Insect Bite Hypersensitivity in Horses

Expression of allergens from *Culicoides nubeculosus,* Cul n 1 and Cul n 2, in insect cells

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Thesis for degree of Bachelor of Science

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HÁSKÓLI ÍSLANDS

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Abstract

Insect bite hypersensitivity (IBH) or summer eczema is a recurrent allergic dermatitis of horses, an IgE- mediated reaction to biting flies of the genus *Culicoides.* Although all breeds of horses can be affected, this condition is especially prevalent in horses born in Iceland and exported to the continent. The present project is a part of a larger project aiming at developing immunotherapy against (IBH). The allergene genes of *Culicoides nubeculosus* originated in the salivary glands of the flies, have been expressed and the proteins produced in *E. coli.* It is of critical importance for monitoring the immune response following immunotherapy to have access to purified allergens that are glycosylated and close to the native form. Two of the most important allergens *Cul n 1* and *Cul n 2* have already been expressed in insect cells but the purification has proven extremely difficult. The aim of this project was therefore to express these two allergens again with the signal peptide of honey bee melittin using the Bac-to-Bac Baculovirus expression system. This is to be able to purify the recombinant proteins for use in diagnostic tests and immunotherapy experiments of insect bite hypersensitivity

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Abbrevations

APCs	Antigen Presenting Cells
B-Cells	B-lymphocytes
DNA	Deoksyribonukleinsyre
ELISA	Enzymed-linked immunosorbent assay
FBS	Fetal bovine serum
IBH	Insect bite hypersensitivity
IL	Interlevkin
MHC	Major histocompatibility complex
NLRs	NOD-like receptors
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PRRs	Pattern recognition receptors
rBAC	Recombinant Baculoviruses
rBacmid	Recombinant Bacmid
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sf-9 cells	*Spodoptera frugiperda*
T-cells	T-lymphocytes
TBS	Tris buffer saline
TBS	Tris buffer saline with tween 20
Th	T helper cells
TLRs	Toll like receptors
Treg	T regulatory cells
WB	Western blot

1 Introduction

1.1 The immune system

The immune system is an advanced system where different types of cells, structures and biological processes are coordinated, to protect against foreign microbes and non-self particles or toxins. Its tasks is to detect the presence of an infectious intruder and deal with the infection through different effector mechanism. It has an immunological memory against reocurring antigens. The system also has regulating mechanism, so it doesn't overreact to harmless environmental factors, or mistake self from non-self (Murphy et al., 2012)

An antigen is any substance that the immune system can register and act against. The immune system is divided into innate and adaptive immunity. The innate is the first line of defense and reacts quickly, but without memory. The adaptive immunity takes longer time to activate, but is highly specific and has memory (Murphy et al., 2012).

Organs of the immune system are divided into primary (central) and secondary (peripheral) organs. Included in the primary lymphoid organs are the bone marrow and the thymus. The secondary tissues are lymph nodes, the spleen, and the mucosal lymphoid tissues of the gut, the nasal and the respiratory tract, the urogenital tract and other mucosa, where antigens are detected and presented to lymphocytes. The cells of the immune system are the white blood cells (leukocytes). They originate from the pluripotent hematopoietic stem cells of the bone marrow. The cells use cytokines and chemokines for communication (Murphy et al., 2012).

1.1.1 The innate immune system

The innate immunity is the first line of defense and acts fast against infection, but can't create immunological memory. The cells included in the immediate defense are granulocytes, monocytes/macrophages, dendritic cells and mast cells. These cells derive from the myeloid lineages (Murphy et al., 2012).

Neutrophils are abundant in the bloodstream, and phagocyte pathogens, and/or destroys them with toxic substances from their granules. Basophils and eosinophils play a role in parasitic infection and allergies, by releasing toxic proteins and enzymes upon activation. Natural Killer cells recognize abnormal cells, and induce

apoptosis by secreting toxic substances from their granules. Monocytes, an early stage of macrophages, ingest and destroy foreign microorganisms and cancer cells, but can also work as antigen presenting cells (APC). Mast cells are granule-rich cells acts in the defense against parasites, and are a big participant in allergic reactions, where they release histamine upon activation. Dendritic cells are present in the skin and inner linings of lungs, stomach and intestines. They phagocytose antigens in the peripheral tissue, degrade them and migrate to the lymph nodes where they present them on Major Histocompatability complexes (MHC) to the T-cells from the adaptive immune system. This creates a bridge between the innate and the adaptive immune system, making them the most important APCs of the immune system (Murphy et al., 2012).

APCs, like dendritic cells, recognize pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs), like the toll like reseptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs). Examples of PAMPs are lipopolysaccaride (LPS), peptidoglycans and RNA from viruses(Murphy et al., 2012).

1.1.2 The adaptive immune system

The adaptive immune system is highly specific because of somatic hypermutation and VDJ recombination, generating many different antigen receptors and making it recognize a tremendous number of molecules. Cells of the adaptive immune system include B-lymphocytes and T-lymphocytes, cells that derive from the lymphoid lineage. The effector cells in the cell-mediated immunity are the T-cells, which recognizes antigens that are presented to them by APCs on their MHCs. T-cells are divided into $CD4^+$ T helper (Th) cells and $CD8^+$ cytotoxic T cells. $CD8^+$ cytotoxic T recognize antigens on MHC class I and kill virus infected cells. $CD4^+$ Th cells recognize antigens on MHC class II. Using cytokines the APCs decide wich subsets of the Th cells to activate according to the invading pathogen. The Th subset then continue the response producing the relevant cytokines (Figure 1). (Murphy et al., 2012).

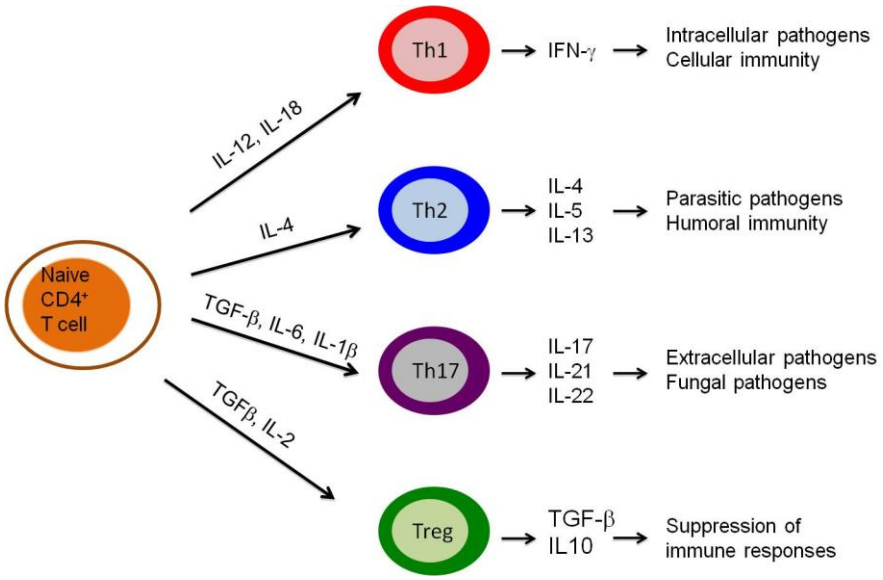


Figure 1. Differentiation of naïve CD4+ T helper cells.
Differentiation of naïve CD4+ T helper cells into different T effector subsets, the main cytokines and the function of the four subsets. Schematic picture: Sigurbjörg Torsteinsdóttir.

B-cells produce antibodies which constitute the humoral response. Upon activation under control by cytokines from Th cells, they produce antibodies (immunoglobulin) with different Isotypes depending on the pathogen. Cytokines from the Th cells can trigger proliferation of B-cells or make them differentiate into memory B-cells, which makes the immunological memory (Murphy et al., 2012).

The immune system of horses uses the same cells, mechanisms and mediators as laboratory mice and men (Lunn et al., 1998). However horses are infected with parasites especially Helminths, causing a T- helper 2 immune response with abundant IgE production and high levels of IL-10(Carvalho et al., 2006; Eydal and Gunnarsson, 1994; Jonsdottir, 2011a; Wilson and Maizels, 2006).

1.2 Allergy

Allergy, or hypersensitivity type 1, is an IgE mediated disorder of the immune system. It occurs when the immune system reacts to a substance in the environment that originally is harmless. Antigens that cause this reaction are called allergens. In allergy, the mast cells and basophils are abundant, mast cells in the tissue and basophils in the blood. At the first encounter with the allergen, signals from Th2 cells, together with cytokines such as IL-4 and IL-13 drive antibody class-switching towards

IgE. After activation, mast cells and basophils also secret IL-4 and IL-13, which also drive the production of IgE by B cells. Mast cells and basophils express an high affinity IgE Fc reseptor(Fcεri). At the first encounter with an allergen, the individuals produce IgE wich binds to the Fcεri, and the cells are sensitized. On the subsequent exposure the allergen cross links the receptors, causing the cells to secret the content of their granules (histamine, proteases and serine esterases), eliciting an allergic reaction (Figure 2).

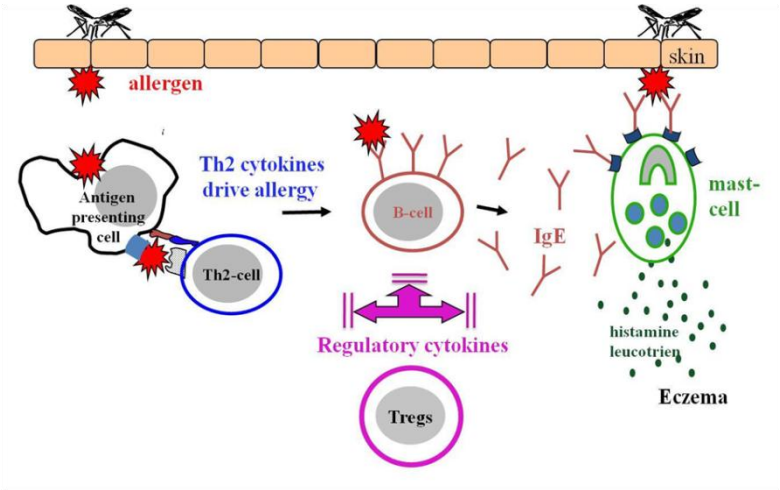


Figure 2. Th2 immune respons in IBH

Eosinophiles are also an active participant in the inflammatory reaction during allergy. They have receptors for cytokines such as IL-5, which activates the cells and stimulate them to degranulate. They can then release toxins that cause tissue damage in allergic reactions, or chemical mediators that amplify the inflammatory response (prostaglandins, leukotrienes and cytokins) (Murphy et al., 2012).

1.3 What is IBH

Insect bite hypersensitivity (IBH) is a recurrent allergic dermatitis that affects the skin of horses (allergic dermatitis) (Bröstrom et al., 1987). Summer eczema as the allergy also is called, is caused mainly by proteins in the saliva from glands of the biting midge of the genus *Culicoides (Baker and Quinn, 1978; Bröstrom et al., 1987; Hellberg et al., 2006). Culicoides* makes a lesion in the skin through their bites and inject proteins that give an IgE mediated response (van der Haegen et al., 2001)

Horses suffering from IBH show strong pruritus (itch) and dermatitis (eczema), that affects mainly the dorsal midline, and the base of the mane and tail (Bröstrom et al., 1987; Townley et al., 1984). Scratching the inflamed area can cause alopecia (hair loss), hyperkeratosis (thickening of the skin) and excoriation (skin lesion). The skin lesion makes them subseptible for secondary bacterial infections (Baker and Quinn, 1978). The symptoms appear during spring to autumn when *Culicoides* spp are active and regress during the winter (Schaffartzik et al., 2012).

The prevalence of IBH varies and principally all horse breeds can be affected. It is prevalent in Icelandic horses exported to the European continent (Anderson et al., 1988; Bröstrom et al., 1987; Lange et al., 2005). *Culicoides* spp are not indigenous to Iceland, and it is thought that the lack of exposure to the *Culicoides* allergens in an early age could be the reason for the high prevalence (Bjornsdottir et al., 2006). IBH is an animal welfare issue. It gives very unpleasant symptoms that needs veterinary attention, making the horses periodically unfit for riding. In severe cases they have been euthanized (Anderson et al., 1988).

IBH is diagnosed mainly on clinical signs but also with intradermal tests and *in vitro* tests. The *in vitro* tests are either serological tests or tests that measure cellular responses after *in vitro* stimulation of allergens from *Culicoides* exracts. There is currently no treatment exept from preventing the horse to get in touch with the allergen, by using insect spray or blankets. Steroids can be used in severe cases. (Baker and Quinn, 1978).

IgE mediate reactions as mast cell degranulation, and are shown in various studies to be involved in IBH (Baselgia et al., 2006; van der Haegen et al., 2001; Wagner et al., 2006b). In Icelandic born horses it has been indicated that there is an imbalance between Th1, Th2 and T regulatory cells, both in the peripheral blood mononuclear cells and the skin (Hamza et al., 2007; Hamza et al., 2008; Heimann et al., 2011).

1.4 *Culicoides*

Culicoides is a genus of biting midges in the family Ceratopogonidae, belonging to the order Dipteria. They are varying from 1 to 3 mm in size, with a wingspan of 2 mm. There are over 1400 species, distributed worldwide except from Iceland, Antarctica, New Zealand and the far South of America (Featherstone, 2010; Mordue and Mordue, 2003). Only the female flies bites, as they need blood meals for egg

production. They are vectors for bluetongue and African horse sickness virus, and can cause decease in horses, sheep and cattle (Mordue and Mordue, 2003). Many studies have reported *Culicoides* allergens as relevant allergens in IBH (Anderson et al., 1993; Hellberg et al., 2006; Van der Meide N. M. et al., 2013). Some of the species of *Culicoides* that have been reported to cause IBH are *C. obsoletus* (Anderson et al., 1991; Townley et al., 1984), *C. pulicaris (Mellor and McCraig, 1974; Townley et al., 1984), C. imicola (Braverman et al., 1983)*, and *C. nubeculosus (Schaffartzik et al., 2010; Schaffartzik et al., 2011)*. When *Culicoides* bite, they secret important pharmacological compounds to overcome physical barriers on the skin, coagulation pathways and other immune responses from the host, so it can feed (Russell et al., 2009).

Culicoides nubecolousus

11 of the salivary proteins from *C. nubecolousus* have been shown to bind serum IgE from IBH affected horses in *in vitro* test. Nine of these have been used in intradermal tests mediating immediate response in the IBH affected horses but not in healty control horses. This shows that 9 of the *C. nubecolousus* allergens are relevant for IBH, possibly 11(Schaffartzik et al., 2010; Schaffartzik et al., 2011). C. *nubecolousus* can breed in captivity, while others, like *C. obsoletus* must be caught in the wild (boorman 1985; van der Meide NM et al., 2012).

1.5 Insect allergens

Innocuous antigens that induce an immune reaction are called allergens. Some define allergenicity as the ability to induce T- helper 2 responses with production of allergen specific IgE antibodies on second exposure. Most of the allergens are proteins and there have been attempts to find similarities among allergens, but nothing is for certain yet. It has been indicated that biochemical appearances like enzymatic activity or lipid-binding activity is important, or their ability to interact with the innate immune system through interaction with TLRs, non-TLR pathogen recognition receptors or to work as dimers to mediate activation of effector cells. An allergen is considered to be a major allergen when it binds IgE antibodies in more than 50 % of allergic individuals. A minor allergen binds IgE in less than 50 % (Deifl and Bohle, 2011).

Insect bite or sting cause nuisance for some human and animals, causing allergic reactions as they injects different immune transforming substances to the host (Arlian, 2002; Russell et al., 2009). An attempt to remove the salivary glands from mosquitos showed that the allergens are in the salivary gland. Though the salivary glands ducts were remove, they could still feed on laboratory rabbits and men, without causing allergy (Hudson et al., 1960)

In vitro testing shows that there is a variation among *Culicoides* species when it comes to create an IgE mediated response. Though some allergens are shared among spescies, as horses affected by IBH that were injected with extract from native and exotic *Culicoides*, reacted to both.

Fifty four novel protein sequences from the salivary glands of *C. nubeculosus* have been identified using mass spectrometry (Russell et al., 2009). Western blot analysis of 11 recombinant *C. nubeculosus* proteins showed that they were recognized by serum IgE from IBH affected horses. These proteins were isolated using phage display technology the proteins, derived from 1400 dissected salivary glands, were isolated and have been named after the systemic allergen nomenclature, Cul n 2-Cul n 11. Cul n 1 was already isolated at that time. Cul n 1- Cul n 4 were reported as major allergens (Schaffartzik et al., 2010; Schaffartzik et al., 2011). Cul n 1 (Antigen 5) and Cul n 2 *(*Hyaluronidase) are the subjects for this project.

Cross-reactivity has been detected by inhibition ELISA-tests, between antigen 5 like protein from *C. nubeculousus* (Cul n1) and *S. vittatum* (Sim v 1) ,and also that the allergens share common IgE binding epitopes (Schaffartzik et al., 2010). Some think that sensitization to *S. vittatum* can be secondary to *Culicoides,* since *S. vittatum* is present on Iceland, but horses living there do not exibit symptoms of IBH before export to the continent, where they are exposed to *Culicoides* (Anderson et al., 1988; Bröstrom et al., 1987; Lange et al., 2005; Schaffartzik et al., 2012).

1.6 Antigen 5

The antigen 5 protein has been reported in insects belonging to Dipteria and Hymenopteria families (King and Spangfort, 2000; Lu et al., 1993). It is a member of the CAP (cysteine-rich secretory proteins (CRISPs), antigen 5, and pathogenesis-related-1 (PR-1)) superfamily, that share amino acid sequence similarity and are found in eukaryotes (Gibbs et al., 2008; King and Spangfort, 2000; Lu et al., 1993)

CRISPs have been seen in snakes and lizards, and reproductive organs of mammals, antigen 5 are abundant in insect venom while PR-1 is seen in plants after infection (Gibbs et al., 2008; Hoffman, 2006). It's function is yet not understood, but it is thought that it suppress the hosts immune system or prevent blood coagulation to prolong bloodfeeding (Ribeiro and Francischetti, 2003).

1.7 Hyaluronidase

Hyaluronidases have been detected in prokaryotes and eukaryotes. They are a family of enzymes that can degrade hyaluronan, chondroitim and chondroitin sulfate and belong to the glycosidase family 56 (Stern and Jedrzejas, 2006). Hyaluronidases are allergens in honey bees and vespids and have been reported in salivary glands of sand flies, black flies, mosquitoes and ticks (Arlian, 2002; Ribeiro et al., 2004; Ribeiro et al., 2000; Soldatova et al., 1998). Hyaluronidase from *C. nubeculosus* binds to serum IgE of IBH affected horses and has been reported as a major allergen in IBH (Schaffartzik et al., 2011).

1.8 Baculoviruses

Baculoviruses are are double-stranded, circular, supercoiled DNA molecules in a rod shaped capsid, widely used to produce proteins in Sf-9 or High Five cells, from the butterfly larvae *Spodoptera frugiperda (O'Reilly et al., 1992; Possee, 1997)*. Baculoviruses infect arthropods and are nonpathogenic to mammals or plants making them safe to use in the laboratory. The most common Baculovirus used in gene expression is *Autographa californica* nuclear polyhedrosis virus (AcMNPV). The nucleocapsids ability to extend provides space for larger inserts(Fraser, 1986). Insects allergens have been successfully produced in the Baculovirus system, expressing proteins with the same antigenicity as the native protein making it a good choice of expression system (Schmidt and Hoffman, 2002; Schmidt et al., 1996; Soldatova et al., 1998; Xu et al., 1998).

The Baculovirus takes advantage of the host cells biological machinery and replicates in the nuclei of the infected host cells. There the large baculovirus DNA is packaged into rod-shaped nucleocapsids, enveloped and budded through the plasma membrane. During the early phase, the infected insect cell releases extracellular virus particles that bud of the cellmembrane. During the late phase occluded virus particles are assembled inside the nucleus. The occluded virus particles are embedded in a homogenous matrix made up mainly of one protein, the polyhedrin

protein. The particles are released during the very late phase of the virus life cycle, when the cells lyse. *In vitro* this can be used to produce a recombinant gene product by replacing the naturally occurring polyhedrin gene in the wild type genome, with recombinant gene or cDNA. During the transcription in the very late phase the inserted genes are controlled by the AcNPV polyhedrin promoter, expressing the recombinant genes, instead of the original polyhedrin protein that normally would be expressed in an unmodified Baculovirus (O'Reilly et al., 1992).

First the system took advantage of homologous recombination, where foreign genes have been cloned into a transfer vector and co-transfected with AcMNPV into Sf-9 cells, allowing recombination between homologous sites transferring the heterologous gene from the vector to the AcMNPV (Luckow et al., 1993). The Bac-to-Bac Baculovirus Expression System from Invitrogen uses site specific transposition instead, making it quicker to identify and purify the protein. The transposition takes place in a special *E. coli* strain DH10Bac, by replacing the polyhedrin gene in the Bacmid (containing the wild type genome), with recombinant gene or cDNA (Figure 3) (Invitrogen, 2009).

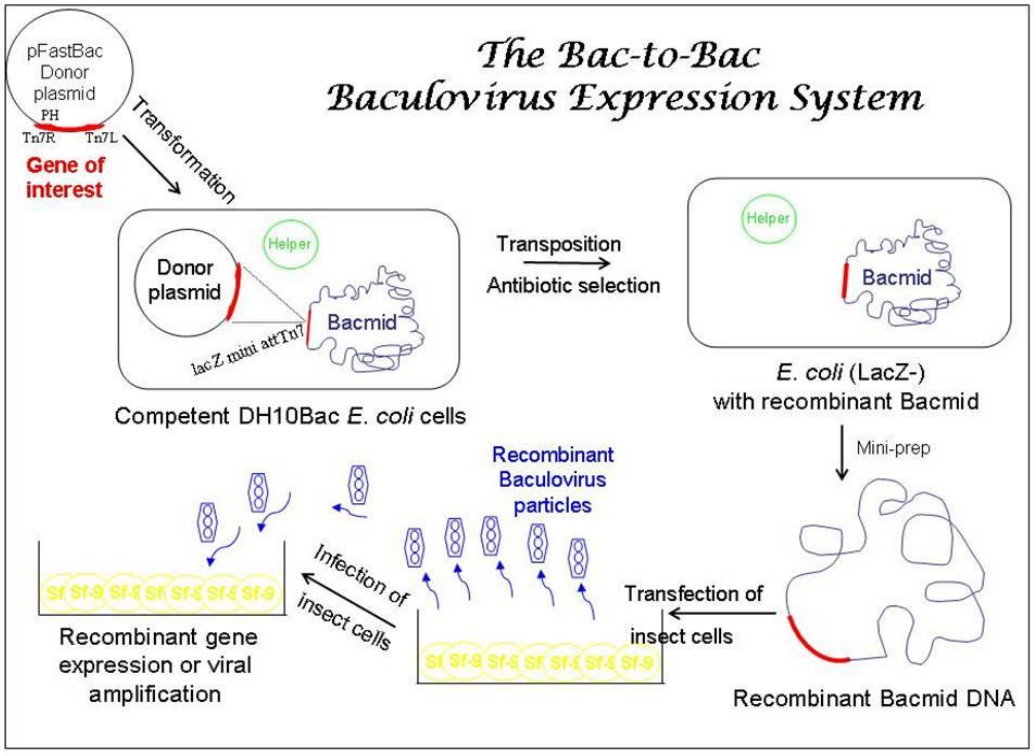


Figure 3. Baculovirus expression system (Invitrogen, 2009)

When an allergen is produced *in vitro* it can become modified, depending on the method, giving it a different structure or function (Kost et al., 2005; Patel and Jones, 1995; Schmidt and Hoffman, 2002). An example of that is when Soldotova et al produced honey bee venom hyaluronidase (hya) in *E. coli* and in a Baculovirus system, and discovered that biologic activity of Hya expressed in Baculovirus was comparable to the native form. Indicating that the conformation of the recombinant protein was similar the native protein. While the enzyme expressed in *E. coli* only showed a little of the same enzyme activity as the native hyaluronidase (Soldatova et al., 1998; Soldatova et al., 2007).

2 Aims of the study

Cul n 1 (antigen 5) and *Cul n 2* (hyaluronidase) are proteins that originate from the salivary gland of the midge *Culicoide nubeculosus* and are important allergens in insect bite hypersensitivity in horses. The aim of the project was to clone the two allergen genes *Cul n 1* and *Cul n 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. This is to be able to purify the recombinant proteins, for diagnostic test and for use in immunotherapy experiments of insect bite hypersensitivity.

3 Material and methods

3.1 Cloning of *Cul n 1* and *Cul n 2*

3.1.1 Origin of the genes

Originally the genes, *Cul n 1* (Antigen 5) and *Cul n 2* (Hyaluronidase), were amplified from λZAP II cDNA library (Schaffartzik et al., 2011). For this project a FastBac vector containing the genes from the λZAP II cDNA library was used. *Cul n 1* was amplified from pFastBac Ag5B and *Cul n 2* was amplified from pFastBac Hya.

3.1.2 PCR

The genes *Cul n 1* and *Cul n 2* were amplified with PCR from a FastBac vector containing the genes from the λZAP II cDNA library. The PCR reactions were performed in DNA Engine® Peltier Thermal Cycler (PTC-200) from MJ Reaserch. The Taq DNA polymerase or Phusion™ Hot Start DNA polymerase was used in the reactions.

PCR with Phusion Hot Start DNA polymerase

Cul n 1 and *Cul n 2* were amplified to make blunt end PCR product for TOPO cloning, using a Phusion™ Hot Start DNA polymerase (Finnzymes).

PCR reaction solution		**PCR program**	
ddH_2O	29.6 µl	1. Denaturing	98 °C for 30 sec
5x phusion buffer	10.0 µl	2. Denaturing	98 °C for 10 sec
dNTP	4.0 µl	3. Annealing	55 °C for 30 sec
Fw primer (appendix I)	2.0 µl	4. Elongating	72 °C for 30 sec
Re primer (appendix I)	2,0 µl	5. Elongating	72 °C for 10 min
DNA	2.0 µl	4 °C forever	
Phusion polymerase	0.4 µl		
Total volume	**50 µl**		

Steps 2 to 4 were repeated 29 times. The annealing temperature depended on the primers and in step 4 the time depended on the size of the gene amplified.

PCR with Taq polymerase

Cul n 1 and *Cul n 2* were amplified to see if the transformation of both One Shot ® Mach1™ T1^{R} *E. coli* (Invitrogen) and DH10Bac™ *E. coli* had been successful and check the orientation of the genes. First in the pFastBac™ /HBM TOPO® Vector, and then in the recombinant Bacmid. The Taq DNA polymerase and 10x buffer was from New England Biolabs.

PCR reaction solution		**PCR program**	
ddH_2O	12.8 µ	1. Denaturing	95 °C for 5min
10x buffer	2.0 µl	2. Denaturing	95 °C for 15 sec
dNTP	2.0 µl	3. Annealing	50 °C for 30 sec
Fw primer(20µM)	1.0 µl	4. Elongating	72 °C for 45 sec
Re primer(20µM)	1.0 µl	5. Elongating	72 °C for 10 min
Culture	1.0 µl	4 °C forever	
Taq polymerase	0.2 µl		
Total volume	**20 µl**		

Steps 2 to 4 were repeated 29 times. The annealing temperature depended on the primers and in step 4 the time depended on the size of the gene amplified.

3.1.4 DNA electrophoresis

The PCR products were run on 1 % agarose gel (Applichem). The agarose was melted in 0.5x Tris borate-EDTA (TBE, appendix II) and 1-3 drops of ethidium bromide (sigma) added. Prior to the PCR products being loaded on the gel, the 10x restriction buffer (RBS, appendix II) was added to each sample, and electrophoresis was carried out at 70 V for 45 min using 400 mA. The size of the products was estimated by comparison to a 2-log ladder (New England Biolabs).

3.1.5 Extraction of DNA from agarose gel

A QIAquick Gel Extraction Kit (Quiagen) was used to extract DNA from agarose gel. The DNA was visualized under UV light and excised from the gel. The gel extraction was performed according to manufacturer's procedures.

3.1.6 DNA quantification

DNA samples were measured in NanoDrop®ND-1000 Spectrophotometer (NanoDrop Technologies Inc.) according to protocol.

3.1.7 TOPO cloning

3.1.7.1 TOPO cloning and transformation

One Shot ® Mach1^{TM} T1^{R} Chemically competent *E. coli* (Invitrogen) were kept at -80 ^{o}C and thawed slowly on ice before use. The TOPO cloning reaction mix (2 µl) was made according to protocol, added to the bacterial cells and kept on ice for 30 min, heat shocked (42 ^{o}C, 30 sec) and incubated on ice for 2 min. SOC (250 µl) medium was added to each vial, before they were incubated (37 ^{o}C, 1 hour) while being agitated. The transformation mix was then plated on preheated LB agars with ampicillin (100µg/mL), and incubated (37 ^{o}C, 16-18 hours).

3.1.7.2 Plasmid purification

After transformation, colonies were picked and added to 2 ml LB medium (appendix II) with ampicillin (100µg/mL) and incubated (37 ^{o}C, 16-18 hours) while being agitated. The cultures were then tested in PCR and plasmids from positive cultures were isolated. To isolate the plasmids cell cultures (2 mL) were centrifuged (3000 RPM, 10 min) and the supernatant discarded. The buffers used were from Quiagen. P1 buffer (250 µl) was added to the pellet, before P2 buffer (250 µl) was added and the tubes were inverted 5x, then added N3 buffer (350 µl) and inverted again. The vials were centrifuged (5000 RPM, 10 min) and the supernatant was transferred to columns, before being centrifuged again (5000 RPM, 1 min), discarding the flow through. PE buffer (750 µl) was added to the column, centrifuged (5000 RPM, 1 min), flow through discarded before being centrifuged again at 14000 RPM for 1 min. DNA was eluted with EB buffer (30 µl) and incubated for 5 min in room temperature. The vials were centrifuged (5000 RPM, 1 min and 14000 RPM, 20 sec).

3.1.7.3 Sequencing

Sequencing was done before cloning, to check blunt end PCR product and after plasmid isolation, to see the orientation of the plasmid and if the sequence was correct.

PCR with Big Dye

Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequensing Kit (Applied biosystems) and sequencing capillary electrophoresis was carried out on an ABI PRISM 310 Genetic Analyzer (Applied biosystems). The buffer was provided with the kit. DNA clones were sequenced with gene specific primers or vector primers, using a concentration of 3-20 ng/µl of the sample for each reaction.

PCR reaction solution		**PCR programme**	
ddH_2O	4.9 µl	1. Denaturing	95 °C for 5 min
5x sequencing buffer	1,5 µl	2. Denaturing	95 °C for 20 se
Big Dye	1.0 µl	3. Annealing	50 °C for 15 sec
Primer	1.6 µl	4. Elongating	60 °C for 4 min
DNA	1.0 µl	4 °C forever	
	Total volume **10 µl**		

Steps 2 to 4 were repeated 29 times. The annealing temperature depended on the primers and in step 4 the time depended on the size of the gene amplified.

DNA precipitation

The PCR product was added to 75 % isopropanol (40 µl), mixed on vortex for 5-10 seconds, incubated in room temperature for 15 min and centrifuged (30 min, 14000 RPM). After discarding the supernatant, 75 % isopropanol (100 µl) was added and the solution mixed on vortex for 5-10 seconds, before they were centrifuged again (5 min, 140000 rpm) and the supernatant discarded. The pellets were dried for one minute at 90 °C, dissolved in 15 µl of HiDi Formamide, mixed briefly, heated at 95 °C for 2 min and mixed briefly before being centrifuged (20 sec, 14000 RPM).

3.2 Expression of recombinant proteins in Sf-9 insect cells

3.2.1 Transformation of DH10Bac™ *E.coli* cells

The pFastBac™ /HBM TOPO® expression cassette is flanked by the left and right arms of the site specific Tn7 transposon. The cassette contains a polyhedrin promoter, a multiple cloning site, a gentamicin resistance gene and an SV40

polyadenylation signal that forms a mini Tn7. The N-terminal Honey Bee Mellitin secretion signal on the plasmid facilitates the secretion of the cloned gene product into the extracellular medium.

The chemically competent MAX Efficiency® DH10Bac™ *E. coli* is used as the host for the pFastBac™ /HBM TOPO® construct containing the genes of interest. The cells contain a Baculovirus shuttle vector (target Bacmid) with a mini-*att*Tn7 target site and a helper plasmid. The target Bacmid contains the 136 kb Baculovirus genome with a kanamycin resistance gene, a LacZα gene that provides confirmation of the transposition at the correct site (gives the Lac^-phenotype), and a mini-F replicon that makes stable replication in *E. coli* possible. The helper plasmid encodes the transposase and tetracycline resistance genes (Bac-to-Bac protocol, Invitrogen, Catalog no. A11339).

The recombinant pFastBac™ /HBM TOPO® construct (2.5 ng) was added to the DH10Bac™ *E. coli* (100 µl) and incubated on ice for 30 minutes. The DH10Bac was transformed using heat shock (45 sec, 42 ^oC). The vials were chilled on ice (2 min) and then 900 µl of room temperature SOC medium was added. The tubes were incubated (37 ^oC, 4 hours) while being agitated. The cells were prepared, using 10-fold serial dilutions (10^{-1}, 10^{-2} , 10^{-3}) of the cells in SOC medium, and plated (100 µl) on separate LB agars containing 50 µg/ml kanamycin, 7 µg/ml of gentamicin, 10 µl/ml tetracyclin, 100 µg/ml X-gal (Appendix II) and 40 µg/ml IPTG (Appendix II). The plates were incubated 48 hours at 37 ^oC.

Colonies containing the $LacZ^-$ recombinant Bacmid were picked, based on their white color, and spread on new LB agars with the same antibiotics and concentration as mentioned above. The plates were incubated 16-18 hours at 37 ^oC.

3.2.2 Isolation of rBacmids

White colonies were picked and cultured in LB medium (2 ml) containing 50 µg/ml kanamycin, 7 µg/ml of gentamicin, 10 µl/ml tetracycline and incubated (48 hours, 37 ^oC) while being agitated. The cultures were tested in PCR with M13 primers and gene specific primers. The positive cultures containing recombinant Bacmids with *Cul n 1* were isolated according to the PureLink™ HiPure Plasmid DNA Purification Kit (Invitrogen). The isolated Bacmid DNA was tested in PCR using the same primers.

3.2.3 Culturing of Sf-9 cells

Sf-9 cells (American Type Collection) are derived from ovaries of the butterfly larvae *Spodoptera frugiperda.* Sf-9 cells were cultured in a closed culture at 27 ^{o}C. The culture medium was Sf-900^{TM} II (Invitrogen) containing penicillin (100 IU), streptomycin (100 IU) and fetal bovine serum (1 %).

3.2.4 Transfection of Sf-9 cells and generation of recombinant Baculovirus

The Sf-9 cells were seeded (0.3x10^{6} cells/ml) in a 12 well plate (Nunc) the day before transfection. To get rid of the antibiotics, the culture medium was removed and the cells washed with 500 µl of Grace's medium (without antibiotics). Then 800 µl of Grace's medium was added to each well. Two solutions were made for the transfection:

Table 1. Solutions for transfection of Sf-9 with rBacmid *Cul n 1*

Well	Solution 1	Solution 2
1	73.7 µl Grace's medium + 26.3 µl rBacmid 6 (1.0µg) *Cul n 1*	8 µl cellfectin + 100 µl Grace's medium
2	87.3 µl Grace's medium + 12.7 µl rBacmid 6 (0.5µg) *Cul n 1*	8 µl cellfectin + 100 µl Grace's medium
3	74.7 µl Grace's medium + 25.3 µl rBacmid 7 (0.5µg) *Cul n 1*	8 µl cellfectin + 100 µl Grace's medium
4	100 µl Grace's medium	8 µl cellfectin + 100 µl Grace's medium
5	100 µl Grace's medium	100 µl Grace's medium

Solution 2 was incubated (5 min, RT) and then added to solution 1. The mixture of solution 1 and 2 was incubated for 20 min in RT. The transfection and control mixtures were added to the Sf-9 cells (Figure 10) and incubated (5 hrs, 27 ^{o}C). The medium was removed and 900 µl of culture medium was added to the cells.

The plate was incubated 6 days before the cells were harvested and prepared for western blot. During the incubation, the cells were observed on a daily basis in a Leitz Transformator Inverted light microscope for cytopathy, viral infection in form of large and irregular shaped cells.

When cytopathy was observed, supernatant (200 µl) from the transfection was transferred to fresh Sf-9 cells in a 12 well plate (first passage) that had been seeded the day before. When cytopathy was observed in them a second passage was done in T25-flasks (Nunc) with 200 µl supernatant into 4.5 ml. The passage is illustrated in figure 4.

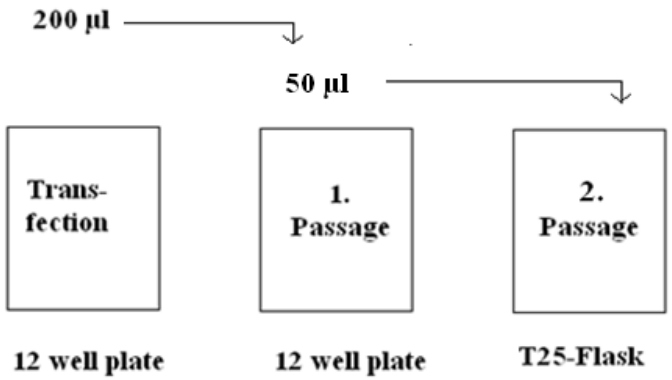


Figure 4. Passage of rBaculovirus

3.2.5 Expression of rBac-HBM-Cul n 1 and testing in western blot

At day 6 the Sf-9 cells were harvested from the plates and spun down in 1.5 ml tubes (14000 RPM, 3 min). The viral supernatant (viral stock) was kept at 4 oC and for longer storage frozen at -80 oC. The cell pellet was resuspended in lysis buffer (50 µl) (appendix II) and 2x Sample buffer (50 µl) (appendix II) then added for western blot analysis. The sampling buffer was also added to 50 µl of the viral supernatant, that also was used in western blot analysis.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The harvested samples were separated by SDS-PAGE in the Mini-protean II system (Bio-Rad), denatured with a sample buffer (appendix II), boiled for 5 min at 100 ^{o}C, spun down for 2 min and run on 14 % acrylamide gels under reducing conditions. Of the molecular mass marker, 4 µl was used, and 11 µl of the samples were loaded to the gel. PageRuler Prestained molecular mass marker from Fermentas #SM0671 was used to estimate the size of the proteins. The proteins were visualized using western blotting on a PVDF membrane (Millipore).

Western blotting

Following SDS-PAGE the proteins were transferred to a PVDF membrane by wet transfer in the Miniprotean II system (Bio-Rad) 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 20 for 30 at RT. The membranes were washed, incubated with monoclonal antibody against *Cul n 1 (Jonsdottir, 2011a)* at 4 ^{o}C over night, washed again and incubated at RT for one hour with the conjugate, goat-anti mouse-AP (Jackson) diluted 1/5000. The membranes were washed with TBS-T between steps, 5x for 5 min. The membranes were developed using BCIP/NBT (appendix II) from Roche diluted 1:50 in alkaline phosphatase buffer (appendix II) after having been washed. The membranes were washed with TBS-T between steps, 5x for 5 min.

4 Results

4.1 Production of allergens from *C. nubeculosus* in Sf-9 cells

4.1.1 Amplification of *Cul n 1* and *Cul n 2*

Cul n 1 and *Cul n 2* were amplified with PCR from a purified FastBac vector containing the genes from a λZAP II cDNA library (Schaffartzik et al., 2011). This was done to produce blunt end PCR product for TOPO cloning. *Cul n 1* was expected to be 504 bp (appendix III) and *Cul n 2* was expected to be 1206 bp (appendix III).

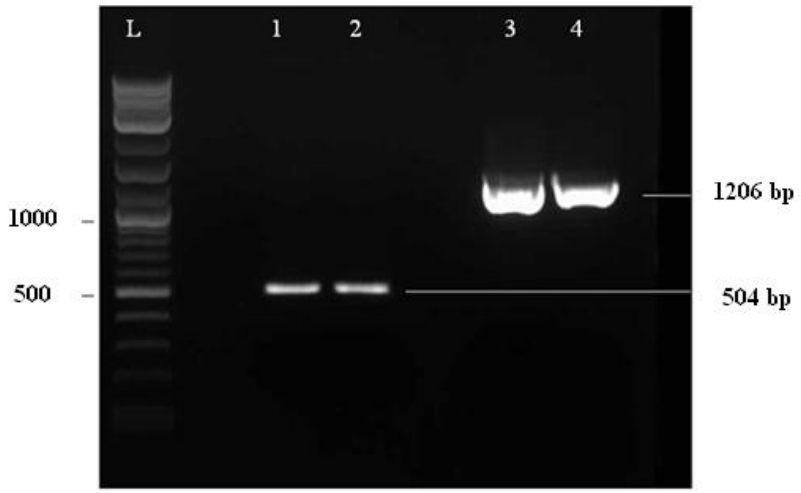


Figure 5. Amplification of *Cul n 1* and *Cul n 2*
Lane L: 2 Log-ladder, **Lane 1 and 2:** *Cul n 1* (504 bp) PCR product, **Lane 3 and 4:** *Cul n 2* (1206 bp) PCR product.

The *Cul n 1* was amplified without the signal sequence and stop codon. The *Cul n 2* was amplified without the stop codon only, as there was no predicted signal sequence. The results, illustrated in figure 5, show the blunt end PCR product, *Cul n 1* is 504 bp (appendix III) and *Cul n 2* is 1206 bp (appendix III).

4.1.2 Sequencing of *Cul n 1* and *Cul n 2* for verification

The blunt end PCR product was extracted from the gel, and the DNA concentration measured (Table 2).

Table 2. DNA concentration of *Cul n 1* and *Cul n 2* after gel extraction

DNA	Concentration (ng/µl)	Purity (260/280)
Blunt end PCR product of Cul n 1	19,37	1,71
Blunt end PCR product of Cul n 2	42,11	1,86

The genes were sequenced to verify the right reading frame. To get the entire sequence, several gene specific primers were used (appendix I). The sequences of *Cul n 1* and *Cul n 2* were as expected and are listed in appendix III, so TOPO Cloning could be performed.

4.1.3 TOPO Cloning and isolation of pFastBac vectors

One shot ® Mach1^{TM} T1^{R} chemically competent *E. coli* were transformed with pFastBac^{TM}/HBM-TOPO® vector after TOPO cloning and tested in PCR to check if the cloning was successful. Forward vector primer and reverse gene specific primer were used in this PCR, to detect the vectors containing *Cul n 1* and *Cul n 2,* in the correct orientation.

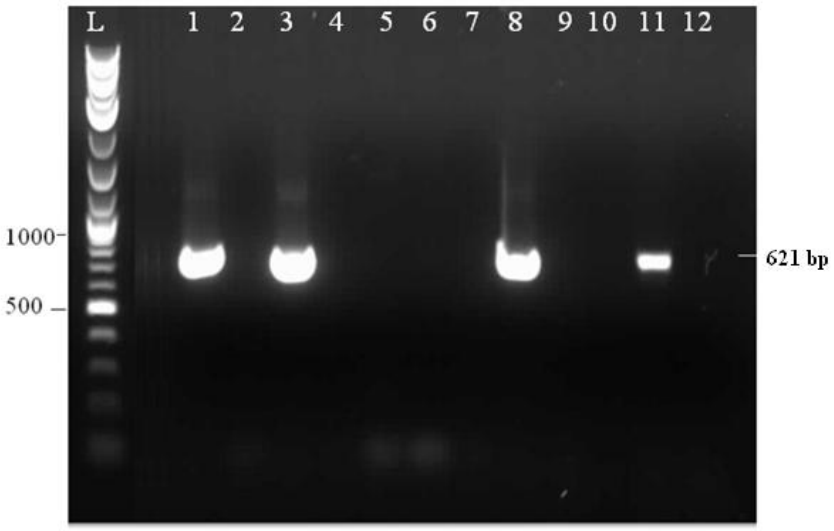


Figure 6. PCR of One Shot® cultures after TOPO cloning of *Cul n 1*
Lane L: 2 Log-ladder, **Lane 1-12:** *E. coli* cultures 1-12

For *Cul n 1* the expected PCR product, using these primers, should be 621 bp or 115 bp longer than the gene alone. Culture number 1, 3, 8 and 11 contained the *Cul n 1* in the correct orientation (Figure 6).

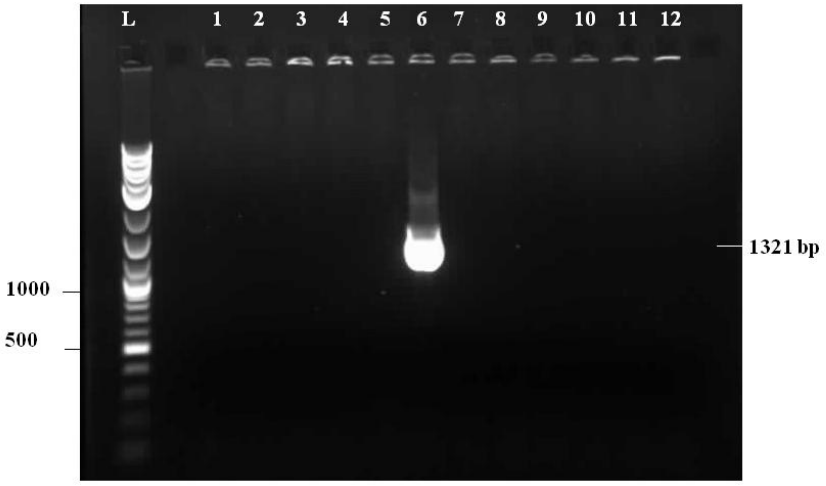


Figure 7. PCR of One Shot® cultures after TOPO cloning of *Cul n 2*
Lane L: 2 Log-ladder, **Lane 1-12:** *E. coli* cultures 1-12

For *Cul n 2* the expected PCR product, using these primers, should be 1321 bp or 115 bp longer than the gene alone. Only culture number 6 contained the *Cul n 2* in the correct orientation (Figure 7). After plasmid purification the DNA concentration was measured (Table 3 and 4).

Table 3. DNA concentration of pFastBac HBM-TOPO_*Cul n 1*

Plasmid	Concentration (ng/µl)	Purity (260/280)
pFastBac HBM-TOPO_Cul n 1 (1)	195.84	1.86
pFastBac HBM-TOPO_Cul n 1 (3)	277.3	1.89
pFastBac HBM-TOPO_Cul n 1 (8)	240.04	1.88
pFastBac HBM-TOPO_Cul n 1 (11)	208.39	1.89

Table 4. DNA concentration of pFastBac HBM-TOPO_*Cul n 2*

Plasmid	Concentration (ng/µl)	Purity (260/280)
pFastBac HBM-TOPO_Cul n 1 (6)	226,65	1,89

For *Cul n 1*, plasmid 3 (Table 3) was used for sequencing and plasmid 6 for *Cul n 2* (Table 4).

Analysing transformants (sequencing)

After plasmid purification the genes were sequenced for verification. Both gene specific primers and vector primers were used (appendix I). The sequencing of *Cul n*

1 showed the right reading frame and orientation (Sequence is listed in appendix III) and could be used for transformation of DH10Bac ™ *E. coli*. The sequencing of *Cul n 2* was not complete, and has to be finished before it can be used in transformation. From now on the results are only relevant for *Cul n 1*.

4.1.4 Transformation of DH10Bac™ *E. coli* and production of Bacmids

DH10Bac™ *E. coli* were transformed with the recombinant pFastBac™/HBM TOPO_*Cul n 1* construct (2,5 ng), and grown with antibiotic selection. Colonies containing the LacZ- recombinant Bacmid (white) were picked and cultured in LB-medium with antibiotics (50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml X-gal, 40 µg/ml IPTG). The cultures were tested in PCR with gene specific primers (appendix I) and M13 primers (appendix I)specific for the Bacmid.

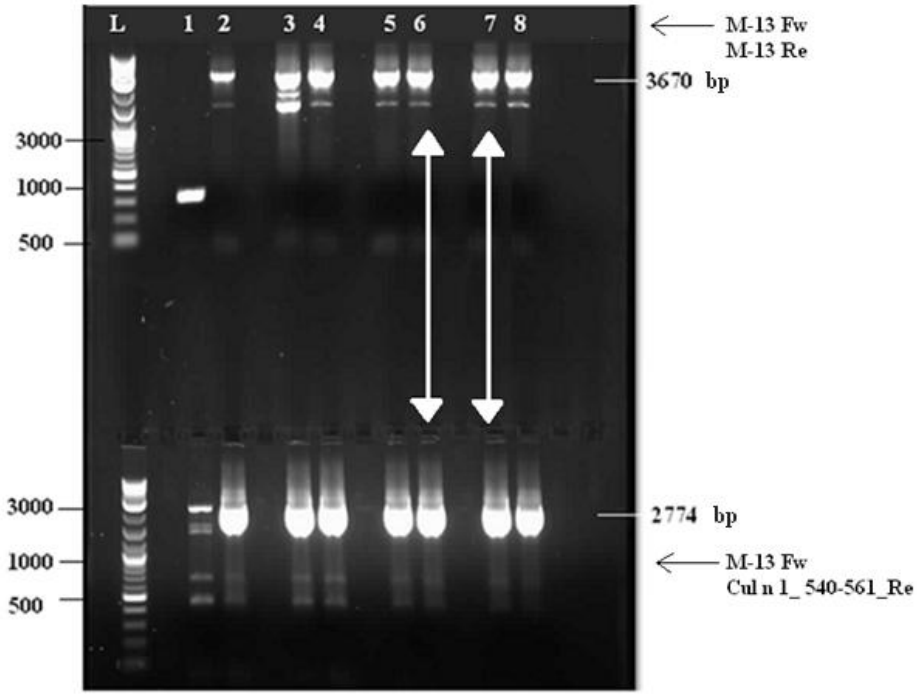


Figure 8. PCR on transformed DH10Bac™
Lane L: 2 Log-ladder, **Lane 1-8:** *E. coli* cultures 1-8, top lanes amplified with M13 Forward and revers primer, bottom lanes were amplified with M13 Forward primer and *Cul n 1*_540-561_Revers primer

The *Cul n 1* gene amplified with M13 Forward and Reverse primers was expected to be 3670 bp, and 2774 bp when using the M13 Forward and Cul n 1_540-561_Reverse primer. As shown in figure 8, cultures number 2, 4, 5, 6, 7 and 8 were positive and contained *Cul n 1*. The rBacmids from cultures number 6 and 7 (shown

by arrows) were purified for transfection of Sf-9 cells. They were picked based on the density in the liquid LB-medium. The DNA concentrations of the recombinant Bacmids are shown in table 5.

Table 5. DNA concentration of rBacmid (Cul n 1)

rBacmid purified from	Concentration (ng/µl)	Purity (260/280)
rBacmid 6	38.23	1.49
rBacmid 7	19.79	1.79

The rBacmids were tested in PCR with gene specific primers and M13 primers (appendix I).

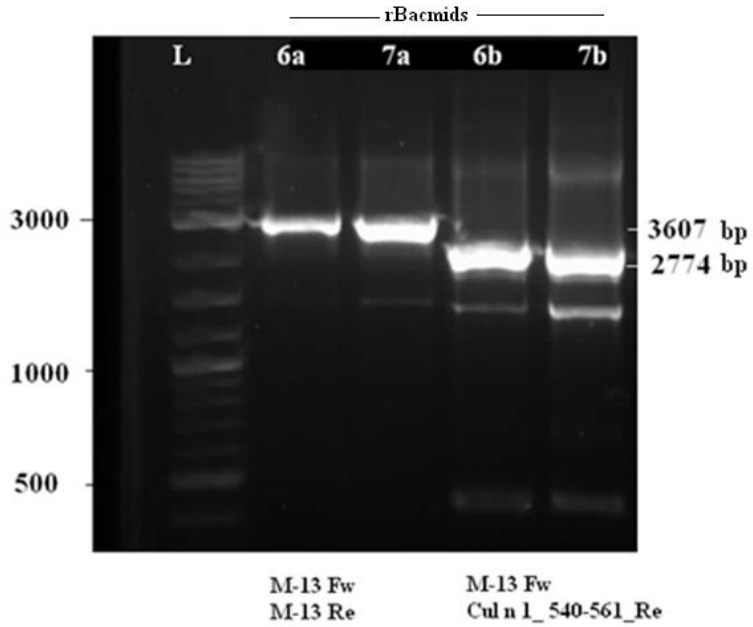


Figure 9. PCR of purified rBacmid (*Cul n 1*)
Lane L: 2 Log-ladder, rBacmid 6a and 7a amplified with M13 primers, rBacmid 6b and 7b amplified with M13 Fw and Cul n 1_540-561_Re primer.

Figure 9 shows that the rBacmids amplified with M13 Forward and Revers primer were 3670 bp, and 2774 bp when using the M13 Forward and Cul n 1_540-561_Reverse primer, so both rBacmid 6 and rBacmid 7 could be used for transfection of Sf-9 cells.

4.1.5 Transfection of Sf-9 cells and production of rBac-HBM-*Cul n 1* virus

Sf-9 cells were transfected according to protocol, with rBacmid 6 and 7, using two different DNA concentrations, shown in figure 10. The supernatant (viral stock) was used to infect new Sf-9 cells to amplify and produce more virus. The infection was done in a 12 well plate (Figure 4). On the second passage, virus from transfection with rBacmid 6 (1.0 µg) and rBacmid 7 (0.5 µg) was used for the infection of T25 flasks (Nunc).

	1 rBacmid 6 1.0 µg	2 rBacmid 6 0.5 µg	
	3 rBacmid 7 0.5 µg	4 Cellfectin control	
	5 Cell Control		

Figure 10. Transfection wells of Sf-9 cells with rBac-HBM-*Cul n 1*
Transfection of Sf-9 in a 12 well plate, using rBacmid 6 (0.5 µg and 1 µg) and rBacmid 7 (0.5 µg). The same set-up was used for first passage.

The cells were examined in a Inverted light microscope (Leitz, Diavert) on a daily basis during incubation for cytopathy and viral infection. The transfection plate was incubated 6 days and first passage 3 days, before harvesting (Figure 4). The observed cytopathy is listed in table 6.

Table 6. Cytopathy of Sf-9 cells transfected with rBac-HBM-*Cul n 1*

Well	Signs of viral infection and cytopathy
1. rBacmid 6 (1.0 µg)	After one day of incubation the cells were slightly enlarged compared to the control cells. Some cells were starting to get a bit elongated or irregular in shape. Viral infection was clear on the second and third day of infection, as the cells were enlarged and varied in size and shape. Also, some cells were small dark clusters (dead/lysed cells) and some were swollen (about to lyse).
2. rBacmid 6 (0.5 µg)	The cytopathy was almost the same as for rBacmid 6 (1.0 µg), but the population was less dense, making it easier to see the different shapes and sizes.
3. rBacmid 7 (0.5 µg)	The cytopathy was almost the same as for rBacmid 6 (1.0 µg), but the population was less dense, making it easier to see the different shapes and sizes.
4. Cellfectin control	Dense growth and round cells, no signs of viral infection. The infected wells had flat cells, while the control cells were rounder.
5. Cell control	Dense growth and round cells, no signs of viral infection. The infected wells had flat cells, while the control cells were rounder.

The cytopathy of the first and second passage showed the same signs of viral infection as listed in table 6.

A B

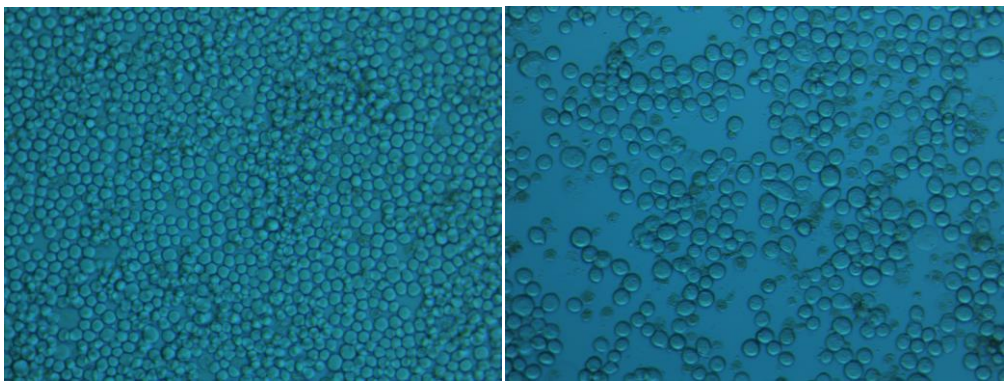


Figure 11. Transfection of Sf-9 cells with rBac-HBM-*Cul n 1*, day 6
Photos taken with inverted light microscope Leica DM IL LED (DFC 425 c), Magnified 400x.
A) Uninfected Cellfectin control, **B)** Sf-9 insect cells transfected with rBacmid 6 (1 µg).

Figure 11 shows the rBacmid *Cul n 1* transfected Sf-9 cells. The control (Figure 11 A) has a crowded, dense population, with small cells, while the transfected cells (Figure 11 B) varied in size and shape, clearly shown by the characteristic elongated cells. In

the figure some of the cells are swollen and about to lyse, while some are dark and small, dead, lysed cells.

A B

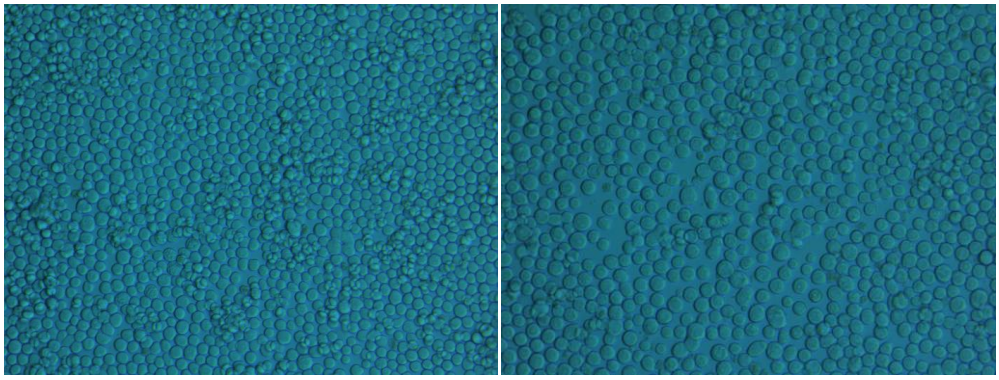
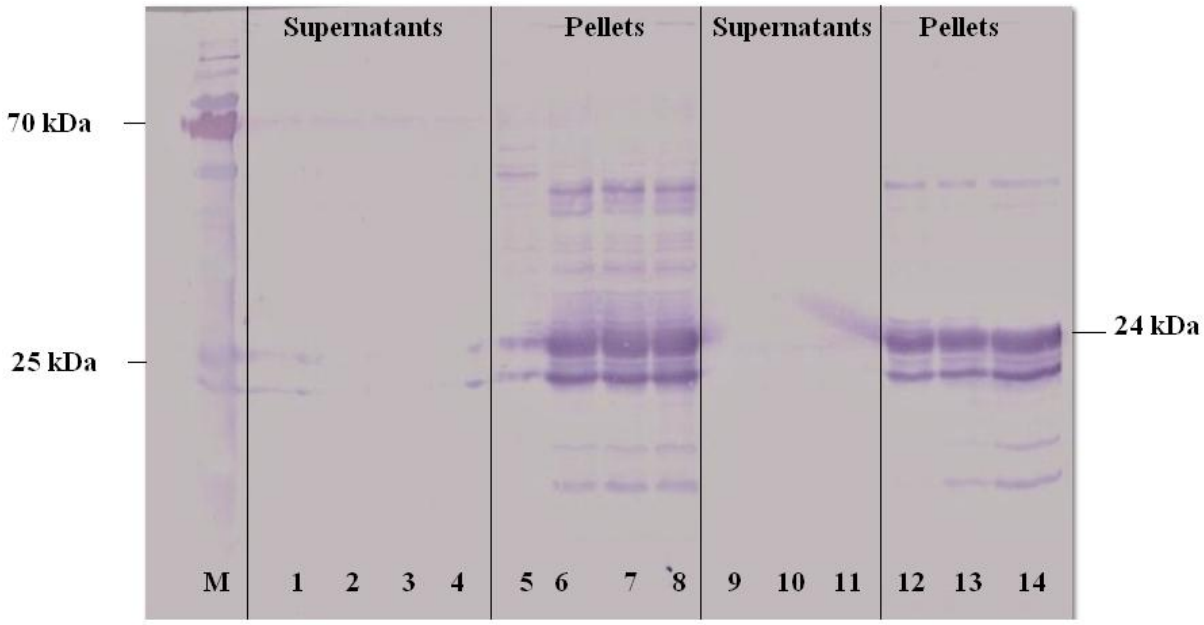


Figure 12. rBac-HBM-*Cul n 1* infected Sf-9 cells, first passage, day 3
Photos taken with inverted light microscope Leica DM IL LED (DFC 425 c), Magnified 400x.
A) Uninfected Cellfectin control, **B)** Sf-9 insect cells infected with rBac-HBM-*Cul n 1*

Figure 12 shows the Sf-9 cells infected with the first passage of rBac-HBM-*Cul n 1*. The infected well (Figure 12 B) was heavily populated, but the cells were bigger than the controls and differed in size. The control (Figure 12 A) showed no cytopathy.

4.1.7 Expression of rBac-HBM-*Cul n 1* in Sf-9 cells

The Sf-9 cells were harvested and tested for protein expression of Cul n 1, in western blot. The transfection was incubated 6 days and first passage 3 days, before harvesting (Figure 4). The pellets were loaded on to the gel in dilution 1:4, but the supernatants were undiluted. To detect the protein a monoclonal antibody directed against Cul n 1, and a secondary goat-anti mouse (Jackson) was used.



Figur 13. Western blot of rBac-HBM-*Cul n 1* expressed in Sf-9 cells
Lanes: Page Ruler (1), Cellfectin control (1, 5), Bacmid 6: 1.0 µg (2, 6, 10, 13), Bacmid: 6 0.5 µg (3, 7, 10, 13), Bacmid 7: 0.5 µg (4,8, 12, 15). Samples from lane 1-8 were harvested on the sixth day of transfection, while lane 9-14 was harvested from the 1.Passage, after three days of infection.

Figure 13 shows that the Cul n 1 protein is efficiently expressed in the Sf-9 cells both in the transfection and the first passage. The size of the Cul n 1 is 24 kDa, and as expected the protein is seen as a band of 24 kD. No expression can be detected in the supernatants and not either in the negative cellfectin control. Only the pellet was positive for the protein of 24 kDa.

5 Discussion

IBH is caused mainly by proteins in the saliva from glands of the biting midge of the genus *Culicoides* spp*.* Since many reports have shown that these proteins are important allergens, they have been the subject of research, with the long term intention to create immunetherapy and use them in diagnostic tests (Fadok and Greiner, 1990; Hellberg et al., 2006; Langner et al., 2009; Quinn et al., 1983; Schaffartzik et al., 2012; Schaffartzik et al., 2010; Schaffartzik et al., 2011; Van der Meide N. M. et al., 2013).

Protein production in *E. coli* is fast and effective, but the proteins are not glycosylated nor correctly folded, and are often biological inactive. Advantages of eukaryotic expression systems are that they can perform many of the posttranslational modifications necessary, like folding or glycosylation. Insect allergens have been successfully produced in the Baculovirus system, expressing proteins with the same antigenicity as the native protein making it a good choice of expression system. (Possee, 1997; Schmidt and Hoffman, 2002). IBH is caused by allergens from insects therefore it is evident to use an insect cell expression system, like the Bac-to-bac®.

Recombinant allergen derivates lacking IgE epitopes are used in treating allergy in humans (Valenta et al., 2012). Therefore *Culicoides* allergens produced in *E. coli* lacking the right conformation and therefore maybe some of the IgE epitopes, are a good source for immunization and desentisation in IBH. However, *E. coli* producing allergens have proven very difficult to use in both diagnostic tests and tests to monitor immunotherapy.

Antigen 5 and hyaluronidase are very important allergens in insects, like the yellow jackets, wasps, yellow hornet, horse fly and white face hornet hornet, as can been seen at the official site for the systematic allergen nomenclature that is approved by the World Health Organization and International Union of Immunological Societies committee (http://www.allergen.org/search.php?TaxSource=Animalia%20Arthropoda).

It has been shown that expressing proteins in the Baculovirus system with the honey bee melittin signal peptide instead of the native peptide, can yield over five times more of the recombinant protein (Tessier DC et al., 1991). At Keldur, *Cul n 1 and Cul*

n 2 were expressed with Bac-to-Bac, but proved difficult to purify. *Cul n 3* and *Cul n 4* were expressed with the HBM signal peptide and have proved easier to purify (Bjornsdottir, 2008; Jonsdottir, 2011b). Therefore it was decided to express *Cul n 1 and Cul n 2* again with HBM.

Sequencing of *Cul n 1* showed that the correct sequence was in the bacmid (appendix III). The Sf-9 cells transfected with rBacmid *Cul n 1* and those infected with rBac-HBM-*Cul n 1* virus showed cytopathy according to protocol. Western blot showed efficient expression of Cul n 1 in the lysed cells. The expression of the protein was strong, and the pellet samples were diluted 1:4 to yield a good blot. The *Cul n 1* spesific monoclonal antibody made at Keldur is also very strong and specific and can be used in high dilutions (Jonsdottir, 2011a).

The end of the sequence of *Cul n 2* has to be verified before it can be used in transformation of DH10Bac and to transfect Sf-9 cells.

The next steps of the project are transfection and generation of rBac-Cul n 2 virus, and cloning of both rBac-HBM-Cul n 1 and rBac-HBM-Cul n 2 viruses in Sf-9 cells. After that production of rBac-HBM-Cul n 1 and rBac-HBM-Cul n 2 proteins in High-5 insect cells, and purification of the hexahistidine tagged proteins must be done, before further studies on the proteins, for diagnostic test and use in immunotherapy experiments in IBH, can proceed.

6 Conclusion

Cul n 1 was cloned and expressed successfully with the Bac-to-Bac® HBM TOPO® Secreted expression system, in Sf-9 cells. The rBac-HBM-Cul n 1 virus is ready for cloning and then infection of High 5 cells for protein production and purification. The end of the sequence of *Cul n 2* has to be verified before it can be used in transformation of DH10Bac® and transfection of Sf-9 cells.

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7 Appendix

7.1 Appendix I - Primers used in this work

Antigen 5	**Nucleotide sequence**
Ag5B-EcoR_ Fw	5’-GGTGGAATTCGCGGCCACCATGATTAAAAAACTTTCG -3’
Ag5NotI_Re	5’- GGTGTGCGGCCGCTCATAGCAGTAAATGTCCAATAAC -3’
M13_Fw	5’- GTT TTC CCA GTC ACG AC -3’
M13_Re	5’- CAG GAA ACA GCT ATG AC -3’
Hyaluronidase	
Hya_1-22_BamHI_Fw2	5’- GGTGTGGGATCCTGCATGTGGTTGAACGTGGTAAATG -3’
Hya_1192-1209_HindIII_Re	5’- CCACACAAGCTTCGCTTAAGACAAATTTGGGGT -3’
Sequencing primers	
Antigen 5	
Cul n 1_ 58-78_ Fw	5’- ACA AAT TTT TGC AAC AAA GAT -3’
Cul n Ag5_411_ Fw	5’- GCA TAC ACA ATG GGC ACA CC -3’
Cul n 1_ 540-561_Re	5’- TAG CAG TAA ATG TCC AAT AAC -3’
FastBac_TOPO_Fw	5’- AAA TGA TAA CCA TCT CGC -3’
FastBac_TOPO_Re	5’- GGT ATG GCT GAT TAT GAT C -3’
Hyaluronidase	
Cul n 2_ 1-22_Fw	5’- ATG TGG TTG AAC GTG GTA AAT G -3’
CnHya_134_Re	5’- GCA TCG TCC TTT GCA TCT AC -3’
CnHya_237_Fw	5’- GGA ACA TCC CAT CAT TTA TG -3’
CnHya_527_Fw	5’- GAG TCA TGG CGA CCG ATT TA -3’
Cul n 2_1186-1206_Re	5’- TGA CAA ATT TGG GGT AAG AA -3’
FastBac_TOPO_Fw	5’- AAA TGA TAA CCA TCT CGC -3’
FastBac_TOPO_Re	5’- GGT ATG GCT GAT TAT GAT C -3’

7.2 Appendix II – Buffers and solutions used in this work

0,5x TBE

0,045 M Tris borate, 0,001 M EDTA

Restriction buffer(Loading dye)

50% glycerol, 15 mM EDTA, 0,25% bromophenol blue

LB medium

1% tryptone, 0,1 % Yeast extract, 1% NaCl

LB agar

1% tryptone, 0,1 % Yeast extract, 1% NaCl, 1.0% Bactoagar

SOC medium

2% Tryptone, 0,5% Yeast extract, 0,05% NaCl, 2,5 mM KCl, 10 mM MgCl, 20 mM Glucose

Lysisbuffer (to check the protein ekspression with western blot)

50 mM NaH_2PO_4, 150 mM NaCl, 0,5 % IgePal

2x sample buffer

0,5 M Tris, pH 6,8, 20 % SDS, Glycerol, Mercaptoetanol, 1 % Bromophenol blue, ddH_2O

10x TBS

75 g Tris, 360 g glycin, 2,5 l H_2O

Transfer buffer (for western blot)

10x TBS, methanol, ddH_2O

Electrophoresis buffer (for western blot)

10x TBS, 20 % SDS, ddH_2O

Wash buffer (for western blot)

5x TBS, ddH_2O, Tween 20

X-gal

Bromo-chloro-indolyl-galactopyranoside

IPTG

Isopropyl β-D-1-thiogalactopyranoside

BCIP/NBT

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

Alkaline phosphatase buffer

10 mM Tris-Hcl, 100 mM NaCl, 5 mM $MgCl_2$, 0.05 % Tween 20, pH 9.5

7.3 Appendix III

7.3.1 Sequences of *C. nubeculosus* used in the Baculovirus expression system

Cul n 1 **(antigen 5) 504**

Nucleotide sequence:

ACAAATTTTTGCAACAAAGATTTATGCAAAAGACAAAATGGACCGCAGTCTTTTACATATTTAAAACACATTGGA
TGTCGTCATACTGGTAAAAATGCTAACACCTGTCCACGCGATGCAAAAATCCTGCCAATGTCAACTAAACGTAAA
AATTTGATTCTTAAAGTACATAATCGATTGCGTAACAAAGTAGCTCTTGGAAAATTACCTGGATTCCCAAAAGCA
GCTCGTATGCCTATTTTACGTTGGGACGATGAATTAGCTTATTTGGCTGAACTTAATGTGAAACAGTGCAAAATG
GAGCATGATCAATGTCGTAATACAGACAAATTTAAATATGCAGGTCAGAATTTAGCATACACAATGGGCACACCT
CAAAAAAATGCAGTTCGAATTAAAAAGCTGATCCGAGCGTGGTTTAAGGAGCATGAAAATGCAACGGCATCATTT
ATTGATAAATATCGGGATCATCCTCAAGGTCGCGTTATTGGACATTTACTGCTA

Translated into amino acid:

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

***Cul n 2* (Hyaluronidase) 1206**

Nucleotide sequence:

ATGTGGTTGAACGTGGTAAATGTCTCACAATTTATGACAGCATGGGCGACCTTTAATTTGATTAATGCACAACAA
CTGATACAAGTGGGACCAGAAAATGTACCATATGAAATTGTAGATGCAAAGGACGATGCATCTGAAAGCAGAGGA
ATATTTTTTAATAACTTCATCACATCAAAAAATAACGATGATAAAAGACATGATTTTACCTTTTACTGGAACATC
CCATCATTTATGTGTTCAAAATACAATGTAACATTCACTGACATGCCTTCATCATATAATATCGTACAAAATAAA
GATGATAAATGGCGTGGTGACCAGATCATAATTTTATATGATCCTGGTAAATTTCCGGCTTTATTAGAGCATCAA
GGAAAATTATATAGACGAAATGGTGGTGTACCACAAGAAGGGAATTTACAAGAACACATCGATTATTTTGCTGAA
AGTGTTAATACCTTGATACCAGATCAAAATTTCTCAGGCATTGGAGTGATTGATCTTGAGTCATGGCGACCGATT
TATCGTCAAAATTCAGGTGTGTTACAGCCATATAAGGATTTATCATATAAATTGGTGCAAAGAAAGCATAGACTA
TGGAGCAAGAAGTTAATTGAAGAAGAGGCAGCTCGTGAATTTGAGACAGCTGGTCGAACATTTGTAGAAGAAACG
GTTAGAGTTGCAAAATATTTACGTCCAAATGCAAAATGGGGCTATTATGGATTCCCGTATTGTTTCAATATGAAT
GGTGGTGCAAATATGAAAGAGGATTGTCCATCTAATGTTAAAGAGGAAAATAATCGTATTAAATGGCTGTGGGAT
ATTGTCGATGTGGTTTTGCCTTCAGTTTATTTGAACAACAAAATAACAGCATCACAAAGAGTCCAATTTGTTCGT
GGGCGAATGCGTGAAGGATGTCGTGTGTCACAATTATCAAAACAACCAGTGAAACCACCAGTATACAGTTATTTG
CGTTATGTTTACACGGACAACCTAAAATACATTTCAAATGAGGATCTCAAACAATCAATTAAAGTACCCAAAGAG
CAAAAGGGTAGTGGATTAATATTTTGGGGCAGTTCATATGATGTCAAAACGAAAGATCAGTGTTTTGATTTTAGA
AATTATGTTGATAATAATTTAGGACCAATTGTACTATCAGCAAATGACAATACACCAAAAATTCTTACCCCAAAT
TTGTCA

Translated into amino acid:

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