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Location of the Fibronectin Binding Domain on the Fibronectin Attachment Protein of Mycobacterium avium subsp. paratuberculosis

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Location of the Fibronectin Binding Domain on the Fibronectin Attachment Protein of *Mycobacterium avium* subsp. *paratuberculosis*

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Abstract

Mycobacterium avium paratuberculosis (MAP) is an acid-fast bacillus that causes a fatal granulomatous ileocolitis called Johne's Disease that affects primarily ruminants. The impact of MAP infection in the US dairy industry is particularly severe, with up to 70.4% of the herds being infected and annual losses as high as $1.5 billion. MAP is capable of persisting for months in the livestock environment and surviving the pasteurization process. While several vaccines are available, none can prevent infection and treatment with antimycobacterial chemotherapy does not result in a cure. Management and control of MAP infection requires characterization of host-pathogen interaction at the earliest stages in pathogenesis. It is known that establishment of infection requires MAP to bind to M cells found in the bovine ileal epithelial layer covering Peyer's patches, a process that is mediated by a fibronectin (FN) protein bridge between host cell and MAP fibronectin attachment protein (FAP-P). Although, MAP and other mycobacteria express several FN binding proteins, interference or blocking FAP-P's ability to bind FN virtually eliminates M cell targeting and intestinal tissue invasion. The binding motif on FN that is recognized by FAP-P has not been demonstrated definitively. Identifying this location may enable the use of peptides that can be added to milk replacer to prevent MAP infection in calves. In this study, we have used a peptide sequence corresponding to the FN binding domain of FAP-P in a luminescence assay to probe a series of FN fragments that together span the length of the FN monomer to identify which of the fragments contained the FAP-P binding site. FN fragments 2, 3, and 4 which contained 550-650 residues and did not overlap. Fragment 1 contained 1959 residues and completely overlapped fragments 2 and 3 and with a partial 78-residue overlap of fragment 4. Significant signal was observed when FN fragments 1 and 4 were probed. This indicated that the site on FN recognized by FAP-P was within this 78-residue region. Further investigation with heat-denatured fragment 4 resulted in reduced probe binding, suggesting that FAP-P binds to a linear sequence of amino acids. Future work will include competition experiments in cell culture.

Chapter 1: Introduction

1.1 Introduction

1.1.1 History

The organism known as *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the pathogen that causes Johne's disease (JD), a granulomatous enteritis and a debilitating wasting disease of ruminants that is chronic and fatal (Twort & Ingram, 1912). Animals are primarily infected through MAP contaminated feces in food and water sources but other routes of infection such as *in utero* are known to occur. Following ingestion of contaminated food or water, the bacteria attach to host epithelial cells of the ileum wall, causing inflammation and inhibition of nutrient transfer to the host. As the immune response is triggered, the host's cells attempt to contain the infection. The immune response walls off the infected area with an influx of immune cells resulting in a thickening of the intestinal wall, leading to eventual loss of nutrient absorption, profuse diarrhea, and extreme weight loss (Twort & Ingram, 1912). This condition is slow, progressive and has no known cure. There are several vaccines and antimycobacterial chemotherapies currently available but none can prevent or cure MAP infection. Diagnostic techniques are expensive and insensitive outside the clinical stages of the disease.

1.1.2 Economic Impact of Johne ’s Disease

By 1996 approximately 21.6% of US dairy operations were infected with MAP (Lombard *et al*., 2013). This infection causes the dairy industry considerable losses in revenue due to early culling of animals, loss of milk production, decreased calving rates and a reduction in consumable beef yield from poor body condition during harvest (Ott *et al*., 1999). Losses to the US dairy industry are as high as $1.5 billion per year due to the disease (Stabel, 1998). A more recent study showed 580 million kg of annual milk production loss was directly associated with Johne ’s Disease (JD) (Losinger, 2005). To date, this disease has infected up to 70.4% of the US dairy herds and is capable of surviving the pasteurization process (Lombard *et al*., 2013).

1.1.3 MAP Pathology

For every clinical cow in a herd, there are 10-15 more infected, asymptomatic animals (Whitlock & Buergelt, 1996). Thus, the clinical cow represents the tip of the “iceberg.” Managing an infected herd is difficult due to the unreliability of diagnostic testing and late detection of the disease until the infected animals are several years of age (Whitlock & Buergelt, 1996). Calves are most likely infected via a fecal-oral transmission during the first couple of weeks of life. Infection may also occur *in utero* or via contaminated milk (R. J. Whittington & Windsor, 2009). An immunocompetent calf will quickly sequester the infection. However, an infected animal will remain asymptomatic for several years and present significant risk to the herd. Stressors associated with calving can alter the host physiology to the point where it impacts the

ability of the immune system to contain the infection, thus, leading to progression of the clinical phase of the disease. Prognosis is poor and death is often imminent. By the time the infected animal exhibits any clinical signs, it has already shed billions of MAP cells via feces and contaminated the surrounding area. Outside the host animal, MAP can survive in the environment for over a year because of its ability to resist heat, cold, and desiccation in open conditions (Whitlock & Buergelt, 1996).

Many infected cattle are culled early due to poor body condition and are destined for slaughter (Ott *et al.,* 1999). Once these infected cattle have been slaughtered and processed, the meat is packaged and shipped out for human consumption. A study conducted by Rossiter *et al.* found that there were visually healthy dairy cows scheduled for slaughter and were found to be MAP positive based on the presence of MAP DNA in their intestinal lymph nodes and feces. MAP has been found in raw milk and in some cases pasteurized milk (Abubakar *et al*., 2007).

1.1.4 MAP link to Crohn's Disease and Transmission

While the involvement of this organism in human disease is controversial, this suggests the possibility of MAP contaminated milk and beef in the food chain and has raised concerns that MAP may pose a risk to humans. One human disease that has been associated with MAP is Crohn's disease (CD), which is a human inflammatory bowel disease that has similar presentation as JD (Abubakar *et al*., 2007). As with JD, there is also no known surgical or pharmaceutical cure that exist. There are similarities between

the two diseases. Both are debilitating, attack the intestinal tracts, involve chronic inflammation of the intestines and progress slowly.

The precise etiology of CD currently remains both uncertain and controversial. The Chiodini *et al*. research study noted remarkable similarities between pathologies of JD and CD (Chiodini, 1989). The commonalities between bot diseases suggest that MAP may be a causative agent (Abubakar *et al*., 2007). However, if MAP is a potential cause for CD, then a reduction in the prevalence of MAP in dairy cattle should lead to a reduction of CD in humans.

Since JD and CD cannot be cured and has no vaccines approved for use within the United States for JD, if we can understand how the organism is able to attach and invade host tissue, we can develop an inhibitory component that can interfere with the establishment of an infection.

1.1.5 Where does the infection start?

In previous research, it was discovered that MAP preferentially invades through microfold cells (M cells) associated with Peyer's patches (Momotani *et al*., 1988). M cells are components of the follicle-associated epithelium (FAE) and they sample antigens that pass through the intestinal lumen and transfer them along to the antigen presenting cells of the immune system presented in the organized lymphoid follicles known as Peyer's patches (Adachi *et al*., 1997; Sigurðardóttir *et al*., 2004). Cattle have numerous Peyer's patches lining the ileum of the intestines. These Peyer's patches are up

to two meters in length along the luminal surface of the intestines, which explains why the bovine ileum is the target organ of MAP (Yasuda *et al*., 2006).

The passage of microorganisms, antigens and nutrients through the M cells are an essential action for proper development of the organism's immune system and it serves as the route of entry for many infectious diseases (Shelkovnikova *et al*., 2021). Numerous pathogenic microorganisms utilize M cells in order to cross the digestive epithelial barrier (Siebers & Finlay, 1996). Like other cells of the epithelium, M cells express β1 integrins that anchor the M cell to the extracellular matrix (Jepson *et al*., 1996). However, M cells are unique in that they display β1 integrins on their apical face. Among the ligands for β1 integrins is fibronectin (FN).

Secott *et al.* demonstrated that, within murine gut loops, MAP targeted and invaded M-cells by forming a FN bridge *in vivo* and was mediated by the integrins located on the M-cell (Secott *et al*., 2004). A previous study by Zhao *et al.* demonstrated that a RWFV motif is crucial for fibronectin attachment protein (FAP) binding to FN and was prominent in the *M. bovis*-FN interaction (Zhao *et al*., 1999). In this study, we aimed to synthetically produce the FN attachment protein on FAP-MAP (FAP-P) by applying the RWFV binding motif within a 12 amino acid sequence polypeptide sequence (GNAQ**RWFV**VWLG) and introducing it to soluble dimerized fibronectin (SDFN).

Because mycobacteria bind to cells in a FN-dependent manner, blocking the ability of FAP-P to bind FN will prevent M cell invasion, thus, preventing establishment of MAP infection. If the MAP's ability to bind to FN is blocked then it will lead to inhibition of FAP-P attachment to FN (Secott *et al*., 2001). In this study we sought to

determine the location of the FN protein region that is recognized by FAP-P. This steers future research to the potential design and introduction of a competing ligand that mimics FN, thus, potentially leading to a reduced likelihood in MAP attachment and infection.

1.1.6 Research Objectives

Secott *et al.* demonstrated that MAP binds to cultured cells and to mouse ileal tissue in a FN-dependent manner, and by blocking FAP-P, we can block the ability of the FAP-P to attach and invade cells. While there may be multiple virulence factors that enable MAP to infect a host, it is the ability of MAP to bind to FN that enables it to target M cells. Previous research has shown that when FAP-P cannot bind FN, MAP does not attach and invade cells (Schorey *et al*., 1996; Zhao *et al*., 1999). It has been reported that FAP from *M. bovis* (FAP-B) recognizes a region on the carboxyl terminus of FN, however, the authors did not offer empirical evidence for this (Ratliff *et al*., 1993). **Therefore, our first objective was to locate the region of FN to which FAP-P binds.**

Once the binding region has been identified, it is important to characterize how FAP-P recognizes FN specifically. **The next objective is to identify if FAP-P recognizes a linear sequence of amino acids or an arrangement of amino acids that results when the peptide is properly folded?** The determination to these questions will enable the design of an analog to compete with FN that will serve as a competitor peptide that can be added to milk replacer to block attachment of MAP to FN.

1.1.7 Predictions and Hypothesis

Ratliff *et al.* mentioned that the FAP-B from *M. bovis* attaches to the C-terminus heparin specific binding region of the FN molecule but did not provide empirical evidence (Ratliff *et al.,* 1993). It is reasonable to predict that FAP-P would bind to the same region of FN to which FAP-B binds, given the sequence similarity among the mycobacterial FAPs. **Our hypothesis is that the location of the fibronectin (FN) binding region recognized by *Mycobacterium avium* subsp. *paratuberculosis* (FAP-P) is located at the C-terminus of the soluble FN molecule.** By presenting intact FN and a series of FN non-overlapping fragments that together span the length of the FN monomer, we will be able to locate the region of FN recognized by FAP-P. We predict that of the ligands presented to the FAP-P probe, we should see binding to the FN fragment containing the heparin binding domain closest to the C-terminus region.

In order to design a FN competitor that can be used to successfully block MAP binding to FN, we must determine if FAP-P recognizes a linear binding site or one that is exposed in a folded protein. It is possible that the FAP-P recognizing region is only detected when the region is properly folded. **Our second hypothesis is that the FAP-P recognizes a linear sequence of amino acids located on the C-terminus of the soluble FN molecule.** We predicted that denaturing the target FN fragment will not prevent binding of the FAP-P peptide sequence. This would offer new insight for continued research and possible future disease management programs, which could involve cost effective disease prevention measures. A possible peptide specific binding agent could then be synthesized and added to calf milk replacer, thus, blocking the attachment to M

cells and preventing MAP infection. This product could be an easy and affordable prevention method for any cattle operation that has a suspected MAP contamination.

Chapter 2: Literature Review

2.1 Johne's disease

2.1.1 History

Johne's disease (JD) is a widespread and endemic diseases caused by *Mycobacterium* subsp. *paratuberculosis* (MAP) that afflicts numerous species of domestic and wild animals, mainly ruminants such as cattle sheep and goats, with the domestic livestock being more commonly infected (Cocito *et al*., 1994). This study's main focus is dairy cattle and interaction between host and MAP. The clinical signs of the disease were first described in detail in late 19th century. In 1895 the German pathologist Dr. Heinrich A. Johne and Dr. Langdon Frothingham a visiting American pathologist worked to identify what appeared to be an atypical case of intestinal tuberculosis (TB) type disease in cattle (Cocito *et al*., 1994). They described a connection between the enteritis and the presence of micro-organisms within the intestinal mucosa of the cattle, lending Dr. Johne's name to the disease (Cocito *et al*., 1994). The condition which became known as JD in other European countries.

With the arrival of early European settlers to the Americas, they brought cattle from their native lands. It is not surprising that a portion of these cattle were previously infected with JD. With the expansion of imported cattle, JD became prevalent among the US herds (Cocito *et al*., 1994). Along with the fast-growing dairy cattle industry, a growing number of sickly cattle began to rise (Cocito *et al*., 1994). As revenue loss began to accumulate in the cattle industry, U.S. veterinarians, ranchers and farmers realized that JD was a significant problem (Cocito *et al*., 1994).

2.1.2 Stages and symptoms

Clinical signs manifest as extreme weight loss and chronic diarrhea, along with severe malnutrition and muscle wasting despite a healthy appetite and access to adequate food supplies (Stabel, 1998). The wasting of the animal starts with the bacteria attaching to host ileal epithelial cells (Secott *et al*., 2001). This eventually leads to mal- absorptive diarrhea that causes severe inflammation, thickening of intestinal wall, inhibition of nutrient transfer to the host and ultimately death (Ott *et al*., 1999). There are four stages characterized with clinically distinct symptoms.

Stage I is known as the silent or subclinical stage. This stage occurs in stock calves and heifers under two years of age (Whitlock & Buergelt, 1996). Tests and diagnostics tests are not capable of detecting MAP at this stage which includes the gold standard of sampling (fecal cultures and serological testing) which is also not capable of MAP detection in animals that young (Whitlock & Buergelt, 1996). This stage has a slow progression of months to years that can last months to years before it develops into stage II (Whitlock & Buergelt, 1996).

In stage II the infected animals are known as subclinical shedders. This typically occurs in heifers and older animals over the age of two years. Animals appear to be in good health and are simultaneously shedding MAP in their manure. Rarely are the organisms shed at levels that are detectable with fecal culture (Whitlock & Buergelt, 1996). This stage may pose the highest threat of intra-herd contamination due to high population density within close quarters (Whitlock & Buergelt, 1996).

Stage III is considered clinical Johne's disease and signs become apparent. These animals have an advanced infection, and the clinical signs are typically triggered by stress from illness, calving, round up health checks ect. (Whitlock & Buergelt, 1996). Clinical signs of disease are acute or transient diarrhea, extreme loss of muscle mass and weight despite maintaining a normal appetite become apparent (Whitlock & Buergelt, 1996). It also includes a decrease in milk and calf production. Most of these animals are shedding billions of bacteria daily and fecal cultures and serologic tests can reliably detect positive results for MAP (Whitlock & Buergelt, 1996). The clinical signs at this stage may last from days to weeks before the animals advance to Stage IV (Whitlock & Buergelt, 1996).

Finally, in Stage IV, known as the terminal stage, the infected animal is extremely emaciated and has fluid diarrhea (Whitlock & Buergelt, 1996). The infected animal develops bottle jaw, a large swelling (intermandibular edema) in the lower mandibular space. This is an accumulation of non-inflammatory fluid that is caused by protein leaching from the bloodstream and is introduced into the digestive tract. This fluid buildup is pronounced due to gravitational forces drawing the fluid into the loose hanging dermal tissue during constant lowering of the head while the animal is attempting to feed (Whitlock & Buergelt, 1996). The bottle jaw manifestation is indicative that death is eminent (Manning & Collins, 2001).

2.1.3 Economic impact

One focus of concern is how MAP affects *Bovinae,* which comprises cattle, bison, African/Asian water buffalo, and horned antelope. Cows are large ungulates that were

domesticated more than 10,000 years ago (Pitt *et al*., 2018). Their domestication has been considered to have been invaluable for the early nomadic hunter-gatherers' transition into more farming and agriculturally based societies across much of Europe, Asia, and Africa (Pitt *et al*., 2018). These animals were and continue to be bred and raised as livestock for meat, milk, hides, and draft animals "beasts of burden" (Pitt *et al*., 2018). With so many uses, this disease has a major impact on numerous agricultural industries, namely dairy operations. The economic losses due to MAP infection are substantial and a pose a significant reason to control the transmission of MAP in a herd.

A detailed survey conducted by Whittington *et al.* showed that of the herds that were tested in 48 countries, more than 20% of herds and flocks were infected with MAP in at least half of the countries. It also detailed that in a number of those countries, more than 40% of the herds tested positive for MAP (R. Whittington *et al*., 2019). In a 1996 report from the US, Johne's positive dairy herds had a minimum of 10% of the cull cows showed clinical disease and lost approximately $200 per cow at slaughter (Ott *et al*., 1999). These MAP positive herd operations also reported a 700 kg reduction in milk production per cow due to the subsequent effects of JD (Ott *et al*., 1999). A study was conducted in 2004 and focused on three American commercial dairy operations. The focus of the data was comparing milk production of MAP infected cattle to uninfected cattle. The results showed, that known JD positive cattle with a positive fecal culture of >30 cfu/g had a 4 kg reduction in milk per day when compared to uninfected cattle (Smith *et al*., 2009).

2.1.4 Transmission

MAP predominately enters the host via the fecal-oral route and can take up residence in the lymphoid tissue of the host's gastrointestinal tract (Sweeney, 1996). Calves browse their environments with their tongues and anything contaminated with feces can be a source of infection. It was previously believed that paratuberculosis was exclusively an enteric infection. Studies conducted during the 1990's have lent supporting evidence and demonstrated that infection in intrauterine and the supra-mammary lymph nodes can take place in cattle with advanced cases of JD (Sweeney, 1996). It was estimated between 1980 to 2013, that 9% of the calves produced from sub-clinically infected dams, 39% of the calves from clinically affected dams and 13% of all cows tested were infected with MAP (R. J. Whittington & Windsor, 2009).

Horizontal transmission of MAP also serves as a source for contamination and occurs when calves' nurse from the infected dam (Arsenault *et al*., 2014). Although calves are typically separated from their dams within 24 hours of birth, during that critical time frame nursing calves are highly susceptible to MAP infection. This is due to their weaker and immature immune system and ingestion of MAP contaminated colostrum/milk being that is being absorbed through their intestinal mucosa along with the natural protective immunoglobulins that aids in their protection (Arsenault *et al*., 2014). The Fecal-oral transmission is caused by ingestion of contaminated food, colostrum, water, and primary contact surfaces such as the cow's teats during nursing (Sweeney, 1996). Calves are usually separated from their dams within a short time period after birth in an attempt to prevent MAP transmission. A fecal-oral transmission is still

possible during the initial hours to days when the newborns are in the calving pen and within close proximity of contaminated surfaces (R. J. Whittington & Windsor, 2009).

Studies conducted on milk from Map-positive cattle revealed that the organisms were present in 12% of the samples. This may indicate that MAP is surviving the pasteurization process and getting into the milk supply (Gao *et al*., 2009; Giese & Ahrens, 2000). In studies that focused on cheese processing in the U.S and Switzerland, revealed that 4-5% of 143 raw milk cheese samples were positive for Map DNA (Ikonomopoulos *et al*., 2005; Jr. *et al*., 2006; Stephan *et al*., 2007). Cheese tested from Greece and the Czech Republic resulted in 31.7% and 3.6% respectively, were positive for MAP by PCR (Botsaris *et al*., 2010).

MAP's prevalence in biofilms and potable water systems has been studied and has been noted that the bacteria is resistant to chlorination during water treatment and processing (Pickup *et al*., 2006). This would indicate that MAP transmission to human via exposure through sources of drinking water is possible (Beumer *et al*., 2010; Pickup *et al*., 2006).

2.1.5 Management

JD has an "iceberg" phenomenon associated with it. In an average herd, for every MAP infected animal exhibiting clinical signs of the disease, there are approximately 10 to 15 other animals that are most likely infected. Animals in stage IV represent the "tip of the iceberg" of herd infection with JD (Whitlock & Buergelt, 1996). Due to this phenomenon, control and management of this disease is difficult. Infected animals that

are in or beyond stage II (clinical/symptomatic) of the disease, shed large quantities of MAP from their feces to the external environment. At more than 10 billion colony-forming units per gram of manure, this presents a huge challenge for on-farm management efforts to control transmission of the organism to other cattle (Whitlock & Buergelt, 1996). Implemented barn protocols for disease control, attempt to break the cycle by removing affected cows from the herd and practicing hygienic rearing of calves. Unfortunately, these programs are not able to account for potential calf infection during gestation (R. J. Whittington & Windsor, 2009).

2.2 Crohn's disease

2.2.1 Links

Crohn's disease (CD) is a chronic, relapsing human systemic type inflammatory disease that mainly affects the gastrointestinal tract and is associated with immune disorders (Chiodini *et al*., 1984). This disease disrupts digestion efficiency. Patients who are afflicted with this disorder will suffer from weight loss, visceral pain, have chronic diarrhea or constipation, vomiting, and general malaise (Chiodini *et al*., 1984).

Although, CD has both genetic and acquired elements to infection, there many arguments that support and refute the link and causation between MAP and CD. Koch's postulates have yet to be satisfied for a true link to CD. CD in humans is similar to JD in animals, both clinically and pathologically and numerous patients with CD have antibodies to MAP in their blood (Chiodini, 1989; Sartor, 2005).

There are just as many arguments that refute the link and causation between MAP and CD. For example, there are many differences in both clinical and pathological responses between JD and CD (Chiodini, 1989). There also isn't any documented evidence of transmission to humans that have been in contact with MAP infected animals or livestock (Sartor, 2005). Due to the serious ethical problems with any attempt to investigate a link between the MAP and CD, experiments to determine whether MAP-exposed test subjects would produce CD symptoms are not possible; however, if there were a connection between CD and MAP, a reduction in JD frequency should lead to a reduction in CD cases.

2.3 *Mycobacterium avium* subsp. *paratuberculosis*

2.3.1 Characteristics

MAP is a mycobactin-dependent, gram positive, obligatory aerobic, acid-fast bacterium that belongs to the genus *Mycobacterium*. It is a small non-motile bacillus that is about (0.5µm by 1.5µm) in size and shape (Cocito *et al*., 1994; Whitlock & Buergelt, 1996). Like *M. bovis* and *M. avium, MAP* is slow to grow *in vitro* and requires a minimum of 20 hours to produce one generation in the most optimum of environments (Lambrecht *et al*., 1988). Like other species of mycobacteria, MAP has a thick protective cell wall that contains 60% lipids (mycolic acids) which is responsible for the organism's chemical and physical resistance and contributes to the bacteria's acid fastness and hydrophobic properties (McNeil *et al*., 1991; Whan *et al*., 2001). It also contributes to an

increased resistance of chemical and physical processes (McNeil *et al*., 1991; Whan *et al*., 2001).

This lipid rich cell wall is an asset and lends MAP its survival advantage but at the cost of having a prolonged generation time (McNeil *et al*., 1991; Merkal & McCullough, 1982; Whan *et al*., 2001). This consequence is caused by MAP's inefficiency of nutrient transfer due to decreased permeability through its cell wall and the inability to produce mycobactin which is a cellular binder and transporter of iron (Merkal & McCullough, 1982).

2.3.2 Environmental Survival

MAP is capable of surviving outside its host for extended periods of time in a variety of environments (Beumer *et al*., 2010). These include but are not limited to farm/ranch housing/holding pens, pastures, dry lots, slaughter houses, water treatment plants, rivers, run off streams, pooled water sources and dairy/meat products. MAP can persist in a dormant state in the environment for approximately 55 weeks and within a water source for 48 weeks (R. J. Whittington *et al*., 2004). Once shed by the host, the bacteria can survive outside a host for up to a year due to its cell wall physiology and characteristics (Sweeney, 1996). As the infected cattle shed the MAP bacteria throughout their immediate area, it leads to a widespread contamination of the dairy barn's internal and external environment (water sheds, feeding corrals, streams, pastures, and stock chutes) (Sweeney, 1996).

The hydrophobic properties of the cell wall make MAP capable of being collected into air pockets within bodies of water and eventually be aerosolized at the air–water interface level (Rowe & Grant, 2006). It has been reported, the presence of viable MAP was detected in dust particles that surrounded the immediate area of infected cattle. This suggests a potential bio-aerosol transmission of MAP infection (Eisenberg *et al*., 2010). These contaminated aerosols can travel by wind currents and be deposited on the majority of surfaces they settle on. For example, if the landing site is a pasture, MAP can be ingested or be transported to other livestock housing/holding areas by grazing animals (Rowe & Grant, 2006). In the areas that are occupied by infected cattle, MAP deposited into the environment has a higher possibility of entering the water system via water run-off. This MAP contaminated water can potentially enter the public water supply at the water treatment plants, where standard chemical processing and sterilization may not have any effect on the bacteria (Dantec *et al*., 2002).

Mycobacteria have more resistance than *Escherichia coli* by two orders of magnitude to chlorination which is commonly utilized in public water treatment processes (Dantec *et al*., 2002). Further, concentrations of chlorine used during water treatment for the U.S. is typically 1ppm, mycobacteria are capable of surviving chlorine concentrations of 2ppm (Whan *et al*., 2001).

2.3.3 Immunology/Pathology

Once inside the host, MAP gains access to the small intestines by using the microfold cell's (M cells) translocation mechanisms across the mucosal barrier. M cells

are located in the gut-associated lymphoid tissue (GALT) area of the Peyer's patch and function as a transporter for the immune system. These cells will sample and transport microbes and particles from the lumen of the gut across the epithelial cell layer and into the organized lymphoid follicles of the ileum known as the Peyer's patch (Ponnusamy *et al*., 2013; Siebers & Finlay, 1996). This mechanism allows the immune system's antigen presenting cells to detect, recognize and destroy any foreign particle that is deemed a threat (Ponnusamy *et al*., 2013). For recognition and processing purposes, M cells have the ability to transport antigens from the lumen of the intestines to the Peyer's patch directly below the M cell. The M cells have been shown to internalize and transport multiple pathogens including MAP without any deleterious effects or degradation to the pathogen (Sigurðardóttir *et al*., 2004). Once transported, MAP can be phagocytosed by surrounding macrophages, where they survive and replicate within the cell (Sigurðardóttir *et al*., 2004).

2.4 Fibronectin Protein

2.4.1 Characteristics

Cells have specific intercellular interactions within the extracellular matrix (ECM) via adhesion proteins. During many biological processes, the majority of the body's cells require adherence to a macromolecule called fibronectin (FN). FN is a pivotal component in cellular activity (Johansson *et al*., 1997). Cells use receptors in conjunction with FN that attract and bind specific ligands that impact morphology and

expression of genes allowing the cell to carry out normal functions (Johansson *et al*., 1997).

The FN protein is a prototypic adhesion protein and is widely distributed amongst vertebrate tissues. It also functions as a potential ligand for the majority of intercellular interactions with the ECM (Johansson *et al*., 1997). FN is complex molecule that normally exists in solution as a homodimer. It is also found as a soluble dimer in serum and bile. It is composed of two, 250 kDa subunit peptide chains that are covalently linked by disulfide bonds near the respective C-terminus (Johansson *et al*., 1997; Pankov & Yamada, 2002). FN is recognized by a minimum of ten cell surface receptors. These specific receptors belong to the integrin family (Johansson *et al*., 1997). Integrins are linker proteins that are structurally and functionally related cell-surface heterodimeric receptors. SDFN can polymerize into a network that interacts with other proteins and glycoproteins that form the ECM, a substrate that anchors cells to tissues (Johansson *et al*., 1997). MAP uses the FN-integrin integration to gain entrance into host tissue.

2.4.2 Peyer's patches and M-cells

Peyer's patches are secondary lymphoid tissues that are distributed among the lower gastrointestinal tract and ileum (Johansson *et al*., 1997). These are pivotal components in the body's immune defense, these tissues consist of localized macrophages, lymphocytes to include follicular dendritic cells (Johansson *et al*., 1997). The Peyer's patch is covered with specialized cells in the FAE (follicle associated epithelium) (Johansson *et al*., 1997).

Microfold cells (M cells) are epithelial cells that are embedded in the FAE (Neutra *et al*., 1996). M cells serve to collect antigens post luminal and pass them along to underlying immune cells (Johansson *et al*., 1997). MAP utilizes the M cell's translocation mechanisms to cross the mucosal barrier into the ileum via the Peyer's patch (Secott *et al*., 2001). M cells can internalize and transport multiple pathogenic microbes including MAP without harming the host cells (Neutra *et al*., 1996; Secott *et al*., 2001).

2.4.3 Fibronectin Attachment Protein

As previously mentioned, MAP requires FN to act as a bridge between pathogen and host in order to begin host infection. The FN attachment protein of MAP (FAP-P) contains a specific RWFV amino acid binding motif, which has been documented in previous research as a pivotal sequence for attachment (Zhao *et al*., 1999). The 1999 Zhao *et al.* study identified that the minimal binding sequence of *M. avium* (FAP-A) for FN, consisted of a 12 amino acid sequence (amino acids 269–280) and contained a RWFV motif. By using this sequence as a base, a panel of synthetic peptides could be utilized to determine which amino acids were required for FAP-A-FN attachment. Their results showed that the RWFV binding motif was critical for FN attachment to FAP-A and that this sequence is entirely conserved within all FAP proteins that have been identified thus far (Zhao *et al*., 1999).

Past research has indicated that the fibronectin attachment protein is necessary for efficient attachment and invasion of epithelial cells and the FAP-P facilitates M-cell

targeting and invasion through a FN bridge with the integrins via host Peyer" Patches (Secott *et al*., 2001, 2002). This interaction provides an optimal route of infection and makes the M-cells a primary target for invasion.

2.5 Bicinchoninic Acid Assay

The Bicinchoninic Acid Assay (BCA) is a protein assay used as a colorimetric detection and quantification of total protein in a given sample (Walker, 1994). In this assay, proteins in the sample can reduce Cu+2 to Cu+1 in an alkaline solution. The reduction of copper is caused by four specific amino acids (cysteine, cystine, tyrosine, and tryptophan) that are present in protein molecules (Walker, 1994). This process is known as the Biuret reaction and results in the formation of a lavender to purple color of the sample solution using the bicinchoninic acid as a reagent with an absorbance of 562nm (Walker, 1994). Since this method is not a true endpoint method, the color will continue to develop with time, however, directly after an incubation period, color development drastically slows down allowing for additional samples to be assayed in the same test (Walker, 1994). Protein concentrations are determined from a series of dilutions of bovine serum albumin (BSA) or bovine serum globulin (BSG) from a known concentration that is assayed in conjunction with an unknown sample (Walker, 1994). The concentration of the unknown samples is calculated by using a standard curve.

Chapter 3: Methods

3.1 Fibronectin Binding Luminescence Assay

SDFN (Met1-Glu2477) (Biomedical Tech Inc.) was added to CCB for a concentration of 1µl/100µl. To detect the FAP-P binding region, under a laminar flow hood (Air Clean systems), SDFN (Biomedical Tech Inc.) was diluted to a working concentration of 1µg/100µL in 4µL of FN to 400µL of 0.1M carbonate coating buffer (CCB) at pH 9.6. FN fragment #1 (Gln32-Pro1991) (BioLegend), FN fragment #2 (Ser607-Pro1265) (Biotechne), FN fragment #3 (Gluc1266-Pro1908) (Biotechne), and FN fragment #4 (Val1913-Glu-2477) (Biotechne) were reconstituted with PBS with a final concentration of 100ug/mL and stored at -20□C. FN fragment samples thawed at room temperature and diluted to a working concentration of 1µg/100µL with CCB at pH 9.6. A 96 well white microplate (Greiner Bio-One) was inoculated with 100µL of FN dimer and FN fragments 1-4 solution. The microplate was sealed with a Top Seal-A microplate sealing film (VWR) and incubated between 2□C-10□C overnight.

After incubation, the SDFN and FN fragment solutions were removed by inversion and the plate was washed with 200µL of washing solution (0.05% Tween20 and PBS) per well for a series of three washes. The remaining free protein binding sites in wells were blocked by adding 200µL of blocking buffer (1% bovine serum albumin (BSA) in phosphate buffered saline tween (PBST)) per well and covered with Top Seal-A microplate sealing film (VWR). The blocked plate was incubated in the dark at room temperature for 2 hours.

After the blocking incubation period, the blocking solution was removed and the plate was washed as before. FAP-P test and control probes were reconstituted with cell culture grade H_2O and dimethyl sulfoxide (DMSO). FAP-P (test: GNAQRWFVVWLG) (Genscript) and control (scrambled: RVAQLVFNWGWG) (Genscript) peptide solution was diluted with acetate, sodium, and Tween (ANT) buffer with a pH 6 and final concentration of 10mM. Control and test FAP-P was added to ANT buffer with a concentration of 4.0μg/100μL. Designated FN coated wells were inoculated with 100μL of FAP-P test/control solution each. CCB, PBS, FAP-P test, FAP-P control, and ANT buffer were added independently to designated non-FN coated wells in volumes of 100μL each well. The inoculated plate was sealed with Top Seal-A microplate sealing film (VWR) and incubated at 37°C in an incubator (Fisher Scientific) for 60 minutes.

After incubation, the microplate was washed as before. Colorimetric substrate consisting of neutravidin-horseradish peroxidase (NHRP) conjugate (Peirce) and tris buffered saline (TBS) at pH 7.4 was added to each well with a working ratio of 1:1000. Designated FN coated wells were independently inoculated with 100μL of TBS and NHRP. The inoculated plate was sealed and incubated at 37°C for 60 minutes.

After incubation cycle, the sealing film, colorimetric substrate all solutions were removed and the plate was washed with 200μL of 1X PBST (peptide diluent) per well for a series of three washes and 100μL of a chemiluminescent substrate of Super Signal ELISA Pico (Peirce) consisting of a 1:1 working ratio of enhancer and stabilizer was added to each FN coated well. The inoculated plate was sealed and incubated at room temperature in a dark environment and developed for 10 minutes. Directly after

development, the film was removed and the plate was placed in a FlouroSkan Ascent Illuminometer machine (Thermo Systems) to be read. Raw data was documented and analyzed for indications of protein binding.

3.2 Effects of Denaturation of FAP-P Probe Binding

Under a laminar flow hood, a SDFN sample was diluted to a working ratio of 1ug/100uL by adding 5µL of FN to 500µL of CCB (pH 9.6). FN fragment #4 samples were distributed into "Native and Denatured" tubes. Samples were diluted to a working ratio of 1ug/100uL with CCB (pH 9.6) and mixed via inversion. Denatured FN tube was treated with 10uL of β-mercaptoethanol (2-ME) and heat treated in a Thermal Master Cycler (Eppendorf) at 95□C for 5 minutes and a holding temperature of 4□C. A 96 well white microplate was inoculated with 100µLper well of SDFN and FN fragment 4 "Native and Denatured" solution. The microplate was sealed with a Top Seal-A microplate sealing film and incubated between 2□C-10□C overnight.

After the incubation, FN solutions were removed by inversion and the plate was washed with 200uL of washing solution (0.05% Tween20 and PBS) as before. The remaining free protein binding sites in wells were blocked by adding 200uL of blocking buffer (1% BSA in PBST) per well and covered with Top Seal-A microplate sealing film. The blocked plate was incubated in the dark at room temperature for 2 hours.

After the blocking incubation period, the blocking solution was removed and the plate was washed as before. FAP-P (test and control) peptide solution was diluted with ANT buffer to a working ratio of 4.0ug/100uL. Designated SDFN and FN 4 fragment

coated wells were inoculated with 100uL per well of FAP-P test/control solution. The inoculated plate was sealed with Top Seal-A microplate sealing film and incubated at 37°C for 60 minutes.

After the incubation cycle, the SDFN/FN fragment 4-FAP-P test/control was removed and the plate was washed as before. Colorimetric substrate consisting of NHRP conjugate and TBS pH 7.4 was added to each well with a working ratio of 1:1000. Designated SDFN and FN fragment 4 coated wells were inoculated with 100uL of TBS and NHRP. The inoculated plate was sealed with Top Seal-A microplate sealing film and incubated at 37°C for 60 minutes.

After the incubation cycle, the colorimetric substrate-SDFN/FN fragment 4 solutions were removed and the plate was washed with 200uL of 1X PBST (peptide diluent) per well for a series of three washes. After the wash cycle was completed, 100µL of a chemiluminescent substrate of Super Signal ELISA Pico consisting of a 1:1 working ratio of enhancer and stabilizer was added to each SDFN/FN fragment 4 coated well. The inoculated plate was sealed with Top Seal-A microplate sealing film and incubated at room temperature in a dark environment and developed for 10 minutes. Directly after development, the film was removed and the plate was placed in a FlouroSkan Ascent Illuminometer machine to be analyzed. Raw data was documented and analyzed for indications of protein binding.

3.3 Data Analyses

A combination of normality test, 1-way ANOVA, Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk, Kolmogorov-Smirnov, and multiple comparisons tests were used to analyze and interpret raw data. All data were treated with an assumption of non-normal distribution and unequal variances. All statistical analyses were calculated with the PRISM Graph statistics program 9.0.

Chapter 4: Results

4.1 Probe Validation: Localizing FN Binding Region of FAP-P

In order to localize the FN binding site for FAP-P, white microplates wells were coated with SDFN and FN fragments 1-4 and probed with either FAP-P control or test peptides in a luminescence assay. Once the binding site was localized, we proceeded to test whether probe binding was different between native or denatured FN.

Initial tests with substrates without the introduction of SDFN, FN fragments 1-4 and FAP-P test/control probes yielded no indication of binding to microplate wells. FAP-P (Cont) alone in the presence and absence of FN and substrate yielded negligible indications of binding. FAP-P (Test) in the presence of FN yielded significant indications of binding (Fig.1). An indication of binding was 2-fold greater with SDFN, FN1 and FN4 than observed with FN2 and FN3 (Fig 1).

Once specificity of probes was established, we next tested the ability of FAP-P probes to bind to SDFN as well as FN fragments 1-4. Although binding was observed with the FAP-P test peptides in the presence of FN2 and FN3, significantly higher probe binding was observed when the microplate wells were coated with SDFN, FN fragments FN1 and FN4 (Fig 1). Results showed values were not of equal variance and had non-normal distribution. A positive significant difference among means with ($P < 0.05$) was indicated with a 1-way ANOVA analysis of the resulting test data.

4.2 Role of FN Conformation in FAP-P Binding in a Luminescence Assay

To establish peptide conformation, FAP-P was introduced to either native FN4 or heat denatured FN4 coated microplate wells in a luminescence assay. Higher binding preference was observed with a greater indication of FAP-P binding preference for FN4 in a native state when compared to FAP-P binding FN4 in a denatured state (Fig.2).

Data was tested for normal distribution and results showed values were not of equal variance and had non-normal distribution. A Kruskal-Wallis test indicated that medians varied significantly amongst the native or denatured treatments. A multiple comparisons analysis test resulted in a significant difference in values between denatured FN4 when compared to native FN4. A 1-way ANOVA indicated that there was a positive significant difference among means with ($P < 0.05$).

4.3 Effect of β-mercaptoethanol in Heat Treated FN Fragment Binding to FAP-P Probes

FAP-P preference for denatured FN4 in the absence or presence of β-mercaptoethanol (2-ME) was determined by heat treating FN4 samples with or without 2-ME. FAP-P probes were introduced to heat treated FN4 with or without the addition of 2-ME coated microplate wells in a luminescence assay. Higher binding preference was observed with a greater indication of FAP-P binding in heat only denatured FN4 when compared to FAP-P binding heat treated denatured FN4 with the addition of 2-ME (Fig.3).

Data was tested for normal distribution and showed values were not of equal variance and had non-normal distribution. A Kruskal-Wallis test indicated that medians varied significantly amongst the heat treatments. A multiple comparisons test resulted in heat treatments had a significant difference in values between FN4 denatured with heat only when compared to FN4 denatured with heat and 2-ME. There was a positive significant difference among means with a ($P < 0.05$) indicated by a 1-way ANOVA statistical test.

Figures

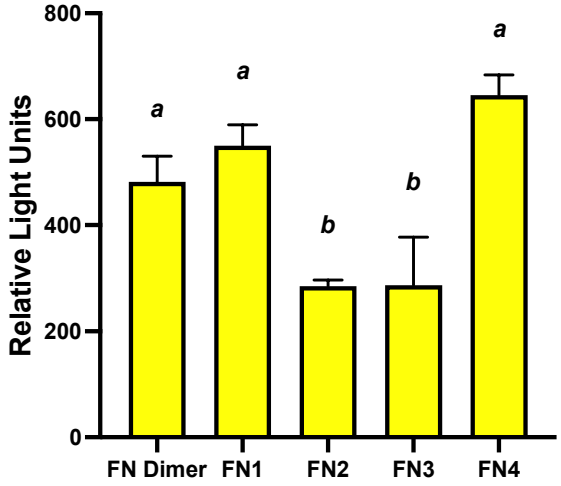

Fig 1. Binding indication of soluble dimerized fibronectin (SDFN) and FN protein fragments in the presence of non-scrambled (Test) fibronectin attachment protein (FAP-P) probes. The FAP-P binding region on FN was localized by determining the FAP-P probe binding preference for FN fragments 1-4. The bar graph represents three non-overlapping/sequential FN fragments (FN2-FN4), one overlapping FN fragment (FN1) and an intact SDFN with the addition of FAP-P (Test) probe. SDFN shown as FN dimer, FN fragments FN1 and FN4 yielded significant indications of FAP-P probe binding preference. Non-normal distribution of data was determined by conducting Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov statistical analysis. A 1-way ANOVA analysis showed a positive significant difference among means with ($P < 0.05$). A Kruskal-Wallis test indicated that medians varied significantly amongst the data. A multiple comparisons test resulted in a significant difference of values between FN2-FAP (Test) and FN3-FAP (Test) vs. FN4-FAP (Test).

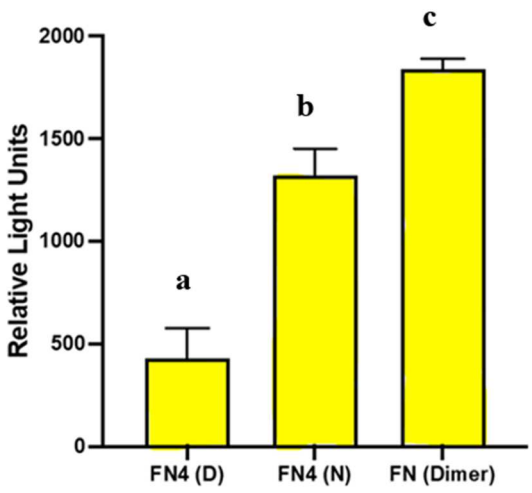

Fig 2. Binding indication of native or denatured fibronectin protein fragment 4 (FN4) and soluble dimerized fibronectin (SDFN) in the presence of a non-scrambled (Test) fibronectin attachment protein (FAP-P) probe. The FAP-P probe binding preference for FN4 in a native state was determined by introducing FAP-P probes to either native or denatured FN4 fragments. The bar graph depicts FAP-P having a higher affinity in binding to the FN dimer and FN4 (N). Non-normal distribution of data was determined by conducting Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov statistical analysis. A Kruskal-Wallis test indicated that medians varied significantly amongst the treatments. A multiple comparisons test resulted in a significant difference in values between FN4-FAP (D) vs. FN (Dimer).

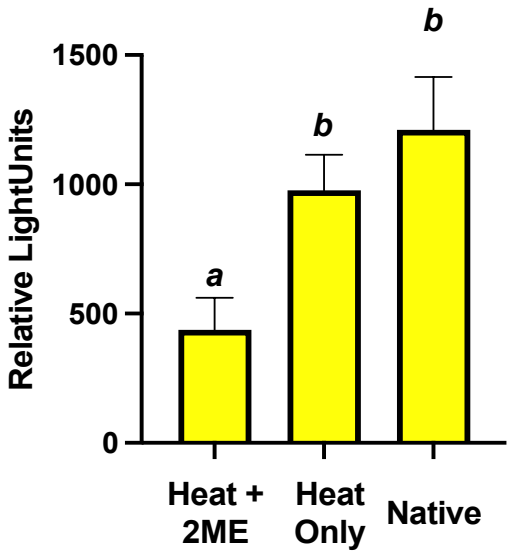

Fig 3. Fibronectin protein fragment 4 (FN4) binding preference within different denaturing heat treatments in the presence of a non-scrambled (Test) fibronectin attachment protein (FAP-P) probe. FAP-P preference for denatured FN4 in the absence or presence of β-mercaptoethanol (2-ME) was determined by subjecting FN4 samples to heat and 2-Me (Heat+2-Me) or heat only (Heat Only) treatments. FAP-P was shown having a higher affinity for binding FN4 in the Heat Only treatments. FN4 processed with Heat + 2ME treatments had a lower affinity for binding FAP-P probes. Non-normal distribution of data was determined by conducting Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov statistical analysis. A Kruskal-Wallis test indicated that medians varied significantly amongst the data. A multiple comparisons test resulted in not having a significant difference in values between FN4 heat treated with 2-ME and FN4 heat treated with heat only. Multiple comparisons tests indicated that there was a significant difference amongst values between heat treatments.

Chapter 5: Discussion

Raising healthy cattle to breeding, milking and harvesting weight for slaughter is a time consuming and costly endeavor for the cattle owners. This study is needed because of the tremendous impact that JD has on cattle ranchers and farmers. In the United States, Johne's Disease (JD) has a high frequency of MAP infection in the cattle industry, with 22% of the herds being contaminated with the disease-causing agent MAP (Speer *et al*., 2006). Of particular interest is the impact on dairy production, with annual losses as high as $1.5 billion due to a reduction of milk production caused by the disease (Stabel, 1998).

JD has no known cure and the use of available vaccines are not capable of preventing the disease. Diagnosis of JD requires there to be positive detection of MAP in a given sample from an infected animal. These tests are expensive and is not feasible to the cattle operation due to the high testing costs and the slow progression of the disease. Also, MAP is non-detectable until the infected animal is in stage III where the disease is considered clinical and terminal (Whitlock & Buergelt, 1996). By this time, the infected animal already has had an extreme loss of body weight, milk and calving production has dramatically decreased, and years of bacterial shedding has occurred.

As MAP is dispersed, it can persist undetected for years in an outside environment, where it can potentially gain access to a new host and continue the host-pathogen infection cycle. The ability to manage and control herd MAP infection requires a characterization of the interaction between the host and the organism on a molecular level at the earliest stages in pathogenesis.

The main problem this study helps address is how to prevent MAP infection. It is known that MAP invades bovine ileal tissue through M-cells via the ileal Peyer's Patches (Momotani *et al*., 1988). Secott *et al.* demonstrated M-cell invasion in murine gut loops in a manner that depends on the interaction of SDFN secreted in the intestinal bile with FAP on the organism (Secott *et al*., 2004). Defining the nature of this interaction between MAP-FAP-SDFN could provide a means to block this interaction. Zhao *et al.* identified the region of FAP that binds to FN but the exact location of FAP-P domain within the 2477 amino acids in the SDFN protein isn't known (Zhao *et al*., 1999). An earlier report suggested FAP recognized a site near the C-terminus of FN, after 30 years; however, experimental evidence was not provided to support this claim (Ratliff *et al*., 1993).

How the binding site is recognized can be answered by determining if the residues are linearly arranged or only accessible when the protein is properly folded. This would help determine the approach needed to identify the binding region. Further, if the peptide blocking of FAP-P to FN interaction and MAP infection is practical and if denaturing this protein makes peptide blocking a consideration.

The outcome of this study has numerous and potentially significant contributions to the fields of agriculture, animal health, livestock husbandry, human medicine, and the production of bovine derived consumable products. Understanding the MAP-FAP-SDFN interaction may lead to the design of competitive ligands to block the MAP attachment to the host cell. This in turn can lead to the development and implementation of novel therapies in disease management. For example, the use of synthesized peptides as a supplement that can be added to calf milk replacer to aid in the prevention of MAP

infection in calves. In order to break the MAP infection cycle, the target demographics need to be the newly born calves where the interaction of the host and MAP organism is at the earliest stages in its pathogenesis.

The main problem this study addresses is that the exact location of the FAP-P binding domain within the 2477 amino acids of the SDFN protein is not currently known. Previous research has been conducted and produced unpublished data that led the researchers to believe that the MAP-FAP attachment region is within the C-terminus on the SDFN protein (Ratliff *et al*., 1993). With this study, we set out to support the previous research and secure a finalized location of the MAP-FAP binding region.

An additional problem that our study concentrated on that has not been previously addressed by other research, is that once the binding region has been established, does MAP have the capability to attach to its target binding region on the SDFN in a denatured or native environment or both. Our final aim for this study that has also not been previously studied is, does FAP-P recognize a linear, conformational sequence of amino acids on SDFN or an arrangement of amino acids that results when the peptide is properly folded.

The results of this study support our primary hypothesis that the C-terminus of the SDFN protein contains the binding domain that *Mycobacterium avium* subsp. *paratuberculosis*-fibronectin attachment protein (MAP-FAP-P) targets to gain access to the host cell. The data indicated that FAP-P had a preference of recognition for a sequence of amino acids on FN fragment 4 (FN4) and suggests that the MAP-FAP-P binding domain is localized with in the SDFN's C-terminus. Positive indication of test

probe binding to FN fragments 2 and 3 was detected. This could be due to some similarities between the molecular nature of FN fragments 2 and 3 binding properties. This does not negate the that there was a greater binding signal of 2-3 times higher when the FAP-P test probe was introduced to FN fragment 4.

Our data indicates that the MAP-FAP attachment region is a linear sequence of amino acids was supported by this study's results. While binding of the FAP-P probe with the denatured FN4 was slightly less than that observed for native FN4, our data demonstrates that FAP-P binds to a linear sequence of amino acids. This would indicate that synthesis of a blocking agent shouldn't be a complicated process.

To identify the location of the FN binding domain on the FAP-P, we used a peptide sequence corresponding to the FN binding domain of FAP in a luminescence assay to probe a series of four protein fragments that together span the length of the 2477 amino acids of the FN monomer. FN fragments 2, 3, and 4 contained between 550-650 non-overlapping residues, whereas FN fragment 1 covered a length of 1959 residues and completely encompassed fragments 2 and 3 and partially overlapped fragment 4 with 78 amino acids. By separating the FN monomer into sequential non-overlapping fragments and exposing each one individually to the FAP binding peptide, we isolated the fragment of amino acids that corresponded to the MAP-FAP-P attachment region. All results were treated statistically as having non-normal distribution of data.

To classify the FN binding domain peptide structure that FAP-P recognizes as linear or conformational, the corresponding peptide sequence to the FN binding domain of FAP was introduced to FN fragment 1 in a native state in a luminescence assay. FN

fragment 4 was subjected to both a native and denatured state in a luminescence assay. The resultant data indicated the FAP-P had a preference of recognition for a sequence of amino acids on FN when the peptide is properly folded or had a nonlinear structure.

Significant signal was seen when FN fragments 1 and 4 were probed. This suggested that the binding site on FN recognized by FAP was within this 78-residue region. This site also included the heparin-binding motif.

Further probe binding was greatest when introduced to FN fragment 4 in a native environment as opposed to a denatured FN fragment. Probing of heat-denatured fragment 4 resulted in a reduction of binding. This indicated that there is a semblance of a three-dimensional structure that is required for the efficient recognition of FN by FAP-P that occurs in its native state.

During the heat-denaturing process of SDFN fragment 4, it was noticed that the samples that contained 2-ME yielded a distinct brown-yellow hue and FAP-P having a higher affinity to bind to the denatured FN fragment 4 with without the presence of 2-ME. This is interesting because the primary use of 2-ME is to denature proteins by breaking disulfide bonds. Since FN fragment 4 does not have any disulfide bonds within its 564-residue length, there should not be any effect on FAP binding preference between heat treatments with or without 2-ME. We see in this study that there is a strong indication that 2-ME has other denaturing properties that affect the binding affinity of FAP-P to FN fragment 4 as seen in. Further investigation into the denaturing effects of 2-ME would be warranted.

In this study, we identified location of the FN binding domain on the fibronectin attachment protein of *Mycobacterium avium* subsp. *paratuberculosis.* Through a combination of luminescence assays, we identified that FN4 contains the binding site recognized by FAP-P, in that FAP-P likely recognizes a linear sequence of amino acids. Soluble FN is a known target of MAP and a major adherence factor for it to gain access to the host via the M-cells and Peyer's patches. A previous study (Ratliff *et al*., 1993) demonstrated that the fibronectin attachment protein of *M. bovis* binds to a sequence of amino acids located on the C-terminus of FN. Our report is consistent with the Ratliff's findings in that FAP-P has a higher preference for binding to the same location on the C-terminus of FN and exhibits a preference for specific sequence of amino acids. Based on this data, we proposed that the location of FN binding region recognized by MAP-FAP is located at the C-terminus of the FN molecule and our results support our hypothesis and previous study.

Based on this study's results, this research has shown that FAP-P has a higher level of binding when introduced to FN fragment 4 and narrowed down to a 78 amino acid overlap sequence between FN fragment 1 and 4. This data paves the way for future studies and research regarding the identification of possible competing ligands to aid in interfering if not blocking MAP's target binding site on SDFN and additional research in the solubility of FAP-P blocking agents within colostrum and milk replacer. Depending on the results of the proposed studies, trials in competition conducted in a cell culture system would be warranted. FAP has a stronger affinity to bind to denatured FN in the

absence of 2-ME. We recommend that a follow-up study be conducted on the properties and effects of 2-ME on the denaturing process of FN fragment 4.

Also, as mentioned previously, there was positive indication of FAP-P test probes binding to FN fragments 2 and 3. The SDFN protein has multiple type III classes in its amino acid sequence. It is reasonable to suspect that there may be similarities in the molecular nature between the two fragments and FN fragment 1 and 4 overlap region that may cause some cross reactivity. We suggest that a future study in these molecular binding properties be conducted.

From an agricultural perspective, the successful completion of this work could reduce disruption to daily practices and animal welfare, while minimizing the potential of transmission of the agent to non-infected cattle. This could also lead to the invention of novel therapies to aid in managing JD infected herds making for easier and more cost-effective herd management practices that are designed to prevent disease outbreak and keep daily operational costs down. Together with lower MAP infection rates, farming practices could remain sustainable and profitable while providing an affordable and safe food supply to the consumers.

Lastly, the outcome of this work may impact CD research. If MAP has an influence on CD occurrence, then if herd infection is decreased then a subsequent decrease in detectable CD should follow. Therefore, with further diminished MAP infection rates, it may be possible to determine if MAP is likely to cause, exacerbate CD or have no effect.

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Appendices

Appendix A: Master materials and Equipment Identification

96 well Micro Test, Clear Plate (Falcon, 35-0072)
.5mL Micro-centrifuge tubes (VWR, 14231-062)
0.2um Bottle Top Filtration Unit (VWR, 97066-200)
1X PBST (phosphate buffered saline, pH 7.4 w/ 0.05% Tween 20,) *see recipe section
2.0mL Micro-centrifuge tubes (VWR, 2017-094)
1.5mL White Snap Lid Centrifuge Tubes (VWR, 14231-062)
1.5mL Brown Snap Lid Centrifuge Tubes (VWR, 20170-036)
15mL Sterile Centifuge Tube (Thermo Fisher, 339650)
Accu-Power Unit (VWR, 300)
Analytical Balance (Denver Instrument, XE-50)
ANT, pH 6 (10mM NH4Ac buffer, pH 6/0.85% NaCl/0.05% Tween 20)
β-mercaptoethanol 25mL (Sigma chemical co., M-1348)
Biotinylated FN (Scrambled: RVAQLVFNWGWG) (Genscript, SC1208) 4mg
Biotinylated FN (Test: GNAQRWFVVWLG) (Genscript, SC1208) 4mg
Blocking Buffer (1% BSA (bovine serum albumin), PBS (phosphate Buffered Saline)
Bottle top filtration unit, 250mL, 2um (VWR, 97066-200)
Bovine Serum Albumin 1G (Sigma Aldrich, 05470-1G)
BCA Protein Assay Kit (Thermo Scientific, 23225 and 23227))
Carbon Coating Buffer pH 9.6
Crystal Violet Staining Solution (Sigma Aldrich, HT901-8FOZ)
Coomasie Staining Solution (BioRad, 1610786)
De-staining Solution (BioRad, 1610438)
Dimethyl Sulfoxide (TCI America, D0798)
F-bottom Chimney well, 96 well Microplate (Greiner Bio-One, 655074)
Fibronectin Fragment #1 (1 Gln32-Pro1991) (BioLegend, 775306)
Fibronectin fragment #2 (Ser607-Pro1265) (Biotechne, 3225-FN-100)
Fibronectin fragment #3 (Gluc1266-Pro1908) (Biotechne, 3938-FN-050)
Fibronectin fragment #4 (Val1913-Glu-2477) (Biotechne, 3624-FN-050)
Fibronectin Dimer, Stabilized Bovine (Met1-Glu2477) (Biomedical Tech Inc., 2265)
Fibronectin Dimer Samples, 0.5mg/ml (Biomedical Tech Inc., 2265)
Fibronectin Dimer Samples, 5mg/ml (Biomedical Tech Inc., 2266)
Filter paper 7.5 x 10cm (Bio-Rad, 1703965)
Freezer -20□C (Frigidaire, WB73751942)
Gel Electrophoresis Assembly (Bio-Rad, Mini-PROTEAN II Cell)
Glacial Acetic Acid (Mallinkrodt AR, 2504)
Impulse Sealer Unit (PFS-200)
Illuminometer, FlouroSkan Ascent FL (Thermo Systems, 5210450)
Incubator (fisher Scientific, 650D)
Lab Quest PH meter (Vernier, LQ2-LE)

Laminar flow hood (Air Clean systems, AC648LFUVC)
LD Poly bags, 4” x 6” (OP, 20-F-0406)
Leammli Sample Buffer 30mL (Bio-Rad, 161738)
LI-COR Imager (Intertek, 120627)
Magnetic stirrer/hot plate (Corning, PC-320)
Mini Protean TGX Gels (Bio-Rad, 4561023)
Mini Vortexer (VWR, MV1-03-030951)
Multi Farrier tips 10uL (Gen3, GEN3-10-L-R-S)
Native sample buffer 30mL (Bio-Rad, 1610737)
Neutravidin-horseradish peroxidase (HRP) conjugate (Peirce, 31001)
Nitrocelulose Membrane Roll 30cm x 3M (Pall Cor., 66485)
Pipet tips 200uL (VWR, 53509-006)
Pipetman 10uL-100uL
Pipetman 0.5uL-5.0uL
Pipetman 100uL-1000uL
Pipette tips 200uL (VWR, 53509-006)
Refrigerator 2□C-10□C (Frigidaire, FFRU17B2QWD)
Refrigerator (walk-in) 2□C-10□C (Controlled Enviro, S275A)
Freezer -20□C-25□C ((Frigidaire, FFU2124PW10)
Nutator Rocking Unit (CLAY ADAMS, 421105)
Running Buffer (Native/Denatured) * see recipe section
Scale/Balance (OHAUS Corp., Scout Pro, SP202)
Super Signal ELISA Pico chemiluminescent substrate (Peirce, 37070)
Super Signal West Pico PLUS chemiluminescent substrate (Peirce, 34577)
Thermal, Master Cycler (Eppendorf AG, 5331 40451)
Methanol (BDH, DDH1135-1LP)
Multiskan Reader (Thermo Electron Corp., 51118750)
Top Seal-A microplate sealing film (VWR, 60941-078)
Transfer Buffer (Native/Denatured)
Tween 20, 100mL (Fisher Scientific, BP337-100)
Wash Solution (0.05% Tween20 and phosphate buffered saline (PBS)
Wash solution (PBST (phosphate buffered saline/Tween20))

Appendix B: Solutions, Reagents, Buffers and Substrates Recipes

Carbonate Buffer
0.925g of N2 Bicarbonate
0.160g of Na Carbonate
100mL of dH2O

- Stir into solution and pH to 9.6.
- q.s to 250mL and autoclave on liquid flask cycle at 121□C for 20 minutes.
- Store at 4-8C.

ANT "acetate sodium Tween" (pH 6.0)
0.385g of NH4Ac
0.016mL of Glacial Acetic Acid
4.25g of NaCl
0.25mL of Tween 20
400mL of dH2O

-Stir into solution and pH to 6.0.
-q.s to 500mL and sterilize with 0.45um filter assy.
- Store at 4C.

30% acrylamide/0.8% bisacylamide
30g of 30% acrylamide
0.8g of bisacrylamide
70mL of dH2O

-Stir into solution and q.s to 100mL.
- Sterilize with 0.45um filter assy and transfer to a sterile amber container wrapped in foil.
- Store at 4C.
-Use appropriate PPE as acrylamide is a neurotoxin.

Stacking Gel (3.9% acrylamide)
0.65mL of 30% acrylamide/0.8% bisacrylamide
1.25mL of 4X Tris-HCl/SDS (pH6.8)
3.05mL of dH2O
25µl of 10% ammonium persulfate
10µl of TEMED

-Cap tube and mix via gentle inversion 4X and cast immediately.
- Store at 4C in dark containment.
- Final volume is for 2-3 stacking gels.

Separating Gel (4X Tris-HCl/SDS pH 8.8)
91g of Tris Base
2g of SDS
300mL of dH2O

-Stir into solution and pH to 8.8.
- q.s to 500mL and sterilize with 0.45um filter assy.
- Store at 4C.

Stacking Gel (4X Tris-HCl/SDS pH 6.8)
6.05g of Tris Base
0.4g of SDS
60mL of dH2O

-Stir into solution and pH to 6.8.
- q.s to 100mL and sterilize with 0.45um filter assy.
- Store at 4C.

2X Sample/Loading Buffer (pH 6.8)
1.52g of Tris Base
20mL of Glycerol
2.0g of SDS
0.001g of Bromophenol Blue
60mL of dH2O

- Stir into solution and pH to 6.8.
- q.s to 100mL and store ate 4-8C.

Running Buffer 10X SDS-PAGE "denaturing sodium dodecyl sµlfate-polyacrylamide discontinuous gel electrophoresis"
30.2g of Tris Base
144g of Glycine
10g of SDS
700mL of dH2O

-Stir into solution and q.s to 1000mL.
-Sterilize with 0.45um filter assy and store at 4C or at room temp.
-pH to 8.5

Native Running Buffer 10X PAGE "polyacrylamide discontinuous gel electrophoresis"
30.2g of Tris Base
144g of Glycine
700mL of dH2O

-Stir into solution and q.s to 1000mL.
-Sterilize with 0.45um filter assy and store at 4C or at room temp.
-pH to 8.5

Transfer Buffer (pH 8.1-8.4)
6.0g of Tris Base
28.8g of Glycine
0.1g of SDS
200mL of Methanol
600mL of dH2O

- Stir into solution and pH to 8.1-8.4.
- q.s to 1000mL and store at 4-8C.
- Solution can be used up to 5X.

Blocking Buffer
5g of BSA "bovine serum albumin"
100mL of 1X PBST "phosphate buffered saline tween"

- Stir into solution and sterilize with .45um filter assy.
- Make solution the day required for test or immediately store at 4-8C.

1X PBST "phosphate buffered saline tween" (pH 7.4)
1.2g of Na2HPO4
.22g of NaH2PO4
8.5g of NaCl
.5mL of Tween 20
500mL of dH20

-Stir into solution and q.s to 1000mL
-Autoclave at STP and store at 4-8C.

20X PBST "phosphate buffered saline tween"
24.0g of Na2HPO4
4.4g of NaH2PO4
170.0g of NaCl
10mL of Tween 20
500mL of dH20

-Stir into solution and q.s to 1000mL
-Autoclave at STP and store at 4-8C.

PBS "phosphate buffered saline"

1X	10X.	20X (40mL)
1X (20mL)		
8g of NaCl for 137mM	80g NaCl for 1.37mM	2mL PBS
1mL PBS		
0.2g of KCl for 2.7mM	2.0g NaCl for 27mM	38mL dH2O
19mL dH2O		
1.44g of Na2HPO4 for 10mM	14.4g of Na2HPO4 for 100mM	
0.24g of KH2PO4 for 1.8mM	2.4g of KH2PO4 for 18mM	
800mL of dH2O	800mL of dH2O	

- Stir into solution and pH to 7.4.
- q.s to 1000mL (1L) and autoclave on liquid cycle for 20mins and store at room temp.

TBS "tris buffered saline" (pH 7.4)
0.484g of Tris Base
5.848g of NaCl
150mL dH2O

-Stir into solution and adjust to pH 7.4.
-q.s to 200mL and sterilize with 0.45um filter assy.
- Sterilize with .45um filter assy and store at 4-8C.

Substrate Solution (super signal pico)
- SuperSignal PICO (Peirce 31070) will be used in this experiment as a suitable substrate solution.
- Make working solution of 50% Pico stable peroxide solution and 50% Pico luminal/enhancer solution and use within 24hours.
- Working solution is light sensitive. Immediately incase in foil or amber bottle. Store at manufactures recommendation at room temp 20-24C.
- For optimal results, use within 30 minutes of introduction to the plate/wells.

If SuperSignal PICO is not available then prepare solution as follows.
10mL of TBS
5mg of DAB
2µl of 30% H2O2

-Stir into solution and use immediately after preparation.
-Use appropriate PPE as DAB is toxic and 30% H2O2 is caustic.

1N NaOH "sodium hydroxide" Stock Solution
40g of pelleted NaOH
500mL of dH2O

- Stir until dissolved and q.s to 1000mL (1L)
- Store at room temp in a designated hazard cabinet.

1N HCl "Hydrochloric acid" Stock Solution
82.80mL of 37% HCl
500mL of dH2O

- Stir until dissolved and q.s to 1000mL (1L)
- Store at room temp in a designated hazard cabinet.

Wash Solution (0.05% Tween 20/PBS)
99.50mL of PBS
.50mL of Tween 20

- Stir until dissolved.
- Store at room temp 4C.

Appendix C: Reconstitution Protocols

Biotinylated FN Protein (Test: GNAQRWFVVWLG) Reconstitution
4.3mg FAP-P protein
3500µl mH20
800µl DMSO (dimethyl sulfoxide)

-Combine into a 5-15ml sterile tube
-Agitate solution with vortex genie until solution is homogenous (5-10 mins)
-Transfer 100µl of reconstituted solution into individual 1.5mL centrifuge tubes until all 4300µl of solution is aliquoted out.
-Store in -20C to -80C freezer until needed.

Biotinylated FN Protein (Control/Scrambled: RVAQLVFNWGWG) Reconstitution
4.6mg FAP-P protein
3200µl mH20
800µl DMSO (dimethyl sulfoxide)

-Combine into a 5-15ml sterile tube
-Agitate solution with vortex genie until solution is homogenous (5-10 mins)
-Transfer 100µl of reconstituted solution into individual 1.5mL centrifuge tubes until all 4300µl of solution is aliquoted out.
-Store in -20C to -80C freezer until needed

Fibronectin Attachment Protein (Scrambled/control) Reconstitution (RVAQLVFNWGWG)
3800µl Molecular H2O
800µl DMSO (dimethyl sulfoxide)
4.6mg FAP scrambled/control peptide (RVAQLVFNWGWG)

Fibronectin Complete Protein (Met1-Glu2477) Reconstitution
5mg/5mL concentration liquid 20mL vial
qs with carbon coating buffer (CCB)
-Transfer 1µl of fibronectin solution per 100µl of (CCB) for a 1:100 ratio or 1µl/100µl.
-Store fibronectin vial at 4C-8C.

Fibronectin Fragment 1 (Gln32-Pro1991) Reconstitution
100ug/65µl FN fragment 1(manufacture made vial)
127µl PBS (phosphate buffer saline)

-Add 35µl PBS to manufacture's vial and mix via inversion.
- Transfer 8µl of homogenized solution to individual 1.5mL snap lid centrifuge tubes.
- Add 92µl PBS to each tube for 8ug/100µl concentration aliquots.
- Store aliquots at -20C.

Fibronectin Fragment 2 (Ser607-Pro1265) Reconstitution
100ug FN fragment 2 (manufacture made vial) *reconstitute at 200ug/mL
500µl PBS (phosphate buffer saline)

-Add 500µl PBS to manufacture's vial and mix via inversion.
- Transfer 40µl of homogenized solution to individual 1.5mL snap lid centrifuge tubes for 8ug/40µl concentration aliquots.
- Store aliquots at -20C.

Fibronectin Fragment 3 (Glu1266-Pro1908) Reconstitution
50ug FN fragment 3 (manufacture made vial) *reconstitute at 100ug/mL
500µl PBS (phosphate buffer saline)

-Add 500µl PBS to manufacture's vial and mix via inversion.
- Transfer 80µl of homogenized solution to individual 1.5mL snap lid centrifuge tubes for 8ug/80µl concentration aliquots.
- Store aliquots at -20C.

Fibronectin Fragment 4 (Val1913-Glu2477) Reconstitution
50ug FN fragment 4 (manufacture made vial) *reconstitute at 100ug/mL
500µl PBS (phosphate buffer saline)

-Add 500µl PBS to manufacture's vial and mix via inversion.
- Transfer 80µl of homogenized solution to individual 1.5mL snap lid centrifuge tubes for 8ug/80µl concentration aliquots. Store aliquots at -20C

Appendix D: Luminescence Assay 1a-1k Protocol

Purpose: To detect fibronectin-fibronectin attachment protein (FN-FAP-P) binding by introducing a biotinylated scrambled (control) RVAQLVFNWGWG FAP-P probe and a biotinylated non-scrambled (test) GNAQRWFVVWLG FAP-P control probe to sequential, non-overlapping fibronectin fragments 1 (Gln32-Pro1991), 2 (Ser607-Pro1265), 3 (Glu1266-Pro1908), 4 (Val1913-Glu2477) and complete fibronectin dimer (Met1-Glu2477) using a luminescence assay.

Materials:
Carbon Coating Buffer pH 9.6
Fibronectin Samples, 0.5mg/ml
Fibronectin Samples, 5mg/ml
Wash Solution (0.05% Tween20 and phosphate buffered saline (PBS)
Blocking Buffer (1% BSA (bovine serum albumin), PBS (phosphate Buffered Saline)
1X PBST (phosphate buffered saline, pH 7.4 w/ 0.05% Tween 20,)
Wash solution (PBST (phosphate buffered saline/Tween20)
Biotinylated FN (Test: GNAQRWFVVWLG) (Genscript, SC1208) 4mg
Biotinylated FN (Scrambled: RVAQLVFNWGWG) (Genscript, SC1208) 4mg
ANT, pH 6 (10mM NH4Ac buffer, pH 6/0.85% NaCl/0.05% Tween 20)
Super Signal Pico ELISA chemiluminescent substrate (Peirce, 31070)
Neutravidin-horseradish peroxidase (HRP) conjugate (Peirce, 31001)
F-bottom Chimney well, 96 well Microplate (Greiner Bio-One, 655074)
Bottle top filtration unit, 250mL, 2um (VWR, 97066-200)
0.45um Filter assy
Top Seal-A microplate sealing film (VWR, 60941-078)
2.0mL Micro-centrifuge tubes (VWR, 2017-094)
Pipetman 0.5µl-5.0µl
Pipetman 10µl-100µl
Pipetman 2000µl-1000µl
Laminar flow hood (Air Clean systems, AC648LFUVC)
Multi Farrier tips 10µl (Gen3, GEN3-10-L-R-S)
Pipet tips 200µl (VWR, 53509-006)
Illuminometer, FlouroSkan Ascent FL (Thermo Systems, 5210450)
Fibronectin monomer (Met1-Glu2477)
Fibronectin Fragment #1 (Gln32-Pro1991) (BioLegend, 775306)
Fibronectin fragment #2 (Ser607-Pro1265) (Biotechne, 3225-FN-100)
Fibronectin fragment #3 (Gluc1266-Pro1908) (Biotechne, 3938-FN-050)
Fibronectin fragment #4 (Val1913-Glu-2477) (Biotechne, 3624-FN-050)
Incubator (fisher Scientific, 650D)

Method:

Part 1

Info Note: Volumes and quantities will vary from trial to trail 1a-1k. Refer to table 1 and table2 for applicable trial volumes and concentration ratios and dilutions for fibronectin samples, fibronectin fragments, fibronectin attachment protein (FAP-P) test and controls.

Trial 1A through 1K

1) Under the laminar flow hood station, transfer recommended volume of CCB solution into individual 2.0mL blue snap lid centrifuge tubes labeled with corresponding fibronectin sample. **See table 1 and 2 for volumes.*

Inoculation

2) Gently invert all sample tubes to ensure even mixture of solutions.
3) Pipette 100µl of each sample to each of the appropriate wells of a 96-well white microplate.
4) Cover plate with an adhesive coated clear plastic film and incubate plate for 18-24 hours at 4□C.

Part 2

Washing

5) After incubation cycle, remove solution from wells by removing plastic film and inverting plate over appropriate waste receptacle.
6) Wash the plate 3X by filling wells with 200µl of wash solution (0.05% Tween20 and PBS). Empty wash solution into appropriate waste receptacle.

Blocking

7) Block remaining free protein binding sites in wells by adding 200µl of blocking Buffer (1% BSA in PBST).
8) Cover plate with an adhesive coated clear plastic film and incubate plate at room temperature 20□C-24□C for 1-2 hours.

Washing

9) After incubation cycle, remove solution from wells by removing plastic film and inverting plate over appropriate waste receptacle.
10) Wash the plate 3X by filling wells with 200µl of wash solution (0.05% Tween20 and PBS). Empty wash solution into appropriate waste receptacle.

Probing

11) Add 100µl of 1.0ug/100µl of biotinylated FAP-P (test) and control (scrambled) peptides to appropriate wells. ** Refer to table 1 and 2 for volumes, Refer to appendix III FAP-P reconstitution for concentrations and dilutions.*

Info Note: For trials 1I and 1J, dilute FAP-P test and controls with (1X) PBS instead of ANT buffer. All previous and future trials FAP-P will be diluted with ANT buffer. Refer to ** Refer to table 1 and 2 for volumes, Refer to FAP-P reconstitution for concentrations and dilutions.*

12) Incubate plate for 60 minutes at 37□C.

Washing

13) After incubation cycle, remove solution from wells by removing plastic film and inverting plate over appropriate waste receptacle.
14) Wash plate/wells 3X with 200µl of 1X PBST (peptide diluent).

Colorimetric Substrate (NHR-PP)

15) Add 100µl of neutravidin-HRP with a working strength of 1:1000 in applicable wells. **Refer to table 2 for volumes of TBS/neutravidin.* Solution must be mixed, kept dark and cold till ready for use. Use within 30 minutes.
16) Cover plate with film and incubate plate for 60 minutes at 37C.

Washing

17) After incubation cycle, remove solution from wells by removing plastic film and inverting plate over appropriate waste receptacle.
18) Wash plate 3X with 1X PBST (peptide diluent).

Chemiluminescent Substrate (Supersignal Pico)

19) Add 100µl of super signal Pico to applicable wells (stabilizer enhancer, mix and kept dark till use. Use within 30 minutes) **Refer to table 2 for volumes and super signal pico recipe for ratio. See figure 1A-1K for microplate well sample lay out.*
20) Incubate plate in the dark for 10 minutes at room temperature 20□C-24□C.
21) Place plate into microplate illuminometer for binding detection.

Table 1. Fibronectin sample volumes, concentrations, and dilutions for trials 1A-1K.

Trial	Fibronectin samples								
	FN (A)	FN (B)	FN (C)	FN (D)	FN (E)	FN1	FN2	FN3	FN4
1A*	1600uL	1600uL	1600uL	1600uL	1600uL	–	–	–	–
1B	–	–	–	3200uL	3200uL	–	–	–	–
1C	–	–	–	3200uL	–	–	–	–	–
1D	–	–	–	1600uL	–	–	–	–	–
1E	–	–	–	3200uL	–	–	–	–	–
1F	–	–	–	3200uL	–	–	–	–	–
1G	–	–	–	–	–	800uL	800uL	800uL	800uL
1H	–	–	–	800uL	–	800uL	800uL	800uL	800uL
1I	–	–	–	800uL	–	800uL	800uL	800uL	800uL
1J	–	–	–	800uL	–	800uL	800uL	800uL	800uL
1K	–	–	–	800uL	–	800uL	800uL	800uL	800uL

* Trial 1A, FN sample A,B and C have a concentration of 0.5mg/1mL. Add 40uL of FN to individual snap lid centrifuge tubes. FN sample D and E have a concentration of 5mg/1mL. Add 20uL of FN sample to individual snap lid centrifuge tubes. Add 1mL of carbon coating buffer (CCB α/β) into each individual sample tube. Trials 1B-1K, Raw FN sample has a premade 1:1 ratio. For every 1uL of raw FN solution, add 100uL of CCB for a end concentration of 1ug/100uL per applicable microplate well.

Table 2. Solution, reagents, and substrate volumes for trials 1A-1K.

Trial	NHR-P		Supersignal Pico							
	Neutravidin	TBS	Enhancer	Stabilizer	FAP (test)	FAP (cont)	CCB α	CCB β	ANT	PBS (1X)
1A	6.4uL	6393.6uL	3200uL	3200uL	2400uL	2400uL	–	–	–	–
1B	5.2uL	5194.8uL	2600uL	2600uL	2000uL	2000uL	2000uL	2000uL	–	–
1C	4.4uL	4395.6uL	2200uL	2200uL	2000uL	2000uL	2800uL	2800uL	–	–
1D	3.6uL	3596.4uL	1800uL	1800uL	2000uL	2000uL	–	4800uL	800uL	–
1E	–	–	–	–	2000uL	2000uL	–	–	–	–
1F	4uL	4396uL	2000uL	2000uL	2000uL	2000uL	–	–	–	–
1G	3.2uL	3196.8uL	1600uL	1600uL	1600uL	1600uL	800uL	–	–	800uL
1H	4.4uL	4395.6uL	2200uL	2200uL	2200uL	2200uL	200uL	–	–	200uL
1I	4.4uL	4395.6uL	2200uL	2200uL	2200uL	2200uL	200uL	–	–	200uL
1J	4.8uL	4795.2uL	2400uL	2400uL	2400uL	2400uL	–	–	–	–
1K	4.8uL	4795.2uL	2400uL	2400uL	2400uL	2400uL	–	–	–	–

Appendix E: Crystal Violet Protocol

Purpose: To verify fibronectin dimer-microplate adherence using a crystal violet staining technique.

Materials:

96 well Micro Test, Clear Plate (Falcon, 35-0072)
1X PBST (phosphate buffered saline, pH 7.4 w/ 0.05% Tween 20,) *see recipe section
1.5mL White Snap Lid Centrifuge Tubes (VWR, 14231-062)
Carbon Coating Buffer pH 9.6 *see recipe section
Crystal Violet Staining Solution (Sigma Aldrich, HT901-8FOZ)
Fibronectin Dimer, Stabilized Bovine (Met1-Glu2477) (Biomedical Tech Inc., 2265)
Fibronectin Dimer Samples, 0.5mg/ml (Biomedical Tech Inc., 2265)
Fibronectin Dimer Samples, 5mg/ml (Biomedical Tech Inc., 2265)
Glacial Acetic Acid (Mallinkrodt AR, 2504)
Incubator (fisher Scientific, 650D)
Laminar flow hood (Air Clean systems, AC648LFUVC)
Pipet tips 200uL (VWR, 53509-006)
Pipetman 10uL-100uL
Pipetman 100uL-1000uL
Pipette tips 200uL (VWR, 53509-006)
Refrigerator 2□C-10□C (Frigidaire, FFRU17B2QWD)
Top Seal-A microplate sealing film (VWR, 60941-078)
Multiskan Reader (Thermo Electron Corp., 51118750)
Wash Solution (0.05% Tween20 and phosphate buffered saline (PBS)
Wash solution (PBST (phosphate buffered saline/Tween20))

Method:

Part 1

FAP-P (test) and control (scrambled) peptide ANT dilution: Under the laminar flow hood station, transfer 768µl of CCB solution into 3 individual 1.5mL white snap lid centrifuge tubes labeled FNS (A), (B), and (C). Prior to step 1, thaw one individual use 100µl FN sample aliquot.

1) Under the laminar flow hood station, transfer 768µl of CCB solution into 3 individual 1.5mL white snap lid centrifuge tubes labeled “CCB Sample A, B, and C”.

2) Add 32µl of 0.5mg/ml FN sample A into CCB sample A.
3) Add 32µl of 0.5mg/ml FN sample B into CCB sample B.
4) Add 32µl of 0.5mg/ml FN sample C into CCB sample C.
5) Gently invert all sample tubes to ensure even mixture of solutions.
6) Pipette 50µl of each sample to each of the 16 wells of a 96-well white microplate.
7) Cover plate with an adhesive coated clear plastic film and incubate plate for 18-24 hours at 4□C.

Part 2

8) After incubation cycle, remove CCB solution by removing plastic film and inverting plate over appropriate waste receptacle.
9) Wash the plate 3X by filling wells with 200µl of wash solution (0.05% Tween20 and PBS). Empty wash solution into appropriate receptacle.
10) Add 100µl of crystal violet dye to each FN coated well.
11) Incubate plate for 30 minutes at 37C.
12) Wash plate/wells 3X with 200µl of 1X PBST (peptide diluent).
13) Add 125µl of 30% glacial acetic acid to each coated well and incubate at room temperature for 15 minutes.
14) After incubation period, transfer 125µl of solution from each filled well to corresponding wells in a clear 96-well microplate.
15) Place plate into microplate in Thermo Electron Mµltiskan Spectrum reader for binding detection.

Appendix F: Bicinchoninic Acid Assay No. 1 and No. 2

Purpose: To verify colorimetric detection and identify intact soluble fibronectin concentrations

Info Note: Bovine Serum Albumin (BSA) protein dilutions were prepared by following the microplate dilution scheme recommended by the Pierce™ BCA Protein Assay Kit (Thermo Scientific) user guide with a working range of 20-2,000µg/ml.

Materials:

15mL Sterile Centifuge Tube (Thermo Fisher, 339650)
Bovine Serum Albumin 1G (Sigma Aldrich, 05470-1G)
BCA Protein Assay Kit (Thermo Scientific, 23225 and 23227))
F-bottom Chimney well, 96 well Microplate (Greiner Bio-One, 655074)
Incubator (fisher Scientific, 650D)
Laminar flow hood (Air Clean systems, AC648LFUVC)
Pipet tips 200uL (VWR, 53509-006)
Pipetman 10uL-100uL
Nutator Rocking Unit (CLAY ADAMS, 421105)
Multiskan Reader (Thermo Electron Corp., 51118750)
Top Seal-A microplate sealing film (VWR, 60941-078)

Method:

Protein standard preparation

1. Label 9 vials A-I.
2. Prepare diluted BSA standard vials per Table 1.

Table 1. Preparation of diluted BSA (bovine serum albumin) standards with final concentrations.

Dilution Scheme for Microplate Procedure {Working Range= 20-2000ug/mL}			
Vial	**Diluent Volme (µl)**	**BSA Source and Volume (µl)**	**BSA Final Concentration (ug/mL)**
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	352	325 of Stock	1000
D	175	175 of vial B	750
E	325	325 of vial C	500
F	325	325 of vial E	250
G	325	325 of vial F	125
H	400	100 of vial G	25
I	400	0	0= Blank

BCA working reagent preparation (WR) for a 50:1 ratio

1. Prepare working agent solution by adding 7.5mL of BCA reagent (A) to a 15mL sterile centrifuge tube and label WR (working reagent).
2. Add 0.15mL (150µl) of BCA reagent (B) to WR tube and mix via inversion and rest for 5-10 minutes to clear turbidity.

Microplate preparation

1. Transfer 25µl of each standard solution to appropriate wells of microplate.
2. Transfer 25µl of FN sample to appropriate wells.
3. Add 200µl of WR to each previously coated well.
4. Cover plate with sealing film and mix plated solutions by placing microplate on a plate shaker for 30 seconds.
5. Incubate plate for 30 minutes at 37C.
6. Allow plate to cool to room temperature for 10-15 minutes.
7. Read absorbance at 550nm in Thermo electron Corp. multiskan spectrum reader.

Appendix G: SDS-Page and Western Blot (Denatured/Native)

Purpose: To visualize protein characteristics of bound fibronectin fragment 4-fibronectin attachment protein (FAP-P) in a native and denatured environment by using gel electrophoresis and western blotting techniques.

Materials:

Fibronectin (fragment 4) 50ug (Val1913-Glu-2477) (Biotechne, 3624-FN-050)
Blocking Buffer (1% BSA (bovine serum albumin), PBS (phosphate Buffered Saline)
Transfer Buffer (Native/Denatured)
Running Buffer (Native/Denatured)
1X PBST (phosphate buffered saline, pH 7.4 w/ 0.05% Tween 20,)
Wash solution (PBST (phosphate buffered saline/Tween20))
Biotinylated FN (Test: GNAQRWFVVWLG) (Genscript, SC1208) 4mg
ANT, pH 6 (10mM NH4Ac buffer, pH 6/0.85% NaCl/0.05% Tween 20)
Super Signal Pico West chemiluminescent substrate (Peirce, 34077)
Super Signal Pico West Plus chemiluminescent substrate (Peirce, 34577)
Neutravidin-horseradish peroxidase (HRP) conjugate (Peirce, 31001)
Bottle top filtration unit, 250mL, 2um (VWR, 97066-200)
.5mL Micro-centrifuge tubes (VWR, 14231-062)
Pipetman 0.5 µl-5.0 µl
Pipetman 10 µl-100 µl
Pipetman 100 µl-1000 µl
Laminar flow hood (Air Clean systems, AC648LFUVC)
M µlti Farrier tips 10 µl (Gen3, GEN3-10-L-R-S)
Pipette tips 200 µl (VWR, 53509-006)
Filter paper 7.5 x 10cm (Bio-Rad, 1703965)
β-mercaptoethanol 25mL (Sigma chemical co., M-1348)
Tween 20, 100mL (Fisher Scientific, BP337-100)
LD Poly bags, 4" x 6" (OP, 20-F-0406)
LI-COR Imager (Intertek, 120627)
Gel Electrophoresis Assembly (Bio-Rad, Mini-PROTEAN II Cell)
Nitrocel µlose Membrane Roll 30cm x 3M (Pall Cor., 66485)
Native sample buffer 30mL (Bio-Rad, 1610737)
Leammli Sample Buffer 30mL (Bio-Rad, 161738)
Sealer Unit (Imp µlse Sealer, PFS-200)
Thermal, Master Cycler (Eppendorf AG, 5331 40451)
Nutator Rocking Unit (Clay Adams, 421105)
Mini Vortexer (VWR, MV1-03-030951)
Lab Quest PH meter (Vernier, LQ2-LE)
Magnetic stirrer/hot plate (Corning, PC-320)
Gel Electrophoresis Assembly (Bio-Rad, Mini-PROTEAN II Cel

AcuPower Unit (VWR, 300)
Pre-stained Protein Ladder (ThermoScientific, 26619)
Rocking Unit (VWR, 490005-696)
Coomasie Staining Solution (BioRad, 1610786)
De-staining Solution (BioRad, 1610438)

Methods:

SDS-Page (Denatured/Native) Trials 1-4

Info Note: When running a native gel; use native only buffers that do not contain SDS, do not add β-mercaptoethanol and do not heat treat fibronectin sample or sample buffer.

Sample and Buffers Prep

1) Prepare buffers. **See Recipes and solutions appendix II and III.*
2) Prepare fibronectin sample by adding 5µl β-mercaptoethanol to 250µl sample buffer.
3) Transfer 10µl of 2X Leammli sample buffer/β-mercaptoethanol solution to 10µl of fibronectin fragment 4 sample and heat solution at 95□99°C for 3□5 min. **Sample will be 20µl of sample with a concentration of 5ug/20µl to later be loaded into the applicable well**.

Gel Electrophoresis

4) Gently remove gel from the package and remove tape from the bottom of the gel.
5) Place gel with wells on top, between a clean Short Plate labeled "UP" (Aft) and a taller 1.0mm Spacer Plate (Fwd) to create the gel cassette.
6) Place the gel cassette into the open slot of the electrode assembly with the Short Plate facing inward toward the green U□shaped gaskets.
7) Place a "Buffer Dam" into the adjacent empty electrode assembly slot.
8) Secure both gel cassette and buffer dam onto electrode assembly by sliding assembly into clamping frame with the cam levers facing forward.
9) Gently remove green comb from gel/cassette and verify that there are no air bubbles in the wells of the gel.
10) Rotate both cam levers of the clamping frame to ensure a proper seal of the inner chamber to short plate.
11) Place the Inner Chamber assembly into the Tank.

12) Fill the Inner Chamber with Running Buffer (1X SDS-PAGE) until the buffer level reaches halfway between the top of the Short /Spacer Plate.
13) Fill the outer chamber of the Tank with Running Buffer (1X SDS-PAGE) to approx. halfway.
14) Load 20µl of FN samples into well receptacles.
15) Load 10µl of (pre□stained) molecular weight (MW) marker "AKA" Ladder" in well receptacle.
16) Place the Lid on the Tank, aligning (red/black) color□coded banana plugs to corresponding jacks.
17) Insert the red/black electrical leads into a power supply and set voltage to 100V and switch power unit to run. **Run for approx. 60 minutes.*
18) Stop electrophoresis by turning off the power supply unit, and disconnect the red/black electrical leads from power supply unit.
19) Remove the Tank lid, Inner Chamber assembly and open the cams of the Clamping Frame to pull the Electrode Assembly out of the Clamping Frame.
20) Remove the Gel Cassette(s) and use the green Gel Releaser to gently pry the glass plates apart.
21) Remove the Short Plate from the Gel Cassette by gently separating the two glass plates.
22) Gently run the sharp edge of the Gel Releaser along each spacer to separate the gel from the spacers on the Spacer Plate.
23) Carefully remove the processed gel from the plates and rinse with dH2O, then immediately place into tray filled with 10mL of transfer buffer and perform Western blot procedure.

Western Blot Transfer (Denatured/Native)

Nitrocellulose Preparation

1. Trim membrane to size with sterilized scissors and label top side of the membrane with applicable information.
2. Immediately place the membrane in tray filled with 10mL transfer buffer for 10 minutes at room temp 20-24C.
3. During 10-minute soak, fill a different tray with 100mL of transfer buffer.

Sandwich

4. During transfer buffer soak, Place two fiber pads and 2 filter papers in transfer buffer tray for complete saturation.

5. Assemble the transfer sandwich by laying out the cassette (black/white with circles) in a tray with the black plate facing down.
6. Place one soaked fiber pad onto the black plate and one soaked filter paper on the fiber pad.
7. Gently roll/slide a glass rod over the surface and remove any air bubbles.
8. Place the processed gel on top of the filter papers and remove any air bubbles. **Place gel upside down with lanes 10 to 1 facing up instead of 1 to 10.*
9. Carefully stack the soaked membrane on the gel with the information side down.
10. Place second soaked filter paper and fiber pad onto the membrane.
11. Clip cassette together by sliding latching lever over the frame.
12. Place the assembled cassette into the transfer tank, black plate next to black electrode.
13. Perform the same sandwich stacking procedure for the native gel, using the same transfer buffer and tray.
14. Place a cooling block in the tank, and fill with transfer buffer till cassettes are covered.
15. Place the lid on the tank, aligning (red/black) color□coded banana plugs to corresponding jacks.
16. Insert the red/black electrical leads into a power supply and set voltage to 100V and switch power unit to run. *Run for approx. 60 minutes.*
17. Stop transfer by turning off the power supply unit, and disconnect the red/black electrical leads from power supply unit.
18. Remove the tank lid and disassemble cassette in reverse order.
19. Place membranes in individual trays filled dH2O and rinse membranes.

Western Blot Block/FAP-P (Denatured/Native)

1. Transfer the membranes into poly plastic envelopes with 10mL blocking buffer (1%BSA in PBST) into an automated rocker set on gentle for overnight at room temp 20C-24C.

<u>Day 2</u>

2. Drain envelope of blocking buffer and rinse membranes in 10mL of ANT buffer (pH 6) two consecutive times.
3. Prepare Fibronectin Attachment Protein (test) sample (FAP-P) by adding 40µl of FAP (test) sample to 5960µl of ANT buffer **for concentration of 40ug/6mL**.
4. Add 3mL of Fibronectin Attachment Protein (test) solution to each envelope and seal.

5. Place sealed envelopes in the automated rocker set on gentle at room temperature 20C-24C for two hours.

Western Blot Wash/NHR-P/Super Signal Pico (Denatured/Native)

1. Remove sealed envelope from rocker and drain solution.
2. Fill envelope with 10mL of 1X PBS-T.
3. Place the envelope with the membrane on the on the rocker for 4 minutes at room temp 20C-24C.
4. Wash the membrane by replacing fluid with 10ml of PBS-T and rock for 10 minutes on gentle. **Repeat this cycle a total of 4X.*
5. After final wash/rock cycle drain the PBS-T and add NHR-P solution, seal and cover. * *NHR-P is light and temperature sensitive with a 1:1000 working ratio.*
6. Place envelope in the rocker on gentle for 1 hour at room temp 20C-24C in the dark.
7. Perform wash/rock cycle in step 4 for a total of 4X.
8. After final wash/rock cycle drain the PBS-T from the denatured membrane and add Super Signal Pico solution, seal, and cover. * *Super Signal Pico is light sensitive and has a 1:1 ratio of enhancer to stabilizer.*

Info Note: Leave the "Native membrane sealed in the 1X PBS-T while the "Denatured" membrane is being processed. Once imaging is complete, make Super Signal pico solution and carry out same steps with the "Native membrane".

9. Place envelope on the rocker on gentle for 10 minutes at room temp 20C-24C in the dark.
10. Rinse membrane with PBS (no Tween). Proceed to imaging. Membrane must remain wet.

Table 1. Trial 1 SDS-PAGE and western blot (denatured/native) solutions and substrate volumes.

Nomenclature	Solutions	Volume (uL)	Incubation (mins)
NHR-P	*TBS*	6000	60
	Neutravidin	6	N/A
Marker		10	N/A
SSPico	*Enhancer*	3000	10
	Stablizer	3000	N/A
Probe	*ANT buffer*	5960	60
	FAP	40	N/A
Block	*BSA1%/PBST*	3000	1440
Note	*No pre-rinse after blocking step*		

Table 2. Trial 2 SDS-PAGE and western blot (denatured/native) solutions and substrate volumes.

Nomenclature	Solutions	Volume (uL)	Incubation (mins)
NHR-P	*TBS*	6000	60
	Neutravidin	6	N/A
Marker		10	N/A
SSPico	*Enhancer*	3000	10
	Stablizer	3000	N/A
Probe	*ANT buffer*	5960	120
	FAP	40	N/A
Block	*BSA1%/PBST*	3000	1440
Note	*1X PBST rinse after block*		N/A

Table 3. Trial 3 SDS-PAGE and western blot (denatured/native) solutions and substrate volumes.

Nomenclature	Solutions	Volume (uL)	Incubation (mins)
NHR-P	*TBS*	6000	60
	Neutravidin	6	N/A
Marker		10	N/A
SSPico	*Enhancer*	3800	10
	Stablizer	3800	N/A
Probe	*ANT buffer*	5960	120
	FAP	40	N/A
Block	*BSA1%/PBST*	3000	1440
Note	*ANT buffer rinse after block*		N/A

Table 4. Trial 4 SDS-PAGE and western blot (denatured/native) solutions and substrate volumes.

Nomenclature	Solutions	Volume (uL)	Incubation (mins)
NHR-P	*TBS*	8000	60
	Neutravidin	1	N/A
Marker		10	N/A
SSPico	*Enhancer*	3800	10
	Stablizer	3800	N/A
Probe	*ANT buffer*	5960	120
	FAP	40	N/A
Block	*BSA1%/PBST*	3000	1440
Note	*ANT buffer rinse after block*		N/A

Appendix H: Native/Denatured Luminescence Assay 2A-2F Protocol

Purpose: To detect binding of fibronectin attachment protein (FAP-P) probe GNAQRWFVVWLG to fibronectin fragment 4 (Val1913-Glu2477) in a native and denatured environment using a luminescence assay.

Materials:

Carbon Coating Buffer pH 9.6
Wash Solution (0.05% Tween20 and phosphate buffered saline (PBS)
Blocking Buffer (1% BSA (bovine serum albumin), PBS (Phosphate Buffered Saline)
1X PBST (phosphate buffered saline, pH 7.4 w/ 0.05% Tween 20)
Wash solution (PBST (phosphate buffered saline/Tween20)
Biotinylated FN (Test: GNAQRWFVVWLG) (Genscript, SC1208) 4mg
ANT, pH 6 (10mM NH4Ac buffer, pH 6/0.85% NaCl/0.05% Tween 20)
Super Signal Pico ELISA chemiluminescent substrate (Peirce, 31070)
Neutravidin-horseradish peroxidase (HRP) conjugate (Peirce, 31001)
F-bottom Chimney well, 96 well Microplate (Greiner Bio-One, 655074)
Bottle top filtration unit, 250mL, 2um (VWR, 97066-200)
0.45um Filter assy
Top Seal-A microplate sealing film (VWR, 60941-078)
2.0mL Micro-centrifuge tubes (VWR, 2017-094)
Pipetman 0.5 µl-5.0 µl
Pipetman 10 µl-100 µl
Laminar flow hood (Air Clean systems, AC648LFUVC)
M µlti Farrier tips 10 µl (Gen3, GEN3-10-L-R-S)
Pipet tips 200 µl (VWR, 53509-006)
Illuminometer, FlouroSkan Ascent FL (Thermo Systems, 5210450)
Fibronectin fragment #4 (Val1913-Glu-2477) (Biotechne, 3624-FN-050)
Incubator (fisher Scientific, 650D)
Freezer -20□C (Frigidaire, WB73751942)
Incubator (fisher Scientific, 650D)
Refrigerator 2□C-10□C (Frigidaire, FFRU17B2QWD)
Refrigerator (walk-in) 2□C-10□C (Controlled Enviro, S275A)

Trial 2A

1) Under the laminar flow hood station, thaw one vial of fibronectin fragment 4 sample. *Sample should be 10µg of fibronectin fragment 4 protein and 10µl of PBS (1:1 ratio). * Refer to appendix III fibronectin fragment 4 reconstitution for concentrations and dilutions.*

Denatured

2) In a 1.7ml snap lid centrifuge tube, add 490µl CCB and 10µl of β-mercaptoethanol and label "denatured"
3) Add 5µl of fibronectin sample to "denatured" vial and heat treat in master thermal cycler set on SDS program.

Native

4) While heat treatment program is running, add 495µl of CCB to the remaining vial of fibronectin fragment 4 sample and label "Native".

Inoculation

5) After heat treat, gently invert sample tubes to ensure even mixture of solutions.
6) Pipette 100µl of each sample to each of the appropriate wells of a 96-well white microplate.
7) Cover plate with an adhesive coated clear plastic film and incubate plate for 18-24 hours at 4□C.

Part 2

Washing

8) After incubation cycle, remove solution from wells by removing plastic film and inverting plate over appropriate waste receptacle.
9) Wash the plate 3X by filling wells with 200µl of wash solution (0.05% Tween20 and PBS) and empty wash solution into appropriate waste receptacle.

Blocking

10) Block remaining free protein binding sites in wells by adding 200µl of blocking Buffer (1% BSA in PBST).
11) Cover plate with an adhesive coated clear plastic film and incubate plate at room temperature 20□C-24□C for 1-2 hours.

Washing

12) After incubation cycle, remove solution from wells by removing plastic film and inverting plate over appropriate waste receptacle.
13) Wash the plate 3X by filling wells with 200µl of wash solution (0.05% Tween20 and PBS). Empty wash solution into appropriate waste receptacle.

Probing

14) Add 100µl of 4.0µg /100µl of biotinylated FAP-P (test) and control (scrambled) peptides to appropriate wells. ** Refer to appendix III FAP-P reconstitution for concentrations and dilutions.*
15) Incubate plate for 60 minutes at 37□C.

Washing

16) After incubation cycle, remove solution from wells by removing plastic film and inverting plate over appropriate waste receptacle.
17) Wash plate/wells 3X with 200µl of 1X PBST (peptide diluent).

Colorimetric Substrate (NHR-PP)

18) Add 100µl of neutravidin-HRP with a working strength of 1:1000 in applicable wells. **Refer to table 1 for volumes of TBS/neutravidin.* Solution must be mixed, kept dark and cold till ready for use. Use within 30 minutes)
19) Cover plate with film and incubate plate for 60 minutes at 37C.

Washing

20) After incubation cycle, remove solution from wells by removing plastic film and inverting plate over appropriate waste receptacle.
21) Remove film and wash plate 3X with 1X PBST (peptide diluent).

Chemiluminescent Substrate (Supersignal Pico)

22) Add 100µl of super signal Pico to applicable wells (stabilizer enhancer, mix and kept dark till use. Use within 30 minutes) **Refer to table 1 for volumes and super signal pico recipe for ratio.*
23) Incubate plate in the dark for 10 minutes at room temperature 20□C-24□C.
24) Place plate into microplate illuminometer for binding detection.

Table 1. Native/Denatured Luminescence Assay trials 2A-2F solutions, substrates and reagents volumes.

	NHR-P		Supersignal Pico							
	Neutravidin	TBS	Enhancer	Stabilizer	FAP (test)	CCB	Beta-merc	FN Frag 1	FN frag 4	FN Dimer
2A	1uL	1000uL	500uL	500uL	1000ul	980uL	10uL	--	1000uL	1000uL
2B	2uL	2000uL	1000uL	1000uL	2000uL	980uL	10uL	5uL	2000uL	2000uL
2C	1.5 ul	1500uL	750uL	750uL	1200uL	3000uL	10uL	--	1200uL	1500uL
2D	1.5uL	1500uL	750uL	750uL	1200uL	3000uL	1uL	--	1200uL	1500uL
2E	1.5uL	1500uL	750uL	750uL	1200uL	3000uL	10uL	--	1200uL	1500uL
2F	1.5uL	1500uL	750uL	750uL	1200uL	3000uL	--	--	1200uL	1500uL

Appendix I: Supplemental Data

Scope: This section contains Luminescence Assay trials 1a-1K Raw Data. "S#" signifies supplemental data 1 and "A-K" signifies the experimental trial.

S1 Luminescence Assay Raw Data for Trials A-K

Table S1A. Trial luminescence raw data results measured in relative light units.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0014	0.0008	0.0000	0.0000	0.0000	0.0001	0.0015	0.0009	0.0000	0.0002	0.0000	0.0001
B	0.0005	0.0028	0.0063	0.0048	0.0000	0.0000	0.0000	0.0019	0.0013	0.0002	0.0011	0.0000
C	0.0015	0.0000	0.0000	0.0023	0.0020	0.0000	0.0010	0.0000	0.0025	0.0000	0.0007	0.0004
D	0.0000	0.0064	0.0013	0.0000	0.0000	0.0016	0.0010	0.0001	0.0008	0.0014	0.0044	0.0006
E	0.0000	0.0009	0.0006	0.0001	0.0002	0.0002	0.0009	0.0008	0.0000	0.0000	0.0005	0.0000
F	0.0022	0.0019	0.0001	0.0028	0.0050	0.0008	0.0000	0.0002	0.0021	0.0002	0.0000	0.0000
G	0.0016	0.0000	0.0019	0.0012	0.0000	0.0022	0.0013	0.0011	0.0002	0.0007	0.0000	0.0016
H	0.0002	0.0000	0.0009	0.0008	0.0039	0.0022	0.0000	0.0020	0.0010	0.0012	0.0015	0.0000

*FlouroSkan program setting: Measurement count: 1 Filter: 0 Scaling Factor : 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	Control	NHRP	α CCB		NHRP	SS Pico	NHRP	SS Pico	SS Pico	NHRP	SS Pico	NHRP
B	Control	NHRP	α CCB		NHRP	SS Pico	NHRP	SS Pico	SS Pico	NHRP	SS Pico	NHRP
C	Control	NHRP	α CCB		NHRP	SS Pico	NHRP	SS Pico	SS Pico	NHRP	SS Pico	NHRP
D	Control	NHRP	α CCB	EMPTY	NHRP	SS Pico	NHRP	SS Pico	SS Pico	NHRP	SS Pico	NHRP
E	Test	SS Pico	β CCB		Test	Cont	Test	Cont	Test	Cont	Test	Control
F	Test	SS Pico	β CCB		Test	Cont	Test	Cont	Test	Cont	Test	Control
G	Test	SS Pico	β CCB		Test	Cont	Test	Cont	Test	Cont	Test	Control
H	Test	SS Pico	β CCB		Test	Cont	Test	Cont	Test	Cont	Test	Control
	(-) FN (-) NHRP (-) SS Pico (+) CCB (+) Wash (+) PBST (+) Block Buffer	(-) FN (+) CCB (+) Wash (+) PBST (+) Block Buffer	(-) FN (-) NHRP (-) SS Pico (+) CCB (+) Wash (+) PBST (+) Block Buffer	(-) FN (-) NHRP (-) SS Pico (-) CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN D (+) NHRP (+) SS Pico (+) α CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN D (+) NHRP (+) SS Pico (+) β CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN E (+) NHRP (+) SS Pico (+) α CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN E (+) NHRP (+) SS Pico (+) β CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN D (+) NHRP (+) SS Pico (+) α CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN D (+) NHRP (+) SS Pico (+) β CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN E (+) NHRP (+) SS Pico (+) α CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN E (+) NHRP (+) SS Pico (+) β CCB (+) Wash (+) PBST (+) Block Buffer

Fig S1A. Trial A microplate well layout.

Table S1B. Trial luminescence raw data results measured in relative light units.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.1772	0.584	279.1	0.6954	0.2874	0.1852	0.1487	0.156	0.1952	0.17	0.1139	0.0927
B	0.242	0.8402	270.4	0.8192	0.399	0.2617	0.2442	0.2826	0.2137	0.1411	0.1646	0.1551
C	0.3365	0.7847	273.4	1.045	0.6287	0.4063	0.4171	0.3866	0.5281	0.3834	0.2988	0.2129
D	0.3027	0.8039	236.9	1.304	1.272	0.9343	1.139	0.9342	1.405	0.9471	0.9747	0.6496
E	0.2861	0.6721	236.8	1.817	592.1	21.62	366.6	16.81	612.5	31.5	414.3	25.81
F	0.2829	0.572	222	1.69	554.6	32.38	362.3	16.65	678.1	25.91	367.8	22.56
G	0.1725	0.3628	21.5	1.561	530.1	29.45	359.6	11.57	657.1	38.73	370.6	30.4
H	0.1022	0.2739	17.11	1.065	534.1	25.74	393.5	12.39	537.2	29.2	554.9	29.73

*FlouroSkan program setting: Measurement count: 1 Filter: 0 Scaling Factor : 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	Control	NHRP	α CCB		NHRP	SS Pico	NHRP	SS Pico	SS Pico	NHRP	SS Pico	NHRP
B	Control	NHRP	α CCB		NHRP	SS Pico	NHRP	SS Pico	SS Pico	NHRP	SS Pico	NHRP
C	Control	NHRP	α CCB		NHRP	SS Pico	NHRP	SS Pico	SS Pico	NHRP	SS Pico	NHRP
D	Control	NHRP	α CCB	EMPTY	NHRP	SS Pico	NHRP	SS Pico	SS Pico	NHRP	SS Pico	NHRP
E	Test	SS Pico	β CCB		Test	Cont	Test	Cont	Test	Cont	Test	Control
F	Test	SS Pico	β CCB		Test	Cont	Test	Cont	Test	Cont	Test	Control
G	Test	SS Pico	β CCB		Test	Cont	Test	Cont	Test	Cont	Test	Control
H	Test	SS Pico	β CCB		Test	Cont	Test	Cont	Test	Cont	Test	Control
	(-) FN (-) NHRP (-) SS Pico (+) CCB (+) Wash (+) PBST (+) Block Buffer	(-) FN (+) CCB (+) Wash (+) PBST (+) Block Buffer	(-) FN (-) NHRP (-) SS Pico (+) CCB (+) Wash (+) PBST (+) Block Buffer	(-) FN (-) NHRP (-) SS Pico (-) CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN D (+) NHRP (+) SS Pico (+) α CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN D (+) NHRP (+) SS Pico (+) β CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN E (+) NHRP (+) SS Pico (+) α CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN E (+) NHRP (+) SS Pico (+) β CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN D (+) NHRP (+) SS Pico (+) α CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN D (+) NHRP (+) SS Pico (+) β CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN E (+) NHRP (+) SS Pico (+) α CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN E (+) NHRP (+) SS Pico (+) β CCB (+) Wash (+) PBST (+) Block Buffer

Fig S1B.1. Trial B microplate well layout.

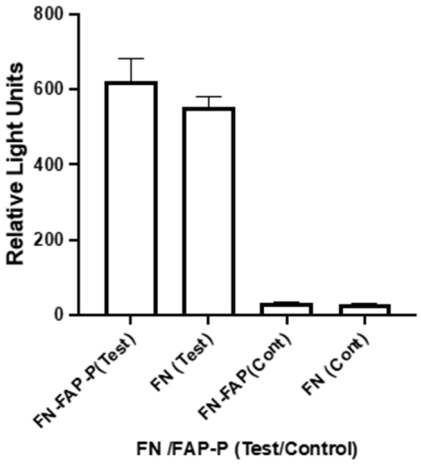

Fig S1B.2. Trial B bar graph with fibronectin + test probe vs. fibronectin + control probe vs fibronectin.

Table S1C. Trial luminescence raw data results measured in relative light units.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0965	0.1811	0.6446	191.9	208.2	0.5895	0.2223	1.047	0.12	0.0946	4.239	0.0668
B	0.1057	0.2417	0.7058	303.9	323	0.8585	0.304	0.5999	0.1734	0.2017	2.767	0.1244
C	0.1128	0.2826	0.8629	289.3	283	0.9534	0.4597	1.637	0.2127	0.2313	2.73	0.2143
D	0.0573	0.1676	0.6111	259.9	277.7	1.083	1.173	1.221	0.3692	0.5787	3.208	0.5217
E	0.0517	0.1494	0.3262	13.67	11.35	1.266	580	17.42	0.6803	1.221	655.2	26.53
F	0.0874	0.1423	0.182	14.16	13.38	1.272	586.7	17.15	0.794	1.374	680.8	26.85
G	0.0638	0.0955	0.1618	11.45	13.02	1.23	601.6	17.61	0.7267	1.232	672.5	7.206
H	0.0353	0.0417	0.1446	13.4	12.9	1.092	597	27.5	0.6389	1.129	611.9	40.27

*FlouroSkan program setting: Measurement count: 1 Filter: 0 Scaling Factor : 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	Control	NHRP	α CCB	Test	Test		NHRP	SS Pico			SS Pico	NHRP
B	Control	NHRP	α CCB	Test	Test		NHRP	SS Pico			SS Pico	NHRP
C	Control	NHRP	α CCB	Test	Test		NHRP	SS Pico			SS Pico	NHRP
D	Control	NHRP	α CCB	Test	Test		NHRP	SS Pico			SS Pico	NHRP
E	Test	SS Pico	β CCB	Cont	Cont		Test	Cont			Test	Control
F	Test	SS Pico	β CCB	Cont	Cont		Test	Cont			Test	Control
G	Test	SS Pico	β CCB	Cont	Cont		Test	Cont			Test	Control
H	Test	SS Pico	β CCB	Cont	Cont		Test	Cont			Test	Control
	(-) FN (-) NHRP (-) SS Pico (-) CCB (+) Wash (+) PBST (+) Block Buffer	(-) FN (-) CCB (+) Wash (+) PBST (+) Block Buffer	(-) FN (-) NHRP (-) SS Pico (-) CCB (+) Wash (+) PBST (+) Block Buffer	(-) FN (+) NHRP (+) SS Pico (+) α CCB (+) Wash (+) PBST (+) Block Buffer	(-) FN (+) NHRP (+) SS Pico (+) β CCB (+) Wash (+) PBST (+) Block Buffer	(-) FN (-) NHRP (-) SS Pico (-) CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN D (+) NHRP (+) SS Pico (+) α CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN D (+) NHRP (+) SS Pico (+) α CCB (+) Wash (+) PBST (+) Block Buffer	(-) FN (-) NHRP (-) SS Pico (-) CCB (+) Wash (+) PBST (+) Block Buffer	(-) FN (-) NHRP (-) SS Pico (-) CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN D (+) NHRP (+) SS Pico (+) β CCB (+) Wash (+) PBST (+) Block Buffer	(+) FN D (+) NHRP (+) SS Pico (+) β CCB (+) Wash (+) PBST (+) Block Buffer

EMPTY (columns 6, 9, 10)

Fig S1C.1. Trial C microplate well layout.

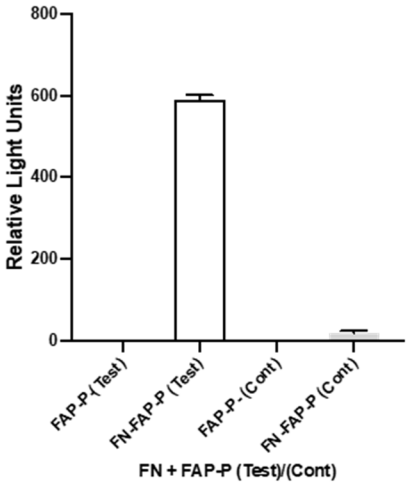

Fig S1C.2. Trial C bar graph with fibronectin + test/control probe +/- fibronectin.

Table S1D. Trial luminescence raw data results measured in relative light units.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0832	0.1524	0.3108	16.42	463.7	0.8687	0.3937	0.1823	0.1692	0.0857	0.0936	0.0759
B	0.057	0.1974	0.3683	14.47	419.4	1.321	0.4137	0.184	0.3017	0.1636	0.1435	0.1428
C	0.0671	0.1335	0.4387	18.53	391.7	1.238	0.5152	0.3308	0.5134	0.3179	0.4517	0.2498
D	0.1006	0.1516	0.4602	15.52	403.6	1.102	0.4832	0.3567	18.8	0.7967	1.564	0.7069
E	0.0891	0.2104	0.4758	9.564	436.3	1.244	0.4558	0.3247	3.814	1.403	884.7	34.79
F	0.0549	0.1738	0.3904	13.37	427.3	1.145	0.5541	0.3803	0.7156	1.771	856.4	17.43
G	0.0674	0.1626	0.4046	11.67	420.5	1.305	0.4668	0.3593	1.15	1.765	879.6	18.99
H	0.06	0.1194	0.3498	15.26	392.2	0.8301	0.3867	0.3271	1.346	1.564	915.8	20.39

*FlouroSkan program setting: Measurement count: 1 Filter: 0 Scaling Factor : 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	Control	Test		Control	Test	(α) CCB	ANT	NHRP	Substrate (pico)		N-HRP	Substrate (pico)
B	Control	Test		Control	Test	(α) CCB	ANT	N-HRP	Substrate (pico)		N-HRP	Substrate (pico)
C	Control	Test	E	Control	Test	(α) CCB	ANT	N-HRP	Substrate (pico)	E	N-HRP	Substrate (pico)
D	Control	Test	M	Control	Test	(α) CCB	ANT	N-HRP	Substrate (pico)	M	N-HRP	Substrate (pico)
E	Control	Test	P	Control	Test	(α) CCB	ANT	NHRP	Substrate (pico)	P	Test	Control
F	Control	Test	T	Control	Test	(α) CCB	ANT	N-HRP	Substrate (pico)	T	Test	Control
G	Control	Test	Y	Control	Test	(α) CCB	ANT	N-HRP	Substrate (pico)	Y	Test	Control
H	Control	Test		Control	Test	(α) CCB	ANT	N-HRP	Substrate (pico)		Test	Control
	(-) FN (+) FAP (-) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (+) FAP (-) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (-) FAP) (-) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (-) FAP) (-) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (-) FAP (-) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (-) FAP) (+) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (-) FAP) (-) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (-) FAP) (-) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB

Fig S1D.1. Trial D microplate well layout.

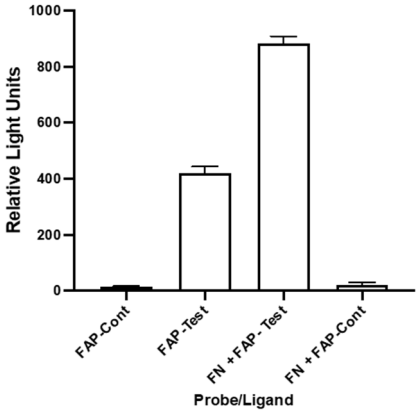

Fig S1D.2. Trial D bar graph with fibronectin + Test/Control probe +/- fibronectin.

Table S1E. No data available. Trial restarted due to possible contamination of microplate.

Table S1F. Trial luminescence raw data results measured in relative light units.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0503	0.0454	0.1285	40.68	100.9	0.3189	0.1544	0.1979	0.4529	1.24	764.6	72.81
B	0.0398	0.0586	0.1209	25.19	65.17	0.3237	0.1796	0.2251	0.5265	1.668	791.1	70.15
C	0.0269	0.1043	0.2151	25.08	81.89	0.2851	0.1835	0.2582	0.5908	1.83	773.9	54.49
D	0.0347	0.0916	0.1928	24.21	98.57	0.3354	0.194	0.3074	0.5536	1.704	783.8	50.3
E	0.0446	0.0663	0.1807	22.83	102.7	0.3701	0.2467	0.2589	0.5245	1.373	812.9	45.07
F	0.0565	0.0483	0.1856	22.18	100.2	0.4115	0.24	0.2491	0.6905	1.578	676.9	37.79
G	0.0215	0.0664	0.1737	22.99	113.4	0.359	0.2082	0.231	0.492	1.585	781.6	40.01
H	0.0422	0.0541	0.1168	14.95	118.8	0.2694	0.2102	14.68	0.4231	1.148	748.9	58.22

*FlouroSkan program setting: Measurement count: 1 Filter: 0 Scaling Factor : 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	Control	Test		Control	Test			N-HRP	Substrate (pico)		Test	Control
B	Control	Test		Control	Test			N-HRP	Substrate (pico)		Test	Control
C	Control	Test		Control	Test			N-HRP	Substrate (pico)		Test	Control
D	Control	Test	EMPTY	Control	Test	EMPTY	EMPTY	N-HRP	Substrate (pico)	EMPTY	Test	Control
E	Control	Test		Control	Test			N-HRP	Substrate (pico)		Test	Control
F	Control	Test		Control	Test			N-HRP	Substrate (pico)		Test	Control
G	Control	Test		Control	Test			N-HRP	Substrate (pico)		Test	Control
H	Control	Test		Control	Test			N-HRP	Substrate (pico)		Test	Control
	(-) FN (+) FAP (-) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (+) FAP (-) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (-) FAP) (-) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (-) FAP) (-) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (-) FAP) (-) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (-) FAP) (+) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (-) FAP) (-) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (-) FAP) (-) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB

Fig S1F.1. Trial F microplate well layout.

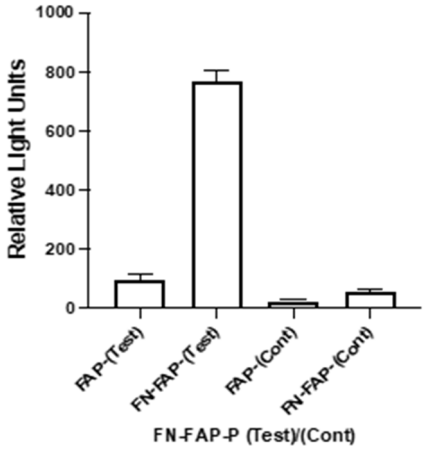

Fig S1F.2. Trial F bar graph with fibronectin + test/control probe +/- fibronectin.

Table S1G. Trial luminescence raw data results measured in relative light units.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.1589	0.3656	57.99	589.2	35.82	374.9	42.17	398	0.9843	0.4116	0.1706	0.1167
B	0.1347	0.5017	41.81	551.7	26.45	297.9	25.95	415.3	1.463	0.6594	0.2756	0.1516
C	0.1317	0.4411	43.01	578	27.9	337.6	22.77	1.866	453.8	0.8947	0.3589	0.2102
D	0.1247	0.4361	55.38	644.1	35.38	288.6	25.77	436.8	1.855	1.572	0.6275	0.2482
E	0.1026	0.3544	0.5356	0.9741	0.8605	0.8397	0.8185	1.184	47.25	678.3	1.326	0.3959
F	0.0599	0.2056	0.2302	0.3921	0.3635	0.4148	0.4622	0.6742	38.6	694.1	1.478	0.5212
G	0.0532	0.0693	0.1441	0.1679	0.1547	0.232	0.3521	0.6776	46.97	732.3	1.618	0.4608
H	0.054	0.0877	0.068	0.1395	0.1268	0.1678	0.2407	0.6139	111.9	704.8	1.215	0.3552

*FlouroSkan program setting: Measurement count: 1 Filter: 0 Scaling Factor : 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	CCB	PBS	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont	FN3/test				
B	CCB	PBS	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont	FN3/test				
C	CCB	PBS	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont		FN3/test			
D	CCB	PBS	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont	FN3/test			EMPTY	EMPTY
E	CCB	PBS							FN4/cont	FN4/test		
F	CCB	PBS							FN4/cont	FN4/test		
G	CCB	PBS							FN4/cont	FN4/test		
H	CCB	PBS							FN4/cont	FN4/test		
	(-) FN (-) FAP) (-) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (-) FAP) (-) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (-) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (-) FAP) (-) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (-) FAP) (-) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB

Fig S1G.1. Trial G microplate well layout.

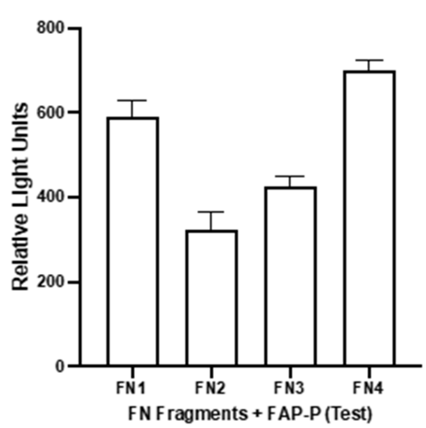

Fig S1G.2. Trial G bar graph with fibronectin fragments 1-4 + test probe.

Table S1H. Trial luminescence raw data results measured in relative light units.

	1	2	3	4	5	6	7	8	9	10	11	12
A	140.6	588	127.1	645.7	112.3	403.9	109.7	504.7	107.8	722.8	1.211	0.4276
B	136.8	502.2	147.8	676.3	122.9	409.7	134.6	564.6	131.9	754.9	1.777	0.4896
C	118.8	490	154.5	677.6	137.8	424.7	133.1	522.8	153.3	770	1.554	0.5656
D	145.4	447.6	121.2	644.3	120	477.7	125.3	495.2	101.7	746.1	1.495	0.4539
E	0.4742	0.8797	1.096	1.228	1.021	1.266	1.105	1.153	1.199	1.235	0.6733	0.2804
F	0.1759	0.3417	0.3455	0.478	0.4275	0.4504	0.4313	0.5154	0.4791	0.4525	0.2652	0.1602
G	0.1081	0.1653	0.192	0.1638	0.2362	0.2775	0.2139	0.209	0.2199	0.1928	0.1576	0.1278
H	0.0991	0.1105	0.1391	0.0945	0.1315	0.1262	0.1631	0.1468	0.1187	0.1394	0.1117	0.1223

*FlouroSkan program setting: Measurement count: 1 Filter: 0 Scaling Factor : 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	FN/cont	FN/test	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont	FN3/test	FN4/cont	FN4/test	PBS/Cont	CCB/Cont
B	FN/cont	FN/test	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont	FN3/test	FN4/cont	FN4/test		
C	FN/cont	FN/test	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont	FN3/test	FN4/cont	FN4/test		
D	FN/cont	FN/test	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont	FN3/test	FN4/cont	FN4/test	PBS/test	CCB/test
E												
F												
G												
H												
	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (+) FAP) (-) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (+) FAP) (-) NHRP (-) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB

Fig S1H.1. Trial H microplate well layout.

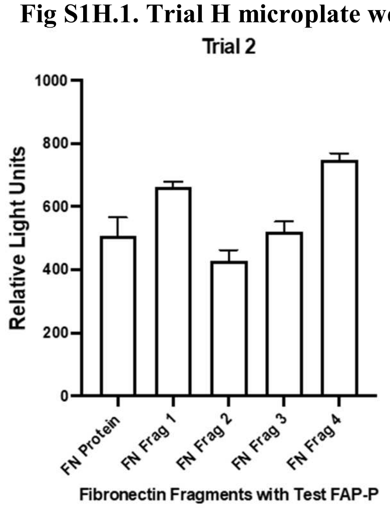

Fig S1H.2. Trial H bar graph with fibronectin fragments 1-4 + Test Probe.

Table S1I. Trial luminescence raw data results measured in relative light units.

	1	2	3	4	5	6	7	8	9	10	11	12
A	68.7	454.9	38.59	399.2	24.21	225.6	30.17	267.8	41.82	299	78.41	121.8
B	28.43	485.1	17.77	455.3	12.74	203.8	14.07	258.6	16.54	400.9	0.8845	0.4601
C	29.74	480.5	21.42	407	12.71	206.4	12.59	299.5	15.11	373.4	1.081	4.763
D	52.77	485.2	42.48	416.9	20.92	235.8	24	323.5	31.46	400.6	135.1	0.3979
E	0.3636	0.7955	0.6703	0.8809	0.539	0.6777	0.5515	0.693	0.5993	0.7035	0.4789	0.2288
F	0.1855	0.2591	0.2725	0.2658	0.2534	0.2145	0.2134	0.2657	0.2224	0.2143	0.1504	0.1373
G	0.0716	0.1095	0.1189	0.1456	0.1493	0.1451	0.1215	0.1256	0.1326	0.1153	0.1257	0.092
H	0.0499	0.0615	0.0631	0.1089	0.0952	0.0587	0.0849	0.0559	0.0776	0.0533	0.0765	0.0397

*FlouroSkan program setting: Measurement count: 1 Filter: 0 Scaling Factor : 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	FN/cont	FN/test	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont	FN3/test	FN4/cont	FN4/test	PBS/Cont	CCB/Cont
B	FN/cont	FN/test	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont	FN3/test	FN4/cont	FN4/test		
C	FN/cont	FN/test	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont	FN3/test	FN4/cont	FN4/test		
D	FN/cont	FN/test	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont	FN3/test	FN4/cont	FN4/test	PBS/test	CCB/test
E												
F												
G												
H												
	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS

Fig S1I.1. Trial I microplate well layout.

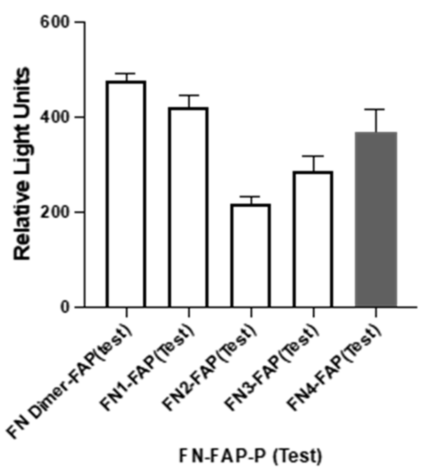

Fig S1I.2. Trial I bar graph with fibronectin fragments 1-4 + test probe.

Table S1J. Trial luminescence raw data results measured in relative light units.

	1	2	3	4	5	6	7	8	9	10	11	12
A	20.99	512.1	17.82	658.9	16.47	477	28.25	484.9	23.58	639.3	16.44	95.4
B	24.18	491.7	21.69	653.8	15.29	425.1	25.99	458.5	24.2	653.1	24.32	80.05
C	24.61	388.6	22.17	629.5	17.46	416.8	20.39	465.7	27.03	632.4	16.53	88.79
D	23.71	388.2	22.72	628.9	19.75	400.5	20.04	472.8	26.82	618.7	20.11	100.5
E	0.312	0.8808	0.8506	1.262	1.004	0.9917	0.9047	1.052	0.9082	1.1	0.6637	0.4692
F	0.1568	0.2362	0.3361	0.3895	0.3578	0.412	0.373	0.422	0.3656	0.4123	0.2647	0.2137
G	0.0866	0.1101	0.1919	0.2232	0.2354	0.2364	0.214	0.1599	0.1496	0.1901	0.2154	0.1027
H	0.0535	0.0792	0.0978	0.0958	0.1343	0.1324	0.1167	0.1339	0.0977	0.0917	0.0872	0.0796

*FlouroSkan program setting: Measurement count: 1 Filter: 0 Scaling Factor : 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	FN/cont	FN/test	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont	FN3/test	FN4/cont	FN4/test	Cont	Test
B	FN/cont	FN/test	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont	FN3/test	FN4/cont	FN4/test	Cont	Test
C	FN/cont	FN/test	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont	FN3/test	FN4/cont	FN4/test	Cont	Test
D	FN/cont	FN/test	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont	FN3/test	FN4/cont	FN4/test	Cont	Test
E												
F												
G												
H												
	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP) (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(+) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(-) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS	(-) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) PBS

Fig S1J.1. Trial J microplate well layout.

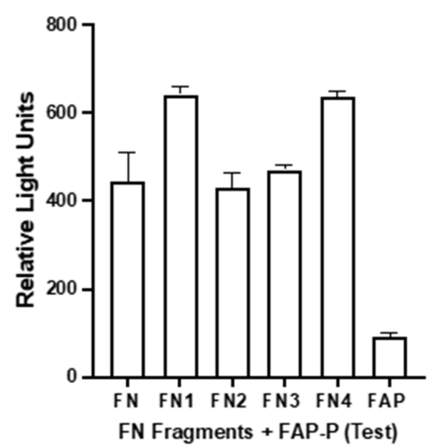

Fig S1J.2. Trial J bar graph with fibronectin fragments 1-4 + test probe.

Table S1K. Trial luminescence raw data results measured in relative light units.

	1	2	3	4	5	6	7	8	9	10	11	12
A	71.99	644.6	98.31	627.3	98.65	409.8	134.1	304.4	72.13	793.1	133.9	139.5
B	58.67	618.5	91.55	722.7	99.8	405.1	85.95	420.4	85.13	816.9	119.7	120.3
C	66.02	640.3	109.4	682.7	119	411.3	114.1	414	103.8	758.9	127.8	137.2
D	59.76	539.4	88.12	684.2	96.32	430.5	124.8	526.1	105.9	730.6	135.1	159.8
E	0.4734	0.9491	1.061	1.305	1.073	1.06	0.9938	1.118	1.189	1.266	0.8778	0.6228
F	0.2077	0.3269	0.372	0.3735	0.5102	0.4132	0.3842	0.4691	0.4765	0.4316	0.3175	0.2734
G	0.104	0.1487	0.1496	0.2528	0.2357	0.2121	0.3017	0.231	0.2303	0.2248	0.1927	0.1661
H	0.0524	0.0796	0.1364	0.1477	0.1481	0.1333	0.1381	0.1153	0.1786	0.1489	0.1336	0.0557

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	FN/cont	FN/test	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont	FN3/test	FN4/cont	FN4/test	Cont	Test
B	FN/cont	FN/test	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont	FN3/test	FN4/cont	FN4/test	Cont	Test
C	FN/cont	FN/test	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont	FN3/test	FN4/cont	FN4/test	Cont	Test
D	FN/cont	FN/test	FN1/cont	FN1/test	FN2/cont	FN2/test	FN3/cont	FN3/test	FN4/cont	FN4/test	Cont	Test
E												
F												
G												
H												
	(+) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(+) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB	(-) FN (+) FAP (+) NHRP (+) SS Pico (+) Wash (+) PBST (+) Block Buffer (+) CCB

Fig S1K.1. Trial K microplate well layout.

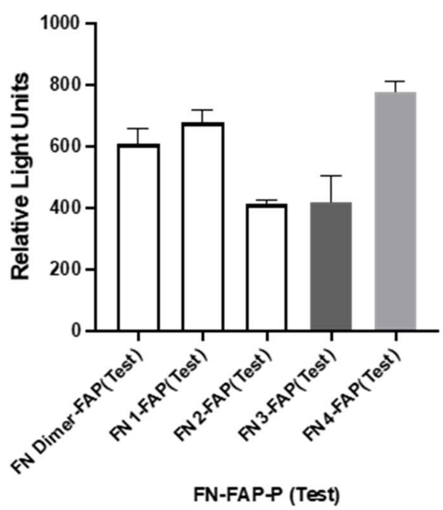

Fig S1K.2. Trial K bar graph with fibronectin fragments 1-4 + Test Probe.

S2 Luminescence Assay 2A-2F Raw Data

Scope: This section contains Luminescence Assay trials 2A-2F Raw Data. "S2" signifies supplemental data 2 and "A-F" signifies the experimental trial.

Table S2A. No data available. Trial restarted due to incorrect program setting of measurement count.

Table S2B. Trial luminescence raw data results measured in relative light units.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.1542	0.7178	463.9	0.875	0.8306	489	0.9017	1.038	448	1.173	319.7	0.5907
B	0.1809	0.757	383.9	1.053	0.924	422.6	0.9368	0.9418	488.6	1.455	382	0.8256
C	0.2199	0.7324	374.5	1.001	1.074	453.8	1.21	1.029	483.9	1.502	416.3	0.8654
D	0.2262	0.6462	408.1	0.9356	0.9287	426.5	1.065	0.9772	434.7	1.511	438.2	0.7338
E	0.1213	0.5727	385.5	0.7609	0.6971	429.2	0.7546	0.6915	440.2	1.098	307.2	0.5135
F	0.1348	0.2336	0.4451	0.3904	0.3477	0.5794	0.4293	0.3419	0.5318	0.5304	0.4656	0.2823
G	0.065	0.1442	0.123	0.1361	0.1531	0.1515	0.2363	0.177	0.1709	0.2021	0.1478	0.1075
H	0.0566	0.0687	0.0608	0.0737	0.0647	0.0872	0.0799	0.0961	0.0415	0.0537	0.0488	0.0558

*FlouroSkan program setting: Measurement count: 1 Filter: 0 Scaling Factor : 1

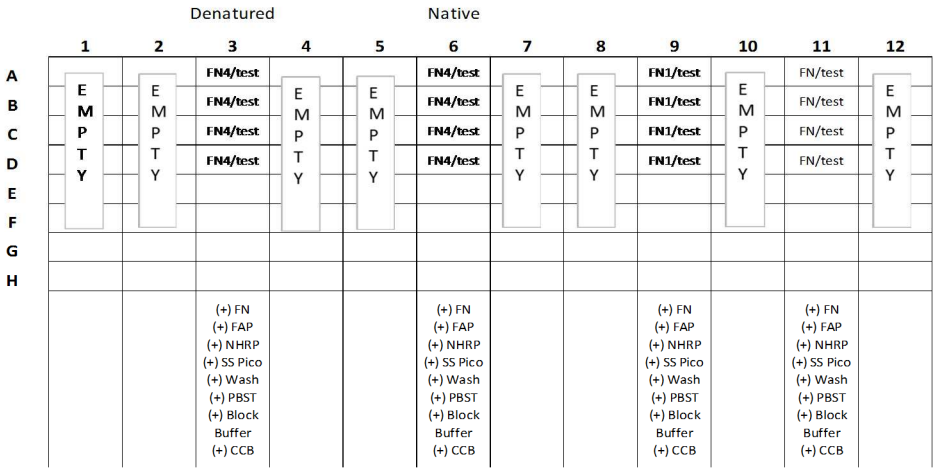

Fig S2B.1. Trial B microplate well layout.

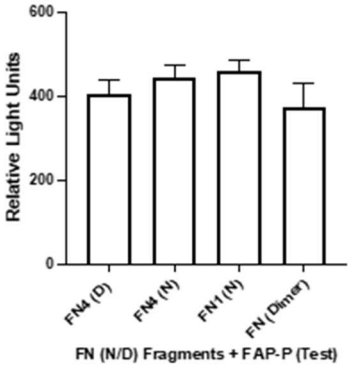

Fig S2B.2. Trial B bar graph with native/denatured fibronectin fragment 4.

Table S2C. Trial luminescence raw data results measured in relative light units.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.1936	0.891	632.9	1.2	1.594	895.8	1.434	0.6402	0.8189	2.515	1830	2.605
B	0.2746	0.9481	422.2	1.522	1.963	1021	2.002	0.8894	1.004	3.208	1864	3.219
C	0.2818	0.926	437.8	1.592	2.02	995.1	2.128	0.96	1.135	3.384	1911	3.362
D	0.3118	0.8463	407.4	1.245	1.719	893.7	1.95	0.7409	1	3.214	1920	3.343
E	0.1633	0.6351	332.5	0.9399	1.501	861.8	1.302	0.6654	0.7406	2.189	1516	2.355
F	0.1306	0.3457	0.5413	0.4055	0.6101	1.202	0.655	0.3355	0.5336	0.8711	1.788	0.9971
G	0.049	0.1127	0.1605	0.2427	0.2443	0.3415	0.2795	0.1939	0.2722	0.3445	0.4447	0.387
H	0.0493	0.0525	0.0855	0.1227	0.1283	0.1926	0.147	0.1128	0.158	0.145	0.1762	0.2109

*FlouroSkan program setting: Measurement count: 1 Filter: 0 Scaling Factor : 1

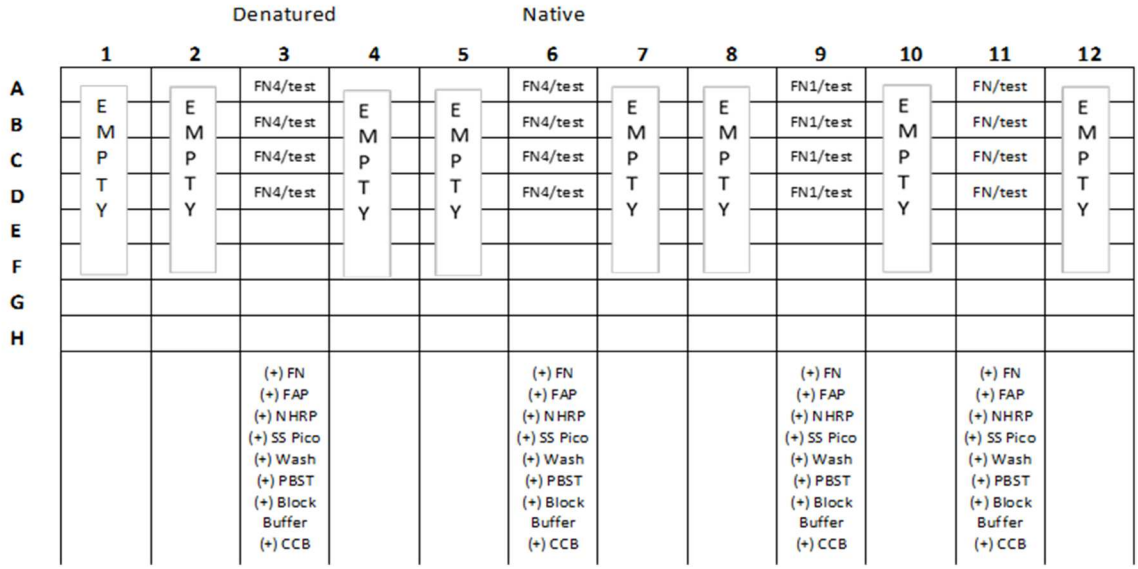

Fig S2C.1. Trial C microplate well layout.

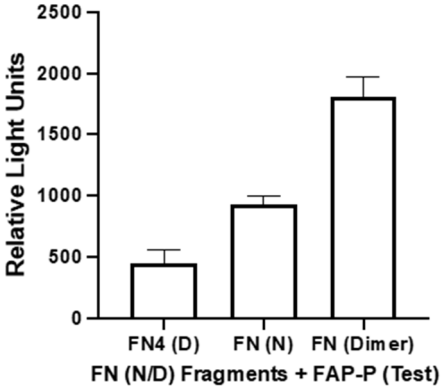

Fig S2C.2. Trial C bar graph with native/denatured fibronectin fragment 4. * New substrate (super signal pico) and neutravidin horse-radish peroxidase used.

Table S2D. Trial luminescence raw data results measured in relative light units.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.1838	0.4908	320.8	0.9435	1.466	1385	3.019	1.385	1.129	2.802	1851	2.552
B	0.1729	0.7476	235.1	1.046	1.208	3.313	1348	2.495	1.508	3.341	1911	3.327
C	0.28	1.044	594.8	1.174	0.9888	2.731	1346	2.67	1.795	3.428	1852	3.45
D	0.2884	0.9671	506.9	1.106	0.8837	2.351	1097	2.4	1.508	3.293	1812	3.193
E	0.2289	0.7706	489	0.8557	0.7547	1.971	1431	2.219	1.222	2.608	1783	2.564
F	0.1597	0.408	0.5558	0.3988	0.4753	0.7506	1.6	0.9752	0.8075	1.329	2.116	1.176
G	0.0996	0.1484	0.1776	0.2134	0.2752	0.3895	0.5115	0.3495	0.32	0.528	0.5848	0.4867
H	0.0538	0.0827	0.1103	0.1588	0.1492	0.1799	0.183	0.1905	0.1789	0.1967	0.2342	0.2226

*FlouroSkan program setting: Measurement count: 1 Filter: 0 Scaling Factor : 1

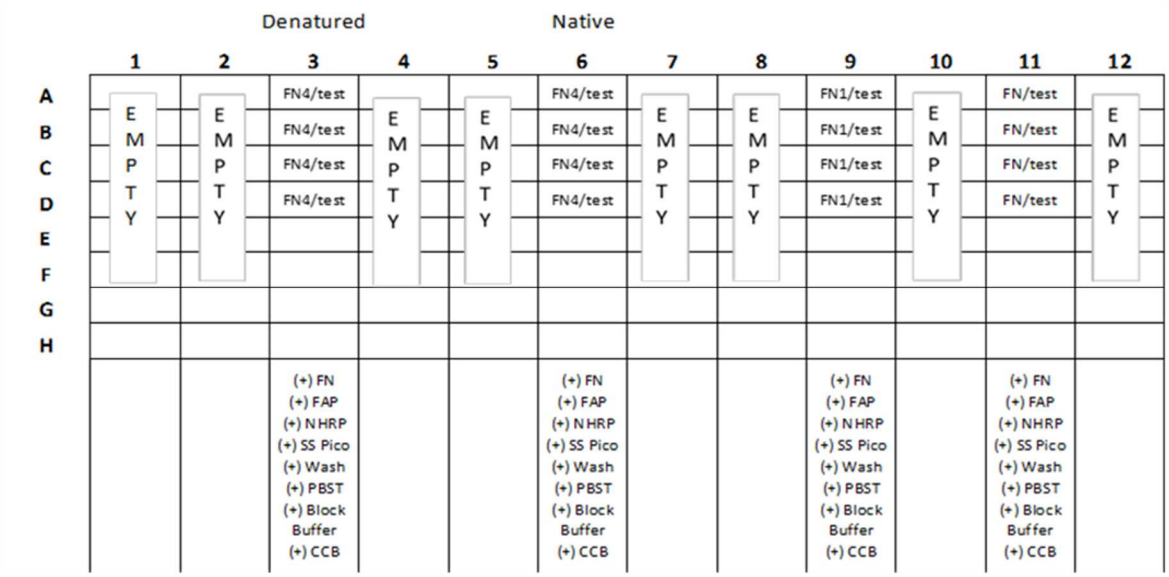

Fig S2D.1. Trial D microplate well layout.

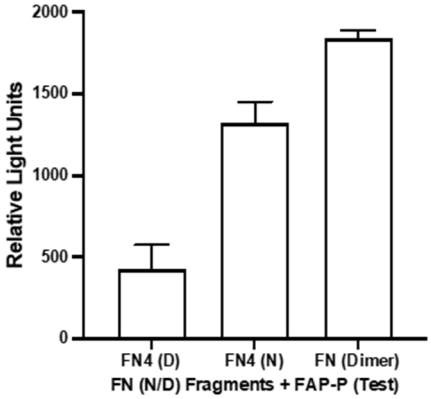

Fig S2D.2. Trial D bar graph with native/denatured fibronectin fragment 4. * New substrate (super signal pico) and neutravidin horse-radish peroxidase used.

Table S2E. Trial luminescence raw data results measured in relative light units.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.4224	1.506	950.9	2.017	2.086	1320	2.422	2.734	1816	2.443	0.7339	0.2859
B	0.585	1.931	1121	2.766	2.875	1386	3.204	3.537	1803	3.139	0.9014	0.3184
C	0.4962	1.985	1027	2.669	3.023	1406	3.379	3.58	1831	3.109	0.8941	0.257
D	0.4237	1.621	1131	2.194	2.407	1443	2.761	2.789	1793	2.456	0.7912	0.2526
E	0.2312	0.7098	1.349	0.9461	1.18	2.083	1.367	1.371	1.831	1.258	0.5038	0.2061
F	0.1879	0.2264	0.3858	0.3972	0.5025	0.5878	0.5254	0.5423	0.5548	0.4266	0.2375	0.1566
G	0.0928	0.1265	0.1637	0.2024	0.2246	0.2058	0.2414	0.2728	0.2271	0.1721	0.1475	0.0878
H	0.1018	0.0877	0.1099	0.1063	0.1254	0.1811	0.1448	0.1727	0.1137	0.169	0.0636	0.0731

*FlouroSkan program setting: Measurement count: 1 Filter: 0 Scaling Factor : 1

Table S2F. Trial luminescence raw data results measured in relative light units.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.3391	1.482	952.7	1.968	2.083	1085	2.548	2.725	1604	2.413	0.8065	0.2824
B	0.4998	1.775	994.4	2.317	2.857	1277	3.082	3.49	1671	2.915	0.81	0.3394
C	0.4486	1.621	685.8	2.212	2.643	1328	3.23	3.523	1569	3.174	0.9249	0.2912
D	0.4027	1.298	947.7	2.038	2.224	1294	2.613	3.26	1668	2.965	0.8663	0.3192
E	0.2587	0.5743	1.281	0.8976	1.083	1.726	1.482	2.324	1530	2.291	0.6253	0.2879
F	0.149	0.2727	0.3481	0.3409	0.4638	0.585	0.6932	1.157	1.753	0.964	0.4257	0.2197
G	0.0555	0.1073	0.1849	0.1581	0.198	0.2903	0.3585	0.5027	0.5082	0.3644	0.2554	0.1508
H	0.0867	0.062	0.0989	0.123	0.1592	0.1389	0.2003	0.2162	0.1328	0.201	0.1186	0.1046

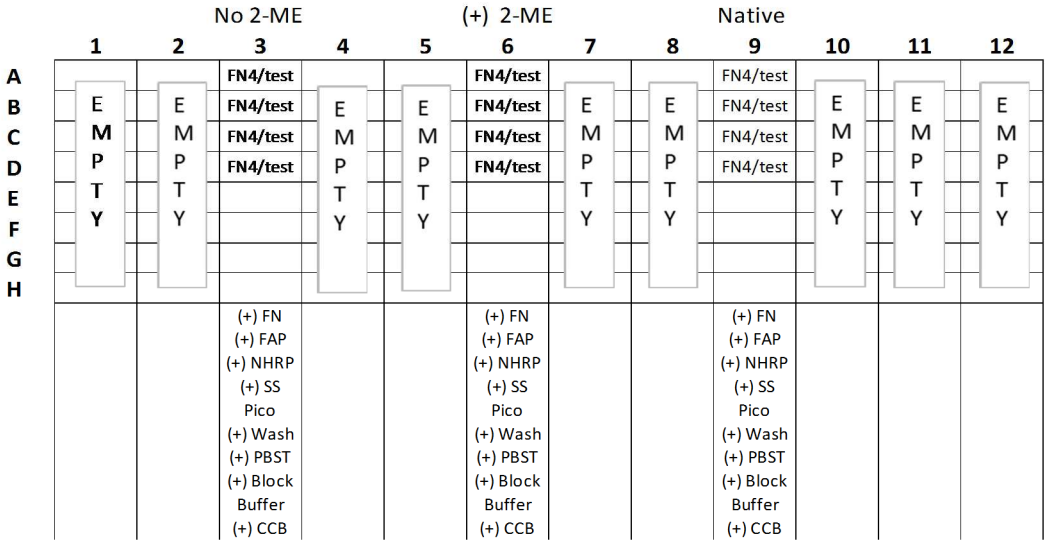

Fig S2E/F.1. Trial E and F microplate well layout. (Heat treatment test) Column 3 FN4 was subjected to a heat treatment without the presence of 2-ME (β-mercaptoethanol). Column 6 FN4 was subjected to a heat treatment with the presence of 2-ME. Column 9 FN4 was not heat treated.

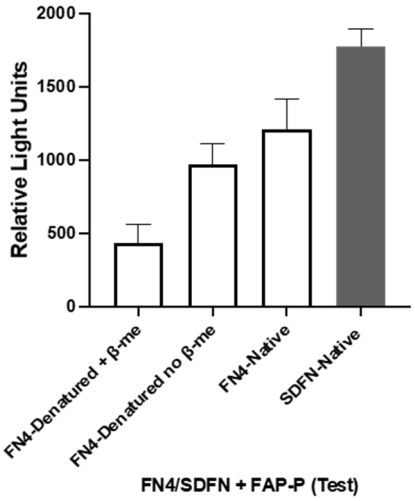

Fig S2E/F.2. Trial E and F bar graph with native/denatured fibronectin fragment 4 with +/- 2-ME (β- mercaptoethanol) heat treatments. Bar data was gathered from pooled values from previous trials A- D. *New substrate (super signal pico) and neutravidin horse-radish peroxidase was used.

S3 Bicinchoninic Acid Assay No. 1 and No. 2 Results and Raw Data

Scope: This section contains Bicinchoninic Assay trials 1-2 Raw Data. "S3" signifies supplemental data 3.

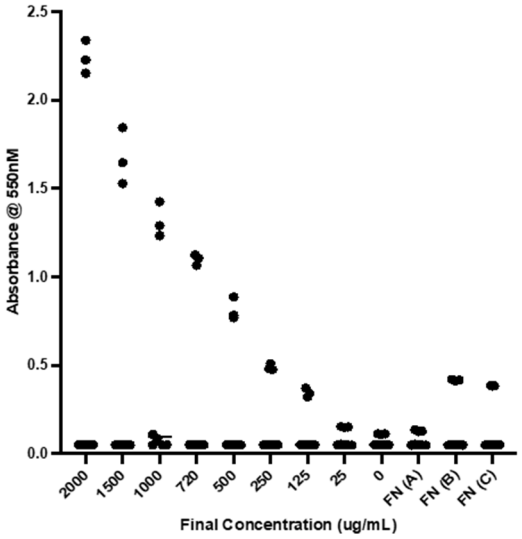

Fig S3.1. Color response curve for BSA protein dilutions and unknown fibronectin protein sample.

Table S3.1. Bicinchoninic Acid Assay #1 raw data for protein concentration in (µg/mL). BSA protein concentrations from 0µg/mL to 2000µg/mL and unknown fibronectin sample concentration.

	2000	1500	1000	720	500	250	125	25	0	FN (A)	FN (B)	FN (C)
A	2.3378	1.5282	1.234	1.1253	0.8866	0.51	0.3712	0.154	0.1136	0.1359	0.4204	0.3848
B	2.1525	1.6464	1.2907	1.1056	0.7841	0.4761	0.3214	0.1511	0.1095	0.1286	0.4107	0.3855
C	2.2269	1.8449	1.4255	1.0658	0.77	0.4806	0.3404	0.1484	0.1132	0.1262	0.4172	0.3831
D	0.0512	0.051	0.0513	0.0504	0.0511	0.05	0.0503	0.0528	0.05	0.0507	0.0499	0.0506
E	0.0514	0.0502	0.0503	0.0512	0.0502	0.0515	0.052	0.0506	0.0516	0.0529	0.0511	0.05
F	0.0514	0.0503	0.05	0.0525	0.0525	0.0506	0.0515	0.0509	0.0522	0.0493	0.0517	0.0515
G	0.0511	0.0498	0.0872	0.0512	0.0508	0.0517	0.0511	0.0501	0.0511	0.0493	0.0506	0.051
H	0.0514	0.0513	0.1104	0.0509	0.0514	0.051	0.0514	0.0498	0.0515	0.0503	0.0499	0.0509

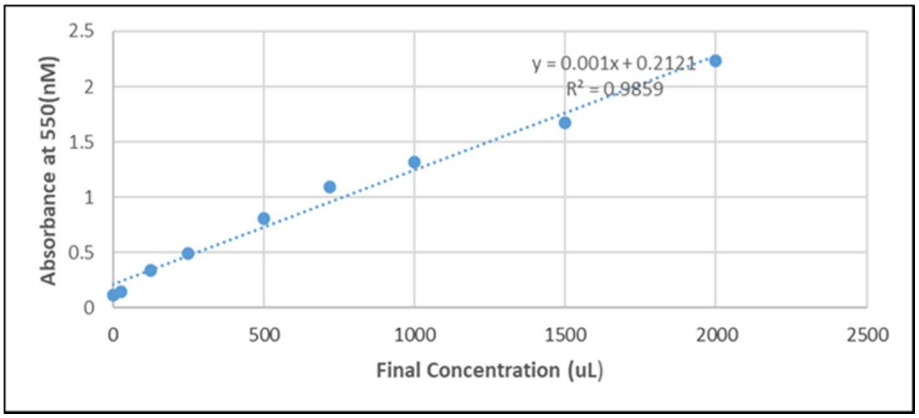

Fig S3.2. Color response curve for BSA protein dilutions and unknown fibronectin protein sample.

Table S3.2. BCA #2 raw data for protein concentration in (µg/mL). BSA protein concentrations from 0µg/mL to 2000µg/mL and unknown fibronectin sample concentration.

	2000	1500	1000	720	500	250	125	25	0		FN (D)	FN (E)
A	1.8144	1.2284	1.1436	0.799	0.6005	0.3701	0.2495	0.1304	0.105	0.0503	0.929	0.976
B	1.8869	1.3784	0.9966	0.7709	0.5062	0.3482	0.2319	0.1253	0.0977	0.0512	0.9948	0.9926
C	2.0661	1.5122	1.0037	0.8313	0.5795	0.3598	0.2354	0.1296	0.1053	0.0531	1.0016	0.9719
D	0.0512	0.0515	0.052	0.0603	0.0629	0.0627	0.0612	0.0566	0.0584	0.0569	0.0515	0.0522
E	0.0526	0.0511	0.0509	0.0523	0.0499	0.0512	0.0511	0.0512	0.0537	0.0518	0.0506	0.0515
F	0.0533	0.0612	0.0535	0.0523	0.0567	0.052	0.0509	0.052	0.0529	0.051	0.6408	0.6017
G	0.0529	0.0526	0.0548	0.0528	0.0556	0.0531	0.0515	0.0538	0.0516	0.0517	0.6567	0.6095
H	0.0526	0.0519	0.0529	0.0507	0.0542	0.0538	0.0541	0.0552	0.0512	0.0528	0.5133	0.1314

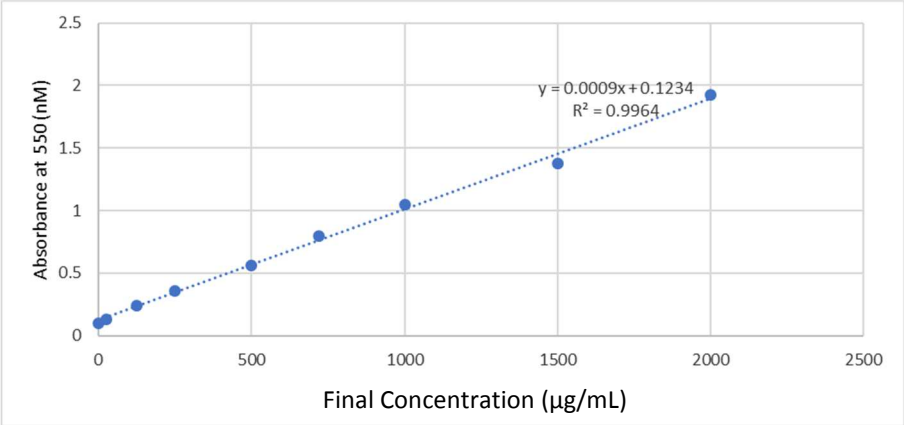

Fig S3.3. Color response curve for BSA protein dilutions and unknown fibronectin protein sample.

S4 Western Blot Images for Trials 2-5

Scope: This section contains Western blot trials 2-5 images. "S4" signifies supplemental data 4 and "a/b" signifies Native or Denatured environment.

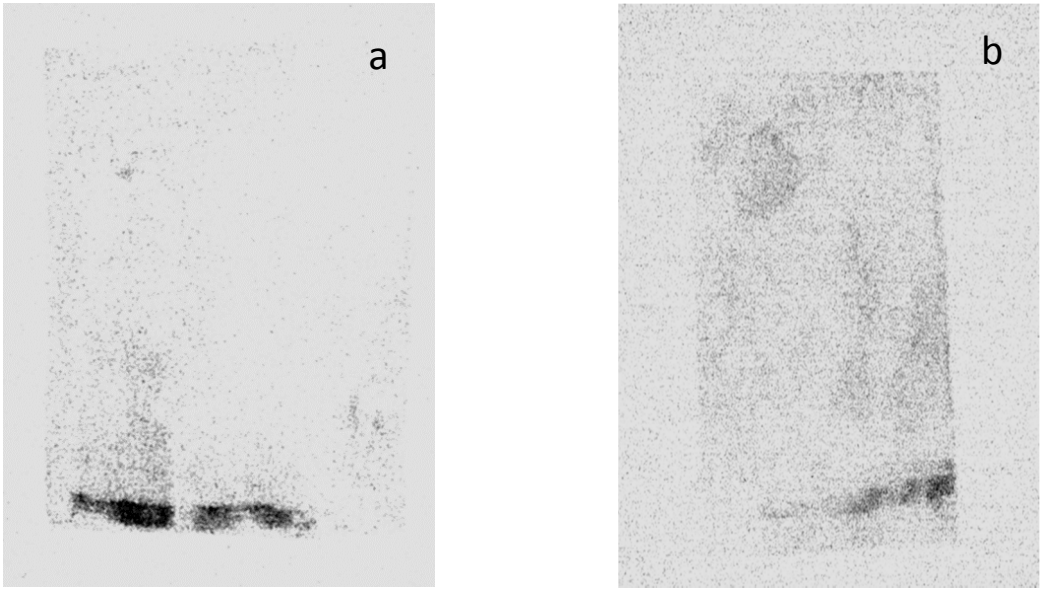

Fig S4.1. Trial 2 Western blot native (a) and denatured (b) fibronectin fragment 4 protein LiCor image post gel-membrane transfer.

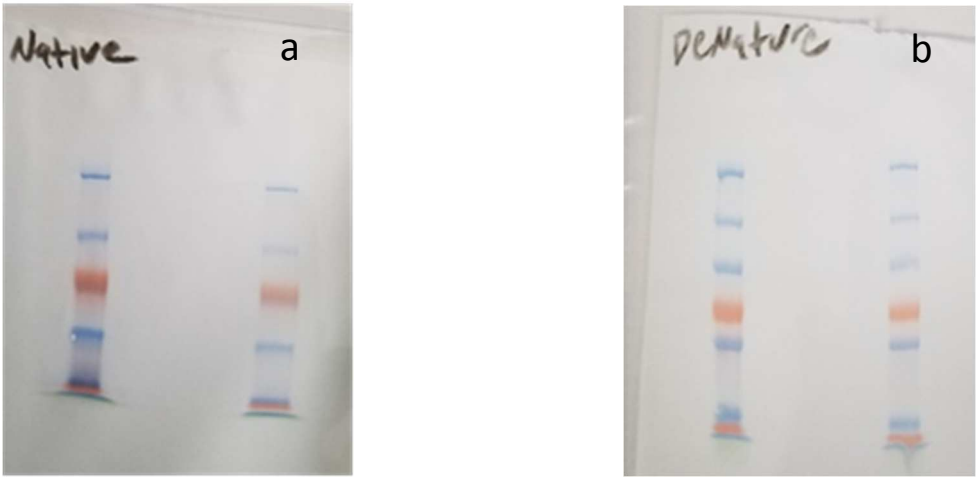

Fig S4.2 Trial 2 Western blot native (a) and denatured (b) fibronectin fragment 4 protein standard gel-membrane transfer.

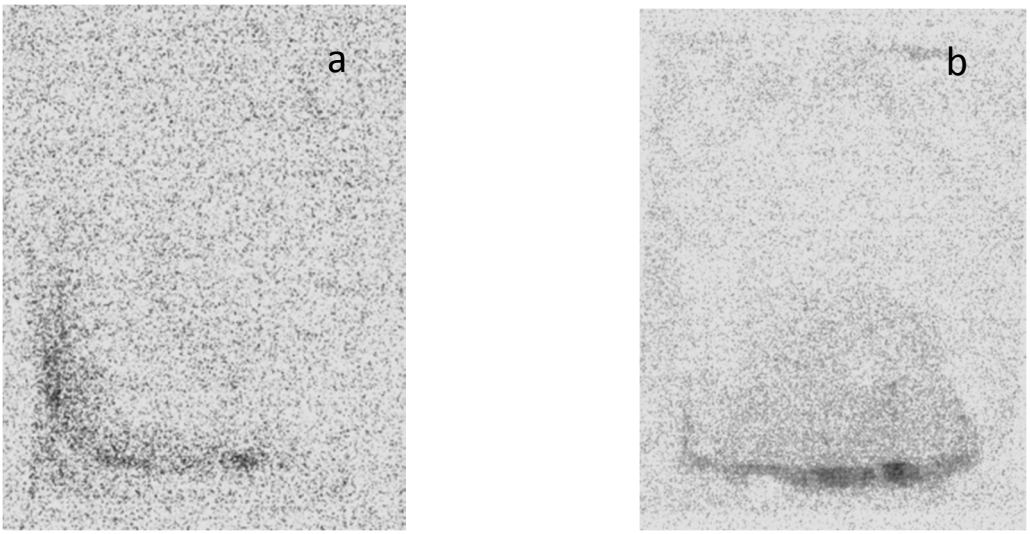

Fig S4.3 Trial 3 Western blot native (a) and denatured (b) fibronectin fragment 4 protein LiCor image post gel-membrane transfer.

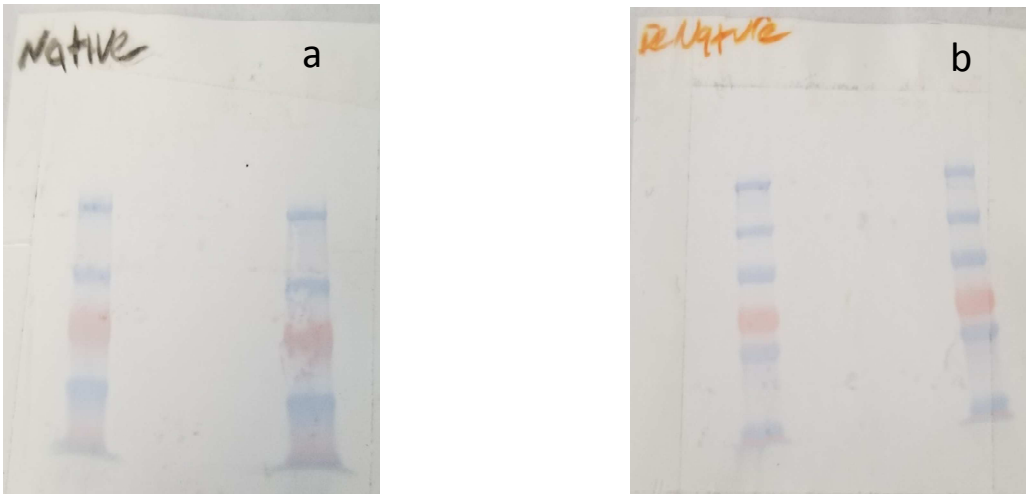

Fig S4.4 Trial 3 Western blot native (a) and denatured (b) fibronectin fragment 4 protein standard gel-membrane transfer.

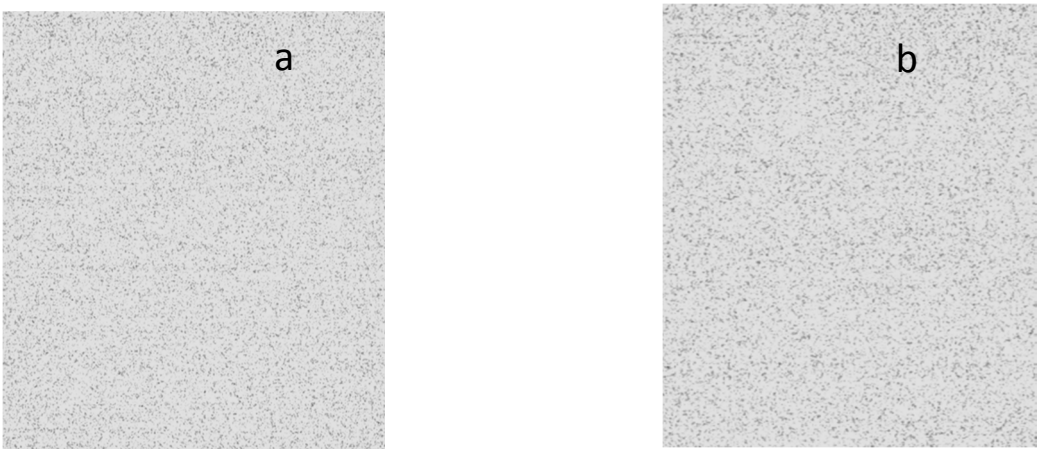

Fig S4.5 Trial 4 Western blot native (a) and denatured (b) fibronectin fragment 4 protein LiCor image post gel-membrane transfer.

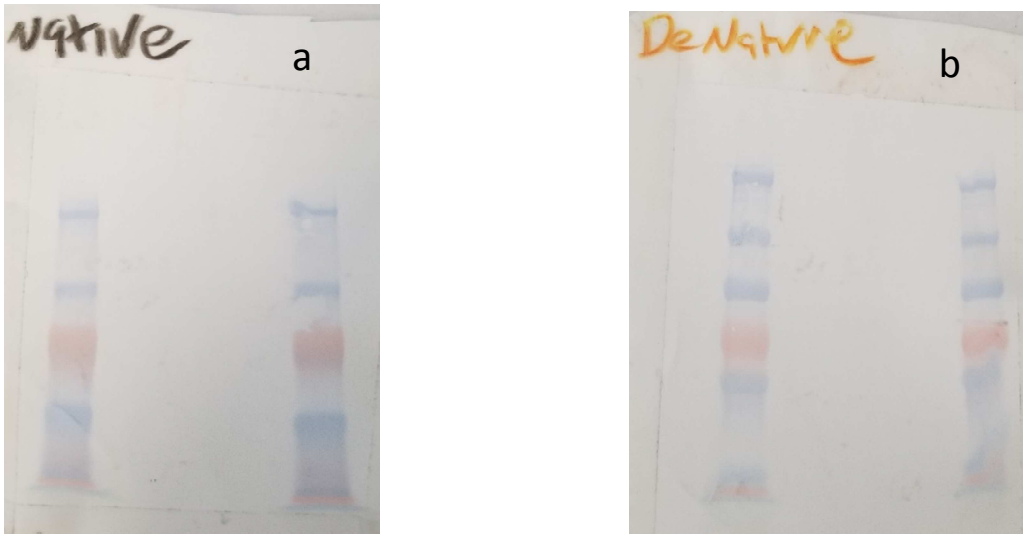

Fig S4.6 Trial 4 Western blot native (a) and denatured (b) fibronectin fragment 4 protein standard gel-membrane transfer.

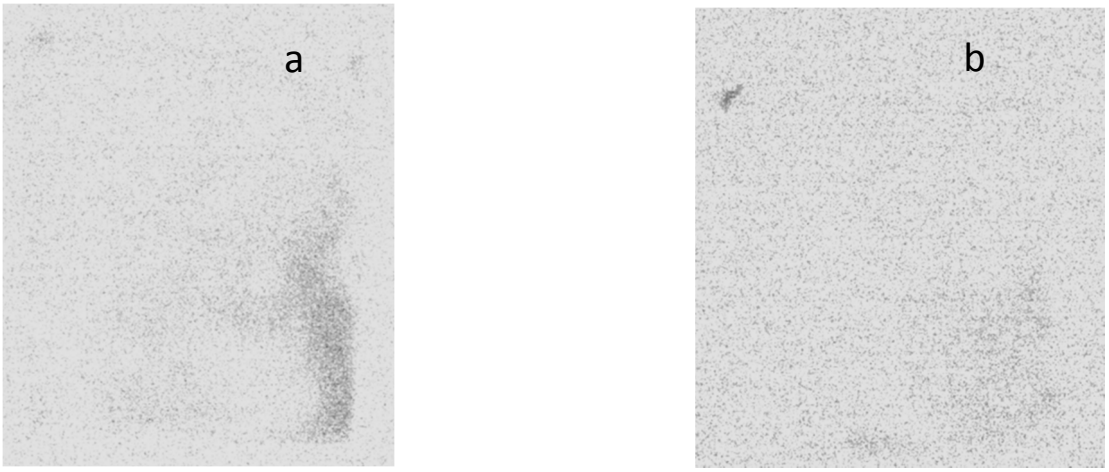

Fig S4.7 Trial 5 Western blot native (a) and denatured (b) fibronectin fragment 4 protein LiCor image post gel-membrane transfer.

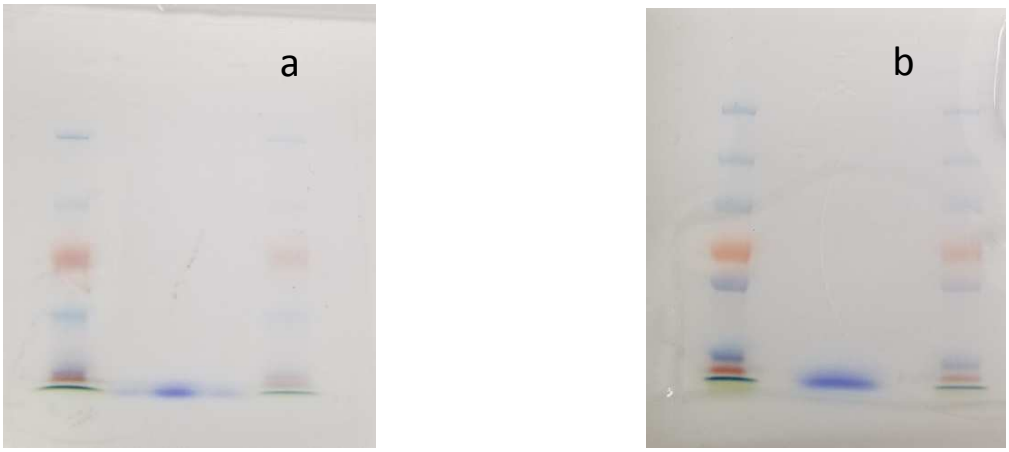

Fig S4.8 Trial 5 Western blot native (a) and denatured (b) fibronectin fragment 4 protein standard gel-membrane transfer.

S5 Fibronectin Protein Amino Acid Sequence and Properties

Fibronectin Protein Amino Acid Properties

Fibronectin monomer Met1-Glu2477
Theoretical pI/Mw: 5.31 / 272320.36
Polararity: 972
Hydrophobic: 484
Charge: (+) 205 (-) 271
kDa: 220

Fibronectin fragment 1 Gln32-Pro1991
Theoretical pI/Mw: 5.16 / 215392.79
Polarity: 20
Hydrophobic: 17
Charge: (+) 156 (-) 213
kDa: 217

Fibronectin fragment 2 Ser607-Pro1265
Theoretical pI/Mw: 4.83 / 71816.90
Polarity: 278
Hydrophobic: 150
Charge: (+) 46 (-) 71
kDa: 73

Fibronectin fragment 3 Glu1266-Pro1908
Theoretical pI/Mw: 4.71 / 69384.54
Polarity: 276
Hydrophobic: 152
Charge: (+) 48 (-) 73
kDa: 70.1

Fibronectin fragment 4 Val1913-Glu2477
Theoretical pI/Mw: 5.64 / 62699.73
Polarity: 204
Hydrophobic: 92
Charge: (+) 54 (-) 60
kDa: 63.5

Fibronectin fragment 1 & 4 overlap Val1913-Pro1991
Theoretical pI/Mw: 5.08 / 9787.91
Polarity: 35
Hydrophobic: 17
Charge: (+) 10 (-) 8
kDa: UNK

Fibronectin fragment 1 no 2,3,4 overlap
Theoretical pI/Mw: 6.54 / 67933.60
Polarity: 35
Hydrophobic: 17
Charge: (+) 58 (-) 62
kDa**:** UNK

Fibronectin Protein Amino Acid Sequence

```
1 mlrgpgpgll llavqclgta vpstgasksk rqaqqmvqpq spvavsqskp gcydngkhyq
   61 inqqwertyl gnalvctcyg gsrgfncesk peaeetcfdk ytgntyrvgd tyerpkdsmi
  121 wdctcigagr grisctianr cheggqsyki gdtwrrphet ggymlecvcl gngkgewtck
  181 piaekcfdha agtsyvvget wekpyqgwmm vdctclgegs gritctsrnr cndqdtrtsy
  241 rigdtwskkd nrgnllqcic tgngrgewkc erhtsvqtts sgsgpftdvr aavyqpqphp
  301 qpppyghcvt dsgvvysvgm qwlktqgnkq mlctclgngv scqetavtqt yggnsngepc
  361 vlpftyngrt fyscttegrq dghlwcstts nyeqdqkysf ctdhtvlvqt rggnsngalc
  421 hfpflynnhn ytdctsegrr dnmkwcgttq nydadqkfgf cpmaaheeic ttnegvmyri
  481 gdqwdkqhdm ghmmrctcvg ngrgewtcia ysqlrdqciv dditynvndt fhkrheeghm
  541 lnctcfgqgr grwkcdpvdq cqdsetgtfy qigdswekyv hgvryqcycy grgigewhcq
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  781 lpgrkyivnv yqisedgeqs lilstsqtta pdappdttvd qvddtsivvr wsrpqapitg
  841 yrivyspsve gsstelnlpe tansvtlsdl qpgvqyniti yaveenqest pvviqqettg
  901 tprsdtvpsp rdlqfvevtd vkvtimwtpp esavtgyrvd vipvnlpgeh gqrlpisrnt
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 1921 itiswrtkte titgfqvdav pangqtpiqr tikpdvrsyt itglqpgtdy kiylytlndn
 1981 arsspvvida staidapsnl rflattpnsl lvswqpprar itgyiikyek pgspprevvp
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 2101 ildvpstvqk tpfvthpgyd tgngiqlpgt sgqqpsvgqq mifeehgfrr ttppttatpi
 2161 rhrprpyppn vgeeiqighi predvdyhly phgpglnpna stgqealsqt tiswapfqdt
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