Insect Bite Hypersensitivity of Horses

Development of ELISA for measuring antibody response against allergens produced in insect cells

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Table of context:

Figures:

Tables:

Abstract

Background: Insect bite hypersensitivity is a recurrent seasonal dermatitis of horses. It is classified as allergy mainly caused by bites of *Culicoides* spp. Allergen genes, originated in the salivary glands of *Culicoides nubeculosus* have been isolated and the proteins expressed in insect cells. The aim of this project was to establish an enzyme linked immunosorbent assay (ELISA) for efficient evaluation of insect bite hypersensitivity immunotherapy in horses.

Material and methods: Blood samples from 12 healthy Icelandic horses from a insect bite hypersensitivity vaccination study was used in optimization of an ELISA procedure. Bac-1 Cul n 4 allergen protein purification from insect cells was conducted with His select nickel affinity gel and used for the ELISA.

Results and conclusions: An ELISA was established through a number of optimization steps. High background noise was observed for the most part of the project, but was efficiently reduced with high-salt buffers and different washing techniques targeting unspecific and weak off-target interactions.

List of abbreviation

ELISA	Enzyme linked immunosorbent assay
NK cells	Natural killer cells
PPR	Pattern recognition receptors
PAMP	Pathogen-associated molecular patterns
APC	Antigen-presenting cell
DAMP	Damage- (or danger) associated molecular patterns
IBH	Insect bite hypersensitivity
PBMC	Peripheral blood mononuclear cell
HRP	Horseradish peroxidase
PBS	Phosphate buffered saline
TBS-T	Tween 20
OD	Optical density
RT	Room temperature
BSA	Bovine serum albumin
pAb	polyclonal antibody

1. Introduction

The objective of this project was to establish an enzyme linked immunosorbent assay (ELISA) using purified allergens produced in insect cells. Subsequently to measure IgG antibody response of vaccinated horses against the relevant allergens.

The work was done with the aim of adapting an ELISA procedure that existed for *E. coli* produced *Culicoides* allergens for insect cell produced allergens.

The project is a step towards developing more efficient tests to evaluate the benefit of insect bite hypersensitivity immunotherapy in horses.

The experimental study was conducted at Keldur's facilities and the material used was attained from on-going IBH studies at Keldur.

2. Theoretical background

2.1 The immune system

The body is protected from pathogens and diseases by the immune system that is composed of a variety of effector cells and molecules that make up the immune system. The immune system discriminates between self and non-self substances called antigens. Its role is to detect those foreign molecules and inactivate or destroy them [1]. The immune system can be divided into the innate immune system and the adaptive immune system. All cells of the immune system secrete cytokines which influences the activity of other cells and often themselves as well [2].

The innate system, also known as the nonspecific immune system, is the first line of defence and responds rapidly without any memory. The cells of the innate system are monocytes, macrophages, dendritic cells, granulocytes (neutrophils, eosinophils, and basophils), mast cells and natural killer cells (NK cells) [3]. The role of the innate system is to take up pathogens, destroy them and display antigens to the cells of the adaptive system. Macrophages, neutrophils and dendritic cells have pattern recognition receptors (PPRs) on their surface, which bind to certain type of pathogen-associated molecular patterns (PAMPs) on the surface of the pathogens. These cells are called antigen-presenting cells (APCs) [3]. Molecules that can initiate and perpetuate immune response in the non-infectious inflammatory response (in contrast to the pathogen-associated) are known as damage (or danger)-associated molecular pattern molecules (DAMPs). DAMPs consist of molecules that are released by cells undergoing stress or abnormal cell death, which are perceived by APCs and that induce the APCs to become activated [4].

The adaptive immune system takes longer to activate but gives very specific response to a specific pathogen. It has immunological memory after an initial response. The effector cells of the adaptive system are B-lymphocytes (B-cells) and T-lymphocytes (T-cells) [5]. APCs present peptides on their surface for T-cell recognition. The T-cells differentiate into $CD8^+$ killer T-cells and $CD4^+$ helper T-cells. $CD8^+$ killer T-cells destroy virus-infected cells and tumour cells [2, 6]. $CD4^+$ helper T-cells assist other white blood cells in immunologic processes, including maturation of B-cells into plasma cells and memory B-cells and activation of $CD8^+$ killer T-cells and macrophages. Once the $CD4^+$ helper T-cells are activated they differentiate further due to cytokines they secrete themselves (Figure 1). Upon re-exposure to a cognate antigen, antigen-specific memory

T-cells expand to large numbers of effector T-cells. These cells persist long-term after an infection [7].

B-cells produce antibodies or immunoglobulins that are proteins specific for antigens. Their role is to bind and neutralize pathogens, promote phagocytosis and activate the complement system. Upon activation, the B-cells differentiate into plasma cells that produce antibodies or long-lived memory B-cells [7]. At subsequent encounter with the same antigen the memory B-cells respond faster and more efficiently. There are five isotypes of immunoglobulins, IgA, IgD, IgE, IgG and IgM with different functions [2, 5].

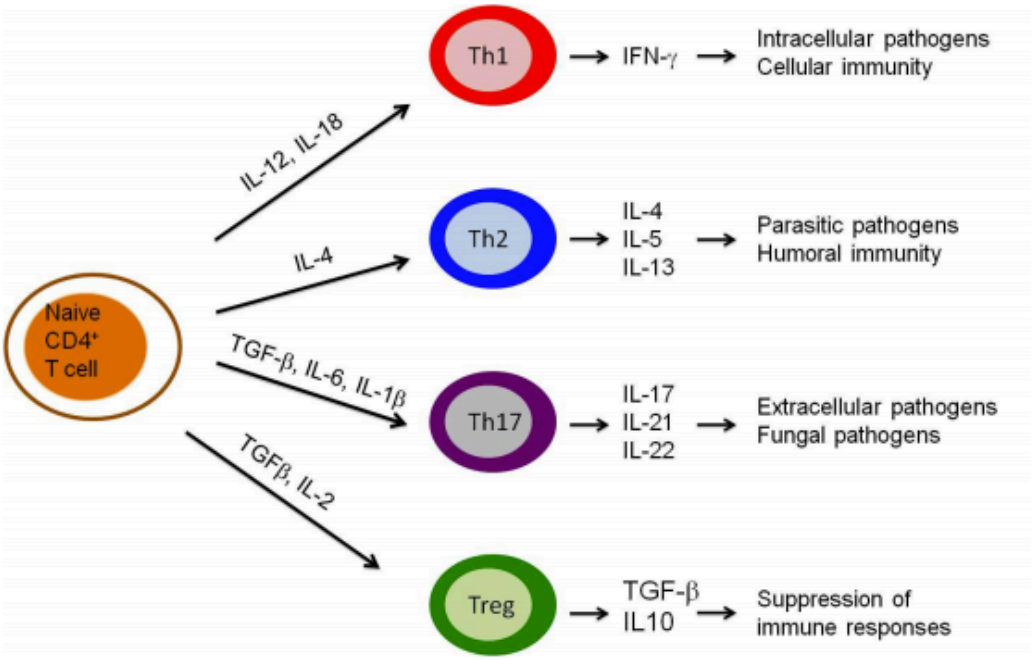

Figure 1: Differentiation of naïve CD4+ helper T-cells. Differentiation of naïve CD4+ helper T-cells into different T effector subsets, the main cytokines and the function of the subsets. Schematic picture: Sigurbjörg Þorsteinsdóttir.

2.1.1 Allergy

Hypersensitivity type I allergy is a Th2 IgE mediated response to innocuous proteins. Reaction occurs because the individual's immune system produces IgE antibodies against an allergen. Subsequent exposure to the allergen triggers the activation of IgE-binding cells, including mast cells and basophils, in the exposed tissue, leading to a series of responses that are characteristic of allergy. Allergies are Th2 responses with production of IL-4, IL-5 and IL-13 cytokines, which are the cell mediators of allergy (Figure 2) [8].

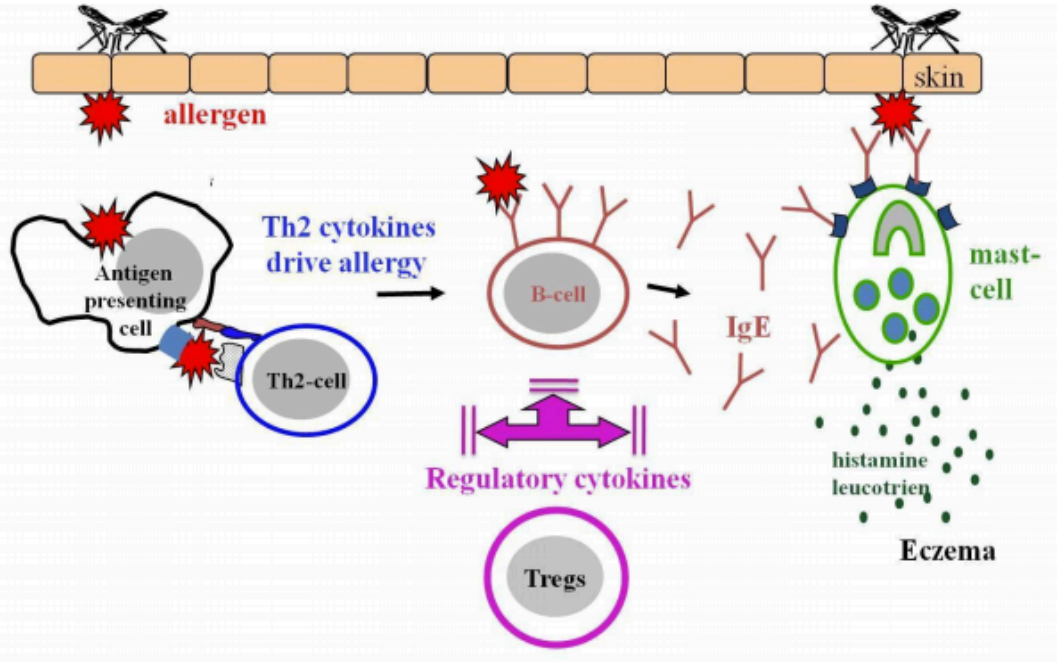

Figure 2. IgE mediated response in IBH. An allergen from the fly saliva is picked up by an APC and shown to naïve CD4+ T helper cell, which differentiates into a Th2 cell. The Th2 cell induces a B-cell to produce specific IgE antibodies against the allergen, The IgE binds to high affinity receptor FcεRI on mast cells and basophils. The horse is now sensitized against the allergen and upon re-exposure it will elicit allergic response due to the release of various inflammatory mediators of mast cells and basophils. Schematic picture: Sigurbjörg Þorsteinsdóttir.

2.2 Insect bite hypersensitivity (IBH)

Insect bite hypersensitivity (IBH) (summer eczema or sweet itch) is a recurrent seasonal dermatitis of horses [9]. IBH is classified as allergy, with production of IgE and inflammatory mediators, mainly caused by bites of *Culicoides* spp. (biting midges) [10-13]. Figure 2 shows in schematic way the interplay between allergens, cells, cytokine and antibodies resulting in allergic reactions. The midges are not indigenous to Iceland and therefore the IBH does not affect horses in Iceland [14]. Horses born in Iceland and exported to the European continent are more strongly affected than other breeds. The frequency of IBH in horses exported from Iceland and not protected from the flies is around 50% after two years or more in *Culicoides* infested areas. In contrast, around 5-10% of Icelandic horses born on the continent from imported parents suffers from IBH, which is similar frequency as most foreign breeds [15-17]. Presently the best way to avoid IBH is to keep the horses away from the biting flies mainly by housing them in the twilight and using blankets to cover the main feeding sites [9].

2.2.1 IBH Allergens

Since the year 2000 there has been an ongoing collaborative project between Keldur and the University of Berne, Switzerland with the final aim to develop immunotherapy against IBH. Salivary gland proteins of *Culicoides* ssp. are the main allergens inducing IgE-mediated IBH in horses [15, 18, 19]. The allergen genes, originated in the salivary glands of *Culicoides nubeculosus* have been isolated and the proteins expressed in *E. coli* (Table 1) [20-22]. The cloning and sequencing of the allergen genes from the laboratory bred midges strain *C. nubeculosus* [21] has made it possible to isolate allergens from common wild *Culicoides* strains.

Table 1. Isolated and expressed allergens originated in the salivary glands of *C. nubeculosus* .

Allergen	Homology to	MW (kD)
Cul n 1	Antigen 5 like protein	25,4
Cul n 2	Hyluronidase	46,7
Cul n 3	Putative cysteine endopeptidase	44,6
Cul n 4	Secreted salivary protein	17,5
Cul n 5	Secreted salivary protein	45,7
Cul n 6	Secreted salivary protein	16,9
Cul n 7	Unknown salivary protein	20,9
Cul n 8	Maltase	68,7
Cul n 9	D7-related salivary protein	15,5
Cul n 10	Secreted salivary protein	47,8
Cul n 11	Trypsin	30,1

In order to be able to evaluate the immunotherapy it is necessary to measure antibody response. *C. nubeculosus* allergens produced in *E.coli* and used for vaccination give too much background when used to stimulate peripheral blood mononuclear cells (PBMC) *in vitro* for cytokine production. This could be due to contamination with lipopolysaccharide or *E. coli* proteins. It is vital to be able to measure the cytokine profile after vaccinations to evaluate the route of the immune response. Therefore the allergens need to be produced in another system and as they originate from insects, Baculovirus and insect cells is the most obvious one to use. Seven of the allergens have already been expressed in insect cells (unpublished results).

2.3 ELISA

The immune system of horses is mediated by the same cells, molecules and mechanisms as in other mammals [23]. In order to measure antibody response (IgG, IgG subclasses, IgE and IgA) of vaccinated horses against the relevant allergens, is necessary to have an efficient enzyme linked immunosorbent assay (ELISA).

ELISAs are plate-based assays designed for detecting and quantifying antibodies. ELISAs are typically performed in 96-well (or 384-well) polystyrene plates, which will passively bind antibodies and proteins. The bottom of each well is coated with a protein to which will bind the antibody to be measured. A sample with antibodies (often blood serum), called primary antibodies is incubated in the wells and after some time, the serum is removed and weakly adherent antibodies are washed off with a series of buffer rinses. To detect the bound antibodies, a secondary antibody is added to each well. The secondary antibody would bind to all the antibodies in the well (species specific). Attached to the secondary antibody is an enzyme that can metabolize colorless substrates into colored products, such as horseradish peroxidase (HRP) or alkaline phosphatase (AP). After an incubation period, the secondary antibody solution is removed and loosely adherent ones are washed off as before. The final step is the addition the enzyme substrate and the production of colored product in wells with secondary antibodies bound. When the enzyme reaction is complete, the entire plate is placed into a plate reader and the optical density; OD (i.e. the amount of colored product) is determined for each well. The amount of color produced is proportional to the amount of primary antibody bound to the proteins on the bottom of the wells (Figure 3).

The ELISA assay can be performed with a number of modifications to the basic procedure. The key step, immobilization of the antigen of interest, can be accomplished by direct adsorption to the assay plate or indirectly via a capture antibody that has been attached to the plate. The antigen is then detected either directly (labeled primary antibody) or indirectly (labeled secondary antibody). The most powerful ELISA assay format is the sandwich assay. This type of capture assay is called a “sandwich” assay because the analyte to be measured is bound between two primary antibodies – the capture antibody and the detection antibody (Figure 3).

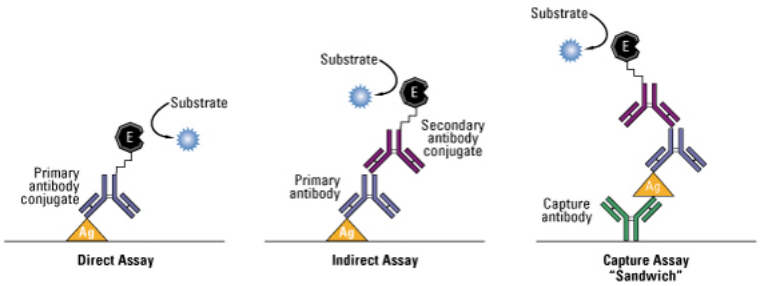

Figure 3. ELISA formats. The antigen of interest is immobilized by direct adsorption to the plate's surface or by first attaching a capture antibody. Detection of the antigen is performed by using an enzyme-conjugated primary antibody (direct) or a matched set of unlabelled primary and conjugated secondary antibodies (indirect) [24].

The direct detection method uses a labelled primary antibody that reacts directly with the antigen. Direct detection can be performed with antigen that is directly immobilized on the assay plate or with the capture assay format. The indirect detection method uses a labeled secondary antibody for detection and is the most popular format for ELISA. The secondary antibody has specificity for the primary antibody. In a sandwich ELISA, it is critical that the secondary antibody is specific for the detection primary antibody only (and not the capture antibody) or the assay will not be specific for the antigen. Generally, this is achieved by using capture and primary antibodies from different host species (e.g., mouse IgG and rabbit IgG, respectively).

At Keldur, immunoblots have been used to measure total IgG responses of vaccinated horses. For this purpose the functional ELISA for the IBH allergens will be of much convenient. Allergens purified on native and denature form will be compaired [21] and IgG (total and subclasses) and IgE response of IBH affected horses against the allergens produced in *E. coli* and insect cells will be compared in ELISA.

3 Materials and methods

3.1 Horses and vaccination treatment

Twelve healthy Icelandic horses, 7-10 years old, were injected into the submandibular lymph nodes with 80 uL of protein (Cul n 3, 4, 8 and 10) [21] three times with a 4-week interval over 8 weeks. The horses were divided into two groups depending on different adjuvants, 1) 50 uL alum in 270 uL PBS and 2) 50 uL alum and 100 uL MPL in 170 uL PBS (total 400 uL, divided to the two lymph nodes). Blood samples were harvested before the first vaccination (week 0) and then two weeks after each vaccination (week 6 and 10). Blood samples were labelled SEA/M-1 – 12, SE = summer eczema, A = alum, M = MPL.

3.2 Protein expression

For production of the allergen, the Bac-to-Bac® expression system (Invitrogen, Carlsbad, CA) was used. Allergen genes were cloned onto a pFastBac plasmid and into the genetic material of the baculovirus (bacmid) using recombination into DH10Bac™ *E.coli*. Production of recombinant viruses was done in Sf-9 (*Spodoptera frugiperda*) (ATCC, Teddington, UK) cells and of recombinant proteins in High-5 cells (*Trichoplusia ni*) (ATCC) (Figure 4).

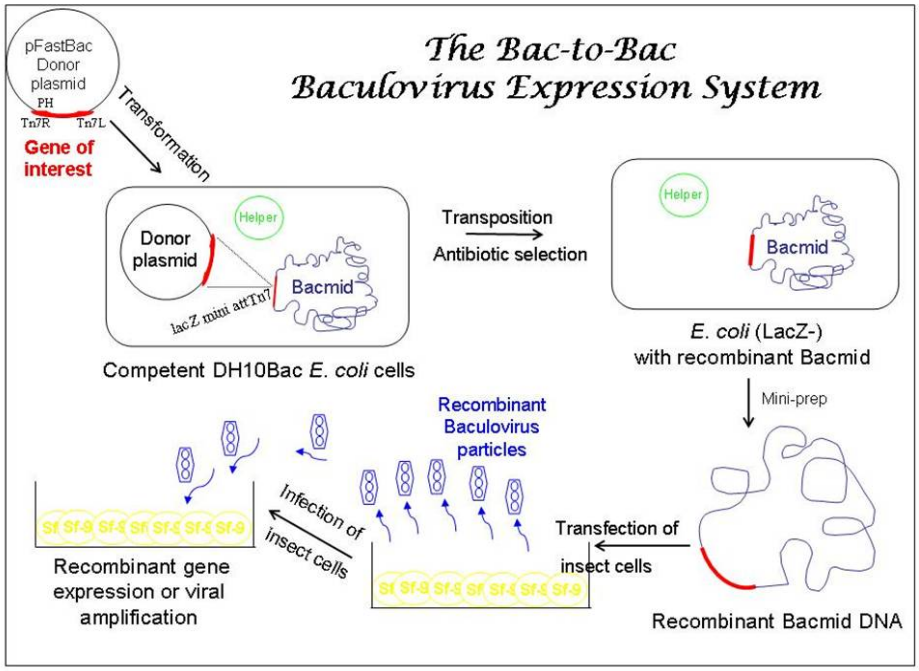

Figure 4: Bac-to-Bac® expression system. Schematic figure of the production of recombinant baculoviruses and allergen expression. . Schematic picture: Sigurbjörg Þorsteinsdóttir.

3.2.1 Protein isolation and purification

Proteins from 100x10^6 High-5 cells were isolated in 8 mL lysis buffer (Appendix A) with 80- 160 µL Protease Inhibitor Coctail (PIC) from Sigma-Aldrich (St. Louis, MO) and sonicated on ice 5x for 10 sec with 20 sec interval. The cell pellet was centrifuged at 18800 x g and the supernatant mixed with His select nickel affinity gel (Sigma-Aldrich) on orbital shaker for 2 hours at 20°C, spun down at 1500 x g for 5 min and the supernatant collected. The pellet was resuspended in wash buffer (Appendix A) (10x the volume of the gel/pellet) and mixed for 5 min and spun down at 1500 x g for 5 min. The pellet was washed 2x in wash buffer with lower concentration of Imidazol and 2x in buffer with higher concentration of Imidazol. The pellet was then dissolved in elution buffer 1 (Appendix A) (in equal volume as the gel/pellet) and applied on a plastic column with 0.2 µm membrane (Sigma-Aldrich) and incubated for 10 min at RT for elution. The elution was repeated with elution buffer 1 and then 2x with elution buffer 2 (Appendix A). Elution fractions were stored in 4°C and tested with coomassie blue staining according to Wong *et al.* [25] and in WB.

The purified protein samples were dialysed in Slide-A-Lyzer® Dialysis Cassette G2 (Thermo Fisher Scientific, Waltham, MA) in appropriate buffer, phosphate buffered saline (PBS) and elution buffer without Imidazol. The dialyse buffer was at a total of 300 times the volume of the sample or more and the sample dialysed for at least 2 hours at 4°C, then the buffer was changed, the sample dialysed overnight at 4°C, spun down at 18800 x g for 3 min and supernatant collected. The protein samples were sterile filtered with 0.2 µm filter (GE Healthcare Life Sciences, Little Chalfont, UK), stored at 4°C and tested with coomassie blue staining.

The proteins were separated by SDS-PAGE in the Mini-protean II system (Bio-Rad, Hercules, CA), the samples were denatured with a sample buffer (Appendix A), boiled for 5 min at 100°C, spun down for 2 min and run on 12% acrylamide gels under reducing conditions. Spectra Multicolor Broad Range Protein Ladder from Thermo Fisher Scientific #26623 was used to estimate the size of the proteins. The proteins were stained with coomassie blue [25].

Following SDS-Page the proteins were transferred to a PVDF membrane by wet transfer in the Miniprotean II system (Bio-Rad) for 1 hour in a transfer buffer (Appendix A). After the transfer the membranes were incubated in Tris buffered saline containing 0.1% Tween 20 (TBS-T) with extra 2% Tween for 30 min at RT. The membranes were washed with TBS-T, 5x for 5 min. Then incubated with primary antibodies overnight at 4°C, washed again and incubated at RT for 1 hour with the conjugate, goat-anti mouse-AP (Jackson) diluted 1:5000 in TBS-T. The membranes were developed using BCIP/NBT (Appendix A) from Roche (Basel, Switzerland) diluted 1:50 in alkaline phosphatase buffer (Appendix A) after having been washed.

The concentration of protein samples was measured using Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific) in VICTOR^3 (Perkin Elmer, Watham, MA) according to manufacturer‘s protocol.

3.3 ELISA

Coating concentration of proteins was obtained and determined from the coomassie measurements. The development and optimization of the ELISA procedure began with a general basic protocol described as follows:

1. Coating (recombinant proteins - allergens)

Allergens diluted in coating buffer (Appenix B) and 100 µL applied per well in a flat-bottomed polystyrene 96-well MaxiSorp™ plate (Nunc, Roskilde, Denmark). Plates were incubated for 2 hours at 37°C (and if to be used later, stored at -20°C).

Washing technique:

Plates were manually washed by pouring washing buffer (Appendix B) over the plates, into the wells, five times and splashed off with no specific interval timing.

2. Primary antibody:

After washing, serum from vaccinated horses was diluted in dilution BSA buffer (Appendix B) and 100 µL applied to wells. Plates were incubated for 90 min at 37°C.

3. Secondary antibody (conjugate):

After washing, an HRP-anti-horse IgG (Jackson Immuno Research Laboratories Inc., West Grove, PA) diluted in dilution BSA buffer was applied in 100 µL per well and plates incubated for 1 hour at 37°C.

4. Enzyme substrate:

After washing, an OPD-substrat (Appendix B) solution was applied in 100 µL per well and light prevented from the plates. The reaction was stopped after 10 min using sulphuric acid (H_2SO_4 (4M)).

The entire plate was placed into a spectrophotometer (VICTOR^3 (Perkin Elmer)) and the optical density (O.D.) was determined for each well at 490 nm.

3.4 Statistical analysis

Two-way ANOVA was used to compare the mean values between weeks of vaccination (week 0, 6 and 10) and between individual horses. A Tukey-Kramer's post-test (all pairwise comparisons) was made in order to compare each vaccination week within each serum dilution and to compare each horse within each serum dilution. A one-way ANOVA was then used to compare the mean values between the two different adjuvants. P-values of <0.05 were considered statistically significant. All statistical analysis was performed using the StatPlus (AnalystSoft Inc., Alexandria, VA) software and Excel (Microsoft, WA).

4 Results

4.1 Protein isolation and purification

Bac-1-Cul n 4 proteins were purified from lysed Cul n 4 baculovirus infected High-5 cells with His select nickel affinity gel. The samples were run on SDS-PAGE and stained with coomassie blue (Figure 5).

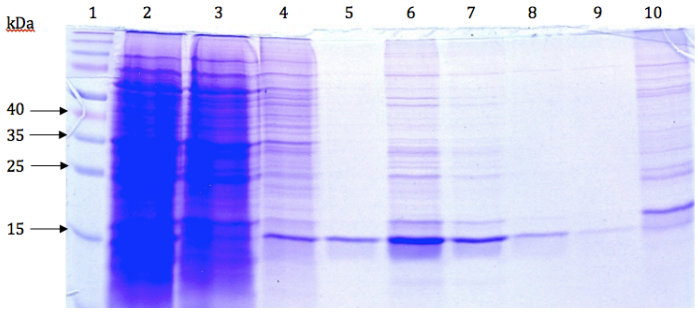

Figure 5: Coomassie blue staining of purified Bac-1-Cul n 4 proteins. Size of protein (kDa) is indicated with arrows. Lane 1 ladder, 2 before purification, 3 after gel binding, 4 wash 1, 5 wash 3, 6 elution 1, 7 elution 2, 8 elution 3, 9 elution 4, gel sample

The purified protein samples were dialysed in appropriate buffer, phosphate buffered saline (PBS) and later elution buffer without Imidazol. The dialysed protein samples were sterile filtered, measured in bradford, and tested with coomassie blue staining finally stored at 4°C Figure 6).

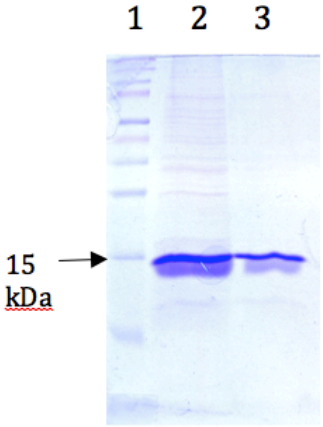

Figure 6: Coomassie blue staining of dialysed Bac-1-Cul n 4 proteins. 1. Ladder, 2. Before dialysis, 3. After dialysis.

Bac-1-Cul n 4 expression was validated with Cul n 4 specific polyclonal antibodies [21] in western blotting (Figure 7).

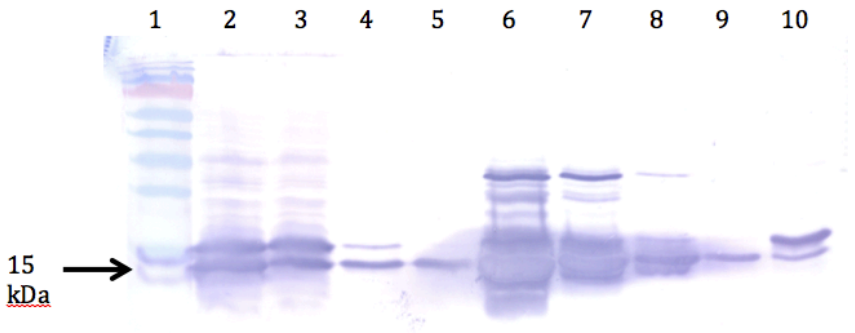

Figure 7: Expression of Bac-1-Cul n 4 proteins in High5 cells. 1. ladder, 2. Before purification, 3. After binding, 4. Wash 1, 5. Wash 3, 6. Elution 1, 7. Elution 2, 8. Elution 3, 9. Elution 4, 10. Gel sample.

4.2 ELISA development

Results from every ELISA conducted can be seen in Appendix C. Results from the ELISA development will be reviewed in the order in which they were conducted.

Dilutions of the coating antigen. For the first ELISA experiment, the general basic procedure described in Chapter 3.3 was used. Protein coating: Bac-1-Cul n 4 concentration was 10, 5, 2, 1, 0.5, 0.2 and 0.1 ug/well (or 100, 50, 20, 10, 5, 2 and 1 ug/ml). Serum sample: SEA/M – 8, week 0 (pre-vaccination) and week 6 (2 weeks after the second vaccination) in 1:100 dilution. Conjugate: 1:2000 dilution. Substrate incubation time: 10 min. This resulted in no difference in O.D. between week 0 and week 6 as can be seen in Figure 8.

Testing of conjugate dilution. Because of the high background signal in the samples from week 0, the conjugate was further diluted to 1:5000. Other variables were maintained as before. Protein coating: Bac-1-Cul n 4 concentration was 10, 5, 2, 1, 0.5, 0.2 and 0.1 ug/well (or 100, 50, 20, 10, 5, 2 and 1 ug/ml). Serum sample: SEA/M – 8, week 0 and week 6 in 1:100 dilution. Substrate incubation time: 10 min. Again, no difference can be seen in O.D. values between serum from week 0 and week 6 (Figure 8). Both samples gave low signals overall.

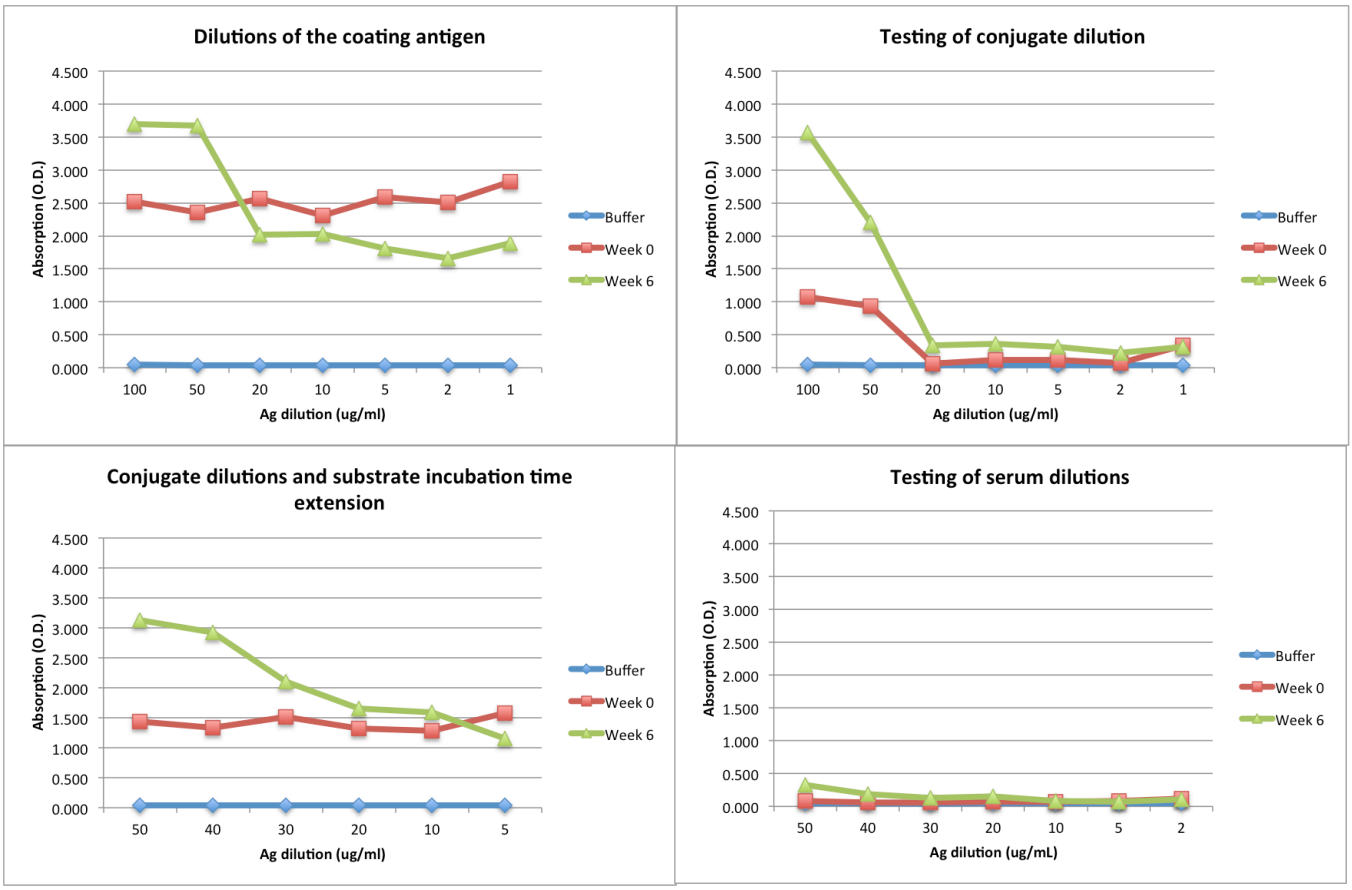

Figure 8: Results from the first four ELISA experiments. Dilutions of the coating antigen: No difference can be seen between serum from week 0 and 6. Testing of conjugate dilution: No difference is observed between serum from week 0 and 6 and both samples give low signals. Conjugate dilutions and substrate incubation time extension: Difference observed at higher Bac-1-Cul n 4 protein concentration. Testing of serum dilutions: No difference between week 0 serum and week 6 detected.

Conjugate dilutions and substrate incubation time extension. Further dilution of the conjugate was then tried, 1:5000, 1:7000 and 1:10.000 and substrate incubation time extended to 20 min. Protein coating: Bac-1-Cul n 4 concentration was 50, 40, 30, 20, 10 and 5 ug/ml. Serum sample: SEA/M–8, week 0 and week 6 in 1:100 dilution. Substrate incubation time: 10 min. Again, this resulted in relatively high signals from week 0 serum. Although slightly more of a difference can be observed between week 0 and week 6 for higher Bac-1-Cul n 4 allergen concentration. Figure 8 shows the results from using conjugate dilution 1:7000 (all data can be seen in Appendix C). Signals become weaker with higher conjugate dilution.

Testing of serum dilution. Serum sample: SEA/M–8 dilutions 1:100, 1:500, 1:1000 and 1:2000. Protein coating: Bac-1-Cul n 4 concentration was 50, 40, 30, 20, 10 and 5 ug/ml. Conjugate: 1:7000. Substrate incubation time: 20 min. For this experiment, all signals

were very low, especially in dilutions 1:500 and above (most of them under 0.200 O.D.). No difference between week 0 serum and week 6 detected. Signals become weaker with higher serum dilutions. Results from serum dilution 1: 1000 can be seen in Figure 8. All data can be seen in Appendix C.

Pre-colostrum foals. Pre-colostrum serum (free of Ig) in 1:100 dilutions was tested from two foals (Dúfa and Eiríkur). Conjugate: 1:7000. Substrate incubation time: 20 min (Figure 9). From this experiment we can reason that there are no antibodies in the foal serum interacting with the plastic or allergen.

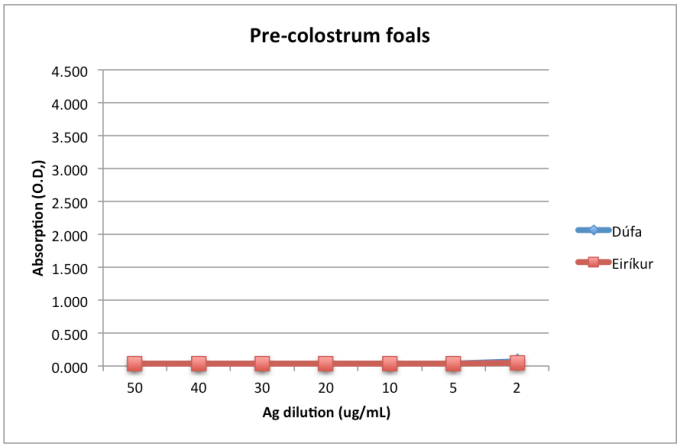

Figure 9: ELISA with serum from pre-colostrum foals. No signals at all.

Testing of dry milk powder blocking and two different conjugates. For the next experiment, a blocking step was added before the serum sample step. 5% dry milk powder and 5% tween 20 in PBS was applied for 2 hours at room temperature (RT). Conjugate: 1:7000 (Jackson) and an older one from Sigma (SAB3700152) at 1:2000. Serum samples: SEA/M–8 dilutions 1:100 and 1:500. Protein coating: Bac-1-Cul n 4 concentration 50, 40, 30, 20 and 10 ug/ml. Substrate incubation time: 20 min. Two plates were run, one with blocking and the other without blocking. No obvious difference was observed between the two plates (i.e. block or no block). The secondary antibody from Sigma gave weaker signals. No difference can be seen in O.D. values between serum from week 0 and 6. Results from using serum samples in 1:100 dilution for both blocking and not blocking, and for both conjugates can be seen in Figure 10.

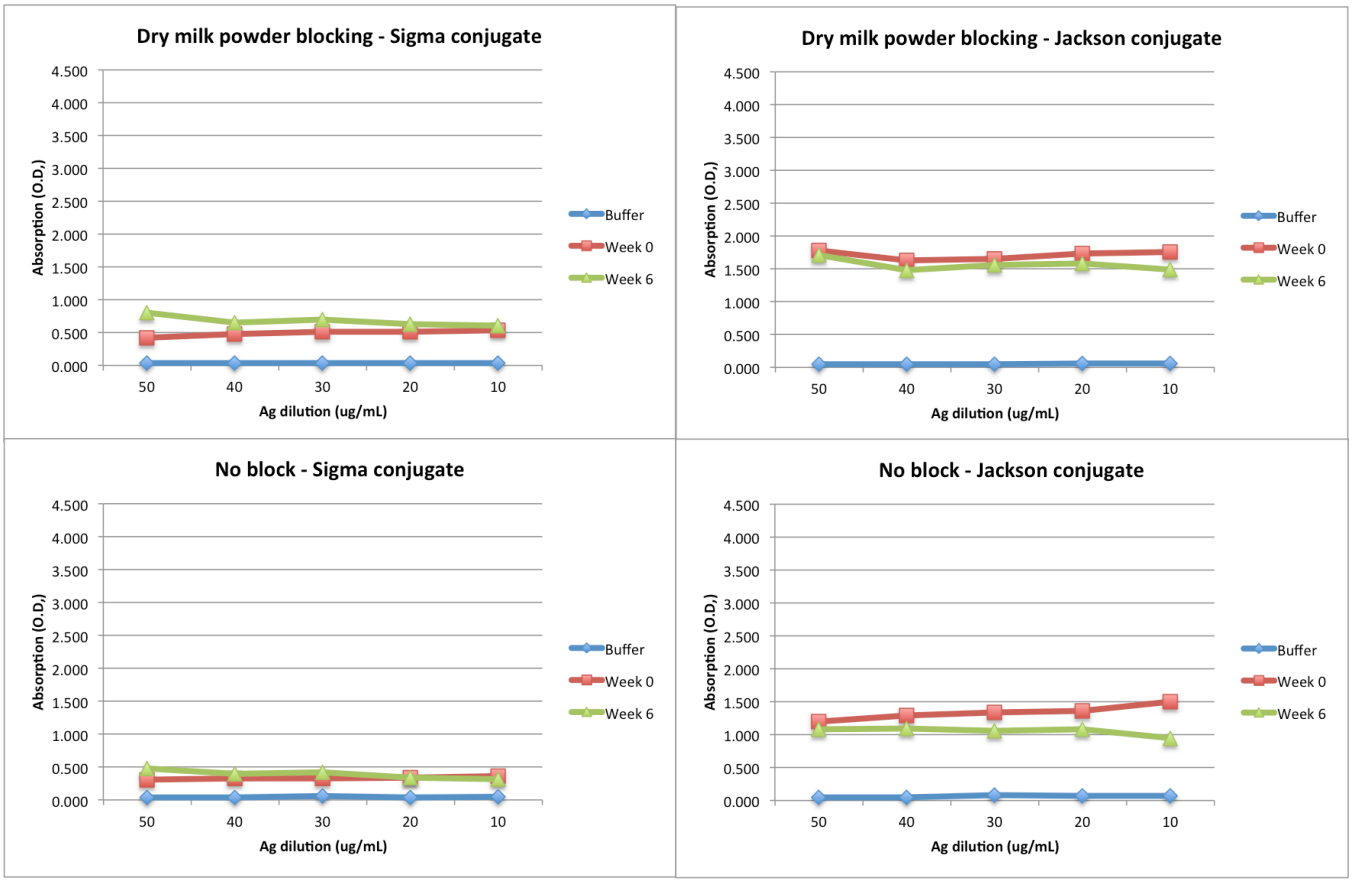

Figure 10: Results from a blocking experimenting ELISA. Sigma no block: No difference is observed between serum from week 0 and 6 and both samples give low signals. Jackson no block: No difference can be seen between serum from week 0 and 6. Sigma block: No difference is observed between serum from week 0 and 6 and both samples give low signals. Jackson block: No difference can be seen between serum from week 0 and 6. No obvious difference was observed between blocking and not blocking.

Capture ELISA with dilution BSA buffer blocking step. Capture (sandwich) ELISA was tried using polyclonal antibodies against Cul n 4 [21] in dilutions 1:2500, 1:5000, 1:10.000 for coating the plates (2 hours at 37°C). Blocking step: the dilution BSA buffer 2 hours, RT. Protein coating: Bac-1-Cul n 4 concentration was 50, 40, 20, 10, 5 and 1 ug/ml but incubation time was shortened to 1 hour. Serum samples: Week 0 serum from another horse (SEA/M - 7) was also tested to ensure that this high background signal in week 0 serum observed is not only in the sera from horse no. 8 all in 1:100 dilution. Conjugate: 1:7000. Substrate incubation time: 20 min. The week 0 serum from horse no. 7 gave lower O.D. values than from horse no. 8, which still had weak signals. Week 6 serum from horse no. 8 gave weak signals. Figure 11 shows the results from using the anti Cul n 4 pAb in 1:5000 dilution. No apparent difference could be observed between Cul n 4 pAb dilutions.

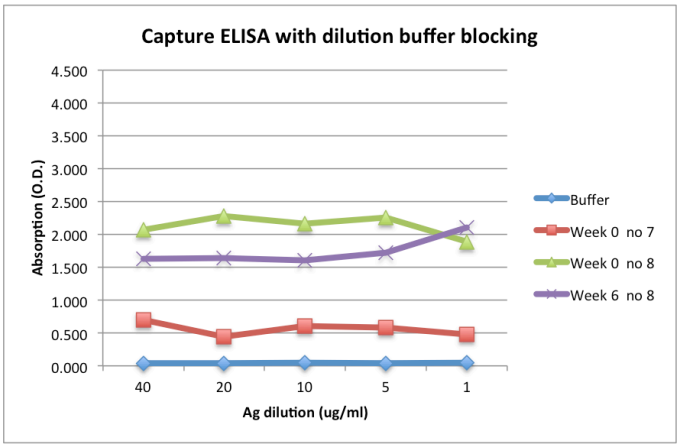

Figure 11: Results from the first capture ELISA. The week 0 serum from horse no. 7 gave lower O.D. values than from horse no. 8, which still had weak signals. Week 6 serum from horse no. 8 gave low signals.

Testing of gelatin blocking and Cul n 4 pAb dilutions. Blocking step: Another blocking agent was tested; gelatin flaked bloom 50 (ICN Biochemicals, Irvine, CA) in comparison to the dilution BSA buffer. Capture step: Cul n 4 pAbs tested at 1:2500, 1:5000 and 1:10000. Protein coating: Bac-1-Cul n 4 concentration was 20, 10 and 1 ug/ml. Serum samples: from SEA/M-10 in dilution 1:200. Conjugate: 1:7000. Substrate incubation time: 20 min. This resulted in low O.D. values for all wells, most of them between 0.2 and 0.3. No difference was seen between blocking agents or Cul n 4 pAbs dilutions. Figure 12 shows the results from using Cul n 4 pAbs in 1:5000 dilution and both blocking agents.

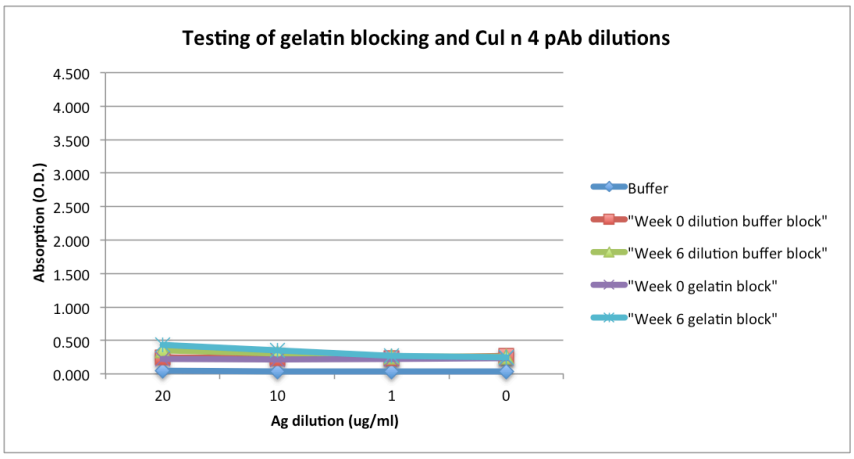

Figure 12: Results from blocking agent testing. Low O.D. values for all. No difference was seen between blocking agents or Cul n 4 pAb dilutions.

Testing of microplate types. Next, a different type of microplate was used; Immulon® 2HB Flat Bottom MicroTiter® Plate (Immuno Chemistry Technologies, Bloomington, MN) in order to test the binding to the plastic. Capture step: Cul n 4 pAb in dilution 1:5000. Blocking step: the dilution BSA buffer incubated for 1 hour, RT. Protein coating: Bac-1-Cul n 4 concentration was 20, 10 and 1 ug/ml. Serum samples: from SEA/M-10 in dilution 1:200. Conjugate: 1:7000. Substrate incubation time: 15 min. No difference could be seen between the two types of plates/surface. Relatively low O.D. values were detected and a slight difference could be observed between O.D. values in serum samples from week 0 and 6 (Figure 13).

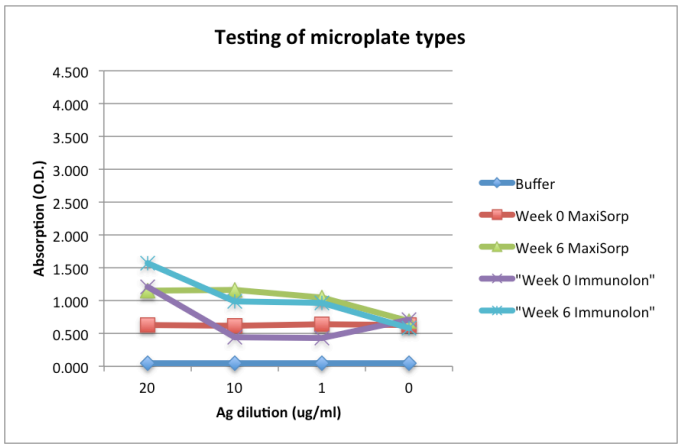

Figure 13. Results from comparing microplate surface types. No difference could be seen between the two types of plates/surface. Relatively low O.D. values were detected.

Capture ELISA with high-salt. A new blocking agent was then tested; a high-salt dilution BSAs buffer, (0.85 M NaCl: addition of 20.45 g NaCl) for 1 hour at RT. A new washing technique was also installed; a Thermo Scientific™ Nunc™ "Immuno Washer", where wells were filled and emptied with the usual washing buffer three times using the washer and then the wells were filled and the buffer left in the wells for 3 min. This washing was repeated 3 times, using high-salt washing buffer (0.85 M NaCl). Capture step: Cul n 4 pAb in dilution 1:5000. Protein coating: Bac-1-Cul n 4 concentration was 40, 20, 10 and 5 ug/ml. Serum samples: from SEA/M-10 in dilution 1:200. Conjugate: 1:2000 and 1:4000. Substrate incubation time: 10 min. This resulted in a clear difference in O.D. values between serum samples from week 0 and 6. Figure 14 shows the results from using secondary antibody in dilution 1:4000.

Capture ELISA with high-salt, optimizing serum and conjugate diltutions. For the next experiment, everything was diluted in high-salt dilution BSA buffer (0.85 M NaCl). Washing technique: "Immuno washer". Capture step: Cul n 4 pAb at 1:5000. Blocking step: high-salt dilution BSA buffer, 1 h RT. Protein coating: Bac-1-Cul n 4 concentration was 40, 20, 10 and 5 ug/ml. Serum samples: SEA/M-10 in 1:200 and 1:400. Conjugate: 1:2000 and 1:4000. Substrate incubation time: 15 min. A difference between serum samples from week 0 and 6 was observed again in the O.D. values. Figure 14 shows the results from using a dilution of 1:2000 for the secondary antibody and 1:200 for the serum samples.

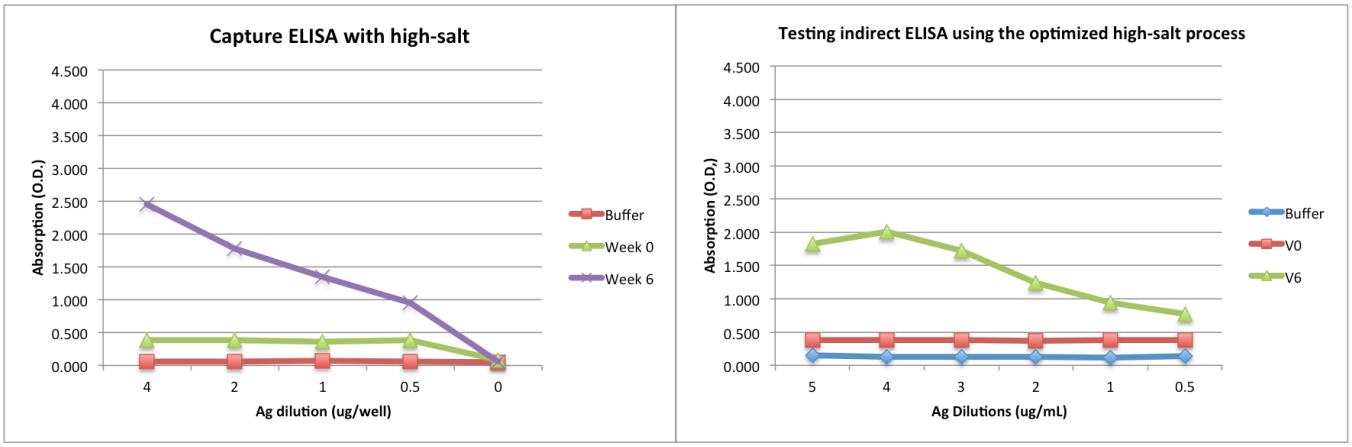

Figure 14: Results from using high-salt buffers. This resulted in a clear difference in O.D. values between serum samples from week 0 and 6 in both experiments.

Testing indirect ELISA using the optimized high-salt process. Using capture proteins is an additional step and therefore, an indirect ELISA was tested again but with the above-mentioned high salt optimized process. Washing technique: "Immuno washer". Blocking step: high-salt dilution BSA buffer, 1 h RT. Protein coating: Bac-1-Cul n 4 concentration was 40, 30, 20, 10 and 5 ug/ml. Serum samples: SEA/M-10 in 1:200. Conjugate: 1:2000, 1:4000 and 1:7000. Substrate incubation time: 10 min. A clear difference between week 0 and week 6 sera was seen. Figure 15 shows the results from using the secondary antibody in 1:7000 dilutions. All data can be seen in Appendix C.

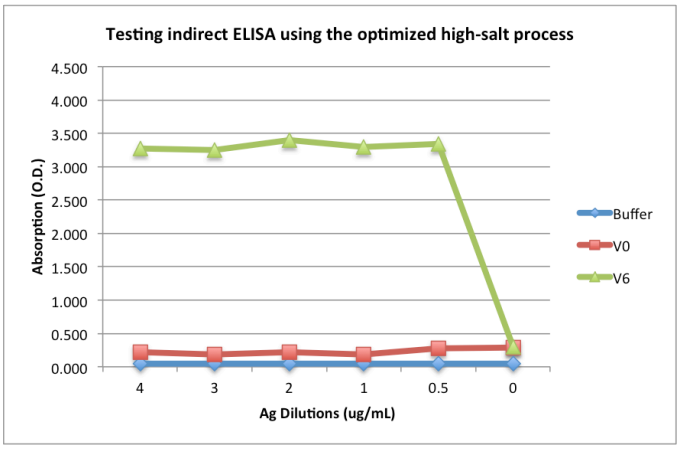

Figure 15: Results from high-salt buffer, indirect ELISA. A clear difference between week 0 and week 6 serums is obvious.

Bac-1 Cul n 4 protein coating serial dilutions. The next step was to make serial dilutions of the Bac-1-Cul n 4 proteins in order to find an optimized concentration to be used for future assays with this protein. Washing technique: "Immuno washer". Blocking step: high-salt dilution BSA buffer, 1 h RT. Protein coating: Bac-1-Cul n 4 concentration was 20, 10, 5, 4, 3, 2, 1 and 0.5 ug/ml. Serum samples: SEA/M-10 and SEA/M-11 1:200. Conjugate: 1:6000 and 1:7000. Substrate incubation time: 10 min. A clear difference between week 0 and week 6 serum was obvious at all Bac-1-Cul n 4 concentrations and 2 ug/mL was chosen for the next experiment. Figure 16 shows the results from using horse no. 10 and secondary antibody dilution 1:7000.

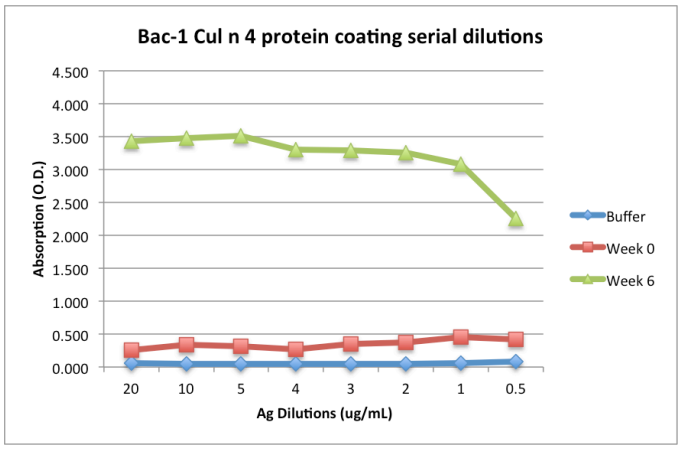

Figure 16: Serial Bac-1-Cul n 4 dilutions. A clear difference between week 0 and week 6 serums was obvious at all Bac-1-Cul n 4 concentrations.

Serial serum dilutions. Serial dilutions for serum samples from all 12 vaccinated SEA/M horses, week 0, 6 and 10 of vaccination were tested (Figure 17). Washing technique: "Immuno washer". Blocking step: high-salt dilution BSA buffer, 1 h RT. Protein coating: Bac-1-Cul n 4 concentration 2 ug/ml. Serum samples: 1:200. Conjugate: 1:7000. Substrate incubation time: 10 min. The difference between the adjuvant groups can be seen in Figure 18.

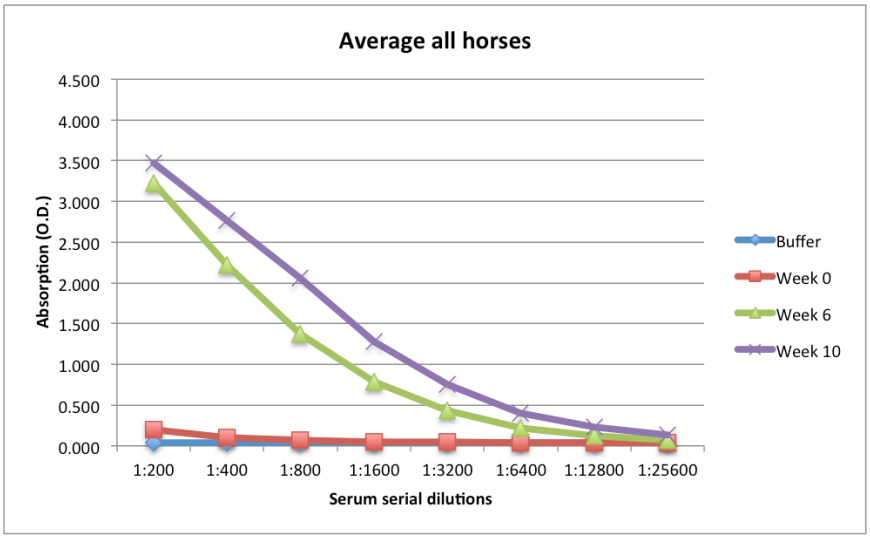

Figure 17: Serum serial dilutions ELISA. Serum serial dilutions were made and tested in ELISA.

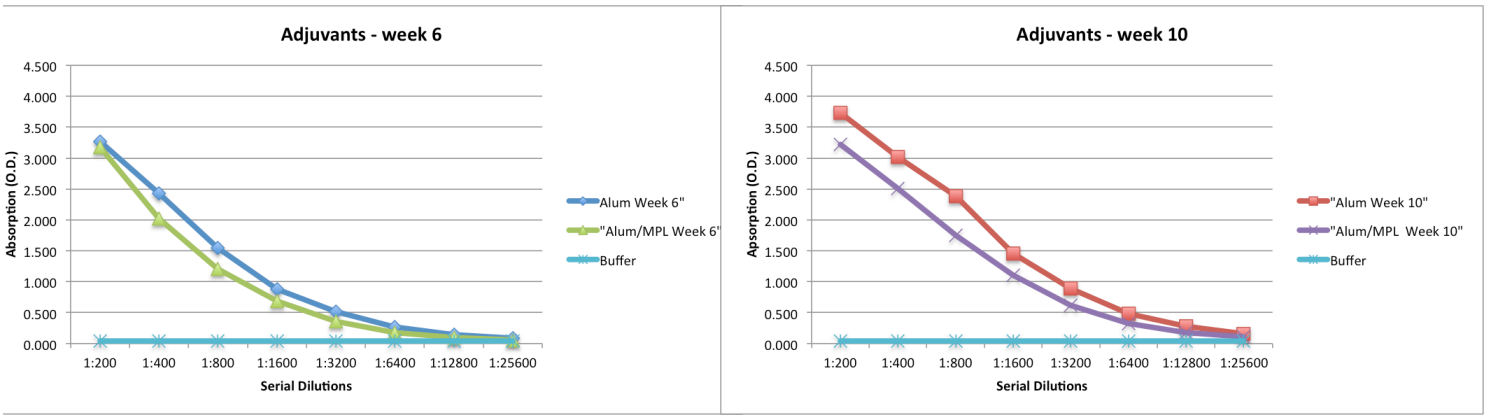

Figure 18: Adjuvants. The difference between using alum alone compared to alum and MPL in combination for week 6 and 10 of vaccination.

4.3 Statistics

Two-way ANOVA was used to compare the mean values between weeks of vaccination in the horses (week 0, 6 and 10) from the serial serum dilution testing (Figure 17). A Tukey-Kramer's post-test (all pairwise comparisons) was made in order to compare each vaccination week within each serum dilution and to compare each horse within each serum dilution (Table 2). All basic statistical data can be seen in Appendix D.

Table 2: ANOVA results. Serial serum dilutions from all 12 horses were tested in ELISA. Individual differences and results from different vaccination time points were analysed with a two-way ANOVA using a Tukey-Kramer post-hoc test for differences between weeks of vaccination. One-way ANOVA was used for analysing differences between adjuvants used.

		p-level							
		1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600
Two way ANOVA	Horses	ns	ns	ns	ns	ns	ns	*	*
	Week	***	***	***	***	***	***	***	***
One way ANOVA	Adjuvant week 6	ns	ns	ns	ns	ns	ns	ns	ns
	Adjuvant week 10	ns	ns	ns	ns	ns	ns	ns	ns
Tukey-Kramer Test for Differences Between Means	Week 0 vs Week 10	***	***	***	***	***	***	***	**
	Week 0 vs Week 6	***	***	***	***	**	*	**	*
	Week 10 vs Week 6	ns	*	**	**	**	**	*	*

ns = non significant, * statistically significant P < 0.05, ** P <0.01, *** P <0.001. P-values of <0.05 were considered statistically significant.

5 Discussion

5.1 ELISA development

The objective of this project was to establish an ELISA using purified allergens produced in insect cells. The work was done with the aim of adapting an ELISA procedure that existed for *E. coli* produced *Culicoides* allergens for insect cell produced allergens.

For a large part of the development, the assays gave high background O.D., which were found to be difficult to decrease (Figures 8-13). This high background was observed in serum from week 0 of vaccination, meaning that the horse had not been vaccinated at that point and shouldn't have shown such a high response to the allergen. Background is preferred to be under 0.2 O.D. units [26]. Factors that are known to influence background include: blocking reagent, diluents, and washing technique, capture and detection antibody concentrations and incubation times. Testing of all possible dilutions and combination of coating, capture proteins, blocking, serum samples, protein coating, conjugate, incubation time and substrate incubation time did not deliver that task.

After testing different dilutions and combinations of coating, serum samples and conjugate, pre-colostrum samples were tested with the Cul n 4 antigen in order to ensure that the antibodies in the serum samples from week 0 of vaccination weren't binding specifically to the conjugate. The pre-colostrum serum samples should not contain any antibodies because the foals do not receive a passive transfer of immunity via the placenta before birth, they to be ingested (with the colostrum). This resulted in no O.D. (Figure 9). This leads to the assumption that the antibodies in the week 0 serum from the vaccination experiment, might be binding the conjugate non-specifically.

To rule out unselective binding to the microplate plastics, a blocking step was added before incubating the serum. This was done in attempt to block the polystyrene/plastic areas that might not be coated with/bound to the antigen. Nonspecific binding (hydrophobic interaction, van der Waals forces, hydrogen bonding and ionic interaction) of conjugate to the plastic could have explained the high background. However, results showed no difference between blocking and not blocking (Figure 10-12). And testing of different microplates did not show a difference (Figure 13).

Capture ELISA was tested to gain a more specific detection of the Cul n 4 antigen. It should have allowed for enhanced O.D. and provide a grater chance of capturing the antigen from a complex solution. This did not deliver in lower background (Figure 11). In that same experiment, week 0 serum from another horse (SEA/M–7) was tested and showed lower O.D. than the SEA/M–8. The O.D. from the SEA/M-7 was still higher than the buffer control and therefore, not sufficient enough.

It was not until testing of high-salt buffers and a different washing technique that the high background appeared to decrease (Figures 14 and 15).

5.1.1 High-salt buffers

Higher salt concentration has been found to help in reducing unspecific and/or weak off-target interactions [26, 27]. Unspecific binding can be caused by substances, which are in excess of the target or with the surface of the plates. Unspecific binding is presumably due mainly to electrostatic interactions, whereas specific binding is dependent also on hydrophobic interactions and hydrogen bonds. The increasing salt concentration separates the unspecific interactions simply due to their ionic properties [27]. The increase in salt concentration in all buffers and solutions in the development of the ELISA are thought to have helped in lowering the O.D. values of the week 0 serum through this process.

5.1.2 Washing technique

The wash steps are necessary because if any unbound material, such as non-specifically bound antibodies, or detection reagent, remains in the microplate wells, it can increase background noise. The change from the first unspecific washing to the “Immuno washer” could have made a difference to minimizing the background observed before. With the “Immuno washer” technique, additional washes and soaking for a few minutes was added. This surely helped in getting rid of unspecific binding to the surface or substances and therefore the background noise of the week 0 serum.

5.2 Difference between weeks of vaccination and adjuvants used

Two-way ANOVA showed a statistically significant difference of the O.D. mean values between week 0 and both week 6 and 10. Confirming that vaccinating the horses did indeed give a higher response in the ELISA compared to no vaccination (week 0). It also revealed that there was a statistically significant difference of the O.D. mean values

between week 6 and 10 (except in the lowest serum dilution 1:200) (Table 2). Week 10 gives higher O.D. values than week 6 (Figure 17 and Appendix C). This could be of great interest for further investigation of whether the vaccination at week 8 (serum week 10) is necessary for a suitable immune response. Statistical testing showed no difference between horses from serum dilutions 1:200 – 1:6400. Indicating that a difference between the individuals could not explain the difference between the weeks of vaccination. The highest serum dilution ratios 1:12800 and 1:25600 did give non-significant results considering the individual difference, meaning that it might be explained by individual differences in those cases. But this needs to be examined further.

A one-way ANOVA was performed to assess the two different adjuvants used (alum and alum/MPL). Testing showed no statistically significant difference in any case Table 2 and Figure 18).

The results from the statistical testing are merely an indication of what could be interesting to investigate further. These testing could and should be done in duplicate or triplicate on each plate and repeated two or three times for greater power and more descriptive results.

5.3 The importance of the ELISA for the IBH project at Keldur and next steps

An ELISA is already being conducted on regular basis at the collaborate laboratory in Bern. That ELISA is unfortunately very time-consuming and labour intensive. This method needed to be updated and set up at Keldur. This project is a step towards developing more efficient tests to evaluate the IBH immunotherapy. The ELISA will be used for detecting more antibodie classes and subclasses and for other IBH antigens. Furthermore, will it be useful for testing antigens that have been produced and isolated differently than those tested for this project. Some antigens have been produced with a different plasmide, pIsecSUMOstar (SUMO: small-ubiquitin-related modifier). SUMOstar is designed for increased protein stability and solubility for isolation from insect cells. Some SUMO-produced antigens are being tested in the optimized ELISA and are showing promising results.

6 Conclusions

The establishment of an enzyme linked immunosorbent assay (ELISA) using purified allergens produced in insect cells was successful through optimization of a procedure that already existed for *E. coli* produced *Culicoides* allergens. For this optimization, the most critical parameters turned out to be salt concentration in buffers and washing techniques that efficiently reduced and the high background noise observed, by impacting unspecific and weak off-target interactions.

Statististical testing on results from serial serum dilutions showed a difference between weeks of vaccination but no difference between adjuvants used. These results are interesting indicators of what could be investegated further.

Overall, this project was a step towards developing more efficient tests to evaluate the IBH immunotherapy experiments conducted at Keldur.

References

1. Murphy K, T.P., Walport M, *Basic Concepts in Immunology*, in *Janeway's Immunobiology* 2008, Garland Science: New York & London. p. 1-38.
2. Murphy K, T.P., Walport M, *Antigen Recognition by B-cell and T-cell Receptors*, in *Janeway's Immunobiology*2008, Garland Sciences: New York & London. p. 111-142.
3. Murphy K, T.P., Walport M, *Innate Immunity*, in *Janeway's Immunobiology*2008, Garland Sciences: New York & London. p. 39-108.
4. Gallucci, S. and P. Matzinger, *Danger signals: SOS to the immune system.* Curr Opin Immunol, 2001. **13**(1): p. 114-9.
5. Murphy K, T.P., Walport M, *Dynamics of Adaptive Immunity*, in *Janeway's Imminobiology*2008, Garland Sciences: New York & London. p. 421-458.
6. Murphy K, T.P., Walport M, *T Cell Mediated Immunity*, in *Janeway's Immunibiology*2008, Garland Sciences: New York & London. p. 323-337.
7. Murphy K, T.P., Walport M, *The Humoral Immune Response*, in *Janeway's Immunobiology*2008, Garland Sciences: New York & London. p. 379-420.
8. Murphy K, T.P., Wallport M, *Allergy and Hypersensitivity*, in *Janeway's Immunobiology*2008, Garland Sciences: New York & London. p. 555-598.
9. Baker, K.P. and P.J. Quinn, *A report on clinical aspects and histopathology of sweet itch.* Equine Vet J, 1978. **10**(4): p. 243-8.
10. Wilson, A.D., et al., *Detection of IgG and IgE serum antibodies to Culicoides salivary gland antigens in horses with insect dermal hypersensitivity (sweet itch).* Equine Vet J, 2001. **33**(7): p. 707-13.
11. van der Haegen, A., et al., *Immunoglobulin-E-bearing cells in skin biopsies of horses with insect bite hypersensitivity.* Equine Vet J, 2001. **33**(7): p. 699-706.
12. Baselgia, S., et al., *Evaluation of an in vitro sulphidoleukotriene release test for diagnosis of insect bite hypersensitivity in horses.* Equine Vet J, 2006. **38**(1): p. 40-6.
13. Fadok, V.A. and E.C. Greiner, *Equine insect hypersensitivity: skin test and biopsy results correlated with clinical data.* Equine Vet J, 1990. **22**(4): p. 236-40.
14. Schaffartzik, A., et al., *Equine insect bite hypersensitivity: what do we know?* Vet Immunol Immunopathol, 2012. **147**(3-4): p. 113-26.
15. Hellberg, W., et al., *Equine insect bite hypersensitivity: immunoblot analysis of IgE and IgG subclass responses to Culicoides nubeculosus salivary gland extract.* Vet Immunol Immunopathol, 2006. **113**(1-2): p. 99-112.
16. Brostrom, H., A. Larsson, and M. Troedsson, *Allergic dermatitis (sweet itch) of Icelandic horses in Sweden: an epidemiological study.* Equine Vet J, 1987. **19**(3): p. 229-36.
17. Bjornsdottir, S., et al., *Summer eczema in exported Icelandic horses: influence of environmental and genetic factors.* Acta Vet Scand, 2006. **48**: p. 3.
18. Langner, K.F., et al., *Comparison of cellular and humoral immunoassays for the assessment of summer eczema in horses.* Vet Immunol Immunopathol, 2008. **122**(1-2): p. 126-37.
19. Hudson, A., L. Bowman, and C.W. Orr, *Effects of absence of saliva on blood feeding by mosquitoes.* Science, 1960. **131**(3415): p. 1730-1.

20. Schaffartzik, A., et al., *Cloning of IgE-binding proteins from Simulium vittatum and their potential significance as allergens for equine insect bite hypersensitivity.* Vet Immunol Immunopathol, 2009. **132**(1): p. 68-77.
21. Schaffartzik, A., et al., *Selective cloning, characterization, and production of the Culicoides nubeculosus salivary gland allergen repertoire associated with equine insect bite hypersensitivity.* Vet Immunol Immunopathol, 2011. **139**(2-4): p. 200-9.
22. Schaffartzik, A., et al., *Cloning, production and characterization of antigen 5 like proteins from Simulium vittatum and Culicoides nubeculosus, the first cross-reactive allergen associated with equine insect bite hypersensitivity.* Vet Immunol Immunopathol, 2010. **137**(1-2): p. 76-83.
23. Wagner, B., *Immunoglobulins and immunoglobulin genes of the horse.* Dev Comp Immunol, 2006. **30**(1-2): p. 155-64.
24. Heyworth, D. *Overview of ELISA*. [cited 2014 12.11.2014]; Available from: http://www.piercenet.com/method/overview-elisa - detectionformats.
25. Wong, C., et al., *Heating greatly speeds Coomassie blue staining and destaining.* Biotechniques, 2000. **28**(3): p. 426-8, 430, 432.
26. R&D Systems, *ELISA Development Guide*, in http://www.rndsystems.com/resources/images/5670.pdf, R.D.S. Inc., Editor: Minneapolis.
27. Tsumoto, K., et al., *Effects of salts on protein-surface interactions: applications for column chromatography.* J Pharm Sci, 2007. **96**(7): p. 1677-90.

Appendix A

Buffers used for protein isolation and purification.

Lysis buffer, pH 8
50mM $NaH_2PO_4xH_2O$, 150mM NaCl, 1% IgePal

Wash buffer, pH 8
50mM $NaH_2PO_4xH_2O$, 300mM NaCl, (20 mM Imidazol or 10 mM Imidazol)

Elution buffer 1, pH 8
50mM $NaH_2PO_4xH_2O$, 300mM NaCl, 250 mM Imidazol

Elution buffer 2, pH 6.5
50mM $NaH_2PO_4xH_2O$, 300mM NaCl, 250 mM Imidazol

2x Sample buffer
0.5% 2-mercaptoethanol, 20% glycine, 2% SDS, 0.1% Bromophenol blue, 130 mM Tris

Transfer buffer
25 mM Tris, 192 mM glycine, 20% methanol

BCIP/NBT
5-bromo-4-chloro-3-indolyl phospate/Nitro blue tetrazoliumchloride

Alkaline phosphatase buffer
100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl2, o.o5% Tween 20, pH 9.5

Appendix B

Buffers used for ELISA

Coating buffer:
0.015M Na_2CO_3, 0.035M $NaHCO_3$, 0.003M NaN_3, H_2O up to 1000 mL

Washing buffer:
0.5000M NaCl, 0.0027M KCl, 0.0015M KH_2PO_4, 0.0065M $Na_2PO_4 2H_2O$, 0.05% Tween 20, H_2O up to 1000 mL

Dilution BSA buffer:
1% BSA (bovine serum albumin), 0.001% Phenol red, Washing buffer up to 1000 mL

OPD substrat (pH 5.0):
4 tablets OPD (orthiophenylenediamine)(DAKO, Glostrup, Denmark), 0.00042% H_2O_2, H_2O up to 12 mL

Appendix C

ELISA results

Dilutions of the coating antigen:

Dato: 220914

	O.D.		
Culn4 protein (ug/mL)	**Buffer**	**Week 0**	**Week 6**
100	0.046	2.516	3.694
50	0.039	2.352	3.670
20	0.038	2.561	2.019
10	0.039	2.310	2.034
5	0.039	2.593	1.807
2	0.037	2.506	1.661
1	0.037	2.822	1.888

Testing of conjugate dilutions:

Dato: 230914

	O.D.		
Culn4 protein (ug/mL)	**Buffer**	**Week 0**	**Week 6**
100	0.046	1.071	3.565
50	0.039	0.939	2.208
20	0.04	0.063	0.339
10	0.041	0.124	0.364
5	0.04	0.115	0.315
2	0.039	0.069	0.227
1	0.039	0.334	0.32

Conjugate dilutions and substrate incubation time extension:

Dato: 240914

	O.D.								
	Sec Ab 1:5000			**Sec Ab 1:7000**			**Sec Ab 1:10000**		
Culn4 protein (ug/mL)	**Buffer**	**Week 0**	**Week 6**	**Buffer**	**Week 0**	**Week 6**	**Buffer**	**Week 0**	**Week 6**
50	0.040	2.183	3.591	0.039	1.437	3.134	0.036	1.156	2.211
40	0.037	1.850	3.243	0.038	1.332	2.927	0.038	1.021	1.839
30	0.039	1.563	2.516	0.038	1.512	2.109	0.038	0.950	1.338
20	0.041	1.516	2.218	0.038	1.321	1.658	0.037	1.082	1.154
10	0.039	1.503	1.772	0.038	1.278	1.587	0.037	0.908	0.977
5	0.039	2.013	1.672	0.038	1.579	1.153	0.043	0.987	0.871

Testing of serum dilutions:

Dato: 290914	O.D.											
	Serum 1:100			Serum 1:500			Serum 1:1000			Serum 1:2000		
Culn4 protein (ug/mL)	Buffer	Week 0	Week 6	Buffer	Week 0	Week 6	Buffer	Week 0	Week 6	Buffer	Week 0	Week 6
0	0.047	1.701	1.200	0.051	0.700	0.542	0.057	0.402	0.322	0.064	0.219	0.199
50	0.048	1.990	2.185	0.040	0.230	0.553	0.042	0.080	0.326	0.043	0.051	0.286
40	0.047	1.165	1.375	0.039	0.139	0.420	0.043	0.063	0.193	0.044	0.044	0.192
30	0.042	1.199	1.184	0.040	0.118	0.269	0.040	0.058	0.129	0.042	0.043	0.11
20	0.042	1.272	1.021	0.047	0.168	0.268	0.046	0.076	0.148	0.047	0.047	0.108
10	0.049	1.476	0.874	0.043	0.177	0.164	0.045	0.069	0.080	0.047	0.051	0.075
5	0.050	1.703	1.107	0.054	0.236	0.163	0.047	0.088	0.071	0.05	0.059	0.068
2	0.054	2.019	1.106	0.053	0.258	0.193	0.049	0.112	0.102	0.065	0.079	0.092

Pre-colostrum foals:

Dato: 290914

	O.D.	
Culn4 protein (ug/mL)	Dúfa	Eiríkur
0	0.086	0.038
50	0.038	0.038
40	0.038	0.038
30	0.037	0.038
20	0.040	0.036
10	0.038	0.038
5	0.038	0.037
2	0.072	0.051

Testing of dry milk powder blocking step and two different conjugates:

Dato: 300914 Blocking with dry milk powder	O.D.											
	Sec Ab Jackson 1:7000						Sec Ab Sigma 1:2000					
	Serum 1:100			Serum 1:500			Serum 1:100			Serum 1:500		
Culn4 protein (ug/mL)	Buffer	Week 0	Week 6	Buffer	Week 0	Week 6	Buffer	Week 0	Week 6	Buffer	Week 0	Week 6
0	0.060	0.091	0.113	0.047	0.060	0.200	0.040	0.047	0.061	0.039	0.046	0.094
50	0.046	1.775	1.711	0.039	0.343	0.545	0.040	0.422	0.798	0.037	0.081	0.183
40	0.045	1.624	1.480	0.040	0.341	0.436	0.038	0.479	0.657	0.038	0.075	0.176
30	0.044	1.654	1.560	0.040	0.342	0.508	0.039	0.508	0.699	0.037	0.077	0.194
20	0.060	1.729	1.578	0.045	0.345	0.445	0.036	0.507	0.629	0.039	0.076	0.150
10	0.054	1.762	1.491	0.043	0.364	0.380	0.036	0.532	0.602	0.039	0.081	0.126

Dato: 300914 No blocking	O.D.											
	Sec Ab Jackson 1:7000						Sec Ab Sigma 1:2000					
	Serum 1:100			Serum 1:500			Serum 1:100			Serum 1:500		
Culn4 protein (ug/mL)	**Buffer**	**Week 0**	**Week 6**	**Buffer**	**Week 0**	**Week 6**	**Buffer**	**Week 0**	**Week 6**	**Buffer**	**Week 0**	**Week 6**
0	0.043	0.147	0.331	0.045	0.085	0.357	0.047	0.070	0.112	0.042	0.060	0.105
50	0.048	1.197	1.087	0.043	0.199	0.397	0.039	0.308	0.476	0.041	0.050	0.149
40	0.053	1.288	1.097	0.042	0.213	0.393	0.038	0.329	0.391	0.039	0.044	0.173
30	0.080	1.337	1.056	0.040	0.211	0.350	0.055	0.323	0.418	0.039	0.058	0.127
20	0.073	1.361	1.080	0.041	0.167	0.274	0.040	0.336	0.344	0.040	0.057	0.087
10	0.073	1.497	0.948	0.044	0.227	0.303	0.042	0.367	0.315	0.042	0.060	0.078

Capture ELISA with dilution buffer blocking step:

Dato: 021014	O.D.											
	Culn4 Asc. 1:2500				Culn4 Asc. 1:5000				Culn4 Asc. 1:10.000			
Culn4 protein (ug/mL)	**Buffer**	**Week 0 no 7**	**Week 0 no 8**	**Week 6 no 8**	**Buffer**	**Week 0 no 7**	**Week 0 no 8**	**Week 6 no 8**	**Buffer**	**Week 0 no 7**	**Week 0 no 8**	**Week 6 no 8**
50	0.060	0.598	2.327	1.510	0.056	0.526	2.114	1.542	0.069	0.571	2.552	1.807
40	0.086	0.669	2.044	1.820	0.041	0.704	2.073	1.627	0.043	0.524	2.214	2.264
20	0.076	0.592	3.062	1.482	0.041	0.441	2.276	1.641	0.044	0.493	2.376	1.970
10	0.077	0.509	3.159	2.154	0.044	0.600	2.160	1.604	0.051	0.591	3.208	2.576
5	0.089	0.496	2.077	1.509	0.041	0.585	2.251	1.717	0.040	0.465	2.580	2.360
1	0.095	0.654	2.149	1.434	0.044	0.480	1.890	2.109	0.044	0.511	2.450	1.493

Testing of gelatin blocking and Cul n 4 pAb dilutions:

Dato: 061014	O.D.								
	Culn4 Asc. 1:2500			Culn4 Asc. 1:5000			Culn4 Asc. 1:10.000		
Culn4 protein (ug/mL)	**Buffer**	**Week 0**	**Week 6**	**Buffer**	**Week 0**	**Week 6**	**Buffer**	**Week 0**	**Week 6**
20	0.045	0.203	0.359	0.043	0.224	0.434	0.044	0.225	0.389
10	0.044	0.214	0.305	0.037	0.216	0.353	0.039	0.214	0.316
1	0.039	0.212	0.246	0.036	0.218	0.272	0.039	0.228	0.265
0	0.040	0.236	0.238	0.038	0.235	0.247	0.045	0.242	0.225
20	0.040	0.239	0.346	0.042	0.248	0.356	0.044	0.228	0.302
10	0.042	0.233	0.304	0.041	0.238	0.308	0.044	0.229	0.282
1	0.040	0.226	0.258	0.039	0.235	0.258	0.044	0.245	0.260
0	0.052	0.294	0.276	0.048	0.266	0.264	0.051	0.299	0.293

Testing of microplate types:

Dato: 091014

MaxiSorp

	O.D.		
	Culn4 Asc. 1:5000		
Culn4 protein (ug/mL)	Buffer	Week 0	Week 0
40	0.045	0.634	1.148
20	0.045	0.617	1.168
5	0.053	0.643	1.053
0	0.047	0.629	0.689

Dato: 091014

Immunolon

	O.D.		
	Culn4 Asc. 1:5000		
Culn4 protein (ug/mL)	Buffer	Week 0	Week 0
40	0.467	1.216	1.567
20	0.293	0.446	0.985
5	0.146	0.426	0.966
0	0.289	0.711	0.577

Capture ELISA with high-salt

Dato: 141014

	O.D.							
	Sec Ab. 1:2000				Sec Ab. 1:4000			
Culn4 protein (ug/mL)	No Asc	Buffer	Week 0	Week 6	No Asc	Buffer	Week 0	Week 6
4	0.104	0.087	0.494	3.136	0.088	0.061	0.380	2.450
2	0.078	0.087	0.511	1.824	0.087	0.054	0.390	1.777
1	0.069	0.109	0.542	1.302	0.141	0.066	0.363	1.352
0.5	0.097	0.066	0.448	0.976	0.080	0.057	0.388	0.951
0	0.129	0.058	0.114	0.086	0.061	0.048	0.085	0.054

Capture ELISA with high-salt, optimizing serum and conjugate dilutions:

Dato: 201014

	O.D.								
	Sec Ab 1:2000						Sec Ab 1:4000		
	Serum 1:200			Serum 1:400			Serum 1:200		
Culn4 protein (ug/mL)	Buffer	Week 0	Week 6	Buffer	Week 0	Week 6	Buffer	Week 0	Week 6
5	0.145	0.385	1.831	0.145	0.301	1.312	0.063	0.158	0.853
4	0.131	0.379	2.006	0.131	0.274	1.267	0.057	0.171	0.988
3	0.128	0.383	1.722	0.128	0.278	1.097	0.056	0.164	0.831
2	0.133	0.363	1.241	0.133	0.274	0.803	0.055	0.159	0.654
1	0.116	0.382	0.947	0.116	0.277	0.611	0.049	0.164	0.455
0.5	0.141	0.383	0.772	0.141	0.285	0.525	0.059	0.175	0.363

Testing indirect ELISA using the optimized high-salt process:

Dato: 211014	O.D.								
	Sec Ab 1:2000			Sec Ab 1:4000			Sec Ab 1:7000		
Culn4 protein (ug/mL)	Buffer	Week 0	Week 6	Buffer	Week 0	Week 6	Buffer	Week 0	Week 6
4	0.087	0.848	3.888	0.063	0.451	3.914	0.045	0.220	3.276
3	0.092	0.903	3.868	0.071	0.449	3.961	0.042	0.181	3.246
2	0.177	0.983	3.897	0.075	0.490	3.781	0.043	0.218	3.399
1	0.155	0.992	3.893	0.097	0.518	3.677	0.042	0.187	3.289
0.5	0.256	1.153	3.976	0.133	0.609	3.759	0.049	0.282	3.343
0	0.163	1.247	1.249	0.071	0.669	0.682	0.042	0.287	0.304

Bac-1 Cul n 4 protein coating serial dilutions:

Dato: 271014	O.D.									
	Sec Ab 1:6000			Sec Ab 1:7000		Sec Ab 1:6000			Sec Ab 1:7000	
Culn4 protein (ug/mL)	Buffer	Week 0 No. 10	Week 6 No. 10	Week 0 No. 11	Week 6 No. 11	Buffer	Week 0 No. 10	Week 6 No. 10	Week 0 No. 11	Week 6 No. 11
2	0.049	0.400	3.773	0.353	3.534	0.055	0.256	3.435	0.223	3.273
1	0.043	0.386	3.878	0.322	3.627	0.042	0.339	3.472	0.182	3.169
0.5	0.041	0.404	3.789	0.307	3.591	0.050	0.320	3.516	0.186	2.565
0.3	0.042	0.443	4.034	0.334	3.551	0.045	0.266	3.297	0.184	2.496
0.2	0.042	0.448	3.854	0.334	3.370	0.047	0.348	3.293	0.194	3.104
0.1	0.045	0.498	3.916	0.362	3.319	0.049	0.372	3.259	0.203	3.082
0.05	0.050	0.542	3.719	0.393	2.988	0.060	0.452	3.084	0.210	2.016

Serial serum dilutions:

	SEA/M - 1			SEA/M - 2			SEA/M - 3			SEA/M - 4			SEA/M - 5			SEA/M - 6		
Serum dilution	**Week 0**	**Week 6**	**Week 10**	**Week 0**	**Week 6**	**Week 10**	**Week 0**	**Week 6**	**Week 10**	**Week 0**	**Week 6**	**Week 10**	**Week 0**	**Week 6**	**Week 10**	**Week 0**	**Week 6**	**Week 10**
1:200	0.123	3.677	3.939	0.150	3.660	3.865	0.410	3.610	3.602	0.210	2.624	3.958	0.188	3.748	3.783	0.264	2.292	3.234
1:400	0.072	2.924	3.393	0.092	3.055	3.718	0.152	2.799	2.460	0.174	1.662	3.378	0.112	2.955	3.381	0.117	1.145	1.772
1:800	0.051	1.904	2.654	0.071	2.044	3.342	0.087	1.891	1.722	0.099	0.903	2.894	0.065	1.845	2.513	0.065	0.663	1.147
1:1600	0.042	1.186	1.752	0.038	1.165	2.261	0.046	1.089	0.950	0.063	0.506	1.781	0.048	1.060	1.393	0.042	0.286	0.628
1:3200	0.066	0.664	1.020	0.038	0.705	1.541	0.039	0.671	0.559	0.045	0.283	1.044	0.042	0.640	0.831	0.035	0.148	0.324
1:6400	0.048	0.352	0.566	0.038	0.364	0.879	0.037	0.323	0.290	0.039	0.142	0.569	0.042	0.329	0.458	0.035	0.068	0.154
1:12800		0.187	0.308	0.038	0.197	0.537	0.037	0.175	0.167	0.041	0.081	0.301		0.178	0.279	0.035	0.044	0.087
1:25600		0.100	0.174	0.042	0.109	0.309	0.041	0.097	0.096	0.045	0.065	0.165		0.094	0.155	0.041	0.041	0.053

	SEA/M - 7			SEA/M - 8			SEA/M - 9			SEA/M - 10			SEA/M - 11			SEA/M - 12		
Serum dilution	**Week 0**	**Week 6**	**Week 10**	**Week 0**	**Week 6**	**Week 10**	**Week 0**	**Week 6**	**Week 10**	**Week 0**	**Week 6**	**Week 10**	**Week 0**	**Week 6**	**Week 10**	**Week 0**	**Week 6**	**Week 10**
1:200	0.195	3.269	3.786	0.157	3.342	3.702	0.168	3.877	3.415	0.154	3.489	3.729	0.218	2.255	1.785	0.163	2.820	2.930
1:400	0.086	2.102	3.105	0.077	2.051	3.431	0.089	2.743	2.308	0.094	2.404	3.685	0.121	1.147	0.797	0.087	1.687	1.740
1:800	0.054	1.230	2.062	0.047	1.227	2.514	0.059	1.857	1.525	0.062	1.277	2.838	0.067	0.673	0.436	0.050	0.942	1.121
1:1600	0.039	0.707	1.301	0.039	0.667	1.617	0.046	1.116	0.856	0.039	0.760	1.972	0.042	0.354	0.227	0.041	0.522	0.630
1:3200	0.040	0.349	0.659	0.039	0.359	0.907	0.042	0.583	0.510	0.037	0.381	1.194	0.037	0.175	0.117	0.062	0.266	0.354
1:6400	0.038	0.178	0.387	0.039	0.183	0.490	0.042	0.308	0.245	0.040	0.201	0.613	0.044	0.086	0.058	0.039	0.131	0.166
1:12800	0.035	0.093	0.205	0.036	0.093	0.263		0.158	0.132	0.036	0.103	0.343	0.038	0.055	0.041	0.037	0.072	0.085
1:25600	0.041	0.055	0.111	0.044	0.055	0.132		0.081	0.078	0.044	0.062	0.204	0.041	0.043	0.039	0.048	0.046	0.056

Appendix D

Statistical data

Two-way ANOVA:

Serum dilution 1:200

ANOVA

Source of Variation	*SS*	*d.f.*	*MS*	*F*	*p-level*	*F crit*
Factor #1 (horse)	5.73533	11	0.52139	1.26975	0.30358	2.77485
Factor #2 (week)	74.41809	2	37.20904	90.61517	2.39208E-11	4.69801
Within Groups	9.0338	22	0.41063			
Total	89.18721	35	2.54821			

Groups	*Difference*	*Test Statistics*	*p-level*
0 vs 10	-3.27732	17.71679	0.00004
0 vs 6	-2.7552	14.89428	0.00004
10 vs 6	0.52212	2.82251	0.13659

Serum dilution 1:400

ANOVA

Source of Variation	*SS*	*d.f.*	*MS*	*F*	*p-level*	*F crit*
Factor #1 (horse)	9.62103	11	0.87464	2.19686	0.05593	2.77485
Factor #2 (week)	44.95735	2	22.47868	56.46058	0.000000002	4.69801
Within Groups	8.75887	22	0.39813			
Total	63.33725	35	1.80964			

Groups	*Difference*	*Test Statistics*	*p-level*
0 vs 10	-2.65778	14.59141	0.00004
0 vs 6	-1.89618	10.41015	0.00004
10 vs 6	0.7616	4.18126	0.01924

Serum dilution 1:800

ANOVA

Source of Variation	*SS*	*d.f.*	*MS*	*F*	*p-level*	*F crit*
Factor #1 (horse)	6.30039	11	0.57276	2.0391	0.07462	2.77485
Factor #2 (week)	24.17427	2	12.08713	43.03165	0.	4.69801
Within Groups	6.17957	22	0.28089			
Total	36.65422	35	1.04726			

Groups	*Difference*	*Test Statistics*	*p-level*
0 vs 10	-1.99908	13.06629	0.00004
0 vs 6	-1.15624	7.55738	0.00009
10 vs 6	0.84284	5.50891	0.00218

Serum dilution 1:1600

ANOVA

Source of Variation	*SS*	*d.f.*	*MS*	*F*	*p-level*	*F crit*
Factor #1 (horse)	2.90951	11	0.2645	1.98943	0.08176	2.77485
Factor #2 (week)	9.18738	2	4.59369	34.55116	0.	4.69801
Within Groups	2.92497	22	0.13295			
Total	15.02187	35	0.4292			

Groups	*Difference*	*Test Statistics*	*p-level*
0 vs 10	-1.23675	11.74958	0.00004
0 vs 6	-0.65391	6.21235	0.00067
10 vs 6	0.58284	5.53724	0.00208

Serum dilution 1:3200

ANOVA

Source of Variation	*SS*	*d.f.*	*MS*	*F*	*p-level*	*F crit*
Factor #1 (horse)	1.21123	11	0.11011	2.06121	0.07166	2.77485
Factor #2 (week)	3.04026	2	1.52013	28.45558	0.	4.69801
Within Groups	1.17526	22	0.05342			
Total	5.42675	35	0.15505			

Groups	*Difference*	*Test Statistics*	*p-level*
0 vs 10	-0.71157	10.66485	0.00004
0 vs 6	-0.33911	5.08252	0.00446
10 vs 6	0.37246	5.58233	0.00193

Serum dilution 1:6400

ANOVA

Source of Variation	*SS*	*d.f.*	*MS*	*F*	*p-level*	*F crit*
Factor #1 (horse)	0.37983	11	0.03453	2.02422	0.07669	2.77485
Factor #2 (week)	0.81032	2	0.40516	23.75156	0.	4.69801
Within Groups	0.37528	22	0.01706			
Total	1.56544	35	0.04473			

Groups	*Difference*	*Test Statistics*	*p-level*
0 vs 10	-0.36636	9.71696	0.00004
0 vs 6	-0.15817	4.19502	0.01883
10 vs 6	0.20819	5.52194	0.00213

Serum dilution 1:12800

ANOVA

Source of Variation	*SS*	*d.f.*	*MS*	*F*	*p-level*	*F crit*
Factor #1 (horse)	0.15871	11	0.01443	3.51588	0.00583	2.77485
Factor #2 (week)	0.20079	2	0.1004	24.46495	0.	4.69801
Within Groups	0.09028	22	0.0041			
Total	0.44979	32	0.01406			

Groups	*Difference*	*Test Statistics*	*p-level*
0 vs 10	-0.19189	4.10904	0.0006
0 vs 6	-0.07097	3.70191	0.00151
10 vs 6	0.12092	2.78424	0.01082

Serum dilution 1:25600

ANOVA

Source of Variation	*SS*	*d.f.*	*MS*	*F*	*p-level*	*F crit*
Factor #1 (hestur)	0.04084	11	0.00371	2.65812	0.02451	2.77485
Factor #2 (vika)	0.04531	2	0.02265	16.21986	0.00005	4.69801
Within Groups	0.03073	22	0.00140			
Total	0.11687	32	0.00365			

Groups	*Difference*	*Test Statistics*	*p-level*
0 vs 10	-0.08783	3.40193	0.00299
0 vs 6	-0.02301	2.82464	0.01083
10 vs 6	0.06482	2.78450	0.01081

One-way ANOVA:

Analysis of Variance (One-Way)

Week 6 serum 1:200

Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
Alum	6	19.61063	3.26844	0.40742
Alum/MPL	6	19.05203	3.17534	0.32012

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	0.02600	1	0.02600	0.07148	0.79462	4.96460
Within Groups	3.63768	10	0.36377			
Total	3.66368	11				

Analysis of Variance (One-Way)

Week 6 serum 1:400

Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
2.92448636977238	5	11.61620	2.32324	0.74637
2.10176508857889	5	10.03213	2.00643	0.38581

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	0.25093	1	0.25093	0.44326	0.52428	5.31766
Within Groups	4.52872	8	0.56609			
Total	4.77965	9				

Analysis of Variance (One-Way)

Week 6 serum 1:800

Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
1.90401529071142	5	7.34631	1.46926	0.40482
1.22995326960667	5	5.97579	1.19516	0.19562

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	0.18783	1	0.18783	0.62565	0.45176	5.31766
Within Groups	2.40176	8	0.30022			
Total	2.58960	9				

Analysis of Variance (One-Way)

Week 6 serum 1:1600

Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
1.18568747815243	5	4.10545	0.82109	0.15838
0.70659659909454	5	3.41911	0.68382	0.08196

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	0.04711	1	0.04711	0.39200	0.54869	5.31766
Within Groups	0.96136	8	0.12017			
Total	1.00847	9				

Analysis of Variance (One-Way)

Week 6 serum 1:3200

Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
0.66372800915235	5	2.44719	0.48944	0.06537
0.3493902153073	5	1.76351	0.35270	0.02324

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	0.04674	1	0.04674	1.05495	0.33442	5.31766
Within Groups	0.35445	8	0.04431			
Total	0.40119	9				

Analysis of Variance (One-Way)

Week 6 serum 1:6400

Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
0.35207534623129	5	1.22523	0.24505	0.01731
0.1781108860505	5	0.90900	0.18180	0.00701

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	0.01000	1	0.01000	0.82227	0.39101	5.31766
Within Groups	0.09729	8	0.01216			
Total	0.10729	9				

Analysis of Variance (One-Way)

Week 6 serum 1:12800

Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
0.18677463644829	5	0.67409	0.13482	0.00462
0.09310036890589	5	0.48112	0.09622	0.00154

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	0.00372	1	0.00372	1.20895	0.30352	5.31766
Within Groups	0.02464	8	0.00308			
Total	0.02836	9				

Analysis of Variance (One-Way)

Week 6 serum 1:25600

Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
0.10004844090232	5	0.40639	0.08128	0.00077
0.05503988203576	5	0.28701	0.05740	0.00023

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	0.00142	1	0.00142	2.85562	0.12952	5.31766
Within Groups	0.00399	8	0.00050			
Total	0.00542	9				

Analysis of Variance (One-Way)

Week 10 1:200

Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
3.93887905936424	5	18.44178	3.68836	0.08174
3.78603469911345	5	15.56091	3.11218	0.65364

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	0.82994	1	0.82994	2.25719	0.17140	5.31766
Within Groups	2.94151	8	0.36769			
Total	3.77146	9				

Analysis of Variance (One-Way)

week 10 1:400

Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
3.39346436437869	5	14.70792	2.94158	0.64670
3.10465534932394	5	11.96055	2.39211	1.43165

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	0.75481	1	0.75481	0.72635	0.41885	5.31766
Within Groups	8.31343	8	1.03918			
Total	9.06823	9				

Analysis of Variance (One-Way)

week 10 1:800

Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
2.65371327121409	5	11.61685	2.32337	0.78613
2.06169628260077	5	8.43432	1.68686	0.98021

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	1.01285	1	1.01285	1.14683	0.31546	5.31766
Within Groups	7.06538	8	0.88317			
Total	8.07823	9				

Analysis of Variance (One-Way)

week 10 1:1600

Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
1.75212534533571	5	7.01263	1.40253	0.42104
1.30067094414543	5	5.30219	1.06044	0.51565

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	0.29256	1	0.29256	0.62468	0.45211	5.31766
Within Groups	3.74675	8	0.46834			
Total	4.03931	9				

Analysis of Variance (One-Way)

week 10 1:3200

Summary

Groups	Sample size	Sum	Mean	Variance
1.02044412602313	5	4.30008	0.86002	0.21900
0.65901650108145	5	3.08197	0.61639	0.18691

ANOVA

Source of Variation	SS	df	MS	F	p-level	F crit
Between Groups	0.14838	1	0.14838	0.73110	0.41740	5.31766
Within Groups	1.62363	8	0.20295			
Total	1.77201	9				

Analysis of Variance (One-Way)

week 10 1:6400

Summary

Groups	Sample size	Sum	Mean	Variance
0.56636528885583	5	2.35109	0.47022	0.07742
0.38714359999511	5	1.57161	0.31432	0.05311

ANOVA

Source of Variation	SS	df	MS	F	p-level	F crit
Between Groups	0.06076	1	0.06076	0.93094	0.36287	5.31766
Within Groups	0.52213	8	0.06527			
Total	0.58289	9				

Analysis of Variance (One-Way)

week 10 1:12800

Summary

Groups	Sample size	Sum	Mean	Variance
0.30758090311668	5	1.37146	0.27429	0.02911
0.20482354240972	5	0.86391	0.17278	0.01596

ANOVA

Source of Variation	SS	df	MS	F	p-level	F crit
Between Groups	0.02576	1	0.02576	1.14329	0.31616	5.31766
Within Groups	0.18026	8	0.02253			
Total	0.20602	9				

Analysis of Variance (One-Way)

week 10 1:25600

Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
0.17373064789836	5	0.77731	0.15546	0.00940
0.1105061202457	5	0.50898	0.10180	0.00449

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	0.00720	1	0.00720	1.03644	0.33845	5.31766
Within Groups	0.05558	8	0.00695			
Total	0.06278	9				