# Insect Bite Hypersensitivity of Horses

Expression of allergens from *Culicoides obsoletus* in insect cells

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**Thesis for the degree of Bachelor of Science**
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HÁSKÓLI ÍSLANDS

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May 2013

This is a thesis for the Bachelor program in Biomedical Laboratory Science



Printed: Prentsmiðjan Leturprent

Reykjavik, Ísland 2013

## Sammendrag

Insektbitthypersensitivitet (IBH), eller sommereksem, er en tilbakevendende allergisk dermatitt hos hester, som i hovedsak er forårsaket av bitt fra knotten *Culicoides* spp. Hester som er født på Island og eksportert til kontinentet har en høyere prevalens av IBH enn andre hesteraser og islandshester født på kontinentet. Det finnes foreløpig ingen effektiv behandling av IBH. Dette prosjektet er en del av et større prosjekt som har som mål å utvikle immunterapi for IBH.

Genene som koder for allergenene fra spyttkjertlene til *Culicoides nubeculosus* (Cul n) har blitt isolert, og proteinene uttrykt og produsert i *E. coli*. For å kunne bruke allergenene i biologiske tester, er det nødvendig å uttrykke dem nær sin opprinnelige form*. C. nubeculosus* ble brukt på grunn av at den kan avles og opprettholdes i laboratoriet, slik at en tilstrekkelig mengde spyttkjertler kunne bli dissekert og innhentet til et cDNA-bibliotek. *C. nubeculosus* er imidlertid ikke en vanlig knott i Europa. Siden artene av *Culicoides* varierer i geologiske regioner, er det viktig for utviklingen av IBH-immunterapi og diagnose, å isolere og uttrykke allergener fra forskjellige arter.

*C. obsoletus* er en knott det finnes rikelig av i Sentral-Europa. I dette prosjektet er målet å isolere og uttrykke to viktige allergener (Cul o 1 og Cul o 2) fra *C. obsoletus* i insektceller, med melittin fra honningbier som signalpeptid, ved bruk av TOPO®-kloning og Bac-to-Bac® Baculovirus ekspresjonssystem. *Cul o 1* ble uttrykt som rekombinant protein og visualisert i western blot med anti-his antistoffer. *Cul o 2* ble klonet i en pFasBac/HBM TOPO-vektor som ble verifisert med sekvensering, og er klar til å bli satt inn i et bacmid for transfeksjon av Sf-9 insektsceller.

De neste trinnene er å uttrykke Cul o 2 i insektceller, å klone begge rekombinante virus, og å uttrykke rekombinante proteiner i HighFive™ insektceller, slik at de kan testes for allergenisitet og brukes i immunterapi.

## Abstract

Insect bite hypersensitivity (IBH), or summer eczema, is a recurrent allergic dermatitis of horses, mainly caused by bites from *Culicoides* spp. Horses born in Iceland that are exported to the continent have a higher prevalence of IBH than other breeds and Icelandic horses born on the continent. There is currently no effective treatment of IBH. The present project is a part of a large project aiming at developing immunotherapy against IBH.

The allergen genes of *Culicoides nubeculosus* (Cul n) originated in the salivary glands of the midges have been isolated, and the proteins have been expressed and produced in *E. coli*. It is of critical importance to express the allergens close to their native form for biological tests. *C. nubeculosus* was used because it can be bred and maintained in a laboratory, so a sufficient amount of salivary glands could be dissected and obtained for a cDNA gene library. However, *C. nubeculosus* is not a common midge in Europe. Since the *Culicoides* species differ between geological regions, it is of importance for the development of IBH immunotherapy and diagnosis to isolate and express allergens from various species.

*C. obsoletus* is an abundant midge in central Europe. In this project, the aim was to isolate and express two important allergens (Cul o 1 and Cul o 2) from *C. obsoletus* with the Honey bee melittin signal peptide in insect cells using TOPO® cloning and the Bac-to-Bac® Baculovirus expression system. *Cul o 1* was cloned and inserted into a bacmid. Sf-9 cells were transfected with recombinant bacmids, and Cul o 1 was expressed as a recombinant protein, visualized in western blot using anti-his antibodies. *Cul o 2* was cloned in a pFastBac/HBM TOPO vector that was verified by sequencing, and is ready to be inserted into a bacmid for transfection of Sf-9 cells.

The next steps are to also express Cul o 2 in insect cells, to clone both recombinant viruses, and to express the recombinant proteins in High Five™ insect cells, so that they can be tested for allergenicity and used in immunotherapy.

## Acknowledgements

I wish to thank, first and foremost, my supervisors Sigurbjörg Þorsteinsdóttir, Sigríður Jónsdóttir and Lilja Þorsteinsdóttir for their guidance, patience and support with lab work and writing this thesis. I consider it an honor to have had the chance to work with them on such an interesting and important project.

I am thankful to the Institute of Experimental Pathology, University of Iceland, Keldur, where the research took place.

I would also like to thank Prof. Eliane Marti at the University of Berne, Switzerland and Prof. Doug Wilson at the University of Bristol, UK for providing the *Cul o 1* and *Cul o 2* allergen genes used in this work.

I am grateful to Høgskolen i Sør-Trøndelag, Avdeling for Teknologi and Martha Á. Hjálmarsdóttir at the University of Iceland for making it possible for me to be a part of the exchange program and participate in this project in Iceland.

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## Abbreviations

| | |
|---|---|
| a.a. | Amino acid |
| APCs | Antigen presenting cells |
| DNA | Deoxyribonucleic acid |
| ELISA | Enzyme linked immunosorbent assay |
| IBH | Insect bite hypersensitivity. |
| IgE | Immune globuline class E |
| MHC | major histocompatibility complex |
| mRNA | Messenger ribonucleic acid |
| NK cells | Natural killer cells |
| PAMPs | Pathogen associated molecular patterns |
| PCR | Polymerase chain reaction |
| rBac | Recombinant baculoviruses |
| rBacmid | Recombinant bacmid |
| RNA | Ribonucleic acid |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| Sf-9 | Spodoptera frugiperda |
| TBS | Tris buffer saline |
| TBS-T | Tris buffer saline with Tween 20 |
| Th | T helper cells |
| Treg | T regulatory cells |

# 1 Introduction

## 1.1 Insect bite hypersensitivity in horses

Insect bite hypersensitivity (IBH), or summer eczema, is an allergic reaction in horses, mainly caused by bites from biting midges of the genus *Culicoides.* Salivary proteins from *Culicoides,* which they inject when they bite horses, initiate IgE mediated response (Baselgia et al., 2006) (van der Haegen et al., 2001). Initial symptoms of IBH are eczema (dermatitis) with intense pruritus, sub-epidermal edemas and skin sensitization. The symptoms make the horse scratch the affected areas, leading to localized hair loss and wounds that are susceptible to infections. As the condition progresses, it may lead to hypertrophy and hyperkeratosis of the epidermal tissue, and the skin forms ridges and folds. (Schaffartzik et al., 2012) (Baker and Quinn, 1978)

In horses affected by IBH, an imbalance between Th1, Th2 and Treg lymphocytes has been observed both in the blood and the skin (Hamza et al., 2007) (Heimann et al., 2011).

The eczema has a distinctive distribution along the dorsal midline, the withers, and the base of the mane and tail, as these are the preferred feeding sites of *Culicoides* spp. Horses show clinical signs from spring to autumn, when *Culicoides* are active. In most cases the symptoms regress during the winter. (Schaffartzik et al., 2012) (Marti et al., 2008)

IBH can affect all horse breeds, but exported Icelandic horses have a significantly higher risk, and this is a big problem for the horse export from Iceland (Bjornsdottir et al., 2006). It is thought that the reason for the high prevalence of IBH in Icelandic horses are caused by lack of exposure to the *Culicoides* allergens from a young age, as *Culicoides* are not found in Iceland (Halldordsottir and Larsen, 1991). IBH is a great nuisance for horses as the symptoms can be very painful. The symptoms persist throughout the summer months, and in this period for time the horse often cannot be trained or ridden. In some cases it might become so severe that the horse must be euthanized. (Anderson et al., 1988)

Having not been exposed to the *Culicoides* allergens from a young age, horses imported to the European continent from Iceland have a >30 % chance of developing

IBH. In comparison, the Icelandic horses that are born on the European continent have a much slighter chance, at approximately 5-10 %. This percentage does not differ from other breeds. (Marti et al., 2008) (Bjornsdottir et al., 2006)

IBH is diagnosed primarily on clinical signs, but it can also be diagnosed using intradermal tests or *in vitro* tests. The *in vitro* tests are either serological tests or tests that measure cellular responses after *in vitro* stimulation of allergens, like histamine release test or sulfidoleukotriene release test. These tests use whole body extract from *Culicoides*, and are therefore not very efficient. The performance of the tests can be enhanced by identifying and using the causative allergens after expressing them as recombinant allergens. (Marti et al., 2008) (Ferroglio et al., 2006)

For the time being, only the symptoms of IBH are treatable. The most effective way to prevent the symptoms is by repelling the *Culicoides* using insect spray and blankets. The symptoms can also be treated with corticosteroids, but it’s rarely completely effective, and may cause serious side effects. (Anderson et al., 1996)

## 1.2 The immune response

The immune system of mammals protects the body from diseases induced by pathogens. It can be divided into the innate and the adaptive system, but the two systems are not separate, as they work closely together to prevent infections. The responses of the immune system are mediated by leukocytes, cells that derive from pluripotent stem cells in the bone marrow. The leukocytes interact with cytokines and chemokines. The primary organs of the immune system are bone marrow and thymus, and the secondary organs are lymph nodes, the spleen, mucosal lymphoid tissue of the gastro intestinal tract, the nasal and respiratory tract, the urogenital tract and other mucosa. (Janeway et al., 2005a)

The innate immune system provides a non-specific protection from pathogens, by recognizing structures on the surface of microorganisms called pathogen associated molecular patterns (PAMP's) (Deifl and Bohle, 2011). Examples of PAMP's are lipopolysaccharides and peptidoglycan from the cell wall of bacteria, and RNA from viruses. Leukocytes associated with the innate immune system are neutrophils, basophils and eosinophils (collectively known as granulocytes), monocytes, macrophages, dendritic cells, mast cells and NK cells. The most abundant cells of the innate system are the neutrophils, short-lived cells that circulate the bloodstream, and can enter inflamed or infected tissue. They ingest pathogens by phagocytosis and destroy them with enzymes contained in granules. The number of basophils and eosinophils increase in cases of infection by parasites, and play an important role in such infections, in addition to allergic reactions. Dendritic cells, monocytes and macrophages are phagocytic cells. The dendritic cells specialize in taking up antigens and later presenting them for recognition by lymphocytes. Dendritic cells are often referred to as the bridge between the innate and adaptive immune system. Cells that present antigens are called antigen presenting cells (APCs). Mast cells release substances that affect vascular permeability when activated, and are central in the allergic response. NK (natural killer) cells are large, granular lymphocyte-like cells that release lytic granules that kill virus infected cells. Unlike lymphocytes, they lack antigen specific receptors. (Janeway et al., 2005a)

The leukocytes of the adaptive immune system are B- and T-lymphocytes. They are able to recognize a vast variety of different antigens and therefore protect against virtually any pathogen. Each naïve lymphocyte bears a unique prototype antigen receptor, so that the population of lymphocytes collectively can bind a huge repertoire of different microbes. The B-lymphocyte antigen receptors bind extracellular antigens, while the T-lymphocyte receptors are adapted to detect MHC-bound antigens either on antigen presenting cells or somatic cells. When activated, the lymphocytes undergo a somatic hypermutation and recombination of the antigen receptors to specialize in binding to a specific antigen, and proliferate. (Janeway et al., 2005a)

B-lymphocytes differentiate into memory cells and plasma cells after activation. The plasma cells produce and release their antigen receptors, called immunoglobulins when not bound to the cell membrane. Immunoglobulines are divided into five classes, IgA, IgD, IgE, IgG and IgM, which have different functions. The memory cells are long lived cells that stay in the body years after the infection, to protect against future encounters from the same pathogen. (Janeway et al., 2005a)

T-lymphocytes recognize antigens presented on the major histocompatibility complex (MHC). Upon activation, the $CD8^+$ T-lymphocytes kill virus infected cells and tumor cells, while the $CD4^+$ T-lymphocytes activate and direct other leukocytes with cytokines. The naïve $CD4^+$ T-lymphocytes can be differentiated into various T-effector subsets (see figure 1). (Janeway et al., 2005a)

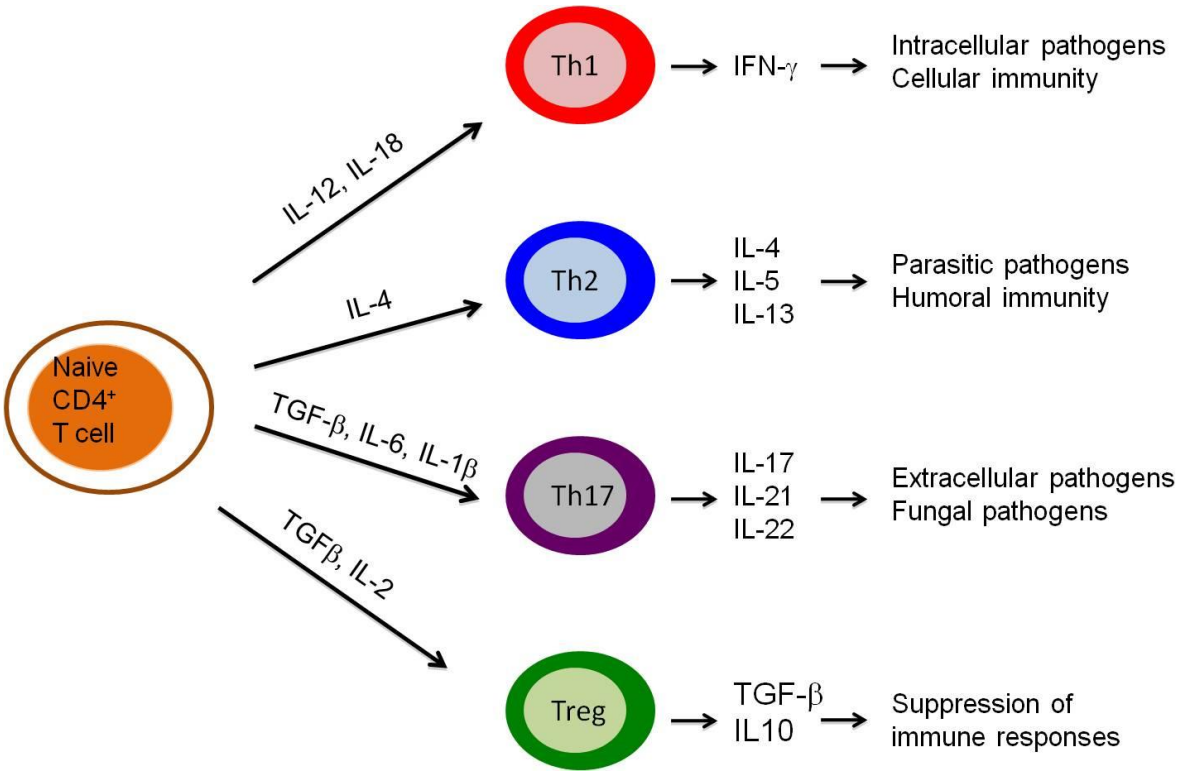


**Figure 1: Differentiation of naïve CD4+ T helper cells**

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

The immune response of the horse is controlled by the same cellular pathways, cells and cytokines as in mice and men (Lunn et al., 1998).

### 1.2.1 Allergy

Allergy, or type I hypersensitivity, is a class of harmful immune system responses initiated by proteins termed allergens, that initially are not harmful to the body. Type I hypersensitivity is a Th2 immune response with production of IgE. IgE-mediated responses involve mast cell degranulation, and the symptoms of allergy vary depending on the dose of the allergen, and how it was ingested. (Janeway et al., 2005b)

The IgE bind to high affinity receptors on effector cells like basophils and mast cells. When the sensitized effector cells encounter the allergen, they release inflammatory mediators such as histamine (see figure 2). (Deifl and Bohle, 2011)

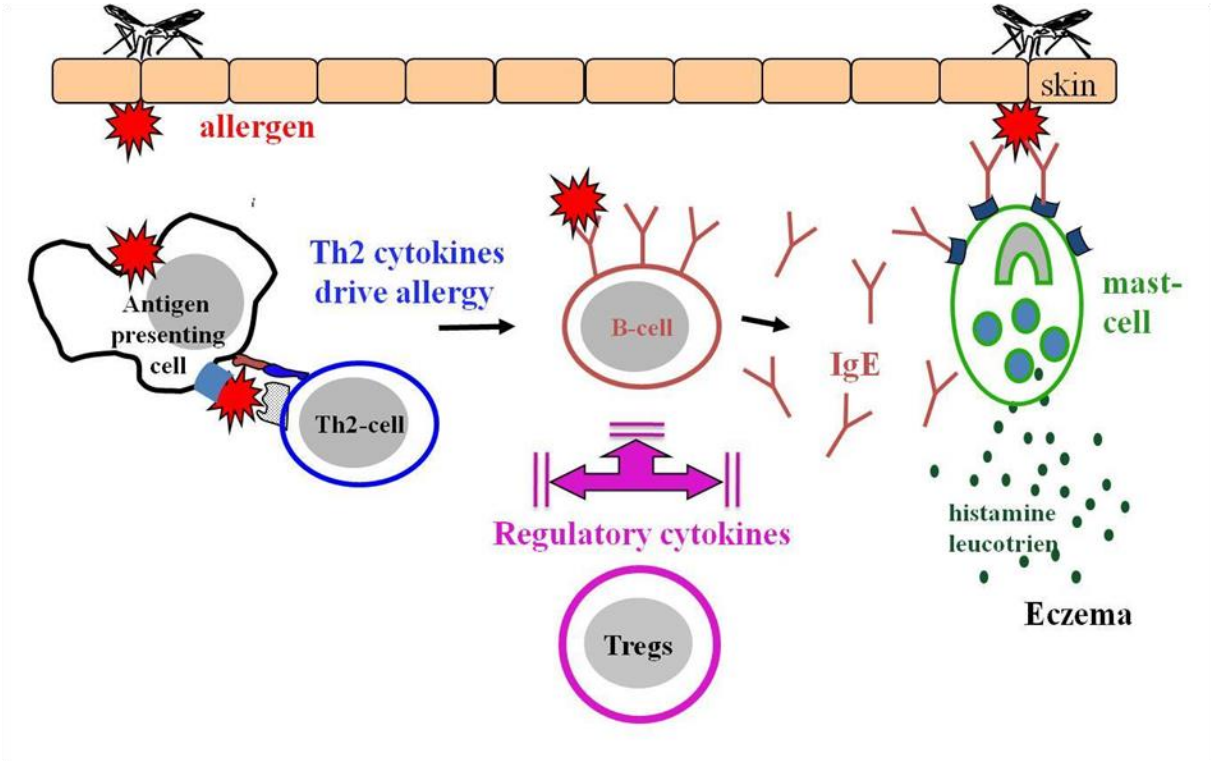


**Figure 2: IgE mediated response in IBH**

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

## 1.3 *Culicoides* spp

*Culicoides* midges are small biting flies, and are the largest genus of the family Ceratopgonidae, which is a family of insects in the Diptera order. The *Culicoides* midges have less than 2 mm wingspan, and weigh approximately 0.5 µg. Their behavior is crepuscular, which means they are most active during dusk and dawn, or on clouded days. There are over 1400 *Culicoides* species, and they are distributed in every continent except Antarctica. (Featherstone, 2010) The most important species involved in IBH vary depending on which species is more common in the region. For example *C. variipennis*, *C. insignis* and *C. stellifer* in the USA, *C pulcaris* and *C. nubeculosus* in France (van der Rijt et al., 2008) and *C. obsoletus* in the Netherlands (van der Meide et al., 2012). *Culicoides* does not exist in Iceland, New Zealand and the far south of South America (Featherstone, 2010).

Once *Culicoides* has found a suitable prey, it will insert its specialized mandibles through an area of soft skin. There it will suck up blood, and secrete compounds into the wound preventing coagulation and encouraging vasodilation. These compounds, proteins found in their salivary glands, may alter the host's innate and adaptive immune responses, and become allergens. (Bishop et al., 2006)

The *Culicoides* eggs, which hatch within a day, are laid during the summer in clusters of 50-100 on moist soil. The larvae bury down into the soil after hatching. In early summer, the larva enters a pupal stage, and goes through a metamorphosis, before emerging as a flying adult with a 20-30 day lifespan. (Featherstone, 2010)

Different species of *Culicoides* are distinguished by dark blotches on their wings, which form a recognizable pattern. The adult *Culicoides* feed mainly on nectar from flowers and sap, but after fertilization, the female needs a meal of mammalian blood for her eggs to develop completely. During the feeding, the *Culicoides* releases a pheromone that attracts other females, and can lead to mass attacks by thousands of individual midges. (Featherstone, 2010)

**Figure 3: *Culicoides* spp.**

## 1.4 Insect allergens

Allergens are non-pathogenic proteins that bind IgE, thus inducing a harmful immune response. The exact common features that separate allergens from other proteins, and give them an allergenic potential, are not known, but allergens are thought to share biochemical properties. A major allergen is regarded as an allergen that binds IgE antibodies in more than 50 % of allergic individuals. A minor allergen is one that bind IgE inn less than 50 %. (Deifl and Bohle, 2011)

Allergens from biting and stinging insects are known to be a cause of allergy, and humans and animals can become sensitized after one or a few encounters with the allergen (Arlian, 2002). The allergens from biting insects are in the salivary glands. If the main salivary duct is removed in mosquitos, they will still be able to feed on mammals, but their bite will no longer cause allergy (Hudson et al., 1960).

*Culicoides* allergens are the main cause of IBH, and this importance has stimulated research to identify the salivary proteins that alter the immune responses (Russell et al., 2009). Even though *in vitro* testing shows that there is some variation in the different *Culicoides* specie's ability to cause IgE mediated response, there are also evidence of allergens shared by the species, as horses suffering from IBH show symptoms when injected with extract from both native and exotic *Culicoides* (Anderson et al., 1993).

With use of mass spectrometry and 2D electrophoresis, mRNA and proteins have been extracted and analyzed from secreted saliva or dissected salivary glands of different *Culicoides spp*. There has been identified 54 novel protein sequences from

*Culicoides nubeculosus* salivary glands, and many of these were identified as candidate allergens that cause IBH in horses (Russell et al., 2009) (Campbell et al., 2005).

Some *Culicoides* midges, such as *C. nubeculosus* and *C. sonorensis* are bred successfully in captivity, while others, like *C. obsoletus* have to be caught in the wild due to limited success in captive breeding (Boorman, 1985) (van der Meide et al., 2012).

The first *Culicoides* allergen to be isolated and published was Cul s 1, maltase from *C. sonorensis*. It was expressed in insect cells using the Baculovirus system. The allergen's allergenicity was tested with intradermal and histamine releasing tests, and Cul s 1 showed basophil degranulation and immediate phase skin reactivity in 7 of 8 IBH affected horses. (Langner et al., 2009)

Then ten allergens were isolated from 1400 dissected salivary glands of laboratory bred female *C. nubeculosus* using a phage display cDNA library. These were termed Cul n 2-Cul n 11. Cul n 1 had been isolated before (Schaffartzik et al., 2010). The allergens were expressed in *E. coli*, and tested intradermally and with IgE ELISA for their ability to bind serum IgE in IBH affected horses. The sensitivity of the allergens ranged from 13-57 %. Major allergens identified were Cul n 1-Cul n 4. (Schaffartzik et al., 2011)

### 1.4.1 *C. obsoletus* allergens

After this project started, a paper entitled "Cloning and expression of candidate allergens from *Culicoides obsoletus* for diagnosis of insect bite hypersensitivity in horses" was published by van der Meide et al. They isolated seven genes from *C. obsoletus* using alignment to *C. sonorensis* and *C. nubeculosus* sequences, and the allergens were expressed as recombinant proteins in *E. coli*. The seven allergens were named Cul o 1-Cul o 7, and they bound IgE from IBH affected horses with a frequency of 38-67 %. The allergen's ability to diagnose IBH affected horses using IgE ELISA and intradermal testing were evaluated, and a combined test with all seven allergens had 92 % sensitivity and 85 % specificity. One of the allergens was a maltase (Cul o 1) and it was also expressed in insect cells using the Baculovirus system along with a maltase from *C. sonorensis* (Cul s 1). IBH affected horses

showed higher IgE levels binding the Cul o 1 allergen than the Cul s 1 allergen. (van der Meide et al., 2013) This indicates that it is important to check for major allergens in the most common wild *Culicoides* species.

The sequences of the *Cul o 1* and *Cul o 2* used in this project were published by Prof. E. Marti and Prof. D. Wilson (http://www.ncbi.nlm.nih.gov/nuccore/JX512273.1, http://www.ncbi.nlm.nih.gov/nuccore/JX512274.1), and are not the same as the *Cul o 1* and *Cul o 2* isolated by van der Meide et al, nor are they the same as any of the other genes in the article. However, they have been shown to be major allergens, as more than 50 % of tested IBH affected horses had IgE response to them with specificity at 95 % (Prof. Eliane Marti, Personal Communications). Therefore it is important to make these allergens available for immunotherapy.

## 1.5 Baculoviruses

Baculoviruses are a group of rod shaped viruses that mainly affect insects. Their capsids are usually 40-50 nm in diameter and 200-400 nm in length, and the genome of the most commonly used Baculoviruses for expression vector work is about 130 kbp. The size of the capsid can vary to accommodate larger genomes, making them capable of carrying large inserts. Baculoviruses have a double stranded, circular DNA genome, and the DNA is condensed into a nucleoprotein structure known as the core. The capsid and the core is called the nucelocapsid. After nucleocapsids are made in the core of infected cells, they are enveloped with the plasma- or nuclear membrane of the host cell. (O'Reilly et al., 1992) (Kost et al., 2005)

The viruses made in the host cell can collectively form crystalline occlusion bodies within the nucleus, which protects the viruses after being released into the environment. The crystalline structure of the occlusion bodies consist of a protein called polyhedrin, and they have an external surface coat called a calyx. The occlusion bodies are released when the infected cells lyse (Kost et al., 2005). Viruses may also be released individually by budding. The budded viruses infect new host cells by adsorptive endocytosis. (O'Reilly et al., 1992)

A schematic overview of the Bac-to-Bac Baculovirus expression system is shown in figure 4.

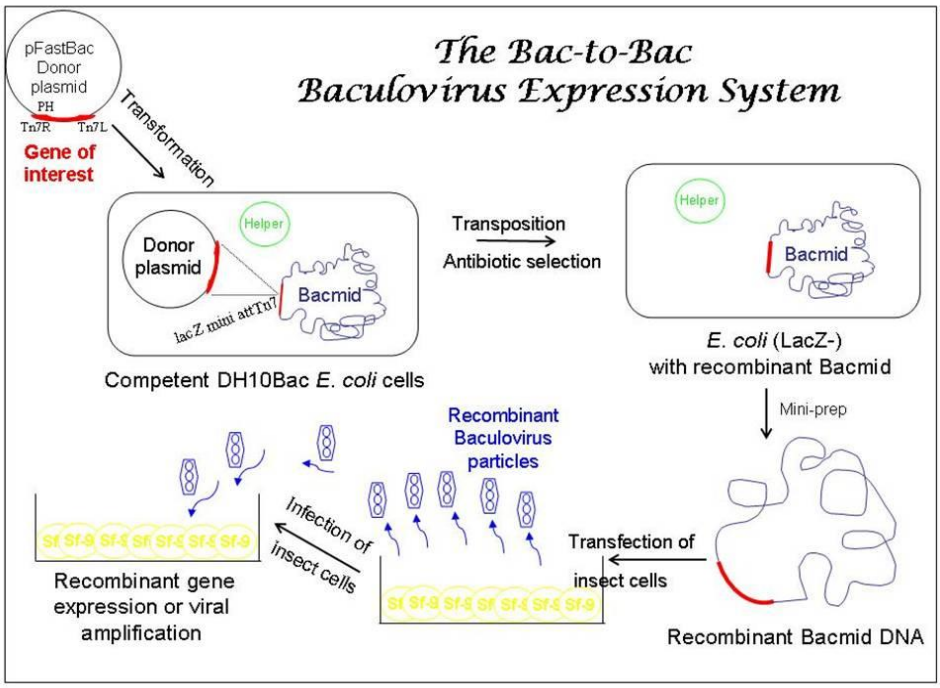


**Figure 4: Schematic overview of the Bac-to-Bac Baculovirus expression system**

After the gene of interest has been cloned into pFastBac HT B vector, the *E. coli* strain DH10Bac is transformed with vector. Homologous recombination occurs between the pFastBac vector and the Bacmid, and with antibiotic selection, recombinant Bacmids are isolated from the *E. coli* culture. Sf-9 insect cells are then transfected with the rBacmid, resulting in recombinant Baculovirus particles. Sf-9 cells are infected for gene expression and viral amplification. Scematic picture: Invitrogen.

## 2 Aims and background of the study

The IBH studies have the long term goal to develop a therapeutic and/or preventive therapy for IBH in horses. Cul o 1 and Cul o 2 are important allergens in IBH in horses. The specific aim of this project was to clone the two allergen genes *Cul o 1* and *Cul o 2*, and express the proteins in insect cells with the signal peptide of honey bee melittin using TOPO® cloning and the Bac-to-Bac® Baculovirus expression system. The expression and purification of the recombinant proteins is necessary for use in diagnostic tests and immunotherapy experiments of IBH.

# 3 Materials and methods

## 3.1 DNA methods

### 3.1.1 Amplification with polymerase chain reaction (PCR)

#### *3.1.1.1 PCR with with Phusion Hot Start polymerase*

Phusion™ Hot Start polymerase was used for amplification of the *Cul o 1* and *Cul o 2* genes, and to achieve a blunt end PCR product to clone into the TOPO® vector.

**Table 1: PCR reaction solution for amplification with Phusion™ Hot Start Polymerase**

| PCR reaction solution | For a 25 µL PCR reaction | For a 50 µL PCR reaction |
|---|---|---|
| $ddH_2O$ | 14.8 µL | 29.6 µL |
| Phusion Buffer (5x) | 5 µL | 10 µL |
| dNTP (2 mM) | 2 µL | 4 µL |
| Forward Primer (20 µM) | 1 µL | 2 µL |
| Reverse Primer (20 µM) | 1 µL | 2 µL |
| Phusion polymerase | 0.2 µL | 0.4 µL |
| DNA template | 1 µL | 2 µL |

PCR-program:

Step 1: Initial denaturation 98 °C for 30 sec

Step 2: Denaturing 98 °C for 10 sec

Step 3: Annealing 60 °C for 30 sec

Step 4: Extend DNA 72 °C for 20 sec

Step 5: Repeat steps 2-4 for 29 cycles

Step 6: Final extension 72 °C for 10 min

The time of the DNA extension (step 4) was based on the length of the DNA sequence. The annealing temperature (step 3) depended on the primers.

### *3.1.1.2 PCR with Taq polymerase*

Amplification with Taq polymerase was used after transformation of One Shot ® Mach1$^{TM}$ T1$^{R}$ chemically competent *E. coli*, and MAX Efficiency® DH10Bac$^{TM}$ chemically competent *E. coli*. This was to test if the bacteria had taken up the vector, and that the insert had the right orientation.

**Table 2: PCR reaction solution for amplification with Taq polymerase**

| PCR reaction solution | For a 20 µL PCR reaction |
|---|---|
| dd$H_2O$ | 12.8 µL |
| Buffer (10x) | 2 µL |
| dNTP (2 mM) | 2 µL |
| Forward Primer (20 µM) | 1 µL |
| Reverse Primer (20 µM) | 1 µL |
| Taq polymerase | 0.2 µL |
| Template (bacterial culture) | 1 µL |

PCR-program:

Step 1: Initial denaturation 95 °C for 5 min

Step 2: Denaturing 95 °C for 15 sec

Step 3: Annealing 50 °C for 30 sec

Step 4: Extend DNA 72 °C for 1:10-3:45 min

Step 5: Repeat steps 2-4 for 29 cycles

Step 6: Final extension 72 °C for 10 min

The time of the DNA extension (step 4) was based on the length of the DNA sequence. The annealing temperature (step 3) depended on the primers.

### 3.1.2 Agarose gel electrophoresis

All products from PCR reactions were run on 1% agarose gel (Sigma). The gel was made by melting agarose powder in 0.5% TBE (appendix II), and adding 1-3 drops of ethidium bromide (Sigma). As loading dye, 10x restriction buffer was added to the PCR product (RBS, appendix II). The electrophoresis was carried out at 70 V for 45 minutes, and a 2-log ladder (100-10000 bp, Biolabs) was used to estimate the size of the DNA fragments.

For visualization and documentation of electrophoresis results, an INGENIOUS Syngene bio imaging UV camera was used with Gene Snap software.

### 3.1.3 Gel extraction

A UV-light was used to visualize the DNA in agarose gel after electrophoresis, and it was excised from the gel with a sharp scalpel. QIAquick Gel Extraction Kit (QIAgen) was used according to protocol to extract the DNA.

### 3.1.4 DNA quantification

To measure the concentration and purity of DNA samples, the NanoDrop®ND-1000 Spectrophotometer (NanoDrop Technologies Inc.) was used according to protocol.

### 3.1.5 Sequencing

For the sequencing reaction, the BigDye Terminator v3.1 Cycle Sequencing Kit (applied biosystems) was used. It was conducted on MJ Research PCT-200 Peltier Thermal Cycler, and the capillary electrophoresis was conducted on ABI PRISM® 310 Genetic Analyzer.

A sequencing reaction was carried out both before and after TOPO cloning. The first sequencing was done with PCR product extracted from agarose gel, and this was done to ensure that the wanted gene had been amplified successfully. When sequencing gel extract, the concentration of Cul o 1 was 15 ng/µL, and Cul o 2 was 8 ng/µL. Primers used for the sequencing were gene specific.

The sequencing reaction following the TOPO cloning was done on a plasmid isolated from OneShot ® Mach1™ T1® transformed *E. coli* cultures. In this reaction, the concentration of DNA was 200-500 ng/µL for both genes. Primers used were both gene specific and vector specific primers.

**Table 3: Reaction solution for sequencing with BigDye**

| Sequencing reaction solution | For a 10 µL PCR reaction |
|---|---|
| $ddH_2O$ | 4.9 µL |
| Buffer | 1.5 µL |
| BigDye | 1 µL |
| 2 µM Primer | 1.6 µL |
| DNA template | 1 µL |

PCR-program:

Step 1: Initial denaturation 95 °C for 5 min

Step 2: Denaturing 95 °C for 20 sec

Step 3: Annealing 50/55 °C for 15 sec

Step 4: Extend DNA 60 °C for 4 min

Step 5: Repeat steps 2-4 for 29 cycles

Annealing temperature (step 3) depended on the primers.

### 3.1.6 DNA sequence

Several primers were used for each gene, since they were optimal for sequencing at different areas of the gene. The data from the capillary electrophoresis was analyzed in Sequencher 4.9.

## 3.2 TOPO cloning

### 3.2.1 TOPO cloning reaction

To clone the gene of interest into FastBac_HBM_Vector, TOPO cloning reaction was performed. The gene was added to a tube containing salt solution, TOPO vector, and deionized and distilled water to a final volume of 5 µL, and incubated for 20-30 minutes, depending in the length of the gene.

**Table 4: TOPO cloning reaction mix**

| Reagent | Volume |
|---|---|
| DNA from gel extraction | 0.5-4 µL |
| Salt solution | 1 µL |
| TOPO® vector (10ng/µL) | 1 µL |
| ddH2O | To a final volume of 5 µL |

For an optimal cloning reaction, the best insert: vector ratio was 1:1 or 2:1, therefore the volume and concentration of added DNA had to be calculated.

### 3.2.2 Transformation into One Shot® Mach1™ T1$^{R}$ competent cells

The chemically competent *E. coli* cells were kept at -80°C, and were thawed slowly on ice. The TOPO® cloning reaction (2 µL) was then added to the cells, and mixed gently by stirring in the solution with the pipette tip. After 30 minutes incubation on ice, the cells were heat shocked on 42°C for 30 seconds. The cells were then incubated on ice for two minutes, before SOC medium (250 µL, Appendix II) was added, and the tube was placed in an agitation incubator at 37°C for one hour. After incubation, volumes of 25, 50 and 100 µL of the bacteria suspension was spread out on LB agar plates containing ampicillin (LB medium, appendix II). The agar plates were incubated for 16-18 hours at 37°C.

### 3.2.3 Selection of recombinant One Shot® Mach1™ T1R

The agar plates contained ampicillin (100µg/mL) as a selection. Since the TOPO® vector contained the gene for resistance to ampicillin, only the bacteria that had taken up the vector could grow. After overnight incubation at 37°C, twelve of the single colonies were picked from the agar plates, placed in liquid LB medium containing ampicillin (2 mL), and incubated for 16-18 hours in an agitation incubator at 37°C.

To see which of the cultures contained TOPO® vectors with the insert in the correct orientation; a PCR was carried out on all colonies with TOPO forward and gene specific reverse primers.

### 3.2.3 Plasmid isolation

A QIAgen MiniPrep kit was used according to manufacturer's protocol, to isolate the plasmids from the cultures that contained the TOPO® vector with insert in correct orientation.

## 3.3 Bac-to-Bac baculovirus expression system

Recombinant baculoviruses are widely used to express heterologous genes in insect cells, and Invitrogen's Bac-to-Bac® HBM TOPO® secreted expression system (Invitrogen) is an efficient and quick method of constructing recombinant baculoviruses. The system is based on site specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) raised in *E. coli*. The *E. coli* strain used in this project was MAX Efficiency® chemically competent DH10Bac™.

### 3.3.1 Transformation into MAX Efficiency® DH10Bac™

The pFastBac expression cassette is flanked by the left and right arms of the site specific Tn7 transposon and contains a polyhedrin promoter, a multiple cloning site, a gentamicin resistance gene and an SV40 polyadenylation signal forming a mini Tn7.

The MAX Efficiency® chemically competent DH10Bac™ *E. coli* strain contains a target bacmid and a helper plasmid. The bacmid is a shuttle vector that contains the baculovirus genome (136bp) with a mini-*att*Tn7 target site, a kanamycin resistance gene, a mini-F replicon which allows stable replication in *E. coli*, and a LacZα gene that makes is possible to confirm a $Lac^-$ phenotype, that means the transposition was at the correct site. The helper plasmid encodes the transposase gene and tetracycline gene. (Bac-to-Bac® HBM TOPO® Secreted Expression System manual, catalog no. A11339, 2009)

The pFastBac_TOPO_HBM construct was transformed by heatshock into the competent DH10Bac™ *E. coli* (Invitrogen) for transposition at the mini-attTn7 site of the bacmid. The cells were then incubated in SOC medium (Appendix II) for four hours, before they were spread out on LB agar plates containing kanamycin (50 µg/mL), gentamycin (7 µg/mL), tetracyclin (10 µg/mL), X-gal (200 µg/mL, Appendix II) and IPTG (30 µL, 100 mM, Appendix II), and incubated in 37 °C for 48 hours.

Colonies were picked using blue/white selection, spread out on new LB agar plates containing the same additives, and incubated at 37 °C for 24 hours. Single colonies from the secondary spread were picked and inoculated in liquid LB medium containing the same antibiotics (2 mL) and agitated at 37 °C for 48 hours.

### 3.3.2 Isolation of rBacmids

A PCR was carried out on the positive colonies using both M13 and gene specific primers. The recombinant bacmids containing the gene of interest were isolated with Invitrogen PureLink™ HiPure Plasmid DNA Purification Kits according to protocol.

## 3.4 Expression of allergens from *C. obsoletus* in Sf-9 insect cells

### 3.4.1 Culturing of Sf-9 insect cells

Sf-9 insect cells (American type culture collection) originate from ovarian tissue from the moth *Spodoptera frugiperda*. The cells were cultured in closed culture at 27 °C. The culture medium used was SF900II medium (Invitrogen), containing penicillin (100 IU), streptomycin (100 IU) and fetal bovine serum (1 %).

### 3.4.2 Transfection of Sf-9 insect cells

The transfection into Sf-9 cells was performed with Cellfectin®II Reagent (Invitrogen) according to protocol. The day before the transfection, cells ($0.3 \times 10^6$ /well) in good condition were seeded into 12 well plates in culture medium. The transfection mix (Table X) was done in Grace's Insect Cell Culture medium (Invitrogen). To get rid of the culture medium, especially the antibiotics, the medium was exported off the wells, and then the cells were washed with Grace's Insect Cell Culture medium (800 µL), beforen Grace's Insect Cell Culture medium (500 µL) was added to the cells.

**Table 5: Solutions for transfection of Sf-9 cells with Cul o 1**

| | Solution 1 | | Solution 2 | |
|---|---|---|---|---|
| Sample | Grace's medium | Bacmid | Grace's medium | Cellfectin®II |
| rBacmid 3 (0.5 µg) | 66.2 µL | 33.8 µL | 100 µL | 8 µL |
| rBacmid 7 (1 µg) | 64.8 µL | 35.2 µL | 100 µL | 8 µL |
| Cellfectin control | 100 µL | - | 100 µL | 8 µL |
| Medium control | 100 µL | - | 100 µL | - |

Solution 2 was incubated in room temperature for five minutes. Solution 2 was carefully added to solution 1, and the mixed solutions incubated in room temperature for 20 minutes. The solutions were slowly added to the wells, and the wells were incubated at 27 °C for five hours. The Cellfectin®II medium was removed and replaced with culture medium (950 µL). The cultures were incubated for three to six days under careful observation for cytopathic effect caused by virus infection.

### 3.4.3 Passage of recombinant baculovirus

When cytopathic effect was observed in a Leitz Transformator Inverted light microscope, supernatant from transfected cells (200 µL) were transferred to healthy Sf-9 cells in a 12 well plate (1. passage). Second passage was done also by infecting Sf-9 cells in T25-flasks (Nunc). Supernatant (200 µL) from cells showing cytopathic effect was transferred into 4.5 mL flasks with healthy Sf-9 cells.

Sf-9 cells were collected from the plates and centrifuged at 14000 RPM for 3 minutes. Sf-9 cells collected from T25 flasks were centrifuged at 2000 RMP for 12 minutes. The viral supernatant (viral stock) was kept at 4 °C, and for long time storage at -80 °C. The cell pellet was resuspended in lysis buffer (50 µL, Appendix II). 2x Sample buffer (50 µL) was added to both the pellet and viral supernatant (50 µL) for western blot analysis.

## 3.5 SDS-PAGE and western blot

Separation of proteins was carried out with SDS-PAGE in the Mini-protean II system (Bio-Rad). The proteins in the samples was denatured by 2x sample buffer (Appendix II), boiled for five minutes at 100 °C, centrifuged for two minutes and run on 14 % acrylamide gel for one hour. To estimate size of the proteins, a PageRuler prestained molecular mass marker (10-170 kDa, Fermentas) was used.

The protein bonds were visualized using western blot on PVDF membrane (Millipore). The proteins were transferred to the membrane using wet transfer in the mini-protean II system (Bio-Rad) for one hour in transfer buffer (appendix II). The membranes were then incubated in TBS-T (appendix II) for 30 minutes, washed 3x five minutes with TBS-T, and then incubated overnight with anti-his antibody (1:1000) at 4 °C. After incubation, the membranes were washed 5x five minutes and incubated for one hour with the conjugate, goat anti-mouse-AP (1:5000, Jackson) or goat anti-mouse-HRP (1:2000, BioRad). The membranes were washed 5x five minutes, and developed with BCIP/NBT (appendix II, Roche) diluted 1:50 in alkaline phosphatase buffer (appendix II) according to protocol.

# 4 Results

## 4.1 Amplification of *Cul o 1* and *Cul o 2*

Blunt end gene products of *Cul o 1* and *Cul o 2* were amplified from cDNA library using gene specific primers (appendix I). A 50 µL PCR solution was made of each gene. A gel electrophoresis was conducted, with the solution dispersed in two wells. Figure 5 shows the PCR products of 557 bp for the *Cul o 1* gene, and 398 bp for the *Cul o 2* gene.

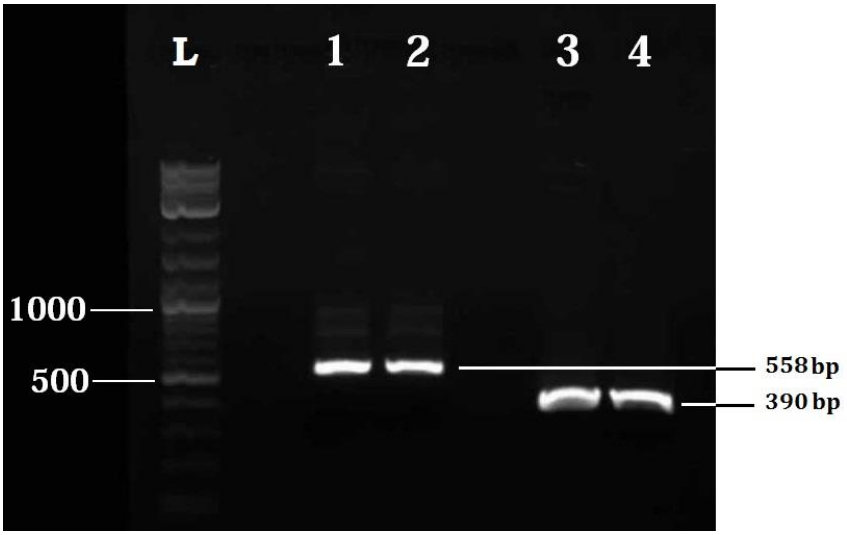


**Figure 5: Amplification of the *Cul o 1* and *Cul o 2* genes**

**Lanes: L** 2-log ladder **1-2** *Cul o 1* (557 bp), **3-4** *Cul o 2* (398 bp)

The DNA was extracted from the agarose gel, and purity and concentration was measured (Table 6).

**Table 6: Concentration and purity of the DNA extracted from agarose gel:**

| Gene | Concentration (ng/µL) | Purity (260/280) |
|---|---|---|
| *Cul o 1* | 28,82 | 2.05 |
| *Cul o 2* | 41.41 | 2.00 |

## 4.2 Sequencing of *Cul o 1* and *Cul o 2* for verification.

Before the TOPO cloning, a sequencing reaction was conducted on the extracted DNA using gene specific primers (Appendix I). The correct sequences were verified for both genes (Appendix III).

## 4.3 TOPO Cloning and isolation of pFastBac vector.

One Shot® Mach1™ T1R cells were transformed with pFastBac™/HBM-TOPO® vector after *Cul o 1* and *Cul o 2* had been TOPO cloned into the vector. Colonies were numbered 1-12, picked and cultured in liquid LB medium. PCR was conducted on the cultures using TOPO forward and gene specific reverse primers (appendix I).

Cultures 7 and 11 were positive for *Cul o 1* in the correct orientation after the transformation (Figure 6)

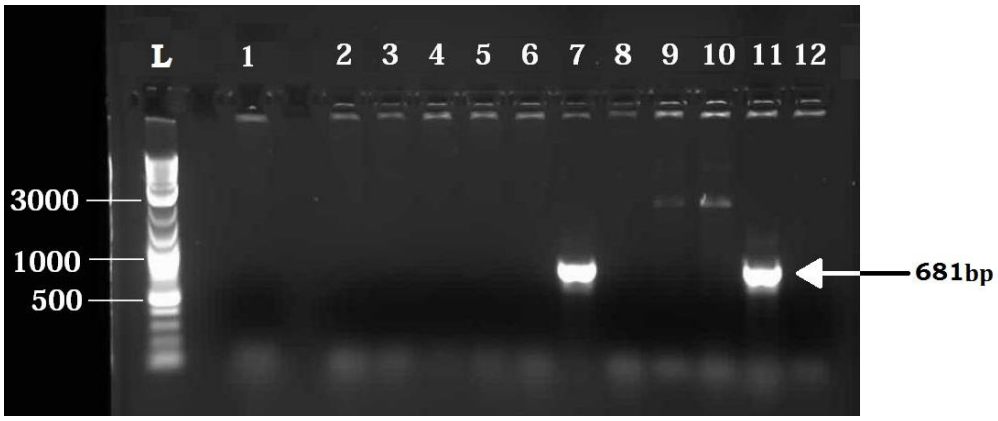


**Figure 6: PCR of One Shot® cultures after TOPO cloning of *Cul o 1***

**Lanes: L** 2-log ladder **1-12** Culture 1-12 (681 bp).

Cultures 2, 4 and 5 were positive for *Cul o 2* in the correct orientation after the transformation (Figure 7).

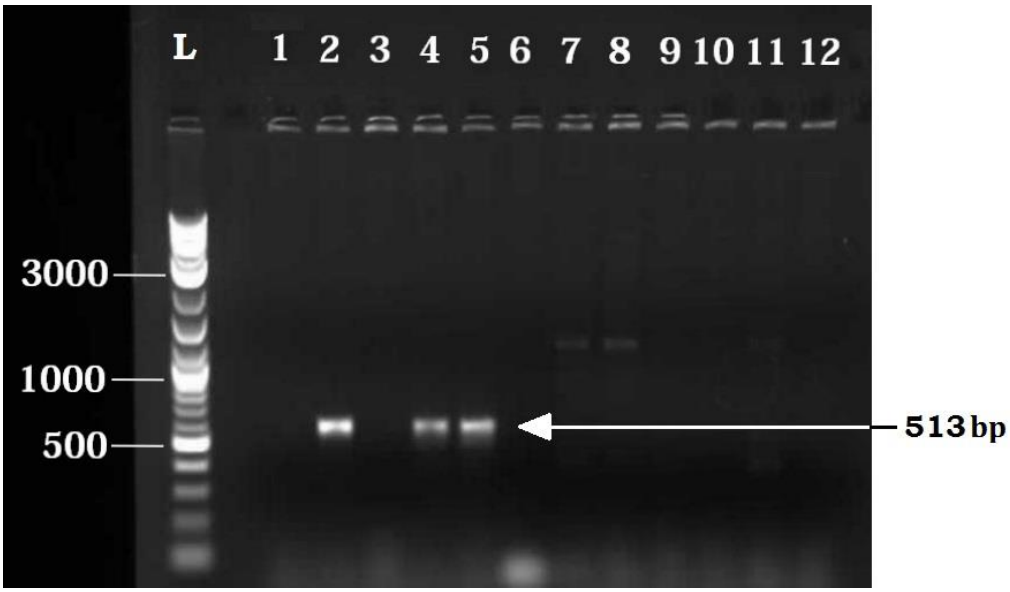


**Figure 7: PCR of One Shot® cultures after TOPO cloning of *Cul o 2.***

**Lanes: L** 2-log ladder **1-12** Culture 1-12 (513 bp).

The pFastBac vectors were isolated with plasmid purification kit, and the DNA concentration was measured (Table 7).

**Table 7: Concentration and purity of the TOPO vectors**

| Vector | Culture | Concentration (ng/µL) | Purity (260/280) |
|---|---|---|---|
| pFastBac/HBM-TOPO *Cul o 1* | 7 | 179.16 | 1.84 |
| | 11* | 223.85 | 1.88 |
| pFastBac/HBM-TOPO *Cul o 2* | 2 | 219.75 | 1.88 |
| | 4 | 229.73 | 1.88 |
| | 5* | 263.34 | 1.88 |

*used for sequencing

A sequencing reaction was conducted on pFastBac/HBM-TOPO *Cul o 1* from culture 11, and on pFastBac/HBM-TOPO *Cul o 2* from culture 5 (Table X). Primers used were FastBac TOPO forward and reverse primers, as well as gene specific primers (appendix I). The sequences were correct for both genes.

This is as far as the work on *Cul o 2* was carried out for this thesis. From now on, all results will be for *Cul o 1*.

## 4.4 Transformation of DH10Bac and production of rBacmid.

Based on the DNA concentration and purity, the miniprep from culture 11 was chosen to transform the DH10Bac.

Eight transformed DH10Bac colonies were picked and numbered 1-8. After secondary spreading of colonies, number 3-8 proved to be lac$^-$, and were transferred to liquid LB medium (appendix II) containing Kanamycin (50 µg/mL), Gentamycin (7 µg/mL) and Tetracycline (10 µg/mL). Results on PCR performed with M13 primers are shown in figure 8A, giving a PCR product of 3716 bp. The M13 Fw and Culo1_595-615_Re primers gave a PCR product of 2820 bp, as shown in figure 8B

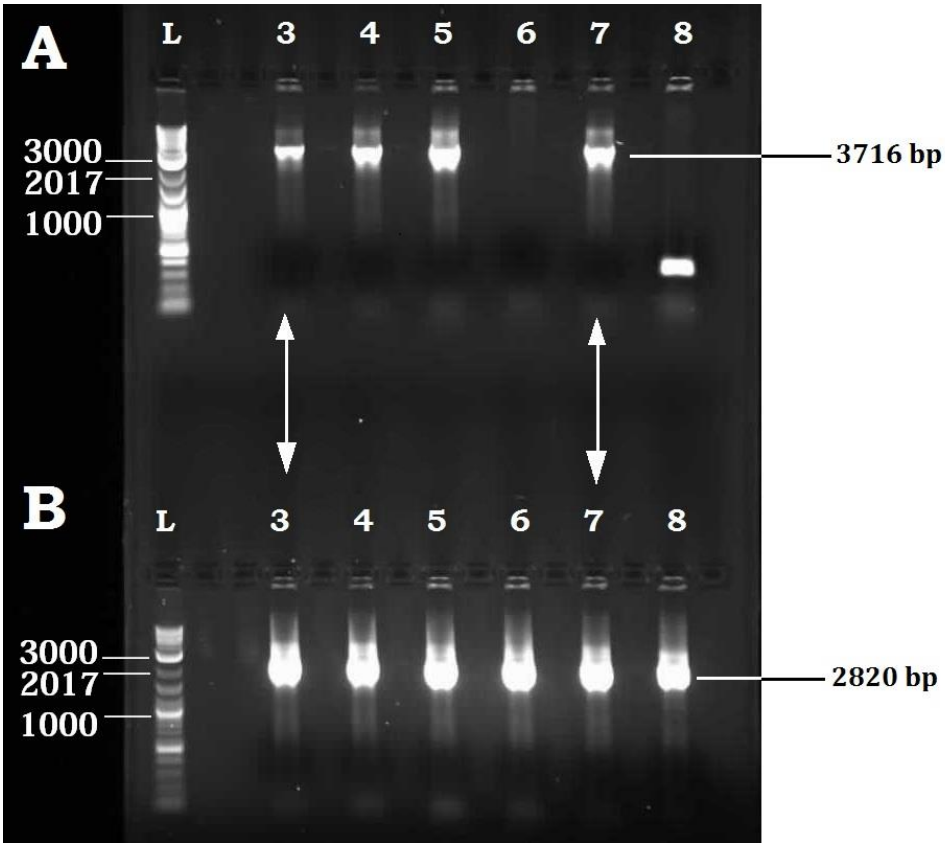


**Figure 8: PCR on DH10Bac transformed with pFastBac/HBM-TOPO *Cul o 1***

**Lanes: L** 2-log ladder **A 3-8** Culture 3-8 (3716 bp), **B 3-8** Culture 3-8 (2820 bp). The arrows point to the cultures that were chosen for isolation of rBacmids.

The rBacmid from cultures 3 and 7 were isolated and tested again with PCR using the same primers. Figure 9 shows the PCR products of rBacmid 3 and rBacmid 7 using both gene specific and M13 primers.

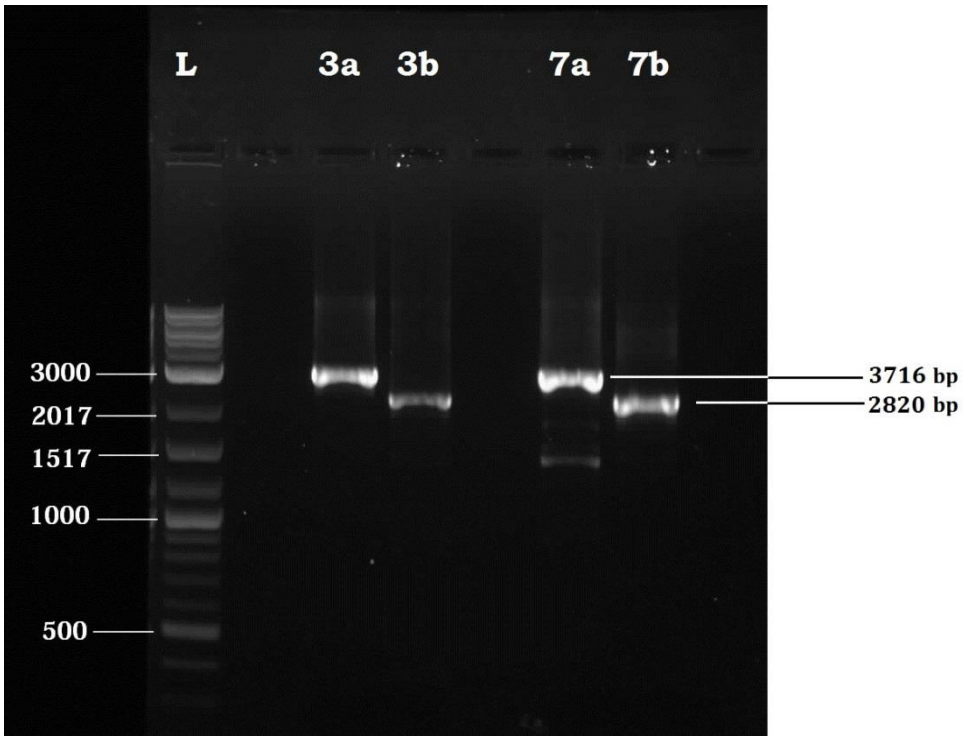


**Figure 9: PCR on isolated rBacmid *Cul o 1***

**Lanes: L** 2-log ladder, **3a** rBacmid 3 (3716 bp), **3b** rBacmid 3 (2820 bp), **7a** rBacmid 7 (3716 bp), **7b** rBacmid 7 (2820 bp).

The DNA concentration of the rBacmids were measured (Table 8).

**Table 8: Concentration and purity of the rBacmids**

| rBacmid | Concentration (ng/µL) | Purity (260/280) |
|---|---|---|
| rBacmid *Cul o 1* (3) | 14.81 | 1.70 |
| rBacmid *Cul o 1* (7) | 28.42 | 1.68 |

## 4.5 Transfection of Sf-9 cells

Sf-9 cells were transfected with the rBacmids to generate rBaculoviruses for expression of the Cul o 1 allergen. Sf-9 cells were transfected with Cellfectin®II using 0.5 µg of rBacmid 3, and 1 µg of rBacmid 7. Negative controls of medium with and without Cellfectin®II were also made. The transfections were done as shown in table 9.

**Table 9: Schematic setup of the transfection of Sf-9 cells with rBacmids in a 12 well plate**

| | | | |
|---|---|---|---|
| | 1. rBacmid 3 (0.5 µg) | 2. rBacmid 7 (1 µg) | |
| | 3. Cellfectin control | 4. Medium control | |

The cell cultures were observed closely in an inverted light microscope (Leitz Diavert) for signs of cytopathic effect (Table 10).

**Table 10: Cytopathy of Sf-9 cells transfected with rBacmid *Cul o 1* and negative control cells observed in a light microscope**

| Well | Signs of cytopathic effect caused by virus infection |
|---|---|
| 1. rBacmid 3 (0.5 µg) | No signs of cytopathy were observed the first 48 hours of infection. Some ghost cells were present, but that did not differ from the Cellfectin and medium control cells. Within 72 hours the cells varied in shape and size. The cells were flat and more dispersedly spread than the uninfected cells. Overall the cell diameter was increased, and there was a much higher number of lysed cells and ghost cells. The population of cells was less dense in the middle of the wells; this indicates that the medium and/or transfection solution had been added to the wells with too much force, thus causing detachment. (Figure X) |
| 2. rBacmid 7 (1 µg) | The cytopathic effects of were similar to that of rBacmid 3, but the cells were even more dispersed. (Figure X) |
| 3. Cellfectin control | The cells were round, uniform in size and shape, and densely populating the wells. A few cells were lysed, and some ghost cells were present. No signs of cytopathy caused by virus infection. The population of cells was less dense in the middle of the wells, as seen in the other wells, indicating medium and/or transfection solution being added with too much force. (Figure X) |
| 4. Medium control | The medium control looked very similar to the Cellfectin control. |

A B

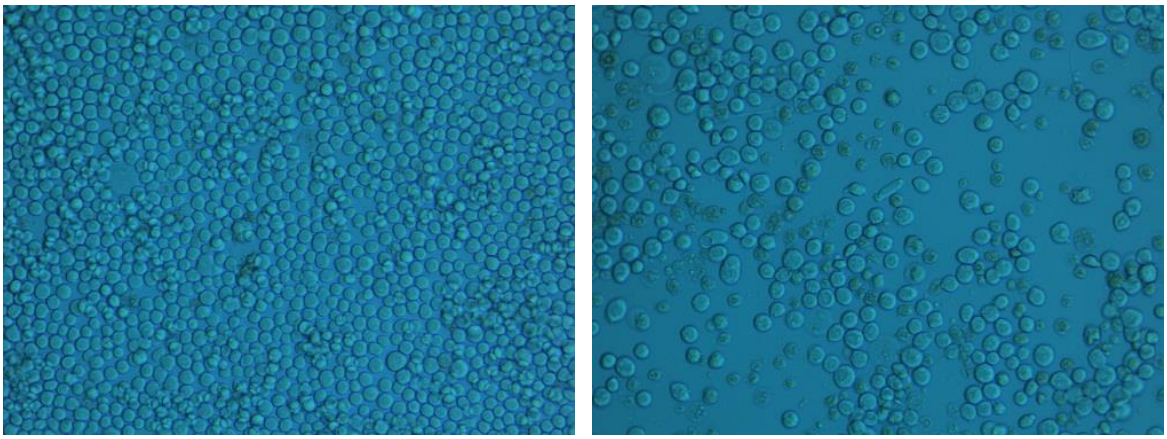

**Figure 10: Sf-9 cells post transfection seen in a light microscope.**

**A:** Cellfectin®II control, **B:** rBacmid *Cul o 1*. Day 6 post transfection, 400x magnification (Leica DM IL LED DFC 425 C).

## 4.6 Passage and production of rBac-HBM-Cul o 1 virus

After cytopathic effect was observed in the transfected cells, supernatant (200 µL) was transferred from the transfection plates into healthy Sf-9 cells in a 12 well plate as seen in table 11. When the first passage Sf-9 cells showed signs of cytopathy, supernatant (200 µL) was transferred into healthy Sf-9 cells in 4.5 mL of medium in T25-flasks (Nunc).

**Table 11: Schematic setup of the first passage of rBacmids in a 12 well plate**

| | | | |
|---|---|---|---|
| | 1. rBacmid 3<br>1st passage | 2. rBacmid 7<br>1st Passage | |
| | 3. Cellfectin control<br>1st passage | 4. Medium control<br>1st passage | |

The cell cultures were observed closely in an inverted light microscope (Leitz Diavert) for signs of cytopathic effect (Table 12).

**Table 12: Cytopathy of Sf-9 cells infected with rBac-HBM-Cul o 1 and negative control cells observed in a light microscope**

| Well | Signs of cytopathic effect caused by virus infection |
|---|---|
| 1. rBacmid 3, first passage | Within 48 hours the overall cell diameter was increased, and the shape of the cells was variable. There was a bigger variation of size than the control cells, and more lysed cells and ghost cells were present. The cells were flat and more dispersedly spread than the uninfected cells. |
| 2. rBacmid 7, first passage | The cytopathic effects of were similar to that of rBacmid 3. (Figure X) |
| 3. Cellfectin control | The cells were round, uniform in size and shape, and densely populating the wells. A few cells were lysed, and some ghost cells were present. No signs of cytopathy caused by virus infection. (Figure X) |
| 4. Negative control | The negative control looked very similar to the Cellfectin control. |

A B

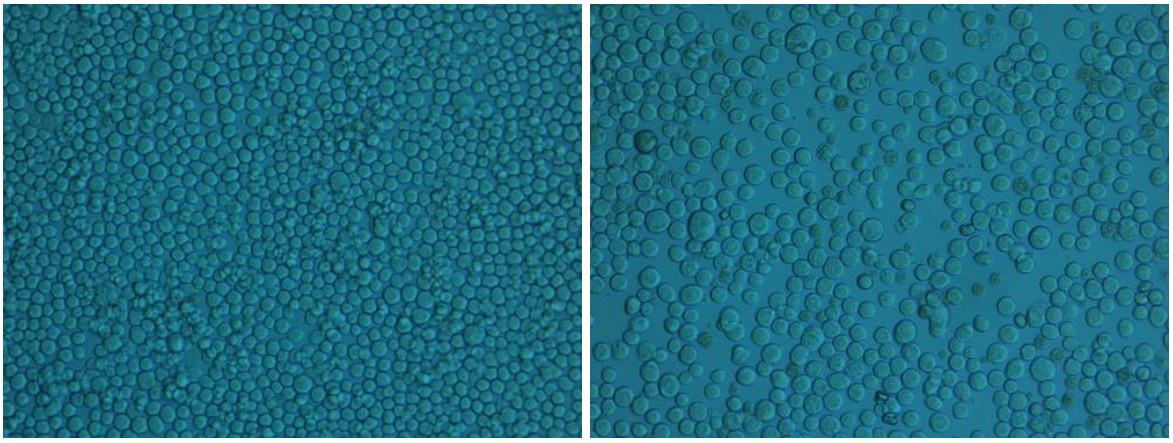

**Figure 11: Sf-9 cells infected with baculovirus seen in a light microscope.**

**A:** Cellfectin®II control, **B:** rBac Cul o 1. Day 3 post infection with first passage, 400x magnification (Leica DM IL LED DFC 425 C).

## 4.7 Expression of rBac-HBM-Cul o 1 protein

The cells and medium from the wells were harvested and spun, and supernatant (virus stock) was separated from the cells. The cell pellets were lysed, and to both supernatant and cell lysate sample buffer was added before the proteins were separated in SDS-PAGE. The proteins were transferred (Western Blot) to a PVDF membrane (Millipore), incubated with primary and secondary antibodies, and then developed. The expression of rBac-HBM-Cul o 1 protein was detected in the pellet samples, as bands of the expected size, approximately 26 kDa (Figure X). The supernatant and Cellfectin®II samples did not show any protein bands.

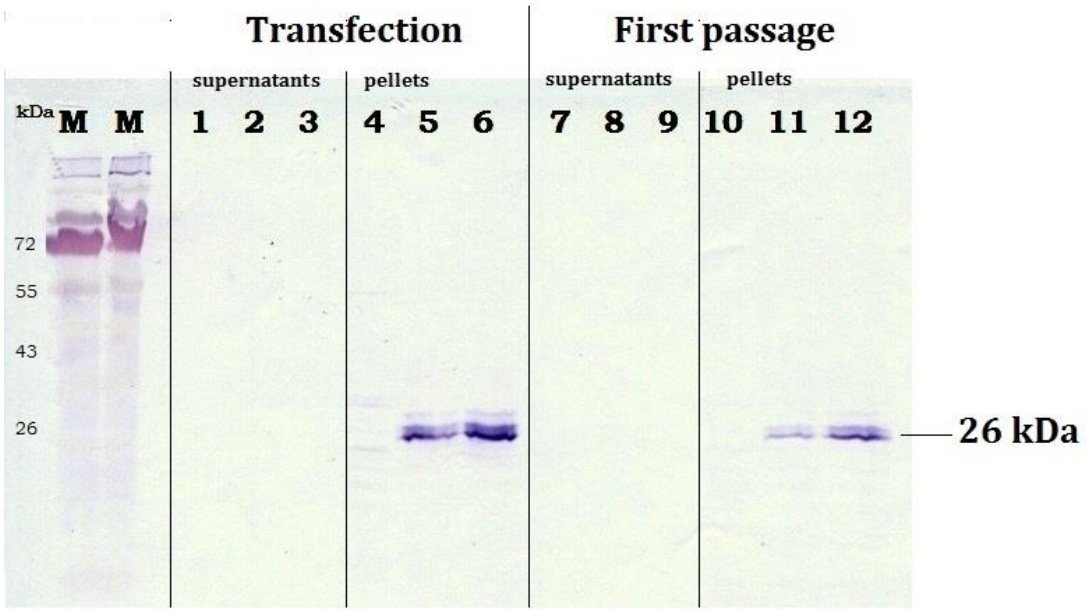


**Figure 12: Western Blot of rBac-HBM-Cul o 1 after transfection and first passage**

**Lane M** PageRuler, **Lanes 1,4,7,10** Cellfectin® control, **Lanes 2,5,8,11** rBacmid 3 (0.5 µg), **Lanes 3,6,9,12** rBacmid 7 (1 µg).

# 5 Discussion

IBH is caused by proteins originated from the salivary glands of *Culicoides* spp. From the salivary glands of *C. nubeculosus,* 54 proteins have been identified (Russell et al., 2009). Some of these proteins were major IBH allergens, and some were minor (Schaffartzik et al., 2011).

Laboratory breeding of *C. sonorensis* and *C. nubeculosus* made it possible to create a cDNA library of *Culicoides, identify and isolate the allergen gene sequences*. Once this had been achieved, allergens from other *Culicoides* species could be isolated by using sequence homology searches (BLAST search). (van der Meide et al., 2013)

Presently, 11 recombinant proteins from *C. nubeculosus* have been expressed and recognized by IgE from IBH affected horses as allergens, with a frequency of IgE sensitization against the single allergens ranging from 13-57 % (Schaffartzik et al., 2011). Seven recombinant proteins have been expressed from *C. obsoletus*, with a percentage of positive tested IBH affected horses ranging from 38-67 % (van der Meide et al., 2013).

Allergenicity testing has shown that IBH-affected horses react to native and exotic species of *Culicoides*, but local species induce a stronger IgE-mediated immune response (Anderson et al., 1993). This indicates presence of both shared and specific allergens in the different species. Van der Meide et al showed that IBH affected horses in the Netherlands have higher IgE levels specific for the Cul o 1 allergen deriving from the local *C. obsoletus*, than the Cul s 1 allergen from the less common *C. sonorensis*, even though they were the same protein, a maltase (van der Meide et al., 2013). It's therefore crucial to isolate allergens from local species for development of diagnostic tests for IBH.

The *Cul o 1* and *Cul o 2* sequences in this project are not the same sequences as any of the *C. obsoletus* genes published by van der Meide et al., but as the proteins are both proven to be major allergens (Prof. Eliane Marti, Personal Communications), it is important to express them.

The aim of this project was to express *Cul o 1* and *Cul o 2* allergen genes in insect cells with the Bac-to-Bac® Baculovirus expression system.

*Cul o 1* has been expressed successfully with HBM. Sf-9 cells were transfected with rBacmids that contained the correct sequence. The Sf-9 cells showed cytopathic effects consistent with efficient expression of rBac-HBM Cul o 1 proteins according to the protocol (Invitrogen). The protein was visualized in western blot using anti-his antibodies as primary antibody (1:1000) and alkaline phosphatase for detection. This gave a rather weak but clear visualization of the protein,

The protein was also visualized with ChemiFast Chemiluminescent Substrate Kit (Syngene), and this gave stronger protein bands (Data not shown).

The his-tag specific antibody is a rather poor antibody compared to the polyclonal protein specific antibodies that have been made against the allergens from *C. nubeculosus* (Schaffartzik et al., 2011).

*Cul o 2* has been TOPO® cloned and verified by sequencing. The TOPO vector is therefore ready to transform DH10Bac *E. coli* cells for production of recombinant bacmid that can transfect Sf-9 insect cells.

The next steps are transfection and generation of rBac-HBM-Cul o 2 virus, and cloning and titration of both rBac-HBM-Cul o 1 and rBac-HBM-Cul o 2 viruses in Sf-9 cells. After that, production of rBac-HBM-Cul o 1 and rBac-HBM-Cul o 2 proteins in High Five™ insect cells, and purification of the hexahistidine tagged proteins. The proteins will then be tested for allergenicity and used in immunotherapy experiments.

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# 6 Appendices

## 6.1 Appendix I - Primers used in this work

| Name of gene | Nucleotide sequence |
|---|---|
| **Cul o 1:** | |
| Culo1_58-81_Fw | 5′-GAGGCATTAGCAAAAAAGAAAAAG-3′ |
| Culo1_181-201_Re | 5′-CAAACCAATGTGGCCCAGTA-3′ |
| Culo1_436-456_Fw | 5′-CACGAGTTAAGCCAGCATAC-3′ |
| Culo1_595-615_Re | 5′-GGGAATCGAACGCAAAACAA-3′ |
| **Culo2:** | |
| Culo2_64-83_Fw | 5′-GGCACTCCAGGAACGACAAG-3′ |
| Culo2_172-192_Re | 5′-GCTGTCACTTACACTGCGTA-3′ |
| Culo2_312-332_Fw | 5′-GAGGATTGCAACATACGGGA-5′ |
| Culo2_433-453_Re | 5′-CAGAGAAACGTTCCGAGAAG-3′ |
| **Vector primers:** | |
| pFastBac-Fw | 5′-GGATTATTCATACCGTCCCA-3′ |
| pFastBac-Re | 5′-CCAATGTGGTATGGCTGATT-3′ |
| M13 Fw | 5′-GTTTTCCCAGTCACGAC-3′ |
| M13 Re | 5′-CAGGAAACAGCTATGAC-3′ |

## 6.2 Appendix II – Buffers and solutions used in this work

**0,5x TBE**

0.045 M Tris borate, 0.001 M EDTA

**RBS (restriction buffer)**

50% glycerol, 15 mM EDTA, 0.25 % NaCl

**LB medium**

1 % Tryptone, 0.1 % yeast extract, 1 % NaCl

**LB agar**

1 % Tryptone, 0.1 % yeast extract, 1 % NaCl, 1.0 % Bacto agar

**SOC medium**

2 % Tryptone, 0.5 % yeast extract 0,05 % NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM Glucose

**2x sample buffer**

0.5 % 2-mercaptoethanol, 20 % glycine, 2 % SDS, 0.1 % Bromphenol blue, 130 mM Tris.

**Transfer buffer**

25 mM Tris, 192 mM glycine, 20 % methanol.

**BCIP/NBT**

5-bromo-4-chloro-3-indolyl phosphate / Nitro blue tetrazolium chloride

**Alkaline phosphatase buffer**

100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl2, 0.05 % Tween 20, pH 9.5

**X-gal**

Bromo-chloro-indolyl-galactopyranoside

**IPTG**

Isopropyl β-D-1-thoigalactopyranoside

**SF900II medium (PEST).**

SF900II, 100 IU penicillin, 100 IU streptomycin, 2 % fetal bovine serum (FBS)

**TBS-T**

Tris buffered saline with Tween 20 (0.1 %)

**Lysis buffer**

50 mM $NaH_2PO_4$, 150 mM NaCl, 1 % Igepal

## 6.3 Appendix III - Nucleotide and amino acid sequences of *Culicoides Obsoletus*

### 6.3.1 *Cul o 1*

618 bp

ATGCGATTTGCAACGATTTTCCTGTTATCAGCTTCAATAATTTTGCTTAGCACTGGCGAGGCATTAGCAAAAAAG
AAAAAGAAGATTGATAAAAGTATGCCACCTGAATGTCTTCCGTTGCCTCCAAAGAAAAGAGCTTCCGAATGCACA
AATCAATCTGGATTCAAGTATTATCCAAAAACAAACCAATGTGGCCCAGTAAGGAATCAATTGTGTCAAGGTCAT
GGTGGTTTCACGACATTAGACGAGTGTGTGTACAAATGTTATGACTATCGGAAAATGTCTACTACCAAGAACGTG
GATGGGTGCAACAAATCAATCAAAGAGGAGGAAATGACAGAAGCCATTCGCGTAGTTAGTCGTGGTGATAGTCCT
GACCTCATTAAAGATAATCGTTGCCGAGAGCCCAATGAACTTAGTCTTAAGAACGGAAGTGCACGAGTTAAGCCA
GCATACAAATTTAACAAGGACACGAATGAATGCGTTGCAATGATGGACAAAGTTTGTCTCGGACGTAATAGATTC
AAGACGAAGGAAGAATGTGTCCATGTCTGTAACTGGAACTTGTCTTCCGGTCGCCATAGACATATTGTCAGGGAA
TCGAACGCAAAACAA**TAA**

205 a.a.

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

Signal sequence

Primer for amplification of the gene without the signal sequence and stop codon (558 bp)

Primer for sequencing the gene

### 6.3.2 pFastBac/HBM-TOPO *Cul o 1*

681 bp

ATGAAATTCTTAGTCAACGTTGCCCTTGTTTTTATGGTCGTATACATTTCTTACATCTATGCGGATCGATCCCTT
GAGGCATTAGCAAAAAAGAAAAAGAAGATTGATAAAAGTATGCCACCTGAATGTCTTCCGTTGCCTCCAAAGAAA
AGAGCTTCCGAATGCACAAATCAATCTGGATTCAAGTATTATCCAAAAACAAACCAATGTGGCCCAGTAAGGAAT
CAATTGTGTCAAGGTCATGGTGGTTTCACGACATTAGACGAGTGTGTGTACAAATGTTATGACTATCGGAAAATG
TCTACTACCAAGAACGTGGATGGGTGCAACAAATCAATCAAAGAGGAGGAAATGACAGAAGCCATTCGCGTAGTT
AGTCGTGGTGATAGTCCTGACCTCATTAAAGATAATCGTTGCCGAGAGCCCAATGAACTTAGTCTTAAGAACGGA
AGTGCACGAGTTAAGCCAGCATACAAATTTAACAAGGACACGAATGAATGCGTTGCAATGATGGACAAAGTTTGT
CTCGGACGTAATAGATTCAAGACGAAGGAAGAATGTGTCCATGTCTGTAACTGGAACTTGTCTTCCGGTCGCCAT
AGACATATTGTCAGGGAATCGAACGCAAAACAAAAGGGCGAAAACTTGTACTTTCAAGGCCATCACCATCACCAT
CACTAG

26 kDa total, 23 kDa without HBM, 226 a.a.

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

HBM

Cloning site

Cul o 1

Cloning site

TEV site

6xHis

STOP

### 6.3.3 *Cul o 2*

456 bp

ATGACAACTTTTAAAACATTTATCGTTCTTTTATCAATTGCTTTGTCGTCGCTTGTCGATGCTGGCACTCCAGGA
ACGACAAGATGTGAGAAGAAAAATAAACTTTCAAAGGAAACAATCGACACCTACCATTCATGGCGGATGCCAACA
TCGTTTCGGACAAAAGCAGAAAGCTGTCACTTACACTGCGTATTAGAGAAAATCGGTTGGATGAAAGGACATAAA
ATATTGGATAAGGAAATAAATTCTGATATTAAAGCATCGAAGGAATCTCCGGAAGCACGATCCTCCCATTTGAGA
ACTCTCTTATTTGAGGATTGCAACATACGGGAAAACAATAAGAAGGATAAGTGCAAGAAAGCTCGCGATGTCTAC
AAATGTCTGGTAGAGAAATTCGAGCCGAGAAGTACCTTTCAGAAAGCATTCGAACGTGCAGAGAAACGTTCCGAG
AAG**TAG**

151 a.a.

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

Signal sequence

Primer for amplification of the gene without the signal sequence and stop codon (390 bp)

Primer for sequencing the gene

### 6.3.4 pFastBac/HBM-TOPO *Cul o 2*

513 bp

ATGAAATTCTTAGTCAACGTTGCCCTTGTTTTTATGGTCGTATACATTTCTTACATCTATGCGGATCGATCCCTT
GGCACTCCAGGAACGACAAGATGTGAGAAGAAAAATAAACTTTCAAAGGAAACAATCGACACCTACCATTCATGG
CGGATGCCAACATCGTTTCGGACAAAAGCAGAAAGCTGTCACTTACACTGCGTATTAGAGAAAATCGGTTGGATG
AAAGGACATAAAATATTGGATAAGGAAATAAATTCTGATATTAAAGCATCGAAGGAATCTCCGGAAGCACGATCC
TCCCATTTGAGAACTCTCTTATTTGAGGATTGCAACATACGGGAAAACAATAAGAAGGATAAGTGCAAGAAAGCT
CGCGATGTCTACAAATGTCTGGTAGAGAAATTCGAGCCGAGAAGTACCTTTCAGAAAGCATTCGAACGTGCAGAG
AAACGTTCCGAGAAGAAGGGCGAAAACTTG TACTTTCAAGGCCATCACCATCACCATCACTAG

20 kDa total, 17.3 kDa without HBM, 170 a.a.

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

HBM

Cloning site

Cul o 2

Cloning site

TEV site

6xHis

STOP