# Immunotherapy for Insect Bite Hypersensitivity in horses

Development of methods for expressing and purifying recombinant allergens in insect cells and their application for evaluating immunotherapy

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**Thesis for degree of Master of Science**
**University of Iceland**
**Faculty of Medicine**
**School of Health Sciences**


HÁSKÓLI ÍSLANDS

# Ónæmismeðferð gegn sumarexemi í hestum

## *Þróun aðferða til að tjá og hreinsa endurraðaða ofnæmisvaka í skordýrafrumukerfi og notkun þeirra við að meta árangur ónæmismeðferðar*

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Ritgerð til meistaragráðu í líf- og læknavísindum

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# Ágrip

Sumarexem er húðofnæmi af gerð I í hestum sem einkennist af framleiðslu á IgE mótefnum. Einkenni sjúkdómsins eru exem og kláði sérstaklega í fax- og taglrótum, jafnvel sáramyndun og sýkingu í sárum. Sumarexem er dýravelferðarmál og vandamál fyrir hrossaútflutning þar sem tíðni þess er mun hærri hjá útfluttum hestum en hjá íslenskum hestum fæddum erlendis. Sumarexem orsakast af biti smámýs (*Culicoides* spp.) en þetta mý lifir ekki á Íslandi. Ofnæmisvakarnir eru prótein sem hestarnir mynda ofnæmisviðbrögð gegn og eru upprunnin í bitkirtlum flugnanna. Þrettán ofnæmisvakar hafa verið einangraðir úr þremur smámýstegundum, *C. sonorensis*, *C. nubeculosus* og *C. obsoletus,* tjáðir í bakteríum (*E.coli*) og hreinsaðir. Ferill sjúkdómsins hefur verið skilgreindur og tilraunir til ónæmismeðferðar eru í gangi. Ofnæmisvakarnir sem hreinsaðir eru úr bakteríum henta illa fyrir sum ónæmispróf sem eru nauðsynleg til að mæla árangur meðferðar. Því er nauðsynlegt að framleiða vakana í heilkjörnungum. Þar sem þeir eru upprunir úr bitkirtlum skordýrs er eðlilegast að tjá þá í skordýrafrumum.

Markmið rannsóknarinnar var að tjá fjóra aðalofnæmisvaka; Cul n 1, Cul n 2, Cul n 4 úr *C. nubeculosus* og Cul o 3 úr *C. obsoletus* í skordýrafrumum og hreinsa þá auk Cul n 3, Cul o 1 og Cul o 2 sem áður höfðu verið tjáðir. Einnig að setja upp ónæmispróf til að meta mótefna- og boðefnasnið í kjölfar meðferðar.

Ofnæmisvakarnir voru tjáðir í skordýrafrumum með Bac-to-Bac baculoveirutjáningarkerfi með þremur mismunandi plasmíðum; pFastBac1, pFastBac-HBM-TOPO (Honey bee melittin seytiröð) og pI-secSUMOstar (Small-ubiquitin-related-modifier sem á að auka stöðugleika og leysanleika próteina). Endurraðaðar baculoveirur voru framleiddar í Sf-9 skordýrafrumum, 6xhis-merkt endurröðuð prótein í High-five skordýrafrumum og próteinin síðan hreinsuð með nikkel perlum og himnuskiljun. Tjáning, framleiðsla og hreinsuð prótein voru prófuð með coomassie litun og í ónæmisþrykki með sérvirkum mótefnum gegn próteinunum. Elísupróf (ELISA) voru sett upp til mótefnamælinga og *in vitro* örvun gerð á hvítfrumum fyrir boðefnaseytingu.

Ofnæmisvakarnir Cul n 3, Cul n 4 og Cul o 2 voru hreinsaðir á náttúrulegu formi. Sett var upp elísupróf fyrir Cul n 3 og Cul n 4 og þeir einnig notaðir við *in vitro* örvun á hvítfrumum úr bólusettum hestum og boðefnaframleiðsla mæld í kjölfarið. Cul n 4 var bæði hreinsaður í fullri lengd og sem SUMOstar prótein. Óklippt Cul n 4 SUMOstar samrunaprótein reyndist nothæft í elísuprófi.

Ekki tókst að hreinsa Cul n 1, Cul n 2 og Cul o 1 tjáða með HBM seytiröð á náttúrulegu formi en Cul n 1 og Cul o 1 voru nothæfir í elísupróf eftir hreinsun á afmynduðu formi. Cul n 1, Cul n 2 og Cul o 3 voru tjáðir sem SUMOstar samrunaprótein og tókst að hreinsa þau á náttúrulegu formi en þau féllu út við himnuskiljun. Örvun hvítfrumna var ekki til lykta leidd hvorki með afmynduðum próteinum né með SUMOstar samrunapróteinum.

Sjö ofnæmisvakar úr smámýi voru tjáðir í skordýrafrumum og sýnt fram á að fimm þeirra séu nothæfir í próf til að meta mótefna- og boðefnaframleiðslu í kjölfar ónæmismeðferðar og/eða sjúkdómsgreiningu. Sýnt var að tjáning ofnæmisvaka sem SUMOstar samrunaprótein getur auðveldað hreinsun þeirra og þau nýst óklippt í mótefnapróf.

# Abstract

Insect bite hypersensitivity (IBH) is recurrent seasonal dermatitis of horses characterized by intense pruritus and eczema leading to excoriations which contribute to secondary infections. The lesions are mainly localized on the head, along the dorsal midline and at the base of the main and tail. IBH is an animal welfare issue and an obstacle in horse exportation as the prevalence of the disease is considerable higher in exported Icelandic horses than in horses born on the European continent. IBH is a type I allergy with production of IgE antibodies, caused by allergens of biting midges (*Culicoides* spp.). The midges are not indigenous to Iceland. Thirteen allergens have been isolated from three midge species, *C. sonorensis*, *C. nubeculosus*, *and C. obsoletus*, expressed in *E. coli* and purified. The pathogenesis of the disease has been studied and development of immunotherapy is ongoing. The allergens produced and purified from *E. coli* are not suitable for some of the immunoassays needed to evaluate immunotherapy. Therefore it is necessary to express them in eukaryotic cells and as they are originated in the salivary glands of insects, it is obvious to express them in insect cells.

The objective of the study was to express four of the major allergens Cul n 1, Cul n 2, Cul n 4 from *C. nubeculosus* and Cul o 3 from *C. obsoletus* in insect cells and purify them in addition to Cul n 3, Cul o 1 and Cul o 2 that had been expressed before as well as to set up immunoassays for evaluation of antibody and cytokine response following immunotherapy.

The allergens were expressed in insect cells using the Bac-to-Bac baculovirus expression system with three different vectors; pFastBac1, pFastBac-HBM-TOPO (Honey bee melittin secretion signal) and pI-secSUMOstar (Small-ubiquitin-related-modifier which increases the stability and solubility of proteins). Recombinant baculoviruses were produced in Sf-9 insect cells, 6xhis-tagged recombinant proteins in High-five insect cells followed by purification with nickel affinity resin and dialysis. Expression, production and purified proteins were tested with coomassie blue staining and Western blot using protein specific antibodies. The purified proteins were used to set up tests for measuring antibodies and cytokines.

The allergens Cul n 3, Cul n 4 and Cul o 2 were purified under native conditions. ELISA was set up with Cul n 3 and Cul n 4 and they used for *in vitro* stimulation of peripheral blood mononuclear cells (PBMC) from vaccinated horses and cytokine production measured. Cul n 4 was purified in full length and as SUMOstar fusion protein. Uncleaved Cul n 4 SUMOstar fusion proteins could be used in ELISA.

Expressed with HBM secretion signal Cul n 1 and Cul o 1 could only be purified under denaturing conditions and subsequently used in ELISA. Cul n 1, Cul n 2 and Cul o 3 were expressed as SUMOstar fusion proteins and purification under native conditions was successful, however, the proteins precipitated after dialysis. *In vitro* stimulation was not accomplished, neither with proteins purified under denaturing conditions nor with SUMOstar fusion protein.

Seven allergens were expressed in insect cells and demonstrated that five of them were applicable for evaluating immunotherapy and/or diagnosis. Expression of allergens as SUMOstar fusion proteins may ease purification and they can be used uncleaved in ELISA.

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# List of abbreviations

| | |
|---|---|
| a.a. | Amino acid |
| AP | Alkaline phosphatase |
| APCs | Antigen-presenting cells |
| B-cells | B-lymphocytes |
| BSA | Bovine serum albumin |
| DCs | Dendritic cells |
| DNA | Deoxyribonucleic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| FoxP3 | Forkhead box 3 |
| FPLC | Fast protein liquid chromatography |
| High-five | Cell line from *Trichopulsia ni* |
| hr | Hour |
| HRP | Horseradish peroxidase |
| IBH | Insect bite hypersensitivity |
| IFN | Interferon |
| Ig | Immunoglobulins |
| IL | Interleukin |
| $iT_{reg}$ | Induced regulatory T cells |
| kDa | Kilo Dalton |
| LPS | Lipopolysaccaride |
| mAbs | Monoclonal antibodies |
| MHC | Major histocompatibility complex |
| NK cells | Natural killer cells |
| $nT_{reg}$ | Natural regulatory T cells |
| OD | Optical density |
| o.n. | Overnight |
| pAbs | Polyclonal antibodies |
| PAMPs | Pathogen-associated molecular patterns |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate buffer saline |
| PCR | Polymerase chain reaction |
| PEST | Penicillin and Streptomycin |
| PRRs | Pathogen recognition receptors |
| r-allergen | Recombinant allergen |
| r-bacmid | Recombinant bacmid |
| r-baculovirus | Recombinant baculovirus |
| RNA | Ribonucleic acid |
| RT | Room temperature |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| Sf-9 | *Spodoptera frugiperda* |
| TBS | Tris buffer saline |
| TBS-T | Tris buffer saline with Tween 20 |
| T-cells | T-lymphocytes |
| $T_{FH}$ | T follicular helper cells |
| TGF | Transforming growth factor |
| $T_H$ | T helper cells |
| TLRs | Toll-like receptors |
| $T_{reg}$ | T regulatory cells |
| WB | Western blot |
| Wo | Without |

# 1 Introduction

## 1.1 The immune system

The immune system is a defense mechanism with different types of effector cells and molecules that coordinate to protect the body against infectious agents and the damage they can cause. It recognizes the presence of infection by distinguishing between self and non-self antigens. Our bodies are constantly exposed to microorganisms and pathogens. Most pathogens we encounter daily do not get past the epithelium of the skin and mucosa, the first line of defense. If the first line of defense fails there are two distinct systems that together eliminate the infection, the innate and adaptive immune systems. Most pathogens are detected and destroyed within minutes or hours after they enter the body by the innate immune system. All cells of the immune system derive from the same pluripotent hematopoietic stem cells in the bone marrow. They give rise to two types of progenitor white blood cells; of myeloid origin (granulocytes, macrophages, dendritic cells and mast cells) and of lymphoid origin (lymphocytes and innate lymphoid cells (ILC)). The cells communicate with proteins/peptides called cytokines and chemokines (Murphy et al., 2012a).

The primary organs of the immune system are bone marrow and thymus where immature lymphocytes develop. The secondary organs are lymph nodes, the spleen and the muscosal lymphoid tissues of the gut, respiratory tract, urogenital and other mucosa. In the secondary organs antigens are localized and exposed to mature lymphocytes (Murphy et al., 2012a).

### 1.1.1 The innate immune system

The innate immune system responds immediately to infection but does not generate long lasting immunological memory. The leukocytes of the innate immune system are of myeloid origin; macrophages, neutrophils, basophils; eosinophils; mast cells, dendritic cells, except for ILC of lymphoid origin. Macrophages are found in most tissues and are the mature form of monocytes. They are relatively long-lived cells and have several roles in the immune response. By phagocytosis, they engulf and kill pathogens and thereby dispose of pathogens and infected cells targeted by adaptive immune response. Macrophages and dendritic cells (DCs) are antigen-presenting cells (APCs). Dendritic cells provide signals that are necessary to activate T lymphocytes that are detecting their specific antigen for the first time, hence are an important link between the innate and the adaptive immune systems. They migrate through the bloodstream from bone marrow to enter the tissues. They take up antigen by phagocytosis like macrophages or macropinocytosis, but their main role in the immune system is not to eliminate infection. Granulocytes have granules in their cytoplasm containing a variety of enzymes and toxic proteins. There are three types of granulocytes; neutrophils, eosinophils and basophils. Neutrophils are the most abundant cells of the innate immune system. They eliminate pathogens by phagocytosis, i.e. take up antigens and destroy them intracellular by degradative enzymes and antimicrobal substances stored in their granules. Eosinphils and basophils release enzymes and toxic proteins upon activation. They are important in defenses against parasites too large to be ingested by macrophages and neutrophils and also play a major role in allergy. The mast cells are found in the connective tissues throughout the body. They contain granules that store

bioactive molecules including histamine, released upon activation. They are involved in defenses against parasites and have a crucial role in allergic reactions (Murphy et al., 2012a). ILCs are a subset of innate cells that are of lymphoid origin but are not antigen-specific as they lack antigen-specific receptor. ILCs play roles in early infection control, adaptive immune regulation, lymphoid tissue development and in tissue homeostasis and repair. ILCs can be divided into three groups depending on the cytokines they produce. Group 1 ILCs produce interferon (IFN)-γ, they include cell population termed ILC1 and natural killer cells (NK cells) that kill virus infected cells and some tumor cells. Group 2 ILCs produce type 2 cytokines interleukin (IL)-4, IL-5, IL-9 and IL-13 that are generally associated with adaptive $T_H2$ cells. Group 3 ILCs are characterized by expression of the transcription factor Rorγt, but their expression of T box transcription factor (T-bet), cell surface markers and cytokines varies (Cortez et al., 2015).

Cells of the innate immune system recognize pathogens through several receptor systems they express, pathogen recognition receptors (PRRs) such as toll-like receptors (TLRs). These receptors recognize molecular structures on the surface of microorganisms known as pathogen-associated molecular patterns (PAMPs), such as lipopolysaccaride (LPS). The innate immune response can either eliminate the infection or contain it while an adaptive response develops (Murphy et al., 2012b).

### 1.1.2 The adaptive immune system

Response by the adaptive system takes days rather than hours to develop. The adaptive immune system is capable of eliminating infections by specific recognition by lymphocytes. These cells have antigen receptors on their surface, highly specialized due to somatic hypermutation and recombination. They recognize and respond to individual antigens. The effector cells of the adaptive immune system are of lymphoid origin, B-lymphocytes (B-cells) and T-lymphocytes (T-cells) (Murphy et al., 2012a).

B-cells originate and develop in the bone marrow. They are the effector cells of the humoral immunity and recognize native antigens in the extracellular environment. When activated, they proliferate and differentiate into plasma cells that produce antibodies or long lived memory B-cells that provide life-long immunity. Antibodies are secreted form of the B-cell receptor and have identical specificity to the receptor. They are known as immunoglobulins (Ig). The role of Ig's is to bind and neutralize pathogens, promote phagocytosis by opsonization and to activate the complement system. Immunoglobulins may be divided into five major classes, IgA, IgD, IgE, IgG and IgM with different functions. Another role of B-cells is to act as APCs (Murphy et al., 2012c). Cross-reactivity is defined as the binding of antigen with antibody developed against another antigen (immunogen). Most antibodies cross-react with closely related antigens but some without any clear relationship to the immunogen (Murphy et al., 2012f).

T-cells originate in the bone marrow but develop in the thymus and are the effector cells of cellular immunity. T-cells detect intracellular infections by recognition of peptide fragments from pathogen proteins presented on major histocompatibility complex (MHC) on the surface of APCs. T-cells are divided into $CD8^+$ T cytotoxic cells and $CD4^+$ T helper cells. $CD8^+$ T cells recognize peptides bound to MHC I, from virus infected cells and tumor cells. Upon antigen recognition they release cytotoxic

proteins, perforin, granzymes and granulysin which lead to apoptosis of the target cell. $CD4^+$ T cells recognize peptides bound to MHC II, they activate and direct other cells of the immune system with cytokines. Both $CD8^+$ T cells and $CD4^+$ T cells form experienced memory cells after primary immune activation enabling a faster and stronger immune response upon second encounter to pathogen (Murphy et al., 2012c).

DCs produce cytokines depending on the pathogens they are presenting, that drive the differentiation of naive T cell into various effector subsets with different immunological functions depending on the type of the pathogen recognized (Figure 1). The main subsets of $CD4^+$ T helper cells are $T_H1$, $T_H2$, $T_H17$, $T_{FH}$ and T regulatory cells ($T_{reg}$). IL-12 and IFN-γ drive the differentiation of naive $CD4^+$ T-cells to a $T_H1$ phenotype. These effector $T_H1$ cells secrete IFN-γ and thereby activate macrophages and enable them to destroy intracellular pathogens. The development of $T_H2$ effector cells is mainly induced by IL-4. Other cytokines that trigger $T_H2$ development are IL-5, IL-9 and IL-13. $T_H2$ effector cells activate mast cells, basophils and eosinophils by secreting IL-4, IL-5 and IL-13. $T_H17$ cells develop in the presence of IL-6 and IL-1β (in humans) and in the absence of IL-4 and IL-12. The effector $T_H17$ cells secrete IL-17 that induces local epithelial and stromal cells to produce chemokines that recruit neutrophils to the infection site. $T_{reg}$ control and suppress adaptive immune responses. They secrete and develop in the presence of IL-10 and transforming growth factor-β (TGF-β). $T_{reg}$ are divided into two groups. Natural regulatory T cells ($nT_{reg}$) are mostly found in lymphoid tissues and discriminate between self- and non-self antigens. They have conventional T-cell receptors and are not considered antigen-specific. Induced regulatory T cells ($iT_{reg}$) are mostly found in the periphery and are not self-reactive as $nT_{reg}$. They are thought to be antigen-specific (Murphy et al., 2012d). The development of $T_{FH}$ is complicated and it is not defined by any single event. In humans the cytokines IL-21, IL-12, IL-23 and TGF-β seem to be dominant in the development and the inducible T-cell costimulator, ICOS (CD278). The $T_{FH}$ effector cells are localized in the B cell follicle and drive B cells to differentiate and produce immunoglobulins. The cytokines $T_{FH}$ cell secrete are characteristic for $T_H1$, $T_H2$ and $T_H17$ (Crotty, 2014; Murphy et al., 2012d). $T_H9$ cells develop in the presence of TGF-β and IL-4, however TGF-β alone generates $T_{reg}$ and IL-4 $T_H2$ cells. These cells have lower $T_H2$-type cytokine production and secrete IL-9 (Dardalhon et al., 2008; Veldhoen et al., 2008). $T_H9$ cells play a role in allergy but it is not know whether they are different from $T_H2$ cells or whether $T_H2$ cells can transform into $T_H9$ during allergic inflammation (Erpenbeck et al., 2003; Staudt et al., 2010). $T_H22$. cells are characterized by the production of IL-22 which functions as proinflammatory or anti-inflammatory cytokine acting primary on nonimmune cells such as those of the skin, the digestive track, the lungs and the kidneys (Akdis et al., 2011; Eyerich et al., 2009). They are also capable of secreting IL-10 and TNF-α (Soyer et al., 2013).

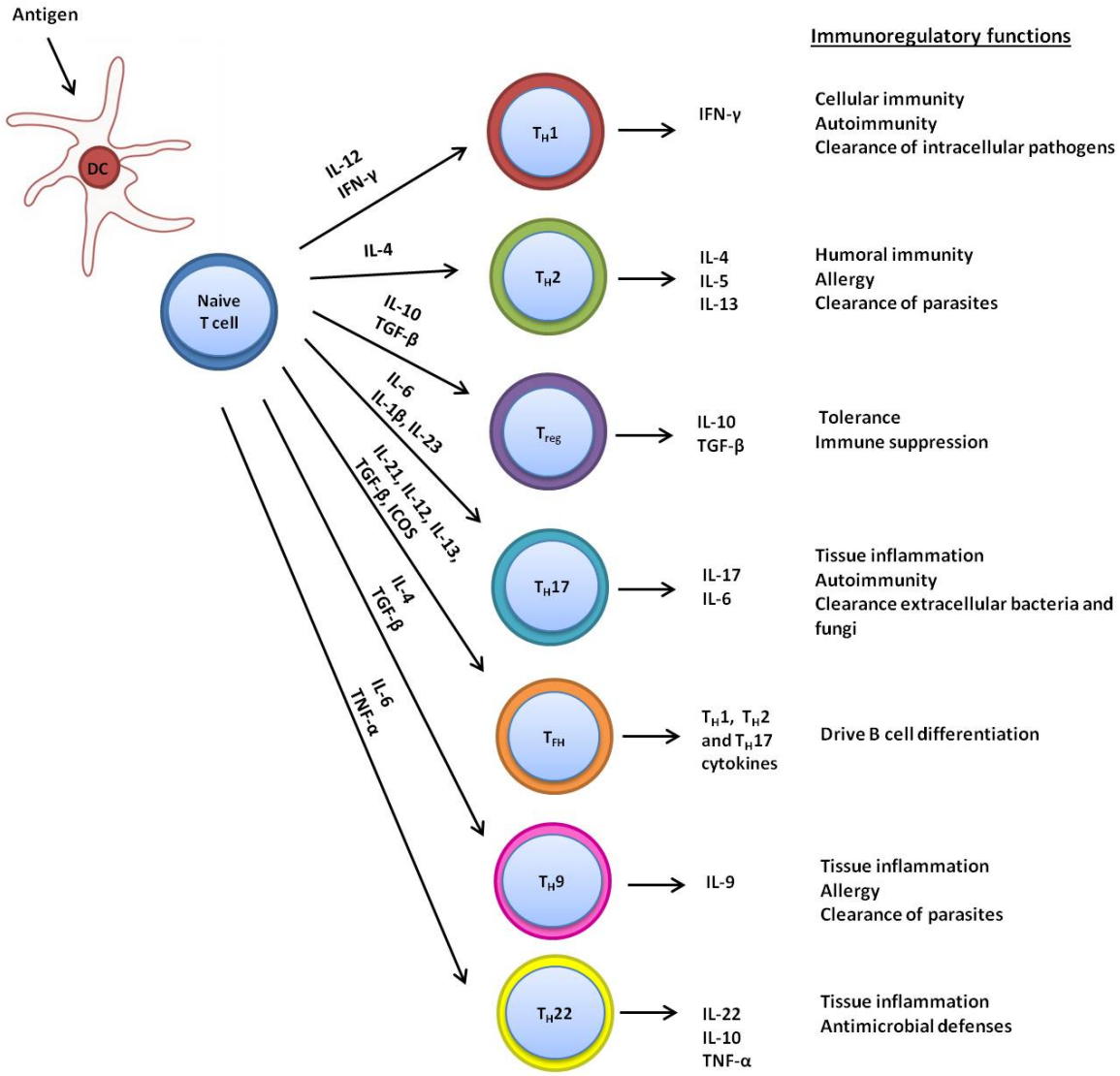


**Figure 1. Differentiation of naive CD4$^{+}$´T cells into effector cells.**
The antigen and environmental factors determine the signals of the DCs in the form of cytokines that induce the development of CD4$^{+}$ T cells. Each effector T cell subset produces unique cytokines and has unique immunoregulatory function (Crotty, 2014; Soyer et al., 2013).

### 1.1.3 The immune system of horses

The immune system of horses consists of the same cells, molecules and mechanisms as those of other mammals. The immunoglobulin classes of horses are the same as in humans and mice, IgM, IgA, IgG, IgE and IgD. However, horses have seven IgG subclasses, IgG1 (IgGa), IgG2, IgG3 (IgG(T)), IgG4 (IgGb), IgG5 (IgG(T)), IgG6 (IgGc) and IgG7 (Wagner, 2006a). Another important difference between horses as compared to laboratory mice and humans is that horses have high parasitic load (Hamza et al., 2010). Therefore, the total concentration of IgE antibodies in the serum of healthy adult horses can be up to 1000-fold higher than in human serum (Wilson et al., 2006).

### 1.1.4 Allergy

An allergy is a hypersensitivity reaction of the immune system to an innocuous antigen or allergen. Allergic reactions are classified into hypersensitivity types I-IV. Type I hypersensitivity is mediated by

IgE antibodies. In some individuals antigen that enters the body becomes an allergen, DCs take it up for antigen-presentation and in the presence of IL-4 it drive the differentiation of the naive T-cell to a $T_H2$ phenotype. The $T_H2$ cell induces B-cell switch to IgE antibody production by secreting IL-4. The IgE antibodies bind to high affinity Fc receptors (FcεRI) on the surface of mast cells (in tissues) and basophils (in the blood). This process is called sensitization. The mast cells and basophils are activated due to re-exposure to the allergen when it cross-links the IgE antibodies bound on their surface. When activated these cells release inflammatory mediators, such as histamine, prostaglandins and leukotrienes that cause allergic symptoms (Murphy et al., 2012e). The innate immune system plays an important role in allergy. After allergen enters the body the epithelium releases Th2-promoting cytokines like thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 which activate different types of cell subsets as DCs and ILC2. Allergic symptoms depend on route of allergen entry. For food allergens the symptoms are in the oral and gastrointestinal mucosa, aeroallergens in the nasal and airway mucosa and subcutaneous entry through skin causes local release of histamine (Palomares et al., 2014). Type II hypersensitivity reactions are mediated by IgG antibodies against cell-surface or allergens in the matrix and type III involves damage caused by complexes formed by antigen and IgG antibodies. Type IV hypersensitivity or delayed type hypersensitivity reactions are T-cell mediated and take 2 or 3 days to develop (Murphy et al., 2012e).

### 1.1.5 Allergens

A protein is classified an allergen when it causes IgE antibody response in at least five individuals according to World Health Organization and International Union of Immunological Societies (Breiteneder H, 2014; Traidl-Hoffmann et al., 2009). Major allergen is generally regarded as one to which more than 50% of patients tested react with the corresponding allergen-specific IgE in the given test system (Breiteneder H, 2014; Larsen & Lowenstein, 1996).

A systematic nomenclature overseen by Allergen Nomenclature Sub-Committee of the World Health Organization (WHO) and International Union of Immunological Societies (IUIS)) is used to name purified allergens. The committee maintains a database of approved allergen names (http://www.allergen.org). Allergens are named using the first three letters of the genus followed by single letter for the species they originate from, and a number indicating the order of allergen purification. For example the first allergen described from the house dust mite *Dermatophagoides pteronyssius* is Der p 1 (Chapman et al., 2007; Radauer et al., 2014; Traidl-Hoffmann et al., 2009).

What makes a protein an allergen is not completely clear. Most allergens derive from animals, plants and fungi. The route of exposure, dose and function of an allergen is important for allergic sensitization. Most allergens are proteins or glycoproteins (Traidl-Hoffmann et al., 2009). Comparison of primary structure (amino acid sequence) of the major protein allergens show that most of them are relatively small (<70kDa), negatively charged with low hydrophobicity and high stability (Chapman et al., 2007; Palomares et al., 2014). Post-translation modification such as glycosylation or disulfide bonds may affect solubility, stability and size of proteins and thereby affect the allergenicity (Aalberse, 2000). However, none of the structural features can distinguish between allergenic and non-allergenic proteins (Aalberse, 2000; Chapman et al., 2007). There are two properties that allergens should have

in common, (i) their ability to cause IgE production and (ii) the capability of causing IgE mediated allergic responses. Allergenic proteins have been divided into two groups, type I allergens are proteases able to promote Th2 responses and type II allergens are nonproteases able to disrupt the barrier by various mechanisms and induce Th2 responses. The cysteine protease Der p 1 the major allergen from house dust mite was the first allergen described to show proteolytic activity and is a type I allergen. The allergen Der p 2 is type II allergen which induces $T_H2$ responses by mimicking components of the TLR4 signaling complex even in the absence of LPS (Palomares et al., 2014).

#### *1.1.5.1 Insect allergens*

Proteins originating from biting and stinging insects are known to cause allergy both in humans and animals (Arlian, 2002; Cantillo et al., 2014; McDermott et al., 2000; Orange et al., 2004; Peng & Simons, 2004; Spillner et al., 2014). Biting insects inject venom with their mouthparts but stinging insects inject venom with their stingers. The mouthparts of biting insects have evolved for handling pray and cause paralysis or even death (Vetter & Visscher, 1998). Some biting insects are blood-feeding and their saliva contains substances that work against the host barriers such as hemostasis, inflammation and immunity (Ribeiro & Francischetti, 2003). The stings of stinging insects have evolved for defense and are therefore designed to cause immediate pain in the hosts. Both mouthparts and stings are designed for venom delivery and have venom glands and ducts (Vetter & Visscher, 1998).

Stinging insects of the hymenoptera family, such as bees (honey bees) and vespids (wasps, hornets and yellow jackets) have venom containing proteins that cause IgE mediated reactions both local and systemic. The venom allergens are proteins of 10 - 200 kDa, some are unique and others have homologous in different species. Insect-allergic patients can be sensitive to multiple species of stinging insects due to cross-reactivity of insect venoms or exposure to multiple insects (King & Spangfort, 2000; Spillner et al., 2014). Twelve honey bee (*Apis mellifera*) venom allergens have been identified and the most prominent and high abundance allergens are phospholipase $A_2$, hyaluronidase, and melittin (Hoffman, 2006; King & Spangfort, 2000; Spillner et al., 2014). It has been demonstrated that the allergen Api m 10, a carbohydrate-rich protein, is one of the major allergens of honey bee venom (Blank et al., 2011b). The most prominent allergens of yellow jackets (*Vespula vulgaris*) venom are phospholipase $A_1$, hyaluronidase and Antigen 5 (Hoffman, 2006; King & Spangfort, 2000; Spillner et al., 2014). An inactive isoform of hyaluronidase in yellow jacket venom has been identified and seems to be the dominating isoform in the venom (Kolarich et al., 2005). These two sets of prominent allergens in honey bees and yellow jacket are found with modification throughout most Hymenoptera species (Spillner et al., 2014).

The allergens of biting insects originate in their salivary glands. When the ducts of mosquito salivary glands are cut, they can still feed and produce eggs but they are unable to cause skin reactions in humans (Hudson et al., 1960). Venom allergens of mosquitoes may cause local or systematic allergic reactions (Cantillo et al., 2014). There are more than 40 mosquito genera and more than 3000 species world-wide. Several allergens have been identified in mosquito whole body extracts but only a few saliva proteins have been characterized to elicit allergic response. Most of the proteins in mosquito saliva are allergenic in humans (Cantillo et al., 2014; Peng & Simons, 2004). Most reported mosquito allergens come from *Aedes aegypti*. Some of the allergens are species-specific

whereas others are conserved between different species. The three major allergens of mosquitoes are apyrase, D7 protein and α-glucosidase. Mosquito allergens may cross-react with allergens originating in other biting insects and even stinging insects (Cantillo et al., 2014; Peng & Simons, 2004).

All the allergens listed in http://www.allergen.org are allergic in humans but allergy is also known in other mammals (Couetil et al., 2007; Jensen-Jarolim et al., 2015). In the attempt to isolate the allergens in insect bite hypersensitivity (IBH) four allergens were isolated from the black fly species *Simulium vittatum* with phage surface display technology from their salivary glands. The allergens were antigen 5- like protein (Sim v 1), Kunitz protease inhibitor (Sim v 2) and two α-amylases (Sim v 3 and Sim v 4). Horses suffering from IBH reacted to all of them (Schaffartzik et al., 2009).

### 1.1.6 Immunotherapy

The only specific disease modifying treatment and desensitization of allergic diseases in humans is allergen specific immunotherapy. It is a long-term treatment that decreases allergic symptoms of many patients. The treatment traditionally consists of the administration of allergen in low doses which are gradually increased over a long period of time. The main routes of administration are injection of allergen in adjuvant subcutaneous (subcutaneous immunotherapy (SCIT)) and tablets or drops under the tongue (sublingual immunotherapy (SLIT)). The outcome of immunotherapy is measured by immune parameters such as antibodies, cytokines and inflammatory mediators. In successful immunotherapy there is generation of allergen-specific $T_{reg}$ and/or $T_H1$ cells, inhibition of $T_H2$ responses, decreases in allergen-specific IgE, increases in allergen-specific IgG and IgA and reduction in infiltrating inflammatory cells (Hanci et al., 2015; Palomares et al., 2014).

## 1.2 Insect Bite Hypersensitivity

Insect bite hypersensitivity (IBH), also known as summer eczema and sweet itch, is a recurrent seasonal dermatitis of horses (Baker & Quinn, 1978; Riek, 1953). IBH is caused by allergic reaction to the bites of biting midges of the genus *Culicoides* (Fadok & Greiner, 1990; Halldorsdottir et al., 1989; Larsen et al., 1988). It is an IgE-mediated type I hypersensitivity with release of inflammatory mediators such as histamine (Marti et al., 1999; Wagner et al., 2006b; Wilson et al., 2001). Few studies have reported delayed reactions but delayed type hypersensitivity is not thought to be the major reaction of IBH (Zeller et al., 2009). The allergens of IBH are originated in the salivary glands of the midges (Hellberg et al., 2006).

### 1.2.1 Epidemiology

IBH is found worldwide where *Culicoides* midges are indigenous and all breeds of horses can be affected. The prevalence of IBH varies between countries and regions within countries (Anderson et al., 1993; Braverman et al., 1983; Brostrom et al., 1987; Halldordsottir & Larsen, 1991; Kurotaki et al., 1994; Larsen et al., 1988; Littlewood, 1998). Due to absence of *Culicoides* in Iceland, IBH is not found in the native horse population. However the overall prevalence of IBH is about 30% in Icelandic horses exported to the European continent. The prevalence is highest two years after export to *Culicoides* infested areas. In Icelandic horses born in infested areas, the prevalence of IBH is around 5-10% which is no higher than in other breeds (Bjornsdottir et al., 2006; Brostrom et al., 1987; Halldordsottir &

Larsen, 1991; Lange et al., 2005). A recent study shows that the likelihood of developing IBH increases with the age of the horse at export. Whereas weanlings at the age 7 to 10 months do not develop IBH in higher frequency than Icelandic horses born in *Culicoides* infested areas (Sommer-Locher et al., 2012). The high prevalence among Icelandic horses exported to the European continent is probably due to lack of exposure to the *Culicoides* midges in early live (Bjornsdottir et al., 2006).

### 1.2.2 Clinical signs, diagnosis and treatment

The disease is characterized by skin sensitization followed by severe itching that leads to localized hair loss, thickening of the skin (hyperkeratosis) and excoriation that can induce secondary infections (Figure 2) (Baker & Quinn, 1978; Brostrom et al., 1987; Riek, 1953). The symptoms appear in the spring and disappear in the late autumn (Baker & Quinn, 1978). Lesions caused by IBH are mostly localized along the dorsal midline of the horse body at the base of the main and tail due to the preferential feeding sites of the biting midge (Townley et al., 1984).

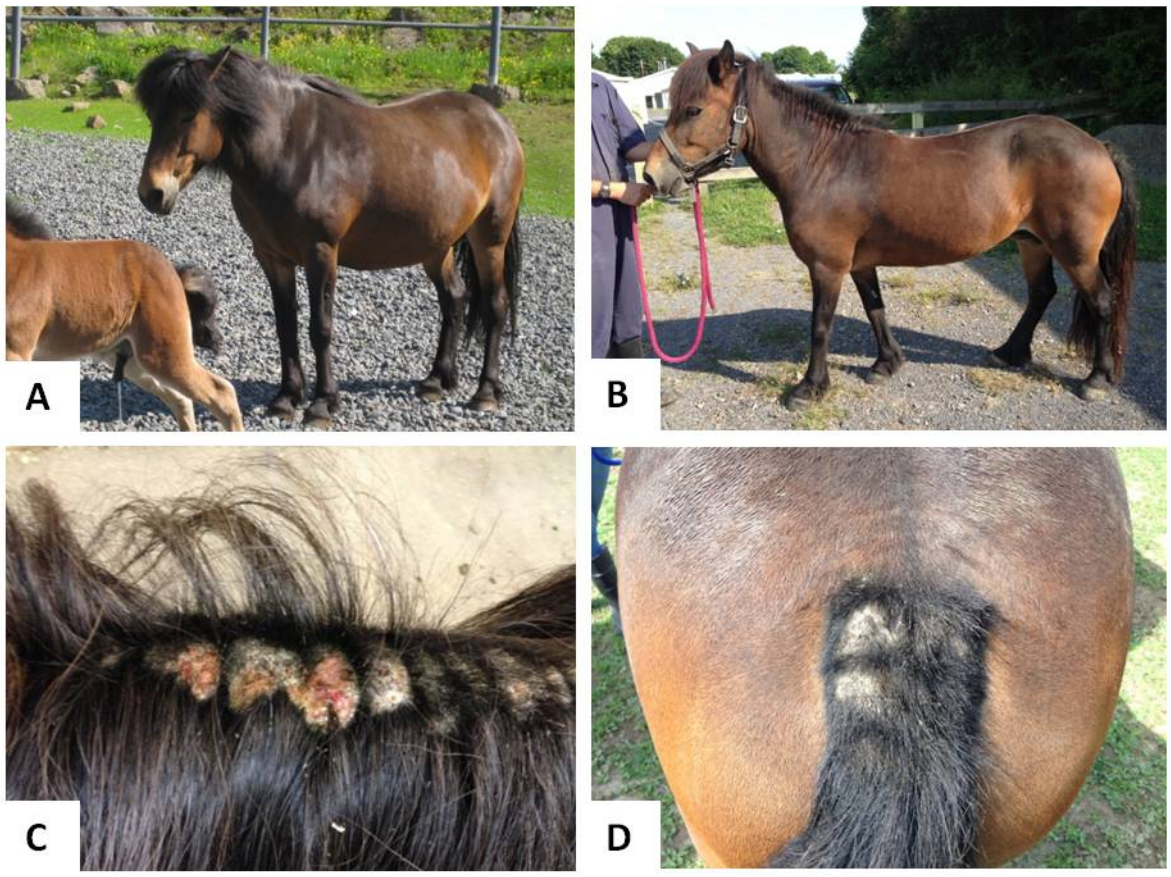


**Figure 2. Clinical signs of IBH**
**A.** The mare Sigga in Iceland July 2011. **B.** Sigga with clinical signs of IBH in USA at Cornell University College of Veterinary Medicine July 2014. **C.** Mane with severe dermatitis and excoriation. **D.** Tail with localized hair loss. Photo A: Sigurbjörg Þorsteinsdóttir, photos B-D: Bettina Wagner.

Diagnosis of IBH is mainly by clinical signs, but various *in vitro* tests have been evaluated as summarized by Marti et al., 2008. These tests are serological or based on cellular response after stimulation *in vitro* with *Culicoides* whole body extract, such as histamine release test and

sulfidoleukotriene release test (Marti et al., 2008). Allergen content of the *Culicoides* whole body extract has not been standardized (Anderson et al., 1993; Braverman et al., 1983; Ferroglio et al., 2006; Marti et al., 2008) and the performance of these tests needs to be improved using high affinity IgE reagents and pure recombinant allergens (Marti et al., 2008).

Attempts for immunotherapy for IBH have been described but presently there is no effective treatment (Anderson et al., 1996; Barbet et al., 1990; Ginel et al., 2014). The only way of preventing IBH is a total avoidance of *Culicoides* midges (Baker & Quinn, 1978; Riek, 1953). Horses are kept in stables around dawn and dusk, the main feeding time of the midge, or dressed in special blankets with hoods to enclose the body (Figure 3) (Littlewood, 1999). Symptomatic treatments like the use of corticosteroids are used in severe cases (Anderson et al., 1996).

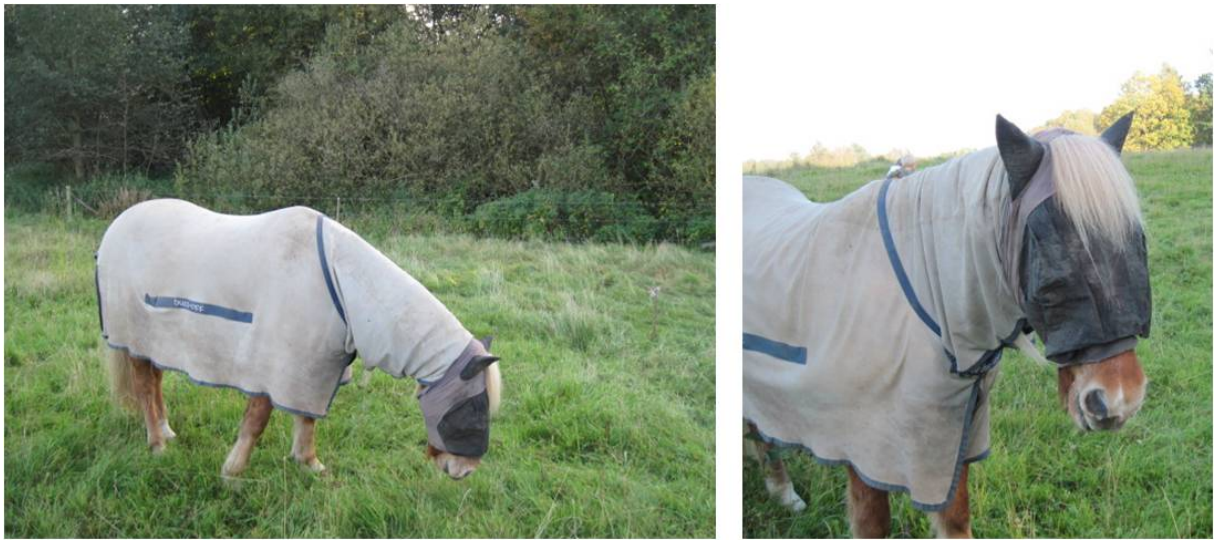

**Figure 3. Horse wearing a blanket as a protection against *Culicoides* bites**
Photos: Þórunn Guðmundsdóttir.

### 1.2.3 Pathogenesis of IBH

IgE antibodies are involved in the allergic reactions of IBH as results show high association between IgE antibodies against salivary gland proteins of *Culicoides* and clinical signs of IBH (Figure 4) (Baselgia et al., 2006; Hellberg et al., 2006; Wagner et al., 2006b; Wilson et al., 2001). Hellberg et al., 2006 showed that 93% of horses affected with IBH had IgE antibodies against salivary gland proteins of *Culicoides* but only 7% of healthy horses. Wagner et al., 2006b have shown with modified Prausnitz-Küstner experiment that the allergic reaction in IBH can be transferred to healthy horses using IgE from IBH affected horses, providing strong evidence that the IgE mediates mast cells degranulation and allergic reactions (Wagner et al., 2006b). Immunoblot analysis showed that IgE, IgG3/5 and IgG1 in sera from IBH affected horses bound significantly more to *C. nubeculosus* salivary gland proteins than in sera from healthy horses (Hellberg et al., 2006). Figure 4 showes the interplay between allergens, cells, cytokines and antibodies resulting in allergic reactions.

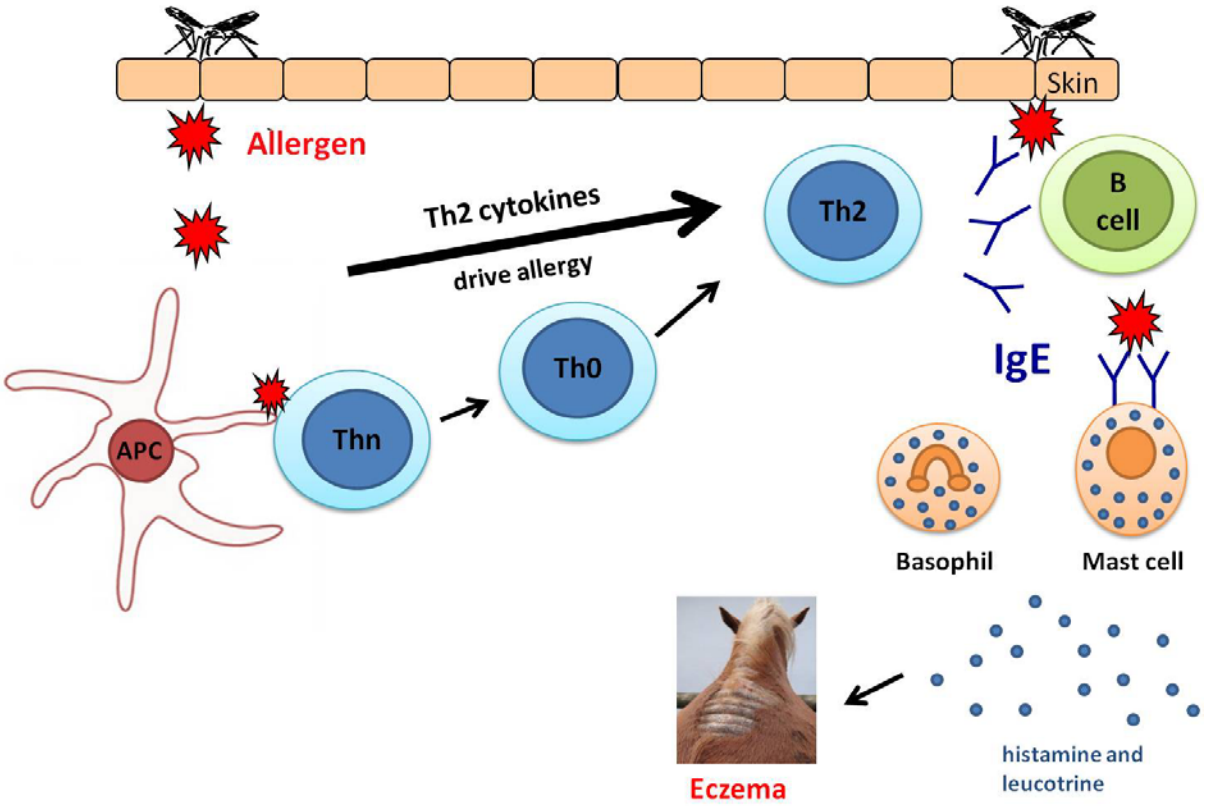


**Figure 4. IgE mediated response in IBH**
The fly bites the horse and secretes proteins (allergens) with its saliva into the skin. The antigen presenting cell (APC) presents the allergen to naive $CD4^+$ T helper cell (Thn) which differentiates into Th2 cell. The Th2 cell induces a B-cell to produce IgE antibodies specific to the allergen. The IgE antibodies bind to high affinity receptor FcεRI on mast cells and basophils. The horse is thereby sensitized against the allergen. Upon re-exposure it will elict allergic response with the release of inflammatory mediators of mast cells and basophils. Schematic picture: Sigurbjörg Þorsteinsdóttir.

The role of T-cells and $T_H1/T_H2$ focus has been investigated both in the skin and the circulation of IBH affected horses. When stimulated with *Culicoides* extract, peripheral blood mononuclear cells (PBMC) from horses born in Iceland and IBH affected horses showed increased IL-4 mRNA levels and higher proportion of IL-4 producing T cells compared to healthy horses (Hamza et al., 2007). Horses born in Iceland and exported to mainland Europe ($1^{st}$ generation horses) acquire IBH at a much higher frequency than horses born on the European mainland ($2^{nd}$ generation horses) (Bjornsdottir et al., 2006). The proportion of IL-4 producing T cells was higher in PBMC from IBH affected $1^{st}$ generation horses as compared to those from IBH $2^{nd}$ generation horses (Hamza et al., 2007). The *Culicoides* specific IL-4 production, rarely detected in healthy $2^{nd}$ generation horses, is suppressed by IL-10 and TGF-β1. Neutralization of these regulatory cytokines resulted in an increased number of IL-4 producing T cells (Hamza et al., 2008).

In lesional skin of IBH affected horses, expression of IL-13 was increased compared to skin of healthy horses but not IL-4 and IL-5 (Heimann et al., 2011). These three cytokines favor the development of $T_H2$ cells. In the initiation of allergic response IL-4 is dominant whereas IL-13 is critical for the effector functions (Corry, 1999). The expression of forkhead box 3 (FoxP3), a marker for $T_{reg}$ cells and IL-10 was significantly lower in the skin of IBH affected horses than in healthy controls (Heimann et al., 2011). In the blood the difference of FoxP3 expression by $CD4^+CD25^+$ cells was also

shown to be significantly higher in healthy than in IBH affected horses following *Culicoides* extract stimulation of PBMC. Also that addition of recombinant IL-4 to PBMC from healthy horses stimulated with the allergen significantly decreased the proportion of FoxP3 expressing cells suggesting that the increased IL-4 production by PBMC of IBH affected horses is associated with a decreased number of allergen-induced $T_{reg}$ (Hamza et al., 2012). Therefore both results from the blood and skin indicate that the IBH is like type I hypersensitivity in humans, $T_H2$ focused and associated with reduced regulatory immune response.

Like allergens, parasite infections induce $T_H2$ immune responses and horses are chronically infected with parasites. Horses living in Iceland have higher helminth burden and higher total serum IgE level than 1$^{st}$ and 2$^{nd}$ generation horses living in Switzerland (Hamza et al., 2010; Wilson et al., 2006). However, in response to parasite extract stimulation they have lower production of IL-4 but higher of IL-10 compared to the counterparts in Switzerland (Hamza et al., 2010). They seem therefore to have helminth infection under stringent immunoregulation. Whether stricter de-worming schedules at the time of importation to the mainland effects their susceptibility to IBH remains a matter of debate.

### 1.2.4 *Culicoides*

*Culicoides* midges (Figure 5) are a genus of small biting flies in the family Ceratopogonidae, a family of Diptera order in the insecta class. There are over 1400 species of *Culicoides* midges, distributed worldwide, except in the Antarctica, New Zealand and Iceland. The midges have less than 2 mm wing span and weight about 0.5 μg. The life cycle of *Culicoides* includes egg, larval, pupa and adult stages. The midges are haematophagous, but it is only the females that suck blood. For some species like *C. nubeculosus* blood sucking is essential for egg production, but other species like *C. impunctatus* are autogenous and therefore opportunistic, feeding on mammals. *C. nubeculosus* feeds mainly on horses, cows and deer. The eggs are laid in a bundle of 30-100 eggs in moist ground 5 days after fertilization, and hatch within a day. Larval state requires moist environment. Adult fly has a lifespan of 20-30 days (Featherstone, 2010; Mordue & Mordue, 2003).

**Figure 5. *Culicoides spp.***
http://www.mikrojezioro.met.pl

The saliva of *Culicoides* midges contains a number of compounds that enable blood feeding by overcoming some of the host defenses like hemostasis and immune responses. When biting, the midges secrete proteins into the host and some become allergens in certain individuals (Ribeiro &

Francischetti, 2003; Russell et al., 2009). Some of the midge species, like *C. sonorensis*, are vectors of arboviruses, such as the bluetongue virus of cattle and sheep and African horse sickness virus of equines (Bishop et al., 2006; Mordue & Mordue, 2003).

Numerous *Culicoides* species have been reported to cause IBH for example *C. obsoletus* (Anderson et al., 1991; van der Meide et al., 2012), *C. sonorensis* (Langner et al., 2009), *C. nubeculosus* (Hellberg et al., 2009), *C. imicola* (Braverman et al., 1983), *C. impunctatus*, *C. chiopterus* (Halldorsdottir et al., 1989) and *C. pulicaris* (Mellor & McCraig, 1974). The distribution of *Culicoides* varies geographically and the dominant species is different between regions. For example *C. impunctatus* is the most abundant species in Scotland (Featherstone, 2010) and *C. obsoletus* in the Netherlands (de Raat et al., 2008; van der Rijt et al., 2008). Anderson et al., 1993 showed with skin test that IBH affected horses can react to *Culicoides* species that they have not been exposed to before (Anderson et al., 1993). However IBH affected horses in the Netherlands had significantly higher IgE ELISA titers against *C. obsoletus* than against *C.nubeculosus* and *C. sonorensis* (van der Meide et al., 2012).

### 1.2.5 *Culicoides* allergens

Some *Culicoides* species like *C. nubeculosus* and *C. sonorensis* can be successfully maintained in laboratory bred colonies and are therefore more accessible for research (Boorman, 1974; Nunamaker & Lockwood, 2001). Other species like *C. obsoletus* have to be collected from the wild (Boorman, 1985).

The first *Culicoides* allergen described was a maltase originated from *C. sonorensis* (Cul s 1) (Table 1), a 66 kDa protein when expressed in the baculovirus expression system. The IgE from serum of 7 out of 8 IBH affected horses bound to the Cul s 1 and 8 out of 8 horses reacted in skin test (Langner et al., 2009). A total of 45 proteins have been identified with mass spectrometry analysis of secreted saliva from *C. sonorensis* including members of D7 family, Kunitz-like protease, maltase and trypsin (Lehiy & Drolet, 2014).

By using mass spectroscopy analysis 54 novel protein sequences including potential allergens were identified from *C. nubeculosus* cDNA salivary gland library (Russell et al., 2009). Western blot analysis of *C. nubeculosus* salivary gland extract showed at least 10 IgE-binding proteins. These proteins were isolated using phage surface display technology and expressed as hexa-histidine tagged proteins in *E. coli*. The proteins are candidate allergens causing IBH and were termed Cul n 2-11 according to the systematic allergen nomenclature. The molecular masses of the allergens range from 15.5-68.7 kDa (Table 1). Western blot analysis showed their ability to bind specific serum IgE of sensitized horses and ELISA tests showed frequency of sensitization ranging from 13 to 57% depending on the allergen (Schaffartzik et al., 2011). Cul n 1 had been isolated before and expressed in the baculovirus expression system (Björnsdóttir, 2008). Specific IgE-binding from serum of IBH-affected horses to Cul n 1 was demonstrated with western blot analysis and 35% of IBH-affected horses were sensitized to Cul n 1 (Schaffartzik et al., 2010). The allergens Cul n 1- Cul n 4 were termed as major allergens of IBH (Schaffartzik et al., 2011).

Cul n 1 shares sequence homology to antigen 5 like protein, a major allergen of vespid venom allergy (Hoffman, 2006; Schaffartzik et al., 2010). Cul n 2 is hyaluronidase a major allergen in honey bee and vespids venom allergy (Arlian, 2002; Hoffman, 2006; King & Spangfort, 2000). Cul s 1 and Cul n 8 share sequence homology to maltase (α-glucosidae) which is also found in the salivary gland of mosquitoes and the sand fly (Jacobson & Schlein, 2001; Marinotti et al., 1996). Cul n 9 is D7-related protein an allergen found in the saliva of mosquitoes (Malafronte Rdos et al., 2003) and sand flies (Martin-Martin et al., 2013).

**Table 1. List of isolated and expressed *Culicoides* allergens.**

| Allergen | Homology to | GenBank accession number | MW (kDa) | % positive sera |
|---|---|---|---|---|
| Cul s 1 [A] | Maltase | Q66UC5_9DIPT | 68.6 | 87,5[A] / 22[E] |
| Cul n 1 [B] | Antigen 5 like protein | EU978899 | 25.4 | 35[B] / 18[D] |
| Cul n 2 [C] | Hyaluronidase | HM145950 | 46.7 | 56,5[C] |
| Cul n 3 [C] | Putative cysteine endopeptidase | HM145951 | 44.6 | 47,8[C] / 25[D] |
| Cul n 4 [C] | Secreted salivary protein | HM145952 | 17.5 | 45,7[C] / 44[D] |
| Cul n 5 [C] | Secreted salivary protein | HM145953 | 45.7 | 43,5[C] / 15[D] |
| Cul n 6 [C] | Secreted salivary protein | HM145954 | 16.9 | 34,8[C] |
| Cul n 7 [C] | Unknown salivary protein | HM145955 | 20.9 | 30,4[C] / 15[D] |
| Cul n 8 [C] | Maltase | HM145956 | 68.7 | 21,7[C] / 4[D] |
| Cul n 9 [C] | D7-related salivary protein | HM145957 | 15.5 | 26,1[C] |
| Cul n 10 [C] | Secreted salivary protein | HM145958 | 47.8 | 15,2[C] / 18[D] |
| Cul n 11 [C] | Trypsin | HM145959 | 30.1 | 13,0[C] |
| Cul o 1 [D] | Kunitz protease Inhibitor | JX512273 | 23.3 | 45[D] |
| Cul o 2 [D] | D7 protein | JX512274 | 17.5 | 40[D] |
| Cul o 3 | Antigen 5 (Cul n 1) | | 29.9 | |
| Cul o 1 [E] | Maltase (Cul s 1) | KC339671 | 66.8 | 43[E] |
| Cul o 2 [E] | Hyaluronidase (Cul n 2) | KC339672 | 42.3 | 62[E] |
| Cul o 3 [E] | Antigen 5 like protein (Cul n 1) | KC339673 | 27.9 | 60[E] |
| Cul o 4 [E] | Trypsin (Cul n 11) | KC339674 | 27.1 | 39[E] |
| Cul o 5 [E] | Unknown saliv protein (Cul n 7) | KC339675 | 17.9 | 67[E] |
| Cul o 6 [E] | D7-related saliv protein (Cul n 9) | KC339676 | 15.2 | 38[E] |
| Cul o 7 [E] | Secreted saliv protein (Cul n 4) | KC339677 | 15.0 | 43[E] |

[A] Langner et al., 2009
[B] Schaffartzik et al., 2010
[C] Schaffartzik et al., 2011
[D] Peeters et al., 2013
[E] van der Meide et al., 2013

Seven allergens originated in *C. obsoletus* were isolated by using sequence homology analysis to *C. obsoletus* specific RNA database with known allergens from *C. nubeculosus* and *C. sonorensis*. The allergens were termed Cul o 1 - 7 (Table 1). All seven allergens were expressed as recombinant

proteins in *E. coli* and Cul o 1 was additionally expressed in the baculovirus expression system. ELISA showed that the IgE from serum of IBH-affected horses bound to the allergens in the frequency of 38-67%. IBH- affected horses showed higher IgE-binding to the Antigen 5 like protein from *C. obsoletus* (Cul o 1) than from *C. sonorensis* (Cul s 1) (van der Meide et al., 2013).

Peeters et al., 2013 had previously isolated two allergens from a *C. obsoletus* salivary gland cDNA library and expressed as recombinant proteins in *E. coli*. They were termed Cul o 1 and Cul o 2 (Table 1) however, they do not share homology to the Cul o 1 and Cul o 2 isolated by van der Meide et al, nor other known *Culicoides* allergens. Cul o 1 shares homology to Kunitz protease inhibitor and Cul o 2 shares homology to D7-related salivary protein (Peeters et al., 2013). An Antigen 5 like protein was also isolated by the same group, expressed in *E.coli* and termed Cul o 3 (Table 1) (unpublished).

Tested with *in vitro* sulfidoleukotriene release assay more than 50% of IBH-affected horses that reacted with *Culicoides* extract also reacted with extract from *Simulium vittatum* (Baselgia et al., 2006). Antigen 5 like protein from *C. nubeculosus* (Cul n 1) and *S. vittatum* (Sim v 1) share sequence homology of about 48% and with ELISA and Western blot it was shown that they are fully cross-reactive and share common IgE binding epitopes (Schaffartzik et al., 2010). Sensitization to *S. vittatum* is likely to be secondary to *Culicoides,* since *S. vittatum* is present in Iceland as horses living there do not exhibit symptoms of IBH before export to the continent, where they are exposed to *Culicoides* (Schaffartzik et al., 2012) and they are negative in cellular antigen stimulation test (CAST) (unpublished).

## 1.3 Production of recombinant proteins

Recombinant proteins are commercially produced with the aid of modern biotechnology and genetic engineering and are used throughout biological and biomedical science. Proteins are both produced in prokaryotes and eukaryotes and the main production systems are genetically modified bacteria, yeast, insect cells and mammalian cells (Demain & Vaishnav, 2009).

### 1.3.1 Protein expression in *Escherichia coli*

*E. coli* is the oldest and most widely used host for the production of recombinant proteins. The advantages of *E. coli* as production system are rapid growth and expression, ease of culture and genomic modifications, high production yields and cost-effectiveness. However the *E. coli* system has some disadvantages, proteins with disulfide bonds (S-S) are difficult to express, the system produces unglycosylated proteins and proteins have to be purified away from bacterial endotoxins and acetate. Some proteins are produced as inclusion bodies in *E.coli.* They are inactive, aggregated and insoluble, making purification difficult as the proteins need to be solubilized with denaturing which unfolds the proteins then refolded again (Demain & Vaishnav, 2009; Schmidt & Hoffman, 2002).

### 1.3.2 Protein expression in insect cells

The most commonly used vector system for recombinant protein expression in insect cells is the baculovirus expression system. Baculoviruses have circular double-stranded DNA, are naturally pathogenic for lepidoteran cells and can easily be grown *in vitro*. The viruses are usually derived from *Autographa californica* multiple capsid nucleopolyhedrovirus (AcMNPV). The host cells are from the moths *Spodoptera frugiperda* (Sf-9) or *Trichoplusia ni* (High-five). The virus genes are expressed in three phases, early, late and very late. The early and late genes are associated with production of virus particles which bud from the cell and spread the infection. The very late gene encoding for polyhederin is under the control of strong promoter and is necessary for the formation of occlusion bodies containing virus particles in the nucleus of the host cells. However, it can be deleted from the genome without affecting the production of infectious virus particles. In the baculovirus vector the polyhederin gene is deleted and replaced with the cloned gene, which is then placed under the strong polyhederin promoter which allows expression of recombinant protein constituting up to 30% of cell proteins (Figure 6). The Baculovirus expression system enables eukaryotic post-translational modifications, including glycosylation, phosphorylation and correct signal sequence cleavage. It usually achieves high expression levels, proper protein folding and S-S bond formation and allows for simultaneous expression of multiple genes. The disadvantage for the expression of mammalian proteins is that the post-translational modification in insect and mammalian cells is not identical (Demain & Vaishnav, 2009; Possee, 1997; Schmidt & Hoffman, 2002). This is however not an issue when expressing insect proteins as in this study.

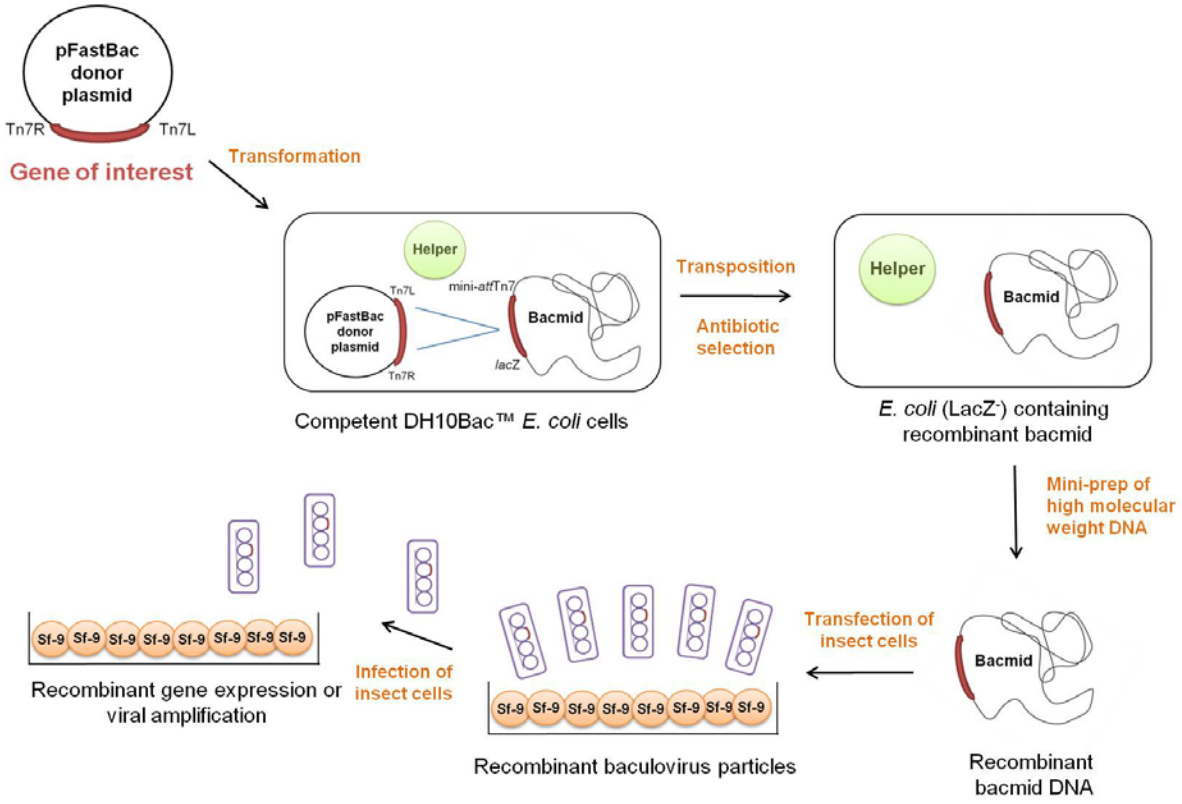


**Figure 6. The Bac-to-Bac Baculovirus Expression System (Invitrogen).**
After the gene of interest has been cloned into pFastBac vector, DH10Bac *E. coli* cells are transformed with the construct. Colonies that contain the gene of interest are selected with antibiotics. Bacmids from positive cultures, containing the gene, are isolated. Sf-9 insect cells are transfected with the recombinant bacmids and baculoviruses produced in Sf-9 insect cells.

Many recombinant allergens have been produced in the Baculovirus expression system. They have similar bioactivities as the native proteins and can bind IgE (Schmidt & Hoffman, 2002). The system has been used to express numerous insect allergens for example Api m 1 (Blank et al., 2011a), Api m 2 (Soldatova et al., 2007) and Api m 10 (Blank et al., 2011b) from honey bee venom, Sol i 3 (Borer et al., 2012) from fire ant venom, Dol m 5 (Tomalski et al., 1993) from baldfaced hornet venom, Cul s 1 (Langner et al., 2009) from *C. sonorensis* venom, Der f 1 (Shoji et al., 1996) from house dust mite, Blo t 11 (Teo et al., 2006) from dust mite, Lep d 2 (Olsson et al., 1998) from dust mite, Per a 5 (Wei et al., 2014) from American Cockroach and Aed a 1 (Peng et al., 2001) and Aed a 2 (Peng et al., 2006) from mosquito. Soldatova et al produced honeybee venom hyalurinodase (Hya) allergen in the baculovirus expression system and *E. coli* to compare with natural Hya for biological activity. They showed that the enzymatic activity of Hya produced in the baculovirus system was equal to natural Hya, whereas the *E. coli* produced Hya had only 20-30% activity of natural Hya (Soldatova et al., 1998).

Some proteins are difficult to express. It has been shown that the fusion of small ubiquitin-related modifier (SUMO) to proteins leads to enhanced expression levels of recombinant proteins in *E. coli* and insect cells. An alternative SUMO-derived tag called SUMOstar has been developed to be used in the baculovirus expression system. The SUMOstar tag can ease the expression of proteins that have been proven difficult and especially those who need native N-terminal residues for function (Liu et al., 2008).

# 2 Aim

The long term aim of the studies of insect bite hypersensitivity is to develop an immunotherapy against the eczema. For evaluation of the immune response following treatment and for diagnostic it is necessary to produce and purify the allergens causing the disease. Thirteen allergens have been isolated from three *Culicoides* species, *C. sonorensis*, *C. nubeculosus* and *C. sonorensis*, expressed in *E. coli* and purified. The allergens purified from *E. coli* are used in experimental treatments but are not suitable for some of the immunoassays needed to evaluate immunotherapy. They can for example not be used to stimulate peripheral blood mononuclear cells (PBMC) *in vitro* for cytokine production due to background. They have also so far not been applicable for diagnostic tests. Therefore it is necessary to have the allergens expressed in a different production system from the proteins that are used for treatment and also to obtain them with the correct post-translational modification. The allergens originate in the salivary glands of insects and hence it was an obvious choice to express them in insect cells.

The specific aims of the project can be divided into three parts

1. Expression and production of four of the major allergens for IBH; Cul n 1, Cul n 2, Cul n 4 from *C. nubeculosus* and Cul o 3 from *C. obsoletus* in insect cells with different baculovirus vector systems.
2. Purification of the four recombinant allergens expressed in insect cells and in addition Cul n 3, Cul o 1 and Cul o 2 that had been expressed before.
3. Establishment of immunoassays with the purified allergens, measuring antibody and cytokine response following immunotherapy.

# 3 Materials and methods

## 3.1 Cloning of *Cul n 1*, *Cul n 2*, *Cul n 4* and *Cul o 3*

### 3.1.1 Origin of the genes

Originally the genes, *Cul n 1, Cul n 2,* and *Cul n 4* were amplified from λZAP II cDNA library made from salivary glands of *Culicoides nubeculosus* (Schaffartzik et al., 2011). In this project the genes *Cul n* 1, *Cul n 2* and *Cul n* 4 were amplified from a pFastBac vector provided by Sigríður Jónsdóttir, The Instituite for Experimental Pathology, University of Iceland, Keldur. *Cul o 3* was amplified from a plasmid containing the gene provided by Dr. Eliane Marti, Universitiy of Bern, Switzerland.

### 3.1.2 Primer design

Primers for amplifying the entire genes of *Cul n 1* (Not published), *Cul n 2* (GenBank accession numbers: HM145950) and *Cul n 4* (HM145952) were designed from known *C.nubeculosus* sequences with appropriate cleavage sites. The sequence of *Cul o* 3 was provided by Dr. Eliane Marti, Universitiy of Bern, Switzerland. All primers were purchased from TAG Copenhagen and are listed in appendix I.

### 3.1.3 Polymerase chain reaction (PCR)

The PCR reactions were performed in DNA Engine® Peltier Thermal Cycler (PTC-200) from MJ Research and Thermal Cycler 2720 from Applied Biosystems. The *Taq* DNA polymerase or Phusion® Hot Start Flex DNA polymerase was used in the reactions.

#### PCR with Phusion®Hot Start Flex DNA polymerase

The genes *Cul n 1, Cul n 2, Cul n 4* and *Cul o 3* were amplified for cloning using a Phusion® Hot Start Flex DNA polymerase (New England Biolabs).

| **PCR reaction solution** | |
|---|---|
| Template DNA | variable |
| 20 µM Forward primer | 1.25 µL |
| 20 µM Reverse primer | 1.25 µL |
| Phusion Hot start Flex 2X Master Mix | 25 µL |
| $ddH_2O$ | to 50 µL |
| Total volume | 50 µL |

| **PCR reaction** | | |
|---|---|---|
| 1. Denaturing | 98°C | 30 sec |
| 2. Denaturing | 98°C | 10 sec |
| 3. Annealing | 50°C - 60°C | 30 sec |
| 4. Elongation | 72°C | 1 min/kb |
| 5. Elongation | 72°C | 10 min |

Steps 2 to 4 were repeated 29 times. The annealing temperature depended on the melting point of the primers and the elongation time depended on the size of the gene amplified (approximally 1 min for each 1000 bp amplified). For the reaction 50-250 ng of template DNA were used.

#### PCR with *Taq* DNA polymerase

*Taq* DNA polymerase (New England Biolabs) was used in PCR's to test bacterial cultures and minipreps after transformation.

| PCR reaction solution | |
|---|---|
| Template DNA | 1 µL |
| 10x Thermo buffer | 2 µL |
| 2 mM dNTP | 2 µL |
| 20 µM Forward primer | 1 µL |
| 20 µM Reverse primer | 1 µL |
| *Taq* polymerase (5 U/µL) | 0.2 µL |
| ddH2O | 12.8 µL |
| Total Volume | 20 µL |

| PCR reaction | | |
|---|---|---|
| 1. Denaturing | 95°C | 30 sec |
| 2. Denaturing | 95°C | 10 sec |
| 3. Annealing | 50°C - 60°C | 30 sec |
| 4. Elongation | 72°C | 1 min/ kb |
| 5. Elongation | 72°C | 10 min |

Steps 2 to 4 were repeated 29 times. The annealing temperature depended on the melting point of the primers and the elongation time depended on the size of the gene amplified (1 min for each 1000 bp amplified).

### 3.1.4 DNA electrophoresis

The PCR products were run on 1% agarose gel. The Agarose Basic (AppliChem) powder was added to 0.5x TBE (Tris borate- EDTA, appendix II) and melted. Ethidium bromide was added to the melted agar before solidification. Before loading the PCR product, a 10x RSB (restriction buffer, appendix II) was added to each sample. Electrophoresis was carried out at 70V for 30-60 min depending on the size of the gene fragments, in 0.5x TBE buffer. The size of the products was estimated by comparison to a 2-log ladder (New England Biolabs). PCR products were visualized under UV light in InGenius (SynGene) and photographed using the GeneSnap program (SynGene).

### 3.1.5 Extraction of DNA from agarose gel and DNA quantification

A Nucleospin® Gel and PCR clean-up Kit (Macherey-Nagel) was used to extract DNA from agarose gel according to manufacturer‘s protocol. The DNA was visualized under UV light and excised from the gel.

The concentration of nucleic acid in the DNA samples was measured in NanoDrop®ND-1000 Spectrophotometer (NanoDrop Technologies Inc.) according to manufacturer‘s user manual.

### 3.1.6 Vectors

The allergen genes *Cul n 1, Cul n 2, Cul n 4* and *Cul o 3* were cloned into different expression vectors either with or without the native secretion signal sequence of the protein. The secretion signal sequence of the allergens was determined by the predictability program SignalP 4.1 Server (Petersen, 2011). The vectors used in this work were pFastBac™/HBM-TOPO® (Invitrogen), pFastBac™ 1 (Invitrogen) and pI-secSUMOstar (LifeSensors) (Figure 7). Before this study the allergens Cul n 1 and Cul n 2 had been expressed using the pFastBac™ HT B vector (Invitrogen) and Cul n 4 at full length using the pFastBac™/HBM-TOPO® vector(Invitrogen) (Figure 7) by Sigríður Jónsdóttir, The Institiute for Experimental Pathology, University of Iceland, Keldur.

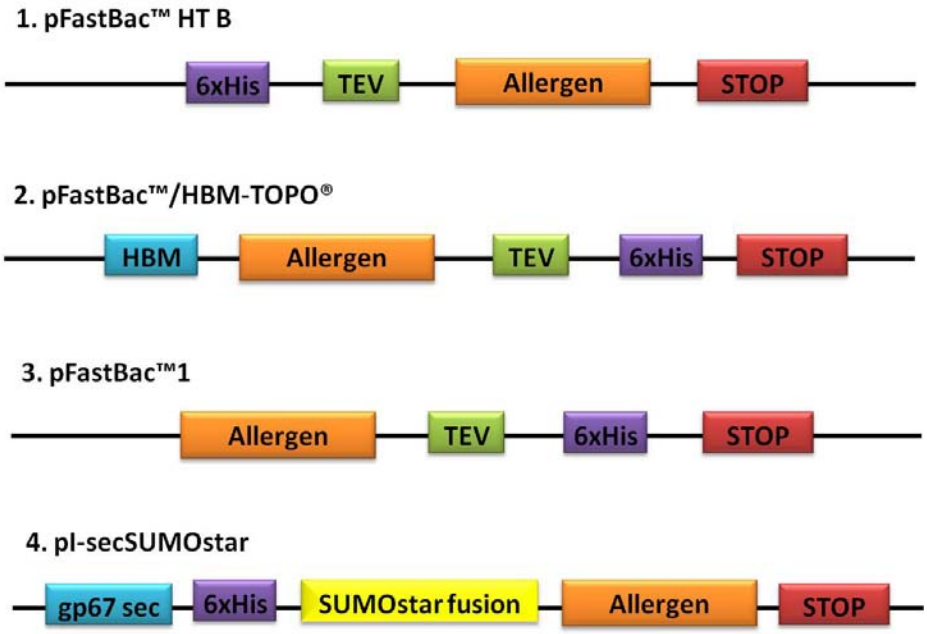


**Figure 7. Schematic picture of the vectors**
**1.** FastBac™ HT B vector contains hexa-histidine tag and TEV protease cleavage site at the N-terminus. **2.** pFastBac™/HBM-TOPO® vector contains Honey-bee melittin secretion signal at the N-terminus of the allergen, TEV protease cleavage site and 6xHis-tag at the C-terminus. **3.** From pFastBac™/HBM-TOPO® vector theTEV protease cleavage site and 6xHis-tag is cloned with the allergen gene into the pFastBac™1 vector. **4.** pI-secSUMOstar vector contains glycoprotein secretion signal (gp67 sec) originated from baculoviruses, 6xHis-tag and a SUMOstar furion protein at the N-terminus of the allergen.

### 3.1.7 TOPO cloning

The genes were TOPO cloned into pFastBac™/HBM-TOPO® vector using Bac-to-Bac® TOPO® Cloning Kit (Invitrogen). The TOPO cloning reaction mix was made according to manufacturer's protocol. The One Shot® Mach1-T1$^{R}$ Chemically Competent *E. Coli* cells, kept in -80°C, were put directly on ice and 2 μL of the TOPO cloning reaction added, kept on ice for 30 min, heat-shocked at 42°C for 30 sec and cooled on ice for 2 min. After that, 250 μL SOC medium (Super Optimal Broth medium with glucose, appendix II) was added to each vial and they incubated at 37°C for 1 hr while being agitated. The transformation mix was spread on preheated LB agar (appendix II) plates containing 100 μg/mL ampicillin and cultured for 16-18 hrs at 37°C.

### 3.1.8 Restriction enzyme digestion and ligation

Restriction sites were incorporated at the 5'-end of the forward and reverse primers to facilitate cloning of the coding sequences into pFastBac™ 1 vector or pI-secSUMOstar vector. For cloning into pFastBac™ 1 vector the genes were amplified with primers containing *Bam*HI and *Hind*III restriction sites. The genes and vectors were double digested with endonucleases *Bam*HI and *Hind*III (New England Biolabs) at 37°C overnight (o.n.), the rest was carried out according to manufacturer's protocol. For cloning into pI-secSUMOstar vector the genes were amplified with primers containing *Bsm*BI and *Xba*I restriction sites. The genes and vectors were digested with endonucleases *Bsm*BI and *Xba*I (New England Biolabs). First with *Xba*I at 37°C o.n. and then with *Bsm*BI at 55°C o.n., and

the rest carried out according to manufacturer's protocol. After restriction digestion the products were run on agarose gel, excised, extracted from the gel and measured in NanoDrop. The genes were ligated into the vectors with T4 DNA ligase (Fermentas) according to manufacturer's protocol, except the ligation reactions were carried out at 16°C for 24-48 hrs with the ratio between gene and vector 3:1 and 6:1.

### 3.1.9 Chemically competent *E. coli* cells and transformation

*E. coli* strain DH5α from stock was plated on LB agar and incubated o.n. at 37°C. Colonies were picked and cultured in SOB medium (appendix II) on a shaker at 16°C until the $OD_{600}$ was between 0.45-0.60, put on ice for 10 min, centrifuged for 15 min at 2000 x g at 4°C. After this step the work was performed on ice in a cold room. The supernatant was discarded and the pellet dissolved with cold HTB buffer (appendix II, 3.2 mL per 10 mL of SOB medium) and kept on ice for 10 min, centrifuged for 15 min at 2000 x g at 4°C. The pellet was resuspended carefully in HTB buffer (0.8 mL per 10 mL SOB medium) and finally DMSO (dimethyl sulfoxide, 60 μL per 10 mL SOB) added. The competent cells were dispensed in 100 μL aliquots, quickly frozen in liquid nitrogen and stored at -80°C.

The chemically competent *E. coli* cells, kept at -80°C, were put directly on ice. Six μL of the ligation mix were added to the cells, they kept on ice for 30 min, heat-shocked at 42°C for 30 sec and cooled on ice for 2 min. Next 250 μL SOC medium was added to each vial and they incubated at 37°C for 1 hr while being agitated. The transformation mix was spread on preheated LB agar plates containing 100 μg/mL ampicillin and cultured for 16-18 hrs at 37°C.

### 3.1.10 Plasmid purification and sequencing

After transformation of chemically competent cells, colonies were picked and cultured in 2.5 mL of LB medium (appendix II) with ampicillin (100 μg/mL) for 16-18 hrs at 37°C while being agitated. The cultures were tested in PCR and plasmid isolated from positive cultures. A Nucleospin® Plasmid Kit (Macherey-Nagel) was used to isolate plasmids according to manufacturer's protocol.

Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and sequencing capillary electrophoresis was carried out on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Isolated plasmids with the gene of interest were sequenced with gene specific primers and vector primers. For each reaction 200-500 ng/μL of sample was used.

| **Cycle Sequencing reaction solution** | |
|---|---|
| Template DNA | variable |
| 5x sequencing buffer | 1.5 μL |
| BigDye | 1.0 μL |
| Primer (2 μM) | 1.6 μL |
| ddH2O | to 10 μL |
| Total volume | 10 μL |

| **Cycle Sequencing reaction** | | |
|---|---|---|
| 1. Denaturing | 95°C | 5 min |
| 2. Denaturing | 95°C | 20 sec |
| 3. Annealing | 50°C - 55°C | 15 sec |
| 4. Elongation | 60°C | 4 min |

Steps 2 to 4 were repeated 29 times. The annealing temperature depended on the melting point of the primers.

After the cycle sequencing reaction 40 μL of 75% isopropanol was added to each product, mixed briefly, incubated at RT for 15 min and centrifuged at 20800 x g (eppendorf Centrifuge 5430) for 30 min. The supernatant was discarded, 100 μL of 75% isopropanol added to each sample, mixed briefly, centrifuged for 5 min at 20800 x g, and the supernatant discarded. The pellet was dried at 90°C for 1 min, dissolved in 15 μL of Hi-Di™ Formamide (Applied Biosystems), mixed briefly, heated at 95°C for 2 min, mixed briefly again and centrifuged at 20800 x g for few sec. Then the samples were ready for sequencing. The data was analyzed using the program Sequencer™ 4.9 from Gene Codes Corporation.

## 3.2 Expression of recombinant allergens in Sf-9 insect cells

### 3.2.1 Transformation of DH10Bac™ *E. coli*

The expression casette on the vectors is flanked by left and right arms of the site specific Tn7 transposon. The casette contains a polyhedrin promotor, a multiple cloning site, a gentamicin resistance gene and the pFastBac vectors contain a SV40 polyadenylation signal that forms a mini Tn7.

The chemically competent MAX Efficiency® DH10Bac™ *E. coli* strain (Invitrogen) contains a Baculovirus shuttle vector (target bacmid) and a helper plasmid. The target bacmid contains 136 kb baculovirus genome, with mini-*att*Tn7 target site, a kanamycin resistance gene, a LacZα gene that provides confirmation of the transposition at the correct site (gives the $Lac^-$ phenotype). It also contains a mini-F replicon allowing stable replication in *E. coli*. The helper plasmid encodes the transposase and confers resistance to tetracycline.

The DH10Bac *E. coli* (75 μL) was transformed using heat-shock (45 sec, 42°C) with vector containing the gene of interest (2.5 ng) for transpositions at the mini-*att*Tn7 site of the bacmid. The vials were chilled on ice for 2 min and then incubated while being agitated in 900 μL RT SOC medium. The cells were prepared in SOC medium, using 10-fold serial dilutions ($10^{-1}$, $10^{-2}$, $10^{-3}$) and plated (100 μL) on separate LB agars containing 50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracyclin, 100 μg/mL X-gal (appendix II) and 40 μg/mL IPTG (appendix II). The plates were incubated for 48 hrs at 37°C.

Colonies containing the $LacZ^-$ recombinant bacmid (r-bacmid) were picked, based on their white color, and spread on fresh LB agars with the same antibodies and concentration as mentioned above. The plates were incubated 16-18 hrs at 37°C.

### 3.2.2 Isolation of r-bacmids

White colonies were picked and cultured in LB medium (2 mL) containing 50 μg/mL kanamycin, 7 μg/mL gentamicin and 10 μg/mL tetracyclin for 48 hrs at 37°C while being agitated. The cultures were tested in PCR with M13 primers and gene specific primers. Positive cultures, containing the gene of interest were isolated with PureLink™HiPure Plasmid DNA Purification Kit (invitrogen) according to manufacturer‘s protocol. The isolated r-bacmids were tested in PCR before being transfected into insect cells. Then the r-bacmids where incubated for 5 min at 72 °C to avoid bacterial growth.

### 3.2.3 Culturing of Sf-9 insect cells

Sf-9 cells (American Type Culture Collection ATCC) are derived from ovaries of the butterfly larvae *Spodoptera frugiperda.* The Sf-9 cells were cultured in a closed culture at 27°C. The culture medium was SF-900™II medium (gibco® by life technologies™) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin (PEST) and 1% fetal bovine serum (FBS) (gibco® by life technologies™), referred to as complete Sf-9 medium.

### 3.2.4 Transfection of Sf-9 insect cells

Transfection into Sf-9 cells was performed with Cellfectin® II Reagent (Invitrogen). The cells were seeded ($0.3x10^6$ cells/mL) in a 12 well plate (Nunc) the day before transfection. The culture medium was removed to get rid of the antibiotics and the cells washed with 500 µL Grace's medium (gibco® by life technologies™) without antibiotics and FBS. Then 800 µL of Grace's medium was added to the cells. Two solutions were made for the transfection. Solution A: r-bacmid and Grace's medium up to 100 µL depending on the concentration of each r-bacmid (0.5 µg – 1.0 µg), medium alone was used for Cellfectin and medium control. Solution B: 8 µL Cellfectin and 100 µL Grace's medium, the Cellfectin was vortexed and mixed with the medium briefly and incubated for 5 min at RT. Then solutions A and B were mixed and incubated for 20 min in RT (Table 2).

**Table 2. Solutions made for transfection of Sf-9 cells with r-bacmids.**

| | **Solution A** | **Solution B** |
|---|---|---|
| **r-bacmid** | r-bacmid (0.5 µg – 1.0 µg) + Grace's medium added to 100 µL | 8 µL Cellfectin + 100 µL Grace's medium |
| **Cellfectin control** | 100 µL Grace's medium | 8 µL Cellfectin + 100 µL Grace's medium |
| **Medium control** | 100 µL Grace's medium | 100 µL Grace's medium |

The transfection and control mixtures were added to the Sf-9 cells and incubated for 3-5 hrs at 27°C. Then the medium was removed and 900 µL of complete Sf-9 medium was added to the cells. Negative controls were Sf-9 cells in medium alone and Sf-9 cells with Cellfectin.

The transfections were incubated for 5-9 days in 27°C and checked regularly in invert light microscope (Leitz DIAVERT) for signs of cytopathic effect (CPE) in form of large and irregular shaped cells. When the transfected cultures showed clear cytopathy, supernatant (200 µL) from the transfection was passaged on fresh Sf-9 cells that had been seeded the day before ($0.4x10^6$ cells/mL) in a 12 well plate (first passage). When infected, supernatant was transferred to fresh Sf-9 cells in T25 flasks (Nunc), 200 µL supernatant into 4.5 mL or in T75 flasks (Nunc), 500 µL supernatant into 14.5 mL (Second passage). All passages were done in complete Sf-9 medium and incubated for 72-96 hrs at 27°C or until clear cytopathy was seen. When harvesting recombinant virus stock, Sf-9 cells from plates were collected, spun down at 18800 x g (HERAEUS PICO 21 Centrifuge, Thermo Electron

Corporation) for 3 min or from flasks at 515 x g (Sorvall RT6000B Refrigerated Centrifuge) for 12 min. The virus stock was kept at 4°C until used.

### 3.2.5 Cloning and production of r-baculoviruses

Cloning of the recombinant baculoviruses (r-baculoviruses) was done from the supernatant harvested from the first passage, in 96 well flat bottom plates (Nunc) with 100 μL/well of Sf-9 cells ($4x10^5$ cells/mL). Seven dilutions were made of the r-baculovirus 1 $x10^{-5}$ – 5 $x10^{-9}$ in complete Sf-9 medium and 100 μL of dilutions added to the wells. The dilutions 1 $x10^{-5}$ and 1 $x10^{-6}$ of r-baculovirus were put into 24 wells and the dilutions 5 $x10^{-7}$, 1 $x10^{-7}$, 5 $x10^{-8}$, 1 $x10^{-8}$ and 5 $x10^{-9}$ were put into 48 wells (Figure 8). The cells were examined regularly for cytopathy and scored finally after 14 days incubation.

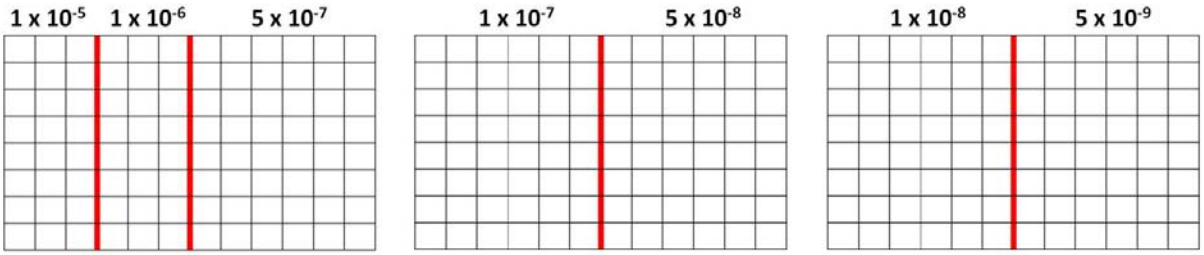


**Figure 8. Distribution of different dilutions of r-baculovirus in cloning plates.**

Clones with cytopathy in the highest dilution were transferred (180 μL) to fresh Sf-9 cells in 24 well plate ($0.4x10^6$ cells/mL) seeded the day before (first passage). When cytopathy was observed in the cells the supernatant was used for a second passage. Production of virus (second passage) was done by using the same procedure in T75 flasks. Second passage virus was used to make large viral stock of third passage virus. Cell pellet from each harvest was lysed for testing in Western blot (WB). Stocks were kept in 4°C and frozen at -80°C for long time storage.

### 3.2.6 Production of r-allergens in High-five insect cells

Recombinant protein accumulation can either be in the supernatant or in the cells. Recombinant allergens (r-allergens) were produced in High-five cells. High-five cells (1.0-1.3 $x10^6$ cells/mL) in 96 mL of SF-900II with PEST in Erlenmeyer flasks were infected with 4 mL of viral stock (3P) and incubated for 72 hr in 27°C while being agitated. After incubation the samples were centrifuged at 515 x g (Sorvall RT6000B Refrigerated Centrifuge) for 12 min. The supernatant was stored at 4°C and the pellet frozen in liquid nitrogen and stored at -80°C.

## 3.3 Protein analysis and purification

### 3.3.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The proteins were separated by SDS-PAGE in the Mini-protean II system (Bio-Rad), the samples were denatured with a sample buffer (appendix II), boiled for 5 min at 100°C, spun for 2 min and run on 12% or 14% acrylamide gels under reducing conditions at 200V in a SDS-PAGE running buffer (appendix II). PageRuler Prestained molecular mass marker from Thermo Scientific #26616 or Spectra Multicolor Broad Range Protein Ladder from Themo Scientific #26623 were used to estimate the size

of the proteins. The proteins were visualized using WB on PVDF membrane (Millipore) or Coomassie blue staining of the gels (Wong et al., 2000).

### 3.3.2 Western blotting

Following SDS-PAGE the proteins were transferred to a PVDF membrane by wet transfer in the Miniprotean II system (Bio-Rad) at 100 V for 1 hr in a transfer buffer (appendix II). 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. Then washed with TBS-T, 3x for 5 min. Next incubated with protein specific antibodies either monoclonal (mAb) or polyclonal (pAb) (Table 3) (Jónsdóttir, 2011; Schaffartzik et al., 2011) o.n. at 4°C, washed again and incubated at RT for 1 hr with the conjugate, Alkaline Phosphatase-conjugated Affinity Pure Goat Anti-Mouse IgG (Fc) (Jackson ImmunoResearch Laboratories, INC. code: 115-055-071) diluted 1:5000 in TBS-T. The membranes were developed using BCIP/NBT (Roche, appendix II) diluted 1:50 in alkaline phosphatase (AP) buffer (appendix II) after having been washed.

**Table 3. Dilutions of protein specific antibodies used in this study.**

| Antibody | Dilution |
|---|---|
| **α-Cul n 1 mAb** | 1:20000 |
| **α-Cul n 2 mAb** | 1:10000 |
| **α-Cul n 3 pAb** | 1:4000 |
| **α-Cul n 4 pAb** | 1:4000 |
| **α-Cul o 1 pAb** | 1:2000 |
| **α-Cul o 2 pAb** | 1:2000 |

### 3.3.3 Coomassie blue staining

Protein samples were run on 12% or 14% SDS-PAGE gel and stained according to Wong et al. with coomassie blue (Wong et al., 2000). After coomassie blue staining the gels were dried between two extra cellophane sheets (Gel drying frames (Sigma-Aldrich)) at RT o.n. in storage solution (appendix II).

### 3.3.4 Dot blot

Dot blot was performed after protein purification for screening of positive protein samples. The dot blot was performed as followed; 2 μL of sample was dotted directly on a nitrocellulose membrane (Hybond™ ECL™), dried for 10 min at 37°C and incubated for 1 hr at RT with protein specific antibody. Then washed five times for 5 min with TBS-T before incubation with goat-anti-mouse-AP (Jackson) diluted 1:5000 for 1 hr at RT. Finally the membrane was developed with BCIP/NBT diluted 1:50 in AP-buffer after having been washed.

### 3.3.5 Bradford protein assay

The protein concentration of samples was measured using Coomassie Plus (Bradford) Assay Kit (Thermo Scientific) at 600 nm in micro-plate spectrometer (VICTOR$^3$ (Perkin Elmer)) according to manufacturer‘s protocol.

### 3.3.6 Protein purification with nickel affinity gel under native conditions

The 6xHis-tagged r-allergens were purified with HIS-Select® HF Nickel Affinity Gel from Sigma-Aldrich. The gel was washed in dd$H_2O$ with 10x the volume of the gel and then equilibrated in lysis buffer with 10x the volume of the gel followed by centrifugation at 800 x g (Sorvall RT6000B Refrigerated Centrifuge) for 5 min. The cell pellet (100x$10^6$ cells) was lysed in 8 mL lysis buffer (appendix II) with 80 - 160 μL Protease Inhibitor Cocktail (PIC) from Sigma-Aldrich (P8340) and sonicated on ice 5x for 10 sec with 20 sec interval. The cell pellet was centrifuged at 10600 x g (eppendorf Centrifuge 5417C) at 4°C for 7 min and the supernatant of the lysed cell pellet mixed with the gel on orbital shaker for 2 hrs at 4°C, spun down at 800 x g for 5 min and the supernatant collected. The pellet was resuspended in wash buffer (appendix II) (10x the volume of the gel) and mixed for 5 min and spun down at 800 x g for 5 min. The pellet was washed four times in different imidazole concentration (appendix III). The pellet was then dissolved in elution buffer 1 (appendix II) (in equal volume to the gel) and applied on a plastic column with 0.2 μm membrane (Sigma-Aldrich) and incubated for 10 min at RT. The elution was repeated once with elution buffer 1 and two times with elution buffer 2 (appendix II). Then the gel was finally washed with buffer F (appendix II) in equal volume to the gel, then dd$H_2O$ and stored in 30% ethanol at 4°C. Elution fractions were stored in 4°C and tested with coomassie blue staining and in WB.

### 3.3.7 Protein purification with nickel affinity gel under denaturing conditions

The same protocol was used as in purification of native proteins except for use of urea in buffers. The nickel affinity gel was washed in dd$H_2O$ and then equilibrated in denature lysis buffer (appendix II). The cell pellet was lysed in guanidine-HCl denature lysis buffer, sonicated on ice and centrifuged. Then the supernatant of the lysed cell pellet was mixed with the nickel affinity gel on orbital shaker for 1 hr at RT. The gel was washed once in denature wash buffer 1 (appendix II, Table 8) and then denature wash buffer 2 (appendix II). The protein was eluted three times with denature elution buffer 1 (appendix II) and three times with denature elution buffer 2 (appendix II). Elution fractions were stored in 4°C and tested with coomassie blue staining and in WB.

### 3.3.8 Protein purification with fast protein liquid chromatography (FPLC)

An attempt was made to purify rBac-HTB-Cul n 2 on HiTrap™ SP Sepharose™ HP (Amersham Pharmacia Biotech) cation exchange column by FPLC using the ÅKTA FPLC system according to the manufacturer’s protocol (Amersham Pharmacia Biotech). The cell pellet (25x$10^6$ cells) kept at -80°C was thawed, 40 μL PIC added, then frozen again in -80°C, thawed and lysed in 5 mL of FPLC lysis buffer (appendix II) followed by sonication on ice 5x for 10 sec with 20 sec interval, spun at 20800 x g at 4°C for 10 min and the supernatant collected. The sample was desalted on Desalt column PD-10 (GE Healtcare) and eluted in 3.5 mL of buffer A (appendix II). Two mL of the sample were loaded to

the column and the protein isolated in 1 mL fractions. The column was washed with 2 column volumes of buffer A. Elution was made with increasing NaCl concentration were the concentration of buffer B (appendix II) was first raised to 20%, then in a gradient up to 100% buffer B in 20 column volume followed by 100% buffer B with flow rate 3 mL/min. As this purification was unsuccessful; HiTrap™ Q HP (Amersham Pharmacia Biotech) anion exchange column was tried. The same protocol was carried out as for the cation exchange column except the cell pellet was lysed in 4 mL of buffer and the sample was not desalted. The elution was made with NaCl gradient from 0% buffer B up to 80% in 10 CV and finally 100% buffer B in the flow rate 2 mL/min. Fraction containing purified protein were pooled and concentrated with Amicon® Ultra Centrifugal Filters (Millipore) with 30 kDa cut off by centrifugion at 800 x g for 15 min at 5°C (Sorvall RT6000B Refrigerated Centrifuge). Purification was also attempted with a HiTrap™ NHS HP column (Amersham Pharmacia Biotech) coupled with α Cul n 2 mAb four years before. Approximately $25x10^6$ cells were lysed in 2 mL PBS and sonicated. The column was washed in PBS (NHS buffer A, appendix II) and the elution was made with decreasing pH (increasing NHS buffer B containing 0.1 M glycine pH 2.3, appendix II). The elutions were collected in 250 µL fractions and 25 µL of neutralizing buffer (appendix II) was added to the collected samples.

### 3.3.9 Dialysis and sterile filtration

The proteins purified with nickel affinity gel were dialyzed in Slide-A-Lyzer® Dialysis Cassette G2 with 7 to 20 kDa cut-off depending on the size of the protein. Native purified proteins were dialyzed in elution buffer without Imidazol or PBS. Denatured purified proteins were dialyzed in buffer with 4M urea at pH 8.0. The samples were dialyzed for 2 hrs in volume at least 300 times the volume of the sample, the buffer changed and dialyzed o.n. for native proteins at 4°C and denature proteins in RT. After dialysis the samples were spun down at 18800 x g (HERAEUS PICO 21 Centrifuge, Thermo Electron Corporation) for 3 min and supernatant collected. The protein samples were sterile filtered with 0.2 µm filter (GE Healthcare Life Sciences) and stored at 4°C.

### 3.3.10 Cleavage with SUMOstar protease 1

Proteins expressed with pI-secSUMOstar vector and purified were cleaved with SUMOstar protease 1 (LifeSensors) according to manufacturer's protocol and tested with coomassie blue staining.

### 3.3.11 Deglycosylation

A PNGase F kit (New England Biolabs) was used for deglycosylation by cleavage of N-linked glycans of purified r-allergens according to manufacturer‘s protocol and tested in WB with protein specific antibodies (Schaffartzik et al., 2011).

## 3.4 Evaluation of immunotherapy – immunoassays

### 3.4.1 Horses and vaccination treatment

#### SEIC vaccination

Twelve healthy Icelandic horses aged 5-8 were vaccinated with four r-allergens (rCul n 1, rCul n 2, rCul n 5 and rCul n 9) produced in *E.coli* and purified (Schaffartzik et al., 2011). The horses were vaccinated three times (week 0, 5 and 9) and blood samples collected before the first vaccination (week 0), and then two weeks after the third vaccinations (week 11). Six horses were vaccinated with 10 µg of each r-allergen in 250 µL IC31® adjuvant (Valneva Austria GmbH, Vienna). The twelve horses were divided into four groups. Group 1 was immunized intradermally without adjuvant and group 2 intradermally with IC31®. Group 3 was immunized in the submandibular lymph nodes without adjuvant and group 4 in the submandibular lymph nodes with IC31® (Jonsdottir et al., Manuscript accepted). The sera from these horses were used to set up an ELISA to measure total IgG.

#### SE A/M vaccination

Twelve healthy Icelandic horses, 7-10 years old, were vaccinated in the submandibular lymph nodes with four r-allergens (rCul n 3, rCul n 4, rCul n 8 and rCul n 10) produced in *E. coli*,and purified (Schaffartzik et al., 2011). The horses were divided into two groups; group 1 was vaccinated with 10 µg of each r-allergen in 500 µg Aluminum hydroxide gel Alhydrogel® 2%, Invitrogen). Group 2 was vaccinated with 10 µg of each r-allergen in a mixture of alum (500 µg) and 50 µg Monophosphoryl Lipid A (Avantilipids). The horses were vaccinated three times (week 0, 4, 8) with a 4 week interval over 8 weeks. Blood samples were collected before the first vaccination (week 0), and then two weeks after the second vaccination (week 6) and the third vaccination (week 10) (Jonsdottir et al., Manuscript). The sera from these horses were used to set up an ELISA to measure total IgG. Four of these horses were later boosted with rCul n 3 and rCul n 4 for in vitro stimulation of PBMC (see section 3.4.2)

### 3.4.2 Isolation and stimulation of PBMC

Blood was collected by jugular puncture into vacutainer tubes (Vacuette, Greiner) containing Lithium Heparin. The blood was mixed, left in the tubes for 30 min at RT and then the leucocyte-rich-plasma layer harvested (3-4 mL from each tube). The plasma was carefully laid on 16 mL Ficoll-Pague (GE healthcare) without mixing and then centrifuged at 515 x g for 20 min at RT (Sorvall RT6000B Refrigerated Centrifuge). The interphase band with the PBMC was harvested into a 50 mL tube, the tube filled up with warm PBS/PEST, centrifuged at 515 x g for 10 min at RT and the supernatant fluid discarded. The cell pellet was resuspended in 12 mL cold PBS/PEST, kept on ice for 15 min, centrifuged at slow speed 130 x g for 12 min at 4°C. Slow speed wash was repeated 4-6 times to get rid of platelets. After the wash the cells are resuspended in 3-5 mL RPMI 1640 Medium GlutaMAX™ (gibco® by life technologies™) supplemented with PEST, non-essential amino acids, MEM vitamins, sodium pyruvate, 10% horse sera and 2-mercaptoethanol (appendix II) and counted. PBMC were resuspended at $5x10^6$ cells/mL. For cytokine measurement at mRNA level, 1 mL ($5x10^6$ cells/well) of

the suspension was added into 1 mL of dilutions of each stimulant in 24 well plates. The stimulants used were rBac-HBM-CuI n 3 and rBac-1-CuI n 4, 10 μg/mL, 5 μg/mL, 2.5 μg/mL and 1.25 μg/mL, Con A as a positive control and medium alone as a negative control. The cells were incubated in humidified $CO_2$ at 37°C. After 24 hrs the PBMC were harvested by centrifuging the plate at 134 x g (Beckman GS-6R Centrifuge) for 3 min without brake, supernatant discarded and 350 μL of RA1 solution from NucleoSpin®RNA II kit (Macherey-Nagel) was applied to each well and the plate shaken for 1 hr at 4°C, the mixture kept at -80°C until the RNA was isolated. For measuring secreted cytokines in the supernatant, 0.5 mL of the cell suspension ($2.5x10^6$ cells/well) was added in 0.5 mL of each stimulant in duplicate in 24 well plates and incubated. After 4 days, 800 μL of the supernatant was harvested and kept at -80°C. They were sent to The Cornell University College of Veterinary Medicine, Ithaca New York were the cytokines IL-4, IL-10 and IFN-γ was measured in Horse Cytokine 5-plex Assay (Wagner & Freer, 2009).

### 3.4.3 Isolation of RNA from stimulated PBMC

RNA was isolated with NucleoSpin®RNA II kit (Macherey-Nagel) according to manufacturer‘s protocol, except the RNA was eluted in 40 μL instead of 60 μL.

### 3.4.4 cDNA synthesis from isolated RNA

A RevertAid™ H Minus First Strand cDNA Synthesis kit (Thermo Scientific) was used according to manufacturer‘s protocol to synthesize cDNA from the isolated RNA. The synthesized cDNA was stored at -80°C and sent to The University of Bern, Switzerland, Department of Clinical Research and Veterinary Public Health for measuring mRNA expression of the cytokines IL-4, IL-10 and IFN-γ in quantitative real time PCR (qPCR) with 18S as reference (Hamza et al., 2007).

### 3.4.5 Indirect ELISA for measuring total IgG

Flat bottomed 96 well MaxiSorp™ plates (Nunc) were coated with 100 μL/well with allergens (0.2 μg/ well) in coating buffer (Carbonate-Bicarbonate Buffer pH 9.6, Sigma-Aldrich) and incubated for 2 hrs then stored at -20°C for later use. The plates were washed in high salt ELISA wash buffer (appendix II) with Immune Washer (Thermo Scientific™Nunc™) where the wells were filled and emptied tree times with the washer and then the third fill was left in the wells for 3 min. This washing was repeated three times. Then the plates were blocked with 200 μL/well of dilution buffer (High salt/BSA, appendix II), incubated for 1 hr at RT and washed as before. The serum from vaccinated horses was diluted in two-fold serial dilutions from 1:200 to 1:25600 in dilution buffer (appendix II), 100 μL applied to appropriate wells and the plates incubated for 1 hr at 37°C followed by wash. The conjugate Peroxidase-conjugated AffinitiPure Goat Anti-Horse IgG (H+L) (Jackson ImmunoResearch Laboratories, INC. Code: 108-035-003) diluted 1:7000 in dilution buffer was applied to the plates, 100 μL/well, incubated for 1 hr at 37°C and then washed. OPD-substrate solution (Dako) (appendix II) was added 100 μL/well incubated in the dark for 10 min at RT. The reaction was stopped by adding 75 μL/well of sulfuric acid (4M $H_2SO_4$). The absorbance (optical density, OD) was measured at 490 nm using microplate spectrometer (VICTOR$^3$ (Perkin Elmer)).

# 4 Results

## 4.1 Amplification and cloning of *Cul n 1, Cul n 2, Cul n 4* and *Cul o 3*

The genes were amplified in PCR with Phusion polymerase from a purified FastBac vectors containing the genes from a λZAP II cDNA library (Schaffartzik et al., 2011). The genes were cloned into the vectors shown in table 4.

**Table 4. List of vectors the allergen genes were cloned into.**

| Allergen gene | Size of insert (bp) | | Vector |
|---|---|---|---|
| ***Cul n 1*** | Without signal sequence | 504 | pI-secSUMOstar |
| ***Cul n 2*** | Full length | 1206 | pFastBac-HBM-TOPO |
| | Full length | 1206 | pI-secSUMOstar |
| | Without signal sequence | 1134 | pI-secSUMOstar |
| ***Cul n 4*** | Full length | 459 | pFastBac-1 |
| | Without signal sequence | 390 | pFastBac-HBM-TOPO |
| | Without signal sequence | 390 | pI-secSUMOstar |
| ***Cul o 3*** | Without signal sequence | 732 | pI-secSUMOstar |

*Cul n 1* was amplified without its signal sequence and cloned into pI-secSUMOstar vector (Figure 9). The *Cul n 1* is 504 bp without its signal sequence and stop codon (appendix IV).

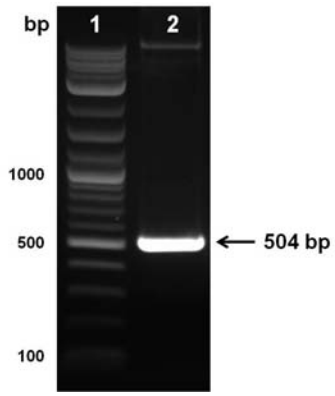


**Figure 9. Amplification of *Cul n 1* for cloning into pI-secSUMOstar vector.**
**Lane: 1** Ladder, **2** *Cul n 1* without signal sequence and stop codon (504 bp).

*Cul n 2* was amplified without stop codon, both with and without its signal sequence and cloned into pI-secSUMOstar vector (Figure 10). *Cul n 2* without stop codon is 1206 bp and without its signal sequence and stop codon 1134 bp (appendix IV). *Cul n 2* had been cloned into pFastBac-HBM-TOPO vector and sequenced before (Arnesen, 2013).

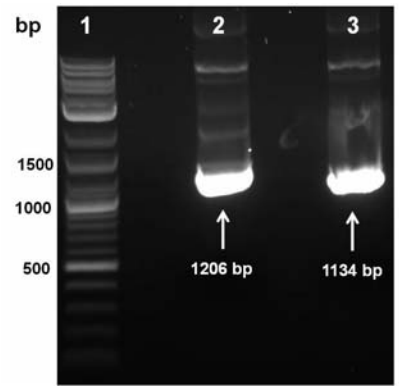


**Figure 10. Amplification of *Cul n 2* with and without signal sequence for cloning into pI-secSUMOstar vector.**
**Lane: 1** Ladder, **2** *Cul n 2* without stop codon (1206 bp), **3** *Cul n 2* without signal sequence and stop codon (1134 bp).

*Cul n 4* was amplified without its signal sequence and cloned into pFastBac-HBM-TOPO vector and pI-secSUMOstar vector (Figure 11). *Cul n 4* without its signal sequence and stop codon is 390 bp (appendix IV). The gene was also amplified with its signal sequence, 6xHis-tag and stop codon from pFastBac-HBM-TOPO-Cul n 4 and cloned into pFastBac1 vector. *Cul n 4* full length is 459 bp and with the 6xHis-tag 508 bp.

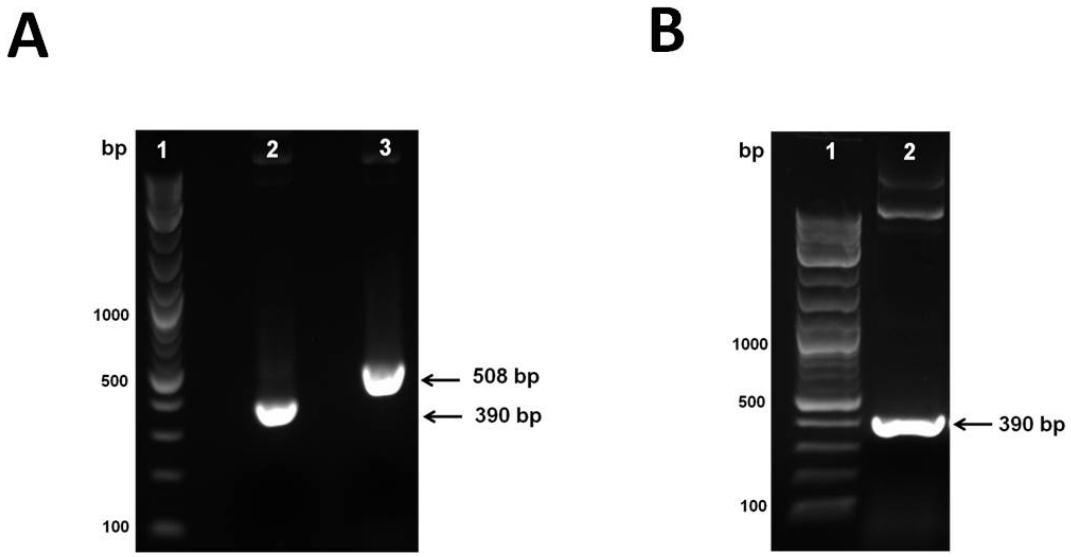


**Figure 11. Amplification of *Cul n 4* with and without signal sequence for cloning into pFastBac-HBM-TOPO vector, pFastBac1 vector and pI-secSUMOstar vector.**
**A Lane: 1** Ladder, **2** *Cul n 4* without signal sequence and stop codon (390 bp), **3** *Cul n 4* at full length (459 bp) with 6xHis-tag (508 bp). **B Lane: 1** Ladder, **2** *Cul n 4* without signal sequence and stop codon (390 bp).

*Cul o 3* was amplified without its signal sequence and cloned into pI-secSUMOstar vector (Figure 12). The *Cul o 3* gene is 732 bp without its signal sequence and stop codon (appendix IV).

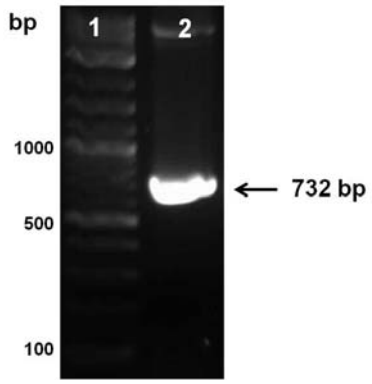


**Figure 12. Amplification of *Cul o 3* for cloning into pI-secSUMOstar vector.**
**Lane: 1** Ladder, **2** *Cul o 3* without signal sequence and stop codon (732 bp).

DH5α *E. coli* cells were transformed and colonies tested for the genes. The vectors containing the gene of interest were purified from the positive cultures and sequenced to confirm the right reading frame of the genes (appendix IV). DH10Bac *E. coli* cells were transformed with the recombinant constructs and r-bacmids isolated. The isolated r-bacmids were analyzed in PCR with gene specific primers and vector primers to verify the presence of the gene on the vector (data not shown).

## 4.2 Expression of recombinant allergens in Sf-9 insect cells and cloning

Sf-9 cells were transfected with r-bacmids to generate r-baculoviruses for expression of the *Cul n 1*, *Cul n 2*, *Cul n 4* and *Cul o 3.* For each construct, one r-bacmid preparation was made and transfected into Sf-9 cells with Cellfectin using 0.5 – 1.0 μg of DNA. The cells were under observation for 7-10 days.

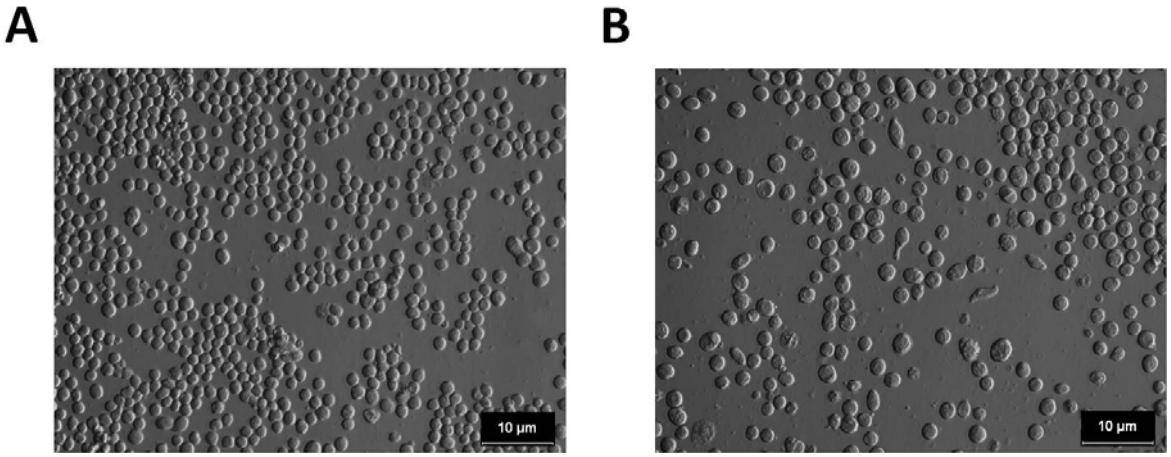


**Figure 13. Sf-9 insect cells.**
**A.** Uninfected Sf-9 cells. **B.** r-baculovirus infected Sf-9 cells.
Photos taken with inverted light microscope, Leica DM IL LED, magnified 200x.

As seen in Figure 13, uninfected Sf-9 cells are round, regular and small (Figure 13A). Signs of infection were large irregular cells with different light refraction (Figure 13B). Supernatant (viral stock) from clearly infected cells was used to infect fresh Sf-9 cells to amplify and produce more virus. The Sf-9 cells from the transfection (T), the first passage (1P), the second passage (2P) and the supernatant from second passage (Sup) were harvested for detection of the recombinant proteins in

WB (Figure 14). The r-baculovirus was cloned and the clones tested in WB, then amplified with 3 passages to get the viral stock used to produce the recombinant allergens in High-five insect cells. List of the clones used for r-allergen production for each construct is shown in Table 5.

**Table 5. List of harvested clones.**

| r-baculovirus | Harvested wells | Highest dilution of Sf-9 supernatant | Clone used for r-allergen production |
|---|---|---|---|
| **rBac-SUMO-Cul n 1** | 3 | $10^{-8}$ | Clone 1 |
| **rBac-HBM-Cul n 2** | 3 | $5x10^{-9}$ | Clone 2 |
| **rBac-SUMO-Cul n 2** | 2 | $5x10^{-9}$ | Clone 2 |
| **rBac-SUMO-wo-Cul n 2**[1] | 2 | $5x10^{-9}$ | Clone 4 |
| **rBac-1-Cul n 4** | 3 | $5x10^{-8}$ | Clone 2 |
| **rBac-HBM-Cul n 4** | 2 | $5x10^{-9}$ | Clone 1 |
| **rBac-SUMO-Cul n 4** | 2 | $5x10^{-9}$ | Clone 2 |
| **rBac-SUMO-Cul o 3** | 3 | $5x10^{-8}$ | Clone 1 |

[1] wo: without its own secretion signal sequence

Figure 14 shows that rBac-1-Cul n 4 is expressed after transfection and the expression is stronger in the first and second passage. The expressed protein is seen as a single band after transfection (T) and first passage (1P) but a double band in second passage (2P) were the smaller band also seen in the first passage is stronger. The predicted size of rBac-1-Cul n 4 is 19 kDa but without its secretion signal sequence 15 kDa. The r-allergen is secreted from the cells into the media. Three rBac-1-Cul n 4 clones expressing the r-allergen were harvested after first passage (Figure 14B). Clone 2 was selected for the production of r-allergen in High-five cells.

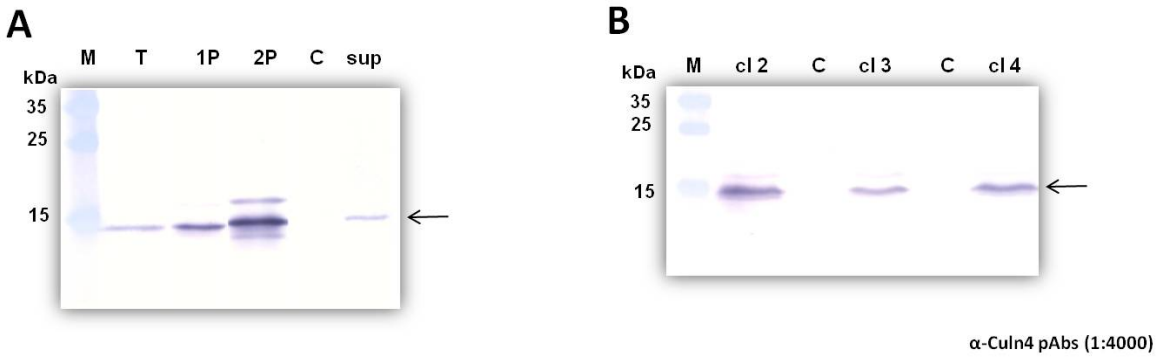


**Figure 14. Western blot showing the expression of rBac-1-Cul n 4 in Sf-9 cells.**
**A** rBac-1-Cul n 4 after transfection and passages, **lane M:** marker, **T:** transfection, **1P:** first passage, **2P:** second passage, **C:** negative control **Sup:** supernatant. **B** r-baculovirus clones positive for rBac-1-Cul n 4, **lane M:** marker, **cl 2:** clone 2, **C:** negative control, **cl 3:** clone 3, **cl 4:** clone 4. The arrows indicate rBac-1-Cul n 4.

The WB of the r-allergen expression and cloning of the r-baculoviruses is shown in appendix V.

## 4.3 Production of r-allergens in High-five insect cells

High-five insect cells were infected with r-baculovirus clones (Table 5) to produce r-allergens.

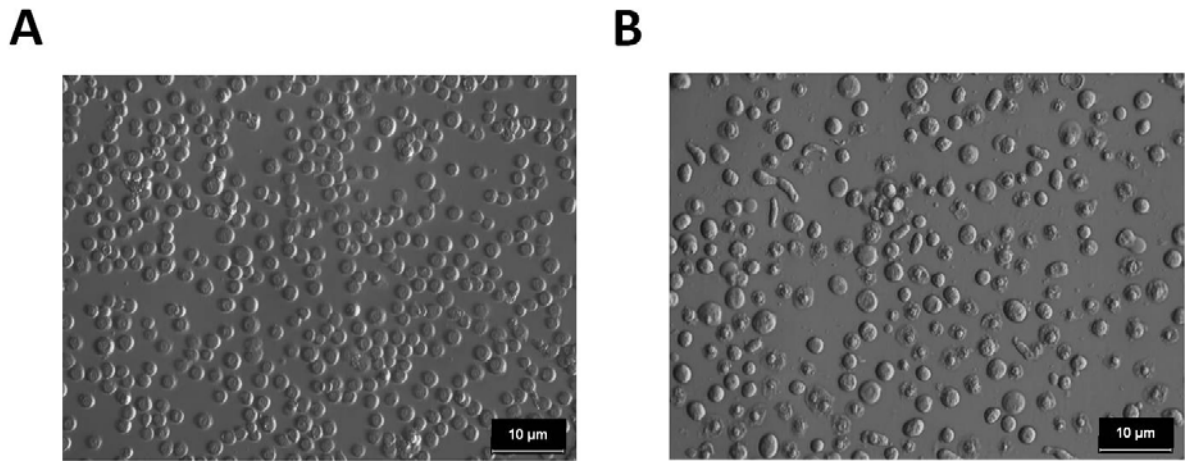


**Figure 15. High-five insect cells.**
**A.** Uninfected High-five cells. **B.** r-baculovirus infected High-five cells.
Photos taken with inverted light microscope, Leica DM IL LED, magnified 200x.

As seen in Figure 15 uninfected High-five cells are round, regular and small (Figure 15A) but infected cells are large irregular and with different light refraction (Figure 15B). Figure 16 shows the expression of recombinant Cul n 4 in High-five cells from three different vector systems, rBac-1-Cul n 4, rBac-HBM-Cul n 4 and rBac-SUMO-Cul n 4.

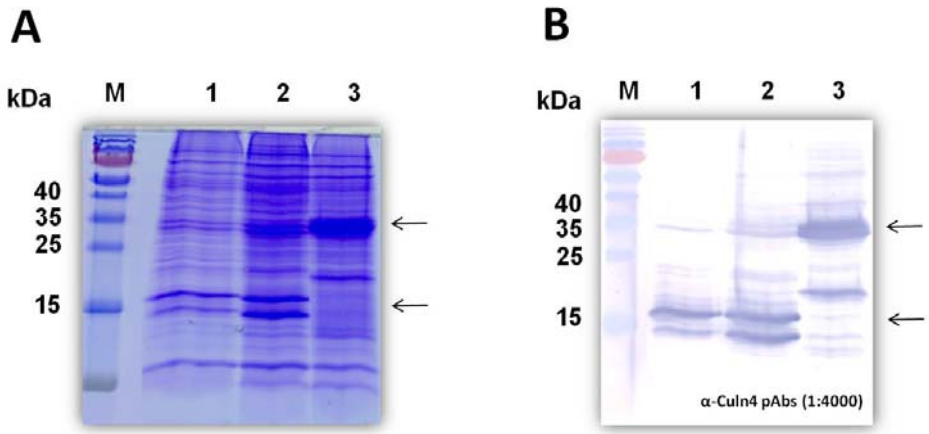


**Figure 16. Production of recombinant Cul n 4 in High-five insect cells.**
**A** Coomassie blue staining, **B.** Western blot**. Lane: 1** rBac-1-Cul n 4**, 2** rBac-HBM-Cul n 4, **3** rBac-SUMO-Cul n 4. The arrows indicate rBac-Cul n 4.

The r-allergens are the strongest protein bands seen in the coomassie staining. rBac-1-Cul n 4 and rBac-HBM-Cul n 4 showed double bands and rBac-SUMO-Cul n 4 a single band. The production of Cul n 1, Cul n 2 and Cul o 3 is shown in appendix VI.

## 4.4 Purification of recombinant allergen

Only the purification results of rBac-Cul n 4 and rBac-Cul n 2 are shown in details but successful purification of the other r-allergens are listed in Table 7 and figures shown in appendix VII. Table 6 shows the r-allergen constructs produced as proteins in High-five cells for purification.

**Table 6. r-allergen constructs produced in High-five cells for purification**

| Vector | | Name of construct | Predicted full size (kDa) |
|---|---|---|---|
| **Cul n 1 – Antigen 5 like protein** | | | |
| pFastBac-HBM-TOPO | Without signal sequence | rBac-HBM-Cul n 1 | 24 |
| pI-secSUMOstar | Without signal sequence | rBac-SUMO-Cul n 1 | 37 |
| **Cul n 2 - Hyaluronidase** | | | |
| pFastBac-HT B | Full length | rBac-HTB-Cul n 2 | 51 |
| pFastBac-HBM-TOPO | Full length | rBac-HBM-Cul n 2 | 51 |
| pI-secSUMOstar | Full length | rBac-SUMO-Cul n 2 | 64 |
| pI-secSUMOstar | Without signal sequence | rBac-SUMO-wo-Cul n 2 | 61 |
| **Cul n 3 – Secreted salivary protein** | | | |
| pFastBac-HBM-TOPO | Full length | rBac-HBM-Cul n 3 | 49 |
| **Cul n 4 – Secreted salivary protein** | | | |
| pFastBac-1 | Full length | rBac-1-Cul n 4 | 19 |
| pFastBac-HBM-TOPO | Without signal sequence | rBac-HBM-Cul n 4 | 19 |
| pI-secSUMOstar | Without signal sequence | rBac-SUMO-Cul n 4 | 32 |
| **Cul o 1 – Kunitz protease inhibitor** | | | |
| pFastBac-HBM-TOPO | Without signal sequence | rBac-HBM-Cul o 1 | 26 |
| **Cul o 2 – D7 related salivary protein** | | | |
| pFastBac-HBM-TOPO | Without signal sequence | rBac-HBM-Cul o 2 | 22 |
| **Cul o 3 – Antigen 5 like protein** | | | |
| pI-secSUMOstar | Without signal sequence | rBac-SUMO-Cul o 3 | 41 |

### 4.4.1 Purification of Cul n 4

Three constructs were made with Cul n 4, listed in Table 6. The proteins from all the rBac-Cul n 4 were successfully purified under native conditions with nickel affinity gel. Figure 17 shows the purification of rBac-1-Cul n 4, double band is eluted in all four fractions E1-E4, the smaller band is stronger in the elutions but more of the bigger band is bound to the nickel gel after elutions. Due to high protein concentration in the elution samples using 500 µL, the purified sample was eluted in 800 µL fractions in later purifications. The average yield of rBac-1-Cul n 4 from purification of $100x10^6$ cells was 600 - 1000 µg.

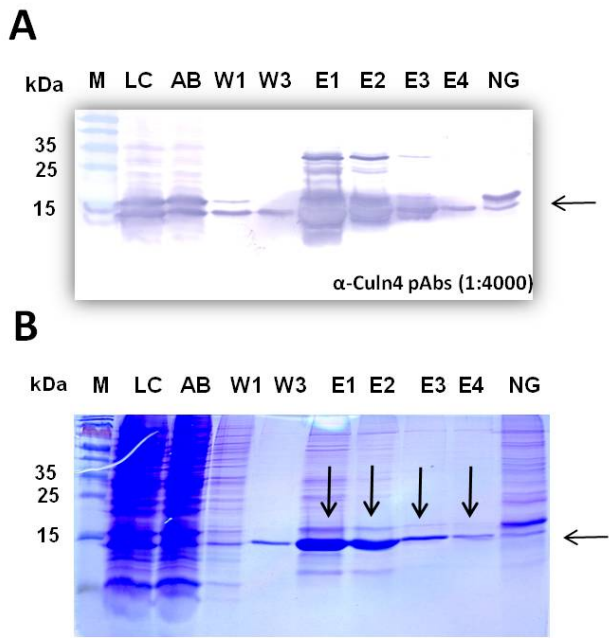


**Figure 17. Purification of rBac-1-Cul n 4 from High-five cells.**
**A** Western blot, **B** Coomassie blue staining**. Lane M:** marker, **LC:** lysed cell pellet, **AB:** after binding, **W1:** wash 1, **W3:** wash 3, **E1 - 4:** elution 1 - 4, **NG:** nickel gel after elution. The arrows indicate rBac-1-Cul n 4.

Figure 18 shows the purification of rBac-HBM-Cul n 4 from High-five cells. Purified r-allergen was found in all elutions (E1 – E4), most in the first two. rBac-HBM-Cul n 4 is seen as a double band and the smaller band is stronger in the elution samples but some of the bigger band bound to the nickel gel after elution. The yield of purified rBac-HBM-Cul n 4 from $100x10^6$ cells was 500 - 750 µg.

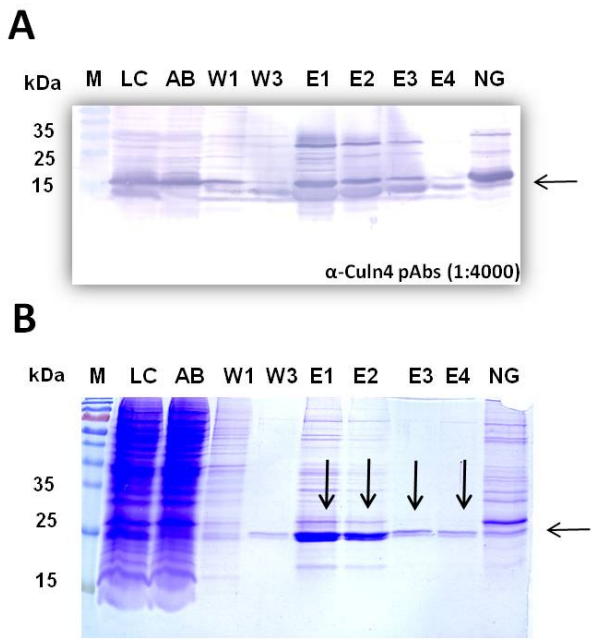


**Figure 18. Purification of rBac-HBM-Cul n 4 from High-five cells.**
**A** Western blot, **B** Coomassie blue staining**. Lane M:** marker, **LC:** lysed cell pellet, **AB:** after binding, **W1:** wash 1, **W3:** wash 3, **E1 - 4:** elution 1 - 4, **NG:** nickel gel after elution. The arrows indicate rBac-HBM-Cul n 4.

Purified rBac-SUMO-Cul n 4 seen as double band in the WB but single band in the coomassie blue staining (Figure 19), was found in all elutions (E1 - E4), most in the first two. The yield of rBac-SUMO-Cul n 4 from 100x10$^6$ cells was around 1000 µg.

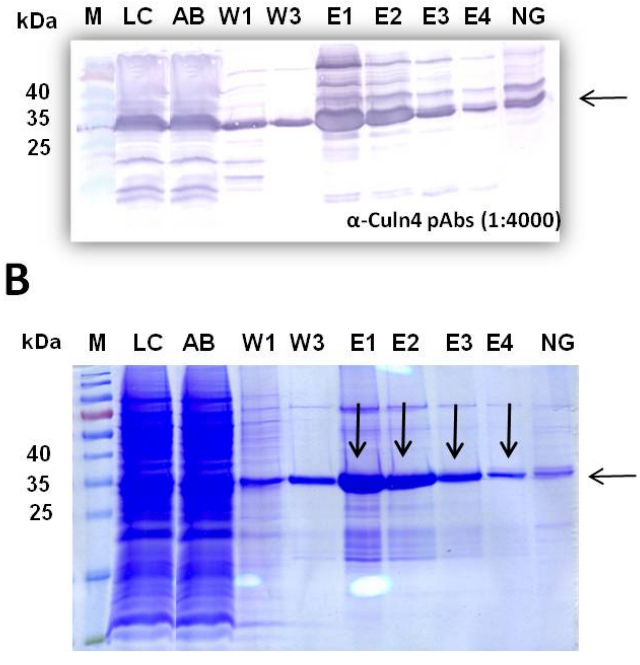


**Figure 19. Purification of rBac-SUMO-Cul n 4 from High-five cells.**
**A** Western blot, **B** Coomassie blue staining**. Lane M:** marker, **LC:** lysed cell pellet, **AB:** after binding, **W1:** wash 1, **W3:** wash 3, **E1 - 4:** elution 1 - 4, **NG:** nickel gel after elution. The arrows indicate rBac-SUMO-Cul n 4.

The purified samples were dialyzed in PBS to remove the imidazole (Figure 20).

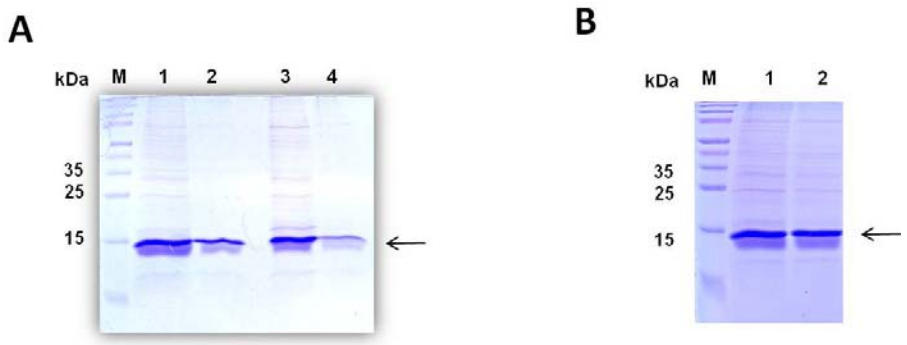


**Figure 20. Dialysis of purified rBac-1-Cul n 4 and rBac-HBM-Cul n 4.**
**A.** Coomassie blue staining, rBac-1-Cul n 4 and rBac-HBM- Cul n 4 before and after dialysis in PBS. **Lane M:** marker, **1 and 3:** rBac-1-Cul n 4 and rBac-HBM-Cul n 4 before dialysis, **2 and 4:** rBac-1-Cul n 4 and rBac-HBM-Cul n 4 after dialysis. **B.** Coomassie blue staining, dialysis of rBac-1-Cul n 4 in elution buffer without imidazole. **Lane M:** marker, **1:** before dialysis, **2:** after dialysis. The arrows indicate rBac-Cul n 4.

After the dialysis in PBS the samples contained less of unspecific proteins but there was also loss of the specific protein (Figure 20). The protein samples were later dialyzed in elution buffer without imidazole where there was less loss of protein (Figure 20). The average yield of purified rBac-1-Cul n 4 from $100x10^6$ cells after dialysis in elution buffer without imidazole was 250 - 500 µg.

Purified rBac-SUMO-Cul n 4 from $100x10^6$ cells was dialyzed in elution buffer without imidazole and the yield was 670 µg. After the imidazole had been removed the SUMOstar was cleaved from the fusion protein with SUMOstar protease 1 according to manufacturer‘s protocol and tested with coomassie blue staining with purified rBac-1-Cul n 4 as a control (Figure 21). After 1 hr incubation at 30°C there was hardly any cleavage and very little after o.n. incubation. Therefore longer incubation and more protease, 15 and 20 units was tried. Cleavage of most of the protein was obtained with 20 U after 7 days (Figure 21).

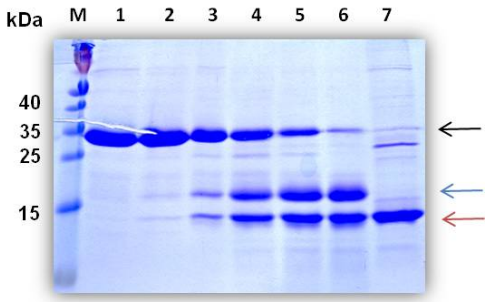


**Figure 21. Cleavage of SUMOstar from rBac-SUMO-Cul n 4 with SUMOstar protease 1.**
Coomassie blue staining. **Lane M:** marker, **1:** rBac-SUMO-Cul n 4, **2:** cleaved with 10 U SUMOstar protease; 1 hr at 30 °C, **3:** over night at 4°C, **4:** 4 days at 4°C, **5:** 15 U for 5 days at 4°C, **6:** 20 U for 7 days at 4°C, **7:** purified rBac-1-Cul n 4 control. The **black** arrow indicates rBac-SUMO-Cul n 4, the **blue** cleaved SUMOstar fusion protein and the **red** rBac-Cul n 4.

The r-allergen Cul n 4 was secreted from infected cells into the supernatant from all three Cul n 4 constructs, rBac-1-Cul n 4, rBac-HBM-Cul n 4 and rBac-SUMO-Cul n 4 (Figure 14 and 48, 49 in appendix V). From $100x10^6$ cells 100 mL of supernatant was collected. Purification with nickel affinity gel was done from 35 mL of supernatant of all constructs with one washing step without imidazole.

The yield of purified rBac-1-Cul n 4 from supernatant (Figure 22) was very poor, bands hardly visible in coomassie blue staining and similar data were obtained for rBac-HBM-Cul n 4 (data not shown).

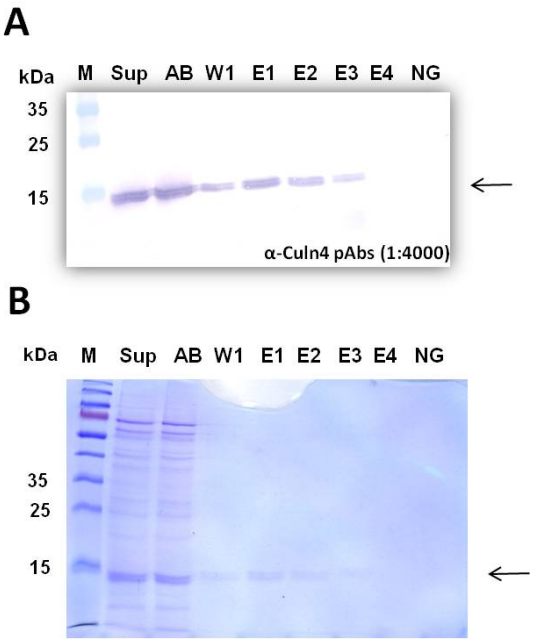


**Figure 22. Purification of rBac-1-Cul n 4 from supernatant of infected High-five cells.**
**A** Western blot, **B** Coomassie blue staining**. Lane M:** marker, **Sup:** supernatant, **AB:** after binding, **W1:** wash 1, **W3:** wash 3, **E1 - 4:** elution 1 - 4, **NG:** nickel gel after elution. The arrows indicate rBac-1-Cul n 4.

On the other hand rBac-SUMO-Cul n 4 was easily purified from supernatant (Figure 23) and the yield exceeding 900 µg from 35 mL.

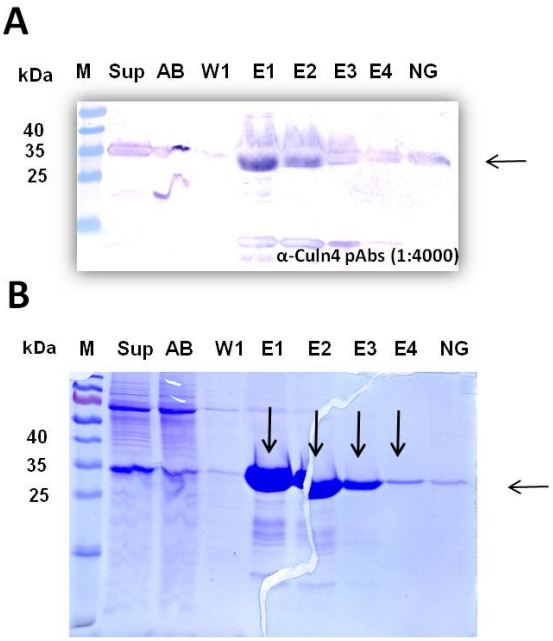


**Figure 23. Purification of rBac-SUMO-Cul n 4 from supernatant of infected High-five cells.**
**A** Western blot, **B** Coomassie blue staining**. Lane M:** marker, **Sup:** supernatant, **AB:** after binding, **W1:** wash 1, **E1 - 4:** elution 1 -4, **NG:** nickel gel after elution. The arrows indicate rBac-SUMO-Cul n 4.

### 4.4.2 Purification of Cul n 2

rBac-HBM-Cul n 2 could not be successfully purified under native conditions with nickel affinity gel. As seen in Figure 24 very little rBac-HBM-Cul n 2 was detected in the elutions (E1 – E4) and left on the nickel gel after elution (NG).

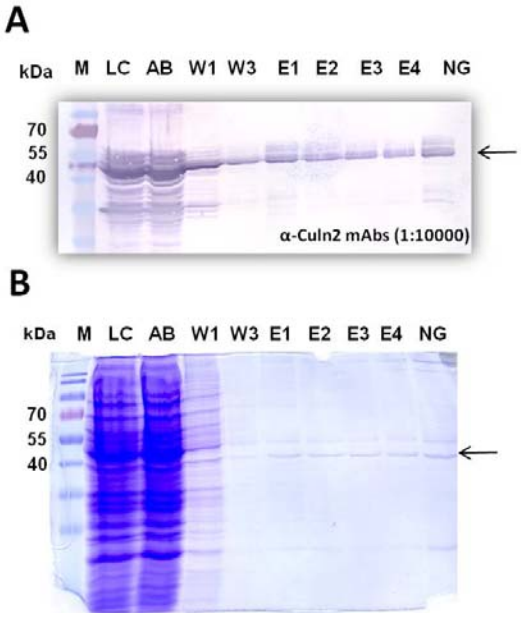


**Figure 24. Purification of rBac-HBM-Cul n 2 from High-five cells.**
**A** Western blot, **B** Coomassie blue staining**. Lane M:** marker, **LC:** lysed cell pellet, **AB:** after binding, **W1:** wash 1, **W3:** wash 3, **E1 - 4:** elution 1 - 4, **NG:** nickel gel after elution. The arrows indicate rBac-HBM-Cul n 2.

Purification of rBac-HBM-Cul n 2 was attempted with cation exchange HiTrap™ SP Sepharose™ HP column in FPLC. About $50x10^6$ cells were lysed, sonicated, centrifuged and the supernatant loaded on the column. Elution was made with increasing salt from 0.2 M to 1 M NaCl, and resulted in three peaks where the first peak indicates the flow through from the wash (Figure 25).

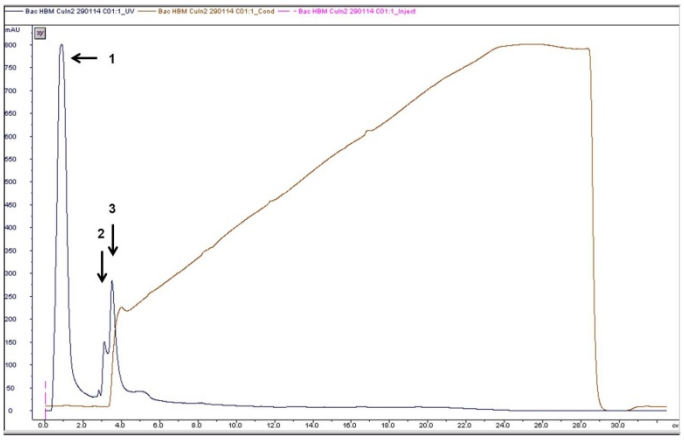


**Figure 25. Purification of rBac-HBM-Cul n 2 on HiTrap™ SP Sepharose™ HP column.**
Chormatographic profile of rBac-HBM-Cul n 2 obtained in HiTrap™ SP Sepharose™ HP column coupled to an ÄKTA-FPLC system. The eluation was under linear gradient from 0.2 M to 1 M NaCl (**brown line**), at a flow rate 3 mL/min collecting fractions of 1 mL. The protein was monitored by measuring absorbance at 280 nm represented as the **blue line**. **Y-axis:** absorption unit (mAU), **x-axis:** column volume (CV: 2 mL), **pink mark:** injection of rBac-HBM-Cul n 2 to the column. The run resulted in three peaks marked 1 - 3.

The elutions from the purification were collected in 1 mL fractions. Fractions 1-13 were collected of the flowthrough (0M NaCl), 14- 32 of the NaCl gradient from 200 mM to 350 mM NaCl, then sample 33 from 400 mM NaCl, 34 from 500 mM NaCl, 35 from 600 mM NaCl and fractions 36-72 from 700 mM to 1 M NaCl gradient. The samples were tested in dot blot with Cul n 2 specific mAb (Jónsdóttir, 2011).

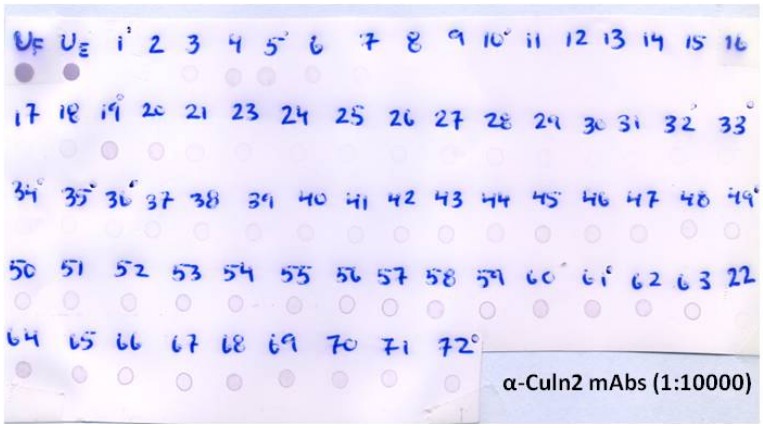


**Figure 26. Dot blot of rBac-HBM-Cul n 2 purification on HiTrap™ SP Sepharose™ HP column.**

According to the dot blot (Figure 26) concentration of rBac-HBM-Cul n 2 was high in the lysed pellet and some washed out with the flow through in the beginning. The protein seems to be distributed in samples 18-12 and 37-72. A few samples were selected to test in WB and with coomassie blue staining (Figure 27).

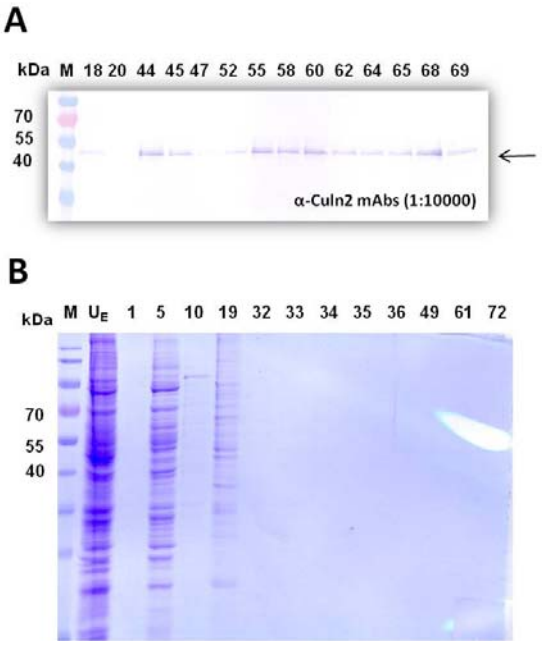


**Figure 27. rBac-HBM-Cul n 2 purification on HiTrap™ SP Sepharose™ HP column.**
**A.** Western blot. **Lane M:** marker, **18-69:** fractions 18-69. **B.** Coomassie blue staining. **Lane M:** marker, **$U_E$:** lysed cell pellet, **1-72:** fractions 1-72. The arrow indicates rBac-HBM-Cul n 2.

As seen in Figure 27A there is some rBac-HBM-Cul n 2 in the fractions, however the concentration is too low to be detected in the coomassie blue staining (Figure 27B).

Two attempts were made to purifiy rBac-HBM-Cul n 2 with HiTrap™ NHS HP column coupled with α Cul n 2 mAb without success (data not shown).

Purification of rBac-HTB-Cul n 2 with anion exchange HiTrap™ Q HP column. About $50x10^6$ cells were lysed, sonicated, centrifuged and the supernatant loaded to the column. Elution was made with increasing salt from 0 M to 0.8 M NaCl then finally 1 M NaCl and resulted in eight peaks where the first peak indicates the flow through from washing (Figure 28).

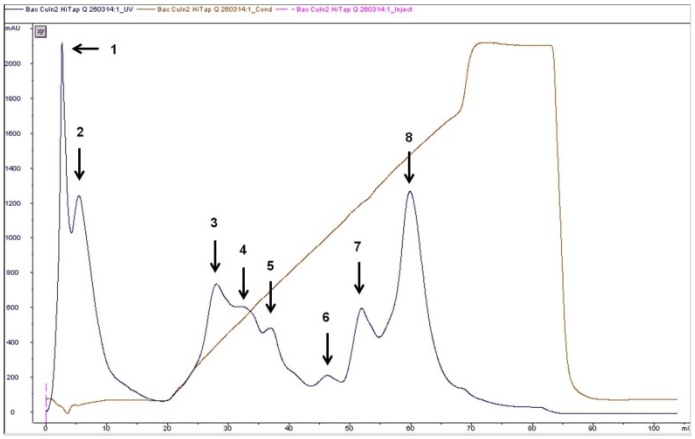


**Figure 28. Diagram from purification of rBac-HTB-Cul n 2 on HiTrap™ Q HP in FPLC.** Chormatographic profile of rBac-HTB-Cul n 2 obtained in HiTrap™ SP Sepharose™ HP column coupled to an ÄKTA-FPLC system. The eluation was under linear gradient from 0 M to 0.8 M NaCl, finally 1M NaCl (**brown line**), at a flow rate 3 mL/min collecting fractions of 1 mL. The protein was monitored by measuring absorbance at 280 nm represented as the **blue line**. **Y-axis:** absorption unit (mAU), **x-axis:** volume (mL), **pink mark:** injection of rBac-HTB-Cul n 2 to the column. The run resulted in eight peaks marked 1 - 8.

The elutions were collected in 1 mL fraction, fractions 1-10 were collected of the flow through (0 M NaCl), 11-60 of the gradient (0 mM to 800 mM NaCl) and 61-64 after the gradient (1 M NaCl). All the samples were tested in dot blot with Cul n 2 specific mAb.


**Figure 29. Dot blot of rBac-HTB-Cul n 2 purification on HiTrap™ Q HP column.**
**U:** lysed cell pellet, **1-10:** flow through, **11-60:** samples from gradient, **61-64:** samples from 100% buffer B.

Some of the rBac-HTB-Cul n 2 does not bind to the column but is washed out with the flow through. In the elution fractions rBac-HTB-Cul n 2 is distributed in samples 21-60, with the most concentration in 52-57 (Figure 29). This shows that the purified rBac-HTB-Cul n 2 was in peak 8 according to the diagram in Figure 28. Fractions 52-57 were tested in WB and with coomassie blue staining together with the concentrate of these samples from a spin column and fraction two from flow through (Figure 30).

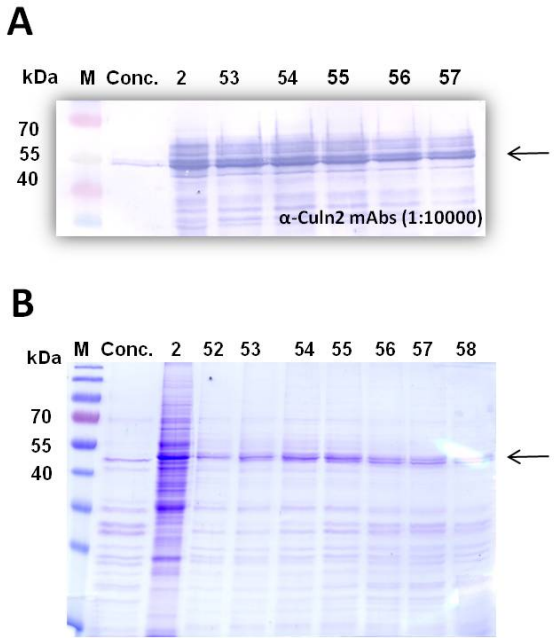


**Figure 30. Samples from rBac-HTB-Cul n 2 purification on HiTrap™ Q HP column.**
**A.** Western blot. **B.** Coomassie blue staining. **Lane M:** marker, **Conc.:** concentration of samples 53-57, **2:** flow through, **52-58:** samples 52-58. The arrows indicate rBac-HTB-Cul n 2.

As seen in Figure 29. Dot blot of rBac-HTB-Cul n 2 purification on HiTrap™ Q HP column. rBac-HTB-Cul n 2 is in fractions 52-58, however, there are a lot of extra bands seen in the coomassie blue staining. Fractions 52-58 were concentrated on spin column and were 255 µg/mL. As the total volume of the final sample was 1 mL, the yield of rBac-HTB-Cul n 2 from $50x10^6$ cells was 255 µg. FPLC purified rBac-HTB-Cul n 2 was not stable in storage at 4°C, within a week most of the protein had precipitated. Different storage in -80°C was tried with the rBac-HTB-Cul n 2 concentrated from fractions 53-57. One sample, 50 µL, was directly frozen, 50 µL with PIC (1:100), 50 µL with 1mM EDTA, 50 µL with PIC (1:100) and 1 mM EDTA and 50 µL with PIC (1:100), 1 mM EDTA and 10% glycerol. All the samples were rapidly frozen in liquid nitrogen and kept at -80°C. Week later the samples were tested with coomassie blue staining and measured in Bradford assay.

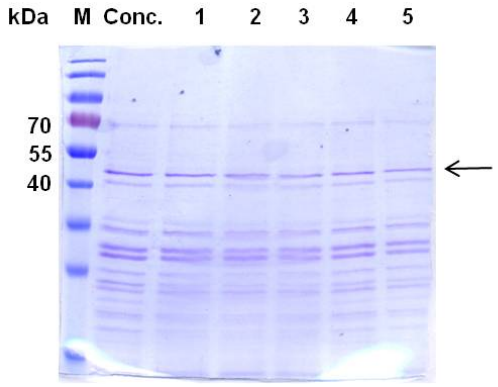


**Figure 31. Different storage of purified rBac-HTB-Cul n 2 tested with coomassie blue staining.** **Lane M:** marker, **Conc.:** samples 53-57 concentrated from FPLC purification, **1-5:** the conc. after 1 week storage in -80°C, **2:** with PIC (1:100), **3:** with 1 mM EDTA, **4:** with PIC (1:100) and 1 mM EDTA, **5:** with PIC (1:100), 1 mM EDTA and 10% glycerol. The arrow indicates rBac-HTB-Cul n 2.

The coomassie blue staining of the storage samples (Figure 31) shows little difference in the purified rBac-HTB-Cul n 2 with the different storage methods. The concentration was 150-175 µg/mL.

rBac-HTB-Cul n 2 was purified under denaturing conditions using guanidium-HCl and urea (Figure 32).

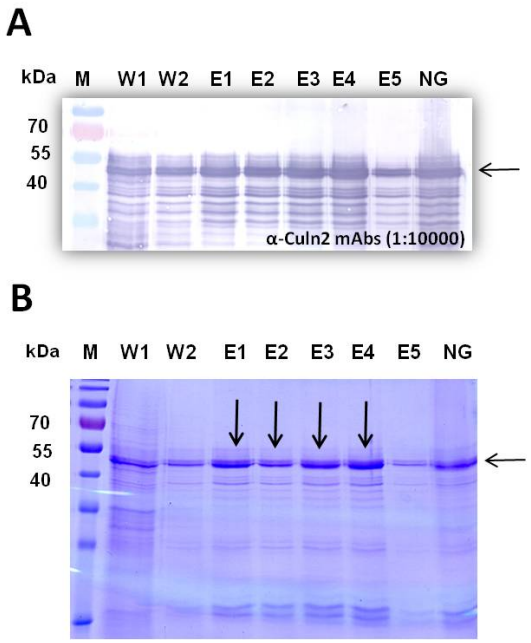


**Figure 32. Denature purification of rBac-HTB-Cul n 2 from High-five cells.**
**A** Western blot, **B** Coomassie blue staining**. Lane M:** marker, **W1:** wash 1, **W2:** wash 2, **E1 - 5:** elution 1 - 5, **NG:** nickel gel after elution. The arrows indicate rBac-HTB-Cul n 2.

As seen in the WB and with coomassie blue staining (Figure 32) purified rBac-HTB-Cul n 2 is detected in all the elutions (E1-E5), some protein is lost in the wash and some still bound to the gel after the elution. Because of high concentration in the elution samples, the elution volume was raised from 500 to 800 µL in later purifications. The yield of purified rBac-HTB-Cul n 2 from $100x10^6$ cells was 1300-2000 µg.

An attempt was made to refold the rBac-HTB-Cul n 2 by removing the urea and raising the pH. First by using denaturing condition in the inition of the purification and native conditions in the second wash and elutions. The purification was tested with coomassie blue staining, which showed no purified rBac-HTB-Cul n 2 in the elution fraction and the protein was still bound to the nickel gel after the elutions (data not shown).

Since the rBac-HTB-Cul n 2 could not be refolded during the purification an attempt was made to refold it with stepwise dialysis, in 6 M urea, 4 M urea, 2 M urea and finally without urea at pH 7. Figure 33 shows rBac-HTB-Cul n 2 could be dialyzed in denaturing elution buffer with 6 M and 4 M urea, but precipitated in 2 M urea.

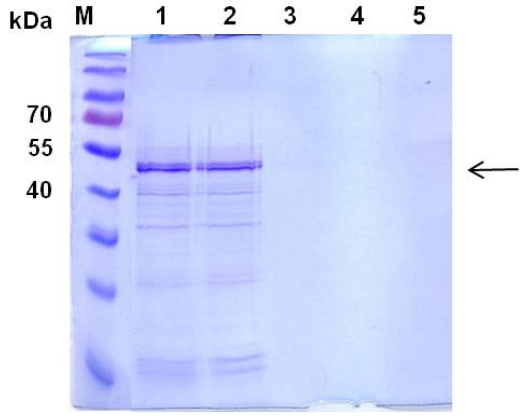


**Figure 33. Coomassie blue staining, the dialysis of denature purified rBac-HTB-Cul n 2.**
**Lane M:** marker, **1:** rBac-HTB-Cul n 2 after dialysis in denaturing elution buffer with 6 M urea at pH 7, **2:** 4M urea, **3:** 2M urea, **4:** 1 M urea, **5:** 0 M urea. The arrow indicates rBac-HTB-Cul n 2.

rBac-SUMO-Cul n 2 and rBac-SUMO-wo-Cul n 2 were purified with nickel affinity gel under native conditions. The purification of rBac-SUMO-Cul n 2 was unsuccessful (data not shown). As seen in Figure 34 the purification of rBac-SUMO-wo-Cul n 2 was successful and purified protein was found in all elutions (E1 - E4), however a large amount of protein was still bound to the nickel gel after elution. The yield of purified rBac-SUMO-wo-Cul n 2 from $100x10^6$ cells was 250 - 580 µg.

Different methods were tried to increase the yield rBac-SUMO-wo-Cul n 2 in the elutions, with more lysis buffer and same amount of nickel gel, at RT with shorter incubation time and with more buffer and more nickel gel. All these attempts were unsuccessful (data not shown).

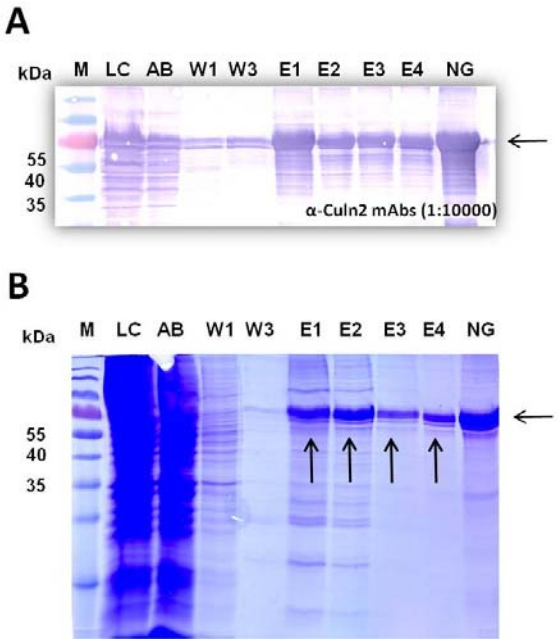


**Figure 34. Purification of rBac-SUMO-wo- Cul n 2 from High-five cells.**
**A** Western blot, **B** Coomassie blue staining**. Lane M:** marker, **LC:** lysed cell pellet, **AB:** after binding, **W1:** wash 1, **W3:** wash 3, **E1-4:** elution 1-4, **NG:** nickel gel after elution. The arrows indicate rBac-SUMO-wo-Cul n 2.

The purified rBac-SUMO-wo-Cul n 2 was dialyzed in elution buffer without imidazol and most of the protein precipitated (data not shown). Dialysis was also tried with increased salt, 500 mM NaCl instead of 300 mM NaCl, with no success (data not shown).

The purification status of all the r-allergens expressed and produced in insect cells is listed in Table 7.

**Table 7. Purification status of the r-allergens expressed in insect cells.**

| Name | Purification status |
|---|---|
| **Cul n 1 – Antigen 5 like protein** | |
| rBac-HBM-Cul n 1 | Purified under denaturing conditions |
| rBac-SUMO-Cul n 1 | Purified under native conditions, precipitates in dialysis |
| **Cul n 2 - Hyaluronidase** | |
| rBac-Cul n 2 | Purified under native conditions with FPLC, unstable |
| rBac-HBM-Cul n 2 | Purified under denaturing conditions |
| rBac-SUMO-Cul n 2 | Purified under native conditions, precipitates in dialysis |
| rBac-SUMO-wo-Cul n 2 | Could not be purified |
| **Cul n 3 – Secreted salivary protein** | |
| rBac-Cul n 3 | Purified under native conditions |
| **Cul n 4 – Secreted salivary protein** | |
| rBac-1-Cul n 4 | Purified under native conditions |
| rBac-HBM-Cul n 4 | Purified under native conditions |
| rBac-SUMO-Cul n 4 | Purified under native conditions |
| **Cul o 1 – Kunitz protease inhibitor** | |
| rBac-HBM-Cul o 1 | Purified under denaturing conditions |
| **Cul o 2 – D7-related salivary protein** | |
| rBac-HBM-Cul o 2 | Purified under native conditions |
| **Cul o 3 – Antigen 5 like protein** | |
| rBac-SUMO-Cul o 3 | Purified under native conditions, precipitates in dialysis, denatured purified |

## 4.5 Deglycosylation

Purified rBac-1-Cul n 4, rBac-HBM-Cul n 4, rBac-SUMO-Cul n 4, rBac-HBM-Cul n 3 and rBac-HBM-Cul o 2 were treated with the PNGase F to test for glycosylation of the proteins.

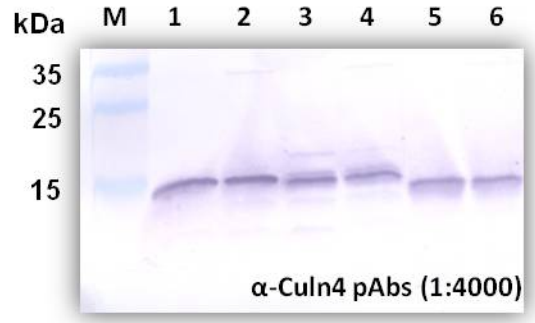


**Figure 35. Deglycosylation of Cul n 4.**
Western blot. **M:** marker, **1:** rBac-1-Cul n 4 untreated, **2:** rBac-1-Cul n 4 PNGase F treated, **3:** rBac-HBM-Cul n 4 untreated, **4:** rBac-HBM-Cul n 4 PNGase F treated, **5:** rBac-SUMO-Cul n 4 untreated, **6:** rBac-SUMO-Cul n 4 PNGase F treated.

All the r-allergens showed one clear band in the untreated sample and one band of the same size in the PNGase treated samples (Figure 35). The treatment did neither show any effect on rBac-HBM-Cul n 3 nor rBac-HBM-Cul o 2 (data not shown).

## 4.6 Immunoassays

PBMC and serum from horses vaccinated with the recombinant proteins purified from *E. coli* mixed in adjuvant (SE A/M 1-6 in alum, SE A/M 7-12 in alum/MPL) and IBH affected horses were used to set up immunoassays with the recombinant proteins purified from insect cells.

### 4.6.1 Stimulation of PBMC with rBac-allergens and measurement of cytokines

Purified rBac-HBM-Cul n 3 and rBac-1-Cul n 4 were titrated by stimulating *in vitro* for 96 hrs PBMC from four vaccinated horses and two controls and secreted cytokines measured in the cell supernatant with Horse Cytokine 5-plex Assay (Wagner & Freer, 2009) at The Cornell University College of Veterinary Medicine, Ithaca New York. Isolation of mRNA and cDNA synthesis was also performed on the lysed cells but expression analysis of cytokines using qPCR is yet to be done.

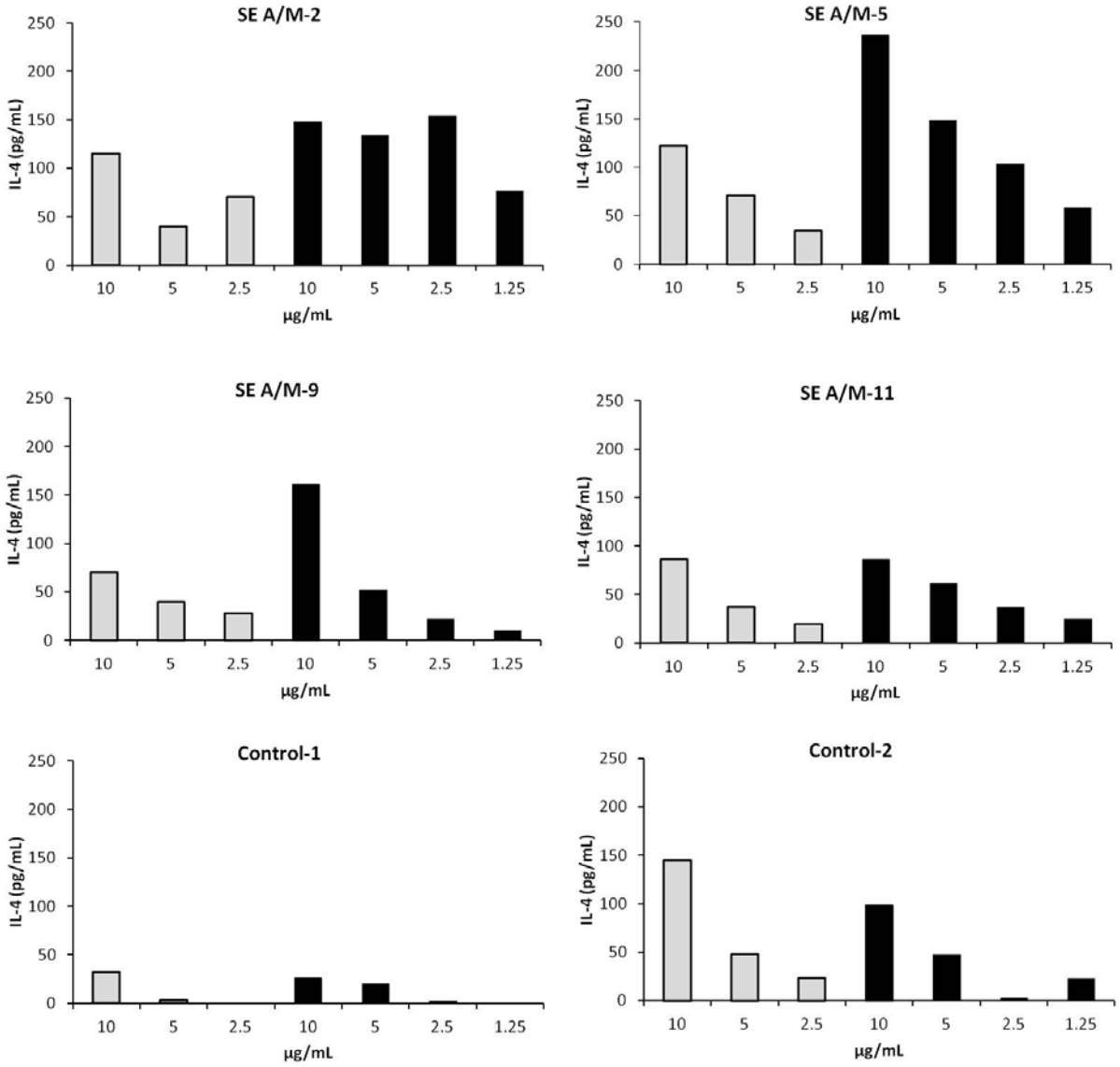


**Figure 36. IL-4 secretion following stimulation of PBMC with rBac-Cul n 3 and 4 in different concentrations.**
**Grey columns:** rBac-HBM-Cul n 3, **black columns:** rBac-1-Cul n 4. One experiment.

All four vaccinated horses produced IL-4 up on stimulation with the proteins at least to 10 μg/mL or 70-123 pg/mL for rBac-HBM-Cul n 3 and 87-237 pg/mL for rBac-1-Cul n 4. Three of the horses (SE A/M-5, 9, 11) responded in a dose dependent manner. IL-4 secretion was higher up on stimulation with rBac-1-Cul n 4 in three vaccinated horses (SE A/M-2, 5, 9) but similar for both proteins in SE A/M-11. Control horse 2 responded with high secretion of IL-4 (144 and 99 pg/mL) in dilution 10 μg/mL both when stimulated with rBac-HBM-Cul n 3 and rBac-1-Cul n 4 and low values in other dilutions but Control-1 was very low or negative in all dilutions.

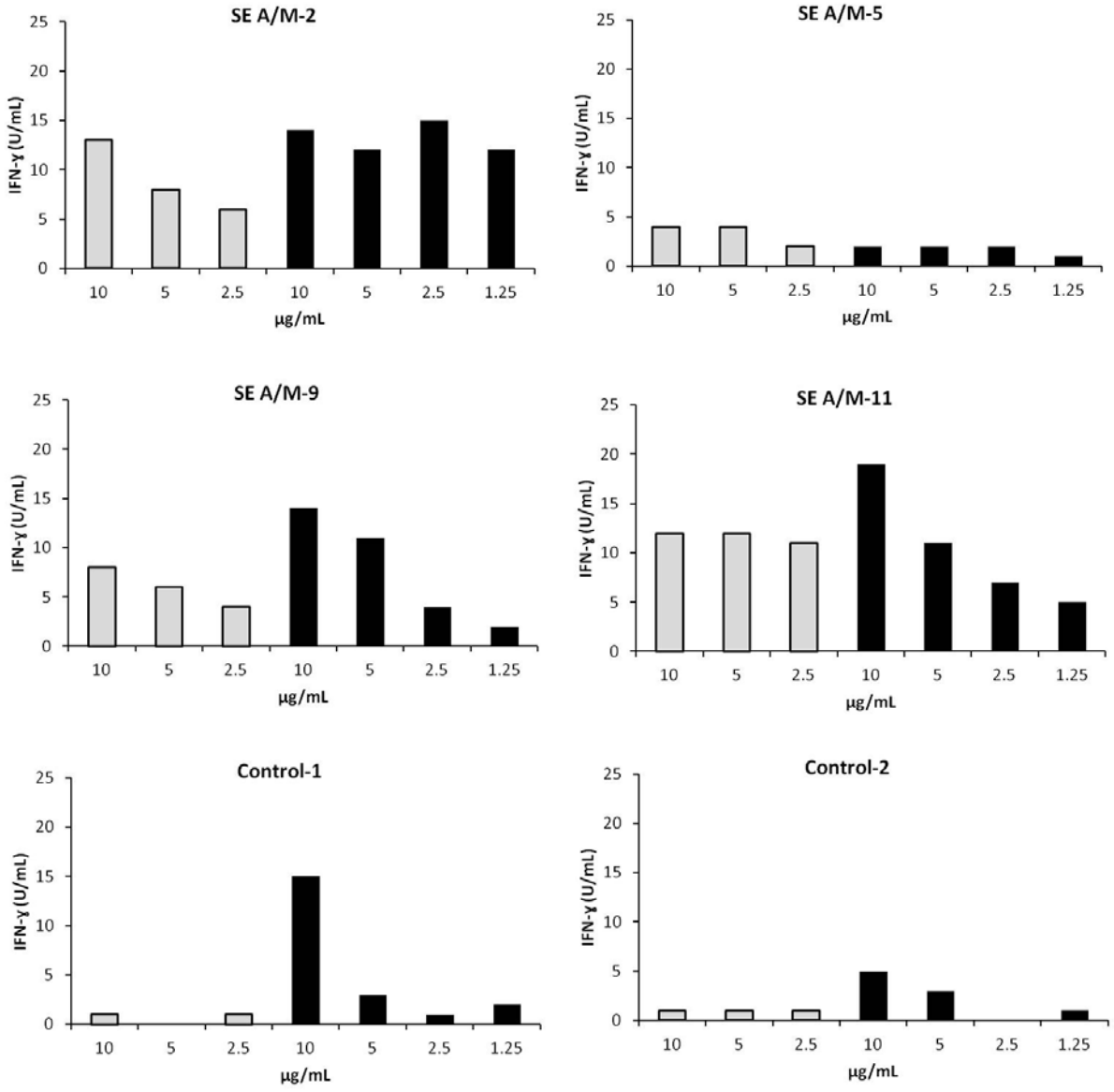


**Figure 37. IFN-γ secretion following stimulation of PBMC with rBac-Cul n 3 and 4 in different concentrations.**
**Grey columns:** rBac-HBM-Cul n 3, **black columns:** rBac-1-Cul n 4. One experiment

Three of the vaccinated horses (SE A/M-2, 9, 11) produced IFN-γ in dose dependent manner up on stimulation with rBac-HBM-Cul n 3. At 10 μg/mL the amount of secreted IFN-γ was 8-13 U/mL. SE A/M-5 showed lower than 5 U/mL in response to all protein concentration and the control horses were negative. Stimulation with rBac-1-Cul n 4 induced IFN-γ production in the same three horses (SE A/M-

2, 9, 11) and the secreted amount was 14-19 U/mL at dilution 10 μg/mL. For horses SE A/M-9 and 11 there was dilution effect whereas in horse SE A/M-2 the secretion was similar in all rBac-1-Cul n 4 concentrations. Control horse 1 had high IFN-γ production (15 U/mL) in 10 μg/mL but other control values were low or negative (Figure 37).

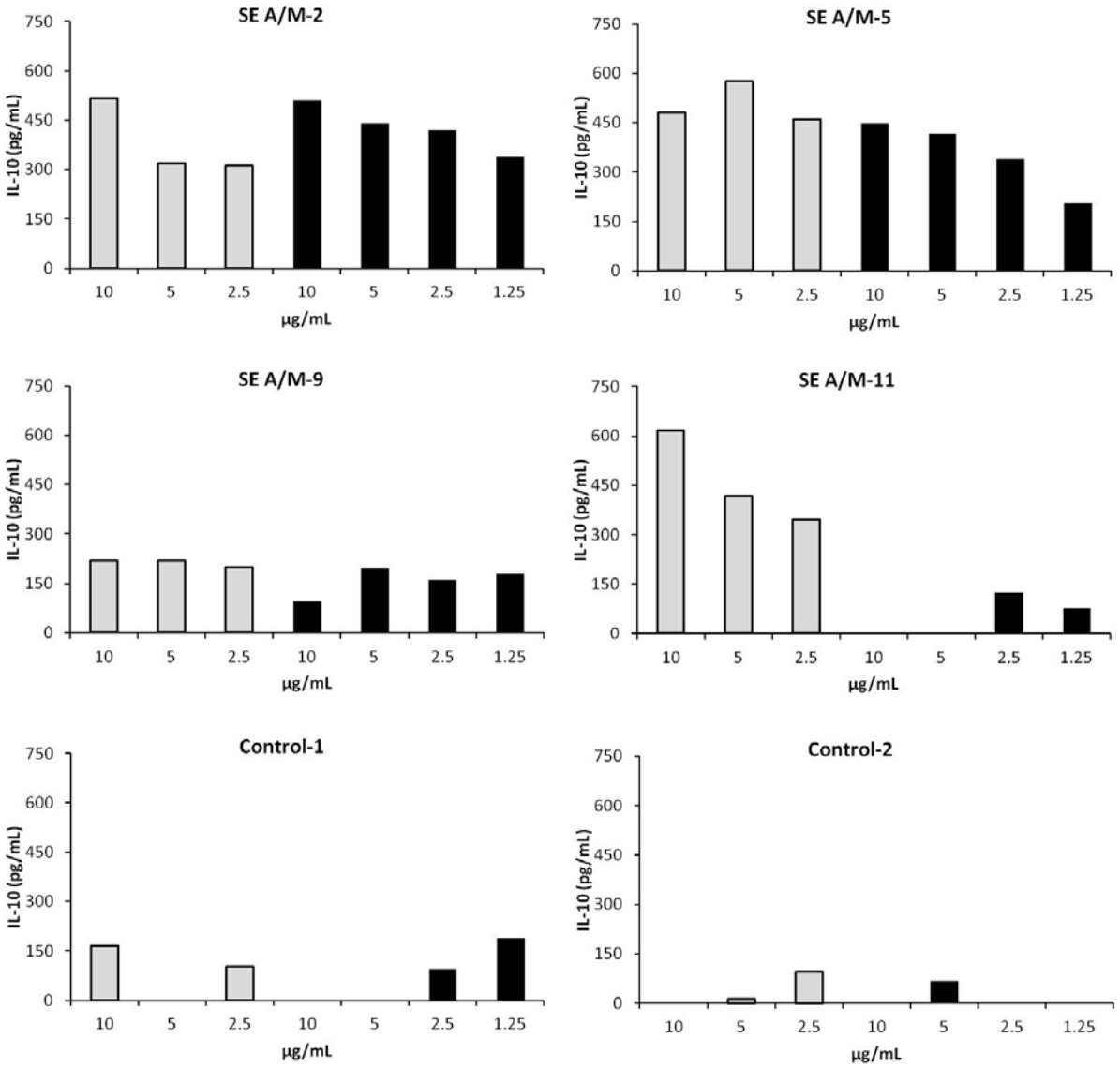


**Figure 38. IL-10 secretion following stimulation of PBMC with rBac-Cul n 3 and 4 in different concentrations.**
**Grey columns:** rBac-HBM-Cul n 3, **black columns:** rBac-1-Cul n 4. One experiment

PBMC from all four vaccinated horses were stimulated to produce IL-10 with all three concentrations of Cul n 3. Titration effect was seen for horses SE A/M-2 and 11 that together with SE A/M-5 responded strongly, 481 – 616 pg/mL in dilution 10 μg/mL. SE A/M-9 had low response and similar in all dilutions (198 – 220 pg/mL). Control horses had minimum or low response. SE A/M-9 had similar low response and not dose dependent (96 – 196 pg/mL) to rBac-1-Cul n 4 and SE A/M-11 was negative. The two other (SE A/M-2 and 5) responded strongly (511 and 447 pg/mL respective in 10 μg/mL) and in a titrated manner (Figure 38).

### 4.6.2 Total IgG response of vaccinated horses to purified rBac-allergens measured in ELISA

The rBac-HBM-Cul n 3 and rBac-1-Cul n 4 were used to set up an ELISA to measure total IgG in serum of the vaccinated horses. The response against rBac-HBM-Cul n 3 and rBac-1-Cul n 4 was compared to response against rCul n 3 and rCul n 4 purified from *E. coli*.

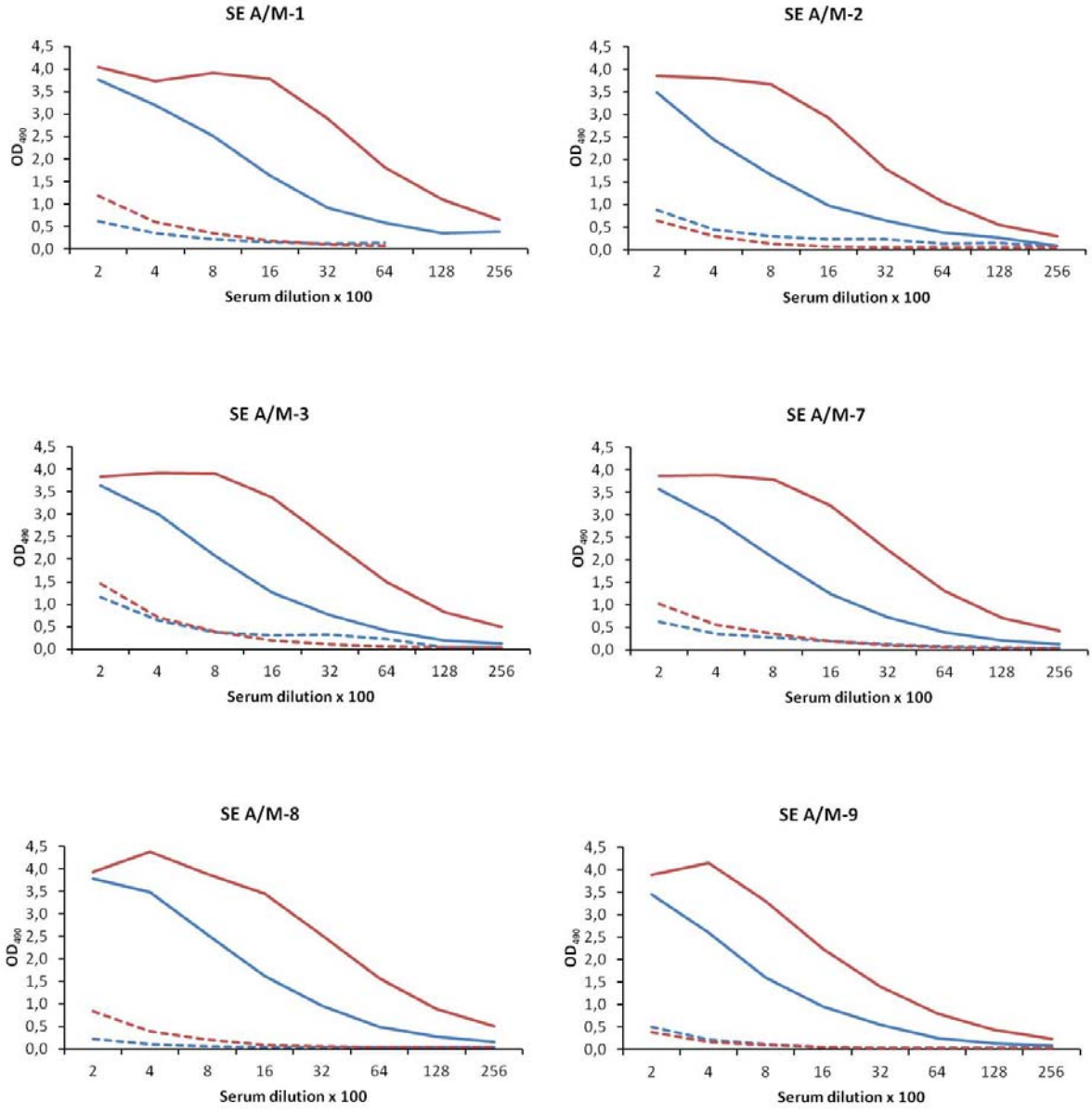


**Figure 39. Cul n 3 specific total IgG response of vaccinated horses measured by ELISA.** **y-axis:** optical density at 490 nm ($OD_{490}$) **x-axis:** serum dilution x 100, **dashed line (- - -)** : serum collected before vaccination (week 0), **continuous line (——) :** serum collected after third vaccination (week 10), **blue line:** rBac-HBM-Cul n 3, **red line:** rCul n 3. One experiment.

All the six vaccinated horses responded strongly to both proteins up to dilution 1/1600. They showed a stronger response to the *E. coli* produced rCul n 3, used for vaccination, than the rBac-HBM-Cul n 3. In four vaccinated horses, SE A/M-1, 3, 7 and 8, the most difference is in serum dilution 1/1600 where the $OD_{490}$ values for rCul n 3 is between 3.2 to 3.8 but between 1.2 to 1.6 for rBac-HBM-Cul n 3. In SE A/M-2 and 9 the greatest difference is in dilution 1/800, $OD_{490}$ values 3.7 and 3.3 for

rCul n 3 but about 1.6 for rBac-HBM-Cul n 3 for both horses. There was background response in the pre-vaccination sera in dilutions 1/200 and 1/400 that differs between horses and between the two proteins. It is slightly higher for rCul n 3 on four horses (SE A/M-1, 3, 7 and 8) $OD_{490}$ values 0.6 – 1.5 in the dilution 1/200 but 0.2 – 1.2 for rBac-HBM-Cul n 3. For SE A/M – 2 and 9 the response is slightly stronger for rBac-HBM-Cul n 3, $OD_{490}$ values 0.5 and 0.8 in the dilution 1/200 but 0.4 and 0.6 for rCul n 3 (Figure 39).

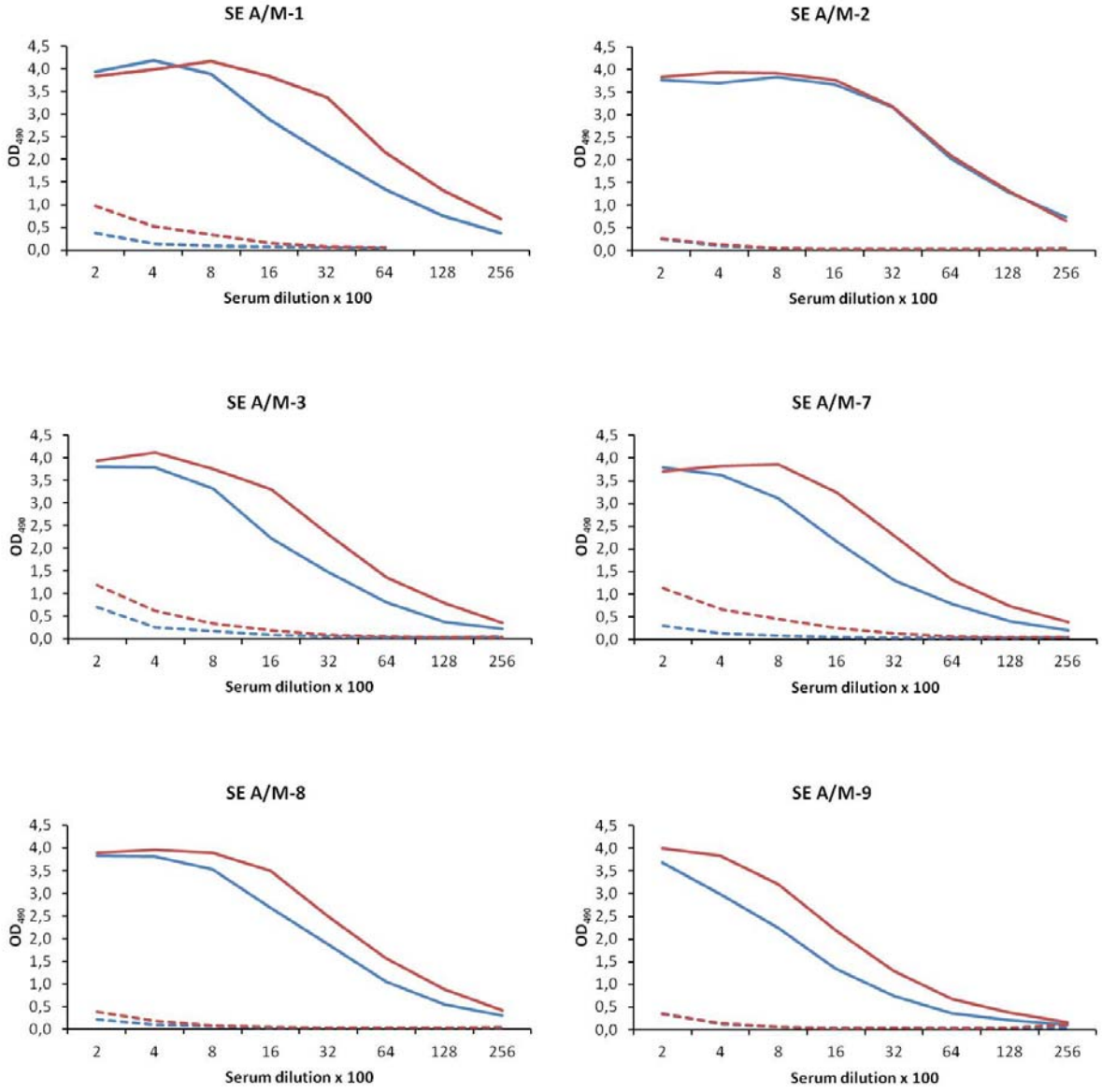


**Figure 40. Cul n 4 specific total IgG response of vaccinated horses measured by ELISA.**
**y-axis:** optical density at 490 nm ($OD_{490}$) **x-axis:** serum dilution x 100, **dashed line (- - -)** : serum collected before vaccination (week 0), **continuous line (——) :** serum collected after third vaccination (week 10), **blue line:** rBac-1-Cul n 4, **red line:** rCul n 4. One experiment.

In general there is less difference between the total IgG response of vaccinated horses to rCul n 4 and rBac-1-Cul n 4 than was for the Cul n 3 proteins and horse SE A/M-2 responds almost the same to both. For SE A/M- 3, 7, 8 and 9 there is most difference between the response to the proteins in serum dilution 1/1600 where the response to rCul n 4 is higher ranging from 2.2 to 3.5 but from 1.3 to

2.7 for rBac-1-Cul n 4. The most difference is in dilution 1/3200 for SE/AM-1 with the values 3.4 and 2.1. In serum collected pre-vaccination, the background is low and similar between rCul n 4 and rBac-1-Cul n 4 for SE A/M- 2 and 9. Sera from horses SE A/M-1, 3 and 7 show background in serum dilutions 1/200, 1/400 and 1/800 for rCul n 4 ($OD_{490}$ 1.0 – 1.2 in serum dilution 1/200) whereas much less and only in dilution 1/200 for rBac-1-Cul n 4 ($OD_{490}$ 0.3 – 0.7). SE A/M-8 has just background in serum dilution 1/200 for rCul n 4 ($OD_{490}$ 0.4) (Figure 40).

In order to examine if uncleaved rBac-SUMO-Cul n 4 could be used in ELISA it was compared to rBac-1-Cul n 4.

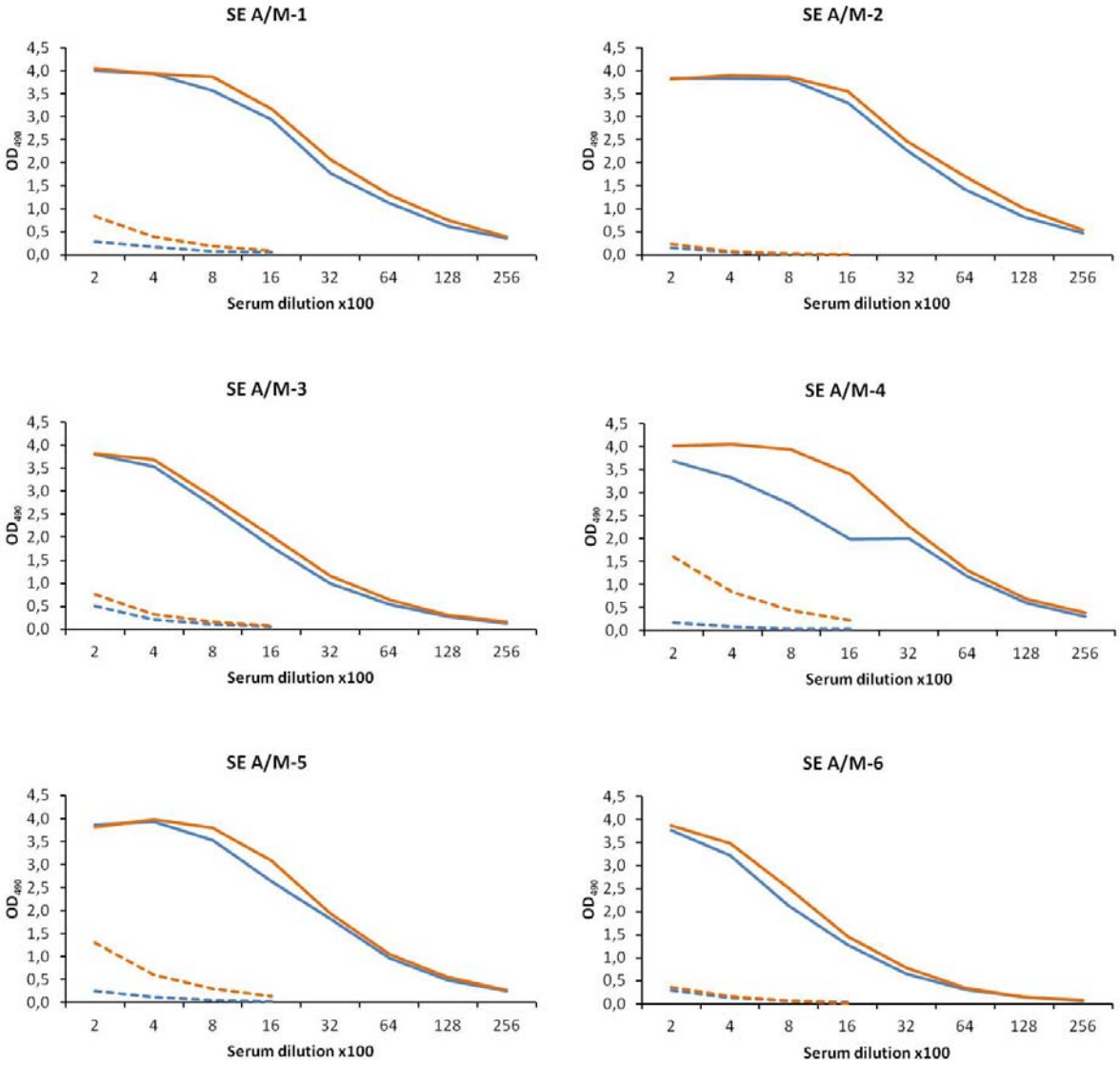


**Figure 41. Comparison of rBac-1-Cul n 4 and rBac-SUMO-Cul n 4 in ELISA.**
Total IgG response of vaccinated horses. **y-axis:** optical density at 490 nm ($OD_{490}$) **x-axis:** serum dilution x 100, **dashed line (- - -)** : serum collected before vaccination (week 0), **continuous line (–––) :** serum collected after third vaccination (week 10), **blue line:** rBac-1-Cul n 4, **orange line:** rBac-SUMO-Cul n 4. One experiment.

Figure 41 shows that the total IgG response of six vaccinated horses to rBac-1-Cul n 4 and rBac-SUMO-Cul n 4 is similar in all serum dilutions except SE A/M-4 where the response to rBac-SUMO-

Cul n 4 is higher in 1/200 - 1/1600 serum dilutions. The response on both proteins in the serum dilution 1/200 had $OD_{490}$ values ranging from 3.7 to 4.0. In pre-vaccination sera the background response is negligible and the same for both proteins in horses SE A/M-2 and 6. The other four have slight background on rBac-1-Cul n 4 in dilution 1/200 ($OD_{490}$ 0.2 – 0.5) but higher values on rBac-SUMO-Cul n 4 both in dilution 1/200 ($OD_{490}$ 0.4 – 1.6) and 1/400 ($OD_{490}$ 0.3 – 0.8) and even two of them in 1/800 ($OD_{490}$ 0.3 – 0.4)

### 4.6.3 IgE response of IBH affected horses to purified rBac-allergens measured in ELISA

rBac-HBM-Cul o 2 (D7-related salivary protein) purified under native conditions and rBac-HBM-Cul n 1 and rBac-HBM-Cul o 1 purified under denaturing conditions were sent to The University of Bern, Switzerland, Department of Clinical Research and Veterinary Public Health. The r-allergens were tested in ELISA measuring IgE in IBH affected horses (Schaffartzik et al., 2011) and compared to r-allergen produced in *E.coli*.

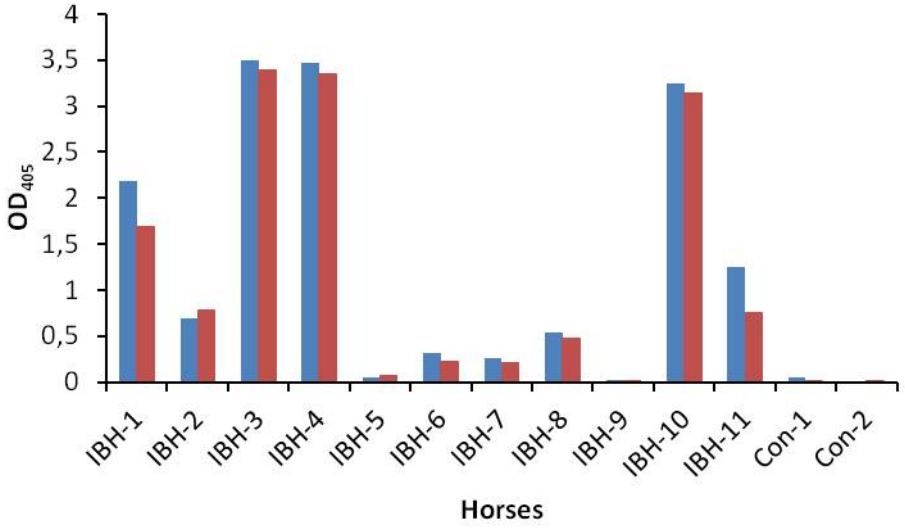


**Figure 42. IgE in serum of IBH affected horses with comparison of rCul o 2 and rBac-HBM-Cul o 2 by ELISA.**
Serum dilution 1:5. **y-axis:** optical density at 405 nm ($OD_{405}$), **x-axis:** IBH: IBH affected horse, Con: Control horse, **blue column:** rBac-HBM-Cul o 2 purified from insect cells, **red column:** rCul o 2 purified from *E. coli*. One experiment.

Six of the IBH affected horses, IBH-1, 2, 3, 4, 10 and 11, have strong IgE response to Cul o 2, especially IBH-3 and 4 with $OD_{405}$ values 3.4 for rCul o 2 and 3.5 for rBac-HBM-Cul o 2. IBH-6, 7 and 8 are low ($OD_{405}$ values 0.2 - 0.5 for rCul o 2 and 0.3 – 0.5 for rBac-HBM-Cul o 2) and IBH-5 and 9 are very low or negative. The response of IBH-1 and 11 is slightly higher on rBac-HBM-Cul o 2 ($OD_{405}$ values 2.2 and 1.3) than on the rCul o 2 ($OD_{405}$ values 1.7 and 0.8) but similar in the other horses. The values were lower than $OD_{405}$ 0.04 in both control horses (Figure 42).

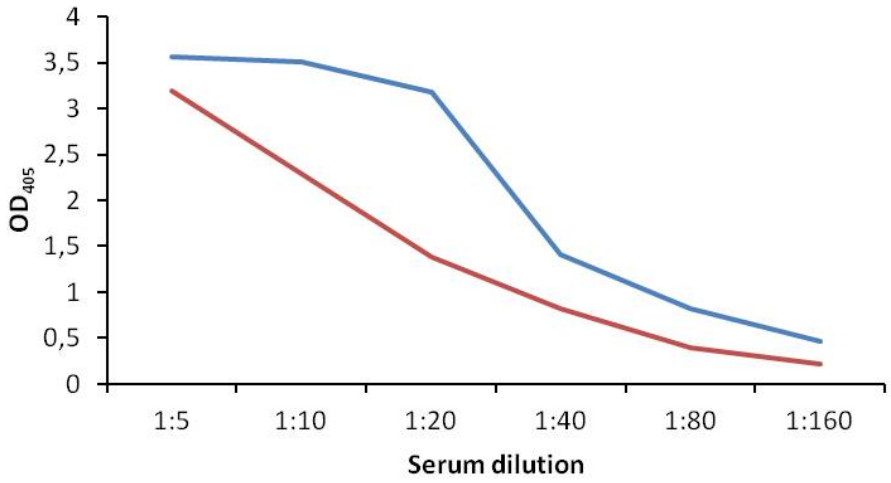


**Figure 43. IgE response of IBH-1 to rBac-HBM-Cul n 1 purified under denaturing conditions compared to rCul n 1 measured by ELISA.**
**y-axis:** optical density at 405 nm ($OD_{405}$), **x-axis:** serum dilution, **blue line:** rBac-HBM-Cul n 1 purified under denaturing conditions, **red line:** rCul n 1. One experiment.

The IgE response of an IBH affected horse (IBH-1) to Cul n 1 (Antigen 5 like protein) produced in insect cells is higher than to Cul n 1 produced in *E. coli* in all serum dilutions. In dilution 1/5 the $OD_{405}$ values are 3.6 for rBac-HBM-Cul n 1 and 3.2 for rCul n 1. There is most difference in dilution 1:20, $OD_{405}$ 3.2 for rBac-HBM-Cul n 1 and 1.3 for rCul n 3 (Figure 43).

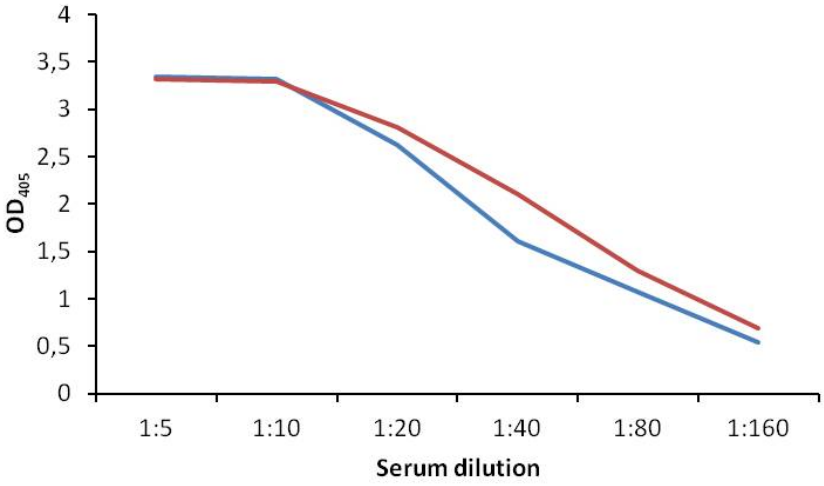


**Figure 44. IgE response of IBH-1 to rBac-HBM-Cul o 1 purified under denaturing conditions compared rCul o 1 measured by ELISA.**
**y-axis:** optical density at 405 nm ($OD_{405}$), **x-axis:** serum dilution, **blue line:** rBac-HBM-Cul o 1 purified under denaturing conditions, **red line:** rCul o 1. One experiment.

The IgE response to Cul o 1 (Kunitz protease inhibitor) was on the other hand similar to both proteins $OD_{405}$ value 3.3 in serum dilution 1/5 and slightly different at 1/40 dilution, 2.1 for rCul o 1 and 1.6 for rBac-HBM-Cul o 1 (Figure 44).

# 5 Discussion

Insect bite hypersensitivity (IBH) is a recurrent seasonal skin disease of the horse which affects all horse breeds, but Icelandic horses exported to *Culicoides* infested areas are more sensitive than Icelandic horses born on the European continent. Two years or more after export roughly 30% of them get IBH if not protected against the flies in heavily *Culicoides* infested areas (Bjornsdottir et al., 2006). In Iceland there is only one horse breed that have been pure bred since it was brought to the country at the time of the Viking settlement. It is unique for its temperance and diligence as well as its five gaits. In the mid 19$^{th}$ century the Icelandic horse was exported to Europe as a working horse, but the export decreased as machines replaced horses (Björnsson, 2006). Export of horses increased again in the mid 20$^{th}$ century as Icelandic horses became popular as riding horses, especially in Scandinavia and Western Europe and the export has increased greatly in the last years. Between 1300-2000 horses were exported anually in the period 1999-2009 (Möller, 2009) and in the year 2014 the number of exported horses was 1269 (Eiðfaxi, 2015). IBH is an animal welfare issue as it causes major discomfort for the horses and the only available treatment is to keep the horses indoors or use blankets to avoid contact with the midges.

From the year 2000 there has been an ongoing collaborative research project on IBH between Keldur and the Department of Clinical Research and Veterinary Public Health, University of Bern, Switzerland. The main objectives of the project are to find and characterize the allergens causing the disease, analyze the immune response that leads to IBH in horses and to develop an efficient immunotherapy.

Thirteen salivary gland proteins of *Culicoides* spp. have been identified as allergens from *C. sonorensis, C. nubeculosus* and *C. obsoletus* cloned and expressed in *E. coli* and purified. Seven of the allergens from *C. nubeculosus* have been expressed in insect cells (Arnesen, 2013; Björnsdóttir, 2008; Jónsdóttir, 2011; Sigurðardóttir, 2011), two from *C. obsoletus* (Foss, 2013; Úlfarsdóttir, 2014). The pathogenesis of the disease has been evaluated both peripheral and in the skin and results indicate that there is an imbalance between $T_H1$, $T_H2$ and $T_{reg}$ cells. Development of immunotherapy is ongoing by three approaches; with purified recombinant allergens in adjuvants, with allergen genes on a viral vector and treatment via the mouth mucosa by feeding horses with barley expressing recombinant allergens.

The main objectives of immunotherapy are to generate allergen-specific $T_{reg}$ and/or $T_H1$ cells, inhibit $T_H2$ responses, decrease allergen-specific IgE, increase allergen-specific IgG and IgA and reduce infiltrating inflammatory cells. It is necessary to be able to measure these immune parameters to evaluate the immunotherapy.

Purification of overexpressed proteins from *E. coli*, leaving no contamination of LPS or *E. coli*, proteins is very difficult (Petsch & Anspach, 2000; Sharma, 1986). LPS is a mitogen that unspecifically stimulates lymphocytes and all horses have immune responses against *E. coli* proteins. Therefore it is essential to have the allergens purified from another system than the proteins used for vaccination. Besides, proteins expressed in bacteria do not have post-translational modifications such as

glycosylation and often have wrong conformation, that can be an important factor for the biological activity of proteins and immune response (Demain & Vaishnav, 2009; Soldatova et al., 1998).

As the IBH allergens are originated from the salivary gland of an insect, major allergens like Cul n 1 - 4 were expressed in insect cells using the Bac-to-Bac protein expression system (Arnesen, 2013; Björnsdóttir, 2008; Jónsdóttir, 2011). However, they had not been successfully purified from insect cells except for Cul n 3 (Figure 55 in appendix VII).

Cul n 4 a secreted salivary protein was expressed in insect cells with different approaches to find a way to ease the purification. It was expressed in the Bac-to-Bac protein expression system using three different vectors, at full length in pFastBac™ 1 and without its own secretion signal in pFastBac™/HBM-TOPO® and pI-secSUMOstar. The allergen was successfully expressed in Sf-9 insect cells and secreted into the supernatant following all approaches, the highest amounts from the SUMO fused Cul n 4 (Figure 4 and 48, 49 in appendix V). All rBac-Cul n 4 proteins could be purified under native conditions after production in High-five cells (Figure 17, 18 and 19). In the purification of rBac-1-Cul n 4 and rBac-HBM-Cul n 4 the purified protein was seen as a double band at 15 kDa. The smaller band was stronger in the elutions but the bigger band was bound to the nickel gel after elutions (Figure 17 and 18). There was some contamination of unspecific proteins seen in the coomassie blue staining that could be minimized by dialysis (Figure 20). Purified rBac-SUMO-Cul n 4 was seen as double band in the WB but a single band in the coomassie blue staining. The detection capacity of coomassie blue staining is 2.5 ng (Wong et al., 2000), indicating that the concentration of the bigger band is less than 2.5 ng.The double band could be explained as degradation, different isoforms or different post-translation modification of the target protein.

An attempt was also made to purify the proteins from supernatant. High yields were obtained in purification of rBac-SUMO-Cul n 4 (Figure 23) but not from purification of rBac-1-Cul 4 and rBac-HBM-Cul n 4 (Figure 22). The pI-secSUMOstar is designed to enhance expression of protein and promote solubility and correct folding. SUMO has a very stable structure and fused to the N-terminus of protein increases the yield by increasing stability (Liu et al., 2008). The vector contains a secretion signal sequence from the envelope surface glycoprotein 67 (gp67) derived from baculovirus (*Autographa californica*) (Figure 7) (LifeSensors). To obtain Cul n 4 without SUMOstar fusion protein, the rBac-SUMO-Cul n 4 was cleaved with specific SUMOstar protease (Figure 21). The cleavage was successful but required both more time and more protease then manufacturer‘s protocaol indicated. After cleavage, the SUMOstar and SUMOstar protease can be removed with nickel affinity gel, but this was not pursued because Cul n 4 was easily purified as rBac-1-Cul n 4. All rBac-Cul n 4 forms were expressed and produced in insect cells. There was more production in the cells and secretion into the supernatant of Cul n 4 fused with SUMO than rBac-1-Cul n 4 and rBac-HBM-Cul n 4.

Hyalurinodase is an important allergen for humans in hymenoptera stings (Arlian, 2002; Hoffman, 2006; King & Spangfort, 2000). In IBH it is also a major allergen and has been isolated from *C. nubeculosus* and *C. obsoletus* (Schaffartzik et al., 2011; van der Meide et al., 2013). Cul n 2 has been used in several vaccination experiments at Keldur so it is important to have it available for immunoassays. Cul n 2 had been expressed in full length using the pFastBac™ HT B vector at Keldur but could not be purified under native conditions (Jónsdóttir, 2011). The allergen was expressed with

HBM secretion signal at full length (Figure 46 in appendix V). rBac-HBM-Cul n 2 could not either be purified under native conditions on nickel affinity gel and the coomassie blue staining indicated that the 6xhis-tag did not bind to the gel (Figure 24). The reason could be that the 6xhis-tag was folded into the protein. Therefore, attempt was made to purify it under native conditions with cation exchange column by FPLC. The purification was tested in dot blot which indicated that the protein was washed out with the flow through (Figure 26). The protein did not bind to a column coupled with specific Cul n 2 mAb, but the column was old which could be the explanation. The purification was successful with anion exchange column (Figure 30), however the purified proteins rBac-HBM-Cul n 2 and rBac-HTB-Cul n 2 were unstable in storage.

The rBac-HBM-Cul n 2 could be purified under denaturing conditions (Figure 32) supporting the theory that the 6xhis-tag is hidden in the tertiary structure of the protein. Antibodies bind either to the conformational shapes on the surfaces of antigens or to peptide fragments, IgE frequently recognizes tertiary structures (Murphy et al., 2012c). Therefore, for the use of the allergens in some immunoassays it is important to be able to purify the proteins under native conditions. The rBac-HBM-Cul n 2 purified under denaturing conditions was dialyzed to remove the denaturing agent, urea in attempt for refolding. However, the protein precipitated with removal of urea.

Since rBac-HBM-Cul n 2 could not be refolded from urea, the allergen was expressed in insect cells using SUMOstar vector that gave good results with Cul n 4, both at full length and without its own secretion signal sequence. The SUMO fused Cul n 2 protein was 64 kDa in full length and 61 kDa without its own secretion signal sequence (Figure 47 in appendix V). rBac-SUMO-Cul n 2 did not bind to the affinity gel and purification was not tried further. On the other hand rBac-SUMO-wo-Cul n 2 was successfully purified under native conditions (Figure 34) but it precipitated in dialysis.

There were also difficulties with Cul n 2 expression in *E. coli*, where it was finally expressed truncated without part of the N-terminus (Schaffartzik et al., 2011). One approach could be to express Cul n 2 truncated in insect cells, if the N-terminus of the protein is causing the unstable product.

Antigen 5 like proteins are common allergens in vespid venom allergy (Hoffman, 2006) and have been isolated from *Simulium vittatum* (Sim v 1), *C. nubeculosus* (Cul n 1) (Schaffartzik et al., 2010) and *C. obsoletus* (Cul o 3) (unpublished). Cul n 1 had previously been expressed in pFastBac™/HBM-TOPO® without its own secretion signal sequence (Arnesen, 2013) and could only be purified under denaturing conditions (Figure 53 in appendix VII). Hence, Cul n 1 and Cul o 3 were expressed without their own secretion signal sequences as SUMO fusion proteins. rBac-SUMO-Cul n 1 was approximately 37 kDa double band (Figure 45 in appendix V) but rBac-SUMO-Cul o 3 41 kDa (Figure 50 in appendix V). Both were purified under native conditions (Figure 54 and 58 in appendix VII) but precipitated during dialysis. Therefore Cul o 3 fused with SUMO was purified under denaturing conditions (data not shown).

It is important to express the major allergens of IBH not only from *C. nubeculosus* but also *C. obsoletus* which is the most common *Culicoides* species in Europe (Bates, 2012; Carpenter et al., 2013). Despite that *C. nubeculosus* is quite rare, 80% of IBH affected horses in Switzerland react with *C. nubeculosus* extract (Baselgia et al., 2006) and the allergens Cul n 1 - 4 expressed in *E. coli* bind IgE from more than 35% of IBH affected horses (Schaffartzik et al., 2011). Cross-rectivity is therefore

common between *Culicoides* species but it cannot be excluded that species specific allergens exist in the native species of the area.

Two other allergens from *C.obsoletus* had been expressed in insect cells with pFastBac™/HBM-TOPO® vector without their own secretion signal sequence, Cul o 1 a Kunitz protease inhibitor (Foss, 2013) and Cul o 2 a D7-related salivary protein (Úlfarsdóttir, 2014). rBac-HBM-Cul o 1 could only be purified under denaturing conditions but rBac-HBM-Cul o 2 was easily purified under native conditions (Figure 56 in appendix VII) with nickel affinity gel (Figure 57 in appendix VII) and dialyzed.

Glycosylation of Cul n 3, Cul n 4 and Cul o 2 purified under native conditions was tested by treatment with PNGase F which cleaves N-linked glycans but there was no effect on any of them (Figure 35) which could indicate that they are not glycoproteins or that the glycans are O-linked, but this has to be tested further.

Of the seven allergens in this study, three were easily purified under native conditions (Cul n 3, Cul n 4 and Cul o 2) but the other four could only be purified in denatured form (Cul n 1, Cul n 2, Cul o 1 and Cul o 3). The SUMO fusion made the purification of the allergens with nickel affinity gel easier but they were anyhow lost during dialysis indicating that they are not stable. Therefore it was important to test whether uncleaved SUMO fusion proteins and/or denatured rBac proteins from urea could be used in immunoassays.

Experimental vaccinations are being tried at Keldur using r-allergens purified from *E. coli* in different adjuvants (Jonsdottir et al., Manuscript accepted; Jonsdottir et al., Manuscript) Immunoassays with rBac-allergens were set up using serum and isolated PBMC from these horses. *In vitro* stimulation of PBMC for cytokine production was only tried once on four vaccinated horses and two controls. These preliminary results were promising. If 5 μg/mL is considered to be optimal protein concentration for stimulation, the controls were very low or negative, whereas the vaccinated horses responded in most cases. Three of the horses responded with IFN-γ and all with IL-10 except one to Cul n 4. Indicating a successful $T_H1$, $T_{reg}$ focus, but two of them also had IL-4 production (Figure 36, 37 and 38). However, this has to be repeated more carefully in additional horses. Stimulation of PBMC neither with denature purified proteins in urea nor with uncleaved SUMOstar proteins was concluded and has to be tested further. Undialyzed proteins with 250 mM imidazole could not be used for stimulation as imidazole is toxic.

ELISA measuring total IgG in serum was set up for the native form purified rBac-HBM-Cul n 3 and rBac-1-Cul n 4 and compared to the corresponding proteins purified from *E. coli*. The vaccinated horses responded efficiently to the rBac proteins although most of the horses less than to the *E. coli* purified counterpart. This was to be expected as the horses were vaccinated with the *E. coli* purified proteins. Background was most often lower with the rBac proteins (Figure 39 and 40). These r-allergens along with rBac-HBM-Cul o 2 and denature purified rBac-HBM-Cul n 1 and rBac-HBM-Cul o 1 were sent to the collaborators at The University of Bern for measuring IgE in serum from IBH affected horses in ELISA. All five proteins worked as well or better than the *E. coli* purified proteins (Figure 42, 43 and 44) (Jonsdottir et al., Manuscript).

The SUMO fused Cul n 4 was compared to rBac-1-Cul n 4 in ELISA to see whether the SUMO fusion part could be left intact. Although background response in low serum dilutions was higher in the rBac-SUMO-Cul n 4 it can be used uncleaved for ELISA (Figure 41).

Based on these preliminary stimulation and ELISA results, Cul n 3 and Cul n 4 from three expression systems, *E. coli*, insect cells and barley are being compared for use in immunoassays.

Cul n 2 could be purified under native conditions as SUMO fusion protein but the imidazole could not be removed without the protein precipitating. Attempts were made to use the purified Cul n 2 in imidazole to set up total IgG ELISA, by using the default protocol and by adding 250 mM imidazole to the coating buffer, but neither worked. Cul n 2 purified under denaturing conditions with or without urea in the coating buffer could not either be used in IgG nor IgE ELISA (data not shown).

Five r-Bac-allergens were successfully purified and they used for immunoassays. All were suitable for measuring IgE in serum of IBH affected horses. Two of the r-Bac-allergens were successfully used for stimulating PBMC from vaccinated horses for cytokine production.

# 6 Conclusion

The long term aim of the IBH research project is to develop an immunotherapy against the disease. For that purpose it is necessary to have access to purified proteins in a suitable form for evaluating immunotherapy. For measuring the T cell response the allergens can be in denatured form but need to be stable in non-toxic solution for *in vitro* stimulations of PBMC. On the other hand it can be important to have the allergens with correct post-translational modifications to measure antibody response and obtain accurate information on the allergenicity. Protein purification can be complicated as each protein has its own character, requiring different purification approaches. Expression of allergens as SUMOstar fusion proteins can enhance the expression and ease the purification of proteins by stabilizing them. However, very unstable proteins will tend to precipitate in physiological buffers. Cleavage of the SUMO part is a tedious and expensive extra step so it is important that SUMO fusion proteins can be used uncleaved in ELISA. For cytokine production, proteins expressed in insect cells and purified in native form are well suited to stimulate PBMC of horses vaccinated with allergens purified from *E. coli*. Proteins that can only be purified under denaturing conditions may be used in ELISA if they do not precipitate in the coating buffer and they should also be applicable in stimulations.

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# Appendix I

## Primers used in this work

| **Cul n 1** | **Nucleotide sequence** |
|---|---|
| Culn1_58-78_Fw | 5’ –ACA AAT TTT TGC AAC AAA GAT- 3’ |
| CnAg5 411 Fw | 5’ –GCA TAC ACA ATG GGC ACA CC-3’ |
| Culn1_540-561_Re | 5’ –TAG CAG TAA ATG TCC AAT AAC- 3’ |
| SUMO_Culn1_Fw | 5’ –CGC GTC TCG AGG TAC AAA TTT TTG CAA A- 3’ |
| SUMO_Culn1_Re | 5’ –CGT CTA GAT TAT AGC AGT AAA TGT CC- 3’ |
| **Cul n 2** | |
| Culn2_1-22_Fw | 5’ –ATG TGG TTG AAC GTG GTA AAT G- 3’ |
| Culn2_1186-1206_Re | 5’ –TGA CAA ATT TGG GGT AAG AA- 3’ |
| SUMO_Culn2_1-18_Fw | 5’ –CGC GTC TCG AGG TAT GTG GTT GAA CGT GGT A- 3’ |
| SUMO_Culn2_73-91_Fw | 5’ –CGC GTC TCG AGG TCA ACT GAT ACA AGT GGG A- 3’ |
| SUMO_Culn2_Re | 5’ –CGT CTA GAT TAT GAC AAA TTT GGG GT- 3’ |
| **Cul n 4** | |
| Culn4_67-87_Fw | 5’ –CGT AGG AAG CAT TTC AGA CA- 3’ |
| Culn4_432-456_Re | 5’ –TAA ACT TTC ATT TAA ACA CTC AGC- 3’ |
| FastBac1_Culn4_BamHI_Fw | 5’ –GCG GAT CCG CAT GAA GTT CCC AAC ATT TTT- 3’ |
| FastBac1_Culn4_HindIII_Rev | 5’ –CGA AGC TTG GCT AGT GAT GGT GAT GGT GAT- 3’ |
| SUMOCuln4BsmBIFw | 5’ –CGC GTC TCG AGG TCG TAG GAA GCA TTT CAG ACA- 3’ |
| SUMOCuln4XBaIRe | 5’ –CGT CTA GAT TAT AAA CTT TCA TTT AAA CAC TCA GC- 3’ |
| **Cul o 3** | |
| Culo3_62_Fw | 5’ –GTG ACC GAA AGC TAT GTA G- 3’ |
| Culo3_387_Re | 5’ –CCA AAT TTT GTC CCG CAT AC- 3’ |
| Culo3_415_Fw | 5’ –GGC GGC GGA AAA GAA CCG AAT G- 3’ |
| Culo3_753_Re | 5’ –CTT CAT CCG GAT CGG GAT TGA A- 3’ |
| Culo3_SUMO_Fw | 5’ –CGC GTC TCG AGG TAC AGA TTT TTG TGA CCG A- 3’ |

**Vector primers**

| | |
|---|---|
| FastBac_TOPO_Fw | 5’ –AAA TGA TAA CCA TCT CGC- 3’ |
| FastBac_TOPO_Re | 5’ –GGT ATG GCT GAT TAT GAT C- 3’ |
| SUMO_Fw | 5’ –CAA GCT GAT CAG ACC CCT G- 3’ |
| SUMO_Re | 5’ –CAG GGG GAG GTG TGG GAG G- 3’ |
| SUMO_polH_Fw | 5’ –GGA TTA TTC ATA CCG TCC CAC CAT- 3’ |
| SUMO_Tn7_Re | 5’ –CTG GGT GTA GCG TCG TAA GCT AAT AC- 3’ |
| M13 Forward | 5’ –GTT TTC CCA GTC ACG AC- 3’ |
| M13 Reverse | 5’ –CAG GAA ACA GCT ATG AC- 3’ |

# Appendix II

## Buffers and solutions used in DNA methods

**10x Restriction buffer (RSB)**
50% glycerol, 15 mM EDTA, 0.25% bromophenol blue

**5x Tris borate-EDTA (TBE) buffer**
0.045 M Tris borate, 0.0001 M EDTA

**LB medium**
1% N-Z amine, 0.1% Yeast extract, 1% NaCl

**LB agar**
1% N-Z amine, 0.1% Yeast extract, 1% NaCl, 1,5% Bacto agar

**SOC medium**
2% Tryptone, 0.5% Yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM Glucose

**SOB medium**
2% Tryptone, 0.5% Yeast extract, 0.05% NaCl, 2.5 mM KCl

**HTB buffer**
10 mM HEPES, 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$, pH 6.7

**X-gal**
Bromo-chloro-indolyl-galactopyranoside

**IPTG**
Isopropyl β-D-1-thiogalactopyranoside

## Solutions for cell culture

**Complete Sf-9 medium**
SF-900™II medium (gibco by life technologies) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (PEST) and 1% fetal bovine serum (FBS)

**Complete RPMI-Glutamax medium**
RPMI-1640 Medium GlutaMAX™ (gibco by life technologies) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (PEST), 1% non-essential amino acids (NEA), 100 μM MEM vitamins, Na-pyruvate, 1mM sodium pyruvate, 10% normal horse sera from Keldur and 50 μM 2-mercaptoethanol

**PBS/PEST**
Phosphate buffer saline (PBS) supplemented with 100 IU penicillin, 100 IU streptomycin (PEST)

## Buffers and solutions used in protein methods

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

**SDS-Page running buffer**
25 mM Tris, 192 mM glycine, 0.1% SDS

**Storage solution**
25% methanol, 7% acetic acid, 3% glycerol

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

**BCIP/NTB**
5-bromo-4-chloro-3-indolyl phosphate/ Nitro blue tetrazoliumchloride

**Alkaline phosphatase buffer**
100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 0.005% Tween 20, pH 9.5

## Buffers used in native protein purification

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

**Wash buffer, pH 8**
50 mM $NaH_2PO_4xH_2O$, 300 mM NaCl, 0, 10, 20 or 30 mM Imidazol

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

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

**Buffer F**
6M Guanidin-HCl, 0,2M acetic acid

## Buffers used in denature protein purification

**Denature lysis buffer, pH 8**
6M Guanidin-HCl, 100 mM $NaH_2PO_4xH_2O$

**Denature wash buffer 1, pH 8**
8M Urea, 100 mM $NaH_2PO_4xH_2O$, 10 mM Tris-base, 100 mM $NH_4Cl$

**Denature wash buffer 2, pH 6.3**
8M Urea, 100 mM $NaH_2PO_4xH_2O$, 10 mM Tris-base, 100 mM $NH_4Cl$

**Denature elution buffer 1, pH 4.4**
8M Urea, 100 mM $NaH_2PO_4xH_2O$, 10 mM Tris-base, 100 mM $NH_4Cl$

**Denature elution buffer 2, pH 4.0**
8M Urea, 100 mM $NaH_2PO_4xH_2O$, 10 mM Tris-base, 100 mM $NH_4Cl$

## Buffers used in protein purification with FPLC

**FPLC lysis buffer, pH 7.4**
50 mM $NaH_2PO_4xH_2O$, 150 mM NaCl

**Buffer A, pH 7.4, filtered and degassed with 0.22 μm filter**
50 mM $KH_2PO_4$, 100mM NaCl, 1mM EDTA, 0.01% Azide

**Buffer B, pH 7.4, filtered and degassed with 0.22 μm filter**
50 mM $KH_2PO_4$, 1 M NaCl, 1mM EDTA, 0.01% Azide

**NHS buffer A, pH 7, filtered and degassed with 0.22 μm filter**
Phosphate buffer saline (PBS), 0.01% Azide

**NHS buffer B, pH 2.3, filtered and degassed with 0.22 μm filter**
0.1 M glycine

**NHS neutralizing buffer, filtered and degassed with 0.22 μm filter**
1 M Tris

## Buffers used in ELISA

**ELISA Wash buffer, pH 7.2**
500 mM NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, 6.5 mM $Na_2PO_4x2H_2O$, 0.05% Tween 20

**Dilution buffer, pH 7.2**
500 mM NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, 6.5 mM $Na_2PO_4x2H_2O$, 0.05% Tween 20, 1% BSA, 0.001% Phenol red

**OPD substrat, pH 5.0**
4 tablets OPD (orthiophenylenediamine) (DAKO), 0.00042% $H_2O_2$, $H_2O$ up to 12 mL

# Appendix III

## Native protein purification with nickel affinity gel – wash steps for different proteins

**Table 8. Imidazole concentration in wash buffer used for protein purification under native conditions.**

| Protein | Imidazol concentration in wash buffer (mM) | |
|---|---|---|
| | First wash | Second wash |
| **rBac-HBM-Cul n 1** | 0 mM | 10 MM |
| **rBac-SUMO-Cul n 1** | 10 mM | 20 mM |
| **rBac-HBM-Cul n 2** | 20 mM | 30 mM |
| **rBac-SUMO-Cul n 2** | 0 mM | 10 mM |
| **rBac-SUMO-wo-Cul n 2** | 20 mM | 30 mM |
| **rBac-HBM-Cul n 4** | 20 mM | 30 mM |
| **rBac-1-Cul n 4** | 20 mM | 30 mM |
| **rBac-SUMO-Cul n 4** | 20 mM | 30 mM |
| **rBac-HBM-Cul n 3** | 10 mM | 20 mM |
| **rBac-HBM-Cul o 1** | 10 mM | 20 mM |
| **rBac-HBM-Cul o 2** | 10 mM | 20 mM |
| **rBac-SUMO-Cul o 3** | 10 mM | 20 mM |

# Appendix IV

## Nucleotide and amino acid sequences used in the Baculovirus expression system

The secretion signal sequence of the proteins is marked as red.

**Cul n 1 (Antigen 5 like protein)**

**564 bp**

**ATG**ATTAAAAAACTTTCGATTGTAATATTATTTTCTTGTATAAGTTTTGTTCTATCGACAAATTTTTG
CAACAAAGATTTATGCAAAAGACAAAATGGACCGCAGTCTTTTACATATTTAAAACACATTGGATGTC
GTCATACTGGTAAAAATGCTAACACCTGTCCACGCGATGCAAAAATCCTGCCAATGTCAACTAAACGT
AAAAATTTGATTCTTAAAGTACATAATCGATTGCGTAACAAAGTAGCTCTTGGAAAATTACCTGGATT
CCCAAAAGCAGCTCGTATGCCTATTTTACGTTGGGACGATGAATTAGCTTATTTGGCTGAACTTAATG
TGAAACAGTGCAAAATGGAGCATGATCAATGTCGTAATACAGACAAATTTAAATATGCAGGTCAGAAT
TTAGCATACACAATGGGCACACCTCAAAAAAATGCAGTTCGAATTAAAAAGCTGATCCGAGCGTGGTT
TAAGGAGCATGAAAATGCAACGGCATCATTTATTGATAAATATCGGGATCATCCTCAAGGTCGCGTTA
TTGGACATTTACTGCTA**TGA**

**187 a.a.**

M I K K L S I V I L F S C I S F V L S T N F C N K D L C K R Q N G P
Q S F T Y L K H I G C R H T G K N A N T C P R D A K I L P M S T K R
K N L I L K V H N R L R N K V A L G K L P G F P K A A R M P I L R W
D D E L A Y L A E L N V K Q C K M E H D Q C R N T D K F K Y A G Q N
L A Y T M G T P Q K N A V R I K K L I R A W F K E H E N A T A S F I
D K Y R D H P Q G R V I G H L L L **Stop**

**Cul n 2 (Hyaluronidase)**

**1209 bp**

**ATG**TGGTTGAACGTGGTAAATGTCTCACAATTTATGACAGCATGGGCGACCTTTAATTTGATTAATGCACAACAA
CTGATACAAGTGGGACCAGAAAATGTACCATATGAAATTGTAGATGCAAAGGACGATGCATCTGAAAGCAGAGGA
ATATTTTTTAATAACTTCATCACATCAAAAAATAACGATGATAAAAGACATGATTTTACCTTTTACTGGAACATC
CCATCATTTATGTGTTCAAAATACAATGTAACATTCACTGACATGCCTTCATCATATAATATCGTACAAAATAAA
GATGATAAATGGCGTGGTGACCAGATCATAATTTTATATGATCCTGGTAAATTTCCGGCTTTATTAGAGCATCAA
GGAAAATTATATAGACGAAATGGTGGTGTACCACAAGAAGGGAATTTACAAGAACACATCGATTATTTTGCTGAA
AGTGTTAATACCTTGATACCAGATCAAAATTTCTCAGGCATTGGAGTGATTGATCTTGAGTCATGGCGACCGATT
TATCGTCAAAATTCAGGTGTGTTACAGCCATATAAGGATTTATCATATAAATTGGTGCAAAGAAAGCATAGACTA
TGGAGCAAGAAGTTAATTGAAGAAGAGGCAGCTCGTGAATTTGAGACAGCTGGTCGAACATTTGTAGAAGAAACG
GTTAGAGTTGCAAAATATTTACGTCCAAATGCAAAATGGGGCTATTATGGATTCCCGTATTGTTTCAATATGAAT
GGTGGTGCAAATATGAAAGAGGATTGTCCATCTAATGTTAAAGAGGAAAATAATCGTATTAAATGGCTGTGGGAT
ATTGTCGATGTGGTTTTGCCTTCAGTTTATTTGAACAACAAAATAACAGCATCACAAAGAGTCCAATTTGTTCGT
GGGCGAATGCGTGAAGGATGTCGTGTGTCACAATTATCAAAACAACCAGTGAAACCACCAGTATACAGTTATTTG
CGTTATGTTTACACGGACAACCTAAAATACATTTCAAATGAGGATCTCAAACAATCAATTAAAGTACCCAAAGAG
CAAAAGGGTAGTGGATTAATATTTTGGGGCAGTTCATATGATGTCAAAACGAAAGATCAGTGTTTTGATTTTAGA
AATTATGTTGATAATAATTTAGGACCAATTGTACTATCAGCAAATGACAATACACCAAAAATTCTTACCCCAAAT
TTGTCA**TAA**

**402 a.a.**

**M** W L N V V N V S Q F M T A W A T F N L I N A Q Q L I Q V G P E N V P Y E I
V D A K D D A S E S R G I F F N N F I T S K N N D D K R H D F T F Y W N I P
S F M C S K Y N V T F T D M P S S Y N I V Q N K D D K W R G D Q I I I L Y D
P G K F P A L L E H Q G K L Y R R N G G V P Q E G N L Q E H I D Y F A E S V
N T L I P D Q N F S G I G V I D L E S W R P I Y R Q N S G V L Q P Y K D L S
Y K L V Q R K H R L W S K K L I E E E A A R E F E T A G R T F V E E T V R V
A K Y L R P N A K W G Y Y G F P Y C F N M N G G A N M K E D C P S N V K E E
N N R I K W L W D I V D V V L P S V Y L N N K I T A S Q R V Q F V R G R M R
E G C R V S Q L S K Q P V K P P V Y S Y L R Y V Y T D N L K Y I S N E D L K
Q S I K V P K E Q K G S G L I F W G S S Y D V K T K D Q C F D F R N Y V D N
N L G P I V L S A N D N T P K I L T P N L S **Stop**

**Cul n 4**

**459 bp**

**ATG**AAGTTCCCAACATTTTTAATACTAGCATTTTTCCTCTCGCTTTACATTAGCTCAACAGCTAGCCG
TAGGAAGCATTTCAGACATTTAAAGCGAATTGAAGCAGCCAACGATTGTCCAGCTAAAAATTCTGGAA
CATATCAAAAAGTATGCAAACAACTCCAAAAATATTATGTCCTTACACCAGATGACAAATTAGGCAGT
TACTTGAAAGGTGGATTACAAGAAGCAGCTAATCGTGTTTTGACGCCTGTTTCAAAATCGGACAAAAT
TACATTTGATATCGTTCAAAATTGTTTAAAGAACTTCCAGGTTATGGTTAACAAGCACAATAAGGAGG
CTCTTAGGAAGTATCGCGAATGCAAGAAGGAATGCTTCACTGAAGTTGGAAAGGAGTTTTCAAGTGCT
CTGGACAAAACAGGTGTTCAAATTGCTGAGTGTTTAAATGAAAGTTTA**TAA**

**152 a.a.**

**M** K F P T F L I L A F F L S L Y I S S T A S R R K H F R H L K R I E
A A N D C P A K N S G T Y Q K V C K Q L Q K Y Y V L T P D D K L G S
Y L K G G L Q E A A N R V L T P V S K S D K I T F D I V Q N C L K N
F Q V M V N K H N K E A L R K Y R E C K K E C F T E V G K E F S S A
L D K T G V Q I A E C L N E S L **Stop**

**Cul o 3 (Antigen 5 like protein)**

**786 bp**

**ATG**TTTCGTATTTGTTTATTTACGGTATTATGTGTGAACTTTGTTGTTGCAACAGATTTTTGTGACCG
AAAGCTATGTAGAAGACAAATTGAGCCAAATGTGTATCAAAATATTCCACACATTGGTTGCAATCATG
ATGGAAGAAATAGTCCCGCATGCCCATCTGACGCCAAAATCCTCCCAATGCCAACTAAACGCAAAAAT
TTAATCCTCCGTGTGCATAATCGCCTGCGGAATAAAGTGGCGCTCGGTCAATTGCCAGGATATCCAAA
AGCCGTGCGAATGCCAATTTTACGGTGGGACGACGAGTTGGCTTACTTGGCGGAATTGAATGTCAAGC
AGTGCGAAATGAAACACGACCAATGTCGTAATACGGATAAATTCAAGTATGCGGGACAAAATTTGGCG
TACATTGGCGGCGGAAAAGAACCGAATGCCGTACGGATTAAAACTCTAGTCCGAGCGTGGTTTGATGA
GTATAAAGATGCAAACTCTTCCTTCATTGATAAGTATCGGAGTCATCCAAATGGAAAAGCCATTGGAC
ACTTTACGGCGATGGTCCAAGATCGCACGGATACCGTTGGATGTGCCATTTTACGTCATACAAAAAAT
ACGTATTTCTTCCTCGCTTGCAACTATTCCTTCACGAATATGGTTAAGGATAATGTTTATACGAGAGG
CGCGAAATCTTGCAGTAAATGCCGCACTGGATGCAGTCCCGTCTACAAGGGCCTGTGCAAGCCTCACG
AGTATGTCAATCCCGATCCGGATGAAGATTTAGAT**TAA**

**261 a.a**

**M** F R I C L F T V L C V N F V V A T D F C D R K L C R R Q I E P N V
Y Q N I P H I G C N H D G R N S P A C P S D A K I L P M P T K R K N
L I L R V H N R L R N K V A L G Q L P G Y P K A V R M P I L R W D D
E L A Y L A E L N V K Q C E M K H D Q C R N T D K F K Y A G Q N L A
Y I G G G K E P N A V R I K T L V R A W F D E Y K D A N S S F I D K
Y R S H P N G K A I G H F T A M V Q D R T D T V G C A I L R H T K N
T Y F F L A C N Y S F T N M V K D N V Y T R G A K S C S K C R T G C
S P V Y K G L C K P H E Y V N P D P D E D L D **Stop**

# Appendix V

## Expression of recombinant allergens in Sf-9 insect cells and cloning

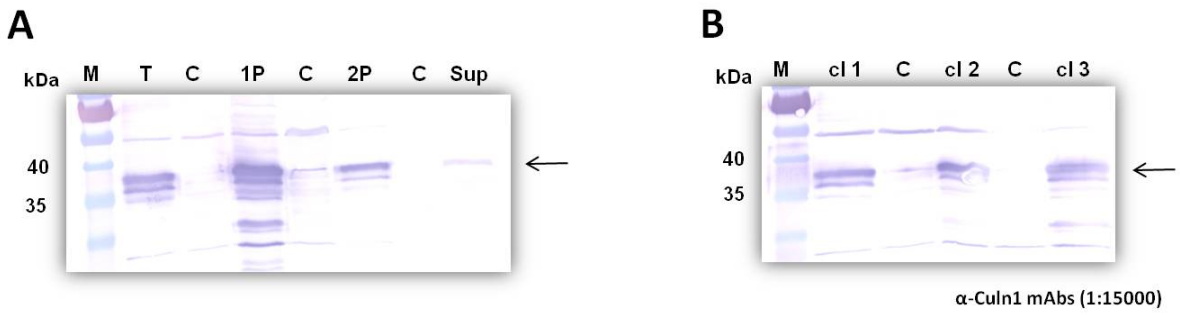


**Figure 45. WB showing the expression of rBac-SUMO-Cul n 1 in Sf-9 cells**
**A** rBac-SUMO-Cul n 1 after transfection and passages, **lane M:** marker, **T:** transfection, **C:** negative control **1P:** first passage, **2P:** second passage, **Sup:** supernatant.
**B** r-baculovirus clones positive for rBac-SUMO-Cul n 1, **lane M:** marker, **cl 1:** clone 1, **C:** negative control, **cl 2:** clone 2, **cl 3:** clone 3.
The arrows indicate rBac-SUMO-Cul n 1.

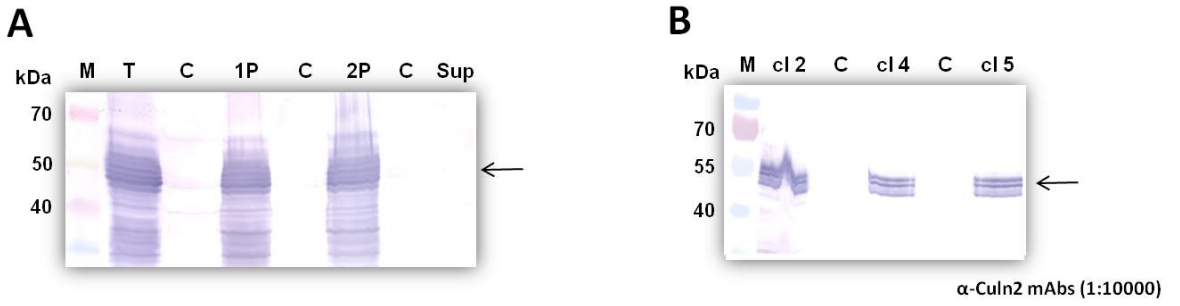


**Figure 46. WB showing the expression of rBac-HBM-Cul n 2 in Sf-9 cells**
**A** rBac-HBM-Cul n 2 after transfection and passages, **lane M:** marker, **T:** transfection, **C:** negative control **1P:** first passage, **2P:** second passage, **Sup:** supernatant.
**B** r-baculovirus clones positive for rBac-HBM-Cul n 2, **lane M:** marker, **cl 2:** clone 2, **C:** negative control, **cl 4:** clone 4, **cl 5:** clone 5.
The arrows indicate rBac-HBM-Cul n 2.

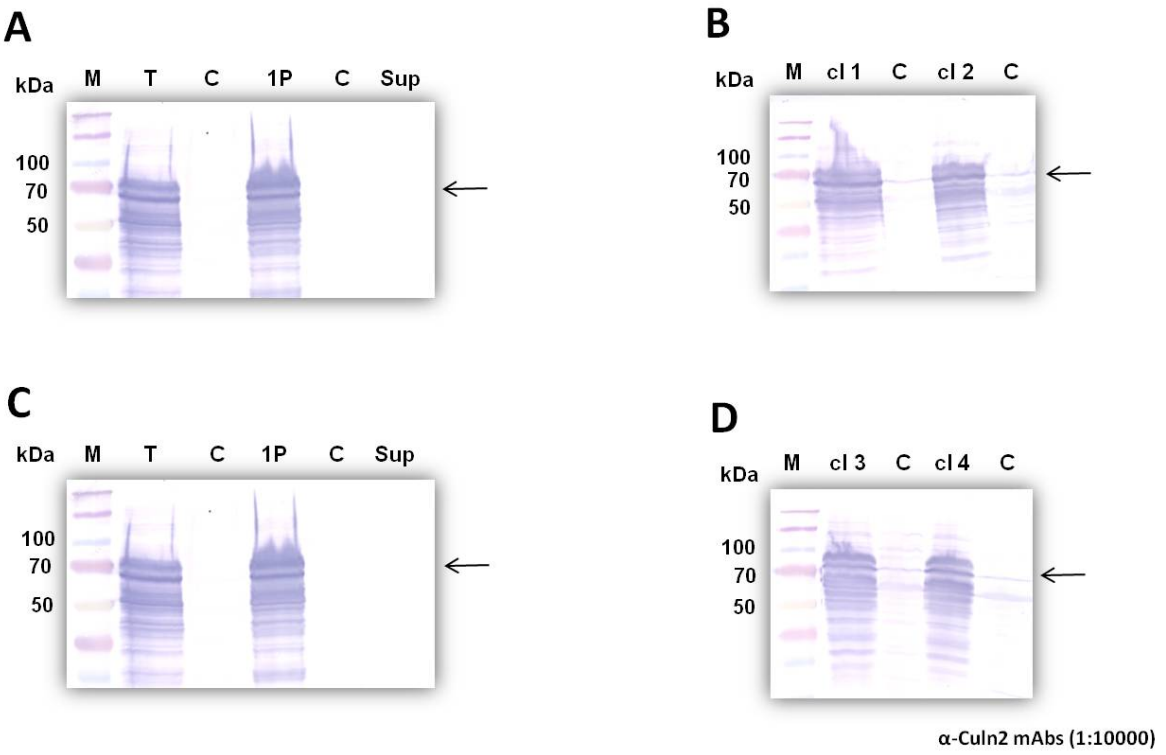


**Figure 47. WB showing the expression of rBac-SUMO-Cul n 2 and rBac-SUMO-Culn2 without its signal sequence in Sf-9 cells**
**A** rBac-SUMO-Cul n 2 after transfection and passages**B** r-baculovirus clones expressing rBac-SUMO-Cul n 2, **C** rBac-SUMO-Cul n 2-without after transfection and passages, **D** r-baculovirus clones positive for rBac-SUMO-Cul n 2 without its signal sequence.
L**ane M:** marker, **T:** transfection, **C:** negative control **1P:** first passage, **2P:** second passage, **Sup:** supernatant, **cl 1:** clone 1, **cl 2:** clone 2, **cl 3:** clone 3, **cl 4:** clone 4.
The arrows indicate rBac-SUMO-Cul n 2.

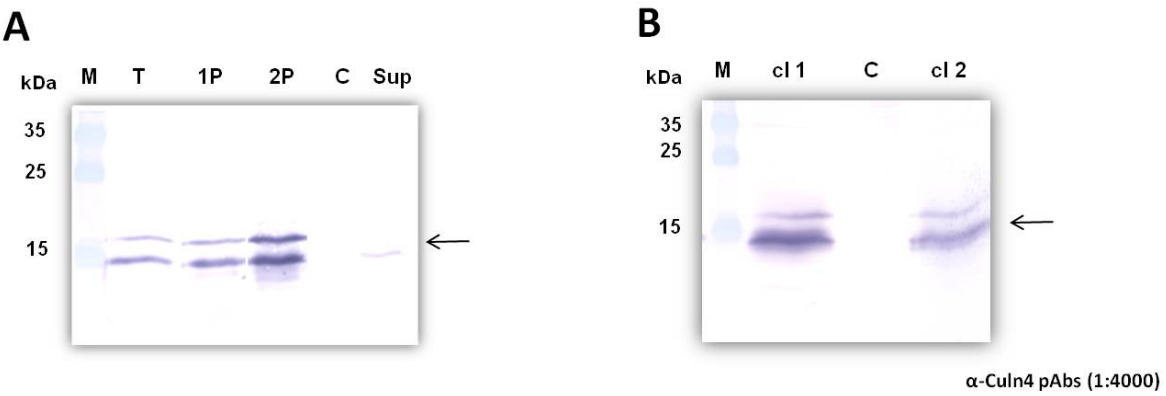


**Figure 48. WB showing the expression of rBac-HBM-Cul n 4 in Sf-9 cells**
**A** rBac-HBM-Cul n 4 after transfection and passages, **lane M:** marker, **T:** transfection, **1P:** first passage, **2P:** second passage, **C:** negative control **Sup:** supernatant.
**B** r-baculovirus clones positive for rBac-HBM-Cul n 4, **lane M:** marker, **cl 1:** clone 1, **C:** negative control, **cl 2:** clone 2.
The arrows indicate rBac-HBM-Cul n 4.

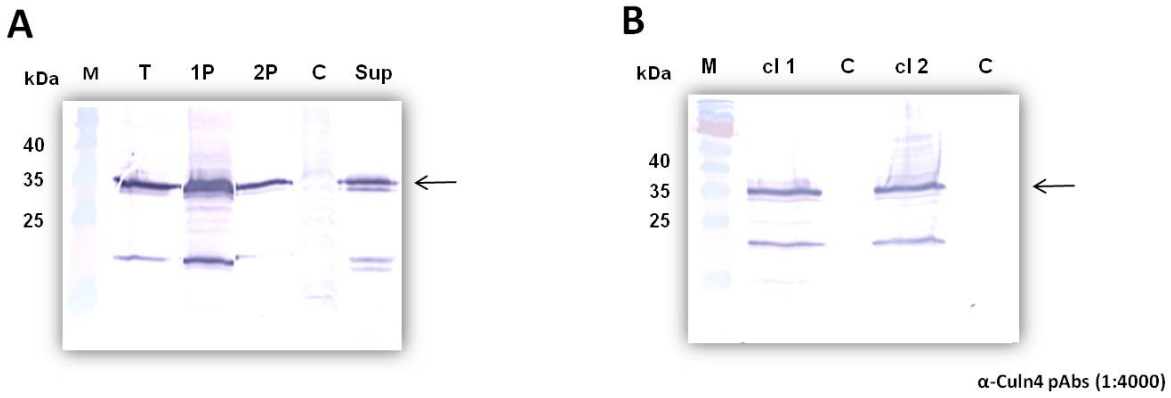


**Figure 49. WB showing the expression of rBac-SUMO-Cul n 4 in Sf-9 cells**
**A** rBac-SUMO-Cul n 4 after transfection and passages, **lane M:** marker, **T:** transfection, **1P:** first passage, **2P:** second passage, **C:** negative control, **Sup:** supernatant.
**B** r-baculovirus clones positive for rBac-SUMO-Cul n 4, **lane M:** marker, **cl 1:** clone 1, **C:** negative control, **cl 2:** clone 2.
The arrows indicate rBac-SUMO-Cul n 4.

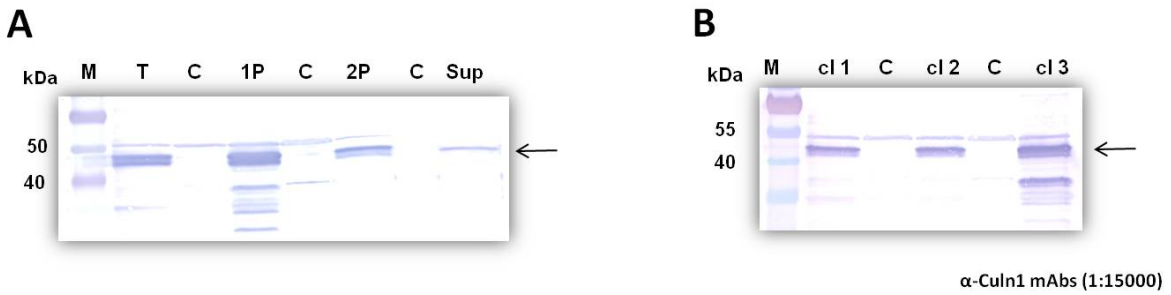


**Figure 50. WB showing the expression of rBac-SUMO-Cul o 3 in Sf-9 cells**
**A** rBac-SUMO-Cul o 3 after transfection and passages, **lane M:** marker, **T:** transfection, **C:** negative control **1P:** first passage, **2P:** second passage, **Sup:** supernatant.
**B** r-baculovirus clones positive for rBac-SUMO-Cul o 3, **lane M:** marker, **cl 1:** clone 1, **C:** negative control, **cl 2:** clone 2, **cl 3:** clone 3.
The arrows indicate rBac-SUMO-Cul n 3.

# Appendix VI

## Production of r-allergens in High-five cells

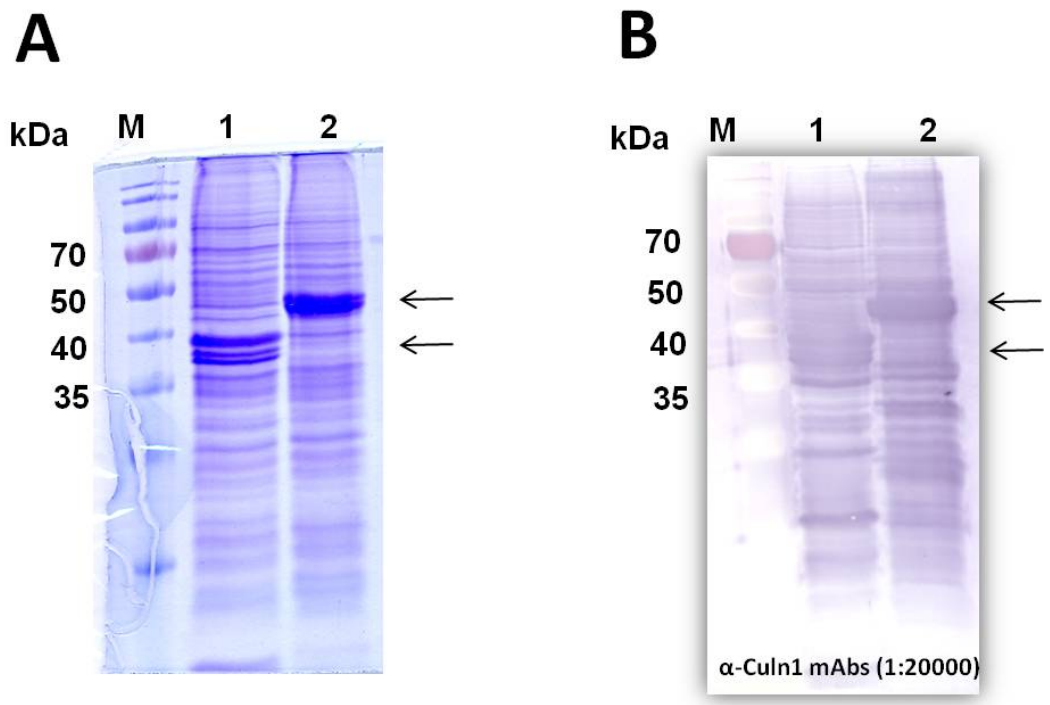


**Figure 51. Production of recombinant Antigen 5 like protein in High-five cells.**
**A** Coomassie blue staining, **2** Western blot. **Lane M :** marker, **1** rBac-SUMO-Cul n 1**, 2** rBac-SUMO-Cul o 3. The arrows indicate Antigen 5 like protein.

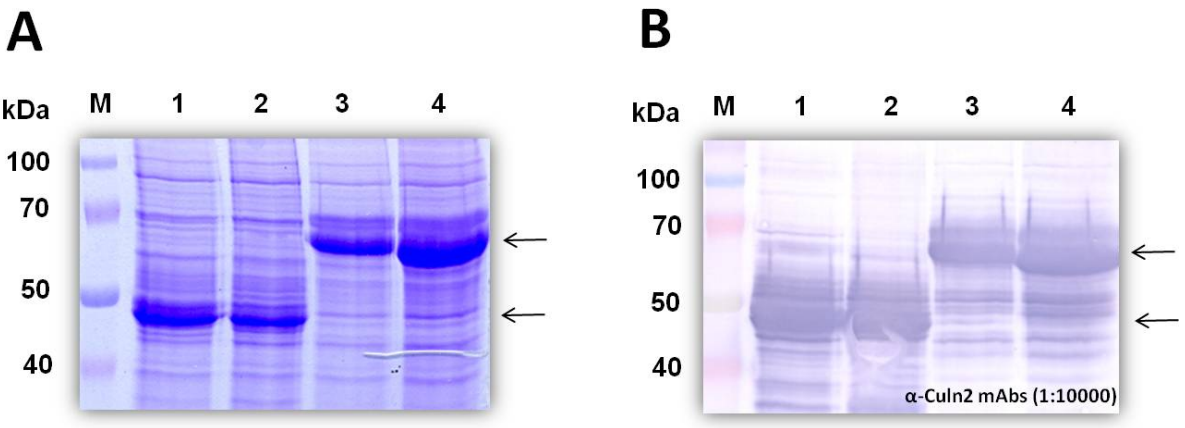


**Figure 52. Production of recombinant Cul n 2 in High-five cells.**
**A** Coomassie blue staining, **2** Western blot. **Lane M:** marker, **1** rBac-HTB-Cul n 2**, 2** rBac-HBM-Cul n 2, **3** rBac-SUMO-Cul n 2, **4** rBac-SUMO-Cul n 2 without its signal sequence. The arrows indicate rBac-Cul n 2.

# Appendix VII

## Purification of recombinant allergens with HIS-Select® HF Nickel Affinity Gel

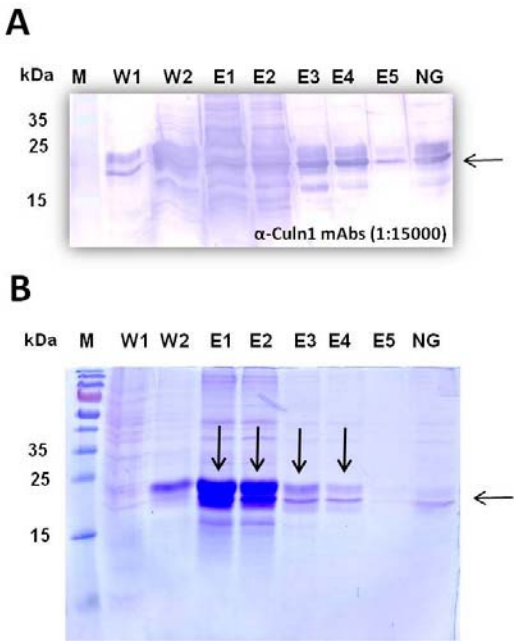


**Figure 53. Denature purification of rBac-HBM-Cul n 1 from High-five cells.**
**A** Western blot, **B** Coomassie blue staining**. Lane M:** marker, **W1:** wash 1, **W2:** wash 2, **E1 - 5:** elution 1-5, **NG:** nickel gel after elution. The arrows indicate rBac-HBM-Cul n 1.

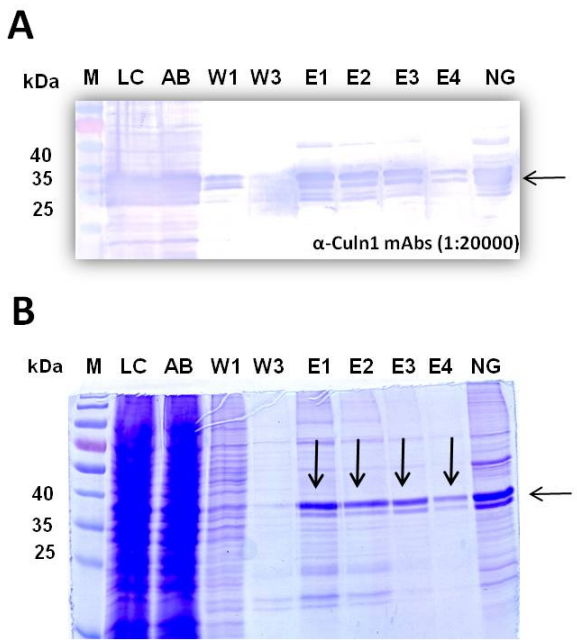


**Figure 54. Purification of rBac-SUMO-Cul n 1 from High-five cells.**
**A** Western blot, **B** Coomassie blue staining**. Lane M:** marker, **W1:** wash 1, **W3:** wash 3, **E1 - 4:** elution 1 - 4, **NG:** nickel gel after elution. The arrows indicate rBac-SUMO-Cul n 1.

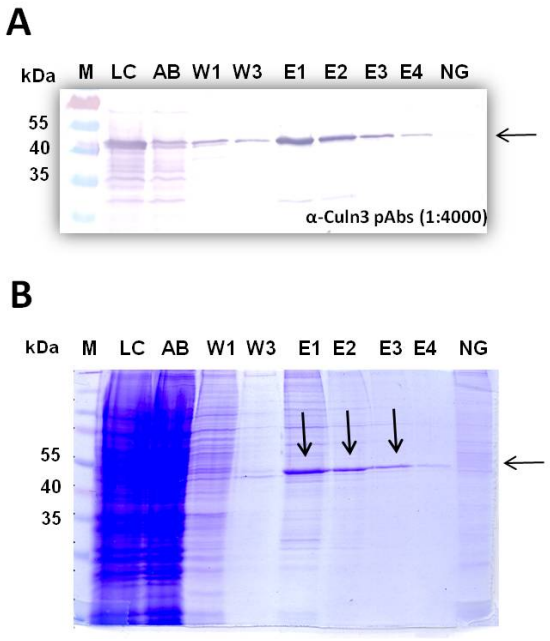


**Figure 55. Purification of rBac-HBM-Cul n 3 from High-five cells.**
**A** Western blot, **B** Coomassie blue staining**. Lane M:** marker, **LC:** lysed cell pellet, **AB:** after binding, **W1:** wash 1, **W3:** wash 3, **E1 - 4:** elution 1 - 4, **NG:** nickel gel after elution. The arrows indicate rBac-HBM-Cul n 3.

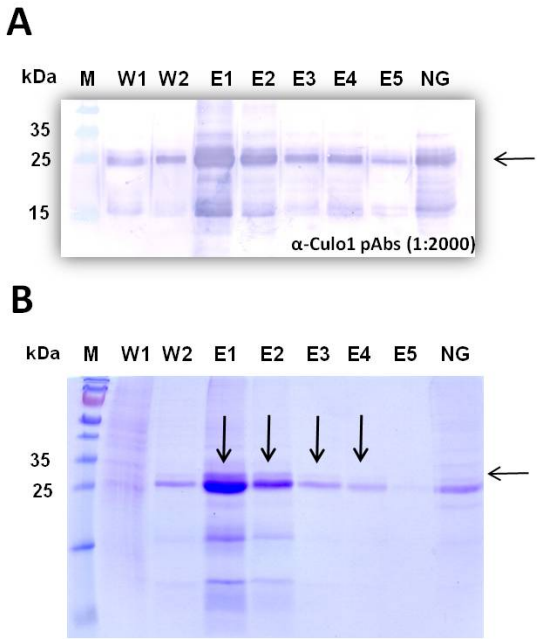


**Figure 56. Denature purification of rBac-HBM-Cul o 1 from High-five cells.**
**A** Western blot, **B** Coomassie blue staining**. Lane M:** marker, **W1:** wash 1, **W2:** wash 2, **E1 - 5:** elution 1 - 5, **NG:** nickel gel after elution. The arrows indicate rBac-HBM-Cul o 1.

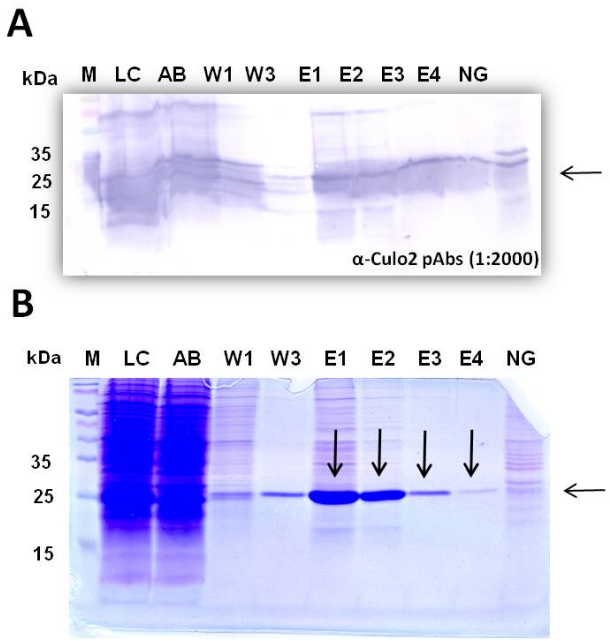


**Figure 57. Purification of rBac-HBM-Cul o 2 from High-five cells.**
**A** Western blot, **B** Coomassie blue staining**. Lane M:** marker, **LC:** lysed cell pellet, **AB:** after binding, **W1:** wash 1, **W3:** wash 3, **E1 - 4:** elution 1 - 4, **NG:** nickel gel after elution. The arrows indicate rBac-HBM-Cul o 2.

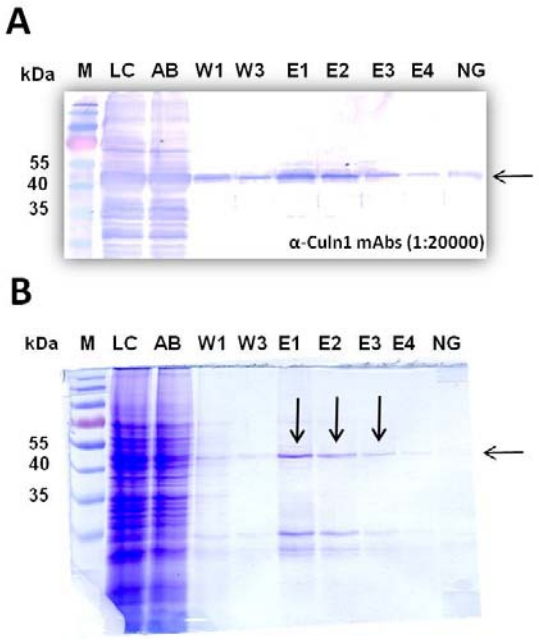


**Figure 58. Purification of rBac-SUMO-Cul o 3 from High-five cells.**
**A** Western blot, **B** Coomassie blue staining**. Lane M:** marker, **LC:** lysed cell pellet, **AB:** after binding, **W1:** wash 1, **W3:** wash 3, **E1 - 4:** elution 1 - 4, **NG:** nickel gel after elution. The arrows indicate rBac-SUMO-Cul o 3