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# Designing a Viral Reduction System for FDA approval in Fibrin Sutures

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# Designing a Viral Reduction System for FDA Approval in Fibrin Sutures

A Major Qualifying Project Report to be submitted to the faculty of Worcester Polytechnic Institute in partial fulfillment of the Degree of Bachelor of Science.

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# Table of Contents

**All sections were written and edited by all team members.

**Table of Figures**

**Table of Tables**

# Acknowledgements

# Abstract

VitaThreads LLC has recently developed an alternative to the current marketed suture that promotes a faster healing process with less induction of fibrotic tissue. The VitaThreads product, a fibrin microthread based suture, is composed of bovine derived fibrinogen and thrombin. To ensure patient safety and gain Federal Drug Administration (FDA) approval, a viral reduction process must be incorporated into fibrin microthread manufacturing to eliminate the risk of viral transmission in patients. The quality of the threads, including mechanical properties, need to be maintained through the reduction process to retain the ability to be implanted. A process was designed in which, thrombin and fibrinogen were initially treated separately at the liquid phase, thrombin was treated with an acid pH alteration and fibrinogen with a solvent detergent combination. Threads were then formed and underwent a secondary treatment utilizing a dry heat procedure. Viral reduction was validated against bacteriophage $\Phi$X174 and quantified using a plaque assay. $\Phi$X174 is commonly used in industry to mimic potential bovine derived viruses. The viral reduction of the acid pH, solvent/detergent precipitation and dry heat was greater than 6, 3.6, and greater than 2, respectively. The viral reduction process did not significantly alter the mechanical properties (ultimate tensile strength) of the sutures. The FDA does not issue a minimum reduction factor for approval, but these results demonstrate process efficiency that is likely to gain FDA approval.

# 1.0 Introduction

Wound healing is a process in which the body repairs itself after a trauma. The process is broken down into six steps: rapid homeostasis, inflammation, proliferation and migration, angiogenesis, re-epithelialization, and synthesis [35]. Scar tissue may form in these steps and leave everlasting damage in the affected tissue. In the cosmetic industry, doctors cannot always limit the presence of scar tissue after a surgical procedure. Scar tissue is much weaker and less tensile than normal, healthy tissue, which can lead to pain and scar visibility on the skin [36].

Sutures are utilized in many surgical procedures and commonly cause an inflammatory response at the wound-site, as well as permanent scarring after removal or degradation. VitaThreads uses bovine derived fibrinogen and thrombin to manufacture VitaSutures, a recently developed alternative to generic sutures, to reduce scarring in wound healing. This new suture reduces the formation of scar tissue, is less inflammatory, and more pliant than others on the market [5,6]. Despite the effectiveness of VitaSutures, they have not reached clinical trials due to lack of a purification system for viral reduction of the starting products. The use of bovine derived materials introduces concern of transmission of bovine-related viruses to the patient. To assure safety, the Federal Drug Administration (FDA) issues requirements for viral reduction, including an overall viral reduction of $10^{12}$ [34]. VitaThreads must meet these standards for their VitaSutures before moving into human clinical trials. The purpose of this Major Qualifying Project is to develop a method to remove bovine-related viruses from the VitaSutures.

# 2.0 Literature Review

## 2.1 Introduction

The literature review section provides an overview of the importance and methods currently employed to remove viruses in biological samples. In the context of the FDA, viral clearance requirements are introduced and are specific to the potential viruses in bovine derived materials. The major viruses found in bovine derived sources are categorized as enveloped or non-enveloped. This characteristic helps determine which purification method can be utilized.

## 2.2 Fibrinogen and Thrombin pH and Temperature Stability

Fibrin microthreads are an engineered skin regenerative suture aimed to reduce scarring during wound closing. As described in Cornwell et al., they are made from the co-extrusion of fibrinogen and thrombin [16]. Fibrinogen is a soluble protein that is convert by thrombin to insoluble fibrin strands forming a fibrin network. Thrombin cleaves the E domain on fibrinogen and allows the D and E domains to overlap and crosslink to form fibrin. The properties of these components are significant to the overall function of the sutures and must not be disrupted during purification. Fibrinogen is both temperature and pH sensitive and follows two steps of irreversible unfolding at different temperatures, influenced by pH (REFERENCE). At neutral pH, the D fragment will unfold at 57.7 °C and the E fragment at 96 °C. The conformation of both is essential to the development of fibrin microthreads. Additionally, at a pH of 3.5, the fibrinogen is slightly less stable at lower temperatures, while the opposite effect is seen at a pH around 8.5 [1]. Thrombin, similar to fibrinogen, will denature at 60 °C and will not be able to function properly. Thrombin is more stable when heated in a dry solid state as compared

to a solution state [2]. Thrombin is in functional form from pH 5-10, but will precipitate at a pH of 5 or less [3]. These characteristics, in terms of pH and heat stability, of fibrinogen and thrombin were considered when designing the reduction system.

## 2.3 VitaThreads Advantages and Formation

The company VitaThreads manufactures fibrin microthreads. Fibrin microthreads, also referred to as VitaSutures, are a designed to be a premium product for physicians to use for patient wound closures. VitaThreads uses bovine derived fibrinogen and thrombin to manufacture VitaSutures. These bovine products are nearly identical in structure and conformation to their human counterparts [4]. VitaSutures are slightly more expensive that other skin closure products on the market, but reduces scar tissue formation in patients [5]. President and CEO of VitaThreads, Adam Collette, mentioned VitaSutures create a "less inflammatory, more pliant suture for patients to allow for a better outcome" [6]. They have demonstrated positive test results in the rat model and are supported by several physicians, including Dr. Frank P. Fechner of Harvard Medical School. However, VitaThreads cannot begin a phase I human clinical trial without FDA clearance. More specifically, the FDA has requirements for confirmed viral reduction before testing in humans.

Initially bovine-derived fibrinogen and thrombin are obtained in powder form and need to be dissolved in HBSS. Once the liquid solutions of fibrinogen and thrombin are developed, 1ml of each solution is drawn up in a syringe and attached to an extrusion system. The extrusion system presses against the syringe pumps at a rate of 0.236mm/min and thin tubing is used to draw out the threads in a pan filled with HEPES buffer solution. Typically each batch of fibrin microthreads made from these solutions

creates approximately thirty threads. The threads form in the shallow buffer solution and are removed from the pan using tweezers after approximately ten minutes. Once removed the threads are hung to dry on a box overnight and removed the following day and placed into aluminum foil for storage if future testing is required.

## 2.4 The Impact of Viral Reduction

Biological samples, derived from animal sources, may contain infectious agents that are harmful to humans. If a patient is exposed to a product that has not been properly purified, they are at risk of contracting diseases due to contamination. For example, the company, Bayer, did not properly sterilize their blood products for hemophiliac patients. These products contained Human Immunodeficiency Virus (HIV), causing thousands of patients over three decades to contract HIV and later die of Aquired Immunodeficiency Syndrome (AIDS) [32]. There are not only scientific reasons to purify products, but also ethical reasons to ensure patients do not contract any life threatening diseases. Certain viruses are more prevalent in human-derived products than other species, including hepatitis A (HAV), hepatitis B (HBV), human immunodeficiency (HIV), and parvovirus (B19) [7].

## 2.5 Bovine Viral Contamination

Since fibrinogen and thrombin are bovine derived, they have the potential to contain viruses specific to the bovine source. These viruses can be transmitted directly to humans and cause illnesses, known as zoonotic diseases. In 2012, 13 of the 56 identified zoonotic diseases killed an estimated 2.2 million people. Cattle, along with chickens and pigs, are the main sources causing these illnesses in humans [8]. While cattle can be infected with different agents, there are specific viruses of concern  for human

4

contraction, such as bovine viral diarrhea (BVD), infectious bovine Rhinotracheitis (IBR), bluetongue (BTV), and parapoxvirus (PPV) [33]. Additionally, researchers at UC Berkeley identified a correlation between bovine leukemia virus (BLV) and human breast cancer; this was determined because milk tanks were not free of viral contamination and many humans who drank that milk later developed breast cancer [9].

## 2.6 Bovine Products on the Market

There are multiple bovine derived medical therapies available for human use. Trasylol (Aprotinin) is a proteinase inhibitor obtained from bovine lung, used in patients undergoing cardiopulmonary bypass surgeries to reduce inflammation and bleeding. The drug was discontinued in 2008 due to associated risks of kidney failure, heart attack, heart failures, and strokes. Although anaphylactic reactions were a potential concern from the product being of animal origin, bovine related reasons were not a factor to discontinue the drug [11]. The company, Lescarden, offers a bovine derived product, referred to as Catrix. Catrix is FDA approved for wound closing applications and is currently in testing for skin care and bio-cartilage applications. In wound closing, it is approved for pressure/stasis/diabetic ulcers, first/second degree burns, post-surgical incisions, radiation dermatitis and more [12]. Another product on the market, is bovine collagen hydrolysate. It is synthesized from the different forms of collagen and contains a high amino acid content to supplement decayed protein [13]. Bovine insulin is also used for human healthcare [14]. The aforementioned shows that FDA clearance of bovine-derived materials is achievable through the proper purification system to remove infectious disease agents, such as viruses.

**2.7 FDA Requirements**

The FDA issues many requirements to ensure the public safety who utilize healthcare products. In the case of viral reduction, they have precise specifications to approve products for use. They assess the appropriateness of viruses used, design of testing, log reduction factor, potential effects of variation in process parameters, and limitations of assays used for detection.

**2.7.1 Viruses Tested**

When analyzing the validity of the viral reduction system, the FDA considers which viruses were tested. The FDA provides a list of major bovine derived viruses that must be eliminated, but does not require every virus be tested. Instead, manufacturers can test one virus that overlaps with another based on their similar characteristics and research showing how they will respond to certain treatments. The FDA classifies the viruses chosen for testing into three types: relevant, model-specific, and model-nonspecific. Relevant viruses are within the same species; model-specific viruses are related (usually the same genus/family); and model-nonspecific viruses are random viruses in nature with a wide range of physico-chemical properties. The purpose of the model-nonspecific type is to demonstrate protection against random viruses in nature that could unexpectedly contaminate the sample.

The FDA recognizes it may be difficult to test certain viruses due to safety concerns. For example, HIV is categorized as a biosafety level 2 (BSL-2) virus and requires certain safety precautions that may not be available at all facilities. In this case, the testing party could use attenuated HIV or bacteriophages, which are accepted by the

FDA as HIV surrogates. An attenuated virus has altered nucleic acid that makes it less dangerous to humans if transmitted. Bacteriophages are a type of virus that infect bacteria and are not harmful to humans. Bacteriophages are becoming increasingly popular in viral reduction tests due to their similarity in physiochemical properties and sizes compared to their mammalian viral counterparts. They are also easier and cheaper to test with little safety concerns for humans [40]. The FDA states, "the choice and number of viruses used will be influenced by the quality and characterization of the [material source] and the production process," which includes mammalian viruses, attenuated viruses, and bacteriophages. After determining which viruses to test and exposing them to steps of the reduction system, there must be an "efficient and reliable assay for detection of every stage of viral reduction." Overall, the FDA issues these requirements to ensure public safety in potentially dangerous consumer products and healthcare treatments.

## 2.7.2 FDA Required Viruses for Reduction

The FDA requires bovine derived products to acknowledge and remove specific viruses that could potentially contaminate them. These viruses can be broken down into bovine-related and non-bovine related viruses. In total, there are fourteen viruses of concern and each virus has their own specific characteristics that can impact the viral reduction process.

**Table 1: FDA Required Viruses for Reduction in Bovine Derived Products [15, 17, 33]**

| | Virus | Size (nm) | Nucleic Acid Type | Genus/Family |
|---|---|---|---|---|
| **Enveloped** | Bovine Parainfluenza Type 3 | 150-200 | RNA (ss) | Paramyxoviridae |
| | Bovine Respiratory Syncytial Virus | 150-200 | RNA (ss) | Paramyxoviridae |
| | Rabies Virus | | RNA | Rhabdoviridae |
| | Infectious Bovine Rhinotracheitis Virus | 120-250 | DNA (ds) | Herpesviridae |
| | Bovine Herpesvirus* | 180-200 | DNA (ds) | Herpesviridae |
| | Bovine Diarrhea Virus (Model for Hepatitis C) | 40-70 | RNA (ss) | Togaviridae (Flavivirdae) |
| | Buynaviruses | 90-100 | RNA (ss) | Buynaviridae |
| **Non-Enveloped** | Reovirus | 60-80 | RNA (ds) | Reoviridae |
| | Bluetongue Virus | 60-80 | RNA (ds) | Reoviridae |
| | Bovine Adenovirus | 90-100 | DNA (ds) | Adenoviridae |
| | Bovine Parvovirus | 18-26 | DNA (ss) | Parvoviridae |
| | Circoviruses | 17-22 | DNA (ss) | Circoviridae |
| | Bovine Polyomavirus* | 40-50 | DNA (ds) | Polyomaviridae |
| | Bovine Retroviruses* | | | |

*Viruses of concern unassociated with bovine derivative

In Table 1, each virus is distinguished based on different characteristics. Most importantly, these viruses were categorized as enveloped or non-enveloped. The enveloping characteristic becomes important when investigating which potential viral reduction techniques to apply. Many viral reduction techniques are either applicable to only enveloped viruses or a combination of enveloped and non-enveloped viruses. Depending on the size of the virus, it allows for the necessary filter to be determined and implemented for viral reduction Furthermore, viruses can be categorized into their genus, which groups them with other viruses with similar properties. Using the genus characterization it will be possible to remove all viruses grouped under that specific genus [18]. Lastly, nucleic acid type becomes important when quantifying viral

concentration after applying the desired virus reduction techniques using polymerase chain reaction (PCR).

## 2.7.3 FDA Reduction Specifications and Testing

In testing the efficacy of the viral reduction system, the FDA requires spiking of pre-products with a known viral concentration. The initial amount is then compared to the concentration after each individual step. A log reduction factor is calculated for each step. Typically, a reduction factor greater than 4 for an individual step is considered effective [34].

$$10^{Ri} = \frac{(v_1 * 10^{a_1})}{(v_2 * 10^{a_2})}$$

$$where\ x = \frac{(v_1 * 10^{a_1})}{(v_2 * 10^{a_2})}$$

$$Ri = log_{10}(x)$$

Ri is the calculated log reduction factor; $v_1$ is the original sample volume (liters); $v_2$ is the final sample volume (liters); $10^{a1}$ is the initial viral titer; $10^1$ is the final viral titer.

The reduction factor can be added together after each step to estimate a total reduction factor for the entire system. However, as the FDA describes, this may overestimate the effectiveness of the system because of potential similarity in two or more reduction steps. For example, if reduction steps A and B each reduce viral contamination by 90% independently, theoretically, only 1% of the virus should remain after moving a sample through both these steps. This is not necessarily the case in practice, because if step B is similar to step A, it may not remove the viruses that step A could not.

The FDA also has standards for low viral concentrations to test reduction. This is usually the case in the actual product process where the pre-product is not spiked with viruses. In some cases, the FDA requires an estimation of the remaining virus based on the equation below.

$$P = \frac{(V - v)}{(V * n)}$$

$$if \ V \gg v$$

$$P = e^{-c*v}$$

P represents the probability the sample contains viruses; V is the overall volume used (liters); v is the volume of sample used (liters), and n is the number of infectious particles distributed in V. When a portion of the whole bulk is assayed for viruses, the P value above shows the probability that specific sample contains viruses based on volume distribution. This metric is required to ensure manufacturers test enough of the bulk material that is representative of the entire production bulk. With both the probability and log reduction factors, the FDA also wants manufactures to explain and justify their approaches for evaluating clearance and to provide two independent studies supporting the results.

## 2.8 Enveloped vs. Non-Enveloped Viruses

Viruses are intracellular pathogens that are composed of a wide range of different properties, including genetic material, function, structure, and more. These pathogens consist of a wide range of cellular molecules that enable their propagation [19]. One classification of viruses is based on their virion structure being enveloped or non-enveloped. Enveloped viruses have a lipid membrane and the structure is broken down into two components: the membrane and the core. The membrane is composed of

10

glycoprotein spikes, a membrane or matrix protein, and a lipid bilayer. The core of this structure either contains an icosahedral capsid or a helical nucleocapsid [20]. The main difference between non-enveloped and enveloped viruses is non-enveloped viruses to do not have a lipid membrane encapsulating the nucleic acids. The structures of non-enveloped viruses are icosahedral capsids that are made up of repeating subunits [20]. This type of virus propagates through cell lysis [19]. These components are important to consider when determining reduction methods.

## 2.9 Reduction Techniques for Enveloped Viruses

There are three different types of reduction techniques that are solely successful in the reduction of enveloped viruses, which are acid, pH, and solvent/detergent. Although each of these methods functions differently to remove the viruses, the fibrinogen and thrombin components cannot be denatured, damaged, or removed. If this occurs, the fibrin microthread VitaSutures will not form. This will be considered when choosing and modifying the techniques for reduction of enveloped viruses.

## 2.9.1 Chemical Treatments

An acidic pH can be used to disrupt the lipid envelope of a virus, which ultimately removes it and destroys the virus [21]. This procedure is typically an easy process to complete and requires a pH less than 6.0, with most studies confirming that the greatest viral reduction occurred at a pH of 4.0 [21]. A study completed by Valdes et al. used a combination of chromatography, low pH, and heating, and concluded that almost all enveloped viruses were inactivated and brought to undetectable levels when exposed to a pH of 3.0 [22]. Another study showed that approximately 90% of the enveloped viruses were removed at a pH of 6.0 [23]. When looking at bovine specific viruses, virus

11

reduction was also completed using 70% Ethanol or 4% NaOH by volume. The use of 70% Ethanol inactivated viruses after 30 minutes, while 4% NaOH removed viruses after only 5 minutes [15]. Depending on the viral characteristics, different pH levels need to be tested to determine the most effective level. Other key factors to take into consideration were the homogeneity of the acid solution, and the temperature and incubation time the product is exposed to the acidic pH [24,21]. Because proteins and enzymes are sensitive to pH changes, this may result in denatured fibrinogen and thrombin.

## 2.9.2 Solvent/Detergent

Solvents and detergents are organic mixtures that disrupt the lipid membranes of viruses, making this method inapplicable to non-enveloped viruses [24]. This disruption does not allow viruses to bind and infect cells [21]. These solvent/detergent reagents, such as Triton X-100 and Tri-n-Butyl Phosphate (TNBP), were added to the product for four to six hours, which includes two different incubation periods to ensure that the entirety of the product is exposed to the reagents [21]. This process occurs at temperatures ranging from 24°C to 30°C and the viruses are typically removed in just a few minutes [24,21]. The reagents must be combined thoroughly to create a homogeneous mixture. They generally do not cause protein denaturation during the process, which ensures proteins maintain functionality [24]. Importantly, after the product is already solvent/detergent treated, this method can be used in combination other techniques such as with nanofiltration and heat treatments [25].

12

## 2.10 Reduction Techniques for Enveloped and Non-Enveloped Viruses

Specific reduction techniques can be used in combination with those mentioned above to ensure the reduction of some non-enveloped viruses, along with enveloped viruses.

### 2.10.1 Nanofiltration

Nanofiltration is method in which viruses are prevented from crossing the membrane filter. Nanofiltration segregates proteins and viruses based on their size. The components larger than the specified pore size are retained and all others are able to flow through. These filters have small pore sizes between 15 and 45 nanometers [24]. The method of nanofiltration is a simple task to carry out and does not require the use of any extensive equipment [24]. When using this method, the protein solution volume, flow rate, filter integrity, protein concentration, and pressure can be altered to best fit the given experiment [24,21]. Studies have shown that nanofiltration is typically able to remove 4 to 6 logs of a variety of viruses in plasma solutions [24]. The nanofiltration process has removed many non-enveloped viruses that are resistant to current inactivation methods [24]. 90-95% of proteins have shown full recovery and upheld biological activity when subjected to nanofiltration [24]. To ensure protein recovery and functionality, the conditions at which this step is processed must be constant and mild with regards to pH, temperature, osmolality, and pressure. Nanofiltration does not have a specific length of time required to carry it out and it can be implemented during the manufacturing process or during the final purification process before thread formation [24].

Currently, nanofiltration techniques are used on human derived thrombin and fibrinogen. Methods for the treatment of solvent-detergent treated/dry-heated fibrinogen

13

and solvent-detergent-treated thrombin has also been performed [25]. Nanofiltration is a viable option for the reduction of bovine viruses in fibrinogen and thrombin solutions.

## 2.10.2 Heating Methods

### 2.10.2.1 Pasteurization

Pasteurization must occur in the liquid phase and begins by filtering the solution to ensure the reduction of particles that could trap and stabilize viruses [24,21]. Filter size depends on the characteristics of the protein and the potential viruses that could be in the source. After filtering, stabilizers are added to the product solutions, such as amino acids, sugars, or citrate [21], to avoid protein denaturation or damage at temperatures above 60°C [21,24]. These solutions are heated in either warm water baths or incubators at temperatures above 60°C for about ten to eleven hours [21,24]. Although this process runs for many hours, after only ten minutes most viruses are inactivated [21]. At the end of pasteurization, the stabilizers are removed by means of filtration, precipitation, or chromatography [24]. Afterwards, tests are conducted to ensure the proteins were not damaged and recovered from heat exposure, and that no viruses remain in the solution [21]. A downside to this viral reduction method is that it may reduce the biological activity of the proteins within the solution by up to 30% [24]. Currently, commercially developed fibrinogen sealants, composed of fibrinogen and thrombin, complete virus reduction using pasteurization at a temperature of 60°C for about ten hours [25].

### 2.10.2.2 Dry Heat

Dry heat is implemented at the final stage of processing and requires an oven with guaranteed homogenous exposure to temperatures ranging from 60°C to 100°C. The product must be exposed to these temperatures for anywhere between a few minutes and

14

a few days, depending on the viruses' characteristics, concentrations, and reduction resistance [24]. Fibrinogen and thrombin-based products are typically exposed to dry heat for about thirty minutes for virus reduction to occur [25]. Similar to that of pasteurization, the dry heat treatment also requires the usage of stabilizers [24]. This method is often deemed less effective than pasteurization, but alternatively it does not need to be applied to the liquid state, rather the final product [24]. Therefore, this method should be used as a secondary virus reduction method to ensure the reduction of more resistant non-enveloped viruses [24].

### 2.10.2.3 Vapor Heat

Vapor heating must occur when the product is in the dry form in order to be effective [24]. By adding water vapor to the product before heating, increased level of viral inactivation can be achieved [21]. This results in an environment with high moisture levels and no oxygen, that is steam-like [24]. Viral inactivation occurs over ten hours, when vapor pressures are between 1190-1375 mbar and the temperature remains between 60°C and 80°C [24]. Currently, commercial products are implementing steam treatment on fibrinogen and thrombin by exposing the dry components to steam at 60°C for approximately thirty hours [25].

### 2.10.2.4 Precipitation

Precipitation purification is a reduction method that is effective on both enveloped and non-enveloped viruses. This process is the fractionalization of the virus into solid and liquid components. Ethanol precipitation is the most prevalent form implemented worldwide [21]. Ethanol functions not only as a precipitant, but also a disinfectant.

However, this is typically only effective at room temperature and the precipitation process must occur at low temperatures [21].

In precipitation, components are separated during the transition between the solid and liquid phases. Once precipitated, the proteins undergo centrifugation to combine together the viral material. From this point, the viral bulk is removed through filtering [21]. This precipitation method must be coupled with other reduction technique to meet FDA standards.

The major drawback of this method is the consistency. It has proved difficult to expose each fractional component to the same amount of precipitate. The proof of viral reduction by precipitation is easy to obtain in small-scale productions, but is difficult to consistently prove in a large scale system [21]. This method has proven to be a great method used in the production of plasma based products, such as intravenous and intramuscular immunoglobulins [21]. Overall, precipitation is primarily dependent on the protein composition and the separation environment, which does not always prove easy to validate [21].

## 2.10.3 Chromatography

Similar to precipitation, chromatography is dependent on the protein composition and separation conditions [21]. The protein purification process of chromatography can remove both enveloped and non-enveloped viruses. Chromatography columns are loaded with an internal resin, which absorbs the protein or virus of interest. The column resin, the protein solution, and the buffer influence viral reduction. A drawback of this process is the inconsistency between viruses. Resins are virus-specific and will not remove all

possible contaminants. The quality of the resin may also affect the ability to purify the biological samples [21].

In ion exchange chromatography there is typically a 2-3-log reduction factor. In specific chromatography processes, such as affinity chromatography, up to a 5 log reduction has been verified [21]. There are a number of factors to consider when using this method:

- Column geometry
- Composition and flow rate of the buffers
- Intermediate wash steps
- Protein composition
- Preparation of the chromatography resin

In a study using ion exchange chromatography, albumin and IgG were purified of a few bovine related viruses, including Bovine Herpesvirus and Bovine Viral Diarrhea. The results showed more than 6.4 log reduction after the reduction. This method was run in combination with other clearance methods to optimize the viral reduction [26].

## 2.11 Viral Reduction Factor Testing Methods

As described in the statistics section (4.2.1) of the literature review, the viral reduction factor is indicative of the reduction system's effectiveness. A testing method must be employed in order to estimate the remaining viral concentration after the clearance process. Based on this value, the viral reduction factor can be calculated. The main testing methods examined here involve microscopy, plaque assays, quantitative polymerase chain reaction (qPCR) and outsourcing.

## 2.11.1 Microscopy

Using electron or fluorescent microscopy is a precise method to estimate remaining viral concentration. The Fluorescent Focus Assay (FFA) is a technique that

utilizes antibody staining for viral detection. Specific antibodies with fluorescent tags will bind to viral antigens. The number of tags seen under the microscope represents the amount of remaining virus for a given sample area. This method is considered more sensitive than many others, but ultimately more difficult and expensive. Many of the tags needed to detect viruses are not commercially available and if available are expensive [27].

Transmission electron microscopy (TEM) approach is another method for viral detection. TEM was one of the first detection techniques used for viral diagnosis, with a detection limit of 105 particles/mL. However, by the 1990s, other techniques were invented and were proven superior. TEM is advantageous for observing molecular characteristics, such as morphology or new viral classifications. Yet, this technique may require years of experience to distinguish viruses and cannot detect a large sample batch. Furthermore, this method was mostly replaced with assays that are cheaper, user-friendlier, and can assess larger quantities [28].

## 2.11.2 Common Industry Tests

TEM has been generally replaced with assays such as qPCR, enzyme-linked immunosorbent assay (ELISA), and hemagglutination assay (HA). The qPCR technique detects specific genetic base pairs on the viral genome. It does this through the use of fluorescent tags that binds to the genome, or a primer that contains a tag. As the genetic material becomes amplified, the tags are tested using cytometry. Their prevalence represents the amount of viral genetic material in the sample and is therefore, proportional to the amount of virus present. This method is highly reproducible, relatively time efficient, and involves moderate prep work. The main drawback is its cost through

purchasing the required tags, primers, and PCR kits. ELISA is another highly sensitive technique and uses antibodies for viral protein detection. The viral protein is extracted from the sample. An antibody will attach to the viral protein (antigen) that is also attached to an enzyme. A substrate is introduced and the activity of the enzyme is translatable to the concentration of virus. It is very sensitive, with a minimum detection range of $10^{12}$ - $10^9$ moles/L depending on the type of ELISA used. The HA test is an indirect way to test viral concentrations. It is based on the principle that specific viruses express the hemagglutinin protein on their surfaces and will form agglutinates with red blood cells. The amount of agglutinate, or clumps of red blood cells, that forms as a result of a known concentration of red blood cells can estimate viral concentration. The drawbacks include tediousness of sample preparation, and maintenance of fresh red blood cells [27].

### 2.11.3 Plaque Assay and Bacteriophages

Plaque assays can be used to test viral concentrations. There are many drawbacks when applying this method with mammalian viruses and it is often more useful when working with bacteriophages (viruses that kill bacteria). The assay works by spreading bacteriophages over a cultured lawn of bacteria. The bacteriophages infect and lyse the bacteria, leaving spotted areas indicating bacterial death. These areas, or plaques, represent the titer of bacteriophage that was in the initial concentration spread over the cultured plate. This method is less expensive than most common industry procedures, but is only available if bacteriophages are used for viral reduction testing.

## 2.11.4 Other Non-Traditional Methods and Outsourcing

The procedures described above are the most commonly used for calculating the remaining virus in a sample. Although not commonly used, flow cytometry is a method with increasing popularity in virology. It works by recording how light scatters as it passes through a sample. Originally, researchers did not believe they could identify a particle as small as a virus with this technique. In the late 1990s, researchers did detect viruses with flow cytometry using a nucleic acid staining dye. Brussaant et al. used the nuclear stain SYBR Green I and was able to detect a wide range of viral families [29]. Western blots have been utilized for viral protein detection as well. Viral detection via western blotting works through identifying, isolating, and quantifying certain proteins that are only expressed in the viral genome [30]. Another option, rather than in-house testing, would be to outsource the samples to a company that performs viral quantification testing. Most of these companies use the same techniques described above. BioReliance performs testing of bovine derived viruses based on the federal 9 CFR rules and regulations. This law overlaps with the FDA requirements for bovine and porcine products. Also, BioReliance can develop testing methods for the customer's needs [31]. There are a number of other companies that will detect and quantify viral contamination. Outsourcing would be easier, but could be the most expensive option depending on several factors.

# 3.0 Design Strategy

## 3.1 Initial Client Statement

The client provided us with the following initial client statement: The fibrin microthread sutures developed by the Gaudette and Pins lab needs to be translated from pre-clinical to clinical trials. To do so, a viral reduction system would need to be incorporated into the manufacturing process for FDA approval.

## 3.2 Design Requirements-Technical

### 3.2.1 Objectives

In this project, the main focus will be the reduction of bovine-related viruses from the Fibrin Microthread product. VitaSutures will need to meet FDA standards in order to ensure patient safety and move into clinical testing. The main objectives of the project are to:

- Identify and meet relevant FDA standards for bovine virus reduction
- Evaluate bovine virus reduction techniques
- Develop a system to quantify viral reduction based on a reduction factor
- Apply viral reduction techniques to various components of the manufacturing process
- Asses the ability of the product to accomplish performance specifications after reduction
  - Test mechanical properties of the threads to ensure 70% of the properties are retained
- Achieve a total viral reduction factor to meet FDA standards for bovine virus reduction

After further research and testing, our team will present a proposed solution and give future recommendations to WPI faculty, students and VitaThreads.

### 3.2.2 Functions & Specifications

In order for this viral reduction process to be developed, certain functions and specifications will be implemented. This project must be completed in a 25-26 week time

21

span, which encompasses the amount of time allotted for a Major Qualifying Project. A virus reduction process must be developed within the $750 budget and kept under an additional $10 cost added to each suture for the consumer market. A fibrin microthread suture with a viral reduction factor must be achieved for each step and ideally a viral reduction factor of 12 for the entire system, while still maintaining 70% of its original mechanical properties. In order to ensure the desired viral reduction factor, an accurate assay with a p-value less than 0.05 will need to be implemented.

### 3.2.3 Constraints

The constraints given by this project are broken up into lower and upper bound categories. The lower bound category is defined by the minimal constraints that need to be met. Constraints in the upper bound group must be met and cannot be compromised. In the lower bound category, the viral reduction must minimally meet the FDA requirements for viral reduction in the entire process. The purified threads must obtain at least 70% of the original mechanical properties. This threshold, provided by Gaudette and Pins labs, was based on the minimum required ultimate tensile strength to maintain functionality in clinical applications. In relation to the product, the fibrin microthreads must form after virus reduction. In the upper bound constraints, there is a limited budget of $750 supplied to the team through the institution. Given the limited budget, there is also a constrained amount of supplies and product available. Time is a very large constraint in this category with a time frame of 25-26 weeks.

### 3.3 Design Requirements-Standards

As described in the literature review, the FDA issues standards for viral clearance systems. These standards must be met in order for the FDA to approve the product for

human clinical trials. The FDA's three principle approaches for evaluating viral reduction systems is as follows:

1. Analyzing viruses tested and how they encompass all other potential viral contaminants.
2. Assessing the capacity of the production process to clear viruses, based on reduction factor calculations.
3. Testing the product at appropriate steps for viruses.

This project mainly focuses on standards 1 and 2. Standard 3 is specific to the full-scale production process. Within standards 1 and 2, the FDA provides more information as to how they judge the effectiveness of the clearance system, as described below:

- Appropriateness and selectivity of test viruses
- Design of the testing study
- Amount of viral reduction achieved
- Time dependence
- Effects of variations in process parameters
- Limitations of different reduction and testing methods

Within the "appropriateness and selectivity of test viruses," the FDA looks for three different classes of viruses to test, that both encompass enveloped and non-enveloped characteristics. These classes are:

- Relevant: the same virus or within the same species
- Model specific: closely related, within the same genus/family
- Model non-specific: no particular viruses, indicative of randomness of viral contamination in nature

Moreover, the second FDA standard encompasses the viral reduction factor. A reduction factor over 4 for one step is considered effective. All together, the total reduction of the clearance system must reach at least 12 [34]. The study must achieve a 95% confidence interval. Lastly, the FDA will look specifically at limitations in the system. These include, but are not limited to:

- Viral resistance
- Summation of reduction steps when calculating reduction factor may overestimate potential of system.
- Steps that too closely resemble one another in reduction technique. Identical procedures cannot be counted twice in reduction factor unless justified.
- Scaled-down process may not accurately reflect full scale.

In all, these FDA standards must be met in order to establish an effective viral reduction system and move towards human clinical trials.

## 3.4 Revised Client Statement

From the Gaudette and Pins labs, it was determined that there was a need to complete viral reduction in the fibrin microthread sutures. Viral reduction is an essential step to obtain FDA approval for translating research into clinical trials. Therefore, it was necessary to develop and design a process to overcome this challenge. The process must meet FDA standards, while maintaining at least 70% of the suture mechanical properties. Additionally, a method must be developed to test both the soluble and fibrin thread to quantify viral reduction.

# 4.0 Design Process

## 4.1 Needs Analysis

The project statement as a whole was further broken down into the specific needs and wants. The development of fibrin microthreads with a reduced virus concentration must be an inexpensive method that does not increase the cost per suture by $10. The viruses present in the fibrin microthreads must be removed while its components are in the powder or liquid phase, or after the suture development. This will ensure that the final product has reached the desired viral reduction factor of 12. In order to confirm the viral reduction factor, an assay with high accuracy ($p < 0.05$) must be implemented. This assay can be either completed in-house or outsourced for confirmation. After the viral reduction procedures, it must be demonstrated that the fibrin microthreads can be fully formed and retain 70% of its original mechanical properties. Without thread formation and at least 70% of the original mechanical properties, the fibrin microthreads will not function as effectively as a suture. All of these needs must be met in order to design a successful virus reduction process, but the incorporation of additional wants (*Table 2*) could improve the outcome of this project for the client.

**Table 2: Needs vs. Wants**

| Needs | Wants |
|---|---|
| As inexpensive as possible | Less than $10 additional cost per suture |
| FDA approval | Viral reduction by a reduction factor of 12 |
| Assay to quantify and confirm viral reduction factor | Ensure the current packaging system for sutures is not compromised |
| Maintain 70% of the original mechanical properties of the sutures | Incorporation of virus reduction process into the manufacturing process (continuous process) |

**4.2 Conceptual Designs**

**4.2.1 pH Alterations**

The first method used to treat both fibrinogen and thrombin was pH alterations. Changing the pH of the solution, to either acidic or basic, can be used to disrupt the lipid envelope of a virus for reduction [21]. The most effective solutions should be treated at a pH of less than 6.0 or greater than 10.0 to remove almost 90% of the present viruses [23]. The trials for pH testing can be seen in Table 3 below, which are in chronological order. All trials began with thrombin and fibrinogen dissolved in HBSS, that were treated to a specific pH level. Five minutes later the pH level was measured to determine if it had remained constant. If the pH level did not remain constant the solution pH was adjusted again to bring it back to the initial pH level. The solution pH was altered using 0.1 M NaOH for basic pH adjustments and 0.1 M HCl for acidic pH adjustments.

Thrombin and fibrinogen were both dissolved in a 20 mM HEPES buffered saline solution (HBSS) for microthread manufacturing. However, since HBSS buffers the pH capacity of a solution, the fibrinogen and thrombin must be dissolved into saline to accurately test the effect of the acid/base treatment. The final product would then be lyophilized and reconstituted in HBSS for microthread manufacturing.

The acid/base treatment began by altering the pH of saline to an acidic or a basic pH and then adding fibrinogen powder (Trials 1 & 2). Saline was utilized, in combination with the fibrinogen and thrombin, in order to provide enough solution for lyophilization and potentially allow for an easier pH alteration to avoid immediate clotting of the product. Within five minutes, the pH changed. Additionally, clotting was present in the solution and could not be brought back to their initial pH. It was then hypothesized that if

fibrinogen dissolved in saline were treated together, potential clotting would not occur (Trials 3 & 4). After bringing the pH to 4.00 or 12.00, both solutions reached an approximate neutral pH after 5 minutes and clotting was present. It was then hypothesized that if the solutions were brought past their isoelectric point (the point at which a molecule carries no net electrical charge) clotting could potentially be avoided. In trials 5, 6, 7, and 8 saline was altered to the highest basic pH (13.80) or the lowest acidic pH (-0.05) that could be reached. When fibrinogen was added to these solutions clotting occurred in the acidic treatments, although the solutions did not return back to a neutral pH after 5 minutes. The trials where fibrinogen was treated with base (Trials 6 and 8) were lyophilized, but could not be redissolved in HBSS. After trials 1-8, it was determined that fibrinogen could not be treated with acidic or basic conditions due to clotting or its inability to re-dissolve.

Next, acid/base treatment was performed on thrombin. Trials 9, 10, and 11 show that 0.4 ml (16 units total) of thrombin were added to 1.6 ml of saline for acidic or basic pH treatment. The pH of all three solutions were easily adjusted and remained constant at a pH of either 4.00 or 12.00. These solutions were then lyophilized, and trials 10 and 11 were able to go back into solution using HBSS and to be used to form fibrin microthreads with untreated fibrinogen.

**Table 3: pH Testing Trials**

| Trial | Treatment Type | Initial Component(s) Treated | Initial pH | Component Added | pH Post 5 minutes | Alteration Attempts |
|---|---|---|---|---|---|---|
| 1 | Acid | Saline | 4.00 | 140 mg Fibrinogen | 6.11 | pH back down to 4.0 |
| 2 | Base | Saline | 12.00 | 140 mg Fibrinogen | 9.10 | pH back up to 12.0 |
| 3 | Acid | Saline + 140 mg Fibrinogen | 4.00 | _____ | 6.47 | pH back down to 4.0 |
| 4 | Base | Saline + 140 mg Fibrinogen | 12.00 | _____ | 6.36 | pH back up to 12.0 |
| 5 | Acid | 3 ml Saline | 2.00 | 140 mg Fibrinogen | 4.00 | _____ |
| 6 | Base | 2 ml Saline | 13.97 | 140 mg Fibrinogen | 13.14 | Lyophilize |
| 7 | Acid | 2 ml Saline | -0.05 | 140 mg Fibrinogen | -0.37 | Neutralize |
| 8 | Base | 2 ml Saline | 13.94 | 140 mg Fibrinogen | 13.89 | Lyophilize |
| 9 | Acid | 1.6 ml Saline + 0.4 ml Thrombin (8 U/200 μL) | 4.00 | _____ | 4.00 | Lyophilize |
| 10 | Base | 1.6 ml Saline + 0.4 ml Thrombin (8 U/200 μL) | 12.00 | _____ | 12.00 | Lyophilize |
| 11 | Acid | 1.6 ml Saline + 0.4 ml Thrombin (8 U/200 μL) | 4.00 | _____ | 4.00 | Lyophilize |

### 4.2.2 Heating Method

The heating viral reduction method was broken down into dry and vapor heat. Dry heat is a method that should be applied to the final product and must be treated in a homogeneous environment. This method should be used as a secondary virus reduction method, to ensure the reduction of the more stable non-enveloped viruses [24]. Vapor heating should be used on the powder product in order to be effective [24]. When the product is exposed to water vapor before heating in an incubator-like system, it may lead to increased viral inactivation [21]. Methods are as follows:

#### 4.2.2.1 Dry Heat

The first heat treatment method encompassed a dry heat step applied to the final microthread product. Dry heat eliminates humidity. In this experiment, threads were placed into an oven at varying temperatures for thirty minutes. Four trials were run, each with a sample size of five threads. The dry heat trials can be seen in *Table 4.* After heat treatment, all threads were mechanically tested using an Instron.

**Table 4: Dry Heat Trials**

| Trial | Specifications |
|:-----:|----------------|
| 1 | 5 threads at 60°C for 30 minutes |
| 2 | 5 threads at 80°C for 30 minutes |
| 3 | 5 threads at 100°C for 30 minutes |
| 4 | 5 threads not exposed to heat treatment (Control Threads) |

#### 4.2.2.2 Vapor Heat

The second treatment tested was vapor heating of the fibrinogen powder. In the first component of the vapor heat testing, fibrinogen powder was exposed to water vapor

at 60°C for thirty hours. This is done prior to the dissolution of the fibrinogen. The next vapor heat experiment executed incorporated both vapor and dry heat, in which fibrinogen powder was first treated with vapor heated for thirty hours at 60°C. The powder was then immediately dry heated for an hour at 60°C. The control for these experiments was untreated fibrinogen powder. Threads were made with untreated thrombin and were all mechanically tested using an Instron.

### 4.2.3 Solvent Detergent and Protein Precipitation

Triton-X100 and TNBP are commonly used solvent detergents that can be utilized as a viral reduction method. Ammonium sulfate and ethanol have both been widely used for protein precipitation [37]. A combination of the solvent detergents and precipitation methods allow for this viral reduction technique to be incorporated into the viral reduction system. This protocol was derived from the Purification of Fibrinogen from Human Plasma by Ayman Ismael and supplemented with the needed alterations [39].

The fibrinogen powder was dissolved in HBSS to a final concentration of 70mg/mL. To begin, TNBP and Triton-X100 were added to the fibrinogen solution to a final concentration of 0.15% and 0.5%, respectively, with continuous stirring overnight. This was also tested at 2X and 5X concentrations of TNBP and Triton-X. The following day, a 4 M ammonium sulfate solution was made and chilled on ice. The solvent detergent treated fibrinogen solution was chilled on ice and then altered to a 1 M ammonium sulfate solution utilizing the 4 M ammonium sulfate solution. The treated fibrinogen solution immediately precipitated and was and stored on ice overnight to continue the precipitation. The next day, the fibrinogen solution was centrifuged at 4000 x g for 20 minutes. Upon completion, supernatant was removed and the remaining

protein was resuspended in an amount of HBSS equivalent to the amount of starting fibrinogen solution. The solution was treated with a second 1 M ammonium sulfate treatment and was chilled overnight for a second time. On the following day, the conical tube containing the fibrinogen product was once again centrifuged at 4000 x g for 20 minutes. The supernatant was then removed and resuspended in the same amount of HBSS as before. The tube containing the precipitated fibrinogen and HBSS was then vortexed and warmed in a water bath to obtain homogeneity and to solubilize the precipitated fibrinogen. Once achieved, threads were made with untreated thrombin and the final product was mechanically tested.

The protein purification process using the ammonium sulfate was repeated using ethanol as a substitute for the ammonium sulfate. To begin, 0.15% microliters of TNBP and 0.5% of Triton-X100 were added to the fibrinogen. Solution was stirred for approximately forty minutes. 10% ethanol was added and chilled at -4°C overnight.

### 4.2.4 Pasteurization

Both fibrinogen and thrombin were heated in their liquid states. There was no noticeable disruption in thrombin, but fibrinogen clotted when heated to 60°C or higher. Stabilizers were used to restrict clotting based on the literature. Literature demonstrated a reduction in clotted fibrinogen exposed to heat at 60°C or higher by using a sugar, amino acid, and magnesium salt cocktail [38]. As shown in Table 6, Trials 1-3 tested sucrose, serine, and magnesium chloride in ranges of 80-100% w/v, 7.5-10% w/v, and 50-100 mM, respectively. Trials 4-6 used xylitol instead of sucrose and glycine instead of serine. In trials 1-6, the concentration of fibrinogen was 70mg/ml, or 7% w/v. It was thought that this concentration was slightly high based on the paper's recommendations for a 0.5-6%

w/v fibrinogen [38]. Thus, 70 mg of fibrinogen was dissolved in at least 2 mL of HEPES or DI water, and trials were repeated with xylitol, serine, and magnesium chloride. All samples were placed in the incubator at 60°C for at least an hour. If there was any visible clotting, the samples were vortexed to see if the fibrinogen would dissolve back into solution.

### 4.2.5 Mechanical Testing

Mechanical testing was performed on threads made after their respective reduction technique. Threads were glued 30 mm apart on their corresponding ends to a piece of velum paper. The glued threads were hydrated in PBS for approximately 20 minutes, imaged, and the diameter measured using ImageJ; 5 measurements were taken at each end of the thread and then averaged. Threads were attached on each end to a tensile tester (Instron) that slowly pulled them apart at a strain rate of 10mm/min until failure occurred. Data was acquired using an Instron for tensile strength and strain, which was then used to calculate the elastic modulus and ultimate tensile strength (UTS). The UTS was the maximum stress before the thread ruptured and the elastic modulus was this stress divided by the respective strain. Results were compared across all threads to see which reduction methods maintained at least 70% of thread performance specifications in relation to the non-treated control. These results were used to propose, verify and select a final design.

### 4.2.6. Plaque Assay

Plaque assays were used to estimate bacteriophage titer after various stages in the reduction process. In this method, bacteriophages will infect and lyse bacteria leaving an area of no growth, referred to as a plaque. A relationship between number of plaques and

bacteriophage concentration can then be developed. The needed materials were purchased from Carolina Biological, which included the basic bacteriophage culture set B (item #121160), plaque assay plating set (item #124730), and additional bacteriophages (item #124425 and #124335). Upon arrival, the bacteriophages and bacterial cultures were kept at 4°C until use.

To validate this assay, plates were prepared by autoclaving the tryptone soft agar provided in the plaque assay plating set for 15 minutes at 121°C. The hot agar was allowed to cool to 50-60°C and then poured into the plates and allowed to solidify before being inverted and transferred to 4°C for storage. The *E. Coli* was in a hardened slant culture and a swab of bacteria was taken from the slant culture and inoculated in LB agar 6 hours prior to conducting the plaque assay. After this time, bacteria concentration was normalized to $10^8$ CFU/ml based on the 1.0 McFarland Standard and 100 µL of bacteria was plated in each trial. The first plaque assay used 1 mL of top agar in combination with the following amounts in *Table 5*. This was repeated without trial 4 during the next plaque assay run. The plates were placed at 37°C for 24 hours to allow plaques to develop.

**Table 5: Components of Plaque Assay Trials**

| Trial | Viral Parts | Viral Concentration* | Bacteria | MgCl2 (10mM) |
|---|---|---|---|---|
| **1** | 10^8 | 890 μL A | 100 μL | 10 μL of 1M solution |
| **2** | 10^6 | 890 μL B | 100 μL | 10 μL of 1M solution |
| **3** | 10^4 | 890 μL C | 100 μL | 10 μL of 1M solution |
| **4** | 10^2 | 890 μL D | 100 μL | 10 μL of 1M solution |
| **5 (negative control)** | 0 | 890 μL only PBS | 100 μL | 10 μL of 1M solution |

*Viral Concentration in serial dilutions:
A: 10 μL viral stock + 990 μL PBS
B: 10 μL A + 990 μL PBS
C: 10 μL B + 990 μL PBS
D: 10 μL C + 990 μL PBS

As no plaques formed, it was determined that the top agar inhibited plaque formation and was not used for the third and fourth attempts. For the third attempt, the solutions in *Table 5* were spread with a swab over the bacterial culture plate. In the fourth attempt, a zone of inhibition procedure was conducted using small paper discs with a diameter of 6 mm. The discs were suspended in the solutions from *Table 5* for 1 hour and placed on the agar plates with already applied *E. Coli*. The plates were incubated at 37°C for 24 hours and images of the plates were taken and were analyzed using ImageJ to determine diameter. From the changes in diameter, a curve was constructed that related the diameter formed to the bacteriophage titer.

This assay was also used for the final dry heat reduction step. The threads were degraded in a 0.5mL solution consisting of 20μL of 0.3 plasmin and 480μL PBS at 37°C overnight. The paper discs were then loaded and the test was performed identically to what was previously described.

## 4.3 Alternative Designs

FDA standards for viral reduction in bovine-derived products require two viral reduction techniques to be applied. Five different techniques were considered for viral reduction in both fibrinogen and thrombin: pH treatment, pasteurization, precipitation, dry heat and vapor heat. A combination of two to four of these methods were used for the final design, dependent on their viral reduction factors and their ease of implementation during the manufacturing process. Although the main goal was to have two methods that can be applied to both fibrinogen and thrombin, it was necessary to have separate methods for each component to achieve the highest possible viral reduction factor. All of these methods were tested to determine if threads were able to form and maintain 70% of their original mechanical properties. The viral reduction methods: dry heat, vapor heat, pasteurization, pH treatment methods, nanofiltration, and solvent detergent were all tested and applied to both thrombin and fibrinogen at their different states. After all of the methods were evaluated based on cost, thread formation, and thread mechanics the final design was determined.

## 4.4 Final Design Selection

A final design system that includes three potential methods was deemed efficient in viral reduction, as well as 70% retainment of the original mechanical properties. Solvent detergent can be applied to fibrinogen in the liquid state, while acid pH (4.00) treatment can be applied to thrombin. Once the fibrinogen and thrombin were treated, as described in the methods above, the liquid solutions were coextruded to make the final product, fibrin microthreads. After the fibrin microthreads were formed, the dry heat treatment method was be applied for 30 minutes at 60°C. The FDA recognizes

ammonium sulfate precipitation and pH alteration as primary reduction methods, while dry heat can only be implemented as a secondary method of viral reduction. *Figure 1* outlines the process for the treatments for viral reduction, as well as our quantification system to measure viral reduction.
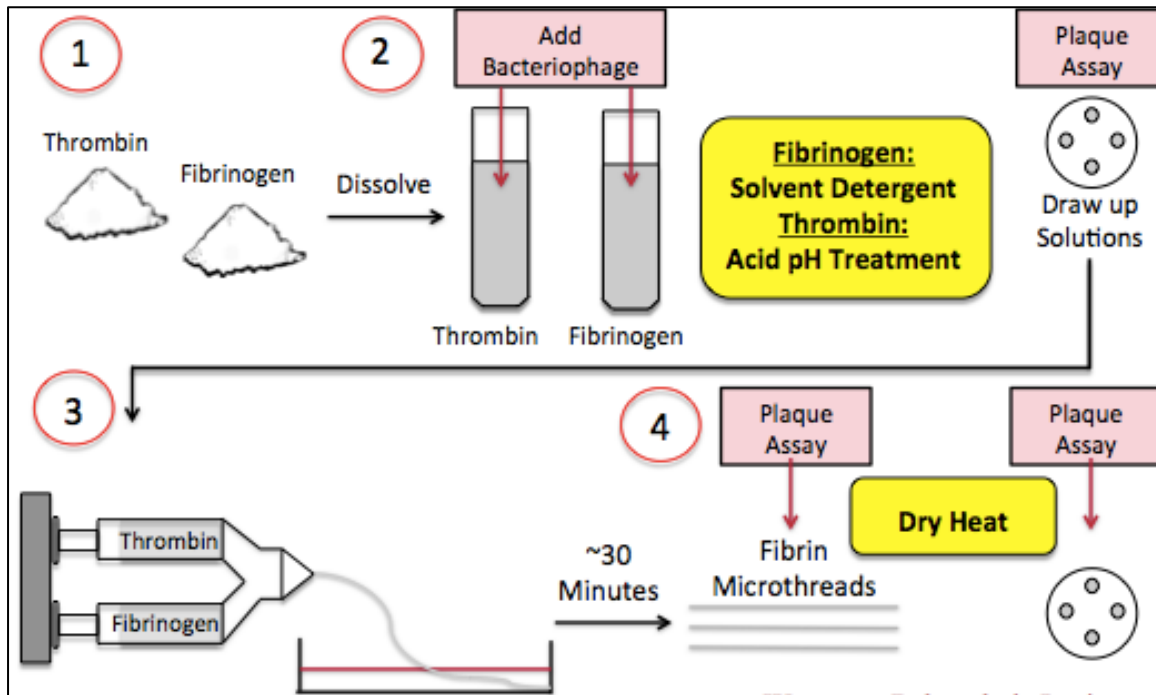

Figure 1: Final Design Process and Testing

## 5.0 Design Verification

### 5.1 pH Alteration Results

After completing the 11 trials mentioned in Section 4.2.1, only trials 10 and 11 were able to return the base products to a solution after pH treatment and lyophilization that could be used to create microthreads. Trials 1-9 either experienced clotting, could not be lypophilized, or failed to return to solution after lyophilization. The solutions needed to be lyophilized and then resuspended to regain the proper concentration to make threads. Trial 10 was base treated saline and thrombin and trial 11 was acid treated saline

and thrombin. Once resuspended in solution after lyophilization, these solutions were used separately to make fibrin microthreads.

The basic pH treated thrombin (trial 10) was used with untreated fibrinogen to make fibrin microthreads. When the threads were drawn out into the HEPES filled pan threads could be visually seen. However, the threads were completely adhered to the pan and could not be removed. The base pH treated threads could not be removed from the pan potentially due to the property changes of thrombin, which may have arose during pH treatment. A second attempt was made to create the threads from the same solution, but the same end result occurred. Therefore, we determined that the basic pH treatment of thrombin does not allow for thread properties to be maintained. The only successful trial from this study was the acid pH treated thrombin. This mixture could be lyophilized and brought back into solution and used to make threads with untreated fibrinogen. The threads formed as expected and the threads were easily removed and dried. After 24 hours of drying, the threads were removed from the drying rack and sections of the threads were used for mechanical testing. Based on these results, it was determined that the thrombin in solution with 850μL of 5 M $CaCl_2$ could be treated to a pH of 4.0 and then lyophilized. After lyophilization, the product was resuspended in 850μL of HBSS and 850μL of 5 M $CaCl_2$. This allowed for threads to be formed as normally expected. Thread properties were determined using mechanical tests. The results obtained from the mechanical testing, can be seen in section 5.6.

## 5.2 Dry Heat Results

The dry heat method applied to fibrin microthreads at varying temperatures did not visibly indicate any change to the threads. The threads did not appear to exhibit any

deformation or degradation. Mechanical properties were tested and results can be viewed in Section 5.6**.**

## 5.3 Vapor Heat Results

Fibrinogen was treated with vapor heat, for 30 hours at 60°C, during the treatment the fibrinogen absorbed water. After treatment, the fibrinogen weighed approximately two-fold more than the original product and could not be dissolved in the HEPES buffer solution. Next, fibrinogen was treated with vapor heat for 30 hours with an additional dry heat step to remove the absorbed water. Similar to vapor heat alone, the fibrinogen was unable to dissolve into the HEPES buffer. As the fibrinogen could not be dissolved, the thread formation step was not performed utilizing this treatment method.

## 5.4 Solvent Detergent with Precipitation Results

The solvent detergent method was successfully applied to fibrinogen, when used in combination with ammonium sulfate precipitation. After the second overnight chilling, the fibrinogen was centrifuged for a second time and resuspended in HBSS. After approximately thirty minutes of vortexing and warming in the water bath the fibrinogen was able to fully dissolve in HBSS. The remaining product was used to make threads with untreated thrombin. Fibrin microthreads were successfully formed when using the normal and 2X (0.3% TNBP and 1.0% TritonX-100), solvent detergent treated fibrinogen. However, the 5X (0.45% TNBP and 2.5% TritonX-100) solvent detergent treated fibrinogen and untreated thrombin did not allow for thread formation. Refer to Section 5.6 for a detailed explanation of the mechanical properties of threads formed after protein precipitation**.**

The solvent detergent method in combination with ethanol precipitation was unsuccessful. The fibrinogen and ethanol solution was stirred for forty minutes and the solution became cloudy (*Figure 2*). The flask was chilled overnight and the next day it was found that the contents had formed into a gel. The gel did not go into solution and threads could not be manufactured from this trial.
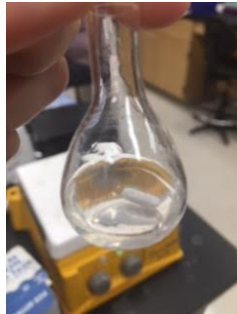


**Figure 2: Ethanol Precipitation Flask**

## 5.5 Pasteurization Results

As described in Table 6, trials 4-6 yielded an excessive amount of clotted fibrinogen, which prohibited thread making. Table 7 shows the trials with diluted fibrinogen concentrations (3.5% w/v or lower). Trial 9 had minimal clotting and was passed through a 27G needle to remove solids and make threads. The threads did not form in the pan, most likely due to a dilute concentration of fibrinogen. Pasteurization was deemed an unsuccessful method for treating fibrinogen for viral reduction.

**Table 6: Pasteurization Trials**

| Trial | Sucrose (% w/v) | Serine (% w/v) | MgCl$_2$ (mM) | Result |
|---|---|---|---|---|
| 1 | 80 | 7.5 | 50 | Clotted |
| 2 | 100 | 10 | 100 | Clotted |
| 3 | 80 | 7.5 | 50 + 90mM CaCl$_2$ | Clotted |
| | Xylitol (% w/v) | Glycine (% w/v) | MgCl$_2$ (mM) | Result |
| 4 | 80 | 7.5 | 50 | Partial clot |
| 5 | 100 | 10 | 100 | Partial clot |
| 6 | 80 | 7.5 | 50 + 90mM CaCl$_2$ | Partial clot |

**Table 7: Further Pasteurization Testing**

| Trial | Specifications | Result |
|---|---|---|
| 7 | Repeat trial 4, but with 3.5% w/v fibrinogen | Partial clot - no noticeable difference |
| 8 | Repeat trial 4, but dissolve xylitol in water prior to adding with other stabilizers and fibrinogen | Clotted |
| 9 | Repeat trial 5, but with 70mg/3ml fibrinogen | Smallest clot out of all trials. Was filtered using 27G needle. Threads did not form most likely from lack of fibrinogen concentration. |
| 10 | Repeat trial 9 to increase fibrinogen concentration. 140mg/3ml fibrinogen | Clotted |

## 5.6 Mechanical Testing

As described previously, fibrin microthreads underwent mechanical testing after their respective viral reduction technique in the absence of viruses. For initial mechanical testing, tested groups were either treated thrombin or treated fibrinogen, but not the combination treatment. This procedure was implemented to determine which methods would most effectively maintain at least 70% of their mechanical properties, analogous with the revised client statement. Additionally, these data informed which treatments would be used going forward to test threads made from treated fibrinogen and thrombin. Tensile strength was tested, because that is the most important mechanical property of the

threads in order to ensure efficiency in clinical applications. Elastic modulus was calculated by dividing ultimate tensile strength (UTS) by strain at the UTS. See Appendix B for the stress/strain curves of each reduction technique. The results were demonstrated that all groups maintained at least 70% of the original mechanical properties in relation to the control threads. The UTS and elastic modulus were variable and not all groups maintained 70% UTS and/or modulus. *Figures 3, 4, and 5* compare strain, UTS, and modulus, respectively. The bars represent mean and the error bars are standard deviation. Table 8 displays which techniques were tested to determine their UTS, strain, and modulus in relation to the original control values. For each method, 6-8 threads were used for mechanical testing.
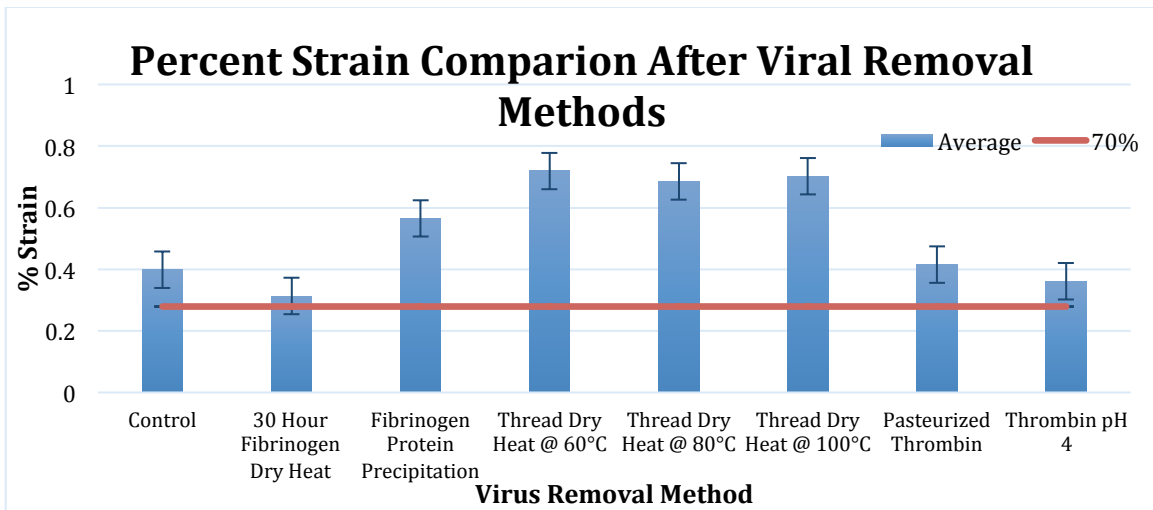


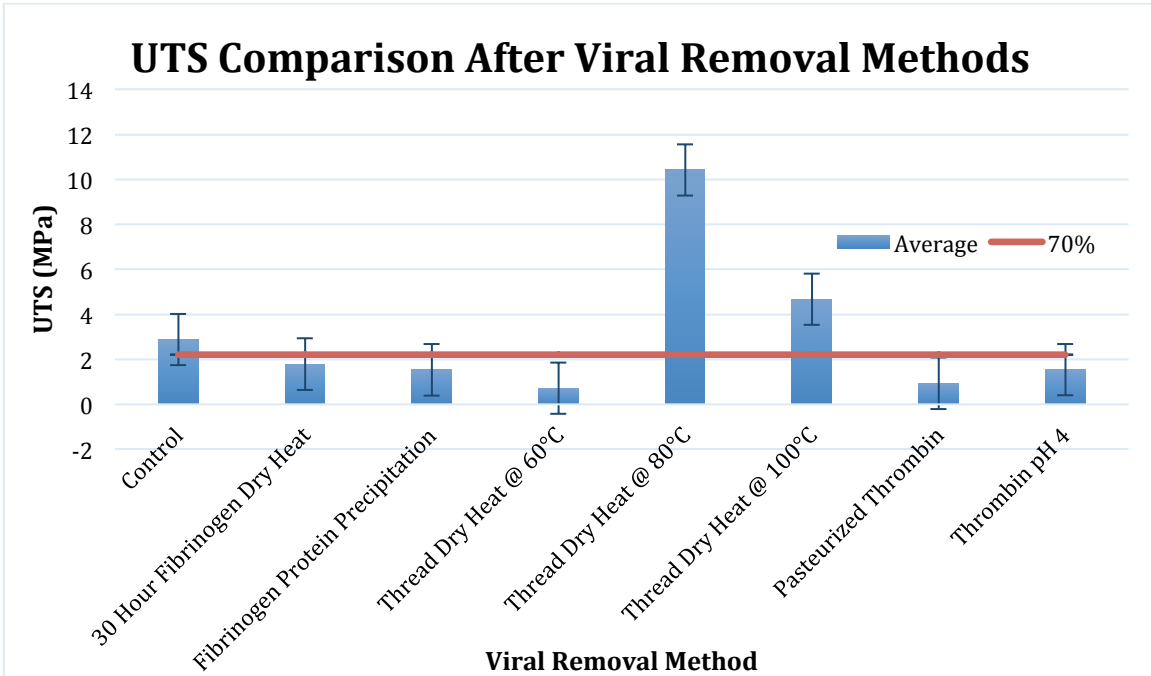**Figure 3: Percent Strain for Each Tested Viral Reduction Method**

**Figure 4: UTS Measurements for Each Tested Viral Reduction Method**
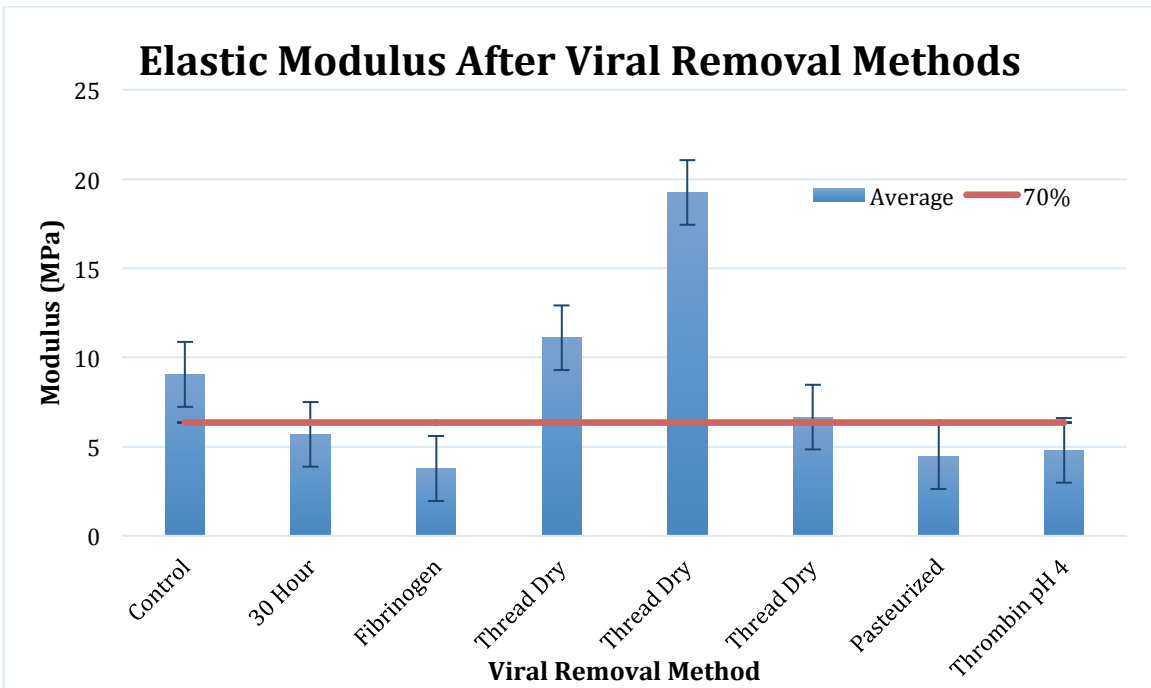


**Figure 5: Elastic Modulus Measurements for Each Tested Viral Reduction Method**

**Table 8: Comparison of UTS, Strain, and Elastic Modulus Values for Tested Viral Reduction Methods**

| | UTS | Strain | Elastic Modulus |
|---|---|---|---|
| **Fibrinogen Dry Heat** | No – 62% | Yes | No – 63% |
| **Fibrinogen Protein Precipitation** | No – 53% | Yes | No – 42% |
| **Thread Dry Heat 60°C** | No – 25% | Yes | Yes |
| **Thread Dry Heat 80°C** | Yes | Yes | Yes |
| **Thread Dry Heat 100°C** | Yes | Yes | Yes |
| **Pasteurized Thrombin** | No – 32% | Yes | No – 49% |
| **Thrombin with pH of 4** | No – 53% | Yes | No – 53% |

The two methods with 70% or more retainment of the original mechanical properties of the fibrin microthreads ere dry heating at 80°C and 100°C. This would only count for one step in the reduction processing according to the FDA. Therefore, using these data it was determined that acid treated thrombin and precipitated fibrinogen yielded the best results and were retested. For the retest, all threads were made on the same day and made by the same person to further control additional variables. *Figures 6, 7, and 8* show the mechanical testing results for the retest, where bars are the mean and error bars are standard deviation. The treated (no heat) group refers to only the fibrinogen protein precipitation and acid thrombin, while the treated (with heat group) includes the former with dry heat, for 30 minutes at 60°C. The red line across each graph refers to 70% of the control.
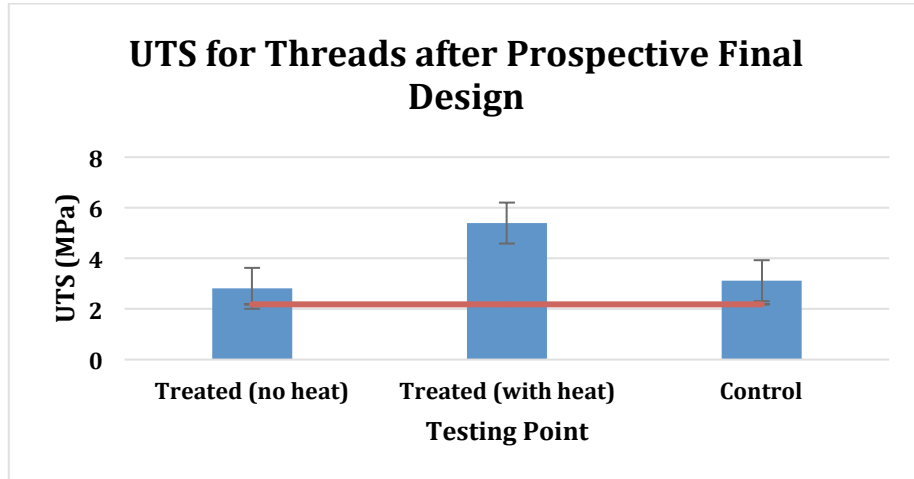
**Figure 6: UTS of Threads after Fibrinogen Protein Precipitation/Acid Thrombin, and after Dry Heat**
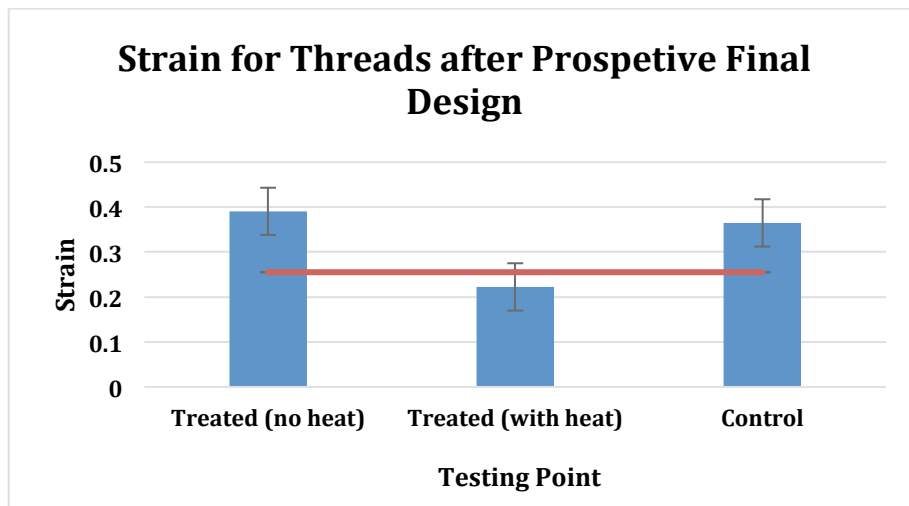


**Figure 7: Strain of Threads after Fibrinogen Protein Precipitation/Acid Thrombin, and after Dry Heat**
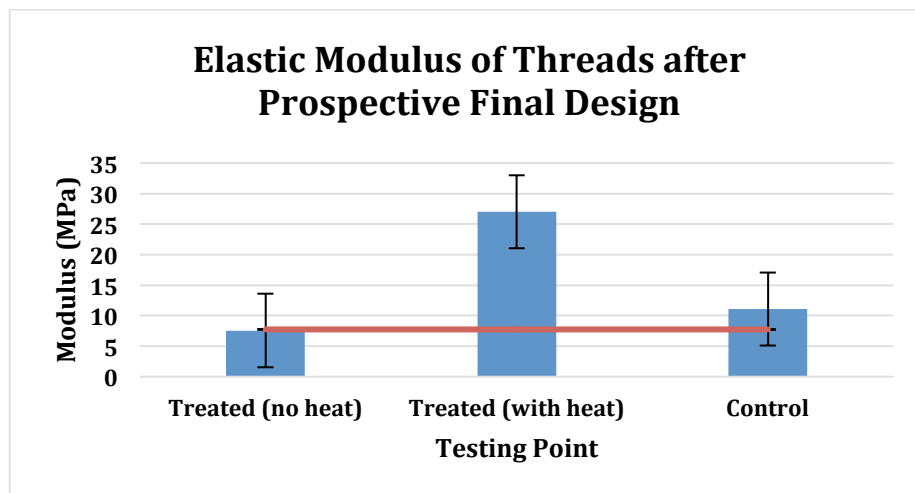


**Figure 8: Elastic Modulus of Threads after Fibrinogen Protein Precipitation/Acid Thrombin, and after Dry Heat**

Fibrin microthreads made from treated fibrinogen and thrombin and subsequently heat-treated maintained UTS and modulus values that were at least 70% of the controls. Confirming that the mechanics of treated microthreads were comparable to the control, the next step was to test these methods for a viral reduction using fibrinogen and thrombin spiked with bacteriophages.

## 5.7 Plaque Assay Validation Results

A plaque assay was run according to Carolina Biologics protocol using a top agar solution and a Φx174 phage with *E.Coli*. This solution contained magnesium chloride for phage attachment, varying bacteriophage concentrations diluted in PBS, $10^6$ CFU of *E. Coli*, and a top agar provided by Carolina Biologics. The top agar solidified before it was plated, so a warming plate was used to liquefy the solution to allow it to be poured onto the base agar. Results from this initial experiment demonstrated that no plaques were formed. However, since no bacteria formed on the control it was hypothesized that rewarming the top agar killed the *E.Coli* (*Figure 9*).
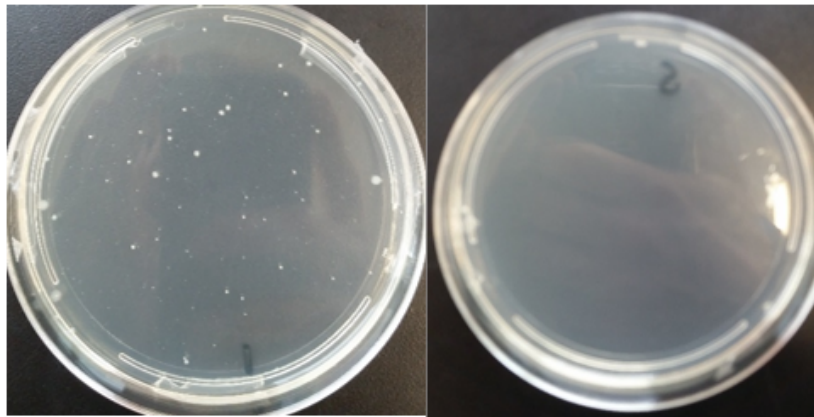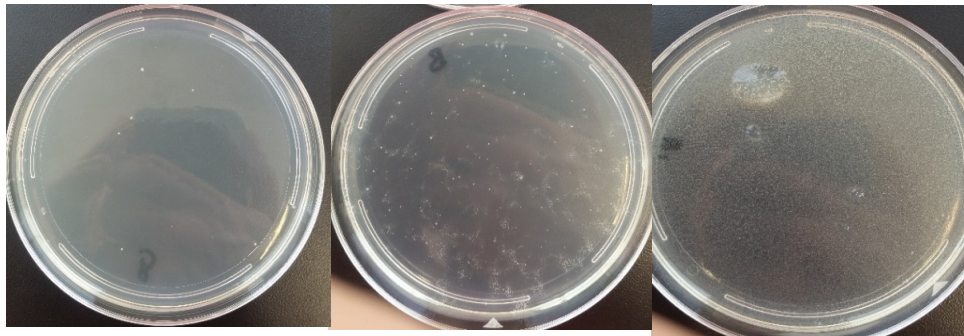


**Figure 9: Plaque Assay Trial 1-The left picture displays the $10^8$ titer plate with bacteria and the right is the positive control with only bacteria.**

In a subsequent attempt, the top agar was maintained in a $50^0$C bath and quickly plated to avoid boiling and killing the bacteria. Three different concentrations of phages were used: $10^8$, $10^6$, and $10^4$. In this trial, the positive control formed a lawn of *E. Coli*. The $10^8$ and $10^6$ plates showed almost no bacterial growth, but $10^4$ showed little bacterial growth with large plaques (*Figure 10*). This data indicates that the high concentration of bacteriophage was spreading and killing the bacteria and lower concentrations of phage need to be used to visualize plaques.



**Figure 10: Plaque Assay Trial 2- The left picture is $10^8$, the center picture is $10^4$, and the right is only bacteria.**

As the top agar demonstrated difficulty in forming plaques, an assay was conducted without top agar using the cotton swab method. Plaques were produced in the $10^4$ and $10^2$ titers, but were difficult to quantify (*Figure 11*). No plaque formation occurred in the $10^8$ and $10^6$ titers, but it is hypothesized that these concentration were too high and kill all bacteria present. To develop a relationship between plaque number and bacteriophage concentration, at least three plates were used to construct a curve, however only two plates produced plaques and the $10^4$ could not be accurately quantified.
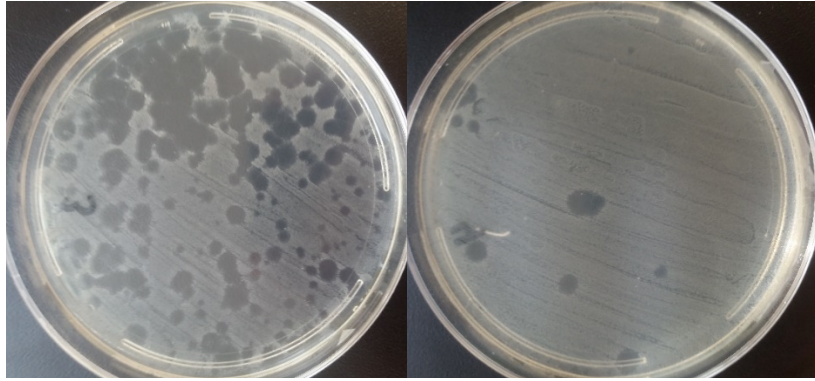
**Figure 11: Plaque Assay Trial 3- The left is $10^4$ titer and the right is $10^2$ titer.**

To further optimize the assay, a zone of inhibition assay was performed as described in the methods section. Briefly 6 mm paper disks were soaked in the respective phage solution for 1 hour and were placed on an agar plate with a lawn of *E. Coli*. As the phage diffuses from the paper it will kill the bacteria forming laques around the paper (*Figure 12*). Greater concentrations of bacteriophage produced larger dead zones (zone of inhibition diameters) around the paper discs.
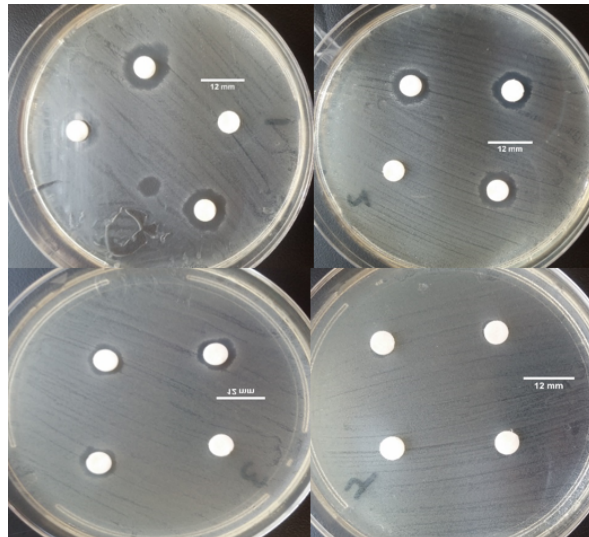


**Figure 12: Plaque Assay Zone of Inhibition Trial 4- The top left and right are $10^8$ and $10^6$ titers, respectively; the bottom left and right are the $10^4$ and $10^2$ titers, respectively.**

These results were quantified to produce a correlation between magnitude of bacteriophage titer and the average diameter of the zone of inhibition trials (*Figure 13*).

47

The exponential curve created was used in the following tests to quantify bacteriophage titer remaining after treating the fibrinogen and thrombin products. The minimum detectable limit was $10^2$ titer of bacteriophage.
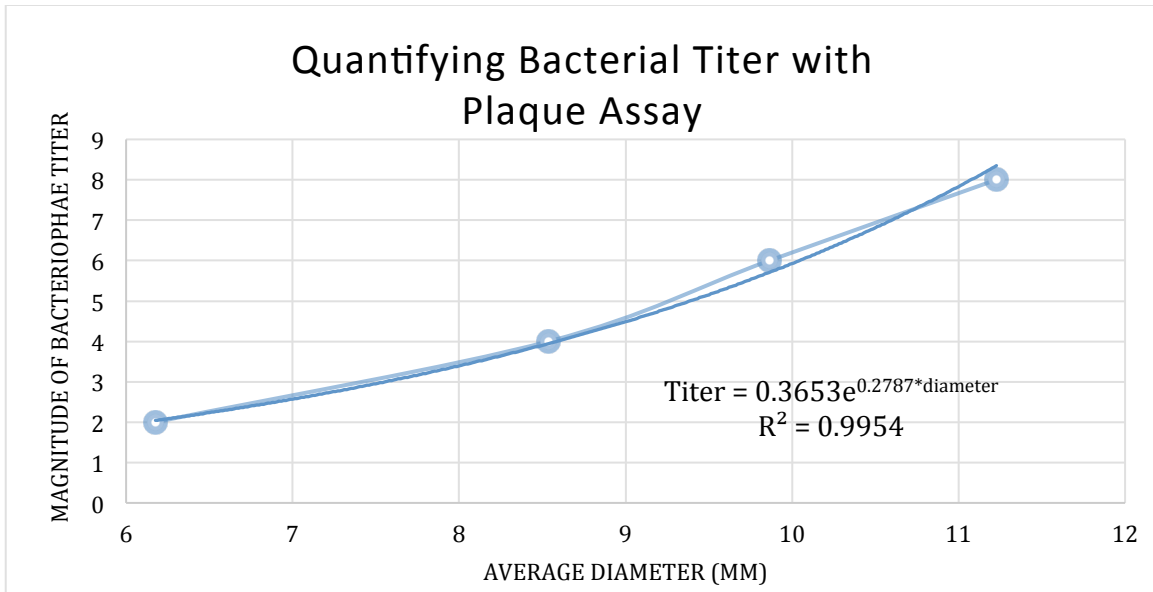


**Figure 13: Relationship Between Plaque Diameter and Titer**

## 5.8 Viral Reduction Results

The zone of inhibition plaque assay was applied to quantify a viral reduction factor for each step in the design. For the acid pH treated thrombin and solvent detergent treated fibrinogen, samples were spiked with $10^8$ particles of bacteriophage and run on the plaque assay after their respective reduction step. In the final dry heat method, bacteriophages were loaded at a titer of $10^8$ in the fibrinogen and thrombin, prior to making threads. It was anticipated that some bacteriophage would be lost when extruding threads. To compensate, the plaque assay was run comparing spiked threads with and without dry heat. *Figure 14, 15, and 16* show plaque assays for final acid thrombin, solvent detergent fibrinogen, and dry heat treatments, respectively.

48
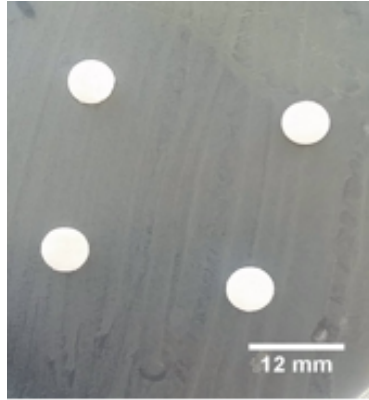
**Figure 14: Acid Thrombin Reduction Step**
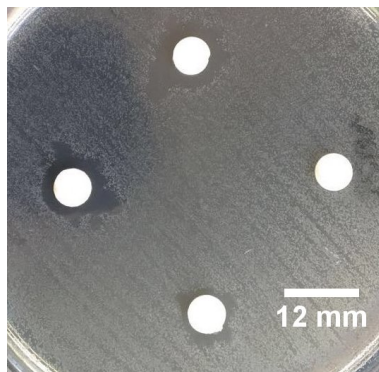


**Figure 15: Solvent Detergent Fibrinogen Reduction Step: 2X Normal Concentration**
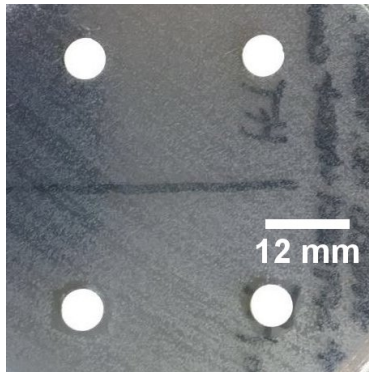


**Figure 16: Comparison of loaded/dry heated threads (top two discs) and loaded/non-dry heated threads (bottom two discs)**

Based on these results, the zone of inhibition diameters were measured and used to calculate subsequent viral concentrations. Twice as much starting material was used in the solvent detergent method to allow for greater viral reduction. *Figure 17* compares the normal, 2-fold increase, and 5-fold increase of Triton-X and TNBP for solvent detergent.

Additionally, the assay reached its minimum detectable limit of $10^2$ bacteriophages in thrombin and the dry heat steps. Therefore, the reduction factor was expressed as greater than the reduction value. *Table 9* shows these results.
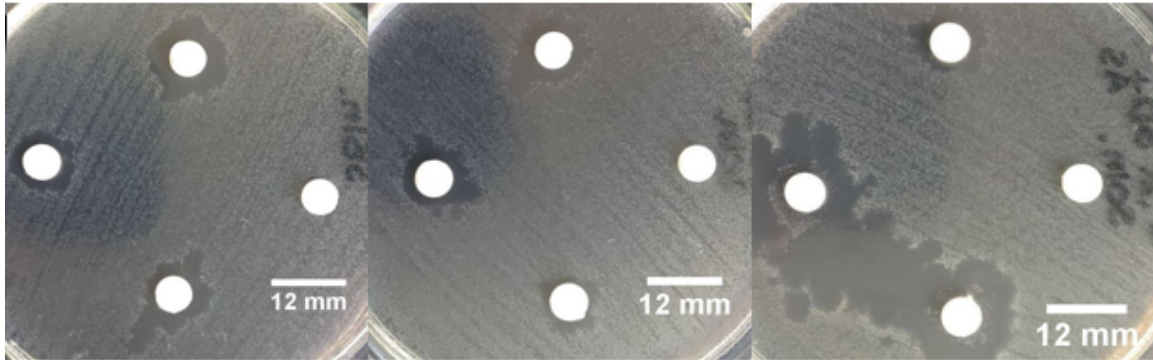


**Figure 17: Solvent Detergent Reduction Results. Normal SD (left), 2x SD (middle), 5x SD (right)**

**Table 9: Viral Reduction Results**

| Reduction Step | Diameter (mm) | Reduction Factor | FDA Effectiveness |
|---|---|---|---|
| **Acid treated thrombin** | 0 – reached minimum detectable limit | Reduction > 6 | Highly effective |
| **Solvent detergent treated fibrinogen** | 8.93 ± 0.91 | Reduction = 3.6 | Moderately effective |
| **Dry heat treated fibrin threads** | Without dry heat: 8.58 ± 0.41 | Reduction > 2 | Moderately effective |

# 6.0 Final Design and Validation

## 6.1 Final Design

After evaluating the different outcomes, such as thread formation, cost, and thread mechanics, the final viral reduction system was determined. The final viral reduction process includes three necessary steps to ensure the desired viral reduction within the final fibrin microthread product. When the fibrinogen and thrombin products were dissolved in HBSS and reach their liquid form, the primary viral reduction steps can be implemented. The thrombin was treated using pH alteration by adding HCl to allow for the pH to reach 4.00. The fibrinogen was treated using a solvent detergent method that

50

includes the usage of Triton-X and TNBP, as well as the incorporation of ammonium sulfate procedures for precipitation of the solvent detergents from the final product after treatment. Once the fibrinogen and thrombin were treated they were co-extruded to form the fibrin microthreads. The fully formed fibrin microthreads were treated using terminal dry heat for thirty minutes as a final viral reduction step. A combination of all three of these processes allowed for the best viral reduction to occur in the fibrin microthread manufacturing process (*Figure 18)*, in order for the threads to meet FDA standards and gain eventual FDA approval.
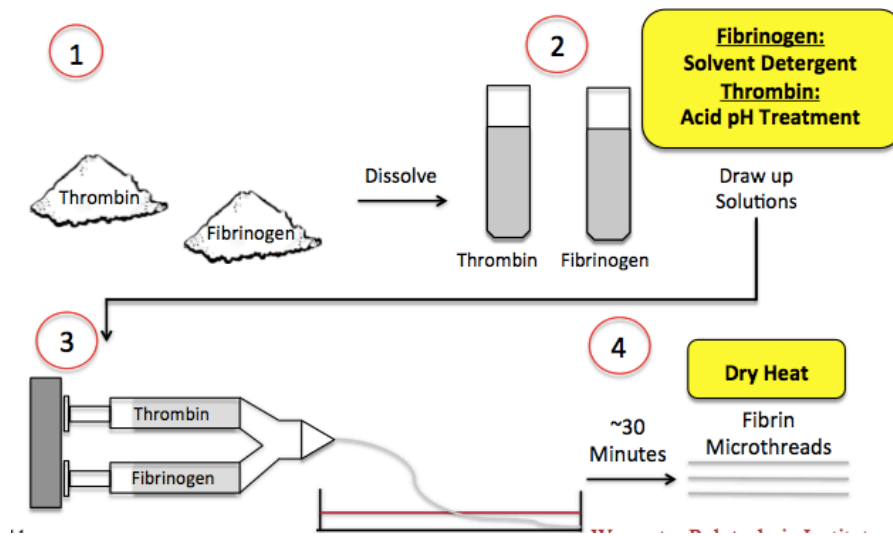


**Figure 18: Integration of final viral reduction system into the existing manufacturing process**

The final design will ideally be integrated into the manufacturing process in the future. There is a strong belief that fibrin microthread sutures will be used universally throughout the healthcare field. The implications that this product will have on the economy, environment, society, politics, ethics, health, safety, manufacturing, and sustainability will need to be considered.

## 6.2 Economics

There are economic factors that must be considered for the eventual implementation of the viral reduction system into the manufacturing process. The economic factors include the cost of the materials needed to execute the viral reduction process, the required machinery to complete the necessary processes, and finally the man-hours required for its successful process fulfillment. It was determined that the cost per 100 sutures would be less than one dollar, based on the cost of materials once the processes are carried out on a larger scale (*Appendix B)*. Additionally, the necessary machinery to complete these procedures would be a machine that adds a required amount of HCl to the fibrinogen to reach the desired pH level, a lyophilizer, a sonicating bath to dissolve the solvent detergent treated fibrinogen, and an oven to provide the final dry heat step. The man-hours would be required for adding the ammonium sulfate precipitation materials, as well as ensuring that the threads are brought through all of the necessary viral reduction steps in a sterile manner. Overall, the addition of this viral reduction process will cost a minimal amount in order to get the manufacturing process to meet FDA requirements and standards.

## 6.3 Environmental Impact

In terms of environmental impact, the viral reduction system for fibrin microthreads will not directly cause a significant impact. However, with the incorporation of this design, FDA approval is likely after the inclusion of this safety precaution. VitaThreads would expect to produce these threads for commercialization not long after FDA approval is achieved. With mass production comes the need for increased products, primarily a bovine source. This increase in demand can have both a positive

and negative impact on the environment. With an increased need increase for bovine sources, an expansion in population, shelter, land, and resources will be required. On the contrary, with this enlargement there is a possibility for the development of renewable energy sources from produced waste. Although minimal and in the long term, the viral reduction system for fibrin microthread sutures will have an impact on the environment.

## 6.4 Societal Influence

With the possibility of future implementation of fibrin microthreads in the healthcare field, there is a significant deal of societal impact. The fibrin microthread was developed to improve patient quality of life, by providing a better alternative to suture products already available on the market. These threads will provide patients with an enhanced quality of life by shortening the wound healing period and inducing less fibrotic tissue formation. With this option also comes a number of potential applications for the sutures throughout the body, whether they be internal or external. All applications would improve the quality of patient treatment and thus have a positive impact on the well being of the patient and society as a whole.

## 6.5 Political Ramifications

There are no influences from the development of this viral reduction process that would have an impact on global politics. Since the usage of VitaSutures has not been introduced to the clinical space yet, the threads are only in the research, development, and manufacturing process at VitaThreads. Therefore, there would be no current political ramifications associated with the fibrin microthreads.

## 6.6 Ethical Concerns

The incorporation of viral reduction steps into the thread formation process does not impose any major ethical concerns. The primary purpose of the design study is to better the lives of patients by ensuring safety and positive health effects from the use of bovine derived fibrin microthread sutures. However, ethical concerns stem from the products used to form these threads, since both the thrombin and fibrinogen components are derived from bovine. Additionally, there are some patient cases in which animal derived products will not wish to be used due to the preservation of animal rights.

## 6.7 Health and Safety Issues

The addition of a viral reduction system in the manufacturing process will improve the safety of the patients treated using fibrin microthread sutures. The microthread product is comprised primarily of bovine derived fibrinogen and thrombin. With the use of any mammalian-based products, the risk of viral transmission is presented to the patient. The fibrin microthreads are currently sterilized to kill bacteria and fungi by means of ethylene oxidation, however this method is not effective against viruses. With the incorporation of the viral reduction system proposed in this design project, viral concentrations are reduced. This viral reduction also minimizes the risk of viral transmission to the patient, ultimately increasing the safety of this product.

Fibrin microthread sutures also improve the health of the receiving patient. These threads have been proven to reduce scar tissue formation, as well as increase the wound-healing rate. By reducing wound healing time, the risk of complications, such as infection and wound reopening, is reduced. Scar tissue often times can negatively affect surrounding tissues by causing pain and limiting functionality. The reduction of the scar

tissue formation, with the use of these fibrin-based sutures, can limit, and in some cases eliminate, the risks of these side effects.

## 6.8 Manufacturability

The process established in the proposal for a viral reduction system was designed for incorporation into the original manufacturing method. The overall steps for producing the fibrin microthreads were not altered, however, additional steps were added to ensure patient safety. The fibrinogen and thrombin initially arrive in powder form are dissolved in a buffered solution, at which point two viral reduction steps are added. Fibrinogen is treated with a solvent detergent and precipitated using ammonium sulfate and the thrombin pH is altered to an acid pH at this phase. These solutions are draw up and extruded together into a HEPES solution where the threads form. The threads are allowed to dry and the viral reduction process is completed with a dry heat treatment. The new design process can be carried out solely by human work or can be executed by a machine-based process, which would ultimately expedite the manufacturing time. This method can also be easily scaled for manufacturing purposes for mass production.

## 6.9 Sustainability

The implementation of the viral reduction system will require the usage of machinery to execute the different procedures, in addition to the equipment already in use for the production of the fibrin microthread sutures. Therefore, the equipment utilized will require a electrical energy input to function, in addition to the man-power to complete additional procedures. All of this equipment can be incorporated into the already developed manufacturing process and thus will have a minor or nonexistent impact on the environment.

# 7.0 Discussion

The purpose of evaluating the different viral reduction methods for fibrin microthread formation, thread mechanics, and cost allowed for the final design to be determined. These three outcomes are crucial for both maintaining the original properties of the fibrin microthreads and ensuring that the cost is low enough for its integration into the current manufacturing process.

The acid pH treatment is applied to the liquid thrombin during the second step of the fibrin microthread manufacturing process. It was recognized that when the thrombin was treated for at least five minutes at a pH of 4.00, viral reduction occurred. This method of viral reduction is an inexpensive method that can be quickly and easily added to the manufacturing process. Typically less than 10 mL of HCl was utilized to pH enough thrombin to make a batch of 100 fibrin microthreads. The plaque assay was run to determine the viral reduction factor of this specific method and it was determined that a viral reduction factor of greater than 6.0 occurred, which is considered to be a highly effective step according to FDA standards. The reason that the exact viral reduction amount could not be determined was due to a limitation in our maximum bacteriophage concentration ($10^8$) the minimum detectable limit of the plaque assay. Therefore, this acid pH treatment can be viewed as an independent reduction step applied to thrombin and can be expected to have a higher viral reduction factor when the method is evaluated by a third party for FDA approval.

The solvent detergent treatment applied to the liquid state of fibrinogen was determined to be most effective when the 2X concentration of Triton-X and TNBP were applied, as opposed to the normal concentrations and the 5X concentrations. This was

further validated using the plaque assay testing, which determined that the solvent detergent method causes a viral reduction of 3.6 occurred. This viral reduction level is considered to be a moderately effective step according to FDA standards. This solvent detergent method can also be viewed as an independent viral reduction step that is applied specifically to the fibrinogen. A specific viral reduction value was able to be determined for this step because it was above the minimal detectable limit of the plaque assay and below the maximum bacteriophage concentration.

The final fibrin microthread products are exposed to terminal dry heat for thirty minutes at 60°C, as a final viral reduction step. This process can likely be implemented at any high dry heat temperature between 60°C and 100°C based on our mechanical properties data, but 60°C was thoroughly tested for this project due to what was available in the lab space. The plaque assay determined that the dry heat procedure results in a viral reduction of greater than 2.0, which is considered to be moderately effective according to FDA standards. Again, due to the limitations that arise from the usage of the plaque assay, the precise viral reduction value is unable to be determined, but it is hypothesized that the reduction factor is significantly higher than 2.

By proving that all three methods incorporated into the final design result in a certain level of viral reduction, it was determined that the FDA requirement of two independent reduction steps must be applied to the product was met. The ease at which these cost efficient methods can be completed, shows that this viral reduction procedures can be readily incorporated into the preexisting manufacturing process.

Lastly, the decision to use a plaque assay for viral reduction quantification was chosen due to its ease of use, accuracy, and cost effectiveness. This method allowed

numerous trials to run at a low cost, which allowed for many of the different viral reduction methods to be tested repeatedly. The team also developed a way to test the total viral reduction that occurred in the fully formed fibrin microthread by dissolving three threads in plasmin, followed by the usage of the plaque assay. The dissolution of the fully formed threads is a newly found method that the team determined functions as desired. Overall, the three methods that comprise the final viral reduction system effectively achieve the aim of this project and can be easily incorporated into the existing production process of fibrin microthread sutures. This process will ensure future patient safety and allow for eventual FDA approval of the fibrin microthread sutures.

# 8.0 Conclusions and Recommendations

This chapter is a summary of the achievements of the design project as well as the impacts the project will have in regards to becoming a material approved by the FDA. The authors will also discuss a number of recommendations and future directions for the advancement of this biomaterial.

## 8.1 Conclusions

The design team initially set an objective to design a process for viral reduction in fibrin microthread sutures. Viral reduction in mammalian derived materials is essential in obtaining FDA approval of biologic materials and to ensure patient safety from transmissible viruses. Ideally, this method will be easily implemented into the current manufacturing system. The group aimed to not only meet FDA standards but also to retain 70% of the mechanical properties shown in threads without viral reduction. There was also a need for viral concentration validation system for the product. The group was successful in designing an effective method to reduce viral concentrations through a multistep process. Fibrinogen was treated with a solvent detergent step and thrombin was treated with a pH alteration. These two proteins, once treated, were used to form full threads. Upon completion, the final product was secondarily treated with dry heat. The combination of these methods proved to enhance mechanical properties. Students were able to determine total viral reduction through a plaque assay. This project was essential for VitaThreads to continue their work with VitaSutures in moving towards clinical studies and eventual FDA approval.

## 8.2 Future Recommendations

Although the viral reduction system developed in this MQP produced significant results that can be incorporated into the fibrin microthread manufacturing process, there are additional procedures that need to be completed in order to reach eventual FDA approval. In this project bacteriophages that mimic bovine-derived viruses were used, since WPI facilities does not allow for biosafety level three and four viruses, such as bovine derived viruses, to be tested. Further research by a third party is not only required by the FDA to prove the accuracy of the laboratory results, but is also necessary for the biosafety level three and four viruses to be tested using this viral reduction system. It has been proven by this project that the viral reduction system reduces the viral presence of bacteriophages and should be further tested to quantify the viral reduction when tested against bovine-derived viruses.

There were also limitations associated with the plaque assay used to quantify the overall viral reduction. There was a minimum detectable limit associated with the plaque assay that we used for quantification, which was set at $10^2$ bacteriophage titer. This limitation did not allow for viral concentration readings below this level to be determined, thus resulting in a range of viral reduction values rather than precise values. In the future PCR could be used instead of a plaque assay to determine exact viral reduction values for each method of the final design. PCR was too costly to utilize due to the budget of this project, although the method for dissolving fibrin microthreads using plasmin can also be implemented when using PCR.

With the focus of the three viral reduction methods, in the future this system can be easily integrated into the current manufacturing process. The current procedures used

for developing fibrin microthreads would remain intact with the addition of the viral reduction system, since all three methods can be incorporated into the existing processes. The pH alteration treatment and the solvent detergent steps can be added into manufacturing when thrombin and fibrinogen are in their liquid-state. Once the treated fibrinogen and thrombin are co-extruded to make the final fibrin microthread, a final terminal dry heat step can be applied to ensure the desired viral reduction. Overall, the current methods utilized for thread formation will not be altered, but rather additional viral reduction steps will be thoroughly incorporated to ensure patient safety.

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# Appendices

## Appendix A: Stress/Strain Curves for Mechanical Testing of Viral Removal Methods

A representative stress/strain curve is provided below for each removal method. The curves show a linear region, followed by an exponential increase, and another linear region before the thread broke at the UTS point. The high frequency noise is a result of the machine used for testing; it was not necessary to filter the data, because the UTS and strain values were still available.
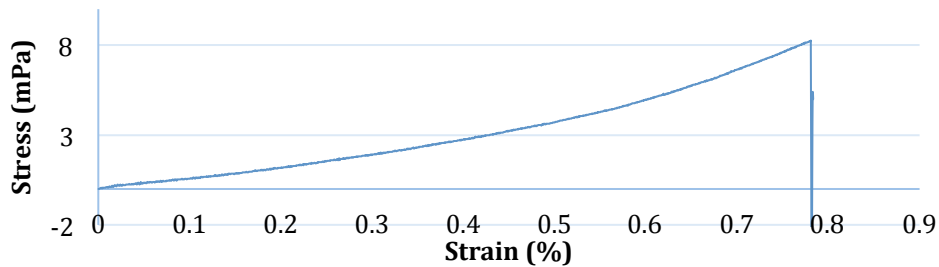


Control Microthreads



Fibrinogen Dry Heated at 60°C
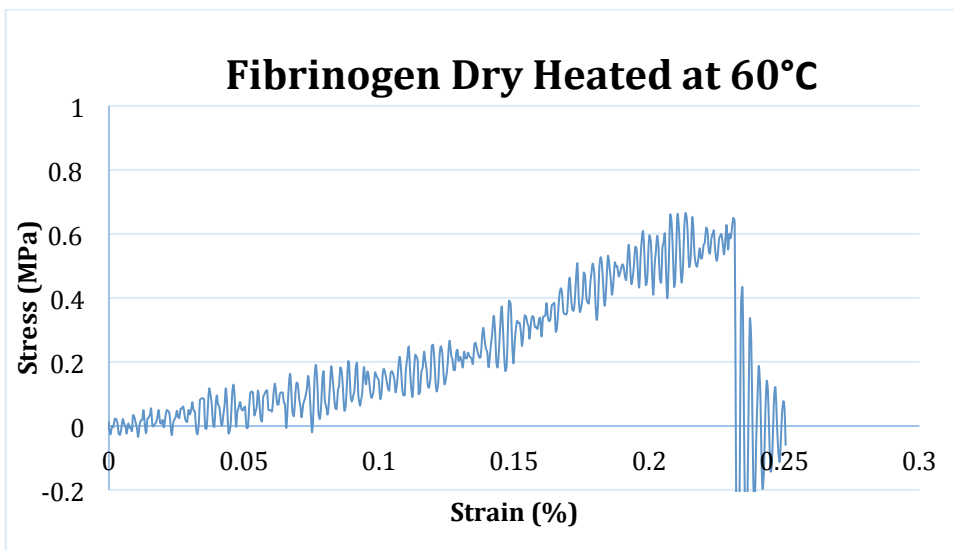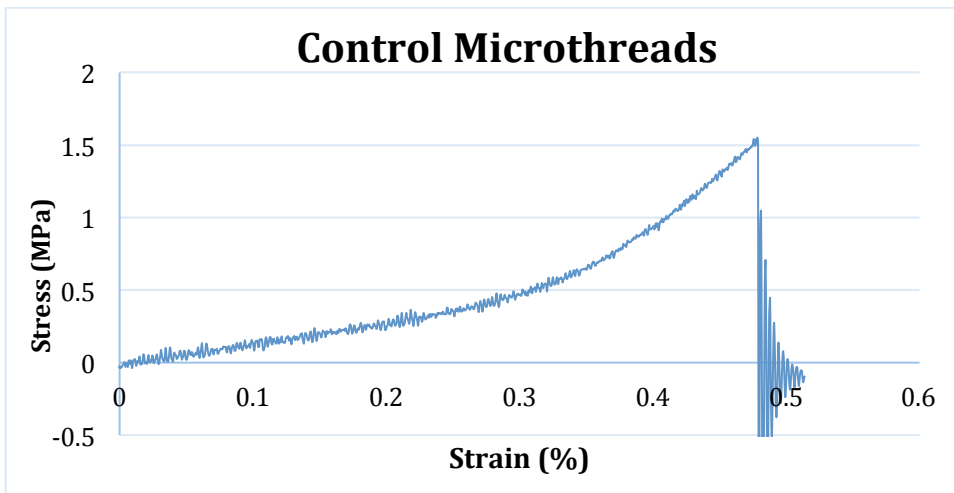
**Microthreads Exposed to Dry Heat at 60°C**
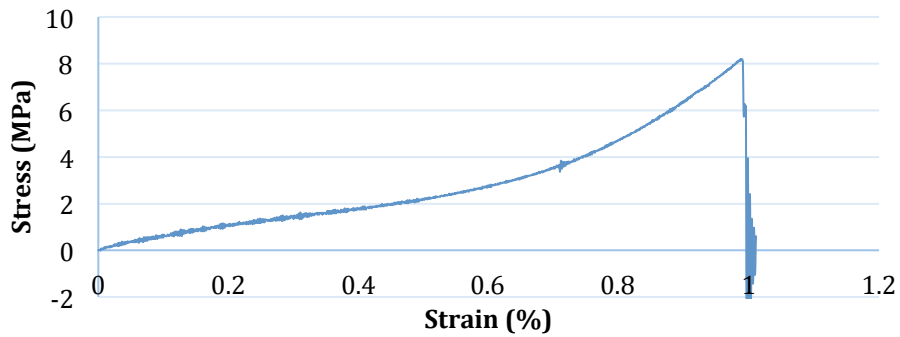


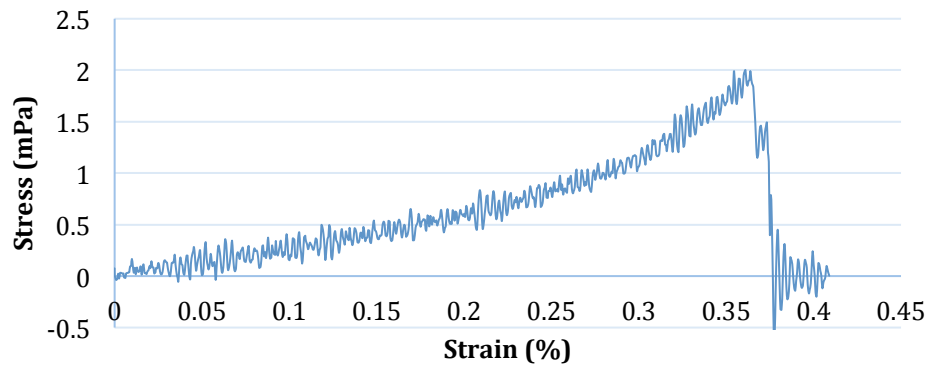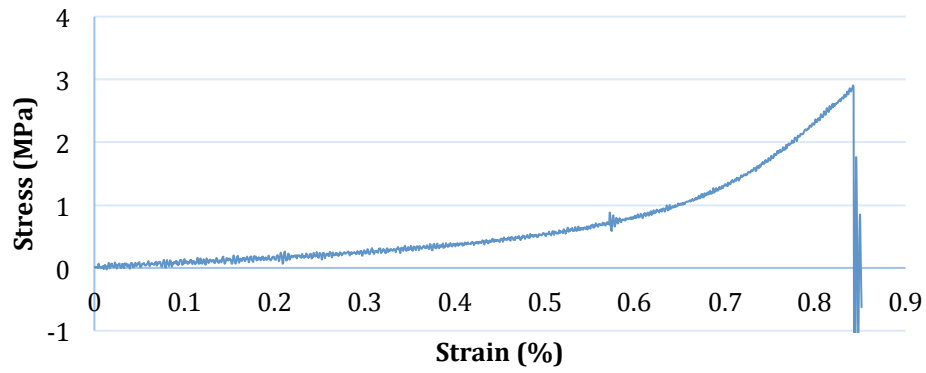**Microthreads Exposed to Dry Heat at 80°C**
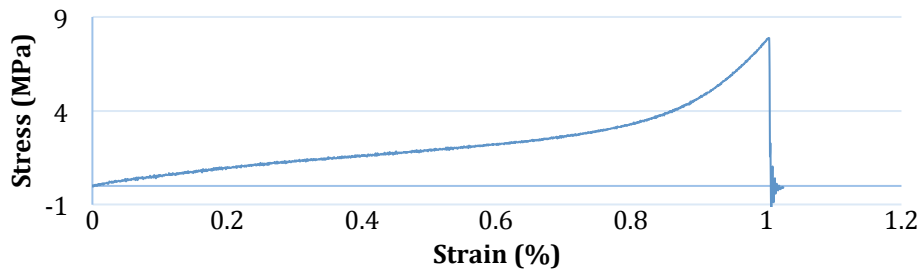


**Microthreads Exposed to Dry Heat at 100°C**

# Thrombin Treated at a pH of 4



# Protein Precipitation Fibrinogen

**Appendix B: Cost of Materials per 100 Sutures**

| Item | Cost |
|---|---|
| TNBP | 1L ($89.95) → $0.02 per batch |
| Triton-X | 500ml ($75) → $0.01 |
| HCl | 2.5L ($63) → $0.47 per batch |
| Ammonium Sulfate | 20lbs ($10) → $0.02 per batch |
| Total Supplies Cost | $0.52 per 100 sutures |