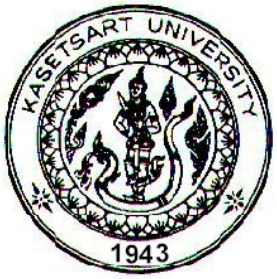

THESIS

TRANSFORAMTION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 ENVELOPE GLYCOPROTEINS IN TOBACCO AND SILKWORM

NUANJUN WICHUKCHINDA

GRADUATE SCHOOL, KASETSART UNIVERSITY

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THESIS

TRANSFORMATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 ENVELOPE GLYCOPROTEINS IN TOBACCO AND SILKWORM

NUANJUN WICHUKCHINDA

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Bioscience)
Graduate School, Kasetsart University
2007

Nuanjun Wichukchinda 2007: Transformation of Human Immunodeficiency Virus Type 1 Envelope Glycoproteins in Tobacco and Silkworm. Doctor of Philosophy (Bioscience), Major Field: Bioscience, Interdisciplinary Graduate Program. Thesis Advisor: Associate Professor Sunanta Ratanapo, Ph.D. 111 pages.

HIV-1 infection which causes AIDS is still be health, social and economical problems in Thailand. Early diagnosis of HIV-1 infected individuals is one of the strategies to confine the spreading of this incurable virus. Every year, commercial kits for HIV-1 infection diagnosis were imported to Thailand at several hundred millions Baht. The development of production system for proteins used as antigens in diagnostic kit is very important for self-depending and decreasing imports of recombinant proteins and commercial kits to Thailand. In this study, two systems, tobacco plant with *Agrobacterium*-mediated and silkworm with piggyBac based transformation were tried for expression of HIV-1 gp120 and gp41.

The successful of transgenesis of gp120 into tobacco plant was demonstrated by the presence of integrated gp120 coding sequence in the genomic DNA of transformed tobacco plants by nested-PCR, and confirmed by sequencing. However, neither gp120 mRNA nor protein was detected. This study did not success for transgenesis of gp41 into tobacco plant, and for neither gp120 nor gp41 into silkworm. Naturally, HIV-1 infects only human and uses advance machinery of human cell to produce all viral proteins. Furthermore, envelope glycoprotein of HIV-1 was expressed as gp160 polyprotein precursor, which later be glycosylated and cleaved into gp120 and gp41 during viral maturation. The research and development of protein production system to produce such special proteins like HIV- 1 gp120 and gp41would be desired.

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Student's signature Thesis Advisor's signature

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Nuanjun Wichukchinda

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LIST OF ABBREVIATIONS

Ab	antibody
Ag	antigen
AIDS	acquired immunodeficiency syndrome
bp	base pair
CRF	circulating recombinant form
DNA	deoxyribonucleic acid
d-NTP	deoxynucleotide triphosphate
ELISA	enzyme link immunosorbent assay
env	envelope
gag	group specific antigen
GFP	green fluorescent protein
GMO	genetically modified organism
gp	glycoprotein
HIV-1	human immunodeficiency virus type 1
ITR	inverted terminal repeat
kb	kilo base pair (1,000 base pair)
kDa	kilo Dalton (1,000 Dalton)
LTR	long terminal repeat
ORF	open reading frame
PBS	phosphate buffer saline
RNA	ribonucleic acid
rpm	round per minute
RT	reverse transcriptase
T-DNA	transfer DNA
Ti	tumor inducing
TSP	total soluble protein
Vir-gene	virulence gene
WB	Western blot

TRANSFORAMATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 ENVELOPE GLYCOPROTEINS IN TOBACCO AND SILKWORM

INTRODUCTION

Human Immunodeficiency Virus (HIV), a causative agent of Acquired Immunodeficiency Syndrome (AIDS), is one of the most terrible viral infections that epidemic worldwide. According to an information on HIV/AIDS epidemic from UNAIDS/WHO (December 2006), there were about 39.5 (34.1-47.1) million peoples living with HIV/AIDS. Among these, 95 percent live in low- and middle-income countries.

In Thailand, the estimated accumulative number of HIV-infected cases since the beginning of epidemic in 1984 to December 2006 is at 1.1 million. Among these, half were deaths, and the remaining is living with HIV/AIDS and requires medical attention. Although Thailand has had a substantial success in HIV prevention and control champagne to decrease the new infected person per year from the peak in 1999 at 142,819 cases to 21,260 cases in 2003, and gradually decreases year by year from 2004 to 2006 at 19,500; 18000, and 17,000 cases, respectively (from Thai Working Groups on HIV/AIDS Projection 2000, AIDS Division, Bureau of AIDS, TB and STDs, Department of Diseases Control, Ministry of Public Health. (www.aidsthai.org). Nevertheless, the new infected cases still are over 10,000 cases per year. Early diagnosis is one of the methods for controlling the spread of the virus.

Current methods to diagnose HIV-1 infection are based on the detection of specific anti-HIV-1 antibody. Every year, Thailand imports commercial kits to diagnose HIV infection for several-hundred millions Baht. Most of these kits are manufactured in European countries, USA, and Japan. There are only two commercial products produced in Thailand, however, they contained imported antigens (personal information). To develop HIV-1 diagnostic kits producing in Thailand, appropriate antigen(s) should be produced in sufficient yields at low cost. Traditional protein

production systems that used microbial fermentation, yeast, and insect and mammalian cell culture have drawbacks in term of cost and scalability. To address the concerns of these drawbacks, simple and inexpensive alternative approaches, which allows the large scale of production would be highly desirable.

OBJECTIVES

Overall objectives

To explore effective HIV-1 antigen production systems for using as a versatile bioreactor to produce recombinant proteins for diagnostic proposes. Two systems, transgenic plant and transgenic silkworm were tried for the possibility to produce the HIV-1 glycoprotein gp120 and gp41.

Specific objectives

1. To construct HIV-1 gp120 and gp41 using transgenic tobacco and transgenic silkworm.
2. To transform tobacco plant and silkworm with HIV-gp120 and gp41 construct.
3. To compare the efficiency of transformation method for transgenic tobacco and transgenic silkworm.

LITERATURE REVIEW

HIV/AIDS

HIV is a member of the Lentivirus genus of the *Retroviridae* family (Varmus 1988). Human Lentiviruses are grouped into two types; HIV-1 and HIV-2, on the basis of sequence analysis and serological properties (Myers *et al.*, 1992). HIV-1 is found worldwide and more virulent (infectious) than HIV-2, which is endemic in West Africa and sporadic in some European countries, Brazil and the United State.

HIV-1 is an envelope virus with 110 nm in diameter and has a cylindrical eccentric core as shown in Fig. 1. This core contains (1) p24 major capsid protein (CA), (2) p7/p9 nucleocapsid protein (NC), (3) diploid (two identical single-stranded) RNA, and (4) three viral enzymes, protease (PR), reverse transcriptase (RT) and integrase (IN) (Turner *et al.*, 1998). The viral envelope is derived from host cell membrane that the virus takes with it when budding. The two viral envelope glycoproteins; gp120 and gp41, are conformationally associated to form a trimeric functional unit. This unit consists of three molecules of gp120 exposed on the virion surface and associated with three molecules of gp41 that inserted into the viral lipid membrane.

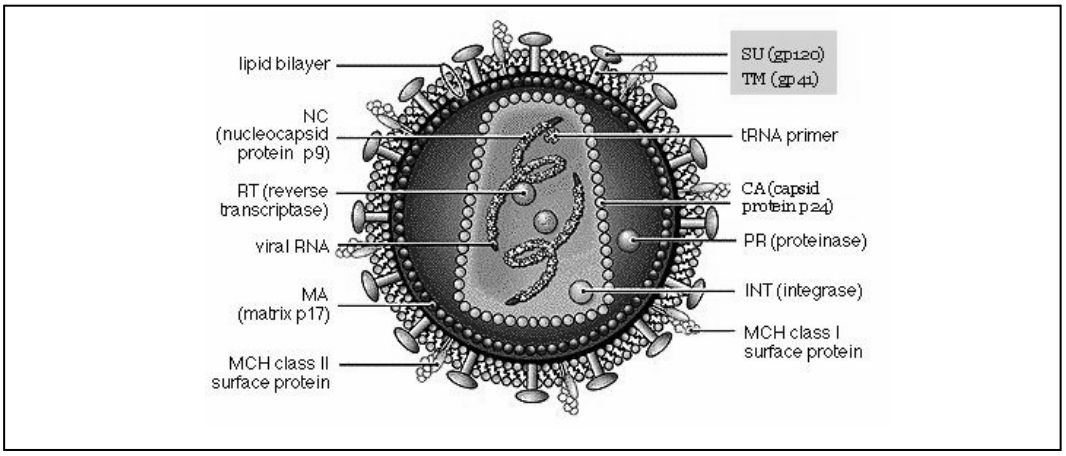

Figure 1 Human immunodeficiency viral particle

Source: Cullen (19991)

As show in Fig. 2, the genetic material of HIV-1 is RNA (9 kb in length), which contains a control region (long terminal repeat: LTR) and nine different genes (gag, pol, env, vif, vpr, vpu, tat, rev, and nef) encoding for 15 proteins.

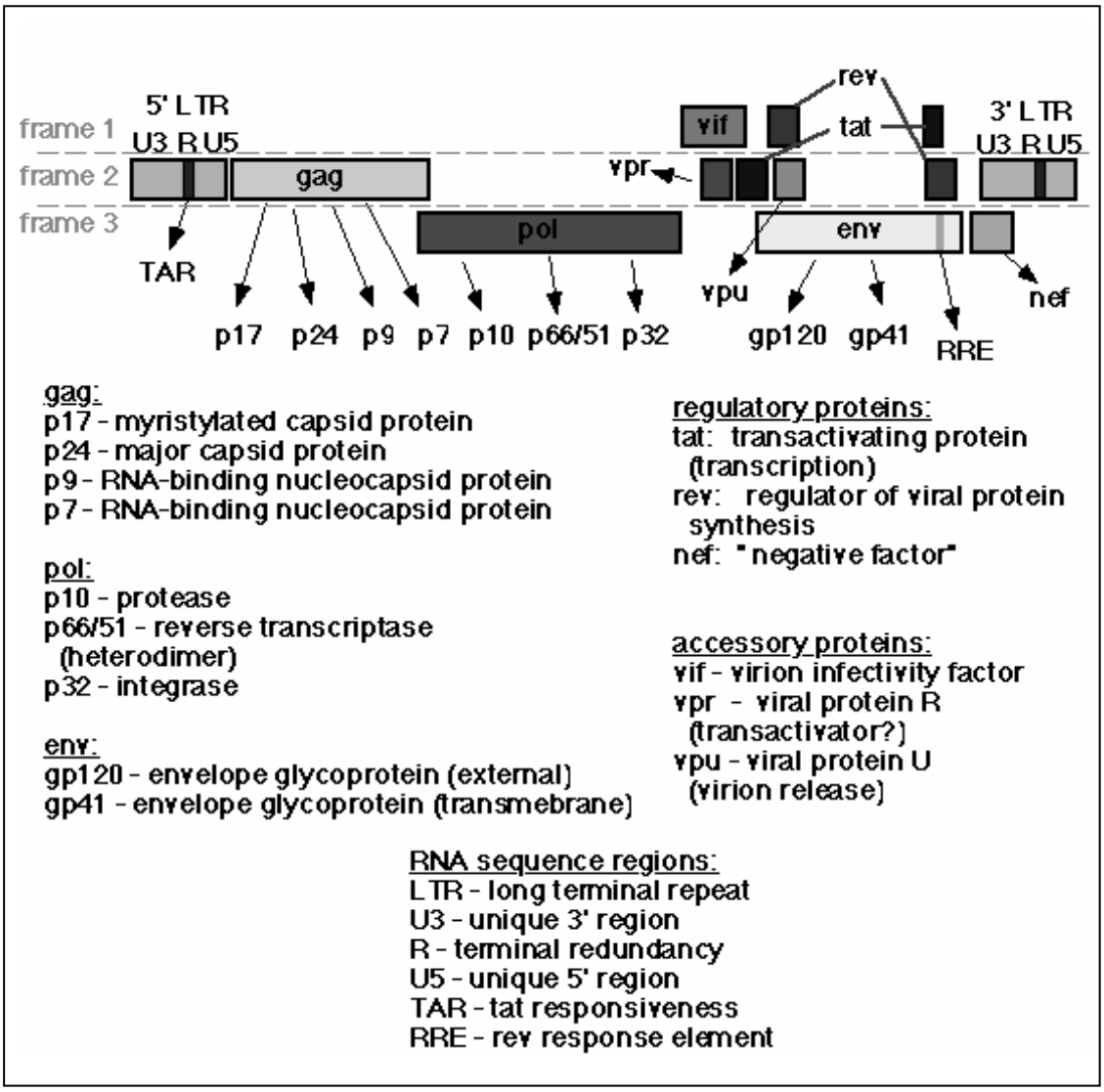

Figure 2 Genomic organization of HIV-1 proviral DNA

Source: Cullen (19991)

To infect a target cell, HIV must introduce its genetic material into the cytoplasm of the cell. The process of viral entry involves fusion of the viral envelope with the host cell membrane, which requires the specific interaction of the viral gp120 with specific cell surface receptors (CD4) and co-receptors (chemokine receptors).

This interaction leads to gp41-mediated fusion, which followed by virion uncoating, reverse transcription of viral genomic RNA into double stranded DNA, nuclear import of the DNA, and then integration into the host chromosome, thus establishing the proviral DNA. The proviral DNA may lie dormant for months to years, which is known as a latent infection.

The major target cells of HIV are T-helper lymphocytes and macrophages, which play important roles in the immune response for protection from infectious organisms. After infection by the HIV-1 2 to 12 weeks, 50-70% of the infected persons usually experience flu-like symptoms (Tindall *et. al.*, 1991). The symptoms generally are fever, headache, fatigue, malaise, decreased appetite, nausea, swollen lymph nodes, muscular stiffness or aching (myalgia), and skin rash (maculopapular). These symptoms may last from a few days to two weeks, and then subside to asymptomatic. However, during the latent period of infection the virus slowly replicates that leads to a gradual decline in T-helper cells. As their numbers decline over a 2-15 year period, then the latency period ends and opportunistic infections appear. Most AIDS patients die from opportunistic infections or cancer. The HIV infected individuals can transmit the virus to contact person at all the stage of infection, especially in latent period that no specific symptoms appeared and they do not recognize their infection status. Transmission routes of HIV-1 are sexual intercourse (heter- or homosexual), contaminated needles using for intravenous drug delivery, and receiving of infected blood or blood products. Infected mother can transmit the virus to her baby during pregnancy or at delivery and also via breast milk.

The first sign of HIV-1 infection may be the appearance of IgM antibodies in the serum, but HIV-1 antigen can be detected in a few days earlier as shown in Fig. 3. IgG antibodies at detectable level appear in most infected persons after 3-12 weeks and IgG antibodies to HIV-1 envelope glycoproteins may be detected much earlier than antibodies to other HIV-1 antigens and last long though the life (Allian *et. al*. 1986).

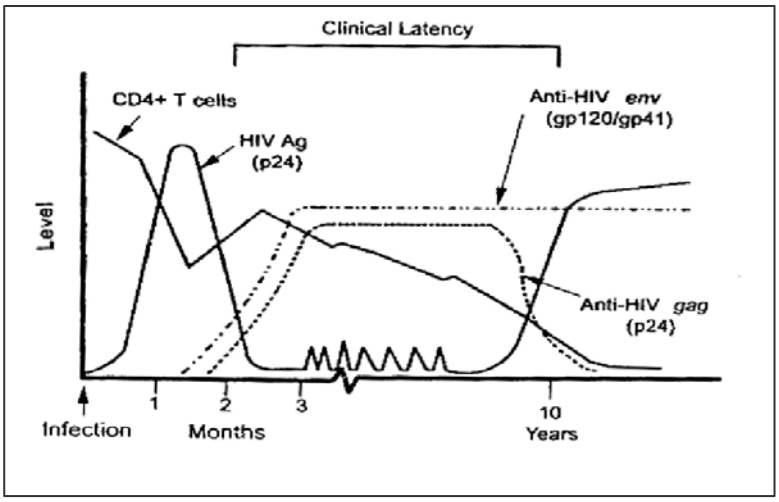

Figure 3 Natural history of HIV-1 infection
Source: Allian *et al.,* (1986)

HIV-1 has extremely genetic variability, which make mutant accumulation of a viral burden over time and lead to a "swarm" or "quasispecies" of the virus within the infected individual. This extensive heterogeneity is the results of the high error rate of viral RT (Preston *et al.,* 1988) and the fast turn over of virions in infected individuals (Ho *et al*., 1995; Wei *et al*., 1995). Furthermore, simultaneous infection in a cell with two different HIV-1 subtypes can generate a new recombination virus. The implications of these variants are a difficulty of drug and vaccine design.

According to whole genome sequences, HIV-1 is differentiated into 11 pure (non-recombinant) subtypes (A1, A2, B, C1, C2, D, F, G, H, J, and K) and at least 18 circulating recombinant forms (CRFs) such as CRF01_AE (recombinants of subtype A and E) CRF02_AG, and CRF0_AB (Robertson *et al.,* 1998) These HIV-1 subtypes/CRFs are circulating at different prevalence in each region of the world. Estimated prevalence of HIV-1 subtypes worldwide is 30% for subtype C, 23% for subtype A, 16% for subtype B, 13% for CRF01_AE, 11% for subtype D, and 7% for others.

Historically, subtype B has been the most common HIV-1 subtype in Europe, the Americas, Japan and Australia. However, other subtypes are becoming more

frequent and now account for at least 25% of new infections in Europe. Subtype A and CRF A/G predominate in west and central Africa, with subtype A possibly also causing much of the Russian epidemic. Subtype D is generally limited to east and central Africa; A/E is prevalent in south-east Asia, but originated in central Africa (Gao *et al.,* 1996); F has been found in central Africa, south America and eastern Europe; G and A/G have been observed in western and eastern Africa and central Europe. Subtype H has only been found in central Africa; J only in central America; and K only in the Democratic Republic of Congo and Cameroon (www. hivdatabase.org; Heydrick. at al., 2000).

In Thailand, HIV-1 CRF01_AE span over 80% of infected individuals and the left is HIV-1 subtype B (Mastro *et al.,* 1996). For route of transmission, sexual contact accounted for the most (83.70%) of reported AIDS cases. The other routes were injecting drug users (4.72%), transmission of HIV from mother-to-child (4.31%), and others and unknown risks (7.27%) (from AIDS Division, Bureau of AIDS, TB and STDs, Department of Diseases Control, Ministry of Public Health. www. aidsthai.org). Whereas AIDS is still being incurable disease, controlling the spread of HIV-1 is important to confine the number of new infected cases. Early diagnosis of HIV-1 infected individuals is one of the strategies to prevent transmission of the virus.

Laboratory methods that are available to screen blood, diagnose infection, and monitor disease progression in HIV infected individuals can be classified into (a) antibody detection, (b) antigen detection, (c) viral nucleic acid (viral genomic RNA or proviral DNA) detection, and (d) T-lymphocyte numbers determination. Among these methods, antibody detection is the most widely used, convenient, and effective way to identify HIV infection. An enzyme linked immunosorbent assay (ELISA) technology is the most commonly used to screen for HIV infection because of its high sensitivity and suitability for testing large numbers of samples (Schochetman *et al.,* 1989). The other technologies such as agglutination, membrane ELISA, and immuno-chromatography, have been applied in many rapid test kits, which can be performed easily without instrumentation.

The antigens used in HIV-1 diagnosis assays are derived from viral structural proteins. The most important antigens are envelope glycoprotein gp120 and gp41 because these antigens induce antibody response earlier than other antigens. Moreover, anti-gp120 and anti-gp41 antibodies are persisting last long throughout the infection. In addition, WHO criteria for interpretation of HIV-1 infection by Western blot assay use the presence of anti-gp120 and anti-gp41 antibody as markers for positive result (World Health Organization, 1990).

Sources of antigens for HIV diagnostic assays included whole viral lysate or purified viral proteins from cell culture, recombinant proteins, and synthetic peptides. However, recombinant proteins and synthetic peptides have been increasingly used because of low risk of exposure to the hazardous virus in production process. In addition, using the selective HIV antigen(s), which has been proved not to cross-reactive with other pathogens or human proteins can increase specificity of the test. There is a limitation in using synthetic peptide for the HIV antigen because it represents only linear antigenic epitope, but not conformational epitope. Principally, recombinant protein is supposed to be the most appropriate representative for the antigen in diagnostic assays.

Recombinant protein production

Proteins of interest can be mass-produced efficiently because of advance in recombinant biotechnology. Usually, foreign proteins can be synthesized in prokaryotic system (microbial cell culture) and eukaryotic system (yeast, mammalian cell, insect cell, plant tissue culture, transgenic plant, and transgenic animal). Selection of the most appropriate method for commercial production requires case-by-case analysis. A wide range of factors must be considered, including cost of production, market volume, efficacy, safety and stability of the product, and whether the foreign protein has different biochemical or pharmacological properties compared with material from existing sources or natural one.

E. coli expression system is a popular and well understood prokaryotic system for protein expression. Prokaryotic expression provides a convenient system to synthesize eukaryotic proteins in cost effective. However, bacteria do not have post translation modification (glycosylation), therefore, recombinant proteins from this system lack many of immunogenic properties, 3D conformation, and other features exhibited by authentic eukaryotic proteins.

Eukaryotic expression, including yeast, insect, mammalian, and plant, overcome many of those limitations in *E. coli* expression system. Yeast expression system provides near native animal glycosylation. However, yeast culture requires expensive complex media and bioreactor. Mammalian cell and insect cell culture provide more authentic proteins, but highly cost and difficult to scale up. Transgenesis in animals, remains a complex technique that is limited to a few laboratory models such as mice, rat, round worm (nematode), and fruit flies (drosophila), and also used on a few large, domesticated animals (cow, sheep, goats, etc.). In addition, there are strong ethical considerations in using transgenic animals and awareness of animal disease transmission.

It is participated that the advances in the genetic engineering and the demand for large quantities of new or improved diagnostics and therapeutics in the healthcare and pharmaceutical markets will lead to a rapid expansion in recombinant protein production. Recently, plant and insect are considered as viable expression systems for an economical alternative in large-scale protein production because they can be cultured easily at low cost.

Foreign protein production in plant

The development of genetic transformation technology for plant has facilitated the study of plant gene expression. As a result, the great progress toward the genetic design of plants with enhanced production traits, such as herbicide, insecticide, and

disease resistance. Consequently, several academic and industrial laboratories have begun experimenting with transgenic plants as a novel manufacturing system to express foreign proteins using in medical sciences. This feature is particular important because of the increase in demands of effective proteins for human scientific or medical purposes. Factors in favor of transgenic plant systems as sources of human-derived protein include: (1) potential of large-scale and low cost biomass production using agriculture, (2) low risk of product contamination by mammalian viruses, blood-born pathogens and bacterial toxins, (3) capacity of plant cells to correctly fold and assemble multimeric proteins, (4) ability to introduce new or multiple transgenes by sexual crossing of plants; and (5) avoidance of ethical problems associated with transgenic animals and the use of animal materials (Fisher *et a*l., 1999; Fischer *et al.*, 2000; Cramer *et al.,* 1996). The successful production of several mammalian and human proteins in plants, including human serum albumin (Sijimons *et al.,* 1989), monoclonal antibody (Hiaat *et al*., 1989), erythropoietin (Matsumoto *et al.,* 1995), and human epidermal growth factor (Higo *et al*., 1993), have been reported. Using genetically engineered plants for the production of immunogenic proteins also provides a new approach for the delivery of a plant-based subunit vaccine (Tacker *et al*., 1998; Kapusta *et al*., 1999). Recent reports have shown that molecular farming in plant has practical, economic and safety advantage compared with conventional systems. A number of plant species have been used for generation and propagation of foreign proteins such as tobacco, potato, sunflower, turnip, soybean, rice, and maize. Recombinant proteins, such as cytokines, antibodies, vaccines, plasma proteins and enzymes, are currently under investigation or licensed for pharmaceutical use (reviewed by Ma *et al*., 2003).

Two basic strategies have been used to produce recombinant proteins in plant host (Hiatt *et al.*, 1989; Ma *et al*., 1994). One is based on the generation of transgenic plants by stable integration of a transgene in the plant genome (transgenic plant). Another uses plants as hosts for transient expression by using engineered plant viruses (chimeric virus). Chimeric virus can present short foreign peptide (9-25 amino acids long) on its surface (Beachy *et al.,* 1996). Some certain plant viruses such as tobacco mosaic tobamovirus (TMV) (Fitchen *et al.,* 1995) cowpea mosaic comovirus

(CPMV) (McLan *et al.*, 1995), and tomato bushy shunt virus (TBSV) (Joelson *et al.*, 1997) have been used specifically to produce short peptide for vaccine epitope. For chimeric plant virus strategy, consideration should be given to appropriate containment measures to prevent transmission to crop plants if potential plants virus are used as vectors for gene transfer.

The generation on transgenic plants often used two core technologies, particle bombardment and *Agrobacterium*-mediated transformation (Horsch *et al*., 1985; Miele 1997). Particle bombardment involves the acceleration of DNA-coated gold or tungsten microparticles, by an explosive or electrical discharge, or by pressurized helium, into the target tissue. This method delivers DNA directly into the tissue and widely used for transformation of cereals and legumes. *Agrobacterium*-mediated transformation is the most popular and widely adopted technique of creating transgenic plant species that are readily amenable to tissue culture. The great advantage of this method is that it offers the potential to generate transgenic cell at relative high frequency, without a significant reduction in plant regeneration rates. Moreover, The DNA transferred to the plant genome is defined and it integrates into the genome as a single copy (Walden *et al.*, 1995).

Agrobacterium tumifaciens is a common soil bacterium that naturally inserts its genes into plants and uses the machinery of plants to express those genes that the bacterium uses as nutrients. This process causes plant tumor called “crown gall disease”. Oncogenic strain of *A. tumifaciens* harbor large plasmid (140-235 kb) called tumor-inducing (Ti) plasmid. A part of Ti plasmid, the transfer DNA (T-DNA), is transferred to the plant, where it is stably maintained in the plant chromosome (Chilton *et al.,* 1980). *Agrobacterium* was initially believed to be restricted to the transformation of certain dicotyledons plants (flowering plants with two cotyledons in their seeds and broad leaves such as tobacco, potato, and tomato), but nowadays transformation of monocotyledonous plants (flowering plants with one cotyledon in their seeds and narrow leaves with parallel veins such as maize and rice) is routinely performed.

The Ti plasmid contains most of the genes required for tumor formation. Wounded plants exude phenolic compounds that stimulate the expression of the virulence gene (*vir*-genes), which are also located on the Ti-plasmid. The *vir*-gene encodes the set of proteins responsible for the excision, transfer and integration of the T-DNA into the plant genome. The genes response for tumor-formation in the T-DNA region is flanked at the both ends by 25 bp of nucleotides called T-DNA borders (left-border and right-border). These borders delimit the transferred DNA fragment. To modify Ti plasmid for gene transfer vector, the tumor-formation genes in T-DNA region were removed (called disarmed Ti plasmid) and replaced by gene(s) of interest. Additionally, some features (such as multiple cloning sites, bacteria and plant selectable markers, reporter genes, and origin of replication in *E. coli* and *A. tumifaciens*) are added into Ti plasmids for making them more applicable in transgenic work.

Two basic vector systems; co-integrative vector and binary vector, are used in *Agrobacterium*-mediated transformation (Walden *et al.,* 1995). Co-integrate vectors are constructed by homologous recombination between bacterial plasmid (called intermediate vector) that contain foreign gene and the T-DNA region of disarmed Ti plasmid in *Agrobacterium.* These vectors are not widely used because of their difficulty to manipulate and inefficiency of transformation. Binary vectors are plasmids that are stable in either *E. coli* or *Agrobacterium*. They contain cloning sites and marker genes flanked by the border sequences. Binary vectors allow manipulations to be made in *E. coli*, followed by transfer to *Agrobacterium* containing a helper plasmid with active virulence region for plant transformation. Binary vectors are increasingly been adopted because of their ease of manipulation.

Tobacco plant has an established history as a model system for molecular farming and is the most widely used species for the production of recombinant pharmaceutical proteins at the research-laboratory level (Fisher *et al.,* 2000). The main advantages of tobacco include the mature technology for gene transfer and expression, high biomass yield, and the potential for rapid scale-up owing to prolific seed production.

The first pharmaceutical relevant protein made in plants was human growth hormone, which was expressed, in transgenic tobacco (Barta *et al.,* 1986). Since then, many other human proteins have been produced in an increasingly diverse range of crops. In 1989, the first antibody was expressed in tobacco (Hiatt *et al*., 1989), which shown that plants could assemble complex functional glycoproteins with several subunits. The structural authenticity of plant-derived recombinant proteins was confirmed in 1992, when plants were used for the first time to produce an experimental vaccine of hepatitis B surface antigen (Mason *et al.,* 1992).

Production of recombinant hepatitis B surface antigen (HBsAg) is a good example for medical application of transgenic plant. HBsAg is heavy used for vaccination and for antigen in diagnostic methods. In this context, the concept of vaccine production by transgenic plant was introduced (Mason *et al.,* 1992) and greatly stimulating research in this field (Domansky *et al*., 1995; Mason and Arntzen 1995; Thanavara *et al.,* 1995; Ehsani *et al.,* 1997; Sojikul *et al.,* 2003). The recent report of Sojikul and coworkers shown the development of transgenic tobacco to produce high accumulation of recombinant HBsAg at 0.226 mg per g of total soluble protein (TSP) from tobacco cell. Although the amount of recombinant proteins produced by transgenic plants were low and vary (0.01 - 6.8% TSP) depending on transformant and induction protocol (Fisher *et al.,* 2003) but can be overcome with buck manufacture of agriculture.

For HIV, there was a report of the production of p24 capsid protein in transgenic tobacco using A*grobacterium*-mediated gene transfer (Zhagg *et al.,* 2002). Southern blot analysis confirmed the present of p24 coding sequence within the genome of transgenic lines and Western blot analysis of protein extracted from transgenic tobacco shown the reactive to p24-specific monoclonal antibody. Estimated yield of p24 protein was 3.5 mg per g of soluble leaf protein. Another study reported a new strategy for plant-based protein production by using fusion molecule of HIV-1 p24-immunoglobulin that gave product 13-fold higher than HIV-p24 expression alone (Obregon *et al*., 2006). Recently, there was a report of expression of HIV nef variants by intracellular targeting in tobacco cells (Marusic *et al*., 2007). For

HIV-1 env glygoprotein, Kim and colleagues reported the expression of fusion protein between cholera toxin B subunit and a fragment of HIV-1 gp120 (103 amino acids) (Kim *et al.*, 2004). So far, there was no report of the expression of the whole HIV-1 gp120 (505 amino acids) or gp41 (353 amino acids) in transgenic plant.

Foreign protein production in silkworm

The mulberry silkworm, *Bombyx mori* (family Bombycidae) has been domesticated for silk production for over 4000 years. The intense study of the silkworm provides knowledge of its biology and genetics, which is the most advance of any lepidopteran species. Therefore, the silkworm has been used as a lepidopteran model for research in physiology, development, endocrinology, biochemistry, genetics, and virology because of its large size, ease of rearing in the laboratory, and economic importance in silk production. It was among the first eukaryotic organisms to serve as a model system for cloning gene and studying the regulation of their expression (Nagaraju, 2002). Advance of biotechnology provides the possibility to create silkworms with heightened immunity to parasites or viruses and to improve the quality of silk. The various fiber proteins could also be transferred to the *Bombyx mori* to produce novel fiber for the textile industry. In addition, the massive production of silk proteins by silk gland in *Bombyx mori* may be used to synthesize proteins for diagnostic and therapeutic purposes.

B. mori is a holometabolous insects, it passes through complete metamorphosis from egg, larva and pupa to adult moth. The silkworm feeds and grows rapidly in only the larval stage (1^{st} to 5^{th} instars). But pupa and moth are non-feeding, therefore, they are not accompanied with increase in body weight and size. At the 5^{th} larva stage, silk gland has DNA replication 17-18 rounds without cell dividing. This phenomenon increases the DNA of the silk gland at 350,000 fold for the main function of silk production, and it can span silk fiber at 300-900 meters long/cocoon.

The silk gland is the specialized salivary gland that produces silk. This gland is composed of two secretary parts, the posterior silk gland (PSG) where fibroin is synthesized and the middles silk gland (MSG) that produces. Fibroin is a three-subunit complex constituted by the heavy chain (H-chain), the light chain (L-chain), and fibrohexamerin (formerly referred to as P25). H-chain is a fiber forming protein (4700 amino acid residues, 350 kDa), which accounts for 85% of the silk mass and its structure determines properties of silk fiber. L-chain is a 26 kDa polypeptide link to H-chain by disulfide bonds (Yamaguchi *et al*., 1989). Light chain is necessary for the secretion of protein. Fibrohexamerin is a glycoprotein (27-30 kDa), which interacts with H-chain/L-chain complex through hydrophobic interaction (Tanaka *et al*., 1999). The molar ratio of H-chain:L-chain:fibrohexamerin in silk threads is 6:6:1 (Inoue *et al.*, 2000). Sericins (65-400 kDa) are a family of hydrophilic peptides encoded by two genes, Ser-1 and Ser-2. Sericin glue the silk threads (Michaille *et al*., 1991) making up 20-30% of the silk protein.

Two systems; transient expression and transgene, are used for foreign protein production in silkworm. Transient expression uses the silkworm-specific baculovirus, *Bombyx mori* nuclear polyhedrosis virus (BmNPV), to carry the gene(s) of interest for infection the silkworm larvae. By using this in vivo expression system, a number of eukaryotic proteins, including human interferon (Maeda *et al.*, 1985; Hirouchi *et al.*, 1987), human growth hormone (Sumathy *et al.*, 1996), human adenosine deaminase (Medin *et al.*, 1990), human interleukin 2 (Pham *et al.*, 1999), bovine interferon (Murakami *et al.*, 2001), mouse interleukin (Miyajima *et al.*, 1987), viral proteins (Tada *et al*., 1988; Kudora *et al.*, 1989; Zhou *et al.*, 1995), and malarial proteins (Pang *et al.*, 2002), have been successfully expressed with biological activities comparable to those of the native counterparts. The expression level of these recombinant proteins are varied, but up to 13 mg/larva had been reported for the expression of luciferase (Palhan *et al*., 1995).

Transgenic organisms are powerful tools for the analysis of gene function. The application of transgenic methods to insects was limited to fruit fly (*Drosophila melanogester*) mainly because of the transposon vector *P element* (Rubin *et al.*,

1982). Gene transfer in insects began in the early 1980s with the transposon vector *P element*, which proved valuable in gene transfer among Drosophila species but was not active in other insects (O'Brochta *et al.,* 1996). Recently, several insect species other than *Drosophila melanogester* have been successfully transformed with other transposable element, such as the medfly, *Ceratitis capitata* has been transformed with *Drosophila Hydei Minos* element (Loukeris *et al*., 1995), *Drosophila virilis* with the *hobo* element (Lozovskaya *et al*., 1995) and *mariner* element (Lohe *et al*., 1996), and *Aedes agyptti* with mariner element (Coates *et al*., 1998) and *herms* element (Jasinskiene *et al.,* 1998). However, an effective stable germline transformation protocol for non-dipteran insects had not been achieved until the year 2000 by using *piggyBac* transposon-derived vector (Tamura *et al.*, 2000).

The *piggyBac* is a short inverted terminal repeat (ITR) transposon element, 2.5 kb long, with 13-bp ITR sequences and a 2.1-kb ORF (Cary *et al*., 1989; Elick *et al*., 1995). It has ability to jump from one place on a chromosome to another by recognizing the unique DNA sequence of TTAA, and then inserts itself and the included gene very accurately. The *piggyBac* was originally discovered as the causative agent of few polyhedra (FP) mutations in baculoviruses passed through the *Trichoplusis ni* TN-368 cell line and was found to have autonomous function based on transposition assay in cell lines (Fraser *et al.*, 1995). Transient excision assay in embryos also supported *piggyBac* function in several insect species with similar rates of movement in lepidopteran and dipteran species (Handler *et al.*, 1998).

In 2000, the international research team headed by Couble (Tamura *et al.,* 2000) had found a way to introduce foreign genes into the silkworm larvae using *piggyBac.* They reported the successful of using microinjection to introduce *piggyBac*-derived vectors, which contains the *B. mori* A3 cytoplasmic actin gene promoter fused to the green fluorescent protein (GFP) coding sequence into newly-laid eggs of *B. mori.* Nearly two percent of the larvae growing from injected eggs expressed GFP and transmitted this characteristic to subsequent generations. Southern blot analysis confirmed that the transgene was integrated into the *B. mori* chromosomes. After that, *piggyBac*-based vector was developed for its more

applicability for silkworm germline transformation (Thomas *et al.*, 2002; Imamura *et al.*, 2003).

Thomas and colleagues used *piggyBac* vector which contained 3xP3-EGFP marker for the screening of transgenic *B. mori*. 3xP3 is eye and nervous tissue-specific promoter, therefore, the transgenic silkworm could be observed for GFP expression in the ocelli and the ganglia of the abdominal nervous system of the all instar stages, and in the eyes of moth. They also demonstrated that the injection position of eggs had percent of positive GFP of the G_0 broods approximately 3.4% and 0.95% by injection at anterior and posterior of egg, respectively (Thomas *et al.*, 2002).

The hope for using silkworm as a bioreactor was lighten up by the report of the successful of transgene expression of the fusion of *procollagen mini* gene with *L-fibroin-GFP* (Tomita *et al.*, 2003). The overall rate of successful transgenesis was approximately 25%. The fibroin-procollagen-GFP fusion protein was synthesized specially in the silk glands and secreted into cocoons. The concentration of fusion proteins extracted from the cocoon was 36.7 µg/mg of total extracted protein or 8.4 µg/mg of dried cocoon or about 70 mg of dry weight protein per one cocoon. The recombinant protein was readily purified to homogeneity in a single step method because the cocoon is greater 95% protein and consists of only a few different proteins. With this method, silkworms could be modified to secrete stronger silk or to add proteins for pharmaceutical value to their silk. The protein could then be harvested directly from the cocoons. This system would cost very little compared to other methods of animal based farming.

Until now, the most efficient method to introduce foreign gene into silkworm eggs is the microinjection (Tamura *et al.*, 2000; Tomita *et al.*, 2003, Imamura *et al.*, 2003). However, this method needs specific equipment (double needle with volume control apparatus) and high experienced person to perform the injection. Gene transformation using electroporation mostly successfully applied in prokaryotic cells was reported in introducing *gfp* gene and artificial synthesized *fibroin-gfp* fusion gene

into silkworm eggs (Zhang *et al*., 1999; Zhao *et al*., 2001). The advantage of electroporation method is that large quantity of silkworm eggs can be transformed in a very short time. However, introducing foreign gene into silkworm eggs by electroporation was reported to be voltage dependent and shown big difference between the tested silkworm strains (Guo *et al*., 2004).

Since the silkworm is an only domesticated insect, which completely controlled by humans. Moreover, the adult moths are unable to fly. Therefore, the sensitive issue of releasing genes from genetically modified organisms (GMO) into the environment does not seem to be relevant for this species. Because of the availability of silkworm larva production in Thailand and the enhanced expression level *in vivo,* use of the *piggyBac*-silkworm expression system to produce protein of interest is an attractive option.

Experimental design

Expression unit for the entire coding sequence of HIV-1 gp120 and gp41 were constructed in separated vectors, which has been previous reported to work in tobacco and silkworm. The entire coding sequence was selected since it has both linear and conformational epitopes cover all HIV-1 envelop glycoprotein. Transformation of these constructs into tobacco and silkworm were checked for the presence of integration and expression.

1. Transgenic tobacco plant

Plant expression vectors are constructed from *pCAMBIA*-based vector to be contained insertion of HIV-1 gp120 or gp41 coding sequence under the control of *CaMV 35S* promoter and *Nos poly A* terminator. Expression unit of both gp120 and gp41 have the 5’end containing 90-bp signal peptide derived form HIV-1gp160 coding sequence, and the 3’ end containing histidine tag. The expression units were introduced into the genome of tobacco plant by *Agrobacterium*-based transformation using leaf-disc transformation technique under the selection of hygromycin. The

transgenic tobacco was checked for the integration and expression of HIV-1 gp120 or gp41.

2. Silkworm transformation

Silkworm expression vectors were constructed from *piggyBac*-based vector to be contained fusion sequence of silkworm *L-fibroin* and gp120 or gp41 coding sequence. The gp120 or gp41 coding sequence was inframed-inserted into *L-fibroin* gene to make fusion protein expression unit under the control of *L-fibroin* promoter and terminator. The enterokinase recognition site (Asp-Asp-Asp-Asp-Lys) was flanked at the both end of gp120 or gp41 for further cleaving of gp120 or gp41 from L-fibroin. The constructions also designed to be contained restriction enzyme recognition site (*Apa*I and *Sac*II) flanking outside of enterokinase recognition site for further using of this construct with other interested proteins. These expression units were inserted into pBac[3xP3-DsRedaf], which had a reporter gene of DsRed under control of the eye and nervous tissue-specific promoter 3xP3. Therefore, Red-eye of moth was used as a marker for positive transformation. The contractions containing expression unit were introduced into the genome of silkworm by *piggyBac*-mediated germline transformation using microinjection or electroporation technique. The helper plasmid pHA3PIG was used to provide the transposase. The transgenic silkworms were checked for the integration and expression of HIV-1 gp120 or gp41.

Research place

1. National Institute of Health, Department of Medical Sciences, Ministry of Public Health.
2. Department of Biochemistry, Faculty of Science, Bangkhen Campus, Kasetsart University.
3. Center for Agricultural Biotechnology, Kamphang Saen Campus, Kasetsart University.

Duration of research

June 2004 – April 2007.

MATERIALS AND METHODS

Materials

1. Plant and animal materials

1.1 Tobacco plant: *Nicotiana tabacum* cv. Xanthi

1.2 The silkworm, *Bombyx mori*, pnd-w1 strain; a nondiapausing mulberry silkworm, which is mutant for non-pigmented eggs and eyes (gift from National Institute of Agrobiological Sciences, Tsukuba, Japan)

2. Other materials

2.1 Plasmid vectors

2.1.1 HIV-1 whole-genomic clone in pCR®-Blunt plasmid (95TNIH022; GenBank Accession number AB032740)

2.1.2 Cloning reagent (Ligation high™, TOYOBO; Cat.LGK-101)

2.1.3 pGEM® T Easy vector System (Promega; Cat.A1360)

2.1.4 pCR8-TOPO cloning system (Invitrogen; Cat. K2520-20)

2.1.5 Plant expression vector (pCAMBIA1303, CAMBIA GPO., Canberra, Australia)

2.1.6 *PiggyBac*-derived vector; pBac[3xP3-DsRedaf] (gift form Professor Toru Shimada, Laboratory of Insect Genetics and Bioscience, Univ. of Tokyo, Japan)

2.1.6 Helper plasmid (pHA3PIG) (gift from Dr. Toshiki Tamura, National Institute of Agrobiological Sciences, Tsukuba, Japan)

2.2 Bacterial strains

2.1.1 Bacterial strain JM109 *E. coli* (Promega; Cat. P9751)

2.1.2 Bacterial strain Mach1™ - T1 Phage resistant (Invitrogen; Cat. C8620-03)

2.1.3 *Agrobacterium tumifaciens* strain AGL1 containing helper plasmid for binary vector system

2.3 Bacterial culture media

2.3.1 Bacterial culture media (Diffco LB, Lennox; Cat; 240230)

2.3.1 Bacto agar (Diffco; Cat. 214010)

2.3.3 S.O.C. medium (Invitrogen, Cat. 15511-034)

2.4 Plant tissue culture media and plant hormone (MS media, Sigma-Aldrich; Cat. M5519)

2.5 Antibiotic (Kanamycin, Amplicilin, Hygromycin, Cefotaxime)

2.6 Specific designed primers (see appendix A, table1 and table 2)

2.7 Restriction enzyme; *Nhe* I, *Nco* I, *Asc* I, and *Fse* I (New England Biolab (NEB, MA, USA)

2.8 DNA polymerase enzyme

2.8.1 Immolase™ DNA polymerase (BioLine; Cat. BIO-21047)

2.8.2 Bio-X-Act™ Long DNA polymerase (BioLine; Cat. BIO- 21050)

2.9 RT-PCR kit

2.9.1 Superscript III™ One-step RT-PCR system with Patinum Taq DNA polymerase (Invitrogen; Cat. 12574-026)

2.9.2 Thermoscript™ TR-PCR system with Patinum Taq DNA polymerase Invitrogen; Cat. 11146-057)

2.10 dNTP (Promega; Cat. U1240)

2.11 DNA size marker (Hyperladder™ I, Bioline; Cat. BIO-33026)

2.11 Protein size marker (TriChromRanger™ Prestained Molecular Weight Marker Mix, Pierce; Cat. 26691)

2.12 Sequencing reagent (The BigDye™ terminator v3.1 Cycle Sequencing kit, Applied Biosystems; Part No. 4337455)

2.13 Sequencing reaction clean-up spin column (DyeEx™ 2.0 Spin Kit, QIAGEN; Cat. 63204)

2.14 RNA purification reagent/kit (TRIzol® Reagent, GIBCOBRL; Cat. 15596-026)

2.15 MasterPure® Plant Leaf DNA Purification kit (Epicenter Madison, Cat. MPP92010)

2.16 Mosquito buffer (see appendix D3)

2.17 Plasmid purification kit

2.17.1 QIAprep Spin Miniprep kit (QIAGEN; Cat. 27106)

2.17.2 PureYield® Plasmid Midiprep System, Promega; Cat. 226358)

2.18 PCR purification kit (QIAquickPCR Purification Kit, QIAGEN, Cat. 28106)

2.19 Gel Extraction kit (QIAquick Gel Extraction kit, QIAGEN; Cat. 28704)

2.20 SDS-PAGE reagent and buffer (see appendix D4.)

2.21 Immuno blot membrane (Immune-Blot® PVDV membrane, BioRad; Cat. 162-0177)

2.22 Protein detection reagent/kit Coomassie® Plus Protein Assay Reagent Kit, Pierce Biotechnology; Cat. 23236)

2.23 HIV WesternBlot Kit (HIB Blot® 2.2 Genelabs Diagnostics, Singapore)

2.24 Agarose

2.24.1 LE agarose (Seakem; Cat. 50004)

2.24.2 Nusive3:1 Agarose (Seakem; Cat. 50090)

3. Equipments

1 Biohazard Class II cabinet (Faster BHD48, Faster, Italy)

2 Autoclave (Tomy, Japan)

3 PCR machine (9700 Applied Biosystems, CA, USA)

4 Refrigerated microcentrifuge (Himac CF15R, Hitachi, Japan)

5 Refrigerated centrifuge (CF16RX, Hitachi, Japan)

6 Microscope (Nikon)

7 –80°C freezer (Sanyo, Japan)

8 –20°C freezer (Revco, NC, USA)

9 Refrigerator (Sharp, Thailand)

10 Vortex (FINEVORTEX, Korea)

11 Heat block (Digital Dry Bath, USA)

12 UV/Vis spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies, LA, USA)

13 Incubator (Yamato, Japan)

14 Orbital shaker (Forma Scientific, UK)

15 Shaker water bath (SWB5050, LabNet, NJ, USA)

16 Electrophoresis set (Mupid, Japan)

17 DNA and protein blotting transfer system (BioRad, CA, USA)

18 Gel documentation (UVIdoc, UVItec, UK)

19 Automate DNA Sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems, CA, USA)

20 Gene Pulser II electroporation system (BioRad, CA, USA)

21 Microapplicator

Methods

Tobacco plant transformation

1. Tobacco plant culture

Tobacco plant (*Nicotiana tabacum* cv. Xanthi) were propagated *in vitro* in Murashige and Skoog (MS) media at 25^{o}C, and subculture every 3-4 weeks.

2. Construction of plant expression vector containing HIV-1 gp120 or gp41 coding sequence

2.1 Sub-cloning of HIV-1 gp120 and gp41 coding sequences

A. HIV-1 gp120

HIV-1 gp120 coding sequence (1545 bp) was amplified from a plasmid clone containing the whole-genome of HIV-1 CRF01_AE [95TNIH022; GenBank accession number AB032740] (Auwanit *et al.*, 2001) with primers NcoI-gp160F and NheI-gp120R (see Table A1 for primer sequences) by using proofreading DNA polymerase (Bio-X-Act long DNA polymerase, see Appendix D1 B for PCR

mixture preparation). The thermal profile for amplification was as follow: 95^{o}C for 5 min; 25 cycles of 95^{o}C for 30 sec, 55^{o}C for 15 sec, 72^{o}C for 2 min; and 72^{o}C for 5 min and then soak at 4^{o}C.

B. HIV-1 gp41

The same plasmid clone of HIV-1 CRF01-AE was used for template to amplify gp-41 coding sequence (1092 bp). To add signal peptide (sequence was derived from the first 90-bp of gp160 coding sequence) into 5' terminal of gp-41 coding sequence, two-step amplification with a proofreading DNA polymerase was used. First, primer 3HSP-gp41F and NheI-gp160R were used in the amplification profile as follow: 95^{o}C for 5 min; 25 cycles of 95^{o}C for 30 sec, 55^{o}C for 15 sec, 72^{o}C for 2 min; and 72^{o}C for 5 min and then soak at 4^{o}C. The 3-ul of the first PCR product was used as template for the second PCR with primers NcoI-5HSP and NheI-gp160R in the amplification profile as follow: 95^{o}C for 5 min; 25 cycles of 95^{o}C for 30 sec, 55^{o}C for 15 sec, 72^{o}C for 2 min; and 72^{o}C for 5 min and then soak at 4^{o}C (see Table A1 for primer sequences and Appendix D1. B for PCR mixture preparation).

PCR products were electrophoresis in 1% agarose gel and 0.5X TBE for the expected size of 1545-bp for gp120 and 1182-bp for gp41 by comparing with DNA size marker (HyperLadder I, see Appendix B). The PCR products were ligated with pGEM® T easy vector using T4 ligase (TA-cloning, Promega, see Appendix D2 A), and then transformed into chemically competent *E. coli* (JM109) by heat shock method. Ten white colonies were selected and checked for the existence of insert by colony-PCR using primer M13-F and M13-R. The gp120-pGEM-T and gp41-pGEM-T plasmid vector were purified by QIAprep Spin Miniprep kit and checked for authentic sequences of gp120 or gp41 using Bigdye terminator v3.1. (see Appendix D1. C for sequencing reaction mixture preparation) and 3100 Prism automate sequencer. The clone that gave no-error sequence for gp-120 or gp-41 was selected for further step.

2.2 Construction of plant expression vectors, pCAMBIA_HIV gp120/gp41

The selected clone was cut with *Nco*I and *Nhe*I, and ligated into pCAMBIA1303 (GenBank Accession number AF234299), which was also cut with same enzymes by using Ligation high reagent™ (see appendix D2. C). The ligation mixture was transformed into chemically competent cell (Mach-1, Invitrogen) by heat-shock method and selected under kanamycin (50 mg/l). The colonies that gave expected product size (1.5 kb for gp-120 and 1.2 kb for gp-41) by colony-PCR using primer pCAMBIA-Sq1 and pCAMBIA-Sq2 (see Appendix D1. A), were then subsequently purified and checked for the entire insert sequences and the joining area.

In this plant expression vector; pCAMBIA_HIV-1 gp120 or gp41, the expression of HIV gp120 or gp41coding sequence was under the control of cauliflower mosaic virus (CaMV) 35S promoter and *Nos* poly-A terminator. The diagram of plant expression; pCAMBIA-HIV gp120/gp41vector was shown in Fig. 4.

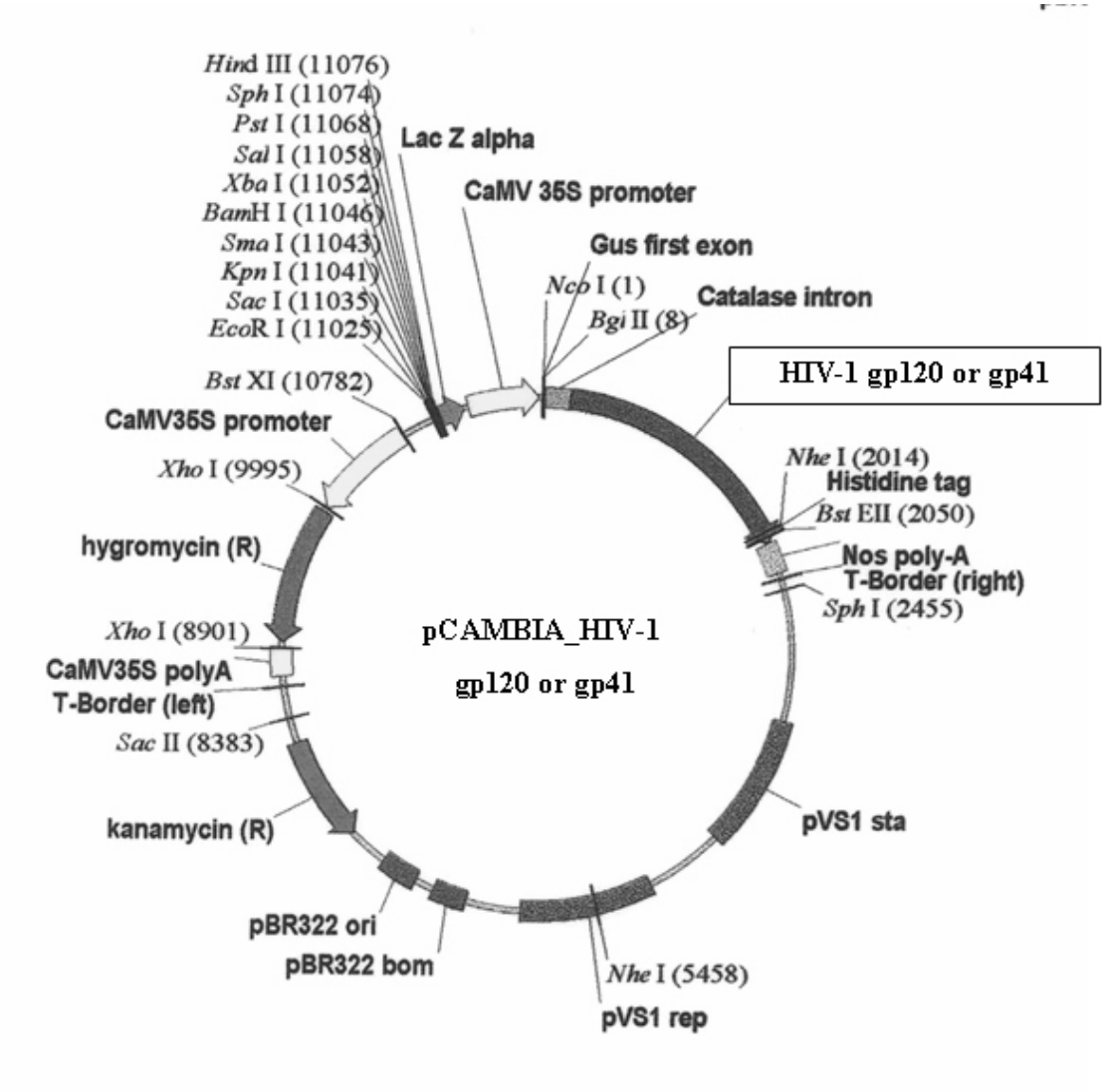

Figure 4 The construction of plant expression vector pCAMBIA_HIV-1 gp120 or gp41

3. Tobacco plant transformation

3.1 Preparation of *Agrobacterium tumefaciens, AGL-1* competent cell for electroporation

From frozen stock culture, *A. tumefaciens* strain AGL1 which containing helper plasmid was streaked on LB medium, and incubated at 28^{o}C and

dark for 1 – 2 days. Single isolate colony was picked for culture in 4 ml of LB at 28^{o}C, 220 rpm for overnight (14-18 hours). The culture was transferred to 30 ml of LB, and incubated at 28^{o}C, 220 rpm for 5 hours (OD_{600} = 0.5). After centrifugation at 3,000 rpm, 4^{o}C for 15 minutes, the supernatant was poured off, and replaced with 30 ml of cold sterile 30 % glycerol. After well mixing, the suspension cells were centrifuged at 3,000 rpm, 4^{o}C for 10 minutes, and then the supernatant was removed. Cells were washed again before adding 500 µl of cold sterile 10 % glycerol. The cell suspension was then aliquot into a volume of 40 µl per vial into a pre-cool microcentrifuge tube and kept at - 80^{o}C.

3.2. Electroporation of plant expression plasmid into *A. tumifaciens, AGL*

For each sample to be electroporated, placed 0.2 cm electroporation cuvette on ice, and placed a 17X100 mm tube with 1 ml of S.O.C. medium at room temperature. *A. tumefaciens, AGL-1* competent cells (40 µl) were thawed on ice,1-2 µl (100 ng) of pCAMBIA_HIV-1 gp120/gp41 was added, and incubated on ice for 1 minute. The mixture of cells and plasmid was transferred to a cold electroporation cuvette, and then placed in the pulse-chamber of Gene Pulser II electroporation system (BioRad), which was pre-setup at a condition as follow: constant resistance at 200 Ω, capacitance at 25 µF, and Voltage at 2.5 kV. After pulsing, the mixture was immediately transferred to a 17 X 100 mm tube containing 1 ml of SOC medium, and incubated at 28^{o}C, 220 rpm for 2-4 hours. After centrifuged at 3,000 rpm for 1 minute and discarded supernatant, 100 µl of LB was added. The cells was then spread onto LB plate containing 100 mg/ml kanamycin and incubated at 28^{o}C, dark for 2 days. Colony-PCR was performed to check for the transformant *A. tumifaciens*, the positive clone was selected for further experiments.

3.3. Leaf-disc transformation.

The *A. tumefaciens* strain AGL-1 containing plant expression vector (pCAMBIA_HIV-1 gp120 or gp41) and helper plasmid was cultured in 50 ml of LB broth with kanamycin (50 mg/l) at 28^{o}C, 220 rpm for 14-16 hours (OD = 0.5). After

centrifuge at 3,500 rpm for 1 minute, pour off the supernatant and resuspend the cells in 50 ml of MS media. From 4-week culture tobacco plants, leave were cut into small pieces of 0.5 X 0.5 cm and soaked in *A. tumefaciens* suspension for 10 minutes. The excess of *A. tumefaciens* was removed by placing the leaf-disc on sterile towel paper, and then placed on MS agar (co-cultivation) at 25 pieces per plate. After incubation at 25-27^{o}C and dark for 2 days, leaf-discs were washed in sterile MS broth for 10 minute, and then washed in MS both with cefotaxime (200 mg/l) for 10 minutes. The leaf-discs were transferred to MS media containing BA (0.5 mg/l) and cefotaxime (200 mg/l), and incubated at 25-27^{o}C and dark for one week to eliminate *A. tumefaciens.* The leaf-discs were ten transferred to MS media containing BA (0.5 mg/l), cefotaxime (200 mg/l), and hygromycin B (200 mg/l), and incubated at 25-27^{o}C and dark. The media were changed every 2 weeks for 2 months, and then subcultured the callus to the MS media for inducing growth (shoot and root) of tobacco plant. The completely elimination of *A. tumefaciens* was checked by incubating the transformed tobacco leaf in LB broth at 28^{o}C, dark for 2 days.

4. Transgene analysis

Genomic DNA from transgenic tobacco leaves were extracted (MasterPure Plant Leaf DNA Purification kit) and subjected for nested PCR using pCAMBIA-Sq1/pCAMBIA-Sq2 primer for outer PCR and HIV-1 specific primers; NcoI-gp160F and NheI-gp120R for gp120 and NcoI-gp160F/NheI-gp160R for gp41 (see Table A1 for primer sequences and Appendix D for PCR mixture preparation). The inner PCR products were purified (QIAquickPCR Purification Kit) and subjected for sequencing to confirm the entire sequences of gp120 or gp41.

5. Gene expression analysis

5.1 mRNA

Total RNA was extracted form tobacco leaves (TRIzol reagent) and used as templates for RT-PCR (Superscript III One step RT-PCR system) with HIV

specific primers; Nco I-gp160F and Nhe I-gp120R for gp120 and Nco I-gp160F/Nhe I-gp160R for gp41. The presence of ubiquitin RNA was used as a house keeping gene expression control (Brunner *et al*., 2004) (See primer sequences in Table A1).

5.2 Recombinant protein analysis

Approximately 300 mg of fresh transgenic tobacco leaves were crushed in 200 µl of extraction buffer (50mM Tris HCL, pH 9.0, 150 mM NaCl, 1 mM EDTA, 20 Mm DTT and 1% PVP). Total soluble protein was determined by Bradford dye-binding procedure (Coomassie Plus Protein Assay Reagent) using bovine serum albumin as a standard.

Dot blot and western blot were performed to determine the immunogenic property of HIV-1 gp120. Extracted proteins were directly blot (dot blot), and also transferred from SDS-PAGE (western blot) onto a membrane (ImmunoBlot PVDV membrane, Bio-Rad), then incubated with serum from HIV-1 infected patient. The immune complex, if presented, would be detected by anti-human immunoglobulin conjugated with horseradish peroxidase (HIV Blot 2.2; Genelabs) which change the substrate into purple substance.

Silkworm transformation

1. Silkworm rearing

The silkworms, pnd-w1 (1st to 5th instar larva) were reared with fresh mulberry leaves at 28°C (see appendix C for silkworm life cycle and detail of rearing). The 5^{th} instar larva was let to spin the cocoon for 4-6 days, and then the cocoon was cut to collect the pupa. Male and female pupa were separated until theirs emergence to moth. After mating for 3-6 hours, female moths were let to lay eggs on the wax paper. These eggs were used for transformation or strain maintenance for the next experiments.

2. Construction of the expression vector containing HIV-1 gp120 or gp41 coding sequence (pBAC_FiL-gp120 and pBAC_FiL-gp41)

2.1 Amplification of L-fibroin promoter, 5'L-fibroin, 3' L-fibroin, and HIV-1 gp120/gp41

A. L-fibroin promoter

Genomic DNA of p50 silkworm was used as a template for amplification of L-fibroin promoter by using proofreading DNA polymerase (Bio-X-Act long DNA polymerase, see Appendix D1.) and primers FiL-P-F and FiL-P-R, in the thermal profile as follow: 95^{o}C for 2 min; 25 cycles of 95^{o}C for 30 sec, 40^{o}C for 30 sec, 72^{o}C for 1 min; and then 72^{o}C for 5 min and soak at 4^{o}C (see Table A2 for primer sequences).

B. 5' L-fibroin

Total RNA were extracted from silkworm pnd-W1 larva stage 5 by TriZol reagent, and were used as template for making cDNA by using Thermascript reverse transcriptase enzyme with specific primer for L-fibroin (FiL-5'-F). Then, 5 µl of cDNA was use to amplify 5' fibroin coding sequence by using proofreading DNA polymerase (Bio-X-Act long DNA polymerase) and primers, FiL-5'-F and FiL-5'-R in the thermal profile as follow: 95^{o}C for 2 min; 10 cycles of 95^{o}C for 30 sec, 45^{o}C for 30 sec, 72^{o}C for 1 min; 10 cycles of 95^{o}C for 30 sec, 50^{o}C for 30 sec, 72^{o}C for 1 min; 10 cycles of 95^{o}C for 30 sec, 55^{o}C for 30 sec, 72^{o}C for 1 min, and then 72^{o}C for 5 min and soak at 4^{o}C (see Table A2 for primer sequences).

C. 3' L-fibroin

Total RNA same as for 5' fibroin amplification were used as template to make cDNA by using Thermascript reverse transcriptase enzyme with specific primer for L-fibroin (FiL-3'-F). Then, 5 µl of cDNA was used to amplify 5'

fibroin coding sequence by using proofreading DNA polymerase (Bio X Act long DNA polymerase) and primers, FiL-3'-F and FiL-3'-R in the thermal profile as follow: 95^{o}C for 2 min; 15 cycles of 95^{o}C for 30 sec, 45^{o}C for 30 sec, 72^{o}C for 30 sec; 15 cycles of 95^{o}C for 30 sec, 50^{o}C for 30 sec, 72^{o}C for 30 sec; and then 72^{o}C for 5 min and soak at 4^{o}C (see Table A2 primer sequences).

D. HIV-1 gp120 and HIV-1 gp41

HIV-1 gp120 and gp41 coding sequence were amplified by using primers gp120-F and gp120R for gp120 coding sequence and gp41F and gp41R for gp41 coding sequence by proofreading DNA polymerase (Bio-X-Act long DNA polymerase) in the thermal profile as follow: 95^{o}C for 2 min; 15 cycles of 95^{o}C for 30 sec, 50^{o}C for 30 sec, 72^{o}C for 1 min; 15 cycles of 95^{o}C for 30 sec, 55^{o}C for 30 sec, 72^{o}C for 1 min 30 sec; and then 72^{o}C for 5 min and soak at 4^{o}C (see Table 2 in Appendix A for primer sequences).

2.2 Joining the fragments

PCR products of 4 fragments (2.1 A to 2.1 D) were joined together to make the fusion coding sequence of L-fibroin and HIV envelope glycoprotein *(FiL-gp120* or FiL-gp41), which the HIV-1 gp120 or gp41 was inserted into L-fibroin between nucleotide sequence position 726/727 (amino acid position 242/243). The purified PCR products of each fragment were joined by using a two-step PCR. First, 2 µl of each four fragments in 25 µl PCR mixture without primers were joined and amplified by using proofreading DNA polymerase in the thermal profile as follow: 10 cycles of 95^{o}C for 1 min, 55^{o}C for 1 min, 72^{o}C for 4 min. Then 25 µl of PCR mixture with primers FiL-P-F and FiL-3'-R was added and amplified for 10 more cycles in the same thermal profile. The diagram of fusion fragments is shown in Fig. 5.

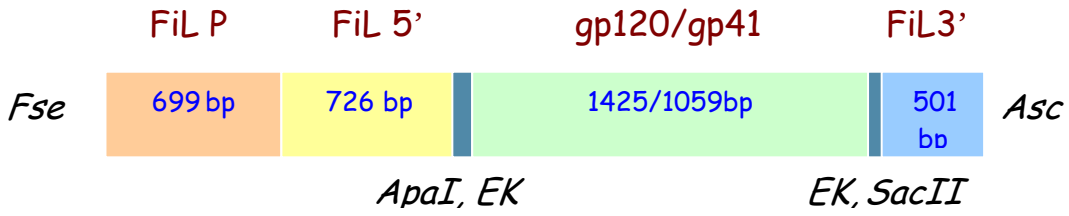

Figure 5 Diagram of fusion *FiL-gp120 or FiL*-gp41, showing insertion site of HIV-1 gp120 or gp41, and restriction enzyme and enterokinase recognition sites.

PCR products were electrophoresis in 1% agarose gel, for checking the expected size of 3286 bp for FiL-gp120 and 2923 bp for FiL-gp41. The PCR products were ligated with plasmid vector (pCR8, TOPO cloning kit), and then transformed into chemically competent *E. coli* (Mach-1, Invitrogen). Twenty four colonies were selected and checked for existence of the insertion by colony-PCR with M13-F and M13-R primers. The pCR8_FiL-gp120 and pCR8_FiL-gp41 plasmid vectors were purified (QIAprep Spin Miniprep kit), and checked for authentic sequences of the fusion of L-fibroin and gp120 or gp41 using Bigdye terminator v3.1 and 3100 Prism automate sequencer. The clone that gave non-error sequence for gp-120 and gp-41 was selected for further step.

2.3 Construction of silkworm expression vectors, pBAC_FiL-gp120 and pBAC_FiL-gp41

The selected clone was cut with *Asc* I and *Fse* I and ligated into pBac[3xp3-DsRedaf] (Tomita *et al.*, 2003), which was also cut with *Asc* I and *Fse* I using Ligation high™ reagent. The ligated vector was transformed into Mach-1 competent cells by heat-shock method and selected with ampicillin resistance. The colonies were checked for the presence of insertion by using primer pBac-F and pBac-R with the thermal profile as follow: 95^{o}C for 5 min; 30 cycles of 95^{o}C for 15 sec, 50^{o}C for 15 sec, 72^{o}C for 3 min, then soak at 4^{o}C. The clones that gave expected size (3.5 kb for FiL-gp120 and 3.2 kb for FiL-gp41 were then subsequently purified

and checked for the entire insert sequences and the joining area (see Table A2 for primer sequences).

In this silkworm expression vector, pBAC_FiL-gp120 and pBAC_FiL-gp41, the expression of the fusion of L-Fibroin and HIV gp120 or gp41 coding sequence was under the control of L-fibroin promoter and SV40 poly-A terminator. Diagram of silkworm expression vector is shown in Fig. 6.

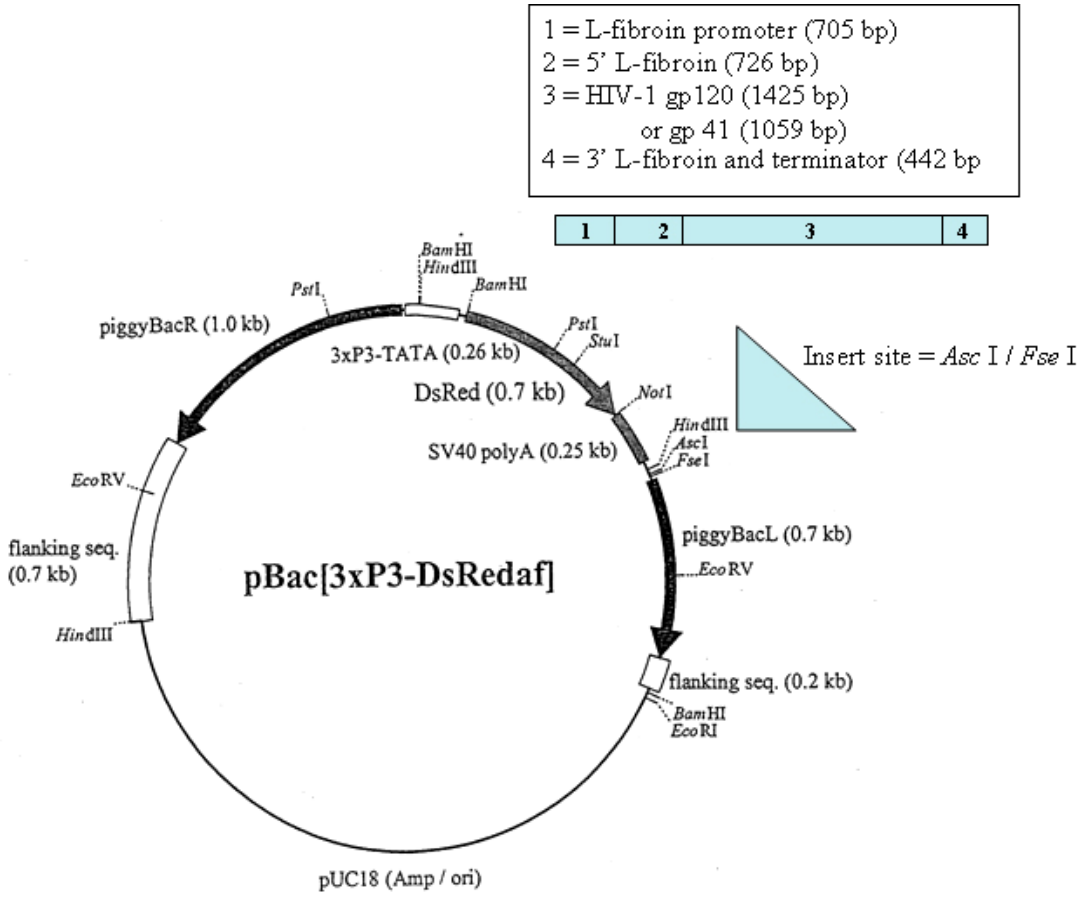

Figure 6 The construction of silkworm expression vector *pBAC_FiL-gp120* or *gp41*. HIV-1 gp120 or gp41 fusion with L-fibroin expression unit was inserted into *pBac*[3xP3-DsRedaf] at *Asc*I/*Fse*I cloning site.

3. Silkworm transformation

3.1 Microinjection

Silkworm expression plasmid; pBAC_FiL-gp120 or gp41 and transposase-producing helper plasmid (pHA3PIG) were purified by PureYield Plasmid Midiprep System (Promega) and eluted with phosphate buffer (0.5 mM phosphate buffer, 15 mM KCl, pH7) (Tamura *et al*., 2000). Equal volume of these two plasmids (each at concentration of 200 ng/µl) were mixed and injected into pre-blastoderm silkworm embryos within 4-6 hours after oviposition (Tamura *et al.*, 2000). Briefly, eggs were washed with cold sterile DW, 3% formaldehyde solution, and cold sterile DW. The eggs were fixed to glass slide with latex glue in the position that let the anterior site (curve side of D-shape) pointed out to the edge of slide. The eggs were injected with the mixture of plasmids by using a syringe with needle no. 30G, which attached with microapplicator (applied from Raksapon, 2007). The plasmid mixture was drawn out at about 200 nl, and let to diffuse into the eggs for a while before sealing with glue. Injected eggs were kept in a dark plastic chamber at 25^{o}C. Hatched larvae (G_0) were allowed to develop to moths by feeding with fresh mulberry leaves. G_0-moths were mated within the same family.

3.2. Electroporation

pBAC_FiL-gp120 or gp41 and transposase-producing helper plasmid (*pHA3PIG*) were purified by PureYield Plasmid Midiprep System (Promega) and eluted with nuclease free water. Equal volume of these two plasmids (each at concentration of 400 ng/µl) was mixed for preparing of electroporation mixture (Guo *et al*., 2004). Within 3-5 hours after oviposition, eggs were washed with cold sterile DW, removed into sterile 5 ml tube, and washed again several times with cold sterile DW. Approximately 400-600 eggs were transferred into a 0.4 cm electroporation cuvette, which pre-cold on ice. After removal of DW as much as possible, 400 µl of electroporation mixture was added. The cuvette was chilled in wet ice for 3-5 min before placed in the pulse-chamber of Gene Pulser II electroporation system

(BioRad), which was pre-setup at a condition as follow: constant resistance at 200Ω, capacitance at 25 μF, and voltage at 1,000 V, and then pulse once. The eggs were dried on towel papers, and then transferred to plastic Petri-Disc, which then placed in a dark plastic box at 25^{o}C. Hatched larvae (G_0) were allowed to develop to moths by feeding with fresh mulberry leaves. G_0-moths were mated within the same family.

4. Transgene analysis

G_0 and G_1-moths were observed for red-eyes by naked eyes screening. Genomic DNA was extracted from the moth after mating and laying eggs. Briefly, the thorax of moths were cut and crushed in 1 ml of Mosquito reagent. After spin down the crude protein, DNA in supernatant was purified by phenol-chloroform extraction. DNA samples was diluted to 50 ng/μl and subjected to PCR. The quality of extracted DNA was determined by the L-fibroin promoter amplification n. Transgenesis was determined by nested PCR using HIV-gp120F/HIV-gp120R or HIV-gp41F/HIV-gp41R as outer primers and HIV-gp120F2/HIV-gp120R2 or HIV-gp41F2/HIV-gp41R2 as inner primers (see Table 2 in Appendix A for primer sequence, and Appendix D1.A for PCR mixture preparation). The amplification profile of both HIV-gp120 and gp41 inner PCR was the same as follow: 95^{o}C for 5 min; 30 cycles of 95^{o}C for 15 sec, 50^{o}C for 15 sec, 72^{o}C for 1min, then soak at 4^{o}C.

Remark**:** In case of commercial kits using, detail procedures were followed the instruction manual provided from the companies.

RESULTS AND DISCUSSIONS

Results

Tobacco plant transformation

1. Construction of plant expression vector

The plant expression vector; pCAMBIA_HIV-1 gp120 and pCAMBIA_HIV-1 gp41, which generated from pCAMBIA1303 as shown in Fig. 4 were constructed. These constructs were sequence checked, and selected for the clones that HIV-1 gp120 or gp41 coding sequence was inframed with histidine tag. The entire sequence did not have any unexpected base as shown in Fig. 7 and Fig. 9. Virtual translation of these expression vectors (Fig. 8 and Fig. 10) did not show any mutations, which generate the stop codon or frameshift.

The correct sequence of HIV-1 gp120 and gp41 and the translation initiation (Kozak sequence) was very important to ensure for the further steps of transcription and translation. In this study, many mutation clones were found (data not shown) that confirmed the necessary of sequence checking for every steps of gene manipulation. Therefore, if there were any failure of expression, the un-correct sequences would be excluded for the cause of unsuccessful.

CCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGAACACGGGGGACTCTTGACCATGGTAGATCT
*GACTAGT*ATGAGAGTGAAGGAGACACAGATGAATTGGCCAAACTTGTGGAAATGGGGGACTTTGATCCT
TGGGTTGGTGATAATTTGTAGTGCCTCAGAAAACTTGTGGGTTACAGTTTATTATGGGGTTCCTGTGTG
GAGAGATGCAGATACCACCCTATTTTGTGCATCAGATGCCAAAGCACATGAGACAGAAGTGCACAATGT
CTGGGCCACACATGCCTGTGTACCCACAGACCCCAACCCACAAGAAATACACATGGAAAATGTAACAGA
AAATTTTAACATGTGGAAAAATAAAATGGCAGAGCAGATGCAGGAGGATGTAATTAGTTTATGGGATCA
AAGTCTAAAGCCATGTGTAAAGTTAACTCCTCTCTGCGTTACTTTAAATTGTACCAATGTCAATGCTAC
CAATGTCAGTAACACAACCGAAGCCCCAAACATAGTAGGAACAGATGAAGTAAAGAACTGTTCTTTTAA
TGTGACCACAGAACTAAGAGATAAAACGCAGCAGGTCCAGGCACTTTTTTATAAGCTTGATATAGTACA
AATGGGAGGTAATGATAGTGGTGAGTATAGGTTAATAAATTGTAATACTTCAGTCATTAAGCAGGCTTG
TCCAAAAGTATCCTTTGATCCAATTCCTATACATTATTGTACTCCAGCTGGTTATGCGATTTTAAAGTG
TAATGATAAGAATTTCAATGGGACAGGGCCATGTAAAAATGTCAGCTCAGTACAATGCACACATGGAAT
TAAGCCAGTGGTATCAACTCAATTGCTGTTAAATGGCAGTCTAGCAGAAGAAGAGATAATAATCAGATC
TGAAAATCTCACAAACAATGCCAAAACTATAATAGTGCACCTTAATAAATCTGTAGAAATCAATTGTAC
CAGACCAACCATATATAAAAGAAAGATAACTATGGGACCAGCACGAGTATACTATAGAACAGGGGAAGC
AATAGGAGATATAAGAAAAGCATATTGTCAGATTAATGGAACAAAATGGAATAAAGTTTTAAAACAGGT
AACTGAAAAATTAAAAGAGCACTTTAATAAGACAATAATCTTTCAACCACCCTCAGGAGGAGATCTAGA
AATTACAATGCATCATTTTAATTGTAGAGGGGGATTTTTCTATTGCAATACAACAAAACTGTTTAATAG
TACTTGGAGAGGAAATGAAACCATTGAATCCAGGGAGGGGTATAATAAAACCATCATACTCCCATGCAA
GATAAAGCAAATTATAAACATGTGGCAGGGAGCAGGACAGGCAATGTATGCTCCTCCCATCAATGGAAC
AATTAATTGTATATCAAATATTACAGGAATACTATTGACAAGAGATGGTGGTGATAACAATAATACAAT
TAATGAGACCTTCAGACCTGGAGGAGGAAATATAAAGGACAATTGGAGAAGTGAATTATATAAATATAA
AGTAGTGCAAATTGAACCACTAGGAATAGCACCCTCCAAGGCAAAGAGAAGAGTGGTGGAGAGAGAAAA
AAGA*GCTAGCCACCACCACCACCACCACGTGTGAATTGGTGA*

Figure 7 Sequence of pCAMBIA_HIV-1 gp120 construction showing the joining region and gp120 coding sequence. Italic = pCAMBIA sequence, Italic with underlined = histidine tag sequence, Underlined = signal peptide sequence, and Black = HIV-1 gp120 coding sequence.

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

Figure 8 Virtual translation of HIV-1 gp120 coding sequence of pCAMBIA_HIV-1 gp120 construction showing the first 30 amino acids of signal peptide and histidine tag that inframed with HIV-1 gp120.

CCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGAACACGGGGGACTCTTGACCATGGTAGATCT
*GACTAGT*ATGAGAGTGAAGGAGACACAGATGAATTGGCCAAACTTGTGGAAATGGGGGACTTTGATCCT
TGGGTTGGTGATAATTTGTAGTGCCTCAGCAGTGGGAATAGGAGCTATGATCTTTGGGTTCTTAGGAGC
AGCAGGAAGCACTATGGGCGCGGCGTCAATAACGCTGACGGTACAGGCCAGACAATTATTGTCTGGTAT
AGTGCAACAGCAAAGCAATTTGCTGAGGGCTATAGAGGCGCAGCAGCATCTGTTGCAACTCACAGTCTG
GGGCATTAAACAGCTCCAGGCAAGAGTCCTGGCTGTGGAAAGATACCTAAAAGATCAAAAGTTCCTAGG
ACTTTGGGGCTGCTCTGGAAAAATCGTCTGCACCACTGCTGTGCCCTGGAACTCCACTTGGAGTAATAA
ATCTTATGAAGAGATTTGGGATAAAATGACATGGACACAATGGGAGAGAGAAATTAGCAATTACACAAG
CACAATATATGAGATACTTACAGAATCGCAAAACCAGCAGGACAGGAATGAAAAAGATTTGTTGAAATT
GGATAAATGGGCAAGTCTATGGAATTGGTTTGACATATCAAGATGGCTGTGGTATATAAGAATATTTAT
AATGATAGTAGGAGGTTTAATAGGTTTAAGAATAGTTTTTGCTGTGCTTTCTATAGTAAATAGAGTTAG
GCAGGGATACTCACCTTTGTCTTTCCAGACCCTCTCCCATCATCAGAGGGATCCCGACAGACCCGAAAG
AATCGAAGAAGGAGGTGGCGAGCAAGGCAGAGACAGATCAGTGCGATTAGTGAGCGGATTCTTAGCGCT
TGCCTGGGACGATCTACGGAGCCTGTGTCTCTTCCTCTACCACCGCTTGAGAGACTTCATCTTGATTGC
AGCGAGGACTGTGGAACTTCTGGGACACAGCAGTCTCAAGGGACTGAGACGGGGGTGGGAAGGCCTCAA
ATATCTGTGGAATCTTCTGGTATATTGGGGCCAGGAACTAAAAATTAGTGTTATTTCTTTGCTTAATGC
TACAGCAATAGTAGTAGCGGGGTGGACAGATAGGGTTATAGAAGTAGCACAAGGAGCTTGGAGAGCCAT
TCTCCACATACCTAGAAGAATCAGACAGGGCTTAGAAAGGGCATTGGCTA*GCCAC*<u>*CACCACCACCACCACA*</u>
<u>*C*</u>*GTGTGAATTGGTGACCAGCTCGAAT*

Figure 9 Sequence of pCAMBIA_HIV-1 gp41 construction showing the joining region and gp120 coding sequence. Italic = pCAMBIA sequence, Italic with underlined = histidine tag sequence, Underlined signal peptide sequence, and Black = HIV-1 gp41 coding sequence.

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

Figure 10 Virtual translation of HIV-1 gp41coding sequence of pCAMBIA_HIV-1 gp41 construction showing the first 30 amino acids of signal peptide (derived from gp160) and histidine tag that inframed with HIV-1 gp41.

2. Tobacco plant transformation

From 300 tobacco leaf-discs of each construct, 4 calli of HIV-1 gp120 transgene, but not gp41, were propagated under a selective of hygromycin resistance (Fig. 11). The contamination of *A. tumefaciens* was completely eliminated after 2-month treatment of the leaf-discs and calli with cefotaxime antibiotic which confirmed by absence of bacterial growth in transformed tobacco leaf cultured in LB broth.

Figure 11 Transformed tobacco plants; 1 = Untransformed tobacco; 2 to 5 = gp120 transgene tobacco line 1-4, respectively.

2.1 Transgene analysis

Genomic DNA extracted from these transformed tobacco leaves shown the presence of the transgene after nested-PCR with specific primers of HIV-1 gp120 coding sequence, whereas untransformed tobacco leaves did not shown this specific band (Fig. 12). This result suggested that the HIV-1gp120 coding sequence had been transformed successfully into tobacco plants.

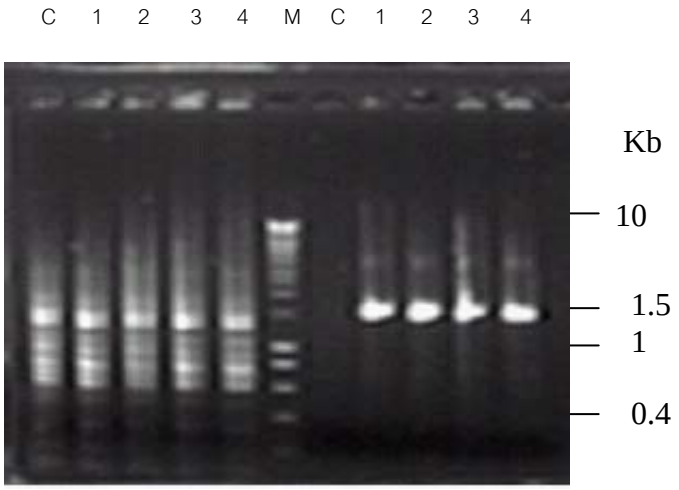

Figure 12 Agarose gel electrophoresis of PCR products of transformed tobacco. C = untransformed tobacco control; 1 to 4 = transformed tobacco line 1 to 4. M = DNA size marker (HyperLadder I). The outer PCR products from pCAMBIA-Sq1/pCAMBIA-Sq2 primers are shown in the left side of M, and the specific inner PCR products (1.5 kb) from HIV-1 gp120 specific primers are shown in the right side of M.

2.2 Expression analysis

A. mRNA analysis

RT-PCR was performed to check the transcription of HIV-1 gp120, and none of the 4 transformed lines gave positive results with HIV-1 specific primers. All of these 4 lines gave positive for RT-PCR of ubiquitin housekeeping gene (Fig. 13)

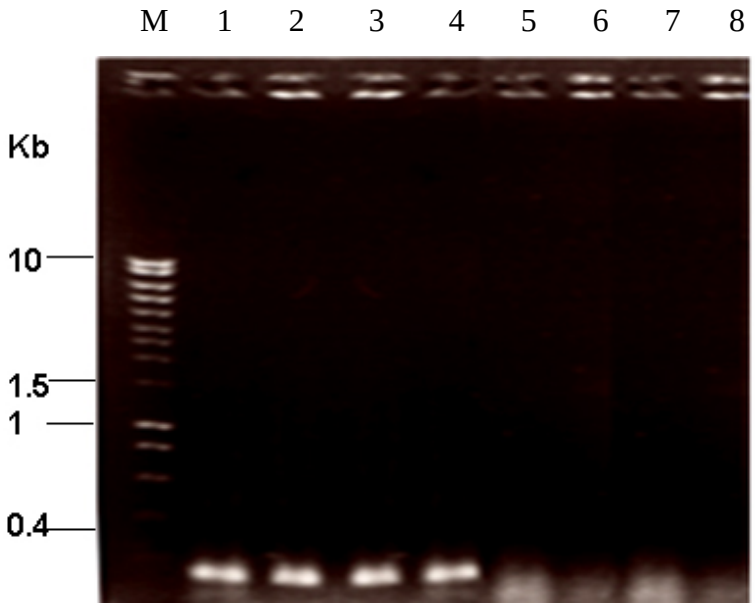

Figure 13 Agarose gel electrophoresis of RT-PCR products of transformed tobacco line 1 to 4; 1 to 4 = Ubiquitin primers that show specific PCR product size at 196 bp. and 5 to 8 = HIV specific primers which do not show specific PCR product size at 1.5 Kb. M = DNA size marker (HyperLadder I)

B. Protein analysis

For protein expression analysis, the total soluble proteins per 300 mg fresh leaf of transformed tobacco line 1 to 4 (1080, 1845, 1922, and 1023 μg/μl; average 1467.5 μg/μl) were not different ($p > 0.05$) from untransformed tobacco (1905 μg/μl). However, all of these 4 lines shown negative results by dot blot and western blot analysis as shown in Fig. 14.

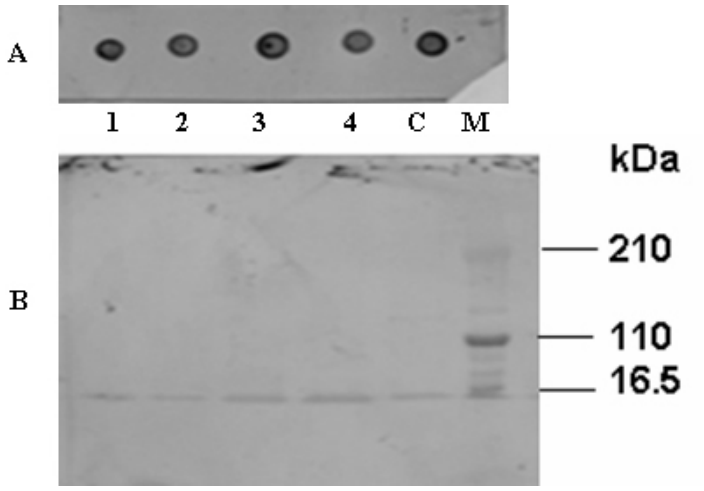

Figure 14 Dot blot (A) and western blot (B) analysis of 4 transformed lines. 1 to 4 = transformed tobacco line 1 to 4, respectively and C = un-transformed tobacco control. M = protein size marker (TriChromRanger Prestained Molecular Weight Marker Mix)

These results confirmed that there was no expression of HIV-1 gp120 in the 4 lines of transformed tobacco.

Silkworm transformation

1. Construction of silkworm expression vector.

The expression unit of HIV-1 gp120 or gp41 was designed to be fused with silkworm L-fibroin. Amplification of each fragment, including L-fibroin promoter, 5' L-fibroin, 3' L-fibroin, and HIV-1 gp120 or gp41 were performed as shown in Fig. 15. Joining the four fragments to make fusion of L-fibroin and gp120 or gp41 under the L-fibroin promoter had been successfully done as showed in Fig. 16.

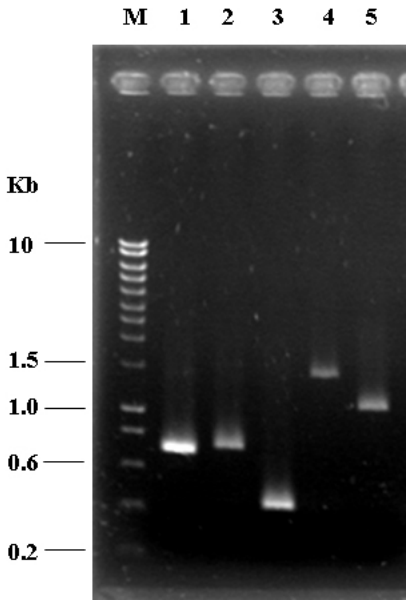

Figure 15 Agarose gel electrophoresis of amplification fragments of L-fibroin and HIV-1 glycoprotein. 1: L-fibroin promoter (705 bp); 2 = 5'L-fibroin (759 bp); 3 = 3' L-fribroin (442 bp); 4 = HIV-1 gp120 (1455 bp); and 5 = HIV-1 gp41 (1092 bp). M = DNA size marker (HyperLadder I)

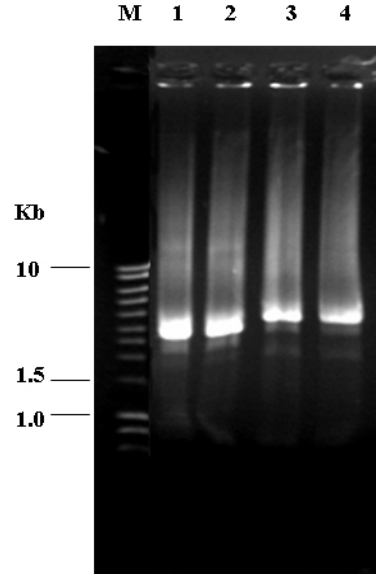

Figure 16 Joining the four fragments of L-fibroin promoter, 5' L-fibroin, HIV-1 gp120 (lane 1 and 2) or gp41 (lane 3 and 4), and 3' L-fibroin. M = DNA size marker (HyperLadder I)

The joining fragments were cloned into a pCR8, and checked for the entire sequence, including the joining region and flanking sequence. The clones that did not contain any unexpected base change were selected for further experiment. The joining fragments were cut with *Fse*I and *Asc*I and ligate with *pBAC*[3xP3-DsRedaf], which cut with the same enzymes to construct a silkworm expression vector; pBAC-FiL-gp120 or gp41 as shown in Fig. 6. These expression units were then sequencing checked again for the selection of correct sequence clones. The stop condon was found in pBAC-FiL-gp41 in 5' L-fibroin fragment and needed new construction, therefore, the first two experiments of transformation (microinjection and electroporation) were performed with pBAC-FiL-gp120 only.

GAAGTGCAGCCAACGTCAAGCGGTAGTGAAATATTAGACGAACAAAATGTTATTGAACAACCAGGTTCTTCATTGG
CTTCTAACAGAATCTTGACCTTGCCACAGAGGACTATTAGAGGTAAGAATAAACATTGTTGGTCAACTTCAAAGTC
CACGAGGCGTAGCCGAGTCTCTGCACTGAACATTGTCAGATCCGAGATCGGCCGGCCTGCATATTGGACATCCCTT
TTCTTGACATCGTATAAATTCGGTAATTCTCGGTACGGTTCGGAAAGTGCACCTGCGGCTATATTCAGACTCGCCA
AGTTACGTCAGTCGTATTGTAATGAGCGATTTAGTGGGCAACTTCATTCTGTTAATTTTGTGTCACGGTGCGCGCG
CATCGTAAAATTTCACTCTCATAGATTTTTCATAACGTGCCTAAAGAAGTATAACTTCAATAATTTAAATTAAAAA
AAAAAACATGCATAGAATAATTATATGAATTATTTAAAATGTCATTTACCGACATTGACATAACAGACGACGTTAA
CACTACAAAACATTTTAATTCCACATTGTTACATATTCAACAGTTAAATTTGCGTTAATTCTCGATGCGAACAAAT
ATAAGAACAATCGGATCAATTAGATCGCTTTGTTTCGAGCAACACTTAGTTTAACTAGAGGCGTACACCTCAGGAA
ATCATCTTCATTAGAAACTAAACCTTAAAATCGCAATAATAAAGCATAGTCAATTTTAACTGAAATGCAAAATCTT
TTGAACGTTAGATGCTGTCAGCGTTCGTTGGTACAGTTGTTTGATATTTATTTTAATTGTCTTTTTATATATAAAT
AGTGGAACATTAATCACGGAATCCTGTATAGTATATACCGATTGGTCACATAACAGACCACTAAAATGAAGCCTAT
ATTTTTGGTATTACTCGTCGCTACAAGCGCCTACGCTGCACCATCGGTGACCATCAATCAATACAGTGATAATGAA
ATTCCACGCGACATTGATGATGGAAAAGCTAGTTCCGTAATCTCACGTGCATGGGACTACGTCGATGACACTGACA
AAAGCATCGCCATCCTCAACGTTCAAGAGATCTTGAAGGACATGGCCAGCCAGGGCGATTATGCAAGTCAAGCATC
AGCGGTGGCCCAAACCGCCGGAATTATCGCCCATCTATCTGCCGGTATCCCCGGTGATGCCTGTGCAGCCGCTAAC
GTCATTAACTCTTACACAGACGGCGTCAGGTCCGGAAACTTCGCCGGCTTCAGACAATCTCTCGGTCCCTTCTTCG
GACACGTGGGACAAAACTTGAATCTTATCAATCAACTCGTCATCAACCCTGGTCAACTCCGATGCTCCGTCGGACC
AGCCCTGGGTTGTGCCGGAGGTGGAAGAATCTATGACTTCGAAGCCGCTTGGGATGCAATCTTAGCCAGCAGTGAC
TCTAGTTTCTTAAATGAAGAGTACTGCATCGTCAAGAGATTGTACAACTCTCGCAACAGCCAAAGCAACAACATCG
CTGCCTACATCACCGCTCACTTACTTCCCCCGGTTGCTCAAGTGTTCCACCAATCAGCTGGATCAATCACAGACCT
CCTGAGAGGCGTTGGCAACGGTAATGACGCGGACGCGGGGCCCGATGACGATGACAAGGAAAACTTGTGGGTTACA
GTTTATTATGGGGTTCCTGTGTGGAGAGATGCAGATACCACCCTATTTTGTGCATCAGATGCCAAAGCACATGGGA
CAGAAGTGCACAATGTCTGGGCCACACATGCCTGTGTACCCACAGACCCCAACCCACAAGAAATACACATGGAAAA
TGTAACAGAGAATTTTAACATGTGGAAAAATAAAATGGCAGAGCAGATGCAGGAGGATGTAATTAGTTTATGGGAT
CAAAGTCTAAAGCCATGTGTAAAGTTAACTCCTCTCTGCGTTACTTTAAATTGTACCAATGTCAATGCTACCAATG
TCAGTAACACAACCGAAGCCCCAAACATAGTAGGAGCAGATGAAGTAAAGAACTGTTCTTTTAATGTGACCACAGA
ACTAAGAGATAAAACGCAGCAGGTCCAGGCACTTTTTTATAAGCTTGATATAGTACAAATGGGAGGTAATGATAGT
GGTGAGTATAGGTTAATAAATTGTAATACTTCAGTCATTAAGCAGGCTTGTCCAAAAGTATCCTTTGATCCAATTC
CCATACATTATTGTACTCCAGCTGGTTATGCGATTTTAAAGTGTAaTGATAAGAATTTCAATGGGACAGGGCCATG
TAAAAATGTCAGCTCAGTACAATGCACACATGGAATTAAGCCAGTGGTATCAACTCAATTGCTGTTAAATGGCAGT
CTAGCAGAAGAAGAGATAATAATCAGATCTGAAAATCTCACAAACAATGCCAAAACTATAATAGTGCACCTTAATA
AATCTGTAGAAATCAATTGTACCAGACCAACCATATATAAAAGAAAGATAACTATGGGACCAGCACGAGTATACTA
TAGAACAGGGGAAGCAATAGGAGATATAAGAAAAGCATATTGTCAGATTAATGGAACAAAATGGAATAAAGTTTTA
AAACAGGTAACTGAAAAATTAAAAGAGCACTTCAATAAGACAATAATCTTTCAACCACCCTCAGGAGGAGATCTAG
AAATTACAATGCATCATTTTAATTGTAGAGGGGATTTTTCTATTGCAATACAACAAAACTGTTTAATAGTACTTG
GAGAGGAAATGAAACCATTGAATCCAGGGAGGGGTATAATAAAACCATCATACTCCCATGCAAGATAAAGCAAATT
ATAAACATGTGGCAGGGAGCAGGACAGGCAATGTATGCTCCTCCCATCAATGGAACAATTAATTGTATATCAAATA
TTACAGGAATACTATTGACAAGAGATGGTGGTGATAACAATAATACAATTAATGAGACCTTCAGACCTGGAGGAGG
AAATATAAAGGACAATTGGAGAAGTGAATTATATAAATATAAAGTAGTGCAAATTGAACCACTAGGAATAGCACCC
TCCAAGGCAAAGAGAAGAGTGGTGGAGAGAGAAAAAAGAGATGACGATGACAAACCGCGGACCGGCACCGGCTTAG
TGGCTAATGCTCAAAGATATATTGCACAAGCAGCCAGCCAGGTTCACGTCTAAATAAGAACTGTAAATAATGTATA
TATATAATTATATAAAAGATATATATAAACCATATACAAACATATATATATCATTATAAGACAATCTACCTATATA
AAAAAAGACTAAAATTAATAATTATGTATACTTTAATTGTGTTTAGGACATTTTATGCAAATTGTGTTTGCGTTAG
GATTTTTTTTGGAAGTTTTTTAGATTATTTATGAATATATAAATAAATATACGTTAATATAATATATATTATATAA
ATCAACGACACGGCTTTTCATTTTGGTGATGATCAATCTTATTGTTCTTCTAATTGATTTTTTTGTACAATAAAGA
TGTATCCAGTTTTCCAGATAAAGAATTTAGGCGCGCCGTACGCGTATCGATAAGCTTTAAGATACATTGATGAGTT
TGGACAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTA
ACCATTATAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAGGTTCAGGGGG

Figure 17 Sequence of FiL-gp120 fusion**.** Green = pBAC, Blue = L-fibroin promoter, Orange = 5'and 3' L-fibroin, Black = HIV- gp120 coding sequence, Red = restriction enzyme cut site, and Red with underline = enterokinase recognition site.

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

Figure 18 Virtual translation of FiL-gp120 fusion. Underline = enterokinase recognition site

GAAGTGCAGCCAACGTCAAGCGGTAGTGAAATATTAGACGAACAAAATGTTATTGAACAACCAGGTTCTTCATTGG
CTTCTAACAGAATCTTGACCTTGCCACAGAGGACTATTAGAGGTAAGAATAAACATTGTTGGTCAACTTCAAAGTC
CACGAGGCGTAGCCGAGTCTCTGCACTGAACATTGTCAGATCCGAGATCGGCCGGCCTGCATATTGGACATCCCTT
TTCTTGACATCGTATAAATTCGGTAATTCTCGGTACGGTTCGGAAAGTGCACCTGCGGCTATATTCAGACTCGCCA
AGTTACGTCAGTCGTATTGTAATGAGCGATTTAGTGGGCAACTTCATTCTGTTAATTTTGTGTCACGGTGCGCGCG
CATCGTAAAATTTCACTCTCATAGATTTTTCATAACGTGCCTAAAGAAGTATAACTTCAATAATTTAAATTAAAAA
AAAAAACATGCATAGAATAATTATATGAATTATTTAAAATGTCATTTACCGACATTGACATAACAGACGACGTTAA
CACTACAAAACATTTTAATTCCACATTGTTACATATTCAACAGTTAAATTTGCGTTAATTCTCGATGCGAACAAAT
ATAAGAACAATCGGATCAATTAGATCGCTTTGTTTCGAGCAACACTTAGTTTAACTAGAGGCGTACACCTCAGGAA
ATCATCTTCATTAGAAACTAAACCTTAAAATCGCAATAATAAAGCATAGTCAATTTTAACTGAAATGCAAAATCTT
TTGAACGTTAGATGCTGTCAGCGTTCGTTGGTACAGTTGTTTGATATTTATTTTAATTGTCTTTTTATATATAAAT
AGTGGAACATTAATCACGGAATCCTGTATAGTATATACCGATTGGTCACATAACAGACCACTAAAATGAAGCCTAT
ATTTTTGGTATTACTCGTCGCTACAAGCGCCTACGCTGCACCATCGGTGACCATCAATCAATACAGTGATAATGAA
ATTCCACGCGACATTGATGATGGAAAAGCTAGTTCCGTAATCTCACGTGCATGGGACTACGTCGATGACACTGACA
AAAGCATCGCCATCCTCAACGTTCAAGAGATCTTGAAGGACATGGCCAGCCAGGGCGATTATGCAAGTCAAGCATC
AGCGGTGGCCCAAACCGCCGGAATTATCGCCCATCTATCTGCCGGTATCCCCGGTGATGCCTGTGCAGCCGCTAAC
GTCATTAACTCTTACACAGACGGCGTCAGGTCCGGAAACTTCGCCGGCTTCAGACAATCTCTCGGTCCCTTCTTCG
GACACGTGGGACAAAACTTGAATCTTATCAATCAACTCGTCATCAACCCTGGTCAACTCCGATGCTCCGTCGGACC
AGCCCTGGGTTGTGCCGGAGGTGGAAGAATCTATGACTTCGAAGCCGCTTGGGATGCAATCTTAGCCAGCAGTGAC
TCTAGTTTCTTAAATGAAGAGTACTGCATCGTCAAGAGATTGTACAACTCTCGCAACAGCCAAAGCAACAACATCG
CTGCCTACATCACCGCTCACTTACTTCCCCCGGTTGCTCAAGTGTTCCACCAATCAGCTGGATCAATCACAGACCT
CCTGAGAGGCGTTGGCAACGGTAATGACGCGGGGCCCGATGACGATGACAAGGCAGTGGGAATAGGAGTTATGATC
TTTGGGTTCTTAGGAGCAGCAGGAAGCACTATGGGCGCGGCGTCAATAACGCTGACGGTACAGGCCAGACAATTAT
TGTCTGGTATAGTGCAACAGCAAAGCAATTTGCTGAGGGCTATAGAGGCGCAGCAGCATCTGTTGCAACTCACAGT
CTGGGGCATTAAACAGCTCCAGGCAAGAGTCCTGGCTGTGGAAAGATACCTAAAAGATCAAAAGTTCCTAGGACTT
TGGGGCTGCTCTGGAAAAATCGTCTGCACCACTGCTGTGCCCTGGAACTCCACTTGGAGTAATAAATCTTATGAAG
AGATTTGGGATAAAATGACATGGACACAATGGGAGAGAGAAATTAGCAATTACACAAGCACAATATATGAGATACT
TACAGAATCGCAAAACCAGCAGGACAGGAATGAAAAAGATTTGTTGAAATTGGATAAATGGGCAAGTCTATGGAAT
TGGTTTGACATATCAAGATGGCTGTGGTATATAAGAATATTTATAATGATAGTAGGAGGTTTAATAGGTTTAAGAA
TAGTTTTTGCTGTGCTTTCTATAGTAAATAGAGTTAGGCAGGGATACTCACCTTTGTCTTTCCAGACCCTCTCCCA
TCATCAGAGGGATCCCGACAGACCCGAAAGAATCGAGGAAGGAGGTGGCGAGCAAGGCAGAGACAGATCAGTGCGA
TTAGTGAGCGGATTCTTAGCGCTTACCTGGGACGATCTACGGAGCCTGTGTCTCTTCCTCTACCACCGCTTGAGAG
ACTTCATCTTGATTGCAGCGAGGACTGTGGAACTTCTGGGACACAGCAGTCTCAAGGGACTGAGACGGGGGTGGGA
AGGCCTCAAATATCTGTGGAATCTTCTGGTATATTGGGGCCAGGAACTAAAAATTAGTGTTATTTCTTTGCTTAAT
GCTACAGCAATAGTAGTAGCGGGGTGGACAGATAGGGTTATAGAAGTAACACAAGGAGCTTGGAGAGCCATTCTCC
ACATACTTAGAAGAATCAGACAGGGCTTAGAAAGGGCATTGGATGACGATGACAAACCGCGGACCGGCTTAGTGGC
TAATGCTCAAAGATATATTGCACAAGCAGCCAGCCAGGTTCACGTCTAAATAAGAACTGTAAATAATGTATATATA
TAATTATATAAAAGATATATATAAACCATATACAAACATATATATATCATTATAAGACAATCTACCTATATAAAAA
AAGACTAAAATTAATAATTATGTATACTTTAATTGTGTTTAGGACATTTTATGCAAATTGTGTTTGCGTTAGGATT
TTTTTTGGAAGTTTTTTAGATTATTTATGAATATATAAATAAATATACGTTAATATAATATATATTATATAAATCA
ACGACACGGCTTTTCATTTTGGTGATGATCAATCTTATTGTTCTTCTAATTGATTTTTTTGTACAATAAAGATGTA
TCCAGTTTTCCAGATAAAGAATTTAGGCGCGCCGTACGCGTATCGATAAGCTTTAAGATACATTGATGAGTTTGGA
CAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCA
TTATAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAGGTTCAGGGGG

Figure 19 Sequence of FiL-gp41 fusion**.** Green = *pBAC*, Blue = L-fibroin promoter, Orange = 5'and 3' L-fibroin , Black = HIV- gp120 coding sequence, Red = restriction enzyme cut site, and Red with underline = enterokinase recognition site

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

Figure 20 Virtual translation of FiL-gp4. Underline = enterokinase recognition site.

2. Silkworm transformation

2.1 Microinjection

The silkworm strain pnd-w1 eggs were injected with the mixture of transposase-producing helper plasmid (*pHA3PIG*) and *pBAC_FiL-gp120* or *pBAC_FiL-gp41* by using modified method. Five experiments had been done. The number of injected eggs, hatched lava, developed moths, G_1 broods was shown in Table 1. All 57 G0 moths from *pBAC_FiL-gp120* injection were not positive for red eye. Female moths in G_0 (n = 26) were mated with G_0-male. All G_1 moths (n = 1140 from 26 broods) were not positive for red eyes, which is a transgene marker.

	Number of injected egg	**Number (%) of G_0 hatched embryo**	**Number (%) of G_0 moth**	**Number of G_1 brood**	**Number of Red-eye positive G_1 moth**
pBAC_FiL-gp120	6,982	75 (1.11%)	57 (0.8%)	26	0
pBAC_FiL-gp41	1,509	1 (0.7%)	0	0	0

Table 1 Injection of pBAC_FiL-gp120 or pBAC_FiL-gp4 in pnd-w1 silkworm. The numbers are from 5 experiments for pBAC_FiL-gp120 and three experiment fro pBAC_FiL-gp41.

2.2 Electroporation

The silkworm strain pnd-w1 eggs were electroporated with the mixture of transposase-producing helper plasmid (pHA3PIG) and pBAC_FiL-gp120 or pBAC_FiL-gp41 using Gene Pulser II (BioRad). pBAC_FiL-gp120 was electroporated for 5 experiments and pBAC_FiL-gp41 was electroporated for 3 experiments. The total number of injected eggs, hatched larva and developed moths

was shown in Table 2. All 7,163 and 3,148 G_0 moths were not positive for red eye. Female moths in G_0 (n = 779 for pBAC_FiL-gp120 and n = 73 for pBAC_FiL-gp41) were mated with G_0-male. All G_1-moths (n = 44,950 for pBAC_FiL-gp120 and n = 3.270 for pBAC_FiL-gp41) were not positive for red eyes.

	Number of electroporated egg	**Number (%) of G_0 hatched embryo**	**Number (%) of G_0 moth**	**Number of G_1 brood**	**Number of Red-eye positive G_1 moth**
pBAC_FiL-gp120	40,050	7,163 (17.8%)	2,283 (6.7%)	779	0
pBAC_FiL-gp41	15,900	3,148 (19.8%)	244 (1.5%)	73	0

Table 2 Electroporation of pBAC_FiL-gp120 or pBAC_FiL-gp4 in pnd-w1 silkworm. The numbers are from 5 experiments for pBAC_FiL-gp120 and three experiment fro pBAC_FiL-gp41.

3. Transgene analysis

Since the piggyBac based vector used for construction of pBAC_FiL-gp120 or pBAC_FiL-gp41 has DsRed marker, which express under the control of the eye and nervous tissue-specific promoter 3xP3, therefore, red-eye of moth was used as a marker for positive transformation. Fig. 21 A and B shown the non-red eyes moths from this study compared with red-eye moths, which published by Tamura and collegues (Tamura et al., 2003) Although there was no any moths gave red eyes positive, extracted DNA from moths were randomly analysis (n = 50 for each construction) for whether there was the integration of transgene or not. The positive of L-fibroin promoter was used to determine the quality of extracted DNA as shown in Fig. 22-A and Fig. 23-A for pBAC_FiL-gp120 and pBAC_FiL-gp41, respectively. Nested PCR for HIV-1 gp120 or gp41 were not positive in all the extracted DNA as

shown in Fig. 22-B, 22-C and Fig. 23-B, 23-C which means there were no transgene in these moths.

Figure 21 Transformed silkworm moths. A and B are silkworm moths from this study which are negative for red eyes marker compared with C, which is red eyes positive silkworm moth published by Tamura et al. (2003)

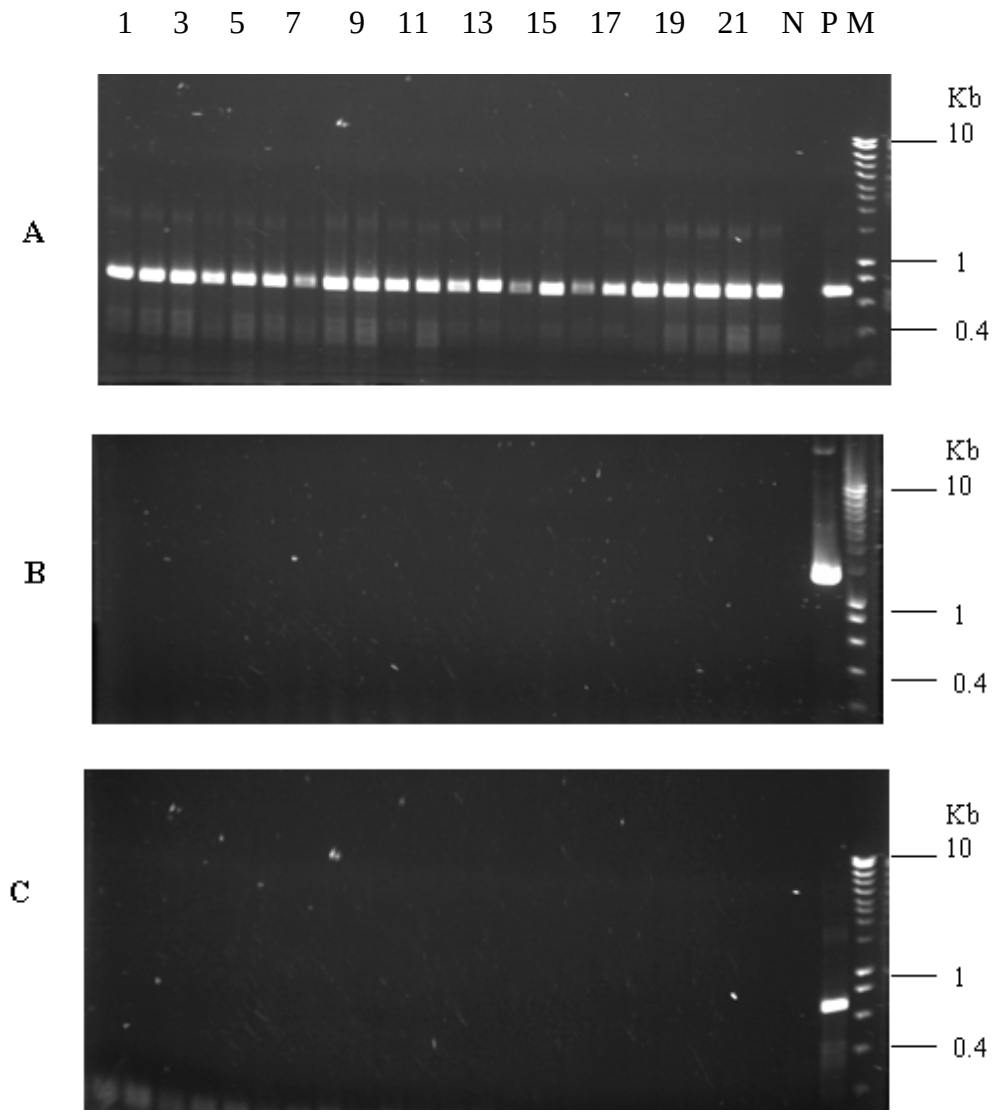

Figure 22 Partial results of transgene analysis of pBAC_FiL-gp120 transformed moths. A = L-fibroin promoter (699-bp), B = outer PCR product of HIV-1 gp120 (1,425 bp) and C = inner PCR product of HIV-1 gp120 (743 bp). Lane 1-22 are extracted DNA extracted from pBAC_FiL-gp120 transformed moths, lane N = negative control, land P = positive control, and lane M = DNA size marker.

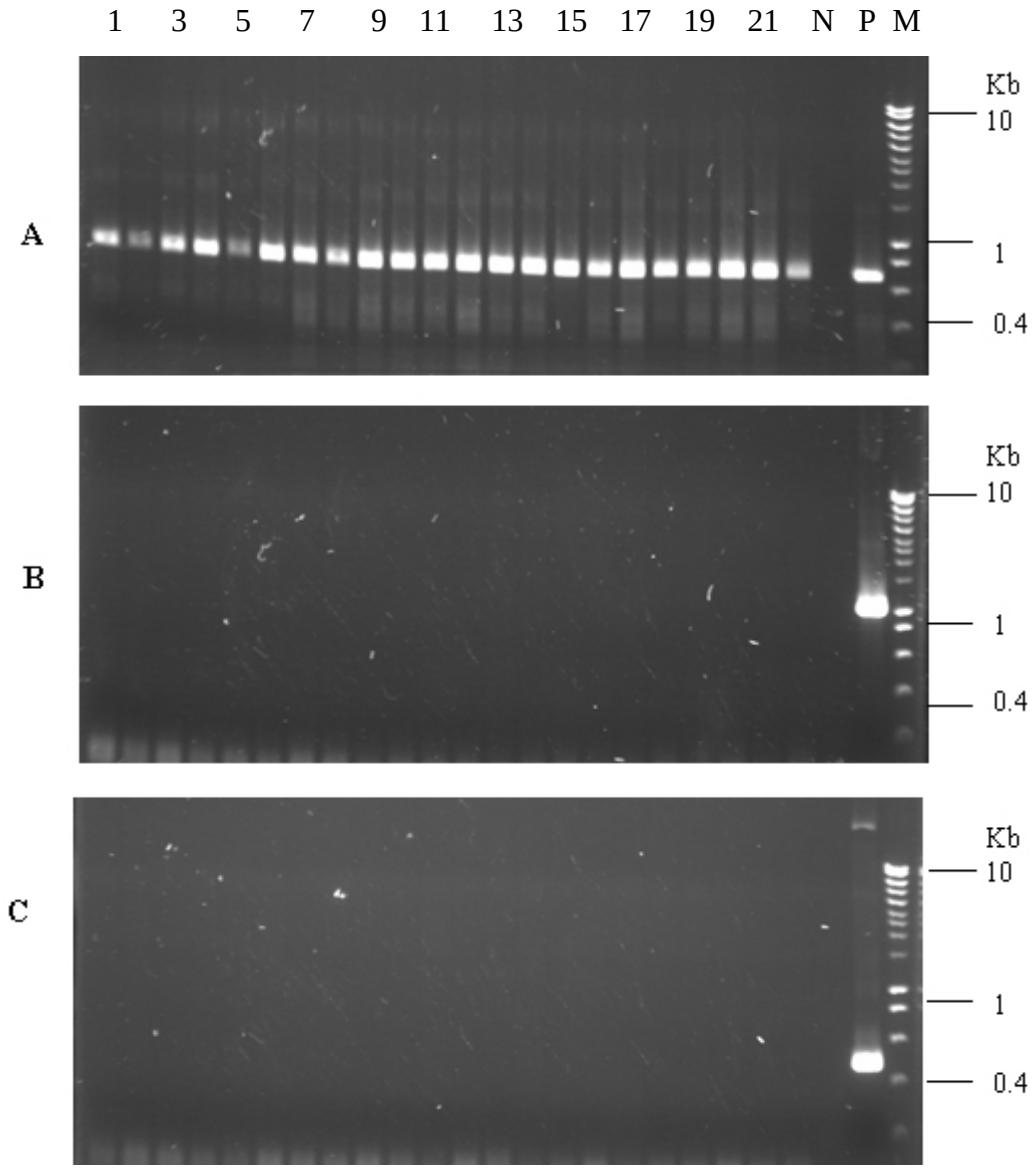

Figure 23 Partial results of transgene analysis pBAC_FiL-gp41 transformed moths. A = L-fibroin promoter (699-bp), B = outer PCR product of HIV-1 gp41 (1,059 bp) and C = inner PCR product of HIV-1 gp41 (461 bp). Lane 1-22 are extracted DNA extracted from pBAC_FiL-gp41 transformed moths, lane N = negative control, land P = positive control, and lane M = DNA size marker.

These results confirmed that there was no transgenic silkworm demonstrated in this study, though the two methods (microinjection and electroporation) were tried.

Discussion

Tobacco plant transformation

In this study, the HIV-1 gp120 coding sequence had been transformed successfully into tobacco plants, but there were no detectable transgene expression at both transcription and translation levels as shown by negative results of RT-PCR and dot blot/western blot. It may due to the absence of transcription at all, or may be possible that there was transcription but the gp120 mRNA in tobacco plant is not stable enough for passing through the whole process of transcription and translation**.** The unsuccessful of gp-41 transformation to tobacco plant may due to gp41 property, which is a transmembrane protein. This property may affect the development of tobacco plant and caused the calli dead.

Transgene expression is influenced by several factors that cannot be controlled precisely through the design of construct design which lead to variable transgene expression and, in some cases, its complete inactivation. Such factors include the position of transgene integration, the structure of transgenic locus, gene-copy number and the presence of truncated or rearrange transgenic copies (Plasterk *et al.,* 2000). Several strategies have been adopted in an attempt to minimize variation in transgene expression. For example, the ability to integrate single-copy transgene into precise locations in the plant nucleus would eliminate position effects and the problems that are associated with variable locus structure.

There were several reports to enhance the high-level of expressions in transgenic plant, such as duplicating the enhancer region (Kay *et al.,* 1987), using specific promoters that allow the expression of a transgene in a particular

environmental, developmental or tissue-specific manner (Conrad *et al*., 1998; Stoger *et al*., 2000), and sub-cellular targeting (Schillberg *et al*., 1999).

There was a report of advantage of sub-cellular targeting, which affects the interlinked process of folding, assembly and post-translation modification (Schillberg *et al*., 1999). To target proteins to the secretory pathway through the inclusion, an N-terminal signal peptide has been added to the expression construct. Comparative experiments with recombinant antibodies have shown that the secretory pathway was more suitable environment for folding and assembly than the cytosol, resulting to higher yield of antibody production. However, most antibodies accumulated to higher levels in the secretory pathway compared with the cytosol, there were some reports of exceptions (De Jaeger *et al*., 1998; Schouten *et al*., 2002), which indicated that the intrinsic features of each antibody (also proteins of interest) might also influence their overall stability. The oxidizing environment of the endoplasmic reticulum (ER), the lack of protease and the abundance of molecular chaperones are important factors for correct protein folding and assembly. Also, protein glycosylation occurs only in the endomembrane system and this modification is required for the correct function of many proteins of human origin. Antibodies yields were demonstrated to be increased if it was retained in the ER lumen by an H/KDEL C-terminal tatrapeptide tag (Conrad *et al*., 1998).

The report on the study of HBsAg production in tobacco had shown the helpfulness of using plant signal peptide to enhance the production yield (Sojikul *et al.,* 2003). In this study, the use of HIV-1 gp160 itself signal peptide, which normally works in human cells may be not suitable for working in tobacco. Another consideration point is the chaperone proteins in tobacco cell may not match for HIV-1 gp120 nascent protein, which may caused the failure of expression. And also, transgenes from heterologous species often have a different codon usage bias to the host plant, which might result in pulsing at disfavored codon and truncation, misincorporation or frame shifting. These points should be evaluated further to develop transgenic tobacco for foreign protein production.

One important thing is HIV-1 infects only human. The virus has tropism for only some specific cells, which have receptor (CD4) and corecptor (CCR5 and/or CXCR4), to complete its replication cycle. Envelope protein of HIV-1 is transcribed and translated as a single polyprotein precursor (gp-160; 858 amino acids), which has heavy glycosylation and then be cleaved later by the host enzyme; furin-like Golgi proteinase, into gp120 (505 amino acids) and gp41 (353 amino acids) during viral maturation (Hallenberger *et al.*, 1997). The process of co-expression and post-synthesis modification of the protein may be necessary to produce these two glycoproteins and make difficulty to produce the entire gp120/gp41. Due to several problems, the other approaches such as production of many short proteins that contain specific antigenic epitopes (see Appendix E for HIV-1 gp160 linear epitopes) may be tried. Pooling of these short proteins may be useful for cover all antigenic epitopes of HIV-1 envelope glycoprotein which can increase sensitivity and specificity of the test kit.

Silkworm transformation

For microinjection, it is likely that the site of injection into the egg, the DNA concentration, the helper to selection vector ratio, the DNA delivery procedure could affect gene transfer efficiency. This study, the position of injection was the anterior site that was reported to give higher percentage of transgenic silkworm than the posterior site (Thomas *et al.*, 2002). However, this study used adapted procedure to inject the egg. This microapplicator adaptation was demonstrated to work with Eri silkworm *(Philosamia recini*), a non-mulberry silkworm (Raksapon, 2007). Anyhow, Eri silkworm egg is 2-3 times bigger than *B. mori*. Although using the smallest needle (no. 30G) that available in Thailand, this method still made a big hole and harm for the embryo. In previous reports, which shown the success of transform the foreign genes, they used microinjection with double needles attached to the specific device for controlling the direction and the volume of injection (at 5-10 nl). The use of double needles that punched the egg with tungsten needle to make a tiny hole before injecting the plasmid with a very fine tip glass needle was nearly harmless to the egg. The process of microinjection was also performed by the high experienced person.

Therefore, the optimization of the microinjection method in available facility is needed to achieve for the successful of transgenic silkworm.

For electroporation, initial experiment was tested with fixed resistance at 200Ω and capacitance at 25 μF, but varies voltage between 300 - 1,800 V. The results shown that at 1,000 V, the survival rate was about 25% (data not shown). Previous report of voltage optimization shown that at 1,000 V, the survival rate was 57% and 50% of these survival larva had positive for gene transformation (Guo *et al.*, 2004). Therefore, the voltage was fixed at 1000 V in the next experiment. Although the high numbers of eggs were tried, but there was no DsRed-eye positive moth had been observed. Previous report of the successful for using this method was performed in different strain of silkworm (Guo *et al*., 2004.) It may be possible that plasmid construct were transferred into silkworm egg, but it was degraded before being transplanted into silkworm larvae ((Nataraju *et al.,* 1996) Also, the optimization of the electroporation in available facility is needed to achieve for the successful of transgenic silkworm.

In this study, silkworm strain pnd-w1, a double mutant for non-pigmented eggs and eyes, was used (Tamura *et al.,* 2000). This silkworm strain also spin white silk thread. These special characteristics make it useful for transgenic experiment. The expression of marker genes like DsRed, GFP, or YFP can be observed under Fluorescent stereomicroscope. Using DsRed marker gene driven by the 3xP3 promoter, make it easier to observe by naked eyes. However, this mutant silkworm strain is weaker than wide type and need specific care of rearing.

Recently, there was a report of the production of germline transgenic silkworm that spin cocoons containing recombinant human serum albumin (rHSA) in the sericin layer (Ogawa, *et al*., 2007). This reports developed a method to produce a recombinant protein as an independent protein in the sericin layer of silkworm cocoon instead of a fusion protein in the fibroin layer. Though the yield of rHSA was 3.0 μg/mg of cocoon compared to 36.7 μg/mg of cocoon by fusion with L-fibroin method (Tomita, 2003), this approach offers the more easier extractable from the

sericin layer than form fibroin layer due to its superficial location ib a cocoon and the hydrophilic nature of sericin. Moreover, the rHSA was secreted from MSG into the lumen.

Since this study did not success in transformation of silkworm, and transformed tobacco plants did not express target proteins, therefore, comparison for the efficiency of transformation methods was omitted.

CONCLUSION AND RECOMMENDATION

Conclusion

In this study, the successful of transgenesis of HIV-1 gp120 into tobacco plant was demonstrated by the presence of integrated gp120 coding sequence in the genomic DNA of transformed tobacco plants detected by PCR. However, neither gp120 mRNA nor protein was detected. This study did not success for transformation of gp41 into tobacco plant, and for both gp120 and gp41 into silkworm. Naturally, HIV-1 infects only human and use human's advance machinery to produce all viral proteins. Furthermore, envelope glycoprotein of HIV-1 was expressed as gp160 polyprotein precursor, which then cleaved into gp120 and gp41 during viral maturation. After that, these two glycoproteins will form tetrameric structure, in which gp120 is outside of the viral particle and attach to the trans-membrane gp41. These may cause the difficulties of the transgenes expression in non-human cells. The research and development of protein production system to produce such special proteins likes HIV-1 gp120 and gp41would be intensively designed to achieve the transgene production in the non-host cell.

Recommendation

1. The improvement of transformation methods or procedures for silkworm, such as using the specific device for microinjection, should be reconsidered.

2. The using of truncated specific coding sequences of antigenic epitope, instead of the entire coding sequence of gp120 or gp41 should be tried, since the long sequence is prone to fail for gene transformation and expression.

3. The using of other organisms to produce such a special protein likes HIV-1 gp120 and gp41 would be considered.

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APPENDICES

Appendix A

Table of primers

Appendix Table A1 Primers for transgenic tobacco

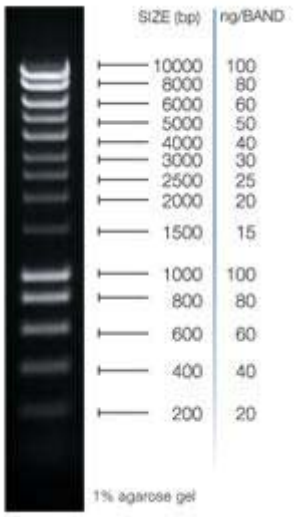
Primer name	5'-3' Sequence	Length (bp)
NcoI-gp160F	CCA TGG TAG ATC TGA CTA GTA TGA GAG TGA AGG AGA CAC AG	41
NheI-gp120R	GCT AGC TCT TTT TTC TCT CTC CAC CAC	27
NcoI-5HSP	CCA TGG ATG AGA GTG AAG GAG ACA CAG ATG AAT TGG CCA AAC TTG TGG AAA TGG GGG ACT TTG ATC CTT GGG	72
3HSP-GP41F	AAA TGG GGG ACT TTG ATC CTT GGG TTG GTG ATA ATT TGT AGT GCC TCA GCA GTG GGA ATA GGA GCT ATG	69
NheI-gp160R	GCT AGC CAA TGC CCT TTC TAA GCC	24
M13F	GTA AAA CGA CGG CCA G	16
M13R	CAG GAA ACA GCT ATG AC	17
pCAMBIA Sq1	CGT CTT CAA AGC AAG TGG	18
pCAMBIA Sq2	AAC ATA GAT GAC ACC GCG	18
UBQ-F	GTT GAT TTT TGC TGG GAA GC	20
UBQ-R	GAT CTT GGC CTT CAC GTT GT	20

Appendix Table A2 Primers for transgenic silkworm

Primer name	5'-3' Sequence	Length (bp)
FiLP-F	GGCCGGCC TGC ATA TTG GAC ATC C	24
FiLP-R	CTT CAT TTT AGT GGT CTG TTA	21
FiL5'-F	TAA CAG ACC ACT AAA ATGAAG	21
FiL5'-R	CTT GTC ATC GTC ATC GGGCCC CGC GTC ATT ACC GTT GCCAAC	42
FiL3'-F	GAT GAC GAT GAC AAG CCGCGG ACC GGC TTA GTT GCT AATGC	41
FiL3'-R	GGC GCG CCT AAA TTC TTT ATC TGG AAA ACT GG	32
GP120-F	GGGCCC GAT GAC GAT GAC AAG GAA AAC TTG TGG GTT ACA	39
GP120-R	CCGCGG CTT GTC ATC GTC ATC TCT TTT TTC TCT CTC CAC CAC	42
GP120-F2	GGC AAA GAA AAA TAG ACA GG	20
GP120-R2	TAC ATT TTA AAA TC(A/G) CAT AAC C	22
GP41-F	GGGCCC GAT GAC GAT GAC AAG GCA GTG GGA ATA GGA GCT ATG	42
GP41-R	CCGCGG GAT GTC ATC GTC ATC CAA TGC CCT TTC TAA GCC	36
GP41-F2	AAT AGA GTT AGG CAG GGA TA	20
GP41-R2	CAA TGC CCT TTC TAA GCC	18
pBac-F	CAG AAT CTT GAC CTT GCC	18
pBaC-R	CAT TCT AGT TGT GGT TTG TCC	21

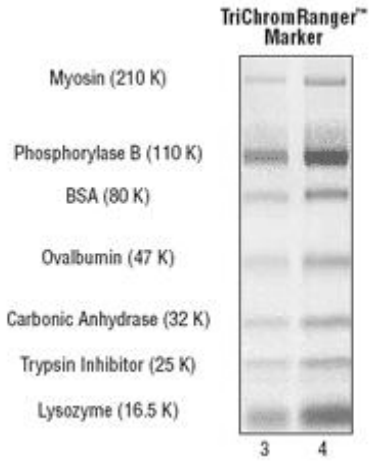
Appendix B

Molecular size marker



Appendix Figure B1 HyperLadder I DNA size marker

Source: Bioline, USA.



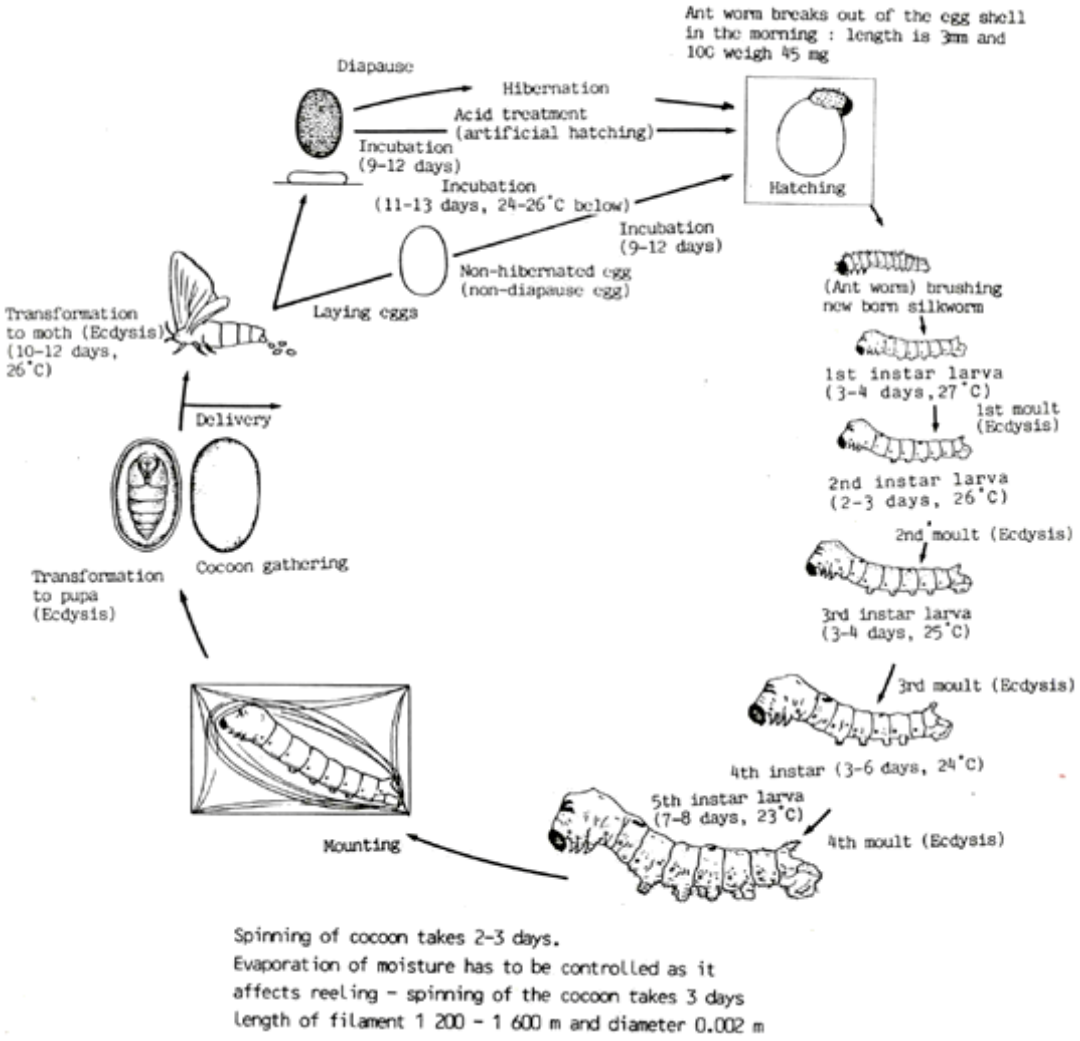
Appendix Figure B2 TriChromRanger Prestained Molecular WeightMarkerMix

Source: Pierce Biotechnology, USA.

Appendix C

Silkworm life cycle and raring

The life cycle of silkworm



Appendix Figure C1 Life cycle of silkworm

Source: Veda *et al*., (1997)

The duration of the whole life cycle of the silkworm is about 54-60 days (depended on strain) at temperatures of 23-25 ºC. The silkworm life cycle are separated into 4 stages (Appendix Figure 1) as follows:

Egg stage: There are two types of eggs, namely the diapause and the non-diapause type. Silkworm races indigenous to temperate regions usually lay diapause

eggs, while those of subtropical regions such as native varieties of Thailand lay mostly lay non- diapause eggs. The eggs of the diapause type aestivate and hibernate and hatch out in early May. The eggs of the diapause will hatch in 11-14 days at temperature of 23-25ºC., whereas the non-diapause type eggs hatch in 9-12 days. However, an artificial hatching method by immersion the eggs in hot hydrochloric acid for 3-5 minutes can make the diapuase type eggs hatching in about the same time.

Larva stage: The newly hatched larvae are black, about 3 mm long, weight about 0.45 mg. The larvae start eating all day and night and will stop eating during molting periods. During about 25-day of larva stage, there are 4 molting times. The body weight of the fifth instar larva is about 10,000 times of that of newly hatched larva (the first instar larva). After the fifth instar larvae reach its maximum weight (called “ripe” or “ mature”) for one or one and half days, it stop eating, raise its head to search for support, and starts spinning cocoons.

Pupa stage: It takes about 4-5 days for the pupa stage. The silk glands of the mature larva are larger because of DNA dividing. This special characteristic is for the heavy expression of silk proteins.

Moth stage: The moth emerges from cocoon after developing in the papa stage for 9 to 14 days which usually takes place in the morning. Soon after that, male and female moths mate. The mating ability of male moths can deteriorate very quickly. To prolong their sexual ability for a week, the male moths can be kept in a refrigerator at 10ºC. The female moth lays eggs shortly after the separation from the male and lays continuously for several hours until almost all eggs are deposited (Tazima, 1978).

Silkworm raring

1. Eggs are incubated in dark and enough humidity at 28 ^{o}C
2. After 10-14 days of incubation, a black dot spot which is usually called “eye spotting” is observed near the polar end of the eggs. These dots correspond to the pigmented larvae heads and are the sign that the eggs are to hatch after 3 days.
3. When the whole egg color turns into pale gray, the eggs will hatch within 1-2 days.
4. If an enough number/percentage of the larvae hatch, remove them to a fresh young mulberry leaf sliced into small pieces on at sheet of paper at the bottom of a plastic box.
5. For the young (1st to 3rd larva) silkworm, giving mulberry leaf, which sliced at 2-5 mm (approximately to the length of the instar). Rear at 27-28^{o}C
6. For the 4th and 5th larva, rear at 25-26^{o}C on plastic-mesh pacer..
7. Between each stage of instar, there is ecdysis, stop feeding and wait until most of the larva complete ecdysis (newly ecdysed larvae are resistant to starvation). Give new mulberry leaf
8. AT the end stage of 5th larva, they will spin silk fiber and become cocoons. Cut to open a cocoon after one week of spinning, and remove the pupa.
9. Determine the sex of pupa, keep in separately box.
10. When the moths hatch; males moths are usually small and active, while female moths are big and behave quietly, mate each couple in separated space for 4 hours.
11. Let the female moth lay eggs on the wax paper in an oviposition chamber (cylinder).

Appendix D

Reagent preparation

D1. PCR mixture

D1.1. Immolase DNA polymerase (For 50 µl reaction)

		final concentration
10X ImmoBuffer	5 µl	1X
50 mM MgCl2	2.5 µl	2.5 mM
5 mM dNTP, each	2 µl	100 µM
10 µM F-primer	1 µl	0.2 µM
10 µM R-primer	1 µl	0.2 µM
5U/10µl enzyme	0.2 µl	1 U
DNA template	1-5 µl	
Nuclease free water up to	50 µl	

Note: - 7 minutes at 95^{o}C pre-heating is needed to activate the enzyme

- In case of colony PCR, total volume of 12.5 µl was used for one reaction.

D1.2. Bio-X-Act Long DNA polymerase (For 50 µl reaction)

		final concentration
10X OptiBuffer	5 µl	1X
50 mM MgCl2	2.5 µl	2.5 mM
5 mM dNTP, each	2 µl	100 µM
10 µM F-primer	1 µl	0.2 µM
10 µM R-primer	1 µl	0.2 µM
4U/10µl enzyme	0.25 µl	1 U
DNA template	1-5 µl	
Nuclease free water up to	50 µl	

D2. Sequencing reaction mixture (10 µl)

DW.	4.4	µl
BigDye terminator v3.1	2	µl
5x Buffer	1	µl
1 µM Primer	1.6	µl
DNA template	1	µl

D3 Ligation mixture preparation

D3.1 TA-cloning by T4 ligase (GEM-T Easy, Promega)

2X Rapid Ligation Buffer, T4 DNA ligase	5	µl
pGEM-T Easy Vector (50 ng)	1	µl
PCR product (with A-overhanging)	X*	µl
Ta DNA Ligase (3 Weiss Units/ µl)	1	µl
Sterile water to a final volume of	10	µl

Mix the reaction gently and incubate overnight at $4^{o}C$

* Molar ratio of PCR product (insert) and vector should be 3-9:1

D3.2 TOPO-TA cloning (Invitrogen)

Fresh PCR product	0.5 – 4	µl
Salt solution	1	µl
Sterile water to a final volume of	5	µl
TOPO-vector	1	µl
Final volume	6	µl

Mix the reaction gently and incubate for 5 minutes at room temperature.

D3.3 Ligation with Ligation High reagent (TOYOBO)

Restriction enzyme cut insert	X*	µl
Restriction enzyme cut vector	Y*	µl
Ligation High reaction	X + Y	µl

Mix well and incubate at $16^{o}C$ for 2 hours.

*Molar ratio of X and Y should be 3-9:1

D4. Mosquito buffer

5 M NaCl	2 ml
Sucrose	6.85 g
Tris base	1.21 g
0.5 M EDTA 9 (pH 8.00)	10 ml
10 % SDS	5 ml
Diethypyrocarbonate	1 ml

Adjust pH to 9.0 with 1 M NaCl

Add DW. to 100 ml

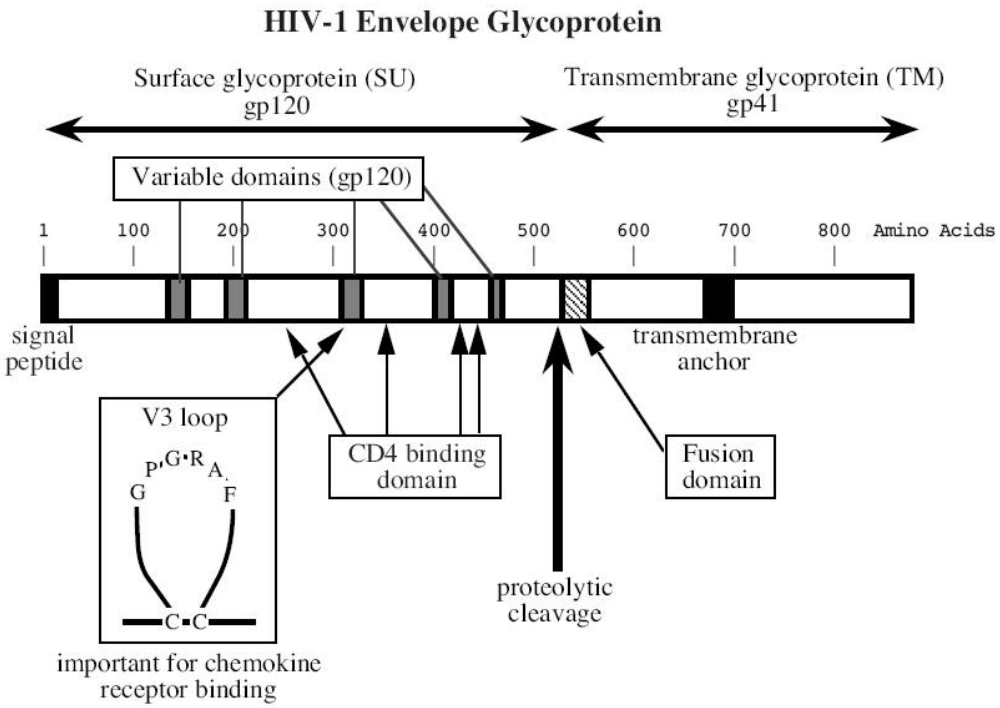
Filter through 0.22 µm filter

D5. Solution for SDS-PAGE

30% acrylamind	Dissolve acrylamind 30 g and bisacrylamind 0.8 g in DW 100 ml
4x Tris-HCl/SDS pH 8.8	Dissolve Tris base 91 g and SDS 2 g in DW 300 ml. Adjust pH to 8.8. Add DW to the final volume of 500 ml
4x TrisCl/SDS pH 6.8	Dissolve Tris base 6.05 g and SDS 0.4 g in DW 40 ml Adjust pH to 6.8. Add DW to the final volume of 100 ml
5x glycine buffer	Dissolve Tris base 51.1 g and glycine 54 g in 10% SDS 50 ml. Add DW to the final volume of 1000 ml
loading buffer	6% mercaptoethanol 6% SDS 0.6% bromophenal blue 20% glycerol
separating gel	30% acrylamind 2.66 ml, 4xTris-HCl/SDS pH8.8 2 ml, DW3.3 ml,10% ammonium persulfate 26.6 µl, TEMMED 5.3 µl
stacking gel	30%acrylamind 0.65 ml, 4xTrisCl/SDS pH6.8 1.25 ml, D.W. 3.05 ml, 10% Ammonium persulfate 25 µl TEMMED 5 µl

Appendix E

HIV-1 envelope glycoprotein



Appendix Figure E1 Diagram of HIV-1 envelope glycoprotein shows the surface glycoprotein (gp120) and the transmembrane glycoprotein (gp41)

Source: Cullen (1991)

Appendix F

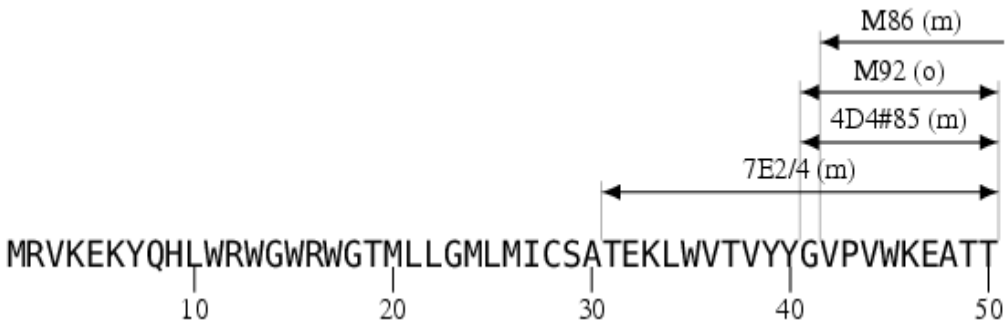
gp160 Ab Epitope Map

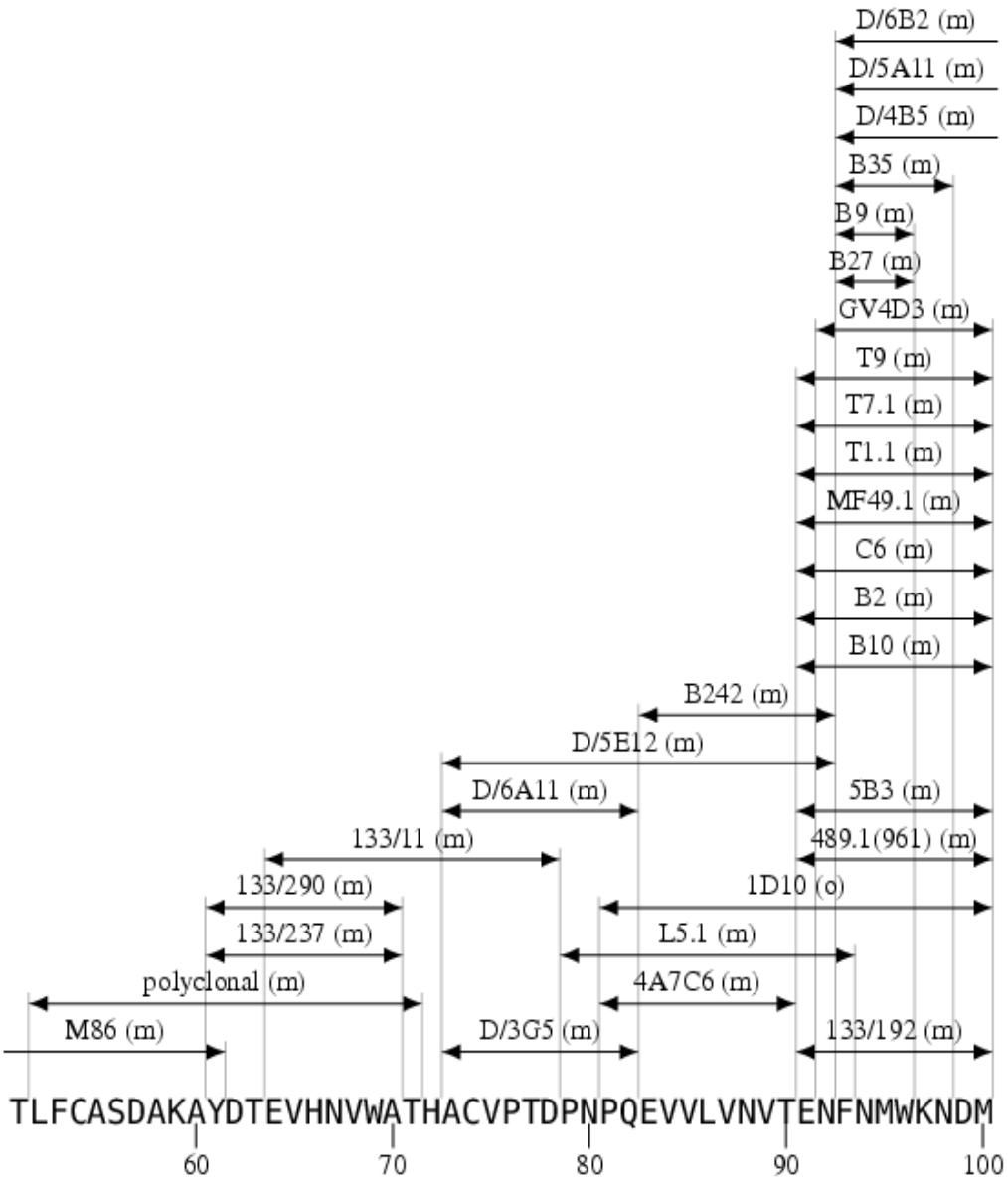
gp160 Ab Epitope Map

The names of MAbs and the location of well characterized linear binding sites of 21 amino acids or less are indicated relative to the protein sequences of the HXB2 clone. This map is meant to provide the relative location of epitopes on a given protein, but the HXB2 sequence may not actually bind to the MAb of interest, as it may vary relative to the sequence for which the epitope was defined. Above each linear binding site, the MAb name is given followed by the species in parentheses. Human is represented by `h', non-human primate by `p', mouse by `m', and others by `o'. More precise species designations for any given MAb can be found using the web search interface.

HIV databases: http://www.hiv.lanl.gov/content/immunology/maps/ab/gp160.html

Last modified: Wed Apr 11 14:48:29 2007

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B9 (m)
B27 (m)
GV4D3 (m)
T9 (m)
T7.1 (m)
T1.1 (m)
MF49.1 (m)
C6 (m)
B2 (m)
B10 (m)
B242 (m)
D/5E12 (m)
D/6A11 (m)
5B3 (m)
133/11 (m)
489.1(961) (m)
133/290 (m)
1D10 (o)
133/237 (m)
L5.1 (m)
polyclonal (m)
4A7C6 (m)
M86 (m)
D/3G5 (m)
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90
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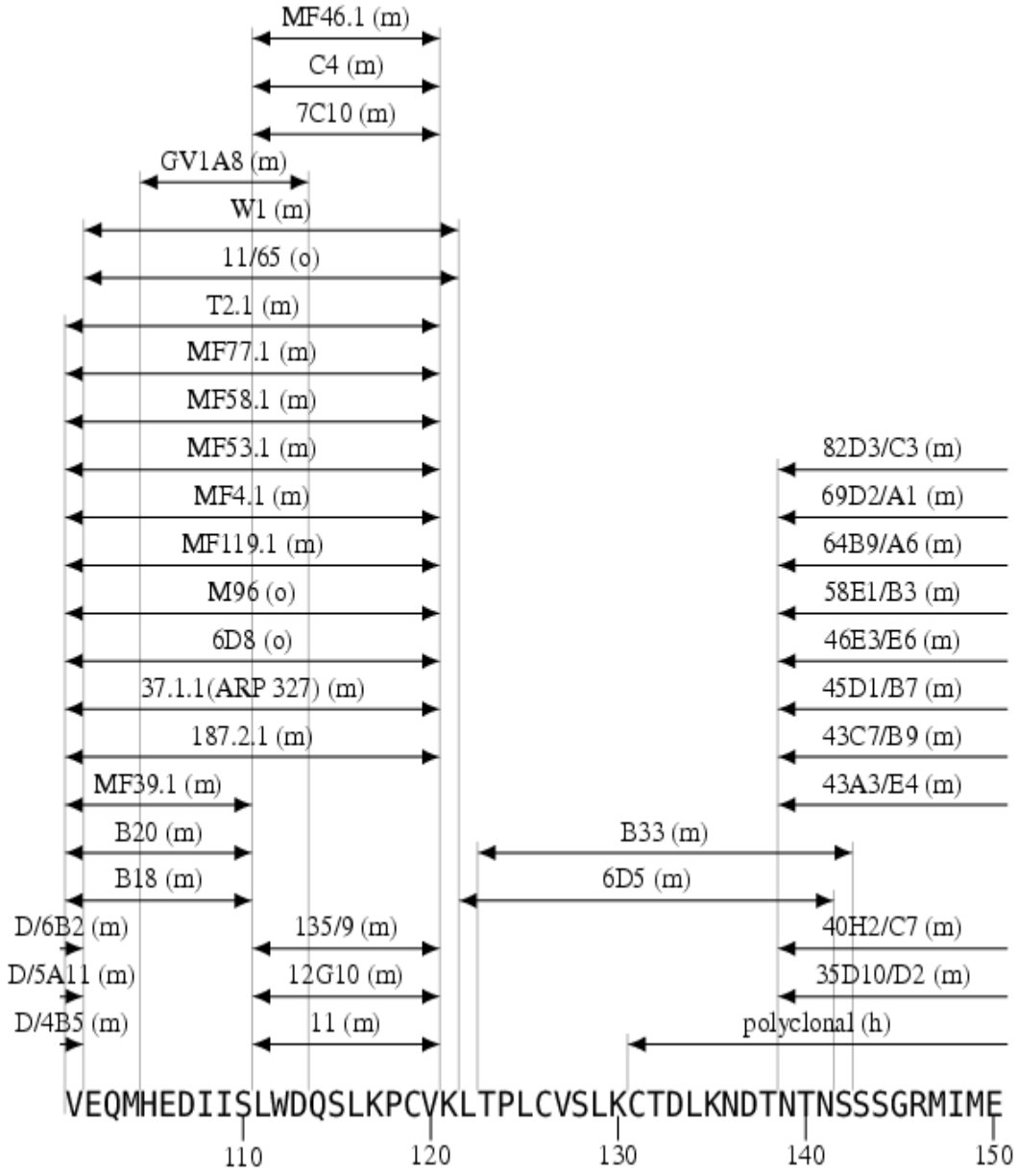
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GV1A8 (m)
W1 (m)
11/65 (o)
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MF77.1 (m)
MF58.1 (m)
MF53.1 (m)
MF4.1 (m)
MF119.1 (m)
M96 (o)
6D8 (o)
37.1.1(ARP 327) (m)
187.2.1 (m)
MF39.1 (m)
B20 (m)
B18 (m)
D/6B2 (m)
D/5A11 (m)
D/4B5 (m)
82D3/C3 (m)
69D2/A1 (m)
64B9/A6 (m)
58E1/B3 (m)
46E3/E6 (m)
45D1/B7 (m)
43C7/B9 (m)
43A3/E4 (m)
B33 (m)
6D5 (m)
135/9 (m)
12G10 (m)
11 (m)
40H2/C7 (m)
35D10/D2 (m)
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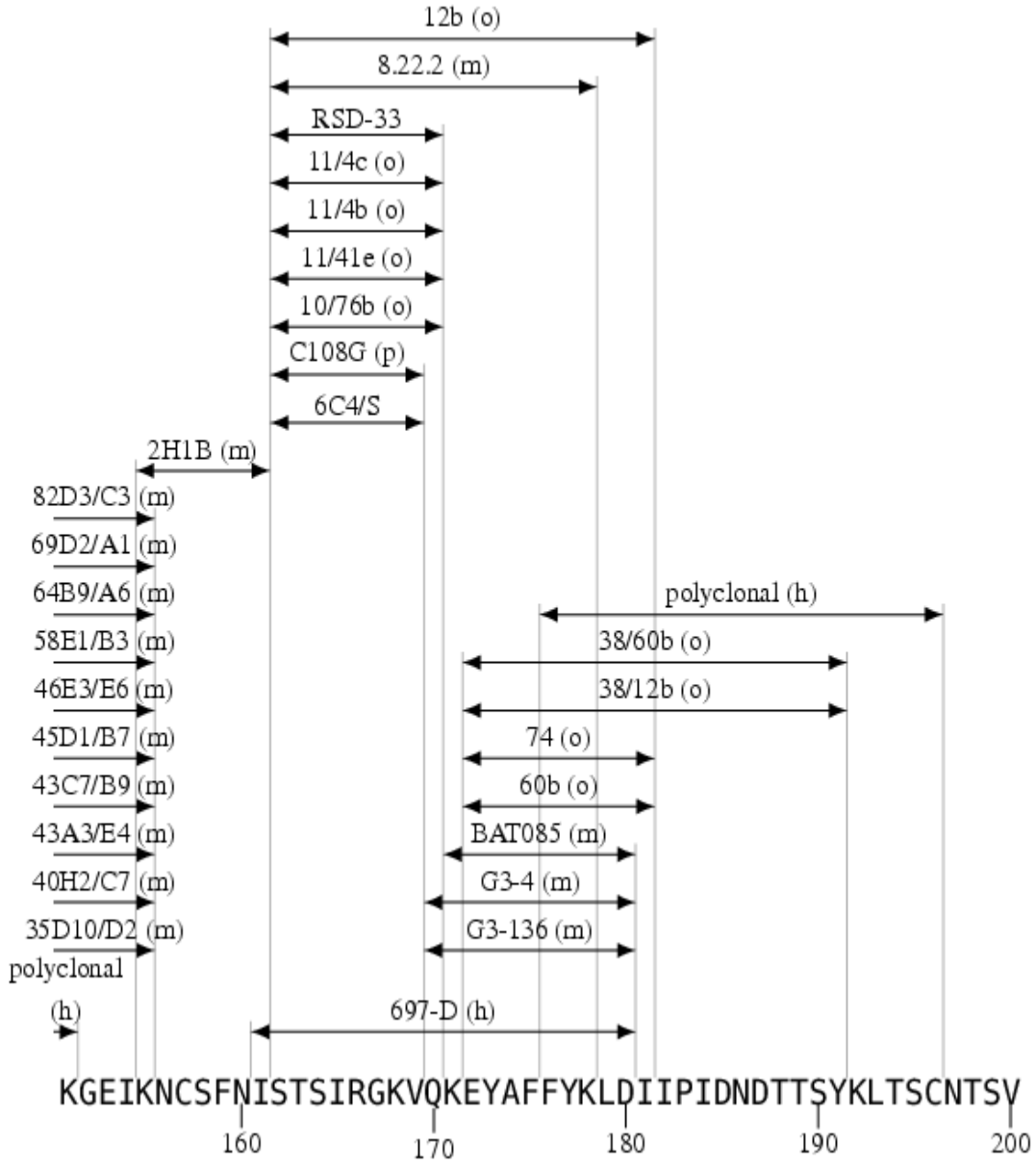
120
130
140
150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11/4b (o)
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10/76b (o)
C108G (p)
6C4/S
2H1B (m)
82D3/C3 (m)
69D2/A1 (m)
64B9/A6 (m)
58E1/B3 (m)
46E3/E6 (m)
45D1/B7 (m)
43C7/B9 (m)
43A3/E4 (m)
40H2/C7 (m)
35D10/D2 (m)
polyclonal (h)
polyclonal (h)
38/60b (o)
38/12b (o)
74 (o)
60b (o)
BAT085 (m)
G3-4 (m)
G3-136 (m)
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180
190
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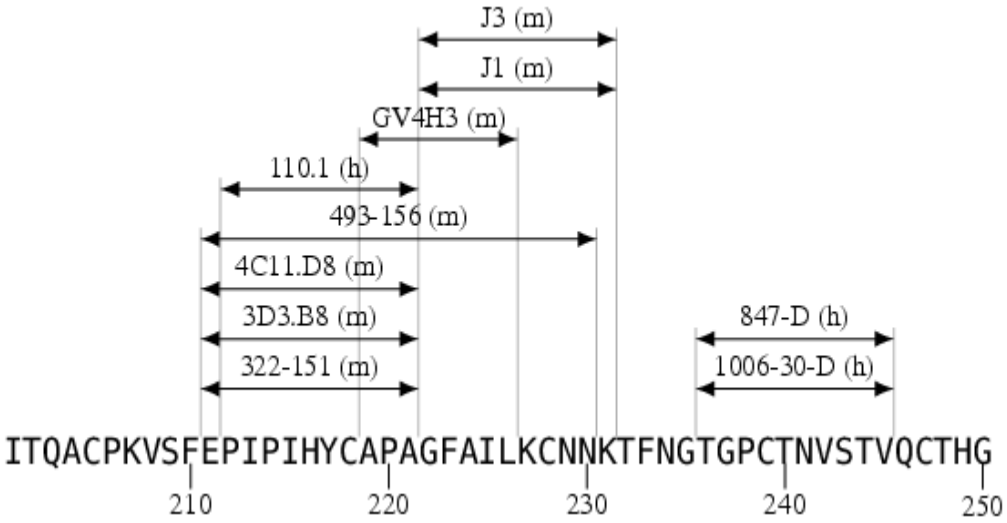
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GV4H3 (m)
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3D3.B8 (m)
322-151 (m)
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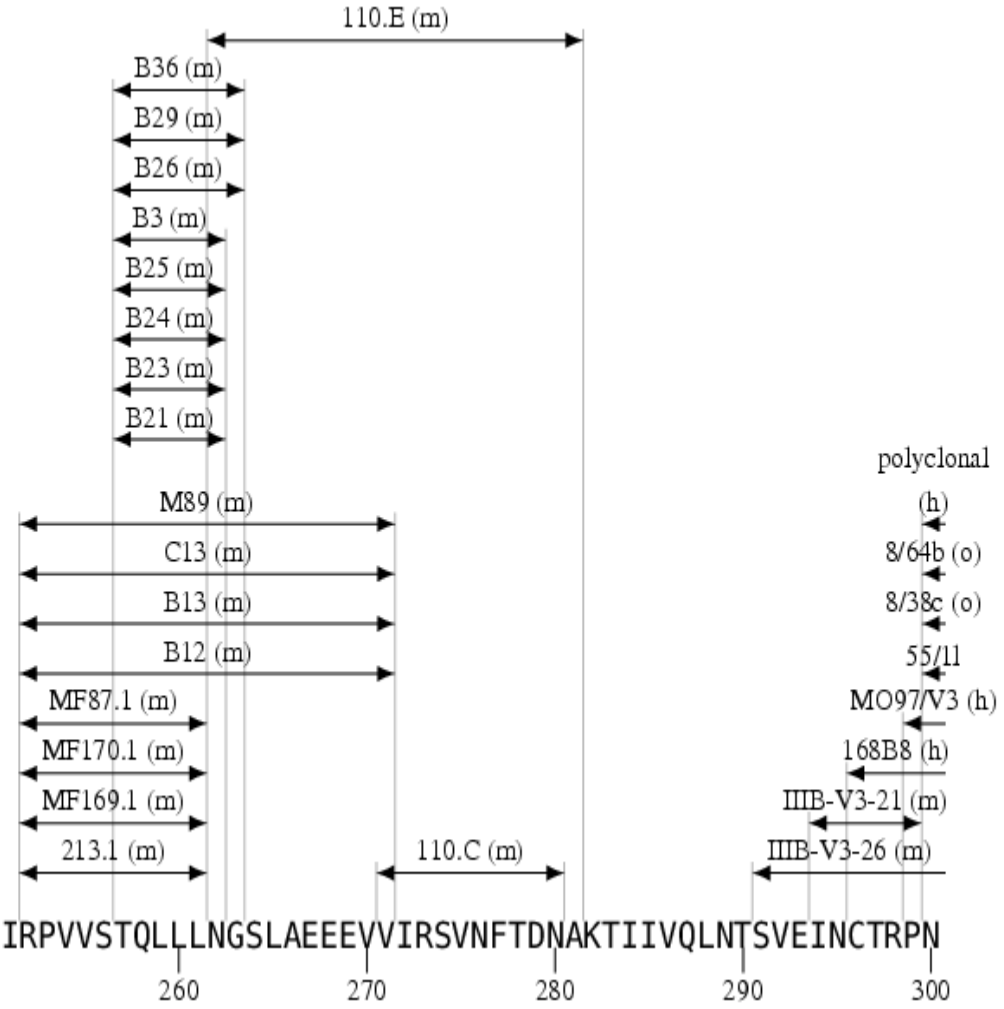
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B23 (m)
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M89 (m)
C13 (m)
B13 (m)
B12 (m)
MF87.1 (m)
MF170.1 (m)
MF169.1 (m)
213.1 (m)
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polyclonal
(h)
8/64b (o)
8/38c (o)
55/11
MO97/V3 (h)
168B8 (h)
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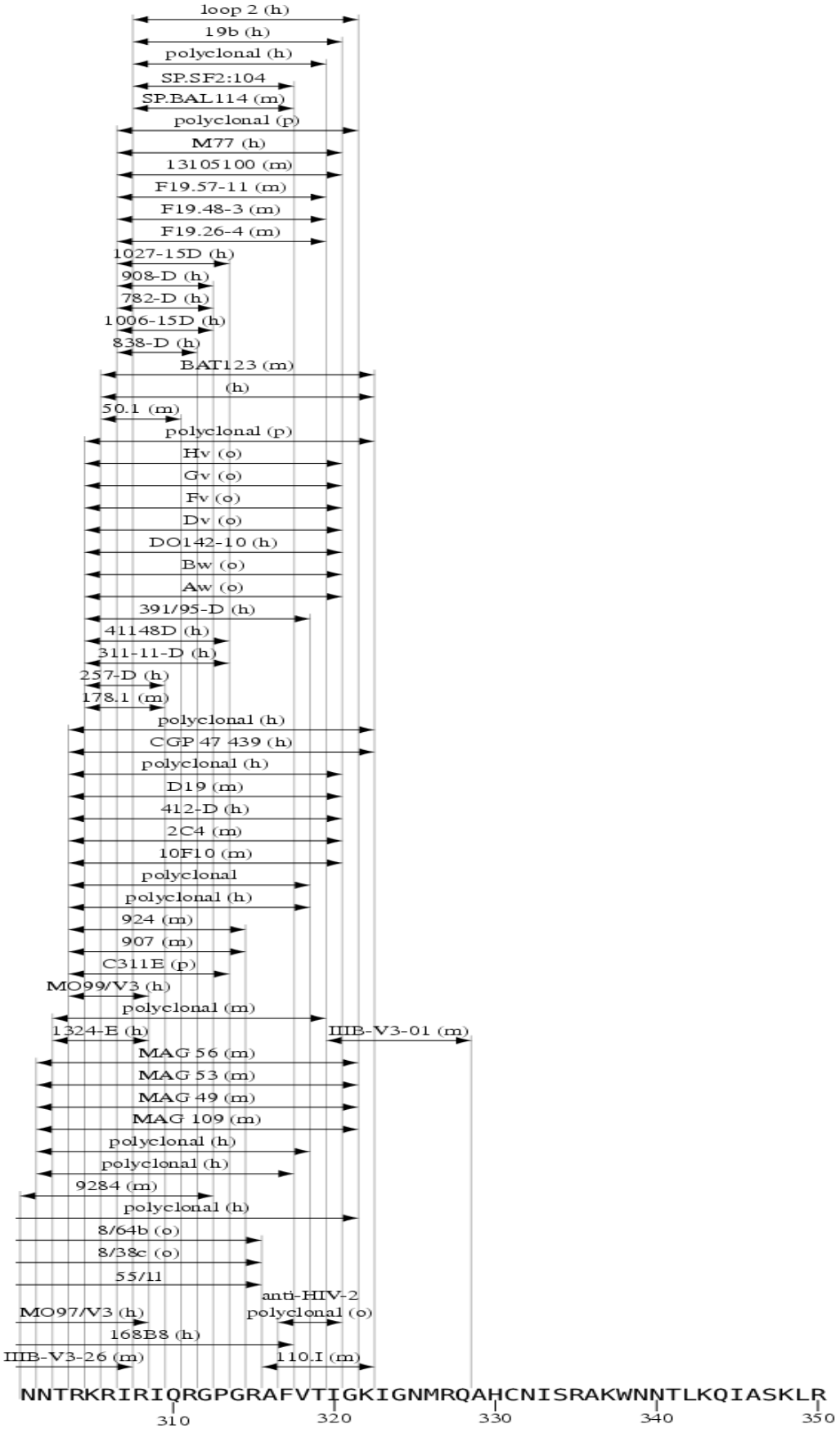
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M77 (h)
13105100 (m)
F19.57-11 (m)
F19.48-3 (m)
F19.26-4 (m)
1027-15D (h)
908-D (h)
782-D (h)
1006-15D (h)
838-D (h)
BAT123 (m)
(h)
50.1 (m)
polyclonal (p)
Hv (o)
Gv (o)
Fv (o)
Dv (o)
DO142-10 (h)
Bw (o)
Aw (o)
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41148D (h)
311-11-D (h)
257-D (h)
178.1 (m)
polyclonal (h)
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polyclonal (h)
D19 (m)
412-D (h)
2C4 (m)
10F10 (m)
polyclonal
polyclonal (h)
924 (m)
907 (m)
C311E (p)
MO99/V3 (h)
polyclonal (m)
1324-E (h)
IIIB-V3-01 (m)
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MAG 53 (m)
MAG 49 (m)
MAG 109 (m)
polyclonal (h)
polyclonal (h)
9284 (m)
polyclonal (h)
8/64b (o)
8/38c (o)
55/11
anti-HIV-2
MO97/V3 (h)
polyclonal (o)
168B8 (h)
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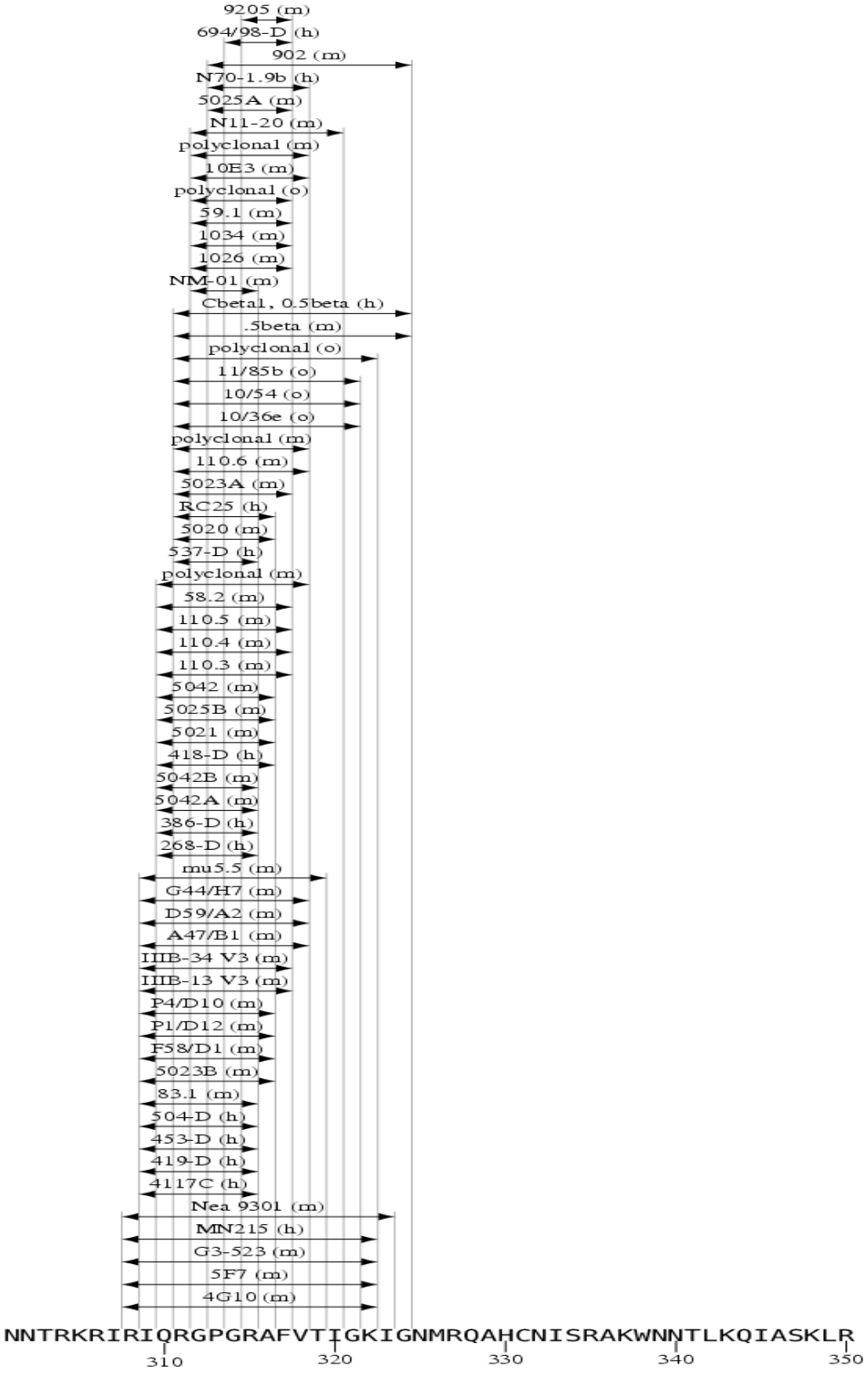
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polyclonal (o)
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1034 (m)
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NM-01 (m)
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5025B (m)
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386-D (h)
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G44/H7 (m)
D59/A2 (m)
A47/B1 (m)
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P4/D10 (m)
P1/D12 (m)
F58/D1 (m)
5023B (m)
83.1 (m)
504-D (h)
453-D (h)
419-D (h)
4117C (h)
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MN215 (h)
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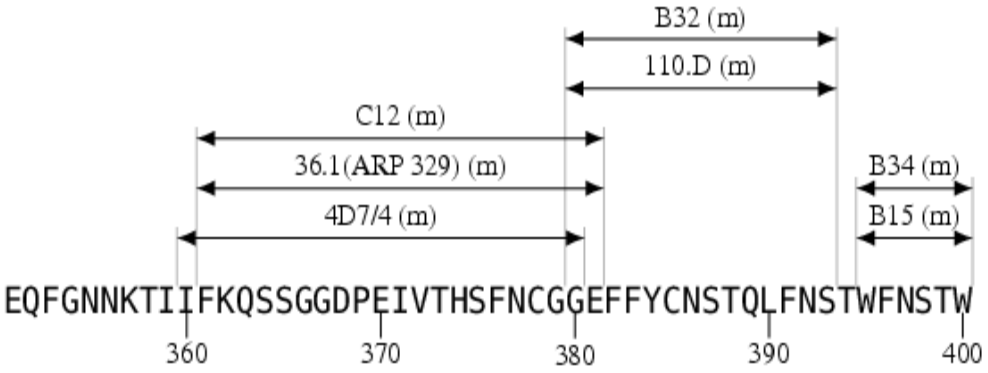
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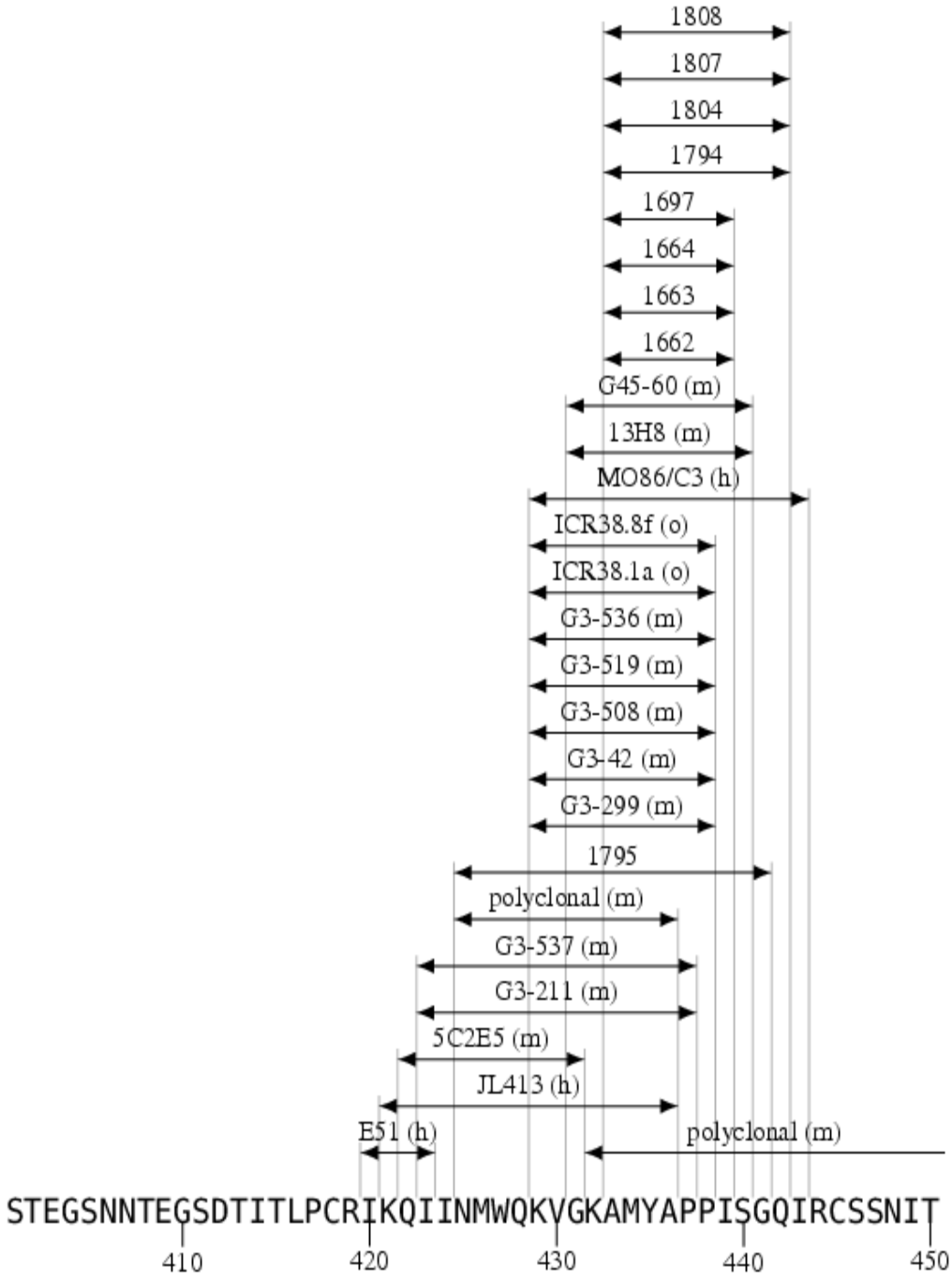
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G3-299 (m)
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polyclonal (m)
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5C2E5 (m)
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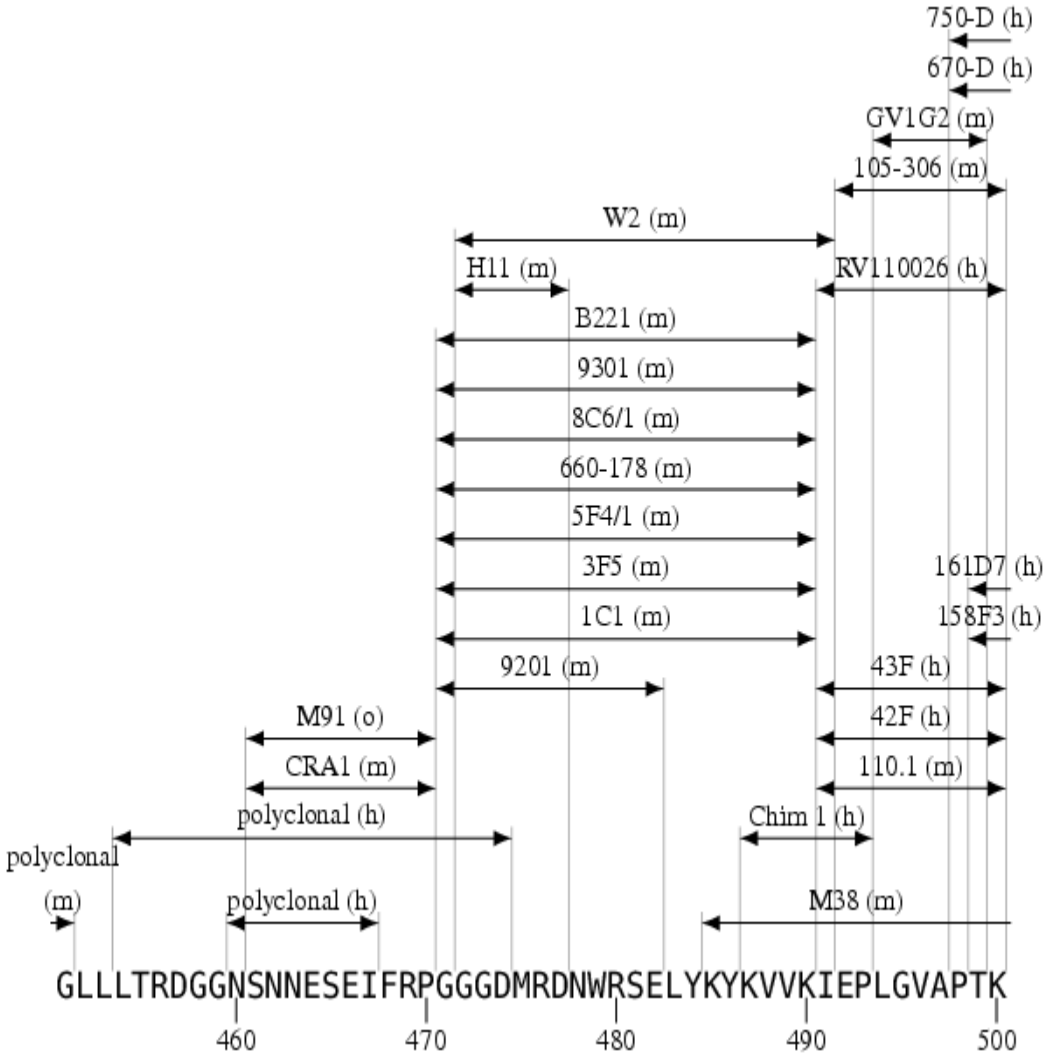
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RV110026 (h)
B221 (m)
9301 (m)
8C6/1 (m)
660-178 (m)
5F4/1 (m)
3F5 (m)
161D7 (h)
1C1 (m)
158F3 (h)
9201 (m)
43F (h)
M91 (o)
42F (h)
CRA1 (m)
110.1 (m)
polyclonal (h)
Chim 1 (h)
polyclonal
(m)
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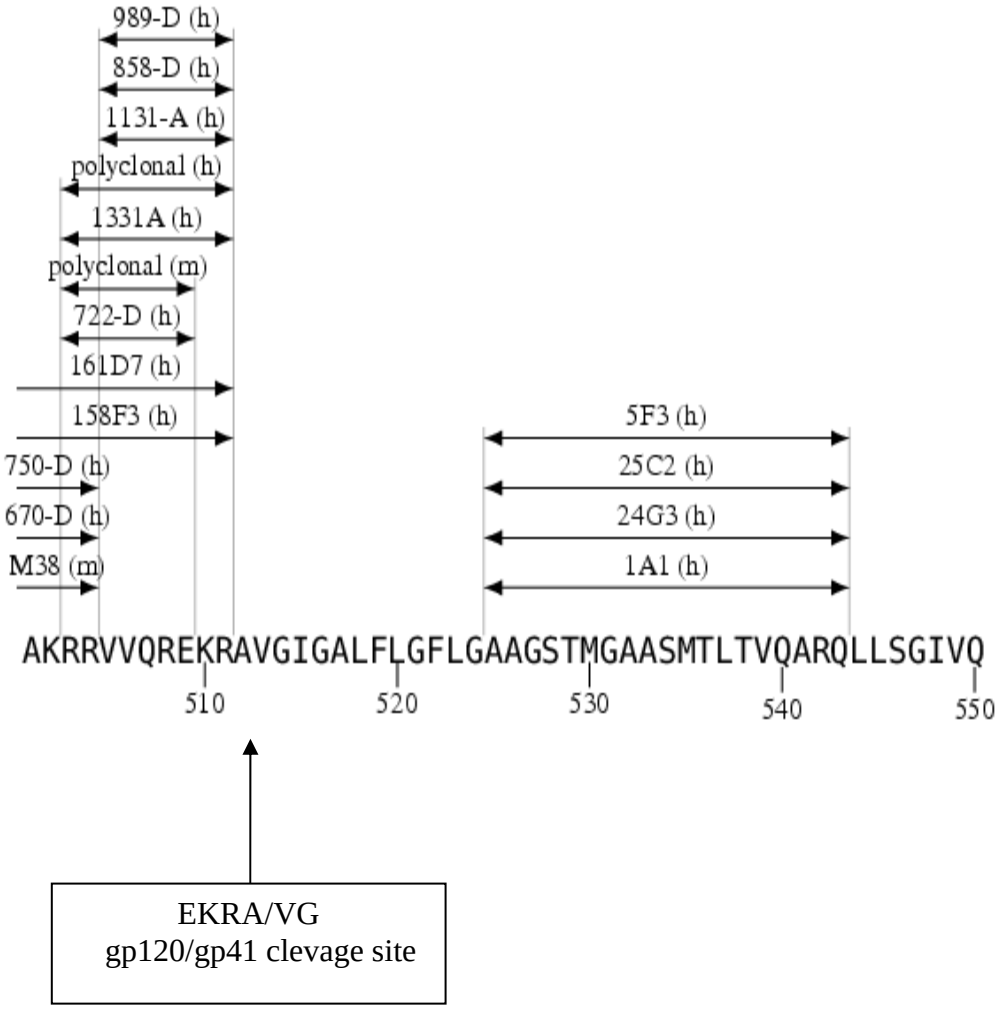
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158F3 (h)
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670-D (h)
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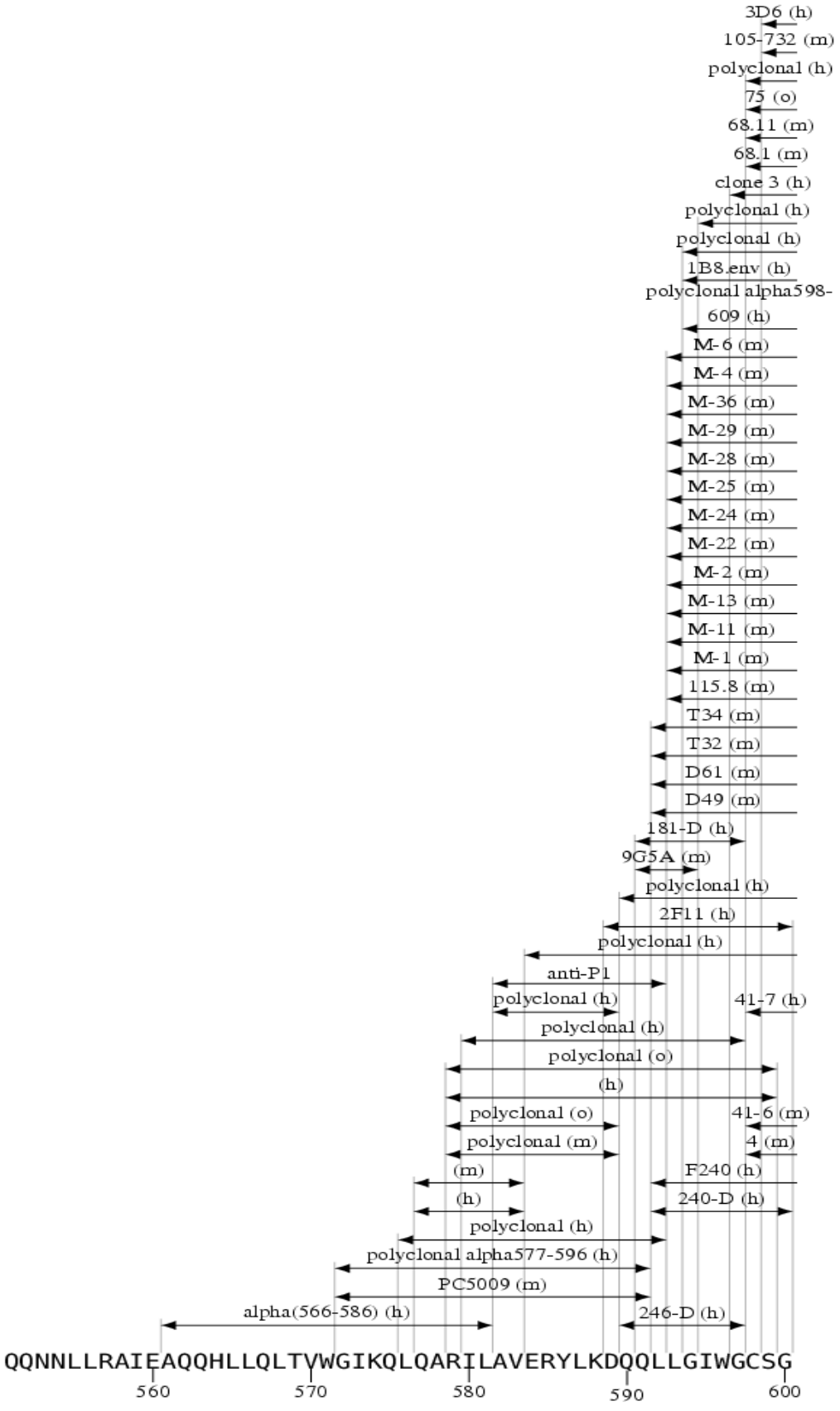
gp120/gp41 clevage site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polyclonal (h)
1B8.env (h)
polyclonal alpha598-609 (h)
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M-4 (m)
M-36 (m)
M-29 (m)
M-28 (m)
M-25 (m)
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M-11 (m)
M-1 (m)
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D49 (m)
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polyclonal (o)
(h)
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polyclonal (m)
4 (m)
(m)
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(h)
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polyclonal (h)
polyclonal alpha577-596 (h)
PC5009 (m)
alpha(566-586) (h)
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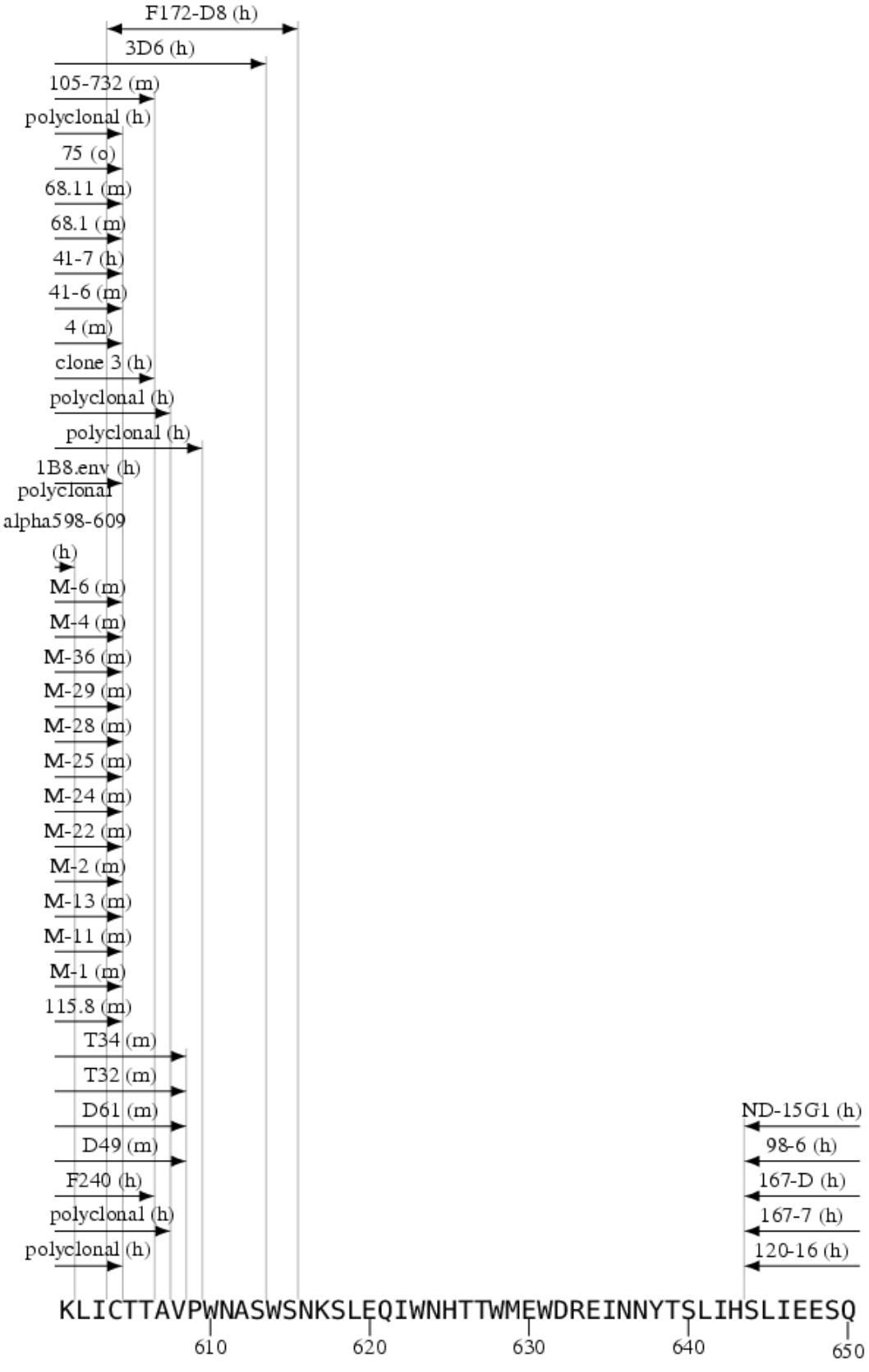
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41-6 (m)
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polyclonal (h)
polyclonal (h)
1B8.env (h)
polyclonal
alpha598-609
(h)
M-6 (m)
M-4 (m)
M-36 (m)
M-29 (m)
M-28 (m)
M-25 (m)
M-24 (m)
M-22 (m)
M-2 (m)
M-13 (m)
M-11 (m)
M-1 (m)
115.8 (m)
T34 (m)
T32 (m)
D61 (m)
D49 (m)
F240 (h)
polyclonal (h)
polyclonal (h)
ND-15G1 (h)
98-6 (h)
167-D (h)
167-7 (h)
120-16 (h)
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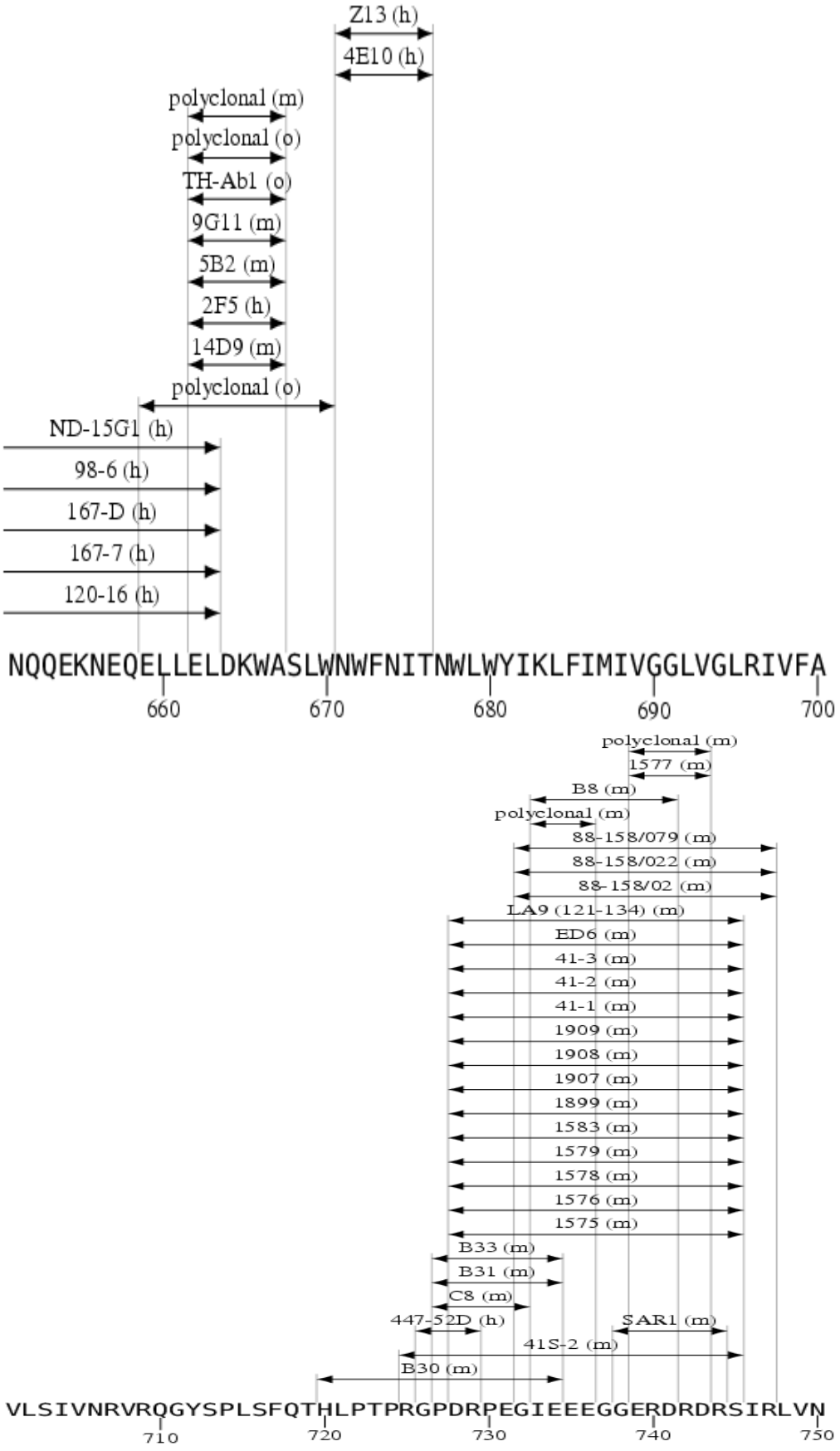
610
620
630
640
650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TH-Ab1 (o)
9G11 (m)
5B2 (m)
2F5 (h)
14D9 (m)
polyclonal (o)
ND-15G1 (h)
98-6 (h)
167-D (h)
167-7 (h)
120-16 (h)
NQQEKNEQELLELDKWASLWNWFNITNWLWYIKLFIMIVGGLVGLRIVFA
660
670
680
690
700
polyclonal (m)
1577 (m)
B8 (m)
polyclonal (m)
88-158/079 (m)
88-158/022 (m)
88-158/02 (m)
LA9 (121-134) (m)
ED6 (m)
41-3 (m)
41-2 (m)
41-1 (m)
1909 (m)
1908 (m)
1907 (m)
1899 (m)
1583 (m)
1579 (m)
1578 (m)
1576 (m)
1575 (m)
B33 (m)
B31 (m)
C8 (m)
447-52D (h)
SAR1 (m)
41S-2 (m)
B30 (m)
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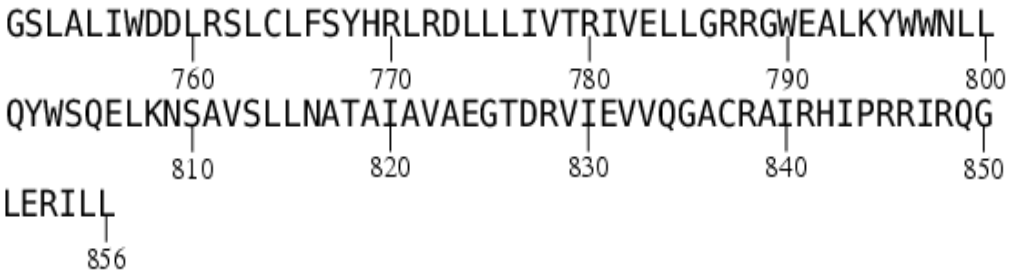
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780
790
800
QYWSQELKNSAVSLLNATAIAVAEGTDRVIEVVQGACRAIRHIPRRIRQG
810
820
830
840
850
LERILL
856

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