the autoclave uses steam sterilization (rather than dry heat) it is imperative that the containers be validated in the autoclave.

Heating Capacity for Potential Containers Preliminary Testing Protocol & Report

TEST DATE: 2/25/08

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- 9.0 CONCLUSIONS AND RECOMMENDATIONS**

REPORT COORDINATOR: DATE: 2/25/08
Emily Hakun

1.0 OBJECTIVE

To determine if the Rubbermaid and Snapware containers can transfer heat effectively to liquid in a Petri dish (within the container).

2.0 SCOPE

This test protocol is aimed at verifying that the containers will transfer heat from a hot plate through the bottom of the container into a Petri dish full of water. The test also aims to verify that the heat of the hot plate will not melt the container.

6 BACKGROUND

2.1 Media must be maintained at 37° C. There must be an established way to heat and maintain the media at this temperature.

2.2 It is challenging to find accurate heat transfer information about the containers being considered; therefore, heat transfer testing is necessary.

7 MATERIALS AND EQUIPMENT

7.3 Test Devices

7.3.1 Rubbermaid Premier 3 Cup Container with lid

7.3.2 Snapware Snap N' Serve 4 Cup Containers with lid – 6081OIL1

7.4 Lab Equipment

7.4.1 VWR III Hot Plate

7.4.2 Water

7.4.3 Plastic Petri Dishes

7.4.4 50 mL Graduated Cylinder

8 PROCEDURE

8.1 Pre-heat hot plate to desired temperature for at least 5 minutes; check to ensure that the temperature light on the hot plate has stopped blinking.

8.1.1 Desired Temperatures:

- Trial 1: 45° C
- Trial 2: 65° C
- Trial 3: 100° C

8.2 Place an open container (Rubbermaid or Snapware) on center of hot plate.

8.3 Place Petri dish in center of container; do not put the lid on the Petri dish.

8.4 Fill Petri dish with 40 mL of cool water (approximately 20° C), record the initial water temperature.

8.5 Snap the lid onto container, make sure it is firmly in place

8.6 Let stand for 5 minutes, visually inspecting for container melting every 2 minutes. Make note of any container melting that occurs. If melting occurs, remove the container from the hot plate and abort the trial.

8.7 After 5 minutes, remove container from hot plate; carefully take off the lid and record the water temperature.

8.8 Immediately replace container on center of hot plate. Put the lid back on the container and ensure that it is sealed.

8.9 Let stand for 5 more minutes, continue checking for container melting every 2 minutes. Make a note of any container melting. If melting occurs, remove the container from the hot plate and abort the trial.

8.10 After 5 minutes, remove container from hot plate; carefully take off the lid and record the water temperature.

5.0 EVALUATION CRITERIA

5.1 Containers shall be visually inspected for melting, approximately every 2 minutes; any evidence of melting shall be noted.

5.2 Temperatures shall be recorded in degrees C at 5 minute intervals. Test conditions that may have affected temperature results shall be noted.

6.0 RESULTS

Trial 1: Heat at 45 ° C for 10 minutes

	Rubbermaid	Snapware
Initial Temp – H ₂ O	18 °C	19 °C
H ₂ O Temp, 5 Min.	20 °C	21 °C
H ₂ O Temp, 10 Min.	21 °C	22 °C
dT/dt	.3 °C/min	.3 °C/min

No container melting occurred during this trial.

Trial 2: Heat at 65 ° C for 10 minutes

	Rubbermaid	Snapware
Initial Temp – H ₂ O	20 °C	20 °C
H ₂ O Temp, 5 Min.	22 °C	25 °C
H ₂ O Temp, 10 Min.	23 °C	28 °C
dT/dt	.3 °C/min	.8 °C/min

No container melting occurred during this trial.

Trial 3: Heat at 100 ° C for 10 minutes

	Rubbermaid	Snapware
Initial Temp - H₂O	20 °C	21 °C
H₂O Temp, 5 Min.	23 °C	28 °C
H₂O Temp, 10 Min.	28 °C	35 °C
dT/dt	.8 °C/min	1.4 °C/min

No container melting occurred during this trial.

7.0 DISCUSSION OF RESULTS

Both containers transferred heat from the hot plate to the water. The temperature of the hot plate needed to greatly exceed the temperature of the water, in order for effective heat transfer to occur. As the temperature difference between the initial water temperature and the hot plate was increased (from 26° C to 79° C), the change in temperature with respect to time increased dramatically (from .3° C/min to 1.4°/min, respectively, with the Snapware container).

The Snapware container was able to transfer heat from the hot plate to the water more effectively during this experiment. The differences in heat transfer capabilities between the two containers increased as the hot plate temperature increased. The design of the base of the Snapware container (with short feet) appears to allow more contact between the container and the hot plate than the Rubbermaid base (with lip) allows, which could contribute to heat transfer differences.

8.0 CONCLUSIONS AND RECOMMENDATIONS

The Snapware container had a much heat transfer rate than the Rubbermaid container; therefore, it is assumed that the Snapware would provide more effective heating for the sterile incubator volume. However, modifications to the bases of either of these containers could alter these properties. For example, removing the lips or feet would probably increase heat transfer capabilities of both containers. Additional testing of temperature capacity (including temperature maintenance with pre-heated water in the Petri dish) is recommended.

Heat Maintenance for Potential Containers Preliminary Testing Protocol & Report

TEST DATE: 2/29/08

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- 2.0 SCOPE**
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- 8.0 DISCUSSION OF RESULTS**
- 9.0 CONCLUSIONS AND RECOMMENDATIONS**

REPORT COORDINATOR: Emily Hakun DATE: 2/29/08
Emily Hakun

1.0 OBJECTIVE

To determine if the Rubbermaid and Snapware containers can adequately maintain the temperature of pre-heated liquid with hot-plate heating.

2.0 SCOPE

This test protocol is aimed at determining the approximate hot plate temperature that will enable the containers to maintain the appropriate temperatures in the media. The test will also verify that the hot plate at the required temperature for temperature maintenance will not melt the container.

3.0 BACKGROUND

3.1 Cell media must be maintained at 37° C. Therefore, the heating method must be capable of maintaining the media at a constant 37° C (+/- 2° C).

3.2 It is difficult to find accurate heat transfer information about the containers being considered for use in this design; therefore, heat transfer testing is necessary.

4.0 MATERIALS AND EQUIPMENT

4.1 Test Devices

4.1.1 Rubbermaid Premier - 3 Cup Container with lid

4.1.2 Snapware Snap N' Serve 4 Cup Containers with lid – 6081OIL1

4.2 Lab Equipment

4.2.1 VWR III Hot Plate

4.2.2 Water

4.2.3 Plastic Petri Dish

4.2.4 50 mL Graduated Cylinder

4.2.5 80 mL Beaker

4.2.6 Digital thermometer

5.0 PROCEDURE

5.1 Pre-heat hot plate to desired temperature for at least 5 minutes, or until the temperature indicator light on the hot plate has stopped blinking (a blinking temperature indicator light indicates that the hot plate is still pre-heating).

5.1.1 Desired Temperatures (temperatures for trials 2 & 3 were determined based on the results of previous trials):

- Trial 1: 45 ° C
- Trial 2: 60 ° C
- Trial 3: 65 ° C

5.2 In the beaker, preheat approximately 40 mL of water to approximately 37° C on the hot plate. Remove beaker with heated water from hot plate

5.3 Place Rubbermaid or Snapware container on center of hot plate.

5.4 Place an empty Petri dish in the center of the container.

5.5 Carefully pour the water into the Petri dish, do not put the lid on the Petri dish.

5.6 Record the water temperature.

5.7 Carefully snap lid onto container, make sure lid is firmly in place and sealed

- 5.8 Let container stand on hot plate for 5 minutes, checking for container melting approximately every 2 minutes. Make note of any container melting that occurs. If substantial melting occurs, the test may be aborted early.
- 5.9 Carefully remove the lid from the container and place the thermometer in the water, be sure that the tip is fully submerged.
- 5.10 Record the temperature in the lab notebook and note any reasons for possible deviations.
- 5.11 Immediately replace the container lid, ensuring that it is sealed.
- 5.12 Repeat steps 5.8-5.11 until a total of 20 minutes has elapsed with the container on the hot plate.
- 5.13 All trials shall be conducted with one container first, for feasibility purposes; if the temperature is not close to being maintained (e.g. far too hot or far too cold) the trial may be aborted early to adjust the hot plate temperature. Likewise, if the temperature is not being maintained well with one container, do not test it with the other container.
- 5.14 This is an iterative testing procedure. After each trial the temperature for the next trial shall be selected, based on logic. For example, if the first trial overheats the media substantially, a much lower temperature shall be used; whereas, if it under heats them slightly a slightly higher temperature for the hot plate shall be used.

6.0 EVALUATION CRITERIA

- 6.1 Containers shall be visually inspected for melting, approximately every 2 minutes; any evidence of melting shall be noted.
- 6.2 Temperatures shall be recorded in °C (to the nearest .1° C) at 5 minute intervals. Test conditions that may have affected temperature results shall be noted.
- 6.3 Data shall be recorded neatly, in ink, in tables in a lab notebook.

7.0 RESULTS

Trial 1: Heat at 45° C for 20 minutes

Time (Minutes)	Water Temperature (°C)	
	Rubbermaid	Snapware
0	N/A	38.0° C
5	N/A	32.0° C
10	N/A	28.0° C
dT/dt (average)	N/A	1° C/min
dT/dt (max. 5min.)	N/A	1.2° C/min

- No container melting occurred during this trial.
- Since the temperature drop was too large with the Snapware container, the trial was aborted after 10 minutes (rather than 20 minutes); the trial was

not conducted on the Rubbermaid container due to poor heat maintenance results with the Snapware.

- The temperature must be increased dramatically to keep the cells around 37° C; try 60° C for the next trial.

Trial 2: Heat at 60 ° C for 20 minutes

Time (minutes)	Water Temperature (°C)	
	Rubbermaid	Snapware
0	N/A	38.8° C
5	N/A	36.0° C
10	N/A	36.2° C
15	N/A	36.1° C
20	N/A	35.5° C
dT/dt (average)	N/A	.165° C/min
dT/dt (max 5 min.)	N/A	.56° C/min

- No container melting occurred during this trial.
- Since the Snapware trial yielded a notable temperature drop the Rubbermaid containers were not tested at this temperature.
- The water temperature is close to the desired temperature (37° C); so the next trial will only need a slight hot plate temperature increase.

Trial 3: Heat at 65 ° C for 20 minutes

Time (minutes)	Water Temperature (°C)	
	Rubbermaid	Snapware
0	37.3° C	37.8° C
5	36.3° C	36.0° C
10	36.3° C	36.8° C
15	35.6° C	37.2° C
20	35.4° C	37.5° C
dT/dt (average)	.095° C/min	.015° C/min

dT/dt (max 5 min.)	.2° C/min	.36° C/min
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- No container melting occurred during this trial.
- Since the Snapware temperatures were within the right range (37° +/- 2° C), this trial was also conducted with the Rubbermaid container.
- The Snapware had a significant drop at the beginning, but leveled out a bit after 10 minutes; the Rubbermaid had fairly a large drop the first 5 minutes, and leveled out more from that point onward.
- Since the trial at 60° C was a little low and this trial was a little high for Snapware temperatures, a temperature between 60° and 65° will probably yield the desired media temperature in the Snapware container.
- The Rubbermaid is still a little low, so a warmer temperature (75° C) will be tested on this container.

Trial 4: Heat at 75 ° C for 20 minutes (Rubbermaid Only)

Time (minutes)	Water Temperature (°C)	
	Rubbermaid	Snapware
0	38.5° C	N/A
5	37.6° C	N/A
10	38.1° C	N/A
15	38.1° C	N/A
20	38.1° C	N/A
dT/dt (average)	.02° C/min	N/A
dT/dt (max 5 min.)	.18° C/min	N/A

- No container melting occurred during this trial.
- The temperature maintenance was very good; despite moderate deviations during the first 10 minutes.
- A slightly lower temperature should be tested for the Rubbermaid container to determine the optimal hot plate temperature to keep the media at 37° C for cell viability.

8.0 DISCUSSION OF RESULTS

Both containers were able to maintain appropriate liquid temperatures, but at different hot plate temperatures. The design specifications desire that the temperature be maintained within 2° C. With the hot plate at 65° C, the Snapware container's media temperature varied by 1.8° C in the first 5 minutes of use; then varied by only .8° C during the remaining 15 minutes of the trial. At the same temperature the Rubbermaid varied by 1° C during the first 5 minutes of use, and

then varied by .9° C in the remaining 15 minutes of the trial (all within the specified 35° to 39° range). Both containers showed that they were capable of maintaining temperatures around 37° C for at least 20 minutes.

9.0 CONCLUSIONS AND RECOMMENDATIONS

Both containers were capable of maintaining the temperature of the media for an extended period of time. This temperature maintenance was much more accurate than the temperature variations in the heating capacity experiment; therefore it is determined that the media should be pre-heated to minimize temperature variation. There was no significant difference in heat maintenance between the two containers.

Lid Watertight Seal Testing Preliminary Testing Protocol & Report

TEST DATE: 2/29/08

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- 9.0 CONCLUSIONS AND RECOMMENDATIONS**

REPORT COORDINATOR: Emily Hakun DATE: 2/29/08
Emily Hakun

1.0 OBJECTIVE

To determine if the Rubbermaid and Snapware containers can maintain a watertight seal with the latex in place.

2.0 SCOPE

This test protocol is aimed at determining if the container lid “frame” is capable of maintaining a watertight seal when the center of the lid is removed and a latex sheet is put in place.

3.0 BACKGROUND

3.1 Sterility must be maintained inside the volume; therefore a lid-container seal is necessary.

4.0 MATERIALS AND EQUIPMENT

4.1 Test Devices

- 4.1.1 Rubbermaid Premier - 3 Cup Container with lid
- 4.1.2 Snapware Snap N’ Serve 4 Cup Containers – 6081OIL1 – with lid
- 4.1.3 15” x 24” Natural Latex Rubber Sheet - .006” thick
- 4.1.4 15” x 24” Natural Latex Rubber Sheet - .012” thick
- 4.1.5 15” x 24” Natural Latex Rubber Sheet - .025” thick

4.2 Lab Equipment

- 4.2.1 Sink (water)

5.0 PROCEDURE

- 5.1 Remove the center from the lid of the container by cutting out a 4” x 4” hole in the center of the lid carefully, with a razor blade.
- 5.2 Cut latex sheets to size. For each thickness you will need:
- 5.3 1 – 7.5” x 7.5” latex sheet (for the Rubbermaid container)
- 5.4 1 – 9.5” x 7.5” latex sheet (for the Snapware container)
- 5.5 Put approximately 1” of water in the container and place the latex sheet over the top of the container, so that it covers all edges.
- 5.6 Use the outside of the container top to “frame” the latex, pressing down on each side to seal the container top to the base. Snap down each of the side snaps on the Snapware. Note difficulty of sealing the top.
- 5.7 Over the sink, tip the container so that each edge of the container is full of water. Note if any water leaks through the top-container-latex interface.
- 5.8 Repeat with all edges of the container being tested.

6.0 EVALUATION CRITERIA

- 6.1 Ease of “framing” to lock down the latex shall be noted on a qualitative scale. Framing can be: easy, fairly easy, fairly difficult, difficult, or overly difficult/not possible.
- 6.2 Leak tests will be conducted on a pass/fail basis. If the container leaks at all, the watertight seal has failed; if there is no leaking it passes.
- 6.3 Data shall be recorded neatly, in ink, in tables in a lab notebook.

7.0 RESULTS

Table 1: Ease of “framing”

Latex Thickness	Rubbermaid	Snapware
.006”	Easy	Easy
.012”	Easy	Fairly Easy
.025”	Fairly Difficult	Not Possible

Table 2: Watertight Leak Test

Latex Thickness	Rubbermaid	Snapware
.006”	Pass (no leak)	Pass (no leak)
.012”	Pass (no leak)	Pass (no leak)
.025”	Pass (no leak)	N/A (can’t seal)

8.0 DISCUSSION OF RESULTS

Both containers passed all the watertight leak tests they were subjected to. The Rubbermaid worked with all three latex thicknesses, but the Snapware could not be sealed with the .025” thick latex; therefore it could not be proven watertight for that thickness of latex.

9.0 CONCLUSIONS AND RECOMMENDATIONS

Both containers would work with latex sheets with thicknesses between .006” and .012” thick; the Rubbermaid will also work with latex sheet thicknesses up to .025”. Thinner sheets were easier to lock down with the container lid “frames”.

Latex Tearing by Thickness Testing Protocol & Report

TEST DATE: 3/6/08

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- 1.0 OBJECTIVE**
- 2.0 SCOPE**
- 3.0 BACKGROUND**
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- 9.0 CONCLUSIONS AND RECOMMENDATIONS**

REPORT COORDINATOR: Emily Hakun DATE: 3/6/08
Emily Hakun

1.0 OBJECTIVE

To determine the tearing potential of the various latex thicknesses if the deposition head is moved to various positions around the container.

2.0 SCOPE

This test protocol is aimed at determining if any of the initial latex thicknesses (.025", .012" and .006") are capable of handling the forces transmitted from a push pin (to represent the deposition head) as it moves around the sterile volume in a specified pattern.

3.0 BACKGROUND

3.0 Sterility must be maintained inside the volume; therefore the latex must not tear when the deposition head is moved around.

3.1 The seal on the container must be maintained while the latex is being pulled.

4.0 MATERIALS AND EQUIPMENT

4.1 Test Devices

- 4.1.1 Rubbermaid Premier - 3 Cup Container
- 4.1.2 Rubbermaid Premier – 3 Cup Lid with center section cut out
- 4.1.3 Snapware Snap N' Serve - 4 Cup Container – 6081OIL1
- 4.1.4 Snapware Snap N' Serve – 4 Cup Lid with center section cut out
- 4.1.5 15" x 24" Natural Latex Rubber Sheet - .006" thick
- 4.1.6 15" x 24" Natural Latex Rubber Sheet - .012" thick
- 4.1.7 15" x 24" Natural Latex Rubber Sheet - .025" thick
- 4.1.8 Craft Foam Sheets – cut into approximately 1" squares

4.2 Lab Equipment

- 4.2.1 Push Pin (thumb tack, with head)
- 4.2.2 Super Glue
- 4.2.3 Scissors
- 4.2.4 Permanent Marker
- 4.2.5 Camera

5.0 PROCEDURE

5.1 Cut the latex into the appropriate sizes, for each thickness you need:

- 2—7.5" x 7.5" sheets for the Rubbermaid containers
- 2—7.5" x 9.5" sheets for the Snapware containers

5.2 To make the reinforced sheets, take one sheet for each container size and each latex thickness. Generously coat one side of the craft foam square with super glue and place it at the center of the latex sheets. Allow to dry for at least 12 hours before testing.

5.3 You should have a total of 12 sheets, with one reinforced and one unreinforced for each size (7.5" x 7.5" and 7.5" x 9") and thickness (.006", .012", .025")

5.4 Place the first latex sheet on top of the container, if it is a reinforced sheet it should be placed foam-side-up. Use the lid frame to snap it in place, ensure

that the lid and latex are sealed securely. Note the thickness, container, and reinforcement status of the sheet.

- 5.5 Gently push the push pin through the latex (and reinforcement, if applicable), holding the surrounding area taught as you do this.
- 5.6 Hold the push pin in place and tilt the container to look inside; make sure that the metal push pin tip is visible (the pin has fully punctured the latex).
- 5.7 Mark approximate midpoints on each side of the frame with the permanent marker or colored tape; place corresponding permanent marker marks on the latex (aligned with the frame marks).
- 5.8 Gently pull the push pin $\frac{1}{4}$ of the way to the right wall (wall A), note tears
- 5.9 Repeat $\frac{1}{4}$ way pulls in the other 3 directions (top – wall B, left – wall C, and bottom – wall D), note tears
- 5.10 If the latex did not tear for the $\frac{1}{4}$ way pulls; repeat step 5.8-5.9 but pulling $\frac{1}{2}$ way to the wall in all 4 directions, again noting tears; otherwise, skip to 5.13
- 5.11 If the latex did not tear for the $\frac{1}{2}$ way pulls; repeat step 5.8-5.9 but pulling $\frac{3}{4}$ of the way to the wall in all 4 directions, again noting tears; otherwise, skip to 5.13
- 5.12 If the latex did not tear for the $\frac{3}{4}$ way pulls; repeat step 5.8-5.9 but pulling all the way to the wall (so the edge of the push pin touches the frame) in all 4 directions, again noting tears; otherwise, skip to 5.13
- 5.13 If the latex tears during any of the above steps, note the tear in the lab notebook and take a picture of the tear. If it does not tear, take a photo of the entire device after the procedure, with and without the push pin in place.
- 5.14 Note the approximate size and shape of the final pushpin (or torn) hole
- 5.15 Repeat steps 5.3 - 5.14 for all containers, latex thicknesses and reinforcement status'.

6.0 EVALUATION CRITERIA

- 6.1 Latex sheets will be evaluated on their tear status (tear or no tear) after each stage of the test.
- 6.2 The general size of the tear will also be noted, and pictures of the tears or end-holes shall be included.
- 6.3 Difficulty of pulling the push pin shall also be noted (easy, medium, or difficult).

7.0 RESULTS

Trial 1: Rubbermaid - .025" Latex - Unreinforced

Stretch	Direction	Tear	Notes
¼ way	A (right), B (top), C (left) & D (bottom)	No	Latex very hard to puncture
½ way	A	Large tear	Test aborted (per protocol)

- Pull Difficulty: difficult
- The Snapware is not compatible with the .025" latex; hence, it was not tested.
- Test results were so poor that reinforced latex was not tested for .025" thickness sheets (since other thicknesses faired much better in unreinforced tests)

Trial 2: Rubbermaid - .012" Latex – Unreinforced

Stretch	Direction	Tear	Notes
¼ way	A (right), B (top), C (left) and D (bottom)	No	Easier to puncture
½ way	A, B, C, & D	No	Fairly difficult to pull
¾ way	A, B, C & D	No	“ “
Full	A, B, C, & D	No	“ “

- Pull difficulty: medium

Trial 3: Snapware - .012" Latex - Unreinforced

Stretch	Direction	Tear	Notes
$\frac{1}{4}$ way	A (right), B (top), C (left) & D (bottom)	No	
$\frac{1}{2}$ way	A, B, C & D	No	
$\frac{3}{4}$ way	A, B, C & D	Minor Tear	Starts to tear, but tear does not expand
Full	A, B, C, & D	Tear Same Size	

- Pull difficulty: medium

Trial 4: Snapware - .012" – Reinforced

Stretch	Direction	Tear	Notes
¼ way	A (right), B (top), C (left) & D (bottom)	No	Much easier to puncture
½ way	A, B, C, & D	No	
¾ way	A, B, C, & D	No	
Full	A, B, C, & D	No	

- Pull difficulty: medium
- Small hole in the craft foam, even smaller hole in latex (approximately the size of 3 push pin holes)

Trial 5: Rubbermaid - .012" – Reinforced

Stretch	Direction	Tear	Notes
¼ way	A (right), B (top), C (left) & D (bottom)	No	Much easier to puncture
½ way	A, B, C & D	No	
¾ way	A, B, C & D	No	
Full	A, B, C & D	No	Lost control, ripped the foam; no tear in the latex

- Pull difficulty: medium
- Smaller hole in craft foam; foam seems to be taking some of the pulling force

Trial 6: Rubbermaid - .006" - Reinforced

Stretch	Direction	Tear	Notes
¼ way	A (right), B (top), C (left) & D (bottom)	No	Much easier to puncture
½ way	A, B, C, & D	No	
¾ way	A, B, C & D	No	
Full	A, B, C & D	No	

- Pull difficulty: easy
- Smallest hole after all tests, looks comparable in size to the initial push pin hole.

Testing Note: Most of the .006" latex was borrowed immediately prior to testing, so there was only enough latex for one trial with this sheet. So, the Rubbermaid reinforced test was the only test initially performed on the .006" latex; this test was selected since it yielded the best results in the other trials.

8.0 DISCUSSION OF RESULTS

The .025" thick latex ripped too easily; most likely due to the large forces exerted on the latex to pull the push pin. The .012" thick latex pulled more easily, and was harder to rip under general conditions; however, there was still a good amount of force required to move the push pin to the extreme positions with this

sheet. The .006" latex, while only tested on the Rubbermaid with reinforcement, was the easiest to pull and puncture.

The reinforced latex sheets were noticeably easier to puncture, probably due to the fact that the latex was held in place by the foam and the super glue. The reinforcement also seemed to help to minimize tearing.

9.0 CONCLUSIONS AND RECOMMENDATIONS

Thinner latex is optimal; .006" latex with reinforcement yielded optimal results. Additional .006" latex shall be purchased, and more testing shall be conducted once septa have arrived.

Evaporation with Water Preliminary Testing Protocol & Report

TEST DATE: 3/24/08

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- 9.0 CONCLUSIONS AND RECOMMENDATIONS**

REPORT COORDINATOR: DATE: 3/24/08
Emily Hakun

1.0 OBJECTIVE

To determine if adequate humidity can be maintained in the sterile incubator volume without an external humidifier.

2.0 SCOPE

This test protocol is aimed at determining if an external humidifier is needed to provide adequate humidity to the sterile incubator volume. The sterile incubator volume humidification will be tested with: no humidity source, a bath of water, and a saturated sponge to determine if one of these methods is adequate.

3.0 BACKGROUND

- 3.1 If the air is not humid enough, media will evaporate. Media evaporation may lead to a change in the media's pH, which could kill cells.
- 3.2 A significant loss of media could adversely affect the printed cells.

4.0 MATERIALS AND EQUIPMENT

4.1 Test Devices

- 4.1.1 Rubbermaid Premier - 3 Cup Container
- 4.1.2 Rubbermaid Premier 3-Cup Container Lid, with center cut out
- 4.1.3 8" x 8" piece of .006" Natural Latex Rubber Sheet
- 4.1.4 Standard Petri Dish
- 4.1.5 6" x 6" x 1" sponges, cut to size

4.2 Lab Equipment

- 4.2.1 VWR III Hotplate
- 4.2.2 Sink (water)
- 4.2.3 50 mL Graduated Cylinder
- 4.2.4 80 mL Beaker
- 4.2.5 Digital Thermometer

5.0 PROCEDURE

- 5.1 If the sponges are not cut, cut them to size. The sponges should fit in the container with a circular cavity the size of a Petri dish.
- 5.2 Preheat the hot plate to 70° C.
- 5.3 Preheat approximately 50 mL of water in the 80 mL beaker, on the hot plate, to a temperature of about 37° C.
- 5.4 Determine which test you will be performing and follow the appropriate sub-procedure:
 - 5.4.1 Sponge:
 - 5.4.1.1 Place sponges in container
 - 5.4.1.2 Saturate sponges with approximately 150 mL of tap water
 - 5.4.1.3 Place empty Petri dish in the center cavity
 - 5.4.1.4 Proceed to step 5.5
 - 5.4.2 No Humidity Source:
 - 5.4.2.1 Place empty Petri dish in center of empty container
 - 5.4.2.2 Proceed to step 5.5
 - 5.4.3 Bath:

- 5.4.3.1 Fill container with warm water to a depth of approximately .25" (~100 mL)
- 5.4.3.2 Place Petri dish in center of container
- 5.4.3.3 Proceed to step 5.5
- 5.5 Place container-Petri dish system on hot plate.
- 5.6 Measure 40 mL of the pre-heated water into a graduated cylinder. Note the exact measurement.
- 5.7 Carefully pour the water into the Petri dish.
- 5.8 Take and record an initial temperature reading of the water.
- 5.9 Place latex and lid on container and seal tightly.
- 5.10 Let sealed container stand on the center of the hot plate for 10 minutes.
- 5.11 Remove lid from container and take the temperature of the water, record this temperature in the appropriate space in the lab notebook.
- 5.12 Replace lid on container and seal tightly.
- 5.13 Let sealed container stand on hot plate for an additional 10 minutes.
- 5.14 Remove lid from container and take the temperature of the water, record this temperature in the appropriate space in the lab notebook.
- 5.15 Carefully lift Petri dish and use a funnel to pour water into graduated cylinder.
- 5.16 Note any spilling or other reasons for possible deviations in results.
- 5.17 Calculate the total change in volume and the percent change in volume change to see how much water was lost due to evaporation.

6.0 EVALUATION CRITERIA

- 6.1 Temperatures will be recorded every 10 minutes to ensure the temperature of the volume is near the desired 37° C. This is to ensure that evaporation losses in the test are as close as possible to accurate.
- 6.2 All volume measurements shall be taken in the same 50 mL graduated cylinder. Measurements shall be made to the nearest .5 mL.

7.0 RESULTS

Test 1: Sponge Humidifier

Trial 1:

Time (minutes)	Water Temp. (°C)	Volume (mL)
0	34.0	40 mL
10	35.8	N/A
20	38.5	38.5 mL
Net Water Loss		1.5 mL
% Water Loss		3.8%

This test was repeated due to the fact that there was a fairly significant water spill (several drops) during the first trial.

Trial 2:

Time (minutes)	Water Temp. (°C)	Volume (mL)
0	32.7	40 mL
10	35.3	N/A
20	37.9	40 mL
Net Water Loss		0 mL
% Water Loss		0%

Average % Water Loss (Trial 1 & 2): 1.9%

Test 2: No Humidity Source

Time (minutes)	Water Temp. (°C)	Volume (mL)
0	33.9	40 mL
10	37.8	N/A
20	39.5	39 mL
Net Water Loss		1 mL
% Water Loss		2.5%

Test 3: Water Bath Humidifier

Time (minutes)	Water Temp. (°C)	Volume (mL)
0	33.0	40 mL
10	35.8	N/A
20	38.5	39 mL
Net Water Loss		1 mL
% Water Loss		2.5%

8.0 DISCUSSION OF RESULTS

All variations of humidifying method (including “no humidity source”) proved to provide adequate humidity to prevent substantial water losses during a 20 minute period. The water losses ranged from 0% to 2.5% (excluding the 3.9% loss for Sponge Trial 1, which was at least partially due to spilling). These water evaporation levels seem reasonable. The final sponge test (Trial 2) did provide the best results, with no notable loss of water.

9.0 CONCLUSIONS AND RECOMMENDATIONS

Since water evaporation trials were successful, testing should be conducted with cell media to see if it evaporates. In the media tests, pH should be recorded in addition to temperature and net evaporation losses. Since the Sponge method was the most successful humidity maintenance method for these trials, it is recommended that the sponge method be tested with the media.

Heater Evaporation with Water Preliminary Testing Protocol & Report

TEST DATE: 3/31/08

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- 2.0 SCOPE**
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- 8.0 DISCUSSION OF RESULTS**
- 9.0 CONCLUSIONS AND RECOMMENDATIONS**

REPORT COORDINATOR: Emily Hakun DATE: 3/31/08
Emily Hakun

1.0 OBJECTIVE

To determine if humidity can be maintained in the sterile incubator volume without an external humidifier using the heater and temperature controller.

2.0 SCOPE

This test protocol is aimed at determining if an external humidifier is needed to provide adequate humidity to the sterile incubator volume when it is heated with the heater instead of the hot plate. The sterile incubator volume will be humidified with a saturated sponge.

3.0 BACKGROUND

3.1 If the air is not humid enough, media will evaporate. Media evaporation may lead to a change in media pH which can kill cells. Adequate humidity prevents excess media evaporation.

4.0 MATERIALS AND EQUIPMENT

4.1 Test Devices

- 4.1.1 Rubbermaid Premier - 3 Cup Container
- 4.1.2 Rubbermaid Premier 3-Cup Container Lid, with center cut out
- 4.1.3 8" x 8" piece of .006" Natural Latex Rubber Sheet
- 4.1.4 Standard Petri Dish (plastic)
- 4.1.5 6" x 6" x 1" sponges, cut to size
- 4.1.6 Custom heater system:
 - 5.7.1.1 Heavy Duty Silicone Rubber Heat Mat; McMaster-Carr
 - 5.7.1.2 Autotuning P-I-D Temperature Controller; McMaster-Carr
 - 5.7.1.3 Type J Bendable Probe Thermocouple; McMaster-Carr

4.2 Lab Equipment

- 4.2.1 VWR III Hot Plate
- 4.2.2 50 mL Graduated Cylinder
- 4.2.3 80 mL Beaker
- 4.2.4 Digital Thermometer
- 4.2.5 Water

5.0 PROCEDURE

- 5.1 If the sponges are not cut, cut them to size. Follow the instructions in the "Evaporation with Water" test protocol to do this.
- 5.2 Preheat the hot plate to 70° C.
- 5.3 Preheat approximately 50 mL of water on the hot plate to a temperature of about 37° C.
- 5.4 Plug in the heater system; place all components of the system securely on the table.
- 5.5 Tape the thermocouple to the heater. Ensure that the tip of the thermocouple is in contact with the heater. A weight can be placed on top of the thermocouple to hold it in place, if desired.
- 5.6 The digital readout screen on the temperature controller should turn on. Set the temperature to 65° C. To set the temperature press the Left arrow ("<")

button) and look at the SV (number lower on the screen), one digit will be brighter, use the up or down arrows to change this value. Press the Left arrow button again to move to a different digit (e.g. ones to tens). Once you have selected the correct set value, press “set”.

- 5.7 Press “set” twice until the display reads “Auto” use the up/down buttons to select “Yes.1”, this will autotune the temperature during the initial phases, leading to a faster desired temperature.
- 5.8 Place the sponges in the container and saturate them with about 150 mL of warm water. Place the empty Petri dish in the center cavity. Place the container on the heater so that the entire bottom of the container is in contact with the heater.
- 5.9 Measure 40 mL of the pre-heated water into a graduated cylinder. Note the exact measurement.
- 5.10 Carefully pour the water into the Petri dish.
- 5.11 Take and record an initial temperature reading of the water.
- 5.12 Place latex and lid on container and seal tightly.
- 5.13 Let sealed container stand on heater for 5 minutes.
- 5.14 Remove lid from container and record the temperature of the water.
- 5.15 Replace lid on container and seal tightly.
- 5.16 Repeat steps 5.13-5.15 until a total of 20 minutes have elapsed.
- 5.17 After 20 minutes, take a final temperature reading. Then, carefully lift Petri dish and use a funnel to pour water into graduated cylinder.
- 5.18 Note spills or other reasons for possible data deviations.
- 5.19 Calculate the total change in volume and the percent change in volume to see how much water was lost due to evaporation.
- 5.20 If the temperatures are not within the desired range (35° to 39° C) this trial can be aborted at any point and re-started with the temperature controller set to a different temperature. The goal is to determine the optimal heater temperature with the heater.

6.0 EVALUATION CRITERIA

- 6.1 Temperatures will be noted every 5 minutes to ensure the temperature of the volume is near the desired 37° C environmental temperature. This is to ensure that evaporation losses in the test are as close as possible to accurate.
- 6.2 All volume measurements shall be taken in the same 50 mL graduated cylinder. Measurements shall be made to the nearest .5 mL.

7.0 RESULTS

Test 1: Heater at 65° C

Time (minutes)	Water Temp. (°C)	Volume (mL)
0	30.9°	40.0 mL
5	35.5°	N/A
10	39.3°	N/A
15	42.7°	39.0
20	N/A	N/A
Net Water Loss		1.0 mL
% Water Loss		2.5%

This test was aborted early due to the fact that the water was heating up too much.

Test 2: Heater at 55° C

Time (minutes)	Water Temp. (°C)	Volume (mL)
0	32.6°	40.0 mL
5	32.1°	N/A
10	N/A	N/A
15	N/A	39.0
20	N/A	N/A
Net Water Loss		1.0 mL
% Water Loss		2.5%

This test was aborted early due to the fact that the water was not heating up enough.

Test 3: Heater at 60° C

Time (minutes)	Water Temp. (°C)	Volume (mL)
0	33.3°	40.0 mL
5	35.1°	N/A

10	37.7°	N/A
15	38.8°	N/A
20	39.8°	39.0 mL
Net Water Loss		1.0 mL
% Water Loss		2.5%

8.0 DISCUSSION OF RESULTS

Humidity was not an issue with the heater any more than it was with the hot plate. Both completed trials had a percent water loss of 2.5%. There were some challenges associated with achieving the appropriate temperature; however, the appropriate heating temperature can be determined through further experimentation. Since this was only the first trial, errors should be alleviated as future trials are performed.

9.0 CONCLUSIONS AND RECOMMENDATIONS

Since water evaporation trials were successful, testing should be conducted with cell media to see if the media evaporates. In the media tests, pH should be recorded in addition to temperature and net evaporation losses. Temperature shall be varied as needed during future trials to determine the optimal heater temperature.

Evaporation and pH Changes with F-12 Media Preliminary Testing Protocol & Report

TEST DATE: 3/24/08

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REPORT COORDINATOR: Emily Hakun DATE: 3/24/08
Emily Hakun

1.0 OBJECTIVE

To determine if appropriate media pH levels and humidity can be maintained in the sterile incubator volume without an external humidifier.

2.0 SCOPE

This test protocol is aimed at determining if an external humidifier is needed to provide adequate humidity to the sterile incubator volume. The sterile incubator volume humidification will be tested with: no humidity source, a bath of water, and a saturated sponge to determine if any of these methods are adequate.

3.0 BACKGROUND

- 3.1 If the air is not humid enough, media will evaporate. Media evaporation may lead to changes in the pH's media, which could kill cells. Adequate humidity prevents excess evaporation of media.
- 3.2 The amount of evaporation should also be recorded to ensure that the Petri dish is not losing too much media.
- 3.3 Cells have a phosphate buffer that enables them to maintain proper pH, provided the external pH stays within their buffering range. The buffering range for this phosphate buffer is from 5.8 to 7.8.

4.0 MATERIALS AND EQUIPMENT

4.1 Test Devices

- 4.1.1 Rubbermaid Premier - 3 Cup Container
- 4.1.2 Rubbermaid Premier 3-Cup Container Lid, with center cut out
- 4.1.3 8" x 8" piece of .006" Natural Latex Rubber Sheet
- 4.1.4 Plastic Petri Dish
- 4.1.5 6" x 6" x 1" sponges, cut to size
- 4.1.6 40 mL F-12 fibroblast media

4.2 Lab Equipment

- 4.2.1 VWR III Hotplate
- 4.2.2 Sink (water)
- 4.2.3 50 mL Graduated Cylinder
- 4.2.4 80 mL Beaker
- 4.2.5 Digital Thermometer
- 4.2.6 Mettler Toledo Seven Easy pH meter
- 4.2.7 F-12 Fibroblast Media

5.0 PROCEDURE

- 5.1 If the sponges are not cut, cut them to size.
- 5.2 Preheat the hot plate to 65° C.
- 5.3 In a beaker, preheat 40 mL of F-12 media on the hot plate to 37° C; remove beaker from hot plate.
- 5.4 Set sponges in container and seat empty Petri dish in its circular cavity.
- 5.5 Place container on the center of the hot plate.
- 5.6 Measure 40 mL of the pre-heated media into a graduated cylinder.
- 5.7 Carefully pour media into the Petri dish.

- 5.8 Take a temperature reading of the media; record this temperature in the appropriate space in the lab notebook.
- 5.9 Remove the pH meter probe from its distilled water bath, gently shake off any excess water and place the pH meter in the media. Press “read” and record the pH. Replace the pH probe in its distilled water bath.
- 5.10 Place latex and lid on container and seal tightly.
- 5.11 Let sealed container stand on hot plate for 10 minutes.
- 5.12 Remove lid from container and record the temperature of the water with the digital thermometer.
- 5.13 Take a pH measurement (see step 5.9), again being sure to replace the probe in the distilled water as soon as it is removed from the media.
- 5.14 Replace lid on container and seal tightly.
- 5.15 Let sealed container stand on hot plate for an additional 10 minutes and take another set of temperature and pH readings. Perform these readings every 10 minutes for 1 hour.
- 5.16 Repeat 5.12-5.16 until 1 hour has elapsed.
- 5.17 After 1 hour, take the temperature and pH readings and then use a funnel to pour the media into the graduated cylinder and record the final media volume.
- 5.18 Calculate the total volume loss and percent volume loss, as well as the total change in pH.

6.0 EVALUATION CRITERIA

- 6.1 Temperatures will be noted every 10 minutes to ensure the media temperature is near the desired 37° C. This is to ensure that evaporation losses in the test are as close as possible to accurate. Temperature readings shall be taken to one decimal place (per the digital thermometer).
- 6.2 All volume measurements shall be taken in the same 50 mL graduated cylinder. Measurements shall be made to the nearest .5 mL.
- 6.3 pH readings shall be taken every 10 minutes (aligned with temperature readings). These shall be recorded to 2 decimal places (per the pH probe).

7.0 RESULTS

Trial 1: Sponge Humidifier

Time (minutes)	Media Temp. (°C)	Media pH	Volume (mL)
0	34.0	7.25	34 mL
10	34.2	7.36	N/A
20	36.4	7.42	N/A
30	37.4	7.50	N/A
40	37.9	7.61	N/A
50	36.8	7.66	N/A
60	37.5	7.70	32 mL
Net Change	N/A	.45	2 mL
% Change	N/A	N/A	5.9%

Trial 2: Sponge Humidifier

Time (minutes)	Media Temp. (°C)	Media pH	Volume (mL)
0	32.3	7.33	40 mL
10	35.4	7.29	N/A
20	36.3	7.40	N/A
30	37.1	7.50	N/A
40	37.7	7.56	N/A
50	37.9	7.62	N/A
60	37.6	7.70	38.5 mL
Net Change	N/A	.37	1.5 mL
% Change	N/A	N/A	3.8%

Trial 3*: Sponge Humidifier

Time (minutes)	Media Temp. (°C)	Media pH	Volume (mL)
0	33.0	7.40	40 mL
10	38.4	7.36	N/A
20	38.6	7.42	N/A
30	39.6	7.50	N/A
40	39.6	7.55	N/A
50	39.7	7.60	N/A
60	39.8	7.67	39 mL
Net Change	N/A	.27	1 mL
% Change	N/A	N/A	2.5 %

*For trial 3 the heater was used instead of the hot plate, the heater was set at 60° C (instead of 65 degrees). All other aspects of the experiment remained the same; hence, the data is being included here rather than as its own test.

8.0 DISCUSSION OF RESULTS

The changes in pH due to evaporation (and other factors associated with the sterile incubator volume), proved to fall within the desired pH range of 5.8 to 7.8. This implies that as long as printing and incubation is conducted within 1 hour, no additional humidification methods are required. The sponge humidifier has proven to be adequate for the purposes of this design. There was a 5.9% media loss during the 1 hour incubation period; the pH changed by .45 (7.25 to 7.70) during the same period.

9.0 CONCLUSIONS AND RECOMMENDATIONS

Since this media evaporation trial was successful, additional testing can be conducted to ensure that this test was accurate. The results of this test suggest that future testing with cells should be conducted, since pH and media maintenance are important for maintaining conditions for cell viability.

Heater Evaporation and pH Changes with High Glucose Media Preliminary Testing Protocol & Report

TEST DATE: 3/31/08 – 4/8/08

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- 9.0 CONCLUSIONS AND RECOMMENDATIONS**

REPORT COORDINATOR: Emily Hakun DATE: 4/8/08
Emily Hakun

1.0 OBJECTIVE

To determine if pH and humidity can be maintained in the sterile incubator volume without an external humidifier.

2.0 SCOPE

This test protocol is aimed at determining if an external humidifier is needed to provide adequate humidity to the sterile incubator volume when High Glucose media is used. The sterile incubator volume humidification will be tested with a saturated sponge to determine if this humidifying method is adequate.

3.0 BACKGROUND

- 3.1 If the air is not humid enough, media will evaporate, this may lead to a change in the media's pH, which could kill cells.
- 3.2 Cells have a phosphate buffer that enables them to maintain proper pH, provided the external pH stays within their buffering range. The buffering range for this phosphate buffer is from 5.8 to 7.8.

4.0 MATERIALS AND EQUIPMENT

4.1 Test Devices

- 4.1.1 Rubbermaid Premier - 3 Cup Container
- 4.1.2 Rubbermaid Premier 3-Cup Container Lid, with center cut out
- 4.1.3 8" x 8" piece of .006" Natural Latex Rubber Sheet (Small Parts, Inc.; Miramar, FL)
- 4.1.4 Standard Petri Dish
- 4.1.5 6" x 6" x 1" sponges, cut to size
- 4.1.6 40 mL HyClone DMEM High Glucose Eagle's Medium (Hyclone Labs; Logan, UT)
- 4.1.7 Silicone Matt Heater (McMaster-Carr; Los Angeles, CA)
- 4.1.8 P-I-D Temperature Controller (McMaster-Carr; Los Angeles, CA)
- 4.1.9 Type J Thermocouple (McMaster-Carr; Los Angeles, CA)

4.2 Lab Equipment

- 4.2.1 VWR III Hotplate
- 4.2.1 Sink (water source)
- 4.2.3 50 mL Graduated Cylinder
- 4.2.4 80 mL Beaker
- 4.2.5 200 mL Beaker
- 4.2.6 Digital Thermometer
- 4.2.7 Mettler Toledo Seven Easy pH meter

5.0 PROCEDURE

- 5.1 If the sponges are not cut, cut them to size.
- 5.2 Preheat the heater (via the temperature controller) to the desired temperature (45 or 60° C). Place the thermocouple tip directly on top of the heater and tape it in place.
- 5.3 Preheat the hot plate to 65° C (for pre-heating the water and media)

- 5.4 In a beaker, preheat 40 mL of High Glucose media on the hot plate to 37° C, once heated, remove from hot plate.
- 5.5 In the 200 mL beaker, preheat 150 mL of water to 37° C.
- 5.6 Set sponges in container; set configuration on heater.
- 5.7 Measure 150 mL of heated water and carefully pour it into the sponges to saturate them.
- 5.8 Place Petri dish in the sponge cavity; place this system on the heater.
- 5.9 Measure 40 mL of the pre-heated media into the graduated cylinder. Note exact initial volume.
- 5.10 Carefully pour media into the Petri dish.
- 5.11 Record the initial media temperature
- 5.12 Remove the pH meter probe from its water bath, gently shake off any excess water and place the pH meter in the media. Press “read” and record the pH. Replace the pH probe in its distilled water bath.
- 5.13 Place latex and lid on container and seal tightly.
- 5.14 Let sealed container stand on heater for 10 minutes.
- 5.15 Remove lid from container and take the temperature of the water with the digital thermometer, record this temperature in the appropriate space in the lab notebook.
- 5.16 Take a pH measurement (see step 5.9), again being sure to replace the probe in the distilled water as soon as it is removed from the media. Record the pH reading in the appropriate space in the lab note book.
- 5.17 Replace lid on container and seal tightly.
- 5.18 Repeat steps 5.14-5.17 until a total of 60 minutes has elapsed or the pH has exceeded 7.9 (whichever comes first).
- 5.19 After the final readings, carefully pour the media into the graduated cylinder (using a funnel to prevent spilling, if desired) and record the final volume of media.
- 5.20 Calculate the total volume loss and percent volume loss, as well as the total change in pH.

6.0 EVALUATION CRITERIA

- 6.1 Temperatures will be noted every 10 minutes to ensure the temperature of the volume is near the desired 37° C environmental temperature. This is to ensure that evaporation losses in the test are as close as possible to accurate. Temperature meetings shall be to one decimal place (per the thermometer), and shall be taken with the digital thermometer.
- 6.2 All volume measurements shall be taken in the same 50 mL graduated cylinder. Measurements shall be made to the nearest .5 mL.
- 6.3 pH readings shall be taken every 10 minutes (at the same time as temperature readings). These shall be recorded to 2 decimal places (per the Mettler Toledo Seven Easy pH meter’s accuracy).

7.0 RESULTS

Trial 1: Heater at 45 °C

Time (minutes)	Media Temp. (°C)	Media pH	Volume (mL)
0	33.7	7.31	39 mL
10	32.6	7.55	N/A
20	31.6	7.78	38 mL
Net Change	N/A	.47	1 mL
% Change	N/A	N/A	2.5%

*This trial was aborted early due to the rapid drop in temperature; this may be partially due to the fact that the initial temperature was below 35.

Trial 2: Heater at 45° C

Time (minutes)	Media Temp. (°C)	Media pH	Volume (mL)
0	37.7	7.25	39 mL
10	33.0	7.51	N/A
20	31.3	7.68	N/A
30	31.3	7.81	38.5 mL
Net Change	N/A	.30	.5 mL
% Change	N/A	N/A	1.4%

The temperature drop was still too large, so the heater temperature was increased for the next trial.

Trial 3: Heater at 60° C

Time (minutes)	Media Temp. (°C)	Media pH	Volume (mL)
0	35.4	7.44	40 mL
10	38.4	7.68	N/A
20	40.3	7.86	N/A
30	40.2	7.99	39.5 mL
Net Change	N/A	.30	.5 mL
% Change	N/A	N/A	1.3%

8.0 DISCUSSION OF RESULTS

The changes in pH due to evaporation (and other factors associated with the sterile incubator volume), were much more dramatic with the high glucose media than with the F-12 media. The pH levels spiked from 7.44 to 7.99 in 30 minutes (versus about 7.3 to 7.7 over an hour with the F-12 media). The high glucose media was outside the desired pH range of 5.8 to 7.8 in a short period of time.

This implies that this media will not maintain the desired pH levels during the specified 1 hour time period, so appropriate accommodations must be made. The net media loss was still quite low (1.3-2.5%), so evaporation losses should be negligible if the changes in pH can be controlled.

9.0 CONCLUSIONS AND RECOMMENDATIONS

Since the high glucose media evaporation trial was not as successful as the F-12 media, additional information was gathered. Careful investigation of the two media types and their contents showed that the F-12 media contains 15mM of Hepes buffer, which would likely yield smaller increases in pH during the testing process.

To test this hypothesis, Hepes buffer shall be added to the high glucose media to achieve a 15mM concentration of buffer in the media. pH tests will be conducted with the modified high glucose media after the buffer has been added.

Appendix D: Container Setup & Cell Printing Protocol

In order to prepare the sterile incubator volume system for cell printing, the following protocol must be adhered to.

In the Lab:

1. Check to ensure that all sterile components have been sterilized (or thoroughly cleaned). These include:
 - Rubbermaid Premier 3 Cup container and Lid
 - Latex sheet with Septum
 - Plastic Petri dish
2. If any of the sterile components are not yet sterile, they must be sterilized (or thoroughly cleaned) before proceeding with the cell printing process.
 - a. Rinse each component with warm water
 - b. Use soft brushes to rub Alconex all over each component, focusing on hard to clean areas (corners, crevices, mated surfaces, etc.)
 - c. Rinse each component with warm water until there is no visible soap residue.
 - d. Spray each component thoroughly with the 10% bleach solution. Be sure that all surfaces area covered.
 - e. Let each component stand for 1 minute with the bleach solution on it.
 - f. Rinse each component with warm water
 - g. Drain excess water into the sink; then, place components on clean paper towels to allow for additional draining.
 - h. Place the components in a pouch and autoclave (if applicable)
 - i. Spray each component thoroughly with the 70% alcohol solution.
 - j. Drain excess solution into the sink; then, place components on clean paper towels for additional draining. Pat the components dry if necessary.
3. Gather the heater configuration components, which should already be wired together:
 - Heavy Duty Silicone Rubber Heat Mat
 - P-I-D Temperature Controller
 - Type J Thermocouple
4. Remove the feedback media volume from the fridge, and place it (in its vial) in a beaker full of warm water.
5. Set up the heater. If the stages are being used, the heat mat should be adhered to a mount and attached to the stages if the stages are not being used the heat mat can simply be placed on a stable print surface. The temperature controller should be placed in a visible location.
6. Plug the power supply into an A/C power source.
7. Check to ensure that the set value (SV) on the temperature controller is set to 37° C. If it is not, use the left arrow button to pick which number to change (e.g. tens or ones), the selected digit will be brighter than the other digits. Use the up arrow and down arrow buttons to adjust the temperature; press “set” again to lock in the set temperature.
8. Place the thermocouple directly on top of the heat mat. Place the feedback container on top of the thermocouple to temporarily hold it in place.

In the Lab:

9. Construct the feedback volume in the same way as the sterile incubator volume, except in open air. Place the Petri dish in the center of the Rubbermaid container and fill it with media (use the same type and volume of media as printing).
10. Thread the bent-thermocouple through the hole in the feedback volume's lid. Pre-position the lid on top of the container to ensure that the thermocouple tip is submerged in the media.
11. Lift off the container lid and place a piece of latex over the top of the container.
12. Place the lid (with thermocouple) over the container and adjust the latex to accommodate the thermocouple (the top should still be mostly sealed).
13. Press down firmly on all sides of the container lid to seal the latex in place.
14. Visually inspect the container to ensure that the thermocouple tip is still submerged in the culture dish.
15. Place the feedback volume in the tray on top of the heat mat. The flat side of the volume should be facing the center.

In the Hood:

16. Carry the sterile components to the hood work area.
 - Rubbermaid Premier 3 Cup container and Lid
 - Latex sheet with Septum
 - Plastic cell culture dish
17. Follow proper hood use protocol to place these items in the hood for construction. Following hood use protocol is imperative for the success of the printed cells and the experiments of all other researchers using the hood.
18. Place the cell culture dish in the center of the Rubbermaid container (see figure 27).

Figure 27: Culture Dish placed in Rubbermaid Container

19. Place the latex sheet over the top of the container, septum-side-up (see figure 28).

Figure 28: Latex placed (septum-side-up) over the container

20. Place the snap fit frame over the container and press down tightly on all sides to seal the lid and latex in place (see figure 29).
21. Apply gentle pressure to the latex to make sure it is fully sealed (it should resist slight pressure). If the latex is not sealed (it does not resist the pressure, or there are visible gaps), remove the frame and replace the latex and repeat steps 11-13.

Figure 29: Press down firmly on all sides of the frame to secure the latex in place

22. Once the latex is sealed, the sterile volume can be removed from the hood.

In the Lab

23. Place the sterile incubator volume next to the feedback volume in the tray, the flat side of the sterile incubator volume should be touching the flat side of the feedback volume.
24. To insert the deposition needle into the sterile incubator volume, hold the septum between your fingers (see figure 30) and gently insert the needle (no larger than 18 gage). Make sure that the needle is inserted far enough to accurately dispense cells.

Figure 30: Support the sides of the septum with 2 fingers while inserting the syringe

25. Deposit the cells as desired.
26. To remove the deposition needle, support the septum with two fingers and gently pull the deposition needle out of the sterile incubator volume. The septum will re-seal to maintain internal container sterility.
27. Turn off the heater by unplugging the temperature controller's power supply.

In the Hood

28. Carefully carry the sterile incubator volume and use proper hood protocol to replace the volume in the hood.
29. Remove the lid and latex from the volume and carefully remove the culture dish.
30. Place a lid on the culture dish, the dish can now be removed from the hood. The cells may be examined using the inverted microscope or immediately placed in the incubator to promote further cell growth.