PRODUCTION OF HUMAN MONOCLONAL ANTIBODY AGAINST CD3ε OF DOMESTIC DUCK *Anas platyrhynchos* BY ANTIBODY PHAGE DISPLAY LIBRARY

INTRODUCTION

Domestic duck, *Anas platyrhynchos*, is an important agricultural species providing for human nutrition (meat and egg). They also serve as a research model for studies viral hepatitis (Mason *et a*l., 1980). In addition, duck is an important host for zoonosis such psittacosis (Saikku *et al*., 1985; Morison *et al*., 1991; Schlossberg *et al*., 1993), chlamydiosis, (Williams, 1989; Anderson *et al.,* 1997; McElnea and Cross, 1999) and avian influenza (Hindshaw *et al.*, 1980; Kawaoka *et al.,* 1988; Webster *et al.,* 1992; Suzuki and Nei 2002). Especially, avian influenza caused by influenza A subtype H5N1 is a serious zoonosis with high mortality rates in Southeast Asia for example Thailand, Vietnam, Cambodia, and Indonesia (CDC, 2007). Highly pathogenic avian influenza H5N1 (HPAI) is a widely spread and deadly disease among avian species especially chickens. It is believed that domestic ducks can harbour the H5N1 virus without showing any clinical signs in most strain (Webster *et al*., 1978; Hinshaw *et al*., 1980; Sharp *et al*., 1997; Webster, 2002; Hulse-Post *et al*., 2005; Sturm-Ramirez *et al*., 2005). Nevertheless recent reports have confirmed that some of H5N1 virus isolated was highly pathogenic in ducks and wild aquatic birds (Chen H *et al*., 2004; Sturm-Ramirez *et al*., 2004; Zhou *et al*., 2006). Consequently, HPAI (H5N1 and H7N7) has infected and caused death in human being (Claas *et al*., 1998; Subbarao *et al*., 1998; Fouchier *et al*., 2004).

Prevention of duck disease and zoonosis calls for knowledge of duck immunology. Nowadays, all study of the avian immune system for more than 50 years, not much has been learned for the duck immune system because all avian immunology is represented by the chicken models (*Gallus gallus*). In fact, the immune system of the duck could be expected to be different from the chicken

because there are the split between duck (*Anseriformes*) and chicken (*Galliformes*) more than 95 million years ago (Rosser, 1976; Olson and Feduccia, 1980). In addition, there are low levels of sequence homology between chickens and ducks.

There are three factors that involve the pathogenesis of infectious disease which are host, environment, and pathogen. Many host factors play a role in the pathogenesis including host defense mechanism against pathogens. The immune system provides protection and pathogen eradication via immune cells and their cytokine. There are two immune cells that derived from lymphoid progenitor cells, B cell progenitor and T cell progenitor. T cell progenitor transforms to T helper cell expressing cluster of differentiation 4 ($CD4^+$) and cytotoxic T cell expressing cluster of differentiation 8 ($CD8^+$) are important cells, because they provide humoral and cell mediated immune responses via T helper cell. In addition, cytotoxic T cell represents cell mediated immune response. Both T helper cell and cytotoxic T cell usually display cluster of differentiation 3 (CD3). CD3 or T3 is expressed by thymocytes, T cells and NK-T cells. CD3 play an essential role in TCR signal transduction and in cell-surface expression of TCR. CD3 is composed of 4 variable chains which are γ, δ, ε, and ζ.

To studies pathobiology and immunology of duck often require lymphocyte subset determination, enumeration and detection of their anatomical affinity. Techniques such as immunohistochemistry and flow cytometry are often used for such purposes. These techniques require monoclonal antibodies specific to subpopulation of duck lymphocytes marker such as CD3 for pan-lymphocyte, CD4 for helper T cell, and CD8 for cytotoxic T cell. Presently, there are many types of monoclonal antibodies against subpopulation of lymphocyte that are available for chickens but not provide for duck lymphocyte except the rabbit polyclonal antibody against intracytoplasmic chain of CD3 (Jeurissen and Janse 1998; Miller *et al*., 2004). However, there are many disadvantages of this polyclonal antibody *i.e.* non-specific and it cannot be applied in living cells due to this polyclonal antibody specific to the intracytoplasmic portion of T cell. Therefore, we produce human monoclonal antibody in single chain variable fragment (ScFv) form and mouse polyclonal

antibody that against duck CD3 epsilon chain (CD3ε). The CD3ε was selected as a candidate because it is only variable chains of CD3 that has been deposited in the GENEBANK (accession number AF378704, AY738731). Both produced monoclonal and polyclonal antibodies are designed for further use for *in situ* localization including immunohistochemistry and flowcytometry. Furthermore, these antibodies may be used for anatomical study of duck-immunology and pathogenesis.

OBJECTIVES

1. To clone, sequence, and express the CD3ε recombinant protein of domestic duck *Anas platyrhynchos*

2. To produce human monoclonal antibody against *Anas platyrhynchos* CD3ε by antibody phage display library technique

3. To produce mice polyclonal antibody against *Anas platyrhynchos* CD3ε

4. To test specificities of the produced monoclonal and polyclonal antibodies by using ScFv ELISA and Western blot analysis

LITERATURE REVIEW

1. Domestic duck (*Anas platyrhynchos*)

1.1 Classification and genetic background

Domestic duck or mallard is classified in Order Anseriformes, Suborder Anseres, Family *Anatidae*, Subfamily *Anatinae*, tribe *Anatini* (Johnsgard 1978; Madge *et al*., 1988; Sibley and Monroe, 1990) Genus and species *Anas platyrhynchos*.

1.2 Distribution

Duck can be found almost anywhere in the world such as Oceana, Asia, Africa, South America and many islands in Thailand, domestic ducks are raised in four different housings i.e. evaporative cooling house, open-housing, backyard and free-grazing (Songserm *et al*., 2006).

1.3 Morphology

The morphology of domestic duck is similar to wild mallard that is an ancestor of them. In addition, the duck vary in body color, body weight etc. For domestic duck, the notable characteristic is the black plumage on the head, breast, and neck, white neck ring, orange bill and foot (Figure 1).

Figure 1 Domestic ducks, the notable characteristic is the black plumage on the head, breast, and neck, white neck ring, orange bill and foot

Source: Songserm (2006)

1.4 Reproductive system

Ducks become sexually mature at 5 months of age. They are polygynous and show no mate loyalty. Ducks copulate in water and exhibit a ritualized pre-copulatory and post-copulatory display. Ducks show marked change in behaviour during the breeding season, with highly aggressive to each other and to other species. The severe feather picking and bruising along the neck and back are common amongst both sexes. Females are sometime drowned by mobs of rival males during mating (Thomus *et al*., 2000).

2. Avian immune system

Previous reviews of the lymphoid system in avian and its function include those by Payne (1971), Szenberg (1976), and Rose (1979). In evolutionary term, it is generally accepted that the Anseriformes (duck, *Anas platyrhyncos* and geese, *Branta Canadensis*) represent primitive bird that have low development than mammal and Galliformes (chiken, *Gallus gallus domesticus*, turkey, *Meleagris gallopavo*, and pigeon, *Columba livia*). The evolution leads to differ in many systems including immune system. Not only is avian immune system different from mammalian immune system but also is in different avian species (Good & Finstad, 1967). There are differences between Anseriformes and Galliformes immune system. An example of differences is the structure of the serum immunoglobulin (Igs). The mammalian IgG consists of two gamma (γ) heavy (H) chains and two light (L) chains which may be kappa (κ) or lambda (λ). In Galliformes, the IgG is replaced by the major low molecular weight (IgY) that has upsilon (υ) H chains. The Anseriformes IgG is a truncated form of IgY that lacks the Fc region, namely IgY (ΔFc). The structures of Igs lead to different immune response such as functional inefficiencies of duck antibodies (Grey, 1967).

The avian immune system is divided into two types *i.e.*, innate and adaptive immunity. In both innate and adaptive immunity operate via immune cells including T lymphoid and myeloid cells. The response of lymphoid cells is divided into cellular

and humoral immune response. The development of the cells that associates with cellular and humeral immune response involves with four lymphoid organs including thymus, spleen, bursa of Fabricius, and thoracic duct.

First, thymus is a paired lobular organ composing of separate lobes of ovoid lymphoid tissue lying in the neck close to the jugular vein and the vagus nerve. It enlarges during young and regresses in adult. In some birds there is evidence of recrudescence, associated with the breeding cycle (Höhn 1956, Kendall & Ward 1974, Bacchus & Kendall 1975) and possibly connected with an erythropoietic function. The thymus involves the maturation of T lymphocyte including positive and negative selection. The maturation of T lymphocyte starts from thymus progenitor cells that come from the yolk sac and bone marrow. The progenitor cells differentiate into immunological competent T cells that regulate cellular immunity. The differentiation of T cell starts from thymus precursor that derives from stem cell near thoracic aorta (Cooper *et al*., 1991). The thymus precursor without CD3 marker is divided into αβ lineage and γδ lineages. The γδ lineages move to skin, intestinal and pulmonary epithelium. At the thymus cortex, the αβ lineage expresses double positive markers ($CD4^+$ and $CD8^+$) and it performs TCR rearrangement. The thymocyte performs positive selection at a thymic epithelial cells and it expresses self major histocompatibility complex (MHC) molecules. Only T cell with receptors binding to self MHC molecules receives a growth promoting signal by the interaction of its receptors with the self MHC molecules. Less than 10% of thymocytes survive because they cannot bind to the self MHC. The negative selection leads to the depletion of self reactive T cells within the thymus. Bone marrow derived thymic and dendritic cells, as well as another cell type, present self peptides in the context of self MHC to T cell in the thymus. Those cells having a high affinity to these peptides are deleted by a process called programmed cell death, or apoptosis. After the selection of T cell, selected T cells migrate into thymus medulla and express $CD4^+$ and $CD8^+$ in individual manner. The T cell resides in thymic medulla for 1-2 days and migrates into peripheral circulation. For the T cell receptor with CD3 marker (TCR)/CD3, it is gradually expressed on thymocytes. The TCR/CD3 expression starts at cytoplasm of thymocyte. After TCR rearrangement, they express low levels of TCR/CD3 molecules

on its surface. The TCR/CD3 molecules increase its expression to become $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ single positive cells at thymic medulla. For the duck lymphocyte, it exert some of the functional characteristics of T cells as they respond to phytohaemagglutinin (PHA)(Higgins and Teoh, 1988), Which has been described to stimulate T cells in mammals.

The spleen is the secondary lymphoid organ that is a round to oval organ lying dorsal of the proventiculus. The structure of spleen is divided into red pulp and white pulp. The red pulp consists of blood filled sinusoids and scattered lymphoid and non-lymphoid cells. For white pulp, it is consists of packed lymphoid and non-lymphoid cell. The spleen of avian dose not serves as a reservoir for blood because it is small but it can show marked enlargement in response to antigenic stimulation.

The bursa of Fabricius or cloacal bursa is a gut associated primary lymphoid tissue. The structure of bursa of Fabricius is a diverticulum on the dorsal surface of the proctodeum. The inner surface of bursa of Fabricius is thrown into a number of folds which partly obscure in the lumen and regresses in adult birds, which is unique to birds. In most species, it is completely involutes about the time of sexual maturity (Riddle 1928; Wolfe *et al*., 1962). The bursa of Fabricius involves the development of B cell. The progenitor cells of B lymphocyte derive from the yolk sac and differentiate into immunological competent B lymphocytes prior to their migration to the periphery as mature B lymphocytes (Glick *et al*., 1956; Cooper *et al*., 1969). In duck, another organ that associates with the development of B cell and ontogeny of duck immunoglobulin are spleen and cervical lymphoid tissue. The B lymphocyte colonizes at secondary lymphoid tissue (spleen, and marrow) which function as the antigen reactive precursors to antibody producing plasma cells. Birds have two thoracic ducts, but lymphatic tubules are less numerous than in mammals. The tubules follow blood vessels and empty into large veins. There are variable amounts of lymphoid tissues occur in virtually all avian tissues and organ.

2.1 Innate immunity

The innate immunity, first line defense of body, is characterized by non-specificity. The innate immunity composes of four types of defensive barriers including anatomic, physiologic, phagocytic, and inflammatory patterns. The example of anatomic barrier is intact body covering (skin and mucous membranes, and mucus secretion). Many disease organisms cannot penetrate into the body by using anatomic barrier. The mechanism of physiologic barrier is the high body temperature in chicken that precludes many diseases. Normal micro-flora is the one of physiologic barrier that provides defense via microbial population stabilizer. The action phagocytes, bactericidal enzymes and interferon are used by phagocytic and inflammatory systems. Macrophage, thrombocyte, and heterophil are the main cell associated with the non-specific immune system. The cells that associated with non-specific immune system are raised from myeloid progenitor cells namely non-lymphoid system. Heterophils and thrombocytes that provide a non-specific immunological defense to the host so called mononuclear phagocytic system. Monocytes in vascular space and tissue macrophages are unique because of their wide distribution throughout the body fluids, organs, and body cavities. The heterophils are stand for mammalian neutrophil. The heterophils are the predominant leukocyte in inflammatory stimuli. The action of heterophil in microbicidal activity is phygocytosis and extracellular granule releasing. The releasing granule contains cationic peptide and lysozyme. Both intact and lysed heterophil exhibit microbial activity, indicate that these cells can performed this function by either phagocytosis or extracellular granule releasing (Harmon, 1992). The one reaction of all inflammatory response is the action of complement system. The aim of complement system is a microbial protection and initiation of inflammatory reaction via glycoprotein interaction. The complement system composes of activation pathway and lytic pathway. The complement system composes of classical pathway alternative pathway, and lectin pathway. There are three mechanism to activate duck's complement system including antibody-mediated activation of the classical pathway, and antibody-mediated and antibody independent alternative pathway. The classical pathway is activated by antibody-antigen complex. The activation of classical pathway requires Mg^{2+} similar to mammalian complement

system. Alternative pathway and lectin pathway associate with innate immunity that is activated by microorganism. The activation of alternative pathway requires Ca^{2+} and Mg^{2+}. There is report about the deficiency of agglutination and precipitation of duck' serum (Higgins & Warr, 1993). The deficiency in agglutination and precipitation is due to ΔIgY that predominant during the later stage of antibody response. The agglutination and precipitation require Fc component of immunoglobulin but ΔIgY is a Cv3 and Cv4 deficiency immunoglobulin.

2.2 Adaptive immunity

Adaptive immune system is characterized by diversity, specificity, heterogeneity, and memory for self/non-self recognition. This system is divided into cellular and non-cellular (humoral) components. These two types need a processed antigen to stimulate response, and their response is to generate a specific antibody for each particular antigen. The bursa produced cells (B cells) are associated with humoral response while the thymus produced cells (T cells) are associated with cell-mediated response. The Cell-mediated immunity is involved in immune mechanism by which cell infected with a foreign agent, such as virus, is destroyed and accomplished *via* a direct effectors cell and target cell contact (Qureshi *et al*., 1998). Both T and B cells are raised from lymphoid progenitor cell in bone marrow like natural killer cell (NK), and dendritic cell (DC). According to adaptive immune system of ducks, this system composes of thymus which is responsible for T cell development (Cantor, 1984) as well as bursa of Fabricius responsible for B cell development (Cooper et al., 1984). About B cell, B cell produces three types of immunoglobulin (Higgins, 1993) such as immunoglobulin M (IgM), and two types of immunoglobulin Y (IgY). In evolutionary terms, the duck is more closely related to amphibious reptiles and the relationship is reflected in the similarity of the immunoglobulins of these species (Magor *et al*., 1994). Duck (IgM) share similar physical, biochemical, and antigenic properties with other vertebrate. The duck IgM has a molecular weight about 900 kDa and μ-like heavy chain about 86 kDa. The two type of IgY including 7-8S and 5-7S in previously name. Presently name is IgY and IgY (ΔFC). The molecular weight of IgY is about 180 kDa and possesses ν H chain of

about 67 kDa which contain a variable (V) and four constant (C) domains. The molecular weight of IgY (ΔFC) is about 120 kDa with ν (ΔFC) H chain of 37-42 kDa which possess a V and only two C domain. The duck light chain resembles the λ chain of chicken and mammal. The two isoforms of IgY rise from single gene. The different of two isoforms process via pre-mRNA processing. About IgY, it occur early and transiently in immune responses while IgY (ΔFC) occur later than IgY and there are protractedly activity in immune response. IgY response by complement fixation, tissue sensitization for anaphylaxis, vehicle of yolk transmitted, and maternally derived antibodies. The all properties of IgY requiring an Fc region. About IgY (ΔFC) that lack two terminal Fc domain, the effective function is restricted to activities not requiring secondary effecter mechanism such as viral neutralization.

T cells diverse functions have been identified in chickens that are similar to described in mammals (Cooper, 1991). Avian T cells have two surface receptors namely T cell receptor (either TCRαβ or TCRγδ). T cell receptor recognize antigen that bound to major histocompatibility complex (MHC) molecules. In addition, T cell receptor is membrane bound receptor that is opposite from B cell receptor because B cells express immunoglobulin on their surface. The T cell receptor molecule is heterodimer composed of either α and β or γ and δ chains. TCR composed of two disulfide linked glycoprotein chain. Avian TCRγδ receptor has higher proportion than mouse indicated that it is an important part of immune system. However, it has not been to determine an exact function of γδ T cell. The γδ T cell activates via the secretion of cytokines by $CD4^+$ *αβ* T cells (Arstila *et al*., 1993; Kasahara *et al*., 1993). Even though, the *αβ* T cells cannot mediate the graft versus host reaction but are attracted to splenic lesion causing by $CD4^+$ *αβ* T cells (Chen *et al*., 1995). The TCR structure is similar to Ig molecules which are composed of extracellular, transmembrane protein. Both TCR and Ig molecules belongs to the Ig superfamily. The extracellular domain is divided into an antigen binding variable (V) region and membrane proximal constant region (C). The structure of extracellular domain of TCR provides cysteine residue that allow the formation of one disulfide bond each within the C and V region. For transmembrane region of TCR, it contains one or two positively change residue that associated with corresponding negatively changes in

transmembrane region of the CD3. The intracytoplasmic portion of TCR is too short to transmit signal after ligation of the V region there for other molecules complex must perform signal transduction. The associated molecules for T cell signal transduction called CD3 complex. The CD3 complex is composed of TCR and CD3 molecules and presented on the cellular membrane of all mature T cells. The CD3 molecules are composed of four chain including γ, δ, ε, and ζ. The CD3 molecules are the member of Ig superfamily that has disulfide-linked at ectodomain portion. In addition CD3 molecules are composed of transmembrane and intracytoplasmid region. Only CD3ζ has few ectodomian portion residues and a large intracytoplasmic domain. The intracytoplasmic domain of CD3 γ, δ, and ε contain one immuno tyrosine base activation motif (ITAM). For CD3 ζ, the intracytoplasmic domain contains three ITAM. The ITAM of CD3 involved signal transduction via phosphorylation.

The CD3γ and CD3δ of chicken are homologous sequences to the mammal CD3. The structure of CD3ζ is indicated that it is important for signal transduction. The individual function of CD3 chain is currently unknown but all chain of CD3 must be intracellularly assembled before it form TCR complex on cell surface. The missing of mutation of any CD3 is resulted TCR complex malfunction. This requirement suggests that each chain within the complex plays a distinct role. The function of TCR in TCR complex is antigen recognition whereas CD3 chain and CD3ζ couple antigen and TCR to intracellular signaling pathway leading to activation of T cell.

In addition, the other membranous glycoproteins of mature T cell are CD4 and CD8 molecule. The CD4 is expressed on the surface of helper T cell and CD8 is expressed on the surface of cytotoxic T cells.

3. CD3 epsilon chain (CD3ε)

CD3 stand for cluster of differentiation 3 that is a protein complex composed of four invariable polypeptide chains *i.e.* γ, δ, and two times ε The CD3 molecule associates with T cell receptors (TCR) that compose of TCRα, TCRβ, TCRγ, and

TCRδ (Figure 2). The associated molecules of CD3 including ζ chain that plays an important role in the stimulation of signal-transduction pathway involved T cell activation. The signaling events induced upon cross-linking of the Fc receptor which contains ITAM in intracytoplasmic region. The signaling initiated the cascade leading to ITAM phosphorylation. CD3, ζ chain, and TCR comprise the T cell receptor complex (TCR complex). In chicken, the CD3ε is a nonglycosylated protein like CD3ζ that is differed from CD3γ and CD3δ glycoprotein. The CD3ε chain is composed of three regions including extacellular domain, transmembrane portion, and intracellular domain. The extracellular domain of CD3ε is consisted of disulfide bond, which associate with corresponding negative charges in transmembrane region of CD3ε. The negatively charged of CD3ε allow these chains to associate with positively charged of TCR.

The CD3ε is a very useful marker for the T cells identification because it can be detected even on immature thymocytes, natural killer T cell (NK-T cell), and represents one of the earliest signs of commitment to the T cell lineage (Higgins *et al*., 1986). In addition, CD3ε is used as a maker for activated NK cell and T cell neoplasm. Although the monoclonal antibody raising agains CD3ε on mouse, human and chicken T cells have been produced but did not cross react with the surface of duck lymphocytes. There is only the polyclonal rabbit antiserum that used for human lymphocyte identification, shows specific activity to duck lymphocytes. The specific activity of this antiserum resulting from conserved antigen at cytoplasmic domain of the CD3ε molecule (Bertram *et al*., 1996). CD3ε is only one from the four invariable polypeptide chains of CD3 molecules that deposited in a GENEBANK. The accession numbers of duck CD3ε encoded DNA sequences are AY738734, AY738731, and AF378704.

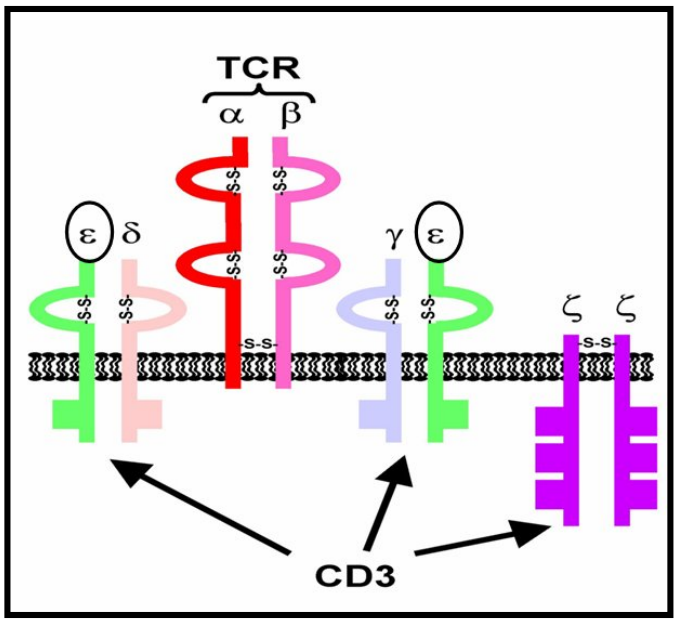


Figure 2 Structure of TCR complex. The TCR complex consists of TCR and CD3. The 4 invariable polypeptide chains of CD3 are gamma (γ), delta (δ), and two times epsilon (ε). The 2 zeta chains (ζ) play an important role in the stimulation of signal-transduction pathway involved T cell activation. The cycle indicated 2 epsilon chains (ε) of CD3.

Source: de Felipe (2004)

4. Protein identification by MALDI-Time of flight (MALDI-TOF)

Protein identification assay is used to assure the protein of interest as a predicted protein. Matrix-assisted laser desorption/ionization Time of flight, MALDI-TOF, is used to identify protein. The concept of MALDI-TOF is providing a protein molecular weight for database blasting. The protein molecular weight is generated Peptide Mass Fingerprints (PMFs) by enzymatic digestion. The protein plug is digested by using digested enzyme, typically with trypsin. The mixture of peptides is measured to produce a characteristic mass profile or PMF. The mass profile is compared with database.

5. The production of antibody

The antibody is a product of B lymphocyte and a part of humeral immune response. There are three ways of antibody response to the one antigen. First is the binding of antibody to the pathological epitope of antigen. Second is the macrophage stimulation to remove antibody coating pathogen. The last is complement activation by using Fc portion of immunoglobulin. The antigen-binding site of antibody is composed of six complementary determining regions (CDRs) or hypervariable region. The three regions reside within the light chain variable domain (V_L) and another regions within the heavy chain variable domain (V_H). The collection of different antibody binding site is created by the combinatorial assembly of germline-encoded segment that composes of more than 10^8 various antibody genes. The lymphocytes express a unique antibody-binding site on their surface. The exposure to antigen selects specific antibody from lymphocyte repertoire. The consequences are the production of antigen-reactive antibodies, and the incorporation of somatic mutation in V genes, allowing subsequent selection of mutation that improves the affinity of the antibody for particular antigen.

5.1 The production of monoclonal antibody

The monoclonal antibody is useful tools in molecular biology such as diagnostic antibody and therapeutic antibody. The way to produce monoclonal antibody is hybridoma technology and selection platform technology. First, the hybridoma technology is used to produce antibody by using tumor cells exploitation. The tumor cells namely myeloma is fused with spleen cells that challenge with relevant antigen. The fusion cells, hybrinoma, multiply rapidly produce a large amount of antibodies. Last, the antibodies can be isolated from recombinant antibody libraries *in vitro*, by using the platform selection. The selection platform is composed of ribosome and mRNA display, microbial cell display, and phage display. The phage display was firstly introduced by G. Smith in 1985. Phage display is currently the most widespread method for proteins expression, including antibodies, protein, or peptides on the surface of filamentous bacteriophages (or simply called phages) of *E. coli, e.g*. M13, fd (Hennie, 2005). The selection process can be adapted to many specific conditions that foil most other display platform, including selection on whole cell, tissue, and even in animal with high affinity and specificity. The essential discoveries for antibody phage display technology are composed of two important things. First is the demonstration of foreign DNA inserted into filamentous phage gene III (g3) that is expressed as a fusion protein. The last is the successful expression of functional antibody fragment in periplasmic space of *E. coli* (Skerra, 1988; Better, 1998). The periplasmic space is located between the two cell membranes of the bacterium. The advantage of antibodies that located in periplasmic space is the correctly folding. By recombinant DNA technology, the large repertoire of antibody, estimated about 10^{11}-10^{12} diversity in human's body, can be fused to g3p and subsequently displayed on the surface of phage particles in the form of a single chain variable fragment (ScFv) which consist of only variable domain of heavy and light chain of antibody. Antigens of interest can be used to select antigen-specific phages from the *in vitro* antibody repertoire (Biopanning). The selected phage can be used to produce ScFv molecule for further analysis (Clackson *et al*., 1991).

There are many advantages of monoclonal antibody from ScFv fragment. Monoclonal antibody from ScFv fragment such as availability of a large number of antibody genes, effective selection of the correct gene from gene pool, and improvement in the affinity and specificity of a selected antibody fragment. In addition its can be saved disadvantage from hybridoma technique such as unstable hybridoma lines or primary fusion cultures, by rescuing the antibody gene, low affinity to low immugenicity substances, Hapten, self-antigen and high toxicity substance by using monoclonal antibody from ScFv fragment.

The gene pool of antibodies can be accessed by using the polymerase chain reaction (PCR). The using primer complement with the termini of the desired antibody gene and can be used to amplify the portion of the inner DNA. The amplicon is not only amplified from individual gene but also entire gene families. The amplicon of heavy and light chain are linked into a single protein by using a peptide of 15-18 amino acid namely peptide linker. The contracted antibodies gene is incorporated into expression vector. The diversity of bacterial antibodies depends on the efficiency of transformation method. The best diversity is about 10^{10} different antibody genes.

6. Antibodies phage display system and Bio-panning

6.1 Filamentous phage

Structure

The bacteriophages or phages in short**,** consist of 11 genes (Figure 3). However, the important structure of phage is capsid and coat protein. The phages express 2700 copies gene 8 protein (g8p or pVIII) that is a major capsid protein. In addition, the phage express 3-5 copies of gene 3 protein (g3p or pIII) that is a one of three minor coat protein on tip of phages. The phages infect a variety of gram-negative bacteria by using pili as a receptor such as F pili of phage strain M13, f1, and fd.

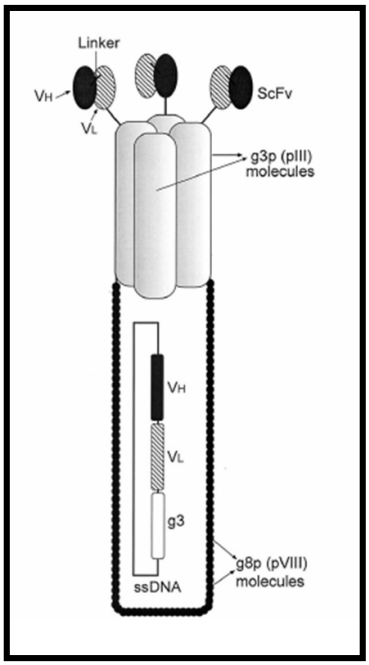


Figure 3 structure of filamentous phage contains ScFv on g3p molecules

Source: Hassan (2002)

Life cycle

The life cycle of phage starts from the attachment of phage g3p to the f pilus of the male *E. coli* (e.g., *E. coli* TG1) without the lytic infection. After the attachment of the circular ssDNA of phage, the ssDNA is converted into double-stranded plasmid like replicative form (RF) by using host DNA replication machinery. The RF undergoes rolling circle replication to make ssDNA and also serves as template for g3p and g8p production. Phage progeny are assembly protein coat and extrude through bacterial membrane. In recombinant phage, the g3p fusion protein displays on the tip of M13 phage.

6.2 Phagemic cloning vector

Phagemid is the most popular vector for display. It is a hybrid of phage and plasmid vector. The one of all DNA in phagemid vector derived from plasmid-like RF of M13. M13 phage contains 2 forms of DNA including single strand DNA template (ssDNA) and double strand DNA (plasmid-like RF). The ssDNA is prepared from phage media and used for sequencing. Double strand DNA is isolated from infected bacteria and used for cloning of target fragment. Phagemid consists of original of replication for both M13 phage and *E. coli*. In addition, it contains multiple cloning site and antibiotic-resistance gene. However, phagemid lacks non-structural and structural gene that required for a generating complete phage therefore it requires all structural protein from helper phage to generate complete phage. The helper phage is phage that contains a slightly defect at origin of replication (such as M13KO7 and VSCM13). The process that added helper phage into phagemid for structural protein adding is called phage rescue. The rations of polypeptide-pIII fusion protein:wild type pIII may range between 1:9 and 1:1000 depending on the type of phagemid, growth condition, the nature of polypeptide fused to pIII, and proteolytic cleavage of antibody-pIII fusions.

6.3 Types of ScFv-phage display library

Non-immune or single pot library

The single pot library is constructed from naïve animals. This library is used to produce monoclonal antibodies against antigen especially low immunogenic antigen. In addition, the library is used to against high toxicity and self antigen that can not perform in conventional technique (Nissim *et al*., 1994). Constructed small-size human single-pot library at $3x10^7$ different antibody clones and used to produce antibodies against self antigen, hapten, and foreign antigen. Moreover, Vaughan *et al*. 1996 constructed a large size library (1.4×10^{10} clones) from over 40 non-immunized human donors and produced antibodies with binding affinities (*Ka*) in the low nanomolar range.

Immune library

The immune library is constructs from immunize animal or human, that is a different point from single pot library. The characteristic of immune library including the enrichment of antigen-specific antibodies, the affinity maturation of some antibodies resulted from immune responses, and class-switching of antibody isotype which is the characteristic of the memory immune response. The example of immune library such as constructed human library from vaccinated donor, the library has been used for surface protein of variola virus monoclonal antibodies selection (Nina *et al*., 2006). In addition, there is the production of immune library from animal such as mice (Andersen *et al*., 1996), chicken (Yamanaka *et al*., 1996), and rabbits (Lang *et al*., 1996).

Synthetic library

The synthetic library is constructed from mutation of complementary determining regions (CDRs) by using mutagenesis or PCR based-strategy. The advantage of this library is most diversity library. There are six CDRs is used to

mutate especially CDR3 of the heavy chain (VH-CDR3) that is the most central to the antigen-binding site of all CDRs and is the most diverse loop in composition and length (Chothia and Lesk, 1987).

Construction of ScFv phage

The ScFv phage library is constructs from B lymphocytes of donor. The variable region of heavy chain (VH) and variable region of light chain (VL)-encoding DNA sequences are amplified from cDNA. The cDNA is reverses transcribed from mRNA of B lymphocytes. Both VH and VL amplicon are linked into a single DNA sequence by using an oligonucleotide linker. The ScFv coding-DNA sequence is inserted into phagemid vector such as pCANTAB5E. The recombinant *scFv*/phagemid vectors are introduced into competent *E. coli*. by using $CaCl_2$ transformation or electroporation. The ligation and transformation step is an influence the size of the library. The antibody phage display library is prepared from *E. coli* transformants by using helper phage (M13VCS or KO7). This process is known as phage rescue, to yield recombinant phages which display scFv antibody fragment as a fusion to one of the phage coat protein. The quality of ScFv phage depend on number of clone that contain scFv insert, number of clone expressing phages carrying scFv, and number of clone expressing soluble ScFv. The quality of ScFv phage can be accessed by a variety method including PCR screening, western blot analysis, and DNA fingerprinting.

6.4 Selection of antigen-specific antibodies from the phage display antibody library: “biopanning”

The biopanning process is used to enrich antigen specific phage clone. The library is incubated with immobilize antigen. After incubation step, the unbound phage is washed with washing buffer. The remaining library that specifically binds the antigen is eluted with elution buffer. The eluate library is introduces into *E. coli* for specific phage amplification. The biopanning should be performed in one time. In

fact, there is the binding limitation of specific phage then several rounds biopanning are necessary. There are four types of biopannig

Immobilize biopanning

The biopanning using immobilize antigen is used to select specific phage library that recognizes immobilize antigen. The interest antigen is trapped by affinity column (Clackson *et al.,* 1991; McCaffterty *et al.,* 1991), plastic surface such as immunotubes (Maxisorb® tubes; Nalge Nunc Intl., Naperville, IL), enzyme link immunosorbant assay (ELISA plate) (Marks *et al.,* 1991; Kang *et al.,* 1991) or BIA® core sensor chips (Malmborg *et al.,* 1996). The important aspect of immobilize biopanning is conformation of immobilize antigen. The conformation of antigen is associated with the phage antibody recognition. The specific phage can be eluted with acidic solution (HCl or glycine buffer) (Kang *et al.,* 1991; Roberts *et al.,* 1992), basic solution (triethylamine) (Marks *et al.,* 1991), enzyme cleavage of protease site constructed between the antibody and g3p (Ward *et al.,* 1996), and competition with excess antigen (Clarkson, 1991).

Antigen in solution biopanning

The advantage of biopanning using antigen in solution is accurate quantification of the antigen used during selection (Hawkins, 1992) and consequently enhance the ability to use lower concentration of the antigen to favor selection of high affinity phage antibodies. The conformational epitope of antigen can be recognized by phage library in this biopanning. The biotinylated antigen and allows binding with library. The specific antibodies are recovered with avidin or streptavidin coated paramagnetic beads. The only disadvantage of this biopanning is that anti-streptavidin antibodies will also be isolated. However, this problem can be solved by a depletion step using streptavidin-coated bead.

Cell biopanning

The phage library selection on cell surface marker is performed on monolayer of adherent cell or cell suspension. Unbound phage can be washed by rinsing tissue culture flask (monolayer) or centrifugation (cell suspension). To optimize specificity of antibodies, a simultaneous positive and negative selection may be applied (de Kruif, 1995). In this approach, the antigen-negative cells "absorber" is used to exclude other surface marker-specific phage in competitive manner. The target cells can be isolated from the absorber by labeling specific antibodies and subsequently sorting by FACS.

In vivo biopanning

This technique is using direct injection of phage repertoires into animal. After injection, tissue are collected and examined for phage bound to tissue specific endothelial cell markers as was demonstrated for peptide phage. Pasqualini and Ruoslahti was a first to isolate phage-display peptide that home to selective vascular beds *in vivo* (Pasqualini and Ruoslahti, 1996). This technique is many advantages such as specificity increase of phage library to intact target, elimination of phage library that recognize ubiquitous plasma and cell surface protein in an inherent blocking step, these peptide may be useful for functional analysis of new receptor and potential identification of novel drug target candidate.

6.5 Production of soluble ScFv molecules

Antigen-specific phage is introduced into specific strain of *E. coli* such as HB2151 *E. coli*. The transformant *E. coli* will produce soluble ScFv. The HB2151 *E. coli* namely non-suppressor recognize amber stop codon that result ScFv expression without g3p protein. The expressing ScFv include E Tag that permitting rapid and simple protein identification and purification by using Anti-E Tag antibody and Anti-E Tag antibody column. The character of expressing protein is depends on isolated clone. The location of expressing protein is culture supernatant in soluble form,

periplasm, and inside bacterial cells by using Anti-E Tag antibody. After localization, the ScFv is purified by using general protein purification including ion-exchange chromatography, gel filtration chromatography, and affinity chromatography (Anti-E Tag column).

7. Western blot

Western blot or immunoblot is a method to detect a specific protein in a given sample of tissue homogenate or extract. The interest protein is separated by gel electrophoresis or isoelectric-focusing. The protein locates in small pore size of the gel that limits penetration by macro-molecular probes. To resolve, the protein is blotted onto an adsorbent porous membrane (usually nitrocellulose or diazotized paper) which gives a mirror image of the gel. The membrane containing protein is used to detect and analyze protein properties by using specific antibody.

MATERIALS AND METHODS

1. Animal

Two khaki Campbell ducks (*Anas platyrhynchos*) were used for all experiment. All ducks had bodyweight approximately 1.6-2.0 kg and 3-4 month-old. Birds were obtained from collaborated slaughterhouse in Nakhon-Pathom province, Thailand.

Institute cancer research (ICR) outbreed mice were used for polyclonal antibody production. Bodyweight of six weeks-old Mice used in this study was approximately 40 g. Animals were obtained from National Laboratory Animal Center, Mahidol University, Thailand.

2. Separation of duck peripheral blood mononuclear cells (PBMCs) from venous blood

Eight milliliters of whole blood was collected from the jugular vein of ducks and added into BD vacutainer™ CPT™ cell preparation tube (REF 362761 BD, Franklin Lakes, NJ) for PBMCs isolation. After mixing with anticoagulant (sodium citrate) by inverting, the blood was centrifuged at 1,500-1,800 x *g*, 25ºC using a horizontal rotor for 20 minutes. After centrifugation, mononuclear cell and platelets was resided in a whitish layer just under the plasma layer that resided in a top layer. Plasma layer was gently removed without disturbing the cell layer. Cell layer was collected by automatic pipette and transferred into a 15-ml size conical centrifuge tube with cap. Five milliliter of 1x phosphate bufferred saline (PBS) pH 7.4 was used to wash PBMCs by gently vortexing. The tube was spun at 500-1,800 x *g*, 25ºC using a fix rotor for 20 minutes and the supernatant was discarded. The cell pellet containing PBMCs was counted by viable cell count method which viable cells were stained with 0.02% tryphan blue dye. The cell pellet was diluted with 1 x PBS pH 7.4. The diluted cell suspension was mixed with equal volume of 0.02% tryphan blue and

filled into sample introduction point of hemocytometer (REICHERT Buffalo, NY.) at the junction of cover glass and counting chamber until the chamber is completely charged. Similarly, the tryphan blue-stained cell suspension was filled at the opposite chamber of the hemocytometer. The cells were allowed to settle for 3 minutes and counted under the low-power magnification and reduce light, focus on the ruled area and observed for even distribution of cell. Count the white cell in the four 1 sq mm corner area. The counting number of cell was calculated by following formula;

$$\text{PBMCs per cu mm} = \frac{\text{average number of chamber WBC x dilution factor}}{\text{Volume of chamber}}$$

3. Extraction of total RNA and generation of cDNA

The 5.7 x 10^7 cells of PBMCs was mixed with 1 ml of TRIZOL® Reagent (Invitrogen, CA, USA). The 0.2 ml of chloroform was added in the mixture and tubes were shaken vigorously by hand for 15 seconds. The mixture was incubated at 25ºC for 3 minutes and spun at 10,000 x *g* at 4ºC for 15 minutes. After centrifugation, the upper aqueous phase containing RNA was collected for RNA precipitation protocol. The aqueous phase was transferred to fresh 1.5-ml plastic tube. The 0.5 ml of isopropyl alcohol was added to the tube and mixed by gently inverting. The mixture was incubated at 25ºC for 10 minutes and spun at 10,000 x *g* at 4ºC for 15 minutes. After centrifugation, supernatant was removed and RNA pellet was washed one time with 1 ml of 75% ethanol by The mixture was mixed by vortexing and spun at 7,500 x *g* at 4ºC for 15 minutes. After discard supernatant, pellet containing RNA was used in cDNA generation protocol. RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Canada) was used for cDNA synthesis. The procedures of cDNA synthesis for PCR amplification was initiated by preparing mixture. The mixture containing 5 µg of total RNA, 1 µl of oligo $(dT)_{18}$ primer (0.5µg/µl) and (diethylpyrocarbonate) DEPC–treated water was added to the mixture to bring final volume to 12 µl. The mixture was incubated at 70ºC for 5 minutes and then mixture was chilled on ice and the drops were collected by brief centrifugation. The tube was placed on ice the mixture containing 4 µl of 5x reaction buffer, 1 µl of Ribolock™

Ribonuclease inhibitor (20u/ µl), and 2 µl of 10 mM dNTP mix was added into the tube. The tube was mixed gently and centrifuge briefly. The tube was incubated at 37ºC for 5 minutes and 1 µl of RevertAid™ H Minus M-MuLV RT (200u/µl) was added into the tube. The tube was incubated at 42ºC for 60 minutes and the reaction was stop by heating at 70ºC for 10 minutes. After stop reaction, cDNA preparation was chilled on ice for further operation.

4. Protein extraction from PBMC

The 5.7 x 10^7 cells of PBMCs were mixed with 1 ml of distilled water. The PBMC was sonicated by using ultrasonic-homogenizer LABSONIC® P (Satorius AG, Germany). The amplitude and the cycle of sonication are 30% and 0.5 cycles respectively. The sonication step is performed for 10 minutes on ice-bath. After sonication, whole cell lysate was centrifuged at 12,000 x *g* for 10 minutes at 4ºC. The supernatant was collected as soluble part. The pellet was collected as insoluble part and solubilized by boiling with 10% sodium dodecyl sulfate (SDS). Both soluble and insoluble parts were subjected to polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

5. Primers design and polymerase chain reaction (PCR)

Anas platyrhynchos T cell antigen CD3 epsilon chain DNA sequences was searched from GenBank (accession number AY738731). Specific primers were designed based on their DNA sequences that split transmembrane region out. In addition, the primers were designed for restriction enzyme (*Hin*dIII and *Kpn*I) of pTrcHis2A expression vector and adding extranucleotide for restriction enzyme cleavage efficiency. cDNA preparation from *Anas platyrhynchos* PBMCs was used as a template in PCR. The PCR mixture containing 1 µl of cDNA template, 18 µl of UDW, 2.5 µl of PCR buffer (10 x *pfu* buffer with $MgCl_2$), 2 µl of dNTPs mix, 0.5 µl of 3'PCR primer, 0.5 µl of 5'PCR primer, and 0.2 µl of *pfu* DNA polymerase enzyme. The reactions were gently mixed and spun down. The mixture was performed PCR under the following condition. The condition is 94ºC for 5 minutes, 30 cycles of 94ºC

for 30 seconds, 55ºC for 1 minute, and 72ºC for 1 minute 30 seconds then 72ºC for 10 minutes and hold at 4ºC. The PCR product was analyzed by agarose gel electrophoresis.

6. Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyze PCR product. The 1% agarose gel was prepared by adding 0.3 g of agarose powder into 30cc of 1x TAE buffer. The mixture was completely dissolved by microwave oven heating. It was heated until the mixture became clear and homogeneous. The mixture was allowed to cool to approximately 50-60ºC before poured it into the gel tray. The gel was allowed to harden for 45 minutes. PCR product was mixed with 10x sample buffer (0.25% bromophenolblue, 0.25% xylenecyanole FF, and 50% glycerol) at 1x final concentration. The mixture was filled to the gel and electrophoresis was performed at constant 100V until the bromophenol blue dye front had arrived about 2/3 of the gel length. The gel was incubated with ethidium bromide buffer (0.5µg /ml ethidium bromide in 1x TAE buffer) for 15 minutes. After incubation, the gel was removed excess ethidium bromide by incubate with 1 x TAE for 10 minutes. The nucleic acid pattern on the gel was directly visualized under ultraviolet (UV) light.

7. DNA purification from agarose gel

Expected band of PCR product, representing putative *Anas platyrhynchos* CD3ε sequences were excised and purified by using a gel extraction kit (Qiagen, Germany). The gel slice was transferred into fresh 1.5-ml tube. The gel was determined weight in microgram. The three volume of NaI was added into the tube containing one volume of gel slice (the 100 mg of gel~100 µl). The mixture was incubated at 55ºC until agarose gel has been dissolve. The appropriate amount of GLASSMILK® was added into the tube according to the protocol and incubated at 25ºC for 5 minutes. The solution was mixed every 1-2 minutes. The solution was centrifuged at 14,000 x *g* at 25ºC for 5 seconds. After discarded supernatant, the 500 µl of NEW Wash was added into the tube and resuspended the pellet. The tube was

centrifuged at 14,000 x *g* at 25ºC for 5 seconds and discarded supernatant. After washing step two times, the pellet was allowed to dry for 10 minutes. The volume of water equal to the amount of GLASSMILK® was added into the pellet and carefully resuspended by gently pipetting up and down. After pellet resuspension, the solution was centrifuged at 14,000 x *g* at 25ºC for 5 seconds. The supernatant was collected as eluted DNA and transferred to fresh 600 µl tube. The purified DNA fragments were inserted into pGEM T-easy vector (Promega, USA) for DNA sequencing and kept it as stock.

8. Ligation of *Anas platyrhynchos* CD3 epsilon chain to pGEM T-easy vector

The purified DNA fragment of CD3ε were added into the mixture of 5 µl of 2x rapid ligation buffer of T4 DNA ligase, 1 µl of pGEM T-easy vector (50ng), 4 µl of PCR product, and 1 µl of T4 DNA ligase (3 Weiss unit/ µl). The mixture was mixed by pipetting and the reaction was incubated at 4ºC for 18 hours. The ligation mixture was used for DNA sequencing and kept it as stock of *Anas platyrhynchos* CD3ε.

9. Transformation of *E. coli* JM 109 strain

The pGEM T-easy vector containing CD3ε vector was introduced into *E. coli* JM 109 strain for kept it as stock. The *E. coli* transformation was performed by chemical transformation and heating shock. The competent cell was prepared by *E. coli* JM 109 bacterial cells, 100 ml of LB broth were inoculated with a single fresh colony of bacterial cells in a 1liter Erlenmeyer flask and incubated in a shaking incubator at 37ºC, 250 rpm until the OD_{600} reached 0.6. The bacterial cells were harvested by centrifugation at 6000 x *g* at 4ºc for 8 minutes. The pellet was resuspended in 0.1 M $MgCl_2$ and centrifuged at 6,000 x g at 4ºc for 8 minutes. The supernatant was discarded and the pellet was resuspended in 0.1 M $CaCl_2$ and incubated on ice for 20 minutes. The bacterial suspension was centrifuged again and the pellet resuspended in 4.3 ml of 0.1 M $CaCl_2$ before mixing with 0.7 ml of glycerol. The competent cells were pipetted in 100 µl aliquots into microcentrifuge tubes, snapped frozen in liquid nitrogen for 5 minutes and kept at -70ºC. The

competent cell preparation was added to ligation mixture. The tube was incubated on ice for 20 minutes. After incubation, the tube was heat shocked at 42ºC for 2 minutes in a waterbath and then incubated on ice for 10 minutes. The LB broth 1 ml was added into cell suspension and the cell was incubated in a shaking incubator at 37ºC, 250 rpm for 30 minutes. The cell suspension was briefly spun and spread on ampicillin LB agar plate and incubated at 37ºC for 18 hours. The single colony was picked and inoculated on 5 ml of LB ampicillin broth. The broth was incubated in a shaking incubator at 37ºC, 250 rpm for 18 hours.

10. Plasmid extraction of *E. coli* JM 109 strain

The plasmid extraction protocol was performed as previously described by Birnboim and Doly in 1979. The LB broth containing pGEM T-easy vector was briefly spun. The pellet was resuspended in 200 µl of solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA) and the solution was transfer into 1.5 ml tube. The 400 µl of solution II (0.1 N NaOH, 1% SDS) was added into the tube and mixed by inverting. The tube was incubated on ice for 5 minutes and 300 µl of solution III (3 M potassium acetate, pH 5.2) was added into the tube. The tube was incubated on ice for 5 minutes and the tube was spun at 12,000 x *g* for 5 minutes at 25ºC. The supernatant was transferred to 1.5 ml tube and 0.6 volume of isopropanol was added to the supernatant. The mixture was mixed by inverting and the mixture was spun at 12,000 x *g* for 5 minutes at 4ºC. The supernatant was discarded and 70% ethanol was added into the tube and the mixture was spun at 12,000 x *g* for 5minutes at 4ºC. Pellet was dried and the pellet was resuspended in 50 µl of ultrapure distilled water (UDW). The plasmid preparation was used for DNA sequencing and enzymatic digestion.

11. Enzymatic digestion of *CD3ε*/pGEM T-easy vector

The *CD3ε* gene was prepared from the recombinant *CD3ε*/pGEM T-easy vector by double digestion with *Hin*dIII and *Kpn*I restriction endonuclease to provide two sticky ends of DNA fragment for ligating into pTrcHis 2A expression vector (Invitrogen, USA). The reaction mixture containing 5 µg of *CD3ε*/pGEM T-easy

vector, five units of *Hin*dIII and *Kpn*I, and 1x Tango buffer (3.3 mM of Tris-acetate pH 7.9, 0.1 M magnesium acetate, 6.6 mM potassium acetate, 0.01mg/ml BSA). The reaction was incubated at 37ºC for 3 hours in water-bath. The digested *CD3ε* fragment was analyzed by using 1% agarose gel electrophoresis and ethidium bromide staining. The digested *CD3ε* fragment was purified from agarose gel by using GENECLEAN® II KIT (Q.BIO gene, USA).

12. Recombinant *CD3ε*/pTrcHis2A vector construction

The pTrcHis2A expression vector was prepared by using double enzymatic digestion with *Hin*dIII and *Kpn*I restriction endonuclease as described in enzymatic digestion of *CD3ε*/pGEM T-easy vector. The digested vector was purified from agarose gel by using GENECLEAN® II KIT (Q.BIO gene, USA). The purified *CD3ε* fragment was ligated into the purified pTrcHis2A vector by using T4 DNA ligase at 16ºC for 18 hours.

13. Screening of Anas *platyrhynchos* CD3ε–expressing DH5α *E. coli* clones

The recombinant *CD3ε*/pTrcHis2A vector was introduced into DH5α *E. coli*. The competent DH5α *E. coli* cell was prepared by using $MgCl_2/CaCl_2$ method as describe in transformation of JM 109 *E. coli*. The single colony of DH5α *E. coli* on LB-A (100 μg/ml of ampicillin) plate was randomly picked and inoculated into 10 ml of LB-A (100 μg/ml of ampicillin) broth at 37ºC for 18 hours. The 500 μl of 18 hours culture was inoculated into fresh LB-A broth and incubated at 37ºC, 250 rpm in shaking incubator until optical density at 600 nm equal to 0.5. The Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added into the broth to final concentration at 1 mM. The broth was incubated at 37ºC, 250 rpm in shaking incubator for 5 hours. The *E. coli* cell was collected by centrifugation at 4,000 x *g* for 10 minutes at 4ºC. The whole cell lysate was prepared by sonication. The bacterial cell pellet was sonicted by using ultrasonic-homogenizer LABSONIC® P (Satorius AG, Germany). The amplitude and the cycle of sonication are 30% and 0.5 cycles respectively. The sonication step is

performed for 10 minutes on ice-bath. After sonication, whole cell lysate was centrifuged at 12,000 x *g* for 10 minutes at 4ºC. The supernatant was collected as soluble part. The pellet was collected as insoluble part and solubilized by boiling with 10% SDS. Both soluble and insoluble parts were subjected to SDS-PAGE, Western blot analysis, and affinity column purification.

14. Measurement of protein concentration

The protein concentration was measured by using Bradford's method with protein assay kit (Bio-Rad, USA). The bovine serum was used as a standard protein at different concentration (0.05, 0.1, 0.25, 0.5, and 1 mg/ml). Ten microliter of standard protein and sample protein were mixed with 200 µl Bradford reagent and gently mixed. The reaction samples were incubated at 25ºC for 5 minutes and the absorbance was measured at 595 nm. A standard curve was generated for the OD values of the standard proteins and the sample protein concentration was calculated from the recorded OD value against the standard curve.

15. SDS-polyacrylamide gel electrophoresis and Western blot

The SDS-polyacrylamide gel electrophoresis was used Laemmli buffer system. A 0.75 mm-thick polyacrylamide gel was prepared on 10x10.5 cm glass plates. A 12% separating gel solution containing 0.65 ml of acrylamide stock solution (46.5% w/v acrylamide and 3% w/v N, N-methylene bis-acrylamide) was mixed with 1.25 ml of 1.5 M Tris-HCl/SDS buffer, pH 8.8, 1.52 ml of deionized distilled water (DDW), 0.05 ml of 10% SDS, 25 µl of 10 % ammonium persulfate and 2.5 µl of N, N, N', N'-tetramethylenediamine (TEMED). The mixture was filled into setting glass plates. Thereafter, the top of the gel was overlaid with the water and left it to polymerize for 1 hour. Following complete polymerization, the water was removed by rinsing. The 4% stacking gel containing 2 ml of acrylamide stock solution (46.5% w/v acrylamide and 3% w/v N, N-methylene bis-acrylamide), 1.25 ml of 0.5 M Tris-HCl/SDS buffer, pH 8.8, 3.05 ml of DDW, 0.05 ml of 10% SDS, 25 µl of 10 % ammonium persulfate and 5 µl of TEMED was poured on top of the resolving gel. The gel was allowed to

polymerize for 45 minutes before the comb was removed. Protein sample was prepared under reducing and non-reducing condition. Protein samples were mixed at a ration of 1:2 with tris-tricine sample buffer (0.1M Tris base, 24% v/v glycerol, 2% w/v SDS, 2% v/v β-mercaptoethanol). For non-reducing condition, the β-mercaptoethanol was not added into sample buffer. The mixture was boiled before loaded into 1 μg/well. Prestained SDS-PAGE standards Broad Range (Bio-RAD CA, USA) was run in parallel with the protein samples as a size reference. Electrophoresis was performed by using electrophoresis buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS, pH 8.25) and The Mini-PROTEAN® 3 cell electrophoresis system (Bio-RAD, CA, USA) at constant current 20 mA per plate. Electrophoresis was stopped when the tracking dye reached the bottom of the gel. The gel was stained with Coomassie blue dye (0.0025% Coomassie® Blue R-250, 40% methanol, and 7% acetic acid) for 18 hours. The exess stain was removed by using high methanol destaining solution (40% v/v methanol, 7% v/v acetic acid) and low methanol destaining solution (5% v/v methanol, 7% v/v acetic acid) until the background was clear. Gel was maintained in 1% glycerol. The soluble and insoluble proteins on SDS-PAGE gel were transferred onto Hybond™-ECL™ Nitrocellulose membrane (Amersham Biosciences, UK) (NC membrane). The Western blot was preformed by using Mini Trans-Blot® Electrophoretic Transfer Cell (BIO-RAD, USA). The protein transfer was performed at constant 100 V for 90 minutes. The transferred proteins were stained with Ponceau S solution (Sigma, USA) by briefly soaking the blotting membrane in the solution ad washing with distilled water until the background became clear. After Ponceau S staining, the empty sites on the NC membrane were blocked by soaking in a blocking buffer [3 % BSA in 0.01 M phosphate buffer saline (PBS), pH 7.4] at 25°C with gentle agitating for 1 hour. The nitrocellulose blot was then washed 3 times for at least 15 minutes with washing buffer [phosphate buffer saline, pH 7.4 containing 0.05% Tween-20 (PBST)] to remove the excess BSA. After washing, the NC membrane was reacted with 1:3,000 diluted HRP-conjugated Nickel probe with gentle agitating at 25°C for 1 hour. After washing, the color reaction was equilibrated in 1/15 M PB, pH 7.6 and the color was developed by 2, 6-dichlorophenolindophenol. The reaction was stopped by washing the membrane with DW and the membrane was allowed to air-dry.

16. Protein purification by using affinity column

The Probond™ purification system (Invitrogen, USA) is used for purification of polyhistidine-containing recombinant proteins including the protein that produced from pTrcHis2A expression vector. The Probond™ purification system provides Ni^{2+}, a electron acceptor, to bind with histidine that is a electron donor. The histidine binding protein is eluted by using replacement imidazole. The CD3ε recombinant protein was purified under native condition. The 2 ml of resin was washed by adding 6 ml of distilled water (DW) and resuspened the resin by inverting. The resin was allowed to settle by using gravity and aspirated supernatant. The six ml of native binding buffer was added into the resin and the resin was allowed to settle by using gravity. Ten milligrams of recombinant protein was added into the resin and the resin was allowed to binding for 30 minutes by using platform shaker. The resin-bounded protein was allowed to settle and the supernatant was collected as unbound fraction. The resin-bounded protein was washed with 8 ml of washing buffer. The resin-bounded protein was allowed to settle and the supernatant was collected as washed fraction. The resin-bounded protein was eluted by using 3 ml of elution buffer containing different concentration of imidazole (50, 100, 150, 200, 250 mM). The purified CD3ε protein was further analyzed by Western blotting

17. Protein identification by MALDI-TOF

The twelve micrograms of 200 mM imidazole-eluate protein was size-separated on 12% SDS-PAGE. Gel plug containing expected band of recombinant CD3ε was subjected to protein identification by using MALDI-TOF.

18. Selection of antigen-specific antibodies from library: "biopanning"

The purified CD3ε recombinant protein was used as antigen to select the human antibody in the form of ScFv molecule from phage display library which was contacted from collaborators (Molecular Immunology Unit, Graduate Program in

Biomedical Science, Faculty of Allied Health Sciences, Thammasat University). In bio-panning process, the 1 µg of recombinant protein dissolved coating buffer (Carbonate-bicarbonate buffer, pH 9.6) was immobilized on an ELISA plate (E.I.A/R.I.A. 8 Well strip Flat bottom Corning, NY USA). The plate was incubated in moist chamber at 37ºC for 2 hours. The mixture was discarded and the 300 µl of 5% skim milk was added into the well and incubated for 30 minutes. The skim milk was discarded and phosphate buffer saline containing 0.1% of Tween 20 (PBS-T) was added into the well and incubated for 5 minutes as a washing step. After PBS-T was discarded, this step was repeated for 3 times. The library was diluted with PBS-T to final concentration of library at 25% v/v. The 200 µl of diluted phage was added into the well and incubated for 1 hour. While incubation period, the mixture was mixed by pipitting every 15 minutes. After discarded the mixture, the washing step was performed. The fifty microlite of 0.1 M HCl-glycine pH 2.2 was added into the well and incubated at 25ºC for 5 minutes. The solution was taken out into 1.5 ml tube containing 3 µl of 2 M Tris-base. The mixture was mixed and incubated with log-phase of HB2151 *E. coli* 200 µl. The mixture was incubated at 37ºC for 20 minutes. After incubation, the bacterial cell was spread into 2XYT-AG plate and incubated at 37ºC for 18 hours.

19. Screening of ScFv-expressing HB2151 *E. coli* clone

The bacterial colonies were used as a template for PCR reaction. The PCR mixture containing 8.4 µl of UDW, 1.25 µl of PCR buffer (10 x *Taq* DNA polymerase buffer with KCl), 0.75 µl of $MgCl_2$, 1 µl of dNTPs mix (2.5 mM for each), 0.5 µl of 3' forward primer, 0.5 µl of 5' reverse primer, and 0.1 µl of *Taq* DNA polymerase (10 U/µl). The mixture was performed PCR under the following condition. The condition is 94ºC for 5 minutes, 30 cycles of 94ºC for 1 minute, 50ºC for 1 minute, and 72ºC for 90 seconds then 72ºC for 10 minutes and hold at 4ºC. The PCR product was analyzed by agarose gel electrophoresis.

20. Production of ScFv against *Anas platyrhynchos* CD3ε

The *scFv*-HB2151 *E. coli* colony from 2XYT-AG plate was picked and inoculated into 2XYT-AG broth at 37ºC for 18 hours. The 5 ml of overnight culture was inoculated into fresh 2XYT-AG broth and incubated at 37ºC, 250 rpm in shaking incubator for 1 hour. The broth was centrifuged at 4000 x *g* for 10 minutes. The cell pellet was resuspended in 2XYT-AI. The broth was incubated at 37ºC, 250 rpm in shaking incubator for 5 hours. The *E. coli* cell was collected by centrifugation at 4,000 x *g* for 10 minutes at 4ºC. The whole cell lysate was prepared by sonication. The bacterial cell pellet was sonicated by using ultrasonic-homogenizer LABSONIC® P (Satorius AG, Germany). The amplitude and the cycle of sonication are 30% and 0.5 cycles respectively. The sonication step is performed for 10 minutes on ice-bath. After sonication, whole cell lysate was centrifuged at 12,000 x *g* for 10 minutes at 4ºC. The supernatant was collected as soluble part. The pellet was collected as insoluble part and solubilized by boiling with 10% SDS. Both soluble and insoluble parts were subjected to SDS-polyacrylamide gel electrophoresis, Western blot analysis.

21. ScFv ELISA

The one microgram of purified CD3ε was coated on an ELISA plate (E.I.A/R.I.A. 8 Well strip Flat bottom Corning, NY USA) as previously described in bio-panning step. After coating step, the excess antigen was discarded and the 300 µl of blocking buffer (3% skim milk in PBS pH 7.4) was added into the well and incubated at 25ºC for 30 minutes. After taking out blocking buffer, the well was washed three times with washing buffer (0.05% of Tween 20 in PBS pH 7.4 [PBS-T]). After washing step, the 100 µl of ScFv solution containing 50 µl of whole cell lysate and 50 µl of PBS-T was added and incubated at 25ºC for 1 hour. After incubation, the well was washed for three times with PBS-T. The CD3ε-bound ScFv was detected by using horseradish peroxidase (HRP)-conjugated anti-E tag diluted in PBS-T at dilution 1:5,000. After incubation step at 25ºC for 1 hour, the well was washed three times with PBS-T. Thereafter washing step, 50 µl of ABTS (2, 2 azino-

bis[3-ethylbenzthiazoline-6-suulphonic acid]) solution (ZYMED CA, USA) was added into the well and incubated at 25ºC for 30 minutes with light protection. The ELISA signal was measured at OD 405 nm by using ELISA reader (Multiskan® EX, Labsystem).

22. Western blot analysis and Immunostaining using ScFv

The highest ELISA signal ScFv was used as an antibody for immunostaining with CD3ε recombinant protein. The NC membrane containing CD3ε recombinant protein was incubated with blocking buffer (3% skim milk in PBS pH 7.4) for 30 minutes. The membrane was washed three times with washing buffer (0.05% of Tween 20 in PBS pH 7.4 [PBS-T]). After washing step, the membrane was incubated with 1 ml of ScFv solution containing 500 µl of whole cell lysate and 500 µl of PBS-T was added and incubated at 25ºC for 1 hour. The membrane was washed three times with washing buffer and incubated with horseradish peroxidase (HRP)-conjugated anti-E tag diluted in PBS-T at dilution 1:400. After incubation step at 25ºC for 1 hour, the membrane was washed three times with PBS-T. The membrane was incubated with 1/15 PB at 25ºC for 10 minutes. The membrane was incubated with mixture of 2, 6-dichlorophenolindophenol (DCIP) and hydrogen peroxide for 5 minutes until color was observed. The excess color was removed by soaking with DW and the membrane was allowed to air-dry.

23. Production of polyclonal antibody

One microgram of CD3ε was electrophoresed on SDS-polyacrylamide gel as previously described. The gel was cut at expected size (23 kDa) and placed into fresh 1.5 ml tube. The gel was ground by using grinder and PBS pH 7.4 was added into the gel to final volume at 500 µl. Gel solution was injected into intraperitoneal area of two mice at days 0, 14, and 28. At pre-immunization and post-immunization period, the blood was collected via orbital sinus. The blood was incubated at 37ºC for 2 hours. The serum was collected used as polyclonal antibody. The polyclonal antibody

was used as an antibody against recombinant CD3ε and native CD3ε in ELISA and Western blot analysis.

RESULTS

1. PBMC isolation, total RNA extraction, and cDNA synthesis

The 5.7 x 10^7 cells/ml of PBMC was isolated from 8 ml of venous blood. The 5.7 x 10^7 cells/ml of PBMC was mixed with 1 ml of TRIZOL® Reagent (Invitrogen, CA, USA) and performed RNA extraction as previously described. The extracted RNA was analyzed by using 1% agarose gel electrophoresis. The gel was stained with ethidium bromide (Figure 4**).** The presence of 5s rRNA, 18s rRNA, and 28s rRNA bands indicates integrity of total RNA preparation. cDNA was synthesized by using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, MD, USA). The synthesis cDNA was used as template for amplification CD3ε gene in PCR.

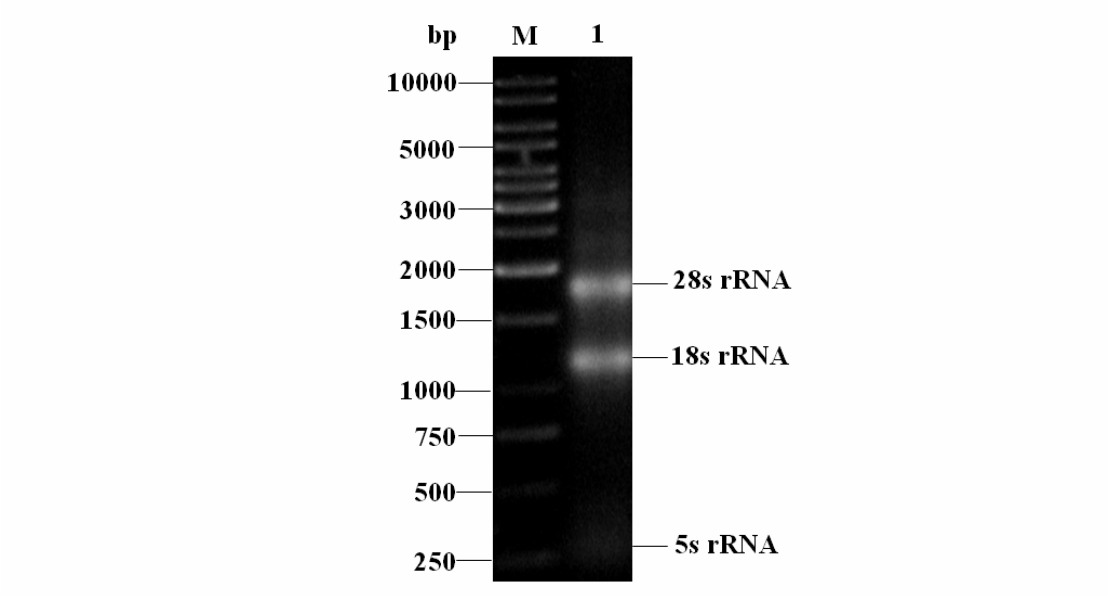


Figure 4 Agarose gel electrophoresis of total RNA extracted from duck PBMC. Lane M, GeneRuler 1 kb DNA ladder. Lane 1, total RNA extracted from duck PBMC was analyzed on 1% agarose gel electophoresis. The bands of 5S rRNA, 18S rRNA, and 28S rRNA are shown (line). The 5S rRNA and 28S rRNA are the part of larger subunit ribosome. For 18S rRNA, it is the small subunit of ribosome. The present of 5S, 18S and 28S rRNA bands indicate the integrity of total RNA extraction.

2. Primer design and polymerase chain reaction (PCR)

The forward and reverse primers were designed to carry recognition sites for *Hin*dIII and *Kpn*I respectively. The nucleotide sequences of primers are shown in figure 5. The amplicon was successfully amplified by PCR with designed primer and was size-separated in 1% agarose gel. The size of amplicon when compared with GeneRuler 100 bp DNA ladder (Figure 6) was approximately 327 bp. The amplicon was purified from agarose gel by using a gel extraction kit (Qiagen, Germany) and cloned into pGEM T-easy vector for DNA sequencing.

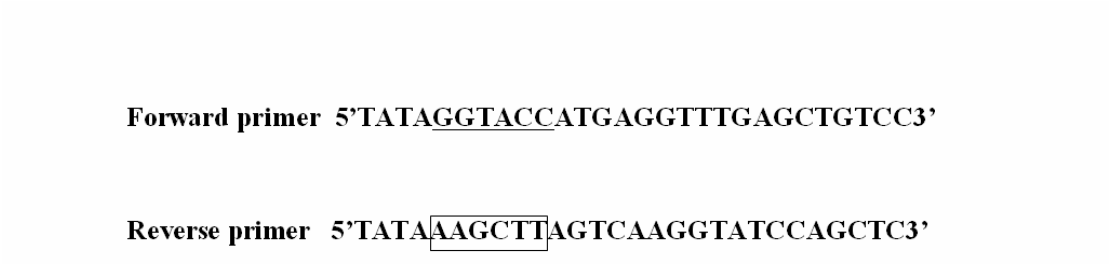


Figure 5 The nucleotide sequences of forward and reverse primers. Line is marked for the recognition site of *Hin*d III restriction enzyme. The recognition site of *Kpn* I endonuclease is boxed.

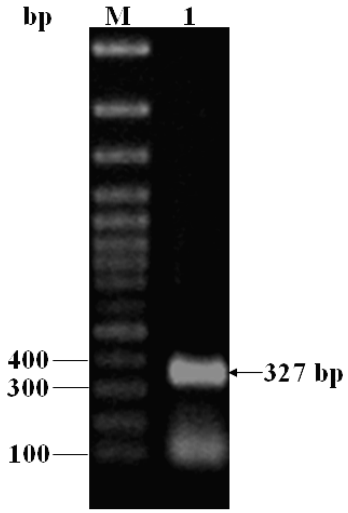


Figure 6 Agarose gel electrophoresis of PCR product. Lane M, GeneRuler 100 bp DNA ladder. Lane 1 shows the PCR amplicon. The amplicon was analyzed on 1% agarose gel. The expected size of PCR amplicon was about 327 bp as indicated by arrow.

3. Ligation of *Anas platyrhynchos* CD3ε gene to pGEM T-easy vector

The amplicon was purified by using a gel extraction kit (Qiagen, Germany). The purified DNA was inserted into pGEM T-easy vector. The ligated pGEM T-easy vector was introduced into chemical competent JM 109 *E. coli*. The pGEM T-easy containing CD3ε gene was analyzed by digesting with *EcoR*I endonuclease. The digested product was size-separated in 1% agarose gel and their size was determined by comparison with a 1 kb DNA ladder (Figure 7)**.** The expected size of DNA insert was approximately 327 bp

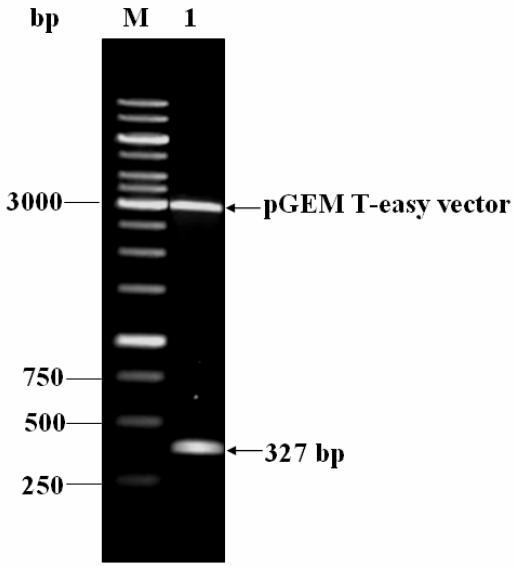


Figure 7 Agarose gel electrophoresis of DNA plasmid. Plasmid extracted from JM109 *E. coli* transformants, was cut by using *EcoR* I restriction endonuclease. The DNA fragment at size~3,000 bp is the linear pGEM T-easy vector. The DNA insert at size 327 bp is expected to be CD3ε-coding DNA sequences.

4. Sequencing results and deduced amino acid sequence analysis

The nucleotide sequence of insert at size 327 bp in pGEM T-easy was analyzed by using M13 forward sequencing primer in DNA sequencing technique. The chromatogram of nucleotide sequence of DNA insert is shown in figure 8 and the profile of DNA insert is displayed in figure 9 by using DNAMAN software. The nucleotide sequence of DNA insert was subjected to homology search with GENEBANK by using BLAST search (Figure 10.) and multiple DNA sequence alignment by using CLUSTAL W (1.74) software (Figure 11). The amino acids were deduced from nucleotide sequences by using DNAMAN software (Figure 12). The deduced amino acid was subjected to homology search and multiple amino acid sequence alignment by using DNAMAN software and CLUSTALW (1.74) software respectively (Figure 13).

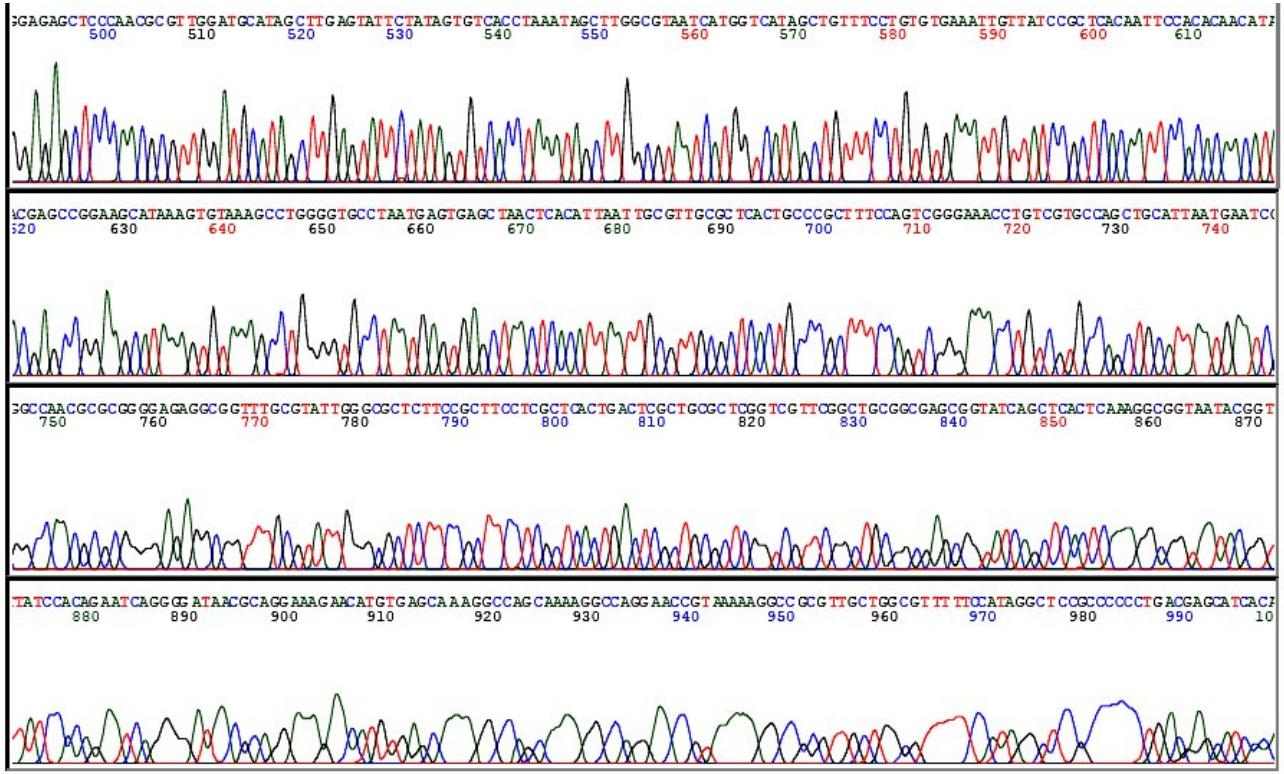
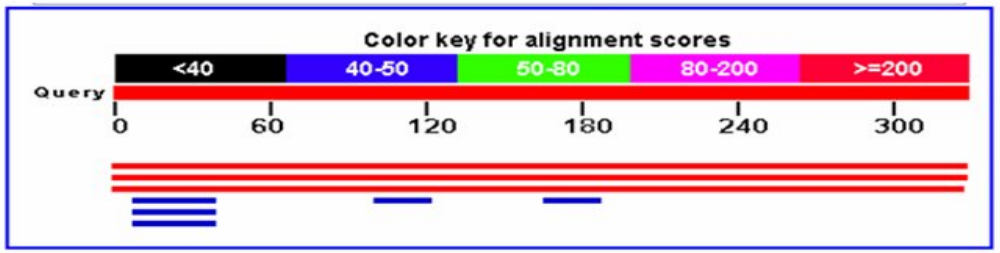


Figure 8 The chromatogram of DNA insert from pGEM T-easy. The nucleotide sequence was analyzed by using M13 forward sequencing primer in DNA sequencing technique

```
SEQ  DNAMAN2: 327 bp;
Composition 87 A; 80 C; 80 G; 80 T; 0 OTHER
Percentage:  26.6% A; 24.5% C; 24.5% G; 24.5% T; 0.0%OTHER

Molecular Weight (kDa): ssDNA: 100.99    dsDNA: 201.6
ORIGIN
1       ATGAGGTTTG AGCTGTCCTT GCCCTTCCTG GGACTCCTGC TGTGTGTGGG TGGCACCGCG
61      GCTCAGGACG TTGCTGATGA AGAACTGACG GCTTCCCCTG AAGAACTCCA GGTGGAAATT
121     TCTGGAACCA CGGTGAAAGT CAGATGTTCC TTGTCTGAGA CAGTACAATG GAAACCATAC
181     AGTCCAGACA CTGAAGACAC TACCTTCATT AAAACGAATC ATGACAGTTC TCCTCTCAAT
241     TTGACTTGTA CAGCGGATAA TAAGAACATC CACATGTACC TGAAGGCCAG AGTGTGCACC
301     AACTGCATGG AGCTGGATAC CTTGACT
```

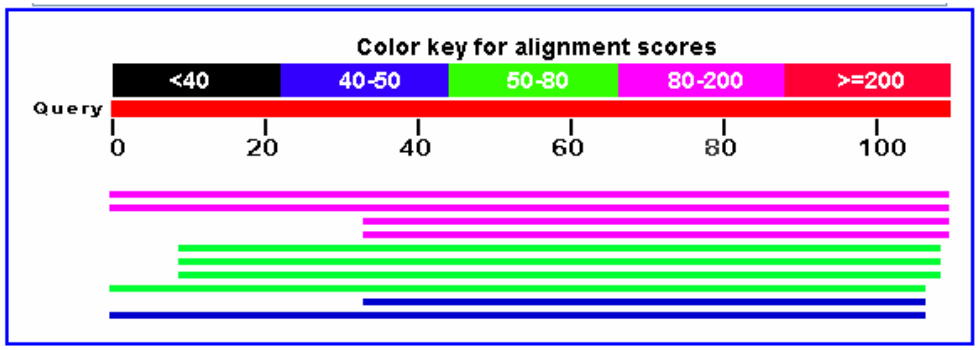
Figure 9 Nucleotide sequence of DNA insert. The gene containing DNA 327 bp and this sequence is shown by using DNAMAN software.



Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF378704.1	Anas platyrhynchos T-cell receptor CD3 epsilon chain mRNA, complet	590	590	100%	8e-166	100%
AY738731.1	Anas platyrhynchos T cell antigen CD3 epsilon chain mRNA, complete	590	590	100%	8e-166	100%
AY738734.1	Cairina moschata clone 3 T cell antigen CD3 epsilon chain mRNA, cor	432	432	99%	5e-118	88%
AC188631.13	Canis familiaris, clone XX-237B24, complete sequence	41.0	41.0	9%	2.9	87%
AC188530.7	Canis Familiaris chromosome 24, clone XX-189D22, complete sequen	41.0	41.0	9%	2.9	87%
XM_542975.2	PREDICTED: Canis familiaris similar to centrosomal protein 2 (LOC48	41.0	41.0	9%	2.9	87%
AL158159.14	Human DNA sequence from clone RP11-498N2 on chromosome 9 Con	41.0	41.0	6%	2.9	100%
AL831712.12	Mouse DNA sequence from clone RP23-118A20 on chromosome 4 Cor	41.0	41.0	6%	2.9	100%

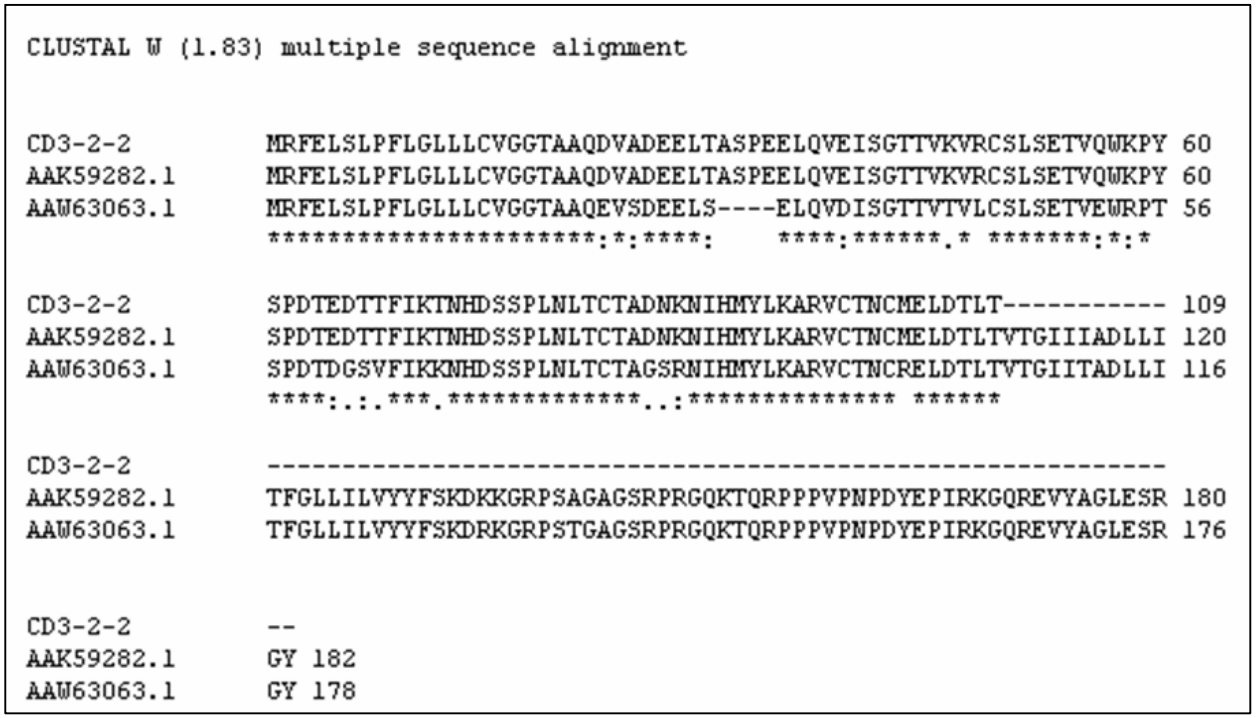
Figure 10 A BLAST search across multiple DNA databases by using BLASTN software showed that CD3ε gene of JM109 *E. coli* clone CD 3-2-2 was homologous to CD3ε gene of *Anas platyrhynchos* and *Cairina moschata* (muscovy duck) at 100% identity (AF 378704 and AY738731) and at 88% identity (AY738734), respectively.

Figure 11 Multiple sequence alignment of CD3ε encoding DNA sequences from JM 109 *E. coli* clone CD3-2-2. AF378304 (*Anas platyrhychos* CD3ε complete code), AY738731 (*Anas platyrhychos* CD3ε non-complete code) and AY738734 (*Cairina moschata* CD3e complete code). The alignment was generated by CLUSTAL W (1.74) software. A dash (-) indicates a missing residue, and a star (*) indicates a residue conserved in all aligned sequences.



Sequences producing significant alignments:	Score (Bits)	E Value
gb|AAK59282.1|AF378704_1 T-cell receptor CD3 epsilon chain [A...	198	8e-50
gb|AAW63063.1| T cell antigen CD3 epsilon chain [Cairina moschat	152	7e-36
ref|NP_996787.1| CD3E antigen, epsilon polypeptide (TiT3 comp...	82.0	1e-14
sp|Q98910|CD3E_CHICK T-cell surface glycoprotein CD3 epsilon ...	81.3	2e-14
sp|Q95LI5|CD3E_MACFA T-cell surface glycoprotein CD3 epsilon ...	53.9	3e-06
ref|XP_001097204.1| PREDICTED: CD3e molecule, epsilon (CD3-TC...	53.5	3e-06
dbj|BAB71850.1| CD3 epsilon FN18- [Macaca fascicularis]	52.4	7e-06
ref|XP_236196.2| PREDICTED: similar to T-cell surface glycopr...	52.4	8e-06
gb|EDL95346.1| CD3 antigen, epsilon polypeptide (predicted) [...	48.9	8e-05
ref|NP_031674.1| CD3 antigen, epsilon polypeptide [Mus muscul...	47.4	2e-04

Figure 12 A BLAST search across multiple amino acid databases by using BLASTP software showed that CD3ε protein of DH5α *E.coli* clone CD 3-2-2 was homologous to CD3ε protein of *Anas platyrhychos* at 100% identity (AAK 59282.1).



```
CLUSTAL W (1.83) multiple sequence alignment


CD3-2-2         MRFELSLPFLGLLLCVGGTAAQDVADEELTASPEELQVEISGTTVKVRCSLSETVQWKPY 60
AAK59282.1      MRFELSLPFLGLLLCVGGTAAQDVADEELTASPEELQVEISGTTVKVRCSLSETVQWKPY 60
AAW63063.1      MRFELSLPFLGLLLCVGGTAAQEVSDEELS----ELQVDISGTTVTVLCSLSETVEWRPT 56
                **********************:*:****:    ****:******.* *******:*:*

CD3-2-2         SPDTEDTTFIKTNHDSSPLNLTCTADNKNIHMYLKARVCTNCMELDTLT----------- 109
AAK59282.1      SPDTEDTTFIKTNHDSSPLNLTCTADNKNIHMYLKARVCTNCMELDTLTVTGIIIADLLI 120
AAW63063.1      SPDTDGSVFIKKNHDSSPLNLTCTAGSRNIHMYLKARVCTNCRELDTLTVTGIITADLLI 116
                ****:.:.***.*************..:************** ******

CD3-2-2         ------------------------------------------------------------
AAK59282.1      TFGLLILVYYFSKDKKGRPSAGAGSRPRGQKTQRPPPVPNPDYEPIRKGQREVYAGLESR 180
AAW63063.1      TFGLLILVYYFSKDRKGRPSTGAGSRPRGQKTQRPPPVPNPDYEPIRKGQREVYAGLESR 176


CD3-2-2         --
AAK59282.1      GY 182
AAW63063.1      GY 178
```

Figure 13 Multiple sequence alignment of CD3ε protein from DH5α *E. coli* clone CD3-2-2. AAK59282.1 (*Anas platyrhychos* CD3ε), and AAW63063.1 (*Cairina moscata* CD3ε). The alignment was generated by CLUSTALW (1.83) software. A dash (-) indicates a missing residue, a colon (:) indicates conserved residues belonging to a CLUTAL 'strong group' and a star (*) indicates a residue conserved in all aligned sequences.

5. Recombinant *CD3ε*/pTrcHis2A vector construction

The pGEM T-easy vector containing CD3ε gene and pTrcHis2A were digested with *Kpn* I and *Hind* III endonucleases. The CD3ε gene was purified from agarose gel by using a gel extraction kit (Qiagen, Germany) and ligated into *Kpn* I and *Hind* III-cut pTrcHis2A expression vector. The CD3ε gene-ligated pTrcHis2A was introduced into DH5α *E. coli* for protein expression. The plasmid of DH5α *E. coli* transformants were prepared by alkaline lysis method and analyzed by double digestion with *Kpn* I and *Hind* III endonuclease. The digested plasmids were size-separated in1% agarose gel (Figure 14).

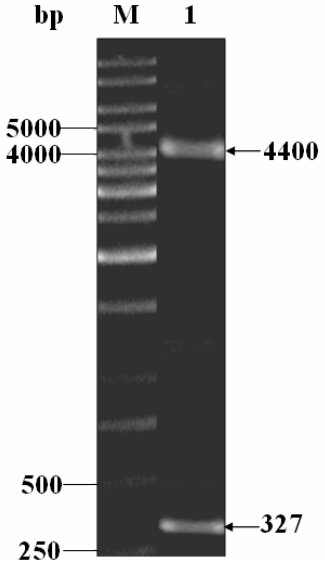


Figure 14 The plasmid pattern of pTrcHis2A containing CD3ε digested with *Kpn* I and *Hind* III endonucleases. Lane M, GeneRuler 1 kb DNA ladder. Lane 1, the DNA fragment after digested with *Kpn* I and *Hind* III endonuclease. The DNA fragment at 327 and 4,400 bp are CD3ε and pTrcHis2A expression vector respectively.

6. Screening of Anas *platyrhynchos* CD3ε–expressing DH5α *E. coli* clones

The DH5α *E. coli* colonies expressing *CD3ε* were screened by 10 ml scale protein expression. After induction with IPTG for 5 hours, the whole cell lysate was prepared and subjected to SDS-PAGE (Figure 15) and Western blot analysis by using HRP-conjugated Nickel probe (Figure 16).

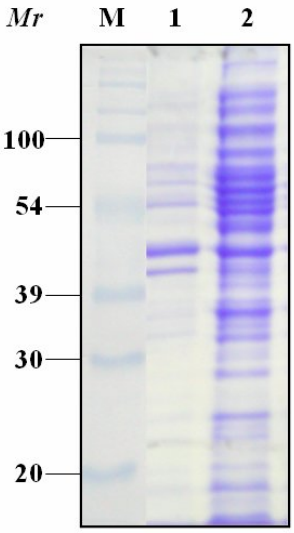


Figure 15 SDS-PAGE-patterns of CD3ε recombinant protein. Ten micrograms of the recombinant protein was separated in a 12% polyacrylamide gel under reducing condition. Lane M, the Prestained Broad Range standard. Lane 1, the insoluble part of CD3ε recombinant protein. Lane 2, the soluble part of CD3ε recombinant protein. Numbers at the left of the figure are relative molecular masses (*Mr*).

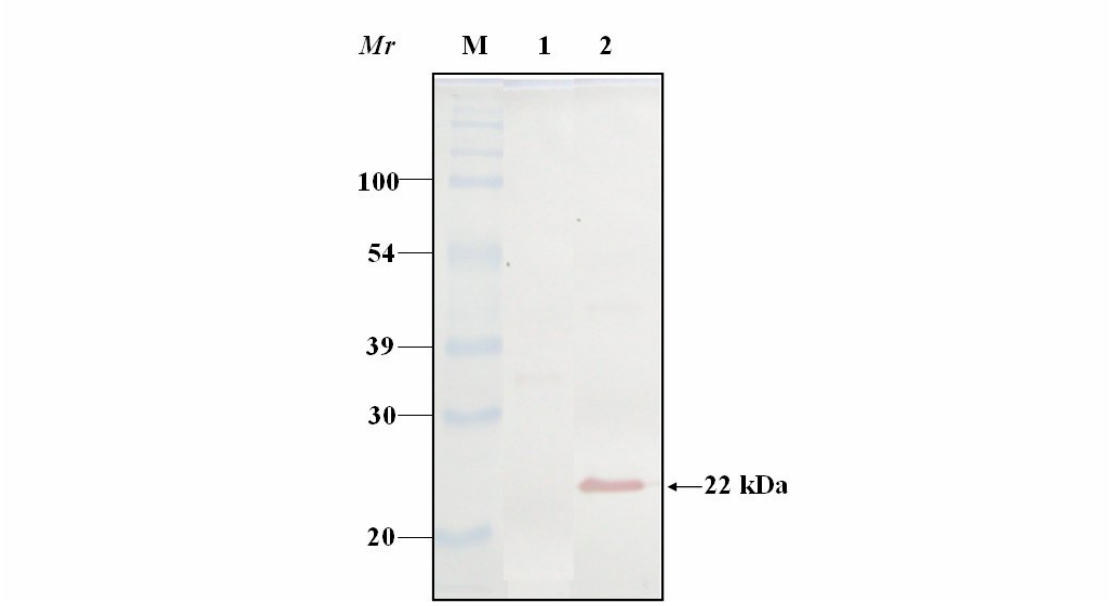


Figure 16 The Western blot-pattern of CD3ε recombinant protein. The NC membrane was incubated with HRP-conjugated nickel probe. Lane M, the Prestained Broad Range standard. Lane 1, the insoluble part of CD3ε recombinant protein. Lane 2, the soluble part of CD3ε recombinant protein. The arrow indicates expected size of CD3ε recombinant protein. Numbers at the left of the figure are relative molecular masses (*Mr*).

7. Protein purification by using affinity column

The soluble part of CD3ε recombinant protein was purified by using affinity column (ProBond™ Nickel-chelating resin, Invitrogen, USA) under native condition without urea. The protein was allowed to bind with resin and eluted with different concentration of immidazole (50, 100, 150, 200, 250 mM). The eluate was analyzed by SDS-PAGE (Figure 17) and Western blot analysis by using HRP-conjugated Nickel probe (Figure 18).

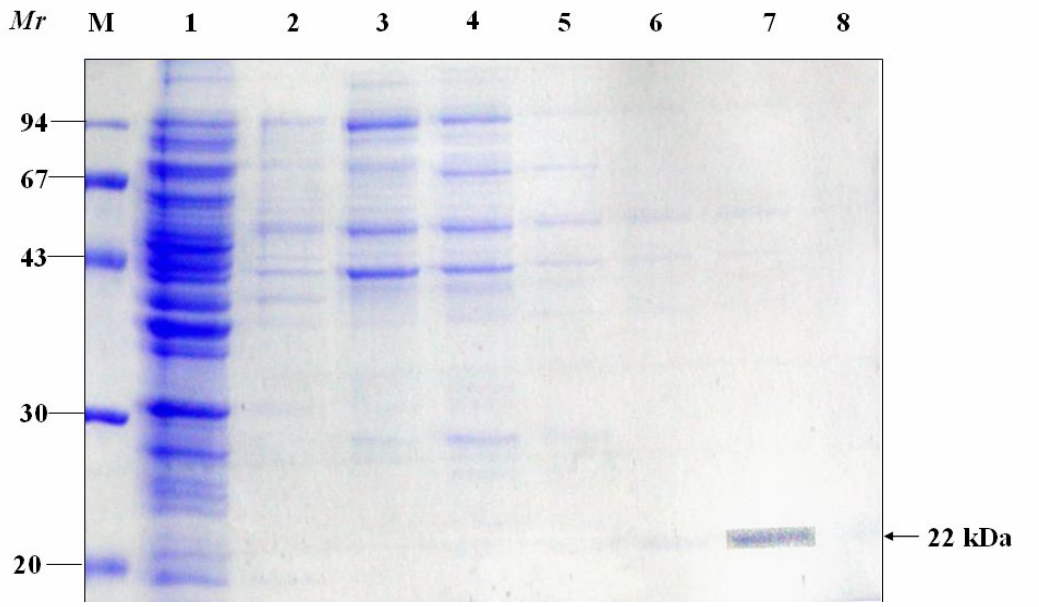


Figure 17 The SDS-PAGE-patterns of CD3ε recombinant protein purification by using affinity column. Twelve micrograms of protein fraction was separated in a 12% polyacrylamide gel under reducing condition. Lane M, the Prestained Broad Range standard. Lane 1, the soluble part of CD3ε recombinant protein. Lane 2, the unbound protein fraction. Lane 3, the unbound protein fraction after washing with 1x native purification buffer containing 20 mM imidazole. Lanes 4-8, the protein fractions were eluted by elution buffer containing 50, 100, 150, 200, and 250 mM imidazole respectively. The arrow indicates the expected size of CD3ε recombinant protein. Numbers at the left of figure are relative molecular masses (*Mr*).

Figure 18 The Western blot analysis of CD3ε recombinant protein incubated with HRP-conjugated nickel probe. Twelve micrograms of protein fraction was separated in a 12% polyacrylamide gel under reducing condition and transfer onto NC membrane. Lane M, the Prestained Broad Range standard. Lane 1, the soluble part of CD3ε recombinant protein. Lane 2, the unbound protein fraction. Lane 3, the unbound protein fraction after washing with 1x native purification buffer containing 20 mM imidazole. Lanes 4-8, the protein fractions were eluted by elution buffer containing 50, 100, 150, 200, and 250 mM imidazole respectively. The arrow indicates the expected size of CD3ε recombinant protein. Numbers at the left of the figure are relative molecular masses (*Mr*).

8. Protein identification by MALDI-TOF

The gel plug of CD3ε recombinant protein at 22 kDa (Figure 17, Lane 7) was subjected to MALDI-TOF. The amino acid sequence was aligned with those of the proteins deposited in the databank. The analysis of CD3e recombinant protein showed that the recombinant protein was homologus to the *Anas platyrhynchos* T-cell receptor CD3ε protein. The recombinant protein had molecular weight at 20114.9 daltons and isoelectric point at 5.67 (Figure 19). The amino acid sequences of analyzed recombinant protein indicated homologous sequences and transmembrane region (Figure 20).

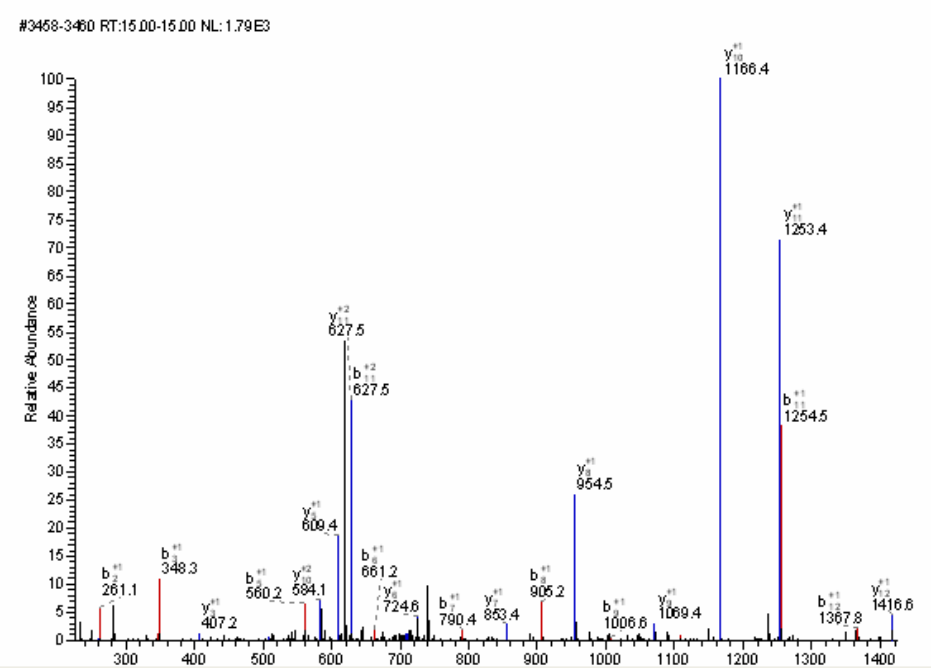


Figure 19 Mass spectra of protein at 22 kDa obtain from MALDI-TOF analysis. The total ion chromatogram (TIC) was collected over a range of *m/z* 300-1400

T-cell receptor CD3 epsilon chain [Anas platyrhynchos]
MW 20114.9 PI 5.67
MRFELSLPFLGLLLCVGGTAAQDVADEELTASPEELQVEISGTTVKVRCSLSETVQWK
PYSPDTEDTTFIKTNHDSSPLNLTCTADNKNIHMYLKARVCTNCMELDTLTVTGIIIA
DLLITFGLLILVYYFSKDKKGRPSAGAGSRPRGQKTQRPPPVPNPDYEPIRKGQREVY
AGLESRGY

Figure 20 The amino acid sequences of CD3ε recombinant protein from MALDI-TOF analysis. The homologous sequences in boxed and line indicate transmembrane region.

9. Selection of antigen-specific antibodies from library: "biopanning"

One microgram of 200 mM imidazol-eluated protein was coated on an ELISA plate (E.I.A/R.I.A. 8 Well strip Flat bottom Corning, NY USA) as previously described. The coated protein was successfully detected by using HRP-conjugated nickel probe. After detection with HRP-conjugated nickel probe, the 5.9 x10^8 cfu of phage library was added into the well and performed bio-panning process as previously described.

10. Screening of HB2151 *E. coli* clone carrying *scFv* gene

The 15 colonies of HB2151 *E. coli* from above were subjected to analyze the *scFv* gene by PCR using primer R1 and R2. The PCR amplicons of *scFv* gene were shown (Figure 21). All fifteen colonies gave DNA band at size 1,000 bp as a positive clone indicating that these *E. coli* carried *scFv* gene. Then, fifteen positive colonies were used to perform ScFv expression**.**

11. Screening of ScFv-expressing HB2151 *E. coli* clone

The HB2151 *E. coli* colonies carrying *scFv* genes were screened by 10-ml scale protein expression. After induction with IPTG for 5 hours, the whole cell lysate was prepared and subjected to SDS-PAGE and Western blot analysis by using HRP-conjugated anti-E tag diluted in PBS-T (Figure 22).

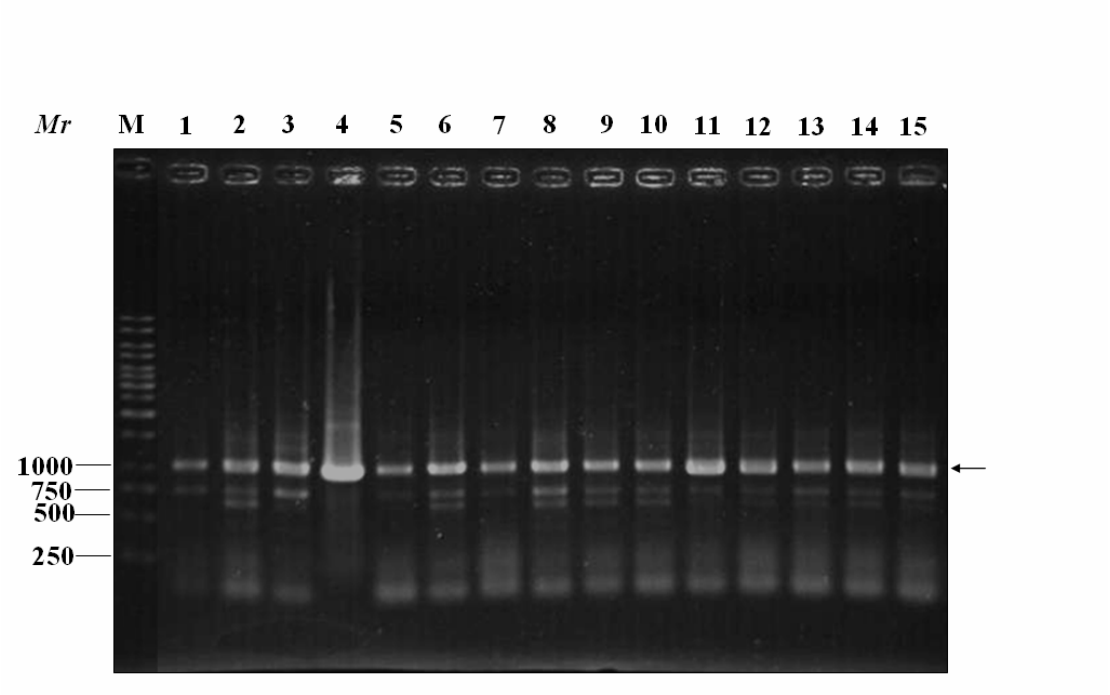


Figure 21 Agarose gel electrophoresis of PCR products. Lane M, GeneRuler 1 kb DNA ladder. Lane 1-15 shows the PCR amplicon. The amplicon was analyzed on 1% agarose gel. The expected size of PCR amplicon was about 1,000 bp as indicated by arrow.

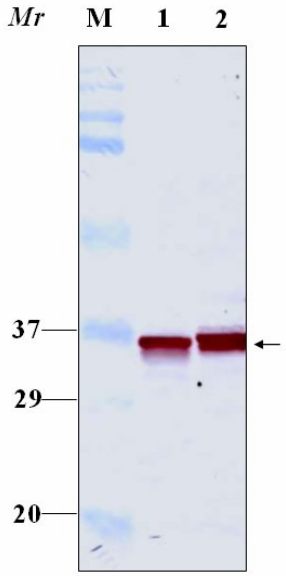


Figure 22 The Western blot analysis of ScFV recombinant protein incubated with HRP-conjugated anti-E tag. The soluble part of ScFv protein was separated in a 12% polyacrylamide gel under reducing condition and transfer onto NC membrane. Lane M, the Prestained Broad Range standard. Lane 1, soluble ScFv protein from HB2151 *E. coli* clone number 4-2. Lane 2, soluble ScFv protein from HB2151 *E. coli* clone number 4-9. The arrow indicates the expected size of ScFv recombinant protein. Numbers at the left of the figure are relative molecular masses (*Mr*).

12. Determine specificity of ScFv antibodies against CD3ε recombinant protein by using ELISA and Western blot analysis

The ScFv antibodies were used in the ELISA to detect specificity of ScFv antibodies against CD3ε recombinant protein. The one microgram of BSA was coated as a baseline control. It was found that clone number 4-2 and 4-9 gave positive ScFv ELISA results (Figure 23). The Western blot analysis using absorbed CD3ε recombinant protein has shown that the ScFv antibodies from HB 2151 *E. coli* clone number 4-2 and 4-9 reacted specifically with CD3ε recombinant protein (Figure 24).

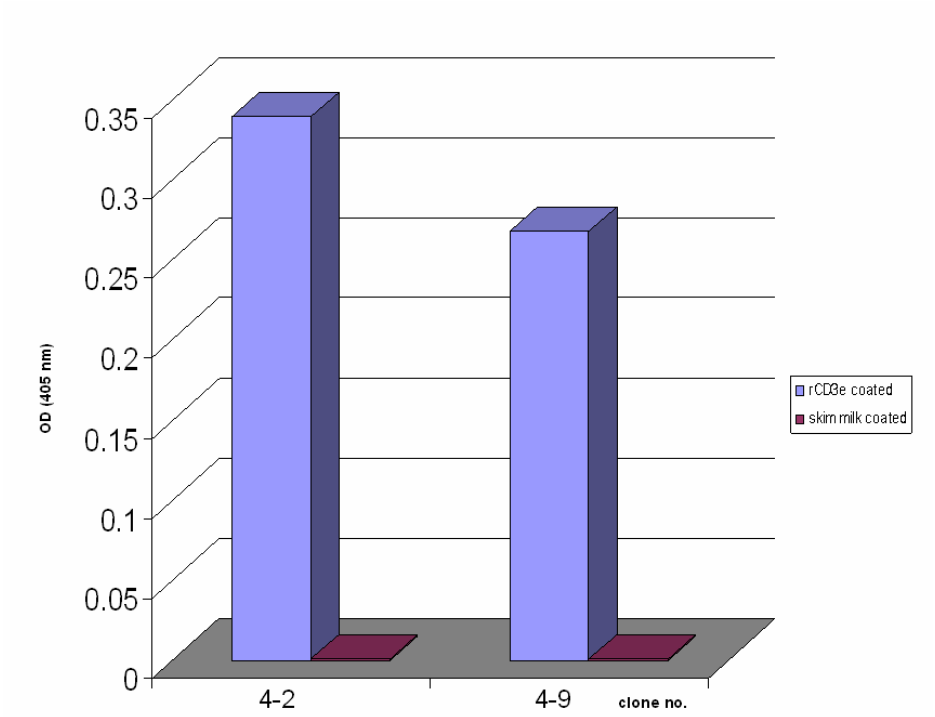


Figure 23 ScFv ELISA results of ScFv antibodies from HB2151 *E. coli* clones against the soluble part of CD3ε recombinant protein. The skim milk was used as negative control to determine non-specific binding of ScFv antibodies.

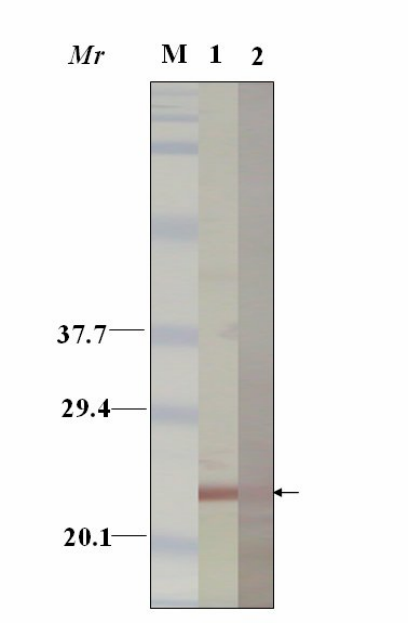


Figure 24 The Western blot analysis of ScFv antibodies incubated with the soluble part of CD3ε recombinant protein. The soluble part of CD3ε recombinant protein was separated in a 12% polyacrylamide gel under reducing condition and transfer onto NC membrane. Lane M, the Prestained Broad Range standard. Lane 1, soluble part of CD3ε recombinant protein was probed with the soluble part of ScFv recombinant protein from HB2151 *E. coli* clone number 4-2. Lane 2, soluble part of CD3ε recombinant protein was probed with the soluble part of ScFv recombinant protein from HB2151 *E. coli* clone number 4-9. The arrow indicates the reactive band of ScFv recombinant proteins against soluble part of CD3ε recombinant protein

13. **Determine specificity of ScFv and polyclonal antibodies against T lymphocyte from lysate duck PBMC by using Western blot analysis.**

The duck PBMC was sonicated and divided into soluble and insoluble fractions. These fractions were blotted onto Hybond™-ECL™ Nitrocellulose membrane (Amersham Biosciences, UK) (NC). This NC membrane was analyzed by using ScFv and polyclonal antibodies as a detection probe. It was found that the ScFv and polyclonal antibodies reacted specifically with soluble fraction of lysate duck PBMC under reducing and non-reducing condition. The reactive band of monoclonal and polyclonal antibodies migrated relative to about 23 kDa (Figure 25).

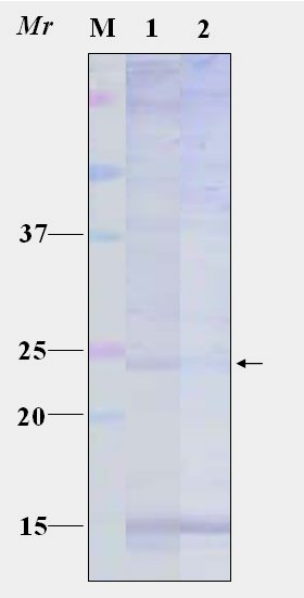


Figure 25 The Western blot analysis of ScFv antibodies incubated with lysate duck PBMC. The soluble part of lysate duck PBMC was separated in a 12% polyacrylamide gel under reducing and non-reducing conditions and transfer onto NC membrane. Lane M, the Prestained Broad Range standard. Lane 1, lysate duck PBMC under non-reducing condition was probed with the soluble part of ScFv recombinant protein from HB2151 *E. coli* clone number 4-2. Lane 2, lysate duck PBMC under reducing condition was probed with the soluble part of ScFv recombinant protein from HB2151 *E. coli* clone number 4-2. The arrow indicates the reactive band of ScFv recombinant proteins against lysate of duck PBMC.

14. **Test specificity of polyclonal antibodies against CD3ε recombinant protein by using ELISA and Western blot analysis**

The polyclonal antibodies were used in the ELISA to detect specificity of polyclonal antibodies against CD3ε recombinant protein. The mouse pre-immune serum was coated as a baseline control. It was found that clone number 1 and 2 gave positive ELISA result (Figure 26). The OD at 492 nm of cut-off of ELISA is 0.05. The Western blot analysis using absorbed CD3ε recombinant protein has shown that the polyclonal antibodies clone number 1 specifically with CD3ε recombinant protein at reciprocal titer 2,000 (Figure 27)

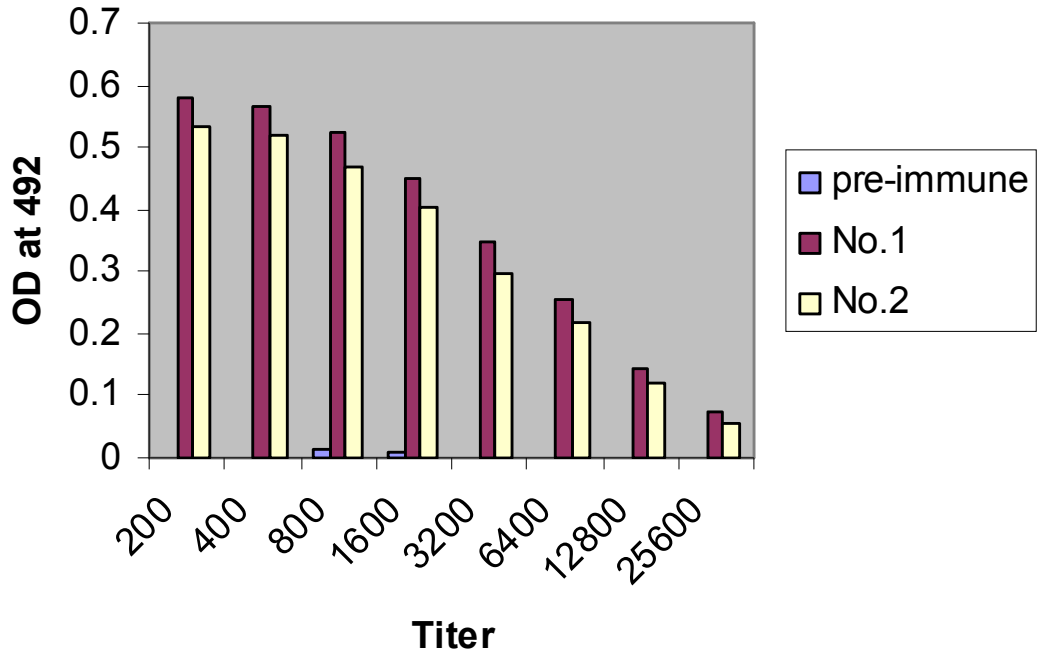


Figure 26 Chart indicated OD_{492} of polyclonal antibodies against CD3ε recombinant protein versus reciprocal titers of polyclonal antibodies. The OD_{492} value of polyclonal antibodies from clone No.1 and 2 were higher than pre-immune serum at maximum reciprocal titer 25,600.

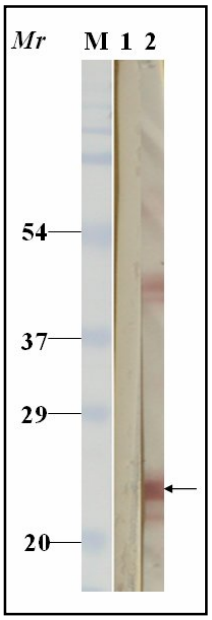


Figure 27 The Western blot analysis of polyclonal antibody incubated with the soluble part of CD3ε recombinant protein. The soluble part of CD3ε recombinant protein was separated in a 12% polyacrylamide gel under reducing condition and transfer onto NC membrane. Lane M, the Prestained Broad Range standard. Lane 1, soluble part of CD3ε recombinant protein was probed with the pre-immune serum from clone number 1. Lane 2, soluble part of CD3ε recombinant protein was probed with the polyclonal antibody from clone number 1. The arrow indicates the reactive band of polyclonal antibody against soluble part of CD3ε recombinant protein

Discussion

Production of CD3ε recombinant protein

T lymphocyte is an important cell that associates with immune response via cellular and humeral immune responses. The T lymphocyte identification is required for pathogenic study and immunological knowledge. The markers that are used for pan T lymphocyte population identification are cluster of differentiation 2 (CD2) and CD3. In vertebrates, the CD2 is identified by using E–rossette formation. The E–rossette formation is the lymphocyte identification method mediated by the binding between CD2 molecule on the T lymphocytes and sheep erythrocytes. Duck T lymphocytes lack CD2 on their surface; therefore, the E–rossette formation can not be used to differentiate T lymphocyte. Hence, the duck CD3, in particular CD3ε will be used as the alternative marker for T lymphocyte identification. To date, the only commercial product that allows for duck T lymphocyte identification is the rabbit polyclonal antibody against human CD3ε (Bertram, 1996). These polyclonal antibodies are not specific to duck CD3ε and detect the intracytoplasmic epitopes that are highly conserved region among the vertebrates. Moreover these antibodies cannot be applied to the living cells. On the other hands, the antibody againsts ectodomain portion, that is the least conserved among the vertebrates, can be applied to living cells for subsequent studies. In this study, we clone ectodomain portion of duck CD3ε gene and produce recombinant protein of duck CD3 ε. In addition, we produce monoclonal antibodies in ScFv form and mouse polyclonal antibodies against the extracellular domain of domestic duck CD3ε.

The extracellular domain of domestic duck CD3ε has been amplified by using specific primers. The CD3ε amplicon showed 100% identity of DNA sequences with *Anas plythyrynchos* T-cell receptor CD3ε chain mRNA (accession number AF378704 and AY738731). Our CD3ε sequencing data also indicated that the domestic duck share the similar ancestor that clearly differs from that of Galliformes. This finding indicated that the chicken immune knowledge cannot be applied to duck. The

recombinant CD3ε has been expressed in soluble form by using pTrcHis2A as an expression vector, although the yield was quite low. The soluble form of recombinant protein is known to provide a protein function and native structure that appropriate for monoclonal antibody production by using selection platform such as phage display library used in this study. The Western blot analysis using HRP-conjugated nickel probe has been shown the recombinant protein migrated relative to about 22 kDa. In order to eliminate high background when using *E. coli* lysate containing recombinant protein in the subsequent antibody production, the recombinant protein was purified by using affinity column. The affinity column provide single band at about 22 kDa from SDS-PAGE while Western blot analysis showing reactive band from the protein fractions were eluted by elution buffer containing 150, 200, and 250 mM imidazol. This SDS-PAGE and Western blot analysis results indicated that the eluate fractions were eluted by elution buffer containing 200 mM imidazol convenient for protein identification because it contain protein purity and quantity. The protein identification results have shown that the recombinant protein was homologous to the *Anas platyrhychos* T cell receptor CD3 epsilon chain protein (accession number AAW 36060). This result showed that recombinant CD3ε was first generated. Although the affinity column provides high protein purity, it provides low protein amount. If the large amount of recombinant protein is required the other protein purification methods such as ion exchange chromatography column and gel filtration should be considered.

Production of monoclonal and polyclonal antibodies

The recombinant protein has been used as an antigen for monoclonal and polyclonal production. The recombinant protein has been coated onto the surface of polystyrene plate (ELISA plate) by using method that used for correctly display conformational epitope. The single round of bio-panning has been used for selection of antibodies from phage library. In general, the several round of bio-panning (average 2-4 cycles) are required to eliminate non-specific phage binding. However, in this study, a single round of bio-panning was used because several round of bio-panning generated the complete and partial deletions of *scFv* gene (data not shown). The result from the amplification of *scFv* gene from HB2151 *E. coli* clone indicated

that all of the HB2151 *E. coli* clones from single round of bio-panning contain the ordinary size of *scFv* gene. Subsequently, HB2151 *E. coli* clones were induced and ScFv proteins were produced. Only 14% of *E. coli* clones were capable of ScFv protein expression. These indicated that, ScFv protein could not be expressed though the eluated phage clones contain ordinary size of scFv amplicon. As confirmed by DNA sequencing analysis these amplicon usually either scFv gene containing the pre-mature stop codon or unknown protein. The latter presumably involved the binding between antigen and phage by using the protein-protein interaction. In this study, ScFv protein is expressed in the soluble form and it has been shown to specifically recognize the recombinant CD3ε in ScFv ELISA. The ScFv ELISA and Western blot analysis results indicated that the ScFv protein can recognize the CD3ε recombinant protein in both conformational and linear epitope. Furthermore, Western blot analysis also suggested that ScFv protein react with the absorbed CD3ε recombinant protein in both reducing and non-reducing forms.

The protein preparation from duck PBMC in both soluble and insoluble forms has been subjected to Western blot analysis and reacted against the ScFv protein. The Western blot analysis has been demonstrated that the ScFv protein from both HB2151 *E. coli* clones reacted with only the soluble form of protein from duck PBMC. There are two evidences supporting specific reaction of monoclonal and polyclonal antibodies against duck CD3ε. The former, the Western blot analysis showed that ScFv protein reacted with duck PBMC protein band with molecular weight about 23 kDa. This 23 kDa protein in duck PBMC protein preparation is corresponded to the duck CD3ε (Bertram EM *et al*., 1996). The latter, the deduced amino acid sequence of recombinant protein indicated that the recombinant protein is identified as hydrophilic ectodomain protein of duck CD3. In addition, the result of Western blot analysis indicated that ScFv protein specific reacted with linear epitope due to the specificity of ScFv protein with duck PBMC under both reducing and non-reducing condition. In addition, the Western blot analysis of native duck PBMC against ScFv protein also showed reactive bands at 15 kDa that might be due to mechanical shearing of native duck PBMC. The CD3ε recombinant protein also used to raise mouse polyclonal

antibodies. The ELISA results and Western blot analysis indicated that the CD3ε recombinant protein exhibits immunogenicity. The Western blot analysis of CD3ε recombinant protein against mouse polyclonal antibodies showed reactive band about 23 and 46 kDa. For size of reactive band at 23 kDa, it was identified to be CD3ε recombinant protein. About size of reactive band at 46 kDa, it might be due to incomplete destruction of disulfide bond within CD3ε recombinant protein. In addition, the reactivity of polyclonal antibodies against CD3ε recombinant protein under reducing condition indicated that native structure of antigen is not required. It is the advantage that monoclonal and polyclonal antibodies react to linear CD3ε epitope. The produced monoclonal and polyclonal antibodies against duck CD3ε still require further investigation for anatomical identification and enumeration of T lymphocyte such as flow cytometric assay and immunohistochemistry. In conclusion, the recombinant ectodomain protein of domestic duck CD3 ε was produce for the first time by using prokaryotic expression system and their specific monoclonal antibodies in ScFv form and polyclonal antibodies were successfully produced.

CONCLUSION

The DNA encoding domestic duck ectodomian protein of CD3ε was amplified by using designed primer. The size of DNA amplicon is 327 bp and the amplicon was successfully clones to pTrsHis2A expression vector.

The ectodomian portion of CD3ε recombinant protein was expressed in soluble part of DH5α *E. coli* lysate. The size of recombinant protein is 23 kDa and the recombinant protein was purified by using affinity column. The analysis of recombinant protein showing the recombinant protein was homologus to the *Anas platyrhynchos* T-cell receptor CD3ε protein.

The purified protein was used as an antigen for polyclonal production and monoclonal production by using biopanning assay. The produced polyclonal antibodies against CD3ε recombinant protein is ELISA titer at reciprocal 25800 when compare with pre-immune serum. For monoclonal antibodies production, there are two clones of HB2151 *E.coli* carrying *scFv* genes specific to the ectodomian portion of CD3ε recombinant protein. These HB2151 *E. coli* clone were expressed ScFv proteins and these proteins can be used as a monoclonal antibodies to detect pan T lymphocyte of duck by using Western blot analysis.

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APPENDICES

APPENDIX A

Reagents and buffers for agarose gel electrophoresis

Reagents and buffers for agarose gel electrophoresis

1. Gel loading buffer (loading dye)

The loading dye buffer composed of 0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol and 35 ml of ultrapure distilled water. The loading dye solution was kept at 4°C.

2. Tris acetate buffer (50x TAE)

The stock 50x TAE was prepared by dissolved 242 grams of Tris-base in 500 ml of distilled water. After the ingredient was completely dissolved, 57.1 ml of concentrate glacial acetic acid and 100 ml of 0.5 M EDTA, pH 8.0, were added into the solution. The final volume was adjusted to 1,000 ml by distilled water. The 50x TAE was stored at 25°C. The 1x working solution was freshly prepared by diluting the stock 50x TAE buffer with distilled water.

3. Working (1x TAE)

Twenty milliliter of 50X TAE was added to 980 ml of UDW. This solution can be reused three times.

APPENDIX B

Media for *E. coli* culture

Media for *E. coli* culture

1. LB broth

This medium consists of:

Bacto tryptone	10	g
Bacto yeast extract	5	g
NaCl	5	g

The broth was prepared by completely dissolving all of the above reagents in DW to final volume of one liter. The preparation was sterilized by autoclaving. The broth was stored at 4°C.

2. LB agar plates

This medium consists of:

Bacto tryptone	10	g
Bacto yeast extract	5	g
NaCl	5	g
Agar	15	g

The agar was prepared by dissolving all of the above reagents in DW to final volume of one liter and then sterilized by autoclaving. The solution was poured into petridishes (25 ml/plate), and allowed for solidification at 25°C and stored at 4°C.

3. LB-ampicillin broth (100 μg ampicillin/ml)

The medium was prepared by mixing 400 μl of 250 mg/ml sterilized ampicillin with 1 liter of the autoclaved LB-broth. The medium was then mixed by swirling.

4. LB-ampicillin agar (100 μg ampicillin/ml)

One liter of LB agar was prepared, autoclaved and cooled down to 55°C. Then 400 μl of 250 mg/ml sterilized ampicillin was added to the agar. After gently mixing by swirling, the preparation was poured into petridishes (25 ml/plate). The plates were stored at 4°C.

APPENDIX C

Reagent for preparation of chemical competent cells and for *E. coli* transformation

Reagent for preparation of chemical competent cells and for *E. coli* transformation

1. TSS buffer

Bacto-tryptone	1.0	g
Bacto yeast extract	0.5	g
NaCl	0.5	g
Polyethylene glycol (PEG, M.W. 3350)	10	g

All ingredients were dissolved in 70 ml of distilled water, and then 5 ml of Dimethylsulfoxide (DMSO) and 5 ml of 1 M $MgCl_2$ were added. The pH was adjusted to 6.5 with HCl or NaOH. The final volume was adjusted to 100 ml with distilled water and the preparation was sterilized by filtering through a 0.22 mm filter. The buffer was stored at 4°C and used within 6 months.

2. **SOB medium**

Bacto tryptone	20	g
Bacto yeast extract	5	g
NaCl	0.5	g

All ingredients were dissolved in distilled water to final volume of 980 ml. The medium was sterilized by autoclaving. Ten ml of 1 M $MgSO_4$ (10 mM final concentration) was added.

3. SOC medium

The medium was prepared by mixing 999 ml of SOB medium with 1 ml of 2 M glucose stock solution. The medium was kept in 5 ml aliquots in 15 ml Falcon tubes and stored at -20°C until use.

APPENDIX D

Reagents for extraction of plasmid DNA from *E. coli*

Reagents for extraction of plasmid DNA from *E. coli*

1. Tris-HCl, pH 8.0 stock solution (2 M)

The solution was prepared by dissolving 242.28 g of Tris base in a volume of UDW. The pH was adjusted to 8.0 with HCl; UDW was added to a final volume of one liter and the preparation was autoclaved.

2. Glucose stock solution (2M)

The solution was prepared by dissolving 36 g of glucose in 100 ml of UDW. The solution was steriled by filtering and it was stored at -20°C until use.

3. EDTA stock solution (0.5 M)

The solution was prepared by dissolving 18.6 g of EDTA Na_2 $2H_2O$ in a volume of UDW. The pH was adjusted to 8.0 with NaOH, added UDW to final volume of 100 ml and autoclaved.

4. Solution I (25 M Tris pH 8.0, 50 mM glucose, 10 mM EDTA)

2.0 M Tris-HCl, pH 8.0	1.25	ml
2.0 M glucose	2.5	ml
0.5 M EDTA pH 8.0	200	µl

All components were dissolved with UDW to final volume of 100 ml.

5. Solution II (0.1 N NaOH, 1% SDS) (freshly prepared)

The solution was freshly prepared by mixing one milliliter of 1 N NaOH with 0.5 ml of 20% SDS and then UDW was added to bring the final volume to 10 ml.

6. Solution III (2.7 M potassium acetate, pH 4.8)

The solution was prepared by dissolving 26.5 g of potassium acetate in a volume of UDW. The solution was adjusted to pH 4.8 with glacial acetic acid and UDW was added to bring the final volume to 100 ml.

APPENDIX E

Reagents, solutions and buffers for recombinant protein purification

Reagents, solutions and buffers for recombinant protein purification

1. Native purification buffer (5x) (250 mM NaH_2PO_4, pH 8.0, 2.5 M NaCl)

Sodium phosphate (monobasic, 7 g) and NaCl (29.2 g) were dissolved in DW and the pH was adjusted with NaOH to 8.0. The final volume was adjusted to 200 ml with DW. The buffer solution was stored at 25°C until use.

2. Stock solution A (10x) of 3 M imidazole pH 6.0 (200 mM sodium phosphate, monobasic (NaH_2PO_4), 5 M NaCl)

Monobasic sodium phosphate (NaH_2PO_4) 27.6 g and 292.9 g of NaCl were dissolved in 800 ml DW. The final volume was adjusted to 1 liter with DW. The buffer solution was stored at 25°C until use.

3. Stock solution B (10x) of 3 M imidazole pH 6.0 (200 mM sodium phosphate, dibasic (Na_2HPO_4), 5 M NaCl)

Dibasic sodium phosphate (Na_2HPO_4, 28.4 g) and 292.9 g of NaCl were dissolved in 800 ml DW. The final volume was adjusted to 1 liter with DW. The buffer solution was stored at 25°C until use.

4. 3 M imidazole pH 6.0 (3 M imidazole, 500 mM NaCl, 20 mM sodium phosphate buffer, pH 6.0)

Imidazole (20.6 g) was dissolved in 90 ml DW, then 8.77 ml of stock solution A (10x) and 1.23 ml of stock solution B (10x) were added and adjusted the pH to 6.0 with HCl or NaOH as necessary. The final volume was brought to 100 ml with DW. The buffer solution was stored at 25°C until use.

5. Native binding buffer (1x)

Eighty milliliters of sterile DW and 20 ml of 5x Native binding buffer were mixed well and the pH was adjusted to 8.0 with NaOH or HCl.

6. Native binding buffer (with imidazole)

Thirty milliliters of 1x Native binding buffer and 100 µl of 3 M imidazole, pH 6.0 were mixed well and the pH was adjusted to 8.0 with NaOH or HCl.

7. Native wash buffer

Fifty milliliters of 1x Native binding buffer and 335 µl of 3 M imidazole, pH 6.0 were mixed well and the pH was adjusted to 8.0 with NaOH or HCl.

8. Imidazole in native wash buffer (1M)

Imidazole (6.8 g) was dissolved in 90 ml of Native wash buffer and the pH was adjusted to 8.0 with HCl or NaOH as necessary. The final volume was brought to 100 ml with native wash buffer. The buffer solution was stored at 25°C until use.

9. Native elution buffers

The native elution buffers contained various concentrations of imidazole in native wash buffer. They were prepared by mixing the following components:

	1 M imidazole dissolved in native wash buffer (µl)	Native wash buffer (ml)
Native elution buffer 1 (50 mM imidazole):	150	2.85
Native elution buffer 2 (100 mM imidazole):	300	2.70
Native elution buffer 3 (150 mM imidazole):	450	2.55
Native elution buffer 4 (200 mM imidazole):	600	2.40

	1 M imidazole dissolved in native wash buffer	Native wash buffer
Native elution buffer 5 (250 mM imidazole):	750	2.25

APPENDIX F

Reagents for SDS-PAGE

Reagents for SDS-PAGE

1. Sample buffer (SDS reducing buffer)

The sample buffer was prepared as a stock solution by combination the following ingredients:

0.5 M Tris-HCL, pH 6.8	1.0	ml
Glycerol	2.0	ml
SDS (10% solution)	1.6	ml
0.05% Bromophenol blue	0.2	ml
2-6-mercaptoethanol	0.4	ml
UDW	2.8	ml

This mixture was stored at 25°C in small aliquots. One part of sample was diluted with equal part of the sample buffer and heated at 100°C for 4 min before loading into gel.

2. Tris-HCl (1.5 M, pH 8.8)

To prepare this solution, 18.15 g of Tris base was dissolved in 50 ml of UDW, then the pH was adjusted to 8.8 with 1 N HCl. The final volume was brought up to 100 ml with UDW. The solution was filtered through sterile a 0.2 μm membrane. This stock solution was stored at 4°C until use for preparing a working solution.

3. Tris-HCl (0.5 M, pH 6.8)

To prepare this solution, 6.05 g of Tris base was dissolved in 50 ml of UDW, then the pH was adjusted to 6.8 with 1 N HCl. The final volume was brought up to 100 ml with UDW. The solution was filtered through a sterile 0.22 μm membrane. This stock solution was stored at 4°C.

4. Sodium dodecyl sulfate (10% SDS; w/v)

This solution was prepared by dissolving 10 g of SDS in 100 ml of UDW.

5. Ammonium persulfate (10%; w/v)

This solution was prepared just before use by dissolving 50 mg of ammonium persulfate (Bio-Rad) in 0.5 ml of UDW.

6. Separating gel (12%)

Polyacrylamide separating gel (12%) was prepared by mixing the following ingredients together:

UDW	3.35	ml
1.5 M Tris-HCl, pH 8.8	2.5	ml
10% SDS solution	100	µl
30% Acrylamide/Bis, 29:1 ratio solution (BioRad)	4	ml

The reagents were gently mixed and degassed under a vacuum for at least 5 min. The polymerization was initiated by adding 50 µl of the 10% ammonium persulfate (freshly prepared) and 5 µl of TEMED (Bio-Rad). The gel was poured into the casting apparatus, over-layered with UDW and allowed to polymerize for at least 20 min at 25°C.

7. Stacking gel (4%)

The stacking gel (4%) was prepared by mixing the following reagents:

UDW	6.0	ml
0.5 M Tris-HCl	2.5	ml
SDS (10% solution w/v)	0.1	ml
30% Acrylamide/Bis, 29:1 ratio solution (BioRad)	4	ml

All reagents were mixed gently and degassed under a vacuum for 15 min, and then 50 µl of freshly prepared 10% ammonium persulfate and 10 µl of TEMED were subsequently added, respectively. After complete mixing and degassing, the upper portion of the gel polymerized in the casting apparatus was rinsed with UDW, the comb was inserted between the glass plates over the polymerized separating gel. The stacking gel was poured and allowed to polymerize for at least 45 min at 25°C before use.

8. Electrode (running) buffer (pH 8.3; 10 x)

The buffer contained the following reagents: 30.3 g of Tris base; 142.9 g of glycine and 10 g of SDS. The buffer was prepared by dissolving all of the above reagents in a volume of UDW. After all ingredients were dissolved, the volume was made up to one liter with UDW. The buffer was stored at 4°C until use for preparing a working electrode (running) buffer.

9. Working electrode (running) buffer (1x)

One hundred ml of the 10x electrode buffer was diluted with 900 ml of UDW. Each preparation of the working running buffer was used for only one electrophoretic run.

10. Coomassie Brilliant Blue stain

10.1 Coomassie® Brilliant Blue R-250 dye solution

Coomassie® Brilliant Blue R-250 dye (USB Corporation, USA) (2.5 g) was dissolved in 454 ml of absolute methanol before 92 ml of glacial acetic acid and 454 ml of UDW were added. This dye was filtered through a Whatman No. 1 paper and kept at 25°C until use.

10.2 Destaining solution

10.2.1 High methanol destain solution

The solution was prepared by mixing 75 ml of glacial acetic acid, 454 ml of methanol and 25 ml of glycerol together. UDW was added to make 1,000 ml. The solution was kept at 25°C until use.

10.2.2 Standard (low-methanol) destain solution

The solution was prepared by mixing 75 ml of glacial acetic acid, 50 ml of methanol and 25 ml of glycerol together. UDW was added to make 1,000 ml. The solution was kept at 25°C until use.

APPENDIX G

Reagents for Western blot analysis

Reagents for Western blot analysis

1. Transfer buffer [25mM Tris, 192 mM glycine and 20% (v/v) methanol]

To prepare 1,000 ml of this buffer, 3.03 g of Tris base and 14.4 g of glycine were dissolved in 800 ml of UDW. Subsequently, 200 ml of methanol was added to yield 20% (v/v).

2. Phosphate buffered saline (0.01 M PBS, pH 7.4)

This solution was prepared by dissolving 1.22 g of anhydrous Na_2HPO_4, 0.17 g of anhydrous NaH_2PO_4 and 8.77 g of NaCl in 1 liter of DW. The pH of this solution was adjusted to 7.4 with 1 N HCl.

3. Phosphate buffer (1/15 M PB, pH 7.6)

The buffer was prepared by dissolving 0.06 g of NaH_2PO_4 and 0.47 g of Na_2HPO_4 in 57.7 ml of UDW. The pH of this solution was adjusted to 7.6 with 1 N HCl.

4. Washing buffer (0.05% Tween-20 in PBS, pH 7.4; PBST)

This solution was prepared by adding 0.5 ml of Tween-20 in one liter of 0.01 M PBS (pH 7.4) and mixed well.

5. Blocking solution (3% BSA, in PBS, pH 7.4)

The solution was prepared by dissolving 3 g of bovine serum albumin (BSA) in 100 ml of 0.01 M PBS, pH 7.4.

6. Diluent solution (0.2% BSA, 0.2% gelatin in PBS, pH 7.4)

The solution was prepared by dissolving 0.2 g of BSA and 0.2 g of gelatin in 100 ml of 0.01 M PBS, pH 7.4.

7. Substrate solution

The solution was freshly prepared by dissolving 0.02 g of 2, 6-dichloro-phenol indophenol (Sigma Chemical Co.) in 10 ml of 1/15 M PB (pH 7.6). Ten µl of 30% H_2O_2 was added immediately before use.

APPENDIX H

Reagents for ELISA

Reagents for ELISA

1. Coating buffer (Carbonate-bicarbonate buffer, pH 9.6)

The buffer was prepared by dissolving 1.26 g of $NaHCO_3$ in 300 ml of DW; then the pH was adjusted to 9.6 with 0.05 Na_2CO_3 (0.53 g in 100 ml of DW).

2. Phosphate buffered saline (0.01 M PBS, pH 7.4)

The solution was prepared by dissolving 1.22 g of anhydrous Na_2HPO_4, 0.17 g of anhydrous NaH_2PO_4 and 8.77 g of NaCl in one liter of DW. The pH of this solution was adjusted to 7.4 with 1 N HCl.

3. Washing solution (PBST)

Washing solution (PBST) was prepared by mixing Tween-20 in PBS, pH 7.4 to a 0.05 % concentration.

4. Blocking solution

The solution was prepared by dissolving 1 g of BSA in 100 ml of 0.01 M PBS, pH 7.4.

5. Diluents solution

The diluents was prepared by dissolving 0.2 g of BSA and 0.2 g of gelatin in 100 ml of 0.01 M PBS, pH 7.4.

6. Substrate buffer (0.1 M citrate buffer, pH 4.5)

The buffer was prepared by dissolving 14.7 g of trisodium citrate ($Na_3C_6H_5O_7.H_2O$) in DW. The volume was made up to 500 ml after the pH was adjusted to 4.5 with 1M HCl.

7. Substrate solution

The substrate solution consisted of 0.05% 1, 4-*p*-phenylenediamine-dihydrochloride (PPD) (Sigma Chemical Co.) in citrate buffer, pH 4.5 and 0.01% of 30% H_2O_2. This solution was prepared freshly before use and always protected from light.

8. Stop solution (1N NaOH)

The solution was prepared by dissolving 20 g of NaOH in 500 ml of UDW.

APPENDIX I

Sequences of R1 and R2 primers

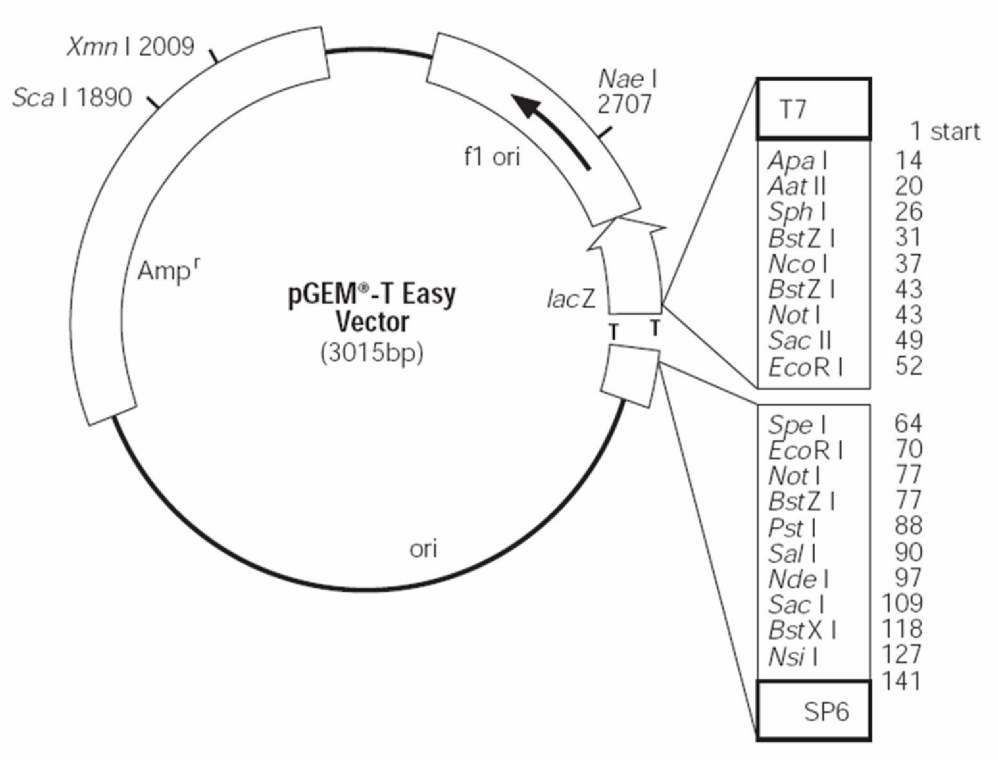
R1 primer 5' CCATGATTACGCCAAGCTTTGGAGCC 3'

R2 primer 5' GCTAGATTTCAAAACAGCAGAAAGG 3'

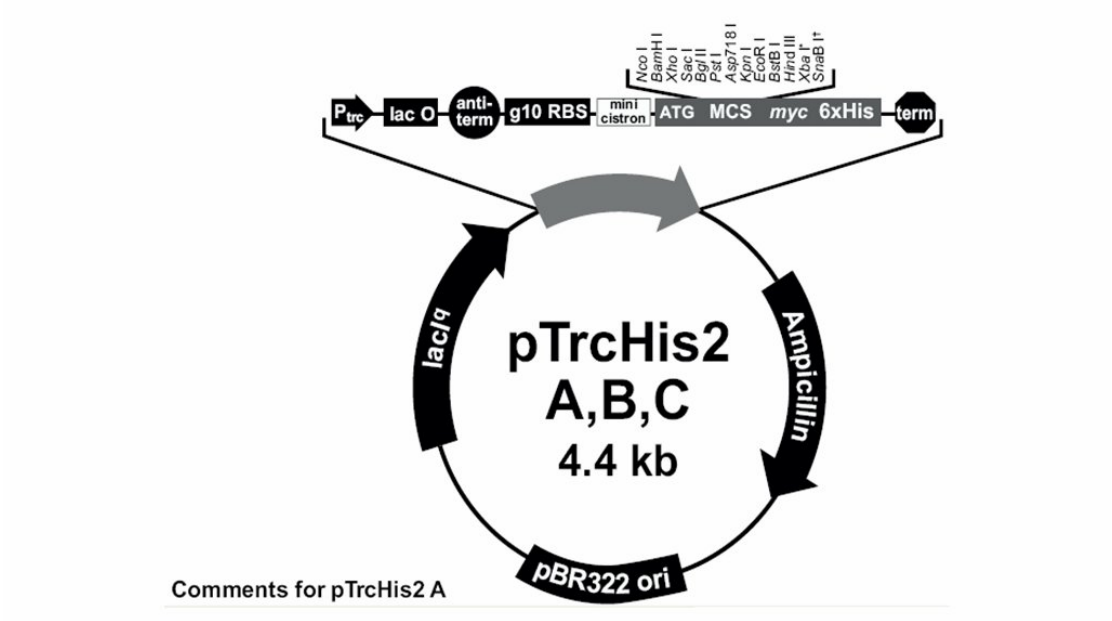
Apppendix Figure I1 The nucleotide sequences of R1 and R2 primers

APPENDIX J

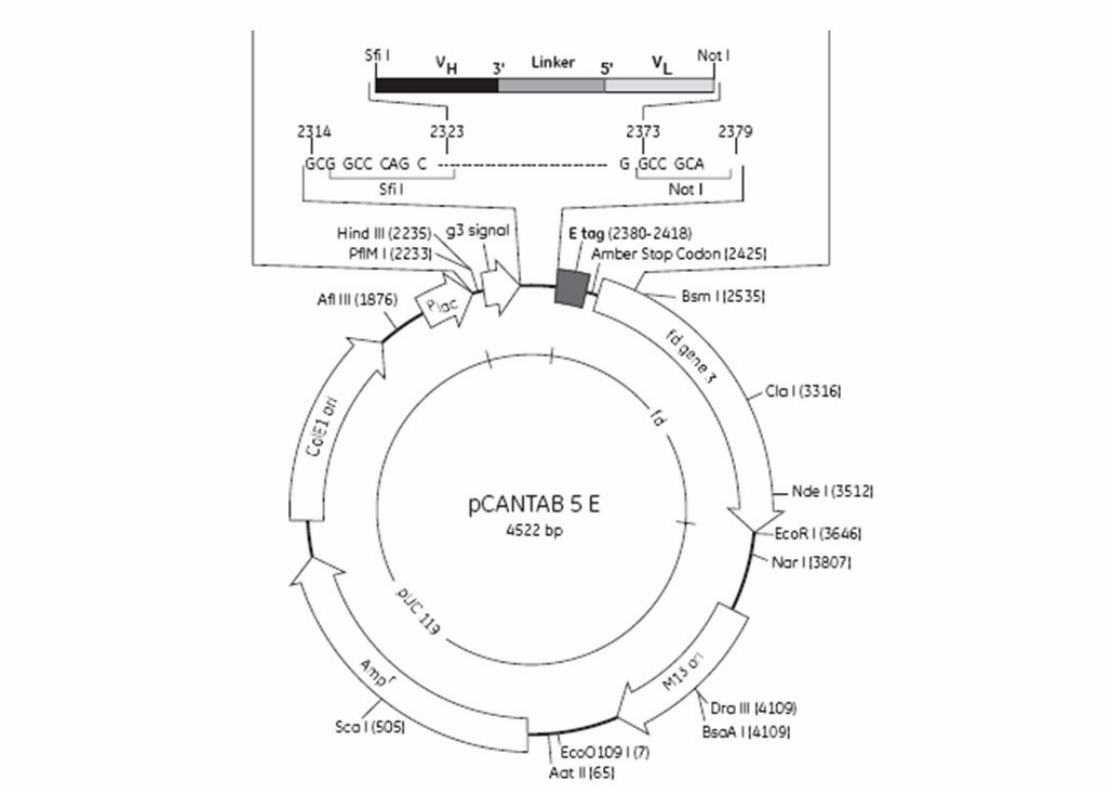
Map of vector



Apppendix Figure J1 pGEM®-T Easy Vector circle map



Apppendix Figure J2 pTrcHis2 Vector circle map



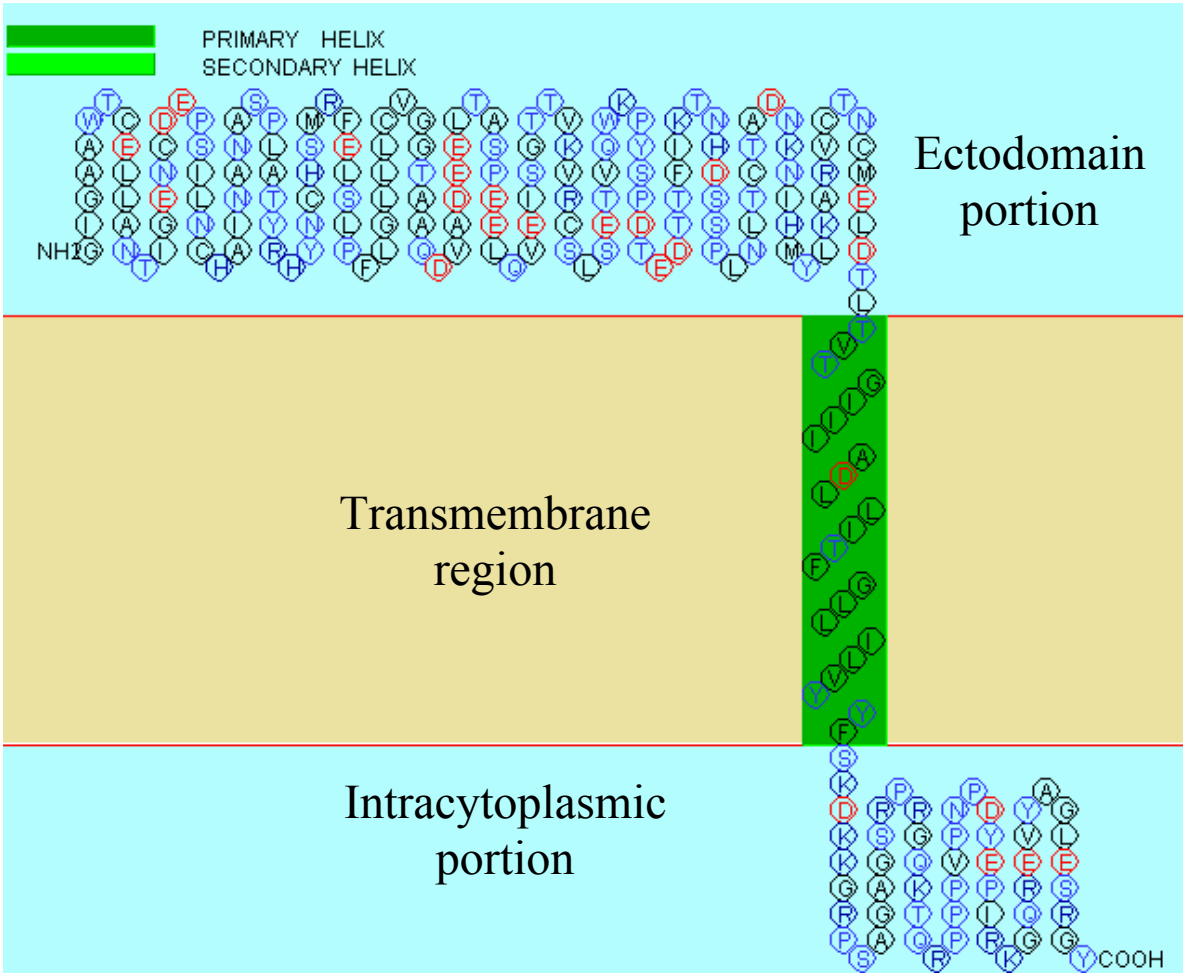
Apppendix Figure J3 pCANTAB 5E Vector circle map

APPENDIX K

Transmembrane region prediction by using SOSUI software

Transmembrane region prediction by using SOSUI software

The webpage that used to predict transmembrane region is http://bp.nuap.nagoya-u.ac.jp/sosui



Appendix Figure K1 transmembrane region of *Anas platyrhychos* CD3ε (AY738731).

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