การพัฒนาวัคซีนไรฝุ่นชนิดดีเอ็นเอวัคซีนต้นแบบโดยใช้ทั้งโมเลกุล และชิ้นส่วนที่กระตุ้นเฉพาะภูมิคุ้มกัน (เอ็ปปิโทป) ชนิด ทีเอช-1 ของเอ็นติเจนของไรฝุ่น “เดอร์ พี-1”

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HOUSE DUST MITE DNA VACCINE CANDIDATE DEVELOPMENT OF FULL LENGTH AND POLY-TH1 EPITOPES

Miss Pinya Pulsawat

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Medical Microbiology
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กิญญา พูลสวัสดิ์ : การพัฒนาวัคซีนไรฝุ่นชนิดดีเอ็นเอวัคซีนต้นแบบโดยใช้ทั้งโมเลกุลและชิ้นส่วนที่กระตุ้นเฉพาะภูมิคุ้มกัน (เอ็ปปิโทป) ชนิด ทีเอช-1 ของเอ็นดิเจนของไรฝุ่น "เดอร์ พี-1" (HOUSE DUST MITE DNA VACCINE CANDIDATE DEVELOPMENT OF FULL LENGTH AND POLY-TH1 EPITOPES) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ศ.นพ.เกียรติ รักษ์รุ่งธรรม, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.ดร.สุรพล พิบูลโกคานนท์, 147 หน้า

โรคภูมิแพ้เป็นโรคที่เกิดจากระบบภูมิคุ้มกันมีการตอบสนองต่อสารก่อภูมิแพ้ในรูปแบบของ T-helper type 2 (Th2) คือ มีการสร้าง IgE antibody แทนการสร้าง IgG ซึ่งสารก่อภูมิแพ้ที่พบบ่อย ได้แก่ ฝุ่นบ้าน, ไรฝุ่น, ละอองเกสรดอกไม้ และ รังแคจากสัตว์เลี้ยง เป็นต้น ไรฝุ่นเป็นสาเหตุที่พบบ่อยที่สุด (60-70%) ในผู้ป่วยโรคภูมิแพ้ หนึ่งในโปรตีนที่สำคัญของไรฝุ่นที่จับกับ IgE คือ Der p 1 (Dermatophagoides pteronyssinus) มีรายงานว่าเป็นสารก่อภูมิแพ้ที่สำคัญที่สามารถกระตุ้นระบบภูมิคุ้มกันในผู้ที่มีแนวโน้มต่อการเกิดโรคภูมิแพ้ให้เกิดปฏิกิริยาภูมิแพ้ได้ง่ายขึ้น ปัจจุบัน นักวิจัยทั้งหลายได้มีการที่จะพัฒนาและค้นหาวัคซีนที่สามารถจะเปลี่ยนระบบภูมิคุ้มกันจากปฏิกิริยาภูมิแพ้ไปเป็นการตอบสนองในรูปแบบของ T-helper type 1 (Th1)

การศึกษาวิจัยเป็นการพัฒนา House Dust Mite (HDM) DNA vaccine เพื่อที่จะกระตุ้นให้เกิดภูมิคุ้มกันในที่สามารถป้องกันการแพ้ต่อไรฝุ่นบ้านได้ โดยใช้ pHIS plasmid vector ที่มี CpG motif อยู่ในโครงสร้างและได้ทำการออกแบบให้มีส่วนของ mature Der p 1 โปรตีนขึ้นมาโดยได้ทำการเปลี่ยนรหัสพันธุกรรมที่ใช้สำหรับการแปลงเป็นโปรตีนไรฝุ่นจากตัวต้นแบบ มาเป็นรหัสพันธุกรรมที่สามารถแปลได้โดยใช้รหัสพันธุกรรมของคนเพื่อเพิ่มประสิทธิภาพในการแสดงออกของโปรตีน มี CpG motif หรือ toll-like receptor-9 ligand เพื่อเพิ่มความสามารถในการกระตุ้น การตอบสนองต่อวัคซีนในรูปแบบ Th1 ได้ดียิ่งขึ้น

การทดสอบการแสดงออกของ Der p 1 โปรตีน ใน HEK cell โดยการใช้ pGFP-mHuDer p 1 plasmid DNA พบว่ามีการแสดงออกของ chimeric GFP-Der p 1 โปรตีนที่มีขนาด 52 kDa และผลการฉีด pHIS-mHuDer p 1 DNA vaccine หรือ liposome-pHIS-mHuDer p 1 DNA vaccine (lipoplexes) เข้าในชั้นผิวหนัง (intradermal) ของหนูทดลองชนิด Balb/c โดยทำการเปรียบเทียบกับกลุ่มควบคุมที่เป็น plasmid DNA ที่ไม่มี Der p 1 gene พบว่า การตอบสนองทางภูมิคุ้มกันหนูทดลองโดยใช้วิธี ELISA หนูทดลองที่ได้รับการฉีด pHIS-mHuDer p 1 vaccine เกิดการสร้างภูมิคุ้มกันแบบ Th1 ซึ่งตรวจพบมีการสร้าง IgG2a ที่จำเพาะต่อ Der p 1 โปรตีน โดยเฉพาะอย่างยิ่ง ในหนูกลุ่มที่ได้รับการฉีดวัคซีนในรูปแบบ lipoplexes นั้น มีการตอบสนองทางภูมิคุ้มกันที่เกิดขึ้นได้เร็วกว่าและดีกว่า คือวัดระดับ IgG2a antibody ได้เร็วขึ้นและสูงขึ้น กว่ากลุ่มอื่นๆ ส่วนผลการทดลองสร้าง allergic model ต่อไรฝุ่นพบว่า การฉีดโปรตีน ProDer p 1 กับ alum เข้าทางช่องท้องของหนู พบว่า มีการตอบสนองแบบ Th2 โดยสามารถตรวจวัดระดับของ IgG1 และ IgE ที่จำเพาะต่อ Der p 1 ได้

กล่าวโดยสรุปคือ pHIS-mHuDer p 1 DNA vaccine สามารถสร้างภูมิคุ้มกันต่อสารก่อภูมิแพ้ Der p 1 การเปลี่ยนรหัสพันธุกรรมเป็นแบบของคนทำให้สามารถเพิ่มการแสดงออกของโปรตีน Der p 1 ได้มากขึ้นเมื่อเทียบกับ wild type Der p 1 ซึ่งเป็นตัวต้นแบบ และกลุ่มควบคุม การตอบสนองทางภูมิคุ้มกันแบบ Th1 ในหนูทดลองหลังจากการฉีด pHis-mHuDer p 1 DNA เข้าในชั้นผิวหนัง โดยสามารถตรวจพบระดับ IgG2a ที่จำเพาะต่อโปรตีนDer p 1 นอกจากนี้ยังพบว่าเมื่อมีการใช้ liposome ร่วมกับ DNA vaccine สามารถเพิ่มประสิทธิภาพของ DNA วัคซีน ในการกระตุ้นการสร้าง IgG2a antibody ในหนูทดลองได้ดีขึ้นหลังจากการฉีดเพียงครั้งแรก ดังนั้น ควรพัฒนาวัคซีนต้นแบบนี้ให้มีประสิทธิภาพที่ดีและปลอดภัยยิ่งๆขึ้น เพื่อนำไปสู่การทดสอบในอาสาสมัครต่อไปในอนาคต

สาขาวิชา.....จุลชีววิทยาทางการแพทย์..... ลายมือชื่อนิสิต.....

ปีการศึกษา .....2551..... ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

##4689677320 : MAJOR MEDICAL MICROBIOLOGY
KEYWORDS : HOUSE DUST MITE ALLERGEN / DNA VACCINE / CODON OPTIMIZATION

PINYA PULSAWAT : HOUSE DUST MITE DNA VACCINE CANDIDATE DEVELOPMENT OF FULL LENGTH AND POLY-TH1 EPITOPES. ADVISOR : PROF. KIAT RAXRUNGTAM, CO-ADVISOR : ASST.PROF. SURAPON PIBOONPOCANUN, Ph.D, SUNEE SIRIVICHAYAKUL, Ph.D., 147 pp.

Allergy is an immune dysregulation against allergens that lead to T-helper type 2 (Th2) immune response and specific IgE production. House dust mite is the most common cause of airway allergic diseases of which 60-70% of patients have detectable anti-house dust mite IgE antibodies. Der p 1 (Dermatophagoides *pteronyssinus*) protein is one of the major HDM allergens that can sensitize the immune system in an allergic prone person for whom develops an allergic disease. Thus developing a safe and effective HDM vaccine to induce Th1 responses to down regulate Th2 responses is warranted.

This study was to developed and optimize a house dust mite (HDM) Der p1 DNA vaccine to generate specific Th1-type responses as a prototype vaccine to be further evaluated its role as a HDM immunotherapy To develop an efficient DNA vaccine, humanized synthetic gene encoding mature Der p 1 allergen was cloned into pHIS plasmid vector containing a CpG motif. The CpG motif, toll-like receptor 9 (TLR9) ligand, served as an adjuvant to activate powerful specific Th1 responses. The protein expression of the construct was tested in pGFP-mHuDer p 1 transfected HEK cells and showed a detection of the chimeric GFP-Der p 1 protein. Intradermally immunizations of pHIS-mHuDer p 1 or control plasmids with and without liposome were performed in Balb/c mice. The immunogenicity was examined by ELISA assays. The results showed mice vaccinated with pHIS-mHuDer p 1 mounted Der p1 specific Th1 immune responses, as evidenced by the detection of specific IgG2a antibody, while it was not detected in control group. Of interest, mice immunized with lipoplexes (liposome-pHIS-mHuDer p 1 DNA) were more efficient to generate faster and higher Der p 1 specific IgG2a responses. An HDM allergic mouse model had also been developed, ProDer p 1 with alum was injected intraperitoneally into mice and had shown a specific Th2 response with IgG1 and IgE antibodies against Der p 1 protein.

In conclusions, a humanized Der p1 DNA vaccine has been developed preclinically. Replacing dust mite codons to mammalian codon usage had increased protein expression and its immunogenicity significantly. Immunogenicity studies in mice, when the recombinant humanized Der p 1 DNA injection intradermally showed specific Th1 immune response. Moreover, the lipoplexes formulation was significantly increased the efficacy of the DNA vaccine. This candidate vaccine is therefore warranted for further development as a therapeutic vaccine for house dust mite allergy.

Field of Study : .MEDICAL MICROBIOLOGY. Student's Signature:.....

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LIST OF ABBREVIATIONS

bp	=	base pair
dNTP	=	deoxynucleotide-5'-triphosphate
^{o}C	=	degree celcious
EDTA	=	ethylenediamine tetraacetic acid
EtOH	=	ethanol
fw	=	forward sequence
g	=	gram
hr	=	hour
kDa	=	kilodalton
L	=	liter
min	=	minute
ml	=	milliliter
MW	=	molecular weight
OD	=	optical density
ng	=	nanogram
NSS	=	normal saline solution
PAGE	=	polyacrylamide gel electrophoresis
PBS	=	phosphate buffer saline
RNase	=	ribonuclease
rpm	=	revolution per minute
rv	=	reverse sequence
sec	=	second
TEMED	=	N,N,N',N'-tetraethyl-ethylenediamine
Tm	=	melting temperature
μg	=	microgram
μl	=	microliter
U	=	unit

CHAPTER I

INTRODUCTION

Allergens are common environmental antigens that induce specific IgE antibody responses and may lead to clinical allergic disorder. Common allergens such as house dust mites and their excretion, pollen, pet dander have been a common cause of allergy. When these sensitized substances are inhaled, swallowed or come into contact with the skin they are recognized by components of the immune system lead to allergic inflammation (1). Upon exposure to an allergen, B cells are induced to produce IgE antibodies. IgE antibodies are secreted and bind to a high-affinity receptor on the surface of mast cells and basophiles. Introduction of allergens and subsequent production of IgE cause allergic sensitization. Reintroduction of foreign antigens result in cross-linking of the IgE antibodies to the cell surface and subsequent activation of mast cells, causing a rapid release of mediators such as histamine, leckotriens, cytokines, chemotactic and enzyme that leads to allergic reaction, such as swelling of tissues, sneezing, wheezing, coughing and other reactions (2). Asthma is a chronic respiratory disease caused by allergens that affect millions of people worldwide, and numerous studies have shown that the prevalence of asthma is increasing (3, 4). House dust mites are important sources of indoor allergens associated with asthma and other allergic diseases such as Allergic Rhinitis and Allergic Dermatitis. In Thailand, approximately 20% and 40% of adults and children

suffer from allergic rhinitis, while approximately 5% to 10% of Thais from all age groups suffer from asthma (5, 6).

Dust mites are members of phylum Arthropoda, class Arachnida, order Acari, suborder Acaridae. The Acaridae are futher divided into three major families, one associated with scabies and the other two with asthma (7). The most common dust mite species around the world include: *Dermatophagoides pteronyssinus* (Dp), *Dermatophagoides farinae* (Df), *Euroglyphus maynei* (Em) and *Blomia tropicalis* (Bt) (7, 8). Voorhorst *et al* has been reported that dust mites are the major source of allergen in house dust (9). Many important allergens from these mite species have been identified and characterized at molecular level. Biological function of several house dust mite allergens has been elucidated, with many of them showing enzymatic activity (3, 7, 8). Up t 23 groups of different *Dermatophagoides* allergens have been identified (10, 11). Group 1 allergens (Der p 1 and Der f 1) are strong immunogens in human. Der p 1 and Der f 1 are closely related (84% amino acid sequence identity) (12, 13). Studies in Thailand found that about 70% of allergic children were sensitized to allergens of Der p 1 and Der f 1, of which these two mite species are most abundant in Thailand (14). Approximately 85% of individuals with asthma have IgE antibodies to house dust mite (HDM) allergens (15).

Der p 1 protein is a cysteine protease (16, 17), which is found in mite feces. A full-length Der p 1 cDNA encodes 320 amino acid residues. When translated, Der p 1 comprises of a signal peptide (18 amino acids), a proenzyme region (80 amino acids), and enzyme (222 amino acids) (17). The amino acid sequence of Der p 1 was showed homology with a group of cysteine proteases (12). Der p 1 induce IgE production by cleaving cell surface molecules such CD25 (18, 19) on T cells and

CD23 on B cells (20-22) . When patients are sensitized by allergen, the specific IgE antibodies were produced and then leading to an immunological sensitization that characterized by a predominance of T helper 2 cells (Th2) mainly producing various cytokines such as IL-4, IL-5 and IL-13 (23, 24). The consequences of the enzymatic properties would be to promote allergic inflammatory responses. Furthermore, by disrupting epithelial tight junctions, Der p 1 facilitates the transport of allergens across the epithelium (25)

Allergen immunotherapy involves the administration of gradual increasing quantities of specific allergens to patients with IgE-mediated conditions until a dose is reached the effective in reducing the severity of the disease from natural exposure (26). The major objectives of allergen immunotherapy are down-regulating Th2 responses to allergic triggers that participate symptoms in the short term, decreasing inflammatory response and preventing the development of persistent disease in the long term (27). The successful and safety of immunotherapy have been shown to be effective in the treatment of stinging-insect hypersensitivity, allergic rhinitis or conjunctivitis, and allergic asthma (26). While immunotherapy is an excellent way to protect against insect venom induced anaphylaxis and the above listed allergies, its side effects and the high frequency of injections over several years may not be the treatment of choice for some patients (28).

To enhance specific T cell response and to increase the safety for House Dust Mite (HDM) immunotherapy, a number of approaches targeting the immune pathway in inflammation have been proposed to reduce the allergic responses. One of the approaches is DNA vaccine. This is a powerful approach to generate better and efficient vaccines. As compared to other vaccine strategies (conventional live

attenuated, whole killed, or subunit vaccines), DNA vaccines have more advantages. For example, injection of plasmid DNA could induce Th1-biased immune responses. DNA vaccine promotes IFN-γ production by CD4+ T-cells (29, 30), elevates production of IgG2a (31), and inhibits of IgE synthesis (29).

To increase the potency of Th1 immune responses, a DNA vaccine model containing immunostimulatory sequence (ISS) has been examined (32-34) The immunostimulatory sequences (ISS) are very rare in vertebrates but are present in many lower, potentially pathogenic organisms. For example, bacterial DNA or oligodeoxynucleotides, containing CpG motifs (CpG-ODN) or an unmethylated CpG dinucleotide, could stimulate antigen-presenting cells (APCs) to produce IL-12 as well as Natural Killer cell activity (34-36). The clinical test using an ISS chemically conjugated to Amb a 1 (Amb a 1-ISS) in a ragweed induced model of allergic asthma showed a reversal of the established manifestations of asthma (37). Thus, the ISS could be a powerful adjuvant to activate of the innate arm of immune response (34-36).

In addition to the ISS, immunogenicity of a DNA vaccine could be improved by optimizing codon usage to improve genetic immunization with allergen genes. It is based on the fact that most amino acids are encoded by more than one codon and codon usage varies from organisms. Differences in the codon usage concerning a heterologous gene and transfected host cells could have a strong effect on protein expression with a significant influence on the immunogenicity of a genetic vaccine (38). Codon-optimization refers to the alteration of gene sequences to make codon usage match the available tRNA pool within the host cell/species of interest.

To increase overall DNA vaccine immunogenicity, two major strategies were included i.e., codon optimization and the use of a plasmid containing a toll-like

receptor (TLR) ligand. The house dust mite DNA sequences as target codons were optimized to increase the efficiency of its protein expression. CpG motifs, known as a TLR9-ligand, were used in this DNA vaccine design. A plasmid vector, pHIS that contains CpG motifs was used to construct with the codon optimized mature Der p1 gene. Taking together, the DNA vaccine comprises of the ISS and codon-optimized allergen gene would become the best model to develop better House Dust Mite (HDM) DNA vaccine. If this candidate is good immunogenic and safe, it will be evaluated clinically in the future project of its role as a therapeutic vaccine for individuals with house dust mite allergic airway diseases. Therefore, this study aims to develop HDM DNA vaccine constructed with the ISS and codon-optimized Der p 1 gene.

Research Questions

Whether DNA vaccine constructed with human codon-usage optimization of Der p 1 DNA and the CpG motif could induce strong Der p 1 specific Th 1 responses and improve allergic immune response in mice.

Objectives

1. To construct full length Der p 1 containing human codon-usage sequence with the CpG motif.

2. To evaluate the immunogenicity of HDM DNA constructs in mice.

CHAPTER II

LITERATURE REVIEWS

Background and Rationale

Allergy is an abnormal sensitivity to a substance which calls an allergen. Genetic and Environmental may be an influence factors to induce Th2-mediated allergic inflammatory response (39). The most common allergens include house dust, dust mites, pet dander, pollen, mold and mildew (40). An allergic reaction occurs when allergens enter to the body. In the allergic-prone person, the first exposure to allergens such as mite allergen, they will produce a large amount of corresponding specific IgE antibodies. These specific antibodies will bind to high-affinity FcεRI receptors on the mast cells or basophils. When predisposing person encounter to the same allergen, the allergens cross-links the IgE molecules bound to mast cells and basophils can cause bridging of the specific IgE on mast cell or basophils. This bridging will initiate the release of chemical (mediators) such as histamine and other chemicals from the mast cells or basophils (Figure 1). These mediators produce the symptoms of an allergic reaction, such as swelling of tissues, sneezing, wheezing, coughing and other reactions (40).

Asthma is a disease in which inflammation of the airways causes airflow into and out of the lungs to be restricted. The muscles of the bronchial tree become tight and the lining of the air passages swells, reducing airflow and producing the characteristic wheezing sound. This is known as an asthma attack, which can occur as

an allergic reaction to an allergen or other substance (acute asthma), or as a part of a complex disease cycle which may include reaction to stress or exercise (chronic asthma). Asthma is common in Thai children with a prevalence of about 4%. The most frequency was affected the boys than girls. Skin test positive to House dust mite was found in these patients (41).

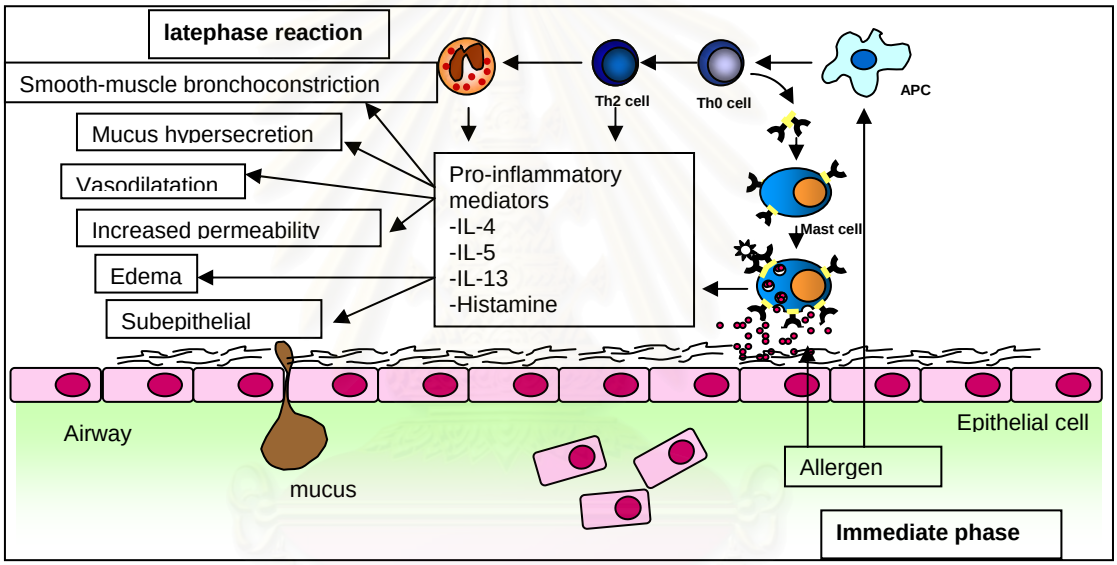

Figure 1 Allergic reactions (42). Genetic, environmental and lifestyle are the factors to promote an allergic immune response. Acute allergic reactions are due to the antigen-induced release of various kinds of mediators such as histamine from activated mast cells. Th2 cells will release the Th2 cytokine such as IL-4, IL-5, IL-9 and IL-13 to induce an inflammatory response by recruitment of eosinophil, Ig-E-induced mast cell degranulation leading to chronic allergic reaction, including late phase reaction.

Dust mite

Mite allergens are divided into specific groups on the basis of their biochemical composition, sequence homology, and molecular weight. The association between asthma and house dust was initially proposed by R.A. Kern in 1921 and the specific agent was identified by R. Voorhorst and colleagues. Thirteen species have been found in house dust, 3 of which are very common in homes worldwide and are the major source of mite allergen. The most common of these species are *Dermatophagoides farinae* (*D farinae* or Df), *Dermatophagoides pteronyssinus* (*D pteronyssinus* or Dp), and *Euroglyphus maynei* (*E maynei*), which are found in temperate climates (7). In tropical climates, the storage mite *Blomia tropicalis* (Family Echymyopodidae) can be a prevalent mite in dwellings, along with other Pyroglyphid mites. The most prevalent mites found worldwide and in the United States are Dp and Df (43). *E maynei* may be prevalent in some temperate geographic area (43). In addition, other astigmatid mites (storage mites) can be found in homes and are a potent source of allergens (43). Group 1 allergens consist of Dp 1 and Df 1 that was the strongest mite allergens in human. Both Dp 1 and Df 1 amino acid sequence are closely related, about 84% of amino acid sequence (12, 13). In Thailand, house dust mite population has been reported in 1995 (14). The house dust mites (HDM) in dust sample from the different parts of Thailand (central, northern and northeastern) were studies (14). House dust mites were identified in 555/630 (88.1%) of collected dust samples. Dp mites were the most abundant species (at mean count 87.2 mites/gram dust) (14).

House dust mites have a thin, permeable skin, which makes them vulnerable to water loss. The mites like to live in places of high humidity ideally 75–80% relative

humidity (RH) (43, 44). House dust mites can also be found in carpets and upholstery. Both are sites that can accumulate skin scales if not clean thoroughly, like mattresses and pillows, allowing dust and mite populations to build up over many years. The average HDM lives for around three months. It goes from egg to larva, to two juvenile stages and developing into a reproductive stage (44). A female mite can produce 2-3 eggs per day, about 100 eggs per life cycle. The mite eats organic materials and takes in oxygen through its outer cover. The mite favorite food is human skin scales (43, 44). House dust mites feed on human skin scales which they shed all the time. Approximately 1g was shaded by human per week. A house dust mite can produce up to 20 droppings a day. The droppings are so small and can be suspended in still air for up to 20 minutes (44) before dropping to the surface. House dust mites can produce around 2000 fecal pellets/life cycle (43), each containing digestive enzymes and product from a diet of mite such as fungus, shed skin scales, pollen, plant fibers, and bacteria to which some people are allergic (43, 44). These small pellets are air-borned and breathed in by humans and stored in the bronchi. In allergic-prone person, these pellets cause a local inflammation in the area of the bronchi where they have stored (44).

Table 1 Molecular characteristic of allergens (10, 11, 45)

Allergen	Frequency of relative (%) IgE binding	MW (kDa)	Function
Group 1 (Der p 1)	>90	25	Cysteine protease
Group 2 (Der p 2)	>90	14	Homology with putative human epididymal protein
Group 3 (Der p 3)	51-90	31	Trypsin
Group 4 (Der p 4)	25-46	60	Alpha Amylase
Group 5 (Der p 5)	55	13	Unknown
Group 6 (Der p 6)	39	25	Chymotrypsin DP5
Group 7 (Der p 7)	53-62	26, 30, 31	Unkown
Group 8 (Der p 8)	40	27	Glutathione transferase
Group 9 (Der p 9)	>90	29	Collagenase-like serine protease
Group 10 (Der p 10)	81	36	Tropomyosin
Group 11 (Der f 11)	82	98	Paramyosin
Group 12 (Blot 12)	50	16	May be chitinase
Group 13 (Lep d 13)	11-23	15	Fatty acid-binding protein

Table 2 Molecular characteristic of allergen (cont.) (10, 11, 45).

Allergen	Frequency of relative (%) IgE binding	MW (kDa)	Function
Group 14 (Der f 14)	84	177	Apolipophorin mag 3
Group 15 (Der f 15)	95	98/109	Chitinase
Group 16w (Der f 16)	35	53	Gelsolin / villin
Group 17w (Der f 17)	35	53	Calcium-binding protein
Group 18w (Der f 18)	?	60	Chitinase
Group 20 (Der p 20)	?	?	Arginine kinase
Group 21 (Der p 21)	?	?	?
Group 23 (Der p 23)	?	14	Unknown function, homology to peritrophin-A domain

Der p 1 protein

A major house dust mite allergen (Der p 1) has been cloned and sequenced (12). Mature Der p 1 is a 25-kDa glycoprotein with cysteine protease activity located in the mite gut and found in high concentration in feacal pellets (12, 46). Full-length cDNA encodes a precursor of 320 amino acid residues, including 18 amino acid of signal peptide, 80 amino acid of pro-enzyme region, and mature enzyme is 222 amino acid residues (12). The N-terminal part of the pro region contains two α-helices (α1 and α2) and the remainder sequence contains two α-helices (α3 and α4) and two β-strands (β1 and β2) (46). The crystal structure of ProDer p 1 was showed in Figure 3. This protein was shown the highly degree of homology between the mature Der p1 with the sequences of the cysteine proteases, papain, actinidin and papaya proteinase f1 by sequence alignment (12, 47) (Figure 2). Der p 1 shares essential structural and mechanistic features with other papain-like cysteine proteinases, including cathepsin B (47). Some study revealed that Der p 1 shown unique mixed cysteine and serine protease activity (21). The proteolytic activity of Der p1 could be inhibited by E64 (l-trans-epoxysuccinyl-leucylamido[4-guanidino]butane) and iodoacetamide (18). Furthermore, serine protease inhibitor, α-antitrypsin that secreted by lung epithelial cells can inactivate Der p 1(48). Der p 1 contain four potential N-linked glycosylation sites, three in the mature sequence and one in pro-peptide (49, 50). Pro-enzyme region of Der p 1 serves as in the correct folding of the mature portions (51) and additional function as reduced allerginicity of this allergen (51, 52) by biologic activities in inducing the release of histamine from allergic person compared with the recombinant mature forms (51).

The proteolytic activity of Der p 1 has been shown to involve in the function of the innate and adaptive immune systems. The proteolytic activity of Der p 1 was showed to cleave cell surface molecules involved in regulation of IgE response i.e. Der p 1 selectively cleaves human CD 25 on T cells (53), cultured mouse spleen T cells (54) and CD 23 on a B cells (55). Der p 1 was showed to disrupt of intracellular permeability by increasing epithelial permeability of tight junction (Tjs) that are the part of the epithelial paracellular permeability barrier (25, 56) and induced significantly higher levels of inflammatory cells in lung (57).

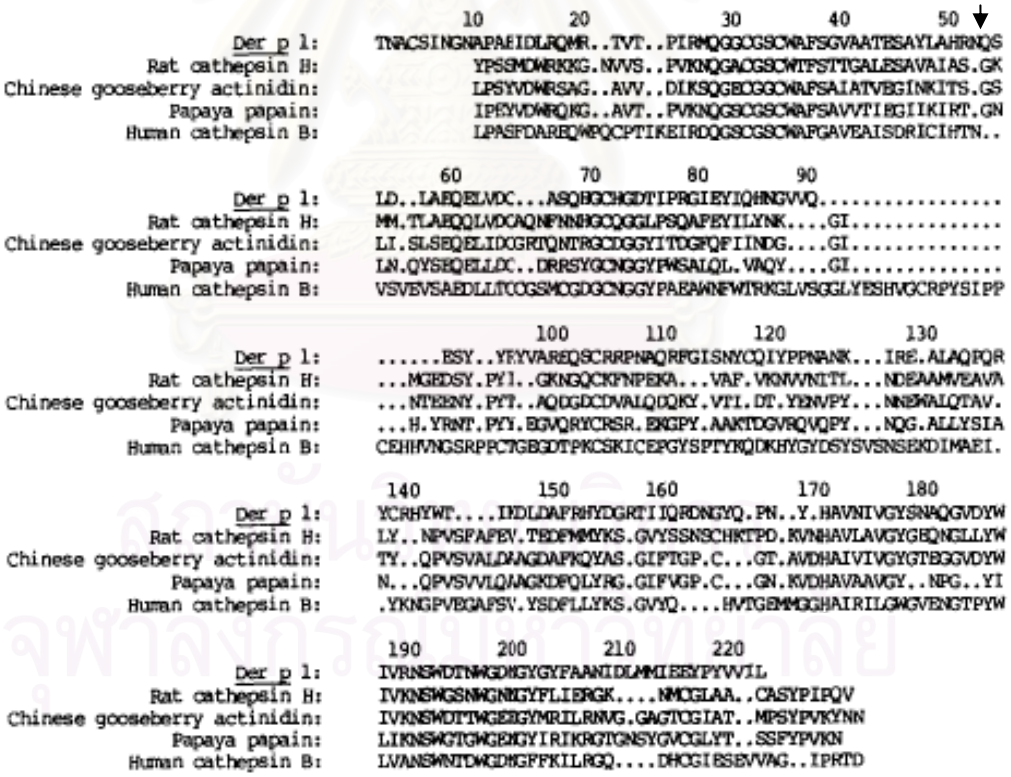

Figure 2 Comparison of alignment sequence of Der p 1, Rat cathepsin, Actinidin, papain, and human cathepsin (12). (▼) amino acid 52-54 are *N*-glycosylation site.

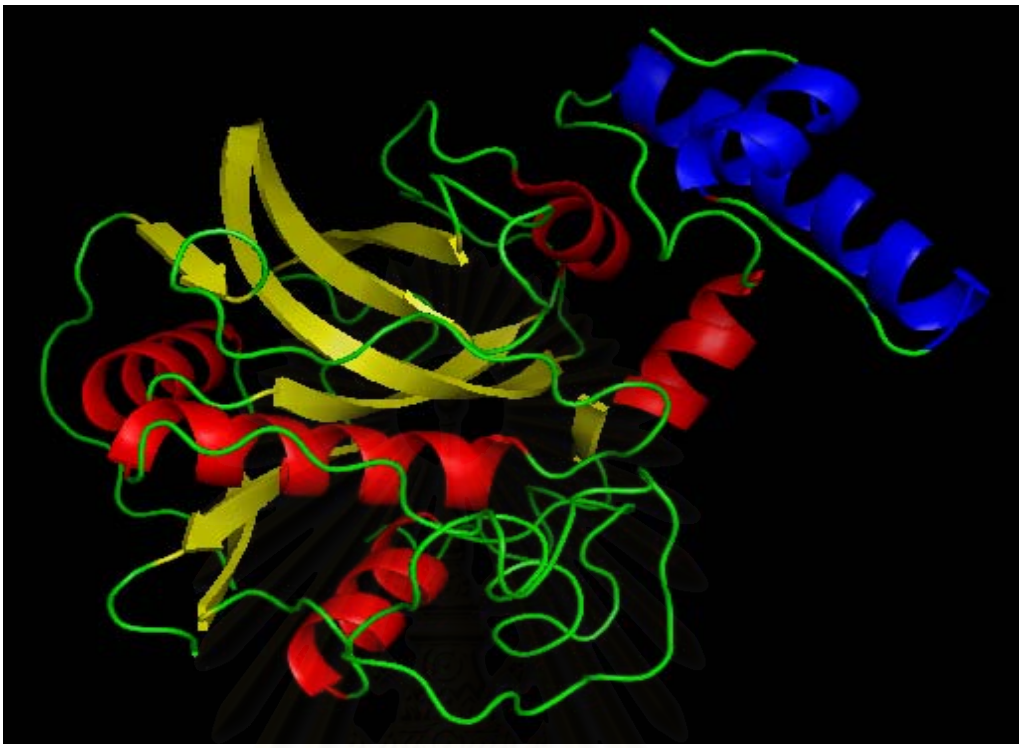

Figure 3 The crystal structure of ProDer p 1 protein (ixkg.pdb) (58). Pro-sequence are shown in blue, α-Helices are shown as red, β-strands are shown as yellow. The model of molecule was generated using modified by PyMol software (DeLano Scientific LLC, USA).

Allergen immunotherapy

Allergen immunotherapy has been used as a method to reduce the allergic responses by giving a small but gradually increasing amount of allergens to an allergic patient, thereby minimizing symptomatic expression of the disease (59). Protocol of Immunotherapy treatment is weekly injections 8-16 weeks during an updosing phase, followed by monthly maintenance injections for a period of 3-5 years (60). This is the

best for treatment in allergic patient with rhinitis, allergic asthma or systemic reactions to hymenoptera/fire ant venom (61, 62). The mechanism of action of allergen specific immunotherapy involved with exposure to an allergen, and then allergen-specific Th2 cells were activated and produced their cytokine such as IL-4 and IL-13, results in an allergen specific immunoglobulin E (IgE) production, crosslink of IgE-prime mast cells, mediator release and recruitment of an inflammatory cells i.e., eosinophil in the site of inflammation such as lung (62). The different propose of allergen immunotherapy development depending on the basic of characteristic of allergen molecule and their epitope that cause allergic responses (63). During desensitized process between courses of allergen-specific immunotherapy, T cell will become tolerate by the induction of regulatory T cells (62). The production of regulatory cytokine such as interleukin 10 (IL-10) and transforming growth factor-β (TGF-β) will suppress the allergic immune response by regulation of T-cell function and immunoglobulin class switching to IgA, IgG1 and IgG4 to block IgE for antigen binding (64). These processes decrease the amount of mast cells and their activation and granulation (64). The limitation of allergen immunotherapy was reported. The treatment of natural allergen that was contamination with other components that the patient was not sensitized before treatment, results in development of new IgE response (65). Using the natural allergen extract that represent some allergic and non allergic compounds are the major disadvantage of current immunotherapy (66). The successfully of allergen-specific immunotherapy was first report in allergic patient with grass pollen in 1911 by Noon and Freeman (67). Recently, many study showed an effective allergen immunotherapy in animal model such as ovalbumin treating mice showed an inhibition of airway eosinophilia and hyperresponsiveness associated with

decreased IL-4 production (68). Allergen immunotherapy has been showed more effective in clinical study such as grass, ragweed, house dust mite, weeds, cat, mold and multiple allergen (69).

Vaccines

Vaccine is a biological preparation that establishes or improves immunity to a particular disease. Vaccines can be prophylactic function such as prevent or ameliorate the disease or therapeutic function to cure the disease. The criteria for effective vaccines were shown in Table 3.

There are four types of tradition vaccine. The comparison of vaccine types were shown in Table 4.

1. Live attenuated vaccine, which prepared from attenuated strain or non-virulent strain. They could stimulated protective immune response and provide continuous antigenic stimulation when they multiply in the host such as yellow fever, measles, rubella and mumps(70). This type of vaccine are highly effective (71). The limitation of this vaccine was the risk of reversion to their pathogenic form that can causing an infection (70).

2. Subunit vaccine, which contains a fragment of organism, composed of antigens purified and inactivated from microbes. This vaccine can stimulated helper T cells and antibody response, but not potent for cytotoxic T cells response (70), such as sub unit vaccine against Hepatitis B virus that contains only surface protein of virus.

3. Vaccines containing killed microorganisms, the virulent micro-organisms have been killed with chemicals or heat such as vaccines against flu, cholera, bubonic plague, and hepatitis A.

4. Toxoids, which are inactivated toxic compounds in cases where these cause illness, such as toxoid-based vaccines include tetanus and diphtheria (57).

Table 3 The several criteria for an effective vaccines (72).

	Properties
Safty	Vaccine must not itself cause illness or death
Protective	Vaccine must protect against illness resulting from exposure to live pathogen
Induces neutralizing antibody	Some pathogens (such as polio virus) infect cells that cannot be replaced (e.g., neurons) Neutralizing antibody is essential to prevent infection of such cells
Induces protective T cells	Some pathogens, particularly intracellular, are more effectively dealt with by cell-mediated responses
Practical considerations	Low cost per dose Biological stability Ease of administration Few side-effects

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Table 4 Comparison types of vaccine (73).

		DNA vaccine	Live attenuated	Killed/protein subunit
Immune response				
Humoral	B cells	+ + +	+ + +	+ + +
Cellular	$CD4^+$	+ + + Th1[a]	+/− Th1	+/− Th1
	$CD8^+$	+ +	+ + +	−
Antigen presentation		MHC class I & II	MHC class I & II	MHC class II
Memory	Humoral	+ + +	+ + +	+ + +
	Cellular	+ +	+ + +	+/−
Manufacturing				
	Ease of development and production	+ + + +	+	+ +
	Cost	+ + +	+	+
	Transport/Storage	+ + +	+	+ + +
Safety		+ + +[b]	+ +[c]	+ + + +

[a]Th1 responses can be induced by gene gun immunization in mice.
[b]Data available only from Phase 1 trials.
[c]Live/attenuated vaccines may be precluded for use in immunocompromised patients and certain infection such as HIV.

DNA vaccine

In the past, gene therapy has been an approach for DNA vaccine such as in 1990 *Wolff et al* reported the plasmid injection for muscular dystrophy into the muscle (74). In 1992, Tang *et al* had reported that gene gun as a new delivery technique for genetic immunization (74). The immunoprotective has been reported in influenza virus mice model in 1993 by Ulmer *et al* and Robinson *et al* (74). The immune response in mice has been reported after injection of genes derived from HIV (Wang *et al*. 1993) or hepatitis B virus (Davis *et al* in 1993) and the successful immune response

to hepatitis B virus surface antigen has been reported in 1993 by Mancini *et al* (74). DNA vaccines are bacterial derived plasmids containing a gene that encoding desired antigen. DNA vaccine is a novel technique that has been used to induction of humoral and cellular immune responses to protein antigens. The direct injection of genetic material into a living host causes a small amount of its cells to produce the introduced gene products. The common feature and advantage/disadvantage of DNA vaccine was shown in Table 5 and 6.

When the plasmid DNA was constructed and introduced into animal tissue with appropriate delivery system (Table 7). Professional antigen presenting cells (APC) uptake the plasmid DNA into its endolysosomal degradation pathway. The protein is produced and processed into small antigenic peptides. The peptides enter to the endoplasmic reticulum (ER) then bind to major histocompatibility complex (MHC class I) molecules. Consequently, cell-mediated immunity is generated. The foreign protein could be presented by major MHC Class II molecules leading to elicit helper T cell response that recognized as exogenous protein, which phagocytosed by APC.

DNA construction

DNA vaccine s elicited the best immune response when highly active expression vectors are used. In generally, DNA vaccine is composed of a bacterial plasmid vector that contains heterologous genes inserted under the control of eukaryotic promoter leading to protein expression in mammalian cells. Expression plasmids used in DNA-based vaccine normally contain two unites: the antigen expression unit and the production unit. For the expression unit, plasmid vector composed of a strong promoter/enhancer sequences, followed by antigen-encoding and polyadenylation sequences (75). The most frequency promoter sequences which have

been used come from the cytomegalovirus (CMV) or simian virus (SV40) (76, 77) or rous sarcoma virus (RSV) (73, 77) to drive the *in vivo* transcription and translation of gene. The insertion gene was cloned into downstream of the promoter that contains a polyadenylation (poly A) sequence for the stability of mRNA transcription. The most frequency used of poly A sequence came from bovine growth hormone gene (BGH) or SV40 polyadenylation sequence(77). For the production unit, this part composed of bacterial sequences necessary for plasmid amplification and selection (75). The most commonly used for selection marker are bacterial antibiotic resistance genes such as the Kanamycin or Ampicilin resistance gene.

Kozak sequence

Kozak sequence is the sequence flanking the AUG initiator codon within mRNA control its recognition by eukaryotic ribosome (76). The Kozak sequence has been identified as -6 GCCA/GCCAUGG +4 located around the initiator codon that is proposed to initiation of translation. This sequence required for optimal translational efficiency of expressed mammalian genes. It has been suggested that, when the -3 position contains purine base or a guanine is on position +4 (76).

Codon usage

The codon usage was biased depend on organism species example mammalian codon usage is different from microorganisms. Such the codon (or tRNA availability) for some species is poor for other species, results in yield of gene expression. This selection of codon in each species will affect the efficiency of gene expression. Consequently, codon optimization can have an effect on protein expression by transfected cell with significant influence on the immunogenicity of a DNA vaccine. As reported in 2003 by Bauer *et al*, they recoded the Art v 1 allergen to be humanized

codon, results in the improvement of the protein expression level and immunogenicity (compared with wild type gene) in mice model (38).

Immunostimulatory DNA

DNA stimulatory DNA sequence (ISS) is act as a powerful adjuvant for activation of the innate immune response. This properties was first recognized as Freund's adjuvant consisting of mycobacterial extract in oil immersion that was characterized more than 60 years ago (32). In 1980, Tokunaga *et al* reported the properties of the mycobacterial genomic DNA that could be a potent antitumor activity (32). Klinman DM *et al* had reported the bacterial DNA specific sequence that contains an unmethylated CpG dinucleotide flanked by two 5'purines and two 3' pyrimidines that was found more frequency in bacteria than vertebrates (78, 79). This sequence was recognized by Toll-like receptor 9 (TLR 9) that expressed by B-cells and plasmacytoid dendritic cells (pDCs) (78) results in Th1 immune response such as induction of Th1 cytokines i.e. IL-12 and IFN-γ. CpG motif can be added to the gene of interest or constructed into the vector backbone (80). Conjugated CpG linked with allergens have been reported such as Amb a1 allergen conjugated with CpG could enhance Th1 bias immunogenicity, reduced the allerginicity and reversed the established allergic responses in the lung of mouse model (37, 81). In human, shot course immunotherapy with Amb a1 linked to immunostimulatory phosphorothioate oligodeoxyribonucleotide had reported that the challenged could be increased Th1 cytokine production and decreased Th2 cytokine production and eosinophilia (82) and increased CD4+CD25+ T cells in the nasal mucosa of subjects with allergic rhinitis (83).

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Table 5 Feature of DNA vaccines (77).

Features	Characteristic
Subunit vaccines	DNA vaccines express only selected component of the antigen
Raise both CMI and HMI	protein synthetized in cells, expresses antigen enter pathways for MHC I and/or MHC II
Can recovery of immunogen by PCR	no requirement for the culture of a pathogen or the production of a protein for vaccine development
Designer genes	Design of gene include single gene, multiple genes or fusion gene with/without leader, ubiquitination, lymphokines, or immunostimulatory sequence
Stability	DNA is stable at room temperature

Table 6 The advantages and disadvantages of DNA vaccines (84).

Advantages	Disadvantages
Able to produce expression of antigen that resemble native form of antigen	Limited of protein antigens (86)
No risk for infection/replication in host cell	Potential for atypical processing of bacterial and parasite proteins
Antigen presentation by both MHC class I and class II molecules	Possibility of inducing antibody production against DNA
Can be polarization of the immune response (85) such as coupling with some adjuvant (86)	Need for codon optimization because obstacle in codon usage in difference species (86)
Immune response focused only on antigen of interest and more than one antigen can coexpression(87)	Risk of affecting genes controlling cell growth
Easy to constructtion and production	Possibility of tolerance to the antigen (protein) produced
Stability for storage at room temperature and transport (86)	Potential for atypical processing of bacterial and parasite proteins
Cost-effectiveness	
Long-term persistence of immunogen	
Low dose for immunization	

Table 7 The advantage and disadvantage of commonly used DNA vaccine delivery methods (84, 88).

Method of Delivery	Advantage	Disadvantage
Intramuscular or Intradermal injection	Direction injection Permanent or semi-permanent expression pDNA spreads rapidly throughout the body	Inefficient site for uptake Sometimes very poor results Relatively large amounts of DNA used
Electroporation (89)	Short pulses of high electric current Enhance DNA vaccine delivery Avoid indirect and inefficient route of endocytosis	Cell damage Non specific transport
Gene Gun	DNA –coated bead bombarded directly into cells Small amounts DNA	Requires inert particles as carrier
Jet injection	No particles required DNA can be delivered to cells mm to cm below skin surface	Significant shearing of DNA after high-pressure expulsion 10-fold lower expression, and lower immune response Requires large amounts of DNA
Complex DNA such as Liposome-mediated delivery	High levels of immune response can be generated Can increase transfection of intravenously delivered pDNA Intranasally delivered liposome-DNA complexes can result in expression in distal mucosa as well as nasal muscosa and the generation of IgA antibodies	Toxicity Ineffectiveness in serum Risk of disease or immune reactions

CHAPTER III

MATERIALS AND METHODS

1. Experimental strategy

The aim of this study was to develop DNA vaccine containing codon-optimized Der p 1 DNA and the CpG motif. All experiments were carried out in sequence as shown in a flow chart (Figure 4). The main sequential steps consist of the design of full length Der p1 humanized codon, PCR-based amplification, cloning into the pHIS plasmid vector, *in vitro* characterization of the recombinant Der p1 plasmid DNA, and finally the recombinant DNA vaccine was evaluated whether it was immunogenic in mice model. As current evidences have suggested that, in general, a DNA vaccine usually requires a booster, such as with a recombinant protein vaccine, to generate sufficient immunogenicity. Thus, in parallel to DNA vaccine development, a recombinant pro-Der p1 candidate protein vaccine has also been developed and was tested for its immunogenicity in mice.

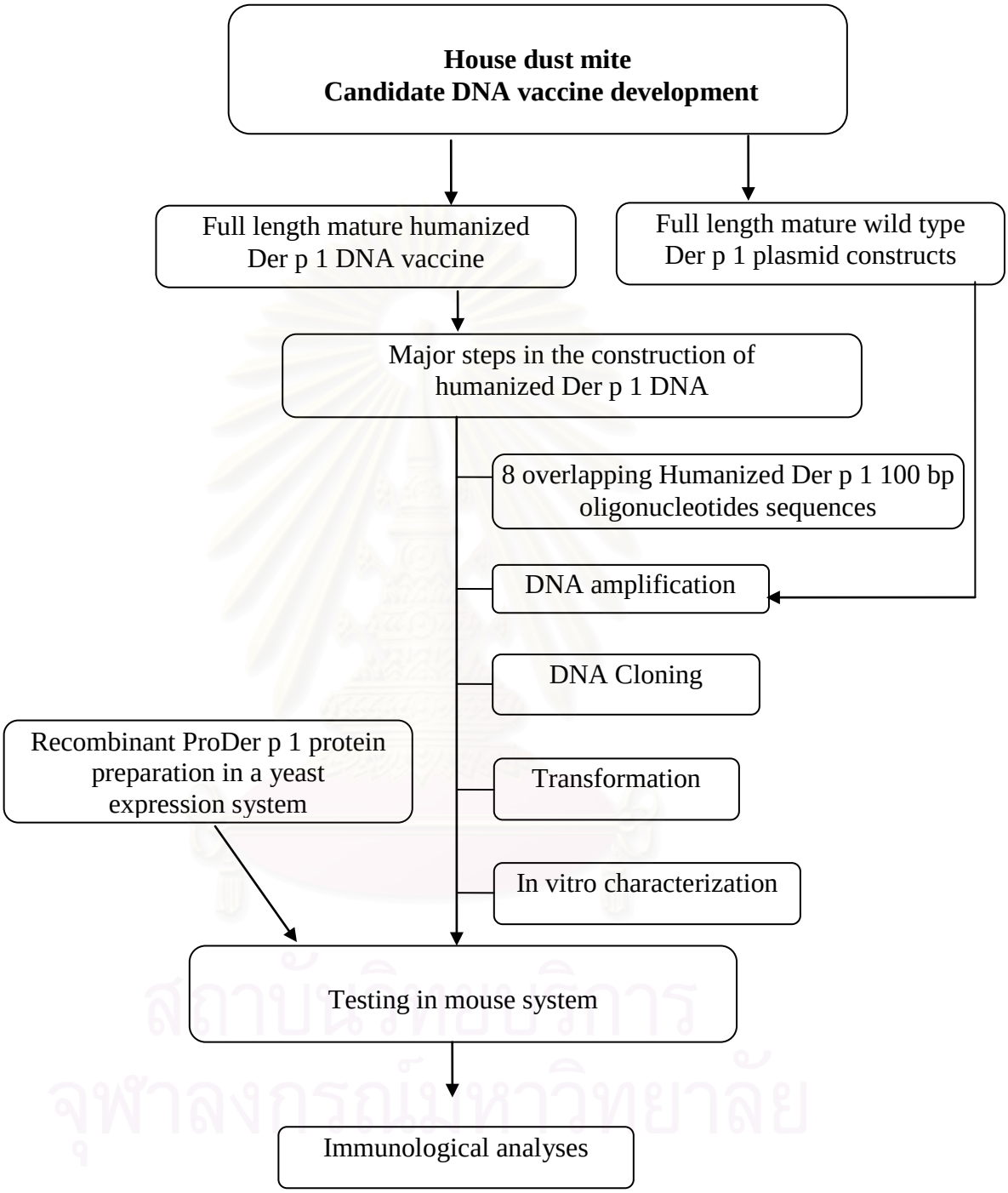

Figure 4 Flow chart of the key experiments

2. Human codon optimization for the Der P 1 full length sequence

To synthesized full length human codon-optimized or humanized Der p 1 DNA, 8 partial overlapping Der p 1 humanized codon oligonucleotides were designed and synthesized for PCR amplification to construct full length mature humanized Der p 1 (mHuDer p 1) DNA. Humanized Der p1 gene was designed by using the human codon usages (www.kazusa.co.jp, Table 8). The amino acid codon that highly expressed in human gene was selected to generated full length mature humanized Der p 1 DNA (mHuDer p 1). The frequency of codon used before and after recoding codon between wild type codon and humanized codon were shown in Table 9 and 10.

Table 8 Human codon usage table (Reproduced from www.kazusa.co.jp).

Amino Acids			Codons frequency per thousand											
			1		2		3		4		5		6	
A	Ala	Alanine	GCC	27.7	GCU	18.4	GCA	15.8	GCG	7.4				
C	Cys	Cysteine	UGC	12.6	UGU	10.6								
D	Asp	Aspartic acid	GAC	25.1	GAU	21.8								
E	Glu	Glutamic acid	GAG	39.6	GAA	29.0								
F	Phe	Phenylalanine	UUC	20.3	UUU	17.6								
G	Gly	Glycine	GGC	22.2	GGA	16.5	GGG	16.5	GGU	10.8				
H	His	Histidine	CAC	15.1	CAU	10.9								
I	Ile	Isoleucine	AUC	20.8	AUU	16.0	AUA	7.5						
K	Lys	Lysine	AAG	31.9	AAA	24.4								
L	Leu	Leucine	CUG	39.6	CUC	19.6	CUU	13.2	UUG	12.9	UUA	7.0	CUA	7.2
M	Met	Methionine	AUG	22.0										
N	Asn	Asparagine	AAC	19.1	AAU	17.0								
P	Pro	Proline	CCC	19.8	CCU	17.5	CCA	16.9	CCG	6.9				
Q	Gln	Glutamine	CAG	34.2	CAA	12.3								
R	Arg	Arginine	AGA	12.2	AGG	12.0	CGG	11.4	CGC	10.4	CGA	6.2	CGU	4.5
S	Ser	Serine	AGC	19.5	UCC	17.7	UCU	15.2	UCA	12.2	AGU	12.1	UCG	4.4
T	Thr	Threonine	ACC	18.9	ACA	15.1	ACU	13.1	ACG	6.1				
V	Val	Valine	GUG	28.1	GUC	14.5	GUU	11	GUA	7.1				
W	Trp	Tryptophan	UGG	13.2										
Y	Tyr	Tyrosine	UAC	15.3	UAU	12.2								
STOP			UGA	1.6	UAA	1	UAG	0.8						

Table 9 The codons and their frequency of mWtDer p 1 DNA and mHuDer p 1 DNA.

Amino acid	WtDp1 codon	No. of wt codon	Frequency of human codon/1000	Optimized codon	No. of codon for the optimized codon
A:Ala	GCC GCT GCA	4 12 6	28.0 18.5 15.9	GCC	22
C:Cys	TGC TGT	3 4	12.6 10.5	TGC	7
D:Asp	GAC GAT	1 11	25.2 21.7	GAC	12
E:Glu	GAG GAA	0 10	39.6 28.7	GAG	10
F:Phe	TTC TTT	1 3	20.4 17.4	TTC	4
G:Gly	GGC GGA GGG GGT	4 2 0 12	22.4 16.6 16.5 10.8	GGC GGA	17 1
H:His	CAC CAT	3 3	15.1 10.8	CAC	6
I:Ile	ATC ATT ATA	8 12 0	20.9 15.8 7.4	ATC	20
K:Lys	AAG AAA	0 2	32.0 24.2	AAG	2
L:Leu	CTG CTC CTT TTG TTA CTA	0 1 1 5 2 0	39.9 19.7 13.1 12.8 7.6 7.2	CTG	9
M:Met	ATG	4	22.1	ATG	4

Table 10 The codons and their frequency of mWtDer p 1 DNA and mHuDer p 1 DNA (cont).

Amino acid	WtDp1 codon	no. of wt codon	Frequency of human codon/1000	Optimized codon	Frequency of human codon/1000 in optimized codon
N:Asn	AAC AAT	8 9	19.1 16.8	AAC	17
P:Pro	CCC CCT CCA	1 0 7	19.9 17.4 16.9	CCC	8
Q:Gln	CAG CAA	0 14	34.2 12.2	CAG	14
R:Arg	AGA AGG CGG CGC CGA CGT	0 0 0 1 8 6	12.0 11.9 11.5 10.6 6.2 4.6	AGA CGG CGC	1 13 1
S:Ser	AGC TCC TCT TCA AGT TCG	2 1 1 5 3 0	19.4 17.7 15.1 12.2 12.1 4.5	AGC	12
T:Thr	ACC ACA ACT ACG	3 1 4 0	19.0 15.0 13.0 6.1	ACC	8
V:Val	GTG GTC GTT GTA	0 8 4 1	28.3 14.5 11.0 7.1	GTG	13
W:Trp	TGG	4	13.2	TGG	4
Y:Tyr	TAC TAT	8 9	15.3 12.1	TAC	17
STOP	TGA TAA TAG		1.6 1.0 0.8		

3. Oligonucleotides and primer design

3.1 Design of overlapping Der p 1 oligonucleotides for codon optimization

Eight partial overlapping oligonucleotides spanning over the mature part of Der p 1 were synthesized (Figure 5). Two pairs of each oligonucleotides with 20 base pair overlapping sequence were amplified per reaction of polymerase chain reaction (PCR) (HuD1:100-mers 1-100, HuD2:100-mers 81-180, HuD3:100-mers 161-260, HuD4:100-mers 241-340, HuD5:100-mers 321-420, HuD6:100-mers 401-500, HuD7:100-mers 481-580, HuD8:106-mers 561-666) as shown in Table 11. The first oligonucleotides (huD1) that covers the 5'end of mature humanized Der p1 (mHuDer p 1) has *Xho* I site included for further subcloning into the pHis vector.

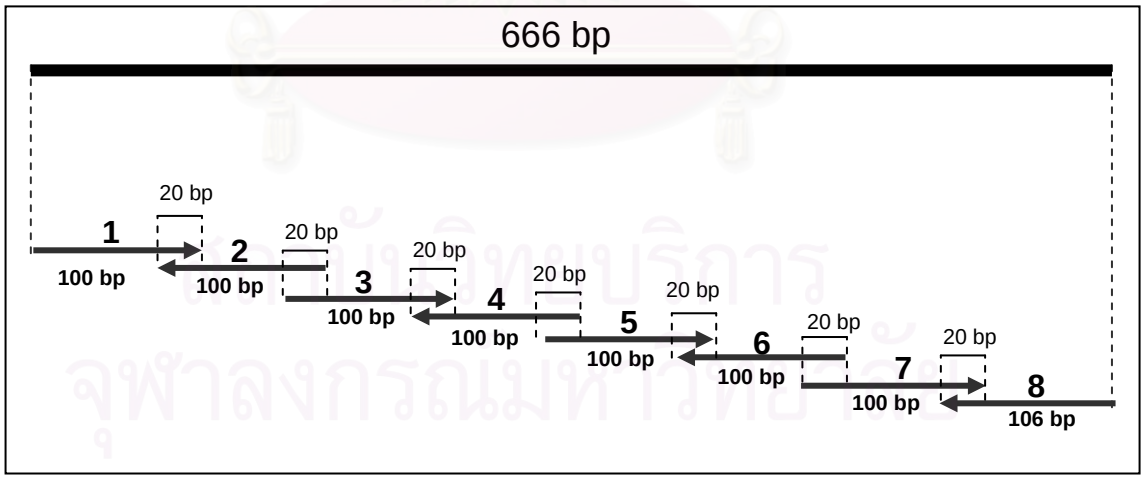

Figure 5 Diagram of 8 partial overlapping oligonucleotides spanning over the mature Der p 1 sequence. Number 1-8 was indicated as primer name (HuD1-8).

Table 11 Eight overlapping oligonucleotides sequences.

Primer Name	Nucleotides sequence	Size (bp)
HuD1	Fw: CCAACGCCTGCAG[1]CATCAACGGCAACGCCCCCGCCGAGATCGACCTGCGG CAGATGCGGACCGTGACCCCCATCCGGAT***GCAAGGCGGCTGCGGCAGCT***[2]	100
HuD2	Rv: ***CTGCTCGGCCAGGTCCAGGC***[2]TCTGGTTCCGGTAGGCCAGGTAGGCGCTCTC GGTGGCGGCCACGCCGCTGAAGGCCCAGC***AGCTGCCGCAGCCGCCTTGC***[2]	100
HuD3	Fw: *GCCTGGACCTGGCCGAGCAG*[2]GAGCTGGTGGACTGCGCCAGCCAGCACGGC TGCCACGGCGACACCATCCCCAGAGGCATC*GAGTACATCCAGCACAACGG*[2]	100
HuD4	Rv: *ATGCCGAACCGCTGGGCGT*[2]*T*GGGCCGCCGGCAGCTCTGCTCCCGGGCCAC GTACCGGTAGTAGCTCTCCTGCACCACG*CCGTTGTGCTGGATGTACTC*[2]	100
HuD5	Fw: *CCCAGCGGTTCGGCATCAGC*[2]AACTACTGCCAGATCTACCCCCCCAACGCCA ACAAGATCCGGGAGGCCCTGGCCCAGA*CCCACAGCGCCATCGCCGTG*[2]	100
HuD6	Rv: *GCTGATATCCGTTGTCGCG*[2]CTGGATGATGGTCCGGCCGTCGTAGTGCCGGA AGGCGTCCAGGTCCTTGATGCCGATGAT*CACGGCGATGGCGCTGTGGG*[2]	100
HuD7	Fw:*CGCGACAACGGATATCAGCC*[2]CAACTACCACGCCGTGAACATCGTGGGCTACA GCAACGCCCAGGGCGTGGACTACTGGAT*CGTGCGGAACTCCTGGGACA*[2]	100
HuD8	Rv:CAGGATCACCACGTAGGGGTACTCCTCGATCATCATCAGGTCGATGTTGGCG GCGAAGTAGCCGTAGCCGTTGTCGCCCCAGTTGG*TGTCCCAGGAGTTCCGCACG*[2]	106

[1] Restriction sites as indicated by underlined, *EcoR* V = GATATC, *Pst* I = CTGCAG, *Sma* I = CCCGGG

[2] Overlapping parts in each pair of oligonucleotides are indicated by italic latter

3.2 Primer designs to amplify wild type and humanized Der p 1 genes

3.2.1. Primer designs to amplified mature wild type Der p 1 (mWtDer p 1), leader Der p 3-mWtDer p 1 (LDp3-mWtDer p 1) and mature humanized Der p1 (mHuDer p 1). The PCR primers were used for amplifying and subcloning of both mWtDer p 1, LDp3-mWtDer p 1, and mHuDer p 1 into the pHIS expression vector were shown in Figure 6 and Table 12. The 5' end of forward primers of both wtDp1 and huDp1 sequences contain *Xho*I restriction site, a Kosak sequence and ATG for start codon. The 3' end of the reverse primers contains *Kpn* I

restriction site and stop codon. The melting temperatures (Tm) of each primer were calculated by using following equation:

$$T_m = 4(\text{number of G + number of C}) + 2(\text{number of A + number of T})$$

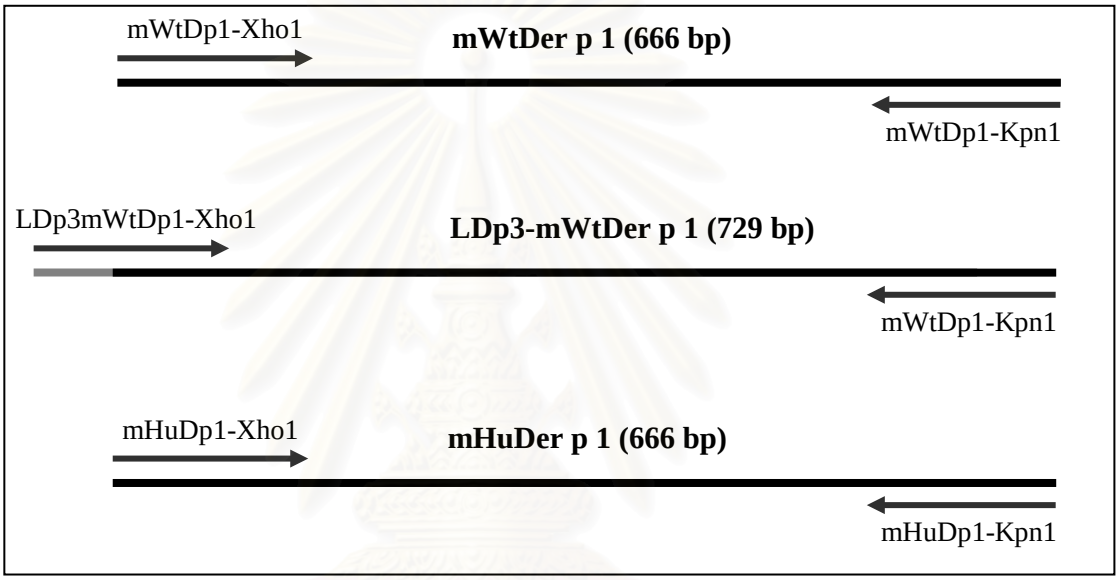

Figure 6 Diagram shows the PCR primers for amplifying Der p 1 cDNA for subcloning into pHIS expression vector.

Table 12 Primers used for the amplifications and subcloning of the mature wild type and mature humanized Der p1 DNA sequences into pHIS vector.

Primer name	Nucleotide sequence (5'→3')	T_m(^oC)
mWtDp1-Xho1	Fw: GATCTCGAG[1]**ATG**[2]ACTAACGCCTGCAGTATCAATGG	66
mWtDp1-Kpn1	Rv: GGTTGGTACC***CTA***[3]GAGAATGACAACCATTGGATATTC	64
LDp3WtDp1-Xho1-F	Fw: GAT**CTCGAG**ATGATCATCTATAATATTTTAATTGT**TTTATTATTGGCCATTAATACATTG**[4]	60
LDp3WtDp1-Xho1-R	Rv: **AAATAATAACCGGTAATTATGTAAC**CGATGATTGCGGACGTCATAGTTAC	50
mHuDp1-Xho1	Fw: GATCTCGAG**ATG**ACCAACGCCTGCAGCATCAACGG	60
mHuDp1-Kpn1	Rv: GGTTGGTACC***CTA***CAGGATCACCACGTAGGGGTACTC	62

[1]Restriction sites underlined, *Xho* 1 = CTCGAG, *Kpn* 1=GGTACC, *Pst* 1=CTGCAG

[2]Start codon as indicated in bold

[3]Stop codon as indicated in Bold-italic

[4]Overlapping sequence of primer to amplified leading part of mWtDp1 gene was indicated by bold font and underline

3.2.2. Primer designs for subcloning into pEGFP-N1 vector. The other set of reverses primers (use with forward primer as described in 3.2.1.) were designed for subcloning target mWtDer p 1, LDp3-mWtDer p 1, and mHuDer p 1 products into pEGFP-N1 expression vector (Figure 7 and Table 13).

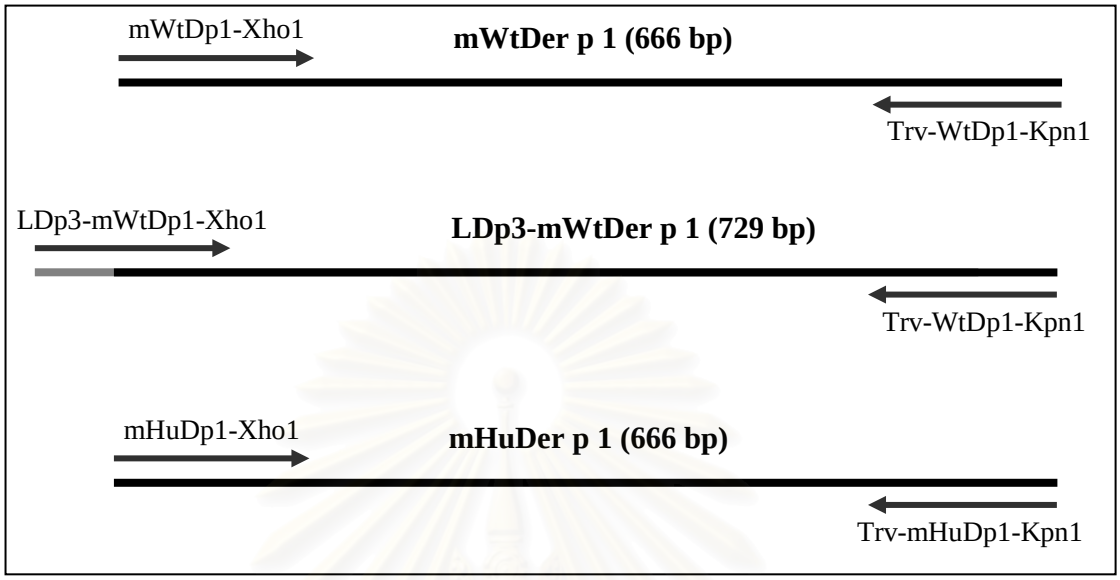

Figure 7 Diagram shows the PCR primers for amplifying Der p 1 cDNA for subcloning into pEGFP expression vector.

Table 13 Primers used for amplifying and subcloning of the mWtDer p 1, LDp3-mWtDer p 1 and mHuDer p 1 DNA sequences into pEGFP-N1 vector.

Primer name	Nucleotide sequence (5'→3')	T_m(^oC)
mWtDp1-Xho1-	Fw: GATCTCGAG[1]**ATG**[2]ACTAACGCCTGCAGTATCAATGG	66
Trv-mWtDp1-Kpn1	TTGGCCATTAATACATTG Rv: TTGGTACCAA***CTA***[3]GAGAATGACAACCATTGGATATTC	68
mHuDp1-Xho1-GFP	Fw: GATCTCGAG**ATG**ACCAACGCCTGCAGCATCAACGG	60
Trv-mHuDp1-Kpn1-GFP	Rv: TTGGTACCAA***CTA***CAGGATCACCACGTAGGGGTACTC	66

[1]Restriction sites underlined, *Xho* 1 = CTCGAG, *Kpn* 1=GGTACC

[2]Start codon as indicated in bold

[3]Stop codon as indicated in Bold-italic

4. DNA amplification

4.1 Wild type Der p 1 gene amplification

To generate the pHIS-mWtDp 1 expression vector, the mature wild type Der p 1 DNA sequence was amplified from a plasmid pPICZα Der p1w1 (gifted from Dr S. Piboonpocanun) by the use of specific primer for mature wild type Der p 1 (Table 12). PCR reaction contained the following:

Stock	Final concentration
DNA template	up to 2 μg
10 x PCR buffer	1/10 Vol
25 mM $MgCl_2$	2.5 mM
10 mM dNTP	1.0 mM
Primer 1	0.2 mM
Primer 2	0.2 mM
pfu DNA polymerase	1.5 Units
Sterile H_20	to a final volume of 50 μl

The PCR condition was performed in a DNA Thermal cycler 9700 (Perkin Elmer, USA) as the following:

Step	Duration/temperature	Cycle
Denaturation	5 min at 94 ^oC	1
Annealing	30 sec at 94 ^oC	29
	30 sec at 60 ^oC	
	1 min at 72 ^oC	
Polymerization	7 min at 72 ^oC	1

After then, the PCR products (mWtDer p 1) were checked by agarose gel electrophoresis.

4.2 Amplification for the LDp3-mWtDer p 1 with a 63 base pairs of Der p 3 leading sequence

To amplified pHIS-Leader Der p3-wtDp1 -plasmid DNA (pHIS-LDp3-mWtDp1), the leading part was amplified by overlapping PCR with the use of specific primers for the leading sequence which is a 63 bp in length of prosequence part of Der p 3 (Table 12).

PCR reaction contained the following:

Stock	Final concentration
10 x PCR buffer	1/10 Vol
25 mM $MgCl_2$	2.5 mM
10 mM dNTP	1.0 mM
Primer 1(LDp3WtDp1-Xho1-F)	3mM
Primer 2(LDp3WtDp1-Xho1-R)	150 pmole
Tag polymerase	1.5 Units
Sterile H_20	to a final volume of 50 μl

The PCR condition was performed in a DNA Thermal cycler 9700 (Perkin Elmer, USA) as the following:

Cycle	Duration/temperature	Cycle
Denaturation	3 min at 94 ^oC	1
Polymerization	15 min at 58 ^oC	1

After then, the PCR products (LDp3-mWtDer p 1) were verified by agarose gel electrophoresis and then purified using QIAGEN purification kits (QIAGEN, USA). The purified LDp3-mWtDer p 1 was prepared for ligation into pHIS-mWtDer p 1 plasmid vector.

4.3 Humanized Der p 1 gene amplification

Eight of the designed partial overlapping codon optimized oligonucleotides (Table 11) were used to amplify in generating mHuDer p 1 DNA sequence. The whole processes to make a final full-length mature humanized Dep1 DNA sequence were included 4 rounds of PCR amplifications as follows:

First, each two pairs of overlapping oligonucleotides were used to amplify for the first round of PCR products (PCR 1-1:HuDp1 and HuDp2, PCR 1-2: HuDp3 and HuDp4, PCR 1-3: HuDp5 and HuDp6, and PCR 1-4: HuDp7 and HuDp8).

Second, each of the first round PCR products was used to amplify for the second round PCR to make longer products (PCR 2-1: HuDp1/2-HuDp3/4 and PCR 2-2: HuDp5/6-HuDp7/8).

Third, each of the second round PCR products were used to amplify for the full length of huDp1 gene (PCR 3-1: HuDp1/4-HuDp5/8).

Finally, the PCR products were amplified by specific primer to mHuDer p 1. Figure 8 was showed as a schematic how to generate final full-length mature humanized Der p 1. Mature humanized Der p 1 was included a restriction site for subcloning into the pHIS plasmid vector (*Xho* I and *Kpn* I). The PCR reaction was followed PCR protocol as described in Materials and Methods 4.1. The PCR cycles were programmed as same as those of the wild type amplification protocol.

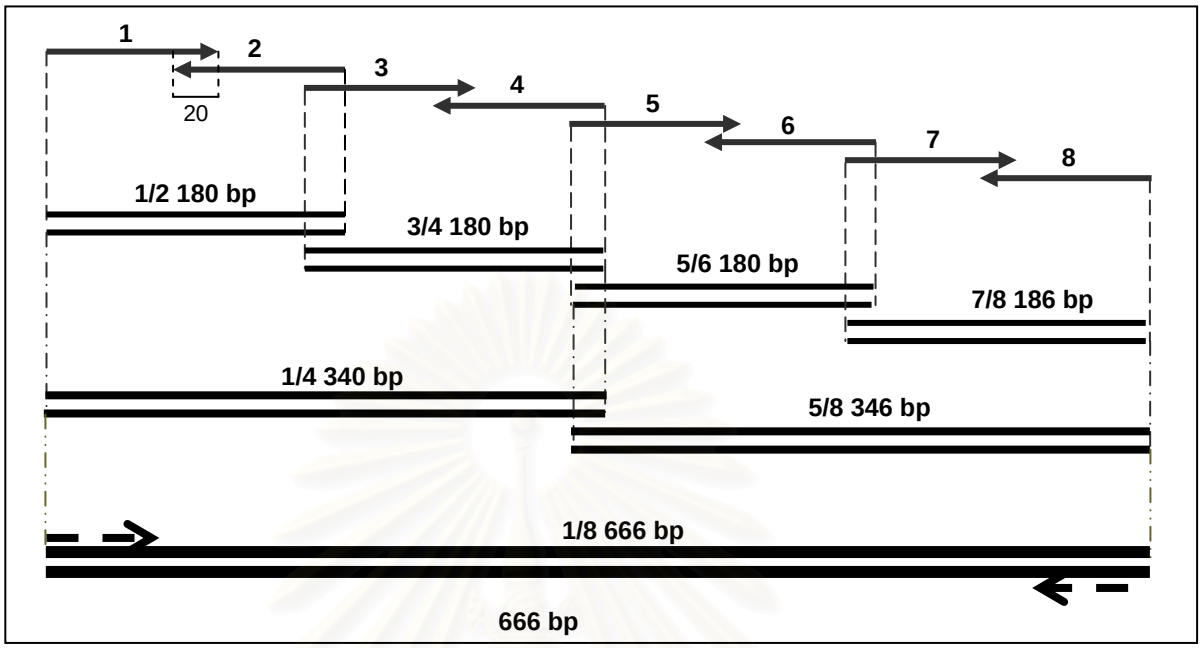

Figure 8 The humanized Der p 1 amplification. The four rounds PCR amplification of 8 overlapping humanized oligonucleotide primers generates a final full-length humanized Der p 1 DNA. The final full-length humanized Der p 1 DNA sequences which will be used for making a recombinant pHIS plasmid construct.

5. DNA preparation and cloning

5.1. Recombinant plasmid DNA construction

5.1.1. pGEM®-T plasmid DNA construct of *humanized Der p1* gene: pGEM® –T Easy vector (Promega, USA) (Figure 9 and 10) was used for subcloning with the mHuDer p 1. To generate an A-tailing for Blunt-ended PCR products for subcloning into pGEM® –T Easy vector, *Taq* DNA polomerase was used to generate

A-tail for blunt-ended fragments during the PCR amplification by using the A-tailing procedure.

Stock	Final concentration
Purified PCR fragment	1-7 μl
10 x PCR buffer with $MgCl_2$	1/10 Vol
100 mM dATP	0.2 mM
*tag*DNA polymerase	5 Units
Sterile deionized H_20	to a final volume of 10 μl

The reaction was incubated for 30 min at 70 ^oC. Subsequently, 1-2 μl of products were then ligated with pGEM® –T Easy vector. The ligation reaction (Promega, USA) contains as following:

Stock	Final concentration
2 x Rapid ligation buffer	1x
pGEM® –T Easy vector	50 ng
An A-tailing-PCR product	40 ng*
T4 DNA Ligase	3 Units
Sterile deionized H_20	to a final volume of 10 μl

*The molar ratio of PCR product : vector = 4:1

The reaction was mixed and incubated at room temperature for 1 hr. The ligation reactions were then transformed into *E.coli* (XL1-B), as describe in materials and methods 5.5.

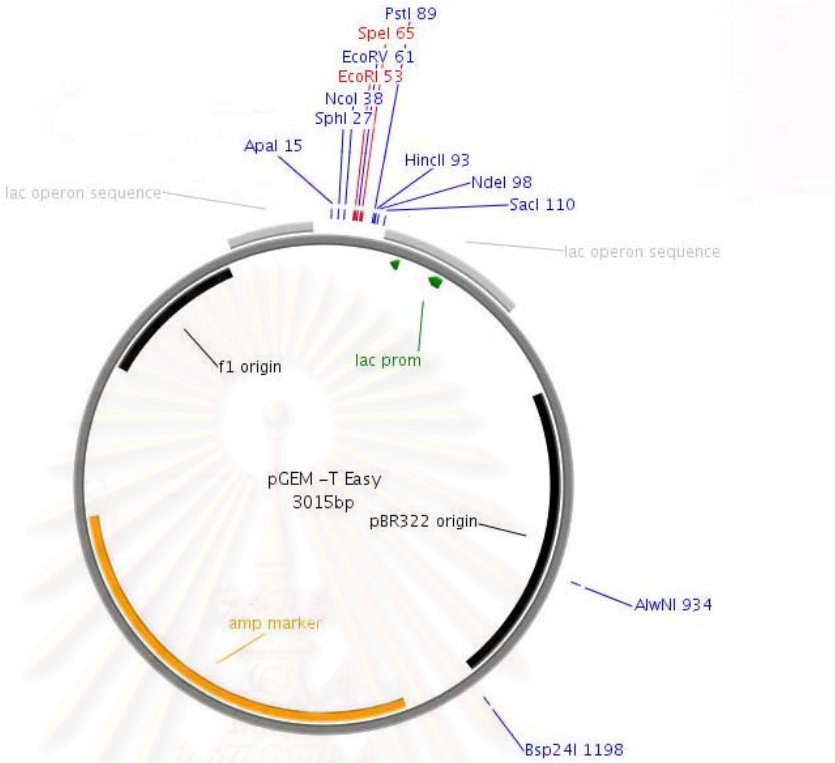

Figure 9 pGEM®-T Easy vector map. This vector containing T7 promoter, SP6 promoter, multiple cloning site, *lac* operon sequence, phage f1 region and ampicilin resistant gene (pGEM®-T and pGEM®-T Easy Vector Systems, Promega, USA) (drawing map was created by PlasMapper software) (90).

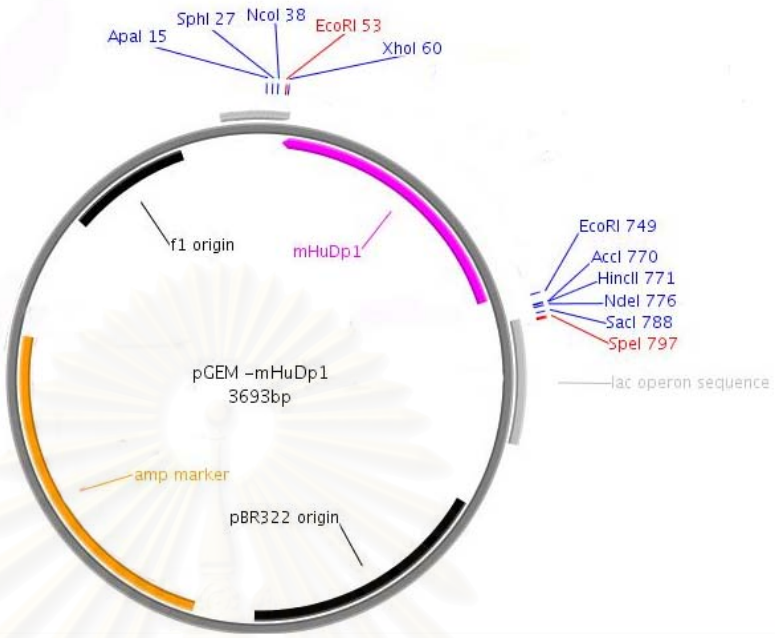

Figure 10 pGEM-mHuDer p 1 vector map. The 666 bp of mHuDer p 1 inserted product represent in pink line (drawing map was created by PlasMapper software) (90)).

5.1.2. Subcloning mWtDer p 1 and mHuDer p1 into the pHIS plasmid vector as the candidate DNA vaccines: The mWtDer p 1 amplified product from pPICZα Dp1w1 vector, and also the mHuDer p 1 amplified product from pGEM®-T Easy vector were digested with *Xho* I and *Kpn* I, The digested product was then purified by QIAGEN purification kits. Then, the purified digested products (mWtDer p 1, LDp3-mWtDer p 1 and mHuDer p 1) were then ligated into the pHIS vector (Figure 11-14) at the multiple cloning sites (*Xho* I and *Kpn* I) by using a rapid ligation reaction kit (Roach, Germany). For the DNA preparation ligation reaction, the

plasmid vector was prepared by using a restriction enzyme digested at the *Xho* I and *Kpn* I site. Dephosphorylation reaction was then performed to remove the 5'-end phosphate from the dsDNA to prevent plasmid religation. The ligation was described in Materials and Methods 5.1.1.

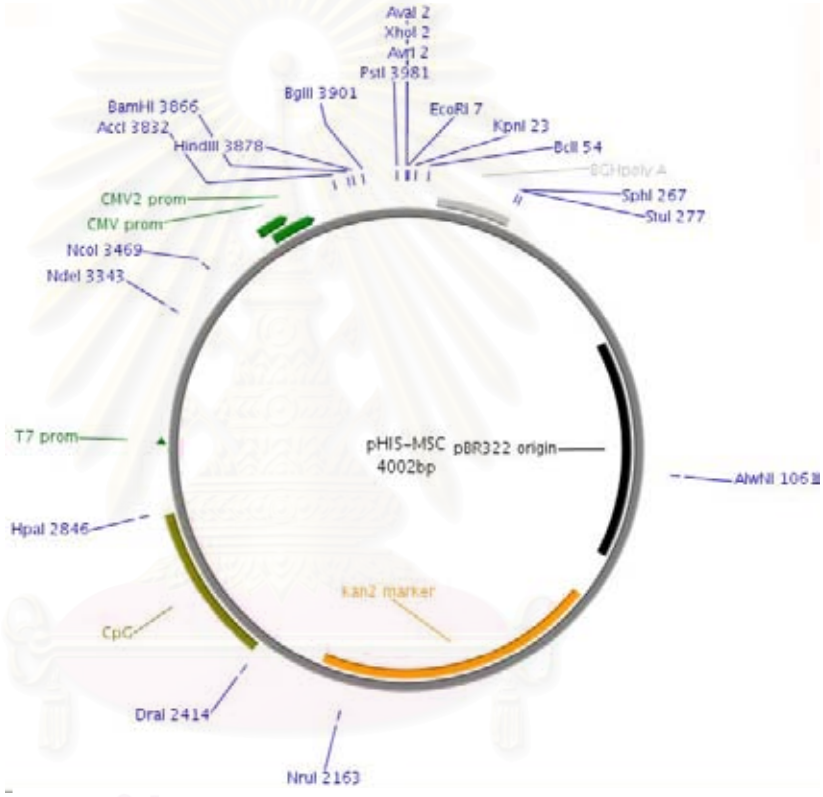

Figure 11 pHIS-MCS vector map. This vector is containing CMV promoter, intron, multiple cloning sites, bovine growth hormone (BGH) poly A sequence (gray), origin of replication of *E.coli* (ColE1) (black), and Kanamycin resistant gene (orange). Both mWtDer p 1 and mHuDer p 1 were cloned at the *Xho* 1 and *Kpn* 1 restriction sites (origin from Coley Pharmaceutical group).

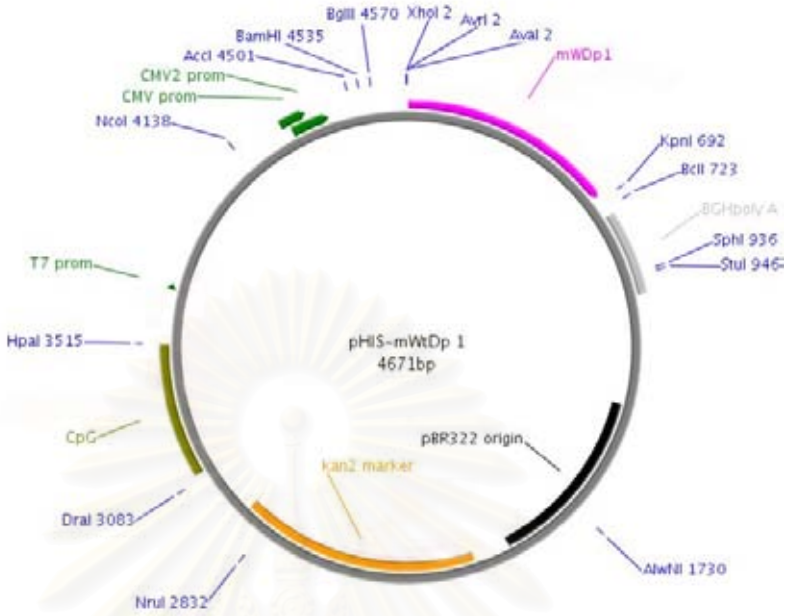

Figure 12 pHIS-mWtDer p 1 vector map. The 666 bp of mWtDp 1 inserted gene represent in pink line (drawing map was created by PlasMapper software (90)).

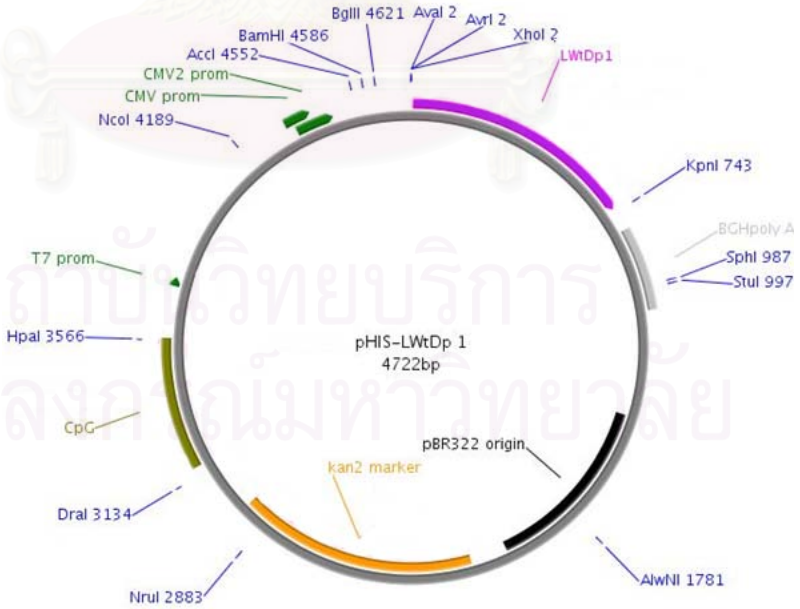

Figure 13 pHIS-LDp3-mWtDer p 1 vector map. The 729 bp of mWtDer p 1 inserted gene represent in pink line (drawing map was created by PlasMapper software (90)).

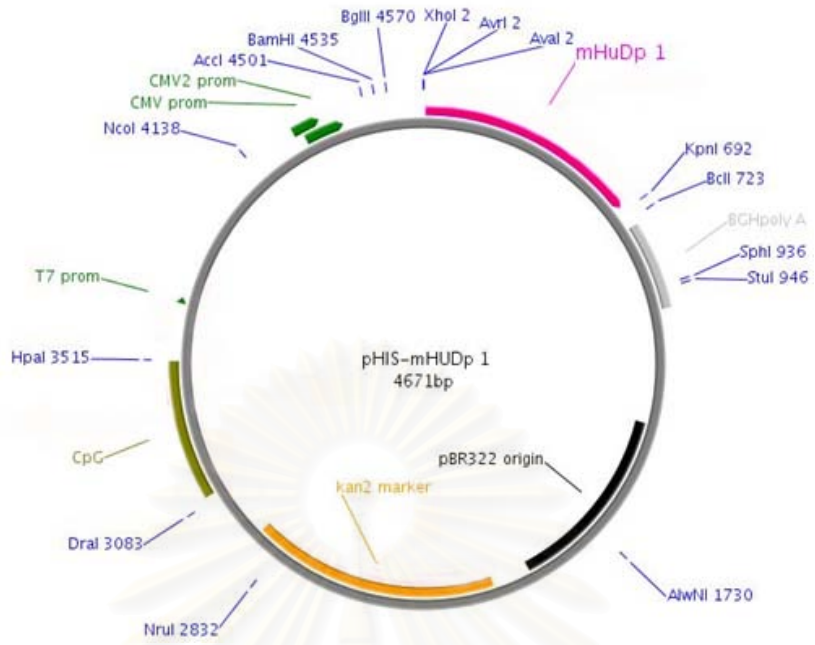

Figure 14 pHIS-mHuDer p 1 vector map. The 729 bp of mWtDp 1 inserted gene represent in pink line (drawing map was created by PlasMapper software (90)).

5.1.3. Plasmid DNA for *in vitro* gene expression assay: pEGFP-chimeric gene was generated for a choice of gene expression assay in the case of limitation in protein expression or conventional detection method such as Western blot analysis. The mWtDer p 1 and mHuDer p 1 PCR products that were amplified with each specific primer (Table 13) for subcloning into pEGFP-N1 vector were digested with *Xho* I and *Kpn* I. The digested PCR products were purified by QIAGEN purification kit. Then, the purified products were ligated into pEGFP-N1 (Figure 15-19) (gifted from Xin KQ, Department of Molecular Biodefense research, Yokohama City University, Japan) at multiple cloning sites (*Xho* I and *Kpn* I). Plasmid DNA ligation reactions were prepared same as described in the protocol to generate the recombinant humanized Der p1 pHIS construct.

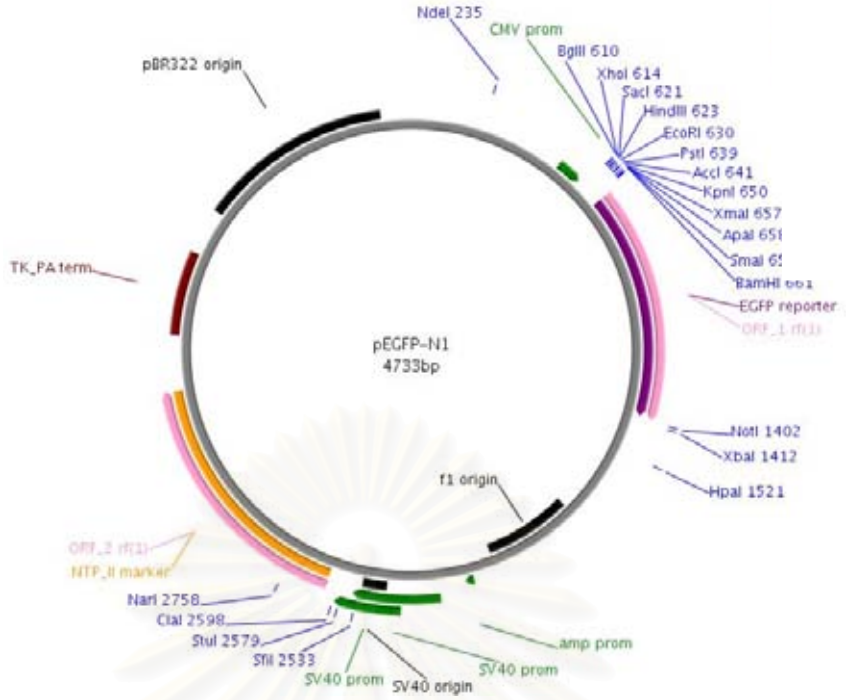

Figure 15 pEGFP-N1 vector map This vector contains SV40 poly A for replication in mammalian cells (green), CMV promoter (green) and neomycin/Kanamycin resistance gene (orange). Gene was cloned into the multiple cloning site (MCS) part as fusion to the N-terminus of pEGFP coding sequences (purple). Mature HuDer p 1, mWtDer p 1, LDp3-mWtDer p 1, and fHuDer p 1 was subcloned to *Xho* 1 and *Kpn* 1 site.

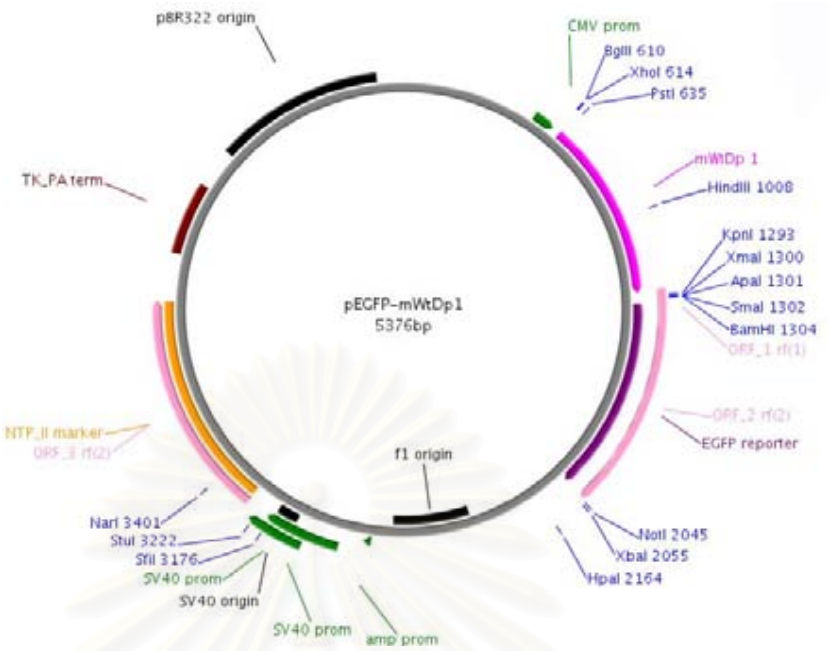

Figure 16 Map of pEGFP-mWtDer p 1 vector map The 666 bp of mWtDer p 1 inserted gene represent in darker pink line upstream of EGFP reporter gene(drawing map was created by PlasMapper software (90)).

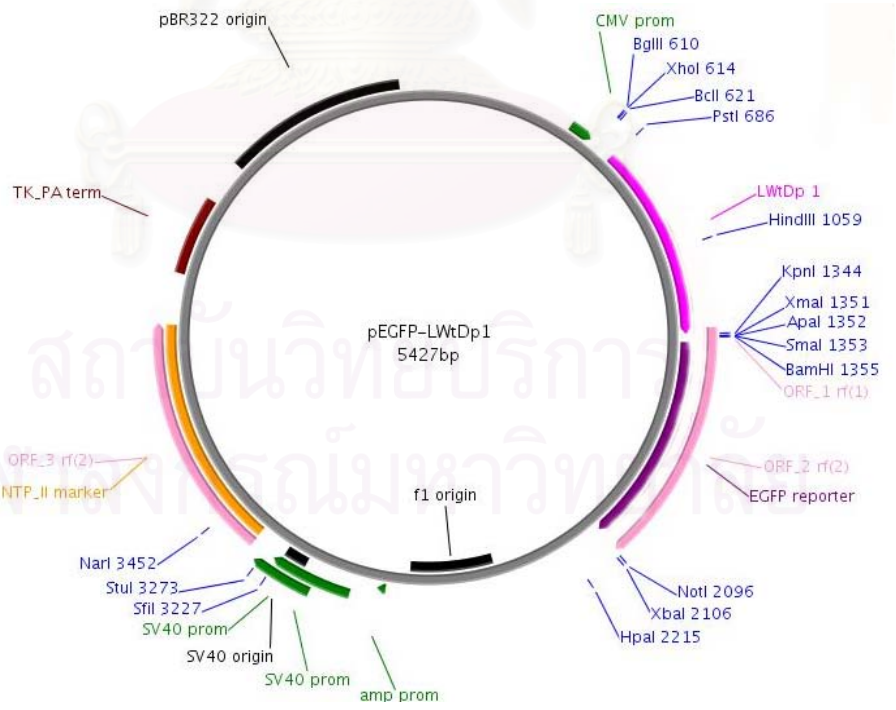

Figure 17 Map of pEGFP-LDp3-mWtDer p 1 vector map The 729 bp of LDp3-mWtDer p 1 inserted gene represent in darker pink line upstream of EGFP reporter gene(drawing map was created by PlasMapper software (90)).

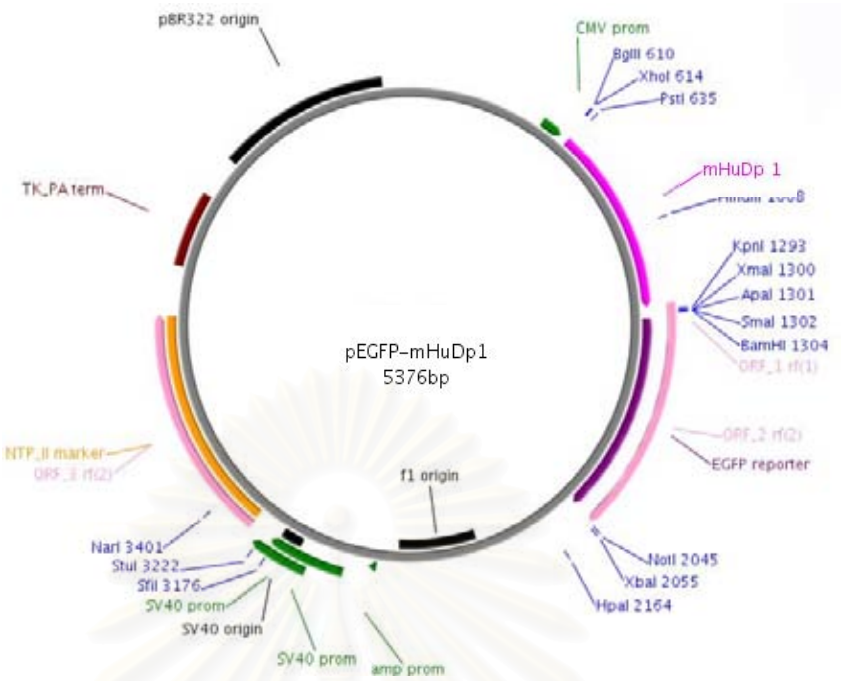

Figure 18 Map of pEGFP-mHuDer p 1 vector map The 666 bp of mHuDer p 1 inserted product represent in darker pink line upstream of EGFP reporter gene(drawing map was created by PlasMapper software (90)).

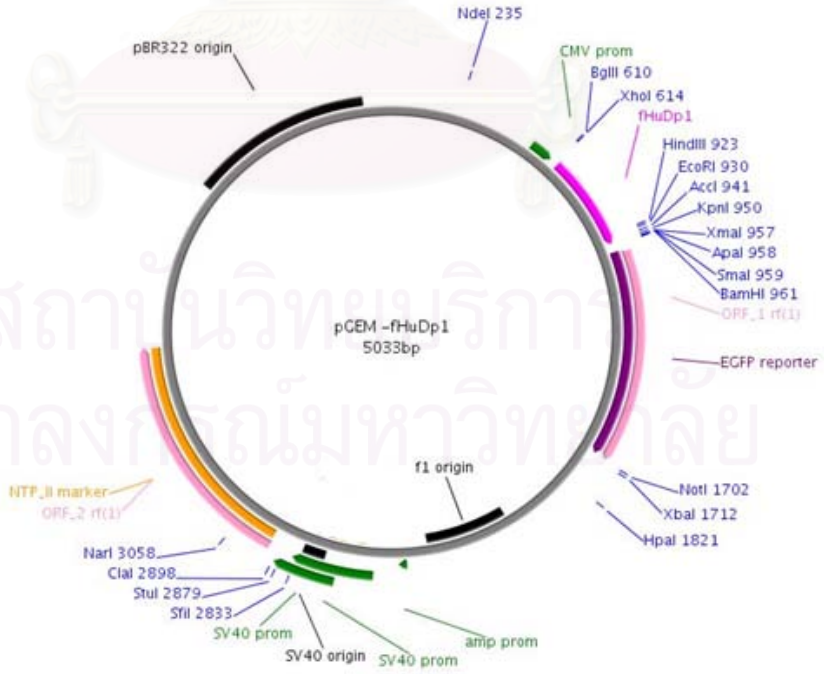

Figure 19 Map of pEGFP-fHuDer p 1 vector map The 297 bp of fHuDer p 1 inserted gene represent in darker pink line upstream of EGFP reporter gene(drawing map was created by PlasMapper software (90)).

5.2. Restriction analysis

The restriction endonuclease digestions were performed to digest the target DNA from plasmid vector. Digested products were used for subcloning or colony screening. The restriction reaction was showed as follow:

Stock	Final concentration
DNA	1 µg
10 x buffer	1/10 Vol
10 x BSA	1/10 Vol
Enzyme	1-3 Units
Sterile deionized H_20	to a final volume of 50 µl

Incubated at 37 oC for 1 hr unless specified by manufacturers

The restriction products were analyzed on Agarose gel electrophoresis in 1% TAE buffer (see Appendix). RNA was removed by adding 10 µl of 20 µg/ml RNAse A (Qiagen, USA) and incubated at 37 ^{o}C for 2 hr.

5.3. Plasmid DNA preparation

5.3.1 Mini-preparation

Single colony of *E.coli* was picked from master plate and cultured in 2 ml LB medium (see appendix) containing 100 µg/ml Kanamycin and incubated overnight at 37 ^{o}C with vigorous shaking for 12-16 h. The bacterial cells were harvested by centrifugation at 4000 x g for 10 min at 4 ^{o}C (Beckman, USA). QIAprep® Miniprep kit (QIAGEN, USA) was used to purify the plasmid DNA as following in QIAprep® Miniprep handbook (QIAGEN, USA).

5.3.2 Endotoxin Free plasmid Giga-preparation

A single colony of *E. coli* was picked and cultured in a starter of 5-10 ml of LB medium containing 100 mg/ml and incubated overnight at 37 ^{o}C with vigorous shaking for 12-16 h. The starter culture was diluted 1/500-1/1000 in LB medium and inoculated in 2.5 liters LB medium. The bacterial cells were grown at 37 ^{o}C for 12-16 h with vigorous shaking. The bacterial cells were centrifuged at 6000 x g for 15 min at 4 ^{o}C then supernatant was removed. QIAGEN® plasmid Giga purification kit was used to preparation of plasmid. For the final step, DNA was dissolved with Endotoxin free H_2O and stored at -20 ^{o}C.

5.4. Competent cells preparation

5.4.1. Bacterial competent cells preparation *E. coli* protocol

Frozen glycerol stock of *E. coli* (XL1-B) was slowly thawed on ice and then inoculated into 2 ml of LB medium and incubated in a 37 ^{o}C for overnight with shaking incubators. Next day, *E.coli* was inoculated into 50 ml of LB medium and incubated for 2-3 hours until the $OD_{600\ nm}$ of 0.25-0.5, at 37 ^{o}C with shaking at 250 rpm. Subsequently, the bacterial cells were incubated on ice for 30 min then the bacterial cells were collected by centrifugation at 4,000 rpm (~3,000 x g) for 10 minutes at 4 ^{o}C. Next, the supernatant was discarded then the cell pellet was resuspended in 10 ml of cold, sterile 50 mM calcium chloride. The mixture was incubated on ice for 30 min then the bacterial cells were centrifuged at 4,000 rpm (~3,000 x g). The supernatant was discarded and the cell pellet was gently resuspended in 1 ml of cold, sterile 50 mM calcium chloride to yield the final competent cell suspension. Competent cells were kept with 15% glycerol at -80 ^{o}C.

5.5. Bacterial transformation

Microcentrifuge tubes were pre-chilled on ice and competent cell (stored at -80 ^{o}C) was slowly thawed on ice. One hundred microliters of competent cells was added into chilled tubes.

The transformation reaction was following as:

Plasmid DNA	1 μl (not more than 50 ng)
Competent cells	100 μl
SOC medium	900 μl

One microliter of 1 μg of plasmid DNA (normally, the DNA amount not more than 50 ng in a volume of 10 μl or less) was added into the competent cell and the contents were gently mixed, and then incubated on ice for 30 minute. The cells were heat shock in water bath at 42 °C for 45 sec and then the tube was immediately placed on ice for 3 minute. Nine hundred milliliters of Super Optimal broth with Catabolite repression (SOC) medium was added into tube and incubated for 1 hr (37 °C) with shaking at 225-250 rpm. Transformants were plate on LB-Kanamycin plate (final concentration = 25 μg/ml) plates.

5.6. Colony screening

5.6.1. Restriction enzyme screening method: To screen that the plasmid contain mWtDer p 1 and mHuDer p 1, transformants colony was selected and pointed for 4-5 dots by sterile toothpick onto master plate (LB-Kanamycin plate) and then incubated at 37 ^{o}C for O/N. The selected colony was cultured in 2 ml LB medium containing 100 mg/ml of Kanamycin (final concentration = 25 μg/ml). The plasmid

DNA was extracted by QIAprep Miniprep and the putative clones was done by restriction enzyme digestion. The restricted DNA patterns were analyzed by electrophoresis on 1 % agarose gel in 1 % TAE buffer.

5.6.2. Colony PCR screening: *E. coli* or *P. pastoris* colonies that contain target DNA could also be selected for protein expression using PCR reaction with each specific primer for mWtDer p 1 and mHuDer p 1. One of four colonies from master plate was picked and resuspened in PCR reaction. The PCR reactions was performed as describe in Materials and Methods 4.1. The expected PCR products were observed by agarose gel electrophoresis.

5.6.3. Blue/white colony screening: This technique screening using LB/Ampicillin/IPTG/x-Gal plates were used to screening and determining the transformants. The transformants culture with pGEM®-T Easy with mHuDer p 1 was plated onto LB/Ampicillin/IPTG/x-Gal plates. The plates were incubated at 37 ^{o}C for overnight. Recombinant clones were identified by color screening indicator plates. Colonies containing mHuDer p 1 insert was generated as white colonies while background containing blue colonies. The *Lac Zα* gene in the multiple cloning sites of plasmid vector to drive the expression of β-galactosidase was disrupted by the targeted DNA resulting in loss of the β-galactosidase production. The bacteria carrying recombinant plasmids will form with white colony.

5.7. Agarose gel electrophoresis

Agarose gel electrophoresis was a method to separate DNA fragments based on size. A 1 % (w/v) Agarose gel mixture in 1x TAE buffer was prepared (the percent gel was prepared depending on the DNA size). The gel was swirled and heated in a

microwave oven to completely dissolve and poured then warm agarose gel mixture into the mold, left the gel set at RT. For the PCR product detection, 5 µl of PCR product were mixed with 6x gel loading buffer to be 1x and then was loaded into the gel by gel electrophoresis apparatus (Bio-Rad, USA) for constant voltage at 100 V for 25 min. After running, the gel was stained in ethidium bromide solution and destained in distilled water for 10 min. The DNA band patterns were visualized under UV light (Geldoc, BioRad, USA) and photographed.

5.8. Sequencing analysis

PCR product was purified by QIAGEN PCR purification kit. The purified PCR product was used for sequencing reaction by using Sequencing Kit (ABI PRISM dideoxy Dye Terminator Cycle Sequencing Kit, BigDye™ Applied Biosystems, USA. The sequencing reaction contained the following:

Stock	Final concentration
DNA template	1 µg
5 x BigDye Sequencing buffer	1/10 Vol
Specific primer	3 mM
BigDye reaction premix	1 µl
Sterile distilled H_20	to a final volume of 10 µl

The sequencing reaction was performed in a DNA Thermal cycler 9700 (Perkin Elmer, USA) as the following:

Step	Duration/temperature	Cycle
Denaturation	1 min at 96 ^{o}C	1
Annealing and extension	10 sec at 96 ^{o}C	
	5 sec at 50 ^{o}C	25
	4 min at 60 ^{o}C	

The amplify product was then precipitate with 3 µl of 3M sodium acetate (3M NaOAc) pH 4.6, 62.5 µl of absolute ethanol and 14.5 µl of distilled water. The reaction was left at RT for 15 min and then centrifuged at 13,000 rpm (~17,900 x g) for 20 min. Supernatant was discarded and the pellet was washed with 200 µl of 70% ethanol (appendix) and then centrifuged at 13,000 rpm (~17,900 x g) for 10 min. Supernatant was removed and the pellet was let the pellet dried at 95 ^{o}C for 1 min. The pellet was resuspended with 12 µl of Hi-Di Formamide (Applied Biosystems, USA). The sample was transferred to 96 well plates and covered lid. The plate was heated at 95 ^{o}C for 2 min and then added into ABI 310 Genetic analyzer (Applied Biosystems, USA).

5.9. Site-direct mutagenesis to correct some point mutations of the amplified full-length Der p1 DNA sequence

5.9.1. Primer design for mutagenesis: Mutagenic oligonucleotides were designed as mutagenic primer by following: the mutagenic primers contained the desired mutation was created by the desired mutation should be put in the middle. The mutagenic primers should be designed between 25-45 bases pair long (Table 14). Melting temperature (*Tm*) should be more than $\geq$78 ^{o}C. Estimating the *Tm* of each mutagenic primer was calculated by: Tm = 81.5+0.41(%GC)-675/N. For calculating:

N = the primer length in bases, Values for **%**GC and % mismatch are whole numbers, as followed the QuikChange® site-direct mutagenesis kit (Stratagene, USA) .

5.9.2. DNA site-direct mutagenesis: Forty microlitters of 1-10 µg plasmids DNA were mixed with 1 M NaOH/1mM EDTA and then incubated at 37 ^{o}C for 15 min. For DNA precipitation, 5 µl of 3M NaOAc (pH 4.8) were added and precipitated with 150 µl of ice-cold absolute EtOH. The mixture was centrifuged at 13,000 rpm (~17,900 x g) for 10 min at 4 ^{o}C then the supernatant was discarded. The pellet was washed with 150 µl of 70% EtOH and then centrifuged at 13,000 rpm (~17,900 x g) for 2 min. The pellet was air-dried and resuspended with 20 µl of distilled water. The PCR reaction preparation was described as in Materials and Methods 4.1 using with mutagenic primers (Table 14). The PCR condition was performed in a DNA Thermal cycler 9700 (Perkin Elmer, USA) as the following:

Step	**Duration/temperature**	**Cycle**
Denaturation	1 min at 94 ^{o}C	1
Annealing	30 sec at 94 ^{o}C	18
	1 min at 40 ^{o}C	
	10 min at 68 ^{o}C	
Polymerization	1 min at 94 ^{o}C	1
	1 min at 40 ^{o}C	
	10 min at 72 ^{o}C	

After PCR performing, 10 Unit of *Dpn* I restriction enzyme (Roach, USA) was added to amplified reaction and mixed thoroughly and then incubated at 37 ^{o}C for 1 hr to digest the parental supercoiled dsDNA. Finally, the reaction was ligated to the pGEM®-T easy vector and transformed into *E. coli* (XL1-B)**.**

Table 14 Mutagenic primers used for the site-direct mutagenesis

Primer name	Nucleotide sequence (5'→3')	T_m(^{o}C)
F-SDM-270	Fw: GCGTGGTGCAGGAGAGCTACTACC	78
R-SDM-270	Rv: CGGTAGTAGCTCTCCTGCACCACG	78
F-SDM-570	Fw: GATCGTGCGGAACTCCTGGGACAC	78
R-SDM-570	Rv: GGTGTCCCAGGAGTTCCGCACGAT	78

6. Recombinant Der p 1 protein expression

6.1. Plasmid DNA preparation

Recombinant plasmid was linearized by *Dra*I restriction enzyme. The digested products were precipitated with 1/10 volume of 3M NaOAc and 3 volume of cold absolute ethanol and then incubated at -20 ^{o}C for overnight. The reaction was centrifuged at 13,000 rpm (~17,900 x g) for 10 min and then washed with 70% ethanol. The supernatant was removed and dried the pellet at room temperature. The pellet was resuspended with 6 µl of sterile distilled water.

6.2. *Pichia pastoris (P. pastoris)* KM71 competent cells preparation

A single colony of freshly streaked *P. pastoris* was cultured in YPD medium (see in appendix) and then incubated at 30 ^{o}C by vigorous shaking for overnight. Small volume of cultured *P. pastoris* was added into 50 ml of YEPD medium and

incubated at 30 ^{o}C by vigorous shaking at 250 rpm until to an OD_{600nm} of 1.3-1.5. The cells were centrifuged at 4,000 rpm (~3,000 x g) for 10 min at 4 ^{o}C and then the pellet was washed with 10 ml sterile water and centrifuged at the same condition. The cells pellet was resuspended in 5 ml of chilled 1 M sorbital and then centrifuged at 4,000 rpm (~3,000 x g) for 10 min at 4 ^{o}C. The washing with 1 M sorbital (see appendix) was repeated again. For the final step, the cell was resuspended in 1 ml of chilled 1 M sorbital and stored at 4 ^{o}C.

6.3. Electroporation of pPICZ α-Dp1w1 vector in *P.pastoris* KM71

Forty microliters of freshly preparation of *P. pastoris* KM71 competent cells were mixed with 5 µl of linearlized plasmid DNA in ice cold microcentrifuge tube. The mixture was transferred to ice-cold electroporation cuvette and kept on ice. The electroporation was performed by Bio-Rad Gene Pulser at condition 200 ohm, 25 µF and 1.5 KV at constant time is 4.5. After electroporation step, one ml of ice-cold 1 M sorbital was immediately added to the cuvette. The mixture was transferred to ice-cold 15 ml tube and then incubated at 30 ^{o}C for 1 hr. After that, the mixture was transferred to microcentrifuge tube and centrifuged at 3,000 rpm for 5 min at room temperature. The supernatant was removed and 400 µl of YPD-Zeocin™ (Invivogen, France) (100 µg/ml) medium was added. One hundred and fifty microliters were plated to YPD plate containing 100 µg/ml of Zeocin™ and then the plate was incubated at 30 ^{o}C for 3 days.

6.4. Colony screening

To screen that the plasmid contain target gene, transformants colony was selected and pointed 4-5 dots by sterile toothpick onto master plate (YPD-zeocin plate)

and then incubated at 30 ^{o}C for O/N. The selected colony was cultured in 2 ml YPD medium containing 100 mg/ml of Zeocin. The plasmid DNA was extracted by following QIAprep® Miniprep handbook (QIAGEN, USA), and then the putative clones was done by restriction enzyme**.**

6.5. Protein expression in *P. pastoris* and purification

The selected clone (pPICZ α-Der p1w1) was grown in 2 ml of YPD/Zeocin™ (100 µg/µl) at 30 ^{o}C for 1-2 days. Seven milliliters of starter culture (starter $OD_{600\ nm}$ of 0.1-0.2) was transferred to BMGY medium (see appendix) until an $OD_{600\ nm}$ of 5-9. Cells were collected by centrifugation at 4,000 rpm for 10 min at 4 ^{o}C. The cell pellets were resuspended in 1 ml of BMMY medium (see appendix) to an $OD_{600\ nm}$ of 25-30). The suspension 1 was induced by daily addition of 0.5 % methanol (MeOH) for 3 days. For the last day of MeOH induction, the mixture was centrifuged at 13,000 rpm (~1,900 x g) for 1 min. Supernatants were collected and analyzed by western blot analysis. The specific bands were detected by specific monoclonal antibody. For column purification of recombinant ProDer p 1 (rProDer p 1) protein, the collected supernatant was concentrated by using dialysis bag (see appendix) in polyethylene Glycol 8000 (PEG 8000) (USb Corporation, USA) to remove excess water. rProDer p 1 protein was purified by AKTA purifier (GE Healthcare, USA) by using Seperdex 75 (GE Healthcare, USA). The protein was detected by SDS-PAGE and western blot analysis.

6.6. Protein electrophoresis

6.6.1. SDS-polyacrylmide gel electrophoresis (SDS-PAGE)

Proteins were analyzed by 8% polyacrylamide SDS-PAGE (Table 15). For denaturing sample, 4x sample loading buffer were added to protein sample for final 1x concentration. The samples were boiled at 95 ^{o}C for 10 min. The mixture was loaded to electrophoresis chamber and ran in 1x running buffer at constants voltage for 100 V, 90 min. After electrophoresis, acrylamide gel was equilibrated in transfer buffer (see appendix). Nitrocellulose membrane and filter paper was cut to gel size and wet in transfer buffer for 10-15 min. The protein was transferred into nitrocellulose membranes (Bio-rad, USA) using Trans-Blot® Semi-Dry (Bio-Rad, USA) with constants voltage at 25 V for 30 min. After blotting, membrane was saturated with 5% skim milk in 1xPBS-T buffer (blocking buffer; see appendix) for over night at 4 ^{o}C. Next, membrane was incubated with 1:1000 of rabbit anti mouse anti-Green Fluorescent Protein (anti-GFP) (Rockland, USA) (supported from Dr. Xin, Yokohama University, Japan) in blocking buffer (5% skim milk in 1x PBS) for 1 h. The horseradish peroxidase-goat (HRP) anti–rabbit antibody (ICN Pharmaceuticals, USA) was diluted 1:3000 in blocking buffer for detection step. The HRP Chemiluminescent reaction is based on the catalyzed oxidation of luminol by peroxide. The membrane was exposed to a suitable X-ray film for an appropriate duration. For positive control of protein expression, membrane was detected by $\tilde{\beta}$-actin antibodies.

Table 15 Preparation of 8%SDS-PAGE

	Resolving gel (ml)	**Stacking gel (ml)**
1 M Tris-HCL (pH 8.8)	5	-
1 M Tris-HCL (pH 68)	-	0.75
40% Acrylamide (37.5:1)	2	0.45
10% SDS	0.2	0.12
Distilled water	2.8	4.68
10% APS	0.075	0.060
TEMED	0.010	0.007

7. *In vitro* characterization of pHIS and pEGFP recombinant plasmid DNA

7.1. Mammalian cell transfection

7.1.1. FuGENE® 6 transfection reagent: One day before transfection, 293 T cells (Human Embryonic Kidney cells) were plated to be 5 x 10^5 cells in 2 ml of Dulbecco's Modified Eagles Medium (DMEM) (GIBCO, USA) with 10% FBS (Biowhittaker®, LONZA, USA). Next day, three microliters of FuGENE® 6 reagent was diluted in 97 µl of Serum Free Medium (SFM), and then vortex and incubate for 5 min. One microgram plasmid DNA (total volume between 0.5-50 µl) was diluted in diluted FuGENE® 6 reagent, vortex once. The FuGENE® 6 reagent-plasmid DNA complexes were incubated at RT for 45 min. The plasmid DNA complex was added into each well containing cell and medium. Then, the cells were incubated at 37 ^{o}C for 48 h. After 48 h, the cells were harvested for protein analysis.

7.1.2. Lipofectamine™ 2000 transfection reagent: One day before transfection, HEK 293 cells (Human Embryonic Kidney cells) (supported from Dr.

Xin KQ, Department of Molecular Biodefense research, Yokohama City University, Yokohama, Japan) were thawed in water bath for 1-2 min. The cells were plate onto culture plate with 10 ml of 10% FBS in DMEM medium and then the cells were cultured at 37 ^{o}C for overnight. After 24 h cultures, cells were washed with 10 ml of steriled PBS (Sigma-Aldrich, USA) and digested with 0.25% trypsin/EDTA for 1-2 min at 37 ^{o}C. The cells were counted by counter chamber. The 5 x 10^5 cells were grown in 2 ml of 10 FBS in DMEM medium and incubated at 37 ^{o} for overnight. Next day, the cells will be 90% confluent at the time of transfection. For each transfection sample, the complex was prepared as follows: plasmid DNA was diluted in 250 µl of serum free medium (DMEM without 10% FBS) and gently mixed. Lipofectamine^{TM} 2000 (Invitrogen, USA) was gently mixed before use, 2.5 µl of Lipofactamine^{TM} 2000 was diluted in 247.5 µl of Opti-MEM I (GIBCO, USA) or serum free medium. Diluted Lipofectamine^{TM} 2000 was incubated at room temperature for 5 minutes. The diluted plasmid DNA and diluted Lipofectamine^{TM} 2000 (total volume = 500 µl) were mixed. The complex was mixed and incubated for 20 minutes at room temperature. The 500 µl of each complex was added into each well containing cells and medium. The plate was gently mixed and incubated at 37 ^{o}C in a CO_2 incubator for 48 hours. The fluorescence images were taken under fluorescence microscope system (Biozero, KEYENCE, Tokyo, Japan).

7.2. Protein sampling

The transfected cells from cultured plate were pipettes, and then spin at 4,000 rpm (3,000 x g) at 4 ^{o}C for 5 min. The supernatant were collected. After that, the cells pellet was washed with steriled PBS for 2 times. One hundred microliters of low salt extract buffer (LESB; see appendix) were added to the cell pellet, vortex and then

incubated on ice for 30 min (vortex each 10 min). The mixtures were centrifuged at 13,000 rpm (~17,900 x g) for 10 min at 4 ^{o}C. One hundred microliters of cell lysate were collected, and then 100 µl of 2xSDS was added. The cell lysates were heated at 95 ^{o}C for 10 min and then left at room temperature until the mixture cold down. The cell lysates were then analyzed by SDS-PAGE and Western blot analysis or kept at -20 ^{o}C for next experiment.

7.3. mRNA analysis

7.3.1. mRNA extraction

Total RNA was extracted from 293 T cells after transfected with each plasmid DNA as following RNeasy® mini kit hand book (QIAGEN, USA).

7.4. Reverse transcriptase-Polymerase chain Reaction (RT-PCR) assay of pHIS-recombinant plasmid DNA constructs

The RT-PCR reactions kit (QIAGEN, USA) were performed as following:

Stock	Final concentration
RNA template (DNase treated)	1 µg
5 x QIAGEN one-step RT-PCR	1/10 Vol
dNTP mix (10 mM each dNTP)	400 µM of each dNTP
Primer 1	0.6 µM
Primer 2	0.6 µM
QIAGEN one step RT-PCR enzyme mix	5-10 U/reaction
RNase free H_20	to a final volume of 50 µl

Program for thermal cycler for RT-PCR step consist of reverse transcription and PCR as following:

Step	Duration/temperature	Cycle
RT	30 sec at 50 ^{o}C	1
	15 min at 95 ^{o}C	
PCR	30 sec at 94 ^{o}C	
	30 sec at 60 ^{o}C*	30
	1 min at 72 ^{o}C	
	7 min at 72 ^{o}C	1

*For a β-action annealing was performed at 52 ^{o}C. The PCR products were detected by Agarose gel electrophoresis.

7.5. SDS-PAGE and Western blot analysis

7.5.1. Protein extraction: The plasmid DNA transfected cells were lysed with lysis buffer (see appendix). The mixture was incubated 30-45 min on ice, mixed and spinned every 5 min. The supernatant was collected by centrifuge at 13,000 rpm (17,900 x g) for 1 min.

7.5.2. Protein measurement: Total proteins were measure by the Pierce BCA Protein assay (Pierce, Rockford, USA) before gel electrophoresis. For microplate procedure, 25 µl of each standard and unknown sample were added into a microplate well (NUNC, DENMARK) (working range = 20-2000 µg/ml). Two hundred microliters of the working reagent (50 parts of BCA reagent A with 1 part of BCA reagent B) were added to each well and mixed thoroughly plate. The plate were

incubated at 37 ^{o}C for 30 min and left plate at room temperature. The absorbance was measured at or near 562 nm on a plate reader (Multiscan EX plate reader, USA).

7.5.3. SDS-polyacrylmide gel electrophoresis: Proteins were analyzed as described in protein electrophoresis. The 8% of polyacrylamide gel preparation was followed in Table 15.

8. Animal study

8.1. Immunogenicity Evaluation in Mice

Six to eight weeks old of female BALB/c mice (from National Laboratory Animal Centre, Mahidol University, Thailand) of 25 in total were used. Mice were randomized into 2 experimental groups.

8.1.1 To evaluate immunogenicity in mice model after pHIS-mHuDer p 1 DNA or rProDer p 1 immunization

8.1.1.1 Control (pHIS empty vector) N=4

8.1.1.2 Vaccine group (pHIS-mHuDer p 1) N=6

8.1.1.3.Allergic model (rProDer p 1+ alum) N=6

8.1.2 To evaluate immunogenicity in mice model after liposome-pHIS-mHuDer p 1 DNA (lipoplex) immunization

8.1.2.1 Liposome control N=4

8.1.2.2 Liposome-pHIS-mHuDp1 DNA N=5

8.2. Liposome preparation

Liposome complex was prepared from 0.467 mg of lipoid S75 (soy bean lecithin), 0.010 mg of DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-

N,N,Ntrimethylammonium methylsulfate) and 0.006 mg of cholesterol. This mixture was dissolved in chloroform in a glass tube and the solvent was evaporated in a rotary evaporator at 60 ^{o}C (instrument supported from Pharmacology Department, Chulalongkorn University, Thailand) until lipid film was dried. Four milliliters of the HEPES buffer (see appendix) was added to the lipid film and swirly mix until lipid film was dissolved. Liposome was then extruded by lipex extruder through a 600 nm polycarbonate filter for 6 times and then through a 200 nm polycarbonate filter for 9 times. The charge and size of liposome was measured (nanoparticle sizer, Malvern, German) and stored at 4 ^{o}C.

8.3. Immunization protocol

Each group of mice were anesthetized with inhalation anesthetic AERRANE (isoflurane) (Baxter, USA) and immunized with each PBS or the particular plasmid DNA (prepared by Giga preparation endotoxin free kit (QIAGEN, USA) by Intradermally (i.d.) 3 points injection for each immunization schedule.

8.3.1. Plasmid DNA vaccination

Mice were vaccinated i.d. at week 0, 1, 2, 3 and 4 (Figure 20) (a total of 5 times immunizations) with 100 µg of endotoxin free purified pHis-mHuDer p1 DNA (50 µl in each point by i.d. at abdominal surface part of mice). The immunogenicity of mice in each group was compared before and after vaccination by ELISA assays to detect Der p1 specific IgE, IgG1 (Th2 responses) and IgG2a (Th1 responses).

8.3.2. Recombinant Der p 1 protein immunization with alum to generate a ProDer p 1-Th2 induction model

For recombinant ProDer p1 (rProDer p 1) immunization, mice were sensitized by intraperitoneal injection (i.p.) with 10 µg of recombinant Der p1 from

purified yeast expression system with 100 µg alum (Sigma, USA) (Figure 20). Recombinant protein was mixed with alum and incubated for 20 min at room temperature. Before injection, the reagent was mixed well before µsed. Mice were injected for a total of 5 times at a weekly interval (wk 0-4). The immunogenicity of mice in each group was compared before and after vaccination by ELISA assays in detection of serum Der p1 specific IgE, IgG1 and IgG2a.

8.3.3. Liposome-recombinant DNA complex (lipoplexes) vaccination

Mice in this group were immunized 3 times at week 0, 1 and 2 (Figure 21) (totally 3 times injection) by intradermal (i.d.) with 50 µg of endotoxin free purified pHis-mHuDp 1 DNA complex with liposome (50 µl in each point by i.d. at abdominal surface part of mice). Liposome-pHIS-mHuDer p 1 complex was prepared before injection, the ratio of liposome and DNA equal to 2:1 respectively. The mixture was incubated at RT for 20 min, not more than 30 min before used. Serum Der p1-specific IgE, IgG1 and IgG2a of mice in each group was compared before and after vaccination by ELISA assay.

8.4. Blood sample collection

Mice were anesthetized and then mice were bled from the retro-orbital plexus and blood was collected into a sterile tube. Each group of mice was bled on days 0, 7, 14, 21, 28, and 36. The blood samples were centrifuged at 4,000 rpm (3,000 x g) for 30 minutes at room temperature (BECKMAN GS-15 R centrifuge, USA). Mice sera were collected for immunoassay and stored at -20 ^{o}C.

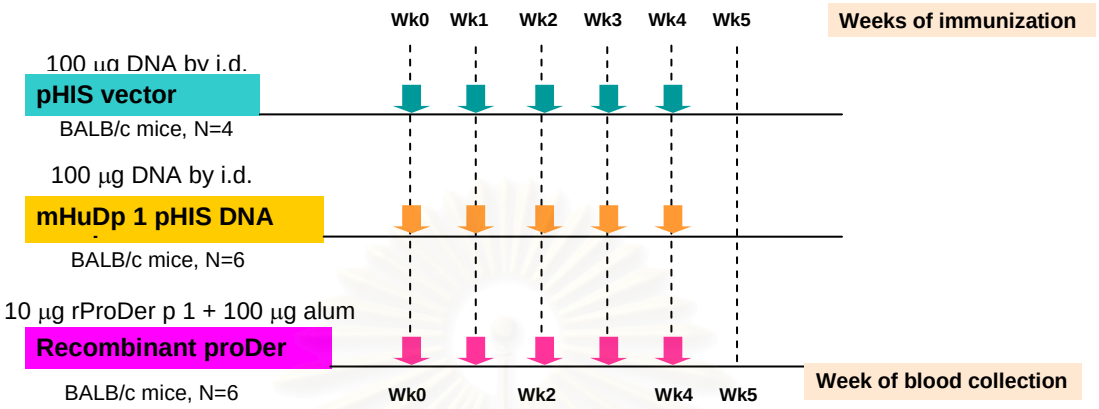

Figure 20 The schematic summary of pHIS-mHuDer p 1 plasmid DNA and rProDer p 1 protein vaccination.

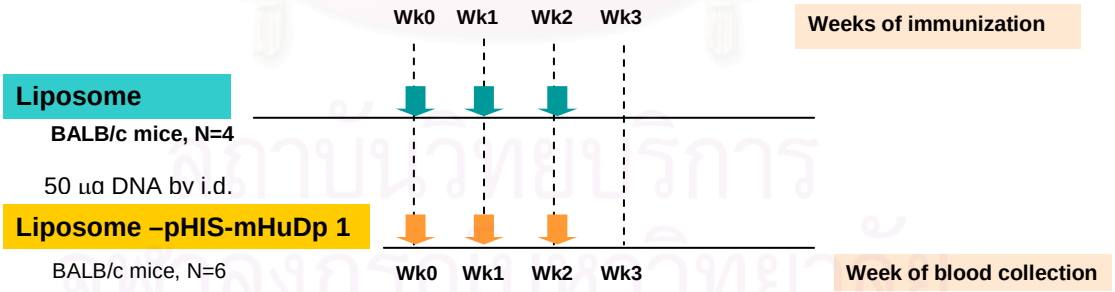

Figure 21 The schematic summary of liposome-pHIS-mHuDer p 1 plasmid DNA (lipoplex) vaccination.

9. Determining total IgE, IgG2a, HDM-specific IgE, and HDM-specific IgG2a

The anti-Der p 1 IgG1, IgG2a and IgE antibodies in mice sera before and after immunization with DNA vaccine were detected by ELISA.

9.1. Specific IgG1/IgG2A and IgE antibodies detection

The 96 wells plate (NuNc maxisorp, Germany) was coated with 500 ng ProDer p 1 protein with coating buffer (see appendix). The plate was sealed and incubated for over night at 4 ^{o}C. The wells were aspirated and washed for 5 times (with wash with 200 µl of wash buffer (see appendix). After last wash, the plate was blotted on paper town to remove any residual buffer. The plate was blocked with 200 µl of blocking buffer (see appendix) for 1 h at 37 ^{o}C. The dilution sera (1:40 for IgG1/IgG2a and 1:20 for IgE) and positive control were prepared in blocking buffer. One hundred microliters was added into appropriate wells and incubated for 1 hr at 37 ^{o}C. The wells were aspirated and washed 6 times with washing buffer. One hundred microliters of 1:250 anti mouse antibody (anti-mouse IgG1 or IgG2a or IgE) (BD Biosciences, USA) was added to each well. The wells were aspirated and washed 6 times with washing buffer. One hundred microliters of 1:250 SAv-HRP ((BD Biosciences, USA) was added to each well and incubated for 30 min at room temperature. The wells were aspirated and washed 6 times with washing buffer. One hundred microliters of TMB Substrate solution (BD Biosciences, USA) was added to each well and incubated at room temperature for 5-10 min. Fifty microliters of stop solution (see appendix) was added to each well. The absorbance was read within 30 min at OD. 450 nm

CHAPTER IV

RESULTS

1. Generation of mature wild type Der p 1 (mWtDer p 1) DNA

Mature wild type Der p 1 (mWtDer p 1) DNA was amplified using ProDer p 1 cDNA as a template. PCR reaction yielded cDNA products with a size 666 bp (mWtDer p 1 cDNA) as shown in Figure 22. The PCR products were purified and confirmed by DNA sequencing analysis. The comparison of the sequence of mWtDer p 1 cDNA and that of Der p 1 cDNA deposited at GenBank P08176, as shown in Figure 23.

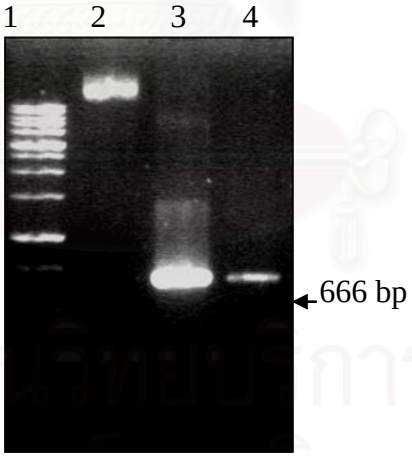

Figure 22 1% agarose gel electrophoresis of mWtDer p 1 cDNA amplified by PCR reaction. Mature WtDer p 1 cDNA mobilized at 666 bp. Lane 1: one kb marker, lane 2: pPICZ α-Der p 1w1, lane 3: mWtDer p 1 cDNA in PCR reaction and lane 4: purified mWtDer p 1 cDNA.

Figure 23 The sequence alignments of Der p 1 cDNA (GenBank P08176) and mWtDer p 1 cDNA. A polymorphism was found at the position 653-654 of the wtDp1, which contains TAT→TCG (Tyr218→Ser218).

2. Construction of mWtDp 1 recombinant plasmid

The purified mWtDer p 1 was digested with restriction enzymes (*Xho* I and *Kpn* I) and ligated with digested pHIS. The ligation reaction was transformed into *E.coli* (XL1-B) and selected by Kanamycin. cDNA from positive clones were digested by *Xho* I and *Kpn* I and analyzed by agarose gel (Figure 24).

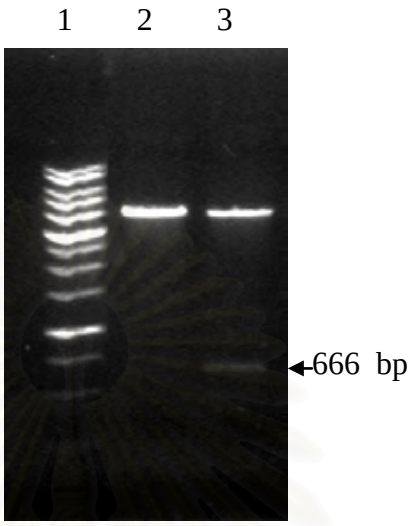

Figure 24 1% agarose gel electrophoresis of colony screening of ligated product of mWtDer p 1 with restriction enzyme. Lane 1: one kb marker, lane 2: pHIS vector digested with Xho I/Kpn I and lane 3: cDNA of positive clone digested with *Xho* I and *Kpn* I. The digested product size was 666 bp.

3. Humanized Der p 1 DNA preparation

3.1 Generation of humanized codon optimization

The 666 bp of humanized Der p 1 gene was generated. The result showed that about 75% (167/222 codons) of codon usages were changed after PCR reaction. The sequence alignment between the mWtDer p 1 and after optimizing codon of the mHuDer p 1 showed that about 73% of nucleotides were changed when compared with mWtDer p 1 DNA (Figure 25).

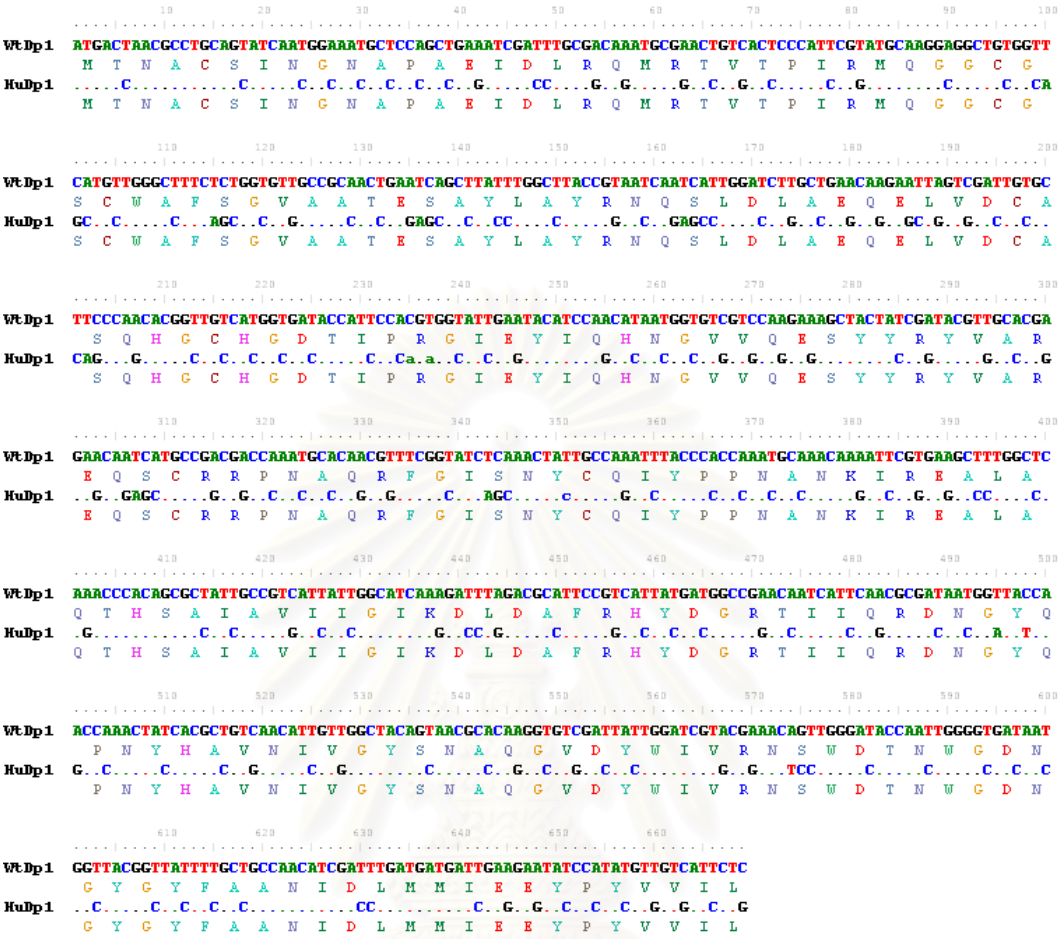

Figure 25 Sequence alignments of Der p 1 DNA (GenBank P08176) comparing with humanized Der p 1 (mHuDer p 1) DNA. Similar in nucleic acid sequence are shown as dot. There were 73% changes from the wild type nucleotides.

Four sets of cDNA products covering the entire *Der p 1* gene (as described in materials and methods) were amplified. The ~200 bp amplified cDNA from each set was analyzed by agarose (HuDp1 and 2:1-2, HuDp3 and 4:3-4, HuDp5 and 6:5-6, and HuDp7 and 8: 7-8 cDNA) (Figure 26). Then, the 2 sets PCR amplified products

(HuDp1 and 2:1-2, HuDp3 and 4:3-4, HuDp5 and 6:5-6, and HuDp7 and 8: 7-8 cDNA) were connected in the same PCR reaction to generate 340 bp cDNA (1-4 and 5-8) (Figure 27). Subsequently, third PCR to generate 1-4 and 5-8 cDNA was done to connect the products from the second PCR (1-8) (Figure 28). Finally, the full length mature HuDer p 1 was amplified by specific primers (mHuDp1-*Xho* I and mHuDp1-*Kpn* I) and the 1-4 and 5-8 cDNA from third PCR reaction. The amplified products of full length HuDp1 (666 bp) were shown in Figure 29.

Figure 26 1% agarose gel electrophoresis of partial humanized Der p 1 cDNA from first PCR. Lane 1: 100 bp marker, lane 2: PCR products from the first pair of humanized oligonucleotides HuDp1 and HuDp2 (1-2), product size 180 bp. Lane 3: PCR products from the second pair of humanized oligonucleotides HuDp3 and HuDp4 (3-4), product size 180 bp. Lane 4: PCR products from the third pair of humanized oligonucleotides HuDp5 and HuDp6 (5-6), product size 180 bp. Lane 5: PCR products from the fourh pair of humanized oligonucleotides HuDp7 and HuDp8 (7-8), product size 186 bp. Lane 6: Negative control.

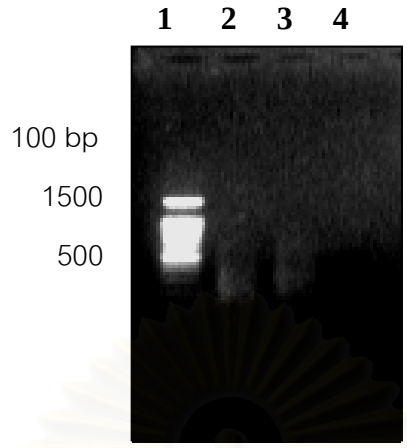

Figure 27 1% agarose gel electrophoresis of the second round PCR for humanized Der p 1 amplification. Lane 1: 100 bp marker, lane 2: PCR product of second HuDp1-2 and HuDp3-4 (1-4), lane 3: PCR product of HuDp5-6 and HuDp7-8 (5-8), lane 4: Negative control.

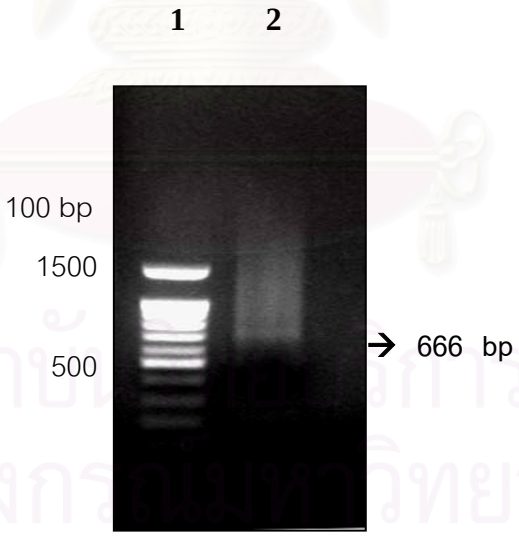

Figure 28 1% agarose gel electrophoresis of the third round PCR for humanized Der p 1 amplification. Lane 1: 100 bp marker, lane 2: PCR product of the third round PCR of HuDp1-4 and HuDp 5-8 (1-8) with the size of 666 bp.

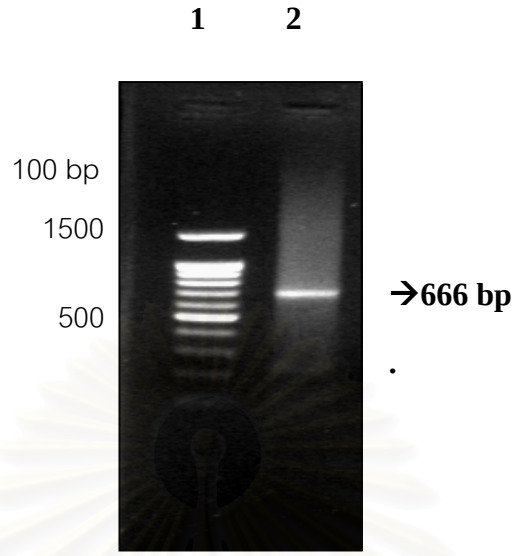

Figure 29 1% agarose gel electrophoresis of the final round PCR for humanized Der p1 amplification. The final PCR products were amplified by specific primers for the mature HuDp1 gene. Lane 1: 100 bp marker, lane 2: A PCR product of full length mHuDp 1, product size is 666 bp.

3.3 Humanized Der p1 sequencing and site-direct mutagenesis

To verify the sequence the cloned mHuDer p 1 in pGEM-T® easy vector, the amplified full length mHuDer p 1 PCR products were then transformed into *E.coli* (XL1-B). The transformed cells were plated and selected by LB-ampicillin/IPTG/xGal plate, as described in material and method. Four colonies were selected for sequencing analyses. The clone c72G-hDp that had the fewest point mutations, and delete (Figure 30).

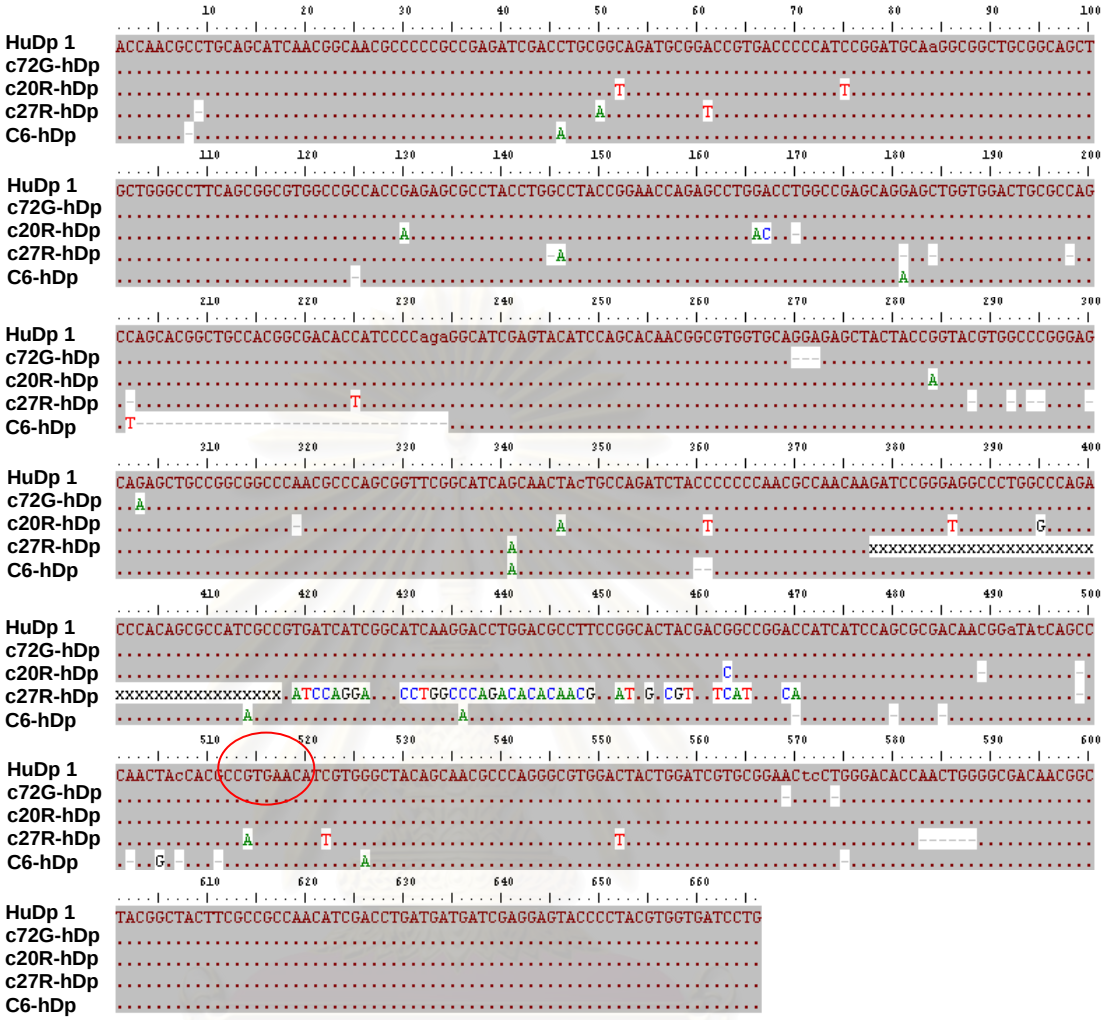

Figure 30 The sequence comparison of 4 colonies contained mHuDer p 1 DNA. The clone that contains a least mutation (c72G-hDp) was selected, define as c72G-hDp.

The sequencing result of the selected c72G-hDp clone was showed 4 mutations were found. One point mutation at position at nucleotide (nt) 303 (arrow pointed Figure 31). Other 3 mutations were detection which was 3-base-deletions at nt 270-273, a deletion at of nt 569 and of nt 574.

Figure 31 Nucleotide sequence of mHuDer p 1 from the selected c72G-hDp clone compared with the reference mHuDer p 1. Four positions of mutations were found from c72G-hDp clone, amino acid deletion at the position 270 and base deletion at position 569 and 574. At position 303 (arrow head), point mutation was found but not affect in the amino acid level (CA**G**101→CA**A**101, glutaine).

The site-direct mutagenesis was performed to correct all these mutations. The detection of nt 270-273 was filled in by PCR reaction with the primer (F-SDM 270/R-SDM 270) (Figure 32). Sequence comparison of the mHuDer p 1 mutated sequence and corrected sequence were shown as an electropherograms in Figure 33. Others two deletions at nt 569 and 574 were filled in by PCR reaction with the primer (F-SDM 570/R-SDM 570) (Figure 32) that covered both deletion mutation. The corrected sequence was shown in Figure 34. After that, the corrected mature humanized Der p 1 was then subcloned into pHis plasmid vector at *Xho* I and *Kpn* I multiple cloning site.

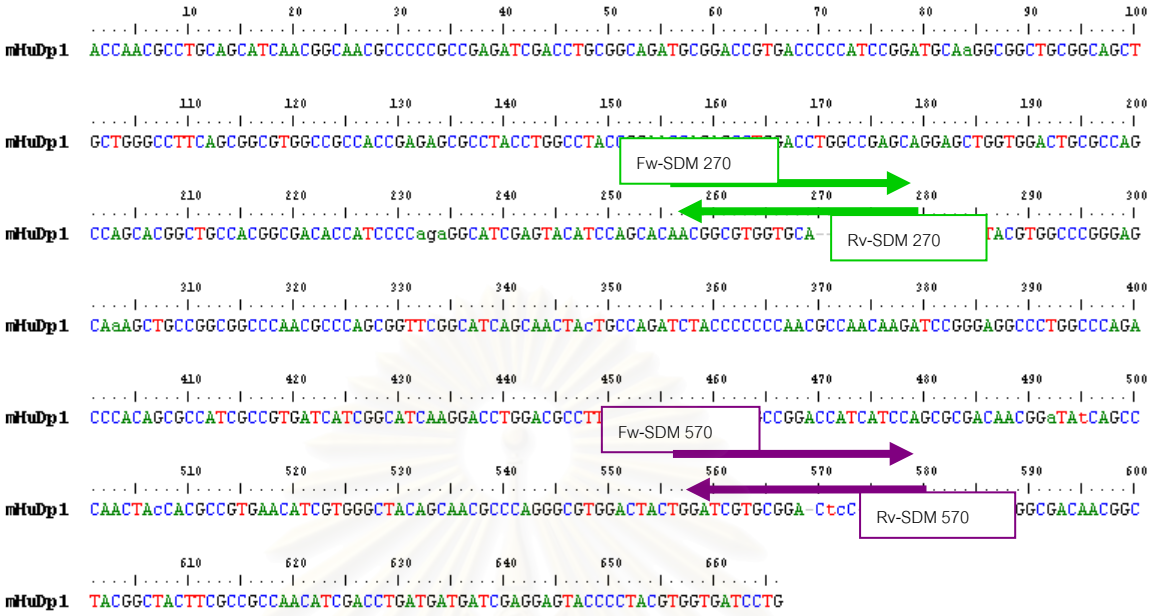

Figure 32 Nucleotide sequence of mHuDer p 1 from the selected c72G-hDp clone compared with the reference mHuDer p 1 and mutagenic primers. Three mutations were found, three-base-deletion at the position 270, one base deletion at position 569 and 574. The primers (Fw and Rv) that were used for site-direct mutagenesis are shown in green and purple line.

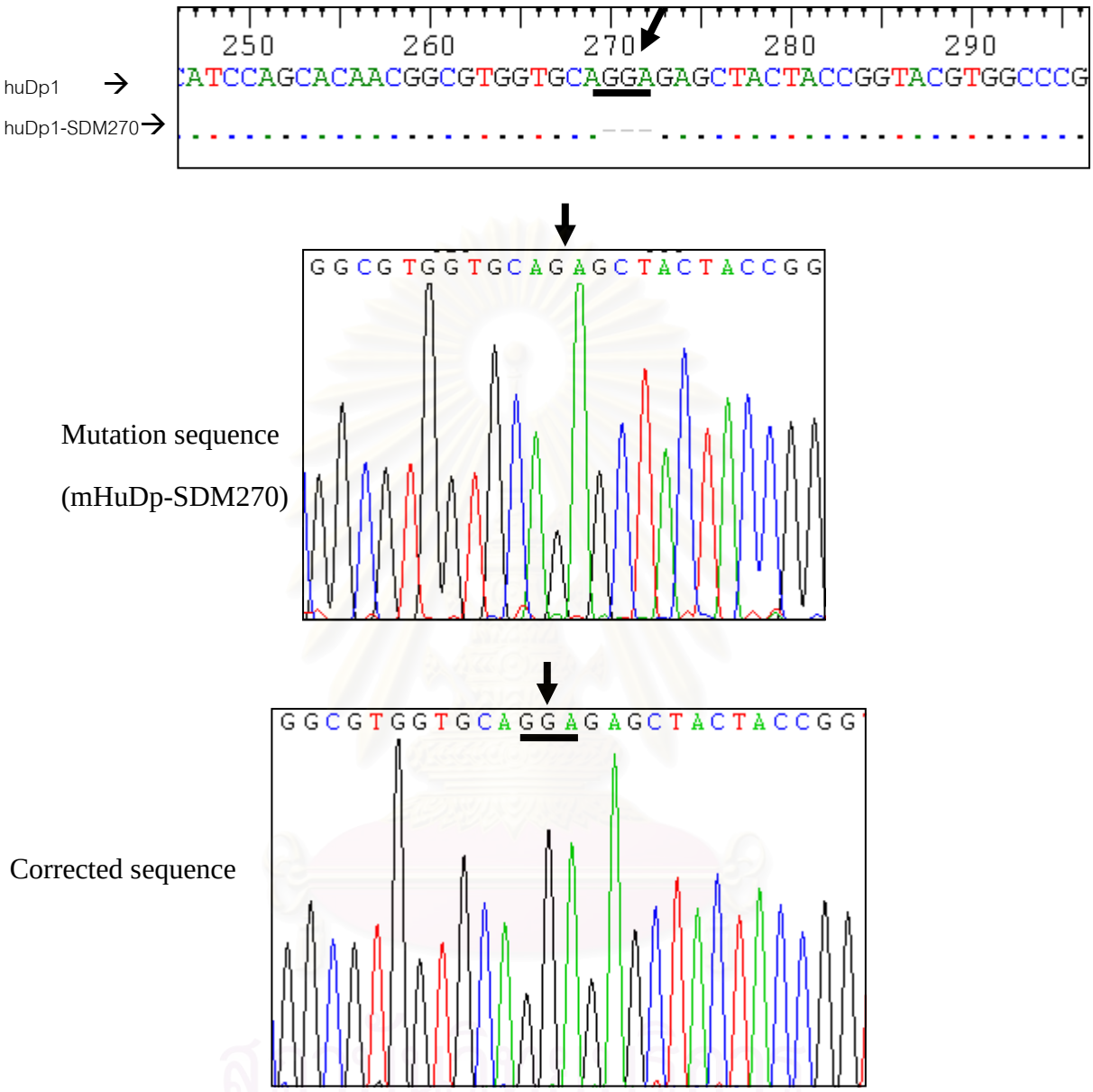

Figure 33 The illustration shows electropherograms of mHuDer p 1. Sequence alignment comparing the sequence obtained from the analysis of the PCR products with reference HuDer p1 showed three bases deletion (nt 270-272), as indicated by arrow, and corrected sequence after filled in.

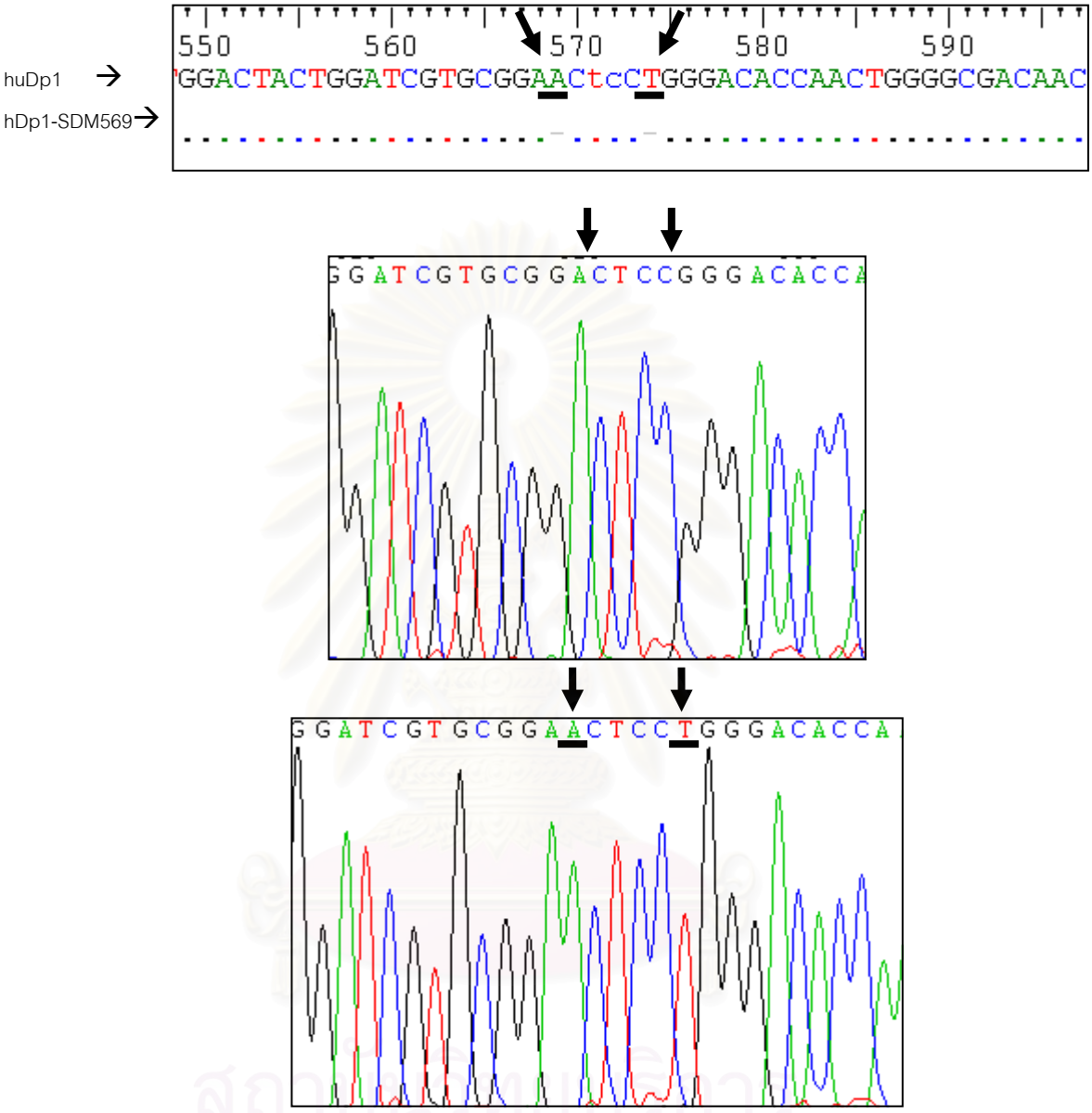

Figure 34 The illustration shows electropherograms of mHuDer p 1. Sequence alignment comparing the sequence obtained from the analysis of the PCR products with reference HuDp1 showed one bases deletion at nt 569 and 574, as indicated by arrow, and corrected sequence after filled in.

4. *In vitro* characterization

To test whether all DNA constructs in this study (Figure 35) would be transcribed and translated after transfection, analysis of mRNA and protein expression were performed using 2 different cell lines: 293 T and HEK 293 cells.

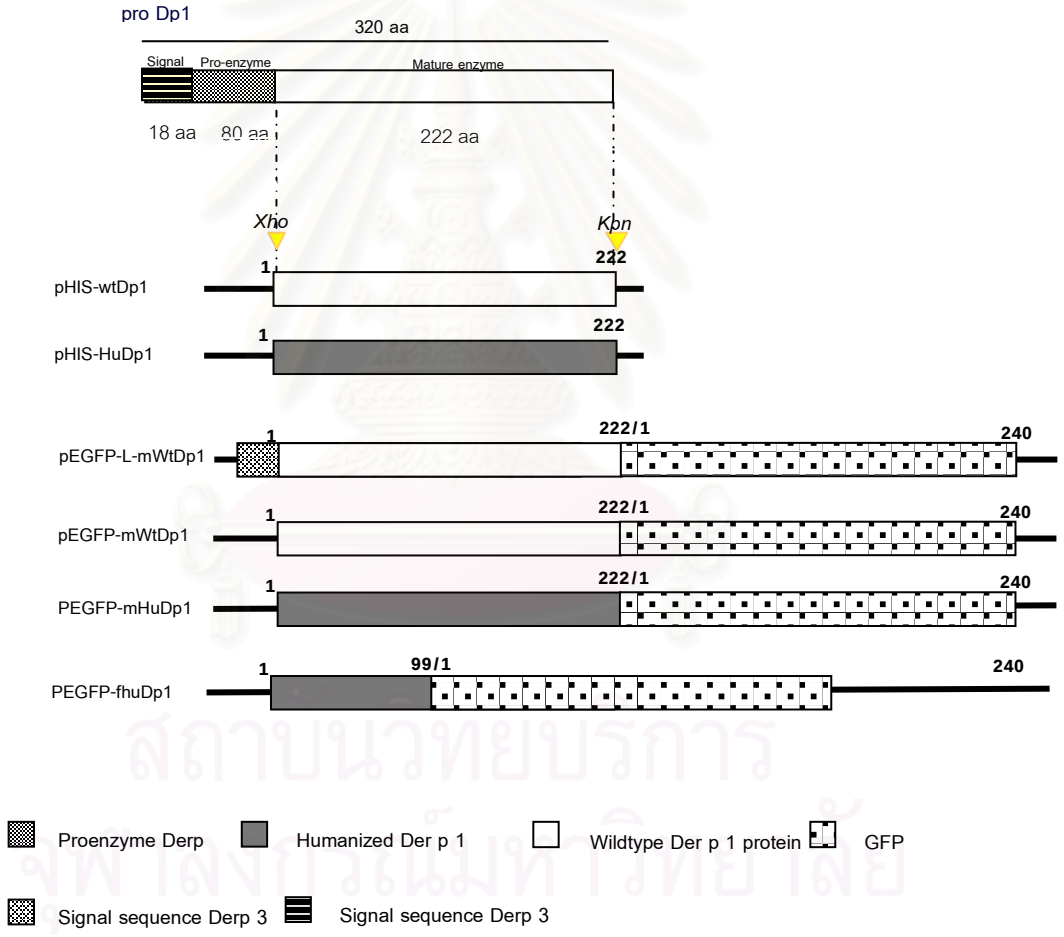

Figure 35 Schematic representation of all constructs.

4.1. mRNA analysis

:5x10^5 of 293 T cells were plated in 6-well plate (costar, Corning, USA) and transfected with pHIS, pHIS-LmWtDer p 1, pHIS-mWtDer p 1, or pHIS-mHuDer p 1 by using FuGENE® 6 reagent, as described in Materials and Methods. Specific Der p1 RNA expression in total RNA (DNase treated) extracted from transformed cells was analyzed by RT-PCR. The amplified RNA product was mobilized at 729 bp for pHIS-LmWtDer p 1, 666 bp for pHIS-mWtDer p 1 or pHIS-mHuDer p 1 and 327 bp for β-actin (Figure 36). The β-actin was used as a positive control for expression level.

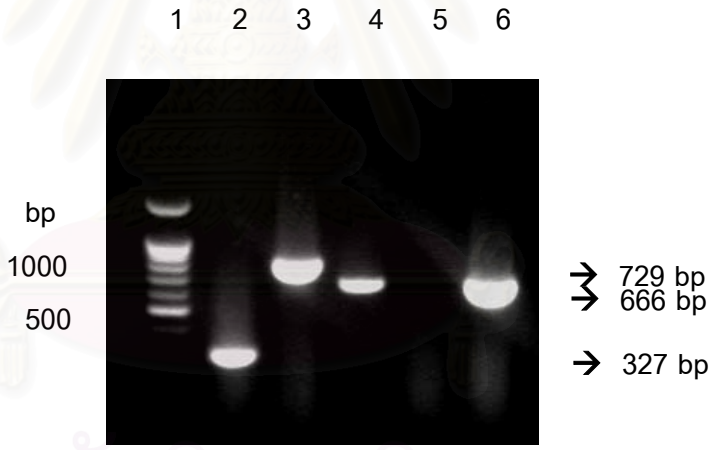

Figure 36 1% agarose gel electrophoresis of mRNA expression by RT-PCR from transfected 293 T cells line. The PCR products were amplified from Der p 1 mRNA (DNase treated sample) of transfected cells lysate with corresponding primers with each gene. Lane 1: 100 bp marker, lane2: 327 bp for β-actin, lane 3: 729 bp for LmWtDer p 1, lane 4: 666 bp for mWtDer p 1, lane 5: negative control and lane 6: 666 bp of mHuDer p 1.

:$5x10^5$ of HEK 293 cells were plated in 6-well plate (costar, Corning, USA) and transfected with pGFP, pGFP-LmWtDer p 1 and pGFP-mHuDer p 1 by using Lipofectamine™ 2000 reagent, as described in Materials and Methods. Specific Der p1 RNA expression in total RNA extracted (DNase treated) from transformed cells was analyzed by RT-PCR. The amplified RNA product was mobilized at 729 bp for pGFP-LmWtDer p 1, 666 bp for pGFP-mHuDer p 1 and 327 bp for β-actin (Figure 37). The β-actin was used as a positive control for expression level.

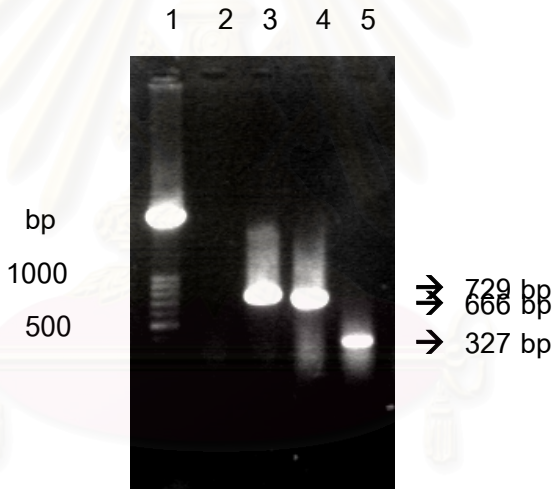

Figure 37 1% agarose gel electrophoresis of mRNA expression by RT-PCR from transfected HEK 293 cells line. The PCR products were amplified from Der p 1 mRNA (DNase treated sample) of transfected cells lysate with corresponding primers with each gene. Lane 1: 100 bp marker, lane 2: negative control, lane 3: 729 bp for LmWtDer p, lane 4: 666 bp of mHuDer p 1, and lane 5: 327 bp for β-actin.

To confirm that the RNA did not contaminated with DNA component during extraction, DNase was used to digest unexpected DNA contaminate. RNA free-DNase treated was then amplified by PCR with each specific primer for each gene. No cDNA products were detected.

4.2 Recombinant protein analysis

4.2.1 pHIS chimeric Der p 1 expression *in vitro*. The transfected 293 T cells were lyzed with lysis buffer to analyze the Der p 1 expression protein. After 48 h cultures, cell lysate was then loaded into polyacrylamide gel. Polyclonal antibody was used to detect Der p 1 protein (dilution 1/10 of human serum skin prick test 4+ against HDM extract). The western blot results showed no proteins were expressed in all plasmid transfected cell (Figure 38).

Figure 38 Western blot analysis of cell lysate containing Der p 1 expressed by transfected 293 T cells. Lane 1: protein marker, lane 2: empty lane, lane 3: medium only, lane 4: pHis, lane5: pHIS-mWtDer p 1, lane 6: pHIS-LmWtDer p 1, lane 7: pHIS-mHuDer p 1 and lane 8: recombinant ProDer p 1 protein (positive control).

Despite specific Der p 1 mRNA was expressed, however transfected protein could not be detected. To determine whether recombinant Der p 1 proteins could be transfected in transfected cells, the pEGFP encoding chimeric GFP-Der p 1 to increase protein detection sensitivity were constructed and chimeric protein expression in HEK 293 cells was performed.

4.2.2. pEGFP chimeric Der p1 expression *in vitro*. The transfected HEK 293 cells were lyzed with lysis buffer to analyze the Der p 1 expression protein. The results showed transfected chimeric GFP-Der p 1 was detected under fluorescence microscope after transfection with the following constructs: pEGFP-LmWtDer p 1, pEGFP-mWtDer p 1, pGFP-mHuDer p 1, and pEGFP-fHuDer p 1 after 48 h post-translation. The fluorescence images were taken under fluorescence microscope (Biozero KEYENCE, Tokyo, Japan), (Figure 39). The high numbers of transfected cells emitting fluorescence were found in cell transfected with pEGFP, pGFP-fHuDer p 1 and pGFP-mHuDer p 1 (Figure 39). However, a few cell transfected with pGFP-mWtDer p 1 or pGFP-LmWtDer p 1 emitted fluorescence.

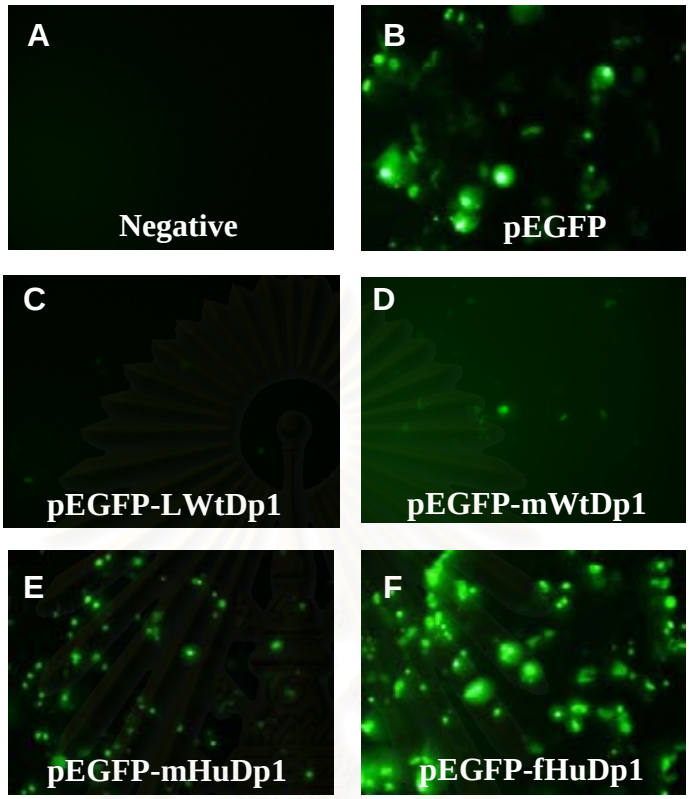

Figure 39 The expression of GFP in HEK 293 cells with pEGFP and pEGFP chimeric mWtDer p 1 or mHuDer p 1. Panal A: non-transfected cells, panel B: transfected pEGFP empty vector, C: transfected with pEGFP-LmWtDer p 1, D: transfected with pEGFP-mWtDer p 1, panel E: transfected with pEGFP-mHuDer p 1 and F: transfected with pEGFP-fHuDer p 1. The results represented one of three experiments.

The cell lysate of chimeric-GFP protein encoded by each targeted DNA was then analyzed by SDS-PAGE and western blot. The GFP and chimeric GFP-Der p 1 were showed in Figure 40A. The GFP was detected at 27 kDa, while full length and humanized Der p 1 peptide were detected at 39 kDa and at 52 kDa, respectively.

However, no wild type Der p 1could be detected. The β-actin was used as an internal control at 47 kDa (Figure 40 B).

To determine whether the chimeric GFP-Der p 1 could be secreted from transfected cell, supernatant from the culture were analyzed by SDS-PAGE and western blot. The western blot result showed proteins mobilized at MW~55-70 kDa (Figure 41A). However, western blot result of supernatant from culture without fetal bovine serum (FBS) showed no detected proteins mobilized at 55-70 (Figure41B).

To study the protein expression level by 293 T cells transfected with pEGFP and pEGFP chimeric DNA as compared with previous experiment with 293 T cell transfected pHIS-Der p 1 plasmid DNA and HEK 293 cells transfected pEGFP-Der p1 plasmid DNA. $5x10^5$ of 293 T cells were transfected with pEGFP, pEGFP-mHuDp1, or pEGFP-fHuDp1 using Lipofectamine^{TM}2000. As a result, the expression level of pEGFP and pEGFP-fHuDp1 was showed in the same way as in HEK 293 transected cells, as shown in Figure 42. The fluorescence images of GFP expression, panel A: GFP expression of nontransfected cells shown 0.18% cell was positive for GFP expression, panel B: transfected pEGFP empty vector shown 21.39 % for GFP expressed cells, panel C: transfected with pEGFP-mHuDp1 shown 14.61 % for GFP expressed cells panel, and panel D: transfected with pEGFP-fHutDp1 shown 5.40% cell expression with GFP (Figure 42). The summarized results were showed in Table 16.

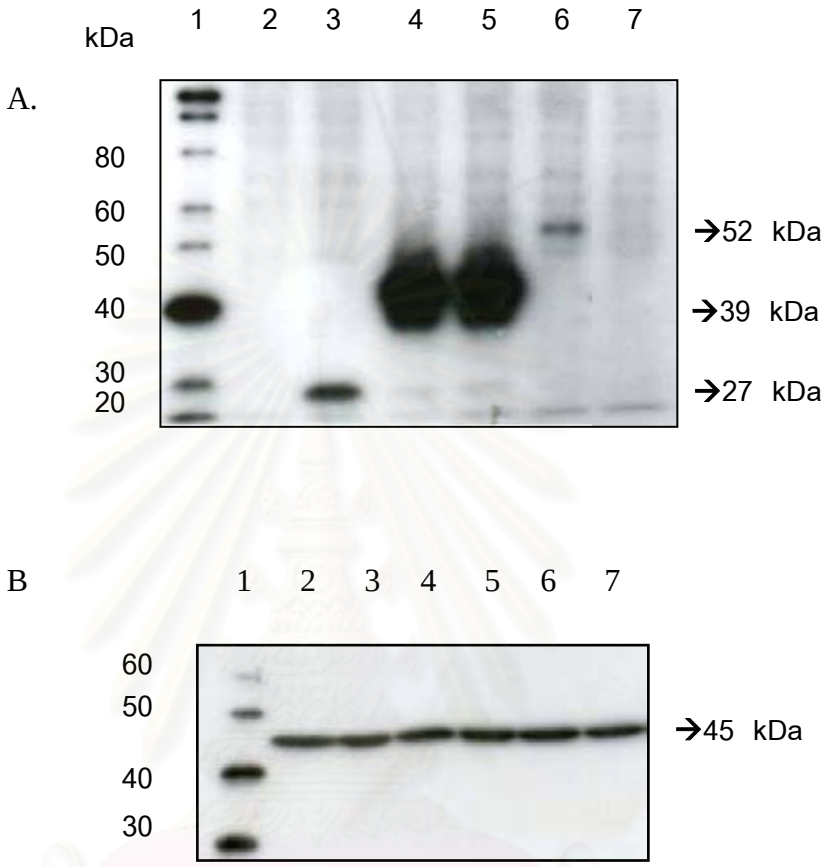

Figure 40 Western blot analysis of Der p 1 protein from HEK 293 cells transfection with chimeric pEGFP plasmid DNA. A shows an immunoblotting of transfected cells lysate with anti-rabbit polyclonal serum against GFP. Lane1: magic marker, lane 2: negative control (non-transfected plasmid), lane 3: pEGFP empty vector, lane 4: pEGFP-fHuDer p 1 clone1, lane 5: pEGFP-fHuDer p 1 clone2, lane 6: pEGFP-mHuDer p 1 and lane7: pEGFP-mWtDer p 1 transfected plasmid. B is β-actin protein expression control.

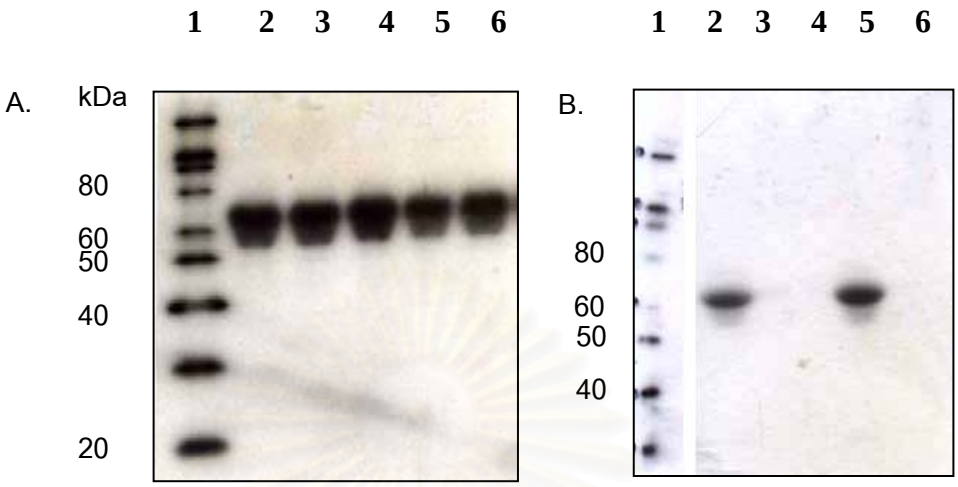

Figure 41 Supernatant analysis from transfected HEK 293 cells with pEGFP and pEGFP chimeric DNA. Supernatants from cell culture with 10% FBS were analyzed by SDS-PAGE and Western blot. The blot was detected by the ECL™ reagent. After that, the blot was exposed with film and then the film was developed. **A:**Lane 1: magic marker, lane 2: nontransfected cells, lane 3: transfected pEGFP empty vector, lane 4: transfected with pEGFP-mWtDer p 1, lane 5: transfected with pEGFP-mHuDer p 1 and lane 6: transfected with pEGFP-fHuDer p 1. **B**: Lane 1: marker, lane 2: nontransfected cells with 10 % FBS in culture medium, lane 3: nontransfected cells without 10 % FBS in culture medium transfected pEGFP empty vector, lane 4: transfected with pEGFP-fHuDer p 1 in culture medium without 10% FBS, lane 5: transfected with pEGFP-fHuDer p 1 in culture medium with 10% FBS and lane 6: transfected with pEGFP-wHuDer p 1 in culture medium without 10% FBS.

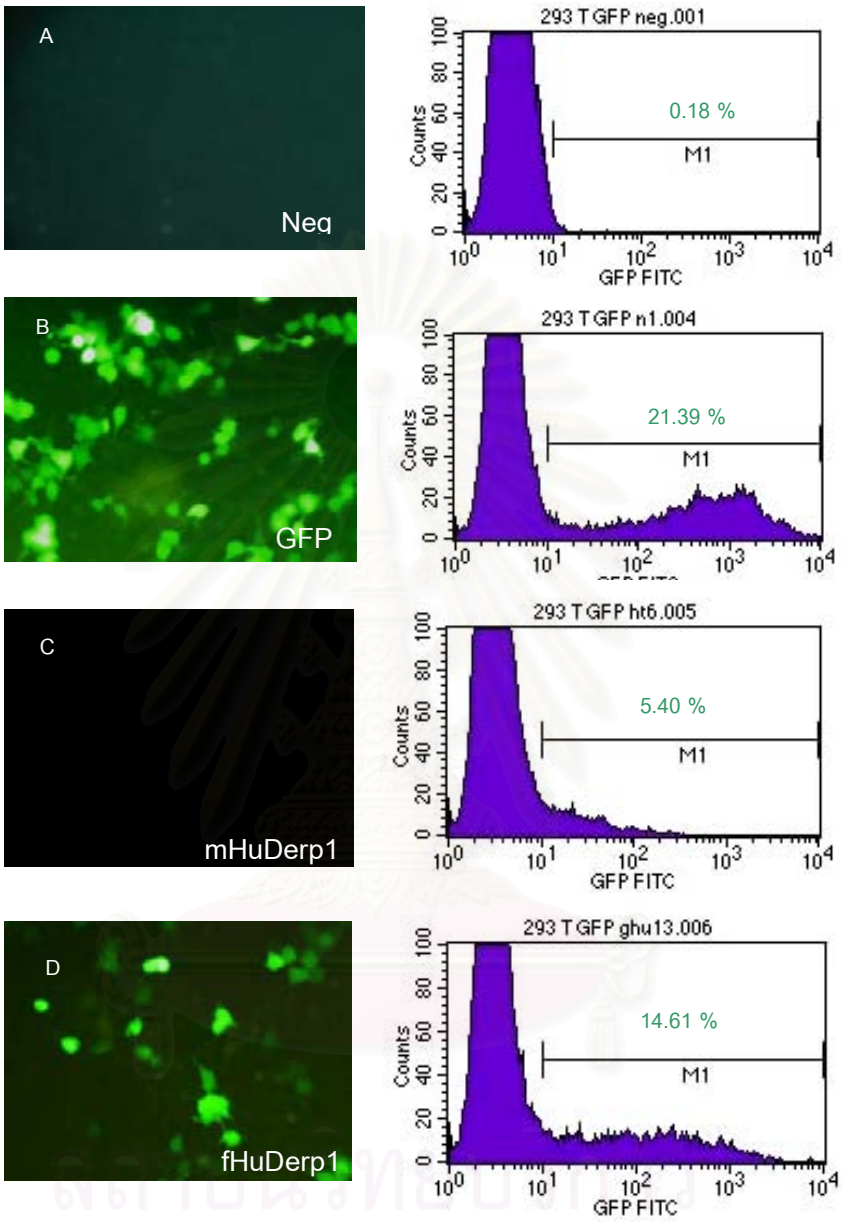

Figure 42 The expression of GFP in 293 T cells after transfected with recombinant pGFP plasmid DNA.

Table 16 Summarized results of *in vitro* characterization.

Cell line	DNA construct	mRNA expresion	Protein
293 T cells	pHIS-mWtDer p 1	Yes	No
	pHIS-LmWtDer p 1	Yes	No
	pHIS-mHuDer p 1	Yes	No
HEK 293 cells	pGFP-mWtDer p 1	nd	No
	pGFP-LmWtDer p 1	Yes	Yes
	pGFP-mHuDer p 1	Yes	Yes
	pGFP-fHuDer p 1	nd	Yes

5. Recombinant ProDer p 1 expression in yeast system

Recombinant ProDer p 1 (rProDer p 1) protein was expressed in yeast *Pichia pastoris* cells to be uses for allergic model immunization. The supernatant from culture of transformed yeast cells was analyzed by SDS-PAGE and western blotting The western blot results showed rProDer p 1 at MW~30 kDa (Figure 43).

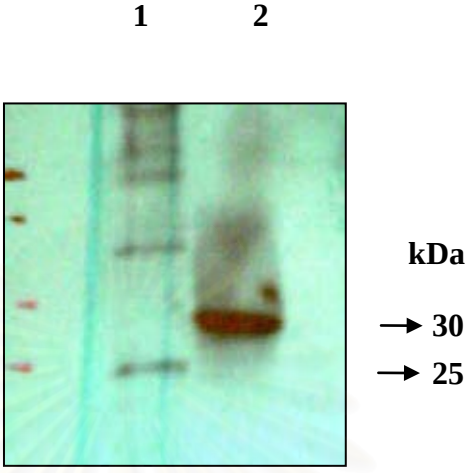

Figure 43 Recombinant ProDer p 1 protein expressed in yeast *P pastoris* transformed with pPIZα-Derp1w1. Supernatant from yeast culture medium transfected with pPICZ α-Dp1w1 was harvested and analyzed. Immunoblotting was showed purified recombinant Der p1 after detected by monoclonal Der p 1. Lane 1: protein marker and land 2: purified recombinant Der p 1, MW~ 30 kDa.

6. In vivo immunogenicity testing in mice

6.1. Mice experimental groups

To test the immunogenicity of all DNA constructs and rProDer p 1, 2 groups of mice were injected:

6.1.1. pHIS-mHuDer p 1 DNA or rProDer p 1 immunization

6.1.1.1 Control (pHIS empty vector) N=4

6.1.1.2 Vaccine group (pHIS-mHuDer p 1) N=6

6.1.1.3.Allergic model (rProDer p 1+ alum) N=6

6.1.2. liposome-pHIS-mHuDer p 1 DNA (lipoplex) immunization

6.1.2.1 Liposome control N=4

6.1.2.2 Liposome-pHIS-mHuDp1 DNA N=5

6.2. Immunogenicity of pHIS-mHuDp1 plasmid

To study the level of immune response in mice serum to plasmid Der p1 DNA vaccine, the level of specific IgG2a, specific IgG1 and specific IgE were measured by indirect ELISA using an individual serum.

6.2.1. IgG2a specific antibodies

IgG2a antibodies against rProDer p 1 were detected in mice immunized by i.d. with pHIS-mHuDer p 1 DNA and liposome-pHIS-mHuDer p 1 DNA compared with control group. Specific IgG2a antibodies against rProDer p 1 protein were detected by ELISA at a collected serum in each time point. In naked DNA vaccine group, IgG2a antibodies were slightly detected at week 4 (at serum dilution 1:40) and the antibody level continuously increased at week 5. Mice from vaccine group (n=6) had specific IgG2a antibody response detectable level (6 from 6 mice), as shown in Table 17 and Figure 44. In liposome-DNA complex group, by use the half amount of pHIS-mHuDp1 DNA as compared with naked DNA injection group, the specific IgG2a antibodies to Der p 1 could detected in 2 mice from 5 mice after one time of injection. At the third week of immunization, specific IgG2a antibodies could be detected in all mice in this group (Table 18 and Figure 45).

Table 17 Specific-IgG2a antibody against rProDer p 1 protein from DNA vaccinated mice and control.

	Mouse specific IgG2a response	
Time	**Control (pHIS)**	**DNA (pHIS-mHuDp1)**
Week 0	0/4	0/6
Week 1	nd	nd
Week 2	0/4	0/6
Week 3	nd	nd
Week 4	0/4	6/6
Week 5	0/4	5/5*[1]

nd = not determination
*[1] = 1 mouse died between experiment

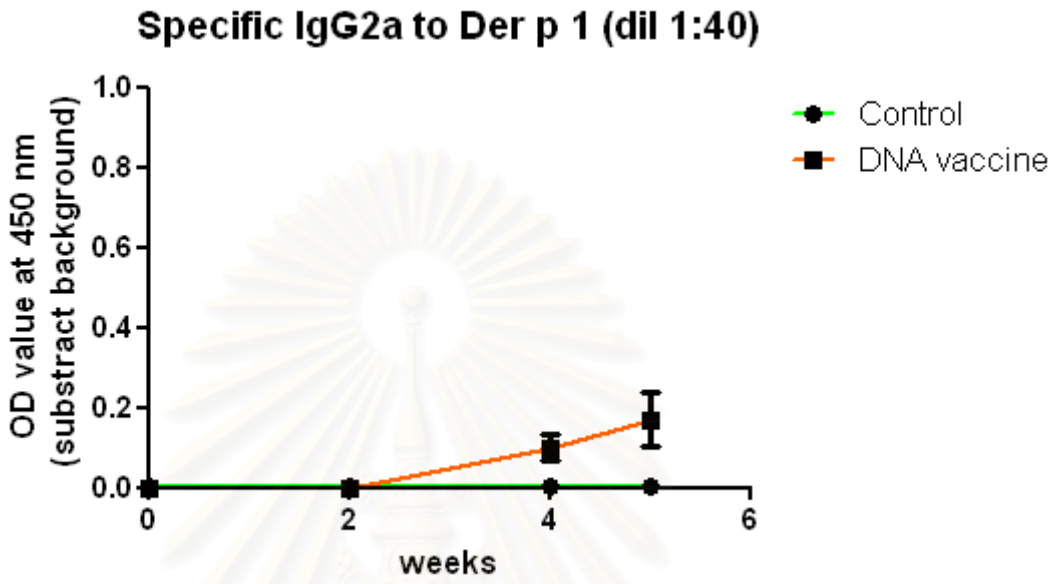

Figure 44 Der p 1-specific antibody responses after naked-DNA vaccination. Balb/c mice were immunized by i.d. with pHIS-mHuDp1 and pHIS empty vector (control). Specific IgG2a against rProDer p 1 protein was measured on wk0, wk2, wk4 and wk5. Der p 1–specific antibody response in DNA vaccine group as indicate by red line and in control group as indicated by green line, data are shown as OD value a 450 nm at dilution 1:40 with subtract background.

Table 18 Specific- IgG2a antibody against rProDer p 1 protein from Liposome-DNA vaccinate mice and control.

	Mouse specific IgG2a response	
Time	**Control (Liposome)**	**DNA (Liposome-pHIS-mHuDp 1)**
Week 0	0/4	0/5
Week 1	0/4	2/5
Week 2	0/4	5/5
Week 3	0/4	3/3*

nd = not determination
* = 2 mice died between experiment

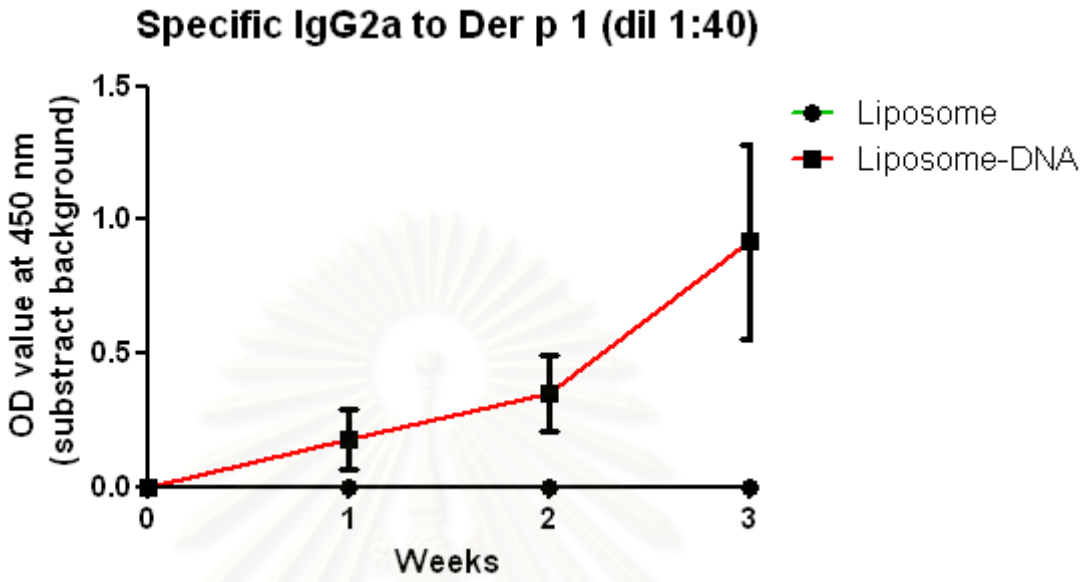

Figure 45 Der p 1-specific antibody responses after Liposome-DNA vaccination. Balb/c mice were immunized by i.d. with pHIS-mHuDp 1 and pHIS empty vector (control). Specific IgG2a against rProDer p 1 protein was measured on wk0, wk2, wk4 and wk5. Der p 1–specific antibody response in DNA vaccine group as indicate by red line and in control group as indicated by green line, data are shown as $OD_{450\ nm}$ at dilution 1:40 with subtract background.

6.2.2. Immunization with rProDer p 1 in mice model

Six mice (allergic model group) were received recombinant ProDer p 1 (rProDer p1) protein induced by i.p. 5 times weekly interval. Specific IgE Abs to rProDer p 1 (characterized as Th2- biased immune response) was measured by indirect ELISA (Figure 46). All mice in this group, 6 from 6 (100 %) contains specific IgE to rProDer p 1 (OD $_{450\ nm}$ with subtract background) compare with DNA vaccine and control group (no detectable specific IgE to Der p1 after subtract background at OD $_{450\ nm}$), as shown in Table 19. However, one mouse was died between experiments. For specific IgG1 against rProDer p 1, 6 from 6 of allergic model (100 %) (Table 20) mice were represent specific IgG1 antibody response (as shown by OD $_{450\ nm}$ after subtract background) (Figure 47) compared with DNA vaccine and control group.

Table 19 Specific-IgE antibody against rProDer p 1 protein from allergic mice model.

	Mouse specific IgE response
Time	**Number of mice**
Week 0	0/6
Week 1	nd
Week 2	6/6
Week 3	nd
Week 4	6/6
Week 5	5/5*[1]

nd = not determination
*[1] = 1 mouse died between experiment

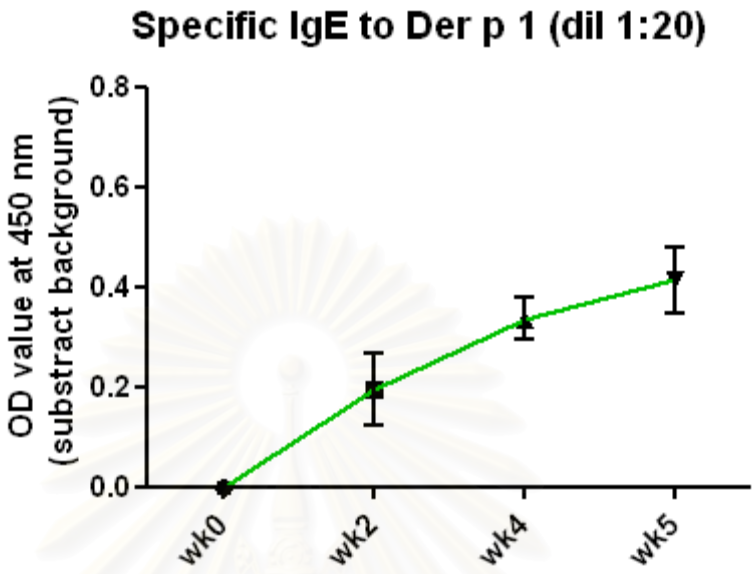

Figure 46 Der p 1-specific IgE antibody responses after recombinant Der p 1 protein immunization. BALB/c mice were immunized by i.p. with rProDer p 1 protein. Specific IgE against rProDer p 1 protein was measured on wk0, wk2, wk4 and wk5. Der p 1–specific antibody response in allergic model group was indicated by green line, data are shown as $OD_{450\ nm}$ at dilution 1:20 with subtract background.

Table 20 Comparison of specific IgG1 antibody against rProDer p 1 protein from vaccinate mice, allergic model and control.

	Number Mouse specific IgG1 response		
Time	**Control group**	**DNA vaccine group**	**Allergic model group**
Week 0	0/4	0/6	0/6
Week 2	0/4	6/6	6/6
Week 4	0/4	6/6	6/6
Week 5	nd	5/5*[1]	5/5*[2]

nd = not determination
*[1] = 1 mouse died between experiment in DNA vaccine group
*[2] = 1 mouse died between experiment in allergic model group

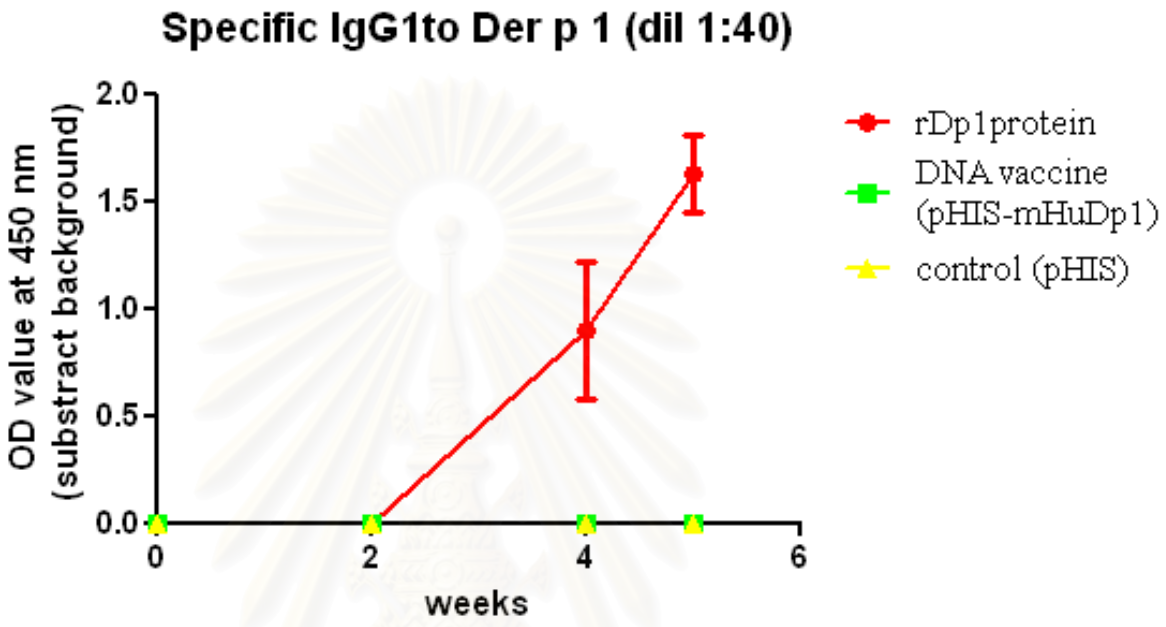

Figure 47 Der p 1-specific IgG1 antibody responses in all experiment groups. All mice were measured specific IgG1 against rProDer p 1 protein on wk0, wk2, wk4 and wk5. Der p 1–specific antibody response in allergic model group was indicated by red line, DNA vaccine group was indicated as green line and control as indicated by yellow line. Data was shown as $OD_{450\ nm}$ at dilution 1:40 with subtract background.

CHAPTER V

DISCUSSION

Allergic diseases are characterized by an inappropriate immune response to various common environmental allergens leading to the production of specific IgE antibodies to an allergen and the cause of allergic reaction and/or inflammation. Currently, allergen specific immunotherapy is an approach to desensitize an allergic reaction. By giving a small amount of allergen extract, at increasing doses, the treatment is performing for several years.

The development of house dust mite vaccine was aimed to deviation the Th2 bias immune response to Th1 bias immune response. DNA based immunization as an effective strategy of vaccine research to induce potent humoral and cellular immune response (91, 92). DNA immunization encoded with allergen could preferentially induce Th1 responses while reduce Th2 responses. Th1 responses are characterized by IgG2a production, induction of IFN-γ production, and suppression of IgE production (30, 52, 85, 93). These events help to prevent the development of airway inflammation or to inhibit STAT 6 and NF-κB expression in mice lung tissue (94).

The DNA components required to yield an effective DNA vaccine for immunization are a strong eukaryotic promoter, a cloning site, a polyadenylation sequence, a selection marker, and a bacterial origin of replication (95). Plasmid vector for immunization should be provided a promoter such as CMV promoter that drives a greater gene expression but should not integrate itself with the host genome, such as

CMV promoter (95). Thus pHIS plasmid vector, containing CMV promoter and intron upstream of promoter, was chosen for this study. The effective pHIS vector was shown in 2003 by Sirivitchyakul *et al* (96). The backbone of this plasmid contains repeated CpG (2006) motifs with the sequence 5' TCGTCGTTTTGTCGTTTTGTCGTT 3'. This CpG motifs is "K" type oligodeoxynucleotides (ODNs) that could activate plasmacytoid dendritic cells and trigger B cell for activation which recognized by Toll-like receptor 9 (TLR9) (97). Consequently CpG motifs act as an adjuvant to boost on cellular or antibody responses elicited by DNA vaccine (98), leading to an induction of Th1 cytokines production such as IL-12 and IFN-γ (99) or elicit regulatory T-cells responses (83). For example, immunization with hen egg lysozyme (HEL) formulated with CpG, results in induction of anti-HEL IgG2a (Th1 responses) (100), or CpG conjugated with ragweed allergen (Amb a 1) (37, 81, 82) results in an increasing specific IgG2a production (37, 81, 82) or $CD4^{+}CD25^{+}$ T cells in nasal mucosa of allergic rhinitis patients (83). However, species specificity could limit the use of CpG motifs because the TLR9 molecule expressed by different species has a difference in the sequence motif recognition which is an important factor in responding to difference CpG include flanking region (97). For example, the TLR9 in mice differ from human by 24% at the amino acid level (97). CpG 2006, which contains 'GTCGTT' motif, was, however, shown as immunostimulatory sequence for leukocytes in both human (83), non-human primates i.e. cattle (101) or monkey (102) and mice (37).

To increase the safety of DNA vaccines against allergy, the DNA or cDNA encoding native allergen should be avoided for prevention of undesired side effect or anaphylactic reaction. The proteolytic activity of allergen was shown to promote potent allerginicity, leading to the increase Th2-bias immune response (25, 53, 55,

103). As described above, Der p 1 protein was considered as less in allerginicity in ProDer p 1 form for vaccine design, which was proposed for several possible reasons that: 1) mature Der p 1 form but unstable; 2) incorrect propeptide cleavage; 3) mature Der p 1 could be autoproteolytically degraded (104); 4) the enzymatically inactive precursor form of HDM allergen, ProDer p 1 such as in yeast expression system (105). In some experimental study, the ProDer p 1 protein was considered to design for an effective and less in allergenicity for HDM vaccine (52, 85). Because of pro-sequence part of Der p 1 serves as a blocking enzymatic activity (blocking at N-terminal B-cell epitope), results in decreasing of allergenicity activity (52, 85). The results demonstrated that DNA vaccine with plasmid encoding ProDer p 1 prevent the development of allergen-specific IgE and airway inflammation after aerosolized with house dust mite allergen (52). Another approach for HDM vaccine design, by replacing the Der p 1 signal sequence with other signal sequences such as Der p 5 signal sequence (93), a mouse Igκ light chain signal sequence (106) or by gp67 signal sequence in pAcGP67A vector to avoid the native structure (104). In this study, leader sequence of Der p 3 (69 bp) was used as a leader sequence for wild type plasmid construct to increase protein expression secretion level because Der p 3 has been showed that it could be automatically processed itself form immature form to be mature form (Dr.S Piboonpocanun, unpublished data). Only wild type plasmid construct was engineered with Der p 3 signal sequence (LDp3-mWtDer p 1), the results when compare with mature wilt type Der p 1 (without any signal sequence, mWtDer p 1). The results showed that no protein expression level could be observed (Figure 40).

In this study, mature Der p 1 without prosequence was constructed into pHIS plasmid vector for two reasons. Firstly, prosequence is important for Der p 1 folding.

Mature Der p 1 protein without pro-sequence that could be results with reducing in IgE reactivity or IgE binding epitopes. Secondly, the pro-sequence could also elicit undesirable immune responses (107), even if many evidences have shown a hypoallergenic response.

To construct a potential DNA vaccine from other species of protein antigen, because each species has a variable requirement for codon references for amino acid coding. Since amino acid can be coded by more than one codon and the codon usage is different in each organism influence the heterologous protein expression level. The codon optimization of gene was required to alternate of gene sequence from original species to match with the codon which available in host cells. In this study, wild type Der p 1 gene was recoding to human codon usage based on the codon usage frequently found and highly expressed in human. Such as GGT (Gly) that codes for wild type Der p 1 gene was changed to GGC (Gly) for humanized Der p 1 gene (Table 8-10). The frequency of human codon per thousand for GGT from wild type Der p1 gene is 12.8 and GGC from humanized Der p 1 is 22.4 (www. kazusa.or.jp/codon). As shown by other studies, in 2003 by Bauer R *et al* demonstrated that codon usage optimization could improve protein expression level in mugwort (*Artemisia vulgaris*), Art v 1 allergen, or in Der p 1 allergen (52). They could induce a strong expression of Art v 1 protein about 180-fold higher when compared with wild type gene in cell culture and could be elicited a strong allergen-specific immune response (38). Analysis of wild type Der p 1 shows, the human codon usage is different with wild type gene about 75 % but results in the same amino acid sequence of wild type protein. To generate mature humanized Der p 1, two general ways could be performed: 1) commercial fully humanized Der p 1 DNA synthesis (price ~120,000 baht) and 2) PCR overlapping

oligonucleotides synthesis (price ~20,000 baht). For this study, humanized Der p 1 was generated by 8 overlapping oligonucleotides PCR amplification. Although overlapping PCR technique is more saved than commercial one but some mutation could be detected, as shown in this study. The selected clone (c72G-hDp1) contains fewest point mutation that contains 3-base-deletion at nt 270-273, a deletion of nt 569 and nt 574, results in reading frame shift (Figure 31). Another base substitution mutation at nt 303 did not have an effect at the amino acid level, it affected only to the reduction of the frequency of human codon usage (i.e., human codon frequency per thousand from CAG = 34.2 → CAA = 12.2).

To facilitate the detection of protein expression, all DNA of interest was cloned into the pEGFP-N1 reporter plasmid. Four Der p 1-derived DNA were used in the present study: wild type Der p 1 (without leader Der p 1 sequence, pGFP-mWtDer p 1), leader-Der p 3-WtDer p 1 (pGFP-LDp3-mWtDer p 1), humanized Der p 1 (pGFP-mHuDer p 1) and fragmented humanized Der p 1 (N-terminal domain, 99 amino acid, pGFP-fHuDer p 1) Once all plasmid was performed transfection in HEK 293 cell line (experimentation in Yokohama city University, Japan). The fusion GFP-Der p 1 proteins could be detected by fluorescence microscopy. The results showed that high number of transfected cell emitted green fluorescence were observed in control group (pEGFP vector) and pEFP-fHuDer p 1, while low number of cells transfected with pEGFP-mHuDer p 1 emitted fluorescence. However, the humanized Der p 1 is expressed in higher yield than wild type Der p 1. Both cell lysate and supernatant from culture was then analyzed by western blot analysis using anti-GFP antibody to GFP protein. The result showed that the Der p 1-GFP chimeric protein could be detected only in cell lysate not in supernatant of cell culture. However, our results

suggested that mature Der p1 protein was expressed and could be detected in the cell lysate as a chimeric with GFP expression.

The direct expression of mature Der p1 was not possible without propeptide sequence (104, 105) while the successful ProDer p 1 expression was reported in insect (104), Chinese hamster ovary (CHO) cells (104), and yeast system (105). The differences in number of transfected cells emitting fluorescence may be due to:

1) Transfected mammalian cells expressed high level of codon-optimized Der p 1 DNA but not dust mite-codon Der p 1. 2) Leader sequence of dust mite protein Der p 5 could increase the protein scretion level in other study (93). However, leader sequence of Der p 3 could not increase Der p 1 expression. 3) The expression level of humanized Der p1 peptides were higher than a whole protein may be due to it is not fully enzymatic function (cysteine protease) because of only N-terminal part of Der p 1 protein (99 amino acid) was used to generate plasmid construct (pGFP-fHuDer p 1)……..

To our knowledge reporting that successfully expression of mature humanized Der p1-GFP fusion protein and fragmented humanized Der p 1-GFP fusion protein. Whereas mature dust mite Der p 1-GFP fusion protein could not be detected. It has been shown that human codon optimized recombinant allergens such as Art v 1 (38), Der p 1 (52) were successfully expressed. Although, detection of GFP fusion protein with anti-GFP antibody was likely from GFP protein, however, the results also indirectly imply that GFP fusion protein had a correct structure. Interestingly, we could not detect the fused mature Der p 1. This may be due to incorrect folding of fused Der p 1. Using other detection methods could be used for detection of fused Der p 1. For example, using Concanavalin A-Sepharose (con A), to capture fused Der p 1

since it has been shown that Con A-sepharose, purification of glycoproteins, polysaccharides and glycolipids (108). Recombinant ProDer p 1 could be purified by incubating with con A to concentrate the secreted protein (105).

In this study, pHIS-mHuDer p 1 was designed for HDM DNA vaccine development although pGFP-fHuDer p 1 induces higher protein expression in transfection assay compared with pGFP-mHuDer p 1 plasmid construct. From the review data, Der p 1-human T cell epitope has been reported such as amino acid 107-119, 110-119 and 110-131 in patient suffering from perennial rhinitis patient (109); amino acid 45-67, 94-14, and 117-143 (110); amino acid 1-56 and 188-222 (111). In murine model, T cell epitope to Der p 1 has been reported in different mice strains such as amino acid 1-56, 5-29, 21-49, 78-100, 93-106, 110-131, 120-143, 144-169 and 197-212 (109). Fragment humanized (pGFP-fHuDer p 1) plasmid DNA (contains amino acid 1-99) did not choice in this study although it contains at least 3 out of 8 human T cell-epitope that has been reported and may be, this N-terminal De p 1 contain less IgE binding epitope also. It can be used as a candidate T cell-epitope DNA vaccine development to avoid to use whole sequence of allergen.

In order to evaluate the immunogenicity of this new Der p 1 vaccine construct, mice were injected with pHIS-mHuDer p 1 plasmid DNA. As control, mice were sensitized with rProDer p 1/alum to drive a typical Th2-based allergic response. The experimental mice were divided into following two groups. The DNA vaccination model (pHis-mHuDer p 1) and allergic model (Figure 20 and 21) were done as described in Materials and Methods. In vaccinate model, Balb/c mice were immunized by intradermal injection with two form of DNA vaccine. For the first group, immunization with naked DNA plasmid constructs (pHIS-mHuDer p 1) another groups vaccinated with plasmid DNA (pHIS-mHuDer p 1) were formulated with

liposome. At least four immunization with pHIS-mHuDer p 1 without liposome were necessary to detect a weak but specific-IgG2a against rProDer p 1 protein antibody response while only one injection of pHIS-mHuDer p 1-liposome formulation was sufficient to measure the high level of specific IgG2a to Der p 1. All mice vaccinated with mature Der p1 DNA vaccine can induce Th1-bias immune response , as shown by specific IgG2a against rProDer p 1 protein, and more effective when combined plasmid DNA with liposome.

Adjuvant is an immunostimulatory molecules which can skew an immune response to Th1 or Th2 bias. Many powerful adjuvants in experimental use are from microbial origin, which elicit strong innate immune reaction and inflammation. For example, immunizing Der p 1 protein with alum, results in specific IgE response while ProDer p 1-liposome complex leading to induce a Th1 biases immune response (85). As mentioned above, plasmid DNA that contains CpG sequence in backbone also has an adjuvant-like activity and which preferentially induce Th1 response.

Liposome is a micromolecule that composes of naturally occurring substances. Liposome was reported as drug carrier system such as in treatment of leishmaniasis (112) or ProDer p 1 allergen (85, 113). As report in house dust mite allergy, ProDer p 1 complexes with cationic liposome, results in a decrease of the allergen immunogenicity (85). For liposome preparation (United States Patent 6355267). In this study, was consisting of liphoid S75 (Lipoid GMBH, Germany), cholesterol (Sigma, USA) and DOTAP (Roach, Germany). The results showed that plasmid DNA-liposome complex induces higher yield of transfection and more protein expression leading to the better immune response. But liposome can also act as adjuvant to activate immune system more efficiently and better immune response (Figure 45).

In the context of the parallel experiment, recombinant ProDer p 1 (rProDer p 1) protein was produced and expressed by yeast *Pichia pastoris*. The recombinant products were purified from yeast culture medium. On SDS-PAGE analysis, purified recombinant Der p 1 protein showed a molecular as about 35-60 kDa. In this study, rProDer p 1 protein were used for allergic model immunization and detection assay by ELISA technique. The correlation of IgE binding between natural and rProDer p 1 protein was tested by ELISA assay. The results showed that glycosylation in recombinant Der p 1 protein did not affect the activity of IgE binding (105). Using competitive inhibition assay, the results showed that rProDer p 1 could be inhibited the IgE reactivity of natural Der p 1 (105). Jacquet *et al*, demonstrated that all IgE binding epitope of rProDer p 1 protein was same as in natural Der p 1. So in this experiment, rProDer p 1 protein was used for ELISA plate coating for specific IgG2a, IgG1 and IgE detection. To generate the allergic model; aluminum hydroxide (alum) was used as an adjuvant to produce Th2-bias immune response. It is well known that alum induces Th2-biased response (114, 115). This study demonstrated that intraperitoneal immunization with recombinant Der p1-adjuvated with alum injected by resulted in Th2 immune response such productions were characterized by specific IgG1 and specific IgE against recombinant Der p 1 protein, not elicited by DNA vaccine group (the result was shown as subtract background at $OD_{450\ nm}$ at serum dilution 1:20).

There are many successful reports about House dust mite vaccine develop work. Many procedures were shown for more effective for allergic vaccine design such as hybridization of Ty virus-like particle with immunodominant epitope of Der p 1 that was reported by Hirscherg S, *et al*. in 1999 (116), plasmid DNA vaccinization

with codon optimization of allergen (38, 52, 96), adding of the leader sequence (93) (104, 106), using immunostimulatory sequence conjugate with allergen (37, 81, 82) or using of effective delivery particle such as liposome for vaccination (85, 113). More interesting for recent report, pilot phase IIa clinical trial with an immunotherapy product, CYT003-QbG10, for the treatment of allergic diseases has been reported. This is comprised of the virus-like particle "Qb" filled with an immunostimulatory DNA (G10) sequence to induce Th1 response which using for treatment the patients suffering from allergic asthma and allergic rhinitis due to house dust mite (rhinoconjunctivitis symptom score reduced 61%) (117). Different approaches for the Der p 1 and/or other HDM DNA vaccine developments were summarized in Table 21. The summary of our Der p 1 DNA vaccine design and the development is shown in Table 22.

The main differences of this current approach include the combinations of human codon optimization of the cDNA sequences, the use of CpG motif containing CMV promoter plasmid, lipoplexes was used to enhance the recombinant DNA delivering system, and the vaccine was injected intradermally for immunization. From the immunization results, its support that this approach is efficient in the induction of Der p1 speicfic Th1 responses and this candidate DNA vaccine is warranted for further development to improve its immunogenicity.

To increase the immunogenicity and evaluate of Der p 1 DNA vaccine (pHIS-mHuDer p 1), our the further experiments could be planned: 1) increase frequency and number of injections, 2) using different liposome 3) using prime boost technique and 4) testing for prophylactic (vaccination first and then sensitize and challenge with Der p 1 protein) and therapeutic (sensitization first then vaccinate and challenge) function.

Table 21 Summerization of Der p 1 DNA vaccine works

	Our vaccine	Wolfowicz B. *et al* Singapore 2003	Chew LJ. *et al* Singpore 2003	Jacquet A. *et al* Belgium 2003	HuangFu T. *et al* Singapore 2005	Kim N. *et al* Korea/USA 2006	Kim CH, Kwon SS (Korea) 2006
Vector	pHIS (CpG)	pCI-neo	pCI-neo	pNIV4868	pVax 1	pCDNA 3.1	NA
Gene/Protein	Mature Der p1	Mature Der p1	Mature Der p1	ProDer p1	Mature Der p1	Dp1,2,3/ Df1,2,3	Murine T cell epitope of Der p
Codon optimization	Human codon	-	-	Human codon	Human codon	-	-
Signal sequence	-	Leading of Dp5	Leading Dp5	-	mouse Ikg light chain	-	NA
Adjuvants/other	CpG 2006 (w/wo liposome)	-	Chitosan	-	-	pertussis	BCG
Injection route	i.d.	i.m. (electroporation)	Oral feeding plus i.m. (electrporation)	i.m.	i.m. (electroporation)	i.m.	x
Protein expression	HEK 293 (chimericGFP)	In vivo protein exp (chimeric GFP)	-	COS cells transfected	-	-	x
Immunogenicity	BALB/c	Sp-IgG2a ↑	Sp-IgG2a ↑	Sp-IgE ↓ Sp-IgG2a ↑ Sp-IgG1 ↑	Sp-IgE (p) ↓ Sp-IgG2a (p) ↑ Sp-IgG1(p) ↑	Total/Sp-IgE ↓ IFN-g mRNA ↑ Inflammatory cell ↓	IL-4 ↓ IFN-g ↑ Eo in BALF↓

Table 22 Summarization of our HDM DNA vaccine development.

	Description
Type of vaccine design	DNA vaccine
Gene design	Mature Der p 1 (house dust mite allergen) with humanized codon optimization
Type of plasmid expression vector for DNA vaccine	pHIS (Coley Phamaceutical company) plasmid vector that contain CpG motif
In vitro characterization of mature humanized Der p 1 DNA	Transfected HEK cells with chimeric pGFP plasmid construct showed low level expression of chimeric GFP-mHuDer p 1
DNAvaccine for vaccination	pHIS-mHuDer p 1 DNA vaccine
Mice model use	Female Balb/c mice, 6-8 wks old (Th2 responder animal)
Route of immunization	Intradermal (i.d.)
Delivery system	With/without Liposome complex (Iipoid S75, cholesterol, and DOTAP)
Type of immune response	Th1 immune response characterized by specific IgG2a antibody against rProDer p 1 protein

CHAPTER VI

CONCLUSIONS

In a context of house dust mite vaccine development in this study, two parallel experiment strategies were created including DNA vaccine and recombinant protein vaccine. The major experiment is the DNA vaccine development. The plasmid constructions consisting of a strong CMV promoter and CpG (2006) motif in plasmid backbone was the selected choice for DNA vaccine design. The pHIS plasmid vector (Coley Pharmaceutical group) was used to engineer with the house dust mite major allergen Der p 1 cDNA. In addition, to increase the DNA vaccine immunogenicity, human codon optimization was performed to increase the level of protein expression.

Eight 100 base-pairs with 20 bp overlapping and human codon-optimized oligonucleotides were created to make a full-length Der p1 cDNA. The 666 bp of mature humanized Der p 1 was generated by PCR overlapping oligonucleotides amplification. For the generation of humanized plasmid DNA constructs, Firstly, the mature humanized Der p 1 gene was subcloned into a pGEM®-T easy vector. The c72G-hDp1 clone that contains the least mutations (4 mutations position) as compared with other 3 sequences (Figure 30). This c72G-hDp1 clone contains one mutation that did not an effect at the amino acid level, but other 3 mutations with base deletion could result in shifting of the amino acid reading frame. Thus, the site-directed mutagenesis was used to correct these 3 mutations. The lesson learned it is the truncated overlapping primers approach to generate a full-length humanized DNA sequence is a

time consuming and has also turned out eventually to be not cost-effective. The pGEM-mHuDer p 1 plasmid vector which contains corrected mature humanized Der p 1 DNA sequence was then subcloned into pHIS vector to generate a pHIS-mHuDer p 1 plasmid DNA candidate vaccine.

To characterize the gene and protein expression level *In vitro,* pHIS-mHuDer p 1 plasmid DNA was firstly performed in a mammalian cells line 293 T cells. The expression of mature Der p 1 protein could not be detected, while specific mRNA expression can be observed in all transfected plasmid constructs i.e., pHIS-mWtDer p 1 (wild type), pHIS-LDp3-mWtDer p 1 (wild type with Der p 3 leading sequence) and pHIS-mHuDer p 1 (humanized mature Der p1) (Figure 36 and 37). To increase the sensitivity of protein detection, a green fluorescent protein reporting system of which pEGFP chimeric plasmid DNA constructs were generated. Mature Der p 1 cDNA was subcloned into upstream of GFP reporter gene (pGFP-mWtDer p 1, pGFP-LDp3-mWtDer p 1, pGFP-mHuDer p 1 and pGFP-fHuDer p 1). The level of chimeric Der p 1-GFP protein expression was then observed by fluorescence emission. The transfection was carried out in HEK 293 cells and 293 T cells, using chimeric pEGFP construct. The GFP expression could be observed in all plasmid constructs. The highest GFP expression was detected in pGFP-fHuDer p 1 plasmid transfected cell. The low expression level was observed in pGFP-HuDer p 1 transfected cell while the lowest expression was detected in pGFP-mWtDer p 1 and pGFP-LDp3-mWtDer p 1 transfected cell (Figure 39 and 42). Western blot analysis was used to confirm the expression of Der p 1-GFP chimeric protein using polyclonal antibody to GFP protein. The results shows that the GFP could be detected as a specific band at molecular size 27 kDa, while mHuDer p 1 –GFP was detected as a thin band at 52 kDa, the large band of fHuDer p 1-GFP protein could be observed at 39 kDa. Although mature Der p 1

protein could not be directly detected by conventional Western blot assay with HDM type-1 skin test positive serum, however, this GFP reporting system has proven for the expression of Der p 1 protein (Figure 39, 40 and 42).

To evaluate the efficiency of pHIS-mHuDer p 1 plasmid DNA *In vivo*, Balb/c mice were used in testing the immunogenicity. As previous consideration the immunization is to induce Th1 immune response by which in mice is to induce Der p1 specific IgG2a antibody. The results showed the specific serum IgG2a was observed in low level in mice injected with pHIS-mHuDer p 1 DNA at week 4 after four immunizations (Table 17 and Figure 44) compared with the control. In contrast, mice immunized with liposome-pHIS-mHuDer p 1 DNA (lipoplexes) vaccine have shown a much higher level of the specific IgG2a antibody in sera against ProDer p 1 protein. More interestingly, the Th1 antibody response (IgG2a) was detectable earlier in these lipoplexes immunized mice as seen by two out of five mice at week 1 (1 week after the first immunization) showed specific IgG2a detection and the antibody was detected in all mice at week 2 (1 week after 2 immunizations) (Table 18 and Figure 45). The results indicate that this recombinant mature Der p1 with human codon optimized DNA vaccine as a lipoplexes formulation has proven immunogenic in inducing Der p1 specific Th1-antibody responses. It is therefore relevant for further development and evaluation.

In parallel, a recombinant ProDer p 1 protein has developed. The pPICZα-Der p1w1 (generated and provided by Dr. S Piboonpocanun) was electroporated into yeast expression system (*P pastoris*). The rProDer p 1 protein was detected and purified at the MW 30 kDa, as shown in Figure 43. The purified rProDer p 1 protein was tested for it's immunogenicity in inducing Der p1 specific IgE and IgG1 (Th2) in mice to set

up a house dust mite sensitized mouse model for further vaccine evaluation in the future. The results show the specific IgE and IgG1 antibody responses (as $OD450_{nm}$ results with the back ground subtracted) were detected in all mice after i.p. immunization with rProDer p 1plus alum as an adjuvant. Thus a HDM allergic mouse model has been established and can be utilized for future preventive and therapeutic vaccine evaluation. More interestingly, with the availability of both a recombinant Der p 1 DNA vaccine and a recombinant protein, will allow us to investigate the potential role of DNA vaccine primed and recombinant protein boosted strategy in HDM allergy therapy.

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APPENDIX

1. Chemicals

1.1. Lipofectamine™ 2000, Invitrogen, USA.

1.2. Fetal bovine serum, Gibco, Invitrogen, USA.

1.3. RPMI 1640, Gibco, Invitrogen, USA.

1.4. One step RT-PCR, QIAGEN, USA.

1.5. QIAGEN Purification kit, QIAGEN, USA.

1.6. QIAGEN Mini, Maxi, Giga preparation, QIAGEN, USA.

1.7. Rapid DNA ligation kit, Roche, USA.

1.8. RNAeasy® mini kit, QIAGEN, USA.

1.9. NuPAGE® Novex® Bis-Tris Mini Gels, invitrogen, USA.

1.10. Zeocin, InvivoGen, France

1.11. Kanamycin

1.12. Amplcilin

2. Instruments

2.1. Advanced Biosafty carbinet class II, Science Tech, UK.

2.2. Autoclave, HICLAVE HVE -25/50, Japan

2.3. Automatic Pipettes, Gilson, France

2.4. Automatic sequencing, Applied Biosystem, USA.

2.5. Balance, Precisa XT 2200 C, Swiss.

2.6. Benchmark Elisa Reader, Bio-Rad, USA.

2.7. Bipfreezer(-70 ^{o}C), Harris, USA.

2.8. Centrifuge 4 ^{o}C GS-15R, BECKMAN, USA.

2.9. CO2 humidified incubator, Forma, USA.

2.10. DNA Thermal Cycler 2400, 9600, Perkin Elmer, USA.

2.11. Electroporation machine, Bio-Rad Gene Pulser, USA.

2.12. ELISA plate reader, Multiscan EX plate reader, USA

2.13. Filter paper 0.6 and 0.2 μm (Millipore, USA)

2.14. Fixed angle rotor, Eppendorf, Germany.

2.15. Gel Doc, Bio-Rad, USA.

2.16. Gel electrophoresis apparatus, Bio-Rad, USA.

2.17. Heat block, Scientific, USA.

2.18. High speed centrifuge Avanti J-25I, BECKMAN, USA.

2.19. Incubator for 37 ^{o}C, Memmert, UK.

2.20. Inverted microscope, Nikon, Japan.

2.21. Microscope CX31, Olympus, Japan.

2.22. pH meter 220, Corning, UK.

2.23. Power supply, Bio-Rad, USA.

2.24. Refrigerated centrifuge, Eppendorf, USA.

2.25. Speed vac DNA, Savant Instrument, USA.

2.26. Shaking incubator, Labcon, South Africa.

2.27. Shaking Waterbath, Memmert, UK.

2.28. Spectophotometer, Bio-Rad, USA.

2.29. UV Transilluminator, Funafuti, Japan.

2.30. Vortex Mixer, Stuart scientific, UK.

2.31. Water bath 37 ^{o}C, Fisher Scientific, USA.

3. Enzymes

3.1. *Dpn* I, Roche, Germany.

3.2. *Dra* I, promega, Germany.

3.3. *EcoR* I, Roach, Germany.

3.4. *Kpn* I, Roche, Germany.

3.5. *Sma* I, Roche, Germany.

3.6. *Xho* I, Roche, Germany.

3.7. *Pst* I, Roche, Germany.

3.8. DNA polymerase, Promega, USA.

3.9. Dnase, Promega, USA.

3.10. Phosphatase Alkaline Shimp, Roche, Germany.

3.11. Ribonuclease inhibitor RNasin®, Promega, USA.

3.12. RNAse A, Qiagen, USA.

3.13. *pfu* DNA polymerase, Promega, USA.

3.14. *Taq* DNA polymerase, Promega, USA.

4. DNA markers

4.1. GeneRuler^{TM} 1 kb DNA Ladder, Fermentas,

4.2. Quick-Load 100 bp DNA Ladder, New England Biolabs, USA.

5. Protein markers

5.1. MagicMark™ XP, Invitrogen, USA.

5.2. SeeBlue® Plus2 Prestained Standard, Invitrogen, USA.

6. Reagents

6.1. 3M Sodium acetate (NaOAc), pH 4.8

16.40% (w/v) Sodium acetate

Dissolved in distilled water and adjusted pH to 4.8 with glacial acetic acid. Distilled water was added to 100 ml. The solution was mixed and autoclaved.

6.2. 4x sample buffer (5 ml)

62.5 mM Tris-HCl pH 6.8

10% (w/v) SDS

20% (v/v) glycerol

0.1% (w/v) bromophenol blue

10% (v/v) β-mercaptoethanol

6.3. 50x Tris-acetate buffer (TAE buffer)

2M Tris-acetate

100 mM Na_2EDTA (pH 8.0)

Distilled water

The solution was mixed and diluted to 1 x before use.

6.4. 70 % ethanol

70% (v/v) ethanol (Merck, Ger)

Sterile distilled water

6.5. Blocking buffer

10% (w/v) Fetal Bovine Serum (FBS) in PBS buffer

6.6. Buffer minimal glycerol complex medium (BMGY)

0.67% (w/v) yeast nitrogen base

1% (w/v) yeast extract

2% (w/v) peptone or tryptone

100 mM potassium phosphate (pH 6.0)

0.00004% (w/v) biotin (Gibco BRL, USA)

1% (v/v) glycerol (Promega, USA)

The solution was steriled by autoclave

6.7. **Buffer minimal glycerol complex medium (BMMY)**

0.67% (w/v) yeast nitrogen base

1% (w/v) yeast extract

2% (w/v) peptone or tryptone

100 mM potassium phosphate (pH 6.0)

0.00004% (w/v) biotin (Gibco BRL, USA)

0.5% (v/v) methanol (Gibco BRL, USA)

The solution was steriled by autoclave

6.8. Coating buffer (0.1 M Sodium Carbonate, pH 9.5)

0.7 % (w/v) $NaHCO_3$	7.13	g
0.2 % (w/v)Na_2CO_3	1.59	g

6.9. Dialysis tube preparation

Dialysis tube was boiled in 10 mM $NaHCO_3$ / 1 mM EDTA for 30 min. Dialysis tube was rinsed with distilled water and then stored in 1 mM EDTA at 4 ^{o}C.

10 mM $NaHCO_3$ / 1 mM EDTA

2% (v/v) 0.5 M $NaHCO_3$

0.2%(v/v) 0.5 M EDTA

6.10. Gel loading buffer

80% w/v formamide

50 mM Tris-borate (pH 8.3)

1 mM EDTA (pH 8.0)

0.1% w/v xylene cyanol FF

0.1% w/v bromophenol blue

6.11. LB medium (Luria-Bertani medium), pH 7.5

1% (w/v) Tryptone or peptone (Gibco BRL, USA)

5% (w/v) Yeast extract (Gibco BRL, USA)

0.05% (w/v) NaCl (BDK, UK)

The medium was steriled by autoclave.

6.12. LB agar plate (Luria-Bertani medium)

1% (w/v) Tryptone or peptone (Gibco BRL, USA)

5% (w/v) Yeast extract (Gibco BRL, USA)

0.05% (w/v) NaCl (BDK, UK)

1.5 % Agar (Becton Dickinson, USA)

6.13. Low Salt Extract Buffer (LSEB)

10 mM Tris-HCl

0.14 M NaCl

1 mM Dithiothreitol (DTT)

3 M $MgCl_2$

Reagents were mixed and adjusted pH to 8.0. Then, 5 g of 0.5% w/v was added. Next, 0.3484 g of 2 mM Penylmethane Sulfonylfluoride (PMSF) that soluted by absoluted EtOH was added and preserved with 0.2 g of NaN3. Adjusted to 1 L with distilled H_2O.

6.14. Lysis buffer

6% (v/v) 5 N NaCl

5% (v/v) 1 M Tris, 7.5

0.5% (v/v) Tritonx100

1% (v/v) PMSF

0.1% (v/v) Leupeptine

0.1% (v/v) Aprotinin

6.15. SOC medium

2% (w/v) tryptone

0.5% (w/v) yeast extract

0.05% (w/v) Nacl

20 mM glucose

6.16. 1 M sorbital

18.21 % (w/v) Sorbital

The solution was steriled by autoclaving.

6.17. TB (terrific broth)

TB powder (Sigma, USA) 47.6 g/L

0.8 % (v/v) glycerol

6.18. Transfer buffer, pH 8.3

25 mM Tris-HCl	30.3	g
192 mM glycine	141.4	g
20% (v/v) Metanol	200	ml

6.19. Wash buffer

0.05% (v/v) Tween -20 in PBS buffer

6.20. YEPD medium

2% (w/v) Tryptone or peptone

1% (w/v) Yeast extract

2% (w/v) glucose (sigma, USA)

6.21. YEPD agar

2% (w/v) Tryptone or peptone

1% (w/v) Yeast extract

1.5 % Agar (Becton Dickinson, USA)

The solution was steriled by autoclaving then added with 2% sterile (w/v) glucose (sigma, USA).

6.22. Phosphate buffer saline (PBS)

1.76 mM KH_2PO_4

8 mM Na_2HPO_4. $2H_2O$

137.9 mM NaCl

2.7 mM KCl

6.23. Running buffer, pH 8.3

25 mM Tris-HCl, pH 8.2

192 mM Glycine

0.1% (w/v) SDS

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