Development of an *in vitro* model system for studying the interaction of *Equus caballus* IgE with its high-affinity FcεRI receptor

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Thesis submitted in part fulfillment of the requirement

for the degree of Doctorate of Philosophy

September 2011

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Abstract

Allergy is a serious life altering disease that is increasingly affecting people in the industrialized countries. Allergic manifestations are not restricted to humans, but are also observed, example, in inbred dogs and horses. Race horses are commonly afflicted by horse allergies and we have developed a cell culture model system for studying horse allergies. We created a genetically engineered rat basophil (RBL-2H3.1) cell line that expresses the ligand binding domain of the horse IgE high-affinity Fc receptor (FcεRI) and complemented this with a mouse B (J558L) cell line that expresses the heavy chain of horse IgE antibody. This allowed assessment of mediator release when the horse IgE binds to the transfected cell surface receptor and initiates downstream signaling upon antigenic challenge. To complement these studies a soluble form of the FcεRI (sFcεRIαD1&2) was expressed which facilitate the measurement of the binding kinetics between the horse IgE and FcεRI receptor. These developments formed the basis for the design of an allergy vaccine strategy, where rats were primed with an IgE-derived peptide followed by subsequent challenge with a chimeric IgE antibody displaying canine self IgE epitopes. The immunization strategy resulted in large polyclonal antibody titer found in the serum of immunized rat. Analysis of the immune response clearly indicated that antibodies were generated which aggregated human, horse and dog IgE on the basophil cell surface indicating that this strategy would be unsafe as an anti-allergic vaccine.

Acknowledgements

With immense pleasure I would like to express my deep and sincere gratitude to all the following people for their help and support during my PhD:

Dr. Birgit Helm
Dr. Julie Gray
Dr. Martyn Gunn
Dr. Lynda Partridge
Dr. Peter Monk
Dr. Hongtu Ye
Dr. Huaying Zhao
Dr. Peter Schuck
Dr. Jonathan Housden
Dr. Amir Rashid
Dr. Ismael Sadroddiny
Dr. Mark Street
Dr. Sandrine Soubes
Dr. Colin Gray
Dr. Arthur Moir
Dr. Susan Newton
Dr. Kay Hopkinson
Dr. Elizabeth Sewards
Dr. Rizwana Lala
Dr. Beining Chen
Dr. Jennifer Louth
Dr. Jim Gilmour
Dr. Andy Wright
Dr. Simon Smith
Will Finnigan
Saud Sabban
Abeer Baghdadi
Dr. Dalal Sabban
Saif Sabban
Dalia Sabban
Badr Sabban
Sultan Sabban
Hanan Naiz
Marzieh Fanaei
Dr. Khalid Al-Abri
Dr. Soleiman Al-Naimat
Sadaa Alssadeq
Charlotte MacPhail
Vaidas Mykolaitis
Tom Minshull
Iman Islam

Abbreviations

IgE	Immunoglobulin E.
FcεRI	High-affinity IgE Fc receptor.
FcεRII	Low-affinity IgE Fc receptor.
FcγR	IgG Fc receptor.
FcεRIα	High-affinity IgE Fc receptor's α chain.
sFcεRIαD1&2	Soluble High-affinity IgE Fc receptor's domain 1 and 2 of the α chain.
Cε	IgE constant domain.
Th_1 Cell	T helper cell type 1.
Th_2 Cell	T helper cell type 2.
IL-4	Interleukin 4.
IFN-γ	Interferon gamma.
B Cell	B lymphocyte.
T Cell	T lymphocyte.
mg ml^{-1}	Milli grams per milliliter.
μg ml^{-1}	Micro grams per milliliter.
ng ml^{-1}	Nano grams per milliliter.
kDa	Kilo Daltons.

k_a	Rate constant of association.
k_d	Rate constant of dissociation.
K_A	Equilibrium constant of association (binding affinity).
K_D	Equilibrium constant of dissociation.
APC	Antigen presenting cells.
E237G	Mutation at position 237 replacing amino acid E (Glutamic acid) by G (Glycine).
TNF-α	Tumor necrosis factor α.
HHoH	Human-Horse-Human IgE chimera.
DHD	Dog-Human-Dog IgE chimera.
HDH	Human-Dog-Human IgE chimera.
Equine IgE anti NIP-HSA	Equine immunoglobulin E that has a variable region binding to NIP-HSA antigen.
NIP-HSA	4-Hydroxy-5-iodo-3-nitrophenylacetic acid conjugated to Human Serum Albumin.
DNP-HSA	2,4-Dinitrophenol conjugated to Human Serum Albumin.
β-hexosaminidase Substrate	4-nitrophenyl N-acetyl β-d-glucosaminide.
Hε	Antibody heavy chain.
FCS	Fetal Calf Serum.
SPR	Surface Plasmon Resonance.
BSA	Bovine Serum Albumin.

BLAST	Basic Local Alignment Search Tool.
$2Fc\varepsilon_{2\text{-}3}$	Peptide used for immunizations.

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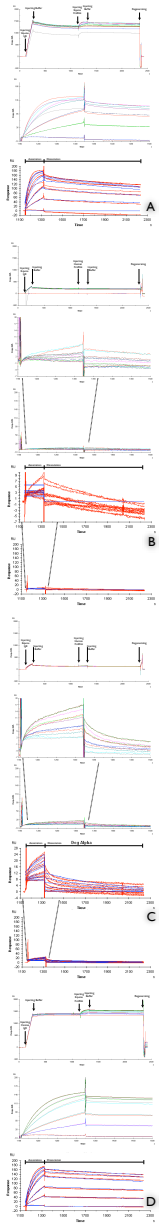
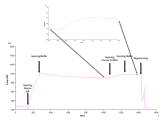
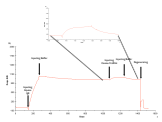
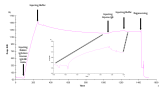
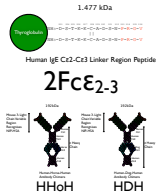
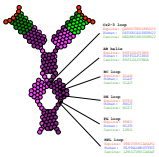


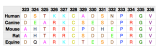












Chapter 1 - Introduction:

1.1 - Overview:

Allergy is an inflammatory disease that has been observed since ancient times, it was first scientifically tested by Charles Blackley in 1873 when he used aqueous pollen extracts on himself (a hayfever allergy sufferer) to prove that pollen is the cause of immediate skin inflammation reaction (Chapman, 1998). But the condition and the allergy causing agents were first defined by Clemens Freiherr von Pirquet who discovered that second injections of horse serum or small pox vaccine caused a severe reaction in some individuals. He termed this condition allergy from the Greek words allos "other" and ergon "works" and thus the allergy causing agent "allergen"(von Pirquet, 1906). Robert Cook in 1916 published a paper showing that allergy has strong familial inheritance, and in the 1960s the Ishizakas demonstrated that allergic reactions are mediated by a new class of antibodies that they called immunoglobulin E (Chapman, 1998; Ishizaka, *et al.*, 1966).

An article dated June 13 2008 in the Healthcare Financial News stated that US$11 billion are spent on allergy annually in the USA alone, which has doubled since the year 2000 (Merrill, 2008). 54.6% of Americans suffer from some type of allergic manifestation (Cohn, *et al.*, 2006) while in the UK all allergies have been on the increase since 1955 (Anderson, *et al.*, 2007), though some data show stability and a slight decrease during the late 1990s and early 2000s (Gupta, *et al.*, 2007), which may be due to awareness of the condition and other health factors. This shows that allergy is a serious lifestyle altering disease where many people suffer from, and the health care industry spends a lot of money on combating. Though many allergies are associated with undesirable and often long-term debilitating side effects, food and insect venom allergies can be lethal.

Allergy is common in males before puberty, but in females after puberty (Almqvist, *et al.*, 2008). This phenomenon has been linked to sex hormones where asthma has shown to increase with irregular menstruation, intake of contraceptives, post menopause, during pregnancy or undergoing hormone replacement therapy (Jensen-Jarolim and Untersmayr, 2008). It was discovered that mast and basophil cells express estrogen alpha receptor (ER-α), an estrogen receptor (Zhao, *et al.*, 2001), which in the presence of estrogen can cause the cells to release mediators (Zaitsu, *et al.*, 2007).

Allergic diseases are not inherited, the tendency of developing an allergic disease is due to deficiencies in the immune system. Atopic patients genetically overproduce IgE even in the absence of allergic disease (Ono, 2000). A study has shown that monozygotic twins have a 60%-70% higher tendency of developing allergy than dizygotic twins, suggesting a strong genetic factor (Duffy, *et al.,* 1990). But identifying the genes responsible for the risk of allergy development proved difficult due to the complex way they function (Ono, 2000). A papery by (Weidinger, *et al.*, 2008) have done a human genome wide scan and identified SNPs and gene sites, such as SNPs within the FCER1A gene which codes for the FcεRI receptor. when these genes are inherited, they cause humans to develop high IgE serum concentrations, which gives them the tendency to develop allergy

There is a hypothesis called the Hygiene Hypothesis and it states that lack of exposure to infectious pathogens in early childhood can lead to inadequate immune system development which can result in an increase in susceptibility to develop allergy (Strachan, 1989; Strachan, 2000). Further study in the immunology pathway has shed light into the viability of this hypothesis, and some groups are researching an allergy cure through it.

1.2 - The Immune Response and The Allergy Pathway:

The allergy pathway is the same pathway as the parasitic immunity. The parasitic immunity pathway is very effective, though it also threatens the host by sensitizing it against innocuous substances, if the pathway makes an error, giving it allergy. Some mechanisms do exits to protect the host organism from autoimmunity, including allergy, which includes compartmentation of B and T cells, within the lymphatic system, so as to minimize interactions with self antigens (Gould *et al.*, 2003) as well as producing large enough quantities of IgE to saturate mast and basophil cells' surfaces and thus protect the host from allergy development (Nielsen *et al.*, 1994).

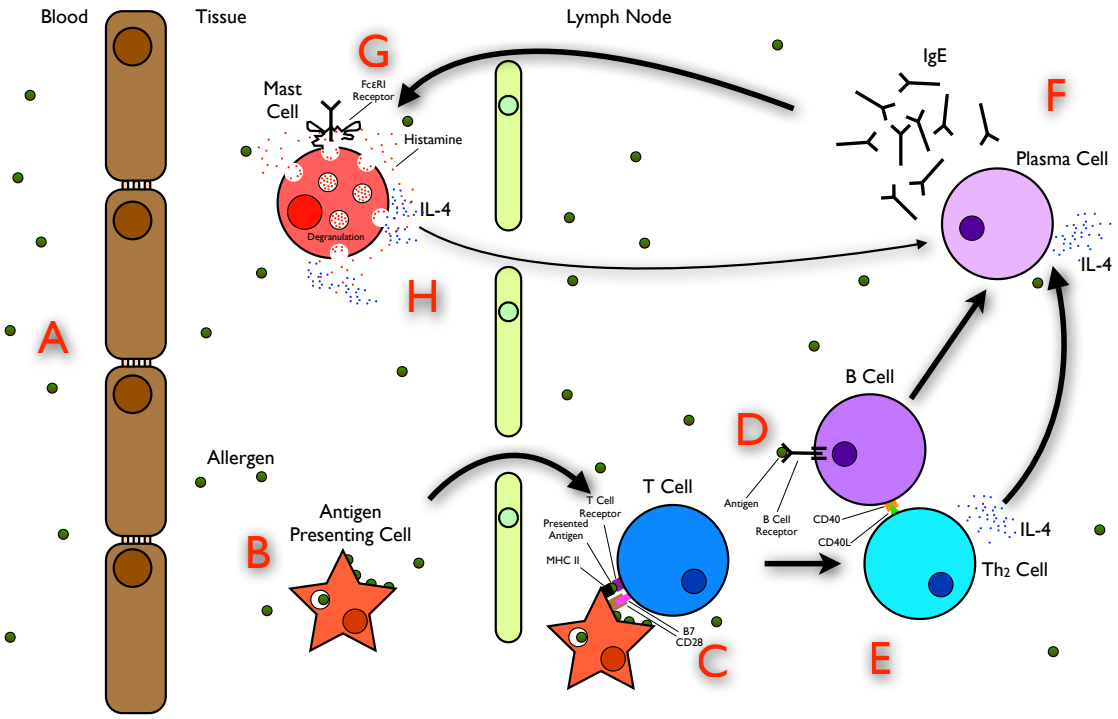


Figure 1: The allergy pathway:
This is a summary diagram that explains how allergy develops. **A.** the allergen enters the body and penetrates the mucosal tissue to the tissue fluid. **B.** in the tissue fluid, an APC (in this case dendritic cells) take up the allergen molecule, breaks it down and present its epitopes through the MHC II receptor. The activated antigen presenting cells then migrate to the nearest lymph node **C.** where they activate T cells that recognize the allergen on the MHC II through their T cell receptor and co-stimulated by the CD28 and B7 receptors. They then give the decision for the T cell to differentiate to T help type 2 (Th_2) cell. **D.** at the same time, B cells recognize the allergen through their B cell receptor and through the activated Th_2 cell **E.** the B cell would be activated through the CD40 and CD40L receptors. **F.** the activated B cell would differentiate into plasma cells that actively synthesize antibodies initially IgM. Cytokines secreted from the Th_2 cell (IL-4) cause the B cell to undergo antibody class switching from IgM to IgE. **G.** the IgE antibody, that now recognizes epitopes on the allergen molecule, circulates around the body through the lymphatic and cardiovascular systems and finally binds to its high-affinity IgE Fc receptor (FcεRI) on mast and basophil cells. **H.** when the allergen re-enters the body at a later time it binds to the IgE's variable region, which is on the cell surface, causing an aggregation of the receptor and thus a downstream signal that results in the cell releasing mediators. Some of these mediators are histamine, causing inflammation, and IL-4, which affects more plasma cells to undergo class switching to IgE and thus the cycle continues.

The pathway starts by the introduction of an allergen to the body (Figure 1 A). Allergens are capable of penetrating the mucosal tissue very effectively and thus enter the tissue fluid. There they are taken up by antigen presenting cells (APC), usually dendritic cells, where they are fragmented by breaking down the protein's covalent and disulphide bridges resulting in smaller peptides (Unanue and Allen, 1987). The processed allergen causes the APC (in this case a dendritic cell) to polarize either into a DC1 or DC2 polarization depending on the type of antigen being processed, bacterial and viral antigens causes DC1 polarization and parasitic extracts causes DC2 polarization. These polarization states is what induces the Th_0 cells to differentiate into Th_1 or Th_2 cells (Gould, *et al.*, 2003; de Jong, *et al.*, 2002). The processed antigen is then presented on the cell's surface using the mega histocompatibility complex II (MHC II) receptor (Figure 1 B) exposing its epitopes. The activated APC cell then migrates to the nearest lymph node, where it is actively directed towards the subcortical zone and interacts with T cells and activates them (Figure 1 C) (Havenith, *et al.*, 1993; Roitt, 2001; Gould, *et al.*, 2003). Some theories state that the APC polarization decision might be coming from the microenvironment (Lambrecht, 2001) and not from the allergen itself. Experiments have shown that dendritic cells found in the spleen produce IFN-γ which causes T cell there to differentiate to Th_1 cells (Iwasaki and Kelsall, 1999), while dendritic cells found in aggregated lymphoid nodules (Peyer's patches) tend to causes T cell there to differentiate to Th_2 cells (Stumbles, *et al.*, 1998). Therefore given a suitable cytokine environment, any molecule can be made to induce an IgE response.

T cell differentiation occurs when a naïve T cell (Th_0) is activated by recognizing an antigen through its T cell receptor when it is presented to it by an ACP (dendritic cell, macrophage or in some occasions B cell) through the MHC II receptor, then co-stimulated though its CD28 receptor by B7 protein on the antigen presenting cell's

surface (Roitt, 2001; Linsley, *et al.*, 1990). In Th_1 cell development, the antigen presenting cell will also release IL-12 which activates STAT-4 (a protein transcription factor) which synthesis T-bet (another protein transcription factor) that results in the synthesis of IFN-γ. The secretion of IFN-γ by Th_1 cells causes B cells to undergo antibody class switching to IgG and inhibits the development of Th_2 cells (O'Shea and Paul, 2002). In the absence of IL-12, the default pathway would be for the Th_0 cell to differentiates to a Th_2 cell when it express GATA-3 (a protein transcription factor) which in turn promotes the cell to express and release IL-3, IL-4, IL-5, IL-9, IL-10, IL-13 and GM-CSF. The secretion of IL-4 causes B cell to undergo class switching to IgE and it also stops Th_1 cell differentiation by inhibiting IFN-γ (Nawijn, *et al.*, 2001; Gould, *et al.*, 2003; Howard and Paul, 1982).

B cells produce the antibody light chain variable region by random organization of the Vκ and Jκ and genes on chromosome 2p11.12, or the Vλ and Jλ genes on 22q11.2 (Tonegawa, 1983; Paul, 1999). The antibody's heavy chain variable region is assembled from V (Variable), D (diversity) and J (joining) genes at chromosome 14q32. Naïve B cells have their heavy chain variable region VDJ recombinant genes joined immediately to Cμ, therefore they express IgM by default. These cells mature in the bone marrow to make sure their new variable region does not recognize self antigens. Once the B cell matures it expresses the variable region through the B cell receptor on its surface it migrates to germinal centers in lymph nodes. B cell receptors are made up of IgM antibodies bound on to CD79 on the surface of the cell, enabling the cell to detect the antigens it is designed for. The B cell is activated when it detects its antigen (allergen in the case of allergy) (Figure 1 D) and receives a signal from activated T cells by binding the T cell's CD40L receptor into to the B cell's CD40 (Figure 1 E). Once the B cell is activated it differentiates into memory cells and plasma cells. The decision for B cells to

differentiate into plasma cells in germinal centers of the lymph nodes occurs in the presence of IL-10 from Th_2 cells which affects B cells to up regulate CD27 on their surface and thus differentiate to plasma cells (Jung, *et al.*, 2000). Activated T cells secrete certain cytokines that affect the activated B cell and cause it to undergo class switching. In cases of allergy, Th_2 cells mainly secrete IL-4 that causes the B cell to premaritally switch from IgM to IgE antibodies (Figure 1 F) (Roitt, 2001; Manis, *et al.*, 2002; Li, *et al.*, 2004), this is called antibody class switching. Plasma cells secrete ~0.1ng of immunoglobulin per day, this is large as antibody synthesis is ~50% of the total protein synthesis by these cells (Hibi and Dosch, 1986).

Antibody class switching occurs when cytokines from Th_2 cells (IL-4 mainly) binds to receptors on the B cell and causes it to remove the Cμ, Cδ, Cγ and Cα genes and thus the VDJ genes bind to Cε genes that code for the Cε1-4 domains of the IgE antibody heavy chain (Roitt, 2001; Gould, *et al.*, 2003**;** Manis, *et al.*, 2002; Li, *et al.*, 2004). *In-vitro* class switching is a slow process, where switching from the μ chain to the ε chain can be achieved by incubating B cells with Th cells and IL-4. The B cells would start to secrete IgE after 10 days of incubation (Gauchat, *et al.*, 1990).

The antibody affinity to the allergen changes over time, usually increasing, this is achieved by somatic hypermutation because activated B memory cells undergo high rates of mutations in areas of the DNA called hyper variable regions. This results in new daughter B cells with slightly different binding affinity to the original antigen. In later activations the B cells producing antibody variable regions with the highest binding affinity would be activated and differentiate much quicker that the other B cells expressing lower binding affinity variable regions. Thus the immune system would better target antigens the more frequent it encounters them (Roitt, 2001; Teng, *et al.*,

2007; Rajewsky, *et al.*, 1987). In terms of allergy, the atopic person becomes more sensitive to the allergen.

Following the active production of IgE by plasma cells, this antibody would circulate the body through the lymph and cardiovascular systems until it finally binds to its high-affinity receptor (FcεRI) found on mast and basophil cells (Figure 1 G) or its low-affinity receptor (FcεRII) found on B cells, T cells and Langerhans cells. For the mast and basophil cell to be maximally sensitized by IgE to an allergen the antibody only needs to bind to 10% of the FcεRI receptors on the cell's surface (MacGlashan and Schroeder, 2000). These cells are the primary mucosal tissue cells that cause immediate hypersensitivity inflammation (Ehrlich, 1877; Metcalfe, *et al.*, 1997). They have granules in their cytoplasm which are vesicles filled with pre-synthesized mediators. Upon the cross linking of the FcεRI receptor on the cell surface, when an allergen binds to several IgE antibodies, a downstream signal cascade occurs resulting in these vesicles migrating and fusing with the plasma membrane, releasing their contents into the surrounding tissue fluid (Figure 1 H) (Roitt, 2001; Gould, *et al.*, 2003; Helm, *et al.*, 1991a). These mediators contribute to the signs and symptoms of immediate-type hypersensitivity (Hypersensitivity Type I). One of these mediators is histamine which causes the five symptoms of inflammation: heat, pain, redness, itchiness and swelling (Herzenberg, *et al.*, 1996). Some mediators are pre-synthesized such as: histamine, proteoglycan, neutral proteases such as β-glucosaminidase, neutrophil chemotactic factor, eosinophil chemotactic factor, platelet activating factor, IL-3, IL-4, IL-5, IL-6, TNF-α and GM-CSF. Therefore these mediators are released immediately when a mast cell degranulates and they cause the immediate-type hypersensitivity symptoms. Later on after activation, other mediators are synthesized, through the lipoxygenase and cyclooxygenase pathways, and thus their release is delayed. Some of these mediators

are: prostaglandins, thrombmoxanes, leukotrienes C_4, D_4 and B_4, and more TNF-α, GM-CSF, macrophage inflammatory protein-1α, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. These mainly cause the recruitment of eosinophils (the parasite fighting neutralizing cell), but also macrophages, neutrophils and Th_2 cells to the site of inflammation and this causes the signs and symptoms of delayed-type hypersensitivity (Hypersensitivity Type IV) (Helm, *et al.*, 1991a; Roitt, 2001; Gould, *et al.*, 2003).

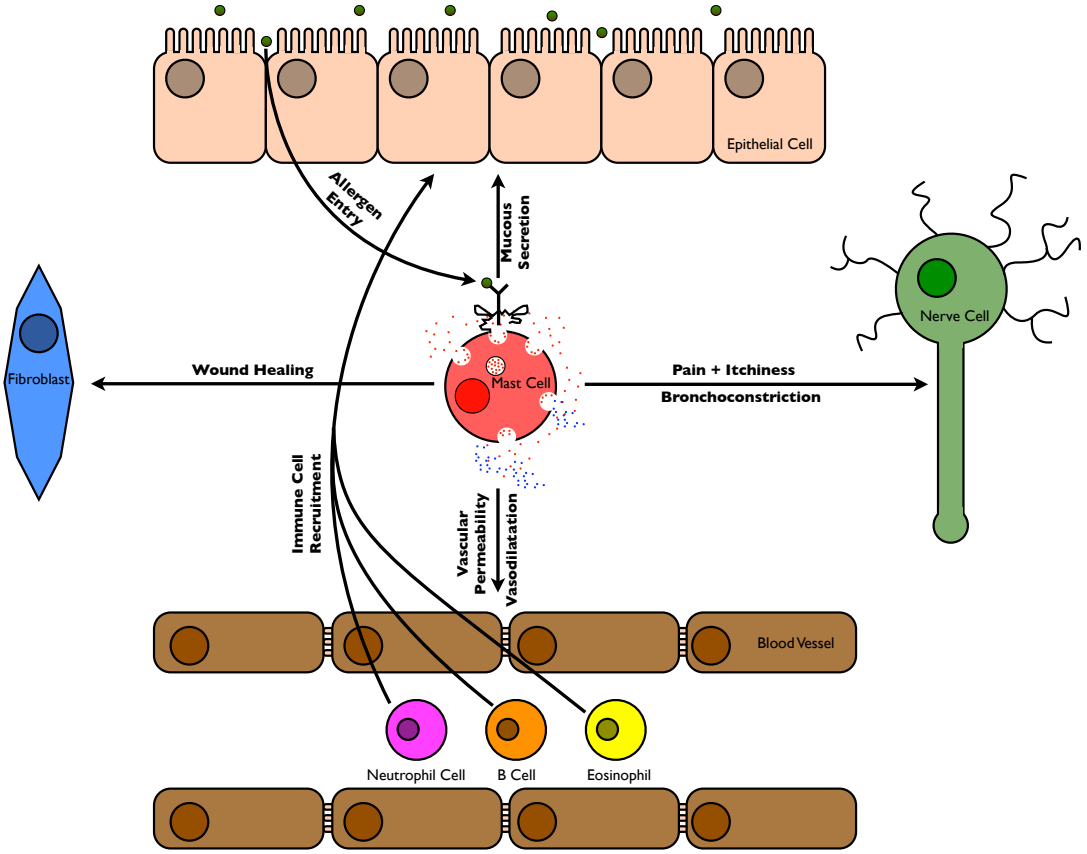


Figure 2: Mast cell effector locations:
Mast and basophil cell mediators affect many other types of cells, they cause the epithelial cells to secrete mucous to wash away the allergen, they cause the nerve cells to signal pain to alarm the host, and cause itchiness to physically remove the allergen from the skin surface, they also cause smooth muscles to contract to localize the allergen and prevent it from spreading around the body, incase of wound development they cause fibroblasts to activate and heal the wound, they also stimulate other leukocytes and recruit them to the site of inflammation to actively neutralize and remove the penetrated allergen and to produce more antibodies (figure inspired from Bischoff, 2007).

Mast and basophil cell mediators affect many nearby cells, which have the receptors for the specific mediators they release. They affect epithelial cells and stimulate them to secrete mucous which washes away the allergen and expel it from the body, for example by coughing. They stimulate nerve cells and cause them to signal pain which alarms the host, and produce the itchiness sensation in the location of the inflammation to physically remove the allergen, the nerve cells would also causes smooth muscles to contract to localize the allergen and prevent it from spreading around

the body. Incase of wound development the mediators would also cause fibroblasts to activate and heal the wound. They also stimulate other leukocytes (B cells, eosinophils ect.) and recruit them to the site of inflammation to actively neutralize and remove the penetrated allergen, and to produce more antibodies (Figure 2) (figure inspired from Bischoff, 2007).

Mast and basophil cell mediators are a combination of molecules each having a different, but overlapping, biological function and they can be categorized into three groups: preformed secretory granule-associated mediators (e.g: histamine, β-hexosaminidase), lipid-derived mediators, and cytokines.(Metcalfe, *et al.*, 1997). One of the cytokines mast cells release is IL-4, which re-stimulates Th_2 cells and causes more plasma cells to undergo class switching to IgE, this is a positive feedback loop which ensures the continual production of IgE (Gould, *et al.*, 2003; Abehsira-Amar, *et al.*, 1992; Mills, *et al.*, 1992; Bieber, *et al.*, 1989) even in the absence of allergen exposure, thus explaining why allergy persist in patients even after prolonged absence of allergic reactions (Smurthwaite, *et al.*, 2001), this can be caused through selective survival of B cells if they undergo many cell divisions (Gould, *et al.*, 2003 and Okudaira, *et al.*, 1981). Many allergens in fact give rise to IL-4 synthesis and secretion prior to IgE synthesis which can contribute to the first stimulus for subsequent development of IgE-mediated allergies (Machado, *et al.*, 1996). The (Okudaira *et al*, 1981) paper has found that the majority of IgE-secreting plasma cells are resistant to large doses of radiation, which makes them resistant to apoptosis. Thus target organs continually have their mast cells sensitized with IgE. The review by (Gould, *et al.*, 2003) discussed that germinal centers appearing in lymph nodes near affected target organs, there plasma cells continually synthesis IgE which binds to the mast cells in the nearby tissue even in the absence of allergen. Receptor occupancy with IgE even in the absence of allergen leads

to an increase in IgE receptor expressing by mast and basophil cells and affects the cytokine pattern of these cells (Kinet, 1999), therefore inhibiting IgE synthesis reduces the quantity of receptor bound IgE and thus down regulates receptors on the mast and basophil cell's surface, which results in reduction in sensitization to allergens. This concept is used later in this project as a basis behind the development of an allergy vaccine (Chapter 8).

Allergy commonly develops during early childhood as a result the human adaptive immune response being shifted towards the Th_2 cell arm when a child is born. This is due to placental cells producing IL-4 to inhibit the mother's cytotoxic leukocytes and natural killer cells from attacking the fetus, which is expressing non-maternal antigens, this has an effect on the fetus' T cells and thus the fetus is born with a polarized immune response towards the Th_2 cell arm. The immune system shifts towards the Th_1 cell arm by the introduction of environmental bacterial and viral infections later on in life (Roitt, 2001; Prescott, *et al.*, 1998).

1.3 - The Allergen Antigen:

Allergens are one of the essential parts of the allergy pathway as their structure is thought to dictate whether the antigen presenting cell would activate the Th_0 cells differentiation into Th_2 cells. These allergen antigens are one of the most well studied proteins in molecular biology due to their significance in medicine (Chapman, 1998). They have a specific nomenclature established by the World Health Organization in 1994 and its basis are the taxonomic name of the source of the first purified allergen, three letters from the genus followed by the first letter of the species followed by an arabic numeral in order of purification from the same source. For example the first allergen to be purified was from Ragweed *Ambrosia artemisiifolia*, thus this allergen is

called Amb a 1 (Chapman, 1998; Ishizaka, *et al.*, 1966) this was the actual allergen that lead to the discovery of IgE.

Allergens have several characteristics that makes them excellent candidates for type I hypersensitivity; they all have low molecular weights (5-50 kDa), and they are strongly hydrophilic, which makes them readily penetrate the mucosal tissue. Some organic molecule allergens, such as penicillin, need to be conjugated to protein carriers (albumin) for them to cross link IgE (Chapman, 1998). The reason allergens are capable of cross linking IgE is because they have multiple repeats of epitopes (Beezhold, *et al.*, 1997; Roitt, 2001) which makes one allergen molecules bind to several IgE antibodies and thus, on the surface of mast and basophil cells, this will aggregate the FcεRI receptor causing degranulation.

Examples of common allergens: peanuts (Ara h 1) vicilin which is a seed storage protein, bee venom (Api m 1) which is phospholipase A_2, pollen (Amb a 1) which is pectate lyase, house dust mite (Der p 1) which is a protease, dogs (Can f 1) which is a salivary protein (Chapman, 1998; Konieczny, *et al.*, 1997). As seen from this small list, most allergens are proteolytic enzymes, and their enzymatic activity further contributes to them readily penetrating mucosal tissues, and causing the immune system to react against them using the Th_2 immune response arm (Dudler, *et al.*, 1995; Machado, *et al.,* 1996**;** Chapman, 1998). But there are also other allergens that are not enzymes such as β-lactoglobulin (found in cow milk), still with an un-determined function (Chapman, 1998).

The allergen enzymatic activity, or lectin type activity, causes dendritic cells to release IL-4 that shifts the immune response towards the Th_2 arm (Machado, *et a.l,* 1996). This has been demonstrated in the paper by (Dudler *et al*, 1995) where an

enzymatically functional bee venom, phospholipase A_2, was introduced into mice in low concentration, these mice developed IgE antibodies against the venom. But when the same molecule with a non-functional enzymatic activity was introduced in the same low concentration, the mice did not developed IgE antibodies against it. At high enough concentration, both the functional and non-functional forms of the venom developed immunological responses.

Patients typically develop asthma if they are exposed to an airborne allergen at levels greater than 1 μg $year^{-1}$, therefore a protocol where infants (0-3 years old) were avoided exposure to allergens showed effectiveness in preventing allergies (Simpson, *et al.*, 2003)

1.4 - The Immunoglobulin E Antibody:

Binding of several allergens onto many IgE molecules and aggregating them initiates allergy symptoms, since the IgE is already bound to its high-affinity FcεRI receptor and acts as sensors on the mast and basophil cells' surface. IgE is the least common antibody in serum and thus it was the last antibody to be discovered by the Ishizakas in 1966 (Ishizaka, *et al.*, 1966). Its size is ~192kDa with a half life in serum of ~3 days, compared with IgG which is ~20 days, the reason for this is that most of the IgE in the body is removed from the serum by binding to basophil and mast cells in tissue (Iio, *et al.*, 1978).

There are 5 antibody classes: IgA, IgD, IgE, IgG and IgM (Roitt, 2001). They all have a basic structure of two light (small) chains, two heavy (large) chains, a variable region, where the antigen binds, and a constant region made up of several domains with internal disulphide bridges (Figure 3). The Fab region contains a variable domain and a constant domain in the light chain and a variable domain and the Cε1 domain of the

heavy chain. The light chain and the heavy chain are connected through two disulphide bridges, and the two variable domains construct the variable region of the antibody where it binds to its antigen, or the allergen in allergic reactions. The Fc region, made up of the two heavy chains connected by two disulphide bridges, is the section of the antibody which determines its identity and thus its effector cell type. Contrary to other antibodies, IgA, IgD and IgG who have a hinge region, IgE does not have one, it is replaced by Cε2, just like IgM (Roitt, 2001). There are two disulphide bridges between the Cε2 domains of the two heavy chains, thus linking the two heavy chains together (Helm, *et al.*, 1991b) The nature of the disulphide bridges arrangement is disputed, early biochemical evidence (Takatsu, *et al.*, 1975) indicates a parallel arrangement, and so do protein chemical studies. There are 5 heavy chain classes: α, δ, ε, γ and μ, but only 2 types of light chains: κ and λ. IgA and IgG have subclasses due to small sequence differences in their heavy chains and their important roles it the wider immune response: IgA1, IgA2, IgG1, IgG2, IgG3 and IgG4 (Gould, *et al.*, 2003), this results in small adjustments in the binding to different types of target cells during different types of infections. The IgE antibody has three domains in its Fc region, Cε2, Cε3 and Cε4, the molecule's Cε3 domain is the site where the antibody actually binds to the FcεRI receptor's α chain where the strongest binding between the IgE and the FcεRI receptor occurs at pH 6.4 and 7.4 (Helm, *et al.*, 1996; Garman, *et al.*, 2000). All heavy chain domains have an internal disulphide bridges that contributes to their shape.

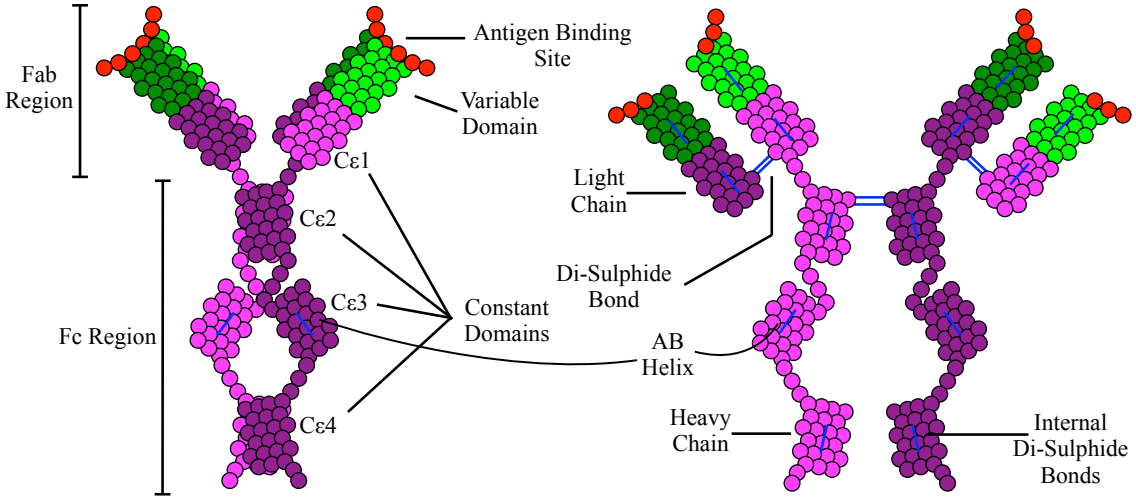


Figure 3: Structure of immunoglobulin E:
The immunoglobulin E (IgE) antibody is composed of two chains, the light (small) chain and the heavy (large) chain. The Fab region contains the light chain and part of the heavy chain connected through a disulphide bridge, they each are composed of two globular domains, and they both compose the variable region where the antibody binds to the antigen it recognizes. The Fc region is composed of the other half of the two heavy chains, where they are bound together by a disulphide bridge. The Fc region contains Cε2, Cε3 and Cε4 constant domains. The Cε3 is the constant domain and the site where the IgE binds to the FcεRI receptor.

Once the IgE binds to its FcεRI receptor, it physically associates and competes with the FcγR receptors (which binds IgG) for their common γ chain, the rivalry ends with the down regulation of the FcγR receptors in favor of FcεRI receptor (Ra, *et al.*, 1989; Kurosaki, *et al.*, 1992; Scholl and Geha, 1993; Gould. *et al.*, 2003). IgE can also bind to its low-affinity Fc receptor (FcεRII, also known as CD23), though this receptor is not classified as an immunoglobulin receptor and more as a lectin receptor (Weis, *et al.*, 1998) and its function is to capture serum IgE, that has bound to an allergen, and transport the allergen to APCs which activates them and allows for the continual maintenance of the Th_2 arm immune response. Back to the high-affinity receptor, once IgE binds to the FcεRI receptor it has a half life of ~20 hours (McDonnell, *et al.*, 2001) but the restricted diffusion in tissue causes the IgE to rebind to the cell's surface

increasing its half life to ~14 - ~21 days (Ishizaka and Ishizaka, 1971; Geha, *et al.*, 1984).

IgE is the least abundant antibody in serum, its concentration is ~150ng ml^{-1} for healthy individuals (King, *et al.*, 1991) ~6ng ml^{-1} of which are allergen specific IgEs (Smurthwaite, *et at.*, 2002), compared to 10mg ml^{-1} for IgG which is the most abundant antibody in serum (King, *et al.*, 1991), though sometimes the level of IgE can increase to up to three times the normal level without any signs of inflammation (Patterson, *et al.*, 1975). On the other hand, atopic individuals have up to ten times the normal IgE level, and if the type of IgE in atopic individuals was analyzed it will show approximately one thousand times the normal level of IgEs that are allergen specific (Smurthwaite, *et at.*, 2002).

The 11 amino acids found in the Cε3 domain Pro^{343}-Ser^{353} (PSPFDLFIRKS) also called the AB helix (Figure 3), were found to be essential to the binding of the IgE molecule to the FcεRI receptor, removal of this sequence results in a complete loss of binding of the IgE molecule to the FcεRI receptor. Though this sequence does not interact nor bind to the FcεRI receptor, but it does provide structural scaffolding that allows the Cε3 domain to be positioned in the right orientation and shape for it to bind to the FcεRI receptor (Helm, *et al.*, 1996). On the other hand this region contain the binding site for FcεRII receptor (Sayers, *et al.*, 2004).

The IgE molecule is N-glycosylated at different sites: three in Cε1, one in Cε2 and two in Cε3 (Herzenberg, *et al.*, 1996). Oligosaccharides mask epitopes otherwise exposed to anti-IgE antibodies, mainly in Cε2 (Björklund, *et al.*, 1999) though the absence of Cε3 oligosaccharides does not affect the binding of the IgE to the FcεRI

receptor (Wurzburg, *et al.*, 2000; Helm, *et al.*, 1996) as the confirmation of the Cε3 structure is maintained by the AB helix (Pro^{343}-Ser^{353}).

The binding affinity between the IgE antibody and its antigen can change through Somatic hypermutation, which is point mutations occurring in the variable region after the B cell has rearranged its variable region and matured. These point mutation usually occur in the sequence RGYW, where R = A or G, Y = C or T, W = A or T (Neuberger, *et al.*, 1998). A single point mutation can cause the variable region to lose affinity to its antigen, or become ten times stronger (Berek and Milstein, 1987). Chronic inflammation results in the local synthesis of IgE in tissue by the development of local germinal centers. As in arthritis (Berek and Kim, 1997) and diabetes (Ludewig, *et al.*, 1998) large quantities of memory cells allow for the activation of naïve B cells and promotes somatic hypermutation (Randen, *et al.*, 1992) which results in the accumulation of long lived plasma cells in this micro-environment. This was found to be the case in lungs after antigenic challenges (Chvatchko, *et al.*, 1996) which explains the reason behind the continues production of IgE even in the absence of antigen exposure.

1.5 - The High-Affinity IgE Receptor:

The high-affinity FcεRI receptor is always expressed on the surface of mast and basophil cells, but they only have an effect in allergy once the IgE molecule binds onto them, this binding is strong and results in cells to be activated against a certain allergen. FcεRI receptor is classified as a Multichannel Immune Recognition Receptor (MIRR) which makes it similar to the T cell receptor, the B cell receptor and the IgG FcγRIII receptor (Keegan and Paul, 1992). The receptor gene is found on the locus 1q23.2 and consists of an α chain (50-60 kDa) containing two immunoglobulin like domains (1 and 2), the site where IgE binds (Garman, *et al.*, 1999), a β chain (32 kDa), which amplifies

the downstream signal, and two disulphide linked γ chains (7-9 kDa each), the site where the downstream signal initiates, thus is it abbreviated $\alpha\beta\gamma_2$ (Figure 4) (Blank, *et al., 1989;* Gould, *et al.*, 2003; Metcalfe, *et al.*, 1997; Donnadieu, *et al.*, 2000). This is the tetrameric form of the receptor which is found on mast and basophil cells with a concentration of ~$2x10^5$ receptor molecules per cell (MacGlashan and Schroeder, 2000). The receptor can also be found in a trimeric form with an $\alpha\gamma_2$ configuration which is found on Langerhans cell (Bieber, *et al.*, 1992), monocyte (Maurer, *et al.*, 1994), platelet (Joseph, *et al.*, 1997) and eosinophil (Gounni, *et al.*, 1994) membranes, at a much lower concentration than mast and basophil cells (Gould, *et al.*, 2003; Garman, *et al.*, 2000). This receptor configuration helps these APCs to recognize allergens which cause them to increase their uptake leasing to their expression and activation of Th_2 cells. (Kraft and Kinet, 2007).

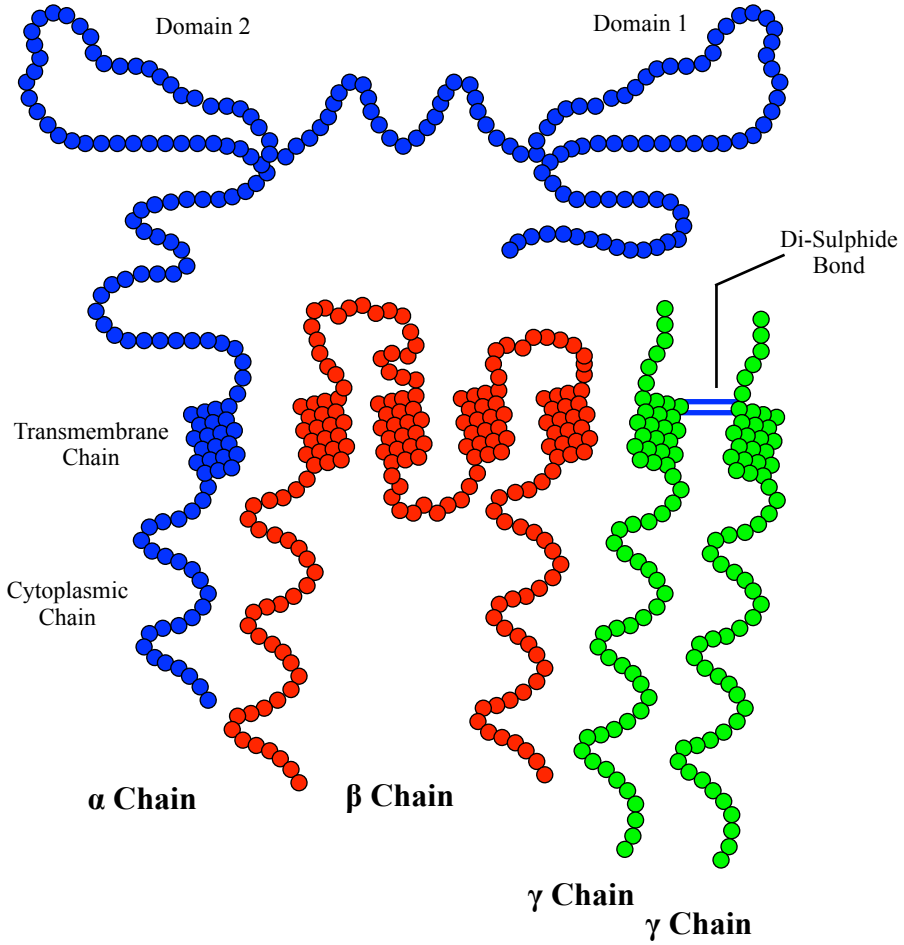


Figure 4: Structure of the high-affinity IgE Fc receptor (FcεRI):
The FcεRI receptor is composed of 3 chains, one α chain with 2 domains, the second domain is capable of binding to the two IgE Cε3 domain heavy chains. One β chain which amplifies the downstream signal, and two γ chains bound together by a disulphide bridge, they are the site where the downstream signal initiates. Figure adapted from (Blank, *et al.*, 1989).

Since the FcεRI receptor has one α chain capable of binding to the heavy chain of the IgE, and the IgE antibody has two heavy chains, the crystal structure of the IgE/ FcεRI complex has shown that the two IgE heavy chains bind at two sites on the FcεRI receptor's α chain thus giving the 1:1 interaction binding ratio (Garman, *et al.*, 2000). From this (Figure 5) it can be determined that the two Cε3 domains of the heavy chain in the IgE molecule interact mainly with the second domain of the FcεRI receptor

(Garman, *et al.*, 2000) thus the first domain acts as scaffolding to position the second domain at the right angle for interaction with the IgE antibody.

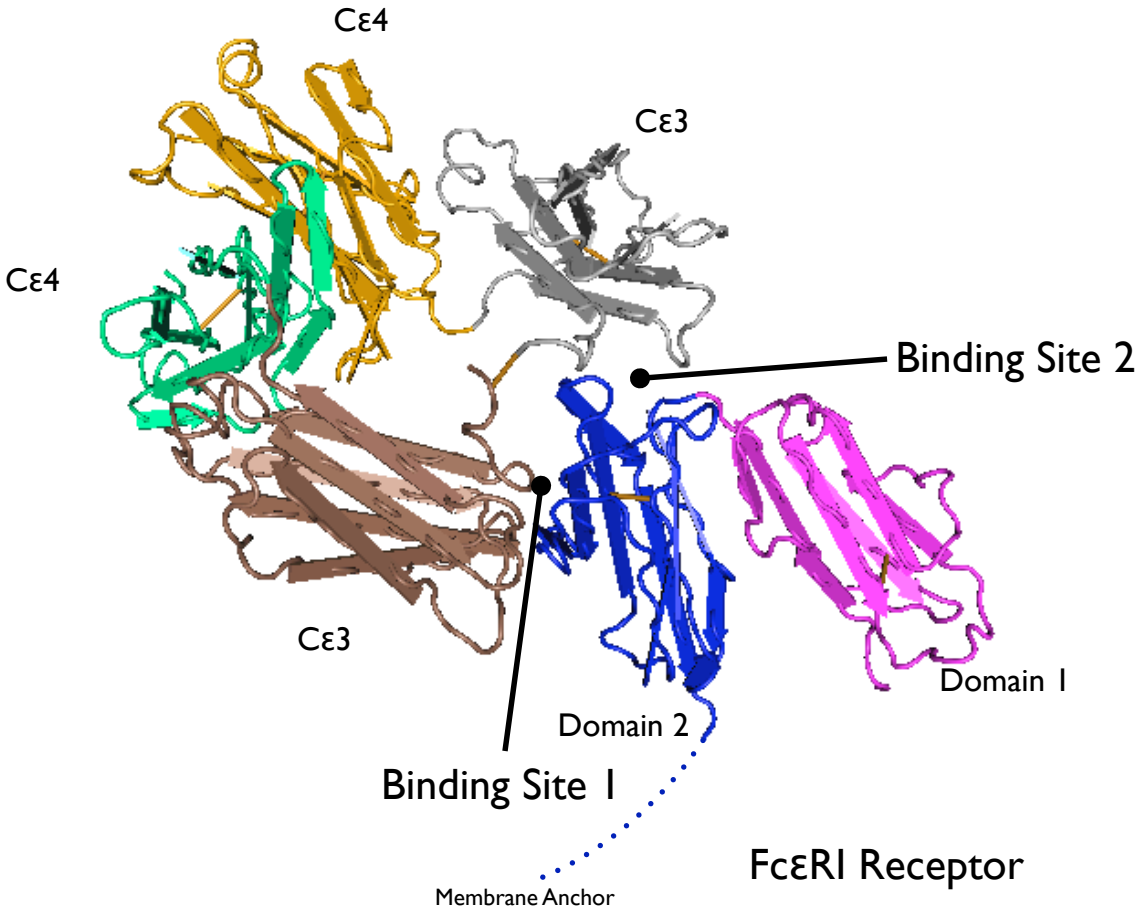


Figure 5: Crystal structure of IgE binding to the FcεRI receptor:
This crystal structure shows that the Cε4 domains of the IgE and the first domain of the FcεRI receptor give structural support to assist in the interaction of the two Cε3 domains, from the IgE heavy chain, with the the second domain of the FcεRI α chain thus giving a 1:1 interaction binding ratio. Figure was adapted from (Garman, *et al.*, 2000).

Human IgE binds to the human FcεRI receptor very strongly with an equilibrium dissociation constant of $K_D = \sim 10^{-10}$ M (i.e: an equilibrium association constant of $K_A = \sim 10^{10}$ M^{-1}) (Sayers, *et al.*, 1998; Nissim and Eshhar 1992; Helm, *et al.*, 1996; Schuck and Minton 1996; Young, *et al.*, 1995; Hakimi, *et al.*, 1990; Miller, *et al.*, 1989; Gould, *et al.*, 2003), compared to IgG1 which is $K_D = 10^{-8}$ M ($K_a = 10^8$ M^{-1}) to FcγRI (Ravetch and Kinet, 1991). The β chain in the FcεRI receptor enhances the strength of signal

transduction up to 12-30 times compared to the $\alpha\gamma_2$ FcεRI receptor with a missing β chain (Lin, *et al.*, 1996; Donnadieu, *et al.*, 2000). It also assists in transporting the receptor into the plasma membrane (Donnadieu, *et al.*, 2000). There are two variants of the β chain in humans, I181L-V183L and E237G, where there are mutations in the β chain, though tests have shown that these mutations do not affect the β chain amplification function (Donnadieu, *et al.*, 2000) but some other polymorphisms might be associated with allergy (Kinet, 1999). The FcεRI, FcγRI, FcγRII and FcγRIII all share the same γ chain (Ra, *et al.*, 1989; Hibbs, *et al.*, 1989; Ernst, *et al.*, 1993; Scholl, *et al.*, 1993; Mesuda, *et al.*, 1993; Pfefferkorn, *et al.*, 1994; Saito, *et al.*, 1995; Morton, *et al.*, 1995) thus they compete for it, and the cell up-regulates or down-regulates these receptors' expressions depending on the immune response conditions. The binding of IgE to the FcεRI receptor on the cell's surface causes the up-regulation of FcεRI receptor expression by ~6 folds (Hsu and MacGlashan, 1996; MacGlashan, *et al.*, 1999), which agrees with the paper by (Kinet, 1999) which states that FcεRI receptor occupancy by IgE increases its expression, or as these papers (Furuichi, *et al*., 1985; Quarto, *et al*., 1985; Yamaguchi, *et al*., 1997; Lantz, *et al*., 1997; MacGlashan, *et al*., 1997; MacGlashan, *et al*., 1998; Saini, *et al*., 1999; Beck, *et al*., 2004) argues that IgE receptor occupancy prevent the receptor from being internalized, and thus degraded, while maintaing its transcription and transportation to the surface, and thus its concentration on the cell surface increases. IgE FcεRII (CD23) receptor occupancy also prevents receptor cleavage by ADAM10 (Kisselgof, *et al*., 1987; Lee, *et al*., 1987). The FcεRIγ is the site of the downstream signal cascade, it also regulates the expression of the FcεRIα chain and prevents its degradation in the endoplasmic reticulum (Ernst, *et al.*, 1993). It also contributes to the correct folding of the FcεRIα (Suzuki, *et al.*, 1998).

The equine FcεRIα chain is N-glycosylated 7 times at amino acid numbers 46, 67, 79, 99, 160, 170, and 195, therefore on domain 1 there are 4 oligosaccharides, domain 2 has 2 oligosaccharides and one oligosaccharide between domain 2 and the transmembrane. These glycosylations are not essential for IgE binding, but they do prevent the receptor from binding with adjacent receptors and aggregate on the cell surface, this was demonstrated where the de-glycosylated of the FcεRIα chain would aggregate in solution (Letourner, *et al.*, 1995; Robertson, *et al.*, 1993; Scarselli, *et al.*, 1993).

When an allergen binds to the IgE's variable region, because of the multi epitope nature of the allergen, it will bind to several IgE molecules at the same time, since one molecule of IgE binds to one molecule of FcεRI receptor, the binding of the allergen causes many FcεRI receptors to come close together and aggregate on the cell surface. This receptor aggregation causes the downstream signal because Lyn kinase, a protein tyrosine kinase, is associated with the β chain. Once the receptor aggregates on the cell surface, Lyn is activated and tyrosine phosphorylates a certain sequence on the β and γ chains called immunoreceptor tyrosine-based activation motif (ITAM). At this moment Syk, also called spleen tyrosine kinase (since it was first discovered in the spleen, but it is an ubiquitous signal transducer), binds to the γ chain where it is phosphorylated by Lyn and thus is activated (Cambier, 1995; Jouvin, *et al.*, 1995). From there, the activated Syk phosphorylates several other proteins which results in three outcomes: stimulation of cytokine synthesis and release, arachidonic acid release and histamine release through the influx of Ca^{2+} ions that promotes vesicles to fuse with the plasma membrane (Siraganian, 2003) (Figure 6).

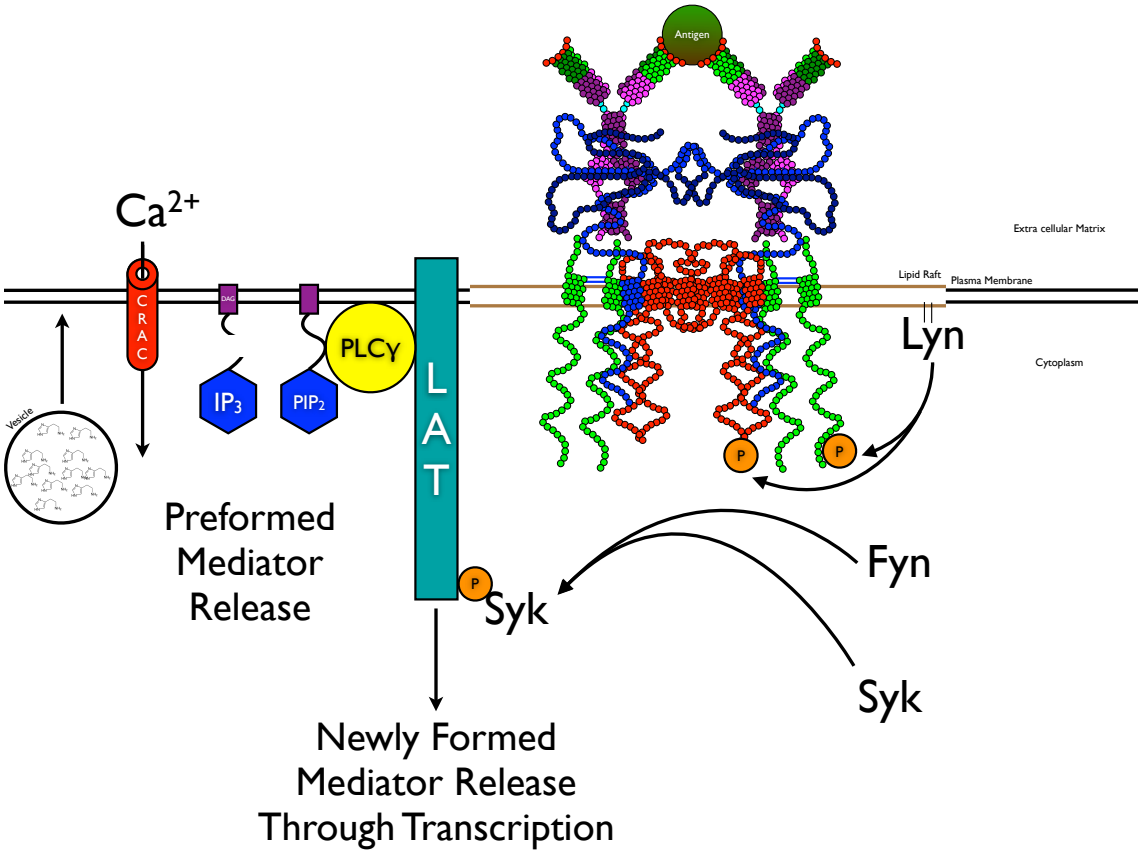


Figure 6: Summary of the IgE/FcεRI mediated downward signaling:
This is a simplified diagram that shows the initial downstream signal protein cascade. When the receptor aggregates due to allergen binding to the IgE antibody on the cell surface, Lyn kinase, which is bound on lipid rafts on the cell surface, phosphorylates the β and the γ chains. Following that, Fyn and Syk kinases are phosphorylated by the phosphate groups from the β and γ chains. The phosphorylation of Syk kinase results in the phosphorylation of LAT that causes the release of preformed mediators, and initiates the expression and release of other delayed mediators.

Lipid rafts were first identified by their ability to resist non-ionic detergents in cell fractions (Simons and Ikonen, 1997). They are thought to be synthesized in the Golgi apparatus (Brown and London, 1998) where they are then transported to the plasma membrane. Lipid rafts are ridged and thus less fluid than the surrounding phospholipids (Brown and London, 1998), and they include a lot of proteins essential for downstream signaling (Simons and Toomre, 2000). The IgE/FcεRI signaling initiates from a lipid raft (Baird, *et al.,*1999), and these rafts are essential for the downstream signally

because if these rafts were removed by depleting plasma membrane from cholesterol, the IgE/FcεRI signaling ceases (Sheets, *et al.*, 1999), therefore these lipid rafts are essential for the signaling as they bring the FcεRI receptors close together that only a small number of receptors needs to be cross linked and thus aggregate for a downstream signal to initiate (Simons and Toomre, 2000), they also bring with them Lyn kinase, which is associated with lipid rafts, from other reactions close to the aggregated receptors so it can phosphorylate their γ chains. 65% of the mast cell surface is made from lipid rafts, and they are assembled once the FcεRI receptor aggregates (Holowka, *et al.*, 2005). The main purpose of lipid rafts is to segregate relevant proteins for a relevant function, thus the FcεRI receptor, whether bound to IgE or not, freely moves in the plasma membrane, but once it aggregates, due to antigen binding, it gains higher affinity to lipids and thus micro lipid rafts accumulate around the aggregated receptors bringing with them relevant signaling proteins one of which is Lyn kinase (Holowka, *et al.*, 2005).

1.6 - Current Therapeutic Interventions:

Currently allergy has no cure, once allergy develops in an organism it is a life long disease, though the severity of symptoms may change with age. The best and oldest way to prevent allergic reactions was to refrain from contact with the allergens, i.e: not keep cats or dogs for mite sensitive individuals (Platts-Mills, *et al.*, 2000). Although this is effective to some extent, people with air-born allergies i.e: pollen sensitive, cannot easily avoid them. Major current allergic treatments aim at alleviating allergy symptoms, thus they do not target the fundamental causes of allergy.

1.6.1 - Pharmacotherapy:

This is the use of drugs to interfere with the inflammation pathway by either halting cell degranulation, block mediator receptors or, during delayed-hypersensitivity,

halting cell chemotaxis. These drug include histamine antagonists (Antihistamines) which are drugs that block the histamine receptors in cells thus rendering them unresponsive to histamine, leukotriene inhibitors acts in a similar way where they inhibit the inflammatory effects of leukotriene and β_2-adrenergic receptor agonists which activates the β_2-adrenergic receptor and thus causes smooth muscle relaxation in bronchioles, these are mainly used against asthma attacks. The most effective current treatment for allergies are glucocorticoids, which are a class of corticosteroids. They are drugs that up-regulate the expression of anti-inflammatory proteins and down-regulate the expression of pro-inflammatory proteins and thus reduce the number of inflammatory cells. At high enough doses they are able to control all types of allergies in all patients, but the limiting factor is their side effects (Barnes, 1999).

Other examples are decongestants, anticholinergics, antagonists, beta-adrenoceptor agonists, theophylline, sodium cromoglycate (Meltzer, 1998). Epinephrine (Adrenaline) is a vasoconstrictive drug, thus effective in reducing inflammation (Simons, 2004), it is mainly used during emergency anaphylaxis to stop swelling. Mast cell stabilizers are drugs that block the Ca^{2+} channels that are essential for cell degranulation (Walsh, *et al.*, 2009).

A new area of research is emerging that targets the Ca^{2+} influx. Ca^{2+} release-activated Ca^{2+} (CRAC) are ion channels that transport Ca^{2+} through the plasma membrane into the cytoplasm. They are important in lymphocyte differentiation, and essential for mast and basophil cell degranulation. An organic molecule called Synta 66 discovered by Synta Pharmaceuticals have shown to be an inhibitor of CRAC (Di Sabatino, *et al.*, 2009), which have shown to inhibit mast cell degranulation (Sweeney, *et al.*, 2009).

1.6.2 - Immunotherapy:

Allergen-specific immunotherapy (SIT) was first used in 1911 (Noon, 1911) to desensitizes patents from allergens. It involves the subcutaneous injection of an allergen extract in an effort to desensitize atopic patients to certain allergens. This was proven successful to treat certain allergies such as anaphylaxis and allergic rhinitis, but it was un-successful in treating asthma (Lewis, 2002). It has remained controversial as it has the potential to sensitize patients even more and thus worsening their condition (Moverare, *et al.*, 2002). The mechanism behind this is that introducing Th_2 cells to allergen-derived peptides renders these Th_2 cells unresponsive, by increasing the intracellular Ca^{2+} ions, they would also not respond to future co-stimulatory signals from antigen presenting cells, this unresponsiveness lasts up to a week *in vitro* (Jenkins, *et al.*, 1987), this treatment is taken subcutaneously and thus called subcutaneous immunotherapy (SCIT). Also immediate mast and basophil degranulation would not occur due to capping of the FcεRI receptor as a result of a high dose of allergens, this was shown by our group as well and discussed in (Chapter 4). Taking this concept to the next level developed a novel immunotherapy called sublingual immunotherapy (SLIT) where the allergen extracts are given to patients under their tongue (Gidaro, *et al.*, 2005) this have been proven to be a very safe treatment with rarely strong reactions. Another study showed that epicutaneous immunotherapy (antigen delivery on to the surface of intact skin) can have a tolerogenic role, as it was was observed that repeated application of ovalbumin (on ovalbumin sensitive mice) on to the surface of their intact skin, down regulated T cell responses (Dioszeghy, *et al.*, 2011) this is a potential novel, non-invasive, therapy.

The use of cytokines to shift the immune system away from allergy associated inflammation is another immunotherapeutic approach to combatting allergy. IFN-γ is a

cytokine that is mainly responsible for the activation of macrophages during an immunogenic reaction (Roitt, 2001; Bancroft, *et al.*, 1991). IFN-γ has shown to counter the effects of IL-4 on effector Th cells, and thus reduce the synthesis of IgE (Gauchat, *et al.*, 1990). Thus the following research paper (Lack, *et al*., 1994) has shown that the introduction of nebulized IFN-γ into the airways of mice that have been previously sensitized to ovalbumin, at closely controlled timing, caused the mice to stop reacting to ovalbumin, this was achieved by reducing the quantity of ovalbumin specific IgE in serum by pushing the immune system towards the Th_1 cell response arm, which resulted in an increase in IgG production. The removal of serum IgE also contributes by down regulating the expression of the FcεRI receptor as discussed earlier by (Kinet, 1999). Another two studies have run similar experiments with inhibiting cytokines: Inhibiting IL-4 by introducing a soluble form of its receptor sIL-4R (Renz, *et al.*, 1994; Conrad, *et al.*, 2010) and inhibiting IL-5 by introducing anti IL-5 antibodies (Hamelmann, *et al.*, 1997) all of which have inhibited immediate and delayed hypersensitivity reaction and thus restored allergen tolerance. Another proposed therapy is using IL-35 to forcefully suppressing Th_2 cells in airway inflammation (Huang, *et al.*, 2011).

Secretory leukocyte protease inhibitor (SLPI) is an enzyme protease that is secreted by leukocytes in mucosal tissue, it has been observed to have anti-inflammatory effects and is a potential immunotherapeutic molecule that shows good promise for asthma treatment (Marino, *et al.*, 2011).

Anti-IgE immunotherapy is a novel immunotherapeutic concept that was first pioneered by (Hook, *et al.*, 1981) and patented by an american company called Tanox Biosystems Inc. in 1987 (Figure 7). This invention involved the direct neutralization of the IgE antibody and thus its active reduction in serum by developing a monoclonal

humanized IgG antibody anti human's Cε3 IgE heavy chain domain, and thus disrupt its binding to the FcεRI receptor (Davis, *et al.*, 1993). The drug they developed was called TNX-901, then the names changed to CGP51-901 when Tanox Biosystems Inc. started collaborating on the research with Ciba-Geigy Ltd. (which later merged with Sandoz to form Novartis International AG), finally the name changed to Talizumab in 1996 when Tanox Biosystems Inc., Novartis International AG and Genentech Inc. resolved a legal dispute, and all three companies started to research the drug on anti-peanut allergy, which showed the successful reduction in patients with severe peanut allergy by the introduction of 450mg of Talizumab (TNX-901, CGP51-901) (Leung, *et al.*, 2003).

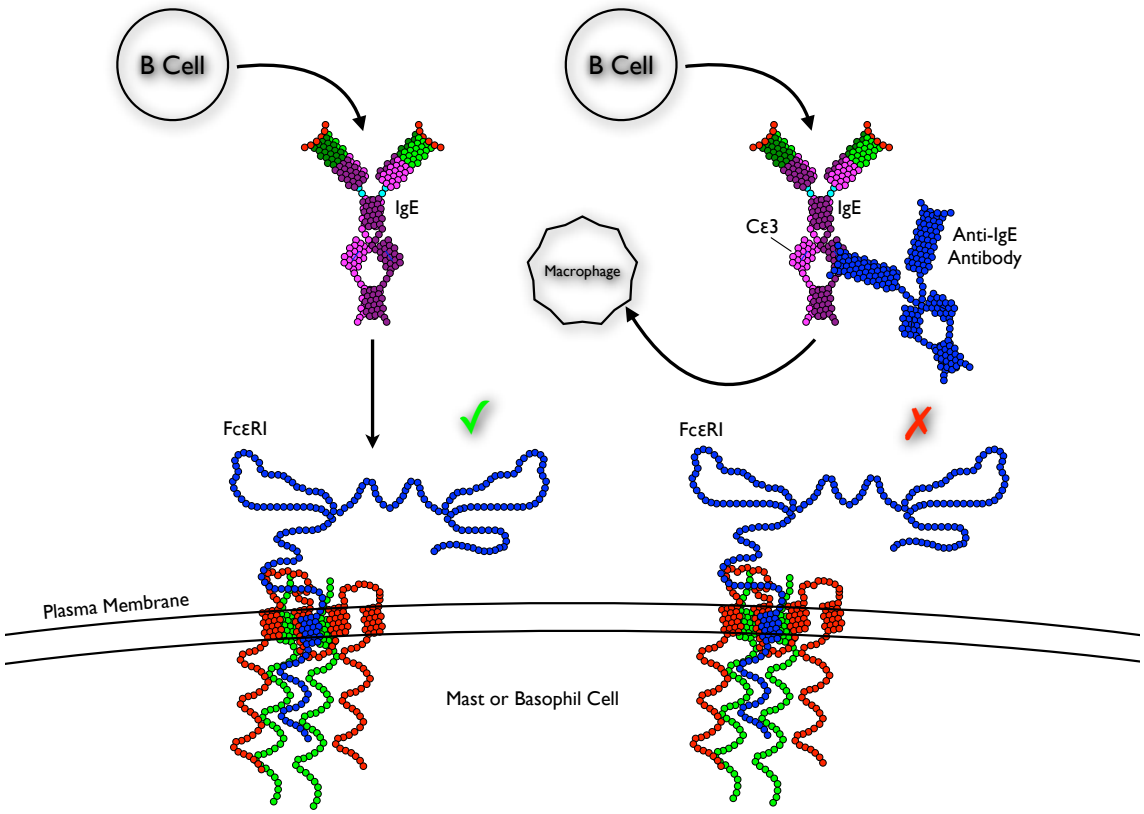


Figure 7: Representation of the novel anti-IgE immunotherapy:
A monoclonal antibody, usually IgG, that binds to the IgE's Cε3 heavy chain domain would prevent it from binding to the FcεRI receptor and thus would be actively removed from serum as it would be digested by macrophage cells.

The three companies developed a better molecule named Omalizumab (trade name Xolair®), this molecule acts in a very similar way to Talizumab but with a better manufacturing process. Omalizumab actively removes non-bound IgE from serum by binding to the HPL loop in the IgE's Cε3 domain (Zheng, *et al.*, 2008), it therefore also contributes to the down regulation of the FcεRI receptor expression on mast and basophil cells (Holgate, *et al.*, 2004; Scheinfeld, 2005). It also reduce the number of inflammation cells, eosinophils, T cell and B cell in tissue (Holgate, *et al.*, 2005). It is administered intravenously or subcutaneously and it results in 98-99% reduction in non-bound serum IgE (Scheinfeld, 2005). The Scheinfeld paper reported that Omalizumab's side effects include viral infection, upper respiratory tract infection, sinusitis, headache and pharyngitis. It also reports malignancy development, but that was observed in the placebo group as well, so it is not yet determined if IgE has a role in cancer development or prevention. Due to the high price of Omalizumab it is prescribed only to patients with moderate to very severe atopic conditions, mainly the sever spectrum of asthma, and for parties who fail to respond to pharmacotherapy (Holgate, *et al.*, 2005) it thus results in a dramatic increase in patients' quality of life. But since IgE synthesis is not inhibited, this treatment has to be repeated every ~3 weeks (Conrad, *et al.*, 2010).

Another drug called mAb12 (Laffer, *et al.*, 2001) used the same concept, but binds to a different epitope from that of Omalizumab's, its added advantage is that it binds and removes IgE already bound to the FcεRI receptor, and therefore reduce the sensitization by IgE of mast and basophil cells (Laffer, *et al.*, 2008) which means that mAb12 has a higher binding affinity to IgE ($>K_A = 10^{10}M$) than IgE has to its FcεRI receptor ($\sim K_A = 10^{10}M$). The down side of these therapeutic strategies is their side effect, which includes the development of serum sickness (Dreyfus and Randolph,

2006) where the body starts to react, with allergy like symptoms, to foreign antibody proteins.

Lumiliximab is a monoclonal chimeric antibody made up of human and *Cynomolgus macaque* that is anti-FcεRII. The FcεRII receptor is found on the surface of B cells and is involved in the regulation and expression of IgE (Rosenwasser and Meng, 2005) by transporting allergens to APC, which would then activate Th_0 Cells. Thus inhibiting this receptor from binding to IgE would result in the down regulation of the IgE synthesis by B cell. This was shown in clinical trials to be effective and reduce the serum IgE level by two-thirds, but this is not sufficient enough to stop the signs and symptoms of allergy. On the other hand, the soluble form of FcεRII have also been demonstrated to lower IgE synthesis as it binds to free IgE antibody in serum (Conrad, *et al.*, 2010).

This concept of synthetic antibodies directed to regulate a biological process *in vivo* branched out and has lead to the development of Trastuzumab by Genentech Inc. which targets the HER2/neu receptor to combat breast cancer development.

All these mAbs (humanized mouse antibodies) are an indication of the use of these antibodies for passive immunization/immunotherapy, i:e they do not give permanent treatment to autoimmune diseases, rather they reduce their symptoms.

1.7 - Equine Allergy:

Horses (*Equus ferus caballus*) were found to develop allergy, this impacts the economy and some high-end industries as discussed later in Chapter 1.9. Horses have also been used as model organisms in physiology, and as a result some advances in medicine were achieved, this is due to their close physiology to humans, at one point in time their serum was used to treat some human diseases (Eyre and Lewis,1973), this

makes horses good model organisms for studying human allergies. In 1887 Stömmer was the first person to compare the asthma symptoms between humans and horses (Cook and Rossdale, 1963), and since then much more physiological similarities were discovered between the two species (Eyre and Lewis,1973). The horse cardiovascular response to inflammation due to histamine release is almost identical to that of humans and other organisms (Eyre and Lewis,1973). Lung physiological measurements were demonstrated to be effective and accurate, which can also be run on conscious horses, with no complications due to sedation, giving more accurate drug pharmacokinetic measurements, therefore they are a good model organism to study the effect of drugs prior to clinical trials (Mirbahar, *et al.*, 1985).

Horses suffer from insect bite hypersensitivity (IBH) (Hellberg, *et al.*, 2006), but horse lung inflammation is usually in the form of recurrent airway obstructions (RAO) which is characterized by noticeable wheezing, repeated coughing and labored breathing usually to mold spores. High levels of IgE in horse serum has shown to be associated with RAO (Künzle, *et al*., 2007). But RAO is not usually suffered by racehorses.

Racehorses usually suffer from inflammatory airway disease (IAD) which can affect horses of any age (Robinson and Hoffman, 2003; Couëtil, *et al.*, 2007). The definition of IAD as proposed in 2002 is discussed in details in the paper by (Robinson and Hoffman, 2003) but some important points are that horses suffering from IAD do not have increased respiration rate at rest, but show poor performance and increased airway mucus after exercise. Also, affected horses do no exhibit signs of illness such as depression. Around 80% of young race horses exhibit IAD at their first year of training, this is because the gene pool of this population is restricted, which result in some

genetic deficiencies such as the tendency of developing allergy, which is a common condition that greatly affects the race horse performance, and thus cause a great deal of money loss to the industry. The IAD illness usually resolves as the horses age, but the condition persists in a minority of them. 70% of race horses that are permanently kept indoors would exhibit IAD. That study showed increased bacterial growth and minimum viral infection in mucus from affected horses, though it still did not conclude the role of allergy in IAD, the paper by (Pirie, *et al.*, 2003) showed that allergens such as mould spores, 3-β-glucan (Riihimäki, *et al.*, 2008), are a major factor in horse IAD. One reason why IAD might have been retained through selective breathing is because it is not very apparent when racehorses develop it, it only becomes clear when the horses have already grown up and started to show signs of reduced performance.

Racehorse IAD due to allergy greatly affects their performance, and since there are very few studies about the molecular basis of equine allergy, this project should give a greater understanding of the allergy kinetics and the condition at the molecular level.

1.8 - Original Antigenic Sin Hypothesis:

This topic is not related to allergy, rather related to vaccine development, which is a concept researched later on in this thesis related to the development of an allergy vaccine.

Thomas Francis first described the hypothesis while conducting research on influenza A virus. There is a schools of thought which stated that the influenza virus' surface antigens change over time by acquiring new antigens from new sources. He noticed, among other researchers, that experimental evidence suggested that all of the virus' antigens are already present, and that different virus variations of the same strain express these antigens at different levels on their surface as to have a dominant antigen

and several recessive antigens. Thomas Francis discusses in his 1960 paper (Francis, 1960) the following observed effect: The first antibodies to be developed in childhood against influenza is that of the strain A. Of course throughout the life time the person would develop antibodies against other influenza strains, but as the population ages, the initial antibody that was developed against influenza A in childhood continues to dominate the antibody in serum during an infection by the same strain with different dominant antigen configuration. This could be the reason why children as well as the elderly are the most susceptible to the influenza virus in a population; children might succumb early to the symptoms of the disease, while the elderly are trapped to produce ineffective antibodies towards the new configuration of the same virus strain (Lambert, *et al.*, 2005). This he called, in 1955, The Doctrine of Original Antigenic Sin (Francis, 1955). The following is a paragraph from his 1960 paper that summarizes the hypothesis: "*The effect is attributed not merely to continuation of initial antibody levels but to repeated stimulation by persistence of the first dominant antigen as a lesser or secondary component of the later* [influenza] *Type A strains*" (Francis, 1960).

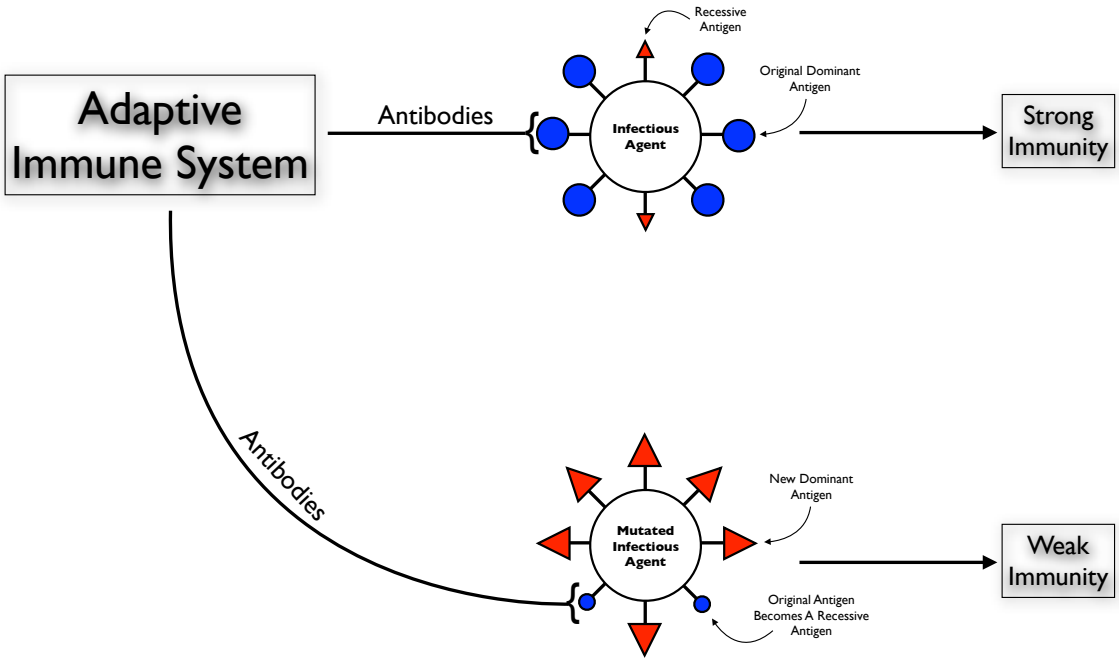


Figure 8: Representation of The Original Antigenic Sin hypothesis:
When the body first encounters an antigen is produces effective antibodies against it's dominant antigens and thus eliminates the infection. But when it encounters the same infection with a dominant new antigen, with the original antigen now being recessive, the immune system will still produce the former antibodies against this old antigen and not develop new antibodies against the new dominant one, this results in the production of ineffective antibodies and thus a weak immunity.

So just to summaries, when the body first encounters an antigen it produces antibodies against it, but when it encounters the same organisms with a new dominant antigen but still has some small traces of the old antigen, due to the pathogen's antigenic drift, the body will produce the former antibodies against this old antigen and not develop new antibodies against the new dominant antigen. This is an attempted short cut where the immune system would react quickly to infections, but, of course, it also works negatively against the immune system where is wastes it energy producing ineffective antibodies (Figure 8). This phenomenon is one of the primary reasons why

vaccines against organisms with highly antigenic instability, such as an anti HIV vaccine and a long term anti influenza vaccine, are not effective using traditional methods. Currently one way to by pass this obstacle is through periodic revaccination (Lambert, *et al.*, 2005).

1.9 - Aims of This Study:

As discussed earlier in chapter 1.7, horse allergy has an effect on some high-end industries. The horse racing industry is worth US$72 billion with a betting turnover of US$115 billion annually (Bruggink, 2009). Since race horses are inbred to select for desirable characteristics such as speed, durability and straight, and since nearly all race horses are descendants of a few arabian horses (*Equus ferus caballus*) which were collected and selectively bread by Lady Anne Blunt in the Crabbet Arabian Stud in Sussex in 1878, they have a great tendency to develop allergy. Therefore this project researched the equine allergy and measured the binding between the equine IgE and its FcεRI receptor in an attempt to establish a baseline, which can be used for further allergy intervention research as a mean of predicting their efficiency in separating the two molecules, this should eventually lead to the development of better equine allergy interventions. The project also went a bit further, it researched a potential allergy vaccine, which involves the host's development of a polyclonal antibody response targeting its own self IgE antibody, the efficiency of the binding between the serum antibodies and the host's self IgE will determine if the binding was greater than the measured value of the binding between the IgE and its FcεRI receptor. The development of a horse allergy vaccine can also pave the way from which the human and canine models can benefit greatly.

Our group was determining the allergy parameters for different model organisms, previous colleagues have established an *in vitro* model system for the human and the

canine allergies, with the human being the most studied. Therefore this project determined the binding kinetics between the equine immunoglobulin E antibody and its high-affinity IgE Fc receptor, as a means of broadening the study of allergy to another model organism and to attempt to reduce the horse racing industry expenditure on expensive treatment for race horses, if a successful treatment was found.

These results were achieved by developing an equine IgE antibody that recognizes 4-Hydroxy-5-iodo-3-nitrophenylacetis acid conjugated with human serum albumin (equine IgE anti NIP-HSA) by synthetically constructing an equine DNA sequenced IgE antibody heavy chain, which was cloned downstream of a mouse λ chain variable region that recognizes NIP antigen in a plasmid called pSV-V_{NP} (Neuberger, *et al.*, 1985). This plasmid was transfected into a mouse B cell line (J558L) and was integrated into the chromosomes of the cells' genome, and thus expressed an equine IgE anti NIP-HSA. Once this was developed, the horse FcεRI receptor's α chain DNA was synthesized and cloned into a plasmid called pEE6 (Stephens and Cockett, 1989) which was transfected into a rat basophil leukemia (RBL-2H3.1) cell line, where it was integrated into the cells' genome and expressed the equine FcεRIα chain receptor, this receptor was combined with the rat endogenous β and γ_2 chains to form a fully functional Fc chimeric receptor capable of binding the equine IgE, and causing a downstream signal in the cells. Form this, the mediator release was investigated on RBL-2H3.1 cells, when they were sensitized with the equine IgE anti NIP-HSA bound to its FcεRI receptor and challenged with NIP-HSA.

Since the cell lines were stable, the mediator release on RBL-2H3.1 cell lines expressing the human, canine and equine receptors was also investigated and the human and canine IgEs were tested on the RBL-2H3.1 cell line expressing the equine receptor,

this was to develop a complete picture of how the equine allergy is related to other model organisms. The mouse IgE was used as a mediator release control.

To measure the kinetic binding between the equine IgE and the equine FcεRI receptor, a soluble form of the FcεRI α chain protein's extracellular domains (sFcεRIαD1&2) were cloned into a yeast expression vector pPIC9k (Invitrogen) where it was cloned into the yeast *Pichia pastoris* and expressed the soluble form of the receptor. The kinetic binding of the two proteins was measured using surface plasmon resonance (SPR). Then the human and canine sFcεRIαD1&2 proteins were also tested with equine IgE, and equine sFcεRIαD1&2 was tested with human and canine IgEs to develop a complete picture of how the allergy is related between model organisms.

Chapter 1.6 mentioned several immunity interventions that target allergy. These treatments are all passive immunizations, where the antibodies give temporary protection from the allergy autoimmune disease. Our novel approach is the development of an active immunization strategy where the patient only needs to take the vaccine once, possibly boosted with it several times, to be protected life long from allergy.

In an attempt to develop an equine allergy vaccine a human-horse-human anti NIP-HSA (HHoH) IgE antibody chimera was developed where the variable region of the antibody was a mouse λ chain that recognizes the NIP antigen, and the heavy chain was a human heavy chain except for the Cε3 domain where it was a horse sequence. The idea was to use this HHoH chimeric antibody as an immune boost, after immunization with a peptide displaying the equine Cε3 domain epitopes, and then test their blood for anti equine Cε3 polyclonal antibodies, which the immune system would hopefully only target, through the original antigenic sin, and not other parts of the IgE chimera. If the affinity of this antibody is greater than that of the IgE/FcεRI complex,

tested earlier in the project, then it can be used as a potential therapeutic treatment where horses can be immunized with this chimeric antibody, which will cause them to develop IgG antibodies against their own self IgE Cε3 domain, this should not only prevents future allergy symptoms, but also knock out the IgE from its FcεRI receptor, which is sensitizing mast and basophil cells, thus permanently treating the organism from any future allergies.

Chapter 2 - Materials and Methods:

2.1 - Materials:

2.1.1 - Equipment:

Equipment	Serial Number	Company
Centrifuge	86319	Sigma
Centrifuge	89/07/346	MSE
Centrifuge	3k15	Sigma Philip Harris
Magnetic Stirrer	B211	Bibby
Balance	PJ3000	Mettler
Microwave	GSS20	Goodmans
Bottle Shaker	Kühner Shaker	Kühner Switzerland
Heating Block	c123813	Grant
Water Bath	8903/2	Nickel Electric
Vortex	WhirliMixer	Fisons
Incubator 37 °C	Compact Incubator	Leec
Incubator 37°C + 5%CO_2 + 90% Relative Humidity	CO_2 Incubator	Galaxy R
Incubator Room	-	LTE scientific
Electrophoresis Tank	-75.710	Continental Lab Product, INC
Class II Laminar Flow Hood	-	BioMAT2
100mm^2 Petri Dishes	-	Sterilin
120mm^2 Petri Dishes	-	Sterilin
24 Well Petri Dishes	-	Sterilin
69 Well Plate Assay Dish	655 180	Cellstar
10ml Pipettes	4101	Corning Incorporated
Gilson Pipettes	Pipetman	Gilson
Multi Tip Gilson Pipette	-	Anachem

Equipment	Serial Number	Company
Universal Tubes 50ml	-	Cellstar
Plate Spectrophotometer	Anthos Htll	Anthos Labtec HT2
Light Microscope	-	Fluovert
Haemocytometer	-	Improved Neubalife
Aspirator	-	Büchi
QIAprep Spin Mini Plasmid Mini Prep Kit	27104	QIAGEN
QIAprep High Speed Plasmid Midi Prep Kit	12643	QIAGEN
Wizard® SV Gel And PCR Clean-Up System	A9281	Promega
30 ml Falcon Tubes Sterile With Label	201172	Greiner Bio-One
Nitrile UltraSense Gloves	US-INT-M	Starlabs
Cryogenic Vile	E3100-0011	Starlabs
50 ml Tubes Sterile	E1450-0200	Starlabs
1.5 ml Test Tubes Sterile	E1415-2231	Starlabs
0.2 ml Test Tubes	I1402-4300	Starlabs
10µl Pipette Tips	S1111-3700	Starlabs
200µl Pipette Tips	S1111-0000	Starlabs
1000 µl Pipette Tips	S1112-1720-c	Starlabs
Duran Bottles 200ml	-	Smiax
Flasks	-	Simax
Parafilm	P7793-1EA	Sigma
Protection Mask	Z300020-1EA	Sigma
96 Well Plates	CLS3595-50EA	Sigma
UV Light Transilluminator	-	UVP
Freeze and Squeeze DNA Gel Extraction Spin Column	732-6166	Bio-Rad

Equipment	Serial Number	Company
Gene Pulser® Cuvette 0.4cm	165-2088	Bio-Rad
Gene Pulser® Cuvette 0.2cm	165-2082	Bio-Rad
Electric Pulse Machine	-	Bio-Rad
0.2 µm Sterilisation Filter	16534K	Sartorius Stedim
0.45 µm Sterilisation Filter	17829K	Sartorius Stedim
Autoclave	-	-
QickChange II Site Directed Mutagenesis Kit	200523	Stratagene
20 ml Syringes	SYR213	Medisave
50 ml Syringes	SYR566	Medisave
Tissues	CMC-717-022U 7308	Fisher Scientific
4°C Fridge	-	-
-20°C Fridge	-	-
-80°C Fridge	-	-
10kDa MW Molecular Filters	UFC901008	Millipore
3kDa MW Molecular Filters	UFC900308	Millipore
BiaCore Tubes	BR-1002-12	GE Healthcare
BiaCore Tube Caps	BR-1002-13	GE Healthcare
Confocal Microscope	-	-
NanoDrop 2000	-	Thermo Scientific
BD FACS Calibur (Flow Cytomerty)	-	BD Biosciences
BD FACS Aria (Cell Sorting)	-	BD Biosciences
Filtered Glass Mesh Funnel	-	Pyrex
3kDa Dialysis Tubings	-	

Equipment	Serial Number	Company
Polypropylene Columns	34964	QIAGEN
SAS-PAGE Mini-protean II Electrophoresis System	-	Bio-Rad
Western Blot Mini Trans-Blot cell	-	Bio-Rad
96 Well ELISA Plates	439454	NUNC

2.1.2 - Standard Chemicals and Kits:

Chemical/ Buffer	Product Number	Company	Contents
Primers	-	Eurofins	-
T4 DNA Ligase	M1804	Promega	-
EcoRI	R6011	Promega	-
HindIII	R6041	Promega	-
BclI	R6651	Promega	-
SphI	R6261	Promega	-
XbaI	R6181	Promega	-
BglII	R6081	Promega	-
NotI	R6431	Promega	-
BamHI	R6021	Promega	-
PstI	R6111	Promega	-
XhoI	R6161	Promega	-
MluI	R6381	Promega	-
Alkaline Phosphatase Enzyme	M1821	Promega	-
REDTaq® ReadyMix™ PCR Reaction Mix	R2523-100RXN	Sigma	-
Tris(hydroxymethyl)aminomethane	154563-1KG	Sigma	-

Chemical/ Buffer	Product Number	Company	Contents
Dulbecco's Modified Eagle's Medium - low glucose	D6046-24X500ML	Sigma	-
Dulbecco's Phosphate Buffered Saline	D1408-24X500ML	Sigma	-
Yeast Extract	LP0021	Oxoid	-
Mycophenolic Acid	M3536-250MG	Sigma	-
HBS-EP Buffer	BR-1001-88	GE Healthcare	-
NaOH Re-Generation Buffer	BR-1003-58	GE Healthcare	-
BiaCore Chips	BR-1000-14	GE Healthcare	-
Agarose	A9539-100G	Sigma	-
Escherichia coli XL1-Blue Supercompetent Cells	200236	Stratagene	-
E.coli JM110 Competent Cells	200239	Stratagene	-
Non-essential Amino Acid Solution	M7145-100ML	Sigma	-
Fetal Bovine Serum	7.01HI	Source Bioscience	-
Sodium Chloride	S7653-1KG	Sigma	-
Escherichia coli XL1-Blue Competent Cells	200249	Stratagene	-
Peptone	P5905-500G	Sigma	-
Yeast Nitrogen Base	51483-100G	Sigma	-

Chemical/ Buffer	Product Number	Company	Contents
Potassium Phosphate Monobasic solution	P8709-1L	Sigma	-
Geneticin G418 Sulphate	11811-031	Invitrogen	-
Sodium Citrate	S1804-500G	Sigma	-
Propanol	18413-0025	FIsher Scientific	-
NIP-CAP-OSu (NIP-ε-Aminocaproyl-OSu)	N-1110-100	Cambridge Research Biochemicals	35mg dissolved in Dimethylformamide
NIP-CAP-OH (NIP-ε-Aminocaproic Acid)	N-1090-1	Cambridge Research Biochemicals	1.5mM in 1xPBS
NIP-HSA	-	-	10mg ml^{-1} Stock Solution
DNP-HSA (Dinitrophenyl Conjugated to Human Serum Albumin)	A6661-100MG	Sigma	15mg ml^{-1} Stock Solution
4-Nitrophenyl N-acetyl-β-D-glucosaminide	N9376-250MG	Sigma	50mM in DMSO 25x Stock Solution
High Grade PCR Master Mix	K0192	Fermentas	-
APC LYNX Rapid Conjugation Kits®	LNK031APC	Serotec	-
CNBr-activated Sepharose™ 4B	17-0430-01	GE Healthcare	-
Rats	-	-	-
Tryptone	LP0043	Oxoid	-
Glycerol	AC15892-0010	Fisher Scientific	-

Chemical/ Buffer	Product Number	Company	Contents
De-Ionized Distill Water	-	Milli-Q	-
Agar (Agarose + Agaropectin)	LP0011	Oxoid	-
Agarose	A9539-250G	Sigma	-
Ethidium Bromide	E7637-1G	Sigma	10mg ml^{-1} Stock Solution
Sodium Acetate	-	AnalaR	
Ethanol	AC39769-0010	Fisher Scientific	
Methanol	A454-1	Fisher Scientific	
Isopropanol	A419-1	Fisher Scientific	
Acetic Acid	SA36-1	Fisher Scientific	
Sulfuric Acid	SA818-1	Fisher Scientific	
Hydrochloric Acid	AC12462-0010	Fisher Scientific	
EDTA	-	AnalaR	
DNA Loading 6x Dye	R1151	Fermentas	
1kbp DNA Ladder	SM1163	Fermentas	
10kDa Protein Loading Dye	SM1811	Fermentas	
Trypsin 10x	59427C-100ML	Sigma	
Triton-X	X100-100ML	Sigma	
Sodium dodecyl sulfate	-	AnalaR	
Citric Acid	C2404-500G	Sigma	
Pipes	P1851-100G	Sigma	
Potassium Chloride	-	AnalaR	
Magnesium Chloride	-	AnalaR	

Chemical/ Buffer	Product Number	Company	Contents
Calcium Chloride	-	AnalaR	
Glucose	-	AnalaR	
Sodium Hydroxide	-	AnalaR	
Hydrochloric Acid	-	AnalaR	
Verkon	-	Verkon	
Liquid Nitrogen	-	-	-
Dimethyl sulfoxide	D2650-100ML	Sigma	
Penicillin/ Streptomycin 100x	P4333-100ML	Sigma	
Erythrosine β	E8886-25G	Sigma	
Non-Enzymatic Cell dissociating Buffer	A7089-100ML	Sigma	
Xanthine	X0626-5G	Sigma	
Hypoxanthine	H9636-5G	Sigma	-
pPIC9k Plasmid	V17520	Invitrogen	-
RBL-2H3.1 (Rat Basophil Leukemia) Cells	-	-	-
J558L (Mouse B Cells)	-	-	-
Protease Inhibitor Cocktail	P8215	Sigma	
Protein Assay Buffer	500-0006	Bio-Rad	
Acrylamide	A3449-100ML	Sigma	

Chemical/ Buffer	Product Number	Company	Contents
Ammonium persulfate	A3678-100G	Sigma	
N,N,N',N'-tetramethyl-ethane-1,2-diamine (TEMED)	161-0801	Bio-Rad	
Coomassie Blue			
β-Mercaptoethanol			
Bromophenol Blue			
Amersham Hybond-LFP Western Blotting Membrane	RPN2020LFP3	Amersham	
Ponceau S stain	P7170-1L	Sigma	
ECL™ Western Blotting Detection Reagents	RPN2109	Amersham	
Light sensitive photographic paper		Kodak	
QuikChange II Site-Directed Mutagenesis Kit	200523	Stratagene	
FuGENE®	11815091001	Roche	
Mouse anti-cat IgE antibody		Serotec	
Mouse anti-dog IgE antibody		Serotec	
TMB (3,3’,5,5’-tetramethylbenzine)	T0440-100ML	Sigma	
Rabbit IgG Anti Rat IgE + Horseradish Peroxidase	A5795	Sigma	

Chemical/ Buffer	Product Number	Company	Contents
Gelatin	G9391-100G	Sigma	

2.1.3 - In-House Prepared Chemicals:

Solution/Buffer	Contents In $1dm^3$
Lysogeny Broth (LB) Media	10g tryptone 10g sodium chloride 5g yeast extract For plates add 15g agar The selective antibiotic ampicillin was added to LB to a final concentration of 50µg ml-1 ampicillin in LB (Sambrook and Russell. 2001)
Ampicillin	Stock solution prepared in H_2O 50µg ml^{-1} in LB
DNA Electrophoresis Agarose Gel	1% Agarose (0.5g agarose in 50ml TAE buffer)
TAE Buffer (Tris Acitic Acid EDTA Buffer) pH 8	4.84g Tris 1.12 ml Acitic acid 0.74g EDTA
70% Ethanol	70ml + 30ml H_2O
3M Sodium Acetate pH 5.2	2.4g sodium acetate in 100ml H_2O
Sodium Acetate pH 4 for BiaCore™ 2000 Ligand Conjugation	10mM sodium acetate pH 4
Tissue Culture Normal Media	Dulbeco's Modified Eagle's media 500ml: 1000mg glucose + 10% FCS + 50000units of penicillin + 50mg streptomycin
Tissue Culture RBL-2H3.1 Cell Selective Media	Dulbeco's Modified Eagle's media 500ml: 1000mg glucose + 10% FCS + 50000units of penicillin + 50mg streptomycin + 0.4g geneticin G418 sulphate
Tissue Culture J558L Cell Selective Media	Dulbeco's Modified Eagle's media 500ml: 1000mg glucose + 10% FCS + 50000units of penicillin + 50mg streptomycin + 0.5mg mycophenolic acid + 25mg xanthine + 7.5mg hypoxanthine
1x Trypsin Solution	1x trypsin protease added to PBS

Solution/Buffer	Contents In $1dm^3$
Phosphate Buffer Saline (PBS)	8g Sodium chloride 0.20g Potassium chloride 1.44g Sodium hydrogen phosphate 0.24g Potassium hydrogen phosphate pH 7.4
Washing Buffer	PBS + 1% FCS
YPD (Yeast extract Peptone Dextrose) Yeast Media	10g Yeast extract 20g Peptone Autoclave then add: 20g Dextrose (Glucose) For solid media add 20g Agar
RDB (Regeneration Dextrose Base) Yeast Media	186g Sorbitol For solid media add 20g Agar Autoclave then add: 20g Glucose 13.4g Yeast nitrogen base 2ml of 0.02% Biotin 10ml of Non-essential Amino Acid Solution
BMGY (Buffered Glycerol-complex Media) Yeast Media	10g Yeast extract 20g Peptone Autoclave then add: 13.4g Yeast nitrogen base 100ml 1M Potassium phosphate Monobasic solution pH 6 10ml Glycerol
BMMY (Buffered Methanol-complex Media) Yeast Media	10g Yeast extract 20g Peptone Autoclave then add: 13.4g Yeast nitrogen base 100ml 1M Potassium phosphate Monobasic solution pH 6 5ml Methanol to give 0.5% final concentration
Coupling Buffer	0.1M Sodium hydrogen carbonate 0.5M Sodium chloride pH 8.3
Regeneration Buffer	0.1M Tris 0.5M Sodium chloride pH 8

Solution/Buffer	Contents In $1dm^3$
Blocking Buffer	0.1M Acetic acid 0.1M Sodium acetate 0.5M Sodium chloride pH 4
Denaturing Buffer	0.1M Tris-HCl pH 8
1mM HCl	From 11M HCl add 91µl to $1dm^3$ H_2O
Elution Buffer	0.2M Glycine pH 2.8
Neutralizing Buffer	1M Tris-HCl pH 8
Chromatography column equilibration solution	300µl Tween 20 in 50ml H_2O (0.6% tween 20)
12% Acrylamide Resolving Gel	4.9 ml H_2O 3.8ml 1.5M Tris pH 8.8 + 0.4% sodium dodecyl sulfate 6ml 30% Acrylamide 150µl Ammonium persulfate 6µl TEMED
5% Stacking Gel	2.7 ml H_2O 500µl 1M tris pH 6.8 40µl 10% Sodium dodecyl sulfate 670µl 30% acrylamide 40µl ammonium persulfate 4µl TEMED
Electrophoresis Running Buffer	14.4g Glycine 3.07g Tris 10ml 10% Sodium dodecyl sulfate
Western Blot Running Buffer	14.4g Glycine 3.07g Tris 200ml Methanol
Non-reducing Protein Loading Dye	60mM Tris-HCl pH 6.8 20% Glycerol 2% Sodium dodecyl sulfate 0.0025% Bromophenol Blue

Solution/Buffer	Contents In $1dm^3$
Reducing Protein Loading Dye	60mM Tris-HCl pH 6.8 20% Glycerol 2% Sodium dodecyl sulfate 0.0025% Bromophenol Blue 5% β-Mercaptoethanol
Coomassie Stain	2g Coomassie Blue 250ml Acetic Acid 1000ml H_2O 1000ml Methanol
Coomassie De-stain	10% Acetic Acid 30% Methanol
Western Blot Blocking Solution	5g Full Cream Milk 50µl Tween 20 100 1xPSB
Western Blot Washing Solution	5g Full Cream Milk 50µl Tween 20
Tris Buffer	1M Tris pH 9
Citrate Buffer	0.2M Citric Acid 0.2M Sodium Acetate pH 4.5
Release Buffer	25mM Pipes 120mM Sodium Chloride 5mM Potassium Chloride 0.04mM Magnesium Chloride 1mM Calcium Chloride pH 7
Triton-X Buffer	5% Triton-X 100
ELISA Immobilization Buffer	15mM Na_2CO_3 35mM $NaHCO_3$ pH 9.6
ELISA Wash Buffer	1x PBS + 0.05% Tween 20
ELISA Blocking Buffer	100ml of (1x PBS + 0.05% Tween 20) + 200mg Gelatin
ELISA Stop Solution	0.2M H_2SO_4

2.2 - Molecular Biology Methods:

2.2.1 - Gene Optimization:

Most amino acids have several codons that code for them, and organisms tend to favor some codons over others, therefore certain codons for amino acids would have higher frequency in some organism's genomes than others, this is termed Codon Usage Bias, and till today has no explanation for it occurrence. Codon usage bias is used here to ensure that the protein of interest is expressed optimally in the host organisms' cells.

The codon frequency tables for the rat (*Rattus norvegicus*), the yeast (*Pichia pastoris*) and the mouse (*Mus musculus*) were obtained from the site: http://www.kazusa.or.jp/codon (See Appendix). The equine FcεRIα receptor (McAleese, *et al.*, 2000) and the IgE antibody heavy chain (Hε) (Navarro, *et al.*, 1995) genes were optimized using the online program http://genomes.urv.cat/OPTIMIZER/. Then the optimized sequences were analyzed in http://gcua.schoedl.de to confirm the correct optimization. The equine FcεRIα gene was optimized for both the rat and yeast codons as the same gene will be used to express the protein in rat cells and in yeast cells, while the Hε was optimized for mouse only and had half a human intron added to the start. The gene sequences were optimized because each organism favors a certain codon for its amino acids, and thus this should increase the tendency of taking up gene by the relevant cells and optimize its protein expression.

2.2.2 - Lysogeny Broth (LB) Media:

LB media was prepared using the following formula: 10g tryptone, 10g sodium chloride, 5g yeast extract and 15g agar for a $1dm^3$ solution. The selective antibiotic ampicillin was added to the LB media to a final concentration of $50\mu g\ ml^{-1}$ ampicillin in LB (Sambrook and Russell, 2001).

2.2.3 - Bacterial Inoculation and Growth:

The bacterial strains used for cloning were *Escherichia coli* XL-1 Blue (for general cloning) and JM110 (for cloning DNA that will later be digested using BclI restriction enzyme, because normal *E.coli* bacteria methylates the BclI TGATCA sequence using the Dam gene, therefore this strain is deficient in it) and TOP 10 for the delivery of synthesized genes from (GenScript). Bacteria were inoculated, using a steel metal loop, into 10 ml LB Media + 50μg ml^{-1} ampicillin and shaken at 37°C for 16 hours to harvest cells, or spread onto a solid LB Media + 50μg ml^{-1} ampicillin plate to isolate one single bacterial colony (Sambrook and Russell, 2001).

2.2.4 - Vectors:

The first of the three vectors used in this experiment was pEE6 (Stephens and Cockett, 1989), this plasmid was sold by CellTech (a defunct UK company since 2004). It integrates into a mammalian cell's chromosome due to sequences from the human herpesvirus 5 and therefore is designed for mammalian protein expression. It contains the ampicillinase gene and the neo gene that codes for aminoglycoside 3'-phosphotransferase which gives resistance to geneticin G418 sulphate (an antibiotic) that inhibits polypeptide synthesis by blocking the elongation step, it works in both prokaryotic organisms and eukaryotic cells. Using the plasmid in bacteria allows for their selection using ampicillin, while using the same plasmid in mammalian cells allows for their selection using geneticin G418 sulphate. The plasmid used already had an insert in it from a previous colleague therefore it was pEE6+CD23α plasmid and the insert was the human low-affinity α chain gene, this insert was removed and replaced with the horse FcεRIα gene. This was helpful as the success of the restriction digest could be quickly determined on a gel.

The second vector used was pSV-V_{NP} plasmid (Neuberger *et al*, 1985). This plasmid is a modified simian immunodeficiency virus (SIV) which infects leukocytes and integrates its genome into their chromosomes, therefore this plasmid is useful for cloning and expressing proteins in leukocytes. The original plasmid name was pSV2gpt because the plasmid contains the ampicillinase gene and the gpt gene that encodes for bacterial xanthine guanine phosphoribosyl transferase (XGPRT), therefore gives resistance to mycophenolic acid. Mycophenolic acid is an immunosuppressant drug that inhibits that *de novo* synthesis of guanine monophosphate (GMP), a purine nucleotides, from xanthosine monophosphate (XMP) by blocking inosine monophosphate dehydrogenase from converting inosine monophosphate (IMP) to XMP. Therefore using this plasmid can select bacteria using ampicillin and mammalian leukocyte cells using mycophenolic acid. The tissue culture selective media should be supplemented with xanthine and hypoxanthine as well because XGPRT, from the gpt gene, allows for the synthesis of GMP by converting IMP to XMP, by passing the inhibited inosine monophosphate dehydrogenase. Therefore XMP continues to be converted to GMP in resistant cells (Figure 9). The hypoxanthine is added to saturate as much of the Hypoxanthine-guanine phosphoribosyltransferase as possible to synthesis IMP which cannot be used by non-transfected cells, and not convert guanine to GMP which can be done by both transfected and non-transfected cells. This selection is therefore weak, but works.

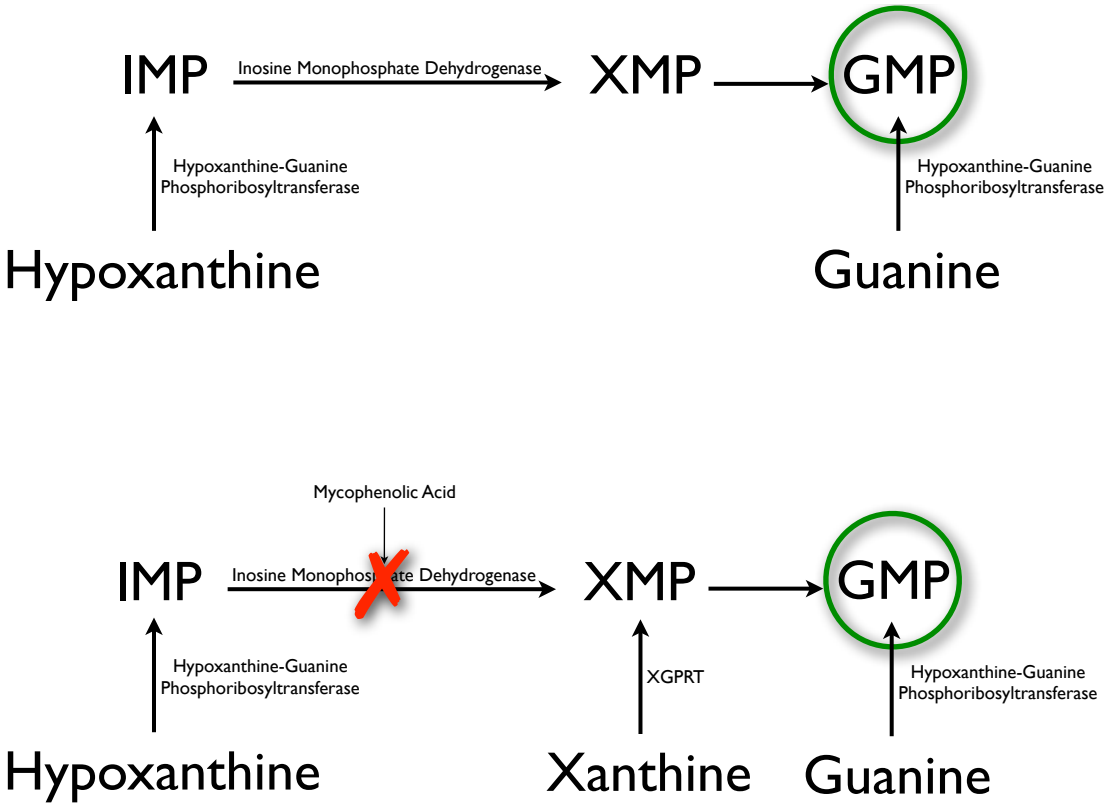


Figure 9: Mycophenolic acid selection:
IMP is converted to XMP by inosine monophosphate dehydrogenase, which in turn is converted to GMP. Mycophenolic acid inhibits inosine monophosphate dehydrogenase and thus cells cannot synthesis XMP from IMP and the GMP in the cell diminishes. The gpt gene codes for XGPRT which converts xanthine to XMP and therefore by passes the inhibited inosine monophosphate dehydrogenase.

The plasmid also called pSV-V_{NP} because it also contains a mouse λ chain variable region that codes for an antibody variable region that binds to the hapten 4-hydroxy-3-nitrophenylacetic acid (NP). Therefore cloning an antibody heavy chain downstream of this sequence results in an antibody that is anti NP. 4-hydroxy-5-iodo-3-nitrophenylacetic acid (NIP) can also be used due to its extreme similarity to NP (Brownstone, *et al.*, 1966a; Brownstone, *et al.*, 1966b) (Figure 10).

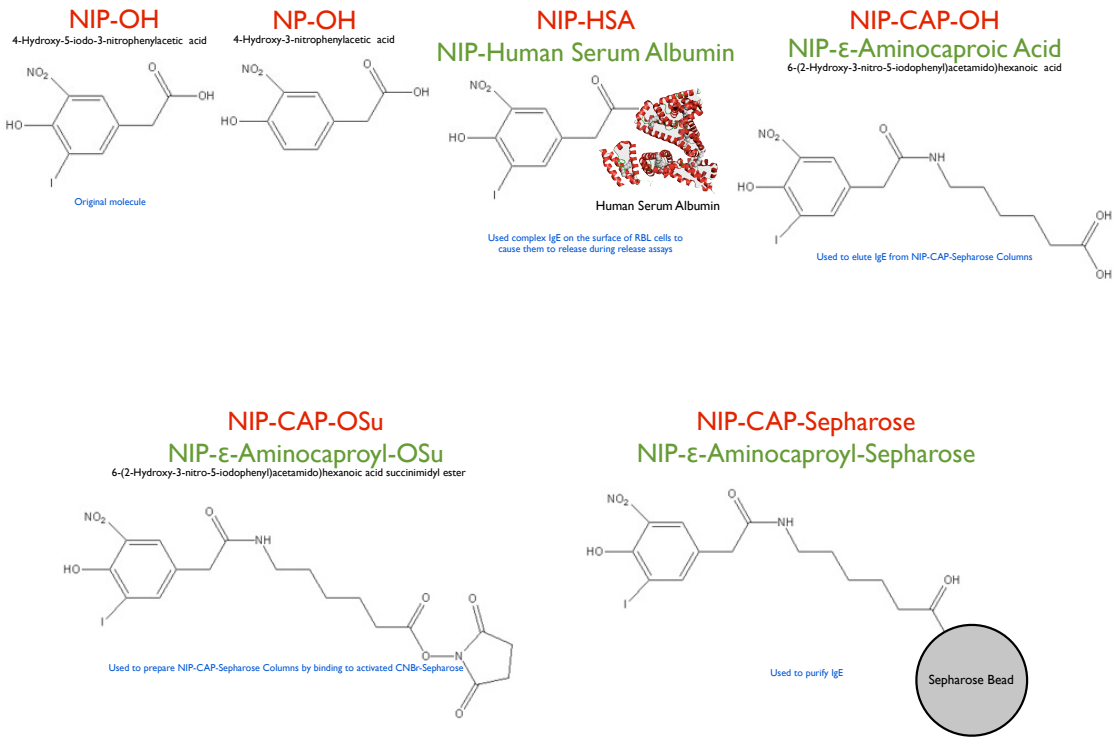


Figure 10: Molecule structure of NP and NIP:
The structure of NP and NIP haptens are very similar therefore both can bind to the antibodies synthesized using the pSV-V_{NP} plasmid. The figure also shows all the NIP structures used in this project.

The pSV-V_{NP}HεF424 plasmid already contained a mutated human IgE heavy chain, the insert was replaced with the horse IgE heavy chain Hε, as previous, which made it easy to determine the efficiency of the restriction enzymes quickly on a gel.

The third vector used was the pPIC9k (Invitrogen). Since this plasmid was purchased from a company and not prepared by another research group, a lot was known about the plasmid's characteristics, also it did not contain an insert. This plasmid was designed and optimized for *Pichia pastoris* protein expression. It contained the ampicillinase gene to allow for bacterial selection and the neo gene to give resistance to geneticin G418 sulphate to allow for yeast selection.

2.2.5 - Plasmid Mini Prep:

After 16 hours of *Escherichia coli* bacteria culture incubation at 37°C in suspension, the cells were mini prepped to extract their plasmid using the QIAprep Mini Plasmid Mini Prep Kit supplied by QIAGEN.

The following is the QIAGEN protocol: 3ml of 16 hours culture incubation at 37°C was centrifuged at 2000xg for 10 minutes, the supernatant was discarded and the pellet re-suspended in 250µl P1 buffer chilled at 4°C. Then 250µl P2 lysis buffer was added followed by 350µl N3 precipitation buffer. The sample was then centrifuges at 10000xg for 10 minuets, the supernatant was transferred to the QIA spin column and centrifuged at 10000xg for 1 minute, and the overflow was discarded. 500µl PB binding buffer was added to the spin column and centrifuged at 10000xg for 1 minute, and the overflow was discarded. 750µl PE wash buffer was added and centrifuged at 10000xg for 1 minute, and the overflow was discarded. The column was then centrifuged dry at 10000xg for 1 minute to remove excess liquid and evaporate the excess ethanol. The bottom tube of the spin column was replaced by a 1.5ml Eppendorf tube, 50µl de-ionized distill water was added directly to the filter of the spin column and centrifuged at 10000xg for 1 minute, the over flow contained the required plasmid at concentration of ~200ng ul^{-1} (QIAgen Mini prep Protocol).

2.2.6 - Polymerase Chain Reaction:

The PCR kit was bought from (Fermentas). 1µl of forward primer and 1µl of reverse primer (both at a concentration of ~100ng ul^{-1}) were used along with to 1µl of mini prep plasmid DNA, the following was the PCR master mix prepared to a final concentration of 50µl:

10X High Fidelity PCR buffer with 15 mM $MgCl_2$	5µl
dNTP Mix, 2 mM each	5µl
Forward primer	1µl
Reverse primer	1µl
Mini prep plasmid DNA	1µl
High Fidelity PCR Enzyme Mix	1µl
H_2O	36µl

The following program was used:

1	94°C	2:30 minutes	Complete DNA melting
2	94°C	30 seconds	DNA melting
3	44°C	15 seconds	Annealing
4	68°C	30 seconds	Polymerization

Program repeated from step 2-4 for 30 cycles. Then the samples incubated at 4°C until removed. The PCR product was purified for cloning purposes using the same protocol from the same kit as the gel extraction of DNA band in chapter 2.2.9.

2.2.7 - Restriction Digest and De-Phosphorylation:

The restriction digests used 8µl of plasmid mini prep with 1µl restriction enzymes, 9µl de-ionized distill water, and 2µl 10x enzyme specific buffer. The samples were incubated in a water bath at 37°C for 1 hour. Vectors were de-Phosphorylated by adding 17µl de-ionized distill water, 1µl alkaline phosphotase enzyme and 2µl alkaline phosphotase buffer that was added directly to the digestion mix and incubated at 37°C

for 1 hour. 4µl of loading dye 6x was added to each sample with a volume of 20 µl before electrophoresis.

2.2.8 - Electrophoresis:

0.5g agarose was added to 50ml TAE 1x buffer to make a 1% gel with 3.5µl ethidium bromide 10mg ml^{-1} stock solution (Sambrook and Russell, 2001). The DNA ladder was prepared as follows: 18µl deionized distill water, 2µl DNA ladder and 4µl loading dye 6x to make a 24µl sample, 4µl of loading dye 6x was added to each DNA sample with a volume of 20 µl before electrophoresis. All gels were run at 100v 80A for 1 hour.

2.2.9 - Gel Extraction of DNA Bands:

The required bands were removed from the agarose gel. 1µl of binding solution was added to every 1mg of gel, heated at 55°C until all the gel has melted. The sample was transferred into the SV Minicolumn and incubated for 1 minute at room temperature. The sample was then centrifuged at 10000xg for 1 minute, overflow was discarded. Column was washed with 700µl Membrane Wash Solution centrifuged at 10000xg for 1 minute then washed again with 500µl and centrifuged at 10000xg for 1 minute, centrifuged dry at 10000xg for 5 minutes. Then samples was transferred to a new Eppendorf tube and the DNA was eluted with 50µl de-ionized distill water (Promega Wizard® SV Gel and PCR Clean-Up System Protocol).

2.2.10 - Ligation:

3µl of the vector and 12µl of the inserts were ligated together using 1µl T4 DNA Ligase enzyme, 11µl de-ionized distill water and 3µl enzyme buffer were all mixed together, to a final 30µl sample volume, and incubated at room temperature for 3 hours.

2.2.11 - Bacterial Transformation:

The ligated samples were transformed into competent *Escherichia coli* XL-1 Blue (or JM110) bacteria using the following protocol: The *Escherichia coli* XL-1 Blue (or JM110) competent bacteria (Stratagen) were thawed on ice for 30 minutes. 50µl of competent bacteria were added into each tube along with 10µl of ligated sample. Mixed very gently and incubated in ice for 30 minutes.The bacteria where then heat shocked at 42°C using a water bath for 45 seconds then placed immediately on ice for 2 minutes. 1ml liquid LB media with no ampicillin was added to the cells and the samples were then placed in a water bath for 45 minutes at 37°C. The samples were then centrifuged gently at 180xg for 10 minutes to collect all the bacteria and the pellet re-suspended in 200µl of the same media. The concentrated bacteria were then spread on solid LB media + 50µg ml^{-1} ampicillin and incubated for 16 hours at 37°C.

2.2.12 - Glycerol Stock:

Cryogenically preserved bacteria was prepared with 20% sterile glycerol and 80% overnight grown bacteria. Vortexed and quickly frozen in -80°C for long term cryopreservation.

2.2.13 - Plasmid Midi Prep:

Using the QIAprep Mini Plasmid Midi Prep Kit supplied by QIAGEN the required colony was inoculated to 50ml of liquid LB media + 50µg ampicillin and incubated at 37°C for 16 hours. The 50ml sample was then centrifuged at 2000xg for 15 min and the supernatant was discarded. The pellet was re-suspended in 6ml chilled P1 suspension buffer and vortexed. 6ml P2 lysis buffer was added and the sample was incubated at room temperature for 5 minutes. 6ml chilled P3 precipitation buffer was added mixed gently then immediately added to QIA Filter Cartridge and incubated at room temperature for 10 minutes. 4ml QTB buffer was added to the High Speed Midi

Tip to equilibrate the filter. After the 10 minute incubation the sample was filtered though the QIA Filter Cartridge and into the High Speed Midi Tip. 20ml QC buffer was added to wash the DNA in the filter. Then the DNA was eluted by adding 5ml Buffer QF into a new tube which was then precipitated by the addition of 3.5ml 100% isopropanol and incubated at room temperature for 5 minutes. The sample was added to QIA Precipitation Filter using a 20ml syringe. The DNA in the filter was then washed with 2ml 70% ethanol, then twice with air. The filter was transferred to a 1.5ml syringe where 1ml deionized distill water was added and pushed through the filter to elute the DNA into an Eppendorf tube. The eluted DNA was again filtered through the same filter to elute more DNA. The final concentration of DNA was ~200μg μl^{-1} (QIAGEN Midi Prep Protocol).

2.2.14 - Quantification of DNA:

DNA was quantified using the NanoDrop 2000 machine where 1μl of DNA sample was added to the machine and measure its absorbance at 260nm. The following formula was used to measure the concentration of DNA:

Double Stranded DNA: OD_{260} of 1 = 50 μg ml^{-1}

Single Stranded DNA: OD_{260} of 1 = 30 μg ml^{-1}

2.2.15 - Ethanol Precipitation:

This method was used to concentrate DNA. For every 1μl of DNA sample, 2μl 70% ethanol and 0.1μl 3M sodium acetate pH 5.2. were added and incubate at room temperature for 1 hour. Centrifuged at 10000xg for 20 minutes, washed with 70% ethanol and centrifuged again at 10000xg for 5 minutes, this step was repeated twice. The pellet was air dried until all ethanol evaporated. The DNA was then re-suspend in the desired volume using deionized distill water.

2.2.16 - DNA Sequencing:

Plasmid DNA was sequenced by diluting the sample down to a final concentration of 100ng μl^{-1} using deionized distill water then sent to Source BioScience Geneservice for sequencing using M13 forward and reverse universal primers, after confirming that these primer sequences are present in the plasmid between the desired gene:

M13 forward: 5'-TGTAAAACGACGGCCAGT-'3

M13 reverse: 5'- CAGGAAACAGCTATGACC -'3

2.2.17 - Point Nucleotide Mutation Generation:

This protocol was from the QuikChange II Site-Directed Mutagenesis Kit (Stratagene): A forward and reverse primer sequence that included the mutated sequence was synthesized. A master mix containing:

Mini prep plasmid DNA	2µl
Reaction buffer	5µl
Forward primer	1.25µl
Reverse primer	1.25µl
dNTP mix	1µl
38.5ddH_2O	38.5µl
PfuUltra HF DNA polymerase	1µl

This gave a final volume of 50µl. The reaction was then run like a PCR reaction with 18 cycles with the following program was:

1	95°C	30 seconds	Complete DNA melting

2	95°C	30 seconds	DNA melting
3	55°C	60 seconds	Annealing
4	68°C	60 seconds	Polymerization

The final product was an un-methylated plasmid containing the mutated sequence and the original methylated plasmid with the un-mutated sequence. Therefore to the sample 1µl of Dpn I restriction enzyme was added and incubated at 37°C for 1 hours. The sample digested all the original methylated plasmid but no the new un-methylated plasmid. Therefore after the digestion the sample was transformed into supercompetent cells, using exactly the same protocol as in chapter 2.2.11.

2.3 - Mammalian Cell Tissue Culture Methods:

2.3.1 - Cell Lines:

RBL-2H3.1 (Rat Basophil Leukemia cells) (Bingham, *et al.*, 1994) are cancerous basophil cells that are well suited for the study of the FcεRI receptor because basophil cell are physiologically very similar to mast cells but are a much more stable cell line in tissue culture. This cell line was therefore used to express the equine FcεRIα chain where it was expressed onto the cell's surface and combined with the rat endogenous β and two γ chains to form a fully functional chimeric receptor capable of binding the equine IgE. An important note to mention is that maintaining this cell line in culture for prolonged periods tends to cause the cells to shift to a non-mediator releasing phenotype which cannot be reversed, therefore it was advised to keep large stocks of non-transfected and cloned functional cells. *Mycoplasma* bacterial infection greatly affects the cell release as well, therefore regular *Mycoplasma* tests were preformed since the bacteria colonized the cell surface and could not be visualized through the microscope.

Mycoplasma tests were usually preformed through PCR, and removing this bacteria from important cell lines were possible but expensive and time consuming.

Since the cells are expressing a protein on their cell surface, isolation of expressing cells was done through Fluorescence-Activated Cell Sorting (FACS), see chapter 2.3.6 which resulted in colonies rich in expressing cells, and were tested again using Flow Cytometry. Inoculating $5x10^5$ cells + 15ml of media in a $100mm^2$ petri dish resulted in plate confluency after ~4 days and must be incubated at 37°C + 5% CO2 + 90% relative humidity. Healthy RBL-2H3.1 cells have a crescent shape and they form a monolayer, therefore harvesting cells requires trypsin digestion to suspend the cells.

J558L cells (mouse B myeloma cells derived from BALB/c strain isolated by M. Bruggeman and M.S. Neuberger) is a stable B cell line used to express antibodies. Selection of this cell line is done through mycophenolic acid selection and therefore the final cell lines are always a mixture of transfected expressing, transfected non-expressing and non-transfected cells, and therefore careful selection of high expressing colonies is essential. Inoculating $5x10^5$ cells + 15ml of media in a $100mm^2$ petri dish will result in plate confluency after ~4 days and must be incubated at 37°C + 5% CO2 + 90% relative humidity. Healthy J558L cells have a round spherical shape and they do not form a monolayer, therefore harvesting cells does not requires trypsin digestion to suspend the cells, simple pipetting will suspend the cells from the bottom of the dish.

2.3.2 - Cryopreservation of Mammalian Cells:

Cryogenically frozen mammalian cell samples were prepared as follows: A cell density of $5x10^6$ ml^{-1} suspended in pure fetal calf serum (FCS). To each tube 900µl of cell and 100µl dimethyl sulfoxide (DMSO) was added. The tubes were gently inverted

and then quickly placed in liquid nitrogen vapor for 2 hours. The samples were then placed in liquid nitrogen for long term cryopreservation.

2.3.3 - Thawing Cryopreserved Mammalian Cells:

Cryopreserved mammalian cells were taken out of the liquid nitrogen and thawed by gently adding tissue culture normal media (Dulbeco's Modified Eagle's media 500ml: 1000mg glucose + 10% FCS + 50000units of penicillin + 50mg streptomycin) using a pipette and transferring the cells into 20ml of normal media. The cells were then centrifuged at 180xg for 3 minutes at 4°C and the media aspirated, this was to remove all traces of DMSO (which is toxic to cells when in solution). The cells were then resuspended on 15ml of normal media and then plated on a $100mm^2$ petri dishes and incubated at 37°C + 5% CO_2 + 90% relative humidity. After 24 hours, it was essential to replace the media with fresh media to remove all dead cells (caused by the cryopreservation), as dead cell debris cause healthy to undergo apoptosis. Careful removal of media was preformed on J558L mouse B cells as they are suspension cells and do not stick to the plastic petri dish surface.

2.3.4 - Maintenance of Mammalian Cell Lines:

Non-transfected mammalian cells were grown in normal tissue culture media (Dulbeco's Modified Eagle's media 500ml: 1000mg glucose + 10% FCS + 50000units of penicillin + 50mg streptomycin). Inoculation of $5x10^5$ cells in 15ml of normal media in $100mm^2$ petri dishes was sufficient to give 50%-75% confluency in ~4 days, at which point the cell population had to be split in order to maintain the cell growth at the log phase, which is when they are most healthy. Chapter 2.3.1 for the description of the two cell lines used here.

In a 75%-90% confluent mammalian cell dish the media was aspirated, the cells were washed with 5ml of 1xPBS then 2ml of trypsin solution was added to the cells and incubated for 5 minutes at 37°C + 5% CO_2 + 90% relative humidity. Cells were then added to 20ml media and centrifuge at 180xg for 3 minutes at 4°C. The cells were washed with 10 ml 1xPBS and centrifuge at 180xg for 3 minutes at 4°C (since J558L cells are suspension cells, no trypsin was needed, simply pipetting the old media several times will cause the cells to get suspended and thus transferred, and therefore no wash step was needed simply 15µl of cells were taken to be counted on the haemocytometer). The washed cells were resuspended in 10ml of normal media and 15µl of cells were taken to be counted on a haemocytometer. Using the calculation at the bottom media was added to the cells to reach a final cell density of $5x10^5ml^{-1}$. 1ml ($5x10^5$) of cells was added 15ml of media and plated into a new petri dish and incubated for another 4 days.

$$\left(\frac{\text{Cell Count 1 + Cell Count 2}}{2}\right) \times 10\,000 = \text{Cell Density in 10ml}$$

$$\frac{\text{Cell Density in 10ml}}{5x10^5} = \text{New Volume}$$

2.3.5 - Transfection of Mammalian Cells:

To a mammalian cell density of $1x10^7$ in $0.8ml^{-1}$ 0.8ml were placed in an test tube and 25µg $50µl^{-1}$ of plasmid DNA was added to them and gently mixed. The cells were transferred to a 0.4cm electrocuvette (Bio-rad) and incubated on ice for 10 minutes. They were then electric pulsed at 250v 960µF using the electric pulse machine (Bio-Rad) and quickly incubated on ice for another 10 minutes. The RBL-2H3.1 cells were grown on four $100mm^2$ petri dishes with normal media for two days followed by changing to selective media (Dulbeco's Modified Eagle's media 500ml: 1000mg

glucose + 10% FCS + 50000units of penicillin + 50mg streptomycin + 0.4g geneticin G418 sulphate) and selecting for proximally 1-2 weeks, changing the media every 2 days, until the cells stopped dying and started to grow, at this point the cells were harvested and sorted using FACS (chapter 2.3.6).

The J558L cells were grown on two 24 well petri dishes with 800µl of normal media for the first two days, then replaced with 800µl selective media (Dulbeco's Modified Eagle's media 500ml: 1000mg glucose + 10% FCS + 50000units of penicillin + 50mg streptomycin + 0.5mg mycophenolic acid + 25mg xanthine + 7.5mg hypoxanthine). The cells where maintained with this selective media, changing it every 4 days with gentle aspiration as to not remove the suspended cells. The cell population decreased very gently and the cell morphology will look very unhealthy (not perfectly spherical cells). After 4-6 weeks the wells started to develop small colonies of healthy resistant cells, once the wells were confluent, the media of each well (totaling 48 wells) was harvested, centrifuged to remove the cells, then tested using SPR for the presence and quantity of IgE expressed (chapter 2.3.7), the well with the highest IgE presence was isolated, grown and frozen to develop the cell line.

In both mammalian cell lines, a control was setup by adding water without DNA to the 0.4cm electrocuvette and selected as discussed. For RBL-2H3.1 cells the control will show no fluorescence during FACS, while J558L cells will show no IgE presence in the SPR test. Though both cell lines most likely, but not always, behave like transfected cells; i.e: will look healthy and be resistant in selective media.

2.3.6 - Sorting Transfected RBL-2H3.1 Cells (Flow Cytometry and Fluorescence-Activated Cell Sorting - FACS):

The RBL-2H3.1 cells were sorted by Fluorescence-Activated Cell Sorting (FACS) to separate non-transfected and non-expressive cells from transfected expressive ones.

Cells were harvested and re-suspended in 1ml cold 4°C washing buffer (PBS + 1% FCS).

1µg of primary IgE antibody (that binds to the novel expressed FcεRIα chain) was added to 1ml of cells and incubated on ice for 30 minutes, then washed twice (by centrifuging at 180xg for 3 minutes and re-suspending in 10ml wash buffer). The cells were then re-suspended in 1ml wash buffer and 1µg secondary antibody (IgG anti primary IgE that was tagged with a florescent dye) was added to the cells and incubated for 30 minutes on ice in the dark to minimize dye bleaching, then washed twice. The cells were assayed and sorted using a flow cytometry machine. A FACS control was setup by adding water instead of primary antibody, and this control was essential to establish the baseline at which the cells were sorted. Another control, where non-transfected cells were tagged with both primary and secondary antibodies, was run to determine the success of the transfection. Chapter 2.4.2 discusses the concept of FACS.

2.3.7 - Selection of Transfected J558L Cells:

The J558L cells were selected as in chapter 2.3.5 and after ~4 - 6 weeks developed healthy cells that dominated the wells. At this point all the cell media in the wells (48 wells) were harvested (800µl) into 48 1ml test tubes, taking care not to remove cells as usual. The test tubes were centrifuged gently at 180xg for 4 minutes to remove the cells and cell debris form the media. Into 48 BiaCore™ 2000 test tubes 180µl HBS-EP buffer (0.01M HEPES + 0.15M NaCl + 3mM EDTA + 0.005% Surfactant P20 (Tween P20) pH 7.4) was added + 20µl of centrifuged cell media, vortexted and loaded into the BiaCore™ 2000 (Surface Plasmon Resonance) SPR machine. The SPR results gave a resonance curve, the presence of a curve determined the detection of the expressed IgE antibody, the hight of the curve determined strong resonance i.e: detection of large quantity of expressed IgE antibody, the resonance units

from the top of the curve is subtracted from the bottom of the curve to give the final resonance units for each well sample which was then plotted on a bar graph to give a visual representation of IgE contents in all the tested wells. The well with the largest resonance unit i,e: greatest quantity of expressed IgE antibody was isolated and the cells grown and frozen to give the final cell line. Chapter 2.4.1 for the concept of SPR and the program used to run BiaCore™ 2000 tests.

2.4 - Proteomics:

2.4.1 - Surface Plasmon Resonance (BiaCore™ 2000 System):

Surface Plasmon Resonance (SPR) is an analytical method that uses light to analyses the contents of a sample for a desired molecule and the quantity of this molecule. The machine that was used is under the brand name of BiaCore™ System model 2000. Following the is concept of SPR:

When light of a certain wavelength (an electromagnetic wave) strikes the surface of a metal (usually silver or gold) it is reflected back at the angle of reflection. But at a certain angle the light will strike the metal surface and its energy is converted into energy used to oscillate electrons within the metal atoms (plasmon), this oscillating wave (plasmon wave) propagates through the outer surface of the metal, thus the light at this angle is absorbed and not reflected (resonance angle). Since the plasmon wave propagates the outer surface of the metal, any molecule added or removed will disrupt this wave and thus change the resonance angle, therefore the sample angle is measured against a reference angle to test the presence of molecules in a sample. The surface of the metal in a CM5 chip supplied by the BiaCore™ System has dextran (a complex and branched glucose polymer) on its outer surface, this molecule is used to bind the amine (NH_2) end of proteins to conjugate them on to the surface of the chip (Figure 11).

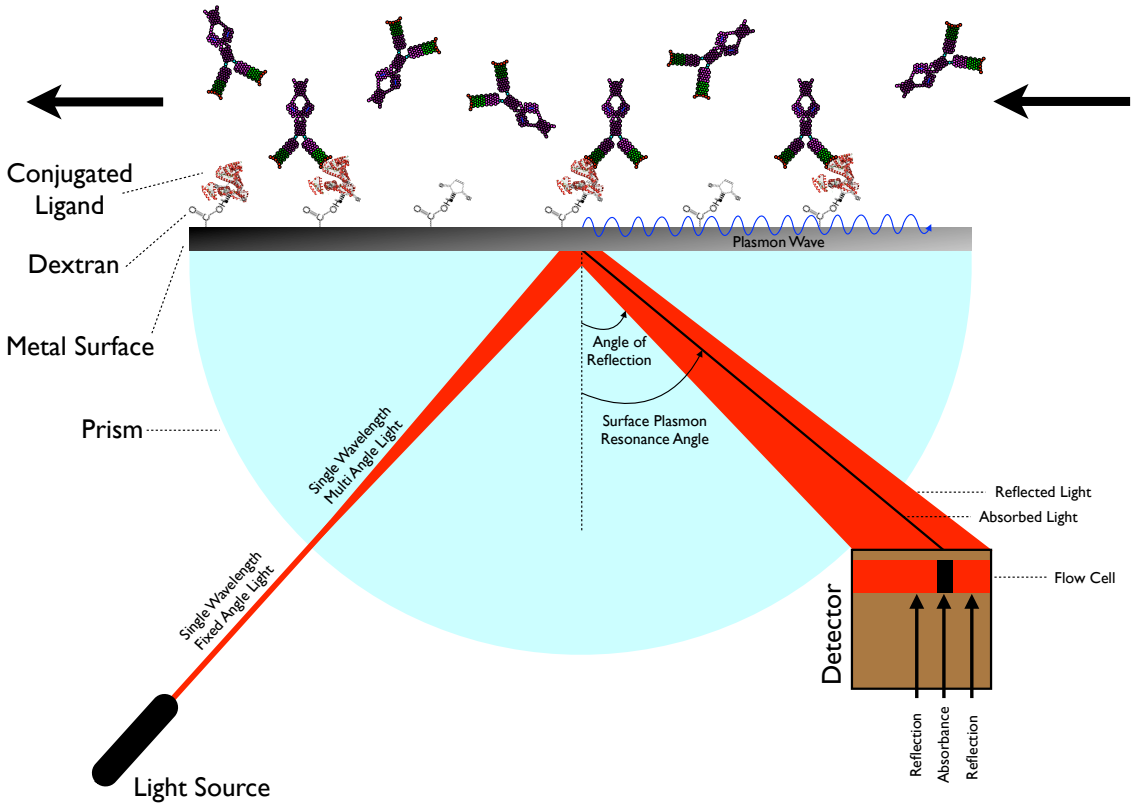
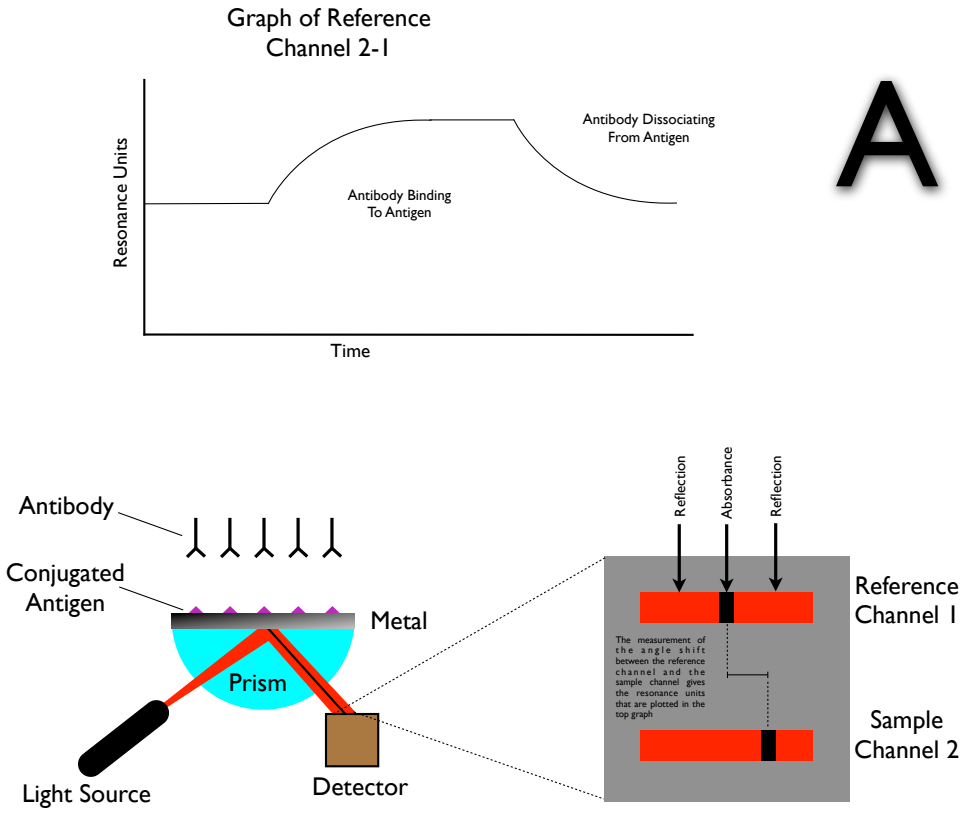


Figure 11: SPR metal surface:
This figure shows the configuration of an SPR chip. The metal chip (silver or gold) is prepared with a dextran surface which can bind the NH_2 end of protein to conjugate them to the metal surface. At the bottom a single wavelength laser beam enters a prism which results in many light angles striking the metal surface, all of them are reflected except for the angle in which the metal will absorb and turn its energy into a plasmon wave onto its outer surface, at this angle no light is reflected and thus appears with very little intensity on the detector. Since the plasmon wave propagates on the outer side of the metal, any interaction with the conjugated protein will change the resonance angle.

Therefore any binding with this protein will change the resonance angle and results in detection of the molecule in question. One chip has 4 channels (usually 2 per reaction), these two channels act as two separate metal surfaces, thus give the freedom to measure two separate resonance angles simultaneously. Since the resonance angle is an absolute value, to measure detection and quantity of the resonance angle the sample channel is referenced against the resonance angle of a reference channel (the control channel) with no protein bound on it, thus the sample channel angle will shift when the

molecule of question is detected, but the reference channel angle will not shift, therefore the difference in angles will give a resonance unit value that is used to measure the presence and the quantity of the molecule in question (Figure 12). Therefore in this project, on a CM5 chip the 1st channel is the reference channel with no conjugated antigen, but the second channel has conjugated antigen on it. When a sample containing antibody against the conjugated antigen is run on the chip surface channel's 2nd resonance angle shift while the 1st channel's angles does not, measuring the difference in angle of channel 2 - channel 1 will give a resonance unit that when plotted in a graph against time will show an upward curve (if another sample containing the antibody's receptor is run on the same chip, another upward curve is plotted as the antibody receptor binds to the antibody). Once the chip is washed the graph curve starts to slope downward as some of the antibody dissociated from its antigen, this small downward curve therefore indicates strong binding between the antibody and the antigen, while a large downward curve indicates weak binding where the bonds between the two molecules can be easily broken when washed with a buffer. The samples in the chip are usually forcefully destroyed by high or low pH to regenerate the chip so it can be reused, this is usually successful enough to allow the chip to be used a multiple of time (Figure 12).


Graph of Reference
Channel 2-1
Resonance Units
Time
Antibody Binding
To Antigen
Antibody Dissociating
From Antigen
A
Antibody
Conjugated
Antigen
Metal
Prism
Light Source
Detector
Reflection
Absorbance
Reflection
Reference
Channel 1
Sample
Channel 2
The measurement of the angle shift between the reference channel and the sample channel gives the resonance units that are plotted in the top graph

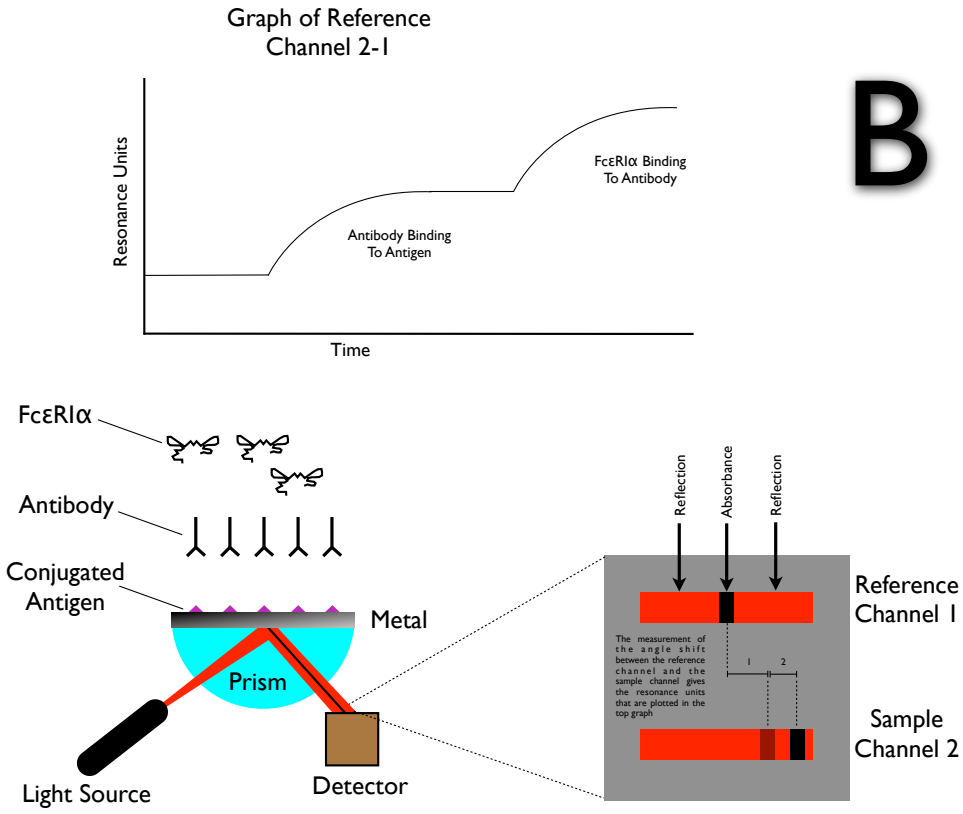

Graph of Reference
Channel 2-1
Resonance Units
Time
Antibody Binding
To Antigen
FcεRIα Binding
To Antibody
B
FcεRIα
Antibody
Conjugated
Antigen
Metal
Prism
Light Source
Detector
Reflection
Absorbance
Reflection
Reference
Channel 1
Sample
Channel 2
The measurement of the angle shift between the reference channel and the sample channel gives the resonance units that are plotted in the top graph
1
2

Figure 12: Diagram explaining Surface Plasmon Resonance (SPR):
This figure shows the workings of the SPR chip. **A.** the chip is conjugated with an antigen, when a sample runs on the sample chip and the antibody binds to its antigen, the sample channel resonance angle shift, while the reference channel (with no antigen conjugated) will not shift. The difference between the two angles (channel 2 - channel 1) will give the resonance units which when plotted on a graph against time will give an upward curve. When the sample is washed out and the antibody starts to dissociate from the antigen this effect is reversed and thus gives a downward curve. This concept can be used to bind more than one protein as in **B.** when the antibody binds to the antigen giving an upward curve, then if another sample containing the antibody's receptor is run on the chip, the antibody receptor binds to the antibody giving another upward curve.

The BiaCore™ 2000 chip used was a CM5 chip and conjugated to channel 2 (flow cell 2 or Fc_2) with human serum albumin conjugated to the antigen, the antigen therefore used was (4-hydroxy-5-iodo-3-nitrophenylacetic acid conjugated to human serum albumin) NIP-HSA, while the reference channel was (flow cell 1 or Fc_1). The final resonance units of the chip was measure to be ~2500 RU and was setup by the surface preparation wizard. The NIP-HSA was at a stock concentration of 15mg ml^{-1} prepared in 1xPBS where 5µl were added to 250µl 10mM sodium acetate pH 4 solution coupling buffer to couple the NIP-HSA to the chip. The concept here is that the human serum albumin has an isoelectric point of 4.7 (therefore at pH 4.7 the charge on this protein is neutral), when the chip is activated its surface is negatively charged therefore to bind the ligand (NIP-HSA antigen) to the surface it should have a positive charge which is achieved by lowering the pH, therefore it is added to the sodium acetate pH 4 solution. The chip is first activated by adding 7.5mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) + 11.5mg of N-hydroxysuccinimide (NHS) dissolved in H_2O. The ligand (NIP-HSA antigen) is then added followed by deactivating the none bound dextran on the chip surface by with 100µl 1.0 M ethanolamine-HCl pH 8.5. The machine running buffer used was HBS-EP

buffer, and the regeneration buffer used to unsure all ligands are securely bound was 10mM glycine at pH 1.7.

Once the chip is made, the setup for the BiaCore™ 2000 to run normal samples was as follows: The machine running buffer used was HBS-EP buffer. The samples were prepared in BiaCore™ test tubes with 180µl HBS-EP buffer and 20µl of sample (1:10 dilution), the tubes were secured with a tube cap and vortexed to ensure total mixing of the sample in it. The samples were loaded into the machine and the regeneration buffer used to remove the protein samples form the chip was 50mM NaOH. The samples were tested using the Binding Analysis wizard, where each samples was tested twice to ensure reproducibility.

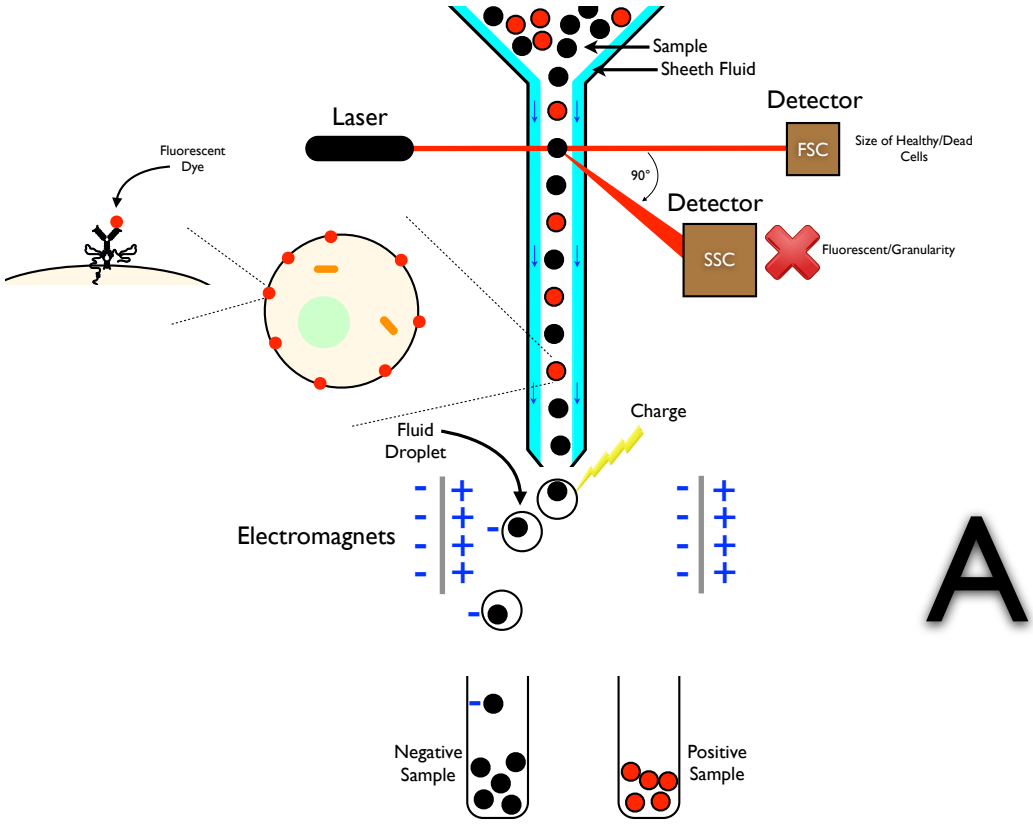
2.4.2 - Flow Cytometry and Fluorescence-Activated Cell Sorting (FACS):

Flow cytometry is a powerful preparatory tool which can allow the identification of the size of cells and their identity which can also incorporate fluorescence when the cells are tagged with a fluorescent dye inside the cell or on its surface. It is used from analyzing the purity of cell lines to biochemical pathways and assays. Flow cytometry can have an extra bit of kit added to them to allow the separation of two cell types in a mixed population when they are tagged with different florescent dyes, this process is called Fluorescence-Activated Cell Sorting (FACS).

The concept of flow cytometry is simple, a population of cells are hydrodynamically-focused to organize random cells from a sample into a fluid stream with ordered cells in a straight line, this is done by having a tube (glass or plastic) with the inner walls built up of a moving stream of fluid, this is called the sheeth flow. The sample of cells is injected into the middle of this sheeth flow where the two liquids will not mix as their density/viscosity are different, thus forming a stable two-layer flowing

fluid. The tube then narrows into a funnel thus constricting the sheeth fluid along with the sample which orders the cells into a single file of cells (Figure 13).

The single file of ordered cells would be passed through a single wavelength laser which analyses the sample using two detectors, the light that is scattered through the sample and collected forward from the cell (Forward Scatter Channel - FSC) gives information on the cells' size, whether they are healthy big round cells, or small dead cell debris. The light that is scattered sideways at a 90° angle form the laser beam due to reflection or fluorescence (Side Scatter Channel - SSC) gives information on the fluorescence of a cell or its granularity (Figure 13) thus enables to identify the type of cell. This information is collected and displayed on a SSC by FSC graph the X-axis gives the cell size and the Y-axis gives their fluorescence/granularity.


Sample
Sheeth Fluid
Detector
Laser
FSC
Size of Healthy/Dead Cells
Fluorescent Dye
90°
Detector
SSC
Fluorescent/Granularity
Charge
Fluid Droplet
Electromagnets
Negative Sample
Positive Sample
A

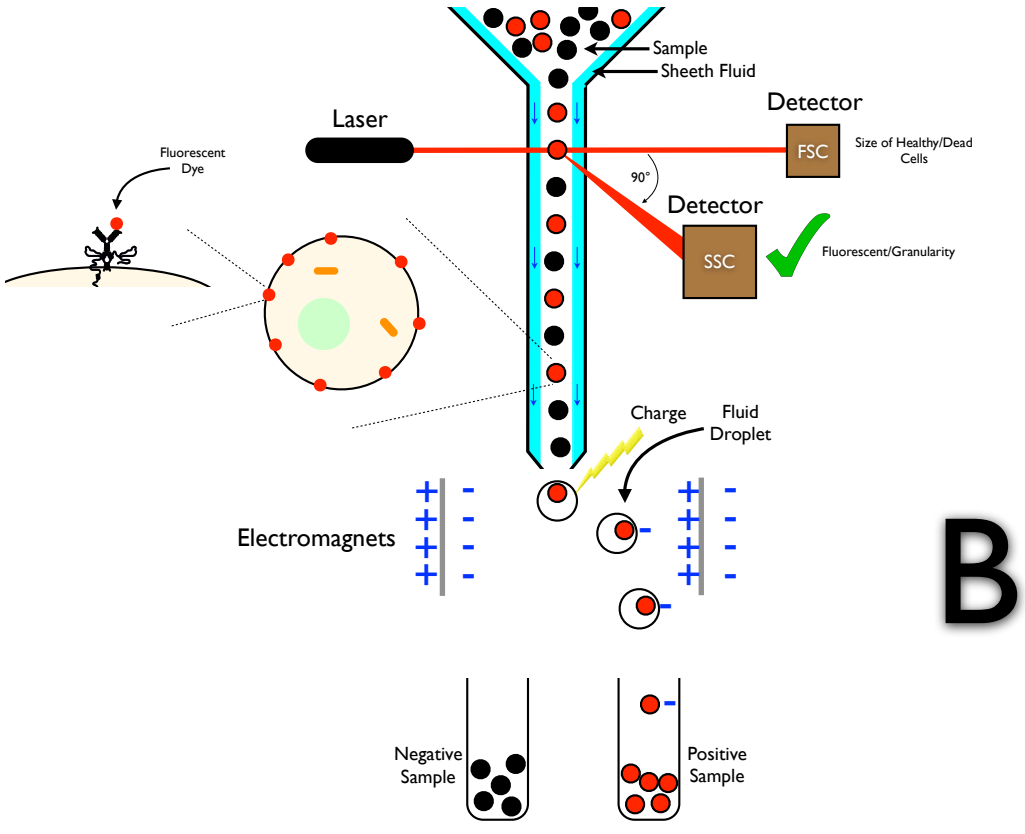

Sample
Sheeth Fluid
Detector
Laser
FSC
Size of Healthy/Dead Cells
Fluorescent Dye
90°
Detector
SSC
Fluorescent/Granularity
Charge
Fluid Droplet
Electromagnets
Negative Sample
Positive Sample
B

Figure 13: Diagram explaining Fluorescence-Activated Cell Sorting (FACS):
This figure is showing a representation of flow cytometry at the top of each diagram, but also shows how additional equipment can enable it to be used for FACS. **A.** the sample containing cells is injected in the middle of a fluid sheeth and funneled to give an single line of ordered cells. The cells are passed through a laser beam that measures the forward scatter (FSC) and side scatter (SSC). Usually this flow cytometry result is sufficient for analytical methods, but for a preparatory cell sorting method (FACS) the cells passed though a nozzle that vibrates and results in cell droplets with approximately one cell per drop, this drop is then electrically charged to give a negative charge on it. The machine computer calculates (since the distance between the laser and the electromagnets is known) when the cell reaches the area between the electromagnets, it will switch them on to divert the cell path so it lands on the correct vial, and thus accumulate the sorted cells. In this case the beam detects no fluorescence and therefore sends the droplet of cell into the negative sample vial. In **B.** the laser beam detects the correct fluorescence and therefore send the charged cell droplet into the correct positive sample vial.

When a sample of cells is injected into the sheeth fluid and funneled to give a single line of ordered cells, it passes through a laser beam where detectors collect the FSC and SSC lights to give a description of the sample, this is flow cytometry, and sometime it is wrongly termed FACS, because FACS is concerned only with a specially constructed machine that uses the concept of flow cytometry to sort a mixed cell populations. In the case of FACS, the cell fluid is not discarded, instead it passes through a vibrating nozzle that is designed to give droplets of fluid with approximately one cell per drop. This drop is then immediately charged to give it an overall negative charge. The information collected from the FSC and SSC detectors will cause the machine computer to calculate which droplets to save and which to discard. Since the distance between the laster beam and the electromagnets in the machine is knows, when the droplet with the positive cells reaches the electromagnets, the computer switch them and deviate the droplet's path to land into the positive sample vial, if the droplet

contains cell that are of no interest, the computer will switch the electromagnets on to deviate the droplet's path so it lands into the waste vial. This results into collecting and sorting the cells of interest so they can grow and form a pure cell line (Figure 13).

2.4.3 - Protein Expression And Quantification in Mammalian Cells:

The J558L cell line was the sole mammalian cell line that was producing a protein (equine IgE antibody) requiring purification. It was realized that an initial cell density of $5x10^5$ cells in 20ml of J558L selective media was the optimal cell density for expression. The cells were incubated in 37°C + 5% CO2 + 90% relative humidity for 5 days which was found to be the ideal length of time where the expressed protein would accumulate enough in the media, but not left long enough for it to start degrading. Therefore after 5 days of expression, the media was harvested by collecting all the cells and media and centrifuged at 180xg for 3 min, which sediments the cells and therefore the media was gently transferred and filter sterilized using a 0.45µm filter to remove all cells from the media. This was essential as mammalian cells, because when they die, or when a bacterial or fungal infection develops in the harvested media, proteases would be released that will degrade the expressed protein. The sterile harvested media was stored at -20°C until the equipment for affinity purification was ready see chapter 2.4.5. The media was concentrated at some points by a 3kDa molecular filter to concentrate the expressed protein and reduce the liquid volume to allow for ease of handling, but it was clear that this will give a low purification yield and results sometimes in albumin impurities, therefore it is recommended not to concentrate the media and use it as it is to purify the expressed protein.

2.4.4 - Protein Expression And Quantification in Yeast Cells:

The protein of interest here was the soluble equine FcεRI α chain's domain 1 and 2 (sFcεRIαD1&2), this gene was cloned into the pPIC9k plasmid. This plasmid was

chosen as the sFcεRIαD1&2 was required to be secreted out of the cell and purified from the media.

The *Pichia pastoris* yeast protocol was taken from Invitrogen's Pichia Multi-Copy *pichia* Expression Kit Version F, *pichia* Fermentation Process Guidelines and *pichia* EasyComp™ Kit. The yeast cells were first streaked on a solid YPD (10g yeast extract + 20g peptone, autoclave then added: 20g dextrose [glucose] + for solid media added 20g agar) media and incubated at 30°C for 2 days until colonies appeared, a single colony was picked and inoculated a10ml liquid YPD media in 250ml braided flasks, to allow for adequate aeration, and incubated at 30°C for ~16 hours until an OD_{600} = 0.1 - 0.2. The cells were pelleted by centrifuging at 500xg for 5 minutes and resuspended in 10ml Solution I. The cells were then centrifuged and resuspended in 1ml Solution I again. At this point the cells were competent and they were aliquoted at 50µl aliquots and stored at -80°C, even though repeated freezing and thawing did not affect cell competency.

3µg of the final plasmid pPIC9K-sFcεRIαD1&2 was linearized by digesting the DNA with SacI restriction enzyme, therefore when transformed the resultant yeast cell phenotype was His^+Mut^+ which was determined to be the best cell phenotype to express this specific protein. The 3µg of linearized DNA was added to 50µl of competent yeast cells then 1ml of Solution II was added to the cells and incubated at 30°C for 1 hour, vortexing every 15 minutes to ensure highest cell transfection. The cells were then heat shocked at 42°C for 10 minutes and the cells pelleted by centrifuging at 3000xg for 5 minutes and re-suspending at 1ml solution III. The cells were then re-pelleted and re-suspended in 150µl of Solution III. All the cells were plated on a solid RDB (186g sorbitol + for solid media add 20g agar, autoclave then add: 20g glucose + 13.4g yeast

nitrogen base + 2ml of 0.02% biotin + 10ml of non-essential amino acid solution) plate which after ~5 days at 30°C revealed ~50 colonies.

The design of the plasmid is such that it allows it to be inserted multiple of times in the yeast genome. The higher copy number of the insert present, the higher the protein expression of that strain, therefore to select the colony with the highest gene copy number 50 transfected colonies from the solid RDB plate were picked and added to 1ml of liquid YPD media and vortexed. A geneticin G418 sulphate solid YPD plates were prepared with the following final concentrations: 0mg ml^{-1}, 0.25mg ml^{-1}, 0.50mg ml^{-1}, 0.75mg ml^{-1}, 1mg ml^{-1}, 1.25mg ml^{-1}, 1.50mg ml^{-1}, 1.75mg ml^{-1}, 2mg ml^{-1}, 3mg ml^{-1}, 4mg ml^{-1}. From the mixture of the cell colonies 10µl were spread on each plate, and the plates incubated at 30°C for ~ 7 days until colonies appear. Colonies will appear in the 0mg ml^{-1} control within 2 days, while colonies in higher geneticin G418 sulphate concentrations will take longer to grow. Once the colonies were large enough to pick, the colony form the highest geneticin G418 sulphate concentration plate was picked (relatively the colony with the highest gene copy number) and protein expression was commenced immediately. It was observed that these colonies would rapidly lose their protein expression if they were frozen for stock. Therefore the colonies were kept in the plates at 4°C if repeated protein expression was required, otherwise the whole protocol needed to be repeated.

The picked yeast colony was added to 25ml BMGY (10g yeast extract + 20g peptone, autoclave then add: 13.4g yeast nitrogen base + 100ml 1M potassium phosphate Monobasic solution pH 6 + 10ml glycerol) media and grown for two days. The cells were harvested by centrifuging at 1500xg for 5 minutes. The cells were then resuspended in BMMY (10g yeast extract + 20g peptone, autoclave then add: 13.4g

yeast nitrogen base + 100ml 1M potassium phosphate Monobasic solution pH 6 + 5ml methanol) media to a final OD_{600}= 1, which will require ~1200ml BMMY media, where 400ml of media was added to 1000ml braided flask to allow for adequate aeration, then all the flasks were incubated at 30°C for 6 days. Every 24 hours the 0.5% methanol in the media would be exhausted as the cells would use it for the expression of the protein in question, therefore it needs to be replenished, therefore every 24 hours 2ml of methanol was added to 400ml of media to result in 0.5% methanol. Noting that adding too much methanol is toxic to the cells.

On the 6th day, the media was harvested by centrifuging at 1500xg for 15 minutes pelleting and discarding the cells. The media was further sterilized by filtering through a 0.45µm filter to removed all traces of the yeast cells, then protease inhibitors (P8215 From Sigma) were added to slowdown the breakdown of the expressed protein, and sodium azide was added to a final concentration of 0.5mg ml^{-1} to inhibit any further microbial growth that might compromise the expressed protein. The media was then concentrated using a 3kDa molecular filter from 1200ml down to 50ml to allow easy of handling during protein purification, chapter 2.4.5.

2.4.5 - Affinity Purification of Proteins:

To purify expressed proteins a chromatography column was setup using CNBr-activated Sepharose™ as the gel medium which the ligand molecule was immobilized in. To prepare the Sepharose™ gel the following protocol was used from GE Healthcare CNBr-activated Sepharose™ 4B manual:

A filtered glass mesh funnel was washed several times with concentrated sulfuric acid to remove all contamination and microorganisms that can affect the quality of the final chromatography gel. Care was taken as to not allow the acid to over heat, which

might lead to an EXPLOSION. 2g of CNBr-activated Sepharose™ was added to 50ml of 1mM HCl and incubated while rotating at room temperature for 60 minutes at which point the powder swelled up. The gel was filtered through the funnel using a vacuum and then washed with further 200ml of mM HCl. This activated the gel at which point it was collected and re-suspended in 50ml of coupling buffer (0.1M sodium hydrogen carbonate + 0.5M sodium chloride pH 8.3). 35mg of the ligand NIP-CAP-OSu (dissolved in dimethylformamide) was added to the gel. The mixture was incubated at room temperature for 2 hours with gentle shaking, at that point the gel colour turned from white to yellow, indicated the coupling of the NIP-CAP to the Sepharose™ beads. The OSu section of the NIP-CAP-OSu molecule was cleaved and the NIP-CAP was then covalently bonded to the Sepharose™ beads to give NIP-CAP-Sepharose™. The gel was then washed in the funnel with 600ml of coupling buffer to remove all the unbound NIP-CAP-OSu. The gel was then collected and re-suspended in 50 ml denaturing buffer (0.1M tris-HCl pH 8) to de-activate all the non-bound sites in the Sepharose™, to prevent binding of unwanted molecules, and incubated at room temperature for 2 hour without shaking. To further block any active groups in the gel it was washed three times with alternating pH starting with 50ml blocking buffer (0.1M acetic acid + 0.1M sodium acetate + 0.5M sodium chloride pH 4) then with 50 ml denaturing buffer. The gel was filtered and washed with 500ml 1xPBS, then collected and stored in 1xPBS + 50mg ml^{-1} sodium azide at 4°C for later use. It is advised to use the gel as soon as possible as the longer it is stored in the fridge the more NIP-CAP dissociates from the Sepharose™ beads giving low purified protein yield. This column was used to purify IgE anti NIP-HSA antibody, but the same protocol was used to couple IgE into the Sepharose™ beads to purify sFcεRIαD1&2 protein, using 10mg of IgE.

To load the gel and make a chromatography column (QIAgen kit Polypropylene Columns) the filers were first equilibrated by chromatography column equilibration solution (0.6% Tween 20 in water) by rotating for 1 hour at room temperature, washed with water and incubate while rotating for 15 minutes at room temperature, this step was repeated twice. When the filters were equilibrated, the bottom filter was placed in the plastic column and added the prepare Sepharose^{TM} gel until it forms, after it settled, a 2cm high gel bed (capable of binding ~6mg of protein). Using too little gel will not result in insufficient quantity of protein, using too much gel will results in very slow fluid migration through the gel resulting is the retainment of other proteins, mainly albumin, which will result in very low protein yield with poor protein purity. When the gel bed settled, the top filter was added leaving ~1mm distance between the gel and the filter to prevent compacting the gel. At this point the gel was washed with 100 ml of 1xPBS to remove all the dissociated NIP-CAP (or IgE antibody) molecules and sodium azide. To re-use the column it was washed with regeneration buffer (0.1M tris + 0.5M sodium chloride pH 8) to remove all bound specific and non-specific proteins.

The media containing the expressed protein was passed through the column three times to ensure saturation of the protein in the column. The column was then washed with 300ml 1xPBS to ensure all non-bound proteins were washed out.

Eluting the IgE antibody: 12ml of 1.5mM NIP-CAP-OH was added to the column to elute the IgE, the eluting liquid was passed only once through the column and collected, and the remaining eluting liquid in the column was pushed by passing 1xPBS until all the eluting liquid has passed. The eluting liquid was then dialyzed using a 3kDa dialysis tubing in 1xPBS for 5 days until all the NIP-CAP-OH (yellow in colour) has been removed and the liquid became clear. The antibody was then concentrated using a

3kDa molecular filter down to 200µl and this resulted in the IgE antibody being dissolved in 1xPBS.

Eluting the sFcεRIαD1&2 protein: three fractions, each of 3ml of elution buffer (0.2M glycine pH 2.8) was added to the column and dropped on to 3ml of neutralizing buffer (1M tris-HCl pH 8). The low pH of the elution buffer dissociated the sFcεRIαD1&2 from the IgE, but when it landed on to the high pH neutralizing buffer the pH equilibrates into ~pH 7 which is ideal for the protein to prevent it from denaturing. The three fractions were then pooled together and washed with 1xPBS by concentrating them through a 3kDa molecular filter, and finally concentrated down to 200µl. This resulted in sFcεRIαD1&2 being dissolved in 1xPBS.

2.4.6 - Quantification of Purified Proteins:

The bradford assay was used: a concentration curve was setup using bovine serum albumin (BSA) as the control protein: 0mg ml^{-1}, 0.1mg ml^{-1}, 0.2mg ml^{-1}, 0.3mg ml^{-1}, 0.4mg ml^{-1}, 0.5mg ml^{-1}, 0.6mg ml^{-1}, 0.7mg ml^{-1}, 0.8mg ml^{-1}, 0.9mg ml^{-1}, 1.0mg ml^{-1}. Using more then 1mg ml^{-1} concentration did not give accurate results, and therefore if the protein of interest was suspected to be more than 1mg ml^{-1}, it was diluted down to a level that was between 0.1 - 1mg ml^{-1}. The concentration curve was setup in 1xPBS, because all protein resulting from the affinity purification in chapter 2.4.5 were dissolved in 1xPBS. Once the concentration curve was setup and the sample diluted down to the proper level, the protein assay buffer (Bio-Rad) was diluted to 1x. in a 96 well plate, 10µl of each of the BSA concentration curve was added to wells, along with the protein of interest, in triplicates. 200µl of 1x protein assay buffer was added to each of the wells with protein and incubated at room temperature for 10 minutes. The intensity of the blue colour that results was read on a plate reader at 620nm. The absorptions of the concentration curve was plotted on a graph of absorbance (optical

density - OD) against the actual concentration. From that a line of best fit was plotted and its equation determined, from this equation the concentration of the protein sample was found using its absorbance. This method was proven to be very accurate if done carefully.

2.4.7 - Protein Separation (SDS-PAGE):

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was the method used to separate proteins for analysis. This method preceded the protein identification (western blot). The protocol was taken from (Sambrook and Russell, 2001) and it was run on the Mini-protean II Electrophoresis System (Bio-Rad):

The resolving gel used was 12% acrylamide (4.9 ml H_2O + 3.8ml [1.5M tris pH 8.8 + 0.4% Sodium dodecyl sulfate] + 6ml 30% acrylamide + 150µl ammonium persulfate + 6µl TEMED) with the ammonium persulfate being freshly prepared. The 5% stacking gel used was (2.7 ml H_2O + 500µl 1M tris pH 6.8 + 40µl 10% Sodium dodecyl sulfate + 670µl 30% acrylamide + 40µl ammonium persulfate + 4µl TEMED), these gels were loaded onto the Mini-protean II Electrophoresis System.

The protein sample was added to a protein loading dye at a 1:1 quantitative ratio and then boiled at 100°C using a water bath for 5 minutes. The non-reducing protein loading dye (60mM tris-HCl pH 6.8 + 20% glycerol + 2% sodium dodecyl sulfate + 0.0025% bromophenol Blue) was used to denature and separate whole proteins, while the reducing protein loading dye (60mM tris-HCl pH 6.8 + 20% glycerol + 2% sodium dodecyl sulfate + 0.0025% bromophenol Blue + 5% β-mercaptoethanol) was used to break disulphide bridges and thus separate different domains of a protein molecule. A 10kDa protein ladder was used as a molecular weight standard. The gel was placed in an electrophoresis running buffer (14.4g glycine + 3.07g tris + 10ml 10% sodium

dodecyl sulfate) and separation carried out at 50v until the protein has passed the stacking gel and entered the resolving gel, at which point the voltage was increased to 120v which took ~2 hours for the protein to run through the entire gel. The gel was stained using coomassie stain (2g coomassie blue + 250ml acetic acid + 1000ml H2O + 1000ml methanol) for 20 minutes at which point the proteins and gel took up the stain, then the gel was de-stained by washing in coomassie de-stain (10% acetic acid + 30% Methanol) for 16 hours at which point the gel lost almost all the blue colour, while the protein maintained it allowing the protein bands to be visualized.

2.4.8 - Protein Identification (Western Blotting):

Protein identity was essential to prove that the purified protein is the protein of interest and not mistakingly another protein of a similar size, this test is very accurate as it incorporates the binding of antibodies to restricted epitopes within the protein of interest, even though there are no results in this project from western blots, due to the commercial scarcity of the required antibodies, non the less this protocol was used in this project.

The proteins were separated using SDS-PAGE as in chapter 2.4.7 but the gel was not coomassie stained, as that would have interfered with the antibody binding to the protein. The clear gel was placed in the Mini Trans-Blot cell cassette (Bio-Rad) with the following configuration: the gel was placed on a (Polyvinylidene fluoride) PVDF membrane (Amersham Hybond-LFP Western Blotting Membrane) and the two were placed between two pieces of filter paper on each side followed by one sponge on each side. This ‘sandwich’ was placed in the Mini Trans-Blot cell cassette with the negative end facing the PVDF membrane and run in western blot running buffer (14.4g glycine + 3.07g tis + 200ml methanol) for two hours at 4°C with 60v. The membrane was then removed and checked for the complete transfer of the protein ladder, the protein of

interest is not visible and therefore to check its presence it was incubated for 5 minute in Ponceau S stain, the stain was washed under distilled water where the protein bands would appear before the stain got completely washed away.

Since the membrane is capable of binding all proteins, it had to be blocked before antibody priming, therefore to allow the protein antibodies to bind only to the protein epitopes and not to the active membrane. The membrane was therefore placed in blocking solution (5g full cream milk + 50µl tween 20 + 100 1xPSB) and shaken for 2 hours at room temperature. To the blocking solution 1µg of primary antibody, that binds to the protein, was added and the membrane was incubated while shaking for 1 hour at room temperature. The membrane was then washed with 200ml of wash solution (50µl tween 20 + 100 1xPSB) to remove all the blocking solution and the primary antibody. 1µg of secondary antibody, that binds to the primary antibody and has a horseradish peroxidase enzyme tagged to it, was added to the membrane in wash solution and incubated while shaking for 1 hour at room temperature. The membrane was washed with 200ml wash solution.

The protein on the membrane was detected according to the (Amersham ECL™ Western Blotting Detection Reagents) protocol which resulted in chemiluminescence from the secondary antibody and thus was photographed on light sensitive photographic paper (Kodak), which was then developed according to the protocol from Kodak. The success of the western blot resulted in a single band in the photographic paper.

2.4.9 - RBL-2H3.1 β-hexosaminidase Release Assay:

This assay was preformed on RBL-2H3.1 cells to determine the percentage of inflammatory mediators released by these cells when their FcεRI receptors binds to IgE.

The RBL-2H3.1 cells were harvested from a 75% confluent petri dish as in chapter 2.3.4. The cell were diluted to a density of $5x10^5$ cell ml^{-1} and 1µg of IgE antibody was added to every 1ml of cells to reach a final IgE concentration of 1ng ml^{-1}, the cells were then gently shaken. To a 96 well plate 100µl of cells + IgE was added to columns 1-6 of the plate. The plate was incubated at 37°C + 5% CO2 + 90% relative humidity for 16 hours. This reaction allowed the interaction of the IgE with the FcεRI receptor for 16 hours, some experiments were done where the IgE was only allowed to interact with the FcεRI receptor for 0.5, 1 and 3 hours. At these experiment the cells were incubated for 16 hours without any IgE, this was to allow the cells to adhere to the plate surface, then the media was discarded and a new warm (37°C) media containing IgE was added to them and incubated for the required amount of time (usually 16 hours for normal, no kinetic studies, release assays).

The plate was flicked up-side-down to discard the media, and all the wells were washed twice with worm (37°C) release buffer (25mM pipes + 120mM sodium chloride + 5mM potassium chloride + 0.04mM magnesium chloride + 1mM calcium chloride). A serial dilution of the antigen (NIP-HSA or DNP-HSA) of 0 ng ml^{-1}, 0.1 ng ml^{-1}, 1 ng ml^{-1}, 10 ng ml^{-1}, 100 ng ml^{-1}, 1000 ng ml^{-1}, 10 000 ng ml^{-1} was prepared in release buffer. 100µl of this serial dilution was added to the washed cell wells form rows A-G with raw H having 100µl of triton-X buffer (5% Triton-X 100) to lyse the cells, this was done for columns 1-6, thus repeating the experiment six times. The plate was incubated at 37°C + 5% CO2 + 90% for 20 minutes, this was the point where the mediators were released.

After the incubation, 50µl of the liquid above the cells form each well was transferred to the corresponding well on the other half of the plate, i.e: columns 7-12.

The remaining 50µl of liquid above the cells was discarded, and replaced with 50µl of triton-X buffer. This setup allowed for each well to measure the amount of released mediators (columns 7-12) as a mean of percentage of the total mediators inside the cell (column 1-6). Raw H was therefore not needed to be used in the calculations, but rather useful to test the workings of the experiment.

One of the mediators released was β-hexosaminidase, and therefore to all the wells 50µl of the β-hexosaminidase substrate (50mM 4-nitrophenyl N-acetyl-β-D-glucosaminide prepared in DMSO diluted down to 2mM by adding it to citrate buffer [0.2M citric acid + 0.2M sodium acetate pH 4.5]) was added. The plates were incubated at 37°C + 5% CO2 + 90% for 2 hours. The chemical reaction that occurred was:

This reaction was stopped by adding 150µl of tris buffer (1M tris-HCl pH9) which, with its high pH, caused 4-nitrophenyl to turn yellow. This allowed its absorbance to be measured at 405nm in a plate reader, and thus the percentage of β-hexosaminidase was calculated using the following formula:

$$\left(\frac{A7 \times 100}{(A7 \times 2) + A1}\right) \times 2$$

A1 and A7 represent the location of the wells in the 96 well pate. This formula was applied to each well, then an average was taken for each row, with the standard deviation calculate as well, then plotted on a graph of percentage of β-hexosaminidase release against antigen concentration.

2.4.10 - Kinetic Analysis Using Surface Plasmon Resonance:

The SPR BiaCore™ System was the machine of choice to measure the kinetic binding between the sFcεRIαD1&2 and the IgE. This was because the SPR uses only micrograms of the required proteins. Another method considered was Isothermal Titration Calorimetry (ITC), but it requires milligrams of the proteins, and the expression levels of the systems used in this project would have been very expensive to reach milligram concentrations, therefore SPR was chosen. Since the CM5 chip used was immobilized with NIP-HSA, IgE anti NIP-HSA was used and added as the capturing molecule until it reached a resonance unit of ~2500 which correspond to ~2μg ml^{-1} of IgE. Then the binding of sFcεRIαD1&2 to the IgE was tested by adding the following concentration to the sFcεRIαD1&2: 0μg ml^{-1}, 1μg ml^{-1}, 2.5μg ml^{-1}, 5μg ml^{-1}, 7.5μg ml^{-1}, 10μg ml^{-1}, (0μM, 0.02μM, 0.05μM, 0.10μM, 0.15μM and 2μM respectively assuming the molecular weight is 51000g mol^{-1}), only 300μl of each sample was required, and the kinetic analysis was preformed at 25°C. All tests were repeated twice and the regeneration buffer used was 10mM glycine pH 1.7. The BiaCore™ software had an application wizard that automatically configured the machine to allow for kinetic binding measurements. The kinetic values were calculated and generated automatically by the BiaCore™ software with the molecular weight of the sFcεRIαD1&2 taken to be 50kDa.

2.5 - Mammalian Immunization:

2.5.1 - Immunization Schedules:

Rats (species: *Rattus norvegicus* also known as Hannovers rats, only males were used) at age ~ 10 weeks old were used for the immunization protocol. Each rat was initially injected subcutaneously, using a 25 gage glass needle, with 100µl of 1mg ml^{-1} of vaccine that was mixed with an equal volume of complete Freund's adjuvant (*Mycobacterium tuberculosis* + mineral oil), which is an immunopotentiator that directs the immune system to attack the vaccine instead of just dissolving it away. Before injection the mixture was votrexted to insure emulsification of the final mixture. 14 days later, and all subsequent injections, another injection was preformed by mixing the vaccine with incomplete Freund's adjuvant (just mineral oil), this was to insure the immune system keeps attacking the vaccine instead of the *Mycobacterium tuberculosis*. 10 days after the injection ~ 200 µl of blood was taken from their tails or leg veins. This was followed by ~20 days recovery before the rats were re-injected again to give an immune boost, then 10 days later another bleed was taken:

1. Pre-immunization bleed (used as a negative control).

2. Rats were immunized with the vaccine ($2Fc\varepsilon_{2\text{-}3}$ peptide + complete Freund's adjuvant).

3. 14 days later, the rats were again immunized with the vaccine ($2Fc\varepsilon_{2\text{-}3}$ peptide + incomplete Freund's adjuvant).

4. 10 days later the first bleed was taken to analyze the immune response.

5. ~14 days later the rats were boosted, boost 1, by a re-injection of ($2Fc\varepsilon_{2\text{-}3}$ peptide or the HDH IgE anti NIP-HSA antibody chimera + incomplete Freund's adjuvant).

6. 10 days later the second bleed was taken to analyze the immune response.

7. Steps 5 - 6 were repeated for boosts 2 and 3.

Each bleed taken was prepared for analysis by incubating at room temperature for ~1 hour for the red and white blood cells to settle. This was followed by incubating at 4°C for ~24 hours for the blood to clot. The bleed was then centrifuged at high speed (10000xg) to remove all blood clots and reveal the bleed serum in the supernatant ~50% of the original bleed volume. The serum was used to test the rat immune response.

Ideally, at the end of the experiment, the rats get bleed out, ~10ml of blood, and treated in the same manner to harvest the serum. And this serum would contain the polyclonal IgG antibodies against what ever antigen used. But this was not used here as our objective was to analyze the state of the immune response.

2.5.2 - Bleed Assay Protocol (ELISA):

The rat bleeds were analyzed using the enzyme-linked immunosorbent assay (ELISA). the ligand ($2Fc\varepsilon_{2\text{-}3}$ or NIP-HSA) was prepared to a final concentration of 1µg ml^{-1} in ELISA immobilization buffer (15mM Na_2CO_3 + 35mM $NaHCO_3$ pH 9.6). To an ELISA flat bottom 96 well plate 100µl of prepared ligand was added and incubated at 4°C for 16 hours. The plate in all incubations was covered with plastic film to prevent evaporation. After the immobilization step, the plate was washed vigorously using a squeeze bottle with ELISA washing buffer (1x PBS + 0.05% Tween 20) 3 times and completely dried. 150µl of blocking buffer (100ml of [1x PBS + 0.05% Tween 20] + 200mg Gelatin) was added to each well and incubated at 37°C for 2 hours and then washed as before. If NIP-HSA was immobilized, 50µl of 1µg ml^{-1} native IgE was added to bind the antigen and incubated at 37°C for 2 hours. Following that prepared rat serum was serially diluted ten steps (from 1:200 - 1:102400) in blocking buffer and 50µl were

added to each well, a control of pre-immunization serum was used with the same dilution strategy. The wells were incubated at 37°C for 1.5 hour and then washed as before. 50µl of the secondary antibody used was rabbit IgG anti rat IgE conjugated with horseradish peroxidase enzyme (Sigma) at 1:1000 dilution factor and incubated at 37°C for 1 hour. The plate was washed for a final time as before followed by an extra 2 washes with distilled water to remove all traces of un-bound secondary antibody. 50µl of TMB substrate (3,3',5,5'-tetramethylbenzine) was added to the plate and incubated for ~ 3 minutes at room temperature, depending on the amount of blue colour generated. The reaction was stopped by adding 50µl ELISA stop solution (0.2M H_2SO_4) where the blue colour changed to yellow due to the low pH. The plate was read on a plate reader at 450nm.

Chapter 3 - Generation of Equine IgE:

3.1 - Introduction:

Since this project is concerned with the study of the interaction between the equine FcεRI receptor and the equine IgE, and since this IgE is not available commercially, this required the synthesis of this antibody *in-house*. The concept of the synthesis was based on the published sequence of the equine IgE heavy chain wild type gene sequence (Navarro, *et al.*, 1995) from the GenBank sequence database (part of the United States' National Center for Biotechnology Information, NCBI), which then had its DNA codons optimized (changed) for mouse codons (*Mus musculus*) to increase the efficiency of protein expression in mouse cells. The optimized gene was constructed digitally and sent to the company (GenScript) which synthesized the gene (Appendix). The equine IgE heavy chain gene was then cloned into a plasmid downstream of a gene sequence that codes for a mouse antibody variable region that recognizes and binds to the antigen 4-hydroxy-5-iodo-3-nitrophenylacetic acid (NIP). Therefore, when expressed, the protein would be a full equine IgE antibody with a mouse variable region that binds to NIP (equine IgE anti NIP). The final plasmid was transfected into mouse B cells, and after antibiotic selection of the cloned cells, a mouse B cell line that expressed equine IgE was developed.

The expressed equine IgE was secreted outside of the cells into the cell media, and therefore harvesting of the media, and running it through an affinity column, resulted in pure equine IgE anti NIP. Just to be clear, the produced antibody binds to the NIP molecule, but since it was an organic molecule, for it to be used in the SPR machine it has to be conjugated to human serum albumin (HSA) therefore the actual antigen used was NIP-HSA and thus this project refers to the antibodies produce as IgE anti NIP-HSA.

3.2 - Results:

3.2.1 - Optimizing The Equine IgE Heavy Chain Gene:

The equine IgE heavy chain (Hε) was searched and found in a paper by (Navarro, *et al.*, 1995), the protein sequence is included in the Appendix. This protein sequence was used to construct a gene optimized for the mouse (*Mus musculus*) as in chapter 2.2.1. The final optimized DNA sequence used had a TAA ending sequence added, two BamHI restriction sites at each end of the sequence to allow for cloning and half a human intron at the start that completed the endogenous intron in the plasmid, this allowed for the cloning of the gene into the pSV-V_{NP} plasmid. The intron with the first restriction was spliced out and the gene of the variable region and the heavy chain were fused to form one single gene, in frame, that was expressed into one single IgE protein. A BglII restriction site was placed right at the end of the gene after the TAA ending sequence but behind the BamHI site to check for the orientation of the gene after its ligation, this was because both ends of the gene were digested with the same restriction enzyme, which allowed for the ligation of the gene in either direction.

3.2.2 - Cloning The Equine IgE Heavy Chain Gene Into Plasmid:

As discussed in chapter 2.2.4 the vector used in this experiment was pSV-V_{NP} plasmid where the equine heavy chain gene (Hε) was cloned downstream of a mouse λ chain variable region that binds to NIP-HSA. As outlined in chapter 3.2.1 the synthesized Hε gene was delivered in the plasmid pUC57 (which had characteristics similar to that of pUC19) and was transformed into *E.coli* TOP 10 bacteria. The bacteria were cultured as described in chapter 2.2.3 and the cells harvested, followed by purifying the pUC57-Hε plasmid as outlined in chapter 2.2.5.

The pUC57-Hε plasmid was digested with BamHI to remove the Hε gene, the plasmid pSV-V_{NP}F424 plasmid was also digested with BamHI to remove the F424 gene

and thus allowed the Hε gene to replace it. The restriction digestion was carried out as shown in chapter 2.2.7 followed by de-phosphorylation of the pSV-V_{NP} plasmid to prevent it from self ligating since both ends of the plasmid had the same restriction sites. Figure 14A shows the restriction digestion results.

The DNA bands of interest were analyzed by agarose gel electrophoresis. see chapter 2.2.8 and then isolated, see chapter 2.2.9 and ligated as shown in chapter 2.2.10. The resulting ligation mixture was transformed into bacterial cells XL-1 Blue as in chapter 2.2.11 and the cells were spread on a plate as in chapter 2.2.3 using the media described in chapter 2.2.2. The colonies that grew after the 16 hour incubation were the transformed colonies with the pSV-V_{NP}Hε plasmid. Six colonies were isolated, grown, harvested and the pSV-V_{NP}Hε plasmids purified to check for the successful insertion of the gene, and since the same restriction enzyme on both end of the gene was used to insert it, the plasmids was also analyzed for the correct orientation of the gene by digesting them with BglII restriction enzyme. Figure 14B shows the gene insertion and the orientation tests, and Figure 15 shows the structure of the pSV-V_{NP} plasmid before and after the Hε insert. The gene and plasmid sizes was calculated to check for the presence of the gene and its orientation, were the pSV-V_{NP} plasmid on its owen had a size of ~7000bp, the F424 gene had a size of 3000bp, the pUC57 plasmid had a size of 2710bp and the Hε had a size of 1302bp. The plasmid with the correct orientation had two bands at 2000bp and 7000bp, while the plasmid with the wrong orientation had two bands at 3000bp and 6000bp.

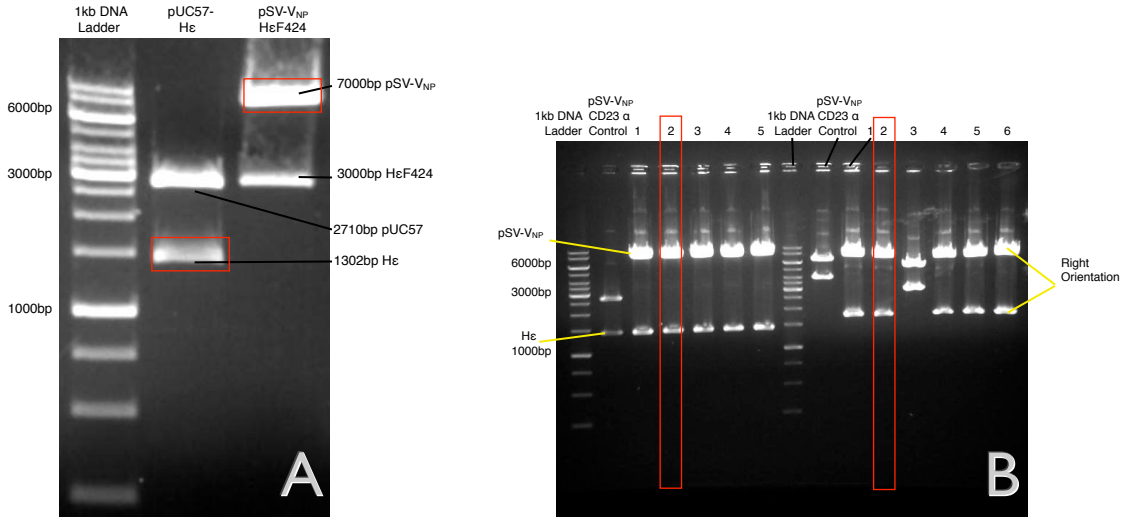


Figure 14: Restriction digestion of pUC57-Hε and pSV-V_{NP}F424:
A. the plasmids were digested with BamHI followed by de-phosphorylation. This was done in preparation to clone the Hε gene into the pSV-V_{NP} plasmid. **B.** the left side of the figure shows the successful insertion of the Hε gene into all of the isolated pSV-V_{NP}Hε plasmids where they were digested with BamHI. The right side of the figure shows the gene orientation test, where the plasmids were digested with BglII, with all the colonies being in the correct orientation, but the gene isolated from the 3rd colony being in the wrong orientation.

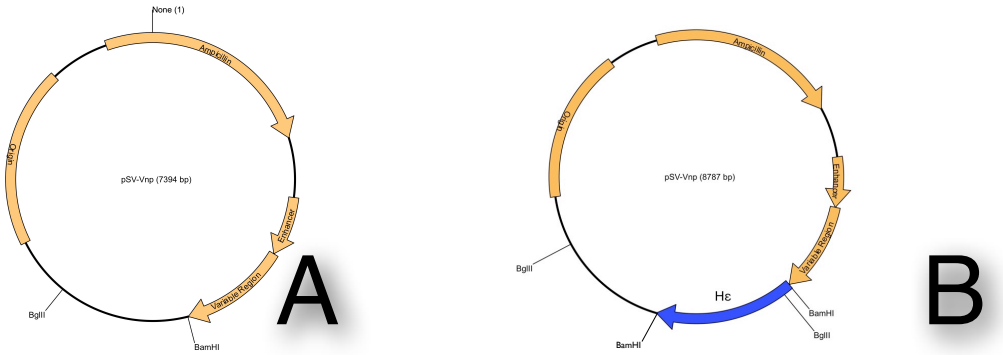


Figure 15: The structure of the pSV-V_{NP} plasmid before and after the insertion of the Hε gene:
A. shows the structure of the pSV-V_{NP} plasmid (without the F424 gene) while **B.** shows the final structure of the pSV-V_{NP}Hε plasmid.

Once the bacterial colony with the pSV-V_{NP}Hε containing the correct Hε gene orientation was identified and a backup bacterial culture was frozen as in chapter 2.2.12,

it was grown and the pSV-V_{NP}Hε plasmid DNA harvested in a larger concentration as in chapter 2.2.13 followed by concentrating the plasmid DNA as in chapter 2.2.15 and the DNA quantified as in chapter 2.2.14 which enabled the required concentration adjusted for mammalian cell transfection.

3.2.3 - Transfecting J558L Cells With Equine IgE Heavy Chain Gene:

The pSV-V_{NP}Hε plasmid was transfected into J558L mouse B cells as outlined in chapter 2.3.5. The cells were then plated onto two 24 well plates, thus dividing them into 48 wells. A control was setup by replacing the plasmid DNA with water and running the experiment normally, this control was plated on to a separate plate.

The first 48 hours after transfection the cells were grown on normal media to allow the transfected cells to express the relevant selected genes. The media was then replaced with selective media and the cells selected as in chapter 2.3.7.

3.2.4 - Selecting Equine IgE Anti NIP-HSA Expressing J558L Cells:

The cells went through a population decline as the majority of the non-transfected cells died. Then after ~5-6 weeks healthy cell colonies, around three to four cells, started to appear, which rapidly grew in number and at ~6 weeks after transfection the well were full of healthy cells, Figure 16 shows what the wells looked like at different selection stages.

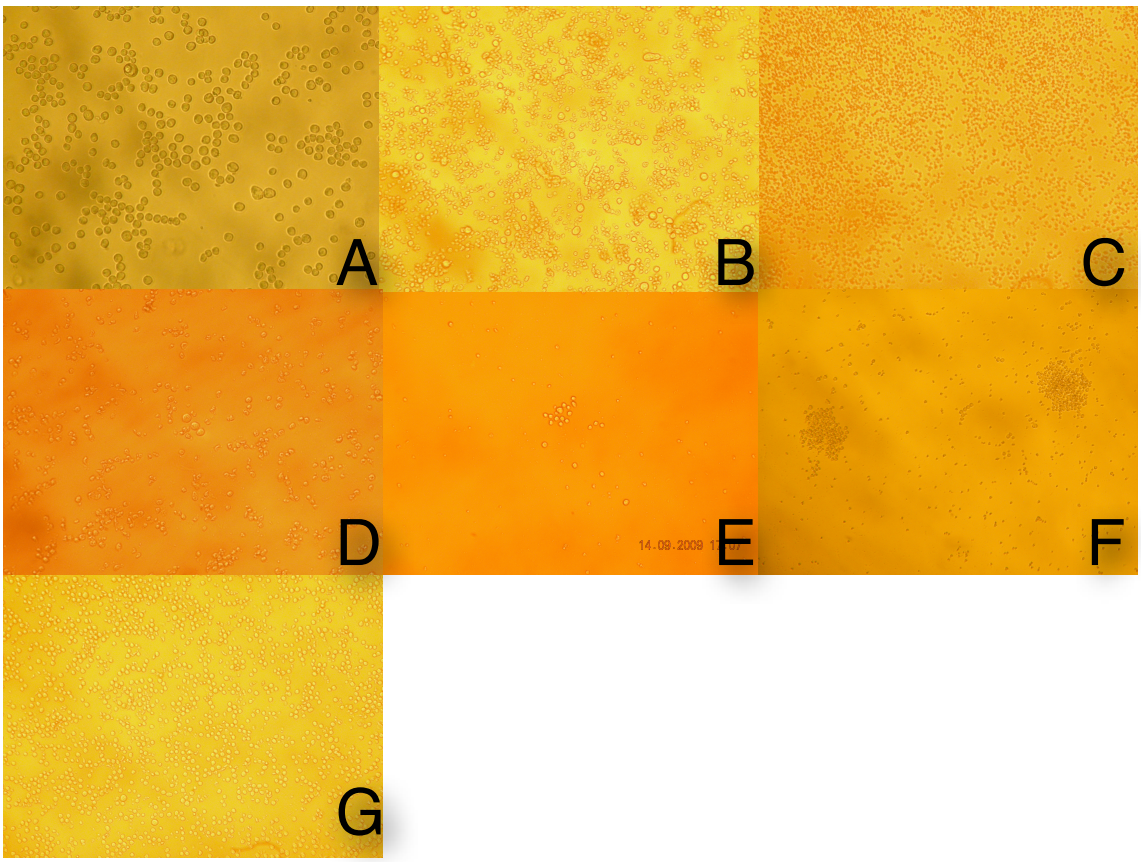


Figure 16: J558L cell morphology during selection:
This figure shows the cell morphology and the general look of the wells at different stages on cell selection. In **A.** the cells were just transfected but not yet selected, therefore their morphology shows very healthy spherical cells that fills the well. In **B.** a week after the introduction of selective media, at this point nearly all the cells in the well look very unhealthy as in **C.** as the healthy cells cannot be distinguished at this stage, wells look like this for the majority of the selection process. In **D.** as the selection progresses, dead cells are removed and the healthy ones remain behind and they start to appear. **E.** The healthy cells then have the freedom to grow and develop very small colonies, which grow quickly as in **F.** until they fill the wells as in **G.**

The media from all 48 wells was harvested and centrifuged at 180xg for 3 minutes to remove all the cells and the supernatant was analyzed by SPR for the presence and quantity of equine IgE as in chapter 2.4.3. Figure 17 shows the SPR results from all 48 wells. The cells in the well with the highest quantity of expressed IgE (in this case well BB3) were collected and grown to develop a J558L cell line that expressed equine IgE anti NIP-HSA. The values for Figure 17 came from the average of the difference between the bottom point and the top point for two SRP well curves, Figure 18 shows

the top actual SPR curve of the BB3 well with the bottom curve being the control media from non-transfected cells and some other selected cells that are not expressing the antibody.

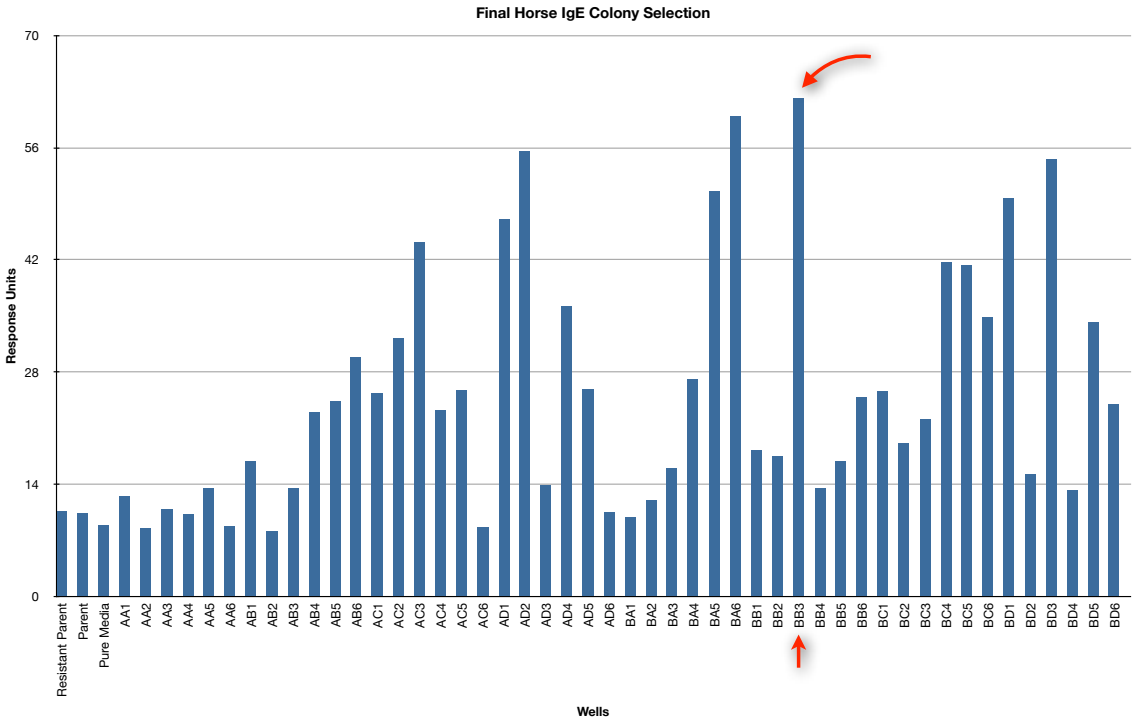


Figure 17: SPR results of J558L selection of equine IgE anti NIP-HSA expression:
This is the SPR result of the transfected and selected J558L cell media from each well. Each bar represents the quantity of equine IgE anti NIP-HSA, therefore well BB3 ,marked with the red arrows, was selected as the well expressing the most IgE, therefore the cells of this well were collected and grown separately where the J558L cell line expressing equine IgE anti NIP-HSA was developed. Actual values for this graph are found in the Appendix.

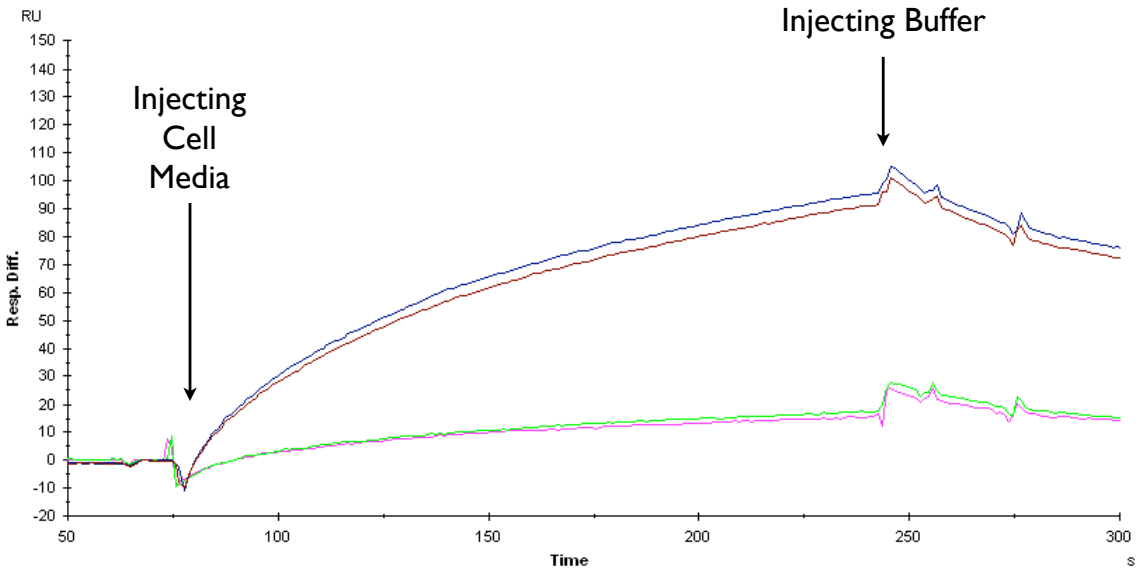


Figure 18: BB3 well SPR curve:
This figure shows the top actual SRP curve of the highest well expressing equine IgE anti NIP-HSA (well BB3 from Figure 17). The bottom curve is the control media from non-transfected cells and some other selected cells that are not expressing the antibody. The values for Figure 14 came from the average of the difference between the bottom point and the top point for the two SRP well curves.

3.2.5 - Collecting And Purifying The Equine IgE Anti NIP-HSA:

A density of $5x10^5$ cells ml^{-1} of the J558L cells expressing equine IgE anti NIP-HSA was plated on a $100mm^2$ petri dish with 20ml of selective media, then incubated for 5 days at 37°C + 5% CO2 + 90% relative humidity as in chapter 2.4.3. This was found to be the optimal cell density and length of time for expression, where the expressed protein accumulated in the media, but not left long enough for it to start degrading. Figure 19 shows that level of IgE relative to incubation time.

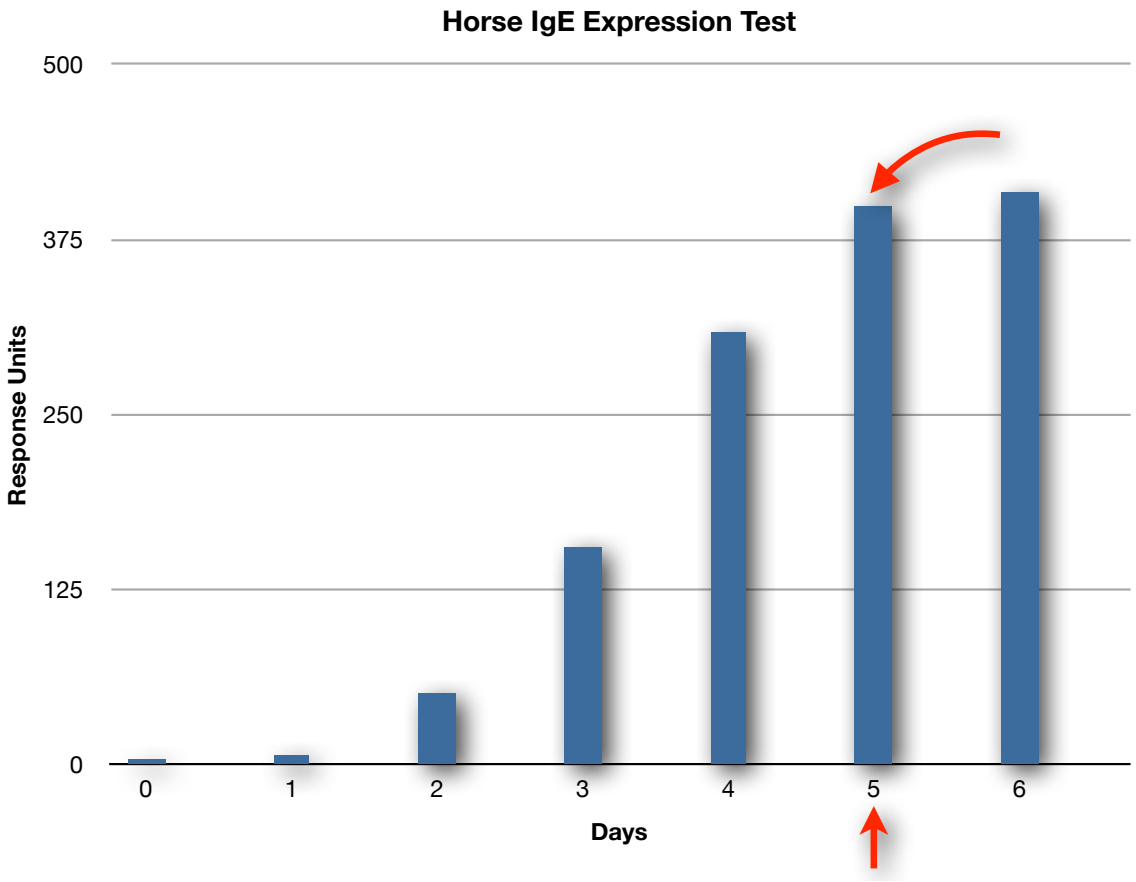


Figure 19: SPR results of the level of IgE against expression time:
This figure is shows the increase in the level of IgE in the cell media against the incubation time. 5 days of expression (marked by the red arrows) was found to be the ideal expression time for this cell line where the level of IgE was at its highest before it started to degrade. Therefore the cell supernatant media was harvested after 5 days of expression.

IgE was expressed in 200ml of media was expressed and collected by centrifuging at 180xg for 3 minutes to remove the cells, then the media was filter sterilized through a 0.45µm filter to remove all cells and debris to prevent dead cell proteases from degrading the IgE. Due to the large volume of the media, it was concentrated using a 3kDa molecular filter to reduce the volume. Figure 20 shows the results of the level of IgE after media concentration, which corresponds to ~3600 resonance units. Values for this graph are in the Appendix. The concentrated media was purified through a chromatography column as in chapter 2.4.5.

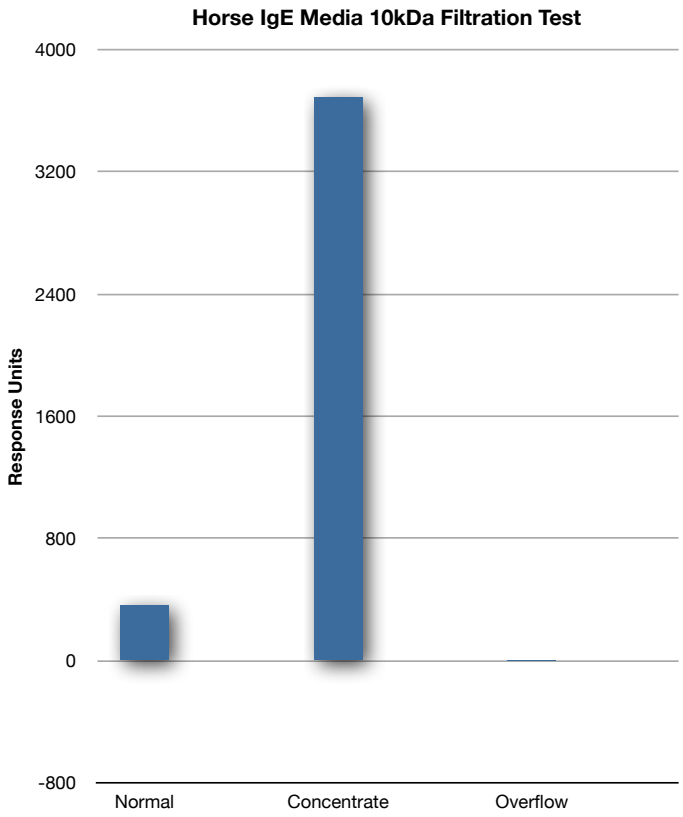


Figure 20: SPR results of the level of IgE after media concentration:
This is the SRP result of the level of the IgE after the media was concentrated through a 3kDa molecular filter. Values for this graph are in the Appendix.

3.2.6 - Checking Equine IgE Anti NIP-HSA Viability And Purity:

The purified equine IgE anti NIP-HSA was concentrated down to ~200µl, using a 3kDa molecular filter, and its concentration was found by using a NanoDrop (Thermo Scientific) spectrophotometer, where the OD_{280} of a 1µl of the protein was measured and the weight extension coefficient of IgE (1.62) used to find the final concentration of the protein, in this case it was 3.64 mg ml^{-1}. The following equation was used to measure the concentration:

$$\left(\frac{OD_{280}}{\text{Weight Extension Coefficient}}\right) \times \text{Dilution Factor} = \text{Concentration mg ml}^{-1}$$

The equine IgE anti NIP-HSA was then tested in an SDS-PAGE for its purity (Figure 21). The SDS-PAGE result showed that the non-reduced IgE had a single strong back band above the level of the ladder as the IgE size was ~192kDa, it also showed very little IgE degradation by the very faint lines under the IgE band, and almost no albumin impurity, which would have shown a band at 65kDa. The equine IgE was also reduced, using β-mercaptoethanol, to break the disulphide bridges between the two heavy chains and between the heavy chain and the light chain, and this resulted in two bands at ~70kDa for the heavy chain, and ~25kDa for the light chain as predicted.

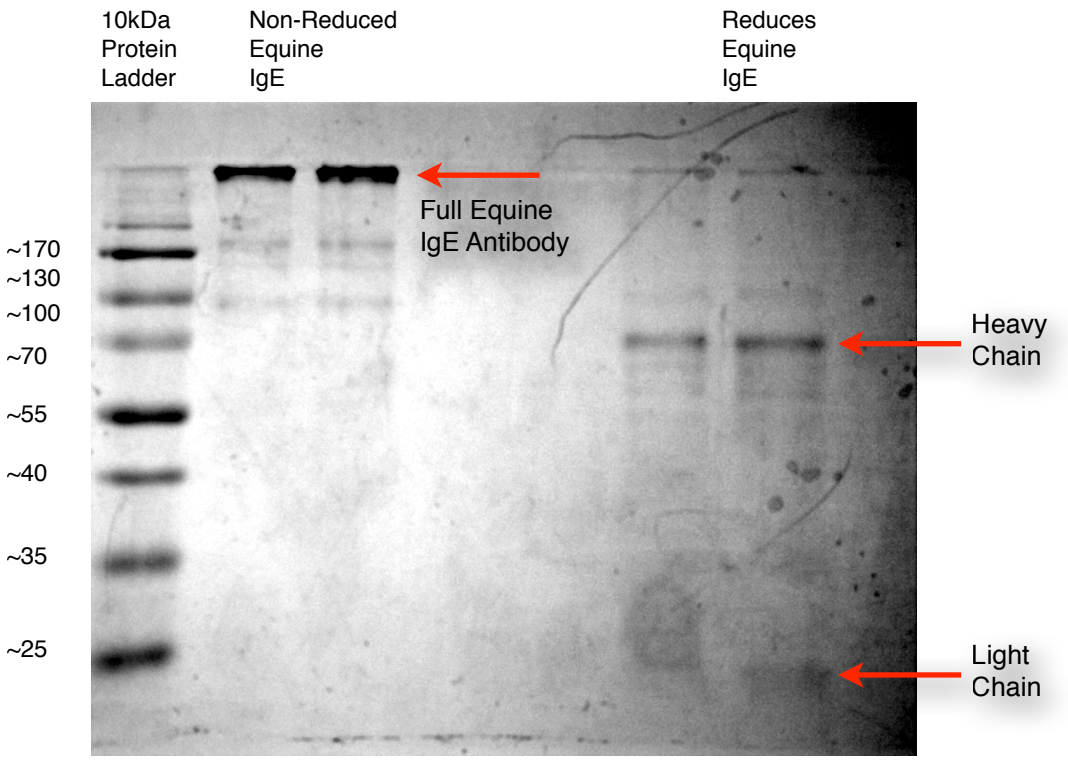


Figure 21: SDS-PAGE of the equine IgE anti NIP-HSA: SDS-PAGE analysis of the equine IgE anti NIP-HSA, on the left is the non-reduced IgE showing a single strong band above the ladder as the IgE size is ~192kDa, the result also show very little IgE degradation by the very faint lines under the IgE band, it also show almost no albumin impurity, which would show a band at 65kDa. On the right is the reduced equine IgE anti NIP-HSA, by β-mercaptoethanol, therefore the disulphide bridges between the two heavy chains and between the heavy chain and the light chain are broken, therefore the top band shows the heavy chain as predicted at ~70kDa, and the bottom line very faintly showing the light chain at ~25kDa as predicted.

The antibody was then tested for viability using SPR, Figure 22 shows the result of the test with a large curve confirming that the antibody recognizes NIP-HSA. It also shows no protein loss during the concentration process.

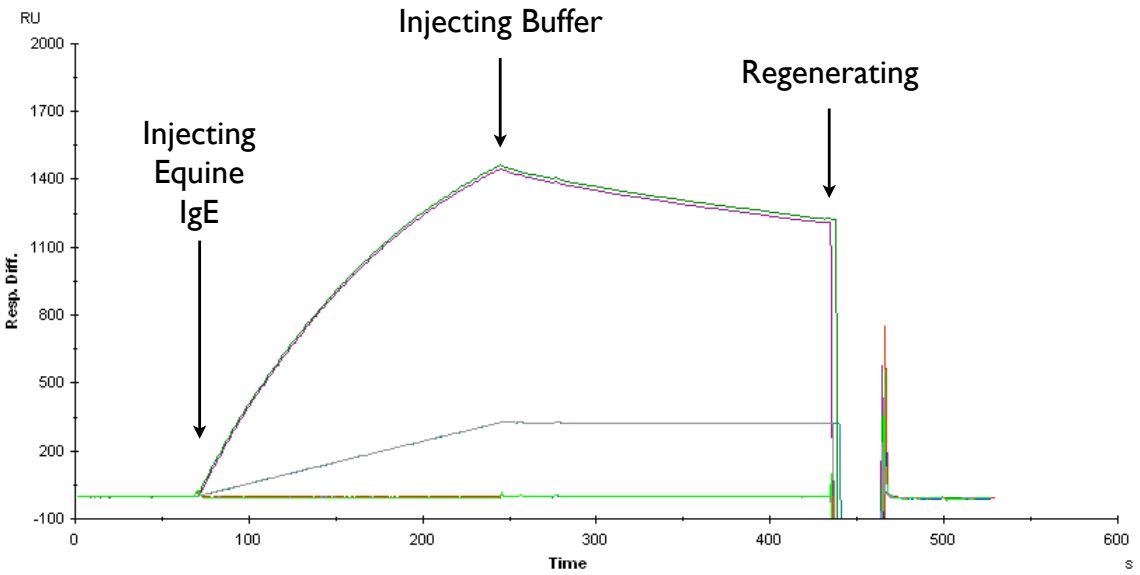


Figure 22: Equine IgE anti NIP-HSA viability test:
This figure is showing the curve of the purified equine IgE anti NIP-HSA. The bottom curve is the overflow from the concentrated purified IgE, the middle curve is the non-concentrated IgE and the top curve is the concentrated IgE. This figure showed no protein loss during the concentration process, it also confirmed that the antibody recognizes its cognate antigen NIP-HSA.

3.3 - Discussion:

This experiment was successful in producing a cell line that expressed equine IgE anti NIP-HSA, purification of the antibody and confirmed its viability in preparation for the next set of experiments. This experiment was necessary as equine IgE anti NIP-HSA was not available commercially. Some shortcuts and efficiencies were also realized while running this experiment.

First of all it was realized that not concentrating the expressed cell media down to a small volume yielded more IgE from the chromatography column, this was because concentrating the media concentrated nearly all the other serum proteins, therefore the washing process did not effectively remove all protein impurities, this should be noted in future antibody purifications by this or similar protocols. The column used was 2cm long, exactly as the protocol stated, this resulted is a large quantity of IgE to be

collected, this is an important note, as in chapter 5 and 7 a larger 12cm column was used and proved to be very inefficient, as the flow rate through it was very slow that it did not remove enough of the protein impurities, and did not elute enough of the protein of interest. It was also realized that using the NanoDrop spectrophotometer did not result in an accurate measurement of proteins, it was better suited for DNA quantification. Therefore the protocol from chapter 2.4.6 was used in later protein quantifications.

Ideally a western blot would have been used to confirm that the antibody was indeed equine IgE, but this was not possible as commercial anti-equine IgE antibodies were not found. An indirect way could have been used where anti mouse λ chain antibodies would bind to the antibody's variable region, but since all the antibodies synthesized in the lab had this chain, this would not have proved anything. A mouse anti-cat IgE antibody was purchased from (Serotec) where the manufacturer stated it might cross react with equine IgE, but when tested by SPR this was shown to be not true and there was no cross reaction, therefore mouse anti-cat IgE antibody would not have been successful in preforming a western blot. The only way the equine IgE anti NIP-HSA identity was confirmed was later on in the project when the kinetic bindings were run and the equine sFcεRIαD1&2 receptor bound to the equine IgE with the highest binding affinity (see Chapter 6).

During the molecular biology, where the plasmids were developed, it was realized that ligating DNA failed when the digested genes were ethanol precipitated after being extracted from agarose gels. This was because the salts from the TAE buffer also precipitated which to a high concentration. Therefore when the final DNA bands were added to the ligation master mix, the T4 DNA ligase enzyme got denatured, and thus the DNA did not ligate, resulting in the cloning process failing. This happened to all the

DNA cloning steps in the following chapters. The problem was overcame by purchasing the Wizard® SV Gel And PCR Clean-Up System (Promega), which purified small DNA strands by binding affinity, ensuring all eluted DNA is in pure de-ionized distilled water. Furthermore, the ethanol precipitation protocol was used later on just to concentrate DNA from samples that had DNA in pure de-ionized distilled water.

A problem was faced when the 1/2 human intron was inserted. Introns have a start sequence of GT and an end sequence of AG, and since the first half of the intron, which is included in the plasmid, was not sequenced, the second half of the intron had to be guessed. Therefore a normal 1/2 human intron was inserted with only the ending sequence of AG.

It was later realized that in humans the G of the AG ending sequence was the first nucleotide of the codon of the amino acid. Thus when the intron was removed, it removed the G from the next condon, but it was replaced by the G behind the starting intron code, therefore in the human wild type IgE gene, the introns are removed normally with no problems. This was not realized, and therefore when the 1/2 human intron was inserted, the ending sequence was AG, i,e: there was an extra G between the intron ending sequence and the first nucleotide of the next codon. This caused a frame shift in the final mRNA sequence and thus did not code for a full equine IgE anti NIP-HSA. This problem was fixed by removing the extra G my point mutation as in chapter 2.2.17, (Figure 23). The primers used were:

```
Forward Primer: 5'-GGTTCTGTCCTCACAGTGAGCAAGCAGGCC-'3
Reverse Primer: 5'-GGCCTGCTTGCTCACTGTGAGGACAGAACC-'3
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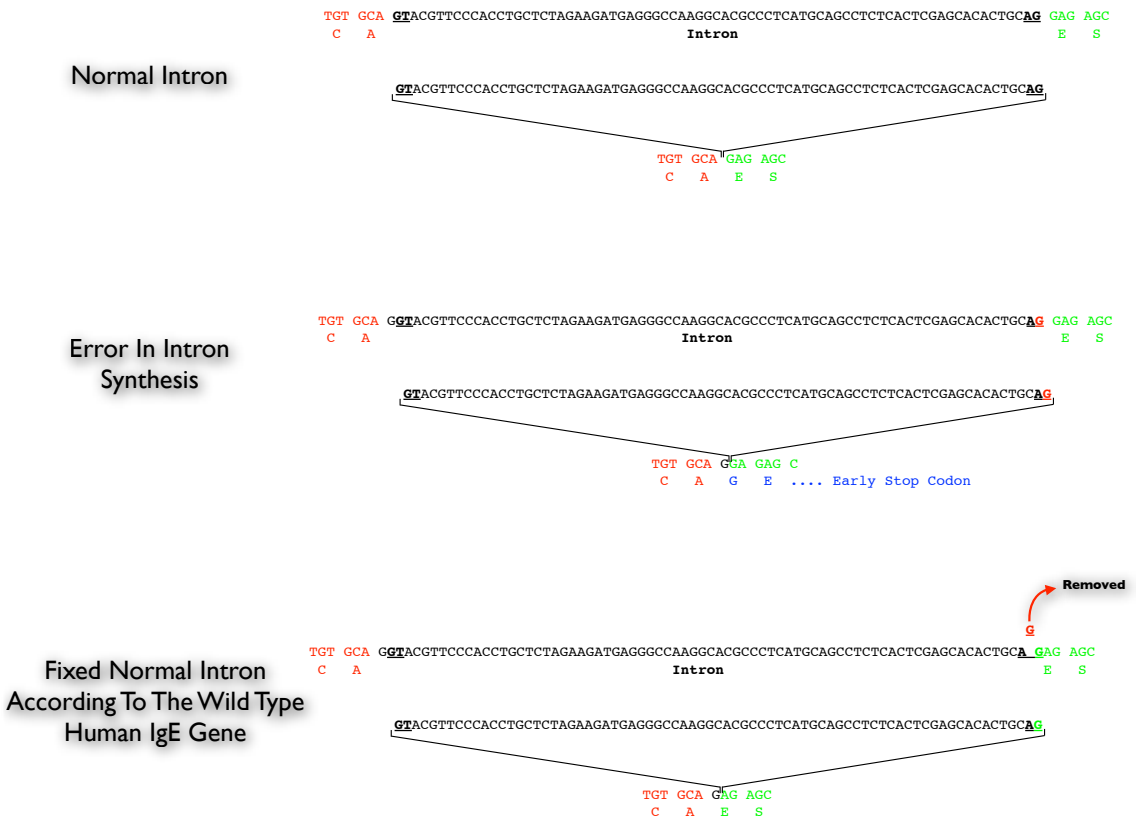

Figure 23: Intron problem and how it was solved:
This figure shows the configuration of a normal human intron, and the human intron found in the wild type IgE gene. This caused a problem when the gene was constructed as an extra G was replaced, since the first half of the human intron was in the plasmid and not sequenced. This was solved by deleting the extra G.

Chapter 4 - Generation of an RBL-2H3.1 Cell Line Expressing Equine FcεRIα:

4.1 - Introduction:

A cell based assay called the β-hexosaminidase release assay (very similar to ELISA) tested the effect of IgE on cell degranulation when it bound to its FcεRI receptor and subsequently challenged with a cognate antigen (allergen). The assay was preformed on rat basophilic leukemia (RBL-2H3.1) cells, this represented and accepted a model system to study IgE mediated degranulation of cellular mediators, which occurs in both mast cells (in the tissue) and basophil cells (in the blood). RBL-2H3.1 cells responses to IgE and antigen provide a key assay representative of the cell biology of allergy in the blood and tissue. As described in chapter 2.4.9 β-hexosaminidase is an enzyme that is released with the mediators, along with histamine and IL-4, during IgE assisted cell degranulation. This works in our favor as the enzyme catalyses a reaction leading to a yellow product that can be measured by a spectrophotometer and the quantity of mediators released can be determined.

For this assay to be used in this project, RBL-2H3.1 cells were transfected with the gene encoding the equine FcεRI receptor's α chain, this chain was translocated into the cell membrane, where it was coupled with the endogenous rat β and two γ chains to make a fully functional FcεRI chimeric receptor capable of binding equine IgE mediated transmembrane signaling via the transfected ligand binding domain. This enabled the investigation of cell degranulation by equine IgE in RBL-2H3.1, and therefore the behavior of IgE and the FcεRI receptor in the equine model was determined.

The equine FcεRI receptor's α chain protein sequence was determined from (McAleese, *et al.*, 2000) using the GenBank sequence database (part of the United States' National Center for Biotechnology Information, NCBI), which then had its DNA codons optimized (changed) for rat codons (*Rattus norvegicus*) and yeast (*Pichia pastoris*) to increase the efficiency of protein expression in rat cells, and also yeast cells for the experiment in chapter 5. The optimized gene was constructed digitally and sent to the company (GenScript) which synthesized the gene (Appendix). The equine FcεRIα gene was then cloned into a plasmid and transfected into RBL-2H3.1 cells which expressed the receptor on its surface.

4.2 - Results:

4.2.1 - Optimizing The Equine FcεRIα Gene:

The equine FcεRI receptor's α chain had been previously published (McAleese, *et al.*, 2000), and the protein sequence is included in the Appendix. This protein sequence was used to construct a gene optimized for the rat (*Rattus norvegicus*) and and the yeast (*Pichia pastoris*) as outlined in chapter 2.2.1. The final optimized DNA sequence used had a TAA ending sequence added along with two restriction sites, HindII at the start and EcoRI at the end, which allowed for cloning into the pEE6 plasmid.

4.2.2 - Cloning The Equine FcεRIα Gene Into Plasmid:

As discussed in chapter 2.2.4 the vector used for this experiment was pEE6 plasmid (sequence in Appendix). Form chapter 3.2.1 the synthesized FcεRIα gene was delivered in the plasmid pUC57 (which had characteristics similar to that of pUC19) and was transformed into E.coli TOP 10 bacteria. Therefore the bacteria were grown as in chapter 2.2.3 and the cells harvested, followed by purifying the pUC57-FcεRIα plasmid as in chapter 2.2.5.

The pUC57-FcεRIα plasmid was digested with HindIII and EcoRI to remove the FcεRIα gene, the plasmid pEE6-CD23α plasmid was also digested with HindIII and EcoRI to remove the CD23α gene and thus allowed the FcεRIα gene to replace it. The restriction digestion was done as in chapter 2.2.7 followed by de-phosphorylation of the pEE6 plasmid to prevent it from self ligating, but this was not really necessary as both ends of the plasmid did not have the same restriction sites. Figure 24A shows the restriction digestion results.

The DNA bands of interest were analyzed on an electrophoresis gel as in chapter 2.2.8 and then isolated as in chapter 2.2.9 and ligated together as in chapter 2.2.10. The resulting ligation mixture was transformed into bacterial cells XL-1 Blue as in chapter 2.2.11 and the cells were spread on a plate as in chapter 2.2.3 using the media from chapter 2.2.2. The colonies that grew after the 16 hour incubation were the transformed colonies with the pEE6-FcεRIα plasmid. Therefore six colonies were isolated, grown, harvested and the pEE6-FcεRIα plasmids purified to check for the successful insertion of the gene. Figure 24B shows the gene insertion, and Figure 25 shows the structure of the pEE6 plasmid before and after the FcεRIα insert. The gene and plasmid sizes was calculated to check for the presence of the gene, were the pEE6 plasmid on its own had a size of 6583bp, the CD23α gene had a size of 1200bp, the pUC57 plasmid had a size of 2710bp and the FcεRIα had a size of 780bp.

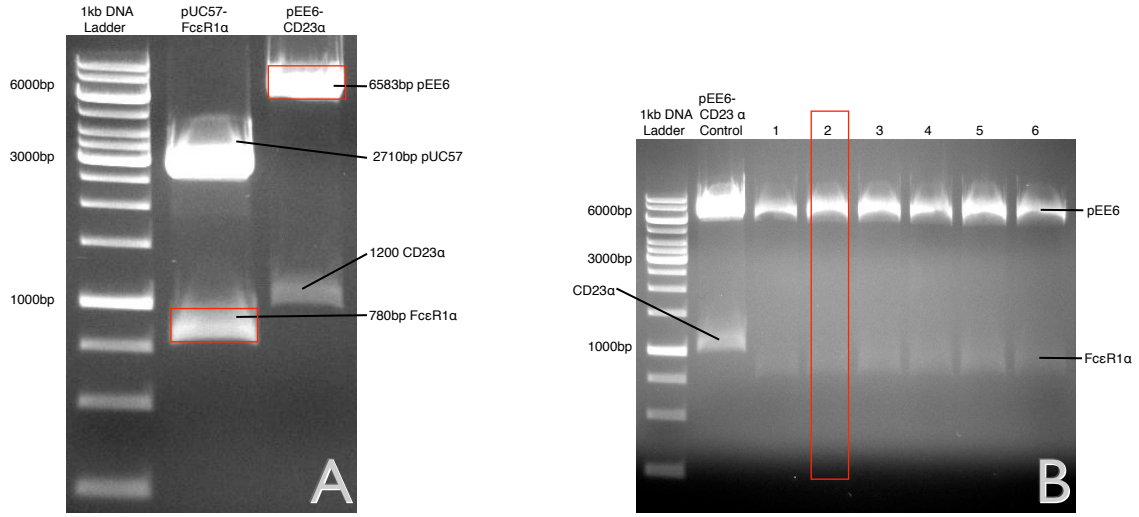

Figure 24: Restriction digestion of pUC57-FcεRIα snd pEE6-CD23α:
A. the plasmids were digested with HindIII and EcoRI followed by de-phosphorylation. This was done in preparation to clone the FcεRIα gene into the pEE6 plasmid. The pEE6 plasmid had a size of 6583bp and the FcεRIα gene had a size of 780bp **B.** shows the successful insertion of the FcεRIα gene into all of the isolated pEE6 plasmids where they were digested with HindIII and EcoRI.

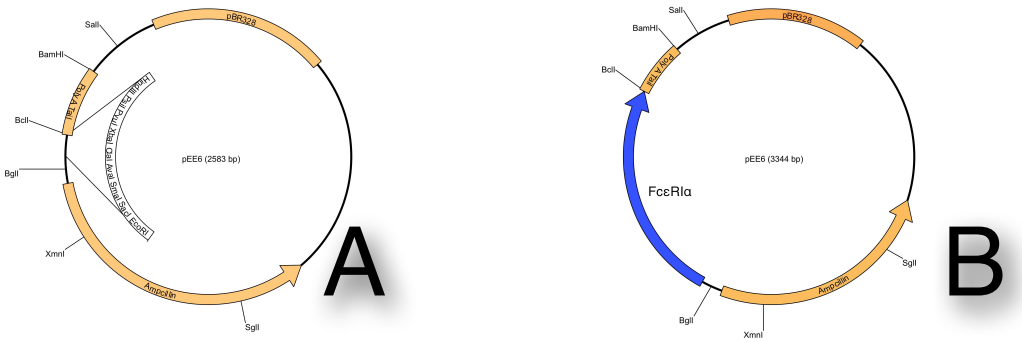

Figure 25: The structure of the pEE6 plasmid before and after the insertion of the FcεRIα gene:
A. shows the structure of the pEE6 plasmid (without the CD23α gene) while **B.** shows the final structure of the pEE6-FcεRIα plasmid.

Once the bacterial colony with the pEE6-FcεRIα was identified and a backup culture was frozen as in chapter 2.2.12, it was grown and the pEE6-FcεRIα plasmid harvested in a larger concentration as in chapter 2.2.13 followed by concentrating the

plasmid DNA as in chapter 2.2.15 and the DNA quantified as in chapter 2.2.14 which enabled the required concentration adjusted for mammalian cell transfection.

4.2.3 - Transfecting RBL-2H3.1 Cells With Equine FcεRIα Gene:

The pEE6-FcεRIα plasmid was transfected into RBL-2H3.1 cells as in chapter 2.3.5. The cells were then plated onto two 100mm^2 dishes. A control was setup by replacing the plasmid DNA with water and running the experiment normally, this was plated out on to a separate dish.

The first 48 hours after transfection the cells were grown on normal media to allow the transfected cells to express the relevant genes. The media was then replaced with selective media and the cells selected as in chapter 2.3.5.

4.2.4 - Selecting And Sorting Equine FcεRIα Expressing RBL-2H3.1 Cells:

The transfected RBL-2H3.1 cells were selected as in chapter 2.3.5 using selective media (Dulbeco's Modified Eagle's media 500ml: 1000mg glucose + 10% FCS + 5000 units of penicillin + 50mg streptomycin + 0.4g geneticin G418 sulphate) and selecting for proximally 1-2 weeks, changing the media every 2 days where the cell population declined as the non-transfected cells died, then the population started to increase, but the cells were harvested early before the dish became confluent, because since the RBL-2H3.1 cells are a monolayer cell line, they can only form a certain colony size before the cells in the middle of the colony starts to die. Therefore the cells were harvested as in chapter 2.3.4 and sorter as in chapter 2.3.6. Figure 25 shows the FACS results, noting that the cells were sorted using canine IgE as the equine IgE was not synthesized at that time. The results show (Figure 26A) very few cells in the negative control (0.3% of the population) that are fluorescent (false auto florescence) these cells were not tagged with any antibody. The cells that were tagged with human IgE (Figure

26B) also showed to be negative as the florescent cells were only 0.7% of the population, therefore it was labeled as negative, these cells were tagged with human IgE - mouse IgG anti human IgE - anti mouse IgG antibody tagged with phycoerythrin. The cells that were tagged with canine IgE showed 1.3% of the population to be florescent positive cells, the rest of the population was transfected cells but not expressing the equine FcεRIα receptor. This percentage was high enough for it to be taken as the positive sample and thus the cells were sorted from it (Figure 26C), these cells were tagged with canine IgE - mouse IgG anti canine IgE - anti mouse IgG antibody tagged with phycoerythrin. It was later realized that the human IgE does not bind to the equine FcεRI receptor, this explained why the human IgE tagged cells showed to be negative (Chapter 6).

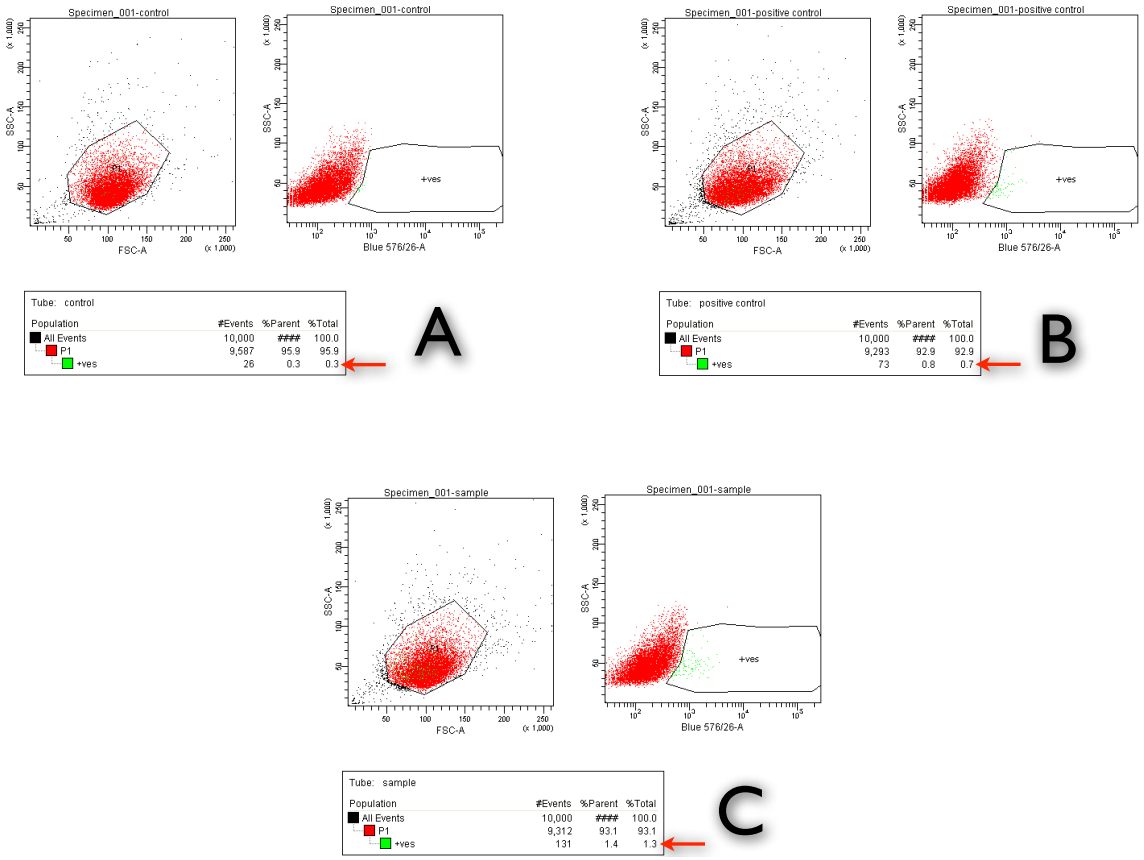

Figure 26: FACS results of RBL-2H3.1 cells transfected with pEE6-FcεRIα:
A. shows the FACS results of the negative control (not tagged with any IgE) showing 0.3% of the population with false auto florescence. The graph on the left is the side scatter against forward scatter graph with the tested population within the circle, and the graph on the right is the side scatter against florescence graph showing the selected population's florescence with the positive florescence within the selected box area. Therefore the 0.3% is the percentage of cells within box in the right graph compared to total number of cells within the total population selected form the left graph. **B.** Shows the FACS results of the cells tagged with human IgE, only 0.7% of the population was positive, therefore it was too little and was considered to be negative. **C.** shows the FACS results of the cells tagged with canine IgE, 1.3% of the population was positive, therefore the positive cells from this sample were sorted and collected, and when grown resulted in an RBL-2H3.1 cell line that expressed equine FcεRIα receptor on its surface.

The sorted cells gave ~10,000 cells back, which were grown up to develop an RBL-2H3.1 cell line that expressed the equine FcεRIα chain. This cell line was tested before the release assays to ensure that the entire population was expressing the receptor

instead of the cells being resistant but not expressing the receptor, which is tends to happen in RBL-2H3.1 cells if they are left in culture for a prolonged period. Figure 27 shows the test results, here the cells were tested with equine IgE tagged with allophycocyanin (APC), and the test showed that almost the entire cell population expressed the equine FcεRIα receptor as there was a complete shift in cell population during the tested between the control and the florescent test.

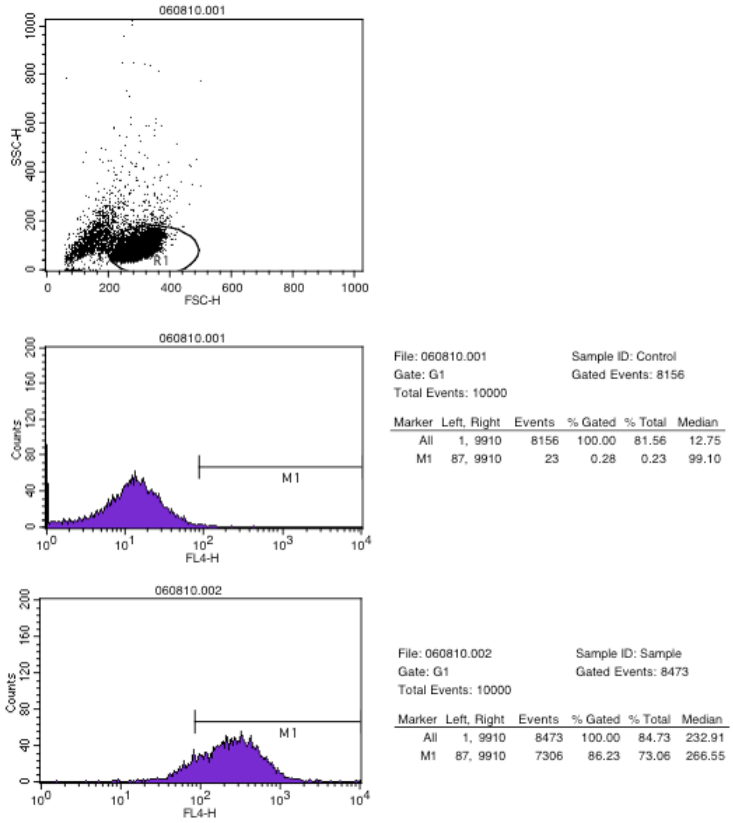

Figure 27: Flow cytometry test of RBL-2H3.1 cells expressing equine FcεRIα:
The top graph shows the side scatter against forward scatter of the cells that represent the cell size and granularity, the circled population of cells are the healthy cells that which the florescence was tested. The middle graph (cell count against florescence) shows the non-florescence of the circled population. The bottom graph (cell count against florescence) shows the florescence of the same circled population. As can be seen almost the entire population shifts from non-florescence to florescence, indicating that almost the entire cell population is expressing the equine FcεRIα receptor subunit. The cell population not circled in the top graph are dead cells due to the preparation process.

To confirm this further, the cells were visualized under a confocal microscope, Figure 28 shows the result of the difference between the control (Figure 28A and B) and the tagged cells (Figure 28B and C).

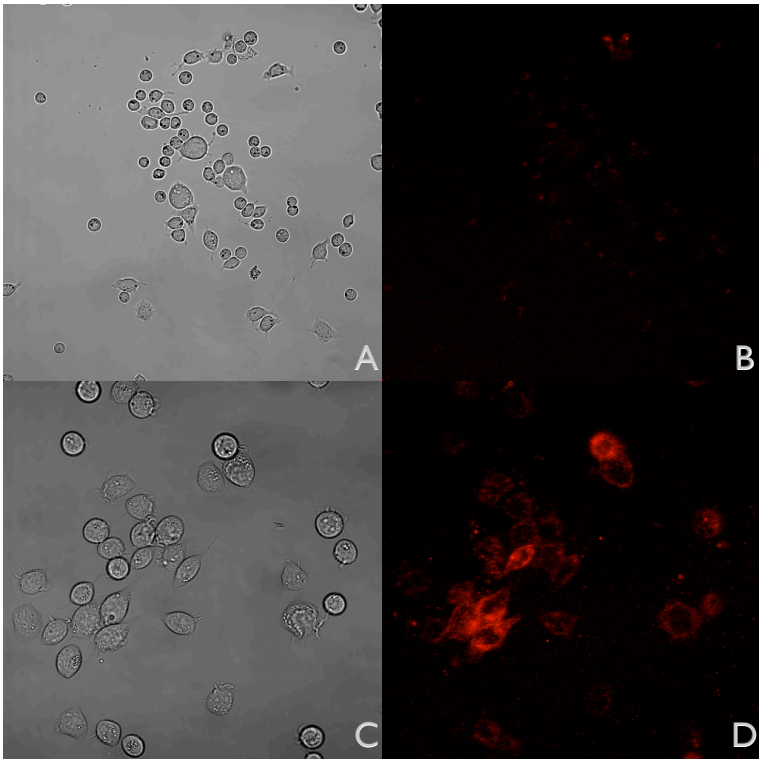

Figure 28: Confocal image of RBL-2H3.1 cells expressing equine FcεRIα:
A. is the figure of the control sample of cells that were not tagged with florescent equine IgE under white light. **B.** is the same cell under 650 nm showing no florescence. **C.** is the positive sample cells tagged with florescent equine IgE under white light. **D.** is the same cell under 650 nm showing florescence.

4.2.5 - β-hexosaminidase Release Assays:

The cells were assayed as in chapter 2.4.9. Figure 29 shows the main β-hexosaminidase release assay data where the RBL-2H3.1 cells expressing equine FcεRIα were tested, using non-transfected RBL-2H3.1 cells as a control, the data for this graph is found in the Appendix, and Figure 30 shows the relative maximum release (at 100ng ml^{-1} of antigen) between the different tests. These cells were tested with mouse, equine, human and canine IgEs to compare equine IgE mediated cell degranulation to them, and also to test how related and similar this is to human and canine IgE mediated release. All positive releases showed the expected sigmoid curve where cells challenged with little antigen concentration would release little mediators,

as there is only little receptor aggregation on the cell surface, but on the other end of the graph, when cells are challenged with a higher antigens concentration, the cells would release little mediators, due to the fact the the receptors would aggregate so much forming a cap on the cell surface, forming a polarized cell, which is not efficient in causing a downward signal cascade. Therefore the maximum release by the cells is in the middle of the graph around 100ng ml^{-1} of antigen, this concentration allows for a uniform distribution of receptor aggregation on the cell surface causing the most efficient downward signal cascade and therefore resulting in the highest mediator release.

The results showed that non-transfected (RBL-parental) cells when tested with mouse IgE anti DNP-HSA showed a maximum release of 51.55% at 100ng ml^{-1} of antigen. This was the control experiment. Transfected cells (RBL-pEE6) tested with mouse IgE anti DNP-HSA showed a similar maximum release of 45.99% at 100ng ml^{-1} of antigen, thus confirming that the cells positively released as the mouse IgE bound to the endogenous rat receptor on the cell surface. When the non-transfected cells were tested with equine IgE anti NIP-HSA a maximum release of only 11.81% at 100ng ml^{-1} of antigen, this was the negative control, as equine IgE was thought not to bind to the rat receptor, and thus showed so due to the very low release.

On the other hand, transfected cells expressing the equine FcεRIα showed a maximum release of 36.68% at 100ng ml^{-1} of antigen when tested with equine IgE anti-NIP-HSA, and therefore showed the successful expression of the receptor and its viable function in causing cells to degranulate. It also showed that the IgE binding to its receptor causes high release, as it was close to the positive control maximum release,

but smaller than the controls as the receptor is competing with the endogenous receptor on the cell receptor and thus its number is small.

The cells were then tested for similarity with human IgE-mediated release, and therefore non-transfected cells were tested with human IgE anti NIP-HSA which showed a flat line with only 7.97% release at 100ng ml^{-1} of antigen, and thus this was the negative control. Transfected cells expressing the equine FcεRIα also showed 8.88% release at 100ng ml^{-1} of antigen. This concluded that the human IgE does not bind to the equine FcεRIα, and thus the human and equine protein sequences are different enough as not to interact with each other. This point is discussed further in the discussion since it was not expected.

The cells were then tested for similarity with canine allergy, and therefore non-transfected cells were tested with canine IgE anti NIP-HSA which showed only 11.50% release at 100ng ml^{-1} of antigen and thus this was the negative control. But transfected cells expressing equine FcεRIα showed 32% release at 100ng ml^{-1} of antigen when sensitized with canine IgE. This concludes that the canine IgE binds to the equine FcεRIα strong enough to cause release, which is almost the same as the equine IgE when bound to the FcεRIα.

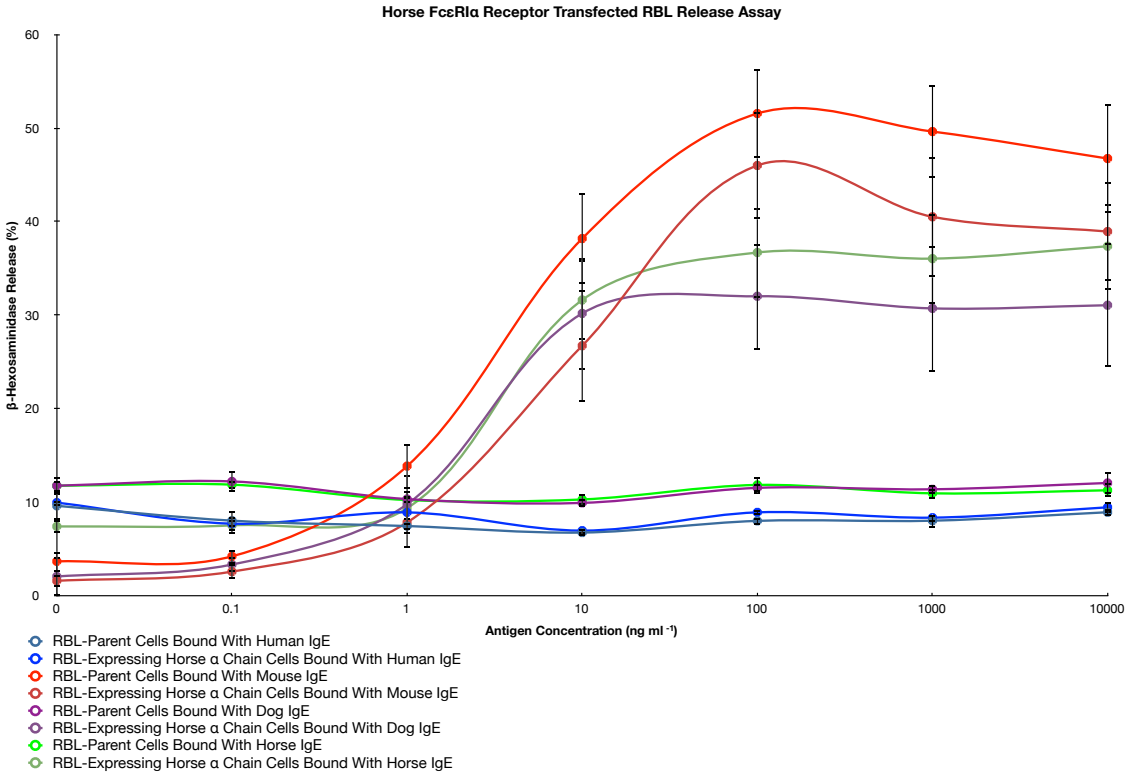

Figure 29: β-hexosaminidase release assays of RBL-2H3.1 cells expressing equine FcεRIα:
This figure shows the release assays of the RBL-2H3.1 cells expressing equine FcεRIα and non-transfected cells (as a control) when tested with mouse,equine, human and canine IgEs. When both cell lines were tested with mouse IgE the cells responded by releasing mediators: maximum 51.55% for non-transfected and 45.99% for transfected cells at 100ng ml^{-1} of antigen, this was the positive control as the mouse IgE binds to the endogenous rat receptor. When tested with equine IgE the non-transfected cells had a maximum release of only 11.81% at 100ng ml^{-1} of antigen, while transfected cells expressing the equine FcεRIα had a maximum release of 36.68% at 100ng ml^{-1} of antigen. When the cells were tested with human IgE both cell lines had very low release, maximum release at 100ng ml^{-1} of antigen of 7.97% for non-transfected cells and 8.88% for transfected cells expressing the equine FcεRIα. When the cells were tested with canine IgE non-transfected cells had a maximum release of 11.50% at 100ng ml^{-1} of antigen, while the transfected cells had a maximum release of 32.00% at 100ng ml^{-1} of antigen. Values for this graph are in the Appendix.

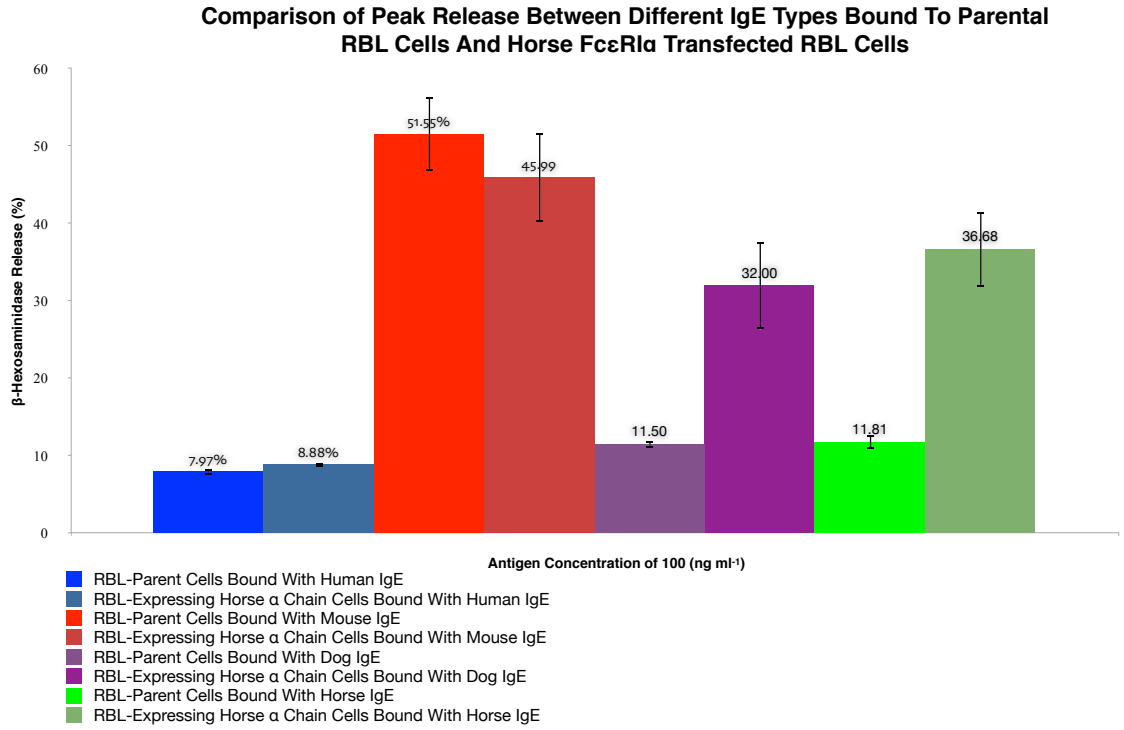

Figure 30: Summary graph of Figure 29's peak release at 100ng ml^{-1} of antigen:
This figure shows only the peak releases at 100ng ml^{-1} of antigen of the cells from figure 29.

Since the canine IgE was found to bind to the equine FcεRIα, the opposite was tested; if the equine IgE binds to the canine FcεRIα. When cells were sensitized with IgE for 16 hours the cells showed no release, while the positive control showed normal release, therefore the cells were tested for 0.5, 1 and 3 hour IgE sensitization times (Figure 31, with the Summary of the peak releases at 100ng ml^{-1} of antigen in Figure 32) and the following was observed:

The equine IgE interacts with the canine FcεRIα but very weakly, as increasing interaction time (0.5, 1 and 3 hours) resulted in no significant higher releases than the negative control (no NIP-HSA antigen added), even when incubated for the full 16 hour. Therefore this result suggests that the equine IgE doe not interacts with the canine FcεRIα. When compared to the mouse IgE control, the increasing interaction time (0.5, 1 and 3 hours) resulted similar non-significant releases, but when incubated for the full

16 hours, the maximum release was 42.42% i.e: very high. The cells were also tested with canine IgE at 16 hours (43.49% maximum release) as a control, since these cells were tested in a similar manner by (Hongtu Ye, PhD Thesis, The University of Sheffield, 2010).

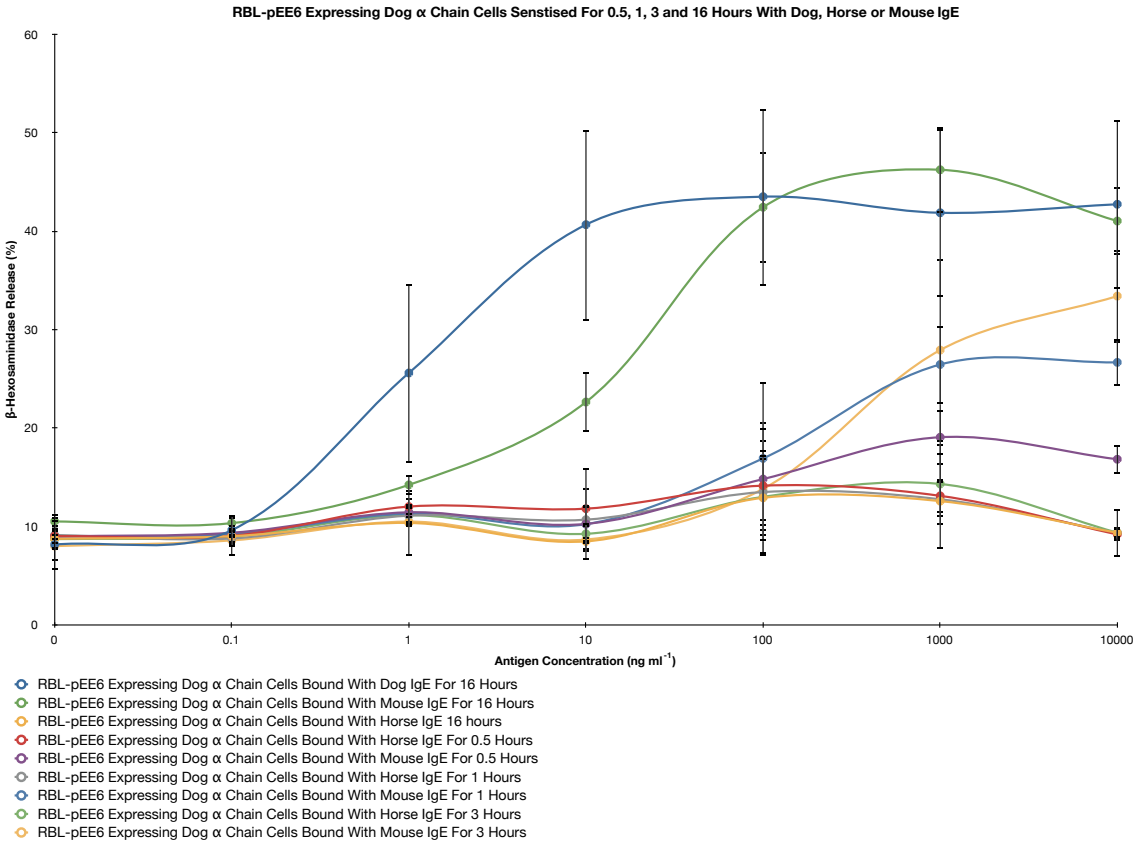

Figure 31: β-hexosaminidase release assays of RBL-2H3.1 cells expressing canine FcεRIα tested with equine IgE:
This figure shows the release assays run on RBL-2H3.1 cells expressing canine FcεRIα sensitized with equine IgE, using mouse and canine IgEs as the control. From this graph it can be seen that the equine IgE does not interacts with the canine FcεRIα, as increasing interaction time (0.5, 1 and 3 hours) resulted in no significant releases, even when incubated for full time (16 hours), compared to the negative control. When these results were compared to the mouse and canine IgE controls, the increasing interaction time (0.5, 1 and 3 hours) resulted in similar non-significant releases, but when incubated for the full 16 hours, the maximum release was very high (42.42% when tested with mouse IgE and 43.49% when tested with canine IgE). Values for this graph are in the Appendix.

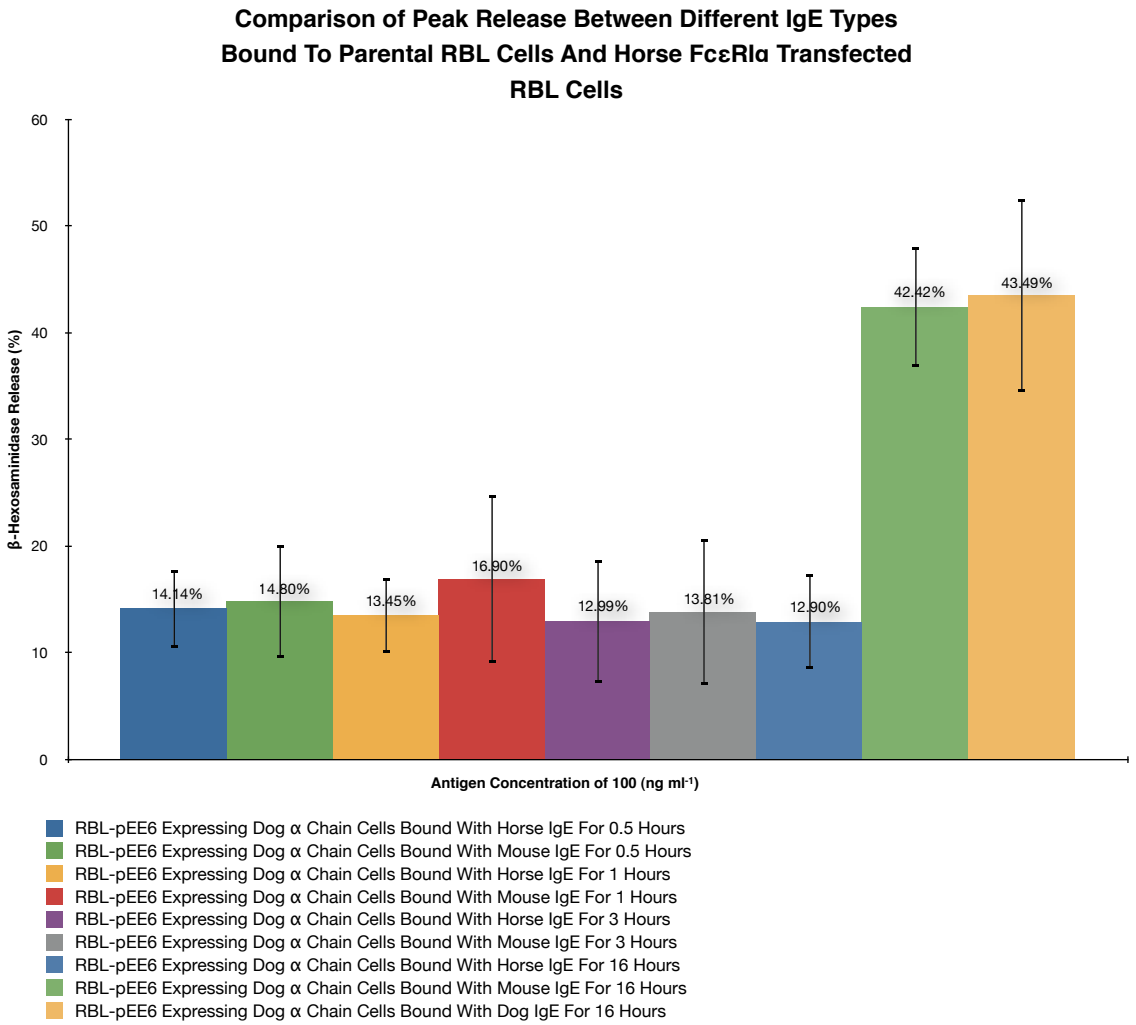

Figure 32: Summary graph of Figure 30's peak release at 100ng ml^{-1} of antigen:
This figure shows only the peak releases at 100ng ml^{-1} of antigen of the cells from figure 31.

4.3 - Discussion:

In conclusion, the cell cloning and cell line development was successful. One point to note is that RBL-2H3.1 cells tend to shift to a non-releasing phenotype if they were maintained in culture for a long time (3-4 months). Therefore a large stock of non-transfected cells that were tested for excellent release, and were free from *Mycoplasma* bacterial infection (which disrupts cell metabolism and release) was frozen and stored. Then from this stock a sample was taken, thawed and quickly transfected with the plasmid. Once the cells were selected, sorted and grown to develop a new cell line, this cell line was again tested for adequate release and infections then a large stock of it was

frozen, in case the cells in culture shift to the non-releasing phenotype, there was plenty of cell stock to complete the experiments.

The release assays tested the mediator release from cells expressing equine FcεRIα when sensitized with equine IgE and challenged with an antigen. The peak release at 100ng ml^{-1} of antigen was 36.68% compared to the control of 45.99% (using mouse IgE on the same cell line) and the negative control was 11.81% (using non-transfected cell with equine IgE). This was within the normal peak release of mediators of these cells under these conditions, and this proved that the equine allergy behaves, *in-vitro*, in a similar manner to that of human and canine IgE-mediated cell degranulation, where they have a peak release at 100ng ml^{-1} of antigen at ~40% (Jonathan E. M. Housden, PhD Thesis, The University of Sheffield, 2007) and ~36% (Athanassios P. Vratimos, PhD Thesis, The University of Sheffield, 2003) respectively.

The RBL-2H3.1 cells are very sensitive to the environment, therefore they tend to release some mediators when they are stressed, such as exposed to a cold environment or the liquid on top of them was pipetted a bit strong. This explains the reason behind the high negative control values, here they are around 10% while they ideally should be around 5%. Nonetheless, the difference between the phenomena tested here is significant enough that this error did not interfere with it.

From the FACS results it can be seen that the human IgE does not bind to the equine FcεRI receptor while the canine IgE does, therefore these IgEs were tested on the RBL-2H3.1 expressing equine FcεRIα cell line. The results showed that is human IgE does not cause any release on the cells, which confirmed that the human IgE does not bind to the equine FcεRI receptor. On the other hand, the canine IgE caused release in the cells with a peak release at 100ng ml^{-1} of antigen at 32.00%, therefore the canine

IgE does bind to the equine FcεRI receptor and causes cells to release mediators. This came as a surprise, and later experiments (Chapter 8) showed indications that the human IgE might be defective, since anti IgE antibodies cause the cells to release mediators, while the human IgE did not. Therefore this result is open to question.

Since the canine IgE binds to the equine FcεRI receptor, the opposite was tested, i,e: if the equine IgE binds to the canine FcεRI receptor. The results showed that no it does not, when tested for a shorter incubation time (0.5, 1 and 3 hours) the released mediator percentage of the peak release at 100ng ml^{-1} of antigen kept steady around 10%, which is the same level as the negative control, and finally when tested for the full 16 hours, there was no significant change in the level of released mediators. Therefore it was determined that the equine IgE does not bind to the canine FcεRI receptor. The positive controls for this experiment were around 40% (mouse and canine IgE).

One explanation of why only the cells would be sensitized enough and thus give off a full release after 16 hours, is because the binding of the IgE to the receptor enhances receptor synthesis which would bind more IgE and this would accumulate after 16 hours which would result in adequate mediator release

Chapter 5 - Generation of Soluble Equine FcεRIα:

5.1 - Introduction:

In preparation for next chapter's kinetic analysis between the equine IgE and the equine FcεRIα, the receptor had to be in solution to be analyzed by SPR, therefore the approach to this was to express only the extracellular domains of the receptor, without the transmembrane region, which would aggregate in aqueous solution. That is why the original equine FcεRIα sequence was codon optimized for both rat and yeast cells.

The equine FcεRI receptor's α chain protein sequence was determined from (McAleese. *et al,*, 2000) using the GenBank sequence database (part of the United States' National Center for Biotechnology Information, NCBI), which then had its DNA codons optimized (changed) for rat codon (*Rattus norvegicus*) and yeast (*Pichia pastoris*) to increase the efficiency of protein expression in rat cells and yeast cells. The optimized gene was constructed digitally and sent to the company (GenScript) which synthesized the gene (Appendix). The equine FcεRIα's extra cellular domains 1 and 2 (Figure 4) were then amplified by PCR and cloned into the pPIC9k plasmid (Invitrogen). The plasmid was then transfected into *Pichia pastoris* yeast cells which expressed the sFcεRIαD1&2 protein extracellularly and in to the media.

5.2 - Results:

5.2.1 - Optimizing The Equine FcεRIα Gene:

The equine FcεRI receptor's α chain was searched and found to be published in a paper by (McAleese, *et al.*, 2000), the protein sequence is included in the Appendix. This protein sequence was used to construct a gene optimized for the rat (*Rattus norvegicus*) and and the yeast (*Pichia pastoris*) as in chapter 2.1.1.

5.2.2 - Amplifying And Cloning The FcεRIα's Domain 1 And 2 Sequence Into Plasmid:

The FcεRIα gene from chapter 4 had its extracellular domains amplified by PCR as in chapter 2.2.6. There was not much freedom in designing the primers as they had to amplify a certain region of the gene, what was added to them was a six base overhang (Red), to allow for the restriction enzymes to bind, followed by a restriction site (Purple), the forward primer had EcoRI and the reverse had NotI, this was to allow for cloning of the PCR product into the pPIC9k plasmid. The reverse primer also had the ending sequence TGA (Black) added to terminate the gene. The resulting PCR product coded for a protein sequence found in the Appendix.

Forward: 5'-CCGCACGAATTCATGGTCCCAGCTGCTATTAGAAAGTC-3'
Reverse: 5'-GATCTTGCGGCCGCTCACAGGTAATCAGAAGTGTATC-3'

The pUC57-FcεRIα was amplified to give a PCR product of sFcεRIαD1&2 as in chapter 2.2.6, the PCR sample was then purified as in chapter 2.2.9 to give a pure sFcεRIαD1&2 PCR product. The sFcεRIαD1&2 was then digested with EcoRI and NotI along with the pPIC9k plasmid as in chapter 2.2.7. The DNA bands of interest were run on an electrophoresis gel as in chapter2.2.8 and then isolated as in chapter 2.2.9. The sFcεRIαD1&2 PCR product and the pPIC9k plasmid were ligated together as in chapter 2.2.10 and the ligation product was transformed into XL-1 Blue bacteria as in chapter 2.2.11 and the cells were spread on a plate as in chapter 2.2.3 using the media from chapter 2.2.2.. The colonies that resulted contained the pPIC9k-sFcεRIαD1&2 plasmid, therefore they were grown and the plasmid purified as in chapter 2.2.5 and digested again with EcoRI and NotI enzymes to check for the successful insertion of the sFcεRIαD1&2 gene. Figure 33A shows the restriction digest results with the pPIC9k

plasmid had a size of 9276bp and the sFcεRIαD1&2 PCR product had a size of 548bp, Figure 33B shows the successful insertion of the sFcεRIαD1&2 gene in to the pPIC9k plasmid and Figure 34 shows that pPIC9k plasmid before and after the insertion of the sFcεRIαD1&2 gene. The colony that contained the sFcεRIαD1&2 gene insert was sent for sequencing, as in chapter 2.2.16, to determine if any mutations occurred during the amplification process, Figure 35 showing the result of the sequence of the chosen colony, with not a single point mutation.

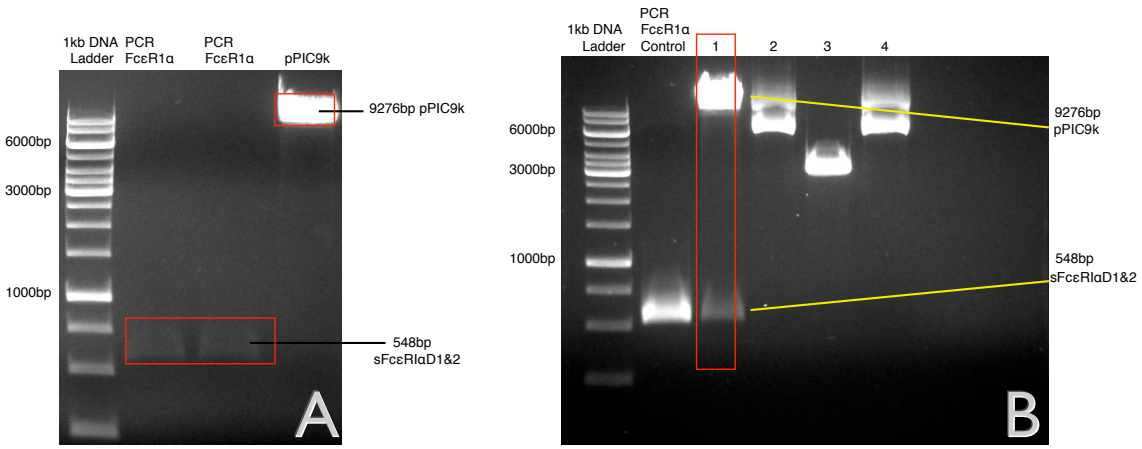

Figure 33: Restriction digestion of sFcεRIαD1&2 and the pPIC9k plasmid:
A. shows PCR product sFcεRIαD1&2 and the pPIC9k plasmid were digested with EcoRI and NotI restriction enzymes, this was done in preparation to clone the sFcεRIαD1&2 gene into the pPIC9k plasmid, the pPIC9k plasmid had a size of 9276bp and the sFcεRIαD1&2 PCR product had a size of 548bp **B.** shows the successful insertion of the sFcεRIαD1&2 gene into one of the isolated pPIC9k plasmids where they were digested with EcoRI and NotI.

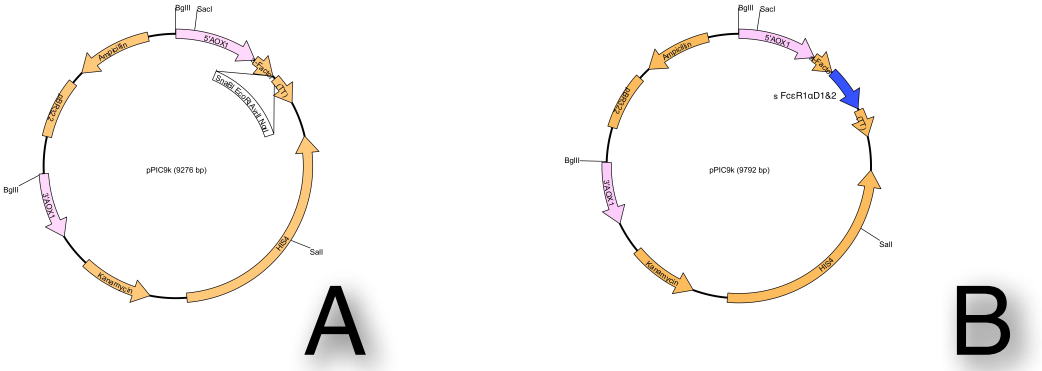

Figure 34: The structure of the pPIC9k plasmid before and after the insertion of the sFcεRIαD1&2 gene:
A. shows the structure of the pPIC9k plasmid (without the sFcεRIαD1&2 gene) while **B.** shows the final structure of the pPIC9k-sFcεRIαD1&2 plasmid.

```
>lcl|60495
Length=1292

 Score =  965 bits (522),  Expect = 0.0
 Identities = 522/522 (100%), Gaps = 0/522 (0%)
 Strand=Plus/Plus

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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  344  ATGGTCCCAGCTGCTATTAGAAAGTCTACTGTCTCTCTGAACCCACCATGGAACAGAATT  403

Query  61   TTTAGAGGTGAAAACGTCACTCTGACTTGTAACAAGAACAAGCCACTGAAGGGTAACTCT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  404  TTTAGAGGTGAAAACGTCACTCTGACTTGTAACAAGAACAAGCCACTGAAGGGTAACTCT  463

Query  121  ACTGAATGGACTTACAACAACACTACTCTGGAAGTCACTACTTCTTCTCTGAACATTACT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  824  TTTAAGAGAACTTCTGAAAGATACACTTCTGATTACCTGTGA  865
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Figure 35: BLAST analysis of the cloned equine sFcεRIαD1&2 gene:
This figure shows the BLAST result of the cloned equine sFcεRIαD1&2 gene (bottom) compared to the original synthesized equine sFcεRIαD1&2 gene (top). The result showed not a single pint mutation in the gene (100% identical is circled in blue).

Once a bacterial colony with the pPIC9k-sFcεRIαD1&2, containing no mutations, was identified and a backup culture was frozen as in chapter 2.2.12, it was grown and the pPIC9k-sFcεRIαD1&2 plasmid harvested in a larger concentration as in chapter 2.2.13 followed by concentrating the plasmid DNA as in chapter 2.2.15 and the DNA quantified as in chapter 2.2.14 which enabled the required concentration adjusted for yeast cell transfection.

5.2.3 - Transfecting And Selecting Pichia pastoris Yeast Cells With The sFcεRIαD1&2 Gene:

The protocol for this experiment is found in chapter 2.4.4. The *Piachia pastoris* yeast cells were streaked on a YPD plate and a single colony was extracted and grown. The cells were harvested and made competent, then a stock of them was frozen. From this stock the competent cells were transformed with the pPIC9k-sFcεRIαD1&2 plasmid and grown in RDB plates. The colonies that grew on the plate had the sFcεRIαD1&2 gene integrated into their genome at various repeats. To select for the colony with the highest sFcεRIαD1&2 repeat 50 colonies were isolated, pooled together and then colony mix was them spread on plates with increasing geneticin G418 sulphate. The colony that grew in the highest geneticin G418 sulphate concentration had the highest number of the sFcεRIαD1&2 gene repeats, therefore it was isolated and used to express the sFcεRIαD1&2 protein.

5.2.4 - Expressing And Purifying the sFcεRIαD1&2 Protein:

The protocol is found in chapter 2.4.4. The isolated colony with the highest sFcεRIαD1&2 gene repeat was added to 25ml of BMGY media. The grown cells were harvested and added to 1200ml BMMY expressing media to a concentration of OD_{600}=1. The cells were incubated in this media and expressed the protein for 6 days, at

which point the media was harvested, filter sterilized and protease inhibitors were added.

The media was tested by SPR by adding equine IgE anti NIP-HSA to a chip immobilized with NIP-HSA, therefore the equine IgE bound and gave a resonance curve. This was followed by the addition of the yeast media. Since the media contained the equine sFcεRIαD1&2 protein, it bound to the equine IgE and thus gave a second resonance curve. The optimal time for expression was 6 days as shown in Figure 36, where the expressed protein accumulated enough in the media, but not left long enough for it to start degrading. After the media was harvested it was tested again to insure the protein did not degrade, Figure 37.

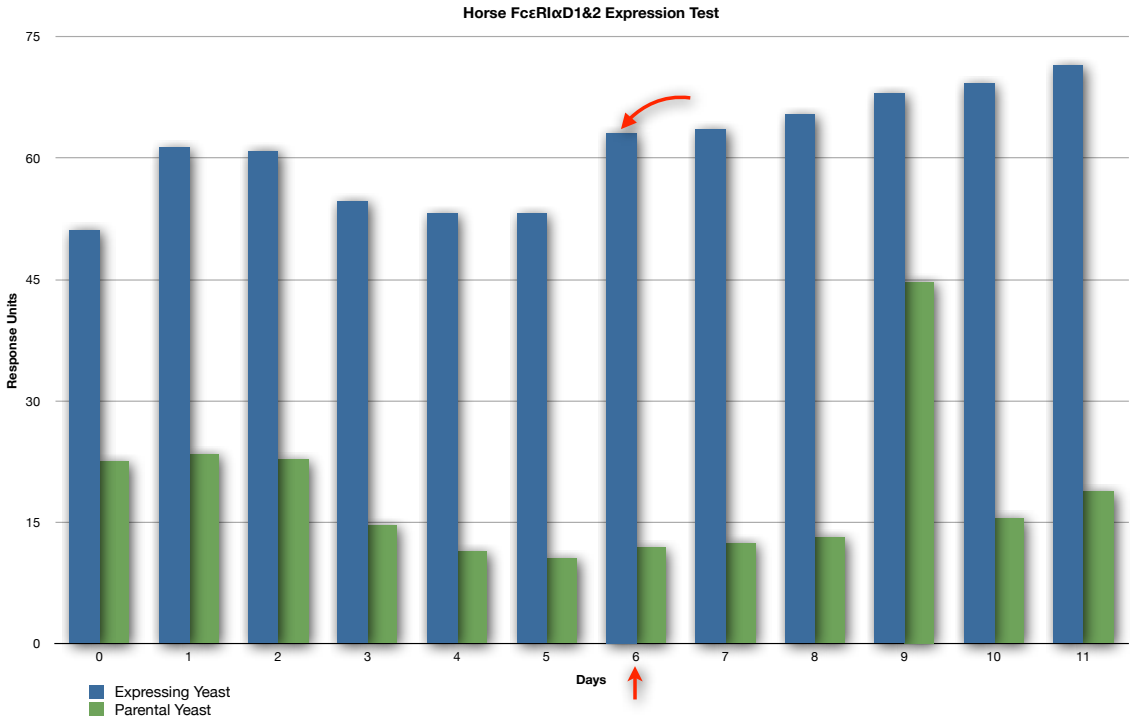


Figure 36: SPR results of the level of sFcεRIαD1&2 against expression time:
This figure shows the level of sFcεRIαD1&2 in the cell media against the incubation time. 6 days of expression (marked by the red arrow) was found to be the ideal expression time for the yeast. Therefore the cell media was harvested after 6 days of expression. Values for this graph are in the Appendix.

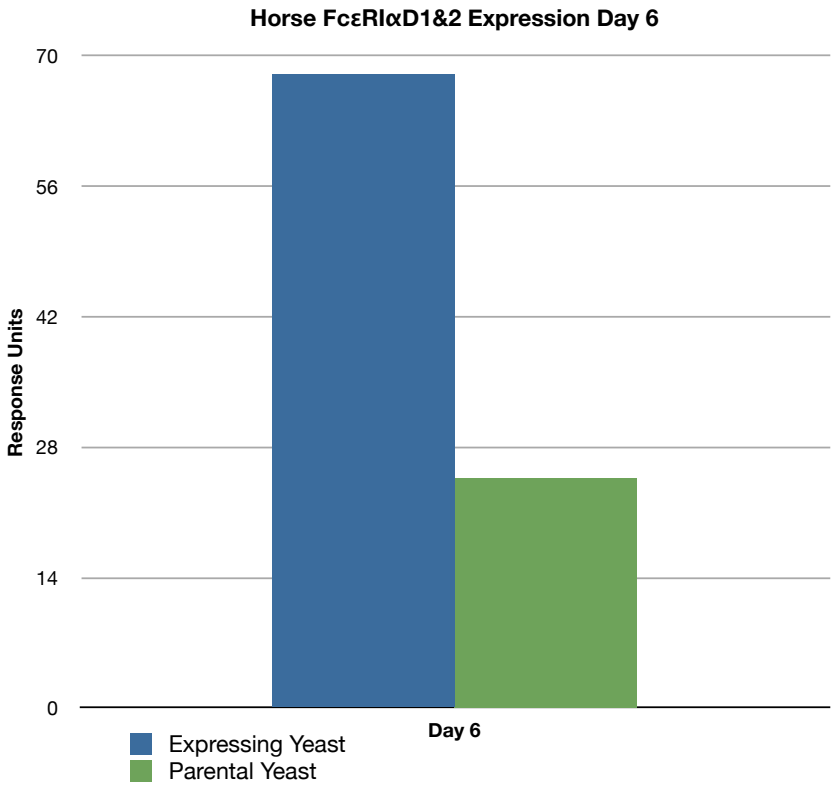


Figure 37: SPR results of the level of sFcεRIαD1&2 after media harvesting:
The figure shows the media right after harvesting still expresses enough of the protein, and that it did not degrade due to the harvesting process. Values for this graph are in the Appendix.

Since the media still contained the equine sFcεRIαD1&2 protein, and it did not degrade due to the presence of proteases, the media was concentrated using a 3kDa molecular filter from 1200ml down to ~50ml to allow for ease of handling during the purification process. After concentration, the media was tested to show the successful concentration of the equine sFcεRIαD1&2 protein and the amount of leakage of the protein in the overflow, Figure 38 shows the level of sFcεRIαD1&2 after media concentration and Figure 39 shows the SPR curve of the concentrated media, indicating that the protein was still viable at that point.

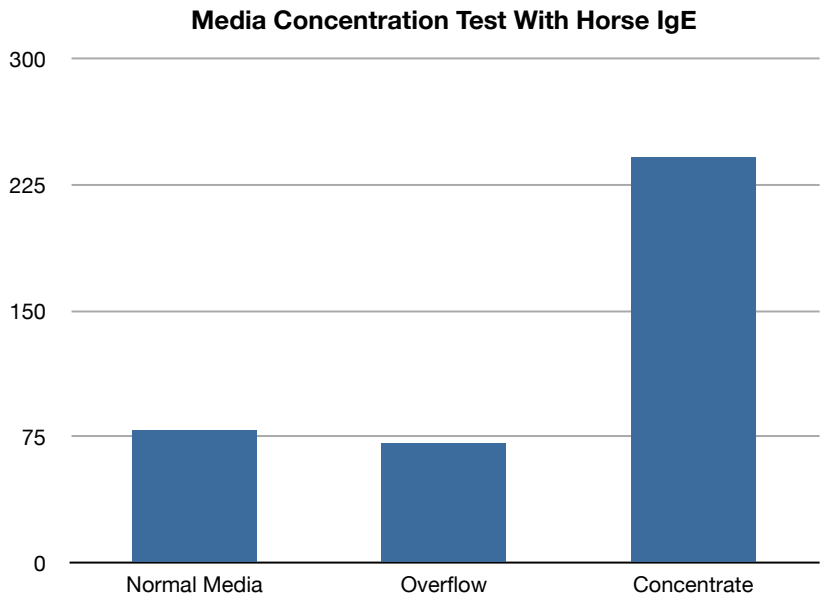


Figure 38: SPR results of the level of sFcεRIαD1&2 after media concentration:
This figure shows the SRP result of the level of the equine sFcεRIαD1&2 protein after the media was concentrated through a 3kDa molecular filter. Values for this graph are in the Appendix.

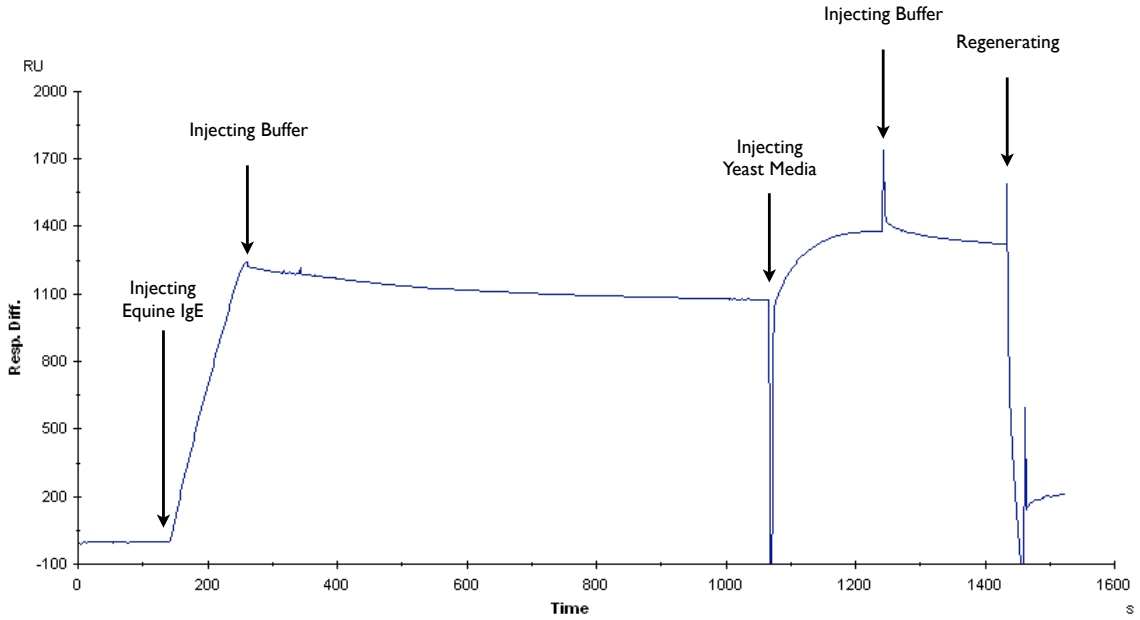


Figure 39: The SPR viability curve of the concentrated media prior to purification:
This figure shows the SPR curve of the concentrated media prior to purification. The curve indicates large quantity of the correctly folded equine sFcεRIαD1&2 protein. The first resonance curve if the equine IgE anti NIP-HSA binding to the NIP-HSA in the chip, the second curve is the equine sFcεRIαD1&2 protein binding to the equine IgE

The media was run through an affinity column, three times, to purify the equine sFcεRIαD1&2 protein as in chapter 2.4.5. The ligand used to purify the equine sFcεRIαD1&2 was canine IgE, that was because the canine IgE was proven to bind to the equine sFcεRIαD1&2 (chapter 4) strong enough to purify the protein, but weak to allow for efficient elution. The equine sFcεRIαD1&2 protein was eluted by passing low pH buffer through the column, and it dropped into a high pH column which neutralized the final buffer mixture. The protein elution was washed in 1xPBS buffer by concentration and dilution twice using a 3kDa molecular filter. The final concentrated purified equine sFcεRIαD1&2 protein was ~200µl and its concentration was determined as in chapter 2.4.6 (Figure 40), in this case the protein concentration was $\underline{0.25\ mg\ ml^{-1}}$ because its OD_{625} = 0.686. The protein was also tested by SPR again for viability, Figure 41 shows the curve of the test.

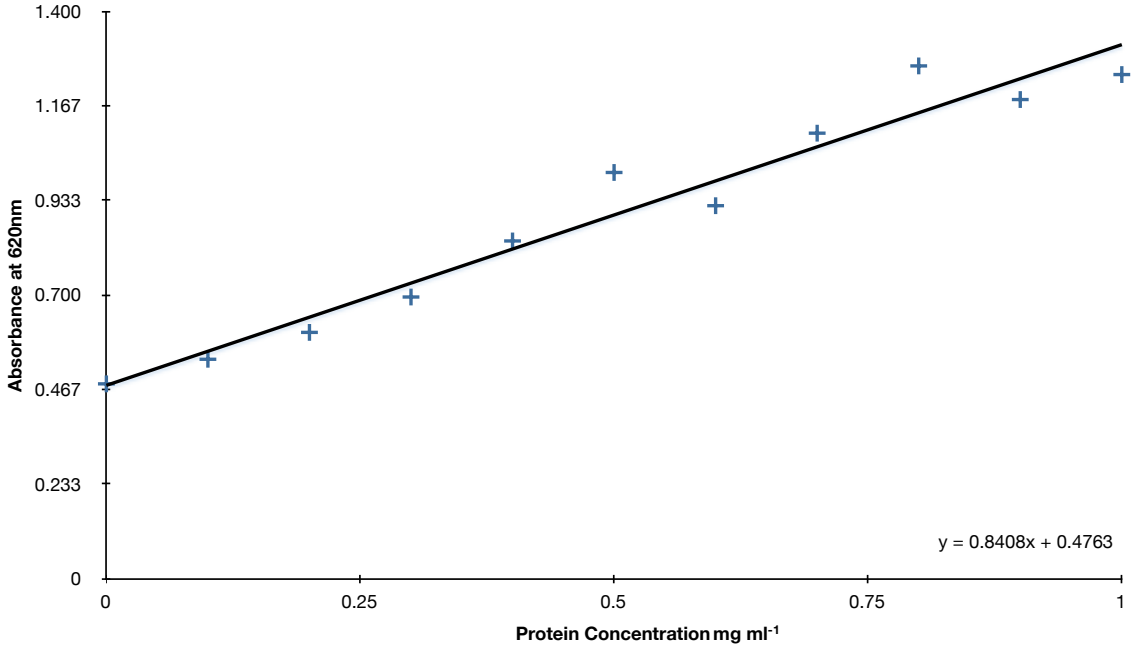


Figure 40: The protein purification graph used to calculate the concentration of the purified equine sFcεRIαD1&2 protein and later on the HHoH IgE anti NIP-HSA:
This figure shows the concentration curve of bovine albumin solutions. At the bottom right of the graph is the equation for the line of best fit, from this equation the concentration of the equine sFcεRIαD1&2 protein was determined. Values for this graph are in the Appendix.

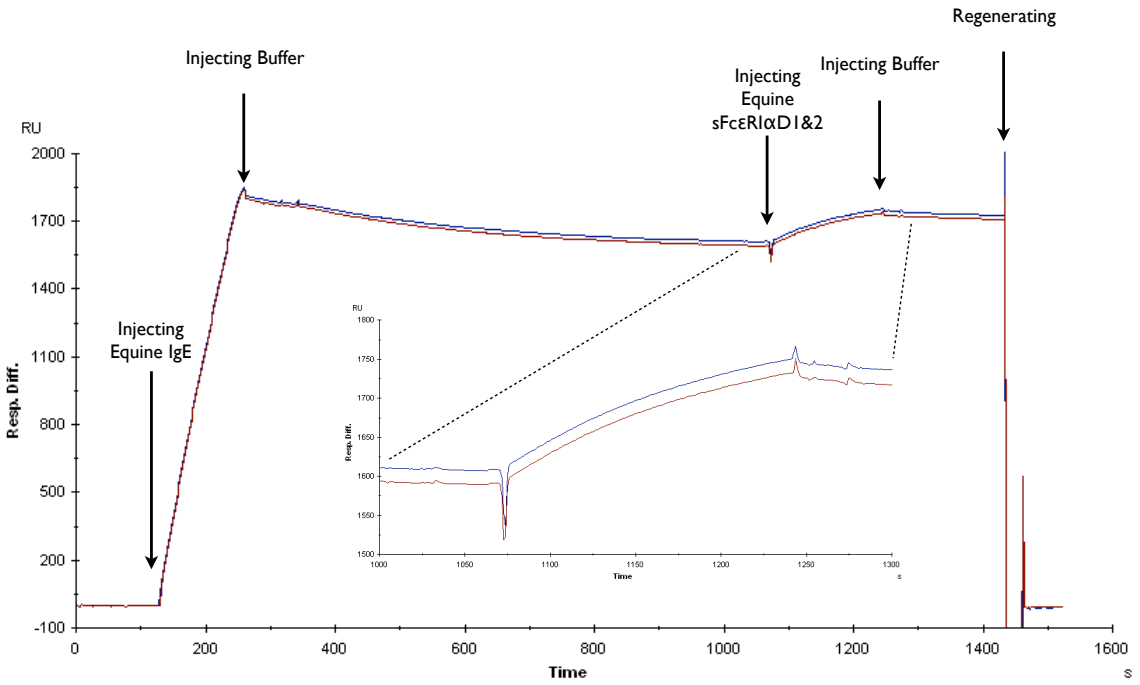


Figure 41: The SPR curve of the purified equine sFcεRIαD1&2 protein:
This figure shows the positive SPR curve of the purified equine sFcεRIαD1&2 protein, the first resonance curve if the equine IgE anti NIP-HSA binding to the NIP-HSA in the chip, the second curve is the equine sFcεRIαD1&2 protein binding to the equine IgE.

The equine sFcεRIαD1&2 protein was then run in an SDS-PAGE to test its purity as in chapter 2.4.7, Figure 42 shows the final gel result with several protein bands, because the equine sFcεRIαD1&2 protein is glycosylated which results in the protein sequence having several different masses. If the protein was de-glycosylated and run on the gel it would have shown a single band at ~22.19kDa. Ideally a western blot would have also been run to confirm the identity of the protein, but no anti equine FcεRIα protein were found commercially, and therefore the western blot was not run. Canine IgE was a good candidate to test for the equine sFcεRIαD1&2 protein in a western blot as it does bind to the protein, and there are commercial anti canine IgE antibodies, but since the laboratory also synthesized canine sFcεRIαD1&2 protein, cross contamination could not have been tested. The only way the identity of the protein was confirmed is

when it showed strong binding kinetics with the equine IgE anti NIP-HSA in Chapter 6 as predicted.

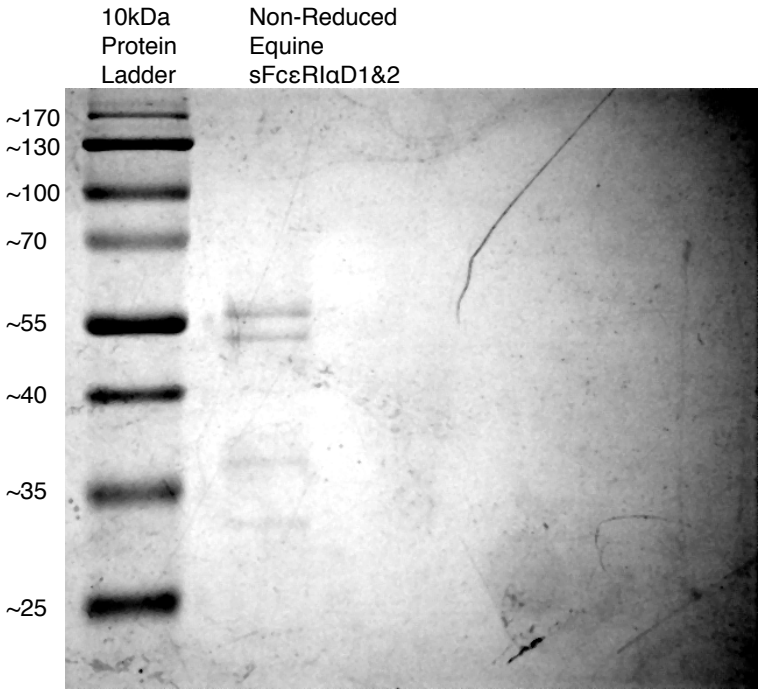


Figure 42: SDS-PAGE of the equine sFcεRIαD1&2 protein: This Figure shows the SDS-PAGE. There are several bands in the gel as the equine sFcεRIαD1&2 protein is glycosylated which results in the protein sequence having several different masses. If the protein was de-glycosylated and run on the gel it would have shown a single band at ~22.19kDa.

5.3 - Discussion:

The sFcεRIαD1&2 protein (Figure 43) was expressed and purified in the end. This experiment protocol appears to be easy, though it was difficult to setup the controls to test its efficiency. Some difficulty was faced during filter sterilizing the media, since it contained many agents that were heat sensitive. Many obstacles were faced but were solved. First of all, after yeast cells were transfected and selected for colonies with the highest equine sFcεRIαD1&2 gene copy number, they were frozen to be kept as a backup as in chapter 2.2.12. This proved fatal as the cells would quickly stop expressing the protein once they were thawed, this problem was solved by not freezing a backup of cells, instead immediately using the isolated colony for expression. This meant that

when more of the protein (Figure 43) was needed, the whole protocol had to be repeated. Therefore in conclusion, even though the protocol seemed easy, its cost was still high, with small protein yield, especially as the selection process required large quantities of geneticin G418 sulphate, which was expensive. For future notice, a paper by (Garman, *et al.*, 1999) showed that the sFcεRIαD1&2 protein can be expressed in large enough quantity for crystallization using the baculovirus expression system. Furthermore, using the FreeStyle™ CHO-S Cells (Invitrogen) kit can yield large quantities of the protein in mammalian (Chinese Hamster Ovary) cells. All these cell lines are stable after they are frozen, and report a high gene expression retention over many generations.

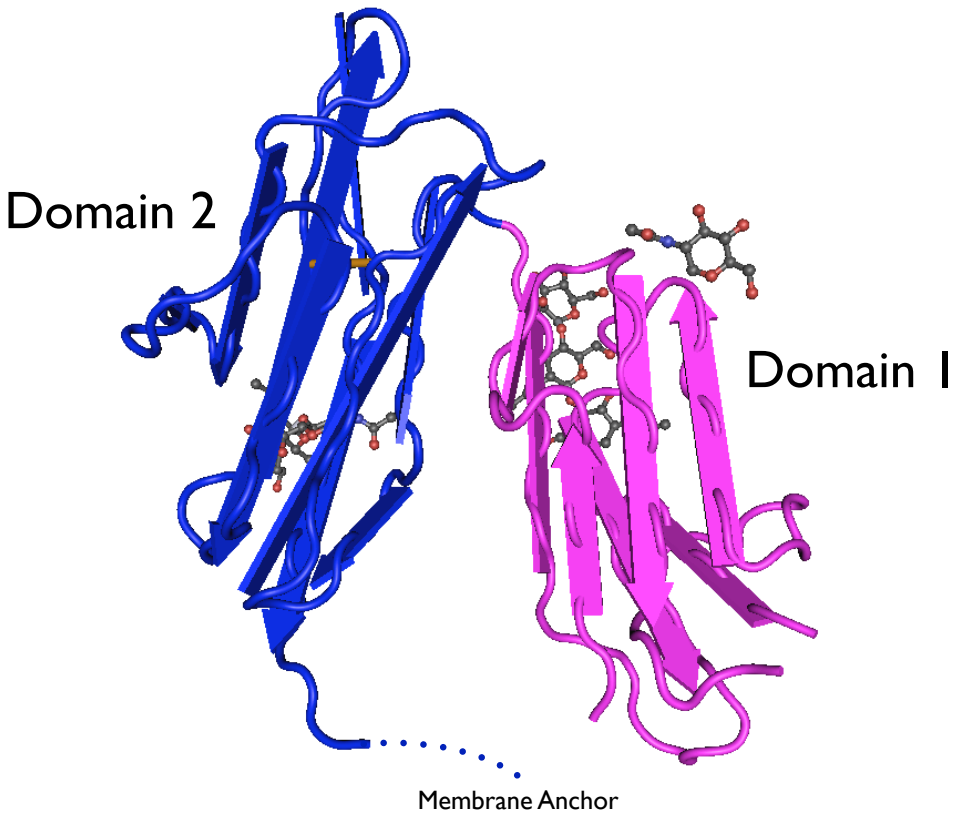


Figure 43: Structure of the sFcεRIαD1&2:
This figure shows the structure of the human sFcεRIαD1&2, the equine sFcεRIαD1&2 would look almost the same as it has the exact same function, but with a different amino acid sequence. This figure was taken from (Garman, *et al.*, 1998).

Second of all, initially when the expressed media was tested for the presence of the equine sFcεRIαD1&2 protein, canine IgE anti NIP-HSA was used, as the equine IgE anti NIP-HSA was not yet synthesized. This did not detect the equine s sFcεRIαD1&2 protein as the canine IgE used was in low concentration. This was solved by using equine IgE anti NIP-HSA, once it was synthesized, or canine IgE in large enough concentration to detect the equine sFcεRIαD1&2 protein.

Finally, low levels of the equine sFcεRIαD1&2 protein were collected after the purification, first because the column was prepared using non-purified media containing canine IgE, therefore much of the column had other proteins (such as albumin) bound to it, which reduced the exposure of the canine antibody to the equine sFcεRIαD1&2 protein, thus bound little of it. Also a large column was used to purify the media, ideally only a 2cm long column is sufficient to purify enough of the protein for many experiments, therefore when a long column (12cm) was used it reduced the fluid flow rate through it. This was not ideal to remove enough of the protein impurities, though the yeast media had little protein impurities, which still resulted in a pure sample. It also was not good to elute enough of the protein. Therefore for future work, only a 2cm long column would be used, and prepared using purified canine IgE.

Chapter 6 - Kinetic Analysis of Equine IgE/FcεRIα Binding:

6.1 - Introduction:

In the present chapter the studies of kinetic bindings between the equine IgE and its sFcεRIαD1&2 receptor was preformed. In previous chapters the synthesis of the equine IgE and the equine sFcεRIαD1&2 were successful, therefore they were run on an SPR machine to measure their kinetic binding as in chapter 2.4.10.

The following is an explanation of the different kinetic constants, note that the explanation will be in such a way where the ligand is bound to an SPR chip surface, while the receptor is injected onto it: in Figure 44 there is a theoretical binding graph between a ligand and its receptor. The association rate constant (k_a) is the time at which half of the total number of receptors in a sample, of a certain concentration, binds to their ligand. Therefore it is the inverse of the time at which half of a receptors bind to their ligand (τ_a) multiplied by the sample's concentration. The dissociation rate constant (k_d) is simply the inverse of the time it takes all the bound receptors to dissociating from its ligand (τ_d) (Figure 44A).

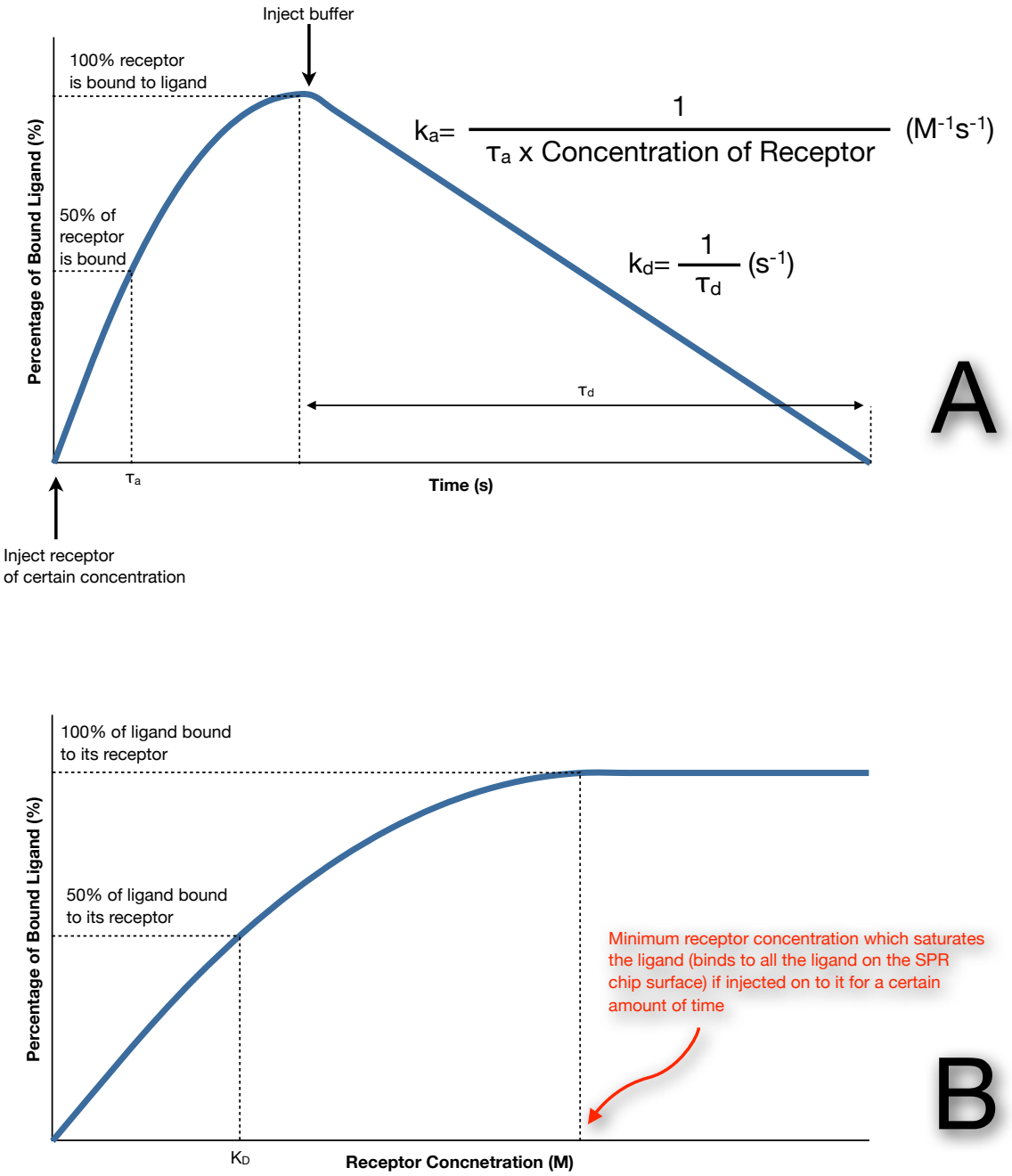


Figure 44: Theoretical binding graph between a ligand and its receptor:
A. this figure shows a mock binding graph between a ligand and its receptor, from a graph like this the k_a and k_d rate constants can be calculated. **B.** shows an alternative way the K_D equilibrium dissociation constant can be calculated from testing different receptor concentrations and injecting them on the a surface conjugated with a ligand for a certain amount of time.

K_D is the equilibrium dissociation constant, it is the equilibrium constant which measures the tendency of two bound molecules to separate, the smaller the K_D value, the least likely the two molecules will separate. It can also be calculated by dividing the

dissociation rate constant (k_d) by the association rate constant (k_a). The equilibrium association binding (K_A), also known as the affinity constant, is simply the reciprocal of the equilibrium dissociation constant K_D value ($1/K_D$), therefore the higher the K_A value the stronger two molecules bind to each other.

$$K_D = \frac{k_d}{k_a}\ (M) \qquad K_A = \frac{1}{K_D}\ (M^{-1})$$

The K_D unit indicate the receptor concentration at which half of the ligand molecules in the SPR chip surface were bound after the receptor solution was injected on to it and allowed to reach equilibrium. Therefore (Figure 45B) shows an alternative way K_D can be calculated.

If the receptor concentration is less than the K_D value then most of the receptors in solution will not bind to the ligand. But if the receptor concentration is greater than the K_D value then most of the receptors in solution will bind to the ligand.

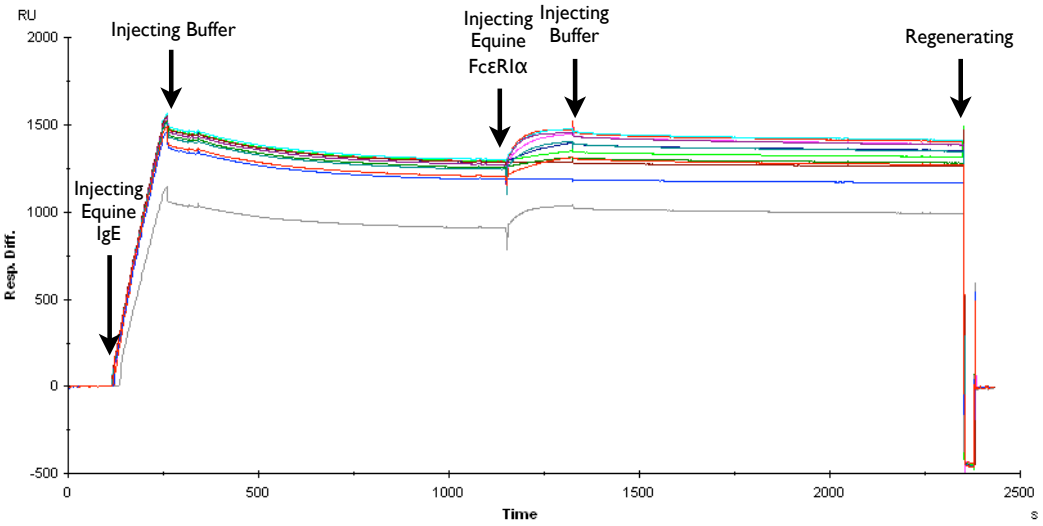
This form of measurements were made through pseudo-first-order reaction. It is difficult to measure binding analysis by monitoring both the concentration of the ligand and the sample (second-order reaction) or by following one reactant's concentration and deducing the other reactant's concentration by their difference is not very accurate. Therefore to solve this problem an approximation called pseudo-first-order is used, where the concentration of a ligand is maintained constant (bound to a surface or presented in a very large concentration), this allows knowledge of one of the reactant's concentration (the ligand) at all time while measuring the other (the sample) accurately: $r = k_a[A][B] \therefore k_a[B]$ where r (previously labeled as τ_a) is the reaction rate, k_a is the

association rate constant, [A] is the concentration of the ligand and [B] is the concentration of the sample.

6.2 - Results:

The BiaCore™ 2000 System SPR machine had the function of automatically calculating the kinetic binding parameters using the serial concentrations of the one protein as it binds to another. In this case the the equine IgE was tested with the equine, human and canine sFcεRIαD1&2. Also the canine IgE was tested with the equine sFcεRIαD1&2 protein. The graph results are on Figure 44, where on the left of the figure is the binding between the IgE (first curve from the left) and the highest sFcεRIαD1&2 concentration of 10μg ml^{-1} (second curve from the left). And right of the figure there can be seen a complex graph where the red lines indicate the actual results of the experiment (showing only the sFcεRIαD1&2 binding portion of the curve), and the blue lines indicate the predicted binding at 1:1 Langmuir binding model (which relates the adsorption of molecules onto a solid surface). It can be seen that once the sFcεRIαD1&2 is added to the IgE it binds (association) until it the chip is saturated (all the IgEs mobilized on the chip have sFcεRIαD1&2 bound to them), then HBS-EP buffer is run through the chip where the curve start to go down slightly (dissociation) as some of the weakly bound sFcεRIαD1&2 would break off the IgE. Figure 45 A shows the kinetic analysis between equine IgE and equine sFcεRIαD1&2, and the binding shows to be as predicted for a 1:1 binding ratio, since 1 molecule of IgE binds to 1 molecule to sFcεRIαD1&2. Figure 45 B shows the binding analysis between the equine IgE and the human sFcεRIαD1&2, they show no binding between the two protein, as the sFcεRIαD1&2 curve is extremely small for the highest concentration of 10μg ml^{-1}, and the blue lines in the graph show that the binding does not fit the 1:1 Langmuir binding model. Figure 45 C shows the binding between the equine IgE and the canine

sFcεRIαD1&2, the graphs show a successful binding in the 1:1 Langmuir binding model, but the association curve us followed immediately by strong dissociation, therefore it can be determined that the equine IgE binds weakly to the canine sFcεRIαD1&2, and from chapter 4 it was determined that this binding is so weak that it does not cause any released in RBL-2H3.1 cells. Because of the weak binding between the equine IgE and the canine sFcεRIαD1&2, the opposite was tested, just as in chapter 4‘s release assays; Figure 45 D shows the binding between the canine IgE and the equine sFcεRIαD1&2 to be even very stable in the 1:1 Langmuir binding model, and this, of course, completes the picture of the observed effects on cell release in Chapter 4. The numerical results of Figure 45 were automatically calculated by the BiaCore™ 2000 System SPR machine software, and they are displayed on Table 1.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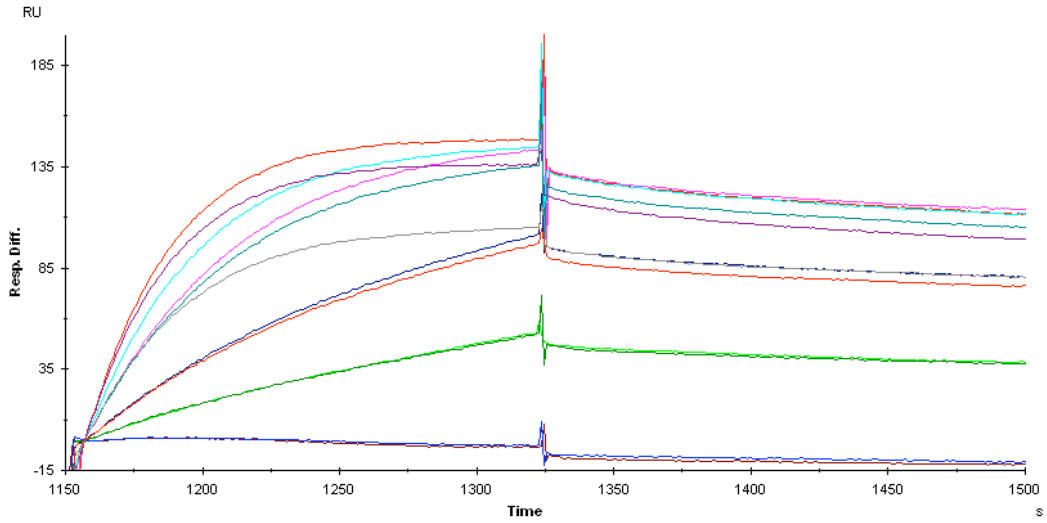
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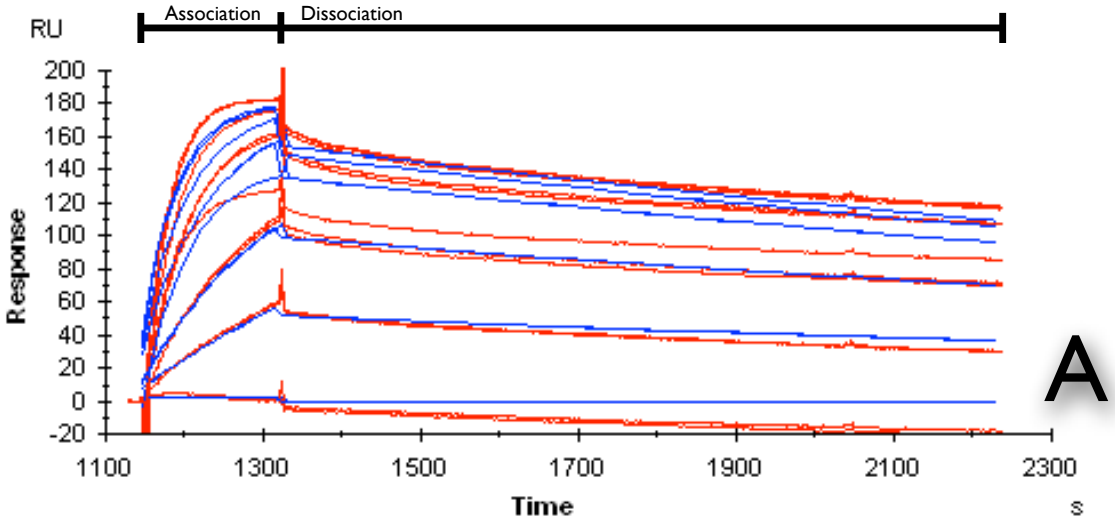
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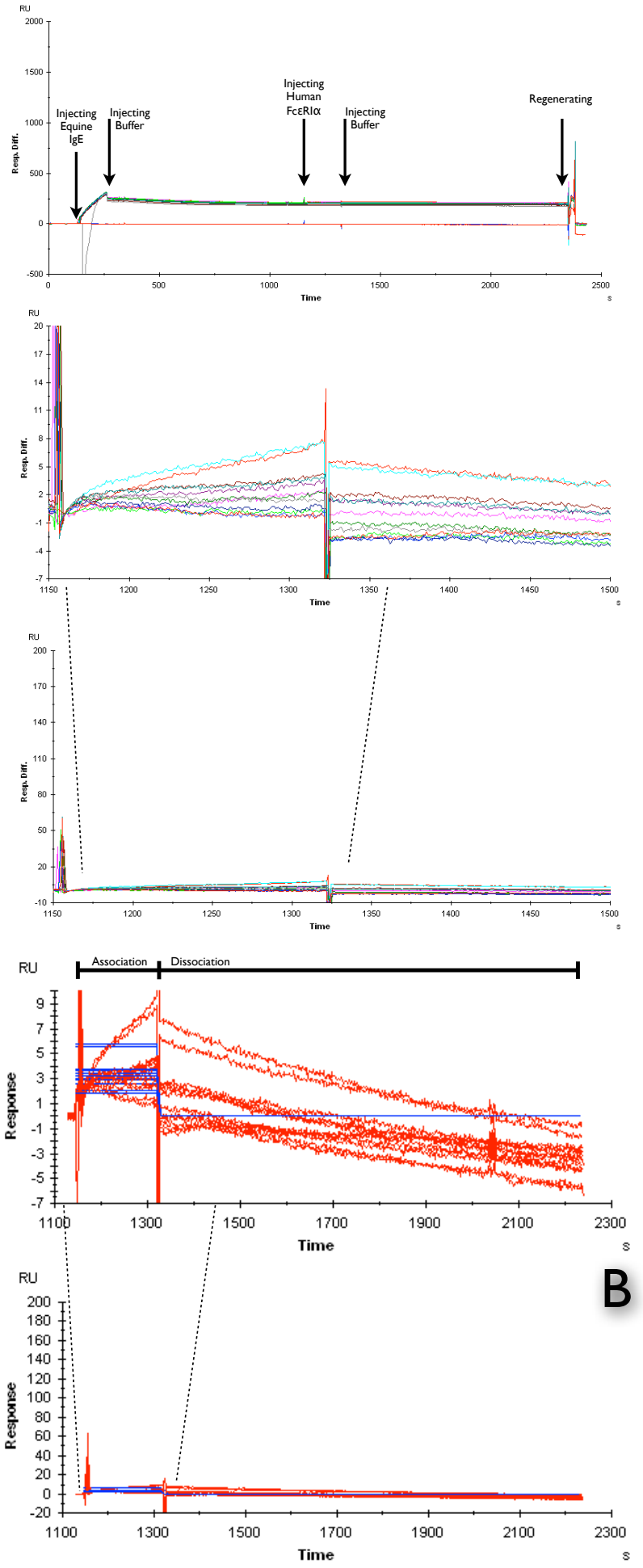
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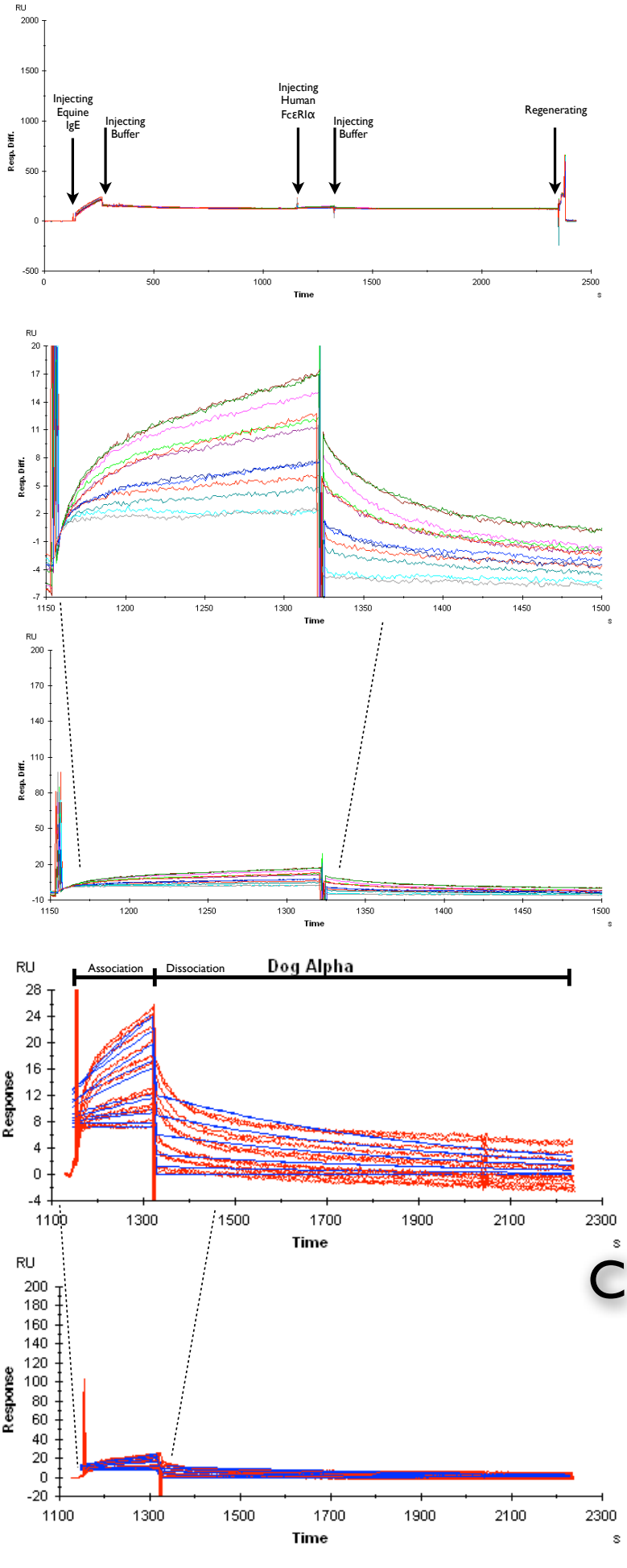
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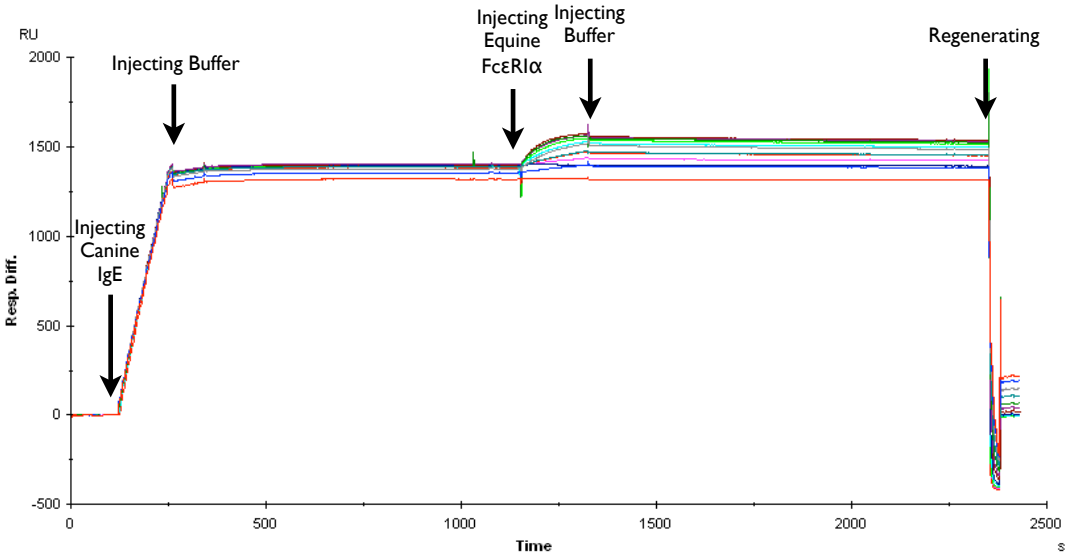
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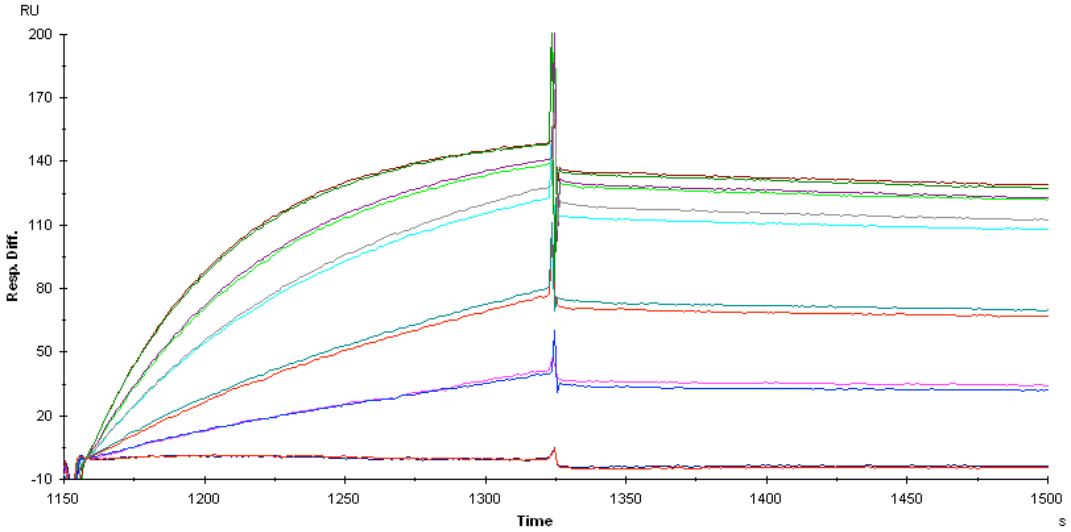
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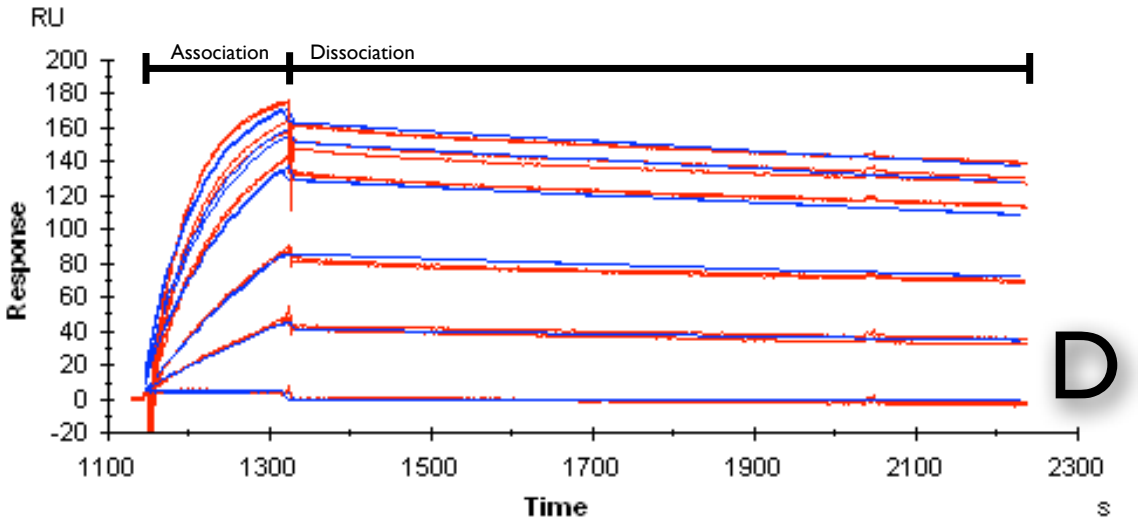
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D

Figure 45: Kinetic analysis graphs:
A. shows the binding between the equine IgE and the equine sFcεRIαD1&2 protein with the left graph showing the entire test, the middle graph only shows the sFcεRIαD1&2 binding and the right graph shows the kinetic analysis graph with the red lines being the actual measurements and the blue lines are the results fitted to the 1:1 Langmuir binding model. The results show a strong binding association between the equine IgE and the equine sFcεRIαD1&2 protein followed by weak dissociation indication their binding stability. **B.** shows the binding between the equine IgE and the human sFcεRIαD1&2 protein with the left graph showing the entire test. The middle graph only shows the sFcεRIαD1&2 binding, with the bottom having the actual axis for comparison, and the top having its axis modified to show the actual curves. The right graph shows the kinetic analysis graph with the red lines being the actual measurements and the blue lines are the results fitted to the 1:1 Langmuir binding model. The results show very week binding association between the equine IgE and the human sFcεRIαD1&2 protein followed by rapid dissociation indication their binding instability. **C.** shows the binding between the equine IgE and the canine sFcεRIαD1&2 protein with the left graph showing the entire test. The middle graph only shows the sFcεRIαD1&2 binding, with the bottom having the actual axis for comparison, and the top having its axis modified to show the actual curves. The right graph shows the kinetic analysis graph with the red lines being the actual measurements and the blue lines are the results fitted to the 1:1 Langmuir binding model. The results show strong binding association between the equine IgE and the canine sFcεRIαD1&2 protein followed by rapid dissociation indication their binding instability. **D.** shows the binding between the canine IgE and the equine sFcεRIαD1&2 protein with the left graph showing the entire test, the middle graph only shows the sFcεRIαD1&2 binding and the right graph shows the kinetic analysis graph with the red lines being the actual measurements and the blue lines are the results fitted to the 1:1 Langmuir binding model. The results show a strong binding association between the canine IgE and the equine sFcεRIαD1&2 protein followed by weak dissociation indication their binding stability.

The research group lead by Dr. Gould (Henry, *et al.*, 1997) discuss a biphasic interaction model between the IgE and its FcεRI receptor to form a successful strong bond; they came to the conclusion that the IgE binds to its FcεRI receptor through low-

affinity first followed by binding through high-affinity, to give the final binding between the two proteins K_A = ~10^{10}M.

Table 1: Kinetic analysis table:
This table shows the kinetic analysis of equine IgE binding to the FcεRI receptor of different species, and that of canine IgE binding to the equine FcεRI receptor. Noting that the original results of Equine IgE - Equine sFcεRIαD1&2 and the Canine IgE - Equine sFcεRIαD1&2 had their concentrations accidentally wrongly inputed into the SPR machine as µM instead of µg ml^{-1}. Also the Equine IgE - Human sFcεRIαD1&2 and the Equine IgE - Canine sFcεRIαD1&2 had their molecular weights for the sFcεRIαD1&2 accidentally wrongly inputed into the SPR machine as 36kDa instead of 50kDa. Therefore these results are raw data without them being re-calculated and fixed.

	Equine IgE – Equine sFcεRIαD1&2	Equine IgE – Human sFcεRIαD1&2	Equine IgE – Canine sFcεRIαD1&2	Canine IgE – Equine sFcεRIαD1&2
k_a ($M^{-1}s^{-1}$)	$2.3x10^6$	$1.8x10^1$	$2.8x10^2$	$1.5x10^6$
K_A (M^{-1})	$5.9x10^9$	$1.4x10^6$	$1.8x10^5$	$8.0x10^9$
k_d (s^{-1})	$3.8x10^{-4}$	$1.3x10^{-5}$	$1.6x10^{-3}$	$1.9x10^{-4}$
K_D (M)	$1.7x10^{-10}$	$7.3x10^{-7}$	$5.6x10^{-6}$	$1.2x10^{-10}$
RU_{Max} (RU)	159	3.00	999	176

It is important to note that these results of Equine IgE - Equine sFcεRIαD1&2 and the Canine IgE - Equine sFcεRIαD1&2 had their concentrations accidentally wrongly inputed into the SPR machine as µM instead of µg ml^{-1}. Also the Equine IgE - Human sFcεRIαD1&2 and the Equine IgE - Canine FcεRIα had their molecular weights for the FcεRIα accidentally wrongly inputed into the SPR machine as 36kDa instead of 50kDa. Therefore the results in figure 45 and their values in table 1 are raw data without them being re-calculated and fixed. These results were sent to Dr. Peter Schuck and Dr. Huaying Zhao where they re-calculated the results and applied them to three kinetic models.

6.3 - Discussion:

6.3.1 - Explanation of The Three Macromolecular Binding Process Models:

The following is the explanation of the three macromolecular binding process models for SPR biosensing according to the research by (Schuck and Zhao, 2010).

When using SPR for measuring rate and affinity constants using the pseudo-first-order reactions, two very common sources of deviation occur, they are the heterogeneity of the surface sites and mass transport limitations. The heterogeneity of the surface sites discusses the concept that, since ligands need to be immobilized onto a surface for a sample to bind to, and measurements can be commenced, the idea of immobilizing a ligand onto a surface raises questions as to the nature of the final bound ligand and the best strategy to go about this immobilization; does the ligand denature and results in a mixture of high-affinity and low-affinity binding epitopes to the sample, which adds errors to the final kinetic constant measurement values? Other questions such as; does the ligand get partially de-natured, and thus its biochemistry changes? Does the sample interact with the actual surface as well as the ligand? The other source of deviation is the concept of mass transport limitations, which explains the actual transfer of sample onto the surface with immobilized ligand; when the sample binds to the ligand on a surface. On the macroscopic scale, does the sample concentration near the surface defers from the actual bulk concentration, resulting in either a local depletion zone? Or incase of washing, a retention zone? On the microscopic scale, does the sample concentration differs through the thickness of the immobilizing matrix (the sensing volume itself.), which is around 100 - 400nm thick. All these result in sample concentration gradients. Therefore to correct for these errors mathematical model were developed that accounts for these phenomenons and thus gives more precise values for kinetic constants measured using SPR.

Discrete One Site Model: This is a model that explains the results in terms of having uniform binding epitopes of the immobilized ligand; i.e: no differences in binding affinity nor configuration (no heterogeneity of the surface sites). This model

usually does not describe SPR bindings because there usually is heterogeneity in the ligand epitope binding sites due to the strategy of binding the ligand to a surface.

Conformational Change Model: This is a model that explains the results in terms of not having all the binding epitopes of the immobilized ligand to be the same; i.e: there are multiples of different confirmations of the same sample binding site on the ligand where the sample can bind at different high and low affinities (having heterogeneity of the surface sites). This model usually describes the majority of protein-protein SPR bindings because of the heterogeneity in the ligand epitope binding sites due to the strategy of binding the ligand to a surface.

Distribution Model: This is a model that explains the results in terms of having a range of binding affinities between the immobilized ligand and the sample due to the fact that the immobilized ligand has different confirmations of its binding site caused by the immobilization process (having heterogeneity of the surface sites), and at the same time the sample interacts at different concentrations with the immobilized ligand due to the effect of mass transport limitations on the interacting surface. This model takes into account both sources of errors found in SPR biosensing and thus gives the most accurate values for the measured kinetic constants.

6.3.2 - Re-Calculated Kinetic Binding Values For Different Macromolecular Binding Process Models:

The results were re-calculated for the correct concentrations of the sFcεRIαD1&2 in μg ml^{-1} and for the molecular weight of 50kDa. They then applied the new re-calculated data to the three macromolecular binding process models described above; the Discrete One Site Model, the Conformational Change Model and the Distribution Model. The software used to apply these models was EVILFIT software. A note, the k_d constant is sometimes referred to as k_{off} and the K_D constant is sometimes referred to as

K_d in the following figures. Of all the three models, the Distribution Model was the one that best fitted and described the data as it had the lowest Root Mean Square Deviation and resulted in two peeks in the $\log_{10} k_d$ against $\log_{10} K_D$ distribution (plots are in the Appendix).

According to Dr. Peter Schuck's and Dr. Huaying Zhao's calculations, the Discrete One Site Model usually did not give reliable results, and so it was not a surprise that this model did not fit the data (Appendix) in the $\log_{10} k_d$ against $\log_{10} K_D$ distribution plot. The Conformational Change Model resulted in a very slow conformational rate constants (k_{cc} = ~10^{-6}), this meant that during the binding there is no structural conformational change.

The Distribution Model was the one that best fitted and described the data (Table 2). The equine IgE binding to the equine sFcεRIαD1&2, equine IgE binding to the canine sFcεRIαD1&2 and canine IgE binding to the equine sFcεRIαD1&2 showed two peaks in the $\log_{10} k_d$ against $\log_{10} K_D$ distribution (plots are in the Appendix), only the equine IgE binding to the human sFcεRIαD1&2 resulted in a single peak. The equine IgE binding to the equine sFcεRIαD1&2 had the value of the 7μg ml^{-1} concentration deviate greatly from the model, it resulted in the model to give an final Root Mean Square Deviation = 2.52. Therefore removing this value resulted in a better fit to this model and a final Root Mean Square Deviation = 0.74.

Table 2: EvilFit Distribution Model:
This table shows the Distribution Model values calculated in the EVIFIT software, note the small Root Mean Square Deviation (compared to the other tables' values) indicating that the results indeed do fit this model.

	EvilFit Distribution Model					
Experiment	**K_D (M)**	**K_A (M^{-1})**	**k_d (s^{-1})**	**Signal (RU)**	**Root mean square deviation (RU)**	**Other Peak**
Horse IgE – Horse sFcεRIαD1&2	$1.58x10^{-10}$	$6.33x10^{9}$	$2.44x10^{-5}$	124.64	0.74	yes
Horse IgE – Human sFcεRIαD1&2	$4.66x10^{-9}$	$2.15x10^{8}$	$1.20x10^{-4}$	7.24	0.61	Broad Distribution
Horse IgE – Dog sFcεRIαD1&2	$1.93x10^{-8}$	$5.18x10^{7}$	$4.81x10^{-5}$	40.67	0.24	yes
Dog IgE – Horse sFcεRIαD1&2	$5.43x10^{-10}$	$1.84x10^{9}$	$4.75x10^{-5}$	161.09	0.63	yes

From the Distribution Model results table (Table 2) it can be seen that the equine IgE binding to the equine sFcεRIαD1&2 has the lowest (weakest) equilibrium dissociation constant $K_D = 1.58x10^{-10}$ M ($K_A = 6.33x10^{9}$ M^{-1}) this indicates that the two proteins have high affinity to each other, are very stable and unlikely to dissociate once bound. Second comes the canine IgE binding to the equine sFcεRIαD1&2 having a similar equilibrium dissociation constant $K_D = 5.43x10^{-10}$ M ($K_A = 1.84x10^{9}$ M^{-1}) this indicates that the two proteins also have high affinity to each other, are very stable and unlikely to dissociate once bound, their K_D is 3.4 times higher (K_A 3.4 times lower) than the equine IgE-equine sFcεRIαD1&2 complex, this is expected since the two molecules come from two different species, this is again expected since the IgE protein sequences between the equine and canine IgEs are 80% similar.

The equine IgE binding to the human sFcεRIαD1&2 and the equine IgE binding to the canine sFcεRIαD1&2 showed to have several magnitudes higher equilibrium dissociation constants $K_D = 4.66x10^{-9}$ M (lower $K_A = 2.15x10^{8}$ M^{-1}) and $K_D = 1.93x10^{-8}$ M (lower $K_A = 5.18x10^{7}$ M^{-1}) respectively than the previous complexes. This was

supported previously by results shown in chapter 4 for flow cytometry and cell mediator release assays, where the equine IgE failed to bind to the canine sFcεRIαD1&2 and the human IgE failed to bind to the equine sFcεRIαD1&2. Even though these predictions can be confirmed by biological studies on living cells, these values should be considered with caution because they way the kinetic measurements were preformed was less than optimal. As can be seen from Figure 45 B and C, the IgE was not bound to the chip in high enough quantity to allow for the gathering for information about the weak binding of the sFcεRIαD1&2 onto it. Ideally ~2500 resonance units needs to be achieved by the IgE when bound to the SPR chip in order to give a complete picture of the sFcεRIαD1&2 binding onto it, this was achieved in the equine IgE binding to the equine sFcεRIαD1&2 and canine IgE binding to the equine sFcεRIαD1&2, but in the equine IgE binding to the human sFcεRIαD1&2 and the equine IgE binding to the canine sFcεRIαD1&2 only ~ 250 resonance units was achieved by the IgE, and this was not adequate. These two kinetic analyses should be repeated with binding more IgE in order to collect more information about the binding kinetics, even though the biological studies indicate low affinity. This was tested as can be seen in Figures 46 and 47, where more equine IgE was added, even though it did not reach ~2500 RU, it was enough to see that the canine and human sFcεRIαD1&2 protein better bind. From these graphs it can be seen, especially with the canine sFcεRIαD1&2 protein, that they will still have a high enough K_D (low K_A) that agrees with the biological results in chapter 4 (and the fact that the IgE protein sequences between the equine and human are only 68% similar.), which showed the equine IgE causes not mediator release on RBL-2H3.1 cells expressing canine FcεRIα or human FcεRI receptors, especially that in this case 20μg ml^{-1} of each sFcεRIαD1&2 protein was added, twice as much as the highest concentration used in the kinetic analysis, and it still gave a slightly smaller curve than

expected. But nonetheless the calculated K_D would be more precise for further research if the kinetic analysis were repeated with the addition of adequate concentration of equine IgE.

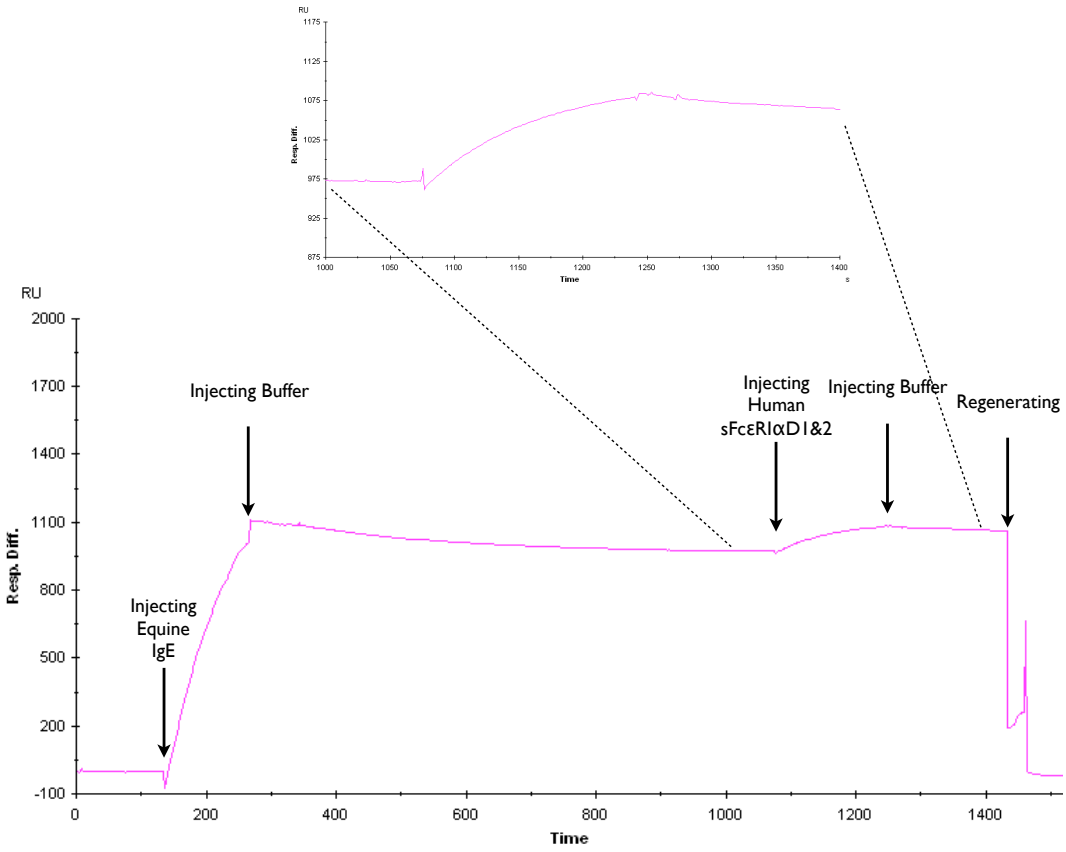


Figure 46: Re-testing the binding between the equine IgE and the human sFcεRIαD1&2:
This figure shows the re-test of the binding between the equine IgE and the human sFcεRIαD1&2 protein. The addition of more equine IgE, even though it did not reach ~2500 RU, was adequate to show clear binding between the equine IgE and the human sFcεRIαD1&2. Though the concentration of the human sFcεRIαD1&2 protein here was (20µg ml^{-1}) twice as much as the highest concentration used in the kinetic analysis, the curve is slightly smaller than expected, this supports the biological results described in Chapter 4 that showed the equine IgE support mediator release from RBL-2H3.1 cell expressing the human FcεRI receptor.

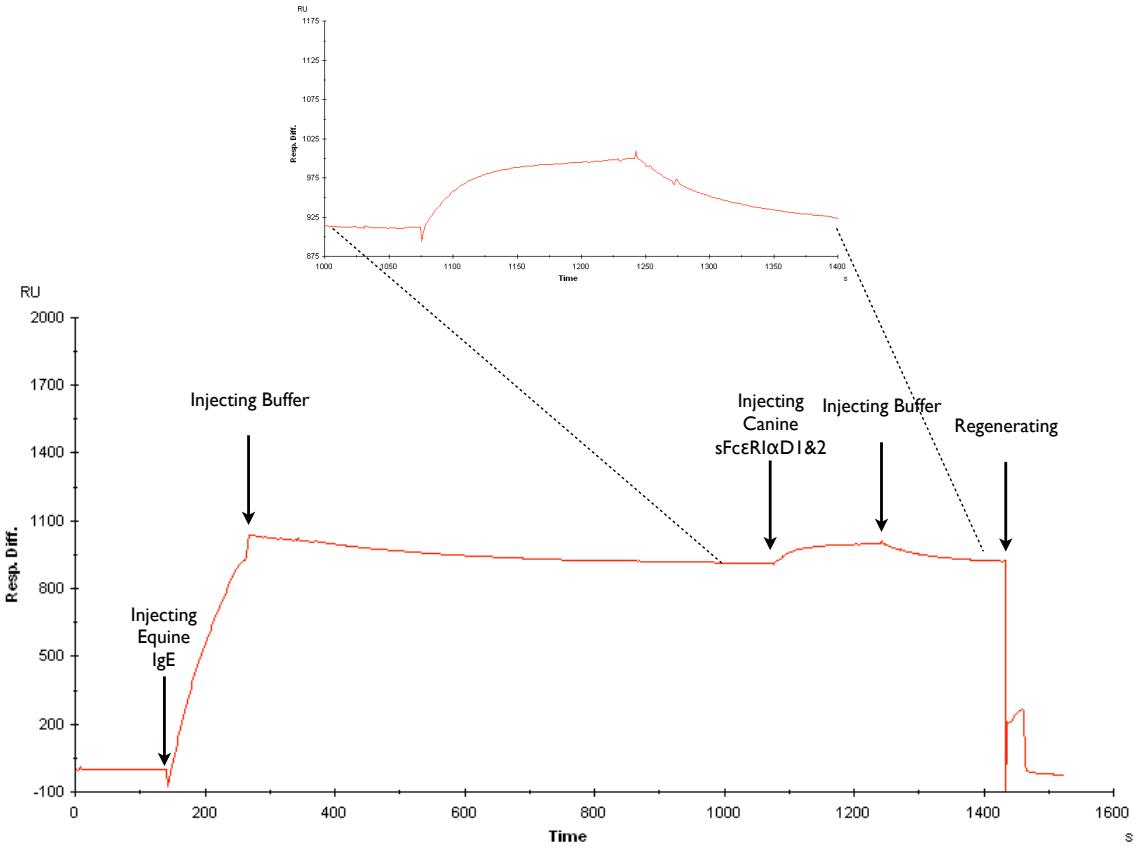


Figure 47: Re-testing the binding between the equine IgE and the canine sFcεRIαD1&2:
This figure shows the re-test of the binding between the equine IgE and the canine sFcεRIαD1&2 protein. The addition of more equine IgE, even though it did not reach ~2500 RU, was still enough to show clear binding between the equine IgE and the canine sFcεRIαD1&2. Though the concentration of the canine sFcεRIαD1&2 protein here was (20µg ml^{-1}) twice as much as the highest concentration used in the kinetic analysis, the curve is slightly smaller than expected, this fits with the biological results obtained in Chapter 4 that showed the equine IgE causes no release in RBL-2H3.1 cell expressing the canine FcεRI receptor.

A rabbit IgG antibody was raised against the human IgE AB helix in order to map the binding site of the low-affinity FcεRII receptor by (Ian Sayers, PhD Thesis, The University of Sheffield, 1997) and the helix's effect on the FcεRI receptor, the detailed concept and protocol can be found in chapter 7 of Ian Sayers' thesis, which showed that immunization with a cyclized peptide is more successful than a linearized peptide. From that study it was discovered that this rabbit IgG anti human IgE AB helix bound to the

IgE antibody and prevented its binding to the FcεRI receptor. When the human and equine IgE sequences were analyzed by BLAST, it was confirmed that the two IgEs are only 68% similar. But when the AB helixes of the two IgEs (equine = PSPLDLYVSKS and human = PSPFDLFIRKS) were analyzed by BLAST, it showed that they are up to 82% similar. Therefore the binding between the rabbit IgG anti human IgE AB helix was tested for binding to the equine IgE, Figure 48.

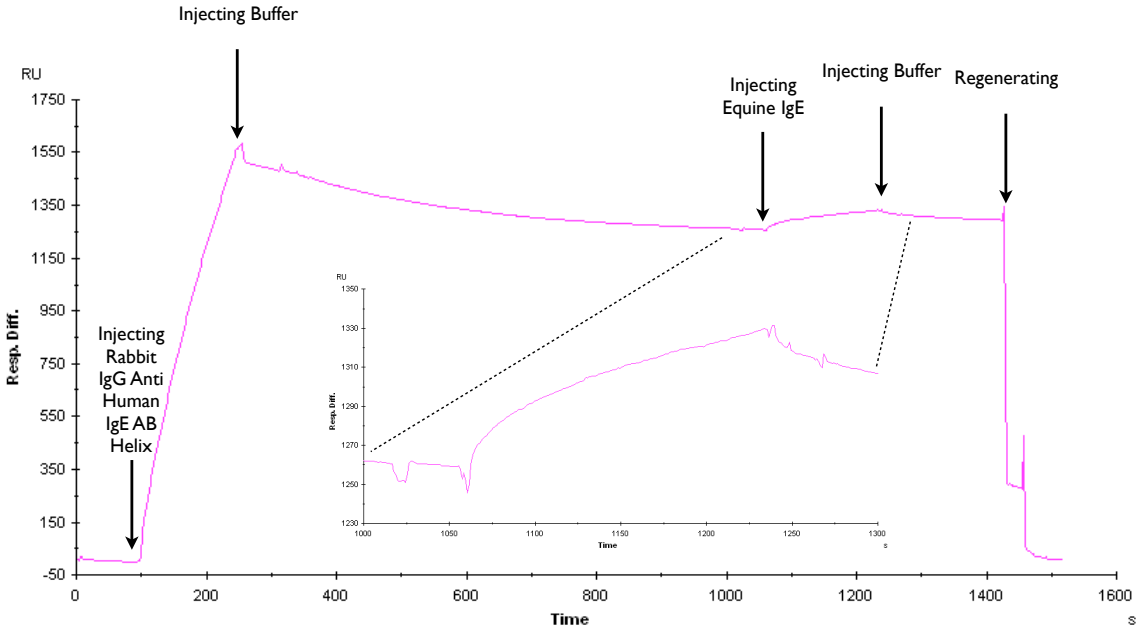


Figure 48: Testing the binding of rabbit serum raised against human IgE AB helix to equine IgE:
This figure shows the successful binding of rabbit serum raised against human IgE AB helix to equine IgE.

This result concludes that antibodies raised against conserved sections of the human IgE can bind to equine IgE, and thus prevent the equine IgE from binding to its FcεRI receptor. This means that the equine organism can be used as a model for developing and studying potential allergy vaccines for humans. Furthermore, it indicates that it might be possible to develop an IgE-derived immunogen that will be relevant to more than one species, e.g: human, horse and dog, where the same

immunogen can be tested in the animal species before clinical trial in human are embarked on.

Chapter 7 - Generation of A Human-Horse-Human IgE Chimera:

7.1 - Introduction:

Since the binding between the equine IgE and the FcεRI receptor was measured in the last chapter to be $K_D = 1.7x10^{-10}$ M, and the crystal structure of human IgE binding to its FcεRI receptor was determined by (Garman, *et al.*, 2000), this chapter will investigate a concept of molecule design, in which an antibody response raised against epitopes in IgE that are involved in receptor binding, and thus prevent receptor binding, provided this antibody could bind to the equine IgE at a stronger affinity than the equine FcεRI receptor, in hopes that this molecule can be used as a cure for allergy. Antibodies can bind very strongly to their antigens, and antibodies can be designed to bind to virtually any type of molecule, therefore the aim here is to develop an IgG antibody anti equine IgE, precisely binding to the Cε2-Cε3 linker region of the IgE, which if it binds strongly enough could separate the IgE from its FcεRI receptor, and prevent it from re-binding, and thus stop the initial step of the IgE mediated cell degranulation that leads to allergy.

In a paper by (Hook, *et al.*, 1981) the research group developed a monoclonal antibody that can bind to human serum IgE, but it did not recognize receptor bound IgE. This concept has been pioneered by other research groups, and a drug called (Omalizumab) was developed by Genentech and Novartis (Chapter 1.6.2) where mice were injected with adjuvants that developed monoclonal IgGs against epitopes within the IgE antibody that are involved in its binding to the FcεRI receptor. Omalizumab targets an epitope called HPL (amino acids 424-426) within the Cε3 domain (Zheng, *et al.*, 2008), and thus binds to free floating human serum IgE and thus causes reduction in its quantity. Another drug called mAb12 (Laffer, *et al.*, 2001) uses the same concept, but

binds to a different epitope from that recognized by Omalizumab. Its added advantage is that it binds and removes IgE already bound to its FcεRI receptor, and therefore reduces the sensitization by IgE of mast and basophil cells (Laffer, *et al.*, 2008) presumably because of its higher binding affinity to IgE than that of IgE to its FcεRI receptor, > K_A = 10^{10} M^{-1}. The side effect of these therapeutics is the development of serum sickness (Dreyfus and Randolph, 2006) because residual mouse antigens still persist in the humanized mAbs (mouse antibodies). These two drugs were developed by immunization with whole IgE antibody molecules and selecting mouse antibodies that inhibit the binding of IgE to its FcεRI receptor, noting that these antibodies are used only for passive immunization, therefore the patients needs to constantly take them. This causes a disadvantage as it is very expensive for patients and insurance companies, since each dose consists of 300 - 400mg of anti IgE antibody, and needs to be taken every 2 - 4 weeks. A paper by (Takahashi, *et al*., 1999) discussed another passive immunisation concept which uses the binding peptide from the variable region of an IgG anti self IgE antibody to bind to serum IgE and thus prevent it from binding to its FcεRI receptor. Their findings were successful in which the peptide did bind to IgE, but the measured binding affinity was around KA = ~104-5 M-1, which is not high enough to remove receptor bound IgE nor prevent serum IgE from binding to its FcεRI receptor.

A way around this is to develop an allergy “therapeutic vaccine”, an immunogen that immunizes an organism against its own self IgE, thus by administering this immunogen, the body would produce IgG anti self IgE which will remove the IgE from serum throughout the rest of the organism’s life. This active immunization would reduce the serum sickness side effect, as well as the cost of the drug, as it only needs to be administered several times, in low quantities, to develop life long immunity. By solving

these two problems this drug can be widely adopted, by passing the side effects and costs which are preventing Omalizumab and mAb12 from gaining world wide use.

Table 3: Anti IgE antibodies:
This table shows previous anti IgE antibodies developed in our lab.

Host	Antibody Isotype	Target	K_A (M)	Reference
Mouse Antibody	IgG3	Human FG loop	$1.3x10^6$	Athanassios Vratimos, PhD Thesis, University of Sheffield, 2003
Mouse Antibody	IgG3	Human AB helix	$2.4x10^5$	Athanassios Vratimos, PhD Thesis, University of Sheffield, 2003
Mouse Antibody	IgG3	Human Cε2-3 loop	$4.7x10^4$	Athanassios Vratimos, PhD Thesis, University of Sheffield, 2003
Mouse Antibody	IgG2A	Canine FG loop	$9.01x10^4$	Athanassios Vratimos, PhD Thesis, University of Sheffield, 2003
Mouse Antibody	IgG1	Canine Cε2-3 loop	$2.88x10^6$	Athanassios Vratimos, PhD Thesis, University of Sheffield, 2003
Rabbit Antibody	Polyclonal	Human Cε2-3 loop	$7.1x10^6$	Ian Sayers, PhD Thesis, University of Sheffield, 1997

Our lab has produced several anti IgE antibodies (Table 3). Serum containing antibodies against human IgE Cε2-3 linker was developed in the PhD thesis by (Mark Street, PhD Thesis, The University of Sheffield, 2010) where an allergy therapeutic vaccine using the structure based peptide approach was researched. Here an immunogen with the protein sequence of the human IgE's Cε2-3 linker (350-363), (the synthetic peptide we made is called here $2Fc\varepsilon_{2\text{-}3}$) Figure 49, was used. This residue is involved in binding of the IgE to the FcεRI receptor, it was used to stimulate the synthesis of natural anti self IgE antibodies that are non-anaphylactic, and capable of binding and removing serum self IgE, and hopefully remove self IgE already bound to its FcεRI receptor. The PRGV amino acid sequence, from IgE's both Cε2-3 linker heavy chains, actually bind to the FcεRI receptor. The thesis by (Mark Street, PhD Thesis, The University of Sheffield, 2010) employed a new (disulphide linked dimer) design for the $2Fc\varepsilon_{2\text{-}3}$ where the Cys 328 amino acid in both chains were bonded together by a disulphide bridge, and

the NH_2 terminal of the peptide was bonded to the adjuvant thyroglobulin to restrict its flexibility, while the COOH terminal was left flexible, this was in an attempt to mimic the conformation of the human self IgE molecule. This idea came about where previous studies showed that unconstrained/circularized peptides did not result in IgG antibodies that bound to the $2Fc\varepsilon_{2\text{-}3}$ strong enough. The $2Fc\varepsilon_{2\text{-}3}$ protein's COOH end also had the sequence (PRGV) which is part of the self IgE's Cε2-3 linker and that binds to the FcεRI receptor (Figure 50), and it was realized that this exact sequence is shared between the human, mouse, rat, canine and equine IgEs (Table 4), therefore developing a polyclonal anti $2Fc\varepsilon_{2\text{-}3}$ response has the potential to cure the human, canine and equine allergies simultaneously. The insoluble thyroglobulin (670kDa) was used as an adjuvant in this study as the peptide itself (~3kDa) was not large enough to trigger an effective immune response. Effective immunogens should have epitopes that can bind to the B cell receptors, and also can cause physical association between B cell and Th_0 cells. Adjuvants that could have been used were bovine serum albumin, ovalbumin, and Keyhole Limpit Hemocyanin, but thyroglobulin was finally chosen as its coupling to the $2Fc\varepsilon_{2\text{-}3}$ protein was efficient. The $2Fc\varepsilon_{2\text{-}3}$ protein was synthesized, bound to thyroglobulin and used to immunized rabbits with three rounds of subcutaneous and intradermal injections, this was effective as previous studies showed that subcutaneous and peritoneal injections resulted in anaphylaxis of 75% of the vaccinated population (Athanassios Vratimos, PhD Thesis, University of Sheffield, 2003). The rabbits developed a polyclonal IgG anti $2Fc\varepsilon_{2\text{-}3}$, and the result of that project showed that the polyclonal IgG antibodies produced by the rabbits successfully bound to human and canine self IgEs. They also did not cause any receptor cross linking on the cell surface, thus did not cause anaphylaxis. This was because the $2Fc\varepsilon_{2\text{-}3}$ protein caused IgG antibodies to target its self IgE epitopes, which are buried into the FcεRI receptor when

the self IgE binds to it, therefore the polyclonal IgG antibodies cannot reach it. But the project showed that these antibodies were still not capable of preventing self IgE from binding to its FcεRI receptor. This was because anti IgG antibodies bound to the self IgE much weaker than the self IgE binds its FcεRI receptor. The self IgE binds to its FcεRI receptor at K_D= ~10^{-10} M, the polyclonal IgGs bound to the self IgE at K_D=~10^{-5}-10^{-6} M (therefore the IgG would dissociate more readily from the IgE than the FcεRI receptor). Thus free floating self IgE would still dissociate from the IgG anti self IgE antibody and bind to its FcεRI receptor.

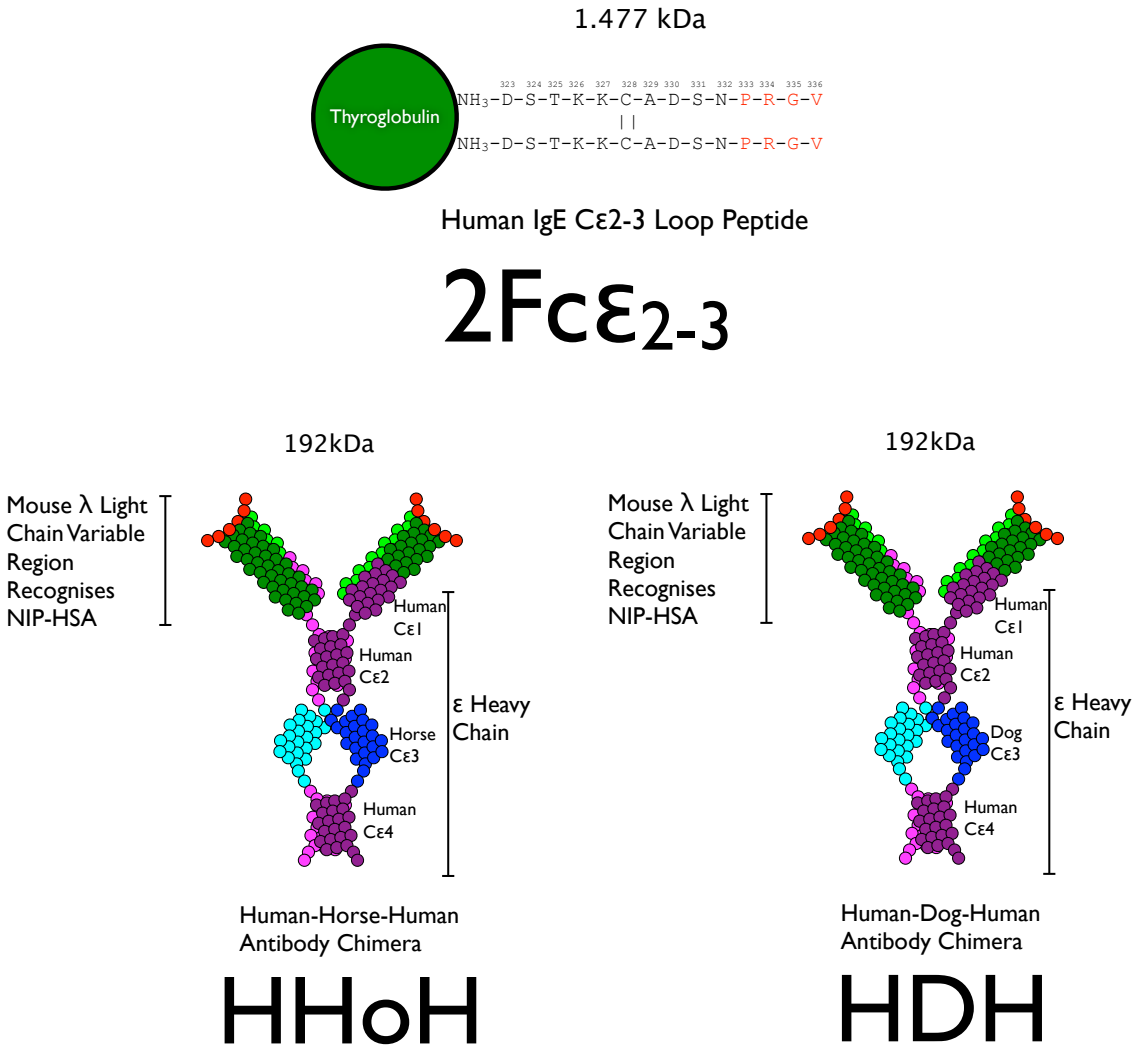


Figure 49: Structure of the 2Fcε$_{2-3}$ protein, the HHoH and HDH antibodies:
This figure shows structure of the 2Fcε$_{2-3}$ protein, the HHoH and HDH chimeric antibodies.

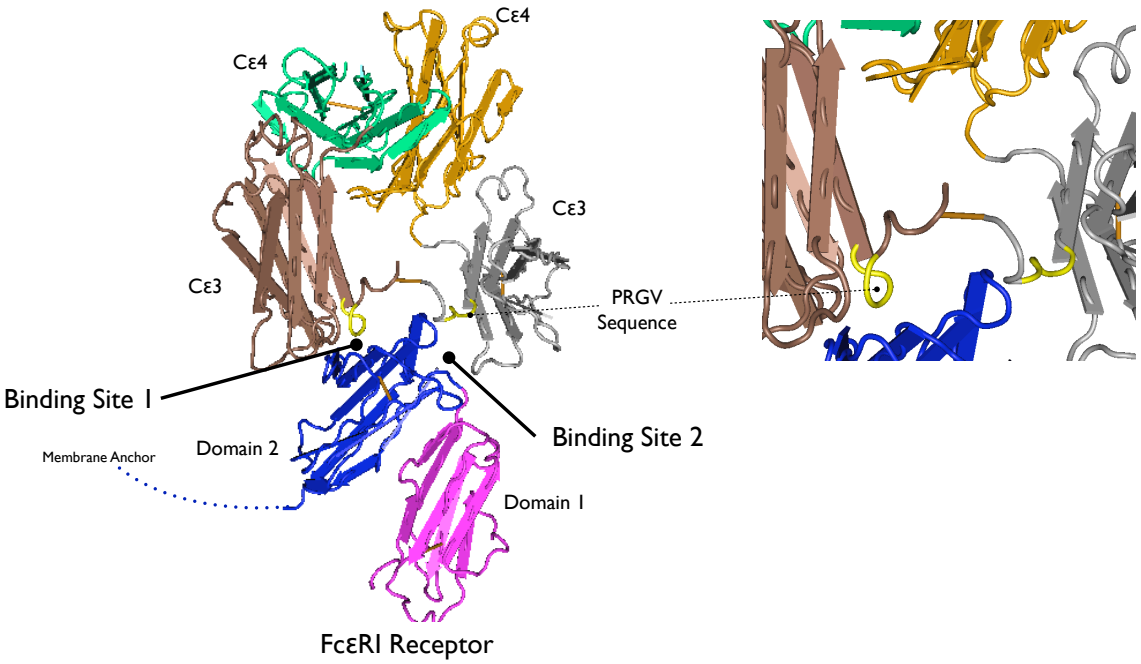


Figure 50: The position of the Cε2-3 linker:
This figure shows the position of the Cε2-3 linker when the IgE binds to its FcεRI receptor. Figure was taken from (Garman, *et al.*, 2000).

Table 4: Sequence of the Cε2-3 linker in different species:
This table shows the homology of the Cε2-3 linker between different organisms. Note that all organisms have the same PRGV sequence.

	323	324	325	326	327	328	329	330	331	332	333	334	335	336
Human	D	S	T	K	K	C	A	D	S	N	**P**	**R**	**G**	**V**
Canine	D	E	A	R	K	C	S	E	S	D	**P**	**R**	**G**	**V**
Mouse	A	H	T	R	R	C	P	D	H	E	**P**	**R**	**G**	**V**
Rat	A	H	T	R	R	C	S	D	D	E	**P**	**R**	**G**	**V**
Equine	Q	A	R	K	C	T	E	S	D	**P**	**R**	**G**	**V**	S

The structure based peptide approach, which was just discussed, had a main limitation which was it developed polyclonal IgG antibodies that bound weakly to the self IgE's Cε3 domain, this was because a small peptide, single or circularized, is still quite different from the actual three dimensional structure of the original protein (Ledin, *et al.*, 2006). (Wang, *et al.*, 2003) explains that this approach was successful in several organisms when the immunogen peptide (self IgE amino acids between 288 and 315 at

the end of the Cε3 domain) was complex with T helper epitopes adjuvants derived from measles virus (UBITh®A), therefore the adjuvants can increase the immunogenicity of the immunogen.

While anti-peptide antibodies commonly are of low affinity when assessed for binding to the integral protein from where the peptide was derived, (Johansson and Hellman, 2007) also argued that vaccinating self antigens is difficult due to the maturation of both B and T cells that are selected not to react against self antigens. Therefore a new concept of therapeutic vaccine design (called here the self/non-self approach) is researched in an attempt to increase the immunogenicity of self IgE. It uses chimeric self and non-self IgE constructs that could induce natural IgG anti self IgE antibodies (Hunter, *et al.*, 2008; Ledin, *et al.*, 2006; Johansson and Hellman, 2007). This has the added advantage over the previous method as having a full IgE antibody as an immunogen allowing the non-self Cε2 and Cε4 domains to maintain the correct three dimensional folding structure of the self Cε3 domain (Hunter, *et al.*, 2008). The use of non-self Cε2 and Cε4 domains is likely to increase the immunogenicity of the final chimeric antibody, which means that the organism's body will most likely develop antibodies against its own self Cε3 domain. This immunogenicity can be further increased if the chimeric antibody was administered several times as boosts to the initial immunization. (Johansson and Hellman, 2007) also argue that immunogenicity can be increased by the use of correct potent adjuvants; such as mineral oil based adjuvants, the vaccine immunogen has to be soluble, properly folded and contain repetitive epitopes, and dimerizing the immunogen increases the immunogenicity by 4 - 8 folds. The self/ non-self concept was shown in the paper by (Ledin, *et al.*, 2006) to be successful in producing antibodies anti self canine Cε3 domain. This was done by inserting the canine Cε3 domain in an opossum IgE heavy chain to make an opossum-dog-opossum

chimeric (ODO) protein, noting this was not a full IgE, rather only a three domain heavy chain construct. The (Ledin, *et al.*, 2006) research, though successful, did not test whether the immunization with a chimeric antibody encompassing the canine Ce3 is likely to give rise to an immune response against epitopes that are not engaged in receptor binding, this might lead the antibodies to binding to receptor bound IgE, cross-linking them and thus initiating mediator secretion. In other words, they did not test if the serum causes anaphylactic. Although the research group did not report such adverse reaction when immunizing dogs with the ODO chimera, this should have been assessed at the molecular level. The generation of our RBL-2H3.1 FcεRIα transfected cell lines (Chapter 4 and from previous colleagues) provide an excellent means to safely test these immune response at the molecular level.

Therefore the current thesis will take this concept of an allergy therapeutic vaccine using the self/non-self approach further to develop active vaccination instead of passive immunization. Instead of immunizing organisms with the $2Fc\varepsilon_{2\text{-}3}$ protein alone, they will be primed with it at first, followed by boost immunizations with a full chimeric antibody, where this antibody will have self Cε3 domain in between non-self Cε2 and Cε4 domains.

The concept of original antigenic sin (Chapter 1.8) will be used here where the immune system would be first primed by the cyclised $2Fc\varepsilon_{2\text{-}3}$ peptide, then boosted using the full 3D Cε3 structure presented to it in the form of a IgE chimera, where the Cε3 domain of the target species is inserted between the Cε1, Cε2 and Cε4 of a distant phylogenic mammal. This should lead the immune system to target the same epitopes in the Cε3 domain and, since the epitopes are now presented in a 3D configuration, the immune system should develop stronger antibodies against them through somatic

hypermutation, thus allow the development of stronger polyclonal antibodies against the self IgE Cε3 domain. It is believed that this immunization protocol would increase the immunogenicity of the self IgE's Cε3 domain and therefore would cause the organism to develop stronger immunity to its own self IgE, where polyclonal antibodies would bind to the self IgE much stronger that the self IgE binds to its FcεRI receptor. The Summary of the immunization protocol is:

1. Subcutaneous and intradermal injections of all immungens.

2. First immunization is by $2Fc\varepsilon_{2\text{-}3}$ protein alone.

3. This is followed by three boosts by the chimeric antibody. Where the Cε3 domain of the IgE is replaced by the organism's own self Cε3 domain protein sequence, and the non-self Cε1, Cε2 and Cε4 domains of a distant phylogenic mammal, which are only there to maintain the correct three dimensional structure of the self Cε3 domain.

The project will be investigating a possible allergy therapeutic vaccine for horses, therefore it started by the synthesis of the human-horse-human (HHoH) IgE anti NIP-HSA chimera antibody, but later used the HDH IgE anti NIP-HSA chimera antibody (Figure 49) due to financial reasons. The human non-self Cε1, Cε2 and Cε4 were used as scaffold with the horse self Cε3 as the human IgE sequence is phylogenically distant than the horse IgE sequence. The human IgE heavy chain wild type sequence (Nishida, *et al.*, 1982) was obtained from the GenBank sequence database (part of the United States' National Center for Biotechnology Information, NCBI). The gene was not optimized and the physical gene was obtained from another colleague in the laboratory. Each domain was amplified, through PCR, with its introns, along with the horse Cε3 domain, and they were constructed to make the full HHoH gene. The HHoH gene was

then cloned into the pSV-V_{NP} plasmid downstream of a gene sequence that codes for a mouse antibody variable region that recognizes and binds to the antigen 4-hydroxy-5-iodo-3-nitrophenylacetic acid (NIP), therefore when expressed the protein would be a full HHoH IgE antibody with a mouse variable region that binds to NIP (HHoH IgE anti NIP). The final plasmid (pSV-V_{NP}HHoH) was transfected into mouse B (J558L) cells, that after selection of the cloned cells, a J558L cell line that expressed HHoH IgE anti NIP-HSA was developed. The protocol for transfection, expression and purification was run exactly as in Chapter 3. The human-dog-human (HDH) IgE chimeric antibody (Figure 49) was used to continue this research as the HHoH was not expressed in enough quantities to allow for vaccination.

7.2 - Results:

7.2.1 - Getting The Human And The Equine IgE Heavy Chain Genes:

The human heavy chain sequence was taken from (Nishida, *et al.*, 1982) while the actual gene was in the lab isolated by a previous colleague (Hongtu Ye, PhD Thesis, The University of Sheffield, 2010). The human gene did not need any optimization nor modification, fist because the introns in the gene are used to insert restriction site where the different domains can be clones, second this gene was already proven to successfully express in J558L cells. The equine heavy chain gene was the same gene from chapter 3.

7.2.2 - Constructing the HHoH Heavy Chain Gene:

The approach to the synthesis of the HHoH gene was through PCR. Each domain was amplified separately and cloned into a separate pUC18 plasmid. That enabled each domain to be sequenced and confirmed that no fatal mutations occurred from the PCR step. The different domains where then put together in a series of clonings and transformations (Chapters 2.2.2 - 2.2.12 and 2.2.16), until the full HHoH gene was

constructed. The final HHoH gene was then cloned into the pSV-V_{NP} plasmid to make pSV-V_{NP} HHoH. Noting that a parallel experiment was run where a GFP gene was inserted downstream of the HHoH to give the plasmid of pSV-V_{NP} HHoHGFP as a means of shortcutting the J558L selection process (discussed later in chapter 7.3). The DNA and protein sequences of the human, equine and the final HHoH IgEs are found in the Appendix. The primers synthesized for this experiment were:

```
Human Cε1Cε2 Primers:
Forward: 5'-AAAAAGCTTGGATCCCTGCCACGGGGTCCC-'3
Reverse: 5'-TTTGAATTCGGATCCGCGGCCGCACGCGTTGATCACTCGAGTGAGAGGCTGCATGAGGG-'3

Horse Cε3 Primers:
Forward: 5'-AAAAAGCTTCTCGAGCACACTGCAGAGAGCGACCCTAGGGG-'3
Reverse: 5'-TTTGAATTCTGATCACCCGTGGCTCACCAGGGGCCTTGGCGATG-'3

Human Cε4 Primers:
Forward: 5'-AAAAAGCTTTGATCACCCAGGGGAGGTGGGC-'3
Reverse: 5'-TTTGAATTCACGCGTAGCTGGATGGAGCCCTGG-'3

GFP Primers:
Forward:5'-AAAAAGCTTACGCGTATGAGTAAAGGAGAAGAAC-'3
Reverse:5'-TTTGAATTCGCGGCCGCTCATTTGTATAGTTCATCCATGC-'3
```

All forward primers had a HindIII restriction site following three adenine nucleotides. And all reverse primers had an EcoRI restriction site following three thymine nucleotides. This configuration was to allow all PCR products to be cloned into the pUC18 plasmid for ease of handling and where they can be used for sequencing to confirm no mutations have occurred. All forward primers had another unique restriction site immediately following the HindIII restriction site, and all reverse primers had another unique restriction site following the EcoRI restriction site. These strategically placed restriction sites allowed the different PCR amplified domains to be added together and constructed the HHoH gene. Figure 51 explains this concept figuratively.

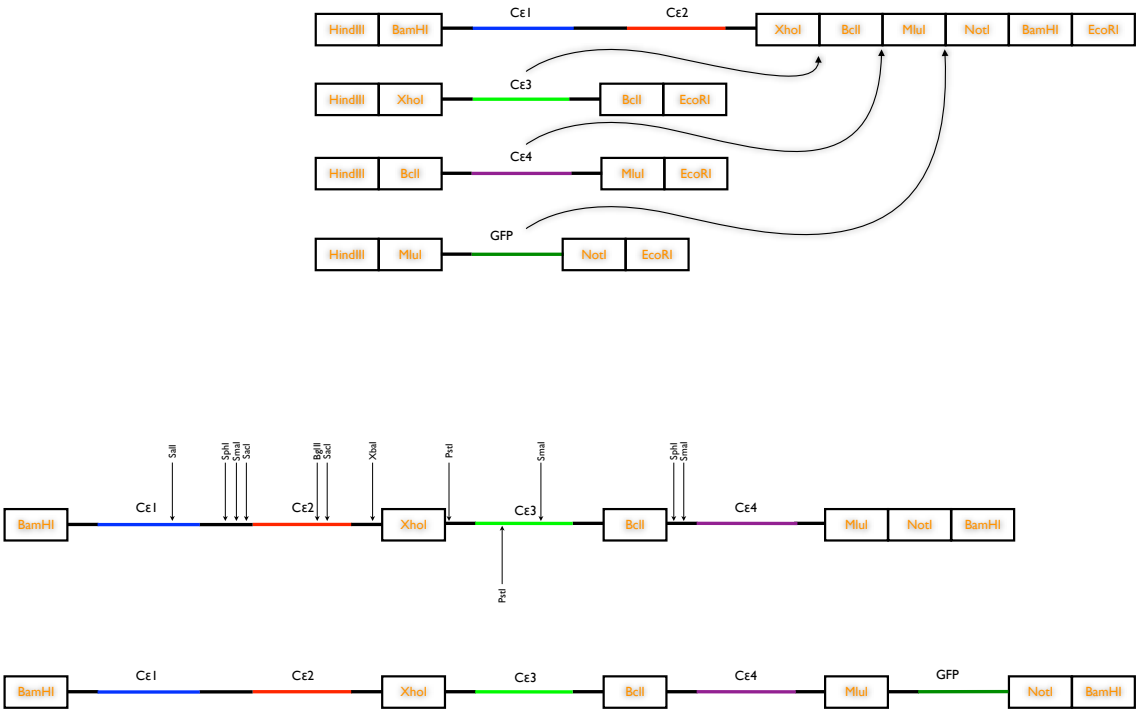


Figure 51: HHoH domain construction concept:
This figure shows the PCR products formed by the above primers and how they were cloned together using the strategically placed restriction sites to make up the full HHoH gene, a copy of this gene had a GFP gene added below it, as an attempt to shortcut the selection period.

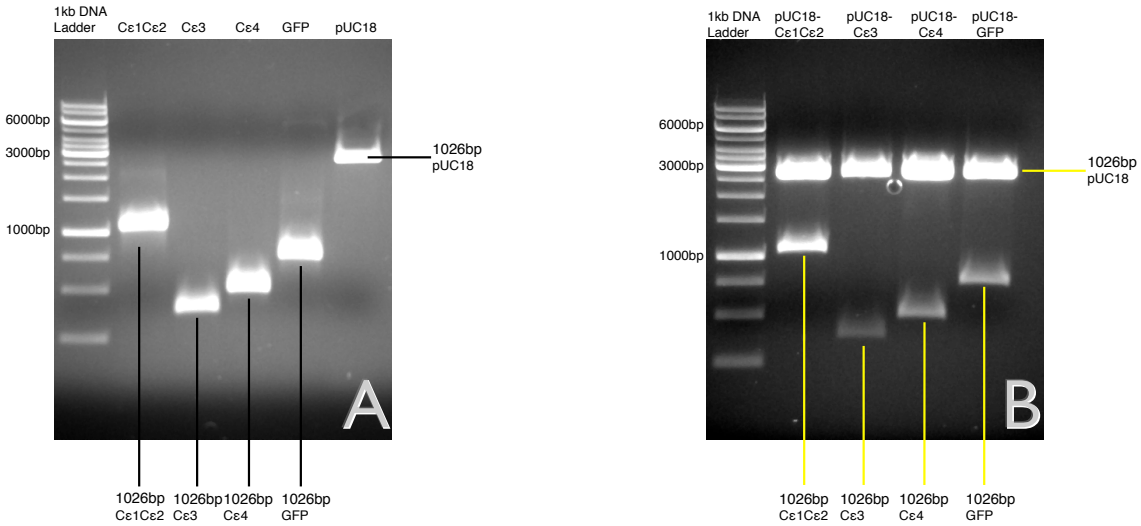


Figure 52: HHoH domain PCR amplifications and cloning into pUC18:
A. shows the PCR product of each domain. **B.** shows the successful cloning of all the PCR products into separate pUC18 plasmids, these plasmids were sent for sequencing to confirm no fatal mutations developed from the PCR step.

All the domains were amplified by PCR as in chapter 2.2.6 where the human Cε1, Cε2 and Cε4 domains where amplified along with the equine Cε3 domain. They were all then cloned into separate pUC18 plasmids and transformed into bacteria. Figure 52A shows the result of the PCR, and Figure 52B shows the result of all the domains successfully cloned into pUC18 plasmids.

Several samples of each plasmid (pUC18-Cε1Cε2, pUC18-Cε3 and pUC18-Cε4) were sent for sequencing, where the results are found in the Appendix, and the plasmids with genes that contained no fatal mutations (silent mutations that either appeared in introns or caused no change in amino acid sequence) were chosen to construct the HHoH gene.

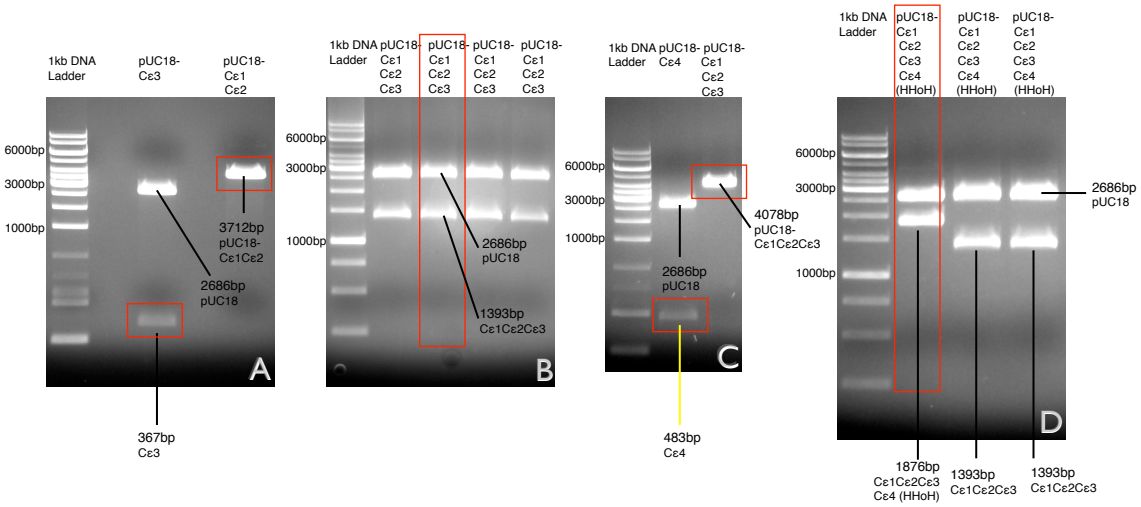


Figure 53: Constructing the HHoH gene:
A. shows the the results of the restriction digest for the addition of Cε3 domain to the Cε1Cε2 domains and **B.** shows the confirmation results of the successful addition to make pUC18-Cε1Cε2Cε3. **C.** shows the restriction digest for the addition of Cε4 domain to the Cε1Cε2Cε3 domains and **D.** shows the confirmation results of the successful addition to make the final HHoH gene represented as pUC18-Cε1Cε2Cε3Cε4 (HHoH).

The domains where then constructed by adding one domain to the other to make the final HHoH gene (Figure 53). The Cε3 domain was added to Cε1Cε2 domains to make a final plasmid of pUC18-Cε1Cε2Cε3 (Figure 53A). The plasmid was

transformed into bacteria, grown, harvested and checked for the successful cloning (Figure 53B). Then the Cε4 domain was added to the Cε1Cε2Cε3 to make the plasmid pUC18-Cε1Cε2Cε3Cε4 ,can also be referred to as pUC18-HHoH, (Figure 53C). The plasmid was transformed into bacteria, grown harvested and checked for the successful cloning (Figure 53D).

7.2.3 - Cloning The HHoH IgE Heavy Chain Gene Into Plasmid:

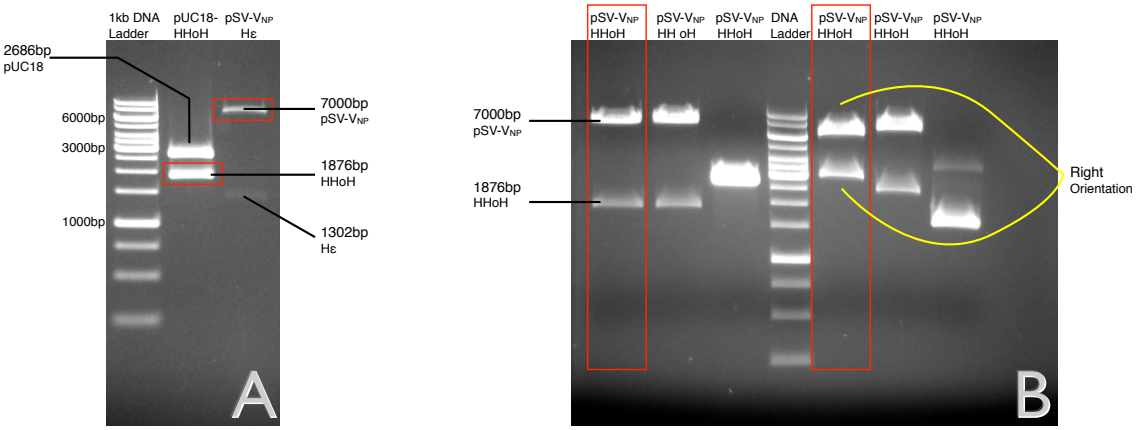


Figure 54: Transferring the HHoH gene from pUC18 plasmid to pSV-V_{NP} plasmid:
A. shows the restriction digest of the HHoH from the pUC18 plasmid. **B.** on the right, shows the successful ligation of the HHoH into the pSV-V_{NP} plasmid, and on the left, shows the orientation tests run to confirm the gene is in the right orientation in the pSV-V_{NP} plasmid, as the gene was digested by the same restriction enzyme from both ends.

The final pUC18-HHoH plasmid was digested with BamHI restriction enzyme, de-phosphorylated and cloned into the pSV-V_{NP} plasmid (Figure 54A). The plasmid was also tested for the successful ligation and orientation of the gene, selecting the plasmid that had the gene in the correct orientation (Figure 54B) as the HHoH gene was digested by the same restriction enzyme from both ends.

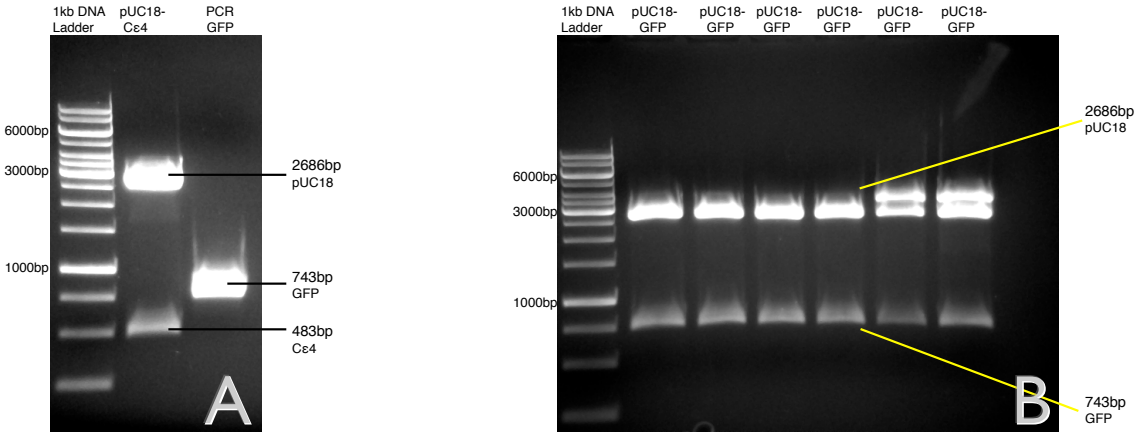


Figure 55: Cloning the GFP gene into pUC18:
A. shows the PCR amplification of the GFP gene . **B.** shows the successful ligation of the GFP gene into the pUC18 plasmid.

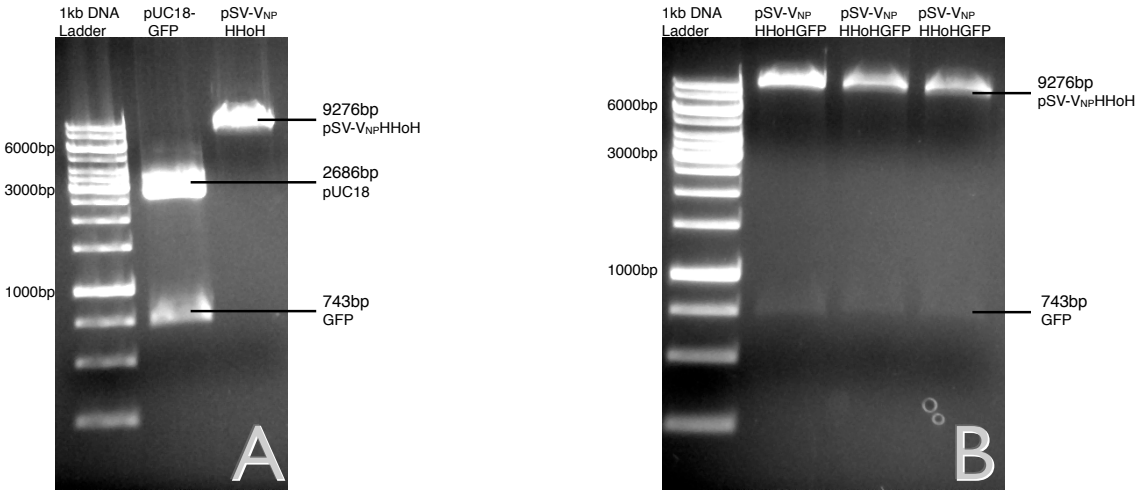


Figure 56: Cloning the GFP gene into the pSV-V$_{NP}$HHoH plasmid:
A. shows the restriction digest of the GFP gene using MluI and NotI restriction enzymes. **B.** shows the successful ligation of the GFP gene into the pSV-V$_{NP}$HHoH to make pSV-V$_{NP}$HHoHGFP.

Some of the pSV-V$_{NP}$HHoH plasmid was used to clone the GFP gene downstream of the HHoH in an attempt to shortcut the J558L selection (Chapter 7.3). The GFP gene was amplified by PCR (Figure 55A) and cloned into the pUC18 plasmid (Figure 55B) then it was sent for sequencing to confirm no fatal mutations occurred in the PCR step, the Appendix contains the sequence and of GFP gene along with the sequencing results. Once a pUC18-GFP plasmid was identified with the gene containing no fatal mutations,

the GFP gene was digested (Figure 56A) and ligated to the pSV-V_{NP}HHoH plasmid to make pSV-V_{NP}HHoHGFP (Figure 56B).

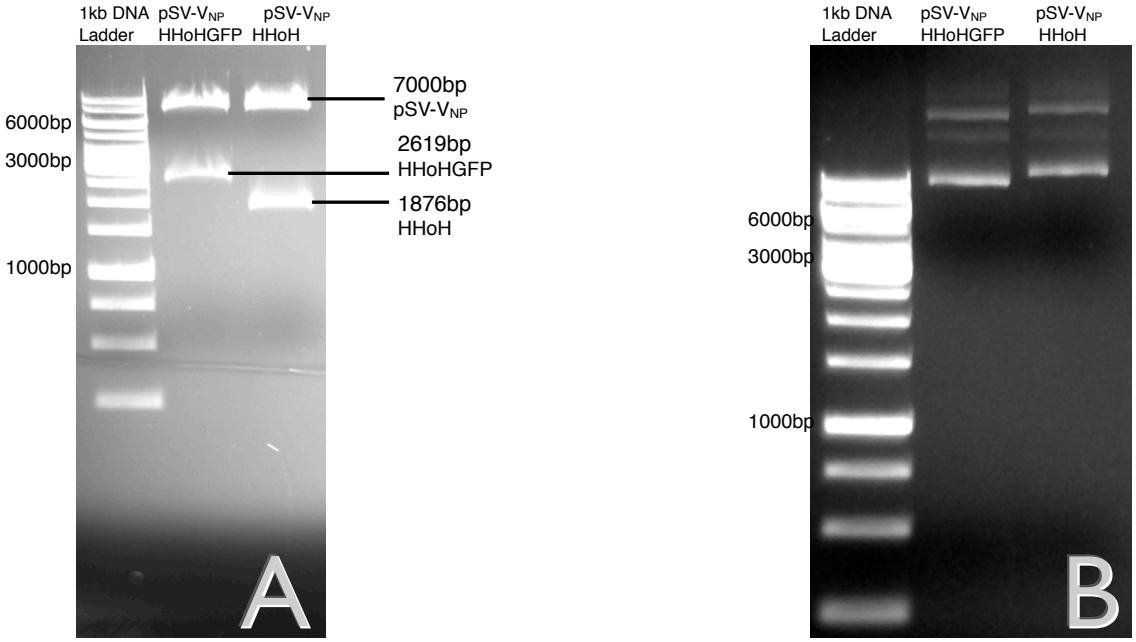


Figure 57: Confirmation of the presence of HHoH and HHoHGFP in their pSV-V_{NP} plasmids:
A. shows a confirmation of the presence of the HHoH and HHoHGFP genes in their pSV-V_{NP} plasmids, HHoH being 1876bp and HHoHGFP being 2619bp. **B.** shows bacterial chromosome impurity test of the final purified pSV-V_{NP}HHoH and pSV-V_{NP}HHoHGFP plasmids, there were no bacterial chromosome impurities.

The final pSV-V_{NP}HHoH and pSV-V_{NP}HHoHGFP plasmid were midi prepped as in chapter 2.2.13 to collect large quantities of the DNA, and then the DNA was concentrated as in chapter 2.2.15 and re-diluted to the required concentration for mammalian cell transfection. They were then run on an electrophoresis gel as a final confirmation for the presence of the correct genes (Figure 57A) and that there is no bacterial chromosome impurity (Figure 57B), which will interfere with the cell selection step as bacterial chromosomes would cause mammalian cells to undergo apoptosis.

7.2.4 - Transfecting And Selecting HHoH IgE Anti NIP-HSA Expressing J558L Cells:

J558L cells were transfected with the pSV-V_{NP}HHoH plasmid as in chapter 2.3.5. Similar selection problems were faced as in Chapter 3 and they are discussed in chapter 3.3. and several attempts, and 6 weeks of selection, the colonies were tested using SPR for the presence and quantity of the HHoH IgE anti NIP-HSA (Figure 58).

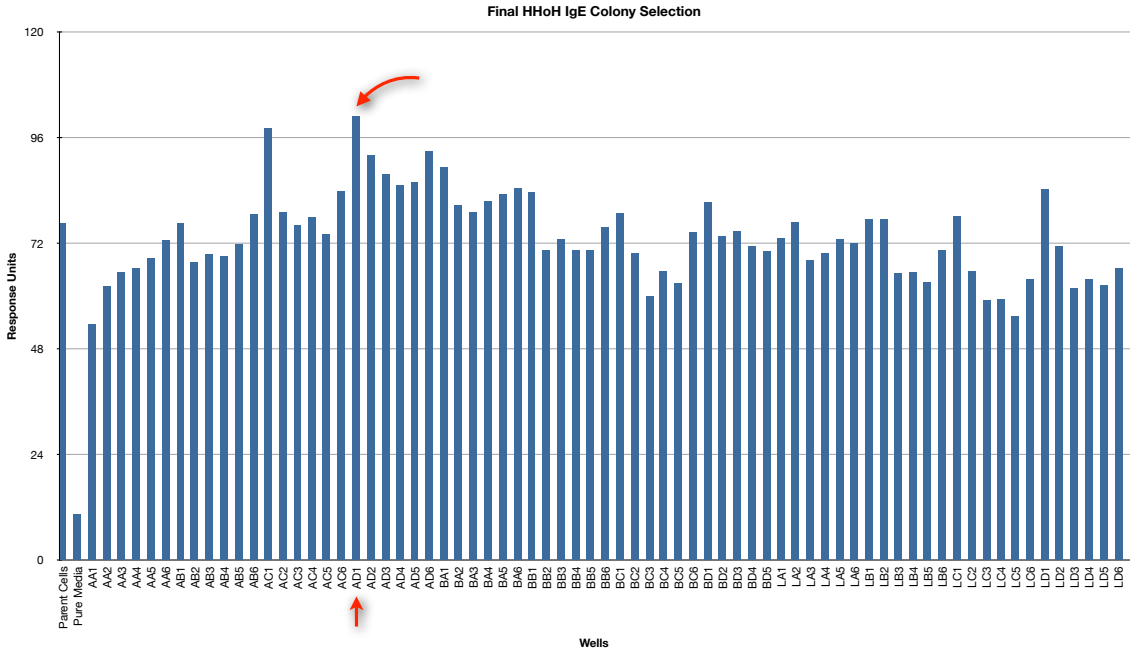


Figure 58: SPR results of J558L selection for HHoH IgE anti NIP-HSA expression:
This figure shows the SPR result of the transfected and selected J558L cell medias from each well. Each bar represents the quantity of HHoH IgE anti NIP-HSA, therefore well AD1 ,marked with the red arrows, was selected as the well expressing the most IgE, therefore the cells of this well were collected and grown separately where the J558L cell line expressing HHoH IgE anti NIP-HSA was developed. Values for this graph are in the Appendix.

The wells labeled A-B where transfected as in chapter 2.3.5 by electroporation, the wells labeled in L are cells transfected using a liposome (FuGENE® From Roche), and here it can be seen that there is comparable results between the two. Yet the selection process still did not go as excepted, compared to results in Figure 17 (Chapter 3.2.4), mainly due to the weak selection process that did not kill enough of the non-

transfected cells, and thus results in colonies that do not have enough expressing cells. This can be determined from the graph where all the bar peaks are close to each other, including the negative control (non-transfected parental cells). Non the less the experiment had to be carried on due to financial reasons, and the AD1 colony was isolated.

7.2.5 - Collecting And Purifying The HHoH IgE Anti NIP-HSA:

A density of $5x10^5$ cells ml^{-1} of the J558L cells expressing the HHoH IgE anti NIP-HSA was plated on a 100mm2 petri dish with 20ml of selective media, then incubated for 5 days at 37°C + 5% CO2 + 90% relative humidity as in chapter 2.4.3. This was found to be the optimal cell density and length of time for expression, where the expressed protein accumulated enough in the media, but not left long enough for it to start degrading.

1200ml of media with expressed HHoH IgE anti NIP-HSA was collected and centrifuged at 180xg for 3 minutes to remove the cells, then the media was filter sterilized through a 0.45µm filter to remove all cell and prevent dead cell proteases from degrading the IgE. Due to the large volume of the media, it was concentrated using a 3kDa molecular filter to reduce the volume. The concentrated media was purified through a chromatography column as in chapter 2.4.5.

7.2.6 - Checking HHoH IgE Anti NIP-HSA Viability And Purity:

The purified HHoH IgE anti NIP-HSA was concentrated down to ~200µl, using a 3kDa molecular filter, and its concentration was determined as in chapter 2.4.6 (Figure 40 from chapter 5.2.4). In this case the HHoH concentration was 0.43 mg ml^{-1} as its OD_{625} = 0.838 and the equation used was y = 0.8408x + 0.4763. The concentration of the HDH IgE anti NIP-HSA was also tested (Figure 59) and came out to be 5.4 mg ml^{-1}

as its OD_{625} = 0.411 at 100x dilution. Later in the discussion section of this chapter (Chapter 7.3) there is an explanation for the reasons behind the use of HDH IgE anti NIP-HSA instead of HHoH anti NIP-HSA in the immunization experiments that followed.

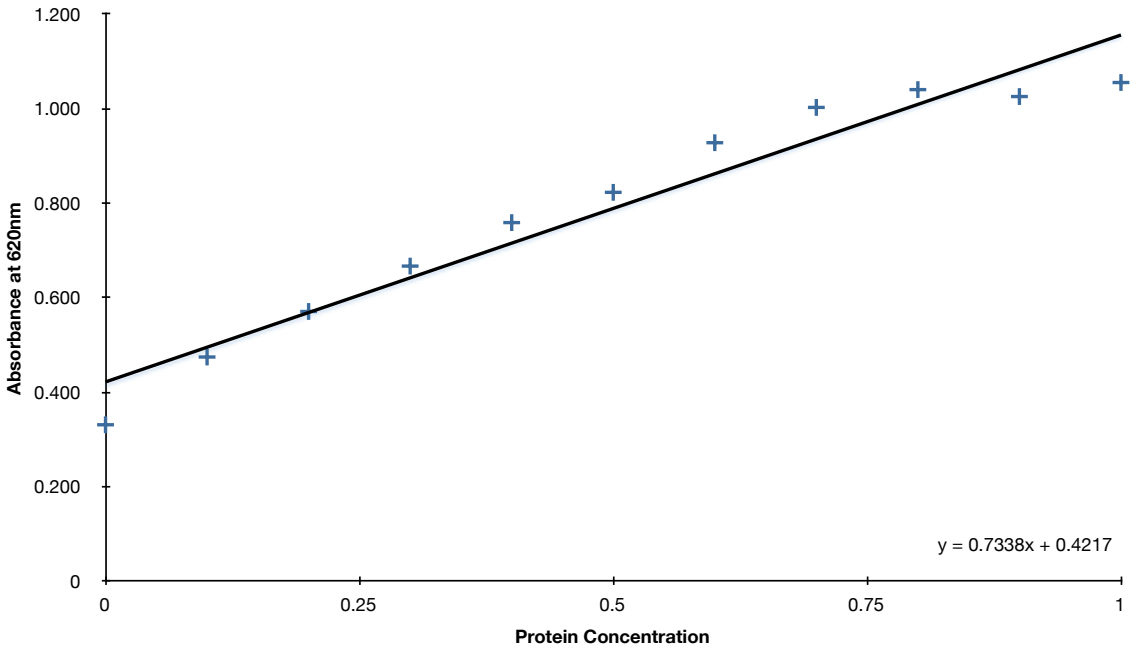


Figure 59: The protein purification graph used to calculate the concentration of the purified HDH IgE anti NIP-HSA: This figure shows the concentration curve of bovine albumin solutions. At the bottom right of the graph is the equation for the line of best fit, from this equation the concentration of the HHoH IgE anti NIP-HSA was determined. Values for this graph are in the Appendix.

The HHoH IgE anti NIP-HSA was then tested on an SDS-PAGE for its purity (Figure 60A). The SDS-PAGE result showed that the non-reduced IgE had a single strong back band above the level of the ladder as the IgE size was ~192kDa, it also showed very little IgE degradation by the very faint lines under the IgE band, and minor albumin impurity, which is shown by a band at 65kDa. The HHoH IgE was also reduced, using β-mercaptoethanol, to break the disulphide bridges between the two heavy chains and between the heavy chain and the light chain, and this resulted should

give two bands at ~70kDa for the heavy chain, and ~25kDa for the light chain, but only the heavy chain band appeared. This was not expected, and the reason for this was not investigated thoroughly. The HDH IgE anti NIP-HSA was also tested in an SDS-PAGE for its purity (Figure 60B) and the results came out as expected.

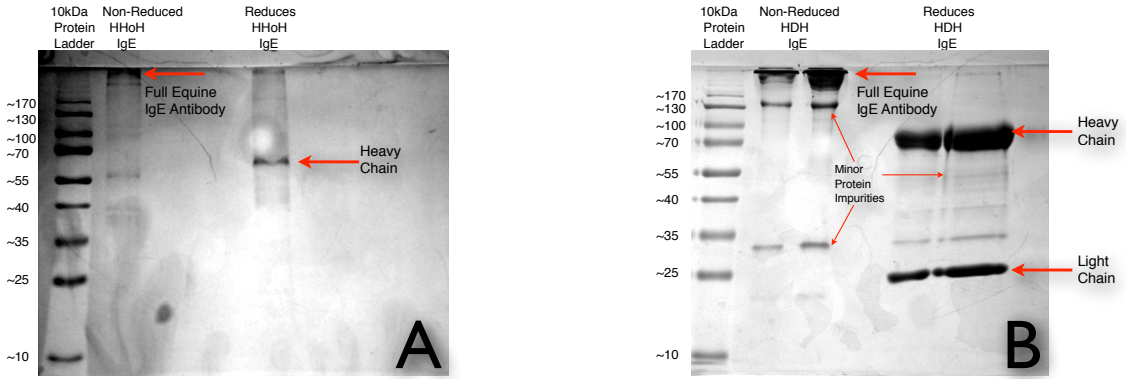
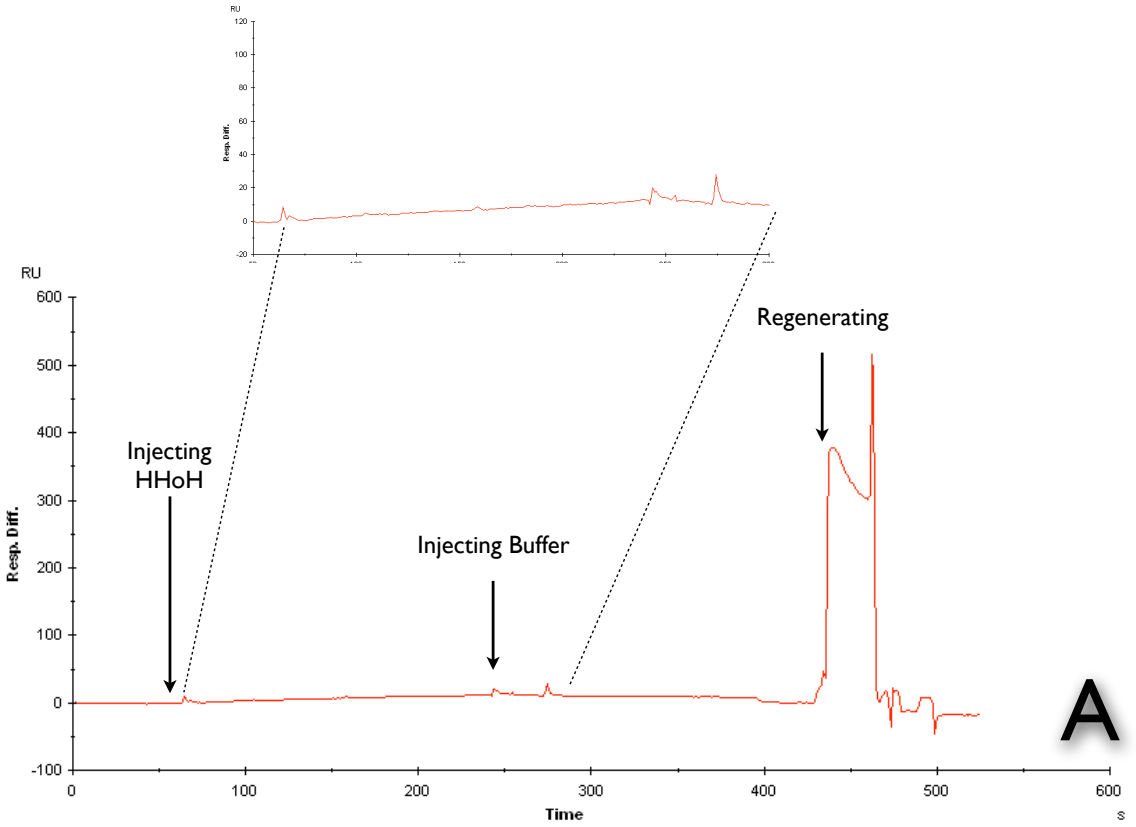


Figure 60: SDS-PAGE of the HHoH and HDH IgEs anti NIP-HSA:
A. is the SDS-PAGE result of the HHoH IgE anti NIP-HSA, on the left is the non-reduced IgE showing a single strong band above the ladder as the IgE size is ~192kDa, the result also show a lot of IgE degradation by the smear of lines under the IgE band, but shows almost no albumin impurity, which would show a band at 65kDa. On the right is the reduced HHoH IgE anti NIP-HSA, by β-mercaptoethanol, therefore the disulphide bridges between the two heavy chains and between the heavy chain and the light chain are broken. The top band shows the heavy chain as predicted at ~70kDa, but no bottom chain is visible, which would show a band at ~25kDa. The reason for this was not investigated thoroughly. **B.** is the is the SDS-PAGE result of the HDH IgE anti NIP-HSA that was prepared by (Hongtu Ye, PhD Thesis, The University of Sheffield, 2010) showing good strong bands at the predicted places, with minor protein impurities and IgE degradation.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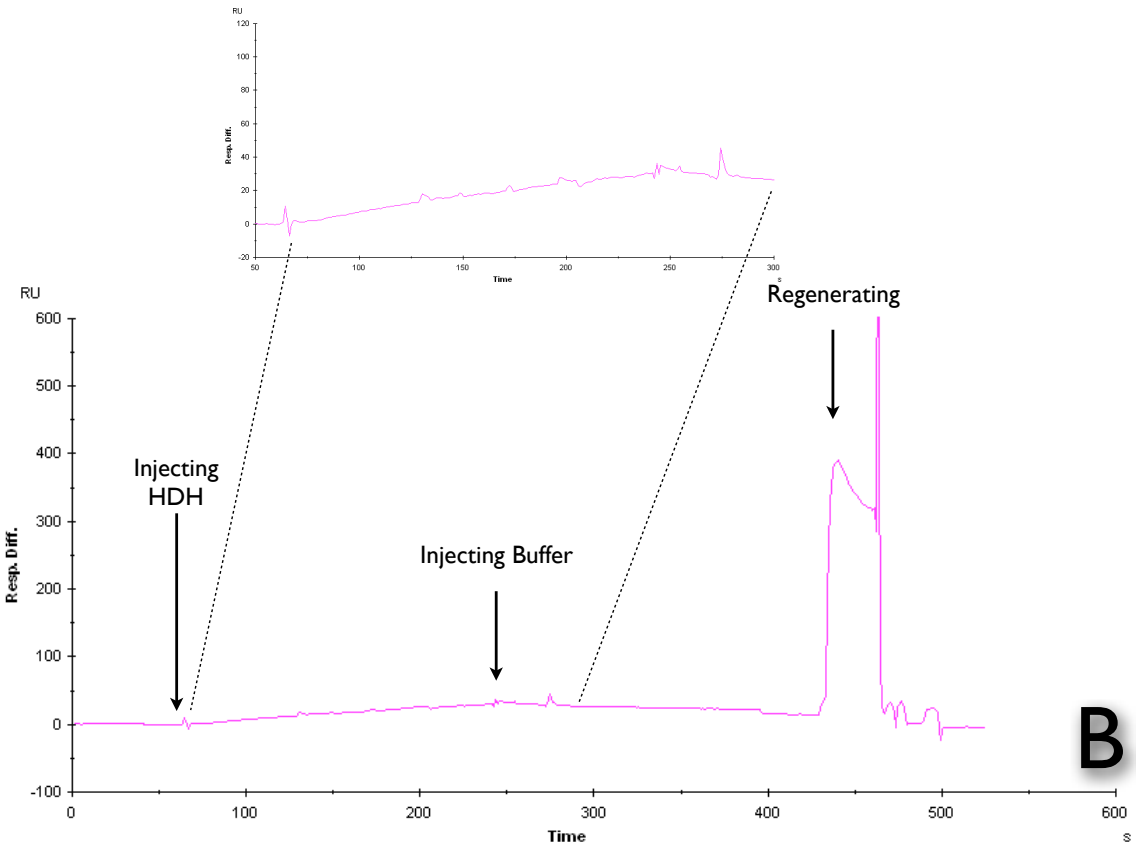
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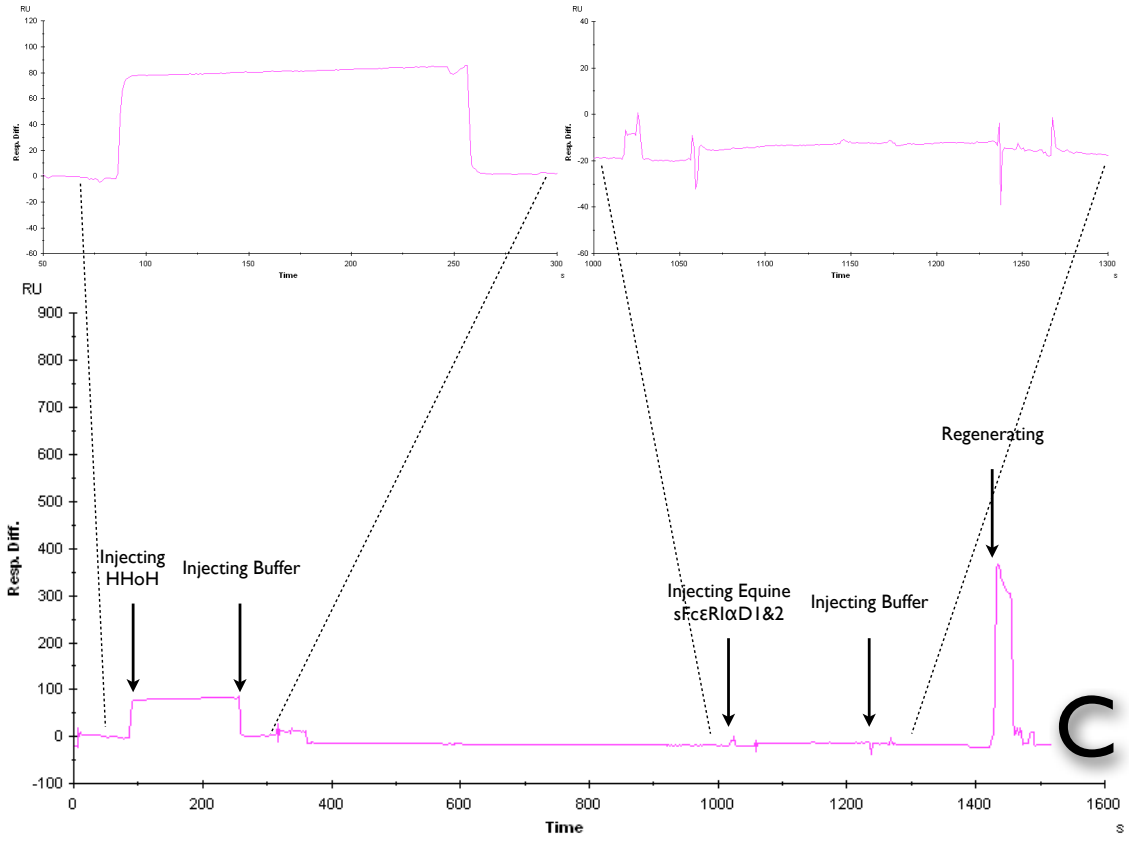


Figure 61: HHoH and HDH IgEs anti NIP-HSA viability test:
A. shows the SPR curve of the HHoH IgE anti NIP-HSA, noting the small curve confirming that the antibody is functional and successfully binds to NIP-HSA. This small curve, compared to the high concentration of the protein in the solution shows that very little of the antibody is functional. **B.** shows the HDH IgE anti NIP-HSA antibody curve to be large as the antibody binds to NIP-HSA as expected. **C.** shows the equine sFcεRIαD1&2 protein binding to the HHoH, the curve are very small therefore due to the small quantities of functional HHoH.

The HHoH and HDH IgEs anti NIP-HSA antibodies were then tested for viability using SPR, (Figure 61) shows the result of the test for HHoH has a small curve (Figure 61A) confirming that the antibody is functional and successfully binds to NIP-HSA. This small curve, compared to the high concentration of the protein in the solution shows that very little of the antibody is functional. The HDH curve (Figure 61B) was normally large as expected. The HHoH IgE anti NIP-HSA was then tested for binding to the sFcεRIαD1&2 protein (Figure 61C), the result shows successful binding as

expected, but the curves were very small due to the low concentrations of functional the HHoH.

7.3 - Discussion:

To take a shortcut, the plasmid pSV-V_{NP}HHoHGFP was synthesized where a GFP gene (Submission by Watkins and Campbell, 1995) was inserted downstream of the HHoH gene, without a linker amino acid region, this was in an attempt to synthesis both IgE outside the cell, and GFP inside the cell, which could have potentially allowed for the cells that express the antibody to be sorted by FACS. This protocol failed as can be seen in Figure 62 because the linker region between the HHoH and the GFP did not have a promoter that allowed the GFP to be synthesized.

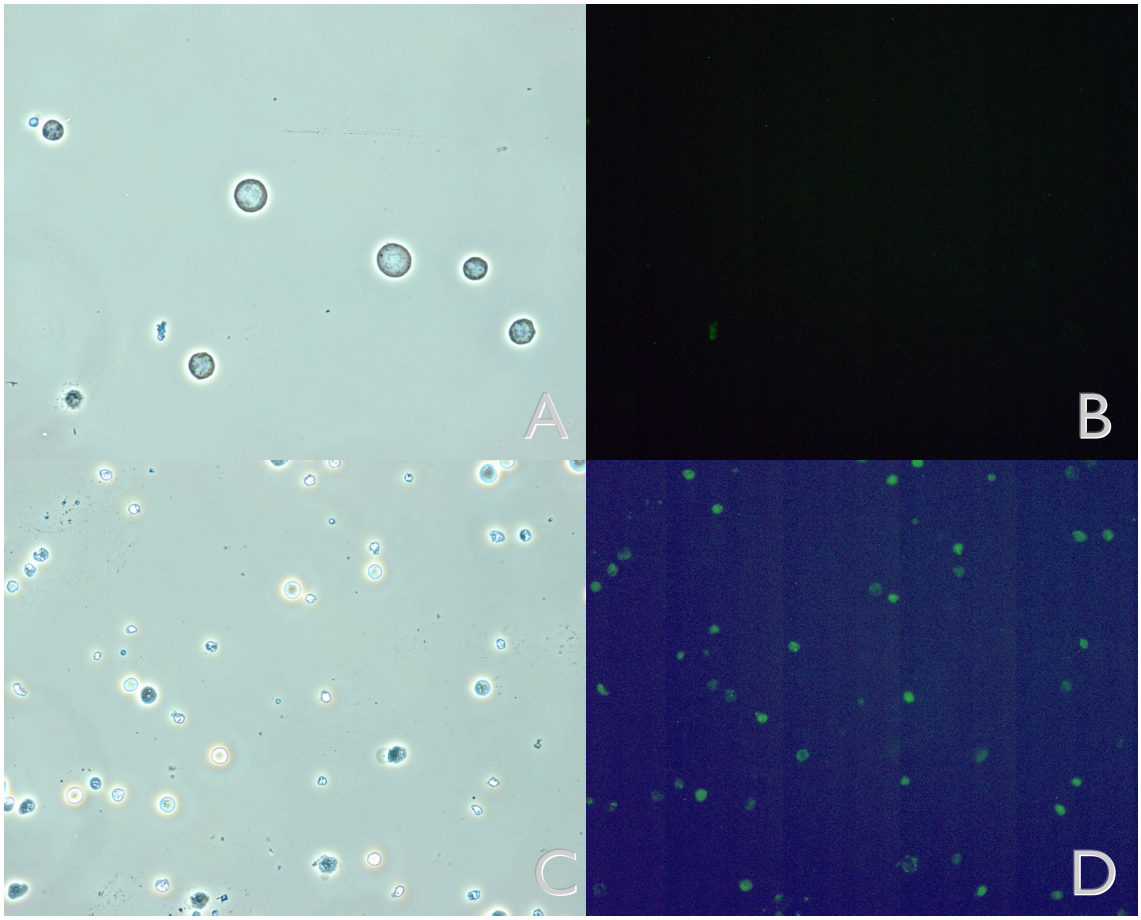


Figure 62: J558L cells cloned with the pSV-V_{NP}HHoHGFP plasmid:
This figure shows the failure of the protocol where GFP was attempted to be expressed inside the J558L cells, along with the HHoH outside the cells, as a mean to allow only expressing cells to be sorted, and hence shorten the selection period. **A.** shows non-transfected cells in white light and **B.** in blue light to allow the GFP to fluoresce **C.** shows the transfected cells in white light and in **D.** in blue light to allow the GFP to fluoresce, noting the green spots here are autofluorescence from dead cell debris and are not an indication of cells expressing GFP.

Digesting the Cε3 domain after the plasmid pUC18-Cε3 was harvested from *E.coli* XL-1 Blue by the restriction enzymes XhoI and BclI did not work, only a single site a was and not the other. The PCR protocol was revised, the gene sequence was analyzed and the restriction digests were optimized (as the BclI enzymes cuts DNA at 50°C while XhoI at 37°C), and still the digestion did not go through completion. Therefore a series of restriction digests revealed the problem (Figure 63).

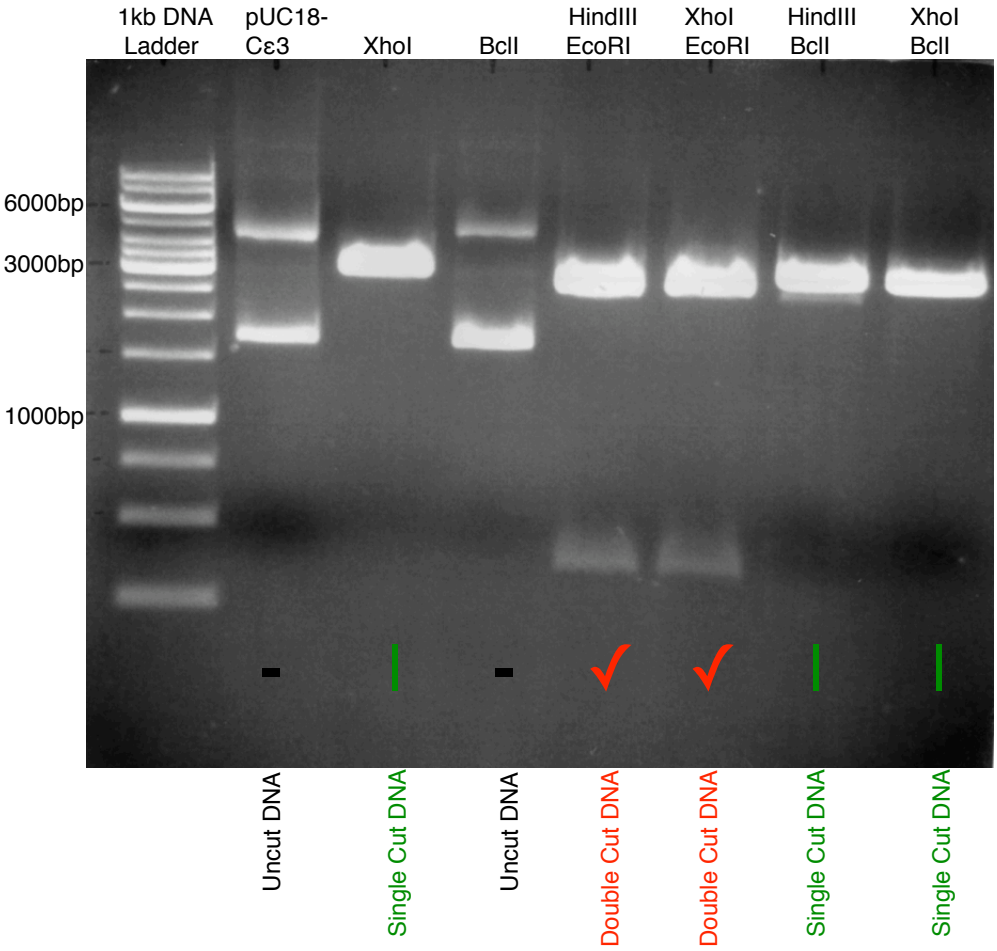


Figure 63: Cε3 restriction digestion investigation:
This figure shows different restriction digests which revealed that only the BclI restriction site is not cutting, when the BclI enzymes was studied further it was revealed that in *E.coli* the BclI restriction site is methylated, there this problem was solved by transforming the pUC18-Cε3 plasmid into *E.coli* JM110 strain, deficient in the *Dam* and *Dcm* genes, therefore it does not methylate DNA at the BclI restriction site.

While all restriction enzymes cut the DNA, once or twice, the BclI restriction site was not being cut at all (the figure shows that the BclI sample is identical to the non-cut control sample). When the BclI restriction enzyme was studied further, it was revealed that *E.coli* bacteria naturally methylate the BclI site through the *Dam* gene (which codes for the Dam methylase), thus the methylated DNA prevents the restriction enzyme from cutting it. This problem was solved by transforming pPUC18-Cε3 plasmid into JM110

E.coli strain which has the *Dam* and *Dcm* genes missing, and thus do not methylate DNA at the BclI position.

In conclusion, the HHoH IgE anti NIP-HSA experiment was not a total success. Up to the construction of the gene and the transfection of the J558L cells everything went well, but during the selection process the cells were not selected properly, the protocol was repeated 4 times and each time the negative control cells would not die, and completely survive in the selection media, this indicates that all the wells with the transfected cells had a large cell impurity with non-transfected cells. That is the reason very few of the HHoH IgE anti NIP-HSA antibody were collected. The protocol was repeated every time with a freshly thawed batch of cells, but the selection process was not strong enough. From Figure 9 in chapter 2.2.4, it can be seen that the selection process greatly depends on hypoxanthine to saturate the hypoxanthine-guanine phosphoribosyltransferase so it will not convert guanine to GMP. If the hypoxanthine is not in large enough quantity in the media, or has degraded or is being metabolized by the cells very fast, the hypoxanthine-guanine phosphoribosyltransferase enzyme will no longer be saturated with hypoxanthine and thus start converting guanine into GMP, and bypassing the selection process. This is the reason why this selection process was sometime low efficient, and an alternative protocol was discussed in chapter 5.3 which uses CHO (Chinese Hamster Ovary) cells to express large quantities of proteins (including antibodies) with a very efficient selection process.

Therefore to continue the next step of the experiment the HHoH IgE anti NIP-HSA, which was supposed to be used to immunize rats, was replaced by the HDH IgE anti NIP-HSA, which should test the exact same allergy vaccine concept and the original antigenic sin hypothesis, but in the canine system. Which if successful can be

said to be relevant for the equine and human systems since all these systems' IgE-mediated responses are almost identical.

Chapter 8 - Development of An Allergy Vaccine:

8.1 - Introduction:

This chapter tested the concept of developing a polyclonal antibody serum against an organism's native IgE that binds at a high enough affinity to remove all non-bound serum IgEs as well as knock off the bound IgE from its FcεRI receptor. A peptide called 2Fcε$_{2\text{-}3}$ was synthesized which includes part of the amino acid sequence within the IgE antibody's Cε3 domain that binds the IgE to its FcεRI receptor (Chapter 7.1 for explanation). This peptide contains a conserved sequence found in human, canine and equine IgEs. The novel concept is using this peptide to immunize the host organisms which are humans, canines and equines, so they can develop IgG antibodies that recognize this sequence, but since the sequence is in a peptide form, even though it is a disulphide linked dimer, will develop IgG antibodies that bind to it at a lower affinity than the IgE binds to its FcεRI receptor. Therefore to increase this affinity, a chimeric antibody, where the native organism's IgE's Cε3 domain is inserted between the Cε1Cε2 and Cε4 domains of a distant evolutionary organism's IgE domains, to make a full IgE chimeric antibody. The organism is given a vaccine boost with this chimera where the immune system, using the Original Antigenic Sin Hypothesis (Chapter 1.8), would target only the sequence, form the 2Fcε$_{2\text{-}3}$ peptide, in the Cε3 domain and develop antibodies with higher affinities to it through somatic hypermutations. If the concept works it will lead to a development of an allergy vaccine where the host organism would remove all serum and bound IgE and thus permanently cure allergy.

8.2 - Results:

8.2.1 - Antibody Titer Analysis By ELISA:

As discussed in the Chapter 7.3, due to limited funds and equipment, the immunization protocols (Chapter 2.5.1) were run on rats using the HDH IgE anti NIP-HSA antibody chimera instead of the HHoH anti NIP-HSA. Therefore rats 1 and 2 were immunized with the 2Fcε$_{2\text{-}3}$ peptide followed by two boosts with the same peptide. Rats 3 and 4 where also initially immunized with the 2Fcε$_{2\text{-}3}$ peptide, but followed by two boosts with the HDH IgE anti NIP-HSA antibody chimera (Table 5).

Table 5: Rat immunization strategy:
This table shows the each rat's immunization strategy and time of each bleed.

	Pre Immunisation Bleed	Bleed 1		Bleed 2		Bleed 3	
		Injected on Day 0 & 14	Bled on Day 24	Injected on Day 42	Bled on Day 52	Injected on Day 92	Bled on Day 102
Rat 1	No immunisation	Immunised with the 2Fcε$_{2\text{-}3}$ peptide		Boosted 1 with the 2Fcε$_{2\text{-}3}$ peptide		Boosted 2 with the 2Fcε$_{2\text{-}3}$ peptide	
Rat 2		Immunised with the 2Fcε$_{2\text{-}3}$ peptide		Boosted 1 with the 2Fcε$_{2\text{-}3}$ peptide		Boosted 2 with the 2Fcε$_{2\text{-}3}$ peptide	
Rat 3		Immunised with the 2Fcε$_{2\text{-}3}$ peptide		Boosted 1 with HDH IgE		Boosted 2 with HDH IgE	
Rat 4		Immunised with the 2Fcε$_{2\text{-}3}$ peptide		Boosted 1 with HDH IgE		Boosted 2 with HDH IgE	

The rat bleeds were initially tested using ELISA (Chapter 2.5.2) for binding to the 2Fcε$_{2\text{-}3}$ peptide (Figure 64A). The results on Figure 65 shows that all rats were successfully developed an immune response to the 2Fcε$_{2\text{-}3}$ peptide, and the pre immunization bleed confirms this. Noting great variability between rats of the sample and control group, which is expected since each individual rat would develop an immune response on its own pace depending its health and environment, though these were kept as constant and optimum as possibly can.

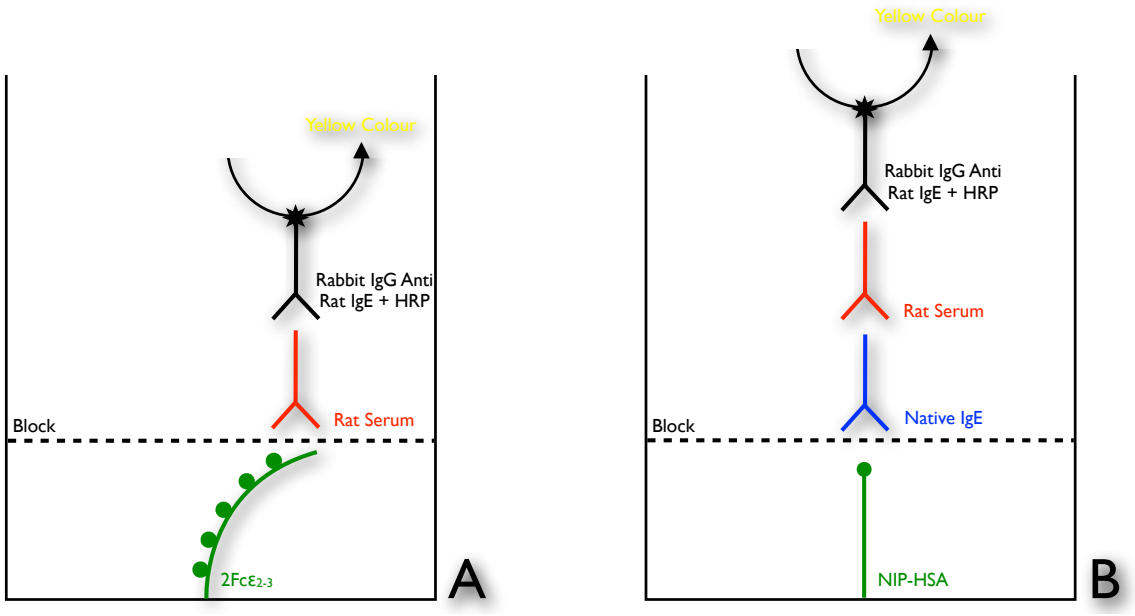
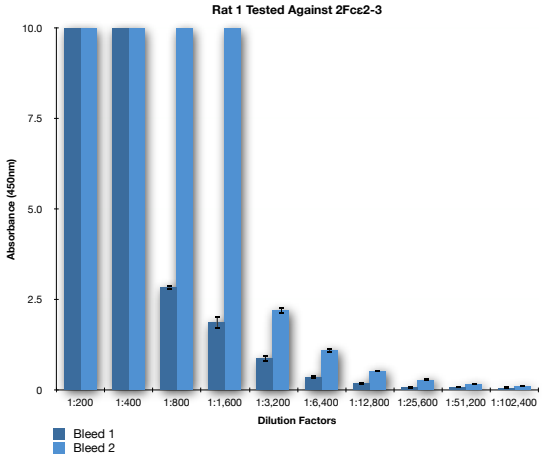
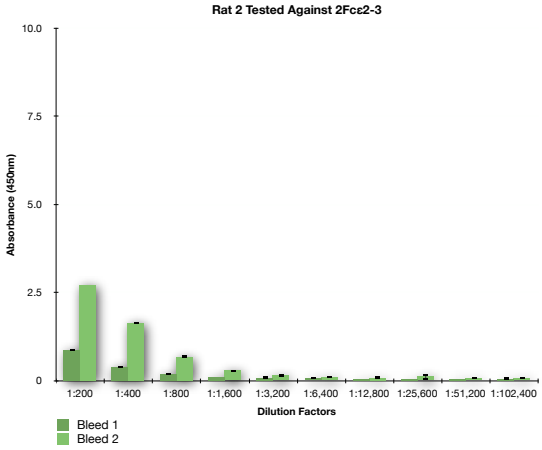
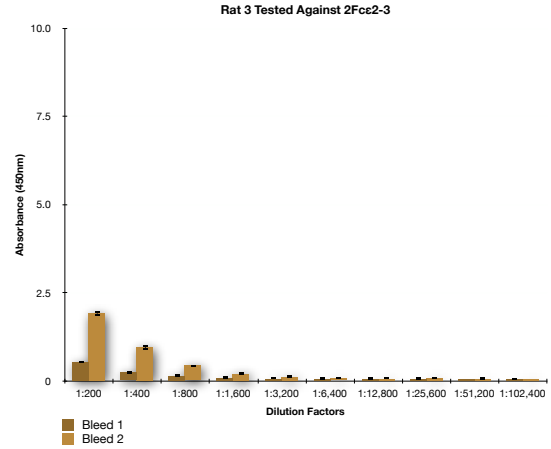
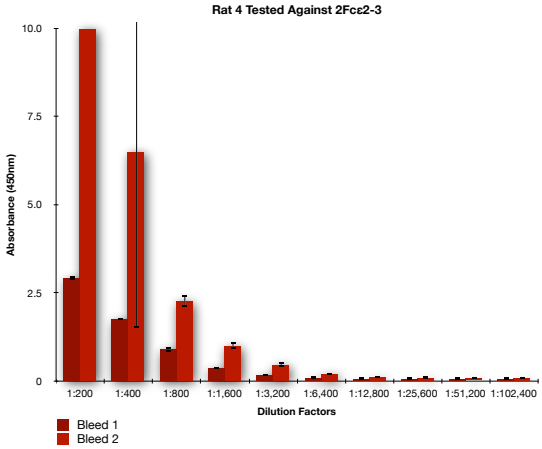


Figure 64: Bleed test using ELISA assay:
This figure shows the two different ELISA assay construction used to test the rat bleeds. **A.** assay tested the difference between the first and second rat bleeds and their antibody titer that bound to the 2Fcε$_{2\text{-}3}$ peptide. **B.** assay tested the difference, in of the second bleed, between the control rats, immunized only with the 2Fcε$_{2\text{-}3}$ peptide, and the test rats, immunized with the chimeric HDH anti NIP-HSA antibody.


Rat 1 Tested Against 2Fcε2-3
Absorbance (450nm)
10.0
7.5
5.0
2.5
0
1:200
1:400
1:800
1:1,600
1:3,200
1:6,400
1:12,800
1:25,600
1:51,200
1:102,400
Dilution Factors
Bleed 1
Bleed 2


Rat 2 Tested Against 2Fcε2-3
Absorbance (450nm)
10.0
7.5
5.0
2.5
0
1:200
1:400
1:800
1:1,600
1:3,200
1:6,400
1:12,800
1:25,600
1:51,200
1:102,400
Dilution Factors
Bleed 1
Bleed 2


Rat 3 Tested Against 2Fcε2-3
Absorbance (450nm)
10.0
7.5
5.0
2.5
0
1:200
1:400
1:800
1:1,600
1:3,200
1:6,400
1:12,800
1:25,600
1:51,200
1:102,400
Dilution Factors
Bleed 1
Bleed 2


Rat 4 Tested Against 2Fcε2-3
Absorbance (450nm)
10.0
7.5
5.0
2.5
0
1:200
1:400
1:800
1:1,600
1:3,200
1:6,400
1:12,800
1:25,600
1:51,200
1:102,400
Dilution Factors
Bleed 1
Bleed 2

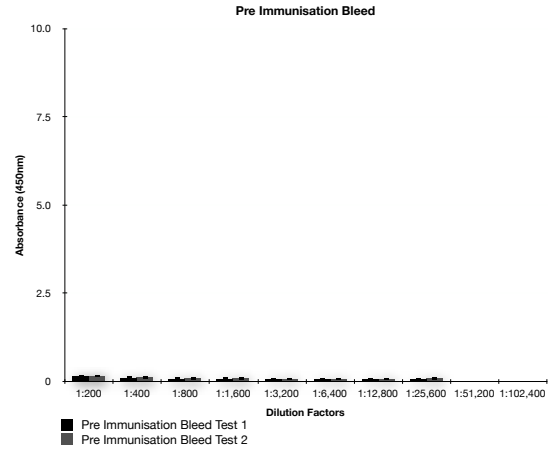
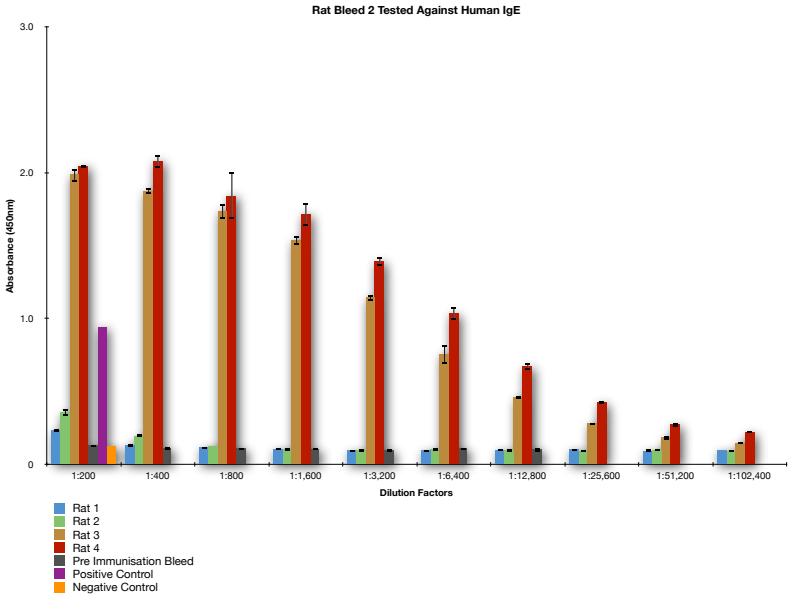
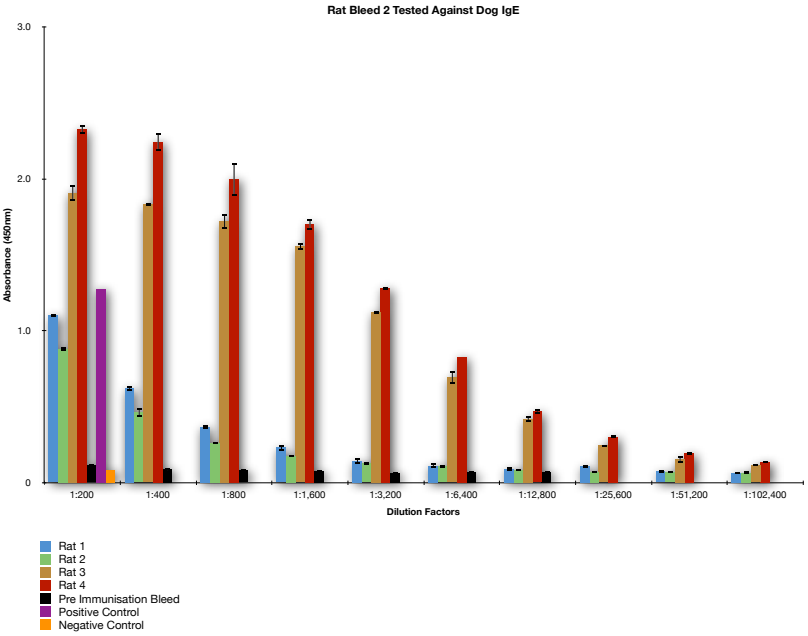

Pre Immunisation Bleed
Absorbance (450nm)
10.0
7.5
5.0
2.5
0
1:200
1:400
1:800
1:1,600
1:3,200
1:6,400
1:12,800
1:25,600
1:51,200
1:102,400
Dilution Factors
Pre Immunisation Bleed Test 1
Pre Immunisation Bleed Test 2

Figure 65: ELISA test of each rat's bleed for binding to the 2Fcε$_{2-3}$:
This figure shows the results of all rats' 1st and 2nd bleeds tested by ELISA for binding to the 2Fcε$_{2-3}$ peptide as in Figure 64A. Noting that rats 1 and 2 were immunized with the 2Fcε$_{2-3}$ peptide and also boosted with it, wile rats 3 and 4 were immunized with the 2Fcε$_{2-3}$ peptide but boosted with the HDH anti NIP-HSA chimeric IgE antibody. The pre immunization serum shows no antibodies binding to the 2Fcε$_{2-3}$ peptide as expected. Noting that variations in antibody titer (quantity of antibodies) is expected between rats since each individual rats reacts differently to the immunization protocol.

Since this initial ELISA test was successful, a further ELISA test was preformed to test whether the rat serum binds and recognizes the native (human, canine, equine) IgE anti NIP-HSA antibody's Cε3 domain (Figure 64B). The results were successful as shown in Figure 66, where rats 1 and 2, that were boosted with the same 2Fcε$_{2-3}$ peptide, had a very low antibody titer that recognizes the native IgE anti NIP-HSA antibody's Cε3 domain, but rats 3 and 4, that were boosted with the HDH IgE anti NIP-HSA antibody chimera, have a higher antibody serum titer that recognize the native IgE anti NIP-HSA antibody's Cε3 domain.


Rat Bleed 2 Tested Against Human IgE
3.0
2.0
1.0
0
Absorbance (450nm)
1:200
1:400
1:800
1:1,600
1:3,200
1:6,400
1:12,800
1:25,600
1:51,200
1:102,400
Dilution Factors
Rat 1
Rat 2
Rat 3
Rat 4
Pre Immunisation Bleed
Positive Control
Negative Control


Rat Bleed 2 Tested Against Dog IgE
3.0
2.0
1.0
0
Absorbance (450nm)
1:200
1:400
1:800
1:1,600
1:3,200
1:6,400
1:12,800
1:25,600
1:51,200
1:102,400
Dilution Factors
Rat 1
Rat 2
Rat 3
Rat 4
Pre Immunisation Bleed
Positive Control
Negative Control

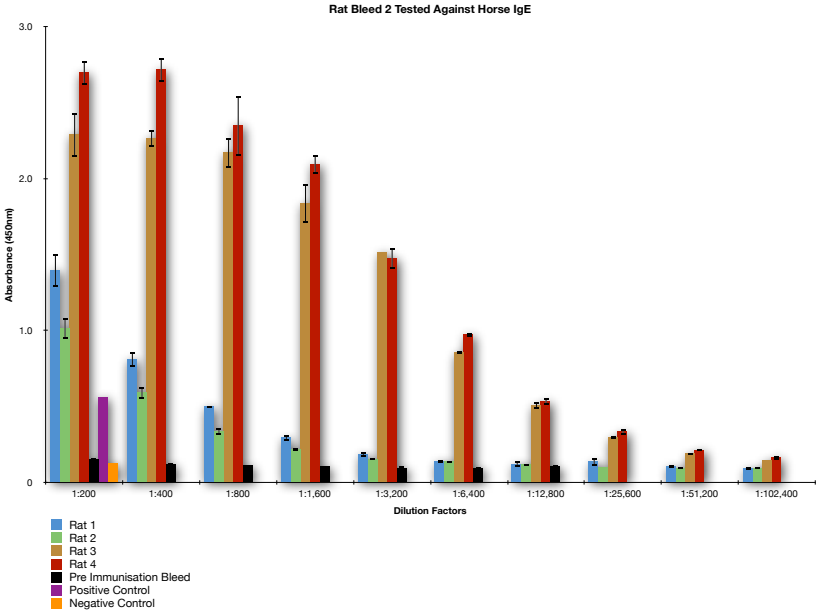
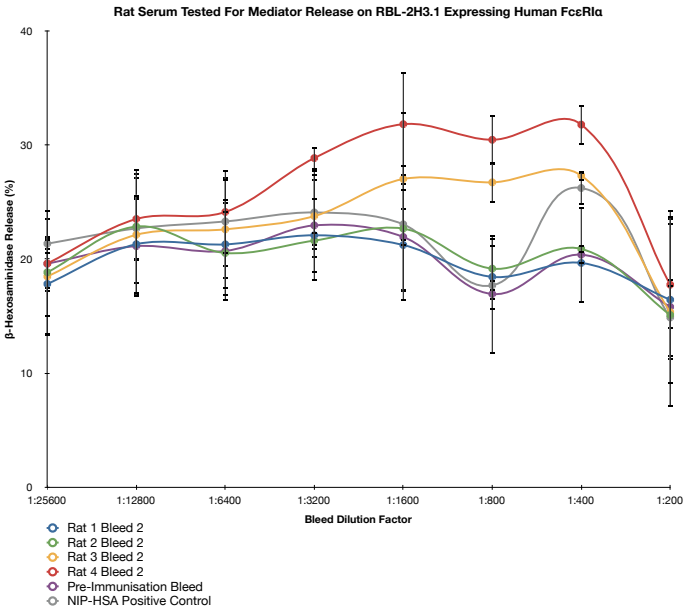

Rat Bleed 2 Tested Against Horse IgE
3.0
2.0
1.0
0
Absorbance (450nm)
1:200
1:400
1:800
1:1,600
1:3,200
1:6,400
1:12,800
1:25,600
1:51,200
1:102,400
Dilution Factors
Rat 1
Rat 2
Rat 3
Rat 4
Pre Immunisation Bleed
Positive Control
Negative Control

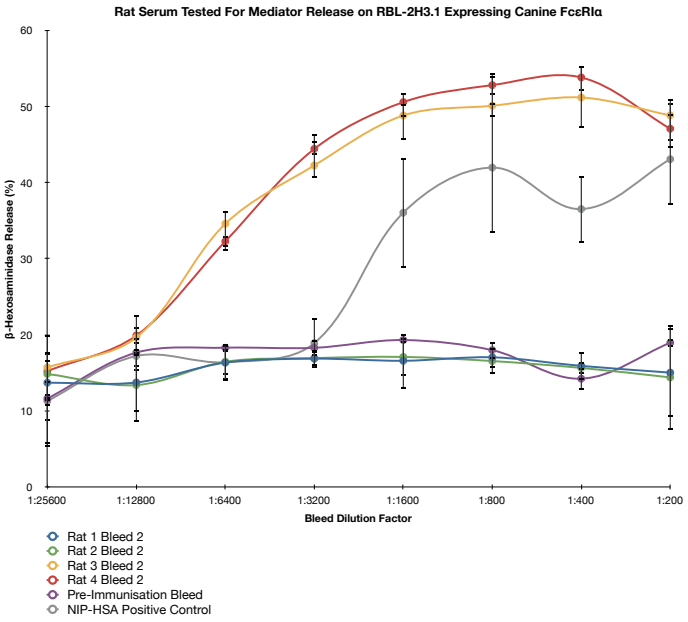
Figure 66: ELISA test of bleed to of all rats for binding to the native IgE antibodies:
This figure shows the ELISA test of all rats' 2nd bleed for binding to the native antibodies (human, equine and canine IgEs), as in Figure 64B. From the figure it can be determined that the rats (3 and 4) that were boosted with the HDH anti NIP-HSA chimeric IgE antibody had a much higher antibody titer than specific to the native IgEs than the rats (1 and 2) that were boosted with the 2Fcε$_{2\text{-}3}$ peptide.

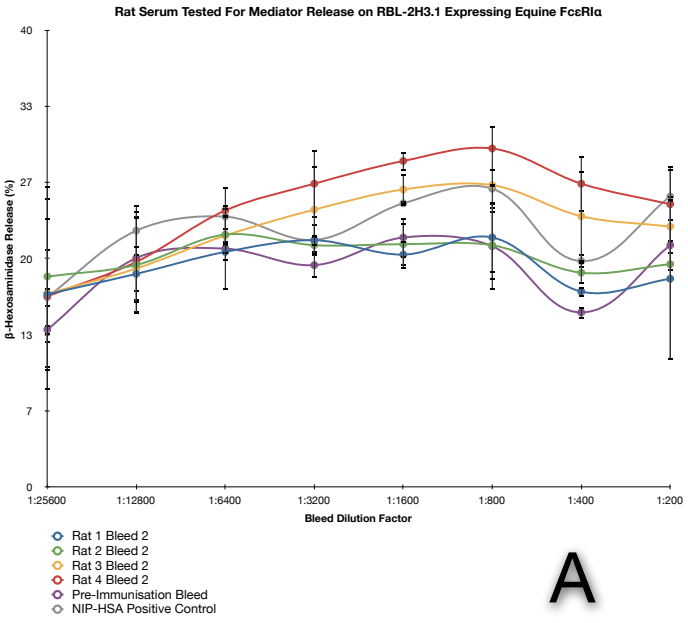
This was expected since displaying epitopes in its native 3D structure would cause the immune system to better target them. The negative pre immunization bleed and no IgE control, along with the positive, rabbit anti human IgE AB helix from chapter 6.3.2, control have verified these results.

8.2.2 -Anaphylactic Shock Test By β-hexosaminidase Release Assays:

So far the immunization protocol looked promising, immunizing rats with the 2Fcε$_{2\text{-}3}$ peptide followed by a boost with the HDH IgE anti NIP-HSA antibody chimera resulted is large serum antibodies that target the desired epitopes. In this experiment the original antigenic sin hypothesis was tested whether or not it worked and the immune system had only targeted the PRGV sequence in the 2Fcε$_{2\text{-}3}$ peptide and not other epitopes in the HDH IgE anti NIP-HSA antibody chimera. We used the β-hexosaminidase release assays (Chapter 2.4.9) that we developed to test whether or not the rat serum causes RBL-2H3.1 cells to release mediators due to the anti IgE antibodies cross linking FcεRI receptor bound native IgE on the cell's surface and thus aggregate the receptor. In this test the antigen (NIP-HSA or DNP-HSA) serial dilution was replaced by a rat serum dilution.


Rat Serum Tested For Mediator Release on RBL-2H3.1 Expressing Human FcεRIα
β-Hexosaminidase Release (%)
0
10
20
30
40
1:25600
1:12800
1:6400
1:3200
1:1600
1:800
1:400
1:200
Bleed Dilution Factor
Rat 1 Bleed 2
Rat 2 Bleed 2
Rat 3 Bleed 2
Rat 4 Bleed 2
Pre-Immunisation Bleed
NIP-HSA Positive Control


Rat Serum Tested For Mediator Release on RBL-2H3.1 Expressing Canine FcεRIα
β-Hexosaminidase Release (%)
0
10
20
30
40
50
60
1:25600
1:12800
1:6400
1:3200
1:1600
1:800
1:400
1:200
Bleed Dilution Factor
Rat 1 Bleed 2
Rat 2 Bleed 2
Rat 3 Bleed 2
Rat 4 Bleed 2
Pre-Immunisation Bleed
NIP-HSA Positive Control


Rat Serum Tested For Mediator Release on RBL-2H3.1 Expressing Equine FcεRIα
β-Hexosaminidase Release (%)
0
7
13
20
27
33
40
1:25600
1:12800
1:6400
1:3200
1:1600
1:800
1:400
1:200
Bleed Dilution Factor
Rat 1 Bleed 2
Rat 2 Bleed 2
Rat 3 Bleed 2
Rat 4 Bleed 2
Pre-Immunisation Bleed
NIP-HSA Positive Control

A

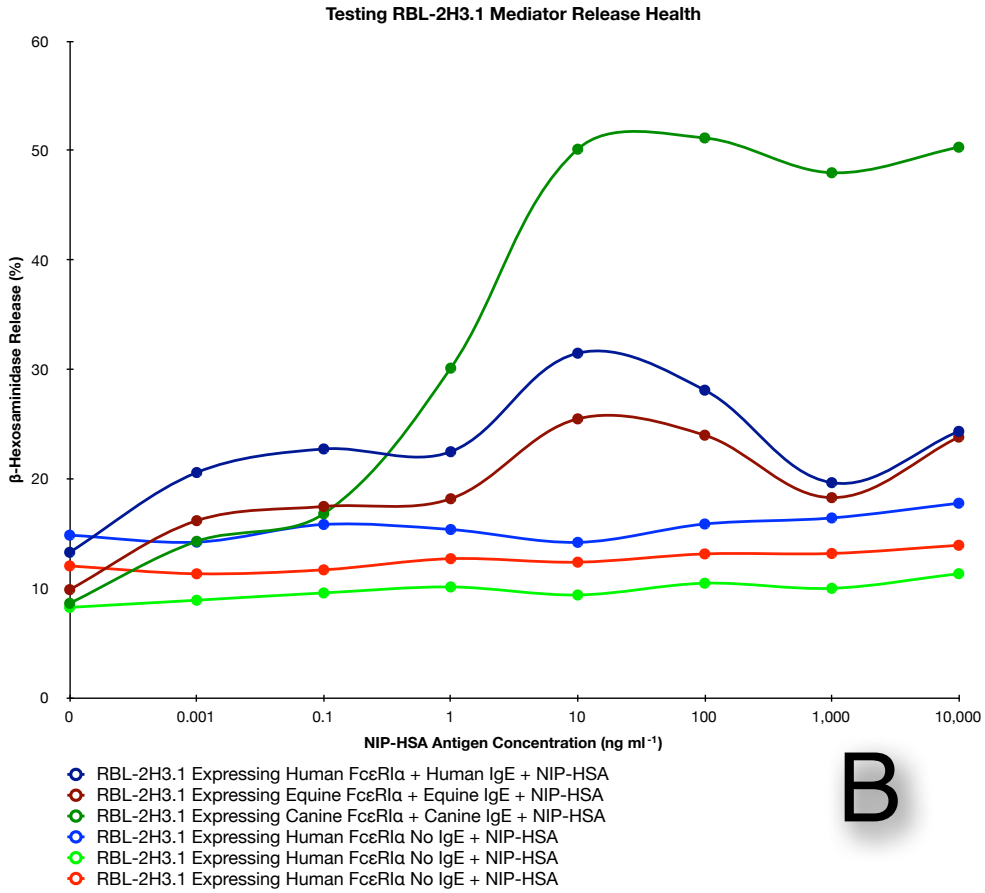


Figure 67: Release assays of bleed 2 from all rats:
A. this figure shows the release assays of the RBL-2H3.1 cells that were expressing human, equine or canine FcεRIα receptors. All cells were sensitized with their corresponding native IgE; RBL-2H3.1 cells expressing human FcεRIα receptor were sensitized with human IgE, ect. The cells were tested for release with the addition of an increasing concentration of all rat's serum from the 2nd bleed, this was to test whether or not the polyclonal antibodies raised against self IgE would bind to receptor bound IgE, aggregating, it and causing an anaphylactic shock which is a body wide cell mediator release. The positive control used NIP-HSA to cause the cells to release, while the negative control used pre-immunization serum. The results show that the rat serum does cross link FcεRI receptor bound IgE and causes mediator release, i.e will cause an anaphylactic shock. **B.** this figure shows a positive and negative control assay to test the mediator release health of the cell lines. In the positive control the cells were sensitized with their corresponding native IgE anti NIP-HSA and challenged with increasing concentration of NIP-HSA, while in the negative control the cells were not sensitized with their corresponding native IgE anti NIP-HSA but still challenged with increasing concentration of NIP-HSA.

The results on Figure 67 have revealed that the original antigenic sin hypothesis did not work as expected and the immune system did target other epitopes in the HDH IgE anti NIP-HSA antibody chimera, which causes the serum antibodies to bind to different sites in the native IgE on the RBL-2H3.1 cell's surface and cause them to release mediators. This is a fatal phenomena, since if this immunization technique was preformed on a live organism it will suffer from a massive anaphylactic shock (Figure 68).

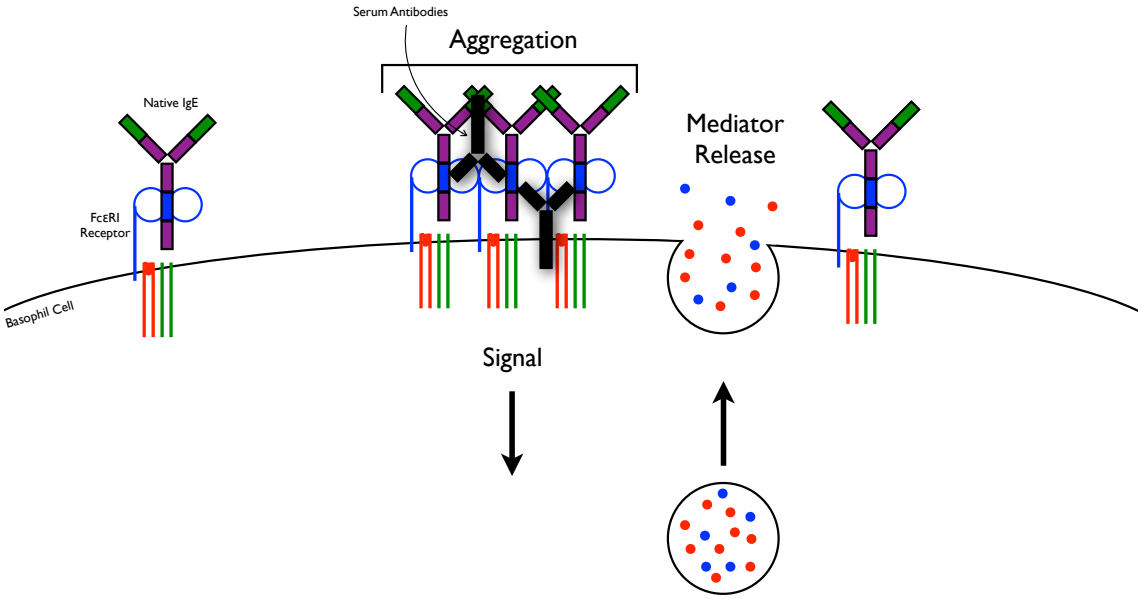


Figure 68: Concept behind immunization leading to massive anaphylactic shock.
This figure shows the concept behind aggregation of FcεRI receptor bound native IgE on the cell's surface and causing mediator release. This proves that if this immunization technique was used it will cause the host organism to undergo a massive anaphylactic shock.

The fact that the RBL-2H3.1 cells expressing the human FcεRIα released mediators when they were sensitized with human IgE and challenged with the rat serum indicate, which the rat immune system targeted epitopes in the Cε1, Cε2 and Cε4 domains of the HDH IgE anti NIP-HSA antibody chimera, is why they cause receptor aggregation of the cell surface. The fact that the RBL-2H3.1 cells expressing the canine FcεRIα released mediators when they were sensitized with canine IgE and challenged with the rat serum indicate that the rat immune system targeted epitopes, different to that from the desired PRGV epitope displayed by the $2Fc\varepsilon_{2\text{-}3}$ peptide, in the Cε3 domain of the HDH IgE anti NIP-HSA antibody chimera, which is why they caused receptor aggregation on the cell surface. The fact that the RBL-2H3.1 cells expressing the equine FcεRIα released mediators when they were sensitized with equine IgE and challenged with the rat serum indicate that the rat immune system that targeted the canine Cε3

domain also targeted the equine Cε3 domain since the two domains are 68% identical (Figure 69).

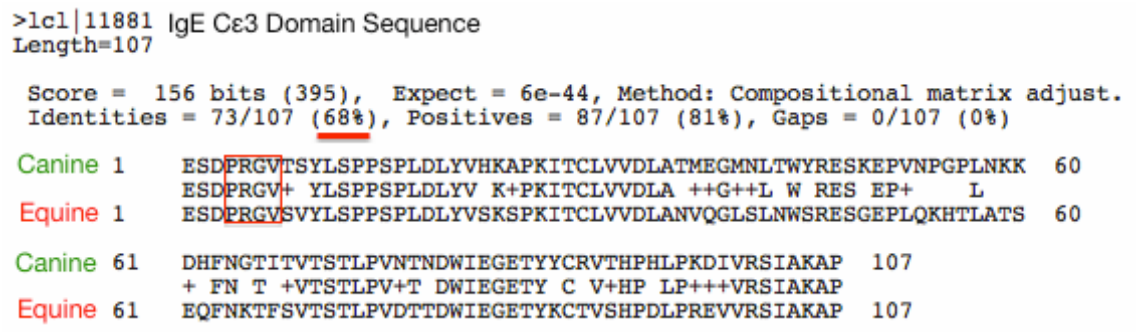


Figure 69: Equine and Canine IgE Cε3 domain sequence: This figure shows the BLAST sequence between the Equine and Canine IgE Cε3 domain. The result shows 68% sequence identity.

8.3 - Discussion:

All the tests were run on bleeds two as the main objective was to identify if the original antigenic sin hypothesis works and the immune system only targets the required PRGV IgE Cε3 epitope. Bleed three was supposed to identify if an extra second boost would increase the immune system antibody's affinity to its self IgE up to the level where FcεRI receptor bound IgE can be knocked off the mast and basophil cell's surface. But since the original antigenic sin hypothesis proved not to work, and the organism would suffer a massive anaphylactic shock if it was immunized using this strategy, testing bleed 3 was abandoned.

The immunization protocol has revealed important information regarding vaccinations; first of all, displaying epitopes in their native 3D structure is the best way to achieve good serum antibody titer against them, much better than a single or a cyclised peptide. Second, the original antigenic sin hypothesis does not work in this immunization and the immune system will recognize the initial epitope from the vaccine peptide, along with other epitopes in the full vaccine boost chimeric antibody

molecule, which will lead to cross linking of FcεRI receptor bound IgE, just like an antigen does, and the cells will respond by releasing mediators that will cause the organism to experience organ wide inflammation, which is basically a massive anaphylactic shock. This is counters the observations in the paper by (Ledin, *et al.*, 2006), where they did not test a potential anaphylactic shock occurring.

But a point to mention is that, at a dilution of 1:200 the mediator release started to fall, this raises the question whether full serum would polarize the basophil cells, though receptor capping, enough to stop them completely from releasing any mediators? This might be the reason why the dogs in the (Ledin, *et al.*, 2006) paper did not die? Immune complexes are theorized not to occur, since the quantity of IgE is considerably lower than that of IgG even in atopic patients.

Chapter 9 - Discussion:

9.1 - General Project Discussion:

In order to study equine allergy, this project developed some essential reagents, all of which allowed these experiments to be preformed. These reagents may also have commercial potential for the assessment of the safety of anti-allergic drugs. The development of an RBL-2H3.1 cell line that expresses equine FcεRIα receptor onto its surface, and an equine IgE anti NIP-HSA, allowed the measurement of the quantity of mediator release when equine IgE binds to its FcεRI receptor (36.68%). It also showed that canine IgE can bind to the equine FcεRI receptor and cause mediator release (32%), while the equine IgE does not recognize the canine FcεRI receptor.

The project also developed a soluble form of the equine FcεRI receptor (sFcεRIαD1&2) which allowed the measurement of the binding between it and the equine IgE (K_D = 1.58x10^{-10}M after calibrating the measured data on the EvilFit Distribution Model). This was taken further and the binding between the canine IgE and the equine sFcεRIαD1&2 was measured (K_D = 5.43x10^{-10}M), this binding affinity is comparable to that between the equine IgE and the equine sFcεRIαD1&2, which explains why the canine IgE causes mediator release in RBL-2H3.1 cells expressing equine FcεRIα.

Since the binding between the equine IgE and its FcεRI receptor was confirmed, and previous members of this laboratory demonstrated the binding of the human IgE/ FcεRI receptor and the canine IgE/FcεRI, this project used this information and tested a hypothesis that attempts to develop a vaccine, where an organism's body would develop antibodies against its own self IgE antibody targeting the complementary sites of interaction between IgE and FcεRI receptor, with the expectation that the binding has a

strong enough affinity to allow the removal of serum IgE and FcεRI receptor bound IgE without aggregating the FcεRI receptor bound IgEs on mast and basophil cell surfaces causing an anaphylactic shock. This hypothesis implements a protocol where rats were initially immunized with an immungen (2Fc$\varepsilon_{2\text{-}3}$ peptide) that caused the organism's immune response to target an essential part of the self IgE antibody, followed by boosts with a full chimeric IgE antibody that displayed the same peptide epitope but in a 3D structure to allow that immune system to develop higher affinity antibodies against it through somatic hypermutations as claimed by other investigators (Ledin, *et al.,* 2006). The hypothesis also useed the concept of original antigenic sin as a basis for a potential immunization strategy, where the immune system is thought to preferentially target, on second exposure, only the epitope displayed by the 2Fc$\varepsilon_{2\text{-}3}$ peptide even if it was displayed in the chimeric IgE antibody, without targeting other epitopes.

The results of this, potential allergy vaccine, was negative. The results showed excellent antibody titers against the target, i.e. the chimeric IgE antibody after the first boost with it, indicating that displaying the 2Fc$\varepsilon_{2\text{-}3}$ peptide epitopes in a 3D structure allows the rat immune system to target them better. But when the rat serum was tested for IgE aggregation on the basophil cell surface, it showed that it does cause the cells to release mediators. This showed that the original antigenic sin hypothesis did not work in this protocol, as hoped, and the rat immune system did target other epitopes in the chimeric IgE antibody which resulted in its cell surface aggregation, i,e: can potentially cause the organism to experience an anaphylactic shock. These results indicate that an anti-allergy vaccine might be possible by targeting sites like the Cε3 interdomain region, which is highly conserved between human, canine and equine, but the challenge is to raise antibodies of a high enough affinity for the receptor binding regions in IgE to inhibit the binding between IgE and FcεR1 receptor.

9.2 - Future Work:

The immunization protocol for the, potential allergy vaccine, caused FcεRI receptor bound IgE aggregation on mast and basophil cell surface resulting in an anaphylactic anti-IgE antibody response. A way around this that can be further researched is to boost the immune system with a chimeric IgE antibody that has the same $2Fc\varepsilon_{2\text{-}3}$ peptide epitope but within a Cε3 domain of an even further remote phylogenic mammal, with an amino acid sequence less than 60%. This might allow that elimination of the dependence on the original antigenic sin hypothesis for the immune system to target only the desired epitope, therefore it would only target this epitope and other in the IgE's Cε3 domain but of different epitopes, ones that are not found in the organism's self IgE antibody. Therefore the final immune response would target only the self IgE antibody epitope without causing FcεRI receptor bound IgE aggregation on mast and basophil cell surface.

The project targeted a sequence called the Cε2-3 linker (amino acids 350-363). The future work can also be to apply these protocols to target other loops: BC loop (362-365), DE loop (393-396), and Omalizumab's suggested target of the FG loop (424-427) "within the HPL loop (424-436)" (Figure 70). All these loops are masked when the IgE binds to its FcεRI receptor. This characteristic makes these loops a good target because, first they interact with the FcεRI receptor, therefore disrupting them will interfere with the IgE binding. Second, they are masked during the IgE binding, thus antibodies that targets them will have no chance of causing degranulation since they will not be able to cross link IgE occupied receptors.

Another loop that can be tested further is the AB helix (343-353) (Figure 70), which is not involved in the IgE/FcεRI receptor binding, but is essential in maintaing

the Cε3 configuration so it can bind to the FcεRI receptor. But is can inhibit binding to the FcεRII receptor.

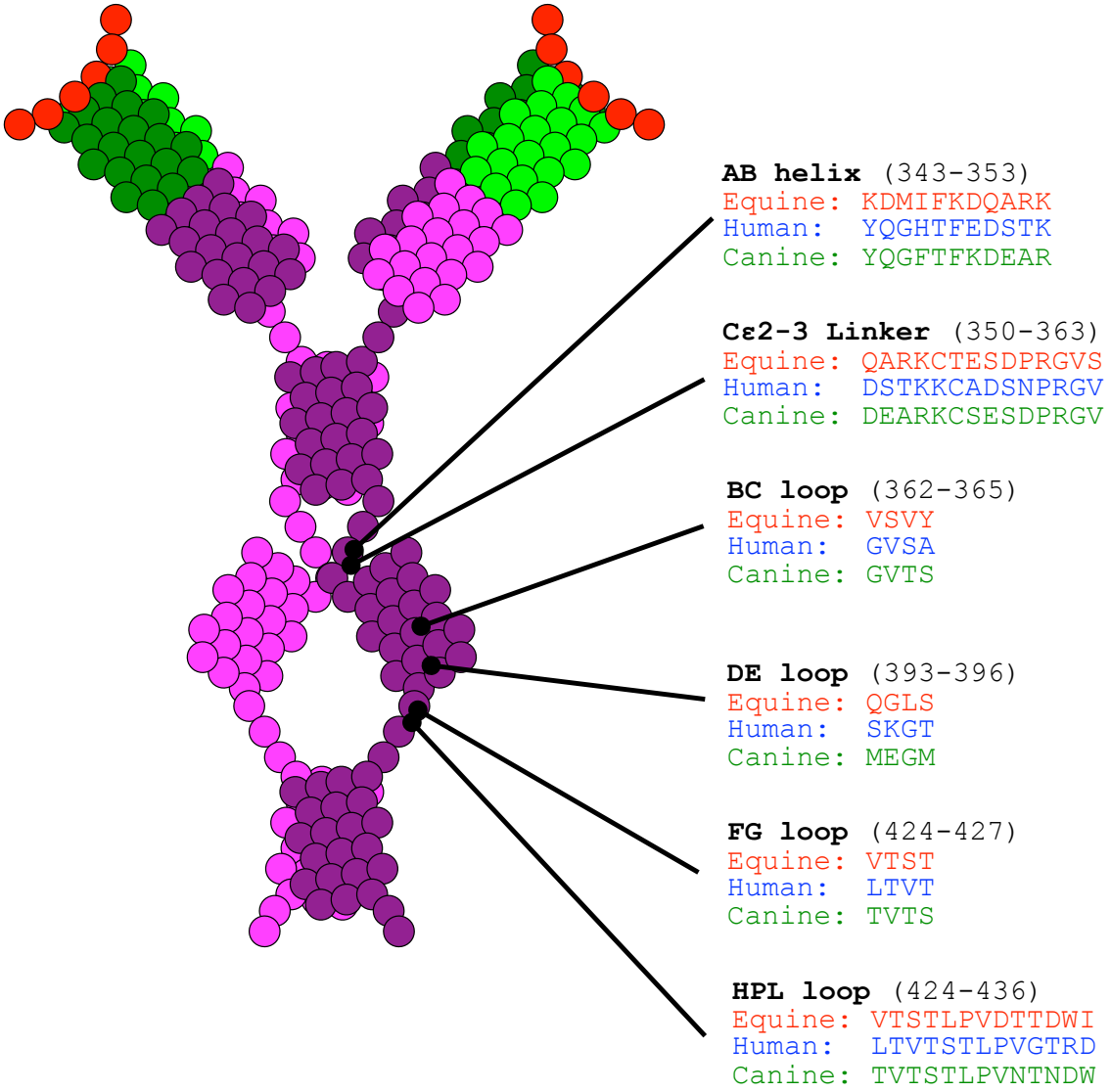


Figure 70: Locations of potentially immogenic sequences to raise non-anaphylactic anti self IgE antibodies:
This figure shows the location of the different essential loops in the full IgE molecule, and their sequences in the equine, human and canine organisms. Noting all these loops are within, or close to, the Cε3 domain.

Since the immunization protocol was not successful in developing a strategy for the development of non-anaphylactogenic anti self IgE antibodies as therapeutic immunogen, further investigations are essential to develop immunization schedules to allow the organism's body to develop non-anaphylactic antibodies against its own self

IgE. One possibility would be to have the vaccine peptide made up of several repeated epitopes encompassing sequences involved in IgE/FcεRI in tandem to further direct the immune system to mount an antibody response against these sequences instead of others within the full IgE antibody, and thus eliminate the possibility of eliciting antibodies that induce IgE/FcεRI receptor aggregation on the cell's surface that results in an anaphylactic shock.

The observed effect, where the mediator release started to decline after the rat serum was at a dilution of 1:200 might be investigated further to see if high enough antibody serum titer would polarize basophil cells and cause them not to release mediators. This might be risky as an organism's response to vaccination cannot be accurately predicted, and might develop only a small antibody titer, not enough to polarize the mast and basophil cells, which would cause the cells to release mediators.

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Appendix:

***Rattus norvegicus* codon usage table:**

First three letters are the codons in the mRNA. The numbers next to them are the frequencies of each codon were 1.00 = 100%, the frequency per thousand and the actual number of the codons in the genome. Taken from: http://www.kazusa.or.jp/codon/

UUU F 0.42 16.5 (115498)	UCU S 0.19 14.8 (103148)	UAU Y 0.40 11.6 (80999)	UGU C 0.45 9.8 (68418)
UUC F 0.58 23.1 (161568)	UCC S 0.23 17.8 (124290)	UAC Y 0.60 17.1 (119192)	UGC C 0.55 11.9 (82751)
UUA L 0.06 5.9 (41454)	UCA S 0.14 10.9 (76435)	UAA * 0.28 0.6 (4473)	UGA * 0.50 1.2 (8137)
UUG L 0.13 12.8 (89203)	UCG S 0.06 4.4 (30458)	UAG * 0.22 0.5 (3637)	UGG W 1.00 13.2 (91958)
CUU L 0.12 12.5 (87332)	CCU P 0.30 17.4 (121358)	CAU H 0.39 9.5 (66664)	CGU R 0.09 5.0 (34788)
CUC L 0.20 20.4 (142093)	CCC P 0.31 18.0 (125645)	CAC H 0.61 14.9 (104027)	CGC R 0.18 9.8 (68486)
CUA L 0.08 7.6 (52955)	CCA P 0.28 16.1 (112364)	CAA Q 0.25 11.1 (77366)	CGA R 0.12 6.8 (47207)
CUG L 0.41 41.1 (286626)	CCG P 0.11 6.3 (43713)	CAG Q 0.75 33.8 (235764)	CGG R 0.20 10.9 (76123)
AUU I 0.33 15.3 (106736)	ACU T 0.24 12.9 (90372)	AAU N 0.41 15.1 (105212)	AGU S 0.15 11.8 (82659)
AUC I 0.52 24.4 (170096)	ACC T 0.36 19.7 (137673)	AAC N 0.59 21.7 (151564)	AGC S 0.24 19.2 (133954)
AUA I 0.15 6.9 (48257)	ACA T 0.28 15.3 (106544)	AAA K 0.38 21.5 (150018)	AGA R 0.20 11.2 (77965)
AUG M 1.00 23.1 (161552)	ACG T 0.11 6.2 (43237)	AAG K 0.62 35.1 (245259)	AGG R 0.21 11.8 (82391)
GUU V 0.16 10.4 (72270)	GCU A 0.28 19.7 (137491)	GAU D 0.43 20.9 (146211)	GGU G 0.17 11.4 (79443)
GUC V 0.25 16.2 (113143)	GCC A 0.39 27.1 (189524)	GAC D 0.57 28.0 (195508)	GGC G 0.33 21.9 (152582)
GUA V 0.11 7.2 (50048)	GCA A 0.23 15.6 (109203)	GAA E 0.39 26.9 (187810)	GGA G 0.25 16.6 (115968)
GUG V 0.47 30.0 (209458)	GCG A 0.10 6.9 (47869)	GAG E 0.61 41.3 (288345)	GGG G 0.24 15.6 (108657)

***Mus musculus* codon usage table:**

First three letters are the codons in the mRNA. The numbers next to them are the frequencies of each codon were 1.00 = 100%, the frequency per thousand and the actual number of the codons in the genome. Taken from: http://www.kazusa.or.jp/codon/

UUU F 0.44 17.2 (422153)	UCU S 0.20 16.2 (398250)	UAU Y 0.43 12.2 (298518)	UGU C 0.48 11.4 (279729)
UUC F 0.56 21.8 (535439)	UCC S 0.22 18.1 (444041)	UAC Y 0.57 16.1 (394074)	UGC C 0.52 12.3 (301384)
UUA L 0.07 6.7 (165150)	UCA S 0.14 11.8 (289799)	UAA * 0.28 1.0 (23403)	UGA * 0.49 1.6 (40148)
UUG L 0.13 13.4 (329668)	UCG S 0.05 4.2 (103815)	UAG * 0.23 0.8 (19126)	UGG W 1.00 12.5 (306619)
CUU L 0.13 13.4 (329757)	CCU P 0.31 18.4 (450637)	CAU H 0.41 10.6 (260637)	CGU R 0.08 4.7 (114854)
CUC L 0.20 20.2 (495018)	CCC P 0.30 18.2 (446868)	CAC H 0.59 15.3 (375626)	CGC R 0.17 9.4 (229758)
CUA L 0.08 8.1 (198032)	CCA P 0.29 17.3 (423707)	CAA Q 0.26 12.0 (293318)	CGA R 0.12 6.6 (161412)
CUG L 0.39 39.5 (969515)	CCG P 0.10 6.2 (151521)	CAG Q 0.74 34.1 (836320)	CGG R 0.19 10.2 (250836)
AUU I 0.34 15.4 (377698)	ACU T 0.25 13.7 (335039)	AAU N 0.43 15.6 (382284)	AGU S 0.15 12.7 (311331)
AUC I 0.50 22.5 (552184)	ACC T 0.35 19.0 (465115)	AAC N 0.57 20.3 (499149)	AGC S 0.24 19.7 (483013)
AUA I 0.16 7.4 (180467)	ACA T 0.29 16.0 (391437)	AAA K 0.39 21.9 (537723)	AGA R 0.22 12.1 (297135)
AUG M 1.00 22.8 (559953)	ACG T 0.10 5.6 (138180)	AAG K 0.61 33.6 (825270)	AGG R 0.22 12.2 (299472)
GUU V 0.17 10.7 (262535)	GCU A 0.29 20.0 (491093)	GAU D 0.45 21.0 (515049)	GGU G 0.18 11.4 (280522)
GUC V 0.25 15.4 (377902)	GCC A 0.38 26.0 (637878)	GAC D 0.55 26.0 (638504)	GGC G 0.33 21.2 (520069)
GUA V 0.12 7.4 (182733)	GCA A 0.23 15.8 (388723)	GAA E 0.41 27.0 (661498)	GGA G 0.26 16.8 (411344)
GUG V 0.46 28.4 (696158)	GCG A 0.09 6.4 (157124)	GAG E 0.59 39.4 (965963)	GGG G 0.23 15.2 (372099)

***Pichia pastoris* codon usage table:**

First three letters are the codons in the mRNA. The numbers next to them are the frequencies of each codon were 1.00 = 100%, the frequency per thousand and the actual number of the codons in the genome. Taken from: http://www.kazusa.or.jp/codon

UUU F 0.54 24.1 (1963)	UCU S 0.29 24.4 (1983)	UAU Y 0.47 16.0 (1300)	UGU C 0.64 7.7 (626)
UUC F 0.46 20.6 (1675)	UCC S 0.20 16.5 (1344)	UAC Y 0.53 18.1 (1473)	UGC C 0.36 4.4 (356)
UUA L 0.16 15.6 (1265)	UCA S 0.18 15.2 (1234)	UAA * 0.51 0.8 (69)	UGA * 0.20 0.3 (27)
UUG L 0.33 31.5 (2562)	UCG S 0.09 7.4 (598)	UAG * 0.29 0.5 (40)	UGG W 1.00 10.3 (834)
CUU L 0.16 15.9 (1289)	CCU P 0.35 15.8 (1282)	CAU H 0.57 11.8 (960)	CGU R 0.17 6.9 (564)
CUC L 0.08 7.6 (620)	CCC P 0.15 6.8 (553)	CAC H 0.43 9.1 (737)	CGC R 0.05 2.2 (175)
CUA L 0.11 10.7 (873)	CCA P 0.42 18.9 (1540)	CAA Q 0.61 25.4 (2069)	CGA R 0.10 4.2 (340)
CUG L 0.16 14.9 (1215)	CCG P 0.09 3.9 (320)	CAG Q 0.39 16.3 (1323)	CGG R 0.05 1.9 (158)
AUU I 0.50 31.1 (2532)	ACU T 0.40 22.4 (1820)	AAU N 0.48 25.1 (2038)	AGU S 0.15 12.5 (1020)
AUC I 0.31 19.4 (1580)	ACC T 0.26 14.5 (1175)	AAC N 0.52 26.7 (2168)	AGC S 0.09 7.6 (621)
AUA I 0.18 11.1 (906)	ACA T 0.24 13.8 (1118)	AAA K 0.47 29.9 (2433)	AGA R 0.48 20.1 (1634)
AUG M 1.00 18.7 (1517)	ACG T 0.11 6.0 (491)	AAG K 0.53 33.8 (2748)	AGG R 0.16 6.6 (539)
GUU V 0.42 26.9 (2188)	GCU A 0.45 28.9 (2351)	GAU D 0.58 35.7 (2899)	GGU G 0.44 25.5 (2075)
GUC V 0.23 14.9 (1210)	GCC A 0.26 16.6 (1348)	GAC D 0.42 25.9 (2103)	GGC G 0.14 8.1 (655)
GUA V 0.15 9.9 (804)	GCA A 0.23 15.1 (1228)	GAA E 0.56 37.4 (3043)	GGA G 0.33 19.1 (1550)
GUG V 0.19 12.3 (998)	GCG A 0.06 3.9 (314)	GAG E 0.44 29.0 (2360)	GGG G 0.10 5.8 (468)

Values for Figure 17:

These are the figures from the SPR analysis of the J558L cell selection.

	Resonance Units	Average Resonance
Resistant Parent	11.40	10.65
Resistant Parent	9.90	
Parent	10.40	10.35
Parent	10.30	
Pure Media	8.90	8.85
Pure Media	8.80	
AA1	12.50	12.45
AA1	12.40	
AA2	8.40	8.55
AA2	8.70	
AA3	11.40	10.90
AA3	10.40	
AA4	10.60	10.25
AA4	9.90	

	Resonance Units	Average Resonance
AA5	13.90	13.55
AA5	13.20	
AA6	8.80	8.75
AA6	8.70	
AB1	17.10	16.90
AB1	16.70	
AB2	8.30	8.15
AB2	8.00	
AB3	14.10	13.55
AB3	13.00	
AB4	23.30	22.95
AB4	22.60	
AB5	25.00	24.30
AB5	23.60	
AB6	30.60	29.85
AB6	29.10	
AC1	25.70	25.30
AC1	24.90	
AC2	32.80	32.20
AC2	31.60	
AC3	45.40	44.20
AC3	43.00	
AC4	23.60	23.20
AC4	22.80	
AC5	26.30	25.75
AC5	25.20	
AC6	8.70	8.60
AC6	8.50	
AD1	48.10	47.05
AD1	46.00	
AD2	56.70	55.55
AD2	54.40	
AD3	13.90	13.85
AD3	13.80	
AD4	37.10	36.15
AD4	35.20	
AD5	26.10	25.80
AD5	25.50	
AD6	10.80	10.55
AD6	10.30	
BA1	10.00	9.85
BA1	9.70	
BA2	11.90	12.05
BA2	12.20	
BA3	16.60	15.95
BA3	15.30	
BA4	28.00	27.10
BA4	26.20	
BA5	51.60	50.60
BA5	49.60	
BA6	60.50	59.95
BA6	59.40	
BB1	19.10	18.25
BB1	17.40	
BB2	17.70	17.45
BB2	17.20	
BB3	63.70	63.20

	Resonance Units	Average Resonance
BB3	60.70	62.20
BB4	13.50	13.55
BB4	13.60	
BB5	17.40	16.80
BB5	16.20	
BB6	25.20	24.90
BB6	24.60	
BC1	25.80	25.60
BC1	25.40	
BC2	19.30	19.05
BC2	18.80	
BC3	22.20	22.15
BC3	22.10	
BC4	42.70	41.75
BC4	40.80	
BC5	42.10	41.35
BC5	40.60	
BC6	35.30	34.85
BC6	34.40	
BD1	51.70	49.65
BD1	47.60	
BD2	15.20	15.20
BD2	15.20	
BD3	56.70	54.50
BD3	52.30	
BD4	12.50	13.20
BD4	13.90	
BD5	34.90	34.25
BD5	33.60	
BD6	24.30	24.00
BD6	23.70	

Values for Figure 19:
These are the SPR values for the equine IgE expression test.

0	4	3.7
0	3.4	
1	7.2	6.7
1	6.2	
2	52.1	51.4
2	50.7	
3	156.5	155.65
3	154.8	
4	311.4	308.6
4	305.8	
5	403.2	398.55
5	393.9	
6	411.1	408.5
6	405.9	

Values for Figure 20:

These are the SPR values for the equine IgE media concentration test.

Normal	359.5	360.35
Normal	361.2	
Concentrate	3735.1	3689
Concentrate	3642.9	
Overflow	0.3	-0.45
Overflow	-1.2	

VSKQAPLILPLAACCKDTKTTNITLGCLVKGYFPEPVTVTWDAGSLNRSTMTFPA
VFDQTSGLYTTISRVVASGKWAKQKFTCNVVHSQETFNKTFNACIVTFTPPTVKLFHSS
CDPGGDSHTTIQLLCLISDYTPGDIDIVWLIDGQKVDEQFPQHGLVKQEGKLASTHSEL
NITQGQWASENTYTCQVTYKDMIFKDQARKCTESDPRGVSVYLSPPSPLDLYVSKSPKI
TCLVVDLANVQGLSLNWSRESGEPLQKHTLATSEQFNKTFSVTSTLPVDTTDWIEGETY
KCTVSHPDLPREVVRSIAKAPGKRLSPEVYVFLPPEEDQSSKDKVTLTCLIQNFFPADI
SVQWLRNNVLIQTDQQATTRPQKANGPNPAFFVFSRLEVSRAEWEQKNKFACKVVHEAL
SQRTLQKEVSKDPGK

Equine IgE Protein Sequence:

This was the equine IgE heavy chain sequence used in this project, noting there is no methionine (M) at the start of the gene as this sequence is only for the 4 heavy chain domains and not including the variable region. Blue represents: Cε1, red: Cε2, green: Cε3 and purple: Cε4.

Optimized Equine IgE DNA Sequence:

This is the final optimized DNA sequence used, noting the addition of a TAA ending sequence, the restriction sites at the ends of the sequence to allow for cloning, and half a human intron that completes the endogenous intron in the plasmid.

MPAPMGSPALLWITFLLFSLDGVPAAIRKSTVSLNPPWNRIFRGENVTLTCNKNKPLKG
NSTEWTYNNTTLEVTTSSLNITNASHRSSGEYRCRNNDLNLSEAVHLEVFSDWLLLQAS
AEEVIEGKALVLRCRGWKDWDVFKVIYYKDGKPLEYWYENKNISIESATTENSGTYYCE
GAFNFKRTSERYTSDYLNITVKKAEQSKRYWLQFIIPLLVVILFAVDTGLFVSTQQQLT
FLLKIKRTRRGRKLMDPHP

Equine FcεRIα Protein Sequence:

This was the equine FcεRIα sequence used in this project. Purple represents: the Signal Peptide, green: Domain 1, red: Domain 2, black: Transmembrane Region and brown: Cytoplasmic Region.

MPAPMGSPALLWITFLLFSLDGVPAAIRKSTVSLNPPWNRIFRGENVTLTCNKNKPLKG
NSTEWTYNNTTLEVTTSSLNITNASHRSSGEYRCRNNDLNLSEAVHLEVFSDWLLLQAS
AEEVIEGKALVLRCRGWKDWDVFKVIYYKDGKPLEYWYENKNISIESATTENSGTYYCE
GAFNFKRTSERYTSDYL

Equine sFcεRIαD1&2 Protein Sequence:

This was the equine FcεRIα sequence used in this project. Purple represents: the Signal Peptide, green: Domain 1, red: Domain 2.

Optimized FcRIα Receptor DNA Sequence:

This is the final optimized DNA sequence used, noting the addition of a TAA ending sequence and the restriction sites at the ends of the sequence to allow for cloning.

GAATTCATTGATCATTAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACCTCCCCCTGAACCTGAA
ACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACA
AATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGGATCCCTGTGGAATGTG
TGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTG
GAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCC
CATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCC
TCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTGGAGATCTGATCAAGAGACA
GGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACT
GGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCT
GTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGAC
GTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGA
AAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCAT
CGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTG
TTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAA
ATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGC
TGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGC
CTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCAACCTGCCATCACGAGATTTCGATTC
CACCGCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTG
GAGTTCTTCGCCCACCCCGGGCTCGATCCCCTCGCGAGTTGGTTCAGCTGCTGCCTGAGGCTGGACGACCTCGCGGAGTTCTACCGGC
AGTGCAAATCCGTCGGCATCCAGGAAACCAGCAGCGGCTATCCGCGCATCCATGCCCCCGAACTGCAGGAGTGGGGAGGCACGATGGC
CGCTTTGGTCGACCTCGGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTC
AGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCC
GCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAG
GTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCA
ACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTT
CTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGA
GTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAG
GATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATT
ATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGAC
AGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGA
TAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGC
AATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAA
GCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTA
TGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCC
TCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCC
GTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGT
CAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGAT
CTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGG
TGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAAT
ATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCG
CACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCC
TGATGGCTCTTTGCGGCACCCATCGTTCGTAATGTTCCGTGGCACCGAGGACAACCCTCAAGAGAAAATGTAATCACACTGGCTCACC
TTCGGGTGGGCCTTTCTGCGTTTATAAGGAGACACTTTATGTTTAAGAAGGTTGGTAAATTCCTTGCGGCTTTGGCAGCCAAGCTAGA
GATCTCTAGCTTCGTGTCAAGGACGGTGACTGCAGTGAATAATAAAATGTGTGTTTGTCCGAAATACGCGTTTTGAGATTTCTGTCGC
CGACTAAATTCATGTCGCGCGATAGTGGTGTTTATCGCCGATAGAGATGGCGATATTGGAAAAATCGATATTTGAAAATATGGCATAT
TGAAAATGTCGCCGATGTGAGTTTCTGTGTAACTGATATCGCCATTTTTCCAAAAGTGATTTTTGGGCATACGCGATATCTGGCGATA
GCGCTTATATCGTTTACGGGGGATGGCGATAGACGACTTTGGTGACTTGGGCGATTCTGTGTGTCGCAAATATCGCAGTTTCGATATA
GGTGACAGACGATATGAGGCTATATCGCCGATAGAGGCGACATCAAGCTGGCACATGGCCAATGCATATCGATCTATACATTGAATCA
ATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATCAATATTGGCTATTGGCCATTGCATACGTTGTATCCATATCAT
AATATGTACATTTATATTGGCTCATGTCCAACATTACCGCCATGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGG
GGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCG
CCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAA
ACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATT
ATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTG
GCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGG
CACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCT
ATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCG
ATCCAGCCTCCGCGGCCGGGAACGGTGCATTGGAACGCGGATTCCCCGTGCCAAGAGTGACGTAAGTACCGCCTATAGAGTCTATAGG
CCCACCCCCTTGGCTTCTTATGCATGCTATACTGTTTTTGGCTTGGGGTCTATACACCCCCGCTTCCTCATGTTATAGGTGATGGTAT
AGCTTAGCCTATAGGTGTGGGTTATTGACCATTATTGACCACTCCCCTATTGGTGACGATACTTTCCATTACTAATCCATAACATGGC
TCTTTGCCACAACTCTCTTTATTGGCTATATGCCAATACACTGTCCTTCAGAGACTGACACGGACTCTGTATTTTTACAGGATGGGGT
CTCATTTATTATTTTACAAATTCACATATACAACACCACCGTCCCCAGTGCCCGCAGTTTTTATTAAACATAACGTGGGATCTCCACGC
GAATCTCGGGTACGTGTTCCGGACATGGGCTCTTCTCCGGTAGCGGCGGAGCTTCTACATCCGAGCCCTGCTCCCATGCCTCCAGCGA
CTCATGGTCGCTCGGCAGCTCCTTGCTCCTAACAGTGGAGGCCAGACTTAGGCACAGCACGATGCCCACCACCACCAGTGTGCCGCAC
AAGGCCGTGGCGGTAGGGTATGTGTCTGAAAATGAGCTCGGGGAGCGGGCTTGCACCGCTGACGCATTTGGAAGACTTAAGGCAGCGG
CAGAAGAAGATGCAGGCAGCTGAGTTGTTGTGTTCTGATAAGAGTCAGAGGTAACTCCCGTTGCGGTGCTGTTAACGGTGGAGGGCAG
TGTAGTCTGAGCAGTACTCGTTGCTGCCGCGCGCGCCACCAGACATAATAGCTGACAGACTAACAGACTGTTCCTTTCCATGGGTCTT
TTCTGCAGTCACCGTCCTTGACACGAAGCTTGGGCTGCAGGTCGATCGACTCTAGAGGATCGATCCCCGGGCGAGCTC

pEE6 Plasmid Sequence:

The pEE6 plasmid sold by CellTech (in defunct UK company since 2004). It integrates into a mammalian cell's chromosome and therefore was designed for mammalian protein expression.

Data For Figures 28 And 29:

	RBL-Non-Transfected Cells Bound With Human IgE											
NIP Concentration (ng ml^{-1})	1	2	3	4	5	6	7	8	9	10	11	12
0	1.633	1.749	1.787	1.773	1.830	1.858	0.074	0.078	0.088	0.124	0.100	0.102
0.1	1.709	1.896	1.909	1.887	1.920	1.868	0.070	0.072	0.069	0.101	0.091	0.084
1	1.737	1.865	1.891	1.904	1.989	1.966	0.076	0.077	0.072	0.073	0.076	0.080
10	1.809	1.897	1.927	1.993	1.984	1.963	0.065	0.064	0.066	0.070	0.079	0.073
100	1.793	2.020	2.023	2.005	1.953	2.107	0.085	0.086	0.083	0.088	0.086	0.086
1000	1.719	1.910	1.967	2.028	1.952	2.010	0.089	0.080	0.085	0.081	0.080	0.086
10000	1.736	1.854	1.963	1.994	1.994	1.970	0.088	0.096	0.092	0.091	0.098	0.096
Total In Cell	0.542	0.688	0.642	0.723	0.742	0.907	0.941	1.220	0.747	1.247	1.207	0.819
	x2 because (I used 50 µl instead of the 100) + Supernatant Value To Find Original Total In Cell						% of Total In Cell					
0	1.781	1.905	1.963	2.021	2.03	2.062	8.310	8.189	8.966	12.271	9.852	9.893
0.1	1.849	2.04	2.047	2.089	2.102	2.036	7.572	7.059	6.742	9.670	8.658	8.251
1	1.889	2.019	2.035	2.05	2.141	2.126	8.047	7.628	7.076	7.122	7.099	7.526
10	1.939	2.025	2.059	2.133	2.142	2.109	6.704	6.321	6.411	6.564	7.376	6.923
100	1.963	2.192	2.189	2.181	2.125	2.279	8.660	7.847	7.583	8.070	8.094	7.547
1000	1.897	2.07	2.137	2.19	2.112	2.182	9.383	7.729	7.955	7.397	7.576	7.883
10000	1.912	2.046	2.147	2.176	2.19	2.162	9.205	9.384	8.570	8.364	8.950	8.881
Total In Cell	2.424	3.128	2.136	3.217	3.156	2.545	77.640	78.005	69.944	77.526	76.489	64.361
	Average	Standard Deviation										
0	9.580	1.51										
0.1	7.992	1.09										
1	7.416	0.39										
10	6.716	0.39										
100	7.967	0.41										
1000	7.987	0.71										
10000	8.892	0.38										

	RBL-pEE6 Cells Bound With Human IgE											
NIP Concentration (ng ml^{-1})	1	2	3	4	5	6	7	8	9	10	11	12
0	1.558	1.607	1.635	1.633	1.530	1.566	0.081	0.089	0.093	0.088	0.085	0.089
0.1	1.538	1.668	1.661	1.829	1.563	1.637	0.068	0.067	0.067	0.070	0.068	0.070
1	1.549	1.720	1.748	1.749	1.629	0.992	0.073	0.072	0.075	0.071	0.070	0.078
10	1.741	1.656	1.748	1.843	1.824	1.869	0.066	0.064	0.066	0.065	0.071	0.065
100	1.742	1.798	1.776	1.870	1.754	1.760	0.087	0.086	0.087	0.086	0.086	0.089
1000	1.766	1.832	1.762	1.758	1.766	1.796	0.083	0.078	0.087	0.079	0.080	0.077
10000	1.722	1.780	1.711	1.794	1.738	1.799	0.100	0.084	0.090	0.091	0.093	0.090
Total In Cell	0.391	0.365	0.290	0.656	0.436	0.615	1.072	0.812	1.109	0.881	0.996	0.623
	x2 because (I used 50 µl instead of the 100) + Supernatant Value To Find Original Total In Cell						% of Total In Cell					
0	1.72	1.785	1.821	1.809	1.7	1.744	9.419	9.972	10.214	9.729	10.000	10.206
0.1	1.674	1.802	1.795	1.969	1.699	1.777	8.124	7.436	7.465	7.110	8.005	7.878
1	1.695	1.864	1.898	1.891	1.769	1.148	8.614	7.725	7.903	7.509	7.914	13.589
10	1.873	1.784	1.88	1.973	1.966	1.999	7.048	7.175	7.021	6.589	7.223	6.503
100	1.916	1.97	1.95	2.042	1.926	1.938	9.081	8.731	8.923	8.423	8.930	9.185
1000	1.932	1.988	1.936	1.916	1.926	1.95	8.592	7.847	8.988	8.246	8.307	7.897
10000	1.922	1.948	1.891	1.976	1.924	1.979	10.406	8.624	9.519	9.211	9.667	9.096
Total In Cell	2.535	1.989	2.508	2.418	2.428	1.861	84.576	81.649	88.437	72.870	82.043	66.953
	Average	Standard Deviation										
0	9.923	0.31										
0.1	7.670	0.39										
1	8.876	2.34										
10	6.926	0.31										
100	8.879	0.27										
1000	8.313	0.43										
10000	9.420	0.60										

	RBL-Non-Transfected Cells Bound With Mouse IgE											
DNP Concentration (ng ml^{-1})	1	2	3	4	5	6	7	8	9	10	11	12
0	1.803	1.779	1.797	1.959	1.730	1.239	0.036	0.064	0.044	0.048	0.054	0.018
0.1	1.910	1.851	1.874	1.936	1.804	1.682	0.032	0.041	0.036	0.043	0.038	0.019
1	1.641	1.733	1.789	1.842	1.744	1.555	0.131	0.157	0.178	0.160	0.185	0.124
10	1.275	1.361	1.350	1.367	1.307	1.175	0.314	0.428	0.554	0.551	0.609	0.510
100	1.349	1.236	1.166	1.080	1.025	0.936	0.433	0.537	0.726	0.704	0.707	0.517
1000	1.173	1.236	1.192	1.156	1.081	1.374	0.385	0.669	0.695	0.716	0.584	0.517
10000	1.284	1.189	1.277	1.492	1.113	1.177	0.414	0.652	0.472	0.574	0.323	0.324
Total In Cell	1.094	0.964	0.888	0.969	1.037	0.933	0.351	0.413	0.463	0.517	0.494	0.410
0	2.165	2.283	2.206	2.189	2.244	2.257	0.039	0.037	0.050	0.036	0.036	0.028
0.1	2.215	2.233	2.221	2.218	2.169	2.265	0.053	0.048	0.051	0.045	0.054	0.033
1	2.133	2.170	2.114	2.049	1.909	1.983	0.168	0.166	0.142	0.161	0.148	0.153
10	1.651	1.693	1.743	1.722	1.747	1.713	0.680	0.586	0.469	0.514	0.435	0.513
100	1.296	1.260	1.356	1.303	1.301	1.283	0.906	0.689	0.699	0.710	0.767	0.774
1000	1.288	1.306	1.285	1.334	1.248	1.285	0.779	0.553	0.672	0.992	0.718	0.545
10000	1.336	1.296	1.459	1.332	1.392	1.416	0.842	0.433	0.641	0.727	0.552	0.697
Total In Cell	0.636	0.653	0.603	0.668	0.658	0.422	1.060	0.713	0.966	0.858	0.680	0.397
0	2.730	2.660	2.686	2.649	2.646	2.674	0.036	0.052	0.044	0.038	0.037	0.038
0.1	2.790	2.668	2.708	2.829	2.667	2.623	0.074	0.063	0.066	0.060	0.056	0.064
1	2.696	2.719	2.698	2.565	2.611	2.540	0.175	0.165	0.168	0.167	0.123	0.162
10	2.339	2.392	2.264	2.295	2.247	2.281	0.653	0.596	0.598	0.585	0.542	0.530
100	1.902	1.917	1.858	1.890	1.824	1.883	0.947	0.849	0.888	0.874	0.870	0.823
1000	2.099	2.062	1.920	1.938	1.911	1.881	0.902	0.879	0.916	0.821	0.793	0.804
10000	2.052	2.091	1.934	2.049	1.856	1.958	0.830	0.777	0.894	0.849	0.784	0.806
Total In Cell	0.356	0.141	0.182	0.173	0.207	0.158	1.940	1.679	1.847	1.482	1.300	1.689
0	2.458	2.438	2.560	2.387	2.265	2.278	0.062	0.045	0.046	0.032	0.047	0.033
0.1	2.584	2.681	2.702	2.686	2.249	2.587	0.076	0.058	0.071	0.043	0.065	0.051
1	2.591	2.600	2.544	2.543	2.434	2.263	0.255	0.212	0.287	0.257	0.208	0.214
10	2.187	2.159	2.254	2.154	1.940	1.915	0.737	0.747	0.713	0.613	0.582	0.529
100	1.875	1.704	1.772	1.839	1.519	1.505	1.024	1.195	1.154	0.745	0.906	0.791
1000	1.929	1.686	1.742	1.720	1.590	1.804	1.001	1.019	1.152	0.791	0.835	0.714
10000	1.936	1.795	1.756	1.768	1.411	1.713	1.036	1.045	1.096	0.871	0.884	0.710
Total In Cell	1.170	0.300	0.250	0.247	0.237	0.198	0.981	1.384	1.601	1.321	1.385	1.538

	x2 because (I used 50 µl instead of the 100) + Supernatant Value To Find Original Total In Cell						% of Total In Cell					
0	1.875	1.907	1.885	2.055	1.838	1.275	3.840	6.712	4.668	4.672	5.876	2.824
0.1	1.974	1.933	1.946	2.022	1.88	1.72	3.242	4.242	3.700	4.253	4.043	2.209
1	1.903	2.047	2.145	2.162	2.114	1.803	13.768	15.340	16.597	14.801	17.502	13.755
10	1.903	2.217	2.458	2.469	2.525	2.195	33.001	38.611	45.077	44.633	48.238	46.469
100	2.215	2.31	2.618	2.488	2.439	1.97	39.097	46.494	55.462	56.592	57.975	52.487
1000	1.943	2.574	2.582	2.588	2.249	2.408	39.629	51.981	53.834	55.332	51.934	42.940
10000	2.112	2.493	2.221	2.64	1.759	1.825	39.205	52.306	42.503	43.485	36.725	35.507
Total In Cell	1.796	1.79	1.814	2.003	2.025	1.753	39.087	46.145	51.047	51.623	48.790	46.777
0	2.243	2.357	2.306	2.261	2.316	2.313	3.477	3.140	4.337	3.184	3.109	2.421
0.1	2.321	2.329	2.323	2.308	2.277	2.331	4.567	4.122	4.391	3.899	4.743	2.831
1	2.469	2.502	2.398	2.371	2.205	2.289	13.609	13.269	11.843	13.581	13.424	13.368
10	3.011	2.865	2.681	2.75	2.617	2.739	45.168	40.908	34.987	37.382	33.244	37.459
100	3.108	2.638	2.754	2.723	2.835	2.831	58.301	52.237	50.763	52.148	54.109	54.680
1000	2.846	2.412	2.629	3.318	2.684	2.375	54.743	45.854	51.122	59.795	53.502	45.895
10000	3.02	2.162	2.741	2.786	2.496	2.81	55.762	40.056	46.771	52.190	44.231	49.609
Total In Cell	2.756	2.079	2.535	2.384	2.018	1.216	76.923	68.591	76.213	71.980	67.393	65.296
0	2.802	2.764	2.774	2.725	2.72	2.75	2.570	3.763	3.172	2.789	2.721	2.764
0.1	2.938	2.794	2.84	2.949	2.779	2.751	5.037	4.510	4.648	4.069	4.030	4.653
1	3.046	3.049	3.034	2.899	2.857	2.864	11.490	10.823	11.074	11.521	8.610	11.313
10	3.645	3.584	3.46	3.465	3.331	3.341	35.830	33.259	34.566	33.766	32.543	31.727
100	3.796	3.615	3.634	3.638	3.564	3.529	49.895	46.971	48.872	48.048	48.822	46.642
1000	3.903	3.82	3.752	3.58	3.497	3.489	46.221	46.021	48.827	45.866	45.353	46.088
10000	3.712	3.645	3.722	3.747	3.424	3.57	44.720	42.634	48.039	45.316	45.794	45.154
Total In Cell	4.236	3.499	3.876	3.137	2.807	3.536	91.596	95.970	95.304	94.485	92.626	95.532
0	2.582	2.528	2.652	2.451	2.359	2.344	4.802	3.560	3.469	2.611	3.985	2.816
0.1	2.736	2.797	2.844	2.772	2.379	2.689	5.556	4.147	4.993	3.102	5.464	3.793
1	3.101	3.024	3.118	3.057	2.85	2.691	16.446	14.021	18.409	16.814	14.596	15.905
10	3.661	3.653	3.68	3.38	3.104	2.973	40.262	40.898	38.750	36.272	37.500	35.587
100	3.923	4.094	4.08	3.329	3.331	3.087	52.205	58.378	56.569	44.758	54.398	51.247
1000	3.931	3.724	4.046	3.302	3.26	3.232	50.929	54.726	56.945	47.910	51.227	44.183
10000	4.008	3.885	3.948	3.51	3.179	3.133	51.697	53.797	55.522	49.630	55.615	45.324
Total In Cell	3.132	3.068	3.452	2.889	3.007	3.274	62.644	90.222	92.758	91.450	92.118	93.952

	Average	Standard Deviation
0	3.637	1.08
0.1	4.177	0.79
1	13.828	2.39
10	38.172	4.83
100	51.548	4.79
1000	49.619	4.97
10000	46.733	5.83

	RBL-pEE6 Cells Bound With Mouse IgE											
DNP Concentration (ng ml^{-1})	1	2	3	4	5	6	7	8	9	10	11	12
0	2.143	2.239	2.136	2.131	2.056	2.069	0.015	0.016	0.011	0.015	0.015	0.009
0.1	2.124	2.222	2.143	2.179	2.147	2.093	0.029	0.021	0.028	0.022	0.019	0.027
1	2.119	2.219	2.166	2.208	2.168	2.095	0.096	0.058	0.077	0.061	0.091	0.088
10	1.730	1.943	1.821	1.913	1.869	1.777	0.453	0.260	0.362	0.228	0.394	0.383
100	1.368	1.518	1.508	1.538	1.492	1.417	0.730	0.510	0.692	0.488	0.765	0.778
1000	1.514	1.680	1.551	1.682	1.552	1.519	0.697	0.504	0.635	0.362	0.661	0.396
10000	1.515	1.597	1.566	1.357	1.583	1.564	0.575	0.475	0.577	0.485	0.647	0.609
Total In Cell	0.075	0.278	0.216	0.259	0.255	0.244	1.323	1.628	1.392	1.108	1.451	1.686
0	2.050	2.132	2.115	2.131	2.145	2.138	0.021	0.023	0.011	0.008	0.010	0.036
0.1	2.052	2.201	2.241	2.245	2.201	2.123	0.028	0.038	0.041	0.023	0.003	0.030
1	1.967	2.173	2.201	2.252	2.177	2.091	0.121	0.093	0.106	0.114	0.156	0.175
10	1.592	1.898	1.873	1.856	1.896	1.716	0.398	0.268	0.314	0.469	0.435	0.547
100	1.387	1.525	1.521	1.498	1.508	1.399	0.605	0.374	0.487	0.743	0.765	0.799
1000	1.479	1.606	1.594	1.583	1.629	1.543	0.572	0.303	0.354	0.619	0.435	0.679
10000	1.467	1.622	1.591	1.570	1.638	1.539	0.507	0.283	0.360	0.485	0.462	0.582
Total In Cell	0.455	0.336	0.445	0.430	0.350	0.225	1.443	0.925	0.711	1.188	0.787	0.930
0	2.032	2.082	2.010	2.123	2.078	2.081	0.016	0.020	0.014	0.025	0.022	0.013
0.1	2.048	2.152	2.124	2.210	2.116	2.058	0.038	0.037	0.029	0.032	0.022	0.037
1	2.010	2.180	2.194	2.158	2.175	2.079	0.094	0.052	0.061	0.056	0.062	0.083
10	1.807	1.919	1.947	2.008	2.021	1.893	0.310	0.228	0.306	0.259	0.261	0.293
100	1.301	1.454	1.438	1.427	1.413	1.433	0.629	0.454	0.654	0.592	0.593	0.664
1000	1.361	1.469	1.455	1.454	1.525	1.487	0.598	0.475	0.593	0.600	0.570	0.538
10000	1.429	1.520	1.529	1.510	1.582	1.531	0.496	0.371	0.618	0.481	0.397	0.538
Total In Cell	0.080	0.940	0.116	0.096	0.185	0.186	1.419	1.351	1.714	1.532	0.905	0.806
	x2 because (I used 50 µl instead of the 100) + Supernatant Value To Find Original Total In Cell						% of Total In Cell					
0	2.173	2.271	2.158	2.161	2.086	2.087	1.381	1.409	1.019	1.388	1.438	0.862
0.1	2.182	2.264	2.199	2.223	2.185	2.147	2.658	1.855	2.547	1.979	1.739	2.515
1	2.311	2.335	2.32	2.33	2.35	2.271	8.308	4.968	6.638	5.236	7.745	7.750
10	2.636	2.463	2.545	2.369	2.657	2.543	34.370	21.112	28.448	19.249	29.658	30.122
100	2.828	2.538	2.892	2.514	3.022	2.973	51.627	40.189	47.856	38.823	50.629	52.338
1000	2.908	2.688	2.821	2.406	2.874	2.311	47.937	37.500	45.019	30.091	45.999	34.271
10000	2.665	2.547	2.72	2.327	2.877	2.782	43.152	37.299	42.426	41.685	44.977	43.781
Total In Cell	2.721	3.534	3	2.475	3.157	3.616	97.244	92.134	92.800	89.535	91.923	93.252
0	2.092	2.178	2.137	2.147	2.165	2.21	2.008	2.112	1.029	0.745	0.924	3.258
0.1	2.108	2.277	2.323	2.291	2.207	2.183	2.657	3.338	3.530	2.008	0.272	2.749
1	2.209	2.359	2.413	2.48	2.489	2.441	10.955	7.885	8.786	9.194	12.535	14.338
10	2.388	2.434	2.501	2.794	2.766	2.81	33.333	22.021	25.110	33.572	31.453	38.932
100	2.597	2.273	2.495	2.984	3.038	2.997	46.592	32.908	39.038	49.799	50.362	53.320
1000	2.623	2.212	2.302	2.821	2.499	2.901	43.614	27.396	30.756	43.885	34.814	46.811
10000	2.481	2.188	2.311	2.54	2.562	2.703	40.871	25.868	31.155	38.189	36.066	43.063
Total In Cell	3.341	2.186	1.867	2.806	1.924	2.085	86.381	84.629	76.165	84.676	81.809	89.209
0	2.064	2.122	2.038	2.173	2.122	2.107	1.550	1.885	1.374	2.301	2.074	1.234
0.1	2.124	2.226	2.182	2.274	2.16	2.132	3.578	3.324	2.658	2.814	2.037	3.471
1	2.198	2.284	2.316	2.27	2.299	2.245	8.553	4.553	5.268	4.934	5.394	7.394
10	2.427	2.375	2.559	2.526	2.543	2.479	25.546	19.200	23.916	20.507	20.527	23.639
100	2.559	2.362	2.746	2.611	2.599	2.761	49.160	38.442	47.633	45.347	45.633	48.099
1000	2.557	2.419	2.641	2.654	2.665	2.563	46.774	39.272	44.907	45.215	42.777	41.982
10000	2.421	2.262	2.765	2.472	2.376	2.607	40.975	32.803	44.702	38.916	33.418	41.273
Total In Cell	2.918	3.642	3.544	3.16	1.995	1.798	97.258	74.190	96.727	96.962	90.727	89.655

	Average	Standard Deviation
0	1.555	0.63
0.1	2.540	0.82
1	7.802	2.72
10	26.706	5.98
100	45.989	5.76
1000	40.501	6.47
10000	38.923	5.27

	RBL-Non-Transfected Cells Bound With Dog IgE											
NIP Concentration (ng ml^{-1})	1	2	3	4	5	6	7	8	9	10	11	12
0	1.165	1.115	1.113	1.14	1.132	1.153	0.079	0.074	0.082	0.073	0.073	0.072
0.1	1.149	1.106	1.16	1.149	1.177	1.12	0.073	0.082	0.07	0.078	0.083	0.09
1	1.178	1.178	1.196	1.176	1.176	1.257	0.07	0.069	0.065	0.069	0.07	0.068
10	1.191	1.154	1.201	1.199	1.19	1.188	0.066	0.063	0.06	0.066	0.067	0.069
100	1.167	1.16	1.203	1.239	1.169	1.16	0.076	0.074	0.073	0.08	0.077	0.081
1000	1.177	1.203	1.242	1.236	1.245	1.167	0.077	0.073	0.077	0.081	0.077	0.08
10000	1.171	1.142	1.205	1.189	1.162	1.094	0.078	0.079	0.069	0.081	0.079	0.089
	x2 because (I used 50 µl instead of the 100) + Supernatant Value To Find Original Total In Cell						% of Total In Cell					
0	1.323	1.263	1.277	1.286	1.278	1.297	11.943	11.718	12.843	11.353	11.424	11.103
0.1	1.295	1.27	1.3	1.305	1.343	1.3	11.274	12.913	10.769	11.954	12.360	13.846
1	1.318	1.316	1.326	1.314	1.316	1.393	10.622	10.486	9.804	10.502	10.638	9.763
10	1.323	1.28	1.321	1.331	1.324	1.326	9.977	9.844	9.084	9.917	10.121	10.407
100	1.319	1.308	1.349	1.399	1.323	1.322	11.524	11.315	10.823	11.437	11.640	12.254
1000	1.331	1.349	1.396	1.398	1.399	1.327	11.570	10.823	11.032	11.588	11.008	12.057
10000	1.327	1.3	1.343	1.351	1.32	1.272	11.756	12.154	10.276	11.991	11.970	13.994
	Average	Standard Deviation										
0	11.730	0.62										
0.1	12.186	1.11										
1	10.303	0.41										
10	9.892	0.44										
100	11.499	0.47										
1000	11.346	0.47										
10000	12.023	1.19										

	RBL-pEE6 Cells Bound With Dog IgE											
NIP Concentration (ng ml^{-1})	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
0	2.022	2.211	2.063	2.021	2.043	2.018	0.012	0.015	0.015	0.012	0.017	0.017
0.1	2.064	2.197	2.147	2.055	2.002	1.951	0.040	0.021	0.040	0.022	0.033	0.048
1	1.978	2.176	2.055	2.092	2.129	2.058	0.110	0.075	0.082	0.059	0.094	0.096
10	1.697	1.839	1.832	1.799	1.846	1.678	0.382	0.145	0.369	0.248	0.268	0.377
100	1.581	1.811	1.661	1.749	1.711	1.699	0.382	0.229	0.316	0.268	0.391	0.409
1000	1.659	1.876	1.738	1.733	1.776	1.733	0.259	0.148	0.364	0.245	0.332	0.416
10000	1.624	1.761	1.693	1.675	1.802	1.614	0.244	0.123	0.305	0.244	0.424	0.391
Total In Cell	0.680	0.560	0.709	0.674	0.657	0.639	1.130	0.500	0.806	0.628	0.907	0.828
0	1.903	1.950	0.194	1.925	2.039	1.963	0.012	0.013	0.011	0.013	0.010	0.013
0.1	1.889	1.930	1.948	1.890	1.875	1.921	0.034	0.035	0.042	0.032	0.038	0.033
1	1.739	1.828	1.876	1.879	1.902	1.920	0.159	0.126	0.129	0.159	0.159	0.157
10	1.464	1.548	1.583	1.640	1.650	1.639	0.422	0.406	0.376	0.404	0.363	0.320
100	1.412	1.461	1.502	1.421	1.527	1.482	0.355	0.267	0.283	0.443	0.507	0.461
1000	1.472	1.493	1.673	1.469	1.553	1.491	0.344	0.260	0.352	0.421	0.457	0.447
10000	1.432	1.409	1.470	1.406	1.505	1.448	0.327	0.325	0.340	0.334	0.442	0.474
Total In Cell	0.347	0.315	0.375	0.324	0.279	0.365	1.208	1.045	1.180	1.106	1.023	1.028
0	1.987	2.134	2.167	2.127	2.052	2.016	0.022	0.020	0.019	0.023	0.022	0.017
0.1	2.073	2.170	2.175	2.192	2.114	2.058	0.043	0.028	0.033	0.032	0.021	0.047
1	2.066	2.142	2.147	2.190	2.180	2.147	0.110	0.085	0.081	0.082	0.086	0.105
10	1.632	1.753	1.746	1.779	1.784	1.700	0.431	0.310	0.426	0.506	0.488	0.447
100	1.660	1.639	1.651	1.637	1.688	1.528	0.422	0.317	0.351	0.455	0.542	0.464
1000	1.524	1.638	1.664	1.676	1.750	1.577	0.431	0.357	0.289	0.504	0.510	0.505
10000	1.529	1.620	1.649	1.606	1.674	1.454	0.445	0.350	0.403	0.373	0.467	0.465
Total In Cell	0.383	0.701	0.507	0.469	0.530	0.507	1.269	1.391	1.412	1.061	1.253	1.180
	x2 because (I used 50 µl instead of the 100) + Supernatant Value To Find Original Total In Cell						**% of Total In Cell**					
0	2.046	2.241	2.093	2.045	2.077	2.052	1.173	1.339	1.433	1.174	1.637	1.657
0.1	2.144	2.239	2.227	2.099	2.068	2.047	3.731	1.876	3.592	2.096	3.191	4.690
1	2.198	2.326	2.219	2.21	2.317	2.25	10.009	6.449	7.391	5.339	8.114	8.533
10	2.461	2.129	2.57	2.295	2.382	2.432	31.044	13.621	28.716	21.612	22.502	31.003
100	2.345	2.269	2.293	2.285	2.493	2.517	32.580	20.185	27.562	23.457	31.368	32.499
1000	2.177	2.172	2.466	2.223	2.44	2.565	23.794	13.628	29.521	22.042	27.213	32.437
10000	2.112	2.007	2.303	2.163	2.65	2.396	23.106	12.257	26.487	22.561	32.000	32.638
Total In Cell	2.94	1.56	2.321	1.93	2.471	2.295	76.871	64.103	69.453	65.078	73.412	72.157
0	1.927	1.976	0.216	1.951	2.059	1.989	1.245	1.316	10.185	1.333	0.971	1.307
0.1	1.957	2	2.032	1.954	1.951	1.987	3.475	3.500	4.134	3.275	3.895	3.322
1	2.057	2.08	2.134	2.197	2.22	2.234	15.459	12.115	12.090	14.474	14.324	14.056
10	2.308	2.36	2.335	2.448	2.376	2.279	36.568	34.407	32.206	33.007	30.556	28.082
100	2.122	1.995	2.068	2.307	2.541	2.404	33.459	26.767	27.369	38.405	39.906	38.353
1000	2.16	2.013	2.377	2.311	2.467	2.385	31.852	25.832	29.617	36.434	37.049	37.484
10000	2.086	2.059	2.15	2.074	2.389	2.396	31.352	31.569	31.628	32.208	37.003	39.566
Total In Cell	2.763	2.405	2.735	2.536	2.325	2.421	87.441	86.902	86.289	87.224	88.000	84.924
0	2.031	2.174	2.205	2.173	2.096	2.05	2.166	1.840	1.723	2.117	2.099	1.659
0.1	2.159	2.226	2.241	2.256	2.156	2.152	3.983	2.516	2.945	2.837	1.948	4.368
1	2.286	2.312	2.309	2.354	2.352	2.357	9.624	7.353	7.016	6.967	7.313	8.910
10	2.494	2.373	2.598	2.791	2.76	2.594	34.563	26.127	32.794	36.259	35.362	34.464
100	2.504	2.273	2.353	2.547	2.772	2.456	33.706	27.893	29.834	35.728	39.105	37.785
1000	2.386	2.352	2.242	2.684	2.77	2.587	36.127	30.357	25.781	37.556	36.823	39.041
10000	2.419	2.32	2.455	2.352	2.608	2.384	36.792	30.172	32.831	31.718	35.813	39.010
Total In Cell	2.921	3.483	3.331	2.591	3.036	2.867	86.888	79.874	84.779	81.899	82.543	82.316

	Average	**Standard Deviation**
0	2.021	2.07
0.1	3.299	0.81
1	9.752	3.19
10	30.161	5.99
100	31.998	5.69
1000	30.699	6.79
10000	31.040	6.62

	RBL-Non-Transfected Cells Bound With Horse IgE											
NIP Concentration (ng ml^{-1})	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
0	1.229	1.183	1.196	1.178	1.143	1.103	0.088	0.074	0.076	0.076	0.069	0.084
0.1	1.206	1.207	1.209	1.189	1.173	1.12	0.083	0.082	0.079	0.08	0.073	0.08
1	1.234	1.232	1.248	1.228	1.201	1.151	0.068	0.068	0.069	0.071	0.068	0.07
10	1.228	1.252	1.242	1.171	1.218	1.141	0.065	0.071	0.068	0.074	0.067	0.069
100	1.24	1.252	1.274	1.155	1.248	1.19	0.076	0.078	0.083	0.09	0.08	0.085
1000	1.153	1.329	1.278	1.254	1.3	1.191	0.075	0.076	0.075	0.082	0.077	0.074
10000	1.245	1.273	1.252	1.211	1.237	1.236	0.073	0.079	0.079	0.088	0.078	0.075
Total In Cell	0.215	0.204	0.233	0.233	0.284	0.222	0.473	0.74	0.693	0.689	0.535	0.474
	x2 because (I used 50 µl instead of the 100) + Supernatant Value To Find Original Total In Cell						**% of Total In Cell**					
0	1.405	1.331	1.348	1.33	1.281	1.271	12.527	11.119	11.276	11.429	10.773	13.218
0.1	1.372	1.371	1.367	1.349	1.319	1.28	12.099	11.962	11.558	11.861	11.069	12.500
1	1.37	1.368	1.386	1.37	1.337	1.291	9.927	9.942	9.957	10.365	10.172	10.844
10	1.358	1.394	1.378	1.319	1.352	1.279	9.573	10.187	9.869	11.221	9.911	10.790
100	1.392	1.408	1.44	1.335	1.408	1.36	10.920	11.080	11.528	13.483	11.364	12.500
1000	1.303	1.481	1.428	1.418	1.454	1.339	11.512	10.263	10.504	11.566	10.591	11.053
10000	1.391	1.431	1.41	1.387	1.393	1.386	10.496	11.041	11.206	12.689	11.199	10.823
Total In Cell	1.161	1.684	1.619	1.611	1.354	1.17	81.481	87.886	85.608	85.537	79.025	81.026
	Average	**Standard Deviation**										
0	11.724	0.94										
0.1	11.841	0.49										
1	10.201	0.36										
10	10.258	0.63										
100	11.812	0.99										
1000	10.915	0.55										
10000	11.242	0.76										

	RBL-pEE6 Cells Bound With Horse IgE											
NIP Concentration (ng ml^{-1})	1	2	3	4	5	6	7	8	9	10	11	12
0	1.734	1.65	1.614	1.587	1.616	1.564	0.068	0.077	0.051	0.068	0.063	0.067
0.1	1.767	1.739	1.695	1.632	1.663	1.556	0.066	0.065	0.07	0.069	0.069	0.074
1	1.733	1.713	1.697	1.617	1.663	1.523	0.088	0.087	0.086	0.087	0.089	0.106
10	1.441	1.441	1.429	1.401	1.411	1.272	0.245	0.292	0.305	0.35	0.401	0.404
100	1.438	1.426	1.392	1.364	1.364	1.304	0.26	0.337	0.376	0.434	0.405	0.488
1000	1.433	1.457	1.43	1.393	1.379	1.302	0.256	0.346	0.401	0.413	0.454	0.466
10000	1.442	1.411	1.389	1.34	1.35	1.237	0.293	0.346	0.393	0.399	0.452	0.428
Total In Cell	0.696	0.675	0.666	0.6	0.687	0.594	0.761	0.763	0.886	0.738	0.769	0.776
0	1.747	1.781	1.722	1.662	1.632	1.484	0.07	0.069	0.071	0.073	0.076	0.073
0.1	1.69	1.763	1.746	1.66	1.543	1.599	0.072	0.082	0.069	0.07	0.083	0.082
1	1.693	1.72	1.694	1.7	1.641	1.613	0.101	0.097	0.096	0.095	0.097	0.107
10	1.477	1.454	1.471	1.425	1.401	1.295	0.355	0.327	0.407	0.386	0.415	0.447
100	1.391	1.374	1.357	1.357	1.326	1.266	0.367	0.45	0.486	0.462	0.534	0.548
1000	1.408	1.396	1.374	1.302	1.335	1.325	0.381	0.402	0.475	0.416	0.5	0.502
10000	1.413	1.357	1.371	1.335	1.375	1.311	0.416	0.421	0.465	0.432	0.541	0.578
Total In Cell	0.672	0.627	0.601	0.63	0.55	0.57	0.877	0.763	0.79	0.504	0.652	0.752
0	1.682	1.719	1.653	1.674	1.684	1.915	0.063	0.062	0.063	0.063	0.061	0.064
0.1	1.725	1.737	1.701	1.658	1.696	1.577	0.061	0.061	0.059	0.057	0.058	0.059
1	1.712	1.704	1.695	1.679	1.707	1.597	0.075	0.079	0.079	0.069	0.066	0.088
10	1.369	1.371	1.455	1.377	1.392	1.357	0.215	0.231	0.338	0.271	0.268	0.335
100	1.349	1.36	1.364	1.317	1.317	1.382	0.32	0.34	0.418	0.319	0.377	0.478
1000	1.321	1.345	1.365	1.334	1.277	1.214	0.303	0.334	0.458	0.334	0.369	0.458
10000	1.316	1.288	1.333	1.218	1.206	1.161	0.353	0.304	0.351	0.303	0.332	0.475
Total In Cell	0.596	0.695	0.607	0.628	0.563	0.651	0.611	0.391	0.675	0.329	0.489	0.542
0	1.696	1.696	1.688	1.69	1.662	1.622	0.063	0.067	0.067	0.067	0.066	0.064
0.1	1.659	1.665	1.643	1.618	1.662	1.585	0.063	0.063	0.061	0.069	0.066	0.068
1	1.675	1.695	1.657	1.614	1.654	1.57	0.075	0.073	0.071	0.079	0.084	0.08
10	1.402	1.465	1.409	1.354	1.567	1.301	0.295	0.287	0.296	0.308	0.361	0.327
100	1.351	1.379	1.369	1.349	1.39	1.269	0.309	0.321	0.296	0.401	0.442	0.377
1000	1.4	1.401	1.419	1.362	1.418	1.463	0.261	0.308	0.305	0.373	0.436	0.394
10000	1.355	1.362	1.349	1.319	1.357	1.223	0.291	0.364	0.336	0.421	0.45	0.443
Total In Cell	0.589	0.639	0.568	0.582	0.665	0.577	0.607	0.672	0.708	0.795	0.71	0.787

	x2 because (I used 50 µl instead of the 100) + Supernatant Value To Find Original Total In Cell						% of Total In Cell					
0	1.87	1.804	1.716	1.723	1.742	1.698	7.273	8.537	5.944	7.893	7.233	7.892
0.1	1.899	1.869	1.835	1.77	1.801	1.704	6.951	6.956	7.629	7.797	7.662	8.685
1	1.909	1.887	1.869	1.791	1.841	1.735	9.219	9.221	9.203	9.715	9.669	12.219
10	1.931	2.025	2.039	2.101	2.213	2.08	25.375	28.840	29.917	33.317	36.240	38.846
100	1.958	2.1	2.144	2.232	2.174	2.28	26.558	32.095	35.075	38.889	37.259	42.807
1000	1.945	2.149	2.232	2.219	2.287	2.234	26.324	32.201	35.932	37.224	39.703	41.719
10000	2.028	2.103	2.175	2.138	2.254	2.093	28.895	32.905	36.138	37.325	40.106	40.898
Total In Cell	2.218	2.201	2.438	2.076	2.225	2.146	68.620	69.332	72.683	71.098	69.124	72.321
0	1.887	1.919	1.864	1.808	1.784	1.63	7.419	7.191	7.618	8.075	8.520	8.957
0.1	1.834	1.927	1.884	1.8	1.709	1.763	7.852	8.511	7.325	7.778	9.713	9.302
1	1.895	1.914	1.886	1.89	1.835	1.827	10.660	10.136	10.180	10.053	10.572	11.713
10	2.187	2.108	2.285	2.197	2.231	2.189	32.465	31.025	35.624	35.139	37.203	40.841
100	2.125	2.274	2.329	2.281	2.394	2.362	34.541	39.578	41.735	40.509	44.612	46.401
1000	2.17	2.2	2.324	2.134	2.335	2.329	35.115	36.545	40.878	38.988	42.827	43.109
10000	2.245	2.199	2.301	2.199	2.457	2.467	37.060	38.290	40.417	39.291	44.037	46.859
Total In Cell	2.426	2.153	2.181	1.638	1.854	2.074	72.300	70.878	72.444	61.538	70.334	72.517
0	1.808	1.843	1.779	1.8	1.806	2.043	6.969	6.728	7.083	7.000	6.755	6.265
0.1	1.847	1.859	1.819	1.772	1.812	1.695	6.605	6.563	6.487	6.433	6.402	6.962
1	1.862	1.862	1.853	1.817	1.839	1.773	8.056	8.485	8.527	7.595	7.178	9.927
10	1.799	1.833	2.131	1.919	1.928	2.027	23.902	25.205	31.722	28.244	27.801	33.054
100	1.989	2.04	2.2	1.955	2.071	2.338	32.177	33.333	38.000	32.634	36.408	40.890
1000	1.927	2.013	2.281	2.002	2.015	2.13	31.448	33.184	40.158	33.367	36.625	43.005
10000	2.022	1.896	2.035	1.824	1.87	2.111	34.916	32.068	34.496	33.224	35.508	45.002
Total In Cell	1.818	1.477	1.957	1.286	1.541	1.735	67.217	52.945	68.983	51.166	63.465	62.478
0	1.822	1.83	1.822	1.824	1.794	1.75	6.915	7.322	7.355	7.346	7.358	7.314
0.1	1.785	1.791	1.765	1.756	1.794	1.721	7.059	7.035	6.912	7.859	7.358	7.902
1	1.825	1.841	1.799	1.772	1.822	1.73	8.219	7.930	7.893	8.916	9.221	9.249
10	1.992	2.039	2.001	1.97	2.289	1.955	29.618	28.151	29.585	31.269	31.542	33.453
100	1.969	2.021	1.961	2.151	2.274	2.023	31.386	31.766	30.189	37.285	38.874	37.271
1000	1.922	2.017	2.029	2.108	2.29	2.251	27.159	30.540	30.064	35.389	38.079	35.007
10000	1.937	2.09	2.021	2.161	2.257	2.109	30.046	34.833	33.251	38.963	39.876	42.010
Total In Cell	1.803	1.983	1.984	2.172	2.085	2.151	67.332	67.776	71.371	73.204	68.106	73.175

	Average	Standard Deviation
0	7.373	0.69
0.1	7.489	0.88
1	9.323	1.25
10	31.599	4.29
100	36.678	4.88
1000	36.025	4.84
10000	37.351	4.62

Data For Figures 30 And 31:

	RBL-pEE6 Expressing Dog α Chain Cells Bound With Horse IgE For 0.5 Hours											
NIP Concentration (ng ml^{-1})	1	2	3	4	5	6	7	8	9	10	11	12
0	2.013	2.052	2.049	2.04	2.048	2	0.09	0.102	0.096	0.094	0.095	0.092
0.1	2.092	2.092	2.013	2.068	2.046	2.036	0.088	0.094	0.092	0.092	0.093	0.097
1	2.06	2.027	1.991	2.056	1.998	1.979	0.136	0.134	0.133	0.143	0.146	0.17
10	1.976	1.988	1.956	1.914	1.914	1.884	0.207	0.163	0.182	0.216	0.206	0.242
100	1.92	1.913	1.974	1.893	1.906	1.893	0.201	0.158	0.183	0.185	0.208	0.227
1000	1.967	1.974	1.964	1.955	1.896	1.886	0.132	0.123	0.142	0.138	0.14	0.164
10000	2.042	2.038	2.021	1.994	2.002	1.955	0.093	0.092	0.092	0.092	0.093	0.088
Total In Cell	0.838	0.896	0.9	0.845	0.861	0.805	0.876	0.918	0.954	0.887	0.926	0.832
0	1.693	1.807	1.804	1.812	1.809	1.833	0.091	0.091	0.088	0.085	0.087	0.078
0.1	1.744	1.779	1.801	1.744	1.772	1.789	0.091	0.096	0.096	0.09	0.091	0.085
1	1.707	1.793	1.803	1.774	1.793	1.783	0.111	0.124	0.121	0.12	0.123	0.112
10	1.721	1.719	1.737	1.752	1.796	1.747	0.094	0.086	0.092	0.091	0.087	0.08
100	1.695	1.762	1.772	1.774	1.796	1.801	0.097	0.095	0.093	0.094	0.092	0.087
1000	1.677	1.636	1.713	1.737	1.695	1.728	0.105	0.105	0.107	0.119	0.116	0.126
10000	1.739	1.746	1.777	1.795	1.756	1.786	0.093	0.091	0.092	0.094	0.091	0.085
Total In Cell	0.753	0.826	0.842	0.808	0.814	0.781	0.748	0.774	0.804	0.715	0.603	0.791
0	1.631	1.553	1.46	1.58	1.674	1.838	0.086	0.094	0.084	0.085	0.084	0.086
0.1	1.485	1.651	1.623	1.738	1.74	1.848	0.086	0.089	0.088	0.088	0.089	0.089
1	1.609	1.57	1.609	1.736	1.772	1.774	0.104	0.118	0.106	0.108	0.113	0.124
10	1.557	1.654	1.725	1.843	1.727	1.756	0.083	0.081	0.082	0.086	0.086	0.084
100	1.578	1.673	1.68	1.626	1.645	1.657	0.145	0.137	0.149	0.155	0.172	0.189
1000	1.654	1.595	1.603	1.603	1.625	1.629	0.137	0.129	0.137	0.141	0.152	0.159
10000	1.684	1.696	1.605	1.698	1.716	1.718	0.091	0.093	0.094	0.092	0.092	0.089
Total In Cell	0.816	0.707	0.857	0.844	0.748	0.865	0.746	0.639	0.695	0.694	0.733	0.805
	x2 because (I used 50 µl instead of the 100) + Supernatant Value To Find Original Total In Cell						% of Total In Cell					
0	2.193	2.256	2.241	2.228	2.238	2.184	8.208	9.043	8.568	8.438	8.490	8.425
0.1	2.268	2.28	2.197	2.252	2.232	2.23	7.760	8.246	8.375	8.171	8.333	8.700
1	2.332	2.295	2.257	2.342	2.29	2.319	11.664	11.678	11.786	12.212	12.751	14.661
10	2.39	2.314	2.32	2.346	2.326	2.368	17.322	14.088	15.690	18.414	17.713	20.439
100	2.322	2.229	2.34	2.263	2.322	2.347	17.313	14.177	15.641	16.350	17.916	19.344
1000	2.231	2.22	2.248	2.231	2.176	2.214	11.833	11.081	12.633	12.371	12.868	14.815
10000	2.228	2.222	2.205	2.178	2.188	2.131	8.348	8.281	8.345	8.448	8.501	8.259
Total In Cell	2.59	2.732	2.808	2.619	2.713	2.469	67.645	67.204	67.949	67.736	68.264	67.396
0	1.875	1.989	1.98	1.982	1.983	1.989	9.707	9.150	8.889	8.577	8.775	7.843
0.1	1.926	1.971	1.993	1.924	1.954	1.959	9.450	9.741	9.634	9.356	9.314	8.678
1	1.929	2.041	2.045	2.014	2.039	2.007	11.509	12.151	11.834	11.917	12.065	11.161
10	1.909	1.891	1.921	1.934	1.97	1.907	9.848	9.096	9.578	9.411	8.832	8.390
100	1.889	1.952	1.958	1.962	1.98	1.975	10.270	9.734	9.499	9.582	9.293	8.810
1000	1.887	1.846	1.927	1.975	1.927	1.98	11.129	11.376	11.105	12.051	12.039	12.727
10000	1.925	1.928	1.961	1.983	1.938	1.956	9.662	9.440	9.383	9.481	9.391	8.691
Total In Cell	2.249	2.374	2.45	2.238	2.02	2.363	66.518	65.206	65.633	63.896	59.703	66.949
0	1.803	1.741	1.628	1.75	1.842	2.01	9.540	10.798	10.319	9.714	9.121	8.557
0.1	1.657	1.829	1.799	1.914	1.918	2.026	10.380	9.732	9.783	9.195	9.281	8.786
1	1.817	1.806	1.821	1.952	1.998	2.022	11.447	13.068	11.642	11.066	11.311	12.265
10	1.723	1.816	1.889	2.015	1.899	1.924	9.634	8.921	8.682	8.536	9.057	8.732
100	1.868	1.947	1.978	1.936	1.989	2.035	15.525	14.073	15.066	16.012	17.295	18.575
1000	1.928	1.853	1.877	1.885	1.929	1.947	14.212	13.923	14.598	14.960	15.759	16.333
10000	1.866	1.882	1.793	1.882	1.9	1.896	9.753	9.883	10.485	9.777	9.684	9.388
Total In Cell	2.308	1.985	2.247	2.232	2.214	2.475	64.645	64.383	61.860	62.186	66.215	65.051

	Average	Standard Deviation
0	9.009	0.76
0.1	9.051	0.70
1	12.010	0.84
10	11.799	4.18
100	14.137	3.62
1000	13.101	1.68
10000	9.178	0.68

	RBL-pEE6 Expressing Dog α Chain Cells Bound With Mouse IgE For 0.5 Hours											
NIP Concentration (ng ml^{-1})	1	2	3	4	5	6	7	8	9	10	11	12
0	1.907	1.785	1.747	1.838	1.883	1.857	0.092	0.089	0.088	0.091	0.091	0.087
0.1	1.893	1.787	1.73	1.863	1.872	1.87	0.09	0.091	0.09	0.095	0.093	0.088
1	1.913	1.762	1.86	1.92	1.964	2.027	0.109	0.123	0.117	0.12	0.123	0.126
10	1.848	1.736	1.753	1.777	1.884	1.924	0.134	0.131	0.135	0.124	0.135	0.135
100	1.775	1.714	1.671	1.717	1.833	1.783	0.211	0.242	0.23	0.254	0.261	0.256
1000	1.774	1.742	1.735	1.844	1.834	1.8	0.244	0.262	0.209	0.235	0.278	0.268
10000	1.804	1.798	1.79	1.849	1.766	1.722	0.209	0.205	0.18	0.196	0.198	0.209
Total In Cell	0.683	0.657	0.751	0.788	0.754	0.733	0.833	0.83	0.837	0.783	0.809	0.76
0	1.679	1.733	1.74	1.805	1.754	1.747	0.096	0.094	0.1	0.094	0.089	0.091
0.1	1.746	1.822	1.769	1.833	1.807	1.728	0.091	0.094	0.097	0.093	0.097	0.097
1	1.681	1.796	1.758	1.728	1.714	1.651	0.106	0.117	0.117	0.115	0.12	0.114
10	1.621	1.788	1.777	1.724	1.716	1.688	0.096	0.083	0.089	0.09	0.082	0.084
100	1.706	1.776	1.756	1.739	1.761	1.688	0.095	0.094	0.095	0.099	0.1	0.09
1000	1.605	1.616	1.668	1.687	1.685	1.632	0.166	0.143	0.14	0.16	0.182	0.176
10000	1.645	1.706	1.739	1.726	1.739	1.635	0.164	0.151	0.162	0.16	0.177	0.176
Total In Cell	0.697	0.763	0.759	0.83	0.871	0.6	0.784	0.78	0.814	0.886	0.858	0.8
0	1.843	1.921	1.873	1.932	1.896	1.812	0.084	0.084	0.084	0.082	0.084	0.092
0.1	1.834	1.837	1.823	1.823	1.795	1.733	0.09	0.099	0.094	0.087	0.096	0.097
1	1.831	1.872	1.805	1.825	1.783	1.723	0.107	0.124	0.115	0.109	0.119	0.12
10	1.797	1.778	1.772	1.796	1.765	1.696	0.087	0.084	0.084	0.083	0.086	0.088
100	1.77	1.722	1.712	1.697	1.703	1.667	0.141	0.118	0.117	0.116	0.13	0.134
1000	1.683	1.62	1.641	1.649	1.662	1.588	0.199	0.18	0.175	0.172	0.199	0.236
10000	1.773	1.664	1.739	1.694	1.745	1.694	0.173	0.154	0.157	0.149	0.156	0.189
Total In Cell	0.792	0.814	0.821	0.762	0.836	0.605	0.801	0.735	0.737	0.668	0.641	0.941
	x2 because (I used 50 µl instead of the 100) + Supernatant Value To Find Original Total In Cell						% of Total In Cell					
0	2.091	1.963	1.923	2.02	2.065	2.031	8.800	9.068	9.152	9.010	8.814	8.567
0.1	2.073	1.969	1.91	2.053	2.058	2.046	8.683	9.243	9.424	9.255	9.038	8.602
1	2.131	2.008	2.094	2.16	2.21	2.279	10.230	12.251	11.175	11.111	11.131	11.057
10	2.116	1.998	2.023	2.025	2.154	2.194	12.665	13.113	13.347	12.247	12.535	12.306
100	2.197	2.198	2.131	2.225	2.355	2.295	19.208	22.020	21.586	22.831	22.166	22.309
1000	2.262	2.266	2.153	2.314	2.39	2.336	21.574	23.124	19.415	20.311	23.264	22.945
10000	2.222	2.208	2.15	2.241	2.162	2.14	18.812	18.569	16.744	17.492	18.316	19.533
Total In Cell	2.349	2.317	2.425	2.354	2.372	2.253	70.924	71.644	69.031	66.525	68.212	67.466
0	1.871	1.921	1.94	1.993	1.932	1.929	10.262	9.787	10.309	9.433	9.213	9.435
0.1	1.928	2.01	1.963	2.019	2.001	1.922	9.440	9.353	9.883	9.212	9.695	10.094
1	1.893	2.03	1.992	1.958	1.954	1.879	11.199	11.527	11.747	11.747	12.282	12.134
10	1.813	1.954	1.955	1.904	1.88	1.856	10.590	8.495	9.105	9.454	8.723	9.052
100	1.896	1.964	1.946	1.937	1.961	1.868	10.021	9.572	9.764	10.222	10.199	9.636
1000	1.937	1.902	1.948	2.007	2.049	1.984	17.140	15.037	14.374	15.944	17.765	17.742
10000	1.973	2.008	2.063	2.046	2.093	1.987	16.624	15.040	15.705	15.640	16.914	17.715
Total In Cell	2.265	2.323	2.387	2.602	2.587	2.2	69.227	67.155	68.203	68.101	66.332	72.727
0	2.011	2.089	2.041	2.096	2.064	1.996	8.354	8.042	8.231	7.824	8.140	9.218
0.1	2.014	2.035	2.011	1.997	1.987	1.927	8.937	9.730	9.349	8.713	9.663	10.067
1	2.045	2.12	2.035	2.043	2.021	1.963	10.465	11.698	11.302	10.671	11.776	12.226
10	1.971	1.946	1.94	1.962	1.937	1.872	8.828	8.633	8.660	8.461	8.880	9.402
100	2.052	1.958	1.946	1.929	1.963	1.935	13.743	12.053	12.025	12.027	13.245	13.850
1000	2.081	1.98	1.991	1.993	2.06	2.06	19.125	18.182	17.579	17.260	19.320	22.913
10000	2.119	1.972	2.053	1.992	2.057	2.072	16.328	15.619	15.295	14.960	15.168	18.243
Total In Cell	2.394	2.284	2.295	2.098	2.118	2.487	66.917	64.361	64.227	63.680	60.529	75.674
	Average	Standard Deviation										
0	8.981	0.72										
0.1	9.355	0.45										
1	11.429	0.61										
10	10.250	1.86										
100	14.804	5.23										
1000	19.056	2.80										
10000	16.818	1.45										

	RBL-pEE6 Expressing Dog α Chain Cells Bound With Horse IgE For 1 Hours											
NIP Concentration (ng ml^{-1})	1	2	3	4	5	6	7	8	9	10	11	12
0	1.932	2	1.987	2.027	2	2.018	0.102	0.104	0.1	0.099	0.098	0.099
0.1	2.076	2.114	2.097	2.081	2.092	2.092	0.098	0.097	0.098	0.092	0.091	0.088
1	2.056	2.056	2.071	2.08	2.051	2.066	0.121	0.123	0.118	0.12	0.117	0.128
10	1.937	2.006	1.999	1.998	1.991	1.956	0.176	0.145	0.158	0.182	0.179	0.206
100	1.975	2.041	2.022	1.995	2.002	1.951	0.201	0.15	0.173	0.189	0.195	0.206
1000	1.955	1.987	2.009	1.939	1.974	1.93	0.135	0.121	0.118	0.13	0.136	0.151
10000	1.994	2.018	2.026	1.963	2.056	1.991	0.096	0.096	0.093	0.108	0.092	0.092
Total In Cell	0.885	0.885	0.942	1.028	1.016	0.863	0.964	1.036	1.087	1.031	0.954	1.009
0	1.712	1.839	1.818	1.868	1.818	1.79	0.097	0.086	0.086	0.086	0.088	0.086
0.1	1.959	1.86	1.827	1.883	1.904	1.807	0.091	0.09	0.09	0.087	0.091	0.093
1	1.932	1.887	1.887	1.863	1.833	1.798	0.107	0.113	0.11	0.107	0.117	0.122
10	1.816	1.809	1.809	1.832	1.814	1.76	0.089	0.082	0.087	0.089	0.093	0.079
100	1.666	1.747	1.715	1.738	1.728	1.709	0.173	0.136	0.142	0.164	0.186	0.162
1000	1.662	1.711	1.702	1.783	1.746	1.7	0.164	0.137	0.134	0.141	0.164	0.16
10000	1.703	1.795	1.731	1.79	1.812	1.8	0.099	0.094	0.091	0.095	0.092	0.086
Total In Cell	0.807	0.88	0.964	0.933	0.978	0.919	0.718	0.807	0.693	0.775	0.729	0.713
0	1.793	1.854	1.818	1.781	1.854	1.783	0.093	0.098	0.088	0.096	0.086	0.099
0.1	1.839	1.86	1.84	1.821	1.86	1.805	0.089	0.097	0.098	0.091	0.093	0.095
1	1.771	1.783	1.746	1.842	1.853	1.75	0.109	0.127	0.121	0.116	0.125	0.133
10	1.874	1.879	1.849	1.865	1.862	1.798	0.085	0.081	0.085	0.085	0.081	0.09
100	1.881	1.863	1.843	1.81	1.857	1.81	0.096	0.091	0.089	0.089	0.09	0.096
1000	1.801	1.794	1.777	1.803	1.803	1.745	0.112	0.113	0.114	0.111	0.127	0.124
10000	1.807	1.841	1.776	1.783	1.788	1.742	0.097	0.097	0.099	0.092	0.094	0.1
Total In Cell	0.743	0.8	0.822	0.824	0.824	0.716	0.846	0.782	0.789	0.673	0.707	0.79
	x2 because (I used 50 µl instead of the 100) + Supernatant Value To Find Original Total In Cell						% of Total In Cell					
0	2.136	2.208	2.187	2.225	2.196	2.216	9.551	9.420	9.145	8.899	8.925	8.935
0.1	2.272	2.308	2.293	2.265	2.274	2.268	8.627	8.406	8.548	8.124	8.004	7.760
1	2.298	2.302	2.307	2.32	2.285	2.322	10.531	10.686	10.230	10.345	10.241	11.025
10	2.289	2.296	2.315	2.362	2.349	2.368	15.378	12.631	13.650	15.411	15.241	17.399
100	2.377	2.341	2.368	2.373	2.392	2.363	16.912	12.815	14.611	15.929	16.304	17.435
1000	2.225	2.229	2.245	2.199	2.246	2.232	12.135	10.857	10.512	11.824	12.110	13.530
10000	2.186	2.21	2.212	2.179	2.24	2.175	8.783	8.688	8.409	9.913	8.214	8.460
Total In Cell	2.813	2.957	3.116	3.09	2.924	2.881	68.539	70.071	69.769	66.731	65.253	70.045
0	1.906	2.011	1.99	2.04	1.994	1.962	10.178	8.553	8.643	8.431	8.826	8.767
0.1	2.141	2.04	2.007	2.057	2.086	1.993	8.501	8.824	8.969	8.459	8.725	9.333
1	2.146	2.113	2.107	2.077	2.067	2.042	9.972	10.696	10.441	10.303	11.321	11.949
10	1.994	1.973	1.983	2.01	2	1.918	8.927	8.312	8.775	8.856	9.300	8.238
100	2.012	2.019	1.999	2.066	2.1	2.033	17.197	13.472	14.207	15.876	17.714	15.937
1000	1.99	1.985	1.97	2.065	2.074	2.02	16.482	13.804	13.604	13.656	15.815	15.842
10000	1.901	1.983	1.913	1.98	1.996	1.972	10.416	9.481	9.514	9.596	9.218	8.722
Total In Cell	2.243	2.494	2.35	2.483	2.436	2.345	64.021	64.715	58.979	62.424	59.852	60.810
0	1.979	2.05	1.994	1.973	2.026	1.981	9.399	9.561	8.826	9.731	8.490	9.995
0.1	2.017	2.054	2.036	2.003	2.046	1.995	8.825	9.445	9.627	9.086	9.091	9.524
1	1.989	2.037	1.988	2.074	2.103	2.016	10.960	12.469	12.173	11.186	11.888	13.194
10	2.044	2.041	2.019	2.035	2.024	1.978	8.317	7.937	8.420	8.354	8.004	9.100
100	2.073	2.045	2.021	1.988	2.037	2.002	9.262	8.900	8.808	8.954	8.837	9.590
1000	2.025	2.02	2.005	2.025	2.057	1.993	11.062	11.188	11.372	10.963	12.348	12.444
10000	2.001	2.035	1.974	1.967	1.976	1.942	9.695	9.533	10.030	9.354	9.514	10.299
Total In Cell	2.435	2.364	2.4	2.17	2.238	2.296	69.487	66.159	65.750	62.028	63.181	68.815

	Average	Standard Deviation
0	9.126	0.52
0.1	8.771	0.53
1	11.089	0.90
10	10.680	3.25
100	13.487	3.47
1000	12.753	1.83
10000	9.324	0.65

	RBL-pEE6 Expressing Dog α Chain Cells Bound With Mouse IgE For 1 Hours											
NIP Concentration (ng ml^{-1})	1	2	3	4	5	6	7	8	9	10	11	12
0	1.907	1.947	1.971	1.975	1.91	1.975	0.091	0.091	0.092	0.085	0.088	0.098
0.1	2.051	1.959	2.022	2.041	1.955	1.983	0.09	0.093	0.097	0.085	0.095	0.1
1	2.07	2.018	2.013	2.06	1.979	1.902	0.11	0.122	0.113	0.116	0.12	0.134
10	1.93	1.887	1.921	1.818	1.939	1.949	0.155	0.131	0.138	0.132	0.136	0.15
100	1.724	1.732	1.739	1.726	1.744	1.662	0.352	0.286	0.315	0.33	0.324	0.349
1000	1.678	1.674	1.775	1.771	1.69	1.665	0.362	0.298	0.312	0.328	0.293	0.385
10000	1.658	1.684	1.759	1.785	1.687	1.697	0.333	0.298	0.304	0.366	0.359	0.352
Total In Cell	0.662	0.738	0.668	0.722	0.694	0.585	1.083	0.975	0.751	1.088	0.993	0.758
0	1.404	1.344	1.369	1.618	1.657	1.762	0.066	0.093	0.091	0.091	0.09	0.081
0.1	1.373	1.558	1.645	1.643	1.716	1.886	0.091	0.093	0.094	0.093	0.094	0.09
1	1.495	1.517	1.623	1.784	1.766	1.834	0.106	0.114	0.115	0.112	0.119	0.109
10	1.612	1.556	1.613	1.752	1.67	1.792	0.086	0.084	0.089	0.085	0.084	0.079
100	1.617	1.506	1.525	1.591	1.739	1.72	0.096	0.094	0.097	0.092	0.096	0.089
1000	1.323	1.412	1.445	1.6	1.676	1.579	0.191	0.169	0.195	0.248	0.276	0.293
10000	1.28	1.384	1.369	1.526	1.542	1.477	0.251	0.224	0.236	0.275	0.29	0.328
Total In Cell	0.502	0.601	0.619	0.791	0.793	0.637	0.67	0.682	0.776	0.838	0.845	0.68
0	1.959	1.983	1.967	1.963	1.932	1.987	0.095	0.09	0.087	0.089	0.085	0.082
0.1	1.979	1.942	1.893	1.889	1.883	1.917	0.092	0.089	0.098	0.097	0.097	0.091
1	1.931	1.893	1.896	1.902	1.864	1.896	0.112	0.125	0.121	0.123	0.129	0.116
10	1.951	1.919	1.891	1.863	1.863	1.88	0.089	0.087	0.093	0.092	0.089	0.086
100	1.875	1.881	1.851	1.833	1.848	1.842	0.138	0.128	0.13	0.137	0.134	0.151
1000	1.742	1.711	1.687	1.657	1.652	1.66	0.29	0.272	0.279	0.343	0.387	0.458
10000	1.753	1.733	1.725	1.676	1.686	1.691	0.288	0.26	0.254	0.257	0.299	0.33
Total In Cell	0.827	0.912	0.915	0.887	0.927	0.932	0.787	0.742	0.77	0.819	0.779	0.696
	x2 because (I used 50 µl instead of the 100) + Supernatant Value To Find Original Total In Cell						% of Total In Cell					
0	2.089	2.129	2.155	2.145	2.086	2.171	8.712	8.549	8.538	7.925	8.437	9.028
0.1	2.231	2.145	2.216	2.211	2.145	2.183	8.068	8.671	8.755	7.689	8.858	9.162
1	2.29	2.262	2.239	2.292	2.219	2.17	9.607	10.787	10.094	10.122	10.816	12.350
10	2.24	2.149	2.197	2.082	2.211	2.249	13.839	12.192	12.563	12.680	12.302	13.339
100	2.428	2.304	2.369	2.386	2.392	2.36	28.995	24.826	26.593	27.661	27.090	29.576
1000	2.402	2.27	2.399	2.427	2.276	2.435	30.142	26.256	26.011	27.029	25.747	31.622
10000	2.324	2.28	2.367	2.517	2.405	2.401	28.657	26.140	25.687	29.082	29.854	29.321
Total In Cell	2.828	2.688	2.17	2.898	2.68	2.101	76.591	72.545	69.217	75.086	74.104	72.156
0	1.536	1.53	1.551	1.8	1.837	1.924	8.594	12.157	11.734	10.111	9.799	8.420
0.1	1.555	1.744	1.833	1.829	1.904	2.066	11.704	10.665	10.256	10.169	9.874	8.712
1	1.707	1.745	1.853	2.008	2.004	2.052	12.419	13.066	12.412	11.155	11.876	10.624
10	1.784	1.724	1.791	1.922	1.838	1.95	9.641	9.745	9.939	8.845	9.140	8.103
100	1.809	1.694	1.719	1.775	1.931	1.898	10.614	11.098	11.286	10.366	9.943	9.378
1000	1.705	1.75	1.835	2.096	2.228	2.165	22.405	19.314	21.253	23.664	24.776	27.067
10000	1.782	1.832	1.841	2.076	2.122	2.133	28.171	24.454	25.638	26.493	27.333	30.755
Total In Cell	1.842	1.965	2.171	2.467	2.483	1.997	72.747	69.415	71.488	67.937	68.063	68.102
0	2.149	2.163	2.141	2.141	2.102	2.151	8.841	8.322	8.127	8.314	8.088	7.624
0.1	2.163	2.12	2.089	2.083	2.077	2.099	8.507	8.396	9.382	9.313	9.340	8.671
1	2.155	2.143	2.138	2.148	2.122	2.128	10.394	11.666	11.319	11.453	12.158	10.902
10	2.129	2.093	2.077	2.047	2.041	2.052	8.361	8.313	8.955	8.989	8.721	8.382
100	2.151	2.137	2.111	2.107	2.116	2.144	12.831	11.979	12.316	13.004	12.665	14.086
1000	2.322	2.255	2.245	2.343	2.426	2.576	24.978	24.124	24.855	29.279	31.904	35.559
10000	2.329	2.253	2.233	2.19	2.284	2.351	24.732	23.080	22.750	23.470	26.182	28.073
Total In Cell	2.401	2.396	2.455	2.525	2.485	2.324	65.556	61.937	62.729	64.871	62.696	59.897
	Average	Standard Deviation										
0	8.962	1.24										
0.1	9.233	0.99										
1	11.290	0.96										
10	10.225	1.98										
100	16.906	7.82										
1000	26.444	4.04										
10000	26.660	2.40										

	RBL-pEE6 Expressing Dog α Chain Cells Bound With Horse IgE For 3 Hours											
NIP Concentration (ng ml^{-1})	1	2	3	4	5	6	7	8	9	10	11	12
0	2.167	2.187	2.174	2.167	2.162	2.097	0.095	0.104	0.099	0.093	0.094	0.089
0.1	2.229	2.201	2.155	2.208	2.187	2.148	0.096	0.096	0.098	0.099	0.1	0.099
1	2.186	2.149	2.161	2.151	2.13	2.125	0.109	0.12	0.122	0.12	0.126	0.13
10	2.157	2.164	2.137	2.127	2.159	2.127	0.096	0.095	0.11	0.109	0.117	0.13
100	2.056	2.075	2.041	2.029	2.033	2.02	0.201	0.175	0.25	0.263	0.288	0.386
1000	2.061	2.054	2.046	2.031	2.023	1.943	0.167	0.148	0.216	0.209	0.227	0.339
10000	2.149	2.155	2.128	2.112	2.102	2.092	0.112	0.102	0.102	0.103	0.099	0.099
Total In Cell	1.142	1.161	1.228	1.104	1.091	1.008	0.774	0.838	0.898	0.852	0.891	0.927
0	1.975	1.928	1.909	1.866	1.922	1.951	0.093	0.095	0.095	0.098	0.095	0.091
0.1	2.002	1.898	1.867	1.846	1.924	1.941	0.098	0.098	0.098	0.094	0.101	0.094
1	1.985	1.893	1.884	1.804	1.939	1.909	0.114	0.128	0.114	0.121	0.122	0.123
10	2.008	1.907	1.834	1.805	1.892	1.898	0.094	0.09	0.095	0.092	0.089	0.092
100	1.941	1.882	1.832	1.835	1.841	1.85	0.1	0.096	0.1	0.097	0.097	0.099
1000	1.854	1.798	1.762	1.733	1.819	1.845	0.17	0.136	0.143	0.162	0.175	0.189
10000	1.943	1.894	1.865	1.85	1.883	1.837	0.109	0.107	0.104	0.104	0.104	0.099
Total In Cell	0.691	0.774	0.744	0.72	0.886	0.816	0.982	0.974	0.84	0.883	0.812	0.825
0	1.939	1.983	2.009	2.022	2.022	1.959	0.1	0.099	0.097	0.099	0.096	0.102
0.1	1.996	1.945	1.971	1.983	1.975	1.963	0.099	0.106	0.104	0.101	0.102	0.106
1	1.978	1.967	1.952	2.009	1.996	1.959	0.117	0.131	0.127	0.141	0.158	0.139
10	1.994	1.976	1.969	1.982	1.968	1.994	0.093	0.093	0.099	0.119	0.104	0.124
100	1.979	1.991	1.971	1.959	1.975	1.928	0.099	0.105	0.103	0.102	0.098	0.1
1000	1.947	1.947	1.928	1.947	1.953	1.914	0.104	0.107	0.107	0.111	0.106	0.121
10000	1.93	1.986	1.964	1.958	1.997	1.975	0.099	0.105	0.102	0.099	0.099	0.097
Total In Cell	1.061	1.05	1.036	0.987	1.06	0.879	0.583	0.807	0.715	0.728	0.785	0.757
	x2 because (I used 50 µl instead of the 100) + Supernatant Value To Find Original Total In Cell						% of Total In Cell					
0	2.357	2.395	2.372	2.353	2.35	2.275	8.061	8.685	8.347	7.905	8.000	7.824
0.1	2.421	2.393	2.351	2.406	2.387	2.346	7.931	8.023	8.337	8.229	8.379	8.440
1	2.404	2.389	2.405	2.391	2.382	2.385	9.068	10.046	10.146	10.038	10.579	10.901
10	2.349	2.354	2.357	2.345	2.393	2.387	8.174	8.071	9.334	9.296	9.779	10.892
100	2.458	2.425	2.541	2.555	2.609	2.792	16.355	14.433	19.677	20.587	22.077	27.650
1000	2.395	2.35	2.478	2.449	2.477	2.621	13.946	12.596	17.433	17.068	18.329	25.868
10000	2.373	2.359	2.332	2.318	2.3	2.29	9.440	8.648	8.748	8.887	8.609	8.646
Total In Cell	2.69	2.837	3.024	2.808	2.873	2.862	57.546	59.076	59.392	60.684	62.026	64.780
0	2.161	2.118	2.099	2.062	2.112	2.133	8.607	8.971	9.052	9.505	8.996	8.533
0.1	2.198	2.094	2.063	2.034	2.126	2.129	8.917	9.360	9.501	9.243	9.501	8.830
1	2.213	2.149	2.112	2.046	2.183	2.155	10.303	11.913	10.795	11.828	11.177	11.415
10	2.196	2.087	2.024	1.989	2.07	2.082	8.561	8.625	9.387	9.251	8.599	8.838
100	2.141	2.074	2.032	2.029	2.035	2.048	9.341	9.257	9.843	9.561	9.533	9.668
1000	2.194	2.07	2.048	2.057	2.169	2.223	15.497	13.140	13.965	15.751	16.136	17.004
10000	2.161	2.108	2.073	2.058	2.091	2.035	10.088	10.152	10.034	10.107	9.947	9.730
Total In Cell	2.655	2.722	2.424	2.486	2.51	2.466	73.974	71.565	69.307	71.038	64.701	66.910
0	2.139	2.181	2.203	2.22	2.214	2.163	9.350	9.078	8.806	8.919	8.672	9.431
0.1	2.194	2.157	2.179	2.185	2.179	2.175	9.025	9.828	9.546	9.245	9.362	9.747
1	2.212	2.229	2.206	2.291	2.312	2.237	10.579	11.754	11.514	12.309	13.668	12.427
10	2.18	2.162	2.167	2.22	2.176	2.242	8.532	8.603	9.137	10.721	9.559	11.062
100	2.177	2.201	2.177	2.163	2.171	2.128	9.095	9.541	9.463	9.431	9.028	9.398
1000	2.155	2.161	2.142	2.169	2.165	2.156	9.652	9.903	9.991	10.235	9.792	11.224
10000	2.128	2.196	2.168	2.156	2.195	2.169	9.305	9.563	9.410	9.184	9.021	8.944
Total In Cell	2.227	2.664	2.466	2.443	2.63	2.393	52.357	60.586	57.989	59.599	59.696	63.268

	Average	Standard Deviation
0	8.708	0.52
0.1	8.969	0.61
1	11.137	1.09
10	9.246	0.89
100	12.997	5.77
1000	14.307	4.14
10000	9.359	0.55

	RBL-pEE6 Expressing Dog α Chain Cells Bound With Mouse IgE For 3 Hours											
NIP Concentration (ng ml^{-1})	1	2	3	4	5	6	7	8	9	10	11	12
0	2.095	2.237	2.161	2.143	2.187	2.155	0.09	0.091	0.097	0.095	0.087	0.085
0.1	2.194	2.208	2.174	2.135	2.187	2.18	0.095	0.095	0.098	0.095	0.1	0.094
1	2.155	2.215	2.174	2.186	2.139	2.134	0.111	0.125	0.123	0.122	0.128	0.127
10	2.141	2.179	2.117	2.032	2.137	2.14	0.104	0.094	0.105	0.101	0.106	0.103
100	1.939	2.022	1.986	1.928	1.934	1.949	0.302	0.21	0.242	0.278	0.333	0.371
1000	1.695	1.735	1.757	1.699	1.711	1.638	0.476	0.34	0.354	0.512	0.582	0.693
10000	1.738	1.772	1.75	1.701	1.731	1.701	0.466	0.379	0.339	0.406	0.571	0.672
Total In Cell	0.895	0.865	1.094	1.02	1.069	1.067	0.91	1.09	0.979	0.951	1.004	0.967
0	1.74	1.947	1.951	2.029	2.004	1.983	0.087	0.085	0.09	0.086	0.084	0.089
0.1	1.959	1.928	1.974	1.975	1.924	1.891	0.091	0.091	0.096	0.092	0.096	0.1
1	2.01	1.936	1.913	1.967	1.896	1.806	0.107	0.115	0.113	0.115	0.113	0.114
10	1.822	1.751	1.891	1.961	1.842	1.853	0.083	0.082	0.087	0.087	0.09	0.091
100	1.811	1.876	1.861	1.83	1.855	1.822	0.115	0.087	0.09	0.085	0.093	0.091
1000	1.755	1.659	1.697	1.706	1.777	1.781	0.19	0.155	0.16	0.188	0.204	0.186
10000	1.462	1.563	1.622	1.515	1.468	1.434	0.382	0.364	0.395	0.38	0.399	0.445
Total In Cell	0.813	0.829	0.855	0.92	0.867	0.739	0.721	0.916	0.794	0.78	0.823	0.831
0	2.092	2.066	1.979	2.004	2.086	2.076	0.09	0.092	0.089	0.09	0.088	0.087
0.1	2.068	2.003	1.975	2.005	2.036	2.022	0.091	0.099	0.097	0.098	0.098	0.098
1	2.076	1.945	1.959	1.928	2.035	2.009	0.11	0.127	0.118	0.119	0.123	0.123
10	2.001	1.951	1.915	1.941	1.993	1.985	0.093	0.089	0.093	0.1	0.095	0.088
100	1.987	1.958	1.947	1.93	1.988	1.967	0.108	0.1	0.098	0.104	0.11	0.101
1000	1.74	1.751	1.655	1.72	1.757	1.725	0.327	0.277	0.332	0.415	0.448	0.482
10000	1.757	1.729	1.693	1.705	1.717	1.705	0.339	0.282	0.35	0.408	0.457	0.512
Total In Cell	0.864	1.018	1.043	0.934	1.008	0.828	0.844	0.731	0.738	0.801	0.814	0.788
	x2 because (I used 50 µl instead of the 100) + Supernatant Value To Find Original Total In Cell						% of Total In Cell					
0	2.275	2.419	2.355	2.333	2.361	2.325	7.912	7.524	8.238	8.144	7.370	7.312
0.1	2.384	2.398	2.37	2.325	2.387	2.368	7.970	7.923	8.270	8.172	8.379	7.939
1	2.377	2.465	2.42	2.43	2.395	2.388	9.340	10.142	10.165	10.041	10.689	10.637
10	2.349	2.367	2.327	2.234	2.349	2.346	8.855	7.943	9.024	9.042	9.025	8.781
100	2.543	2.442	2.47	2.484	2.6	2.691	23.751	17.199	19.595	22.383	25.615	27.573
1000	2.647	2.415	2.465	2.723	2.875	3.024	35.965	28.157	28.722	37.606	40.487	45.833
10000	2.67	2.53	2.428	2.513	2.873	3.045	34.906	29.960	27.924	32.312	39.749	44.138
Total In Cell	2.715	3.045	3.052	2.922	3.077	3.001	67.035	71.593	64.155	65.092	65.258	64.445
0	1.914	2.117	2.131	2.201	2.172	2.161	9.091	8.030	8.447	7.815	7.735	8.237
0.1	2.141	2.11	2.166	2.159	2.116	2.091	8.501	8.626	8.864	8.522	9.074	9.565
1	2.224	2.166	2.139	2.197	2.122	2.034	9.622	10.619	10.566	10.469	10.650	11.209
10	1.988	1.915	2.065	2.135	2.022	2.035	8.350	8.564	8.426	8.150	8.902	8.943
100	2.041	2.05	2.041	2	2.041	2.004	11.269	8.488	8.819	8.500	9.113	9.082
1000	2.135	1.969	2.017	2.082	2.185	2.153	17.799	15.744	15.865	18.060	18.673	17.278
10000	2.226	2.291	2.412	2.275	2.266	2.324	34.322	31.777	32.753	33.407	35.216	38.296
Total In Cell	2.255	2.661	2.443	2.48	2.513	2.401	63.947	68.846	65.002	62.903	65.499	69.221
0	2.272	2.25	2.157	2.184	2.262	2.25	7.923	8.178	8.252	8.242	7.781	7.733
0.1	2.25	2.201	2.169	2.201	2.232	2.218	8.089	8.996	8.944	8.905	8.781	8.837
1	2.296	2.199	2.195	2.166	2.281	2.255	9.582	11.551	10.752	10.988	10.785	10.909
10	2.187	2.129	2.101	2.141	2.183	2.161	8.505	8.361	8.853	9.341	8.704	8.144
100	2.203	2.158	2.143	2.138	2.208	2.169	9.805	9.268	9.146	9.729	9.964	9.313
1000	2.394	2.305	2.319	2.55	2.653	2.689	27.318	24.035	28.633	32.549	33.773	35.850
10000	2.435	2.293	2.393	2.521	2.631	2.729	27.844	24.597	29.252	32.368	34.740	37.523
Total In Cell	2.552	2.48	2.519	2.536	2.636	2.404	66.144	58.952	58.595	63.170	61.760	65.557

	Average	Standard Deviation
0	7.998	0.42
0.1	8.575	0.46
1	10.484	0.58
10	8.662	0.38
100	13.812	6.81
1000	27.908	9.28
10000	33.394	4.71

	RBL-pEE6 Expressing Dog α Chain Cells Bound With Horse IgE For 16 Hours											
NIP Concentration (ng ml^{-1})	1	2	3	4	5	6	7	8	9	10	11	12
0	1.611	1.764	1.799	1.83	1.823	1.793	0.093	0.088	0.086	0.089	0.09	0.085
0.1	1.492	1.829	1.824	1.854	1.829	1.842	0.09	0.091	0.09	0.089	0.093	0.086
1	1.506	1.836	1.831	1.85	1.856	1.853	0.107	0.117	0.113	0.114	0.122	0.114
10	1.397	1.762	1.769	1.806	1.84	1.807	0.081	0.077	0.083	0.083	0.081	0.08
100	1.353	1.719	1.696	1.734	1.762	1.721	0.12	0.124	0.133	0.178	0.195	0.2
1000	1.261	1.668	1.712	1.752	1.793	1.657	0.104	0.115	0.112	0.134	0.145	0.152
10000	1.356	1.679	1.774	1.83	1.832	1.833	0.092	0.089	0.088	0.087	0.085	0.083
Total In Cell	0.626	0.954	0.921	0.961	1.009	0.943	0.568	0.606	0.682	0.791	0.837	0.778
0	1.234	1.433	1.505	1.564	1.529	1.499	0.095	0.092	0.088	0.091	0.087	0.085
0.1	1.345	1.53	1.578	1.561	1.565	1.527	0.094	0.098	0.093	0.091	0.095	0.089
1	1.357	1.445	1.566	1.552	1.552	1.501	0.111	0.122	0.119	0.12	0.122	0.116
10	1.499	1.472	1.546	1.532	1.564	1.533	0.092	0.087	0.09	0.091	0.087	0.087
100	1.135	1.363	1.461	1.447	1.448	1.433	0.114	0.109	0.135	0.133	0.133	0.138
1000	1.067	1.399	1.447	1.431	1.487	1.393	0.122	0.13	0.15	0.154	0.16	0.17
10000	1.29	1.424	1.534	1.413	1.534	1.529	0.097	0.097	0.101	0.106	0.096	0.093
Total In Cell	0.548	0.703	0.669	0.699	0.68	0.635	0.566	0.694	0.659	0.702	0.704	0.643
0	2.682	2.205	2.143	2.162	2.152	2.11	0.07	0.074	0.07	0.071	0.074	0.075
0.1	2.686	2.192	2.132	2.179	2.069	2.034	0.071	0.072	0.078	0.081	0.09	0.088
1	2.11	2.535	2.152	2.173	2.03	2.022	0.065	0.066	0.065	0.071	0.07	0.075
10	2.137	2.25	2.157	2.14	2.053	2.077	0.066	0.064	0.068	0.076	0.083	0.077
100	2.112	2.62	2.152	2.148	2.051	2.047	0.073	0.077	0.079	0.087	0.095	0.099
1000	2.24	2.355	2.226	2.266	2.13	2.186	0.07	0.072	0.075	0.078	0.084	0.088
10000	2.25	2.232	2.152	2.194	2.097	2.045	0.07	0.074	0.077	0.078	0.079	0.091
Total In Cell	0.973	1.016	0.894	0.918	0.834	1.043	0.539	0.996	1.171	1.164	1.134	1.249
	x2 because (I used 50 µl instead of the 100) + Supernatant Value To Find Original Total In Cell						% of Total In Cell					
0	1.797	1.94	1.971	2.008	2.003	1.963	10.351	9.072	8.727	8.865	8.987	8.660
0.1	1.672	2.011	2.004	2.032	2.015	2.014	10.766	9.050	8.982	8.760	9.231	8.540
1	1.72	2.07	2.057	2.078	2.1	2.081	12.442	11.304	10.987	10.972	11.619	10.956
10	1.559	1.916	1.935	1.972	2.002	1.967	10.391	8.038	8.579	8.418	8.092	8.134
100	1.593	1.967	1.962	2.09	2.152	2.121	15.066	12.608	13.558	17.033	18.123	18.859
1000	1.469	1.898	1.936	2.02	2.083	1.961	14.159	12.118	11.570	13.267	13.922	15.502
10000	1.54	1.857	1.95	2.004	2.002	1.999	11.948	9.585	9.026	8.683	8.492	8.304
Total In Cell	1.762	2.166	2.285	2.543	2.683	2.499	64.472	55.956	59.694	62.210	62.393	62.265
0	1.424	1.617	1.681	1.746	1.703	1.669	13.343	11.379	10.470	10.424	10.217	10.186
0.1	1.533	1.726	1.764	1.743	1.755	1.705	12.264	11.356	10.544	10.442	10.826	10.440
1	1.579	1.689	1.804	1.792	1.796	1.733	14.060	14.446	13.193	13.393	13.586	13.387
10	1.683	1.646	1.726	1.714	1.738	1.707	10.933	10.571	10.429	10.618	10.012	10.193
100	1.363	1.581	1.731	1.713	1.714	1.709	16.728	13.789	15.598	15.528	15.519	16.150
1000	1.311	1.659	1.747	1.739	1.807	1.733	18.612	15.672	17.172	17.711	17.709	19.619
10000	1.484	1.618	1.736	1.625	1.726	1.715	13.073	11.990	11.636	13.046	11.124	10.845
Total In Cell	1.68	2.091	1.987	2.103	2.088	1.921	67.381	66.380	66.331	66.762	67.433	66.944
0	2.822	2.353	2.283	2.304	2.3	2.26	4.961	6.290	6.132	6.163	6.435	6.637
0.1	2.828	2.336	2.288	2.341	2.249	2.21	5.021	6.164	6.818	6.920	8.004	7.964
1	2.24	2.667	2.282	2.315	2.17	2.172	5.804	4.949	5.697	6.134	6.452	6.906
10	2.269	2.378	2.293	2.292	2.219	2.231	5.818	5.383	5.931	6.632	7.481	6.903
100	2.258	2.774	2.31	2.322	2.241	2.245	6.466	5.552	6.840	7.494	8.478	8.820
1000	2.38	2.499	2.376	2.422	2.298	2.362	5.882	5.762	6.313	6.441	7.311	7.451
10000	2.39	2.38	2.306	2.35	2.255	2.227	5.858	6.218	6.678	6.638	7.007	8.172
Total In Cell	2.051	3.008	3.236	3.246	3.102	3.541	52.560	66.223	72.373	71.719	73.114	70.545
	Average	Standard Deviation										
0	8.739	2.23										
0.1	9.005	1.95										
1	10.349	3.36										
10	8.475	1.85										
100	12.900	4.40										
1000	12.566	4.89										
10000	9.351	2.40										

	RBL-pEE6 Expressing Dog α Chain Cells Bound With Mouse IgE For 16 Hours											
DNP Concentration (ng ml^{-1})	1	2	3	4	5	6	7	8	9	10	11	12
0	1.581	1.776	1.815	1.842	1.821	1.83	0.106	0.106	0.102	0.098	0.097	0.115
0.1	1.579	1.78	1.821	1.824	1.812	1.868	0.105	0.093	0.095	0.102	0.108	0.111
1	1.709	1.81	1.833	1.857	1.838	1.846	0.151	0.138	0.139	0.15	0.153	0.172
10	1.511	1.721	1.76	1.838	1.771	1.779	0.264	0.183	0.232	0.272	0.295	0.276
100	1.151	1.422	1.533	1.542	1.543	1.464	0.455	0.36	0.456	0.647	0.684	0.645
1000	1.192	1.281	1.429	1.415	1.331	1.368	0.518	0.418	0.541	0.626	0.7	0.686
10000	1.2	1.243	1.382	1.373	1.362	1.334	0.517	0.405	0.409	0.444	0.45	0.527
Total In Cell	0.566	0.621	0.707	0.69	0.697	0.757	0.888	0.895	0.831	0.94	0.938	0.8
	x2 because (I used 50 µl instead of the 100) + Supernatant Value To Find Original Total In Cell						% of Total In Cell					
0	1.793	1.988	2.019	2.038	2.015	2.06	11.824	10.664	10.104	9.617	9.628	11.165
0.1	1.789	1.966	2.011	2.028	2.028	2.09	11.738	9.461	9.448	10.059	10.651	10.622
1	2.011	2.086	2.111	2.157	2.144	2.19	15.017	13.231	13.169	13.908	14.272	15.708
10	2.039	2.087	2.224	2.382	2.361	2.331	25.895	17.537	20.863	22.838	24.989	23.681
100	2.061	2.142	2.445	2.836	2.911	2.754	44.153	33.613	37.301	45.628	46.994	46.841
1000	2.228	2.117	2.511	2.667	2.731	2.74	46.499	39.490	43.090	46.944	51.263	50.073
10000	2.234	2.053	2.2	2.261	2.262	2.388	46.285	39.454	37.182	39.275	39.788	44.137
Total In Cell	2.342	2.411	2.369	2.57	2.573	2.357	75.833	74.243	70.156	73.152	72.911	67.883
	Average	Standard Deviation										
0	10.500	0.89										
0.1	10.330	0.87										
1	14.218	1.00										
10	22.634	3.05										
100	42.422	5.61										
1000	46.227	4.38										
10000	41.020	3.44										

	RBL-pEE6 Expressing Dog α Chain Cells Bound With Dog IgE For 16 Hours											
NIP Concentration (ng ml^{-1})	1	2	3	4	5	6	7	8	9	10	11	12
0	2.565	2.893	2.495	1.992	2.228	1.839	0.068	0.073	0.072	0.073	0.068	0.074
0.1	2.471	2.602	2.565	2.068	1.893	1.731	0.115	0.098	0.092	0.094	0.11	0.118
1	1.726	1.749	1.698	1.67	1.671	1.531	0.395	0.338	0.381	0.464	0.524	0.472
10	1.881	1.461	1.463	1.399	1.307	1.254	0.587	0.447	0.591	0.771	0.875	0.765
100	1.502	1.509	1.434	1.35	1.353	1.185	0.693	0.488	0.61	0.822	0.952	0.657
1000	1.484	1.56	1.472	1.428	1.418	1.204	0.623	0.542	0.713	0.791	0.837	0.607
10000	1.982	1.479	1.393	1.369	1.4	1.219	0.642	0.52	0.707	0.786	0.896	0.706
Total In Cell	1.312	0.751	0.753	0.781	0.823	0.807	1.024	1.062	0.938	0.986	0.922	0.843
0	1.632	1.713	1.812	1.764	1.698	1.654	0.096	0.094	0.096	0.105	0.095	0.111
0.1	1.684	1.8	1.835	1.756	1.738	1.724	0.102	0.1	0.097	0.103	0.102	0.107
1	1.661	1.693	1.776	1.776	1.779	1.74	0.179	0.174	0.164	0.17	0.185	0.227
10	1.272	1.471	1.521	1.538	1.546	1.481	0.319	0.265	0.332	0.439	0.447	0.47
100	1.271	1.399	1.477	1.451	1.473	1.396	0.368	0.307	0.321	0.465	0.534	0.552
1000	1.183	1.43	1.506	1.444	1.543	1.426	0.32	0.313	0.337	0.344	0.475	0.531
10000	1.132	1.376	1.443	1.42	1.443	1.398	0.352	0.334	0.374	0.365	0.398	0.544
Total In Cell	0.548	0.748	0.838	0.78	0.896	0.799	0.651	0.75	0.919	0.875	0.867	0.858
	x2 because (I used 50 µl instead of the 100) + Supernatant Value To Find Original Total In Cell						% of Total In Cell					
0	2.701	3.039	2.639	2.138	2.364	1.987	5.035	4.804	5.457	6.829	5.753	7.448
0.1	2.701	2.798	2.749	2.256	2.113	1.967	8.515	7.005	6.693	8.333	10.412	11.998
1	2.516	2.425	2.46	2.598	2.719	2.475	31.399	27.876	30.976	35.720	38.544	38.141
10	3.055	2.355	2.645	2.941	3.057	2.784	38.429	37.962	44.688	52.431	57.246	54.957
100	2.888	2.485	2.654	2.994	3.257	2.499	47.992	39.276	45.968	54.910	58.459	52.581
1000	2.73	2.644	2.898	3.01	3.092	2.418	45.641	40.998	49.206	52.558	54.140	50.207
10000	3.266	2.519	2.807	2.941	3.192	2.631	39.314	41.286	50.374	53.451	56.140	53.668
Total In Cell	3.36	2.875	2.629	2.753	2.667	2.493	60.952	73.878	71.358	71.631	69.141	67.629
0	1.824	1.901	2.004	1.974	1.888	1.876	10.526	9.890	9.581	10.638	10.064	11.834
0.1	1.888	2	2.029	1.962	1.942	1.938	10.805	10.000	9.561	10.499	10.505	11.042
1	2.019	2.041	2.104	2.116	2.149	2.194	17.732	17.050	15.589	16.068	17.217	20.693
10	1.91	2.001	2.185	2.416	2.44	2.421	33.403	26.487	30.389	36.341	36.639	38.827
100	2.007	2.013	2.119	2.381	2.541	2.5	36.672	30.502	30.297	39.059	42.031	44.160
1000	1.823	2.056	2.18	2.132	2.493	2.488	35.107	30.447	30.917	32.270	38.107	42.685
10000	1.836	2.044	2.191	2.15	2.239	2.486	38.344	32.681	34.140	33.953	35.552	43.765
Total In Cell	1.85	2.248	2.676	2.53	2.63	2.515	70.378	66.726	68.685	69.170	65.932	68.231

	Average	Standard Deviation
0	8.155	2.53
0.1	9.614	1.64
1	25.584	9.12
10	40.650	9.74
100	43.492	9.01
1000	41.857	8.56
10000	42.722	8.58

Values for Figure 35:

These are the SPR values for the equine sFcεRIαD1&2 expression test.

Days	Expressing Yeast	Parental Yeast	Resonance	Samples
0	51.1	22.55	45.3	0
1	61.3	23.35	56.9	0
2	60.85	22.75	61.4	1
3	54.65	14.65	61.2	1
4	53.15	11.35	61.5	2
5	53.15	10.5	60.2	2
6	63.1	11.85	55.5	3
7	63.5	12.4	53.8	3
8	65.35	13.1	53.8	4
9	68	44.7	52.5	4
10	69.25	15.5	53.8	5
11	71.4	18.85	52.5	5
			63	6
			63.2	6
	Standard Deviation		63.8	7
0	8.20	0.35	63.2	7
1	0.14	0.07	65.7	8
2	0.92	0.49	65	8
3	1.20	0.35	68.5	9
4	0.92	0.21	67.5	9
5	0.92	0.14	69.3	10
6	0.14	0.35	69.2	10
7	0.42	0.14	71.5	11
8	0.49	0.00	71.3	11
9	0.71	0.00	22.3	P0
10	0.07	1.98	22.8	P0
11	0.14	0.21	23.4	P1
			23.3	P1
			23.1	P2
			22.4	P2
			14.9	P3
			14.4	P3
			11.5	P4
			11.2	P4
			10.4	P5
			10.6	P5
			12.1	P6
			11.6	P6
			12.5	P7
			12.3	P7
			13.1	P8
			13.1	P8
			44.7	P9
			376.6	P9
			14.1	P10
			16.9	P10
			18.7	P11
			19	P11

Values for Figure 36:
These are the SPR values for the equine sFcεRIαD1&2 expression test at day 6.

	Expressing Yeast	Parental Yeast		
Day 6	68	24.7	67.3	Sample
	Standard Deviation		68.7	Sample
	0.99	1.84	26	Parent
			23.4	Parent

Values for Figure 37:
These are the SPR values for the equine sFcεRIαD1&2 media concentration test.

	Cycle 1	Cycle 2	Average
Normal Media	64.1	93	78.55
Overflow	66.6	75.1	70.85
Concentrate	303.8	178.2	241

Values for Figure 40:
Dilution curve Bradford assay for equine sFcεRIαD1&2 and HHoH IgE anti NIP-HSA.

μg ml^{-1}	Dilution Curve Average	Dilution Cure 1	Dilution Cure 2	Dilution Cure 3
0	0.480	0.419	0.449	0.572
0.1	0.541	0.516	0.496	0.610
0.2	0.607	0.550	0.629	0.642
0.3	0.694	0.681	0.710	0.692
0.4	0.833	0.836	0.876	0.786
0.5	1.002	0.895	1.031	1.079
0.6	0.920	0.943	0.945	0.871
0.7	1.099	1.118	1.271	0.907
0.8	1.265	1.082	1.505	1.207
0.9	1.182	1.131	1.259	1.155
1.0	1.243	1.248	1.256	1.226

Chapter 6.3.2 Supplementary Table: EvilFit Discrete One Site Model:

This table shows the Discrete One Site Model values calculated in the EVIFIT software, note the large Root Mean Square Deviation indicating that the results do not fit this model and deviate from it.

	EvilFit Discrete One Site			
Experiment	K_D (M)	k_d (s^{-1})	Signal (RU)	Root mean square deviation (RU)
Horse IgE – Horse sFcεRIαD1&2	1.09×10^{-9}	1.51×10^{-4}	166.8	1.66
Horse IgE – Human sFcεRIαD1&2	5.77×10^{-9}	2.24×10^{-4}	6.4	0.60
Horse IgE – Dog sFcεRIαD1&2	6.89×10^{-9}	3.50×10^{-4}	14.8	0.64
Dog IgE – Horse sFcεRIαD1&2	1.37×10^{-9}	1.22×10^{-4}	168.3	0.90

Chapter 6.3.2 Supplementary Table: Original Fit Using One Site Model:

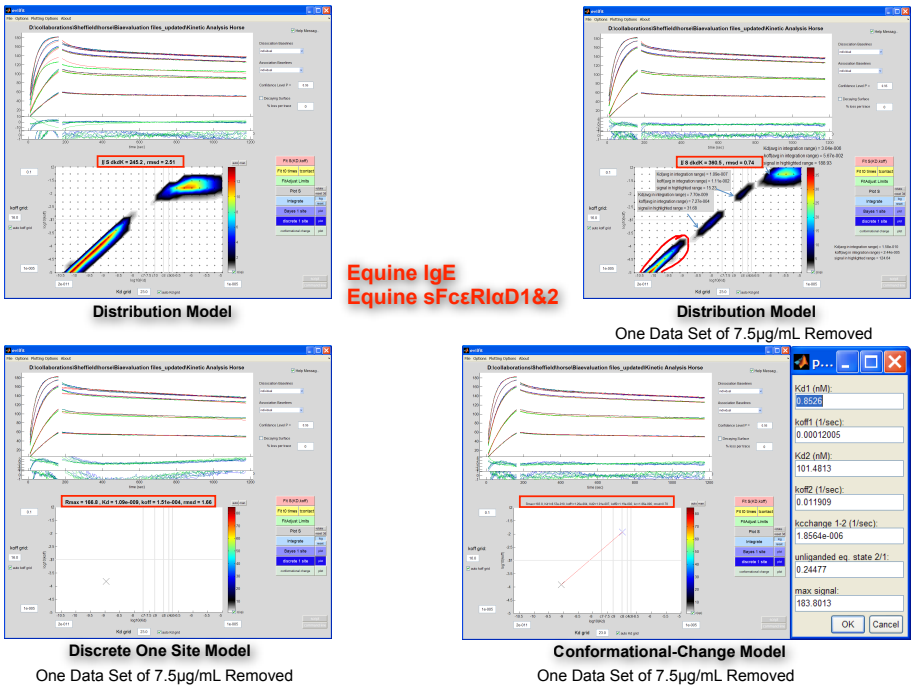
This table shows the original fit using the Discrete One Site Model values, note the extremely large Root Mean Square Deviation indicating that the results do not fit this model and greatly deviate from it.

	Original Fit Using One Site Model			
Experiment	K_D (M)	k_d (s^{-1})	Signal (RU)	Root mean square deviation (RU)
Horse IgE – Horse sFcεRIαD1&2	1.70×10^{-10}	3.80×10^{-4}	159	5.11
Horse IgE – Human sFcεRIαD1&2	5.26×10^{-7}	1.30×10^{-5}	3	0.75
Horse IgE – Dog sFcεRIαD1&2	4.03×10^{-6}	1.60×10^{-3}	999	0.94
Dog IgE – Horse sFcεRIαD1&2	1.20×10^{-10}	1.90×10^{-4}	176	1.90

Chapter 6.3.2 Supplementary Table: Conformational Change Model values:

This table shows the original fit using the Conformational Change Model values , note the small conformational rate constants (k_{cc}) indicating that the results do not fit this model and the binding between the two molecules does not result in co conformational change.

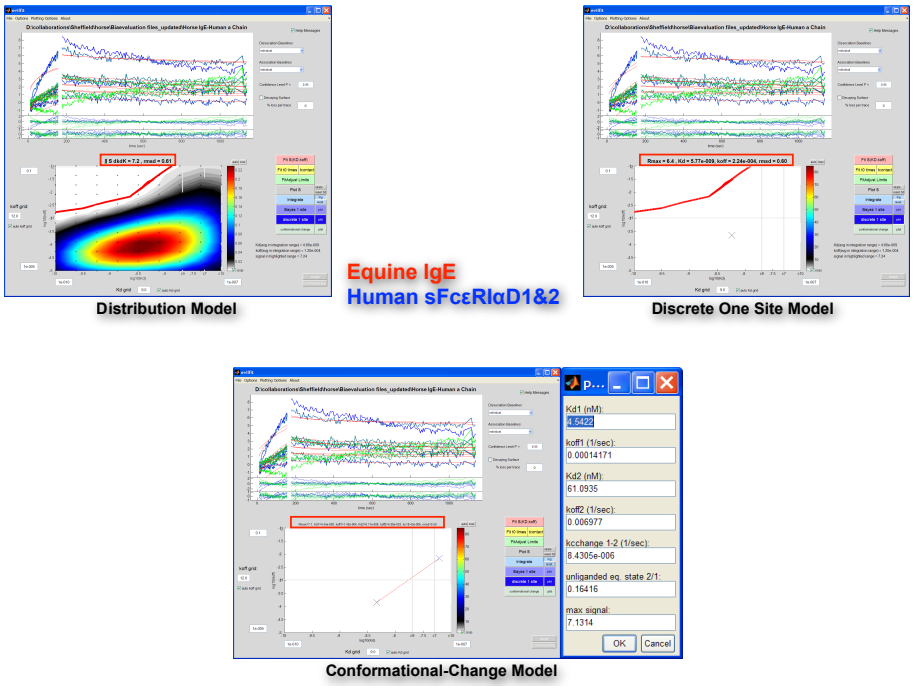
	Conformational-Change Model							
Experiment	Root mean square deviation (RU)	Conclusion	K_D 1	k_d 1	K_D 2	k_d 2	k_{cc}	Unliganded Equilibrium State 2/1
Horse IgE – Horse sFcεRIαD1&2	0.78	No cc	$8.53x10^{-10}$	$1.20x10^{-4}$	$1.01x10^{-7}$	$1.19x10^{-2}$	$1.86x10^{-6}$	0.24
Horse IgE – Human sFcεRIαD1&2	0.59	No cc	$4.54x10^{-9}$	$1.42x10^{-4}$	$6.11x10^{-8}$	$6.98x10^{-3}$	$8.43x10^{-6}$	0.16
Horse IgE – Dog sFcεRIαD1&2	0.25	No cc	$2.31x10^{-8}$	$1.74x10^{-4}$	$1.61x10^{-7}$	$1.59x10^{-2}$	$2.94x10^{-6}$	0.57
Dog IgE – Horse sFcεRIαD1&2	0.64	No cc	$1.26x10^{-9}$	$1.13x10^{-4}$	$2.67x10^{-7}$	$1.59x10^{-2}$	$1.10x10^{-7}$	0.16



Chapter 6.3.2 Supplementary Figure: Diagram representing the three models applied to the binding between the equine IgE and the equine sFcεRIαD1&2:

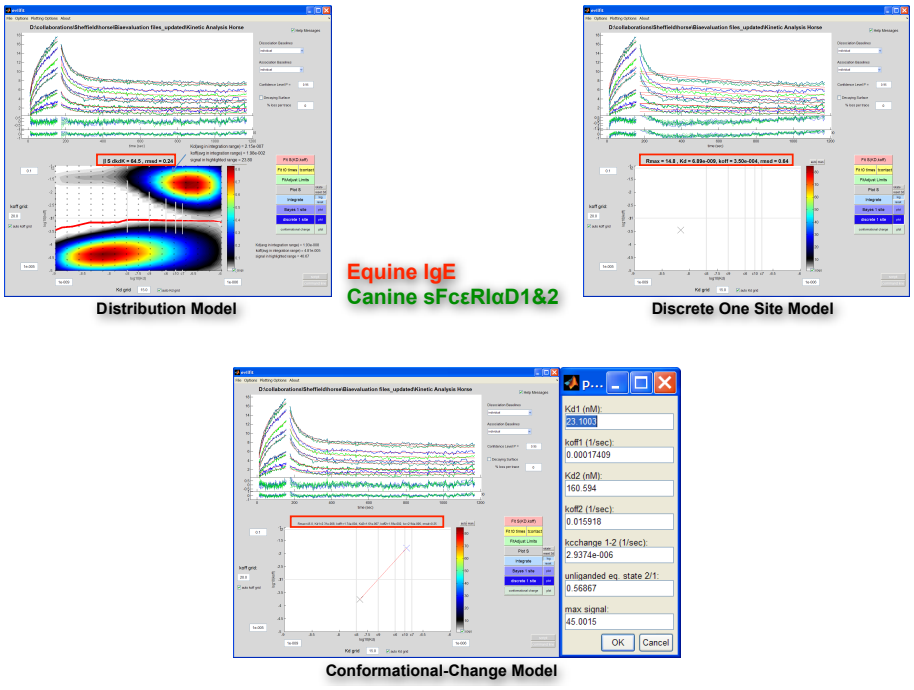
This figure shows at the top the plot of Resonance Units against Time of the actual equine IgE binding to the equine sFcεRIαD1&2 binding analysis, the red lines indicate the predicted best-fit trace of model's fit. The lines in this plot have the start of the association and dissociation lines cut off as there was great deviation in the measurements, this was due to buffer

shifts during the injections, these buffer shifts can be seen in the red lines of Figure 45. The figure also shows at the bottom the EVILFIT analysis of the equine IgE binding to the equine sFcεRIαD1&2 and applied it to the three models. The best model to fit the data is the Distribution Model after the values for the 7µg ml^{-1} concentration was removed. At the right bottom of the window of the Distribution Model there can be seen the K_D and k_d values of the main peak, which is circled in red. The kinetic properties, thermodynamic properties and binding capacity of each of the small peaks in the $\log_{10} k_d$ against $\log_{10} K_D$ distribution plot of the Distribution Model is labeled in the grey boxes. The final Root Mean Square Deviation is marked inside the red rectangle. The dots indicate the grid points for the calculated parameter. The grid spacing is selected logarithmically in both k_d and K_D directions, so that lines of constant k_a are diagonal. The height of the peak values can be read from the colour bar at the right. The vertical grey lines are the experimental sFcεRIαD1&2 receptor concentrations. The horizontal grey line is the inverse of the longest time constant for which the data would permit observing a (e^{-1})-fold decay.



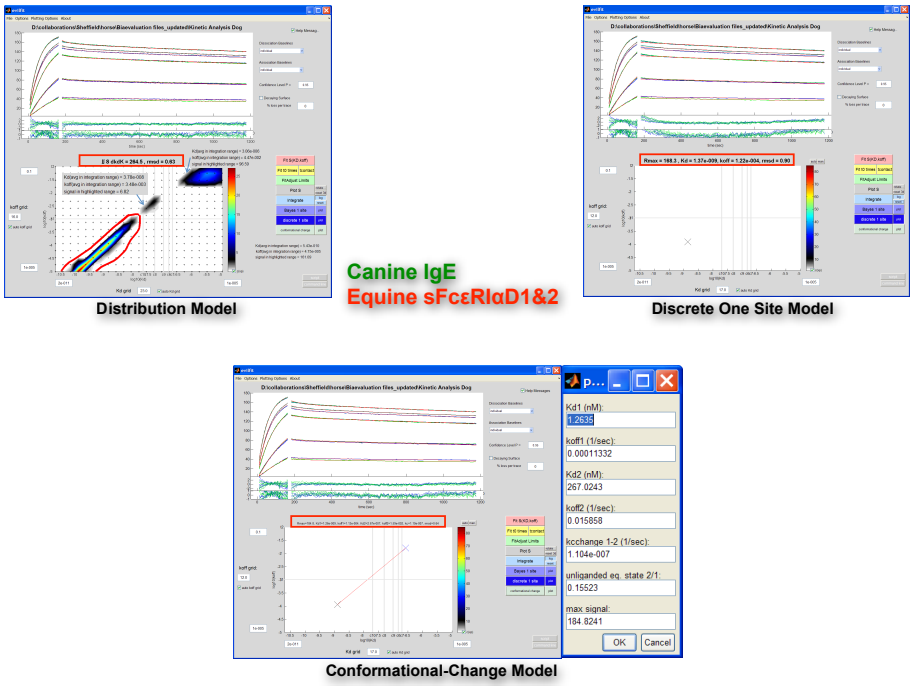
Chapter 6.3.2 Supplementary Figure: Diagram representing the three models applied to the binding between the equine IgE and the human sFcεRIαD1&2:
This figure shows at the top the plot of Resonance Units against Time of the actual equine IgE binding to the human sFcεRIαD1&2 binding analysis, the red lines indicate the

predicted best-fit trace of model's fit. The lines in this plot have the start of the association and dissociation lines cut off as there was great deviation in the measurements, this was due to buffer shifts during the injections, these buffer shifts can be seen in the red lines of Figure 45. The figure also shows at the bottom the EVILFIT analysis of the equine IgE binding to the human sFcεRIαD1&2 and applied it to the three models. The best model to fit the data is the Distribution Model after the values for the 7µg ml^{-1} concentration was removed. At the right bottom of the window of the Distribution Model there can be seen the K_D and k_d values of the main peak, which is circled in red. The kinetic properties, thermodynamic properties and binding capacity of each of the small peaks in the $\log_{10} k_d$ against $\log_{10} K_D$ distribution plot of the Distribution Model is labeled in the grey boxes. The final Root Mean Square Deviation is marked inside the red rectangle. The height of the peak values can be read from the colour bar at the right. The vertical grey lines are the experimental sFcεRIαD1&2 receptor concentrations. The horizontal grey line is the inverse of the longest time constant for which the data would permit observing a (e^{-1})-fold decay.



Chapter 6.3.2 Supplementary Figure: Diagram representing the three models applied to the binding between the equine IgE and the canine sFcεRIαD1&2:
This figure shows at the top the plot of Resonance Units against Time of the actual equine IgE binding to the canine sFcεRIαD1&2 binding analysis, the red lines indicate the

predicted best-fit trace of model's fit. The lines in this plot have the start of the association and dissociation lines cut off as there was great deviation in the measurements, this was due to buffer shifts during the injections, these buffer shifts can be seen in the red lines of Figure 45. The figure also shows at the bottom the EVILFIT analysis of the equine IgE binding to the canine sFcεRIαD1&2 and applied it to the three models. The best model to fit the data is the Distribution Model after the values for the 7µg ml^{-1} concentration was removed. At the right bottom of the window of the Distribution Model there can be seen the K_D and k_d values of the main peak, which is circled in red. The kinetic properties, thermodynamic properties and binding capacity of each of the small peaks in the $\log_{10} k_d$ against $\log_{10} K_D$ distribution plot of the Distribution Model is labeled in the grey boxes. The final Root Mean Square Deviation is marked inside the red rectangle. The height of the peak values can be read from the colour bar at the right. The vertical grey lines are the experimental sFcεRIαD1&2 receptor concentrations. The horizontal grey line is the inverse of the longest time constant for which the data would permit observing a (e^{-1})-fold decay.



Chapter 6.3.2 Supplementary Figure: Diagram representing the three models applied to the binding between the canine IgE and the equine sFcεRIαD1&2:
This figure shows at the top the plot of Resonance Units against Time of the actual canine IgE binding to the equine sFcεRIαD1&2 binding analysis, the red lines indicate the

predicted best-fit trace of model's fit. The lines in this plot have the start of the association and dissociation lines cut off as there was great deviation in the measurements, this was due to buffer shifts during the injections, these buffer shifts can be seen in the red lines of Figure 45. The figure also shows at the bottom the EVILFIT analysis of the canine IgE binding to the equine sFcεRIαD1&2 and applied it to the three models. The best model to fit the data is the Distribution Model after the values for the 7μg ml^{-1} concentration was removed. At the right bottom of the window of the Distribution Model there can be seen the K_D and k_d values of the main peak, which is circled in red. The kinetic properties, thermodynamic properties and binding capacity of each of the small peaks in the $\log_{10} k_d$ against $\log_{10} K_D$ distribution plot of the Distribution Model is labeled in the grey boxes. The final Root Mean Square Deviation is marked inside the red rectangle. The height of the peak values can be read from the colour bar at the right. The vertical grey lines are the experimental sFcεRIαD1&2 receptor concentrations. The horizontal grey line is the inverse of the longest time constant for which the data would permit observing a (e^{-1})-fold decay.

Human IgE heavy chain DNA sequence (Nishida *et al*, 1982):

GGATCCCTGCCACGGGGTCCCCAGCTCCCCCATCCAGGCCCCCCAGGCTGATGGGCGCTGGCCTGAGGCTGGCACTGA
CTAGGTTCTGTCCTCACAGCCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
AATGCCACCTCCGTGACTCTGGGCTGCCTGGCCACGGGCTACTTCCCGGAGCCGGTGATGGTGACCTGGGACACAGGC
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ACCGTCTCGGGTGCGTGGGCCAAGCAGATGTTCACCTGCCGTGTGGCACACACTCCATCGTCCACAGACTGGGTCGAC
AACAAAACCTTCAGCGGTAAGAGAGGGCCAAGCTCAGAGACCACAGTTCCCAGGAGTGCCAGGCTGAGGGCTGGCAGA
GTGGGCAGGGGTTGAGGGGGTGGGTGGGCTCAAACGTGGGAACACCCAGCATGCCTGGGGACCCGGGCCAGGACGTGG
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CTGTGCCTCGTCTCTGGGTACACCCCAGGGACTATCAACATCACCTGGCTGGAGGACGGGCAGGTCATGGACGTGGAC
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CCTGGAGGTGACCAGGGCCGAATGGGAGCAGAAAGATGAGTTCATCTGCCGTGCAGTCCATGAGGCAGCGAGCCCCTC
ACAGACCGTCCAGCGAGCGGTGTCTGTAAATCCCGGTAAATGACGTACTCCTGCCTCCCTCCCTCCCAGGGCTCCATC
CAGCTGTGCAGTGGGGAGGACTGGCCAGACCTTCTGTCCACTGTTGCAATGACCCCAGGAAGCTACCCCCAATAAACT
GTGCCTGCTCAGAGCCCCAGTACACCCATTCTTGGGAGCGGGCAGGGC

Human IgE heavy chain protein sequence:

ASTQSPSVFPLTRCCKNIPSNATSVTLGCLATGYFPEPVMVTWDTGSLNGTTMTLPATTLTLSGHYATISLLTVSGAW
AKQMFTCRVAHTPSSTDWVDNKTFSVCSRDFTPPTVKILQSSCDGGGHFPPTIQLLCLVSGYTPGTINITWLEDGQVM
DVDLSTASTTQEGELASTQSELTLSQKHWLSDRTYTCQVTYQGHTFEDSTKKCADSNPRGVSAYLSRPSPFDLFIRKS
PTITCLVVDLAPSKGTVNLTWSRASGKPVNHSTRKEEKQRNGTLTVTSTLPVGTRDWIEGETYQCRVTHPHLPRALMR
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HHoH domain amplifications, top sequences are the originals and bottom sequences are the sample sequences:

Cε1Cε2:

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Cε1Cε2 forward sequencing

Cε1Cε2 reverse

Cε3:

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AGGGAGAGCGGCGAGCCTCTGCAGAAGCACACCCTGGCCACCAGCGAGCAGTTCAACAAGACCTTCAGCGTGACCAGC
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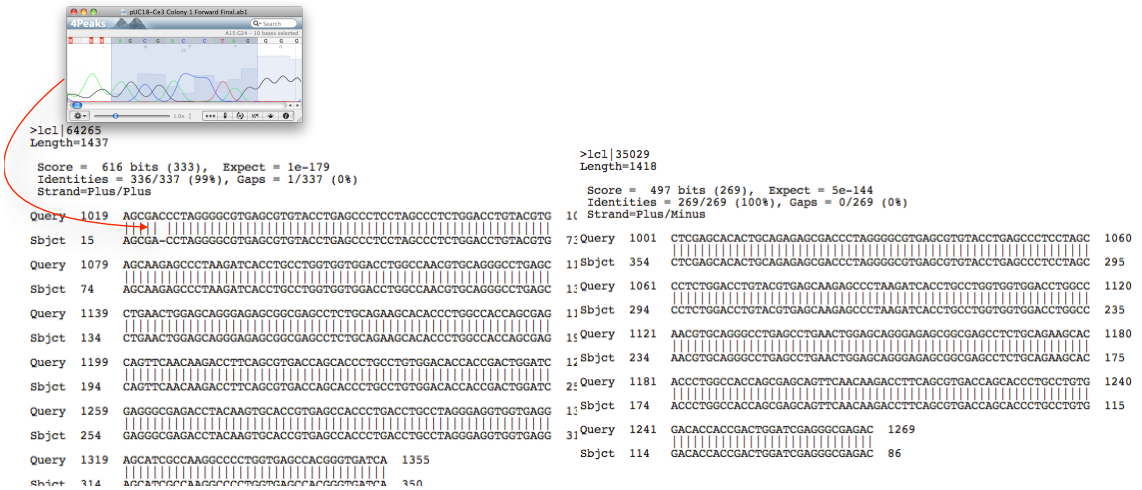

Cε3 forward sequencing

Cε3 reverse sequencing

Cε4:

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CGGGACAAGCGCACCCTCGCCTGCCTGATCCAGAACTTCATGCCTGAGGACATCTCGGTGCAGTGGCTGCACAACGAG
GTGCAGCTCCCGGACGCCCGGCACAGCACGACGCAGCCCCGCAAGACCAAGGGCTCCGGCTTCTTCGTCTTCAGCCGC
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Length=1401

 Score =  837 bits (453),  Expect = 0.0
 Identities = 453/453 (100%), Gaps = 0/453 (0%)
 Strand=Plus/Plus

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Query  1788  CCCTCCCTCCCAGGGCTCCATCCAGCTACGCGT  1820
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Sbjct  434   CCCTCCCTCCCAGGGCTCCATCCAGCTACGCGT  466
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Cε4 forward sequencing

GFP:

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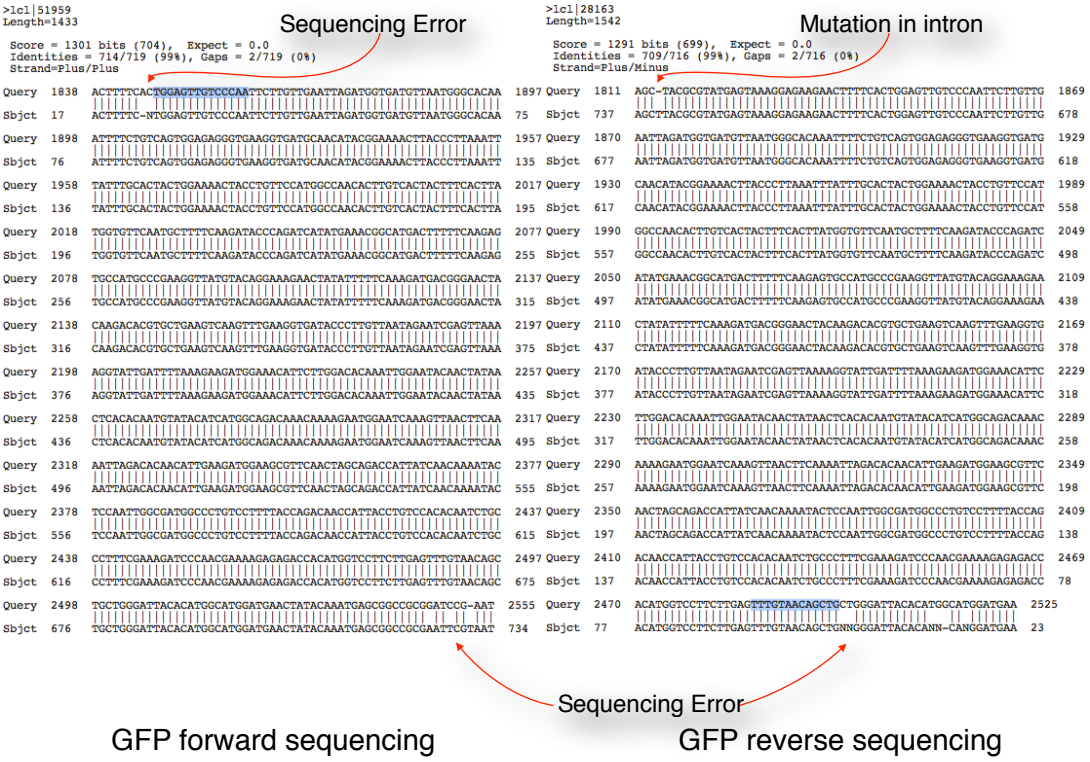

Final HHoH gene:

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HHoH protein sequence:

ASTQSPSVFPLTRCCKNIPSNATSVTLGCLATGYFPEPVMVTWDTGSLNGTTMTLPATTLTLSGHYATISLLTVSGAW
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VTRAEWEQKDEFICRAVHEAASPSQTVQRAVSVNPGK

Final HHoHGFP gene:

AAGCTTGGATCCCTGCCACGGGGTCCCCAGCTCCCCCATCCAGGCCCCCCAGGCCTGATGGGCGCTGGCCTGAGGCTG
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GCTGCTGGGATTACACATGGCATGGATGAACTATACAAATGA**GCGGCCGCGGATCCGAATTC**

Values for Figure 58:

These are the figures from the SPR analysis of the J558L cell selection expressing HHoH.

	Resonance Units	Average Response
Parent Cells	76.60	76.65
Parent Cells	76.70	
Pure Media	10.70	10.50
Pure Media	10.30	
AA1	49.70	53.65
AA1	57.60	
AA2	62.50	62.20
AA2	61.90	
AA3	64.90	65.50
AA3	66.10	
AA4	66.50	66.45
AA4	66.40	
AA5	69.60	68.65
AA5	67.70	
AA6	73.00	72.70
AA6	72.40	
AB1	78.00	76.50
AB1	75.00	
AB2	67.90	67.65
AB2	67.40	
AB3	70.30	69.50
AB3	68.70	
AB4	69.50	69.15
AB4	68.80	
AB5	72.00	71.75
AB5	71.50	
AB6	80.10	78.65
AB6	77.20	
AC1	99.70	98.20
AC1	96.70	
AC2	78.60	79.05
AC2	79.50	
AC3	76.20	76.15
AC3	76.10	
AC4	78.70	77.95
AC4	77.20	
AC5	75.10	74.10
AC5	73.10	
AC6	83.30	83.95
AC6	84.60	
AD1	103.70	100.80
AD1	97.90	
AD2	93.20	92.10
AD2	91.00	
AD3	89.10	87.65
AD3	86.20	
AD4	85.80	85.30
AD4	84.80	
AD5	86.20	85.95
AD5	85.70	
AD6	93.50	92.90
AD6	92.30	
BA1	90.10	89.40

	Resonance Units	Average Response
BA1	88.70	89.40
BA2	82.00	80.70
BA2	79.40	
BA3	80.00	79.15
BA3	78.30	
BA4	82.80	81.70
BA4	80.60	
BA5	83.80	83.10
BA5	82.40	
BA6	85.40	84.50
BA6	83.60	
BB1	84.30	83.65
BB1	83.00	
BB2	70.80	70.40
BB2	70.00	
BB3	74.00	72.95
BB3	71.90	
BB4	71.00	70.35
BB4	69.70	
BB5	71.90	70.40
BB5	68.90	
BB6	76.00	75.65
BB6	75.30	
BC1	80.40	78.90
BC1	77.40	
BC2	70.30	69.75
BC2	69.20	
BC3	59.80	59.90
BC3	60.00	
BC4	65.50	65.60
BC4	65.70	
BC5	63.00	62.85
BC5	62.70	
BC6	75.80	74.60
BC6	73.40	
BD1	82.20	81.35
BD1	80.50	
BD2	74.30	73.60
BD2	72.90	
BD3	76.10	74.75
BD3	73.40	
BD4	72.20	71.40
BD4	70.60	
BD5	71.00	70.25
BD5	69.50	
LA1	73.60	73.20
LA1	72.80	
LA2	76.90	76.75
LA2	76.60	
LA3	69.20	68.25
LA3	67.30	
LA4	70.30	69.70
LA4	69.10	
LA5	73.90	72.85
LA5	71.80	
LA6	74.40	72.15
LA6	69.90	
LB1	78.80	77.60

	Resonance Units	Average Response
LB1	76.40	77.00
LB2	77.90	77.45
LB2	77.00	
LB3	66.10	65.15
LB3	64.20	
LB4	66.50	65.45
LB4	64.40	
LB5	64.10	63.15
LB5	62.20	
LB6	71.20	70.50
LB6	69.80	
LC1	79.10	78.25
LC1	77.40	
LC2	66.40	65.65
LC2	64.90	
LC3	60.30	59.15
LC3	58.00	
LC4	60.70	59.35
LC4	58.00	
LC5	55.50	55.45
LC5	55.40	
LC6	64.50	63.75
LC6	63.00	
LD1	84.80	84.25
LD1	83.70	
LD2	72.50	71.30
LD2	70.10	
LD3	63.40	61.90
LD3	60.40	
LD4	64.90	63.80
LD4	62.70	
LD5	64.10	62.55
LD5	61.00	
LD6	67.90	66.40
LD6	64.90	

Values for Figure 59:
Dilution curve Bradford assay for HDH IgE anti NIP-HSA.

µg ml^{-1}	Dilution Curve Average	Dilution Cure 1	Dilution Cure 2	Dilution Cure 3
0	0.331	0.321	0.337	0.335
0.1	0.474	0.459	0.483	0.481
0.2	0.571	0.550	0.596	0.566
0.3	0.667	0.633	0.675	0.692
0.4	0.759	0.739	0.774	0.763
0.5	0.823	0.736	0.854	0.878
0.6	0.928	0.788	1.056	0.939
0.7	1.002	0.954	1.035	1.018
0.8	1.040	0.939	1.076	1.105
0.9	1.025	0.925	1.046	1.105
1.0	1.055	0.916	1.098	1.151

Values for Figure 65:
ELISA test of each rat's bleed for binding to the 2Fc$\varepsilon_{2\text{-}3}$.

Test 1

	Rat 1 Bleed 1		Rat 2 Bleed 1		Rat 3 Bleed 1		Rat 4 Bleed 1		Pre Immunisation Bleed	
1:200	9.999	0.000	0.871	0.030	0.532	0.006	2.915	0.040	0.136	0.016
1:400	9.999	0.000	0.395	0.028	0.239	0.014	1.747	0.005	0.089	0.001
1:800	2.845	0.069	0.185	0.012	0.140	0.004	0.902	0.071	0.070	0.006
1:1,600	1.878	0.178	0.108	0.000	0.091	0.003	0.357	0.003	0.062	0.002
1:3,200	0.877	0.100	0.078	0.002	0.067	0.002	0.163	0.011	0.054	0.002
1:6,400	0.369	0.055	0.067	0.001	0.053	0.001	0.094	0.005	0.052	0.004
1:12,800	0.188	0.016	0.052	0.000	0.052	0.001	0.061	0.002	0.050	0.002
1:25,600	0.079	0.050	0.046	0.000	0.050	0.001	0.054	0.001	0.051	0.003
1:51,200	0.072	0.004	0.048	0.000	0.043	0.000	0.051	0.001		
1:102,400	0.059	0.001	0.050	0.003	0.047	0.001	0.053	0.001		

Test 2

	Rat 1 Bleed 2		Rat 2 Bleed 2		Rat 3 Bleed 2		Rat 4 Bleed 2		Pre Immunisation Bleed	
1:200	9.999	0.000	2.708	0.000	1.918	0.074	9.999	0.000	0.151	0.011
1:400	9.999	0.000	1.640	0.010	0.955	0.072	6.487	4.967	0.099	0.004
1:800	9.999	0.000	0.677	0.001	0.437	0.022	2.269	0.167	0.080	0.008
1:1,600	9.999	0.000	0.284	0.021	0.209	0.027	0.999	0.096	0.077	0.008
1:3,200	2.211	0.100	0.142	0.001	0.114	0.002	0.464	0.054	0.067	0.014
1:6,400	1.105	0.064	0.096	0.003	0.070	0.006	0.202	0.017	0.060	0.001
1:12,800	0.538	0.030	0.076	0.001	0.066	0.001	0.103	0.005	0.067	0.011
1:25,600	0.284	0.005	0.117	0.078	0.074	0.004	0.085	0.001	0.072	0.007
1:51,200	0.155	0.002	0.060	0.001	0.057	0.006	0.069	0.001		
1:102,400	0.101	0.008	0.066	0.006	0.060	0.000	0.072	0.001		

Values for Figure 67:
ELISA test of bleed to of all rats for binding to the native IgE antibodies.

Human IgE test

	Rat 1		Rat 2		Rat 3		Rat 4		Pre Immunisation Bleed		Positive Control	Negative Control
1:200	0.233	0.008	0.354	0.021	1.986	0.045	2.043	0.009	0.127	0.004	0.941	0.120
1:400	0.132	0.011	0.195	0.001	1.874	0.019	2.081	0.043	0.108	0.002		
1:800	0.114	0.006	0.123	0.000	1.737	0.049	1.841	0.159	0.107	0.004		
1:1,600	0.104	0.002	0.102	0.003	1.535	0.028	1.714	0.076	0.103	0.003		
1:3,200	0.093	0.004	0.094	0.001	1.143	0.019	1.393	0.032	0.096	0.004		
1:6,400	0.093	0.004	0.101	0.001	0.756	0.063	1.034	0.043	0.108	0.008		
1:12,800	0.097	0.003	0.094	0.001	0.461	0.006	0.670	0.021	0.102	0.013		
1:25,600	0.100	0.006	0.092	0.002	0.278	0.004	0.424	0.007				
1:51,200	0.092	0.001	0.096	0.002	0.182	0.010	0.271	0.014				
1:102,400	0.094	0.000	0.092	0.002	0.147	0.005	0.218	0.001				

Canine IgE test

	Rat 1		Rat 2		Rat 3		Rat 4		Pre Immunisation Bleed		Positive Control	Negative Control
1:200	1.102	0.009	0.881	0.009	1.907	0.050	2.326	0.030	0.114	0.003	1.276	0.085
1:400	0.622	0.012	0.463	0.027	1.832	0.004	2.243	0.058	0.091	0.007		
1:800	0.368	0.013	0.260	0.003	1.721	0.047	1.998	0.109	0.081	0.004		
1:1,600	0.232	0.019	0.174	0.001	1.555	0.023	1.704	0.037	0.076	0.004		
1:3,200	0.142	0.018	0.128	0.004	1.123	0.011	1.280	0.011	0.066	0.003		
1:6,400	0.112	0.016	0.108	0.006	0.694	0.043	0.826	0.000	0.070	0.004		
1:12,800	0.093	0.013	0.083	0.003	0.421	0.018	0.469	0.015	0.071	0.002		
1:25,600	0.109	0.009	0.070	0.002	0.245	0.008	0.304	0.001				
1:51,200	0.075	0.004	0.068	0.001	0.157	0.023	0.195	0.006				
1:102,400	0.064	0.001	0.065	0.001	0.114	0.001	0.138	0.007				

Equine IgE test

	Rat 1		Rat 2		Rat 3		Rat 4		Pre Immunisation Bleed		Positive Control	Negative Control
1:200	1.397	0.112	1.014	0.071	2.288	0.146	2.696	0.076	0.148	0.010	0.560	0.123
1:400	0.810	0.049	0.591	0.037	2.265	0.054	2.716	0.076	0.119	0.001		
1:800	0.500	0.008	0.337	0.025	2.170	0.095	2.349	0.198	0.110	0.006		
1:1,600	0.293	0.021	0.218	0.006	1.835	0.127	2.094	0.062	0.103	0.000		
1:3,200	0.186	0.013	0.154	0.004	1.511	0.000	1.475	0.068	0.095	0.001		
1:6,400	0.138	0.010	0.136	0.004	0.854	0.001	0.973	0.013	0.095	0.001		
1:12,800	0.121	0.018	0.117	0.005	0.507	0.023	0.533	0.023	0.103	0.002		
1:25,600	0.135	0.025	0.099	0.000	0.296	0.003	0.333	0.021				
1:51,200	0.105	0.002	0.094	0.001	0.189	0.004	0.212	0.002				
1:102,400	0.091	0.002	0.094	0.001	0.142	0.000	0.163	0.011				

Values for Figure 71:
Release assays of Bleed 2 from all rats, along with the positive and negative control graph.

Human

Bleed Dilution Factor	1	2	3	4	5	6	7.00	8.00	9.00	10.00	11.00	12.00
1:25600	1.43	1.41	1.32	1.39	1.61	1.29	0.18	0.21	0.19	0.18	0.11	0.12
1:12800	1.32	1.32	1.32	1.28	1.68	1.37	0.11	0.12	0.11	0.13	0.17	0.17
1:6400	1.39	1.39	1.42	1.34	1.65	1.41	0.16	0.18	0.17	0.18	0.18	0.18
1:3200	1.41	1.45	1.48	1.36	1.63	1.41	0.17	0.18	0.19	0.21	0.17	0.19
1:1600	1.40	1.48	1.41	1.27	1.65	1.43	0.16	0.16	0.17	0.22	0.20	0.21
1:800	1.37	1.39	1.31	1.24	1.66	1.36	0.16	0.18	0.23	0.27	0.19	0.20
1:400	1.29	1.37	1.26	1.22	1.69	1.35	0.15	0.16	0.23	0.25	0.14	0.14
1:200	1.22	1.36	1.23	1.17	1.76	1.39	0.10	0.12	0.17	0.22	0.21	0.22
1:25600	1.18	1.17	1.17	1.14	1.14	1.19	0.12	0.1	0.1	0.1	0.1	0.1
1:12800	1.12	1.15	1.1	1.12	1.11	1.16	0.15	0.2	0.2	0.2	0.2	0.2
1:6400	1.11	1.14	1.11	1.12	1.21	1.17	0.18	0.2	0.2	0.2	0.2	0.2
1:3200	1.12	1.31	1.08	1.09	1.13	1.16	0.17	0.2	0.2	0.2	0.2	0.2
1:1600	1.13	1.16	1.1	1.06	1.16	1.18	0.19	0.2	0.2	0.3	0.2	0.2
1:800	1.13	1.16	1.09	1.03	1.16	1.28	0.17	0.2	0.2	0.3	0.2	0.2
1:400	1.09	1.14	1.02	1.04	1.15	1.19	0.12	0.1	0.2	0.2	0.1	0.1
1:200	1.16	1.2	1.06	1.1	1.55	1.19	0.19	0.2	0.3	0.3	0.2	0.2
	% of Total In Cell						x2 because (I used 50 µl instead of the 100) + Supernatant Value To Find Original Total In Cell					
1:25600	20.47	22.87	22.63	20.84	12.31	15.58	1.80	1.83	1.71	1.76	1.84	1.53
1:12800	14.73	15.28	14.62	16.99	16.58	19.41	1.55	1.56	1.55	1.54	2.01	1.70
1:6400	18.52	20.21	19.04	20.90	18.07	19.89	1.71	1.74	1.75	1.69	2.01	1.76
1:3200	18.97	19.98	20.00	23.42	17.26	21.23	1.74	1.81	1.85	1.78	1.97	1.79
1:1600	18.60	17.69	19.61	25.64	19.59	23.04	1.72	1.80	1.75	1.71	2.05	1.86
1:800	19.22	20.75	25.74	30.26	18.23	22.73	1.70	1.75	1.76	1.78	2.03	1.76
1:400	18.46	18.93	26.40	29.23	13.95	16.67	1.58	1.69	1.71	1.72	1.96	1.62
1:200	14.45	15.21	21.46	27.15	19.04	23.79	1.43	1.60	1.57	1.61	2.17	1.82
1:25600	16.43	15.09	15.34	17.75	15.81	14.88	1.41	1.38	1.38	1.39	1.35	1.40
1:12800	20.90	22.40	22.32	22.22	22.59	23.28	1.42	1.48	1.42	1.44	1.43	1.51
1:6400	24.08	25.49	25.20	26.12	24.19	25.48	1.46	1.53	1.48	1.52	1.60	1.57
1:3200	23.60	21.08	25.21	24.83	24.16	25.35	1.47	1.66	1.44	1.45	1.49	1.55
1:1600	25.56	25.55	27.92	32.05	26.30	25.13	1.52	1.56	1.53	1.56	1.57	1.58
1:800	23.23	24.58	28.29	33.38	25.64	23.35	1.47	1.54	1.52	1.55	1.56	1.67
1:400	18.41	19.38	27.04	31.67	19.92	18.72	1.34	1.41	1.40	1.52	1.44	1.46
1:200	24.87	26.56	33.17	36.42	21.72	28.66	1.54	1.63	1.59	1.73	1.98	1.67
Average	Rat 1 Bleed 2	Rat 2 Bleed 2	Rat 3 Bleed 2	Rat 4 Bleed 2	Pre-Immunisation Bleed	NIP-HSA Positive Control	Standard Deviation					
1:25600	17.82	18.84	18.47	19.61	19.59	21.35	2.85	5.50	5.15	2.19	2.47	0.49
1:12800	21.30	22.85	22.12	23.51	21.13	22.68	4.37	5.04	5.44	3.70	4.25	2.74
1:6400	21.28	20.53	22.60	24.13	20.71	23.29	3.93	3.74	4.36	3.69	4.32	3.95
1:3200	22.08	21.62	23.76	28.85	22.95	24.08	3.28	0.78	3.68	0.99	4.88	2.92
1:1600	21.23	22.66	27.01	31.82	21.93	23.04	4.92	5.56	5.87	4.53	4.75	1.48
1:800	18.44	19.16	26.72	30.45	16.93	17.69	2.84	2.70	1.80	2.20	5.24	0.44
1:400	19.66	20.89	27.31	31.78	20.38	26.23	0.03	0.31	0.45	1.72	4.22	1.45
1:200	16.43	15.09	15.34	17.75	15.81	14.88	7.37	8.02	8.28	6.55	1.89	3.44

Canine

Bleed Dilution	1	2	3	4	5	6	7.00	8.00	9.00	10.0	11.00	12.00
1:25600	1.84	1.90	1.81	1.79	1.63	1.80	0.17	0.19	0.21	0.18	0.11	0.09
1:12800	1.74	1.77	1.60	1.58	1.69	1.66	0.11	0.10	0.17	0.19	0.17	0.16
1:6400	1.77	1.78	1.44	1.42	1.60	1.70	0.15	0.16	0.36	0.35	0.18	0.15
1:3200	1.72	1.75	1.24	1.20	1.61	1.78	0.18	0.19	0.50	0.51	0.17	0.18
1:1600	1.73	1.74	1.08	1.06	1.61	1.25	0.14	0.16	0.54	0.56	0.19	0.44
1:800	1.74	1.79	1.05	1.03	1.62	1.14	0.17	0.16	0.59	0.61	0.17	0.53
1:400	1.79	1.79	1.04	1.01	1.61	1.24	0.15	0.16	0.62	0.56	0.12	0.41
1:200	1.75	1.80	1.08	1.06	1.73	1.20	0.11	0.09	0.55	0.43	0.20	0.54
1:25600	1.77	1.81	1.74	1.76	1.76	1.82	0.12	0.1	0.13	0.1	0.12	0.1
1:12800	1.7	1.79	1.63	1.68	1.75	1.75	0.17	0.2	0.23	0.2	0.21	0.2
1:6400	1.72	1.74	1.45	1.51	1.71	1.74	0.19	0.2	0.4	0.4	0.2	0.2
1:3200	1.71	1.74	1.27	1.29	1.75	1.7	0.17	0.2	0.42	0.5	0.21	0.2
1:1600	1.79	1.83	1.21	1.22	1.78	1.57	0.21	0.2	0.55	0.6	0.22	0.4
1:800	1.77	1.86	1.2	1.1	1.79	1.27	0.19	0.2	0.54	0.6	0.21	0.4
1:400	1.72	1.82	1.13	1.01	1.76	1.29	0.18	0.2	0.53	0.6	0.16	0.3
1:200	1.73	1.87	1.2	1.13	1.8	1.32	0.21	0.2	0.54	0.6	0.22	0.4
	% of Total In Cell						x2 because (I used 50 µl instead of the 100) ÷ Supernatant Value					
							To Find Original Total In Cell					
1:25600	15.83	16.89	18.76	16.90	11.41	9.44	2.19	2.29	2.23	2.15	1.84	1.99
1:12800	11.04	9.96	17.53	19.06	16.34	15.82	1.96	1.97	1.94	1.95	2.02	1.97
1:6400	14.66	14.83	33.46	32.83	18.03	15.25	2.07	2.09	2.16	2.11	1.95	2.01
1:3200	17.55	17.69	44.49	45.80	17.52	16.59	2.09	2.13	2.23	2.21	1.95	2.13
1:1600	14.02	15.37	49.86	51.47	19.10	41.20	2.01	2.06	2.15	2.18	1.99	2.13
1:800	16.10	15.33	52.96	54.02	17.18	48.04	2.07	2.11	2.23	2.24	1.96	2.19
1:400	14.60	15.09	54.19	52.72	13.16	39.69	2.10	2.11	2.27	2.14	1.85	2.06
1:200	10.90	9.54	50.41	44.68	18.63	47.32	1.96	1.99	2.18	1.92	2.13	2.28
1:25600	11.59	12.81	12.65	13.56	11.82	13.17	2.00	2.08	1.99	2.04	2.00	2.10
1:12800	16.42	16.82	21.79	20.75	18.98	18.60	2.03	2.15	2.08	2.12	2.16	2.15
1:6400	18.02	18.08	35.73	31.74	18.57	17.54	2.10	2.12	2.26	2.21	2.10	2.11
1:3200	16.18	16.18	40.04	43.07	19.06	21.22	2.04	2.08	2.12	2.27	2.16	2.16
1:1600	19.15	18.81	47.80	49.71	19.53	30.90	2.21	2.25	2.32	2.43	2.21	2.27
1:800	17.98	17.77	47.28	51.63	18.78	35.92	2.16	2.26	2.28	2.27	2.20	1.98
1:400	17.23	16.21	48.21	54.95	15.30	33.37	2.08	2.17	2.18	2.24	2.08	1.94
1:200	19.16	19.26	47.28	49.51	19.28	38.83	2.14	2.32	2.28	2.24	2.23	2.16
Average	Rat 1 Bleed 2	Rat 2 Bleed 2	Rat 3 Bleed 2	Rat 4 Bleed 2	Pre-Immunisation Bleed	NIP-HSA Positive Control	Standard Deviation					
1:25600	13.71	14.85	15.71	15.23	11.62	11.30	3.00	2.88	4.32	2.36	0.29	2.64
1:12800	13.73	13.39	19.66	19.91	17.66	17.21	3.80	4.85	3.01	1.20	1.87	1.97
1:6400	16.34	16.46	34.59	32.28	18.30	16.39	2.38	2.30	1.61	0.77	0.38	1.61
1:3200	16.86	16.94	42.27	44.44	18.29	18.91	0.97	1.06	3.15	1.93	1.09	3.28
1:1600	16.58	17.09	48.83	50.59	19.31	36.05	3.63	2.43	1.46	1.24	0.31	7.29
1:800	17.04	16.55	50.12	52.82	17.98	41.98	1.33	1.73	4.02	1.69	1.14	8.57
1:400	15.91	15.65	51.20	53.83	14.23	36.53	1.86	0.79	4.22	1.58	1.51	4.47
1:200	15.03	14.40	48.84	47.09	18.95	43.08	5.84	6.87	2.22	3.42	0.46	6.00

Equine

Bleed Dilution	1	2	3	4	5	6	7.00	8.00	9.00	10.00	11.00	12.00
1:25600	1.27	1.26	1.28	1.28	1.39	1.11	0.18	0.20	0.19	0.16	0.11	0.11
1:12800	1.18	1.23	1.19	1.24	1.49	1.16	0.12	0.12	0.13	0.12	0.16	0.15
1:6400	1.23	1.23	1.22	1.22	1.46	1.18	0.15	0.16	0.18	0.20	0.16	0.17
1:3200	1.26	1.25	1.19	1.15	1.45	1.20	0.18	0.17	0.22	0.23	0.17	0.16
1:1600	1.18	1.23	1.13	1.08	1.47	1.19	0.14	0.15	0.21	0.22	0.19	0.20
1:800	1.26	1.26	1.13	1.11	1.51	1.17	0.15	0.15	0.20	0.22	0.17	0.19
1:400	1.24	1.24	1.14	1.12	1.45	1.16	0.13	0.15	0.21	0.22	0.13	0.15
1:200	1.19	1.18	1.09	1.08	1.57	1.24	0.09	0.09	0.15	0.16	0.19	0.21
1:25600	1.26	1.26	1.32	1.25	1.31	1.23	0.09	0.1	0.08	0.1	0.1	0.1
1:12800	1.2	1.25	1.29	1.22	1.24	1.17	0.15	0.2	0.17	0.2	0.18	0.2
1:6400	1.17	1.22	1.23	1.22	1.25	1.18	0.16	0.2	0.17	0.2	0.19	0.2
1:3200	1.22	1.25	1.22	1.21	1.38	1.19	0.17	0.2	0.17	0.2	0.18	0.2
1:1600	1.42	1.28	1.27	1.22	1.3	1.32	0.19	0.2	0.21	0.2	0.2	0.2
1:800	1.06	1.19	1.14	1.09	1.21	1.11	0.17	0.2	0.2	0.2	0.19	0.2
1:400	1.15	1.28	1.22	1.18	1.27	1.2	0.12	0.1	0.16	0.2	0.12	0.1
1:200	1.19	1.28	1.27	1.29	1.37	1.28	0.18	0.2	0.21	0.2	0.2	0.2
	% of Total In Cell						x2 because (I used 50 µl instead of the 100) ÷ Supernatant Value					
							To Find Original Total In Cell					
1:25600	21.60	24.10	22.89	19.60	14.09	16.04	1.62	1.66	1.66	1.59	1.62	1.32
1:12800	16.90	16.44	17.70	16.55	17.41	20.87	1.42	1.47	1.45	1.49	1.80	1.47
1:6400	20.03	20.95	22.49	24.60	18.34	21.85	1.54	1.56	1.57	1.62	1.79	1.51
1:3200	21.93	21.78	26.99	28.66	18.63	20.63	1.61	1.60	1.63	1.61	1.78	1.51
1:1600	19.51	19.92	27.00	29.13	20.63	24.68	1.47	1.54	1.55	1.52	1.85	1.58
1:800	19.64	19.02	26.53	28.29	18.38	24.90	1.57	1.56	1.54	1.55	1.85	1.56
1:400	17.44	19.48	26.55	28.30	14.91	20.22	1.50	1.54	1.55	1.56	1.70	1.45
1:200	13.24	13.62	21.13	22.41	19.57	25.39	1.37	1.37	1.38	1.39	1.95	1.66
1:25600	12.16	12.77	10.96	13.79	13.47	17.23	1.43	1.44	1.48	1.45	1.51	1.49
1:12800	20.42	22.46	20.57	22.88	22.69	24.03	1.51	1.61	1.62	1.58	1.60	1.54
1:6400	21.16	23.27	21.56	23.85	23.41	25.51	1.48	1.59	1.57	1.60	1.63	1.58
1:3200	21.29	20.58	21.59	24.47	20.23	22.63	1.55	1.57	1.56	1.60	1.73	1.54
1:1600	21.20	22.61	25.12	27.98	23.08	25.00	1.80	1.65	1.70	1.69	1.69	1.76
1:800	24.07	23.32	26.36	31.01	23.80	27.36	1.40	1.55	1.55	1.58	1.59	1.53
1:400	16.79	18.05	20.88	24.84	15.67	19.35	1.38	1.56	1.54	1.57	1.51	1.49
1:200	23.23	25.41	24.49	27.12	22.77	25.49	1.55	1.72	1.68	1.77	1.77	1.72
Average	Rat 1 Bleed 2	Rat 2 Bleed 2	Rat 3 Bleed 2	Rat 4 Bleed 2	Pre-Immunisation Bleed	NIP-HSA Positive Control	Standard Deviation					
1:25600	16.88	18.43	16.92	16.70	13.78	16.63	6.68	8.01	8.44	4.10	0.44	0.84
1:12800	18.66	19.45	19.14	19.72	20.05	22.45	2.49	4.25	2.02	4.47	3.74	2.23
1:6400	20.59	22.11	22.02	24.22	20.88	23.68	0.80	1.64	0.66	0.53	3.58	2.58
1:3200	21.61	21.18	24.29	26.56	19.43	21.63	0.45	0.84	3.82	2.96	1.13	1.41
1:1600	20.35	21.27	26.06	28.56	21.85	24.84	1.19	1.90	1.33	0.82	1.73	0.22
1:800	21.86	21.17	26.44	29.65	21.09	26.13	3.13	3.04	0.12	1.92	3.84	1.73
1:400	17.12	18.77	23.71	26.57	15.29	19.79	0.46	1.01	4.01	2.44	0.54	0.61
1:200	18.23	19.51	22.81	24.77	21.17	25.44	7.06	8.34	2.38	3.33	2.27	0.07

Positive Control

NIP-HSA Concentration (ng ml^{-1})	1	2	3	4	5	6	7	8	9	10	11	12
0	1.19	1.19	1.96	2.17	1.71	1.71	0.09	0.1	0.09	0	0.09	0.09
0.001	1.23	1.18	1.95	2.12	1.7	1.72	0.15	0.2	0.17	0	0.16	0.17
0.1	1.23	1.14	1.93	1.94	1.72	1.69	0.16	0.2	0.19	0	0.18	0.18
1	1.16	1.17	1.66	1.71	1.68	1.66	0.16	0.2	0.36	0	0.18	0.19
10	1.08	1.14	1.34	1.4	1.59	1.61	0.24	0.3	0.66	1	0.27	0.27
100	1.14	1.11	1.22	1.19	1.64	1.63	0.21	0.2	0.62	1	0.25	0.27
1,000	1.18	1.12	1.2	1.26	1.61	1.67	0.13	0.2	0.62	1	0.18	0.19
10,000	1.24	1.24	1.3	1.44	1.67	1.69	0.18	0.2	0.7	1	0.26	0.26
	ecause (I used 50 µl instead of the 100) + Supernatant V To Find Original Total In Cell						% of Total In Cell					
0	13.11	13.45	8.73	8.52	9.79	9.95	1.37	1.4	2.15	2	1.9	1.9
0.001	19.40	21.75	14.47	14.10	15.92	16.42	1.53	1.5	2.28	2	2.02	2.06
0.1	21.05	24.40	16.45	17.16	17.62	17.32	1.56	1.5	2.31	2	2.09	2.04
1	21.62	23.33	30.43	29.80	17.65	18.71	1.48	1.5	2.39	2	2.04	2.04
10	30.41	32.54	49.43	50.81	25.56	25.39	1.55	1.7	2.65	3	2.14	2.16
100	26.45	29.75	50.20	52.09	23.29	24.68	1.55	1.6	2.45	2	2.14	2.16
1,000	18.17	21.13	50.82	45.12	17.94	18.62	1.44	1.4	2.44	2	1.96	2.05
10,000	22.31	26.37	51.85	48.75	23.88	23.74	1.6	1.7	2.7	3	2.19	2.22
	Human	Canine	Equine									
0	13.28	8.62	9.87									
0.001	20.57	14.29	16.17									
0.1	22.73	16.81	17.47									
1	22.48	30.12	18.18									
10	31.48	50.12	25.48									
100	28.10	51.15	23.98									
1,000	19.65	47.97	18.28									
10,000	24.34	50.30	23.81									

Negative Control

NIP-HSA Concentration (ng ml^{-1})	1	2	3	4	5	6	7	8	9	10	11	12
0	1.39	1.42	2.26	2.35	1.62	1.68	0.13	0.12	0.1	0.11	0.11	0.1
0.001	1.61	1.45	2.32	2.16	1.65	1.76	0.13	0.13	0.11	0.11	0.1	0.1
0.1	1.41	1.43	2.19	2.38	1.7	1.63	0.14	0.13	0.11	0.13	0.11	0.1
1	1.61	1.44	2.19	2.11	1.6	1.63	0.14	0.14	0.12	0.12	0.12	0.1
10	1.61	1.4	2.12	2.39	1.58	1.65	0.13	0.12	0.11	0.12	0.12	0.1
100	1.61	1.4	2.18	2.12	1.61	1.63	0.13	0.15	0.12	0.13	0.12	0.1
1,000	1.43	1.37	2.24	2.42	1.63	1.65	0.14	0.14	0.12	0.14	0.12	0.1
10,000	1.41	1.34	2.16	2.16	1.65	1.65	0.15	0.15	0.13	0.14	0.14	0.1
	ecause (I used 50 µl instead of the 100) + Supernatant V To Find Original Total In Cell						% of Total In Cell					
0	15.45	14.25	7.94	8.56	11.67	12.41	1.6	1.66	2.45	2.57	1.83	1.9
0.001	13.72	14.71	8.95	8.86	10.91	11.74	1.9	1.7	2.55	2.37	1.85	2
0.1	16.37	15.28	9.43	9.71	10.99	12.37	1.7	1.69	2.42	2.64	1.91	1.9
1	14.45	16.28	10.10	10.14	12.57	12.83	1.9	1.72	2.44	2.35	1.83	1.9
10	13.44	14.95	9.71	9.06	13.57	11.19	1.9	1.65	2.35	2.63	1.83	1.9
100	14.00	17.74	9.69	11.22	12.97	13.30	1.9	1.7	2.41	2.39	1.85	1.9
1,000	16.28	16.57	9.68	10.30	12.65	13.70	1.7	1.64	2.48	2.7	1.87	1.9
10,000	17.54	17.99	11.04	11.62	14.42	13.43	1.7	1.63	2.43	2.44	1.93	1.9
	Human	Canine	Equine									
0	14.85	8.25	12.04									
0.001	14.21	8.90	11.32									
0.1	15.83	9.57	11.68									
1	15.37	10.12	12.70									
10	14.19	9.38	12.38									
100	15.87	10.46	13.14									
1,000	16.42	9.99	13.18									
10,000	17.77	11.33	13.93									