Harris, Melanie, Advanced Problem Solving in the Biotherapeutics Industry: Parameters influencing the delivery of a novel cell therapy product and exploration of a new method for determining activity of *Clostridium histolyticum* collagenase, a wound debridement enzyme. Master of Science (Biotechnology), May, 2015, 112 pp., 37 tables, 14 figures, references, 32 titles.

ABSTRACT

Biotechnology is a multi-faceted industry with many unique challenges that require knowledge in a broad range of topics. When working in the wound care field it is necessary to not only create a product in the laboratory, but also effectively bring it to the patient. This task requires many skilled people who can test it for efficacy, design and conduct clinical trials, confirm quality and consistency, design packaging, consider transportation issues and so on. The following investigation focuses on the testing of a cellular product and its accompanying device under various conditions as well as the exploration of a new assay capable of the activity of a wound debridement enzyme. The results of the product/device testing have generally confirmed the comparability of the cellular product devices as well as their resistance to various temperatures encountered in the clinical environment. A new modified assay for the testing of collagenase has been established as precise and comparable to current methods, though it requires more testing to confirm robustness.

Advanced Problem Solving in the Biotherapeutics Industry:

Parameters influencing the delivery of a novel cell therapy product

and exploration of a new method for determining activity of *Clostridium histolyticum* collagenase, a wound debridement enzyme

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ADVANCED PROBLEM SOLVING IN THE BIOTHERAPEUTICS INDUSTRY: PARAMETERS INFLUENCING THE DELIVERY OF A NOVEL CELL THERAPY PRODUCT AND EXPLORATION OF A NEW METHOD FOR DETERMINING ACTIVITY OF *Clostridium histolyticum* COLLAGENASE, A WOUND DEBRIDEMENT ENZYME

INTERNSHIP PRACTICUM REPORT

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF BIOMEDICAL SCIENCES

with a focus in BIOTECHNOLOGY

By

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Fort Worth, Texas

May 2015

ACKNOWLEDGEMENTS

I would like to thank everyone at Smith & Nephew for allowing me the opportunity to complete my internship at their Biotherapeutics facilities, especially Lei Shi, Ph.D., who was always willing to help when I needed it. I have been honored to work with such a talented Research and Development group here and am grateful for my lab mates Jason Campbell, M.S. and Sumith Kottegoda, Ph.D., in particular who were always available to answer any questions that I had with patience and kindness.

Special thanks go to my mentor and committee member Aleksa Jovanovic who helped me every step of the way through the ups and downs of my internship journey.

I would also like to thank the University of North Texas Health Science Center Team for all of their advice and guidance: Patricia Gwirtz, Ph.D., FACC, Jerry Simecka, Ph.D. and Derrick Smith.

I would not have been able to accomplish anything without my wonderful friends and family. Thanks especially to my husband, Michael Harris, for things too numerous to list.

I would also like to thank my friends, readers of thesis, advisors and confidants: Christine Hajovsky, Haydee Izurieta Munoz, and Virginie Coindre.

I could not have done this without you all. Thank you so much.

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LIST OF ABBREVIATIONS

CPT1F	Fibrinogen containing component of HP-802-247
CPT2C	Thrombin containing placebo component of HP-802-247
CPT2V	Thrombin and cell containing component of HP-802-247
VEGF	Vascular endothelial growth factor
GM-CSF	Granulocyte-macrophage colony-stimulation factor
TGF-β	Tumor growth factor beta
IGF-1	Insulin-like growth factor 1
TGF-α	Tumor growth factor alpha
PDGF	Platelet-derived growth factor
HBSS	Hank's balanced salt solution
SEM	Scanning electron microscope
EDTA	Ethylenediaminetetraacetic acid
FALGPA	N-[3-(2-Furlylacryloyl)]-L-Leucyl-Glycyl-L-Prolyl-L-Alanine
FITC	Fluorescein isothiocyanate
LOQ	Limit of quantitation
LOD	Limit of detection
CAB	Collagenase assay buffer
ANOVA	Analysis of variance
RSD	Relative Standard Deviation

CHAPTER I

INTRODUCTION TO THE STUDY

Device and formulation for HP-802-247

The following research performed at Smith & Nephew Biotherapeutics will aid in the establishment of comparability between the current spray delivery system for HP-802-247 and the future model at conditions mimicking those of the clinical settings. The goal of the comparability studies is to determine whether the current and future spray pumps will deliver equal amounts of drug product at a range of product temperatures. The number of actuations it takes to prime the current and future sprayers will also be examined as well as the polymerization of the fibrin clot that will be formed upon the application of the drug at various temperatures. The spray pump equivalency will be established for spray area, ovality, and dosage. To be equivalent, the spray pumps must deliver the drug product with a comparable number of viable cells, and with statistically equivalent potency.

The main objective of comparability tests is to establish equivalence of parameters at a range of temperature from 4-25°C. The comparability experiments include a test of the spray area of component 1 (CPT1F, Fibrinogen component), component 2 (CPT2V, Thrombin component vehicle), and surrogate (CPT2V equivalent component) at different temperatures that

may possibly be encountered in clinical settings. Surrogate component equivalency to CPT2V behavior will also be confirmed.

The drug product is maintained in an -80°C environment at all times prior to use. Just before use, the drug product is placed in a 37°C water bath for 5 minutes. It is postulated that the final product temperature is in the range of 4-25°C in current clinical setting. These comparability tests will provide insight into what delivery parameters for HP-802-247 are affected by this temperature range of 4-25°C.

Another comparability experiment will be the testing of the drug products efficacy and potency after dispensing with both the current (Lot 004) sprayer model as well as the future (Lot 005) model. The model of the new sprayer is the same design, but composed of a new type of resin in Lot 005. One method for testing efficacy will be determining the cell count percent viability after dispensing the drug product. Potency of cells will be tested by examining the secretion of growth factors after dispensing the drug product. Currently, the growth factors vascular endothelial growth factor (VEGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are assayed for this test. The potency and efficacy of HP-802-247 must be validated for any new device being used.

Enzyme activity assessment

The development of new assays is an important process in industry. The purpose of this assay exploration is to develop a robust and simplified test for the activity of *Clostridium histolyticum* collagenase, the active ingredient in Collagenase SANTYL® Ointment (CSO), Smith & Nephew's wound debridement product. Comparability with current methods used by Smith & Nephew R & D will also be explored. The potential uses for the assay include product

quality validation as well as compatibility studies using SANTYL® in conjunction with other wound care products, including novel formulations that might be used with the CSO.

PROBLEMS/HYPOTHESIS

Smith & Nephew is a global medical device and pharmaceutical organization dedicated to helping improve people's lives through application of advanced surgical and wound healing devices and drugs.

One way Smith & Nephew-Biotherapeutics can achieve better quality of life for patients is improving the therapeutic outcome of chronic wounds. HP-802-247 is a revolutionary new cellular wound therapy product for the treatment of venous leg ulcers. HP-802-247 is a two component cell delivery system administered by spray pump. The current product consists of two vials containing CPT1F (component 1, fibrinogen component) and CPT2C (component 2, thrombin and cell containing component), respectively. At clinical sites, the vials are removed from storage in -80°C and placed in a 37°C water bath for 5 minutes to thaw. After thawing, the health care provider removes the caps, replaces them with our current model sprayer, and primes both pumps simultaneously. CPT1F is sprayed first directly onto the wound from a distance of 10 cm, followed by CPT2C in the same manner. The unique ability of this cellular product could provide patients with a better option for the treatment of their chronic wounds.

Problem 1: Product temperature

Pharmaceutical products must be consistent in their dosage and performance. In previous studies conducted by Smith & Nephew-Biotherapeutics, product temperature range after thawing in the above described manner using new secondary thawing packaging has been between 4 and 25°C. Because the drug product has a range of temperatures after thawing, it is important to determine experimentally if the temperature range has any significant effects on sprayer performance parameters such as spray area, ovality (a measurement describing the degree of deviation from circularity using maximum diameter/minimum diameter), dosage, actuations, and/or polymerization time. Temperature is one of many factors that can impact the efficacy and viability of cell-based solutions (Katare et al. 2012, He 2011, 47-73, Pasch et al., 10).

In order to ensure that the specifications of dosage and efficacy are met, it must be determined that the product delivery is consistent given the possible variation in drug product temperature during application. It is hypothesized that the range of product temperature in question does not significantly affect the efficacy and delivery of HP-802-247. In comparability tests, this practicum project will be investigating the influence of temperature fluctuation on spray area, dosage applied, actuations (also known as 'pumps to prime') and clotting for HP-802-247 product components. These data will be used to ensure quality and consistency of the new spray device, new thawing device, and other packaging (e.g. secondary packaging) as compared to the current device.

Problem 2: Sprayer pathway and new sprayers

In order to begin using a new device for clinical trials, there are stringent consistency and quality assessments that must be performed to ensure equivalence from trial to trial. Aptar Pharma, the manufacture of the VP-7 pumps, made the decision to change the resin used for the cannula, actuator head, hinged nozzle, and terminal to prevent possible mechanistic issues. The new pumps (Lot 005) use this new resin and must be qualified as being equivalent to previous devices used in clinical trials. It is hypothesized that any lot-to-lot differences resulting from the usage of a new resin in the manufacturing process of the sprayers will not be significant.

Another factor that may affect cell potency and viability is the pathway through the sprayer itself. Cellular product will be subjected to mechanical forces of the sprayer during the spraying process. Historically, one way to test the effect of spraying with regards to the performance of the drug product has been by testing the viability of cells after spraying. The potency of cells in the drug product are tested by performing assays for vascular endothelial growth factor (VEGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) production by cells after spraying in-vitro with current and future spray pumps. These tests help to confirm that the drug product is still potent after the spraying process. Moreover, such experiments will ensure the comparability of both lots of spray pumps with regard to drug product quality and potency. It is hypothesized that the new sprayers will not significantly affect the drug product qualities of potency and viability.

The drug product cells must have comparable viability and potency. If not equivalent, further action regarding qualifications for clinical trials must be pursued. These experiments are

important steps towards ensuring a smooth transition from clinical trial to clinical trial and onward to commercial use.

Problem 3: Assay development

In order to effectively test product compatibility with Smith & Nephews collagenase containing ointment SANTYL® there must be a robust and reliable method for analysis of collagenase activity. Current available methods lack similarity to in-vivo conditions, use dangerous chemicals such as cyanide, involve complex preparation steps, and are expensive and time consuming. A new method would ideally be simpler, less expensive, and be a more accurate in-vitro model for collagen degradation. This new method should also be accurate, precise and a suitable for simulating in-vivo conditions which SANTYL® is exposed to in clinical usage.

CHAPTER II

PARAMETERS INFLUENCING THE DELIVERY OF A NOVEL CELL THERAPY PRODUCT

BACKGROUND AND LITERATURE

HP-802-247 and chronic venous leg ulcers

Chronic venous leg ulcers are open, long-lasting, difficult to treat and often recurring wounds resulting from a venous insufficiency located on the lower leg or ankle (Ylönen et al. 2014, 194-202, Kapp, Miller and Donohue 2013, 189-198). These wounds have the potential to cause great pain and discomfort, both physically and socially for the patient (Ylönen et al. 2014, 194-202, Kapp, Miller and Donohue 2013, 189-198). Much work has been done to find better options for treating chronic wounds and products including advanced dressings, growth factor containing ointments and skin grafts are examples of the technologies employed in the treatment of chronic wounds. There is an ongoing effort to create better options for treating chronic wounds.

Chronic wounds do not heal normally and exhibit several differences when compared to acute wounds (Schultz et al. 2003, S1-S28). The typical acute wound goes through a well-defined process of coagulation, inflammation, cell proliferation, and epithelialization (Schultz et

al. 2003, S1-S28). Chronic wounds do not progress normally through all the typical healing steps and appear to be 'stalled.' (Schultz et al. 2003, S1-S28). Not all chronic wounds get stalled in the same phase, and this is why careful monitoring of the wound is essential to proper treatment (Schultz et al. 2003, S1-S28). Each wound has different needs and there is not a one size fits all approach to wound care for successful healing of chronic wounds (Kirsner et al. 2012, 977-985).

The coagulation phase of wound healing begins immediately after injury as a result of platelets that release many different growth factors (Schultz et al. 2003, S1-S28). Growth factors attract inflammatory cells to the wound site and can encourage cell division (Schultz et al. 2003, S1-S28). They are also involved in directing the movement of various cells involved in wound healing and regulating the re-building of extracellular matrix (Schultz et al. 2003, S1-S28). Some typical examples of growth factors involved in wound healing include platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and granulocyte macrophage colony-stimulating factor (GM-CSF) (Schultz et al. 2003, S1-S28). Typically the inflammatory phase of wound healing occurs concurrently with or after coagulation (Schultz et al. 2003).

Inflammation, regulated mainly by cytokines, attracts immune system cells, such as macrophages and leukocytes (Schultz et al. 2003). These immune cells mode of action is still not completely understood. It is postulated that they play a role in several key processes during the wound healing stages: (a) They are capable of destroying bacteria and breaking down extracellular matrix by the action of released proteases (removal of the damaged extracellular matrix will allow new tissue to be built unimpeded across the wound); and (b) Growth-factors are also released by the cells during this phase such as basic fibroblast growth factor (bFGF),

TGF-α and TGF-β (Schultz et al. 2003). Normally these influential proteins and polypeptides are released near the wound site and communicate locally to regulate the stages of wound healing (Schultz et al. 2003). The inflammation begins to subside during the cell proliferation phase of wound healing (Schultz et al. 2003).

The proliferation of cells, formation of extracellular matrix and the necessary vasculature to supply new cells with oxygen and nutrients dominates the cell proliferation phase (Schultz et al. 2003, S1-S28). The extracellular matrix provides a place for new epithelial cells to migrate across and attach, forming the new mature tissue (Schultz et al. 2003, S1-S28). The fibrin and fibronectin matrix acted as a temporary scaffold over the fresh wound site (formed in previous phases), and is now replaced by a new extracellular matrix (Schultz et al. 2003, S1-S28). In order for the new matrix to form, it is essential that the old one be broken down by special proteases, including collagenase and gelatinase. (Schultz et al. 2003, S1-S28)

The final phase of wound healing is the remodeling of the scar tissue formed in the previous phases. This phase also involves proteases breaking down excess matrix and the apoptosis of fibroblasts whose primary purpose was to produce new extracellular matrix (Schultz et al. 2003, S1-S28).

HP-802-247 is a revolutionary cellular product designed to heal chronic venous leg ulcers. This drug product contains growth-arrested, viable keratinocytes and fibroblasts cultured from neonatal foreskin. HP-802-247 consists of a liquid Fibrinogen component (CPT1F) and a liquid Thrombin-containing cell component (CPT2C). Fibrinogen and Thrombin combine to form a Fibrin matrix, an integral part of the temporary wound matrix. The Fibrinogen component is first sprayed on the wound and will form a matrix upon contact with the Thrombin-

containing cell component, which is sprayed second. This Fibrinogen-Thrombin mechanism is the same one used to form a blood clot (Kirsner et al. 2012, 977-985). In this way, the fibrin clot forms a temporary barrier across the open wound. This temporary matrix is useful because cells can adhere to this fibrous matrix or utilize it for migration across the wound (Kirsner et al. 2012, 977-985). The fibroblast and keratinocytes are able to produce growth factors (such as VEGF, GM-CSF and many others) and have been shown to accelerate the healing and increase wound closure in chronic venous leg ulcers when compared to compression bandaging and placebo treatments (Kirsner et al. 2012, 977-985). The final skin tissue is not comprised of the growth-arrested allogeneic keratinocytes and fibroblasts (Kirsner et al. 2012, 977-985). These cells are believed to serve only as custom growth-factor producers that aid in the progression through the wound healing process. Because HP-802-247 contains living cells, it has the potential to react to the environment unlike topical growth factors alone (Kirsner et al. 2012, 977-985).

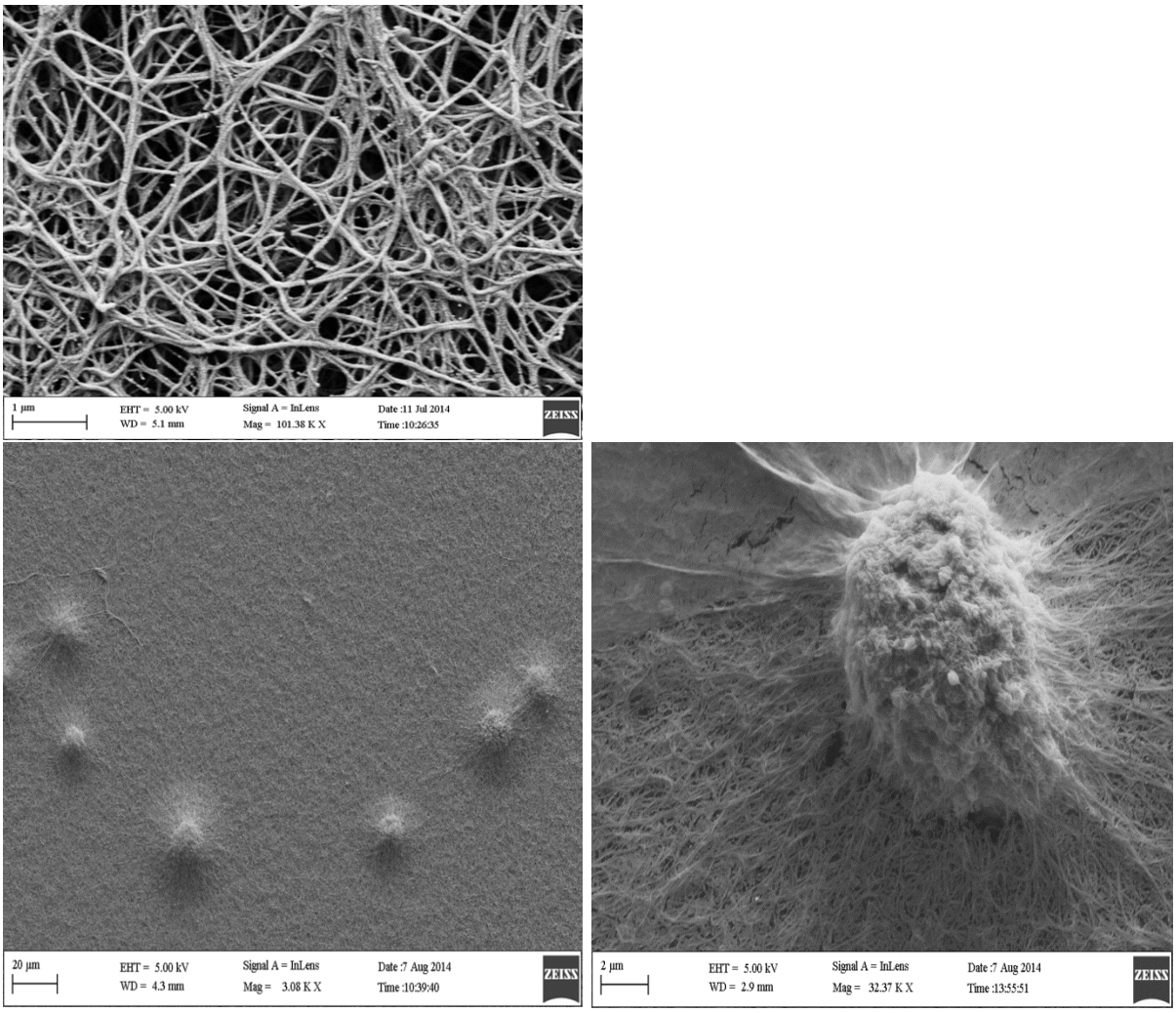

Figure 1. SEM photographs of HP-802 products (sample preparation by Jason Campbell, M.S. and images taken by UT Southwestern Electron Microscopy Core Facility)

Drug delivery and Sprayer dynamics

Ensuring the accurate delivery and dosage of pharmaceutical products dispensed using a device can be especially challenging due to additional variability of device performance (Trows et al. 2014, 195-219, Guo et al. 2008a, 417-426). Many studies have been designed to assess the delivery of sprays (nasal and throat in particular) to ensure proper delivery and regulatory requirements. Typically these studies have focused on two main parameters: device and

formulation. Results of device quality testing are usually indicated by the manufacturer in the technical description of the product and usually include approximate dosage, spray area, ovality, and droplet size distribution. Formulation effects on device performance should be tested with the placebo and drug product in-house to better understand the physical properties of the product and therefore ensure accurate drug delivery.

Because these sprayers are manufactured for use with a variety of products, the manufacturer quality tests are typically done using water. Much of the pharmaceutical sprayer literature focuses on the effect of formulation type on the sprayer performance. Formulation factors of importance in spray dynamics include viscosity and surface tension (Trows et al. 2014, 195-219). It is logical to conclude that the more similar a formulation is to water, the closer its parameters will be to those specified by the manufacturer. Unfortunately, most of the pharmaceutical formulations are far more complex than water and do not always behave as such. Typical formulations include emulsifiers/surfactants, dissolved solutes, thickeners and more (Trows et al. 2014, 195-219). For example, nasal sprays need to be thin enough to spray but thick enough to adhere to nasal passages (Pennington et al. 2008, 923-929). This challenge can be overcome with non-Newtonian fluids, which exhibit shear-thinning behavior under the pressure of the spraying device (Pennington et al. 2008, 923-929). Complex fluid dynamics is just one of the many reasons it is important to test sprayers in house using placebo and drug products to assure accuracy and quality.

HP-802-247 is a novel cell-delivery product. It consists of two components which both include ingredients which may alter the solutions physical characteristics so the behavior is significantly different from that of water. HP-802-247 has the extra challenge of not only being a mixture of varying viscosity and ingredients but having temperature fluctuations (received

frozen at -80ºC before being thawed in a 37ºC water bath) that may alter the behavior of the fluids significantly.

SPECIFIC AIMS

As previously stated, the goal of this project is to establish equivalence and comparability of the current and future spray pumps made with a new resin for HP-802-247 delivery at various product temperatures. The performance of the spray pumps will be tested for spray area, ovality, and dosage within the product temperature range of 4°C to room temperature (22ºC in the lab where experiments are conducted). The performance of the drug product will also be established by determining the polymerization of the components at 4-22°C and by testing the viability and potency of cells after spraying. Because this is an evolving project, methods and techniques may be revised based on new insights that may result from the on-going analysis of data and collaboration of the research and development (R & D) group at Smith & Nephew. The summary of aims is stated as follows:

1. Establish effect of product temperature on spray area, dosage, and ovality
2. Confirm comparability between different sprayer lots (manufactured with a new type of resin in Lot 005) for spray area, dosage, and ovality
3. Establish polymerization time at various product temperatures
4. Establish comparability of Lot 004 and Lot 005 sprayers regarding cell count and viability

SIGNIFICANCE

Venous leg ulcers are believed to affect over 1.65% of adults over the age of 65, and the number is expected to increase along with the aging of the baby-boomer generation (Kirsner et al. 2012, 977-985). These chronic wounds are economically and socially costly (Kucharzewski, Kozka and Urbanek 2013). HP-802-247 has been shown to significantly reduce wound area and increase closure of wounds in chronic venous leg ulcers when compared to placebo in previous Phase II clinical trials (Kirsner and Slade 2013, 372). During the HP-802-247 clinical trials, Smith & Nephew-Biotherapeutics has received feedback from clinical sites and is working on new and improved packaging for the commercial market. This packaging is being designed carefully to be more intuitive, elegant, user friendly and equivalent to the current clinical trial packaging. The new packaging must be shown experimentally to be comparable to the current packaging in terms of dosage and spray area. Lack of comparability between products could lead to the need for additional clinical trials, which could significantly slow down the future commercial distribution. This study will provide important insights on the nature of the drug product at various temperatures and its effect on packaging and delivery. The investigational techniques used and developed here as well as new insight and knowledge gained could be applied to future cellular products research and developments. As the technology of cell-based medicine develops at a fast pace globally, the demand to determine the best ways to store, preserve, and deliver these complex biologics does as well (He 2011, 47-73).

MATERIALS AND METHODS

Spray Area and Ovality

The BSA-surrogate solution was prepared by Smith & Nephew protocol standard using bovine serum albumin, NaCl, glycerin, FD&C Blue, and purified water. Surrogate solution was stored at 4°C. Vials of CPT1F & CPT2V were removed from the -80°C freezer and placed in a 37°C water bath for 5 minutes to thaw. 20 µL of FD & C blue solution (15 mg/mL) was added to each vial to visualize spray area. Vials were placed in 4°C refrigerator, water bath set to 10°C or 15°C, or left at room temperature (RT) as indicated. (Note: The difference between RT for spray performance experiments (22°C) and polymerization experiments (20°C) was the difference in the RT for two different labs where the experiments were performed). Vials were then removed one at a time and the cap was replaced with VP-7 sprayer (Lot 004 or Lot 005 as indicated). Vial was then pumped until primed, then attached to the spray apparatus set at a 10 cm distance from a sheet of Whatman #3 paper. Four sprays from each were sprayed onto Whatman #3 paper and the spray perimeter was quickly outlined with indelible ink pen. The filter paper was then set aside to dry. The same process was repeated for all vials at all temperatures. The dried Whatman papers were scanned as jpeg images and uploaded to computer. ImageJ (public domain software available free via National Institutes of Health, imagej.nih.gov) was used to determine the spray area and minimum and maximum feret diameters. Averages for each temperature group and ovality (maximum feret axis/minimum feret axis) were calculated with Microsoft Excel. Each group was statistically compared by

ANOVA using Microsoft Excel and/or JMP statistical software. Further statistical tests, such as Tukey HSD for comparing multiple means, Student's t-test for groups of two, fit least squares, and simple linear regressions were also utilized.

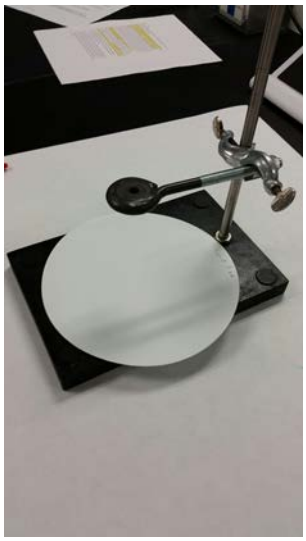

Figure 2. Simple spray distance apparatus used in experiments (experiment performed by Jason Campbell, M.S. at S&N Biotherapeutics)

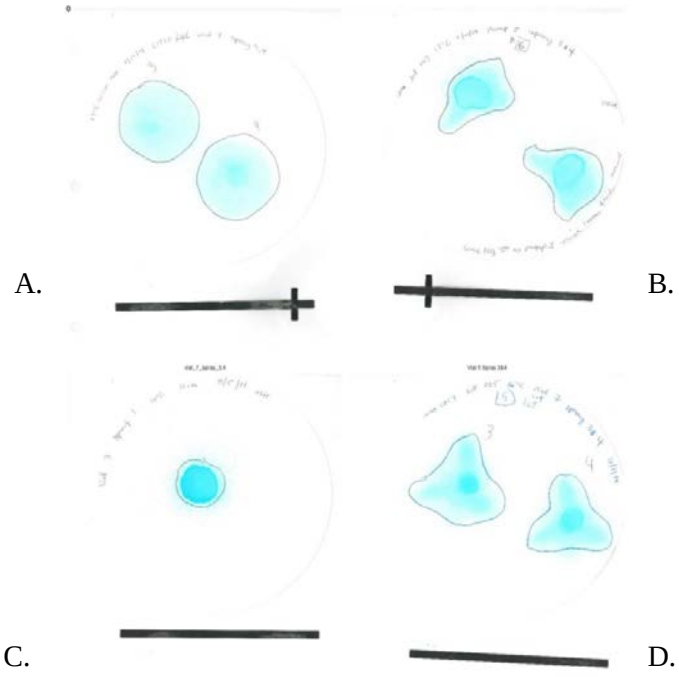

Figure 3. Examples of spray area experiment (A) Ideal ovality and flawed area (B) Ideal area and flawed ovality (C) Ideal ovality and flawed area (D) Flawed ovality and adequate area

Spray Dosage

Surrogate solution was stored at 4°C. Vials of CPT1F & CPT2V were removed from the -80°C freezer and placed in a 37°C water bath for 5 minutes to thaw. 20 µL of FD & C blue solution (15 mg/mL) was added to each vial to visualize component for consistency with previous experiments testing spray area and dosage simultaneously. Vials were then removed one at a time and the cap was replaced with VP-7 sprayer. Scale was prepared with a stand to hold the centrifuge tube during weighing and tarred. Each component was sprayed into the centrifuge vial (including priming sprays) and the weight recorded after each spray. Spraying followed by weighing continued until all product was discharged completely from each vial. This process was repeated for all solutions at 4, 10, 15, and 22°C. Raw data were then transcribed into Microsoft Excel and JMP statistical software for analysis of weight per spray.

Based on previous experiments and also the clinical application procedure, the pump was considered primed when a spray weighed over 60 mg as this ensured the next actuation would deliver a full spray. The next 4 sprays after priming were averaged for each vial. The average number of pump actuations for priming and the average of the 4 spray weights after priming were calculated for each temperature group. Temperature groups were statistically compared using Microsoft Excel and JMP statistical software utilizing multiple tests such as ANOVA, Student's t-test, fit least squares, and simple linear regression.

Polymerization Assay

Vials of CPT1F & CPT2V were removed from the -80°C freezer and placed in a 37°C water bath for 5 minutes to thaw. CPT2V was then diluted 1:20 with 1X HBSS. Assays were performed at 4, 10, 15, 20, and 37°C and all dilutions were stored at each temperature for at least 15 minutes using water bath or refrigerator as needed. ST-art 4 instrument was turned on and cuvettes prepared by placing 1 magnetic ball into each cup. For 37°C assays, cups and balls were pre-warmed in incubation chamber for 2-3 minutes before performing polymerization experiment. 100uL of CPT1F was pipetted into each cup in cuvette with magnetic ball and then placed in the quantification chamber. The ST-art4 instrument electronically attached pipette (fitted with disposable Finnpippette syringe tips) was then used to pipette 50uL of diluted CPT2V into each cup of the cuvette and polymerization time was recorded. This was repeated for each group at each temperature. A control experiment was run with Fibrinogen CPT1F control and various CPT2V/Thrombin Controls (Lot 174, Lot 337, and Thrombin Control). Results were transcribed into Microsoft Excel and then JMP statistical software for averaging, charting, and analysis.

Cell Viability

The number and percent of viable cells present in CPT2C after dispensing from the current (Lot 004) and future (Lot 005) sprayer (manufactured using a new resin) will be determined with the NucleoCounter 3000 instrument. This experiment must be performed in a Biosafety cabinet and all materials used must be cleaned with 70% ethanol. CPT2C was removed from -80C°C and thawed in a 37°C water bath for 5 minutes as performed. CPT2C was then sprayed into a new tube using either Lot 004 or Lot 005 sprayers as necessary. Unsprayed component will be transferred directly into a new tube by pipetting and will serve as the 'pre-spray' control sample. Cell count was then analyzed with the NucleoCounter 3000. Data includes total cell number, percent viability, and cell concentration. Data were averaged and compared statistically for any significant differences among and between groups using Microsoft Excel and/or JMP statistical software ANOVA.

Potency Assay

The potency of cells sprayed with current (Lot 004) and future (Lot 005) sprayers (manufactured using a new resin) was compared using the VEGF and GM-CSF ELISA. Serum-free medium (SFM) was pre-warmed to 37°C for 15-30 minutes prior to experiment. CPT1F and CPT2C were then thawed according to clinical method as mentioned previously. This experiment must be performed in a Biosafety cabinet and all materials used must be cleaned with 70% ethanol. CPT1F was then gently inverted and 1 mL per well was added to 24 well tissue culture plates by pipette. CPT2C was sprayed into vials (using both lots of sprayers into respective tubes) and then pipetted into the culture plate on top of CPT1F in order to form a fibrin clot. After polymerization (3 minutes), serum-free medium was added to each well and

plate was placed in the incubator (37°C, 5% CO_2) for 24 hours. After 24 hours, the medium from the plates is carefully removed and reserved for the assay. The SFM is then replaced with fresh medium and then plates are again incubated for 24 hours. After second 24-hour incubation period (total 48 hours) the medium is again removed and reserved for use in the assay.

ELISA

The ELISA (enzyme-linked immuno assay) was performed using R&D Systems Quantikine ELISA kits (Human GM-CSF and Human VEGF). First the appropriate standards of growth factor must be prepared by dilution. A 96-well plate provided with kit was then used for placement of samples, standards, and blanks. The diluent was added to each well, followed by the appropriate samples, standards and blanks in order and quantity as indicated by the kit. The plate was then covered and incubated at room temperature for 2 hours.

The plate was washed 3 times with wash buffer before adding growth factor conjugate (VEGF conjugate or GM-CSF conjugate), covering, and allowing to incubate at room temperature for 2 hours if assaying VEGF, 1 hour for GM-CSF. It is very important to protect the samples from light from this point forward. After incubation plate was washed as in previous steps 3 times before adding substrate solution to each well. Plate was then incubated and covered for 20 minutes. After last incubation, stop solution was added to each well and the plate was placed in the plate reader to determine absorbance at 450 nm with a wavelength correction at 540 nm. Softmax Pro software was used to organize the data and calculate the concentrations of VEGF and GM-CSF secreted.

RESULTS

Spray Area

The area of spray was modeled using JMP statistical software fit of least squares and regression with area as the dependent variable and temperature, lot, and type of solution as independent variables. *R-squared* value for the model is below 0.5 (see Table 1) which suggests that there are more effects influencing the area parameter that are not included in this particular model.

Table 1. Summary of Fit for Area Model with effects of temperature, type of solution and lot

RSquare	0.318132
RSquare Adj	0.314555
Root Mean Square Error	4.005691
Mean of Response	20.19812
Observations (or Sum Wgts)	576

These findings are in agreement with the significant Lack of fit analysis. Cross effect model designs did not improve *R-square* value and were therefore not further investigated. Therefore, based on our available data and model, the mean is a better predictor of performance than the model. Although the model is incomplete the leverage effects are still valid and of interest. Significant leverage effects include type of solution and sprayer lot. The leverage effect of temperature is not significant on the spray area of any solution. Because of the lack of temperature effect on area the average areas have been plotted without it by sprayer lot and

solution type for reference (see Figure 3). The average area of each solution at various temperatures using Lot 004 and 005 sprayers are given in Table 2.

Table 2. Average spray area of HP-802-247 solutions at various temperatures

Type	Temperature (ºC)	Lot 004 Average Area (cm^2)	Lot 004 SD	Lot 005 Average Area (cm^2)	Lot 005 SD
CPT1F	4	25.72	3.39	20.26	3.45
	10	22.06	2.27	21.65	3.56
	15	25.64	3.31	22.56	3.03
	22	21.93	5.91	22.61	3.23
CPT2V	4	19.31	3.31	24.66	3.06
	10	17.59	2.33	24.91	4.28
	15	16.33	4.88	23.20	3.57
	22	18.13	2.49	21.05	4.71
Surrogate	4	14.69	4.22	18.94	3.30
	10	16.08	3.11	19.27	2.96
	15	13.71	3.94	17.47	2.05
	22	16.12	2.48	17.99	2.27

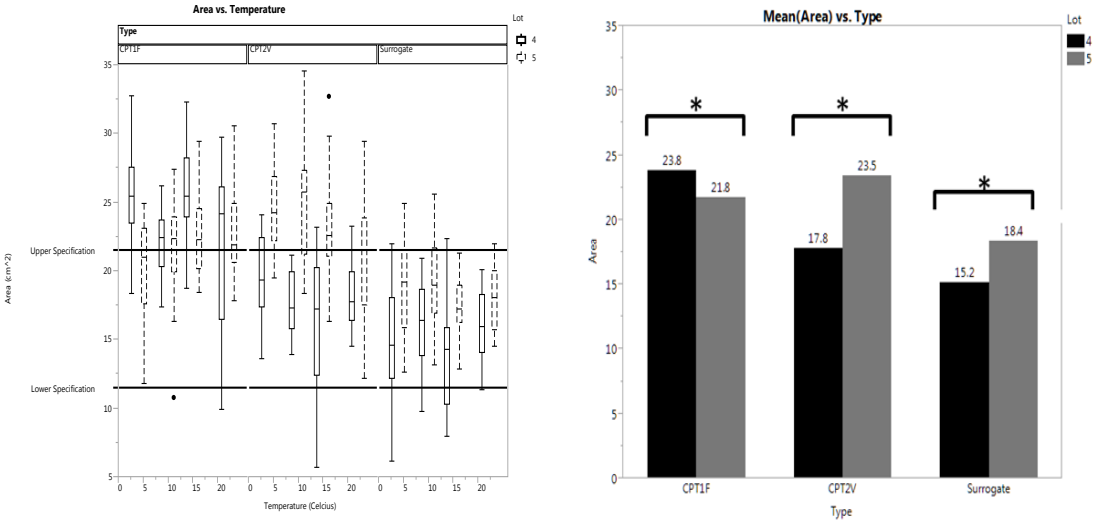

Figure 3. Box plot analysis for variation in spray area using two lots of Aptar VP7 sprayers at various product temperatures (left); Comparison of Aptar VP-7 spray areas based on type of solution and lot of sprayer (right); *(p-value < 0.05 significant)*

Table 3. Means and standard deviations for Lot 004 and Lot 005 by solution type

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
CPT2V						
4	112	23.8372	4.31527	0.40775	23.029	24.645
5	112	21.7701	3.41373	0.32257	21.131	22.409
CPT2V						
4	80	17.8407	3.50872	0.39229	17.06	18.622
5	80	23.456	4.17567	0.46685	22.527	24.385
Surrogate						
4	96	15.1505	3.59488	0.3669	14.422	15.879
5	96	18.4157	2.74776	0.28044	17.859	18.972

Table 4. Ordered differences report for Lot 004 and Lot 005 by solution type using Tukey-Kramer HSD comparison

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
CPT1F						
4	*5*	*2.067134*	*0.519917*	*1.04253*	*3.091738*	*<0.0001*
CPT2V						
5	*4*	*5.61535*	*0.6097889*	*4.410961*	*6.819739*	*<0.0001*
Surrogate						
5	*4*	*3.265229*	*0.461805*	*2.354306*	*4.176152*	*<0.0001*

(Italics indicate significance using Tukey-Kramer HSD, p-value < 0.05)

Table 5. Sorted parameter estimates for effects of spray parameters on spray area

Term	Estimate	Std Error	t Ratio	Prob>|t|
Type[CPT1F]	*2.725275*	*0.228096*	*11.95*	*<0.0001*
Lot[4]	*-0.922172*	*0.166525*	*-5.54*	*<0.0001*
Type[CPT2V]	*0.5699812*	*0.248064*	*2.3*	*0.0219*
Temperature	-0.047826	0.025194	-1.9	0.0582

(Italics indicate significance using Fit of least squares parameter estimates, p-value <0.05)

Table 6. Ordered differences report for significance between solutions within lots

	Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Lot 004	*CPT1F*	*Surrogate*	*8.686713*	*0.5381952*	*7.418674*	*9.954752*	*<0.0001*
	CPT1F	*CPT2V*	*5.996536*	*0.5664341*	*4.661963*	*7.331108*	*<0.0001*
	CPT2V	*Surrogate*	*2.690177*	*0.5857718*	*1.310043*	*4.070311*	*<0.0001*
Lot 005	*CPT2V*	*Surrogate*	*5.040298*	*0.5219668*	*3.810494*	*6.270101*	*<0.0001*
	CPT1F	*Surrogate*	*3.35435*	*0.4795724*	*2.224431*	*4.484268*	*<0.0001*
	CPT2V	CPT1F	1.685948	0.5047354	0.496743	2.875153	0.0027

(Italics indicate significance using Tukey-Kramer HSD, p-value <0.05)

As shown in Figure 3 when comparing average areas of solutions by lot all means are significantly different. The solutions spray results did not appear to follow any comparable trends when comparing both sprayer lots (see Table 6 for differences in solutions). Although significantly different, all averages are within the specifications set by the Smith & Nephew (16.5 ± 5 cm^2).

Average area of spray and the relationship with the lower and upper specification for area are described by Box plot (see Figure 3). All solution area means are all above lower specifications and 75% of individual sprays are within specifications. CPT1F and CPT2V spray area means at all temperatures are well above lower specification. Surrogate solutions at all temperatures performed as described and predicted based on sprayer specifications with averages within both the lower and upper specifications. It should be noted that the Lot 004 sprayer data were non-normally distributed but closely fit the extreme value distribution as a result of the left-skewed tails (see Figure 3). Lot 005 spray areas were all normally distributed (p-value >0.05).

Ovality

Ovality (D_{max}/D_{min}) is another indicator of spray performance but may be more linked to sprayer parameters such as nozzle orifice, diameter, shape, etc. than formulation (Trows et al. 2014, 195-219). All means for Lot 004 and Lot 005 were below the specification (< 1.3) in all types of solution (see Table 7). Higher ovality is indicative of more oval or irregular shaped spray plumes. No relationship to temperature was observed upon fit of least squares regression (see Table 8) and regression line was a poor fit. Table 9 shows the effects on ovality as described by regression model with Lot 004 being the only significant effector on ovality. Average ovality for all solutions was < 1.3. Ovality was then grouped without using temperature as a variable to spot any further trends like formulation based or lot differences (see Figure 5 and Tables 10, 11, and 12). Significant differences were found in Lot 004 versus Lot 005 with CPT1F and with Surrogate solutions. Within lot groups, CPT1F and Surrogate solutions had significant differences but only in Lot 004 group.

Table 7. Average spray ovality of HP-802-247 solutions at various temperatures

Type	**Temperature (ºC)**	**Lot 004 Ovality**	**Lot 004 SD**	**Lot 005 Ovality**	**Lot 005 SD**
CPT1F	4	1.18	0.08	1.25	0.12
	10	1.24	0.10	1.24	0.08
	15	1.22	0.10	1.22	0.07
	22	1.21	0.07	1.24	0.13
CPT2V	4	1.19	0.08	1.21	0.11
	10	1.20	0.08	1.26	0.11
	15	1.20	0.07	1.28	0.15
	22	1.20	0.09	1.24	0.10
Surrogate	4	1.14	0.04	1.18	0.07
	10	1.14	0.05	1.26	0.17
	15	1.25	0.19	1.24	0.14
	22	1.20	0.08	1.17	0.08

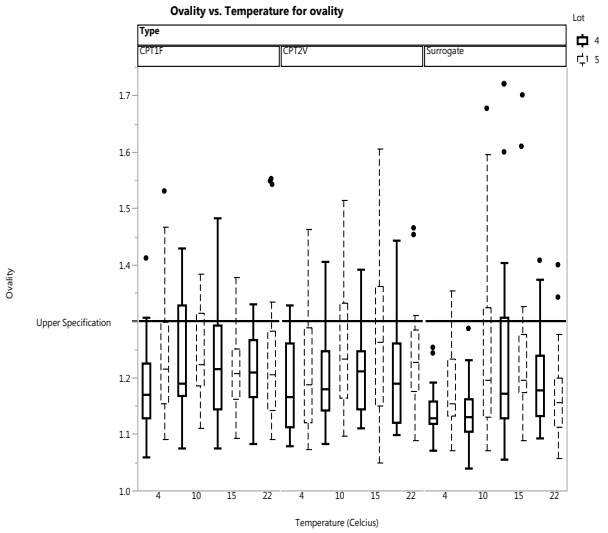

Figure 5. Box plot of solution temperature versus spray pattern ovality for Lot 004 and Lot 005 Aptar VP-7 sprayers

Table 8. Summary of Fit for ovality model with effects of temperature, type of solution and lot

RSquare	0.042138
RSquare Adj	0.035428
Root Mean Square Error	0.106349
Mean of Response	1.214696
Observations (or Sum Wgts)	576

Table 9. Sorted parameter estimates predicted by least squares fit analysis of ovality including all variables

Term	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
Lot[4]	*-0.01686*	*0.004431*	*-3.8*	*0.0002*
Type[CPT1F]	0.0105832	0.00607	1.74	0.0818
Temperature	0.0010098	0.00067	1.51	0.1326
Type[CPT2V]	0.007269	0.006601	1.1	0.2713

(Italics indicate significance, Fit Least Squares parameter estimates, p-value <0.05)

Table 10. Mean and standard deviations for solution ovality sprayed with Lot 004 and Lot 005 sprayers

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
CPT1F						
4	112	1.21307	0.088702	0.00838	1.1965	1.2297
5	112	1.23712	0.101603	0.0096	1.2181	1.2561
CPT2V						
4	80	1.1978	0.078599	0.00879	1.1803	1.2153
5	80	1.24577	0.118989	0.0133	1.2193	1.2722
Surrogate						
4	96	1.18009	0.115687	0.01181	1.1567	1.2035
5	96	1.21323	0.128684	0.01313	1.1872	1.2393

Table 11. Ordered differences report comparing ovality variation within solution groups separated by Lot

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
CPT1F						
5	4	0.0240445	0.0127445	-0.001071	0.0491601	0.0605
CPT2V						
5	*4*	*0.0479699*	*0.0159438*	*0.0164795*	*0.0794603*	*0.0031*
Surrogate						
5	4	0.0331323	0.0176609	-0.001704	0.0679689	0.0622

(Italics indicate significance, Tukey-Kramer HSD, $p<0.05$)

Table 12. Ordered differences report comparing ovality variation between solution groups separated by Lot

Lot 004						
Level	**- Level**	**Difference**	**Std Err Dif**	**Lower CL**	**Upper CL**	**p-Value**
CPT1F	*Surrogate*	*0.0329792*	*0.0133683*	*0.001482*	*0.0644763*	*0.0377*
CPT2V	Surrogate	0.0177024	0.0145501	-0.016579	0.0519838	0.4443
CPT1F	CPT2V	0.0152769	0.0140698	-0.017873	0.0484266	0.5237

Lot 005						
Level	**- Level**	**Difference**	**Std Err Dif**	**Lower CL**	**Upper CL**	**p-Value**
CPT2V	Surrogate	0.03254	0.0175663	-0.008848	0.0739278	0.1547
CPT1F	Surrogate	0.0238914	0.0161395	-0.014135	0.0619176	0.3019
CPT2V	CPT1F	0.0086486	0.0169864	-0.031373	0.0486701	0.867

(Italics indicate significance, Tukey-Kramer HSD, p<0.05)

Weight (dosage)

Average dosage was calculated recording the weight of 4 sprays after priming for all sprayers based on established protocols. Pump was considered primed after recording visible spray of at least 60 mg. The average dosage was modeled using JMP statistical software Fit of least squares model with lot, temperature and type of solution as effects. The low *r-squared* value of the linear regression model indicates that the data do not have a linear relationship based on temperature (see Table 14). Lack of fit significance (p-value <0.05) supports this theory. The leverage effects of lot and type were significant; although the ranking of parameter estimates shows that the effect of CPT1F type has the greatest influence on dosage (see Table 15). Temperature had an insignificant effect on the dosage of solution delivered.

Specifications for dosage are 117-143 mg per spray. The box plots of dosage at various temperatures feature many outliers (See Figure 6), the majority of which were on the lower side

of specification for Lot 004 sprayers. These outliers contribute to the left skewness and non-normal distribution of dosage. Lot 005 consistently outperforms Lot 004 and the means of CPT2V and surrogate are within specifications. CPT1F mean dosage is consistently below specification for both lots. Surrogate spray mean is above specification for Lot 004. In testing mean dosage of CPT2V, Lot 004 sprayers were slightly below specifications (see Table 13).

When comparing solution dosage independent of temperature of product, Lot 005 performed more closely to specifications than Lot 004 (Figure 6 and Table 19). When comparing each solution lot to lot, average dosages are significantly different (Student's t-test, $p < 0.05$, see Figure 6). The average dosages of Surrogate solution versus the CPT2V solution also proved significantly different (Tukey-Kramer HSD, $p < 0.05$). Although the differences in CPT2V and surrogate solution are statistically significant, the estimated differences from CPT1F were greater for both solutions (see Table 18).

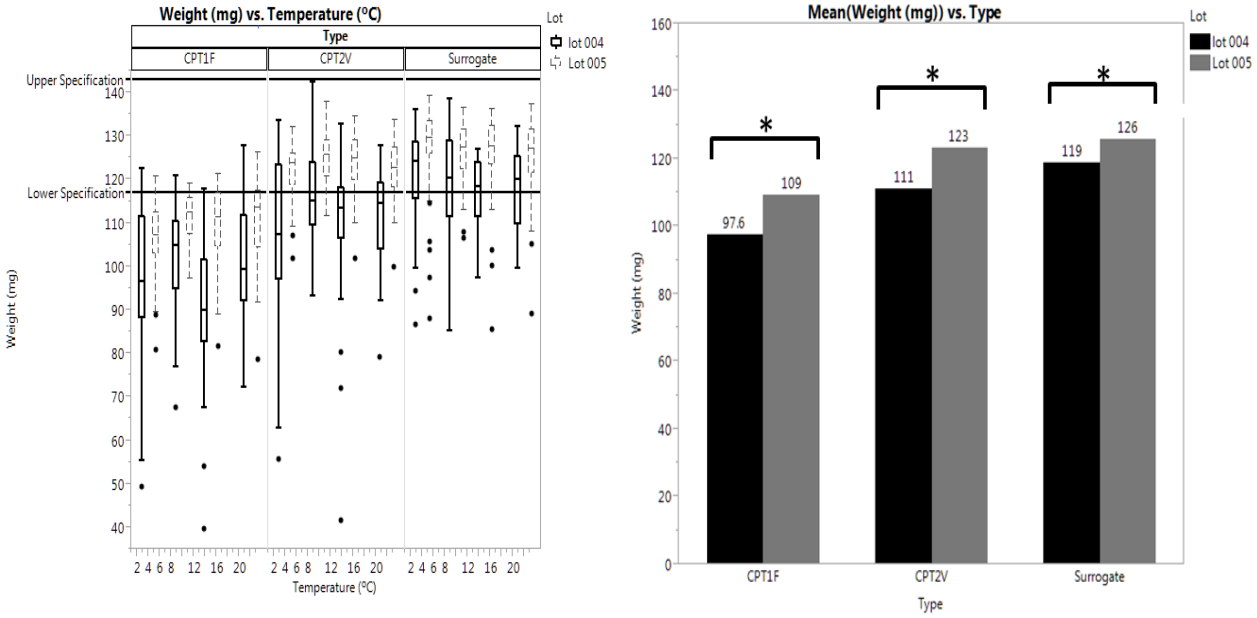

Figure 6. Box plot of dosage per spray of solutions at various temperatures using two lots of Aptar VP-7 sprayers (left) Aptar VP-7 spray dosage based on type of solution and lot of sprayer, significance indicated by asterisk (right, $p<0.05$)

Table 13. Average spray dosage of CPT1F, CPT2V, and Surrogate solution at various temperatures

Type	**Temperature (ºC)**	**Lot 004 Average weight (mg)**	**Lot 004 SD**	**Lot 005 Average weight (mg)**	**Lot 005 SD**
CPT1F	4	*96.17*	17.78	*106.17*	8.29
CPT1F	10	*102.56*	11.93	*111.36*	5.65
CPT1F	15	*90.20*	15.72	*109.61*	8.97
CPT1F	22	*101.64*	13.72	*110.80*	9.26
CPT2V	4	*107.72*	18.07	122.06	6.46
CPT2V	10	*116.52*	9.83	124.58	6.36
CPT2V	15	*109.60*	15.93	124.00	6.76
CPT2V	22	*111.36*	10.89	122.19	6.64
Surrogate	4	121.41	11.97	126.87	11.30
Surrogate	10	119.08	12.14	126.05	7.18
Surrogate	15	117.31	8.31	125.63	10.44
Surrogate	22	117.65	9.83	125.24	9.14

(Italics indicate results below specifications)

Table 14. Actual by predicted summary of fit for effects of temperature, type and lot on dosage of product delivered

RSquare	0.421287
RSquare Adj	0.417643
Root Mean Square Error	11.27942
Mean of Response	114.4063
Observations (or Sum Wgts)	960

Table 15. Sorted parameter estimates for effects on spray dosage

Term	Estimate	Std Error	t Ratio	Prob>|t|
Type[CPT1F]	*-12.23*	*1.200966*	*-10.18*	*<0.0001*
Lot[lot 004]	*-4.966667*	*0.849211*	*-5.85*	*<0.0001*
Type[CPT2V]	1.49125	1.200966	1.24	0.2156
Temp	-0.067182	0.12848	-0.52	0.6015

(Italics indicate significance, fit least squares parameters, p<0.05)

Table 16. Means and standard deviations for dosages of solutions by Lot

Lot 004						
Level	**Number**	**Mean**	**Std Dev**	**Std Err Mean**	**Lower 95%**	**Upper 95%**
CPT1F	*160*	*97.641*	*15.6146*	*1.2344*	*95.2*	*100.08*
CPT2V	*160*	*111.298*	*14.3487*	*1.1344*	*109.06*	*113.54*
Surrogate	160	118.861	10.703	0.8461	117.19	120.53
Lot 005						
CPT1F	*160*	*109.484*	*8.33901*	*0.65926*	*108.18*	*110.79*
CPT2V	160	123.208	6.58956	0.52095	122.18	124.24
Surrogate	160	125.946	9.56899	0.7565	124.45	127.44

(Italics indicate performance below specifications)

Table 17. Ordered differences report between solution types (Lot 005 sprayers)

Level	**- Level**	**Difference**	**Std Err Dif**	**Lower CL**	**Upper CL**	**p-Value**
Surrogate	*CPT1F*	*16.46187*	*0.9231443*	*14.29152*	*18.63223*	*<0.0001*
CPT2V	*CPT1F*	*13.72438*	*0.9231443*	*11.55402*	*15.89473*	*<0.0001*
Surrogate	CPT2V	2.7375	0.9231443	0.56714	4.90786	0.0089

(Italics indicate significance, Tukey-Kramer HSD, p<0.05)

Table 18. Ordered differences report between solution types (Lot 004 sprayers)

Level	**- Level**	**Difference**	**Std Err Dif**	**Lower CL**	**Upper CL**	**p-Value**
Surrogate	*CPT1F*	*21.22062*	*1.533317*	*17.61572*	*24.82553*	*<0.0001*
CPT2V	*CPT1F*	*13.6575*	*1.533317*	*10.05259*	*17.26241*	*<0.0001*
Surrogate	*CPT2V*	*7.56312*	*1.533317*	*3.95822*	*11.16803*	*<0.0001*

(Italics indicate significance, Tukey-Kramer HSD, p<0.05)

Table 19. Ordered differences report for variance of solution dosage between lots

Level	**- Level**	**Difference**	**Std Err Dif**	**Lower CL**	**Upper CL**	**p-Value**
CPT1F						
Lot 005	***Lot 004***	*11.84312*	*1.399451*	*9.089772*	*14.59648*	*<0.0001*
CPT2V						
Lot 005	***Lot 004***	*11.91*	*1.248267*	*9.454093*	*14.36591*	*<0.0001*
Surrogate						
Lot 005	***Lot 004***	*7.084375*	*1.135012*	*4.851294*	*9.317456*	*<0.0001*

(Italics indicate significance, Tukey-Kramer HSD, p<0.05)

Priming

While doing some experiments, actuations (also known as pumps to prime) were recorded. The clinical information suggests pumping the sprayer 4 times until primed before application of the drug product onto the wound. Actuations to prime were determined by this examiner to be described in the weight analysis as pumps needed to visible spray product with a minimum weight of 60 mg. The model fit does not indicate any linear or significant relationship with temperature (Table 21). Type of solution and lot were the most significant effects on pumps to prime (Table 22). Although there was a significant effect, that effect was minute and all averages were very close in range (see Tables 20, 22)

The analysis of this relationship does not indicate that priming pumps are affected by any of the tested product temperatures (Table 22). Within sprayer lots, Surrogate and CPT2V average actuations to prime were not significantly different but CPT1F average actuation to prime was significantly different than both Surrogate and CPT2V (see Table 25). Testing each component between lots using the Student's t-test indicates significant differences between the lots for all solutions, and Lot 005 has consistently lower average priming number. The figures of pumps to prime also show this relationship visually (see Figure 7).

Table 20. Average actuations to prime for CPT1F, CPT2V, and Surrogate solutions at various temperatures using two lots of Aptar VP-7 sprayers

Type	Temperature (ºC)	N	Lot 004 Average Actuations to prime	Lot 005 Average Actuations to prime	Lot 004 SD	Lot 005 SD
CPT1F	4	10	5.8	5.1	0.4	0.6
CPT1F	10	10	5.2	5.0	0.4	0.0
CPT1F	15	10	6.3	5.3	0.9	0.5
CPT1F	22	10	5.8	4.8	0.6	0.8
CPT2V	4	10	5.3	4.3	0.5	0.5
CPT2V	10	10	5.1	4.4	0.7	0.5
CPT2V	15	10	5.7	4.4	0.5	0.5
CPT2V	22	10	5.2	4.5	0.4	0.7
Surrogate	4	10	5.4	4.6	0.7	0.5
Surrogate	10	10	5.2	4.6	0.4	0.8
Surrogate	15	10	5.1	4.7	0.3	0.7
Surrogate	22	10	5.4	4.8	0.5	1.0

Table 21. Actual by predicted summary of fit for effects of temperature, type and lot on dosage of product delivered

RSquare	0.34335
RSquare Adj	0.332173
Root Mean Square Error	0.621722
Mean of Response	5.083333
Observations (or Sum Wgts)	240

Table 22. Parameter estimates for response of pumps to prime by lot, type and temperature

Term	Estimate	Std Error	t Ratio	Prob>|t|
Lot[Lot 004]	*0.375*	*0.040132*	*9.34*	*<0.0001*
Type[CPT1F]	*0.3291667*	*0.056755*	*5.8*	*<0.0001*
Type[CPT2V]	*-0.220833*	*0.056755*	*-3.89*	*0.0001*
Temp	0.0047687	0.006072	0.79	0.433

(Italics indicate significance; Fit least squares parameter estimates, p<0.05)

Table 23. Means and standard deviations of pumps to prime for each solution type and lot

Means and standard deviations

	Level	Number	Mean	Std Dev
CPT1F	Lot 004	40	5.775	0.733362
	Lot 005	40	5.05	0.552384
CPT2V	Lot 004	40	5.325	0.572332
	Lot 005	40	4.4	0.545377
Surrogate	Lot 004	40	5.275	0.505736
	Lot 005	40	4.675	0.764182

Table 24. Ordered differences summary for variance of solution actuations to prime between lots

	Level	- Level	Difference	p-Value
CPT1F	Lot 004	Lot 005	0.725	<0.0001
CPT2V	Lot 004	Lot 005	0.925	<0.0001
Surrogate	Lot 004	Lot 005	0.6	<0.0001

(Tukey-Kramer HSD ordered differences, p<0.05 indicates significance)

Table 25. Ordered differences summary for variance of solution actuations to prime within lots

	Level	- Level	Difference	p-Value
Lot 004	*CPT1F*	*Surrogate*	*0.5*	*0.0011*
	CPT1F	*CPT2V*	*0.45*	*0.0037*
	CPT2V	Surrogate	0.05	0.929
Lot 005	*CPT1F*	*CPT2V*	*0.65*	*<0.0001*
	CPT1F	*Surrogate*	*0.375*	*0.0236*
	Surrogate	CPT2V	0.275	0.128

(Italics indicate significance p<0.05, Tukey-Kramer ordered differences,)

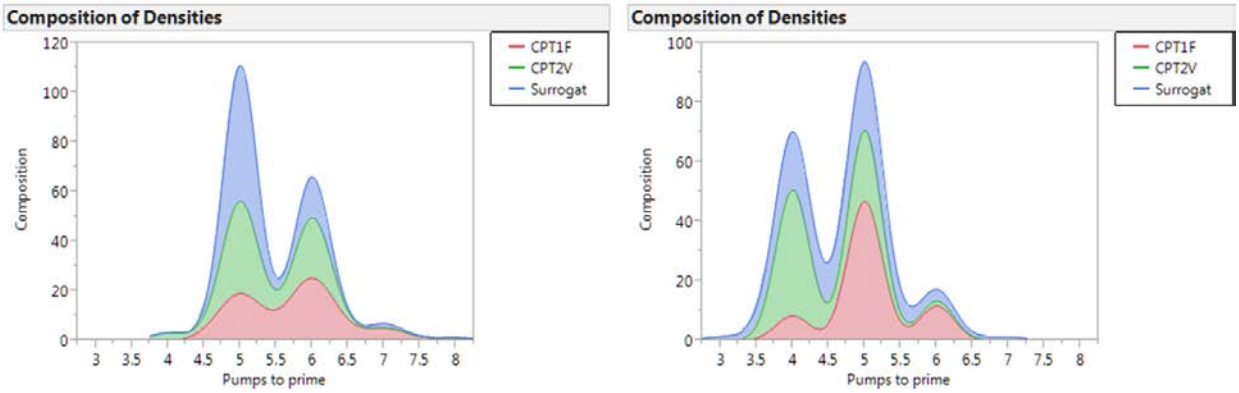

Figure 7. Composition of densities for pumps needed to prime Aptar VP7 sprayers (Lot 004 [left] and Lot 005 [right])

Table 26. CPT1F and CPT2V average time to polymerize at various temperatures

Temperature (°C)	**Average Polymerization Time (seconds)**	**SD**
4	29.31	8.80
10	28.02	8.69
15	25.46	5.38
20	29.81	4.01
37	27.09	4.96
Difference of means	None	

(ANOVA, $p > 0.05$ significance)

Polymerization

Table 26 shows results of polymerization tests at various temperatures with no significant difference (Tukey-HSD test results, $p>0.05$). Table 27 shows the control experiment for the previously untested (for polymerization times) lot of R & D grade CPT1F (01MAY14). Although the polymerization times were not significantly different from one another, they were higher than previous experiments suggest they should be. To control this outcome, a previously tested CPT1F (lot 337), Fibrinogen control, and Thrombin control were tested (see Table 27). The average polymerization times using these controls along with the same CPT2V used in the

previous experiment showed a much faster polymerization time than the polymerizations seen when using CPT1F (lot 01MAY14). The results are more consistent with previous experiments.

Table 27. Summarized ANOVA data for polymerization time of Fibrinogen Control with CPT2C Lot 174, 337, and Thrombin Control

Dilution		**Temperature (°C)**	**Lot**	**Average polymerization time (seconds)**	**SD**
CPT1F (fibrinogen control)	**CPT2C (vehicle only)**				
undiluted	1:20	20	174	12.3	0.1
			174		
			Thrombin (Control)	13.2	0.7
			Thrombin (Control)		
			337 (control lot)	11.9	0.1
			337 (control lot)		
Overall				**12.48**	**0.6**
Difference of means				**None**	

(ANOVA, $p < 0.05$)

Viability and Potency

The viability of sprayed cells (as described using number of viable cells) was not significantly different when comparing Lots 004 and 005 see Table 28). VEG-F production at 48 hours post spray was significantly different for Lot 004 and Lot 005 (see Table 29). GM-CSF production at 48 hours post-spray was not significantly different between lots (see Table 30). All groups were tested using Student's t-test with significance at $p < 0.05$.

Table 28. Means, standard deviations, and Student's t-test comparing viability of HP-802-247 cells post-spray using Lot 004 and Lot 005 sprayers

Level	Number	Mean (E5)	Std Dev
4	3	6.78041	0.486605
5	3	6.22779	0.530675

t Test	
Assuming equal variances	
Difference	-0.5526
Std Err Dif	0.4157
Upper CL Dif	0.6015
Lower CL Dif	-1.7068
Confidence	0.95
t Ratio	-1.3294
DF	4
Prob > |t|	0.2545
Prob > t	0.8728
Prob < t	0.1272

(Significance at $p < 0.05$)

Table 29. Means, standard deviations, and Student's t-test comparing VEGF production at 48 hours of HP-802-247 cells post-spray using Lot 004 and Lot 005 sprayers

Level	Number	Mean (pg/mL)	Std Dev
4	12	96.6581	10.0582
5	12	70.4409	5.4811

t Test	
Assuming equal variances	
Difference	-26.217
Std Err Dif	3.307
Upper CL Dif	-19.36
Lower CL Dif	-33.075
Confidence	0.95
t Ratio	-7.92854
DF	22
Prob > |t|	<.0001
Prob > t	1
Prob < t	<.0001

(Significance at $p < 0.05$)

Table 30. Means, standard deviations, and Student's t-test comparing GM-CSF production at 48 hours of HP-802-247 cells post-spray using Lot 004 and Lot 005 sprayers

Means and Std Deviations

Level	Number	Mean (pg/mL)	Std Dev
4	12	63.8728	22.2571
5	12	59.0181	27.8076

t Test

Assuming equal variances	
Difference	-4.855
Std Err Dif	10.282
Upper CL Dif	16.469
Lower CL Dif	-26.178
Confidence	0.95
t Ratio	-0.47215
DF	22
Prob > |t|	0.6415
Prob > t	0.6793
Prob < t	0.3207

(Significance $p < 0.05$)

DISCUSSION

The influence of product temperature on spray device performance and polymerization time of HP-802-247 components was tested at four different temperatures to mimic the range of previously determined thawing outcomes with new Thawing device as seen in Figure 8. Sprayer performance must be reliable and reproducible to ensure proper treatment of patients. Dosage has been tested to various ranges in clinical trials and is extremely important.

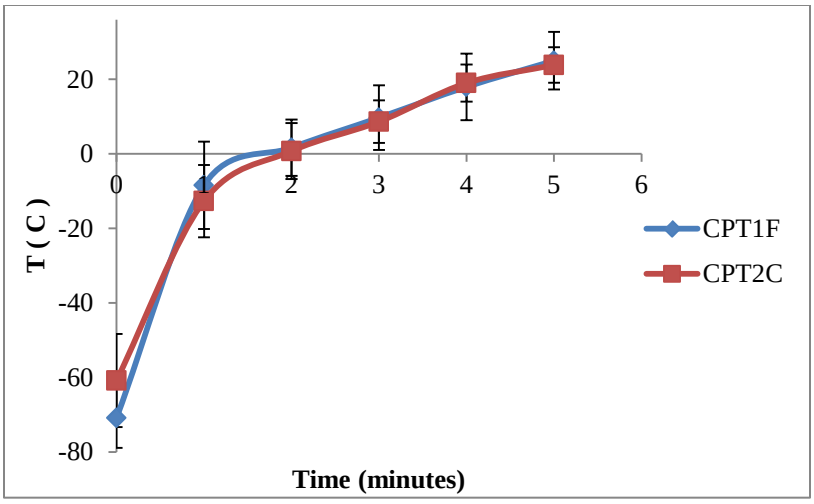

Figure 8. The thawing curves for HP802-247 components in the current clinical setting (previous experiment by Aleksa Jovanovic, Ph.D. and Jason Campbell, M.S., Smith & Nephew Biotherapeutics)

Figure 9. Visible inspection of sprayer heads from different lots (Photograph by Jason Campbell, M.S.)

Area

The main purpose of these experiments was to compare sprayer parameters and the effect of product temperature variation at 4-22ºC (Specific Aim 1). The results of these spray area experiments suggest that temperatures from 4-22ºC do not significantly affect the spray area of HP-802-247 components. The statistical analysis of the spray area data indicates that the type of solution and the lot and are both significant contributors to the differences of the mean, with lot being the dominant effect over type.

The low *R-square* value for the model including temperature as a variable suggests that product temperature does not have a linear relationship to spray area (Specific Aim 1). A non-linear model of the relationship of product temperature and spray area did not significantly improve the *R-square* value. Taking this into consideration it can be concluded that the variability of spray area is not significantly affected by product temperature.

Another purpose of this experiment was to determine if sprayer performance was comparable between different lots (Specific Aim 2). The spray area averages for CPT1F, CPT2V, and Surrogate solution, although statistically different from one another were all above the specifications set by Smith & Nephew (16.5 ± 5.5 cm^2). In both lots, surrogate solution had the lowest area. This could be explained by differences in viscosity, which are known to affect droplet size and spray angle which contribute to spray area and ovality. There are between lot differences but all data were within ideal range so further investigation of this parameter is unnecessary.

Viscosity data for the surrogate solution is unavailable, its intended purpose was to model the physical qualities of CPT2C, the active pharmaceutical component of HP-802-247, which it

does well in all areas tested (see Table 31). CPT2V viscosity was measured by an outside source to be greater than water at an average of 2.1025 cPs (at 31ºC). The difference in viscosity is small in magnitude, previous studies by Dayal *et al.*, which used sodium carboxymethyl cellulose (NaCMC) to increase viscosity of nasal spray solutions, determined that more viscous nasal spray solutions had decreased plume angles and spray area (Dayal et al. 2005, E573-85, Dayal, Shaik and Singh 2004, 1725-1742). This spray behavior is hypothesized to be a result of normal Newtonian fluid behavior (no-shear thinning properties) in which more viscous solutions exhibit an increased droplet size upon spraying.

Not all viscous fluids can be expected to exhibit Newtonian fluid dynamics. In the Dayal study, some viscous fluids, such as Carbopol have shear-thinning behaviors meaning that with increased pressure, the viscosity is temporarily decreased and fine spray can be achieved while enabling the spray to become more viscous upon contact with the nasal cavity and stay in place (Dayal, Shaik and Singh 2004, 1725-1742, Pennington et al. 2008, 923-929). This quality can be useful in pharmaceutical products in which dripping is an issue. HP-802-247 components are designed to quickly form a fibrin clot, so shear-thinning attributes are not important.

Table 31. Summary spray parameter averages and their relationship to specifications

Type	**Area**		**Ovality**		**Dosage**	
	Lot 004	Lot 005	Lot 004	Lot 005	Lot 004	Lot 005
CPT1F	**(+)**	**(+)**	**(=)**	**(=)**	**(-)**	**(-)**
CPT2V	**(=)**	**(+)**	**(=)**	**(=)**	**(-)**	**(+)**
Surrogate	**(=)**	**(=)**	**(=)**	**(=)**	**(+)**	**(+)**
Lot vs. Lot	significantly different for all types		significantly different for CPT2V		significantly different for all types	

Note: (+) exceeds specification, (-) below specification, (=) within specification

Ovality

Ovality of 1.00, exhibited by a perfect circle, is considered ideal for pharmaceutical sprayers. For HP-802-247, the coverage of the wound is an important factor for healing and dosage purposes. In previous trials wound sizes of no greater than 10 cm in diameter were treated. For proper treatment, it must be ensured that the wound is completely covered with drug product for optimum results. So although a spray has an area significantly larger than the wound, if it has flawed ovality and/or asymmetry, the coverage of the wound may be unpredictable and incomplete.

As detailed in the results section ovality does not appear to be affected by any of the tested parameters such as temperature, solution type, or lot of sprayer (Specific Aims 1 and 2). Average ovality was within specifications < 1.3 for all groups in all conditions. It is more likely that other device specific parameters affect ovality but they have not been tested in this study. Data were not normally distributed and when transformed using Log_{10}, the data fit the lognormal distribution well ($p > 0.05$). Log distributions are common in biological and physical processes with data that are non-negative or data that are a result of multiplicative processes of many independent random variables (Feng et al. 2014, 105-109, Limpert, Stahel and Abbt 2001, 341). This fit is not surprising because ovality calculations are always greater than one (maximum diameter/minimum diameter always ≥ 1). But it should be noted that statistical tests like regression and analysis of variance (ANOVA) are robust for non-normal data (Curran-Everett 0406).

Dosage

Dosage testing is integral to proper drug delivery from both, patient optimal therapy and safety standpoints. As with spray area parameters a least fit squares model was created using JMP statistical analysis software. The *R-squared* value for the spray area model (0.4020) is low which suggests that the parameters included in the model (temperature, lot, and solution) do not predict spray dosage in a reliable linear manner (Specific Aims 1 and 2). Robustness of model was tested by crossing effects to provide a possible non-linear model, but *R-squared* was not significantly improved (note that this did cause Lack of Fit to became insignificant). Lack of linear relationship implies that spray dosage is not significantly affected by temperature of solution. Fit of least squares on temperature of solution and dosage was not shown to be significant, confirming the regression analysis interpretation. Fit of least squares analysis does indicate that the type of solution and lot of sprayers did have statistically significant effects on the dosage delivered by the sprayers but the effects are relatively small (< 8.5%). Use of CPT1F had the strongest effect on dosage and resulted in dosage averages below specification (see discussion below). Use of Lot 004 sprayers were shown to negatively affect dosage to a lesser degree and CPT2V had the lowest significant effect on dosage. Lot 004 sprayers were older than Lot 005 and this may possibly have contributed to their lack of consistency in performance.

As shown on the box plot in Figure 6 there are a large number of outliers, with the majority of extremely low values linked to Lot 004 sprayers. Non-normality can be expected for non-negative data distributions such as weight. The upper boundary of dosage is constrained by the length of sprayer tubing in the displacement pump. Typically these Weibull or Extreme Value type distributions are seen in length of life, fragmentation, and product failure rate studies

(Brown and Wohletz 1995, 2758, Freund 1961, 971-977). What is intriguing about these findings is that CPT1F, while having the largest average area, has the lowest average dosage.

CPT1F has an average viscosity of 0.98 cPs at 50-100 RPM, which is less than that of CPT2V (2.23 cPs at 50 RPM, 1.98 cPs at 100 RPM, at 31ºC). In previous studies testing spray pump parameters for use in pharmaceutical nasal spray, that dosage is mainly affected by device parameters, not formulation parameters such as viscosity and surface tension (Trows et al. 2014, 195-219). But another previous study by Guo *et al.* found that significant differences in dosage did occur with varying surface tension and viscosity but they were only 'slight differences' (Guo et al. 2008, 417-426)

Viscosity, density and surface tension (as exhibited by surfactant/emulsifier content) could be contributing factors to these formulation-based differences. Clearly, the argument could be made that some formulation characteristics may be affecting the performance of the sprayer with regards to dosage. Protein content, known to decrease surface tension because of its hydrophilic and hydrophobic portions, is greater in CPT2V than CPT1F. Therefore, the higher surface tension in CPT1F could affect the rate of flow into the sprayer tubing, explaining the greater number of pumps required to prime. The difference between types is a convincing argument for formulation-based factors effecting spray output.

If necessary, the increase in dosage of CPT1F without changing the formulation could be achieved using the device with a longer stroke length or larger metering chamber. The most important dosage is that of CPT2C (modeled using CPT2V in this study) because it contains the cellular product. The average dosage for CPT2V was well within range of 130 ± 13 mg using the Lot 005 sprayers. It is beyond the scope of this investigation to determine what ratio of

CPT1F to CPT2C is necessary for optimum performance of HP802-247. Future studies could attempt to elucidate this relationship. It should be noted that the Clinical Phase 2 trials used Aptar sprayers that were composed of the same resin as Lot 004 and achieved statistically significant clinical outcomes for wound healing. Theoretically, the area and distribution of CPT1F coverage could be more important for treatment than the weight or dosage but this theory is so far untested.

Priming

There are no product specifications to be met for priming. Priming pumps were included in this study because they were hypothesized to be a predictor of spray performance. Pumps to prime were not linked to any other performance parameter tested. The experiments also did not provide any evidence for a temperature range of 4-22ºC affecting the number of actuations needed to prime. Although there were statistically significant differences for types of solutions and lots, the differences in average number of primes were all <1, which for the purpose of HP-802-247 is not large enough to warrant further investigation.

Polymerization at various temperatures

The polymerization time results showed that the kinetics of the reaction is relatively unaffected in the tested (4-20°C) range, and the polymerization times are similar to reaction time at 37°C (target temperature). The simple mixing experiment of the two HP-802-247 components at 4°C and 20°C on 37°C surface (Figure 10) further confirmed aforementioned conclusions on relative temperature independence of polymerization kinetics on temperature. The only difference observed between 4°C and 20°C temperatures is that the components kept at 20°C polymerized within five seconds, while it took ten seconds for polymerization of components

kept at 4°C but this difference was not statistically significant using ANOVA test (p-value > 0.05).

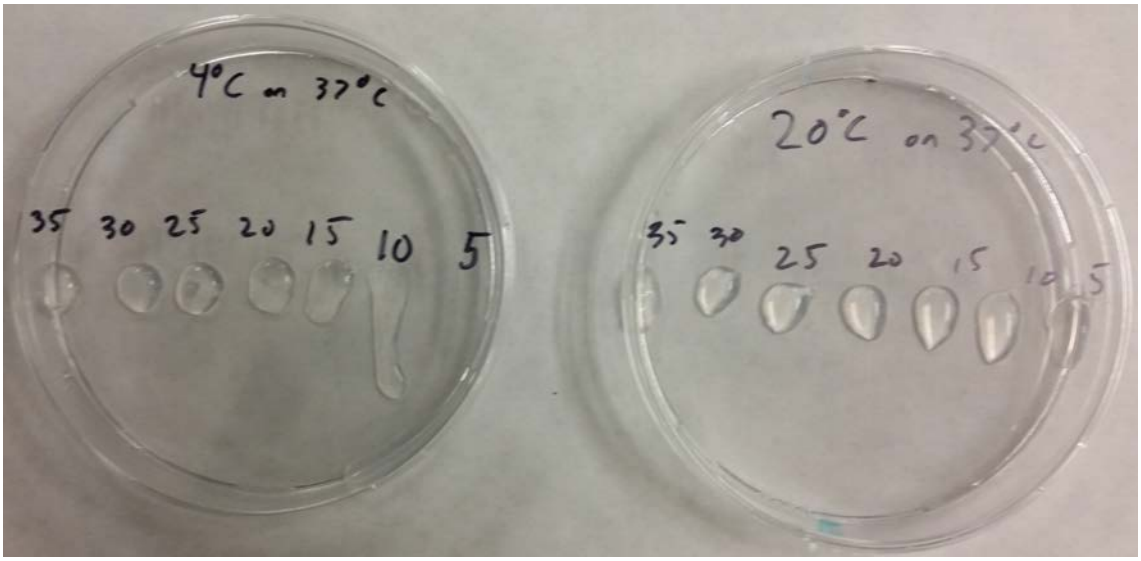

Figure 10. Simple polymerization assay (Experiment and photograph by Jason Campbell, M.S., Smith & Nephew Biotherapeutics)

In the polymerization (clotting) assays, no significant difference in clotting time was observed at any temperature, including 37°C. In an experiment controlling for differences in CPT1F, it was shown that the CPT2V used in previous experiments was polymerizing at the usual rate (~15 seconds) and suggests that CPT1F.01MAY14 may have been the underperforming component that contributed to slower polymerization times (~30 seconds) in the previously described polymerization assay. This may indicate that the longer polymerization times may have been caused by difference, variation, or expiration of the R & D CPT1F material (lot 01MAY14). It is suggested that more research be done to explain the variability and/or stability of fibrinogen components used. Reconstituted fibrinogen has a shelf life of 2 years at 2-

8º C (as determined by the manufacturer stability studies). It is hypothesized that frequent freeze-thaw cycles also be studied in stability experiments.

Viability and Potency

The means for number of viable HP-802-247 cells post-spray did not differ significantly between Lot 004 and Lot 005. Therefore the sprayers can be considered equivalent in this regard (Specific Aim 2). The VEGF detection at 48 hours was significantly different. Fibroblasts are "mechanoresponsive" cells, meaning they are reactive to mechanical stress such as the stress found at sites of injury (Frairia and Berta 2012, 138-147). Shock wave therapy has been shown to increase the production of various tissue healing factors including VEGF based on this mechanoresponsive mechanism (Frairia and Berta 2012, 138-147). Differences in pressure or mechanical force of Lot 004 sprayers acting on HP-802-247 are hypothesized to potentially increase production of VEGF.

The specification for VEGF is 72-416 pg/mL/day and Lot 005 average VEGF value was slightly below this level [70.44 (5.48)]. Previous studies have shown VEGF averages for this lot of CPT2C to be higher than these results. The aging of the cells (>12 months old) may have contributed to the lower levels of VEGF. Stability has only been studied for up to 12 months at this time. It is hypothesized and in agreement with previous studies that VEGF for both lots would likely be above specification for product <12 months old. These findings must be further investigated using newer product as well as further stability testing of product if use past 1 year is of interest.

GM-CSF was not significantly different between groups. There is no current specification for expression of this growth factor by HP-802-247 cells. Currently, mechanisms

for HP-802-247 are under investigation and many growth factors, including GM-CSF, are being considered for use as a quality standard for the product, hence its inclusion in this study.

SUMMARY AND CONCLUSIONS

The purpose of this work was to show the HP-802-247 product delivery as a function of product temperature (Specific Aim 1). Spray parameters (spray area, dosage, ovality and priming) as well as polymerization kinetics of components at different temperatures revealed relative insensitivity of the drug delivery system with regards to product temperature. The average spray area and dosage of Surrogate and CPT2C solutions show no significant differences at various product temperatures. Surrogate solution behavior is an adequate model for CPT2V and should be used in future experiments as a substitute for CPT2V.

Statistically different averages encountered do not appear to have a linear trend in relation to product temperature. This implies that the differences may be related to some factor other than product temperature, such as normal variation in the device, variation associated with the usage (pressure applied to the sprayer, velocity of spray), and/or formulation differences. Sprayer lots did show some significant differences (Specific Aim 2) but none of those differences are concerning because in general, Lot 005 (future sprayers composed of new resin) outperform Lot 004 (current sprayers).

The differences in averages and dosages found were small, but the only concerning difference occurred in CPT1F with average dosage below the specifications (130 ± 13 mg). The smaller dosage of CPT1F may not be clinically significant because it is not the drug-containing portion of the product and the spray area is within specifications therefore providing adequate

coverage of the wound. The purpose of CPT1F is to provide fibrinogen to the wound, which will form a clot when CPT2C is added. Further investigation of this mechanism may be of interest for future study.

Polymerizations were unaffected by the temperature ranges tested (Specific Aim 3). It should also be noted that the CPT1F (lot 01MAY14) was not performing as expected in the polymerization assays. When used for this experiment, it was observed that the component was sometimes too viscous to be pipetted. It is possible that there may have been some issue beyond the scope of this examination with the CPT1F R & D lot being used in polymerization assays. Future stability studies could investigate these findings.

Viability and potency of the HP-802-247 product does not appear to be majorly affected by the usage of future sprayers (Lot 005) and performance is mostly comparable (Significant Aim 4). Significant differences in VEGF production are evident but hypothesized to be below specifications based on the age of the product. It is suggested that future studies include fresher product and a larger sample size to re-examine the effect of sprayers on potency of the cellular product.

This study indicates that product temperature at the range of 4°C to room temperature is unlikely to have any significant effect on spray area, ovality, dosage, or sprayer performance and that Lot 005 is more consistent than Lot 004. Although some statistically significant differences were observed, they do not appear to implicate product temperature as the source of variance. The variance does not appear to be linked to any one temperature and does not appear to trend with temperature. The link to CPT1F differences found during polymerization studies is puzzling, but may be an artifact of the product lots used (such as the CPT1F lot 01MAY 14 was

shown to have longer polymerization times than previous experiments). Other variation between products, such as formula surface tension and/or viscosity, likely contributes to some of the differences in spray area and dosage. More tests with CPT1F of known quality may be performed to confirm this hypothesis. The data gathered in this study will help set the specifications for the development of a new HP802-247 paradigm.

CHAPTER III

Exploration of a New Method for Determining Activity of *Clostridium histolyticum* Collagenase

BACKGROUND AND LITERATURE

Collagen quaternary structure has been of great scientific interest for decades and complete understanding is still elusive in part because the breakdown of collagen fiber quaternary structure involves the breaking of cross-linked chains (Shoulders and Raines 2009, 929-958). This invariably changes the structure of the individual fibrils themselves, making native structure analysis extremely difficult. Current high-resolution studies of collagen structure rely on collagen-related peptides (Shoulders and Raines 2009, 929-958).

Collagen, the most abundant protein in mammals, makes up most of the extracellular matrix in tissues (Eckhard et al. 2014, 102-114, Shoulders and Raines 2009, 929-958). The polypeptide formation consists of repeating triplets of amino acids with Glycine in position three (except in nonfibrillar forms) that twists in a left-handed polyproline II-type helix (Eckhard et al. 2014, 102-114). The most common amino acids in collagen are glycine, proline and hydroxyproline (Eckhard et al. 2014, 102-114, Shoulders and Raines 2009, 929-958). In animals, tropocollagen complex molecules are made of three polypeptide strands twisted into a right-handed, rigid, triple helix (also called a 'super-helix') conformation (Eckhard et al. 2014, 102-114). Tropocollagen helixes may reach a diameter of < 2 nm, a length of about 300 nm and

can self-assemble forming intertwined fibrils (Eckhard et al. 2014, 102-114). These fibrils can have a diameter of up to 500 nm, 1 cm in length and have multiple intermolecular interactions including hydrogen bonding and cross-linkage between strands (Eckhard et al. 2014, 102-114, Shoulders and Raines 2009, 929-958).

It is necessary to break these fibers down for various biological maintenance and renovation purposes (Eckhard, Schonauer and Brandstetter 2013, 20184-20194). For this reason there are endogenous proteases including mammalian matrix metalloproteinases (MMPs) capable of breaking down collagens. MMPs are highly specialized proteases and their cleavage of collagen is site-specific (Zhang et al. 2013, 46-48). Dysfunctional MMPs have been implicated in the breakdown of the basement membrane involved with the process of tumor metastasis, making them a target for therapeutic treatments (Zhang et al. 2013, 46-48). These proteases have a wide range of beneficial and sometimes harmful functions that may play an important role in the treatment of disease.

Bacteria can be particularly effective at breaking down human tissue for use as a source of carbon (Eckhard, Schonauer and Brandstetter 2013, 20184-20194). Unlike mammalian collagenases, *Clostridium histolyticum* collagenase has a very robust collagenolytic capability with little specificity regarding types of collagen (Eckhard, Schonauer and Brandstetter 2013, 20184-20194). *C. histolyticum* collagenase (also called *Clostridium histolyticum* collagenase) is an approximately 115 kDa zinc-metalloprotease that consists of 6 domains in which structure is much conserved (Eckhard, Schonauer and Brandstetter 2013, 20184-20194) The domain organization consists of a collagenase unit (made of activator unit and peptidase unit) and a collagen recruitment unit (Eckhard, Schonauer and Brandstetter 2013, 20184-20194). *Clostridium histolyticum* collagenase consists of various proteases as well as the main

components, Collagenase G (ColG) and collagenase H (ColH) (Eckhard et al. 2014, 102-114, Eckhard, Schonauer and Brandstetter 2013, 20184-20194). These two enzymes have structural similarity in 5 out of 6 domains (Eckhard, Schonauer and Brandstetter 2013, 20184-20194). The structure differences of the enzymes results in differing modes of action and targets (Eckhard, Schonauer and Brandstetter 2013, 20184-20194). Together these enzymes work in tandem to efficiently degrade collagen in its many forms (Eckhard, Schonauer and Brandstetter 2013, 20184-20194). ColG is able to attack multiple strand sites and is largely responsible with breaking down collagen quaternary structure, while ColH acts mainly on breaking peptide bonds within single strands (Eckhard, Schonauer and Brandstetter 2013, 20184-20194). The attack on interior peptide bonds can only occur after ColG has helped to expose the inner portions of collagen normally buried within the structure (Eckhard, Schonauer and Brandstetter 2013, 20184-20194). The robust activity exhibited by *C. histolyticum* collagenases can be utilized for therapeutic purposes including wound debridement, which is the removal of necrotic tissue from a wound (Eckhard, Schonauer and Brandstetter 2013, 20184-20194).

As described in a previous chapter, for wound healing to occur necrotic tissue must be removed for several reasons. Dead tissue provides a carbon-source for bacteria and its removal helps prevent infection (Eckhard, Schonauer and Brandstetter 2013, 20184-20194). Its removal allows cells to begin building new temporary scaffolding matrix and prevents excess fluid buildup under the tissue (Schultz et al. 2003, S1-S28). The body has its own autolytic methods for removal of dead tissue, but this often progresses slowly, increasing the length of the inflammatory phase and the total time to heal (Schultz et al. 2003, S1-S28). In wound care, the necrotic tissues can be removed by various techniques including sharp surgical (scalpel) and mechanical (e.g. wet-to-dry dressing and water pressure) techniques (Schultz et al. 2003, S1-

S28). Surgical methods are usually reserved for large and highly infected wounds requiring drastic and immediate attention. Mechanical methods can cause considerable pain and trauma to surrounding healthy tissue. High-pressure water therapies have been implicated in forcing some bacteria further into the wound and causing future infection of the wound (Schultz et al. 2003, S1-S28). Enzyme mediated debridement is a gentler and less traumatizing method of removing necrotic tissue from chronic wounds.

Collagenase SANTYL® Ointment (CSO) is a useful enzyme-based alternative to more painful and invasive measures such as scalpel and mechanical debridement (Shi et al. 2010, 456-462, Jovanovic et al. 2012). SANTYL® consists mainly of white petrolatum, while the active pharmaceutical ingredient is *Clostridium histolyticum* collagenase at a concentration of 250 collagenase units per gram of ointment.

Clostridium histolyticum collagenase actively breaks down collagen while leaving nearby tissue such as fat, keratin, or fibrin intact (Schultz et al. 2003, S1-S28). Application of SANTYL® to the wound enables *Clostridium histolyticum* collagenase to gently breakdown necrotic tissue without traumatizing nearby viable tissue (Shi et al. 2010, 456-462). For R& D and quality purposes it is useful to have a way to assess the activity of bacterial collagenase for product compatibility and quality assessment.

PROBLEMS/HYPOTHESIS

There are many different ways to assess enzyme activity, and each method has merits and drawbacks. These limitations must be carefully weighed and considered based on the goals of the study and the resources available. A useful method for the measurement of enzyme activity

is the quantitation of the reactions end products. For collagenase activity this method would focus on the breakdown of collagen into peptide fragments. There are many options for quantifying proteins, but not all are effective for analyzing the products of collagen breakdown because of its unique structure and makeup.

The fluorescent methods are useful, but require expensive reagents and equipment to read the fluorescence (Zhang et al. 2013, 46-48). The labeling of collagen with fluorescein isothiocyanate (FITC) is labor intensive and inefficient (Zhang et al. 2013, 46-48). FITC labelled collagens are available for purchase but are prohibitively expensive.

The bicinchonicic acid assay (BCA) method is an inexpensive and easy way to quantify protein but is not accurate for the main constituents of protein (glycine, hydroxyproline, and proline) and so not useful for collagenase activity assessment of collagen breakdown.

The synthetic peptide N (3-[2-furanacryloyl])-Leu-Gly-Pro-Ala (FALGPA) is a commonly substrate used to test the activity of collagenase. The breakdown product of FALGPA is chromogenically active and can be observed in real time using a spectrophotometer. This method is effective for measuring the activity of ColH (also known as type II collagenase), known to mainly attack exposed peptide bonds on single strands, but is not likely to provide insight into the first step of native collagen degradation wherein the activity of ColG (also known as type I collagenase) provides access to these normally buried sites (Eckhard, Schonauer and Brandstetter 2013, 20184-20194). Another drawback of this costly substrate is that some reagents cannot be analyzed using FALGPA. Anything with similar absorption maxima or that is insoluble in aqueous solution are not compatible with the FALGPA method. Substrates that have similar absorption maxima to FALGPA (~345 nm) are not conducive to accurate kinetic

measurements when analyzing real-time spectra because of the interference of the substrate in spectrophotometric assessment. Insoluble substrates are not compatible with spectrophotometry because it requires a homogeneous and clear solution for reproducible and accurate analysis. Therefore FALGPA has limited applications with many solutions commonly encountered in the wound care field (Jovanovic et al. 2012). There is great interest in more flexible methods of measuring *Clostridium histolyticum* collagenase activity.

Traditional ninhydrin methods of detecting peptides (modified from that of Stein and Moore (Moore and Stein 1954, 907-913) as well as Rosen (Rosen 1957, 10-15)) are accurate techniques that benefit from their ability to utilize non-soluble collagen as a substrate. The downside of this procedure is the use of a hazardous potassium cyanide-containing buffer as well as the time-consuming and complex nature of the process.

A new modified ninhydrin method under investigation is a simplified modification of the older Moore and Stein method (Zhang et al. 2013, 46-48). This method eliminates the usage of potassium cyanide and uses PEG to stabilize Ruhemann's purple, the colorimetric molecule of analysis. This modified method does not require extensive preparation steps for the sample before analysis. Whether this method can be adapted to be more similar to an in-vivo environment using insoluble bovine tendon collagen fibers instead of solubilized collagen or gelatin is one of the main factors being investigated in this study.

The usage of collagen fibers ensures that the mechanisms of both ColG and ColH can be effectively assessed in a way more similar to in vivo approaches. In methods using peptides such as FALGPA, it is primarily only the mechanism of ColG that is being tested. The modified ninhydrin method is a cheaper, safer, and faster alternative to the previously mentioned methods

of collagenase activity. In order to validate the process and declare comparability to current methods, it must be established to be precise, sensitive, reliable and robust.

SPECIFIC AIM

1. Investigate a new collagenase activity assay and its comparability with current assay methods using tests for precision, working range, sensitivity, and robustness

In order to effectively test products compatibility with collagenase containing ointment SANTYL® there must be a robust and reliable method for analysis of collagenase activity. Current available methods are incomplete models for in-vivo, use dangerous chemicals such as cyanide, involve complex preparation steps, are expensive and/or time consuming. The new method should be comparable to current methods while being simpler, less expensive, and a more accurate model of in-vivo collagen degradation

SIGNIFICANCE

Typically a wound is cleansed, debrided and covered with anti-microbial dressings and/or ointment (Shi et al. 2010, 456-462, Jovanovic et al. 2012). An important step in the healing of wounds is debridement, the removal of necrotic or damaged tissue from the wound bed. Collagenase SANTYL® Ointment (CSO) is a Smith & Nephew *Clostridium histolyticum* collagenase containing debridement ointment. Frequently, clinicians and nurses will use Collagenase SANTYL® Ointment (CSO) in conjunction with other wound care products such as wound dressings, cleansers and antibacterial preparations. Therefore, it is imperative for the research and development (R & D) department at Smith & Nephew-Biotherapeutics to test the compatibility of these wound care products with collagenase in order to provide scientific opinion on such use of SANTYL® Ointment (CSO).

Smith & Nephew Biotherapeutics R & D team has previously done extensive studies on the effects of various products on *Clostridium histolyticum* collagenase (Shi et al. 2010, 456-462, Jovanovic et al. 2012). Because *Clostridium histolyticum* collagenase is an enzyme it is possible for some products to inhibit its activity and therefore not be recommended for use in tandem with SANTYL® Ointment (CSO). Therefore it is of utmost importance to have a sensitive and robust method of detecting collagenase activity. Current methods of collagenase activity assessment are complex, time-consuming, dangerous, or incomplete models for enzyme activity. The aim of this assay development experiment is to create a safe and simple method that more closely models the in vivo activity of *Clostridium histolyticum* collagenase on collagen fibers.

MATERIALS AND METHODS

Buffer preparation

Collagenase assay buffer (CAB) consists of 50mM Tricine, 400 mM NaCl, and 10 mM $CaCl_2$ then adjusted to a pH of 7.4. Citrate buffer is comprised of 20.5 mM citric acid and 29.5 mM sodium citrate and adjusted to pH of 5. Ninhydrin reagent (280 mM ninhydrin in DMSO) is prepared as in the method of Zhang et al. (Zhang et al. 2013, 46-48) combining $SnCl_2$ solution (7.1 mM in citrate buffer) with ninhydrin solution at a 1:1 ratio. Stannous chloride solution may be stabilized for long-term storage by sonication under vacuum or degassing with N_2. Quench buffer was prepared using 12% PEG 4000 in 25 mM EDTA. Ninhydrin reagent is prepared fresh for each experiment.

FALGPA method (Jovanovic et al. 2012)

Different preparations must be utilized to test different types of product. For dressings a 2' square of the product was placed in a 50 mL conical centrifuge tube and 25 mL of collagenase assay buffer (CAB) was added. Tubes were then placed on rotator for 2 hours to allow any natural component leaching to occur. Extract was then ready for use in FALGPA assay. Water-based cleansers were used as is. Powdered products as well as gels were shaken for solubilization or emulsification in collagenase assay buffer (CAB) for 2 hours in similar fashion to dressings. After rotation, tubes were spun down and supernatant was used as extract. Collagenase powder was prepared at a concentration of 0.5 mg/mL for a final concentration of

0.25 mg/mL. Collagenase powder was combined with appropriate volume of collagenase assay buffer (CAB) and mixed on rotator for ≥ 15 minutes to ensure solubilization and even distribution in solution. FALGPA was removed from freezer and allowed to reach room temperature before opening. FALGPA was then measured and solubilized with CAB in a manner similar to that of collagenase powder but with a final concentration of 1 mg/mL and solubilization time of ≥30 minutes.

Reactions were as follows:

Table 32. Reactions for FALGPA method

Type	**Extract**	**Buffer (CAB)**	**Collagenase (0.5 mg/mL)**	**FALGPA (1 mg/mL)***
(+) Control	n/a	1 mL	1 mL	150 µL
Unknown	1 mL	n/a	1 mL	150 µL
Blank	n/a	2 mL	n/a	150 µL

*FALGPA to be added last after all samples are plated

Reactions (excluding FALGPA) were then combined and allowed to rotate for 25 minutes. After rotation samples were plated on a 96-well plate (4 wells per sample). FALGPA was then added to all wells and plate was immediately read by spectrophotometer using kinetic settings for 35 minutes at 345 nm.

Data were then analyzed using Microsoft Excel and/or JMP statistical software for averaging and differences of mean using Student's t-test for groups of two, and ANOVA (Tukey HSD) for groups of 3 or more.

Modified ninhydrin method (Zhang et al. 2013, 46-48)

Experiments for evaluating the activity of collagenase in various solutions were performed using collagenase assay buffer (CAB) as the positive control, various formulations (A-D) as the unknowns, and EDTA, a known inhibitor of collagenase, as the negative control. These reactions (see Table 33) were monitored for a total of 7 hours with samples extracted at 2, 4, 6, and 7 hour time points. Samples were then analyzed to determine the amount of collagen digested (achieved by removing a sample from the aqueous layer and determined by quantity of peptide in spectrophotometric assay). Quantitative values obtained for each reaction were then plotted to obtain a linear equation for total amount of liberated peptide over time.

Bovine tendon collagen was weighed out (20 mg ± 1 mg) and placed in 10 mL Pyrex tubes. Collagenase solution was then prepared by dissolving collagenase powder in CAB (0.5 mg/mL solution, final concentration 13 µg/mL) and mixed until soluble using a rotator. Leucine standards serial dilutions were prepared (0.2 mM, 0.1 mM, 0.05 mM). Reactions were prepared as shown in Table 33:

Table 33. Reactions for modified ninhydrin method

Type	**Unknown**	**Buffer**	**Collagenase (mg/mL)***
(+) Control (collagenase assay buffer)	n/a	5.85 mL (CAB)	0.156 mL
Unknown (buffers A-D)	5.85 mL	n/a	0.156 mL
Blank	n/a	2 mL (CAB)	n/a
(-) Control (EDTA, serial dilution 10-0.1 mM)	n/a	5.85 mL (EDTA in water)	0.156 mL

*Collagenase to be added last after all samples are plated

Reaction tubes were then placed in a rack in a 37ºC water bath and samples were removed at 120, 240, 360, and 420 minutes. A 150 µL sample was removed from each reaction, placed in a 0.650 mL Eppendorf tube and spun at 15,200 rpm for two minutes. 100 µL of centrifuged sample was placed in 2.0 mL Eppendorf lo-bind tube and combined with 100 µL of stop buffer. 500 µL of ninhydrin reagent was added to each tube under ventilation hood and tubes were placed in an 80ºC thermal plate. Tubes were removed after 10 minutes and allowed to cool to room temperature (note samples can be refrigerated at this point for up to 2 hours if needed) before diluting with 500 µL purified water (color should be evident at this point). Samples were then ready to be plated on a standard 96-well plate for spectrophotometric analysis. 200 µL of sample was plated per well with 4 total replicates. Spectrum was examined at 450-600 nm with expected peak at 570 nm. Resulting concentrations were then calculated on Spectramax software using leucine of known molarity to calibrate a standard curve. Regression plots for each solution were determined using JMP statistical software. Means, standard deviation, relative standard deviation (%), variance, standard error and analysis of means were calculated using Student's t-test for groups of two, or ANOVA (Tukey-HSD) for groups of 3 or more. Limits of detection, and quantitation were calculated. Limits of detection were determined using the following equations at each time interval and averaged (Vial and Jardy, 1999, 2672-2677):

$$\text{Limit of Detection (LOD)} = 3.3 * SD_{\text{blank}} / \text{Slope}_{\text{calibration curve}}$$

$$\text{Limit of Quantitation (LOQ)} = 10 * SD_{\text{blank}} / \text{Slope}_{\text{calibration curve}}$$

RESULTS

In the modified ninhydrin method, known concentrations of leucine were used to create a standard curve for quantification purposes using the concentration of leucine (mM) for the y-axis and signal detection of absorbance (OD, optical density) from the spectrophotometer for the x-axis. Optical density (OD) is directly proportional to color concentration in solution. In a precise assay that is within proper range for the instrument, method, and the enzyme, the standard curve should very closely follow a linear equation. When plotted, the leucine standards all had an *r-squared* value >0.96 (see Table 34 and Figure 11), which indicates a close fit of data points with the linear equation. The limit of detection and limit of quantification as defined in the MATERIALS AND METHODS section help determine the approximate sensitivity and range for the assay (see Table 34).

Table 34. Results determined from modified ninhydrin method calculated using one set of experimental data with samples of reactions taken over several hours Limit of detection and quantitation (in µM & OD with SD in parenthesis), *r-squared* values, and slope of standard curves (see METHODS section for calculation of LOD and LOQ)

Reaction Time (minutes)	R-squared value	Slope	LOD (µM/mL)	LOQ (µM/mL)	LOD (OD)	LOQ (OD)
120	0.99	1.305	24 (4.5)	41 (4.5)	0.007 (0.001)	0.02 (0.0029)
240	1.00	1.288				
360	0.99	1.783				
420	0.96	1.461				

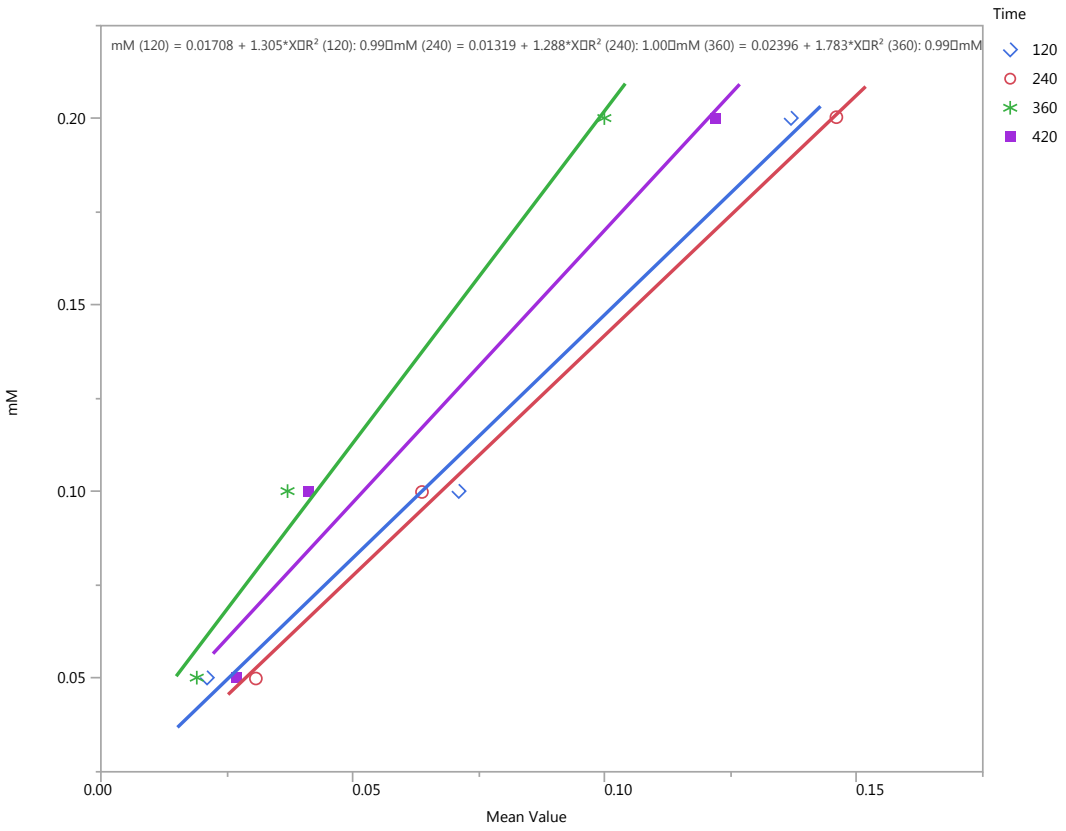

Figure 11. Standard curves calculated for one collagenase experiment with several sample readings over the course of seven hours using leucine at a concentration 0.05 to 0.2 mM (y-axis) and mean value of absorbance from spectrophotometric reading (x-axis) at various time points during collagenase analysis. Linear equations for each leucine standard curve with *r-squared* values ranging from 0.96 to 1.00 (see upper left) and legend (upper right) indicating time point of experiment from which standard curve samples were prepared.

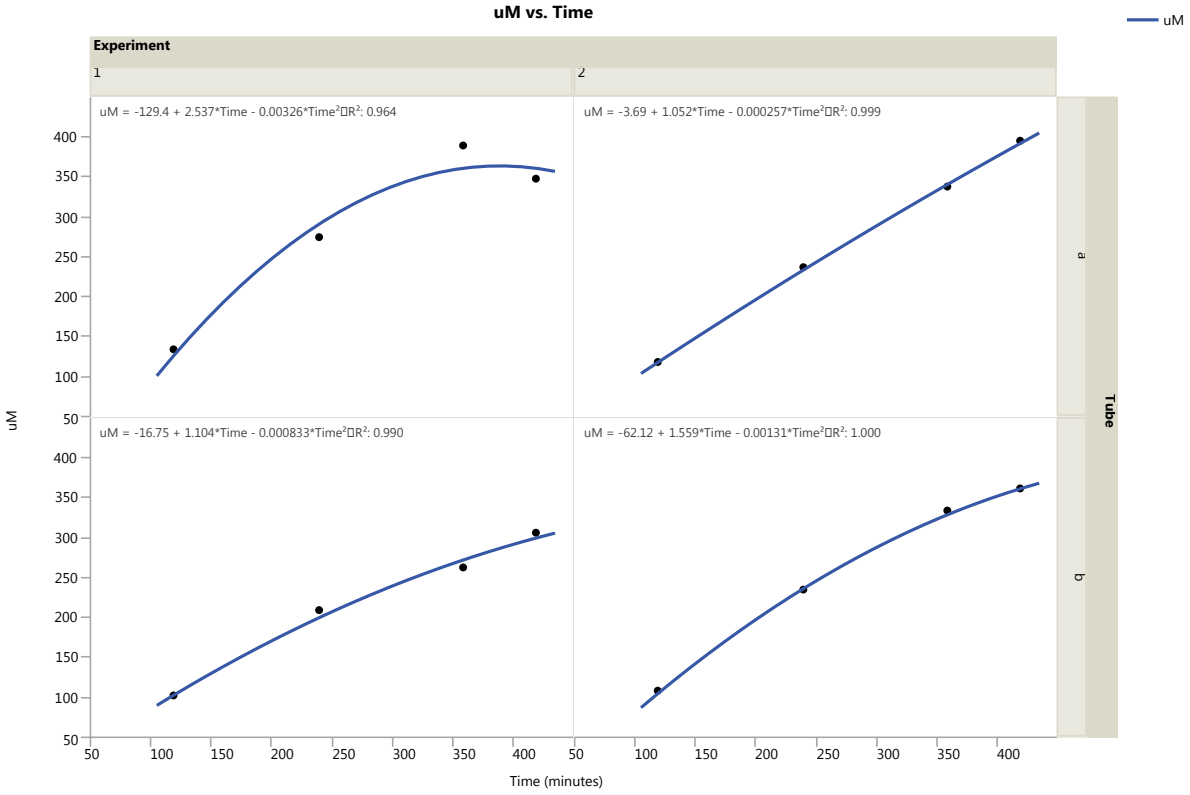

Figure 12. Collagenase activity (described by peptide concentration in µM, y-axis) in 2 experiments using positive control buffer (CAB, collagenase assay buffer) with samples taken from digestion over several hours (in minutes digested, x-axis). 2 experiments (1 & 2) with *n=2* tubes of reaction (a & b). Upper right of each quadrant shows quadratic equations of the line with *r-squared* values ranging from 0.96 to 1.00.

It is important to use a positive control in all experiments to confirm the validity of a test. Collagenase assay buffer (CAB, used as a positive control) experimental data values (from two experiments with two replicates each) were fitted with both linear and quadratic equations and had greater *R-squared* value fit for quadratic as shown above (see Figure 12). Along with these positive controls, the unknowns in this experiment, solutions A-D fit both linear and quadratic equations (*R-squared* > 0.97) but were plotted using the quadratic fit for comparability with control (see Figure 13 and Table 37). Formulations A-D collagenase reactions all had *R-squared*

values greater than 0.97 (see Figure 13). The positive control, collagenase assay buffer (CAB) and collagenase reactions had r-square values of 0.96 and 1.00 (see Figure 12).

Experiments were repeated and results and variance were compared both days, within and between (intra-day and inter-day), to test the robustness and the precision of the new assay method. Low levels of variation between replicates can confirm the precision of the method. Robustness, which is a resistance to small variation in day-to-day technique, can be supported with low levels of intra and inter-day variation. Means, SD, CV, variance and SE for intra-day and inter-day precision of control and other formulations were calculated using JMP statistical software (see Tables 35 & 36). Intra-day precision of 90% of values had an RSD below 5. Inter-day precision for 75% of values had an RSD below 20.

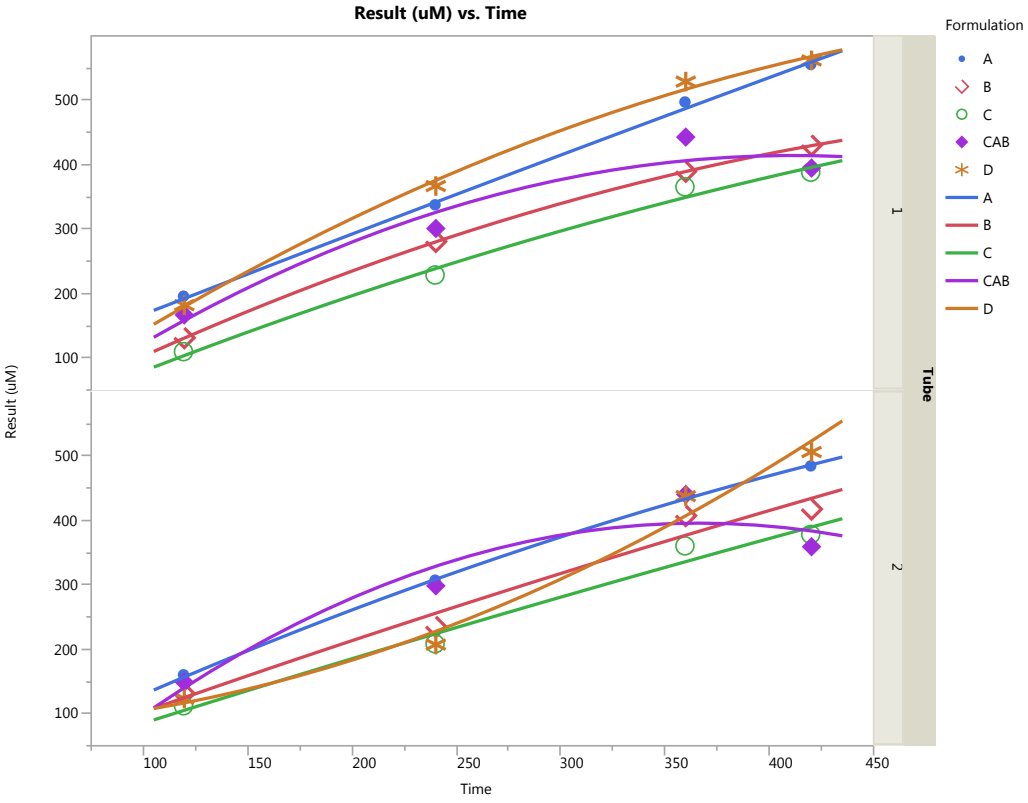

Figure 13. Liberated peptide concentration (µM, y-axis) over time (minutes, x-axis) from collagen/*C. histolyticum* collagenase reaction suspended in various formulations and positive control (CAB) over 7 hours (sample identification in upper right legend, *r-squared* values for each reactions quadratic equation shown in upper left corner of each graph).

Table 35. Intra-day variation (mean, SD, variance, SE, and RSD) for experimental values of liberated peptides from collagenase/collagen reaction suspended in various solutions over 7 hours using modified ninhydrin method. Low RSD % confirms the low amount of intra-day variation and robustness of the method.

Solution	Tube	Time	N	Value (mM)	SD	Variance	SE	RSD %
A	1	120	4	0.19	0.0019	0.0000	0.0009	0.97
A	1	240	4	0.34	0.0024	0.0000	0.0012	0.70
A	1	360	4	0.50	0.0038	0.0000	0.0019	0.77
A	1	420	4	0.55	0.0091	0.0001	0.0046	1.64
A	2	120	4	0.16	0.0019	0.0000	0.0009	1.20
A	2	240	4	0.31	0.0022	0.0000	0.0011	0.73
A	2	360	4	0.44	0.0025	0.0000	0.0013	0.57
A	2	420	4	0.48	0.0176	0.0003	0.0088	3.63
B	1	120	4	0.13	0.0060	0.0000	0.0030	4.59
B	1	240	4	0.28	0.0050	0.0000	0.0025	1.78
B	1	360	4	0.39	0.0094	0.0001	0.0047	2.42
B	1	420	4	0.43	0.0150	0.0002	0.0075	3.49
B	2	120	4	0.13	0.0029	0.0000	0.0015	2.25
B	2	240	4	0.24	0.0101	0.0001	0.0051	4.29
B	2	360	4	0.41	0.0112	0.0001	0.0056	2.77
B	2	420	4	0.42	0.0138	0.0002	0.0069	3.29
C	1	120	4	0.11	0.0046	0.0000	0.0023	4.26
C	1	240	4	0.23	0.0069	0.0000	0.0035	3.05
C	1	360	4	0.37	0.0157	0.0002	0.0078	4.29
C	1	420	4	0.39	0.0289	0.0008	0.0145	7.47
C	2	120	4	0.11	0.0030	0.0000	0.0015	2.71
C	2	240	4	0.21	0.0029	0.0000	0.0015	1.42
C	2	360	4	0.36	0.0126	0.0002	0.0063	3.49
C	2	420	4	0.38	0.0194	0.0004	0.0097	5.14
D	1	120	4	0.18	0.0073	0.0001	0.0037	4.01
D	1	240	4	0.37	0.0165	0.0003	0.0082	4.48
D	1	360	4	0.53	0.0162	0.0003	0.0081	3.06
D	1	420	4	0.56	0.0105	0.0001	0.0053	1.88
D	2	120	4	0.12	0.0030	0.0000	0.0015	2.43
D	2	240	4	0.25	0.0934	0.0087	0.0467	36.75
D	2	360	4	0.44	0.0101	0.0001	0.0050	2.31
D	2	420	4	0.56	0.1303	0.0170	0.0651	23.07
CAB	1	120	4	0.17	0.0039	0.0000	0.0020	2.36
CAB	1	240	4	0.30	0.0014	0.0000	0.0007	0.47
CAB	1	360	4	0.44	0.0099	0.0001	0.0049	2.24
CAB	1	420	4	0.40	0.0042	0.0000	0.0021	1.07

(Highlighted values with RSD ≥5%)

Table 36. Inter-day variation (mean, SD, variance, SE, and RSD) for experimental values of liberated peptides from collagenase/collagen reaction suspended in various solutions over 7 hours using modified ninhydrin method. RSD ≥20% as highlighted indicates high variation between inter-day experiments.

Solution	Time	N	Result Mean (mM)	SD	Variance	SE	RSD%
A	120	16	0.22	0.05	0.003	0.013	22.8
A	240	16	0.37	0.05	0.002	0.012	13.6
A	360	16	0.45	0.03	0.001	0.008	6.8
A	420	16	0.58	0.07	0.005	0.018	12.3
B	120	16	0.14	0.02	0.001	0.006	16.4
B	240	16	0.28	0.03	0.001	0.008	12.0
B	360	16	0.36	0.04	0.002	0.010	11.6
B	420	16	0.46	0.05	0.003	0.013	11.1
C	120	16	0.12	0.02	0.001	0.006	19.9
C	240	16	0.25	0.05	0.002	0.012	19.8
C	360	16	0.30	0.08	0.006	0.020	26.5
C	420	16	0.50	0.13	0.016	0.032	25.2
D	120	16	0.16	0.03	0.001	0.007	16.6
D	240	16	0.33	0.06	0.004	0.016	19.4
D	360	16	0.38	0.11	0.013	0.028	29.5
D	420	16	0.61	0.08	0.007	0.021	13.6
CAB	120	16	0.16	0.02	0.000	0.005	11.2
CAB	240	16	0.31	0.03	0.001	0.008	10.6
CAB	360	16	0.53	0.12	0.015	0.031	23.2
CAB	420	16	0.35	0.03	0.001	0.008	9.6

(Highlighted values with RSD ≥20)

Table 37. Equations and r-squared values calculated using experimental values of liberated peptides from collagenase/collagen reaction suspended in various formulations (A-D) over 7 hours using modified ninhydrin method

Tube	Solution	Equation						R^2
1	A	40.4	+	1.281	*Time	- 0.000109	*Time^2	1.00
	B	-56.9	+	1.731	*Time	- 0.001365	*Time^2	1.00
	C	-54.44	+	1.42	*Time	- 0.000829	*Time^2	0.99
	D	-68.54	+	2.297	*Time	- 0.001861	*Time^2	1.00
	CAB	-94.63	+	2.473	*Time	- 0.003004	*Time^2	0.95
2	A	-19.85	+	1.585	*Time	- 0.000904	*Time^2	1.00
	B	-14.82	+	1.205	*Time	- 0.000325	*Time^2	0.97
	C	-19.64	+	1.07	*Time	- 0.000227	*Time^2	0.98
	D	74.98	+	0.06116	*Time	+ 0.002394	*Time^2	0.98
	CAB	-167.6	+	3.075	*Time	- 0.004195	*Time^2	0.92

It is important to have a negative control as well as a positive control in order to confirm the validity of an experiment. EDTA is a known inhibitor of *C. histolyticum* collagenase and therefore an excellent choice for a negative experimental control. If the assay is valid, the negative control should produce no discernible result. EDTA reactions (concentrations >1 mM) with collagen and collagenase resulted in sample readings that were all below the limit of quantitation and detection (LOQ & LOD); but reactions with 0.1 mM concentration EDTA exhibited low but quantifiable activity (see Figure 14).

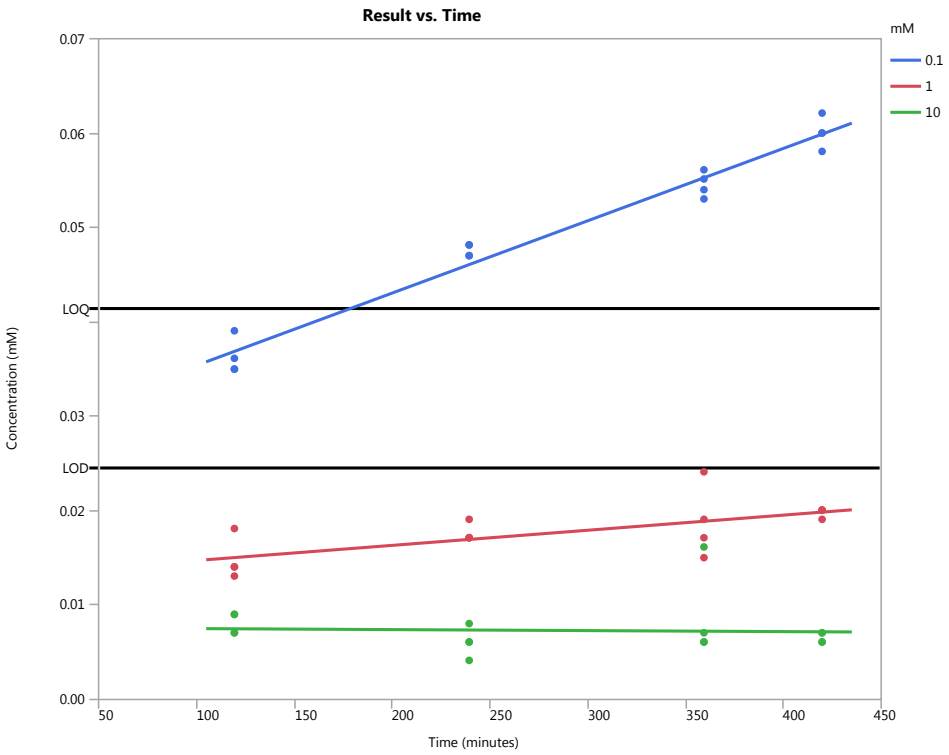

Figure 14. Liberated peptide concentration (µM, y-axis) over time (minutes, x-axis) from collagen/*C. histolyticum* collagenase reaction suspended in EDTA (negative control) over 7 hours confirms expected inhibition of collagenase with most values below the limit of detection (concentration in mM in upper right legend, *r-squared* values for each reactions quadratic equation shown in upper left corner of each graph, LOD line (0.024 mM) and LOQ (0.41 mM))

DISCUSSION

The precision of the modified ninhydrin method was examined in several ways. Leucine standard curves fit linear standard curve with *r-squared* values of >0.96 indicating good precision of the method. Limit of detection (LOD) and limit of quantitation (LOQ) calculations of 24 (4.5) and 41 (4.5) µM/mL describe the sensitivity of the assay. Positive control results were as expected with high *r-square* values to confirm precision and validity of method.

Positive control samples confirmed the validity of the experiment with consistent and well-defined results as described by the high *r-squared* value following the expected linear enzymatic reaction similar to previous studies at Smith & Nephew. The quadratic equation could be a result of the saturation effect in enzyme kinetics that describes a point at which enzyme reaches a saturation point and the velocity of the reaction reaches its maximum.

Negative control (EDTA) tests reveal different outcome compared to previous experiments. Previous experimental data published by Jovanovic et al. using the FALGPA method (Jovanovic et al. 2012) indicated that 0.1 mM EDTA solution inhibits collagenase activity on FALGPA by 5% versus control, but results of the modified ninhydrin method show that collagenase activity is inhibited 85% by the same EDTA concentration solution compared to control. This suggests that the effect of 0.1 mM EDTA on collagenase is different when using whole collagen fibers versus FALGPA method. It should be noted that there are many differences between the methods including concentrations of substrates and collagenase as well as the type of substrate and therefore comparisons must be interpreted cautiously. The modified

ninhydrin method may reveal a broader picture of *Clostridium histolyticum* collagenase activity than other methods. Repeated tests must be completed to further investigate these results.

The different inhibition results are interesting but not unexpected as FALGPA kinetic studies examine only the peptidase activity of collagenase on small peptides not large macromolecular collagen fibrils (mainly Col H). It is hypothesized that this inhibition model using FALGPA is incomplete and the method using collagen fibers may be more close to the in-vivo model as it has revealed different results at this concentration of EDTA. Although collagenase did show activity when using EDTA at 0.1mM, the average of all values was 6 times less than that of collagenase in collagenase assay buffer (CAB).

Precision and robustness of the new ninhydrin method was also confirmed by comparing relative standard deviation (RSD) for intra- and inter-day experiments. Intra-day experiments show promise by having low variation overall with 90% of replicates below 5% RSD. Inter-day experiments show a greater variation with 75% of replicates below 20% RSD. This finding is unsurprising because of the instability of ninhydrin reagent and will need to undergo further development if the experiment is intended to be used for quantitative results. Currently this method may be more suited to examining relative relationships as opposed to quantitative or quality purposes. It should be noted that purging the stannous chloride solution with N_2 can further stabilize ninhydrin reagent, but this option was not available for these experiments at this time. It is hypothesized that removing oxygen from the solution could increase the stability of ninhydrin solution and decrease the inter-day RSD values. Future studies should attempt to stabilize inter-day RSD values and also investigate the accuracy of the assay.

SUMMARY AND CONCLUSIONS

The modified ninhydrin method has proved to be a promising assay for future experiments involving *Clostridium histolyticum* collagenase assessment. The precision has been established, but more experiments must be repeated to ensure the results described previously. Accuracy may need to be tested by using a collagen reference standard (available from various manufacturers) to confirm the effectiveness of the assay. Accuracy must be established if the method is to be used for quality purposes. It should also be noted that the collagen fibers used were over a year old. Older collagen fibers are known to be more variable in their breakdown and could have affected the rates of collagenase activity. Stability studies could further elucidate this hypothesis.

The modified ninhydrin assay has also been validated with its more complete picture of collagenase inhibition in the presence of EDTA. Inter-day variance was found to be somewhat higher than expected and future experiments should confirm whether degassing of stannous chloride might help to stabilize the solutions used and eliminate some variance. Because the experiments performed were mainly testing relative relationships the intra-day accuracy is less of an issue than if the test was for quantification or quality purposes. Preliminary studies have shown the modified ninhydrin assay is comparable to the collagen-FITC method that is currently used. The modified ninhydrin method of assaying collagenase activity continues to be of great interest but will require more testing to confirm or improve the accuracy and robustness before further adoption of the assay for other purposes.

CHAPTER IV

DESCRIPTION OF INTERNSHIP SITE AND EXPERIENCE

Smith & Nephew Biotherapeutics, Fort Worth, Texas, is a part of the Advanced Wound Management division which focuses on innovation in the chronic wound care field. The building houses labs for Analytical Chemistry and Formulation, Cell Science, Microbiology, and Biochemistry. This location also includes offices for various clinical, regulatory, and quality divisions. An animal lab for in-vivo testing is located at the nearby University of Texas Health Science Center.

Working at Smith & Nephew Biotherapeutics has been more fulfilling than I could have imagined. From the beginning I was welcomed as a part of the team and was able to participate in many different meetings and activities with the employees. I got to learn new techniques and become familiarized with the day-to-day rhythm of industry.

Research & Development provides freedom to explore, investigate, and improve processes and products. Creative thought is highly valued and encouraged. It was a pleasure to work here and I am so grateful for the time that I have had with these inspirational scientists. I have grown more confident in my abilities as a scientist because of the freedom and trust that I received.

JOURNAL SUMMARY

My main project at Smith & Nephew-Biotherapeutics was analysis of HP-802-247 product and sprayers as outlined in Chapter I. As my skills progressed I was encouraged to explore more topics and also shadow other employees to pick up new experiences including use of statistical software, creation of data reports, observing cell culture techniques, and using instruments for spectrophotometry and fluorescence reading. When the fluorescence reader was out for service, I was tasked with finding alternatives for the time we were lacking a fluorescence reader and that began my exploration of novel methods for collagenase assessment. The project grew and it was then decided that it should be incorporated into my practicum.

APPENDIX

DAILY JOURNAL

8/4/14
Arrived and received workspace and equipment.
Read required SOP's for training
Received ID badge
Read wound paper, "Wound bed preparation: a systematic approach to wound management." Schultz et. al. [Wound Rep Reg 2003; 11: 1-28]

8/5/14
Continued to read wound paper
Watched blood borne pathogen training video and received certificate
Received computer credentials
Assisted with preparation of media (surrogate solution—BSA-glycerol)
Watched spill cleanup safety video
Autoclaved media for cold storage experiments with Jason
Observed cell line (keratinocytes) media change and filtering of media for cold storage experiment
Trained to do experimental spray testing at 10cm with surrogate solution (4, 10, 15, and 25C)
Scanned spray data papers onto computer for analysis of spray area

8/6/14
Read protocols and memos on various topics including previous spray testing
Used ImageJ to analyze scanned testing papers from the spray testing experiment done on 8/5/14
Observed cell work (removing cells, part of keratinocyte protocol, resuspending in various storage media (1, 2, 3, and control) to compare viability
Produced spreadsheet with data analysis of spray testing experiment described above and discussed results

8/7/14
Repeated spray testing experiment with surrogate solution at 4, 10, 15, 25°C (3 vials each today for a total of 6 vials), prepared data spreadsheet and discussed results. Previous conclusion that there is no significant difference at various temperatures still holds.
Read memos regarding various component solutions past studies

8/8/14
Spray test area (10cm) experiment using CPT2 (5 vials each) at 4, 10, 15, 25°C and data analysis using scanner, Image and excel and discussed results.
Attended quarterly Smith & Nephew-Biotherapeutics 'Open-mic' meeting

8/11/14
Spray test testing regarding product weight per spray (dosage) using surrogate solution at 4, 10, 15, 25°C and discussed results.
General file and lab notebook organization and labeling
Repeating Stericycle training (online version)

8/12/14
Repeated spray testing regarding product weight per spray (dosage) using surrogate solution at 4, 10, 15, 25°C
Measured surrogate density at 4, 10, 15, 25°C

8/13/14
Spray area testing (10cm) with CPT1F 4, 10, 15, 25°C
Checked keratinocyte cell viability/cell number counting using the NC 3000

8/14/14
Spray testing weight per spay (dosage) CPT2.179 4, 10, 15, 25°C and data analysis
Discussed results

8/15/14
Spray testing weight per spray (dosage) with CPT1.150 4, 10, 15, 25°C
Journal club meeting presentation by Jason Campbell on "Release of a Wound-Healing Agent from PLGA Microspheres in a Thermosensitive Gel"

8/18/14
Organizing spray testing data from previous week (compiling charts on Excel for CPT1 data)
Lab cleanup

8/19/14
FDA briefing
Lab cleanup
Literature review (various cryobiology, wound healing technology, etc.)
FDA visit

8/20/14
FDA visit
Literature review continued
Hepatitis B vaccine series 1 at Concentra provided by Smith & Nephew-Biotherapeutics
Discussed current and future projects
Feedback on data presentation

8/21/14
FDA visit
Checked viability and number of cells in various media plated 15 days previous. All cells appeared to be doing well. Prepared new bottles to grow stored cells. Protocol for keratinocytes.
Prepared some preliminary statistical analysis on Excel for the spray testing data. May need to repeat the experiments for more data points.
We will be working on a memo to present the data next week.
Prepared documentation for first committee meeting

8/22/14
FDA visit
Literature review
Reviewed proposal requirements, began draft
Entered literature into RefWorks file
First committee meeting—discussed proposal, toured the facility, signed paperwork for internship

8/25/14
Literature review for proposal background
Polymerization assay techniques
Went to UNTHSC for PPD testing
Checked keratinocyte viability—Doing literature search to see if there is any indication of how long cells can survive in media.
35-placebo formulation

8/26/14
Literature review for cell culture
Reviewed old proposal examples
Made in-house placebo formulation
Read protocol for fibrinogen assay (STA-art 4)

8/27/14
R&D team meeting. Each group described their prior work and future directions with the 802 projects.
Went to UNTHSC to get TB test checked
Cell count/viability
Planned clotting experiment (modified for STA-Fibrinogen assay)
Continued to stir placebo formulation

8/28/14
Fibrinogen Assay with CPT1 & 2 at various temperatures (4°, 10°, 15°, 25°C). This test run helped me determine the new guidelines for this modified assay.
Set up CPT1 & 2 vials with dilution buffers in preparation for repeat of experiment tomorrow.

8/29/14
Clotting assay with CPT1 & 2 repeat with modified protocol and 1-day old refrigerated samples.
Data analysis
9/1/14
Labor Day holiday—OFF

9/2/14
Clotting assay and complete a first draft of proposal.
Proposal Draft work
CPT1 & CPT2 clotting assay at 37C with 'old' vials (8/28/14)
Data analysis using Excel
Will repeat with fresh vials CPT1 & 2 placebo tomorrow

9/3/14
Clotting assay with old and new CPT1 & CPT2 at 37°C
Also reversed order CPT1 and CPT2 at 37°C with old and new vials.
Data analysis with Excel
Worked on proposal

9/4/14
Patent review. Confirmed that chemicals were ordered
Town hall meeting, 9:30-10:30 am
Repeat 37°C experiment with newer CPT1 & CPT2 placebo
Worked on proposal
Data Analysis with excel

9/5/14
Finished first draft of proposal
4°, 10°, 15°, 20°C experiment with cpt1 & 2 placebo (new, unopened)
Analyzed data with excel

9/8/14
Got Report to use as a template on the data gathered from spray and clotting tested since 8/4/14.
Began to write report and gather data in correct format.
Repeated more clotting assays at various temps. Data still not similar to previously collected data.
Will test with new dilutions tomorrow and see if this the is source of differences

9/9/14
Performed new clotting assays at 4-37C with new dilutions. Data still not similar for 37C clotting times. Scheduled shadow on Friday to see if we can figure out what we are doing differently.
Received revisions on proposal. Reviewed and applied revisions
Continued work on Report.

9/10/14
Report and revision of data with new averaging method (spray area of CPT2 and surrogate only ones needed to be modified)
Observed cell viability and growth factor analysis (new pump comparability)
Discussed proposal, confirmed with committee that we need to add protocols for growth factor secretion and cell viability but leave side projects out of the proposal
Revising draft proposal

9/11/14
Read protocols for Cell counting/viability and potency assays; received ELISA kit booklet for future experiments
Proposal revisions and additions of more sections on cell viability test and potency assays
ImageJ work on spray area of new sprayers (experiments done by AJ and JC) and excel analysis of data

9/12/14
Worked on fibrinogen assay protocol. Protocol was good, but there were still differences with our results. Ran controls for CPT2 and found that CPT1F.lot01MAY14 to be the differing variable.
Re-ran experiments for 4, 10, 15, 20, 37C and entered data into spreadsheet
Sent proposal draft to committee
Worked on Report

9/15/14
Updated lab notebook
Further analyzed clotting data from previous week and charted together
Formulations for comparisons—examples 30 and part of 31

9/16/14
Continued to add data to Report
Formulation completion for comparison 31

9/17/14
Continued additions of data and commentary for sprayer report
Began plating formulations for comparison testing

9/18/14
Worked on charts for sprayer testing data
Literature Review
Analysis of Data—clotting times
Plated sample formulation for comparison test

9/19/14
Literature review
Plated last sample formulation for comparison test
Excel statistical tests on sprayer data for 4 and 22C (t-tests), determined that more powerful software is needed to properly interpret data
JMP training

9/22/14
JMP uploaded
Shadowed during potency ELISA assays
Began dilutions for film viscosity comparisons (1:10 and 1:15)
JMP tutorial

9/23/14
Continued dilutions for film viscosity comparisons (1:20 and 1:25)
Received proposal feedback from committee and began revision
Worked on JMP data analysis

9/24/14
Product compatibility tests (with collagenase) and assayed for collagenase activity
Viscosity comparisons of formulations

9/25/14
Repeated compatibility tests and assay for collagenase activity
Data analysis for product compatibility and for viscosity
Photographed various dilutions for viscosity report 9/25/14
Proposal adjustments

9/26/14
Data analysis (JMP) of spray tests

9/29/14
Revising spray test report
Began formulations

9/30/24
Continued formulations
Review of proposal draft and forwarded

10/1/14
Continued formulation and troubleshooting, research on preservatives
Hep B shot (second in series)
Literature search on various statistical analyses
Data analysis (spray studies)

10/2/14
Revised report
Data analysis (spray studies)
Lab cleanup

10/3/14
Revised report
Chemical Inventory

10/6/14
Formulation work
Chemical Inventory

10/7/14
Revised report
JMP statistics
Chemical Inventory
Lab Cleanup

10/8/14
Chemical Inventory
Lab Cleanup
Formulation work

10/9/14
Chemical Inventory
Lab Cleanup
pH formulations

10/10/14
Osmolality of formulations
Formulation work/compatibility
Data analysis

10/13/14
Repeat formulation experiments with improved protocol
Meetings re: Clinical Trial Phase 3 results
Report revisions

10/14/14
Brainstorming meeting—802, all R&D
Formulations
Report revisions

10/15/14
Formulation assay and analysis
Report revisions

10/16/14
Formulation of new dilution for assay
Report

10/17/14
Assay for formulation and analysis
Flu shot day
Report

10/20/14
Sprayer priming tests with lot 005 vp7 sprayers, surrogate solution, room temp
Finished report draft

10/21/14
“Blank” formulation
Repeated priming tests for lot 005 vp7 sprayers, surrogate solution, room temp, this time also recording dosage
Analyzed priming and dosage information using excel and JMP

10/22/14
Continued formulation explorations
Needed to analyze full length collagen-fitc, but the fluorescence reader is oos
Devised and performed test for using spectrometer for fitc analysis
Analyzed peaks

10/23/14
Attempted to use new spec technique to analyze formulations with collagen fitc
Analyzed after 2 hour digestion, may not be sufficient for analysis of enzyme activity
Placed experiment back for 24-hour digestion and analysis

10/24/14
Analyzed 24 hour digestion, but determined there were insufficient controls for quantification
24 hours seems good for digestion in the future

10/27/14
Started repeat of previous formulation assay with additional blanks for quantification
Product compatibility assay with collagenase
Received and reviewed proposal notes

10/28/14
Performed assay on formulation (after 24 hour digestion)
Proposal edits

10/29/14
Test formulations to determine what is causing precipitate, titration with CaCl2 suspect
Research for collagen specific protein assays

10/30/14
Continued precipitate analysis
Began 24 hour digest of collagen for BCA analysis test
Report revisions
Proposal revisions

10/31/14
BCA assay
Formulation analysis, continued
Research paraben solubility

11/3/14
Finished formulation analysis, determined buffer was precipitating
Determined BCA not appropriate test for collagenase activity
Researched more proteinase analysis methods

11/4/14
Researched proteinase analysis and collagen structure
Prepared modified ninhydrin reaction methods
Received new lab notebook

11/5/14
Requested chemical for ninhydrin reaction
Worked on organization of literature and materials
Lab notebook update

11/6/14
Lab notebook update
Researched buffer systems
Began preparing new test formulation

11/7/14
Finished test formulations
Research de-oxygenation methods

11/10/14
Precipitation issues continued
Surrogate area spray testing at all temperatures

11/11/14
Continued VP7 lot 005 spray testing (dosage)

11/12/14
New ‘blank’ formulation
Assay

11/13/14
Assay diagnostics

11/14/14
Assay diagnostics

11/17/14
Assay diagnostics

11/18/14
Assay diagnostics
Product compatibility

11/19/14
Formulation assay

11/20/14
Assay diagnostics
Repeat formulation assay

11/21/14
Product compatibility

11/24/14
Repeat 11/20 experiments with different concentrations

11/25/14
Begin test of new protocol

11/26/14
Continue new protocol test

11/27-12/1
Thanksgiving break (and one sick day)

12/2/14
Lab notebook update
VP7 lot 005 Sprayer CPT1 spray area experiment

12/3/14
Sprayer data analysis (excel and JMP)

12/4/14
Sprayer data analysis (excel and JMP)

12/5/14
New collagenase analysis

12/8/14
Kinetics study on collagenase activity from new assay

12/9/14
Preparation of solutions and media for collagenase assay

12/10/14
New assay for formulations

12/11/14
Analyzed results from formulation analysis

12/12/14
Journal Club
Continued analysis of data and experimental design for next formulation/collagenase analysis

12/15/14
Experimental design for collagenase assay
Preparation of media for collagenase assay

12/16/14
Collagenase assay (scale up)

12/17/14
Data analysis from previous days experiment

12/18/14
Repeat collagenase assay with formulations

12/19/14
Data analysis from yesterday's assay
Formulation evaluation

12/22/14
Data analysis
Lab notebook

12/23/14
Data analysis
Lab notebook
Collagenase assay

12/24/14
Data Analysis
Lab notebook

12/25-1/1
Christmas Break

1/2/15
Preparing Thesis

1/5/15
Preparing Thesis
Shadow for viability/potency experiment

1/6/15
Sprayer Testing (CPT1 dosage, new sprayers)

1/7/15
Sprayer testing (CPT2 dosage, new sprayers)

1/8/15
Data entry/analysis (sprayer reports)

1/9/15
Sprayer testing (CPT2 spray area, new sprayers)
Data entry/analysis (sprayer reports)

1/12/15
Preparing thesis outline
Set date and time for defense
Data analysis for sprayer results
Research viscosity info

1/13/15
Lab notebook update results
Thesis outline work
Finish sprayer stats

1/14/15
Finished notebook update
Statistical arrangement and data analysis comparing sprayer lots all parameters
Statistical research

1/15/15-2/1/15
Writing thesis

2/2/15
Potency analysis for HP-802 experiment begun. Plating product and incubations
Writing thesis

2/3/15
Potency analysis continued, media change at 24 hours
Writing thesis

2/4/15
Writing thesis

2/5/15
Writing thesis

2/6/15
Potency of HP-802 ELISAs for VEGF and CM-GSF and analysis
Viability of HP-802 post-spray tests and analysis

2/9/15
Planning and preparing for collagenase assay
Writing thesis

2/10/15
Collagenase assay all day

2/11/15
Preparing for collagenase assay
Writing thesis

2/12/15
Collagenase assay all day

2/13/15
Data analysis for collagenase assays
Editing thesis

2/14/15 – 3/12/15
Editing thesis, new sections, continued analysis of results

3/13/15
Turned in thesis draft to committee
Collagen FITC experiment

3/14-3/27

Thesis and presentation work. Turned in final draft of thesis

LABORATORY TECHNIQUES AND PROTOCOLS

Molecular Methods

Title: Transformation of competent E. coli with cDNA construct of GAPDH

Date: 7/7/14

Introduction:

Transformation of bacteria is a technique in which a foreign segment of DNA is inserted into a bacterial cell. Bacteria can be made to take up foreign DNA by a procedure to make them "competent." This competency can be achieved by treating with ice-cold solution of $CaCl_2$ and given a brief heat shock. This can help the bacterial wall be more neutrally charged and willing to uptake the foreign plasmid. There are multiple ways to induce competency.

We have used in this experiment pUC-GAPDH13 (3.99 kb). All plasmids must have 3 components, the ORI (replication origin), MCS (multiple cloning site), and antibiotic resistance site. The MCS is the site that allows a place for amplification and cutting sites and also contains cDNA of interest. The antibiotic resistance helps us to select colonies of bacteria that have been successfully transformed.

Ultimately, we will cut the insert of GAPDH and use it as a probe for the specific RNA transcript expressed in cells (of GAPDH). We can analyze this by Northern Blot. GAPDH is a so-called 'house-keeping' gene. This means it is present in all cells for normal maintenance and upkeep.

Method:

1. Filled ice bucket
2. Labelled 1.5 mL tube with initials, date and title of experiment
3. Note—professors thawed and provided vial (50 uL) of frozen competent E. coli cells (DH5α)
4. Place 25 uL of competent E. coli cells into labeled vial
5. Added 2 uL (~100 ng) of plasmid DNA (2 uL of pUC GAPDH, 0.1 ug/uL)
6. Gently mixed the plasmid DNA and competent cells by tapping the tube
7. Tubes are incubated for 30 minutes, then placed in 42°C water bath to eat shock cells for 1 minute
8. Cells then placed on ice for 2 minutes before adding 500 uL LB (room temperature)
9. Placed vial in 37°C water bath (professors will move to the shaker after 1 hour)

10. After 1 hour, plated 10 uL & 50 uL of cells (respectively) onto two different agar plates (carbenicillin), and placed in incubator overnight
11. After overnight incubation, counted colonies

Transformation efficiency calculation:

$$\frac{(\#\ of\ colonies)}{Amount\ DNA\ plated\ (ng)} * 1000\ (\frac{ng}{ug})$$

$$\frac{132}{20\ ng} * \frac{1000ng}{ug} = 6.600\ ug$$

Title: RNA isolation (HNPE cells)

Date: 7/8/14

Introduction:

In the previous experiment we transformed bacteria to contain a plasmid of interest, GAPDH. This cDNA is to be used as a probe to look for RNA expression of this gene in future experiments. In particular, we will be looking at expression in HNPE cells (human non-pigmented ciliary epithelial cells). In this experiment we have received a tissue culture of HNPE cells and will be extracting the RNA from them.

By analyzing the RNA content of a cell we can see how much expression a cell is undergoing.

The procedure of RNA isolation is begun by lysing a cell (in this case using Trizol reagent) and then separating out the various components with a combination of protein denaturants (for example, phenol and/or chloroform) as well as centrifugation. RNases are inactivated by this process, which is important because RNases degrade RNA. Once the treatments have been applied, we should have a tube with 3 layers: the upper, aqueous layer contains RNA, the interface contains proteins, and the lower organic phase contains DNA. If the cells are too dense, sometimes the aqueous layer sinks to the bottom of the tube. This can be remedied by adding more Trizol and further incubation.

Trizol—guanidine thiocyanate (chaotropic agent); disrupts plasma membrane and causes organelle lysis and inhibits RNases by binding to them; *Very corrosive, use proper PPE

Method:

1. Received a 100mm tissue culture dish containing HNPE cells and removed 1 mL of liquid from the dish, dumped excess liquid into a waste bucket, and re-plated reserved media in dish
2. Used a scraper in petri dish to scrape cells into the media for about 1 minute

3. Removed the liquid media and scraped cells and placed in 1.6 mL Eppendorf tuve and placed on ice
4. Placed cell tube in centrifuge and spun down for 5 minutes at 3000 rpm, 4°C
5. Removed medium from tube and leave pellet (leave on ice)
6. Added 1 mL of Trizol B reagent and aspirate until the cell pellet dissolved, keep on ice for 10 minutes
7. Added 200 uL of chloroform and inverted 3 time (under the hood); note—pink (organic phase) was at the top of the tube, indicating excess of cells and need for further treatment)
8. Added 200 uL Trizol B and incubated tube 5 minutes on ice
9. Centrifuged tube at 14,000 rpm at 4°C for 5 minutes; note—phases now in proper orientation
10. Aspirated aqueous phase into a clean 1.6 mL tube (note: ~ 600 uL) and added equal amount of isopropanol
11. Centrifuged for 10 minutes at 14,000 rpm, 4°C
12. Removed isopropanol and added 70% ethanol (1 mL) without disturbing RNA pellet, then centrifuged at 4°C, 14,000 rpm for 10 minutes
13. Removed ethanol and let air dry for about 10 minutes
14. Added 50 uL autoclaved water
15. Placed tube in -80°C for 1 hour
16. Placed samples in water bath (65°C) for 3 minutes
17. Aliquot 3 uL for analysis with nanodrop
18. Turn on nanodrop, use 1 uL water as blank, set to analyze RNA
19. Measure 1 uL sample
    a. ug/mL = 1.5859
    b. 260/280 = 1.87

Title: Plasmid DNA isolation using the Wizard Plus SV miniprep DNA purification system

Date: 7/9/14

Introduction:

In this experiment our goal is to recover the plasmid cDNA that was incorporated into pUC vector during experiment 1. The basic steps to purify plasmid DNA from bacteria are as follows:

1. Growth of bacterial culture (from agar plates from previous experiment)
2. Harvest and lysis of bacterial cells
3. Purification of plasmid DNA

Mini-prep kits provide reagents and easy to follow instructions for plasmid purification.

Methods:

1. Grew pUCGAPDH from experiment 1 (see page 1) overnight in 3 mL LB by touching a colony with pipette tip and dropping into culture tube containing LB. Placed in 37°C shaker overnight.
2. Pipetted 1.5 mL culture into 1.6 mL tube and spun down at room temperature for 2 minutes
3. Discarded supernatant and repeated step 2 with the rest of the culture
4. Discarded supernatant with small pipet to get all LB removed and added 250 uL resuspension solution (from kit) and resuspend pellet
5. Add 250 uL of lysis solution and invert 4 times to mix
6. Add 350 uL of neutralization solution and invert 4 times to mix
7. Centrifuge at top speed (14k rpm) for 6 minutes at room temperature
8. Transfer supernatant to Wizard pro kit column and centrifuge at 14k rpm for 1 minute at room temperature. Discard flow through
9. Added 750 uL wash solution and centrifuged at 14k rpm for 1 minute at room temperature. Discard flow through
10. Repeat step 9 with 250 uL of wash solution but centrifuge for 2 minutes
11. Dry spin column for 3 minutes and transfer column to a new clean 1.5 mL tube
12. Resuspend in 50 uL nanopure water
13. Nanodrop
    a. Concentration = 242.4 ng/uL
    b. 260/280 = 1.87

Title: Restriction digestion of cDNA clone encoding GAPDH gene

Date: 7/9/14

Introduction:

Once we have purified our plasmid of interest we need to cut it (it is circular) and purify out our cDNA insert (in this case the GAPDH gene). To accomplish this, we have special enzymes that cut the DNA at special (usually palindromic) sequences.

By adding the Pst1 enzyme to a restriction digest we can cut out our fragment of interest. The digest reaction consists of water and buffer (buffer H, specially designed to optimize the activity of Pst1) and the restriction enzyme Pst1

Method:

Calculating the dilution for restriction digestion

Concentration of DNA: 242.4 ng/uL = 0.2424 ug/uL

Need 1 ug/10 uL

$$1\frac{ug}{0.2424\frac{ug}{ul}} = 4.12\ ug\ in\ 10\ uL$$

$$4.2\ uL\ DNA + 5.8\ uL\ nanopure\ H2O = 10\ uL\ total$$

Master mix (10 reactions):

Water	70 uL
10X Buffer H	20 uL
Pst1 (10 ug/uL)	10 uL

1. Prepare DNA as calculated above
2. Prepare master mix as shown above
3. Add 10 uL master mix to 10 uL DNA
4. Placed reaction in 37°C water bath for 1 hour

Title: Agarose gel electrophoresis
Date 7/9/14
Introduction:
Agarose—polysaccharide derived from seaweed; co-polymer of D-galactose & 3,6-anhydro-L-lactose
TAE—gel running buffer; Tris, acetic acid, and EDTA
40 mM Tris
20 mM acetic acid
1 mM EDTA
Agarose gel is made by combining agarose and gel running buffer (in this case, 1X TAE) and melting to combine. The cooled gel forms a semi-solid matrix with which we can use to visualize DNA.

Note: Boil using microwave and use caution because molten agarose can cause severe burns while hot

Ethidium Bromide (EtBr) can be added to gel or gel can be soaked in buffer with EtBr after running to visualize DNA. EtBr is mutagenic, carcinogenic, teratogenic. Use proper PPE when handling. When exposed to UV light, EtBr bound to DNA fluoresces orange.

A ladder is used as reference.

When gel is poured and solidified with wells, samples can be loaded. Voltage applied will cause negatively charged DNA to move towards positive charges at the bottom of the gel. Voltage stopped depending on size of the fragments being investigated. They can run off the gel so use caution.

Method:

1. Weighed 1 gm agarose into a 250 mL conical flask and added 100 mL of 1X TAE buffer (1% mixture)
2. Microwaved 1 minute with intermittent swirling until boiling (CAUTION: swirl gently because hot agarose can boil over and cause serious burns)
3. Added 10 µL of 10 mg/mL ETBr and rocked gently for about 10 minutes until it is cool to the touch on undersurface of arm
4. Assembled the gel casting unit with taped edges and comb
5. Poured molten agarose into the casting
6. Allowed to solidify for >20 minutes (this can vary depending on thickness and size of gel) then after removing tape placed in gel box with fresh 1X TAE buffer
7. Prepared samples:
    a. Restriction enzyme reaction from last experiment
       20 µL of reaction
       4 µL of 6X loading dye
    b. Uncut plasmid DNA
       2 µL DNA
       3 µL purified water
       1 µL 6X loading dye
8. Loaded samples into gel
    a. Well 2: uncut
    b. Well 3: cut
    c. Well 1: 1 Kb ladder
9. ~150 volts are ideal for gel of this size
10. After yellow dye reached the bottom of the lane voltage was removed
11. Carefully removed gel with gloves and placed in biorad imager
12. Saved image of gel
13. Moved gel to UV light box and used a clean blade to cut out band of interest (size ~350 bp) and placed in sterile 1.6 mL tube (CAUTION: UV light can damage eyes and requires UV protective goggles)
14. Discarded remainder of gel and froze cut DNA at -80ºC

Title: Designing primers (with Primer3)

Date: 7/10/14

Introduction:

The purpose of this experiment is esigning primers for use in RT-PCR (reverse transcriptase PCR.

DNA → RNA

Reverse transcriptase (viral origin)

What should be considered for designing primers?

1. 3' OH to build on
2. Primer length
    a. 20 bases ideal, based on [$4^x > y$], where x is the number of primer nucleotides and y is genome complexity
3. 40-50% GC content
4. Avoid self-hybridization and primer dimers
5. Max length (dependent on purpose, ex: qPCR only needs ~75 bases)
6. Include introns or not?
    a. Software can help answer questions quickly

Primer 3 primer design software

1. Find sequence of interest at NCBI (mRNA, cDNA, etc.)
2. Paste into Primer 3 softwarre
3. Adjust options to your preference

Title: cDNA synthesis

Date: 7/10/14

Introduction:

Continued process to investigate gene exprerssion. Previously HNPE cells had total RNA extracted, produced complimentary cDNA using AMV or MMLV reverse transcriptase to isolate total genomic mRNA transformed to sscDNA

Methods:

For cDNA synthesis:

1 mM dNTP's

5 mM Mg

20 units RNasin

50 mM KCl

Total RNA

TT oligo primers

Random hexamer primers (or 5X RT tRNA)

1. Thawed total RNA from HNPE RNA extraction experiment (project 2); RNA concentration at 1.59 µg/µL
2. Combined nanopure water (14.3 µL), RNA (1.7 µL), and 5X inscript RT enzyme (4 µL) into labeled PCR tube in that order
    a. Note: enzyme should be used with caution. Fluctuation temperature must be avoided and enzyme should be out of freezer for as little time as possible. Fingers should be placed far above the meniscus of enzyme to prevent excess warming of enzyme. Replace in freezer ASAP and keep on ice at all times when out of freezer. Avoid frost-free freezers since they go through heating/cooling cycles.
3. Placed PCR tubes in thermocycler for 1 cycle:
    a. 65ºC for 5 minutes (denaturing)
    b. 25ºC for 5 minutes (annealing)
    c. 42ºC for 30 minutes (RT optimal working temperature)
    d. 85ºC for 5 minutes (kills RT activity)
4. Placed cDNA in -80ºC freezer until further use

Title: Polymerase chain reaction (PCR)

Date: 7/11/14

Introduction:

DNA thermal cycling was invented by Kary Mullins who was awarded the nobel prize for this discovery. DNA in which the investigator wanted to amplify is combined with $MgCl_2$, nucleotides (dNTPs), upstream and downstream primers and Taq polymerase (named after organism of origin, Thermus aquaticus) and placed in a machine capable of controlled temperature changes. These changes in temperature help the amplification to proceed.

Primers help the polymerase determine which portions of the DNA to amplify by binding to their complementary sequence and giving the Taq a place to begin its enzymatic activity of binding a complementary DNA strand.

Thermo cycler steps:

1. Denaturation
    a. 94ºC, 0.5-1 minute
    b. High temperature, low salt, high pH favorable
2. Annealing/hybridization
    a. 60ºC, 0.5 minutes
    b. Depends on melting temperature [(GC content * 4) + (AT content * 2)]; annealing temperature is usually 2-3ºC below melting temperature
    c. Low temperature, high salt, low pH favorable
3. Extension/elongation
    a. 72ºC, 0.5 minutes

Note: In 30 cycles using Taq polymerase, 1 fentogram of DNA could be amplified to 1 µg

Purpose: To amplify the gene of interest in cDNA from previous experiments, primers flanking the gene (GAPDH) were chosen and will be used in this reaction to amplify that section of cDNA.

Methods:

1. Prepared master mix for PCR reaction as follows:

Nuclease free water	297.5 µL
goTaq 5X buffer	100 µL
$MgCl_2$ (25 mM)	60 µL
dNTPs (10 mM each)	10 µL
(S) upstream primer (GAPDH)	10 µL
(A) downstream primer (GAPDH)	10 µL
Taq Polymerase	10 µL
Total volume (enough for 10 rxns)	2.5 µL

2. Combined 49 µL master mix and 1 µL cDNA in PCR tube
3. Placed in thermocycler at the following settings:

Temperature (ºC)	Time (minutes)	
95	2	
95	0.5	} 30x
58	0.5	
72	0.5	

72	10	
4	∞	

4. Prepared agarose gel (2%) and assembled as described in Experiment 5 (Agarose gel)
5. Ran gel at 60 volts for 1 hour
6. Visualized gel on BioRad Chemidoc
7. Quantified DNA based on software analysis of band intensity (379.268 ng)

Title: TA cloning

Date: 7/11/14

Introduction:

Taq polymerase adds a single "A" to the end of the PCR product. The TA cloning vector utilizes this "A" to ligate with the "T" overhang (on the vector)

The vector includes:

1. Lac promoter—allows bacterial expression of lacZ α fragment for α-complementation (blue-white screening)
2. LacZ α—encodes for β-galactosidase
3. Kan^R –provides resistance to Kanamycin
4. Amp^R—provides resistance to Ampicillin
5. pUC ori—for replication, selection and maintenance in E. coli
6. x-gal—plates with X-gal will turn colonies without inserts blue
   (no insert means an uninterrupted, functional lacZ gene; insert will interrupt lacZ gene and colony will appear white)

The goal of this procedure is to insert PCR product from Experiment 8 (GAPDH gene) into a vector. This vector allows bacterial expression of the gene of interest. Once a positive colony with insert is isolated, a culture of the colony can be developed that includes many copies of GAPDH gene.

Methods:

1. Determined the amount of PCR product needed to ligate 50 ng of PCRII vector (final ratio 3:1 with excess PCR product to drive reaction)
   a. PCR vector—660 daltons = 2.57×10^6 Daltons
   b. PCR product = 2.31×10^5 Daltons
2. Mixed ligation reaction

Nuclease free water	3 µL

5X ligation buffer	2 µL
PCRII vector (25 µg/µL)	2 µL
Fresh PCR product	2 µL
T4 ligase	1 µL
Total	10 µL

3. Incubated at room temperature for 30 minutes and then stored at -20ºC
4. When ready, take 2 µL of ligation reaction and add to TA clone kit tube of competent cells, stir gently with pipette tip and allow to sit on ice for 30 minutes
5. Store remaining ligation reaction mixture at -20ºC
6. Heat shock the cells in water bath (42ºC) for 30 seconds without shaking
7. Placed cells on ice at least 1 minute
8. Pippetted 250 µL SOC medium to each vial for all recovery phase
9. Placed cells in 37ºC shaker for 1 hour at 225 rpm
10. Under sterile conditions, plated 50 µL and 250 µL respectively on 2 carbenicillin and x-gal treated plates (you can purchase pre-treated plates)
11. Incubated plates overnight at 37ºC
12. Examined plates, looking for white colonies (blue colonies indicate no insert)
13. Picked and labeled 2 colonies
14. Placed tip in colony and placed into 3 mL LB broth laced with antibiotic in culture tube and allowed to grow overnight at 37ºC
15. Culture now ready for mini-prep

Title: Northern Blot (RNA denaturing gel)

Date: 7/14/14

Introduction

Northern analysis separates RNA on a denaturing gel to be transferred to a membrane.

Considerations when working with RNA:

1. Cleanliness—RNases are ubiquitous
2. Fresh reagents—less than 1 year old

Methods:

1. Prepared denaturing gel

Agarose	1.2 g
10X MOPS	10 mL
Autoclaved water	75 mL

   a. Microwaved about 2 minutes with intermittent swirling until boiled

   b. Cooled on stirrer about 10 minutes
   c. Added 15 mL formaldehyde under hood (toxic fumes)
2. Poured gel into mold, casting box with 8-toothed comb (holds 50 µL per well)
3. Prepared 15 ng of RNA. Starting concentration 1.58 ng/µL, final solution 14 µL
4. Added 21 µL RNA sample buffer to 14 µL RNA
5. Placed in 65ºC water bath for 10 minutes, then placed on ice for 3 minutes
6. Removed sample from water bath and added 7 µL 6x loading dye and 1 µL EtBr
7. Loaded sample into denaturing well (43 µL)
   a. No ladder necessary, bands from 28S and 18S serve as ladder
8. Ran gel at 80V for 3 hours
9. Took picture on Biorad Chemidoc
10. Assembled transfer components: Tray, support, wick, gel, filter membrane, Whatman 3mm paper, stack of paper towels, weight
11. Applied parafilm to gel border
12. Applied tape to put pressure on paper towels
13. Allow gel to transfer overnight

Title: Southern Blot Analysis

Date: 7/14/14

Introduction:

The Southern Blot analysis is similar to the Northern blot analysis, except that instead of binding RNA to an RNA probe (of RNA:DNA hybridization) we will be examining DNA:DNA hybridization.

Most steps are very similar to that of the Northern Blot (see experiment XXXX). The only difference in this case being that we will have to soak the gel in denaturing solution because the gel was not a denaturing gel. The gel must then be washed and neutralized before setting up the apparatus as in experiment XXXX.

Methods:

1. DNA gel from experiment 8 (PCR amplifying GAPDH cDNA was removed from the freezer and placed in a large pyrex dish
2. Right top corner of gel was cut for orientation purposes
3. Added enough denaturing solution to cover and place on rocker for 45 minutes
   a. Denaturing solution is comprised of 1.5 M NaCl and 0.5 M NaOH)
4. Discarded denaturing solution and rinsed 2 times with di water

5. Added enough neutralization buffer to cover gel and rocked for 30 minutes (1 M Tris HCl pH 7.4, 1.5 M NaCl)
6. Discarded neutralizing solution and rinsed the gel with 20X SSC and transferred to nylon membrane (see exp. XXX for apparatus setup)
7. Allowed transfer to proceed overnight.
8. Removed all tape, paper towels and extra paper to expose nylon membrane, flip DNA side up and place in UV crosslinker oven for about a minute at 254-352 nm.
9. Remove from oven, carefully labeled with pencil name and orientation of gel, continue to experiment XXX "Northern and Southern Blot hybridization"

Title: Southern and Northern Blot hybridization

Date: 7/15/14

Introduction:

Procedure for Northern and Southern Blot from this point on is the same. Both hybridization preparation work will work in tangent. At this phase of the blotting experiments we are preparing the nylon membranes for probing

Methods:

Buffers

Prehybridization		Hybridization
Formamide	10 mL	10 mL
20X SSC (3 M NaCl, 0.3 M sodium citrate)	5 mL	5 mL
50X Denhart solution	2 mL	800 µL
1M sodium phosphate buffer, pH 6.5	1 mL	650 µL
10% SDS	200 µL	400 µL
Nanopure water	1.2 mL	2 mL
Heat-denatured salmon sperm	600 µL	400 µL
Dextran sulfate	n/a	2 g (shaker to incorporate)

1. Prepare buffers (add salmon sperm DNA and probes just prior to hybridization)
2. Place UV-crosslinked membranes in respective labeled roll tubes
3. Added 19 mL of prepared prehybridization buffer to each tube
4. Placed tubes in hybridizing oven at 42ºC for about 4 hours
5. Discarded solution

6. Carefully, using forceps, removed nylon from tubes and placed in clean plastic Tupperware faceup
7. Poured 2X SSC (enough to cover) and prewash membranes about 5 minutes (repeat once, discarding solution after each wash)
8. Repeat washing with 1X SSC and discard wash after 5 minutes
9. Wash with 0.2X SSC for 5 minutes and discard wash
10. Warm blocking buffer and 4X wash buffer in 37-50ºC water bath
11. Add blocking buffer (enough to cover) and wash for 15 minutes
12. Add 333 µL streptavidin-horseradish peroxidase conjugate (1:300 dilution). At this point cover the box with aluminum foil because the streptavidin-horseradish peroxidase is light sensitive
13. Discard blocking buffer after 1 hour
14. Poured 1X wash buffer (enough to cover)
15. Repeat for a total of 4 washes at 5 minutes each with gentle shaking
16. Transferred membranes to new containers and added 50 mL of 1X wash solution and incubated for 5 minutes with gentle shaking
17. Prepared substrate working solution by adding 6 mL luminol/enhancer solution to 6 mL stable peroxide solution just before applying to the membrane
18. Removed membranes from buffers and blot an edge to remove excess buffer, place in clear container
19. Brought both membranes to biorad chemidoc imager and took exposure pictures.

Title: Immunocytochemistry

Date: 7/17/14

Introduction:

Immunocytochemistry involves the experimental process of visualizing proteins inside of fixed, intact cells. The means of achieving this involve antigen-antibody interactions and tags to visualize the location of this interaction. Fluorochrome-labeled anti-immunoglobin antibodies are widely used for this interaction. The basic steps involved in visualizing proteins in an intact cell via immunocytochemistry are as follows:

1. Prepare sample (cell culture, etc.)
    a. Single cell layer (cytospin or grow directly on plate)
2. Fixation (PFA, acetone-methanol, freeze)
    a. Preserves cell structure and prevents loss of antigen
3. Permabilization
    a. Creates pores for antibodies to enter cell membrane, usually using solvents, saponins, nonionic detergents

4. Blocking
    a. Serum from secondary antibody used
5. Application of primary antibodies
6. Application of secondary antibodies
7. Stain nucleus using DAPI or other dye
8. Mount to slide with fluorosafe
9. Visualize with microscope

Methods:

Preparation

1. Obtained HNPE cells in media (cells should be grown, treated, fixed and stained directly in multi-welled plates, chamber slides or on coverslips
2. Aspirated media and washed 2 times with 1X PBS, taking care to not let cells dry in between washes
3. Aspirate PBS and quickly place 1 mL ice cold methanol in plates and placed in the freezer for 5 minutes
4. Aspirated fixative (methanol) and rinsed 3 times in PBS for 5 minutes each time

Immunostaining

1. Permabilized cells using permabilization buffer for 5 minutes at room temperature
2. Rinse 3 times in PBS quickly
3. Block specimen by removing PBS and plated 800 µL of blocking buffer for 1.5 hours
4. Prepared 1:500 dilution of antibodies to antibody solution
5. Prepared secondary antibody buffer solution right before usage
6. Placed primary antibody solution (~1 mL) onto plates and placed on orbital rotator for 1 hour
7. Removed antibody buffer and rinsed 3 times with PBS at 5 minutes a rinse
8. Incubated cells with 5 µg/mL DAPI for 5 minutes
9. Rinsed with PBS
10. Mounted coverslip with fluorosafe
11. Sealed with nailpolish (clear)
12. Stored in dark at -20ºC or 4ºC
13. Visualized by software and microscope

Title: RNAi (RNA interference)

Date: 7/21/14

Introduction:

RNAi involves techniques in which siRNA is introduced into a cell to suppress a targeted gene of interest. RNA interference begins with:

1. dsRNA corresponding to a target is introduced into cells and is processed by DICER into short interfereing (si) RNA
2. siRNA unwinds and is incorporated into RNA-induced silencing complexes (RISC)→endonuclease rich complexes
3. siRNA in complex binds complementary RNA and ultimately degrades it..suppressing gene expression

Methods

Note: all done under bio-hood

1. Obtained HNPE cell culture, removed media and rinsed with 1 mL serum-free DMEM
2. Discarded media and added 2 mL SF-DMEM
3. In separate tubes:
    a. 15 µL lipofectamine and 300 µL SF-DMEM
    b. 30 µL of c-Jun siRNA (100 pmol/10 µL) and 300 µL SF-DMEM
4. Kept at room temperature for 5 minutes
5. Added nucleotide to lipofectamine solution from previous step and allow to combine for 15 minutes at room temperature (mix well)
6. Changed out 2 mL SF-DMEM to plate
7. Added 200 µL of mixture (lipo-siRNA) drop by drop to the plate and mixed by swirling in all directions
8. Allowed to incubate at 37ºC for 6 hours and then changed media and continued incubation overnight
9. Aspirated media from HNPE RNAi plates
10. Added 1 mL Trizol and shook for 10 minutes at room temperature
11. Used scraper to remove cells from plate and placed in 1.5 mL tube
12. Added 0.2 mL chloroform and shook vigourously for 15 seconds then allowed mixture to sit at room temperature for 3 minutes
13. Centrifuged the homogenate at max speed 16,000g for 15 minutes
14. Carefully removed the upper colorless aqueous phase (contains RNA) and placed in a clean tube
15. Added 0.5 mL of isopropanol to RNA and inverted the tube to gently mix and allowed to sit at room temperature for 10 minutes
16. Centrifuged sample at max g for 10 minutes at 4ºC
17. Removed supernatant carefully and washed RNA pellet 1X with 75% EtOH by vortexing and centrifuging at 7.500g for about 5 minutes at 4ºC
18. Dried the RNA pellet by removing the EtOH with pipet and allowing to air dry for about 10 minutes

19. Dissolved RNA pellet in 45 µL water
20. Mixed the following:
    a. 45 µL RNA
    b. 5 µL 10X DNase I buffer
    c. 1 µL DNase I

    And placed in 37ºC water bath for 30 minutes

21. Added 10 µL DNase I inactivation reagent, mixed well and left at room temperature for 2 minutes
22. Spun tube at max g for 1 minute
23. Transferred supernatant to new tube and incubated tube at 65ºC for 15 minutes, then quenched on ice for 2 mimutes
24. Nanodrop RNA
    a. Concentration 201.3 ng/ µL
25. Made cDNA synthesis mix for Rt-PCR
    a. 5 µL RNA
    b. 4 µL Super mix
    c. 11 µL water
26. Placed in thermocycler
    a. 25ºC for 5 minutes
    b. 42ºC for 30 minutes
    c. 85ºC for 5 minutes
    d. 4ºC ∞

Title: qPCR

Date: 7/24/14

Methods

1. Prepared master mix for 8 samples:
    a. 80 µL 2X Supermix
    b. 8 µL cDNA
    c. 24 µL water

(will add 6 µL primer to each 18 µL mastermix for a total of 24 µL per well)

2. Primer mix
    a. 8 µL primer
    b. 16 µL water
3. Primer only mix
    a. 50 µL 2X Supermix

    b. 16 µL water
4. Loaded 19 µL master mix and 6 µL primer to each well, also did primer only wells
5. Professors ran qPCR and distributed results
6. Formula:
   ΔCt_1 = target – reference
   ΔCt_2 = target- experimental
   $\Delta\Delta Ct = \Delta Ct_1 - \Delta Ct_2$

Title: Blast of TA clone

Date: 7/24/14

Method

1. After TA cloning and restriction digest, PCR product was submitted for sequencing
2. Downloaded ApE (a plasmid editor)
3. Opened sequence file using ApE
4. Excluded first 111 bp from sequence (primers included promoter)
5. Went on NCBI website and clicked “BLAST”
6. Submitted ApE file
7. Results aligned with Human GAPDH mRNA

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