การโคลนและการแสดงออกของชิ้นส่วนแปรผันสายเดี่ยว
ของมอโนโคลนอลแอนติบอดีต่อนอร์ฟลอกซาซินใน *Pichia pastoris*

นายจิรวัฒน์ มาลา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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CLONING AND EXPRESSION OF SINGLE CHAIN VARIABLE FRAGMENT OF MONOCLONAL ANTIBODY AGAINST NORFLOXACIN IN *Pichia pastoris*

Mr. Jirawat Mala

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biotechnology
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Thesis Title	CLONING AND EXPRESSION OF SINGLE CHAIN VARIABLE FRAGMENT OF MONOCLONAL ANTIBODY AGAINST NORFLOXACIN IN *Pichia pastoris*
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จิรวัฒน์ มาลา : การโคลนและการแสดงออกของชิ้นส่วนแปรผันสายเดี่ยวของมอโนโคลนอลแอนติบอดีต่อนอร์ฟลอกซาซินใน *Pichia pastoris* (CLONING AND EXPRESSION OF SINGLE CHAIN VARIABLE FRAGMENT OF MONOCLONAL ANTIBODY AGAINST NORFLOXACIN IN *Pichia pastoris*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร.ศรินทิพ สุกใส, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.กิตตินันท์ โกมลภิส, รศ. ดร.ธนาภัทร ปาลกะ, 177 หน้า.

ในปัจจุบัน เทคโนโลยีแอนติบอดีลูกผสม เป็นเทคโนโลยีทางเลือกสำหรับผลิตแอนติบอดีที่มีต้นทุนต่ำ ซึ่งมีสัมพรรคภาพและจำเพาะตามที่ต้องการ ในงานวิจัยนี้ ชิ้นส่วนแปรผันสายเดี่ยว (scFv) ที่จดจำต่อยาปฏิชีวนะนอร์ฟลอกซาซินได้ถูกออกแบบและสร้างขึ้นมาได้สำเร็จ สายดีเอนเอคู่สมถูกสังเคราะห์มาจากอาร์เอ็นเอทั้งหมดของกลุ่มเซลล์ไฮบริโดมาที่ผลิตสารต้านนอร์ฟลอกซาซิน (Nor155) ดีเอนเอที่ถอดรหัสพันธุกรรมบริเวณ V_H และ V_Lของเซลล์ไฮบริโดมานี้ถูกเพิ่มปริมาณและตรวจสอบลำดับนิวคลีโอไทด์ ชิ้นส่วนดีเอนเอบริเวณของ V_H และ V_L มีขนาด 402 คู่เบส และ 363 คู่เบส ตามลำดับ ชิ้นส่วนแปรผันสายเดี่ยวของ Nor155 ถูกสร้างโดยการเพิ่ม $(Gly_4Ser)_3$ ซึ่งทำหน้าที่เป็นตัวเชื่อมระหว่างบริเวณ V_H และ V_L และถูกถ่ายโอนเข้าสู่ pPICZ**α**A ซึ่งเป็นดีเอนเอพาหะแสดงออกของ *Pichia pastoris* เพื่อสร้างเป็น pPICZ**α**A-scFv (pJM01) หลังจากตรวจสอบความถูกต้องของลำดับนิวคลีโอไทด์ ชุดของยีนชิ้นส่วนแปรผันสายเดี่ยวแอนติบอดีแสดงให้เห็นกรอบการอ่านยีนมีขนาด 1161 คู่เบส ซึ่งมีศักยภาพในการแปลรหัสเป็นสายพอลิเพปไทด์ได้ 386 กรดอะมิโน โดยน้ำหนักโมเลกุลที่คาดการณ์คือ 42.5 กิโลดาลตัน (ภายในเซลล์) และ 32.67 กิโลดาลตัน (หลั่งออกภายนอกเซลล์) ตามลำดับ จากนั้น pJM01 ถูกรวมเข้ากับโครโมโซมของ *Pichia* ที่ลำดับการถอดรหัสของ *AOX1* และเซลล์ที่ได้รับการถ่ายโอนถูกยืนยันด้วยวิธี Southern blot ปฏิกิริยาลูกโซ่พอลิเมอเรส และการวิเคราะห์ลำดับนิวคลีโอไทด์ ในการวิเคราะห์ด้วย Southern blot ของ 9 เซลล์ที่ได้รับการถ่ายโอน ซึ่งแสดงให้เห็นว่ายีนของชิ้นส่วนแปรผันสายเดี่ยวถูกแทรกอยู่ในโครโมโซมของ *Pichia* จากการทำการแสดงออกของชิ้นส่วนแปรผันสายเดี่ยวในการเลี้ยงแบบเขย่า เซลล์ที่ได้รับการถ่ายโอน O5 แสดงให้เห็นว่าสามารถแสดงออกชิ้นส่วนแปรผันสายเดี่ยวได้สูงที่สุดในจำนวน 9 เซลล์ที่ได้รับการถ่ายโอน ระดับการถอดรหัสของอาร์เอ็นเอนำรหัสของเซลล์ที่ได้รับการถ่ายโอน O5 แสดงให้เห็นว่า การแสดงออกที่ชั่วโมงที่ 24 มีระดับการแสดงออกสูงที่สุด เท่ากับ 688 เท่า เมื่อเปรียบเทียบกับตัวควบคุม แอนติบอดีชิ้นส่วนแปรผันสายเดี่ยวลูกผสม ถูกทำให้บริสุทธิ์ด้วยคอลัมน์จับแบบจำเพาะ (IMAC) จากผลของ SDS-PAGE และ Western blot แสดงให้เห็นว่า โปรตีนหลักของชิ้นส่วนแปรผันสายเดี่ยวที่ถูกทำให้บริสุทธิ์มีน้ำหนักโมเลกุล 32.67 กิโลดาลตัน ซึ่งจากการทดลองนี้ชี้ให้เห็นว่า แอนติบอดีชิ้นส่วนแปรผันสายเดี่ยวถูกผลิตได้สำเร็จจาก *P. pastoris* การตรวจวัดการจับกันแบบจำเพาะใน ELISA และ Surface plasmon resonance (SPR) ชี้ให้เห็นว่า ชิ้นแปรผันสายเดี่ยวที่ถูกทำให้บริสุทธิ์ยังคงคุณสมบัติในการจับกับแอนติเจน จากผลการทดลองทั้งหมดสามารถสรุปได้ว่า ยีนที่ถอดรหัสพันธุกรรมชิ้นส่วนแปรผันสายเดี่ยวของมอโนโคลนอลแอนติบอดี Nor155 สามารถถูกผลิตได้ในยีสต์ นอกจากนี้ความสามารถในการจับของชิ้นส่วนแปรผันสายเดี่ยวต่อแอนติเจนที่สอดคล้องยังคงคุณสมบัติเดิม

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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5472879023 : MAJOR BIOTECHNOLOGY
KEYWORDS: CLONING / EXPRESSION / SCFV / PICHIA PASTORIS / NORFLOXACIN / RECOMBINANT ANTIBODY / SOUTHERN BLOT

JIRAWAT MALA: CLONING AND EXPRESSION OF SINGLE CHAIN VARIABLE FRAGMENT OF MONOCLONAL ANTIBODY AGAINST NORFLOXACIN IN *Pichia pastoris*. ADVISOR: SARINTIP SOOKSAI, Ph.D., CO-ADVISOR: ASST. PROF. KITTINAN KOMOLPIS, Ph.D., ASSOC. PROF. TANAPAT PALAGA, Ph.D., 177 pp.

Currently, recombinant antibody technology has provided an alternative approach for engineering low-cost antibodies with desirable affinities and specificities. In this research, a single chain variable fragment (scFv) that recognizes norfloxacin antibiotic was successfully designed and constructed. The cDNA was synthesized from total RNA of antinorfloxacin-producing hybridoma clone (Nor155). DNA encoding V_H and V_L regions of this hybridoma were amplified and sequenced. DNA fragments of V_H and V_L regions were approximately 402 bp and 363 bp, respectively. The scFv of Nor155 was constructed by addition of $(Gly_4Ser)_3$ as a linker between V_H and V_L regions and subcloned into pPICZ**α**A, an expression vector of *Pichia pastoris*, to generate pPICZ**α**A-scFv (pJM01). After sequencing analysis, the scFv antibody gene cassette revealed an open reading frame of 1161 bp with the potential to encode a polypeptide of 386 amino acids and a predicted molecular weight of 42.5 kDa (intracellular) and 32.67 kDa (secreted), respectively. The pJM01 was incorporated into the *Pichia* chromosome at *AOX1* promoter and transformants were confirmed by Southern blot analysis, PCR and sequencing analysis. Southern blot analysis was carried out in 9 transformants, which showed that the scFv gene was integrated into the *Pichia* chromosome. By performing the scFv expression in shaker culture, transformant O5 showed the highest scFv expression, among 9 transformants. The mRNA transcript level of transformant O5 showed highest relative normalized expression at 24 h, 688 times compared to the control. The recombinant scFv antibody was purified by immobilized metal affinity chromatography (IMAC). SDS-PAGE and Western blot revealed a predominant protein at 32.67 kDa of the purified scFv, thus indicating the scFv antibody was successfully expressed by *P. pastoris*. The specific binding and affinity in ELISA and surface plasmon resonance (SPR) indicated that the purified scFv still functional in term of antigen binding. From all results, it could be concluded that scFv of monoclonal antibody Nor155 could be produced in yeast. In addition, the binding ability of the obtained scFv to the corresponding antigen remains functional.

Field of Study: Biotechnology
Academic Year: 2015

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBRIVATIONS

%	Percentage
°C	Degree Celsius
µg	Microgram(s)
µL	Microliter(s)
µg/mL	Microgram per milliliter
µg/µL	Microgram per microliter
×g	Multiply by gravitational force ($\times 9.80665\ m.s^{2}$)
AOX1	Alcohol oxidase 1 gene
AOX2	Alcohol oxidase 2 gene
ELISA	Enzyme linked Immunosorbent assay
e.g.	Example (exempli gratia, Latin)
FQs	Fluoroquinolones
g	Gram(s), (Unit of mass)
h	Hour(s)
His^{-}	Histidine auxotroph
HPLC	High performance liquid chromatography
Ig	Immunoglobulin
kb	Kilobase pair(s)
$lb.in^{-2}$	Pound per square inch
M	Molar
MAb	Monoclonal antibody
mg	Milligram(s)
mL	Milliliter(s)
MMH	Minimal methanol histidine
Mut^{+}	Methanol utilization plus phenotype
Mut^{s}	Methanol utilization slow phenotype
ng	Nanogram(s)
nm	Nanometer

OD	Optical density
PAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RAb	Recombinant antibodies
RNase	Ribonuclease A
rpm	Revolution per minute
SPR	Surface plasmon resosnance
T	Temperature
TAE	Tris-acetate-EDTA
Tm	Melting temperature (°C)
UV	Ultraviolet
w/	With
w/o	Without
w/v	Weight by volume
WT	Wild type strain
YNB	Yeast nitrogen base
YPD	Yeast extract peptone dextrose
YPG	Yeast extract peptone glycerol

CHAPTER I
INTRODUCTION

1.1 Importance and Rationale

During the last two decades, various immunoassay methods have been developed and reported based on polyclonal antibody (PAb) and monoclonal antibody (MAb). The MAb, most commonly used in immunology reactions, can be generated by a modified hybridoma technology. To produce the MAb through this technology, there are various limitations such as MAbs preparation which is extremely complicated, time-consuming (4 and 6 months to produce), highly expensive serum, unstable hybridoma clone and requires expertise, respectively [1, 2]. Moreover, it is well known that hybridoma cells fail to grow to higher cell densities than the order of 10^6 cells/mL. The reason for this is either that essential nutritional components are consumed or that the cells produce some kind of inhibitory or toxic compound. In addition, hybridoma derived antibodies cannot be improved until they are first converted into recombinant antibodies [3, 4]. Finally, the preparation of high quality antibodies is still a bottleneck issue when establishing immunoassay methods [2, 5].

To avoid these problems, an alternative production system is required. Nowadays, recombinant antibody (RAb) technology has provided an alternative approach for engineering low-cost antibodies with desirable affinities and specificities [5-7]. Single-chain antibody fragment (scFv) is developed via this technology in which to replace the monoclonal antibody to some extent. The obvious advantages of scFv are that it retains the original antigen-binding site, allowing it to maintain its specific affinity for the antigen, and it can be produced in large scale in microorganisms at low cost [2].

Normally, RAb fragments are usually produced in *Escherichia coli* as expression system and purified from the cell extract. However, bacteria often are unable to fold properly or to secrete eukaryotic protein such as antibodies, thus leading to low yields of soluble and active proteins even after denaturation and

refolding of intracellular accumulated inclusion bodies. Therefore, other expression systems such as yeasts, mammalian, insect, plant and *in vitro* translation systems have been used to produce RAb fragment.

Among of these expression systems, the fermentation of antibody-producing yeasts is a promising method. The main advantage of the yeast, especially methylotrophic yeast *Pichia pastoris*, is that it is eukaryotic of which genetic information is well understood. Its genetic can be manipulated. It can be cultivated easily and inexpensively. Proteins produced in yeast also are subjected to typical eukaryote-specific, post-translational modification mechanism (proteolytic processing, folding, glycosylation and formation of disulfide bridges). Due to the high yields obtained, yeast has become a popular protein expression system [8].

The RAb obtained from yeasts is cheaper than the hybridoma derived MAbs. It is also easier to maintain the eukaryotic clone of recombinant antibodies than the hybridoma clones. Hence, it is reasonable to obtain recombinant antibodies from hybridoma clones of MAbs with well-proven diagnostic use of their application in diagnostic assays as a cheaper substitute for MAb [9].

The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University recently succeeded in the generation of monoclonal producing antibody against norfloxacin, an antibiotic used to treat microbial infection in human, domestic animals, poultry and fish. The obtained monoclonal antibody was intended for an application in drug residue detection in which a large amount of antibody is required in the development of the test kit. Therefore in this research, the scFv fragment of antinorfloxacin-producing hybridoma was constructed and expressed in *Pichia pastoris* GS115 as a model of recombinant antibody expression against antibiotic in methylotrophic yeast.

1.2 Objectives of the Dissertation

1.2.1 To obtain scFv antibody gene from the antinorfloxacin-producing hybridoma clone 155 (nor155)

1.2.2 To construct the yeast expression plasmid that harboring scFv antibody gene for expression in the methylotrophic yeast *Pichia pastoris*

1.2.3 To use methylotrophic yeast *Pichia pastoris* as the host model for expression the recombinant scFv antibody

1.3 Expected Beneficial Outcomes of the Dissertation

1.3.1 Genetic information of Norfloxacin–specific antibody gene

1.3.2 Norfloxacin–specific antibody producing yeast

CHAPTER II
THEORETICAL BACKGROUND AND LITERATURE REVIEWS

2.1 Antibody

Antibodies are a modular defense system that identifies and neutralizes foreign objects like bacteria and viruses. Each of them could recognize a specific antigen unique to its target as they possess the antigen-binding sites, a paratope (a structure analogous to a lock) located at the upper tips of the "Y" shape antibody molecules. This paratope is specific for one particular epitope (analogous to a key), displayed on the particular antigen, allowing these two structures to specifically bind together. Thus, this mechanism could allow an antibody to tag a microbe as well as an infected cell to be attacked by other parts of the immune system and also to directly neutralize its target [10, 11].

2.1.1 Structure of Antibody

Antibody (or immunoglobulin) molecules are glycoproteins composed of one or more units, each containing four polypeptide chains: two identical heavy chains (H) and two identical light chains (L). The amino terminal ends of the polypeptide chains show considerable variation in amino acid composition and are referred as the variable (V) regions to distinguish them from the relatively constant (C) regions. Each L chain consists of one variable domain VL and one constant domain CL. The H chains consist of a variable domain, VH, and three constant domains CH1, CH2, CH3. Each heavy chain has about twice the number of amino acids and molecular weight (~50,000 Da) as each light chain (~25,000 Da), resulting in a total immunoglobulin monomer molecular weight of approximately 150,000 Da [12].

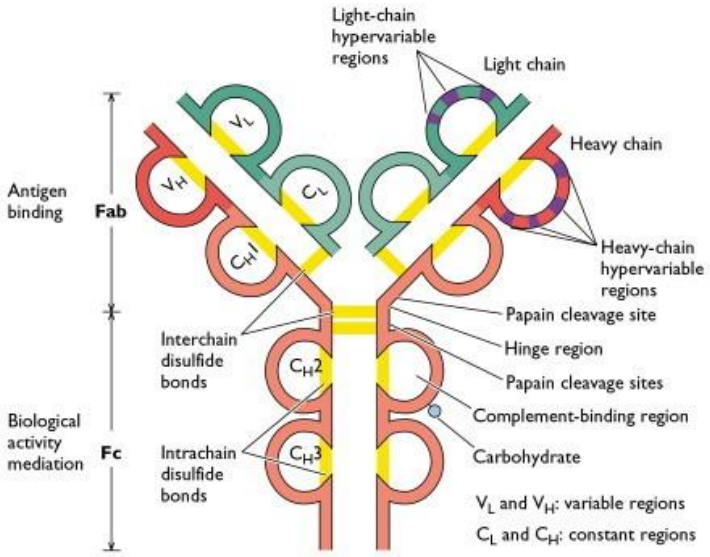

Figure 2.1 Generalized structure of an immunoglobulin (IgG) [13].

Heavy and light chains are held together by a combination of non-covalent interactions and covalent interchain disulfide bonds, forming a bilaterally symmetric structure. The V regions of H and L chains comprise the antigen-binding sites of the immunoglobulin (Ig) molecules. Each Ig monomer contains two antigen-binding sites and is said to be bivalent. The hinge region is the area of the H chains between the first and second C region domains and is held together by disulfide bonds. This flexible hinge (found in IgG, IgA and IgD, but not IgM or IgE) region allows the distance between the two antigen-binding sites to vary [14].

2.1.2 Classes of Immunoglobulins

The five primary classes of immunoglobulins are IgG, IgM, IgA, IgD and IgE. They are distinguished by the type of heavy chain found in the molecule. IgG molecules have heavy chains known as gamma-chains; IgMs have mu-chains; IgAs have alpha-chains; IgEs have epsilon-chains; and IgDs have delta-chains. Differences in heavy chain polypeptides allow these immunoglobulins to function in different types of immune responses and at particular stages of the immune response. The polypeptide protein sequences responsible for these differences are found primarily

in the Fc fragment. While there are five different types of heavy chains, there are only two main types of light chains: kappa (κ) and lambda (λ) [15].

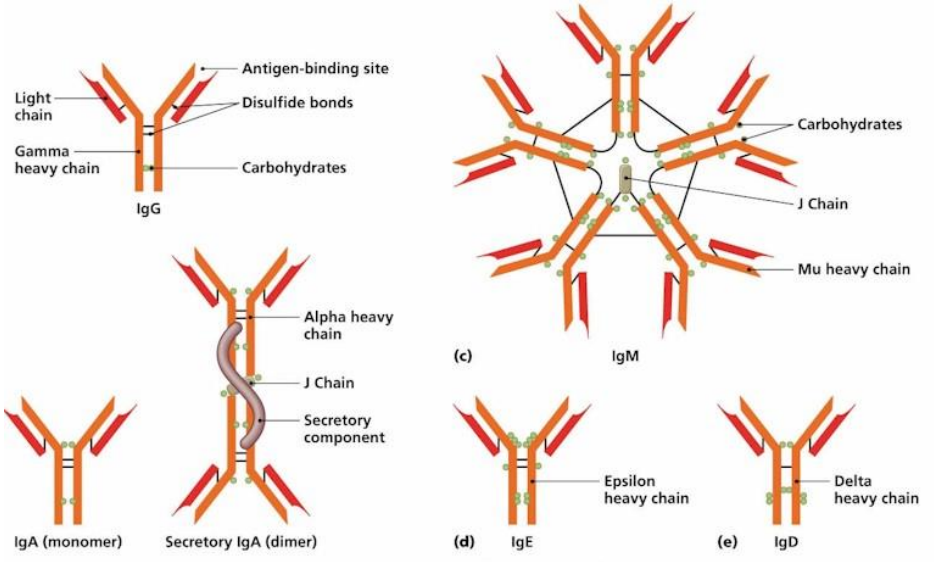

Figure 2.2 Five classes of antibodies or immunoglobulin molecules [16].

2.2 Antibody Production

When an antigen is introduced into an animal, one aspect of the immune response is the secretion by plasma cells of antibodies: immunoglobulin molecules with binding site that recognize the shape of particular determinants on the surface of the antigen and bind to them. The combination of antibody with antigen sets in train processes that can neutralize and eliminate the antigen [17].

2.2.1 Polyclonal Antibodies Production

Naturally, an immune system of animal produces several thousands of different antibodies at one given time. This diversity in antibody specificity allows the animal to respond rapidly to most threats such as disease causing organisms. Upon immunization with most antigens such as a recombinant protein, the immune system of animal detects the antigen and results in a polyclonal response and the accumulation of many different antibodies in the serum. The serum is a source of

polyclonal antibodies which may contain immunoglobulins of varying isotypes, affinities, specificities and biological activities. Polyclonal antibodies are easy to produce and can lead to very sensitive immunoassays since various antibodies recognize and bind the antigen by different epitopes or structures present on the surface of antigen. However, since polyclonal antiserum consists of numerous antibodies with differing specificity, it can bind to other antigen with the same or similar epitopes on their surfaces and may lead to cross-reactivity that may not be desirable. For polyclonal antibody production, typically a purified protein is mixed with an adjuvant and saline buffer and injected into animals (mice, rabbits, etc.). Following several immunizations and regular monitoring of the immune response against the antigen, blood from the animal is retrieved and the antiserum containing the antibodies is collected for further analysis and assay development [11].

2.2.2 Monoclonal Antibodies Production

Monoclonal antibodies, as opposed to polyclonal antibodies, are issued from one single clone of cells. To obtain monoclonal antibodies, individual B cells are fused to myeloma cells and isolated by serial dilution, resulting in a fusion product, or hybridoma cell line, each of which can produce one specific antibody for extended periods of times via tissue culture [11].

For monoclonal antibody, typically mice are immunized with the purified protein following the same immunization protocols as for polyclonal antibody production. However, mouse splenocytes are collected following the immunization process and fused with myeloma cells, thereby forming hybridomas. These hybridomas can then be screened, and the best clones cultured in standard tissue culture facilities. Ascetic fluid used to be the method of choice for monoclonal antibody production. The hybridoma cells line obtained for a given antibody was injected into the peritoneal cavity of mice, where it grew and simultaneously produced the antibodies. After a given length of time, ascetic fluid containing the antibodies was harvested from the peritoneal cavity. However, due to animal care issues associated with this technique, it is not as often used nowadays for standard

monoclonal antibody production. Hybridoma technology via tissue culture now tends to be main source of monoclonal antibodies [14].

2.3 Hybridoma Technology

Hybridoma technology is a technology of forming hybrid cell lines (called hybridomas) by fusing an antibody-producing B cell with a myeloma (B cell cancer) cell that is selected for its ability to grow in tissue culture and for an absence of antibody chain synthesis. The antibodies produced by the hybridoma are all of a single specificity and are therefore monoclonal antibodies. The production of monoclonal antibodies was invented by César Milstein and Georges J. F. Köhler in 1975. They shared the Nobel Prize of 1984 for Medicine and Physiology with Niels Kaj Jerne, who made other contributions to immunology [18].

The basic of hybridoma technology is the immortalization of B-lymphocytes with antibody-producing capacities, but limited in vitro growth characteristics. The lymphocytes are fused with cells from a non-antibody-producing and continuously growing tumor cell line or myeloma cell line so that hybrids continue to secrete antibodies while gaining the immortality of the parent tumor cell. Because each B-lymphocyte produces an immunoglobulin molecule with a fixed specificity, clones derived from hybridized cell populations are homogeneous in nature, and each of the clones secretes an immunoglobulin with a single molecular structure and antigen specificity. Thus, all the antibody molecules secreted by the same cell line exhibit identical specificity, affinity and isotype (class or subclass). The antibodies produced by the fusion procedure are known as monoclonal antibodies, distinguishing them from the heterogeneous polyclonal antibodies obtained by conventional antibody production. Antibody-producing clones may be stored in liquid nitrogen in the viable state and retrieved at will for culture and production of the corresponding antibody [19-21].

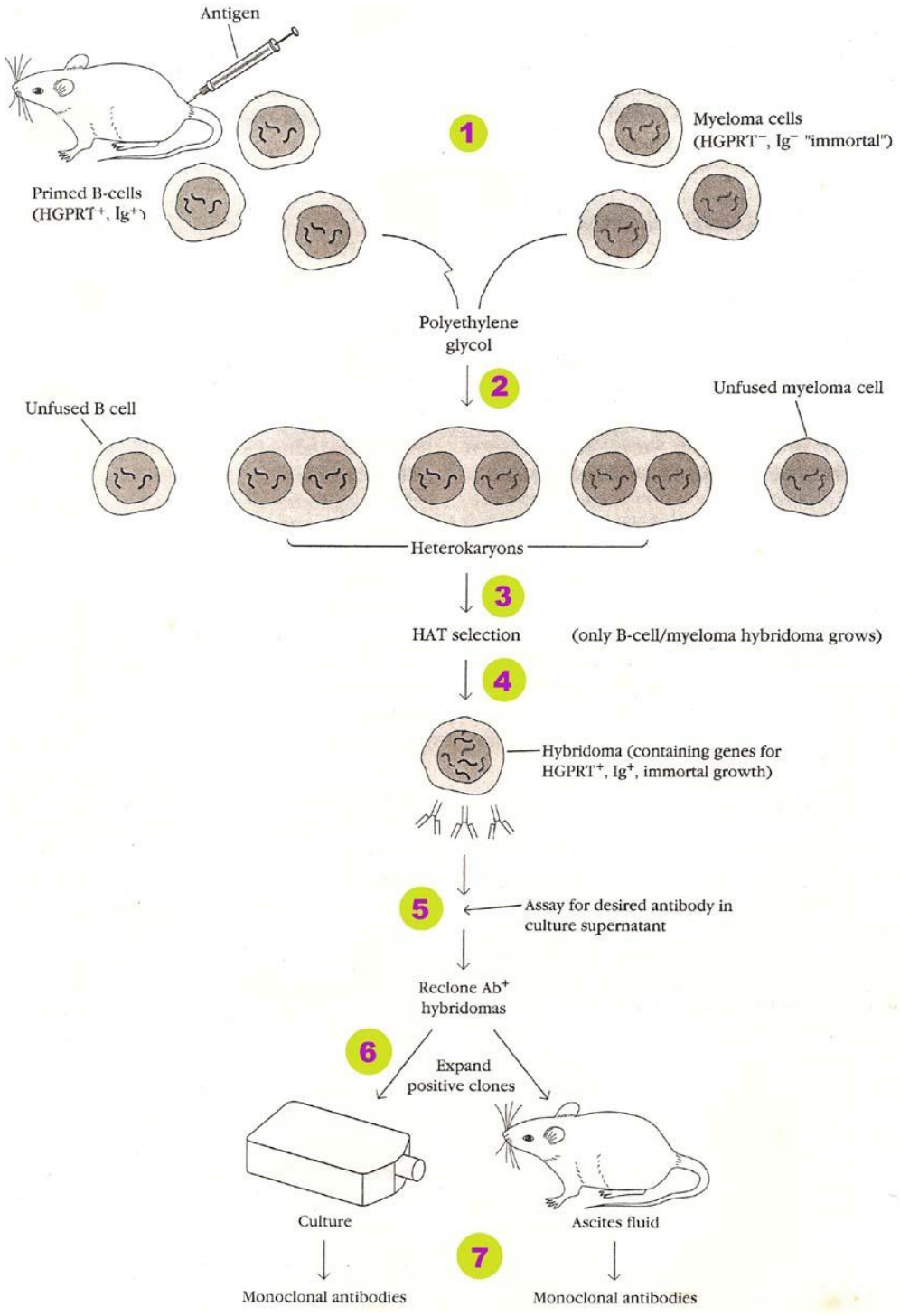

Figure 2.3 Schematic diagram of the production of hybridoma clones capable of screening monoclonal antibodies [21] [22].

Hypoxanthine-aminopterin-thymidine (HAT) medium is often used for preparation of monoclonal antibodies, which relies on the combination of aminopterin, a drug that acts as a powerful folate metabolism inhibitor by inhibiting dihydrofolate reductase, with hypoxanthine and thymidine which are intermediates in DNA synthesis. The trick is that aminopterin blocks DNA *de novo* synthesis, which is absolutely required for cell division to proceed, but hypoxanthine and thymidine provide cells with the raw material to evade the blockage (the "salvage pathway"), provided that they have the right enzymes, which means having functioning copies of the genes that encode them. The enzyme dihydrofolate reductase, which produces tetrahydrofolate (THF) by the reduction of dihydrofolate, is specifically blocked by aminopterin. THF, acting in association with specific proteins, can receive single carbon units that are then transferred to specific targets. One of the important targets for cellular reproduction is thymidylate synthase, which creates thymidine monophosphate (TMP) from deoxyuridine monophosphate (dUMP). By additional phosphorylation reactions, TMP can be used to make thymidine triphosphate (TTP), one of the four nucleotide precursors that are used by DNA polymerases to create DNA. Without the THF required to convert dUMP, there can be no TTP, and DNA synthesis cannot proceed, unless TMP can be produced from another source. The alternative source is the thymidine present in the HAT medium that can be absorbed by the cells and phosphorylated by thymidine kinase (TK) into TMP. The synthesis of IMP, (precursor to GMP and GTP, and to AMP and ATP) also requires THF, and also can be bypassed. In this case hypoxanthine-guanine phosphoribosyltransferase (HGPRT) reacts hypoxanthine absorbed from the medium with PRPP, liberating pyrophosphate, to produce IMP by a salvage pathway. Therefore, the use of HAT medium for cell culture is a form of artificial selection for cells containing working TK and HGPRT. Many useful refinements to the scheme are made possible by poisons that kill cells, but to which they are immune if they lack one of these genes. Thus, a cell lacking TK is resistant to bromodeoxyuridine (BrdU) and a cell lacking HGPRT is resistant to 6-thioguanine (6-TG) and 8-azaguanine. Thus, selection with one of the latter two drugs, followed by HAT medium, will yield revertant colonies. [14, 21, 23].

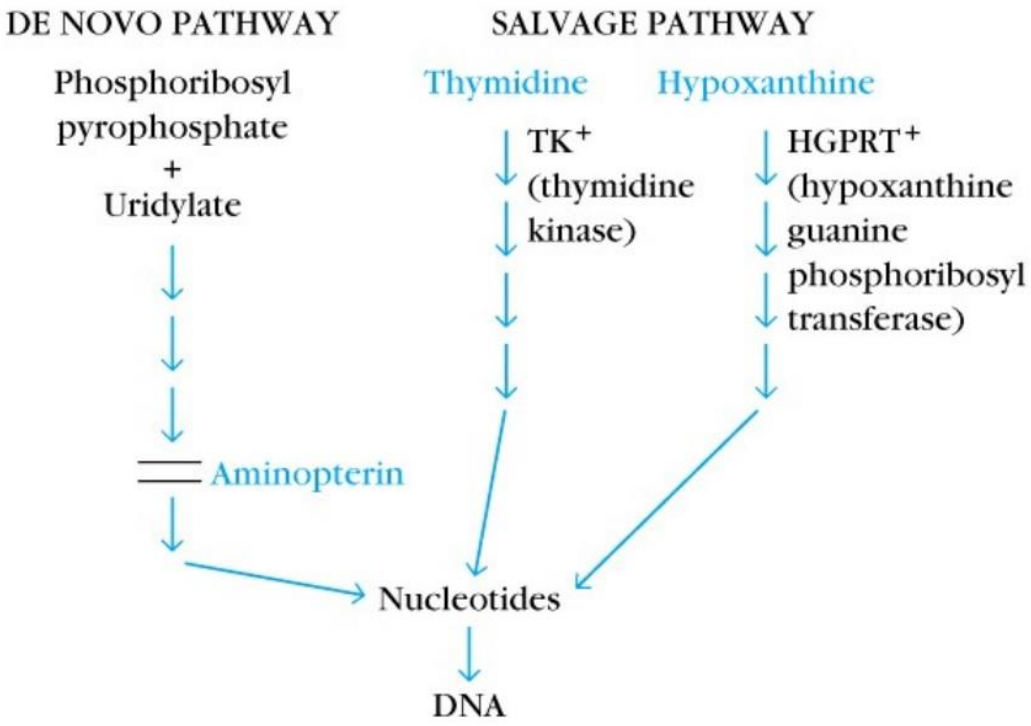

Figure 2.4 HAT selection medium [23]

2.4 Recombinant Antibody Technology

Recombinant antibody fragments are essential tools for research, diagnostics and therapy [24]. This technology facilitates the production of a range of novel antibodies and antibody derivatives and offers several advantages over both polyclonal and monoclonal antibody production. These advantages include the speed of antibody generation, the ability to produce numbers of different antibodies from a library and the possibility of altering the antibody affinity and specificity to suit the requirements of the application. These advantages enable the isolation of specific antibodies tailored for the particular application of interest to the research [25, 26]. Moreover, advances in recombinant antibody technology have greatly facilitated the genetic manipulation of antibody fragment. The genetic manipulation of recombinant antibodies thus improved our understanding about the structure and functional organization of immunoglobulins. Further, these advances have led the development of a large variety of engineered antibody molecules for research, diagnosis, and therapy with specificities out of reach of conventional antibody technology [27].

Recombinant DNA technologies have been wildly used for allowing the engineering of recombinant antibodies. The main requirement of recombinant antibody technology is the reliable cloning of functional light and heavy chain genes through the using hybridomas or immune repertoires as the source of DNA [25, 28].

The single-chain variable region (scFv) antibody is considered to be the smallest antigen-binding moiety. Since genes of scFvs are easily constructed from cDNA obtained from hybridoma cells or animal spleens [29]. ScFv has a wide range of therapeutic and diagnostic applications because of small size and its retained antigen binding properties. Recently, the scFv strategy has become one of the most popular methods in antibody engineering [28].

2.4.1 Single-Chain Variable Fragment (scFv)

Single-chain variable fragment (scFv) is a smallest engineered antibody consist of the variable regions of the antibody heavy (V_H) and light (V_L) chains fused together into a single polypeptide chain via short polypeptide flexible linker [10, 30, 31]. The resulting small antibody fragment (minimal size ~30kDa) still retains the binding specificity and affinity comparable to that of its parent antibody [32, 33]. The scFv is a popular structural format as it can be rapidly constructed [27]. In addition, it has a monomeric structure that remains stable event at low concentration and physiological temperature [34]. Moreover, this antibody fragment can be expressed by microorganisms and amenability to vitro evolution (affinity and specificity maturation) [35]. These advantages of scFvs over full-length antibodies make them an attractive antibody format [34].

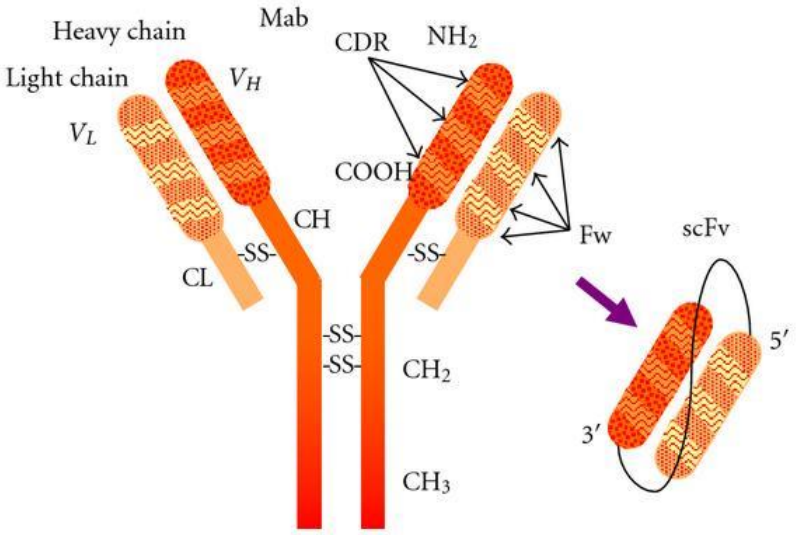

Figure 2.5 Antibody model showing subunit composition and domain distribution along the polypeptide chains. Single-chain fragment variable (scFv) antibody generated by recombinant antibody technology appears in the shaded area [10].

2.4.2 The Generation of scFv Antibodies

scFv antibodies have been constructed mainly form hybridoma, spleen cells from immunized mice, and B lymphocytes from human. scFv is a noncovalent heterodimer comprised of the V_H and V_L domains in which can then be used in the construction of recombinant scFv antibody. In order to attain these domains, mRNA is first isolated from hybridoma (or also from the spleen, lymph cells and bone morrow) followed by reverse transcribed into cDNA to serve as a template for antibody genes amplification (PCR). With this method, large libraries with a diverse range of antibody V_H and V_L genes could be created [11].

In the scFv construction, the order of the domains can be either VH-linker-VL or VL-linker-VH and both orientations have been applied. One of the most popular methods used is through PCR assembly. In this method, it allows the V domains of antibody to be cloned without any prior information about the nucleic acid as well as amino acid sequence of the particular antibody. Moreover, the V domains of antibody can be combined by in vitro recombinant directly after the PCR of V_H and V_L genes into plasmids or phagemid [11].

In a typical scFv, the linker peptide is generally designed with glycine and serine residues to provide flexibility and protease resistance [36, 37]. The most commonly used linker of scFv composed of three repeated units of a combination of glycine and serine residues $(GGGGS)_3$. The length of the linker between V_H and V_L affects the affinity of the scFv [30]. The Fv domains are stabilized through this amino acid long, flexible and hydrophilic linker [38]. The scFv usually shows monovalent antigen binding affinity similar to the Fab fragment of the parent antibody [37]. The flexible polypeptides, such as $(G_4S)_3$, have been used in various scFvs, because such linkers do not contribute to the formation of any specific secondary structure [39].

The effects of flexible linker lengths on the molecular association of scFvs have recently been reported. When the V_H and V_L domains are linked using flexible linkers longer than 12 residues, the scFvs are predominantly monomers. However, the scFvs with the shorter flexible linkers form dimeric or multimeric molecules [39].

2.5 Production of Recombinant Antibody

2.5.1 *Escherichia coli* Expression

Antibody fragments can be readily expressed in *E. coli*, allowing low-cost production and purification, important advantage for many applications [32]. However, most scFvs tend to form inclusion bodies when expressed in bacteria, especially in *E. coli* [32, 40, 41]. In the cytoplasm, which constitutes a much larger compartment, the scFvs are often unfolded, but the yield can reach 30-50% of total protein [40]. Although *E. coli* is frequently used as a host strain for scFv production, most scFvs that are over-expressed in *E. coli* form inclusion bodies, inactive and insoluble aggregates [39].

2.5.2 Yeasts Expression

Yeasts constitute the other main hosts used for recombinant antibody expression. Several species have already been examined for their heterologous expression abilities [40].

2.5.2.1 *Saccharomyces* cerevisiae

Yeast *Saccharomyces cerevisiae* has been used extensively as a host for the expression of foreign genes in a number of cases, especially in fermentation, and there is a comprehensive body of knowledge on its genomic and biological background. However, the product yields of secreted proteins are low, and there are difficulties in secreting some protein. Many of the secreted proteins of *S. cerevisiae* are not found free in the medium, but rather in the periplasmic space [42].

2.5.2.2 *Hansenula* polymorpha

The methylotrophic yeast *H. polymorpha* provides an alternative expression system and has already been successfully developed for several recombinant antibodies. *H. polymorpha* is able to utilize methanol as sole carbon source resulting in over-expression methanol utilization enzymes namely, methanol oxidase (MOX)

[43-45]. In recent years, the methylotrophic yeast *H. polymorpha* has gained increasing attention as a promising host for expression of heterologous proteins [46, 47]. *H. polymorpha* has some specific advantages over other methylotrophic yeasts being more thermotolerant and capable to grow at highter rates on simple, defined media. The relative high optimal growth temperature for *H. polymorpha* (37-43°C vs. 30°C for *P. pastoris* and *S. cerevisiae*) may be favorable for the production of mammalian proteins and furthermore has the advantage that it allows a better cooling management and reduces the risk of contaminations in large scale fermentations [48, 49].

2.5.2.3 *Pichia pastoris*

The methylotrophic yeast *P. pastoris* is powerful systems for the heterologous expression which has been widely used for the high-yield expression by secretion into the culture supernatant or by intracellular localization [34] [50]. This yeast enables the production of different antibody fragment such as diabodies (bispecific), Fab fragment and scFvs. As in *E. coli*, the efficiency of scFvs secretion has proved to be very dependent on their sequences [40]. As a eukaryote, *P. pastoris* has many of the advantages of higher eukaryotic expression system such as protein processing, protein folding and post-translational modification, while being as easy to manipulate as *E. coli* or *S. cerevisiae*. It is faster, easier, and less expensive to use than other eukaryotic expression system and generally gives higher expression levels. As yeast, it shares the advantages of molecular and genetic manipulations with *Saccharomyces*, and it has the added advantage of 10- to 100-fold higher heterologous protein expression levels. These features make *Pichia* very useful as a protein expression system. Many of the techniques developed for *Saccharomyces* may be applied to *Pichia*. These include: transformation by complementation, gene disruption and gene replacement. In addition, the genetic nomenclature used for *Saccharomyces* has been applied to *Pichia*. For example, histidinol dehydrogenase is encoded by the *HIS4* gene in both *Saccharomyces* and *Pichia*. There is also cross-complementation between gene products in both *Saccharomyces* and *Pichia*. Several wild-type genes from *Saccharomyces* complement comparable mutant

genes in *Pichia*. Genes such as *HIS4*, *LEU2*, *ARG4*, *TRP1*, and *URA3* all complement their respective mutant genes in *Pichia*.

2.6 Pichia pastoris

2.6.1 Background

The Phillips Petroleum Company was the first to develop media and protocols for growing *P. pastoris* on methanol in continuous culture at high cell densities (>130 g/dry cell weight [51]. During the 1970s, *P. pastoris* was evaluated as a potential source of single-cell protein due to the ability of this yeast to utilize methanol as sole carbon source. Unfortunately, the oil crisis of the 1970s caused a dramatic increase in the cost of methane (the source of the methanol). Simultaneously, the price of soybeans, the major alternative source of animal feed, fell. Therefore, the economics of single cell protein (SCP) production from methanol became highly unfavorable. The ICI equivalent product, Pruteen (*Methylophilus methylotrophus*), underwent a very similar pattern of development and economic non-viability in the same time period [52].

However, in the following decade, Phillips Petroleum, together with the Salk Institute Biotechnology/Industrial Associates Inc. (SIBIA, La Jolla, CA, USA), studied *P. pastoris* as a system for heterologous protein expression. The gene and promoter for alcohol oxidase were isolated by SIBIA, who also generated vectors, strains and corresponding protocols for the molecular manipulation of *P. pastoris*. What began more than 20 years ago as a program to convert abundant methanol to a protein source for animal feed has developed into what are today two important biological tools: a model eukaryote used in cell biology research, and a recombinant protein production system. *Pichia* has gained widespread attention as an expression system because of its ability to express high levels of heterologous proteins. As a result, recombinant vector construction, methods for transformation, selectable marker generation, and fermentation methods have been developed to exploit the productive potential of this system [52].

Research Corporation Technologies (Tucson, AZ, USA) are the current holders of the patent for the *P. pastoris* expression system, which they have held since 1993, and the *P. pastoris* expression system is available in kit form from Invitrogen Corporation (Carlsbad, CA, USA) [51-53].

2.6.2 Pichia pastoris as a Methylotrophic Yeast

P. pastoris is methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde using molecular oxygen by the enzyme alcohol oxidase. In addition to formaldehyde, this reaction generates hydrogen peroxide. To avoid hydrogen peroxide toxicity, methanol metabolism takes place within a specialized cell organelle, called the **"peroxisome"**, which sequesters toxic by-products away from the rest of the cell. Alcohol oxidase has a poor affinity for O_2, and *P. pastoris* compensates by generating large amounts of the enzyme. The promoter regulating the production of alcohol oxidase is the one used to drive heterologous protein expression in *Pichia* [54].

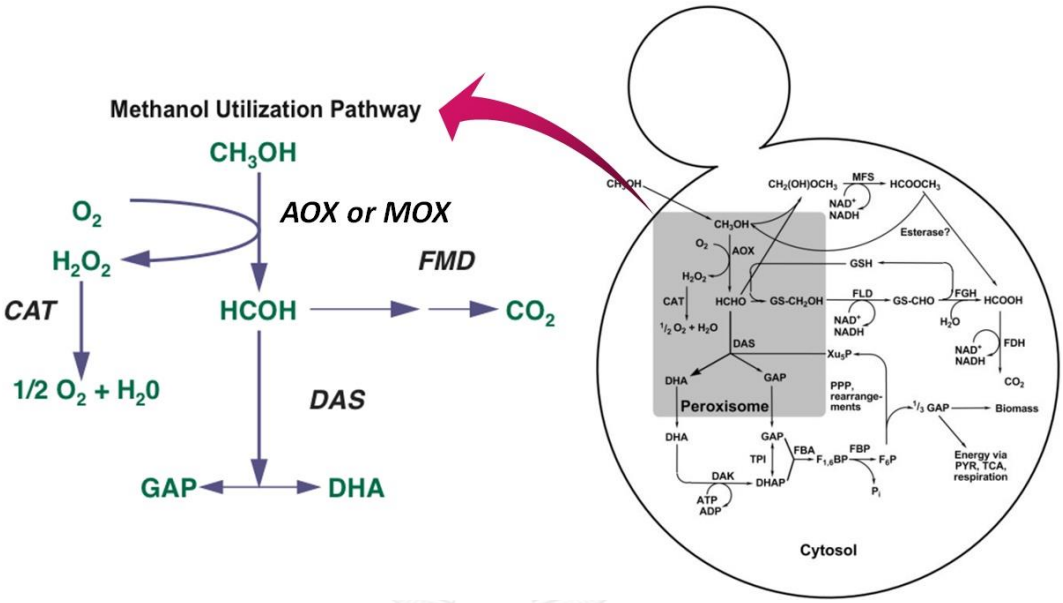

Figure 2.6 The methanol utilization pathway in methylotrophic yeasts [55, 56]

AOX: alcohol oxidase, CAT: catalase, FLD: formaldehyde dehydrogenase, FGH: S-formylglutathione hydrolase, FDH: formate dehydrogenase, DAS: dihydroxyacetone synthase, TPI: triosephosphate isomerase, DAK: dihydroxyacetone kinase, FBA: fructose 1,6-bisphosphate aldolase, FBP: fructose 1,6-bisphosphatase, MFS: methylformate synthase; DHA: dihydroxyacetone, GAP: glyceraldehyde 3-phosphate, DHAP: dihydroxyacetone phosphate, F1,6BP: fructose 1,6-bisphosphate, F6P: fructose 6-phosphate, Pi: phosphate, Xu5P: xylulose 5-phosphate, GSH: glutathione, PYR: pyruvate; PPP: pentose phosphate pathway, TCA: tricarboxylic acid cycle.

2.6.3 Two Alcohol Oxidase

Two genes in *P. pastoris* code for alcohol oxidase are *AOX1* and *AOX2*. The majority of alcohol oxidase activity in the cell is attributable to the product of the *AOX1* [57]. Expression of the *AOX1* gene is tightly regulated and induced by methanol to vary high levels, typically ≥ 30% of the total soluble protein in cells grown with methanol. The *AOX1* gene has been isolated and a plasmid-borne version of the *AOX1* promoter is used to drive expression of the gene of interest encoding the desired heterologous protein. While *AOX2* is about 97% homologous to *AOX1*, growth on methanol is much slower than with *AOX1*. This slow growth on methanol allows isolation of Mut^S strains (*aox1*) [52, 58, 59].

2.6.4 Phenotype of *aox1* Mutants

Loss of the *AOX1* gene, and thus a loss of alcohol oxidase of cell activity, results in a strain that is phenotypically Mut^S (Methanol utilization slow). This has in the past been referred to as Mut^-. The Mut^S designation has been chosen to accurately describe the phenotype of these mutants. This results in a reduction in the ability of cells to metabolize methanol. The cells, therefore, exhibit poor growth on methanol medium. Mut^+ (Methanol utilization plus) refers to the wild type ability of strains to metabolize methanol as the sole carbon source. These two phenotypes are used when evaluating *Pichia* transformants for integration of gene [52, 54, 57, 58, 60]. The GS115 strain, for example, is defective in the histidine dehydrogenase gene (*his4*). Their use allows transformants to be selected based on their ability to grow in non-histidine containing media.

The genotype and phenotype characteristics of a number of the more useful strains are summarized in the Table 2.1.

Table 2.1 Genotype and phenotype of *P. pastoris* starins

Strain	Genotype	Phenotype
X-33	Wild-type	Mut^+
GS115	*his4*	His^-, Mut^+
KM71H	*arg4*, *aox1*:: *ARG4*	Mut^S, Arg^+
GS115/albumin*	*HIS4*	Mut^S
GS115/pPICZ/*lacZ***	*his4*	His^-, Mut^+

*secretion control, ** Intracellular control

2.6.5 Transformation/Integration into the *Pichia* genome

P. pastoris is transformed by integration of the expression cassette into the chromosome at a specific locus to generate genetically stable transformants. Chromosomal integration is more desirable than the use of episomal plasmid expression systems as episomal plasmids tend to have low copy number, and this will affect the amount of product expressed. The size of the plasmid may also affect the stability in the host since large episomal plasmids can be lost during repeated generations as they are mitotically unstable. In addition, transformants containing episomal plasmids need to be cultured under continual selection-based media conditions in order to maintain the transformed population of cells. This procedure may require the use of additives such as antibiotics, which in turn result in increased production costs. Development of genetically stable expression strains is therefore highly desirable with a rate of selectable markers usually set as the target. Moreover, such integration vectors usually contain selectable markers that enable detection of the transformants. Some vectors allow for direct analysis of tandem multiple integration events, but otherwise further analysis of the integration number is required [54, 58].

Integration into the genome can occur via homologous recombinant when the vector/expression cassette contains regions that are homologous in the *P. pastoris* genome and hence integration can occur via gene insertion or gene replacement (Figure 2.7) [60]. Integration by gene insertion can result in tandem multiple integration events due to repeated recombinant events at a ratio of 1-10% of transformation. Transformations that target gene replacement generally result in single copy transformants; however, gene replacement transformations are usually more genetically stable. Gene replacement is achieved by digesting the expression vector such that the 5’ and 3’ ends of the vector correspond to the 5’ and 3’ *AOX1* regions of the *AOX1* chromosomal locus. Transformation, therefore, results in site-specific eviction of the *AOX1* gene [Figure 2.7 (C)]. This event occurs at a frequency of 5–25% of the transformants. The other transformants are either His^+ conversions or of the Mut^+ phenotype as a result of gene insertion events at either the *his4* or *AOX1*

locus [Figure 2.7 (A, B)]. The site of integration and the type (insertion or replacement) events can be confirmed by southern blot analysis hybridized with a probe generated from the *AOX1* promoter region [54].

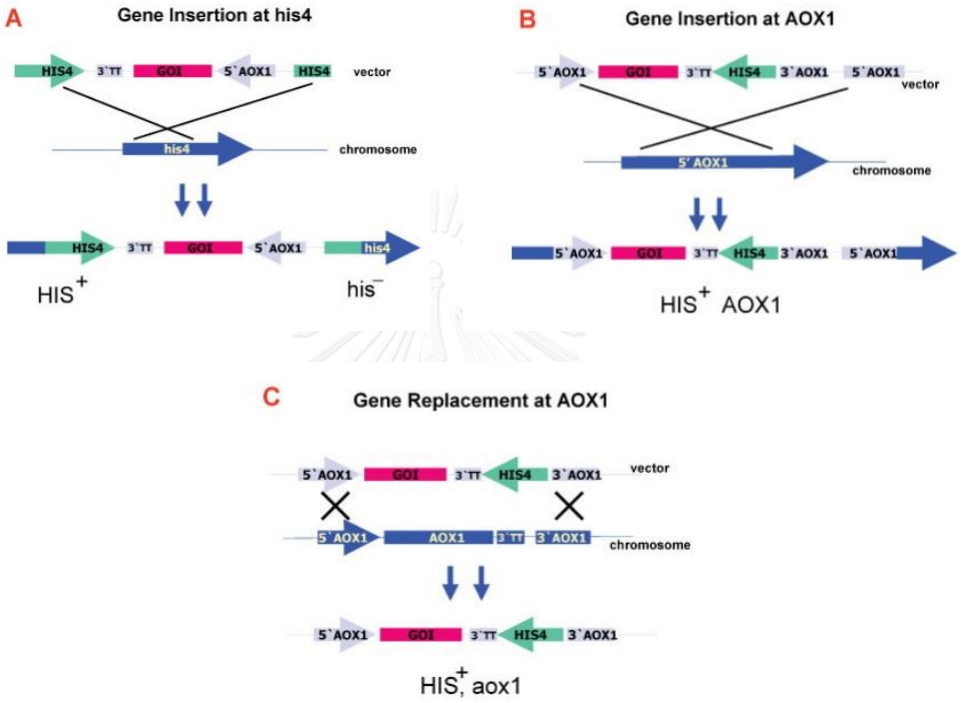

Figure 2.7 Integration into the *P. pastoris* genome, by gene insertion (A, B) and gene replacement (C) [57].

The introduction of the expression cassette into the yeast chromosome can be achieved in a variety of ways including spheroplast formation, electroporation and lithium chloride treatments. The spheroplast transformation method has been used to generate multi-copy transformants by using vectors such as pPIC9K and pPIC9. This method requires several steps with the risk that contamination of the yeast may occur. Also, over-digestion with the cell-lysing enzyme, zymolyase, can reduce cell viability. Electroporation has become increasingly popular and can be used successfully with zeocin-resistant vectors. This method requires fewer steps and the

risk of contamination is reduced. Experience in this laboratory has shown that very efficient expression systems can be constructed through application of this strategy, enabling a diverse range of mature and correctly folded proteins to be prepared and readily purified, particularly when they contain a peptide tag, such as hexahistidine, at the N- or C-terminal positions [54, 60].

2.6.6 *Pichia* Expression Systems

Expression of the *AOX1* gene is controlled at the level of transcription. In methanol-grown cells approximately 5% of the polyA$^+$ RNA is from the AOX1 gene. The regulation of the *AOX1* gene is a two-step process: a repression/de-repression mechanism plus an induction mechanism. Briefly, growth on glucose represses transcription, even in the presence of the inducer methanol. For this reason, growth on glycerol is recommended for optimal induction with methanol. Note that growth on glycerol alone (de-repression) is not sufficient to generate even minute levels of expression from the *AOX1* gene. The inducer, methanol, is necessary for even detectable levels of *AOX1* expression [52].

2.6.7 Intracellular and Secretory Protein Expression

Heterologous expression in *P. pastoris* can be either intracellular or secreted. Secretion requires the presence of a signal sequence on the expressed protein to target it to the secretory pathway. While several different secretion signal sequences have been used successfully, including the native secretion signal present on some heterologous proteins, success has been variable. The secretion signal sequence from the *S. cerevisiae* α-factor prepro peptide has been used with the most success [54, 61]. The major advantage of expressing heterologous proteins as secreted proteins is that *P. pastoris* secretes very low levels of native proteins. Since there is very low amount of protein in the minimal *Pichia* growth medium, the secreted heterologous protein comprises the vast majority of the total protein in the medium and serves as the first step in purification of the protein. However, there are

recognized glycosylation sites (Asn-X-Ser/Thr) in protein's primary sequence, glycosylation may occur at these sites [54].

2.6.8 Post-translational Modification of Secreted Proteins

P. pastoris, unlike bacterial expression systems, has the ability to perform many of the post-translational modifications usually performed in higher eukaryotes, e.g. correct folding, disulfide bond formation, O- and N-linked glycosylation and processing of signal sequences.

Glycosylation is the most common post-translational modification to proteins secretion. Approximately 0.5-1.0% of the translated proteins in eukaryotic genomes is glycoproteins. Glycosylation occurs in the lumen of the endoplasmic reticulum after protein translation. In comparison to *S. cerevisiae*, *Pichia* may have an advantage in the glycosylation of secreted proteins because it may not hyper-glycosylate. Both *S. cerevisiae* and *P. pastoris* have a majority of N-linked glycosylation of the high-mannose type; however, the length of the oligosaccharide chains added post-translationally to proteins in *Pichia* (average 8–14 mannose residues per side chain) is much shorter than those in *S. cerevisiae* (50– 150 mannose residues). Very little O-linked glycosylation has been observed in *Pichia [52, 60, 61]*.

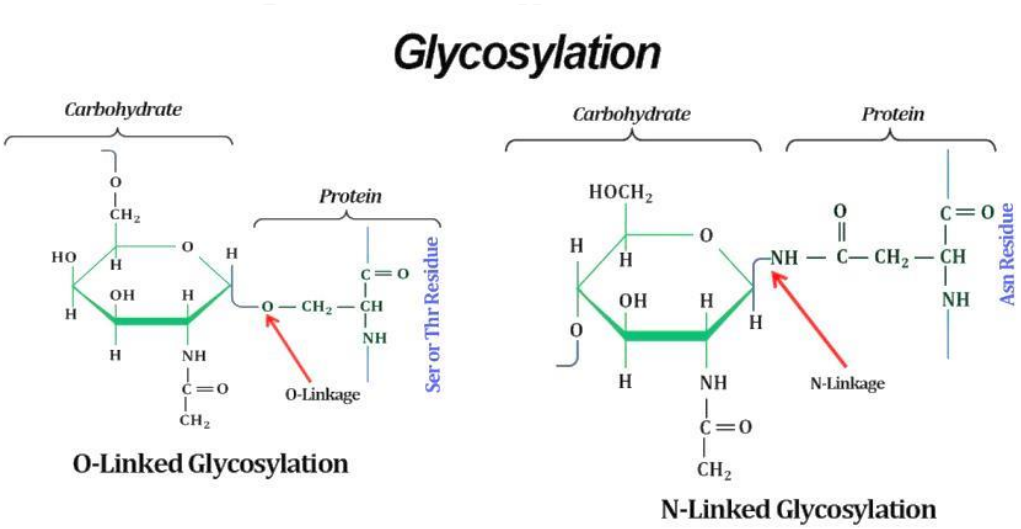

Figure 2.8 Type of glycosylation were found in yeast

In addition, *S. cerevisiae* core oligosaccharides have terminal α-1, 3 glycan linkages whereas *P. pastoris* does not. It is believed that the α-1, 3 glycan linkages in glycosylated proteins produced from *S. cerevisiae* are primarily responsible for the hyper-antigenic nature of these proteins making them particularly unsuitable for therapeutic use. Although not yet proven, this is predicted to be less of a problem for glycoproteins generated in *P. pastoris*, because it may resemble the glycoprotein structure of higher eukaryotes [52].

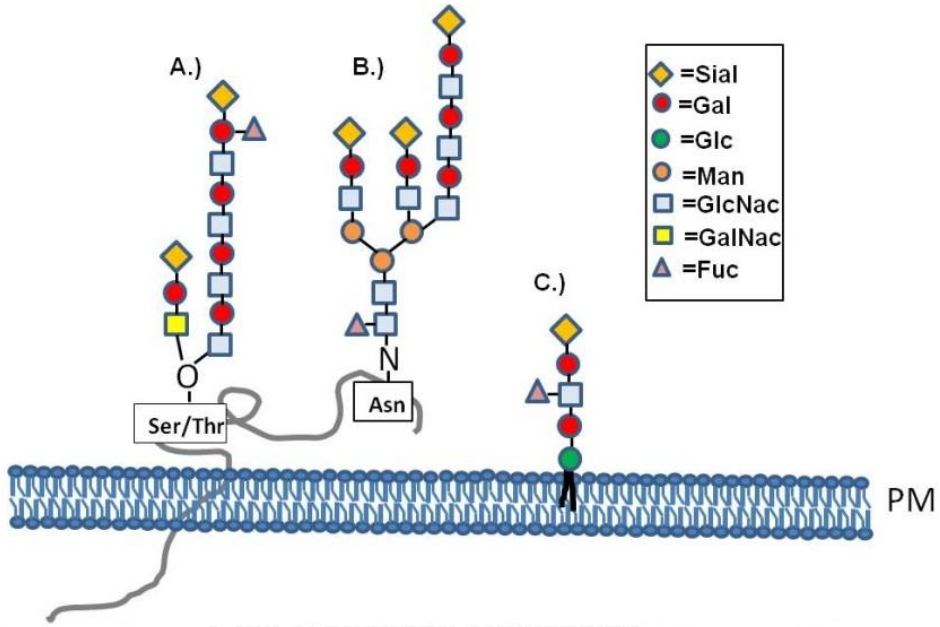

Figure 2.9 Protein glycosylation patterns which are recognized at Asn and Ser/Thr glycosylation sites [54].

2.7 Norfloxacin

Norfloxacin is a synthetic, orally absorbed antibacterial agent. It is derived from a chemical interaction between the starting material 1-ethyl-6-fluoro-7-chloro-1, 4-dihydro-4-oxoquinoline-3-carboxylic acid and piperazine. Norfloxacin is extensively used in both human and animals. In recent years, norfloxacin ranked as the second most prescribed fluoroquinolones (FQs) antibacterial just next to levofloxacin in China [62].

Norfloxacin is a 4-quinolone, 3-carboxylic acid with a fluorine atom, a piperazine moiety and a nitrogen atom at the 6-, 7-, and 8-positions, respectively. The piperazine ring imparts anti-pseudomonal activity and the fluorine atom is responsible for increasing potency against Gram-negative bacteria and broader antibacterial spectrum [63].

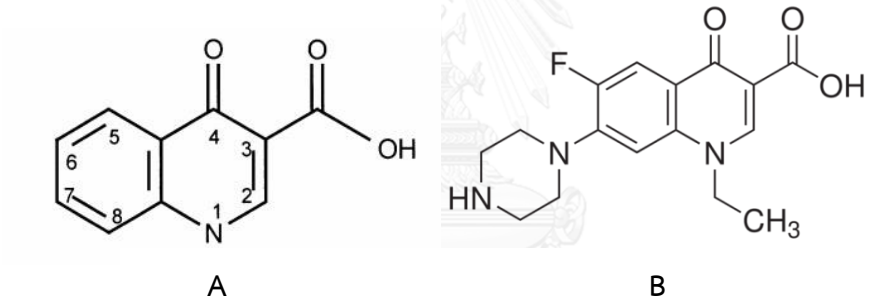

Figure 2.10 The Basic structure of the quinolone (QN) (A) and the structure of norfloxacin (B) [64]. Molecular formula of norfloxacin: $C_{16}H_{18}FN_3O_3$. Molecular weight of norfloxacin: 319.34

The norfloxacin belong to the second generation of quinolones and their characteristic is the greater effectiveness against both Gram-negative and Gram-positive pathogens that are resistant to other antibacterial agents. FQs are potent, broad-spectrum antibiotics that have been used in medical practice for the treatment of severe or resistant infections since the late 1980s. As their name suggests, they are derived from the quinolone family of antibiotics; quinolones themselves are synthetic constructs, developed by modification of 1-alkyl-1, 8-naphthyridin-4-one-3-carboxylic acid [65]. FQs differ from QNs by the replacement of

the eighth carbon atom of the backbone with a nitrogen atom and the addition of a fluorine atom at the sixth position, giving them more potent antibiotic action and a broader spectrum of activity [65] [66]. Their spectrum of efficacy against a wide range of Gram-positive and Gram-negative pathogenic bacteria has led to widespread use worldwide, although, in an attempt to maintain their effectiveness, current UK prescribing guidelines largely recommend this class as second-line agents for use when narrow-spectrum antibiotics have failed.

Table 2.2 The (fluoro)quinolones licensed for clinical use and their current status [65]

Generation	Drug	Use in clinical practice
First generation	Nalidixic acid	Generic form available
	Cinoxacin	Discontinued
Second generation	Norfloxacin	Available as Noroxin
	Ciprofloxacin	Available as Cipro and generic form
	Lomefloxacin	Discontinued
	Ofloxacin	Available as Floxin and generic form
	Levofloxacin	Available as Levaquin and generic form
Third generation	Sparfloxacin	Discontinued
	Gatifloxacin	Discontinued
	Grepafloxacin	Discontinued
Fourth generation	Trovafloxacin	Discontinued
	Moxifloxacin	Available as Avelox
	Gemifloxacin	Available as Factive

2.9.1 Mechanism of Action

FQs act by inhibiting two enzymes involved in bacterial DNA synthesis, both of which are DNA topoisomerases that human cells lack and that are essential for bacterial DNA replication, thereby enabling these agents to be both specific and bactericidal. DNA topoisomerases are responsible for separating the strands of duplex bacterial DNA, inserting another strand of DNA through the break, and then resealing the originally separated strands [65, 67].

In both prokaryotes and eukaryotes, DNA exists as double strands that intertwine around each other to form a double-helix structure. However, in bacteria further twisting of the double-strand structure can occur whereby torsional stresses force the double helix to cross over on it to produce a plectonemic arrangement. This process, also known as supercoiling, enables bacterial DNA to exist in a complicated condensed state in which the DNA can be condensed into compact supercoils allowing large amounts of DNA to be packed into the cell. The degree of supercoiling of DNA is not fixed and there is continuous remodeling of DNA topology within bacteria in response to environmental stress, growth stage, and cellular processes such as transcription, DNA replication, and recombinant. Topoisomerase I and topoisomerase II enzymes work in opposition to control the level of twisted DNA. Topoisomerase I reduces the number of negative supercoils. By contrast, topoisomerase II introduces negative supercoils, which unwind over-twisted DNA into a relaxed state and can further change the DNA topology into an under-twisted plectoneme [68, 69].

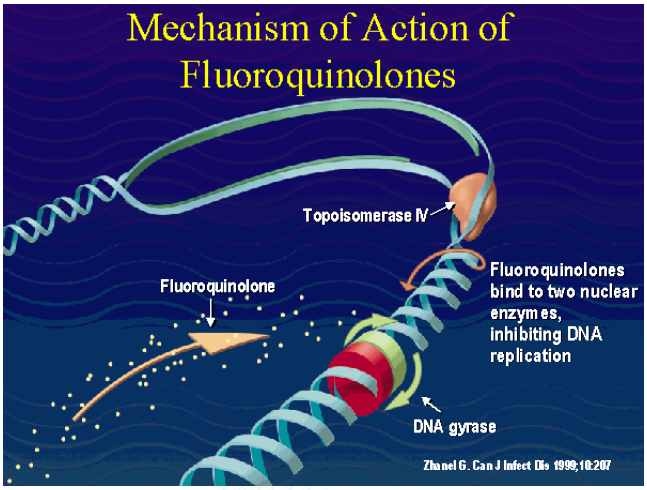

Figure 2.11 Mechanism of action of FQs

In multiple species of bacteria, early biochemical evidence indicated that FQs damage bacterial DNA and lead to defects in negative supercoiling. This effect was linked to inhibition of DNA gyrase activity, an enzyme found in all bacteria. When susceptible DNA gyrase is exposed to FQs, the drug interacts at the surface of an alpha-helical domain of the enzyme involved in DNA cleavage and re-ligation. The toxic effects result from the irreversible formation of a trapped intermediate consisting of fluoroquinolone, gyrase and cleaved DNA. This prevents progression of replication forks and transcription complexs, leading to fragmentation of the chromosome and the cell death [70].

The effect of FQs on bacterial proliferation suggests three mechanisms of cell killing:

I) *Mechanism A*: common to all FQs. This requires RNA and protein synthesis and is only effective against dividing bacteria. Mechanism A appears to involve the blocking of replication by the gyrase-FQs complex on DNA.

II) *Mechanism B*: does not require RNA and protein synthesis and can act on bacteria that are unable to multiply. Mechanism B can be correlated with dislocation of the gyrase subunits that contain the ternary complex.

III) *Mechanism C*: requires RNA and protein synthesis but does not require cell division. Mechanism C may correlate with trapping of topo IV complexs on DNA [71].

2.9.2 The Problems of FQs

The use of antibiotics, especially FQs, in food-producing animals has recently become a very important public health issue. These agents are widely used for the prevention and therapy of infectious disease in animals, an important measure when raising animals under intensive husbandry methods of production. In addition, they are routinely used at sub-therapeutic levels as animal feed additives for their growth-promoting properties.

According to a report by the Union of Concerned Scientists (UCS), 16 million kg of antibiotics are used annually in the US, and approximately 70% are used for non-therapeutic purpose. Antibiotic use in animal feeding has increased from nearly 91000 kg in 1950 to 9.3 million kg in 1999. In the European Union (EU) in the 1990s, a total use of 5 million kg of antibiotics has been reported. Of these, 3.5 million kg were used for therapeutic purpose, while the remaining 1.5 million kg were used as feed additives for growth promotion [72].

The increasing demand for high agriculture productivity, related to the extremely competitive environment of today's market, has led farmers to make use of abusive practices. However, the extensive abuse of antibiotics has caused severe food safety problems which carry many disadvantages including the stimulation of pathogen resistance to antibiotics with the possible transfer of resistant pathogens from animals to human. Meanwhile, some research indicates that low-level does of antibiotics for long periods could result in bacteria resistance [5]. Moreover, the presence of drug residues in animal products may pose a potential health risk to the public [73]. Furthermore, antibacterial residues in animal derived product have consequences in human health, including transfer of drug resistant pathogens from animals to human giving rise to the continuous need for new drugs. In addition, contaminated food sources with agent residues that can cause long-term anomalies such as allergic reactions and hypersensitivity reactions [74]. To preserve efficacy, antibiotics such as FQs should be used judiciously and for appropriate indications [75].

2.1.4 Adverse Effects

The extensive use of FQs in veterinary has promoted the accumulation of their residues in food derived from animals, which may trigger allergic reactions in some hypersensitive individuals and most importantly induce pathogens resistant to clinical drugs in human [76]. Moreover, these residues may possible transfer from either animal food product to animals or animal food product to human [77]. Thereby, the residues are accumulated in the human body as well as in the environmental habitat and possible passed through to food chain of human. In the final, the residues may possible highly accumulate in the human body.

In general, fluoroquinolones are well tolerated, with most side-effects being mild to moderate. On occasion, serious adverse effects occur. Common side-effects include gastrointestinal effects such as nausea, vomiting, and diarrhea, as well as headache and insomnia [78, 79]. As noted above, under licensed use, norfloxacin is also now considered to be contraindicated for the treatment of certain sexually transmitted diseases by some experts due to bacterial resistance. Moreover, norfloxacin has been reported to rapidly cross the blood-placenta and blood-milk barrier, and is extensively distributed into the fetal tissues [65]. For this reason norfloxacin and other fluoroquinolones are contraindicated during pregnancy due to the risk of spontaneous abortions and birth defects. In addition, fluoroquinolones have also been reported as being present in the mother's milk and are passed on to the nursing child, which may increases the risk of the child suffering an adverse reaction even though the child had never been prescribed or taken any of the drugs found within this class [71].

2.1.5 Regulations

The evolution of food production systems from small farming units to large scale intensive production systems has been accompanied by an increasing administration of antimicrobials to food producing animals, to prevent and control the spread of infections in the farm. The use of these pharmaceuticals is restricted in many countries, especially those commonly applied in human medicine, which are forbidden for animal use [80].

FQs are widely used in veterinary medicine, and they are also subjected into the regulation. To minimize risks in human health by the consumption of FQs residues in foods, the European Union (EU) by the Council Regulation No. 2377/90 has established maximum residue limits (MRLs) of veterinary medicinal products in foodstuffs of animal origin for several FQs, which are listed in Table 2.3 [81]. For example, the MRLs according to this regulation in chicken muscle are 200 μg/kg for danofloxacin, 300 μg/kg for difloxacin, 100 μg/kg for enrofloxacin and 200μg/kg for flumequine, respectively. Another provision according to this directive is that the use of FQs is prohibited in animals from which eggs are produced for human consumption. Since the beginning of year 2000, an MRL is required for every new substance the might be commercialized in the EU for veterinary use [82].

Although antibiotics are approved for use in animal husbandry, there is extensive regulatory oversight to ensure the safety of our foods. The use of antibiotics is strictly regulated by the Food and Drug Administration (FDA) and the USDA. The FDA is the regulatory agency responsible for approval of antibiotic usage in poultry [83]. Once approved, both the FDA and its sister agency, the USDA, have active surveillance and compliance programs to ensure the proper use of antibiotics and the safety of the food supply.

FDA has banned the use of enrofloxacin, member in FQs, for the treatment of bacterial infections in poultry since 2005, as they was scientific evidence of the emergence of *Campylobacter* resistant species in chickens and turkeys treated with the antimicrobial. These finding have also prompted the European Commission to initiate a referral procedure for all veterinary medicinal products containing QNs and

FQs, for all animal producing species, to promote their prudent use across the EU [80].

Table 2.3 MRL Values Established by the EU for FQs of Veterinary use [64]

compound	species	MRL value (μgkg-1)		
		muscle	kidney	egg
danofloxacin	all species other than hereafter	100[a]	200	b
	bovine, ovine, caprine	200	400	
	poultry	200	400	
enrofloxacin + ciprofloxacin	all species other than hereafter	100[a]	200	b
	bovine, ovine, caprine	100	200	
	porcine, rabbit	100	300	
	poultry	100	300	
flumequine	all species other than hereafter	200	1000	b
	bovine, ovine, caprine	200	1500	
	poultry	400	1000	
	fish	600[a]		
sarafloxacin	fish (salmonidae)	30[a]		
oxolinic	bovine	100	150	b
	porcine	100	150	
	chicken	100	150	
	fish	100[a]		
difloxacin	all species other than hereafter	300[a]	600	b
	bovine, ovine, caprine	400	800	
	porcine	400	600	
	poultry	300	800	
marbofloxacin	porcine, bovine	150	150	

[a] For fin fish, this MRL relates to “muscle + skin in natural proportions”

[b] Not for use in animals from which eggs are produced for human consumption

2.9.5 Analytical Methods

In order to ensure human food safety and the entire ecosystem security, analytical methods as sensitive as possible are required in order to check food samples before their disposal to the markets for human consumption. Recently EU has issued the decision 2002/657/EC which concerns the performance of analytical methods and the interpretation of results in the official control of residues in products of animal origin [81]. Many published papers have been devoted to the analysis of FQs residues in animal products over the last 10 years. The increasing number of published papers concentrating on the determination of FQs' residues in food is illustrating the seriousness of this state. To facilitate the monitoring of FQs concentration level in poultry and foodstuff, the development of sensitive and efficient methods for the quantification of FQs in animal derived foods is necessary [84].

2.9.5.1 Chemical Technique

Traditionally, FQs residue analysis has relied upon classical analytical techniques, such as high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), LC-MS/MS, or in a few cases by gas chromatography (GC), high-performance thin-layer chromatography (HPTLC) or even a non-chromatographic method [66, 84].

Since FQs are polar compound and most of them are fluorescent, reversed-phase liquid chromatography with fluoremetric detection is the determination technique mainly used for routine residue analysis. However, several methods dealing with LC-MS have recently been reported for confirmatory analysis. In contrast, sample treatment varies greatly among the methods proposed, often irrespective of the sample matrix and the analyte. Moreover, few authors have reported optimization of both the extraction and the clean-up steps and, in most of the reviewed papers, there is a lack of information concerning the criteria used for the selection of the experimental conditions [85].

High-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) have been applied for FQs analysis. Among them, HPLC is the most popular analytical technique for FQs due to the merits of good resolution, high reproducibility, and easy automation [86]. Compared with the mass spectrometry (MS) and ultraviolet (UV) detector, fluorescence detection (FLD) is more widely used due to the advantages of high sensitivity, low cost and easy combination with HPLC for FQs analysis. However, FQs in animal derived foods are at trace level and the real sample matrix is quite complicated. Therefore, a suitable sample pretreatment technique to remove sample matrix and enrich FQs before instrumental analysis is highly demanded. Liquid-liquid extraction (LLE) and solid phase extraction (SPE) are commonly used sample preparation methods for FQs analysis, while LLE and SPE consume large amounts of organic solvent and often require complex time-consuming multi-step procedures which may cause contamination or losses of target analytes and deteriorate the accuracy [76].

Capillary electrophoresis (CE) has emerged as a useful technique for pharmaceutical analysis because it offers high separation efficiency, high resolution and speed and small sample volume requirements, necessary in the analysis of complex mixtures [74, 86].

Liquid chromatography (LC), with UV, fluorescence and mass spectrometric detection, has been a major technique used for the determination in various biological matrices. Most of them apply extraction of analytes from the matrix with organic solvents of medium polarity, and the clean-up usually involves different treatments such as liquid-liquid extraction or solid phase extraction (SPE), and a few cases, dialysis has been proposed [74, 84].

However, the entire instrument as mentioned above generally requires highly skilled personnel, high-cost complex equipment and laborious sample pretreatment. Moreover, these techniques are generally time-consuming methods and often require extensive sample preparation prior to analysis and data interpretation *post*-analysis. As such, they may be considered as less cost-effective for primary screening. Therefore, chromatographic techniques are not practical for screening large number of food samples [87].

2.9.5.2 Immunological Techniques

Immunological techniques are increasingly considered as interesting alternative and/or complementary methods for FQs residue analysis. Compared with instrumental analysis methods, immunoassay technology has the advantages of rapid, sensitive, cost-effectiveness, portability, and high throughput characteristics [87]. Among these, Enzyme-linked immunosorbent assay (ELISA) is the most suitable method for rapid screening of FQs residues in the veterinary field. It is known that ELISA has been developed for primary screening of drugs, pesticides and others in different matrices such as muscle, kidney, liver, serum, milk, and egg, respectively [62]. These methods have shown great potential as a screening tool, although most only allow semi-quantitative analysis of the sum FQs [86, 88].

CHAPTER III

METERIALS AND METHODOLOGY

3.1 Materials

3.1.1 Microorganisms

- *Escherichia coli* strain TOP10F' was used as a host for all recombinant plasmid constructions.
- *Pichia pastoris* GS115 (Mut^+, His^-) was used as a host for recombinant scFv antibody expression.

All microorganisms as above were purchased for Invitrogen™ (USA).

3.1.2 Chemicals and Reagents

Chemicals and Reagents	Company, country
Absolute ethanol	Merck, U.S.A.
Absolute methanol	Merck, U.S.A.
Acetic acid (glacial) 100% anhydrous	Merck, Germany
Agar (Microbiology grade)	Merck, Germany
Agarose (Molecular biology grade)	Research organics, Inc., U.S.A.
Bacto™ Peptone powder	Becton, Dickinson and Company, France
Biotin	Fluka, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, U.S.A.
Calcium chloride anhydrous	Merck, Germany
D(+) Glucose anhydrous	Carlo Erba Reagenti, Italy
di-Potassium hydrogen phosphate	Carlo Erba Reagenti, Italy
di-Sodium hydrogen phosphate	Merck, Germany
Dimethyl sulfoxide (DMSO)	Fluka, Switzerland
Dithiothreitol (DTT)	Bio basic, Inc., Canada
DNA ladder (1 kb), M11	SibEnzyme, Ltd., Russia
D-Sorbitol	Fluka, Germany

Ethidium bromide	Bio basic, Inc., Canada
Ethylenediaminetetraacetic acid (EDTA)	Carlo Erba Reagenti, Italy
Glutaraldehyde 50% in water	Merck, U.S.A.
Glycerol	Ajax Chemicals, Australia
Histidine	Fluka, Germany
Hydrochloric acid 37%	Merck, Germany
Hydrogen peroxide	Merck, Germany
Nitrocellulose membrane (NitroBind™)	Life Science Products, Inc., U.S.A.
Potassium acetate	Fluka, Switzerland
Potassium *di*-hydrogen phosphate	Merck, Germany
Skim milk	Difco, U.S.A.
Sodium chloride	Ajax Chemicals, Australia
Sodium dodecyl sulfate (SDS)	Bio basic, Inc., Canada
Sodium hydroxide	Ajax Finechem Pty, Ltd., Australia
Sulfuric acid	Merck, Germany
TAE buffer premix powder	Bio basic, Inc., Canada
TMB (3,3',5,5'-tetramethylbenzidine)	Sigma-Aldrich, U.S.A.
Tris (Molecular biology grade)	Research organics, Inc., U.S.A.
Tryptone powder	Bio basic, Inc., Canada
Tween-20	Sigma Aldrich, Germany
Yeast nitrogen base powder (w/o amino acid with ammonium sulfate)	Bio basic, Inc., Canada
Yeast extract powder	Bio Springer, France

3.1.3 Equipment and Supplies

Equipment and Supplies	Company, Country
Autoclave (HV-50)	Hirayama manufacturing Corp., Japan
Balance (Adventurer™, ARC 120)	Ohaus Corp., U.S.A.
Balance (Adventurer™, AR 2140)	Ohaus Corp., U.S.A.
Bench-top centrifuge, WiseSpin® (CF-10)	Dihan scientific Co., Ltd., South Korea
Biological safety cabinet (Heal force®, HFsafe-1200)	Shanghai Lishen Scientific equipment Co., Ltd., China
E.Z.N.A.® Gel extraction kit	Omega Bio-Tek, Inc.,U.S.A.
Freezer (-20°C) (SF-C697)	Sanyo Commercial Solution, Ltd., Thailand.
Gene Pulser® Cuvette, 0.2 cm.	Bio-Rad Laboratories, Inc., China
High speed micro refrigerated centrifuge (MTX-150)	Tomy Seiko Co., Ltd., Japan
High speed refrigerated centrifuge (6500)	Kubota Corp., Japan
Hot plate (PC-101)	Corning, U.S.A.
Incubator (MIR 152)	Sanyo Electric Co., Ltd. Japan
Microplate reader (Multiskan FC, Type 357)	Thermo Fisher Scientific Instruments Co.,Ltd., China
MicroPulser™	Bio-Rad, U.S.A.
Microwave oven (National®)	Matsushita Electric Industrial Co. Ltd., Japan
Mupid®-EXU Submarine electrophoresis system	Advance Co, Ltd., Japan
pH meter (Accumet® AB15)	Fisher Scientific, Singapore
Refrigerator	Panasonic Appliances Lights Action Alliance Co., Ltd., Thailand
Refrigerated incubator shaker (Innova™	New Brunswick Scientific Co., Inc.,

Equipment and Supplies	Company, Country
4330)	U.S.A.
T100™ Thermal cycler	Bio-Rad, Singapore
Ultra low refrigerator (MDF 79OAT)	Sanyo Electric Co., Ltd. Japan
UV transilluminator	UVItec, U.K.
UVitec platinum gel documentation system	UVItec, U.K.
UV-Visible recording spectrophotometer (UV-160)	Shimadzu Corp., Japan
Vortex mixer (KMC-1300V)	Vision scientific Co., Ltd., Korea
Water pro plus	Labconco Corp., U.S.A.

3.1.4 Antibodies, Enzymes and Antibiotics

Enzymes, Antibiotics and Antibodies	Company, Country
Mouse anti-c-myc antibody (9E10)	Invitrogen
Mouse anti-His antibody (c-term)	Invitrogen
Mouse anti-His antibody (c-term)-HRP	Invitrogen
Goat anti-Mouse IgG, (H+L) HRP conjugate	Jackson Immuno Research Laboratories Inc., U.S.A.
*Bam*HI	Roche, Germany
*Bgl*II	Roche, Germany
*Eco*RI	Roche, Germany
i-*Taq* DNA polymerase	iNtRON Biotechnology, Korea
*Kpn*I	Roche, Germany
Lyticase	Invitrogen, U.S.A.
RNase A (Ribonuclease A)	New England Biolab, U.K.
*Pme*I	Roche, Germany
T4 DNA ligase	Promega, U.S.A.
Anhydrous ampicillin	Sigma, U.S.A.
Zeocin™	Invitrogen, U.S.A.

3.1.5 Primers

Primer ID	Sequence (5'-3')	Purposes	References
VHFwMH1	GAATTCSARGTNMAGCTGSAGSAGTC	VH amplification	[89]
VHRwIgG1	GAATTCATAGACAGATGGGGGTGTCGTTTTGGC		
VLFwMk	GGGGAGCTCGAYATTGTGMTSACMCARWCTMCA	VL amplification	
VLRwKc	GGGGAGCTCGGATACAGTTGGTGCAGCATC		
N1	GAATTCGAAGTAGAGCTGGAGGAGTCTGGG	VH-linker amplification	This study
N2	GGATCCACCTCCGCCTGAACCGCCTCCACCATA GACAGATGGGGGTGTCGTTTTGG		
N3	GGCGGAGGTAGATCTGGCGGTGGCGGATCGGA CATTGTGATGACACAGTCTACA	Linker-VL amplification	This study
N4	CGAGGTACCGGGAGCTCGGATACAGTTGGT		
5'AOX1	GACTGGTTCCAATTGACAAGC	Integrated Confirmation	Invitrogen
3'AOX1	GCAAATGGCATTCTGACATCC		
TJ01F	AAGGAAGCTGCCCTGTCTTAAACCTTT		This study
TJ01R	TAGGTTACAAATAAAAAAGTATCAAAA		
P1	GTTGACTCATGTTGGTATTGTGAA		
P2	GCAGCAATGCTGGCAATAGTA		
P3	AACGAAAACTCACGTTAAGGGA		
P4	CCACCACCTAGAACTAGGATATCA		
TJ03F	ATGAGATTTCCTTCAATTTTTACTGCT	Sequencing	This study
TJ04F	AGAGGCTGGAGTGGGTCGCAA		
TJ05F	CCGCCACCGCCAGATCCACCT		
TJ06R	GCCTCCCCCAGACTCCTCCAG		
AOX1-F	GATCTAACATCCAAAGACGAAAGGTTG	AOX1 probe Amplification	This study
AOX1-R	CGTTTCGAATAATTAGTTGTTTTTTGA		
Actin1Fw	GGTATTGCTGAGCGTATGCAAA	RT-qPCR	[90]
Actin1Rw	CCACCGATCCATACGGAGTACT		
TJ05R	CCGCCACCGCCAGATCCACCT		This study

Degenerate bases: R= (A/G), Y=(C/T), M= (A/C), S=(C/G), W= (A/T), N= (A/G/C/T)

The underline letter represents the restriction enzyme

3.1.6 Media

- Luria-Bertani (LB) medium was used as a growth medium for competent *E. coli* cultivation.
- Low salt LB/Zeocin™ medium (LB with 25 μg.mL^{-1} of Zeocin™ final concentration) were used for recombinant *E. coli* cultivation and screening of *Zeo*^{R} transformants.
- YPD medium and YPD Zeocin™ (YPD with 100 μg.mL^{-1} of Zeocin™ final concentration) medium were used for recombinant yeast cultivation and screening.
- YPG medium was used for cell manipulation in cell production phase.
- MMH medium with 0.5% methanol was used for recombinant scFv antibody induction in an expression phase.

The media compositions and preparation were summarized and described in an appendix A.

3.1.7 Vectors

- pGEM® T-easy vector (Promega) (Appendix D)
- pPICZαA (Invitrogen™) (Appendix D)

3.1.8 Kits

- NucleoSpin® RNA II kit (Macherey-Nagel)
- RevertAid™ First strand cDNA synthesis (Fermetas)
- MasterPure™ Yeast DNA purification Kit (epicentre)
- Frozen-EZ Yeast Transformation II™ (Zymo Research)
- E.Z.N.A.® Yeast RNA kit (Omega)
- Tetro cDNA synthesis kit (Bioline™)
- Pierce™ BCA protein assay kit (Thermo Scientific)
- QIAquick PCR purification kit (QIAGEN)
- SilverQuest™ Staining Kit (Invitrogen)

3.2 Methods

3.2.1 Preparation of first-strand cDNA

The norfloxacin-producing hybridoma clone 155 (Nor155) was kindly obtained from the Institute of Biotechnology and Genetic Engineering (IBGE), Chulalongkorn University. This clone was used for RNA extraction. Briefly, total RNA extraction from 5×10^6 Nor155 using a NucleoSpin® RNA II (Macherey-Nagel) was carried out according to the manufacturer's instruction. First strand cDNA coding for the variable heavy (V_H) chain and variable light (V_L) chain was synthesized from the total RNA extract (approximately 1 µg RNA) by using a RevertAid™ First strand cDNA synthesis kit (Fermentas).

3.2.2 Amplification of V_H and V_L Genes

The 1st strand cDNA fragments encoding the variable heavy chain (V_H) were amplified by using VHFwMH1 as forward primers and VHRwIgG1 as a reverse primer while those encoding the variable light (V_L) chian were amplified by using VLFwMk as a forward primer and VLRwKc as a reverse primer, respectively (Table 3.1.5). Each PCR reaction contains: 2 µL of 1st cDNA, 20 pmol of 5' and 3' primers, 5 µL of 2.5 mM dNTPs, 5 µL of Taq polymerse buffer, and 0.5 µL of *i-Taq* DNA polymerase (Intron Biotechnology). The final volume was brought to 50 µL with nuclease-free water. Cycling conditions were: initial melt at 94°C for 3 min followed by 30 cycles of three-step program (94 °C, 1 min; 45°C, 1 min; and 72°C, 2 min). The reactions were then held at 72°C for 10 min and cooled to 4°C. Then, the amplicons were electrophoresed through 1% low-melting point agarose gel and visualized by staining with ethidium bromide. Each of the amplified DNA fragments corresponding to the predicted size was excised from the gel and was purified by using QIAquick PCR Purification Kit (QIAGEN).

3.2.3 Preparation and Transformation of Competent *E. coli* TOP10F'

3.2.3.1 Preparation of Fresh Competent E. coli Using Calcium Chloride (86)

A fresh single colony of *E. coli* Top10F' was inoculated in 10 mL of LB medium in 250 mL Erlenmeyer flask and cultivated with shaking at 180 rpm at 37°C for overnight. Two percent of overnight culture was transferred to 50 mL LB medium and cultivated with shaking at 250 rpm at 37°C for approximately 3 hours. The cell culture was aseptically transferred to sterile 50 mL polypropylene tube and stored on ice for 10 minutes to cool the cell culture to 0°C. The cells were harvested by centrifugation at 4,000×g at 4°C for 10 minutes, decant the media. The cells were re-suspended in 10 mL of sterilized ice-cold 0.1 M $CaCl_2$ and chilled on ice for 5 minutes. The cells were recovered by centrifugation at the same condition followed by decanting the supernatant. One milliliter of sterilized ice-cold 0.1 M $CaCl_2$ was added into the tube and mix by pipetting. Then, the cell suspension was aliquot to new microfuge, 200 µL per tube, and stored on ice until use.

3.2.3.2 Ligation Reaction

The purified fragments (V_H and V_L fragments) which obtained from 3.2.2 were used in ligation reaction according to the manufacturer's instruction. The reaction was carried out by individual ligation of V_H and V_L fragments into the pGEM® T-easy vector (Promega). T4 DNA ligase was then used as an enzyme for this ligation. The reaction was gently mixed by pipetting and was incubated either at room temperature for 1 hour or, alternative, overnight at 4°C for maximum number of transformants.

3.2.3.3 Transformation of E. coli by Heat Shock

The ligation reaction (no more than 10 µL) was added to 200 µL of freshly prepared *E. coli* competent cells and gently mixed by pipetting, stored on ice for 30 minutes. The tube was immediately heated in water bath at 42°C for 90 seconds (do not shake the tube). Then, the tube was rapidly transferred to chill on ice for 5

minutes. Eight-hundred microliters of LB medium were added into the tube and incubated at 37°C for 1 hour. The cell suspensions (with the volume of 50, 100, 200, 300 µL per plate) were spreaded on LB agar plates containing 100 µg/mL ampicillin, 50 µL X-gal (20mg/ml) and 30 µL IPTG (0.1 M) respectively, and incubated at 37°C for overnight. By this procedure, the white colonies which appeared on plate were selected as positive clones for next experiments.

3.2.4 Extraction of Plasmid DNA

This protocol was modified from extraction and purification of plasmid DNA in molecular cloning: a laboratory manual, second edition (1989) **[91]**.

3.2.4.1 Growth of the Bacterial Culture

A single colony of recombinant *E. coli* which obtained from 3.2.3.3 was inoculated in LB medium supplied with 100 µg/mL ampicillin and was shaken vigorously at 37°C for overnight.

3.2.4.2 Harvesting and Alkaline Lysis

Three milliliters of bacterial cell culture were collected by centrifugation at 10,000×g at 4°C for 30 s. The cell pellet was washed with 1 mL of sterilized TE buffer (pH 8.0) and centrifuged again followed by decanting the supernatant. The cell pellet was re-suspended in 100 µL of ice-cold of Solution I and mixed vigorously by vortex. The solution was chilled on ice for 5 minutes. Then, 200 µL of freshly prepared Solution II was added into the tube and mixed by inverting the tube for five times, then stored the tube on ice for 5 minutes. An ice-cold Solution III (300 µL) was added into the tube and gently mixed by inverting the tube for ten times and followed by storing on ice for 5 minutes. The lysate cells were centrifuged at 12,000×g at 4°C for 5 minutes and the supernatants (400 µL) were transferred by pipetting to a new microfuge tube.

The obtained supernatant was precipitated with 2 volumes of ice-cold 95% ethanol (800 µL) and mixed by vortex. The solution was centrifuged at 12,000×g at 4°C for 5 minutes followed by decanting the supernatant. The plasmid DNA was rinsed with 1 mL of ice-cold 70% ethanol and carefully removed the supernatant.

Next, the pellet of plasmid DNA was dried in a vacuum desiccator up to 30 minutes. The plasmid DNA was re-dissolved in 50 µL of sterilized ultrapure water or sterilized TE buffer (pH 8.0) containing RNAase A (20 $\mu g.mL^{-1}$ final concentration).

The quantity and quality of the plasmid DNA were determined by electrophoresis through 1% agarose gel and visualized by staining with ethidium bromide. Each of the plasmid DNA revealed the corresponding to the predicted size was selected for next experiments.

3.2.5 Construction of scFv Antibody Gene

3.2.5.1 Construction of V_H and V_L Gene with Flexible Polypeptide Linkers

A 10 amino acid linker encoding $(Gly_4Ser)_2$ was introduced behind the V_H domain and in front of the V_L domain to further produce a full-length scFv which was oriented as V_H-$(Gly_4Ser)_3$–V_L by using specific designed primer. The N1 and N2 primers were amplified VH-linker and N3 and N4 primers were used for linker-VL, respectively (Fig. 3.1). These primers were designed based on the V_H and V_L gene sequences as shown in 3.1.5. After PCR, the VH-linker and linker-VL amplicons were separated by 1% (w/v) agarose gel electrophoresis and continuous purified. Afterward, the synthesized genes were cloned separately to the pGEM® T-easy vector and were selected as above. Plasmid of the ampicillin-positive clones were extracted and analyzed by restriction enzyme digestions and electrophoresis. The plasmids containing the fragments of the specified sizes were confirmed by DNA sequencing (Macrogen, Korea). Finally, the transformant clones with the accurate sequence were then used to generate the expression plasmid.

3.2.5.2 Construction of Recombinant scFv Antibody Expression Plasmid

The VH-linker and linker-VL clones having the *Bam*HI–*Bgl*II restriction enzyme sites and *Eco*RI-*Kpn*I linearized pPICZαA vector (Fig. 3.1) were assembled by ligation reaction and then introduced into *E. coli* TOP10F' to generate the pPICZαA-scFv construct named pJM01. The pJM01 was cloned in frame with the α-factor secretion signal sequence open reading frame of the pPICZαA vector.

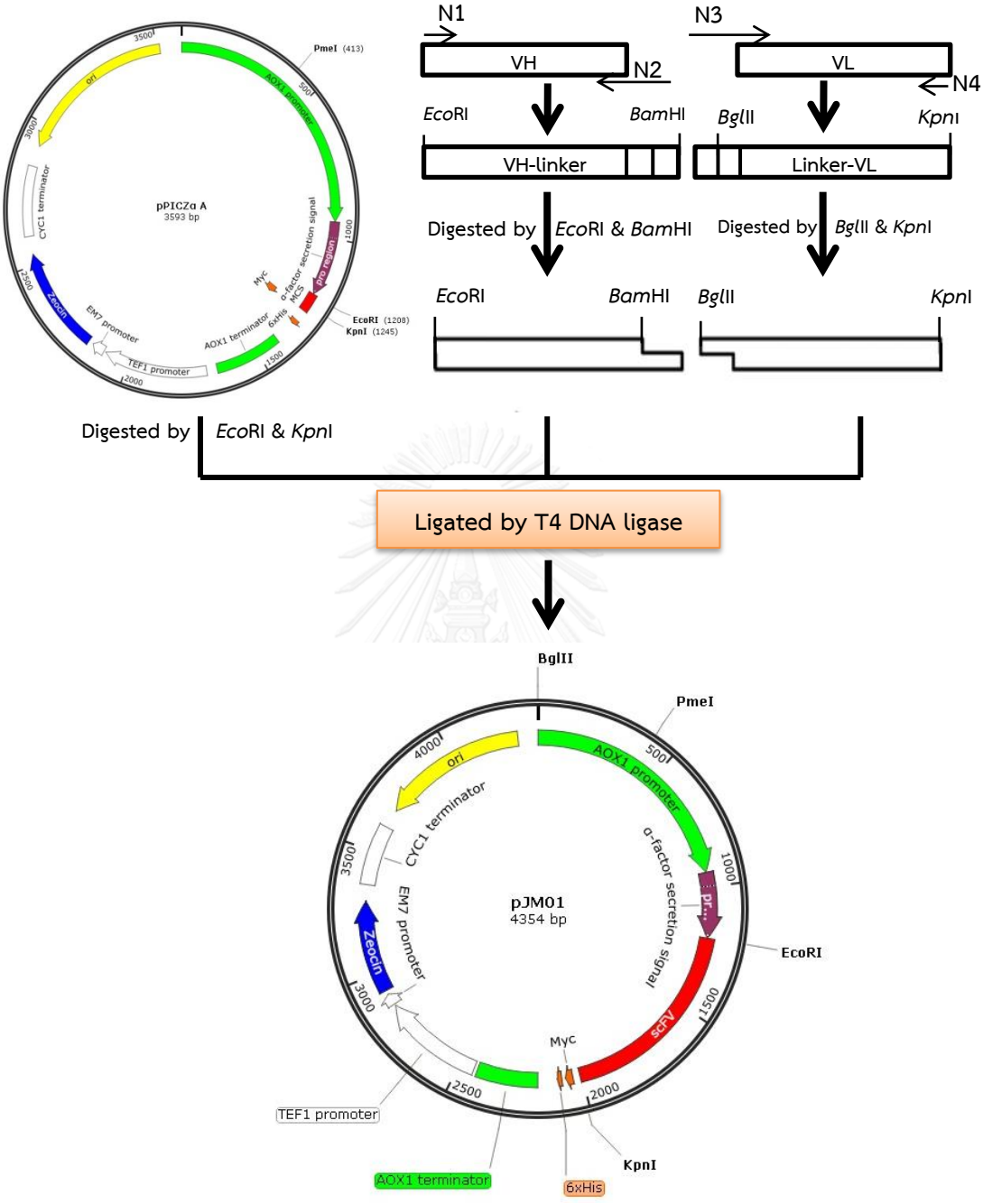

Figure 3.1 Construction of recombinant scFv antibody expression plasmid

3.2.6 Verification of pJM01 Plasmid

To confirm the construct of scFv expression plasmid, pJM01 plasmid, the following three methods were carried out.

3.2.6.1 PCR Analysis of Positive Transformants

Five zeocin-resistance transformant clones were analyzed by using PCR with designed primers TJ01F/TJ01R and N1/N4, respectively based on the pJM01 sequences (fig.3.2). To obtain the DNA template, plasmid DNA was extracted. The PCR product could detect the ligation of the scFv antibody gene in to the pPICZαA, while pPICZαA vector was used as the negative control. PCR components and conditions were as follows: AmpliTaq Gold® 360 Master Mix (AB Applied Biosystem), 0.5 µM of each primer, 1 cycle of 95°C for 5 min, 30 cycles of three-step program (95°C, 15 sec; 55°C, 30 sec and 72°C, 30s). The reactions were then held at 72°C for 7 min and cooled until used at 4°C. The PCR products were determined by agarose gel electrophoresis.

3.2.6.2 Restriction Enzyme Digestion

The pJM01 plasmid of positive clones with the specified size according to the expected map was double digested with *Eco*RI and *Kpn*I, respectively. After digestion by these restriction enzymes and run though the gel electrophoresis, the DNA fragment of scFv could be showed the expected size with 800 bp.

3.2.6.3 Sequencing Analysis

To consider the scFv antibody gene was cloned in frame with the α-factor secretion signal sequence in pPICZαA vector. The positive clones, which showed the expected size from 3.2.6.1 and 3.2.6.2 were sequenced with BigDye® terminal sequencing kit and ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems, USA) using four oligonucleotide primers, T03F, T04F, T05F, and T06R which were designed based on pJM01 sequence as shown in 3.1.5.

The schematic representation of all pJM01 verification (PCR, enzyme digestion and sequencing analysis) was shown in Figure 3.2

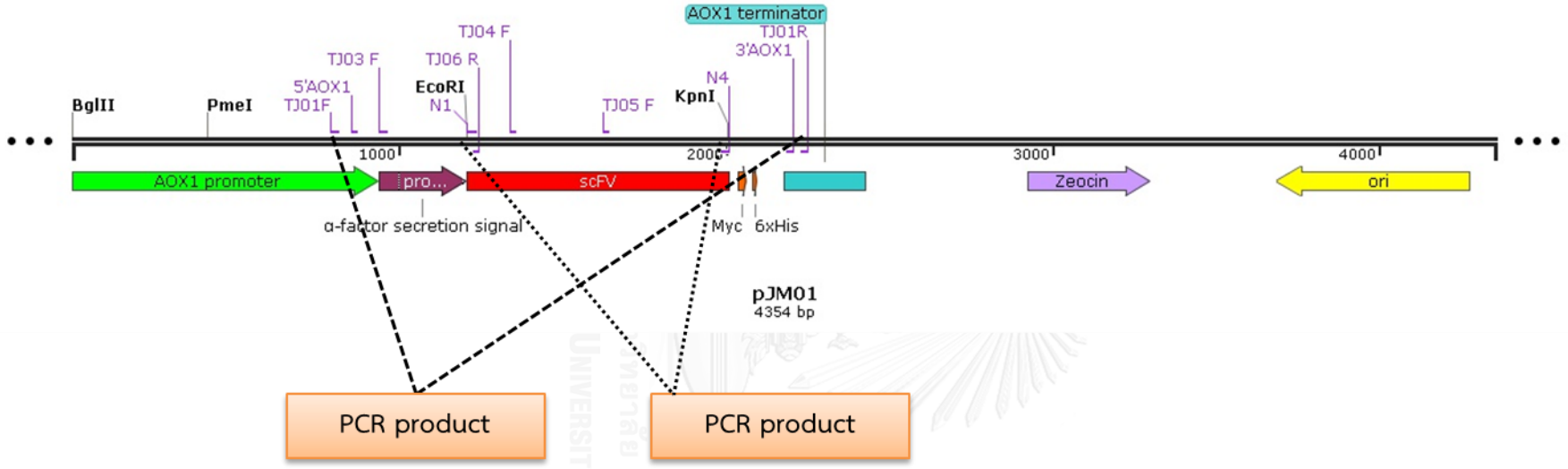

Figure 3.2 The schematic representation of pJM01verification

3.2.7 Preparation and Transformation of Competent Yeast

3.2.7.1 Preparation of Fresh Competent Yeasts (86)

A fresh single colony of yeast *P. pastoris* GS115 was inoculated in 20 mL YPD medium in 250 mL Erlenmeyer flask and cultivated with shaking at 200 rpm at 30°C for overnight. Five percent of the overnight culture was transferred to 50 mL YPD medium and cultivated with shaking at 300 rpm at 30°C until the OD_{600} reached to 1. Subsequently, cells were harvested by centrifugation at 2,000×g at 20°C for 5 minutes, decant supernatant. The cells were re-suspended in 10 mL of YPD medium with 2 mL of 1 M HEPES buffer (pH 8.0). After that, 250 µL of 1 M DTT (250 µL) was added into the tube and gently mixed. Then, the cell suspension was incubated without shaking at 30°C for 15 minutes. Cells were collected by centrifugation at 2,000×g at 4°C for 5 minutes. The cell pellets were washed two times with 25 mL of sterilized ice-cold double distilled water and washed again with 10 mL of sterilized ice-cold 1 M D-sorbitol. The cells were recovered by centrifugation at 2,000×g at 4°C for 5 minutes and re-suspended with 500 µL of sterilized ice-cold 1 M D-sorbitol. Eighty microliters of the cell suspension was then aliquoted into 1.5 mL sterilized microfuge tube and store the tubes on ice for transformation by electroporation.

3.2.7.2 Transformation of Yeasts by Electroporation

This protocol was modified from DNA-mediated transformation in method in molecular biology, second edition [92] and EasySelect™ *Pichia* Expression Kit [93]. The *Pme*I (Takara)-linearized pJM01 was transformed into fresh competent *P. pastoris GS115* by electroporation. Freshly prepared competent cells (80 µL) were mixed up with 5-10 ng of plasmid DNA and transferred to an ice-cold 0.2 cm gap electroporation cuvette, which stored on ice. The cells were pulsed with 2.5 kV by MicroPulser™ Electroporator (Bio-Rad). Then, 500 µL of sterilized ice-cold 1 M D-sorbitol was immediately added to the cuvette, gently mixed by pipetting and transferred the cell suspension to new microfuge tube, stored on ice for 30 minutes. The cell suspension was incubated without shaking at 30°C for 1 hour. Afterward, 500 µL of YPD medium was added and continuously incubated with shaking at 200 rpm

at 30°C for 1 hour. The cells were spreaded and selected onto selective plate, YPD plate with 100 μg.mL^{-1} of Zeocin™, and incubated at 30°C for 2-4 days until zeocin resistant (*Zeo*^{R}) colony appeared.

3.2.7.2.1 Screening of Zeo^{R} *Transformant by Colony PCR*

For confirmation integration of P*me*I-linerarized pJM01 has integrated into the *P. pastoris* GS115 genome. The following protocol was carried out. Ten microliter of each *Zeo*^{R} transformant culture was placed into a 1.5 mL microcentrifuge tube. For relative dense cultures, one microliter of the culture was diluted with 9 μL of sterilized nuclease-free water. Alternatively, a single colony was picked up and re-suspended in 10 μL of sterilized nuclease-free water. Then, 10 μL of a 5 U/μL solution of lyticase was added and incubated at 30°C for 10 minutes. Next, this sample was either placed at -80°C for 10 minutes or immersed in liquid nitrogen for 1 minute. Sample was then centrifuged at 2,000×g at 4°C for 5 minutes and the obtained supernatant was directly use as template in PCR. PCR amplification was performed in a 25 mL volume containing 2.5 mL 10× PCR buffer, 2.0 mM of each primer (5'AOX1 paired with 3'AOX1 primers or N1 paired with N4 primers) , 25 mM dNTPs, 1.25 U Taq DNA polymerase (TaKaRa). The thermocycle conditions were: 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 62°C (5'AOX1/3'AOX1 primer) or 55°C (N1/N4 primer) for 30 s, extension at 72°C for 1 min, and a final elongation step at 72°C for 10 min. The PCR products were examined on 1.0% w/v agarose gels.

3.2.7.2.2 Screening of Zeo^{R} *Transformants by PCR*

The six to ten *Zeo*^{R} transformants were analyzed. Firstly, genomic DNA of these *Zeo*^{R} transformants was extracted by using MasterPure™ Yeast DNA purification Kit (Epicentre). The quantity and quality of genomic DNA were examined via 1.0% w/v agarose gels. Afterward, amplification of the scFv gene was done with N1 and N4 primers (scFv- specific primers) as shown in 3.1.5. The PCR reaction and PCR condition

were mentioned as above in 3.2.7.2.1. The pJM01 plasmid and pPICZαA (plasmid without insert) were used as positive and negative control, respectively.

3.2.7.3 Transformation of Yeasts by Frozen-EZ Yeast Transformation II™

The yeast transformation by Frozen-EZ Yeast Transformation II™ (Zymo Research) was carried out according to the manufacturer's instruction. Firstly, yeast cells were grown at 30°C in 10 mL YPD broth until mid-log phase (~5 x 10^6 - 2 x 10^7 cells/ml or OD600 of 0.8-1.0). The cells were pelleted at 500 x g for 4 minutes and discarded the supernatant. This pellet was added 10 ml of EZ-1 solution to wash the pellet, and discarded the supernatant. A 1 ml EZ-2 was added into the solution to re-suspend the pellet. At this point, the competent cells were used for transformations directly or stored frozen at or below -70°C for future use. In transformation, 50 µl of competent cells were gently mixed with 0.2-1 µg DNA and 500 µl EZ-3 solutions and mixed thoroughly. After that, the mixture was incubated at 30°C for 45 minutes. During this incubation, the mixture was mixed by vortexing 2-3 times. 50-150 µl of the above transformation mixture was spreaded on selective plate, YPD plate with 100 µg.mL^{-1} of Zeocin™ and incubated the plates at 30°C for 2-4 days to allow for growth of *Zeo*^{R} transformants. The *Zeo*^{R} transformants was picked up to freshly YPD-Zeocin™ agar medium and was screened as mentioned in 3.2.7.2.2.

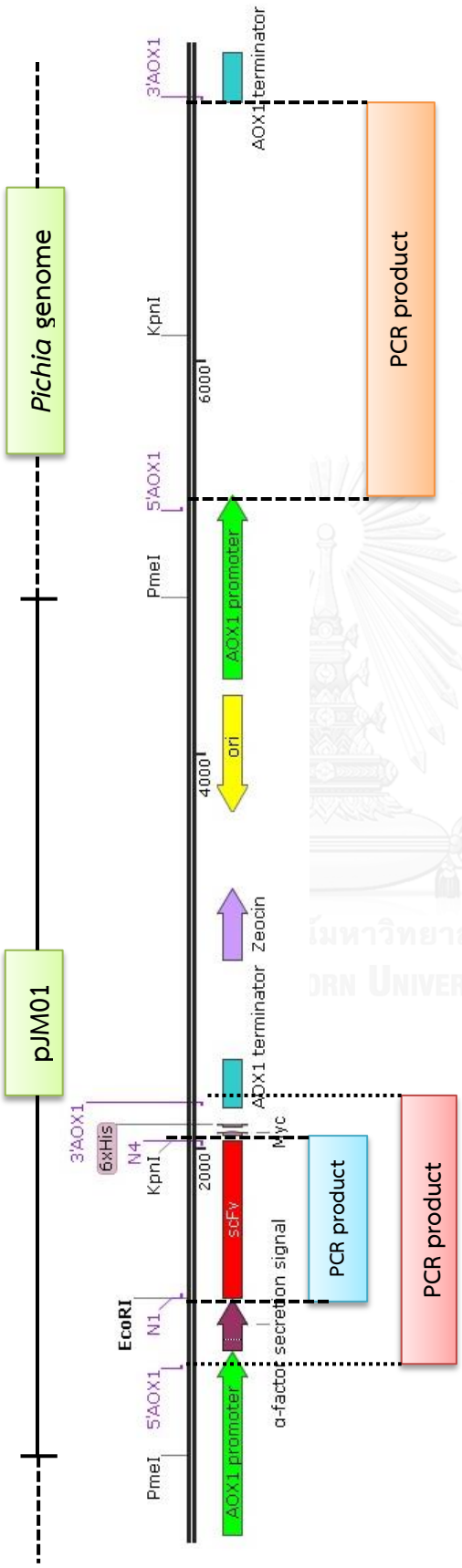

Figure 3.3 The schematic representation of screening of Zeo^{R} transformation by colony PCR and PCR

3.2.8 Confirmation of pJM01 Integrated into *Pichia* Genome

3.2.8.1 Southern Blot Analysis

Genomic DNA of the selected clones was extracted from the freshly grown YPD cultures of *P. pastoris* strains expressing scFv using the MasterPure™ Yeast DNA Purification kit (Epicenter). Chromosomal DNA concentrations were quantified using Qubit® 3.0 Fluorometer (Life Technologies). A 2.5 µg of chromosomal DNA were digested individual with restriction enzymes *Eco*RI and *Kpn*I, respectively. These digested DNA were separated on 0.8 % TAE agarose gel. Through capillary action, the DNA was transferred to a Hybron-N^{+} positively charged nylon membrane (GE Healthcare). The *AOX1* promoter was used as probe in hybridization solution to estimate scFv located. This probe was amplified using AOX1F and AOX1R primers and subsequent labeling of the probe. The pre- and post-hybridization of membrane were performed according to the protocol of the manufacturer and followed by CDP-Star™ detection was employed for capturing chemiluminescence signal.

3.2.8.2 Sequencing Analysis

The integration and the direction of *Pme*I-linearized pJM01 plasmid integrated into *Pichia* genome were confirmed by sequencing. The *Zeo*^{R} transformants yeasts that harboring the scFv antibody from 3.2.7.2 and 3.2.7.3 were used to extract genomic DNA as template for analysis and the nucleotides sequence of scFv was sequenced as described in 3.2.6.3.

3.2.8.3 Verification of pJM01 Integrated into Pichia Genome at AOX1 Promoter

There are several publications and scientific papers reported that *AOX2* showed the similarity to *AOX1* with about 97% homologous. For this reason, the confirmation of pJM01 integrated into the genome of *Pichia* at *AOX1* promoter is needed to prove. The P1 paired with P2 primers and P3 paired with P4 primers were specific designed based on the *AOX1* promoter in the *Pichia* genome [94]. These primers was proved that they could specific anneal with only AOX1 promoter. The PCR reaction and PCR condition were carried out as described in 3.2.7.2.1.

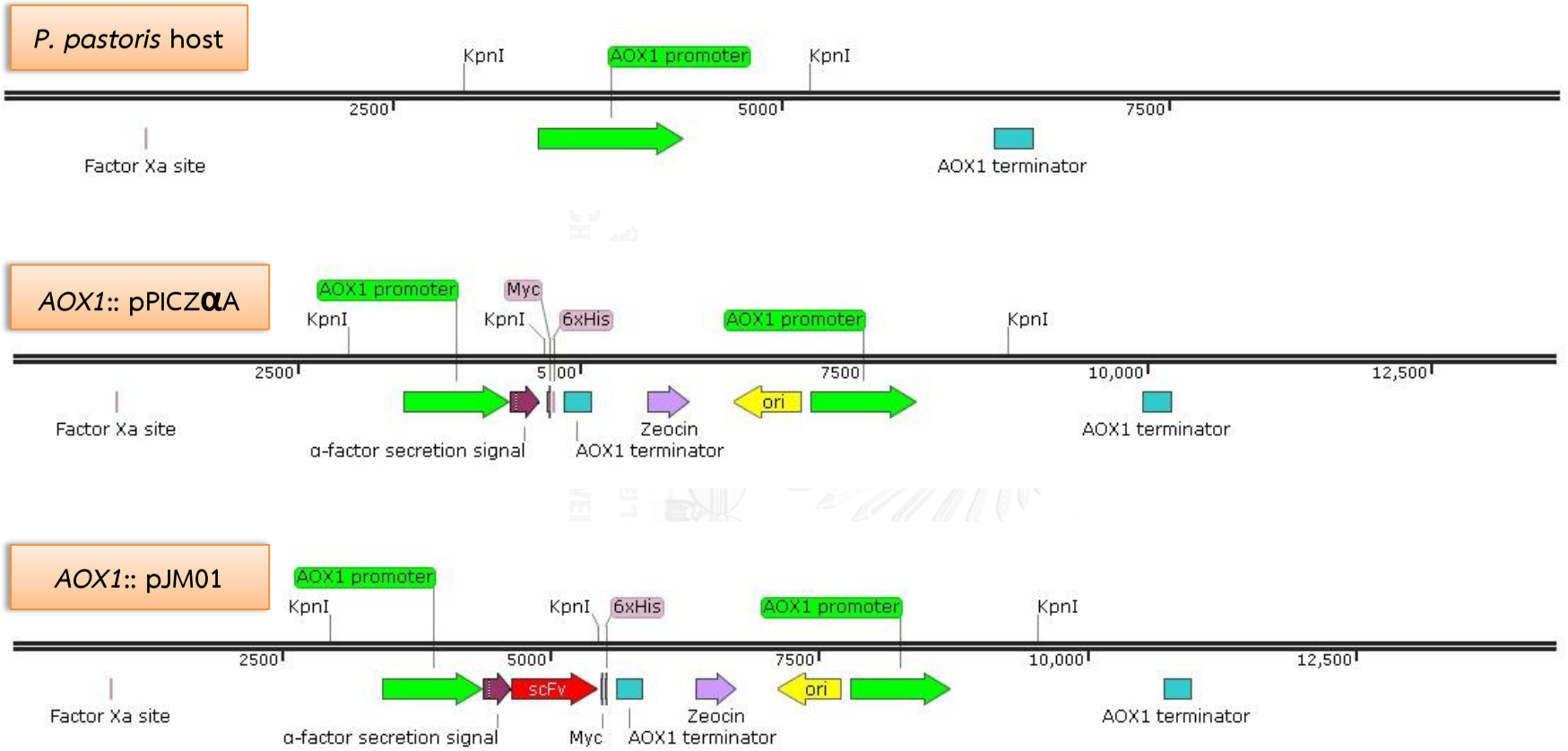

Figure 3.4 The schematic representation of Southern blot analysis by using *Kpn*I digestion

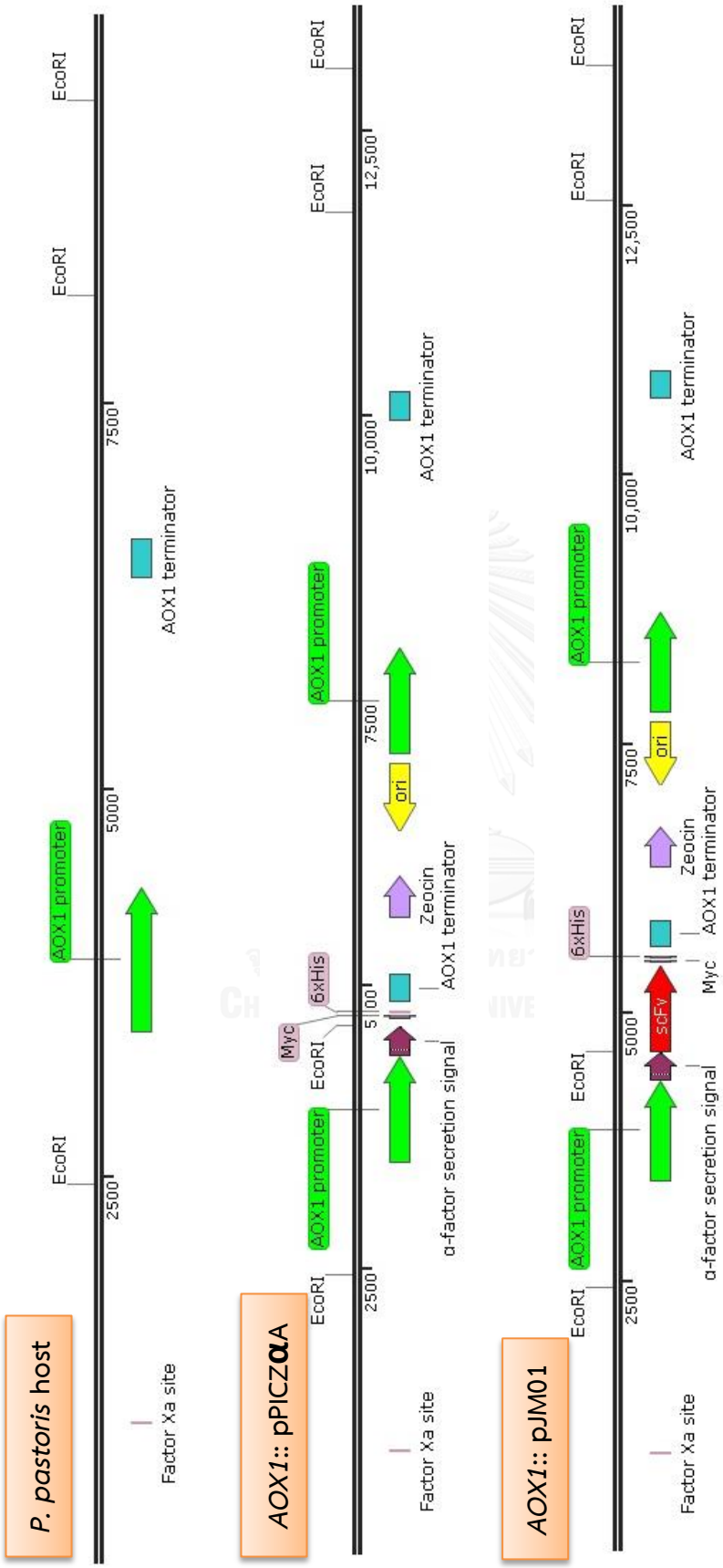

Figure 3.5 The schematic representation of Southern blot analysis by using *Eco*RI digestion

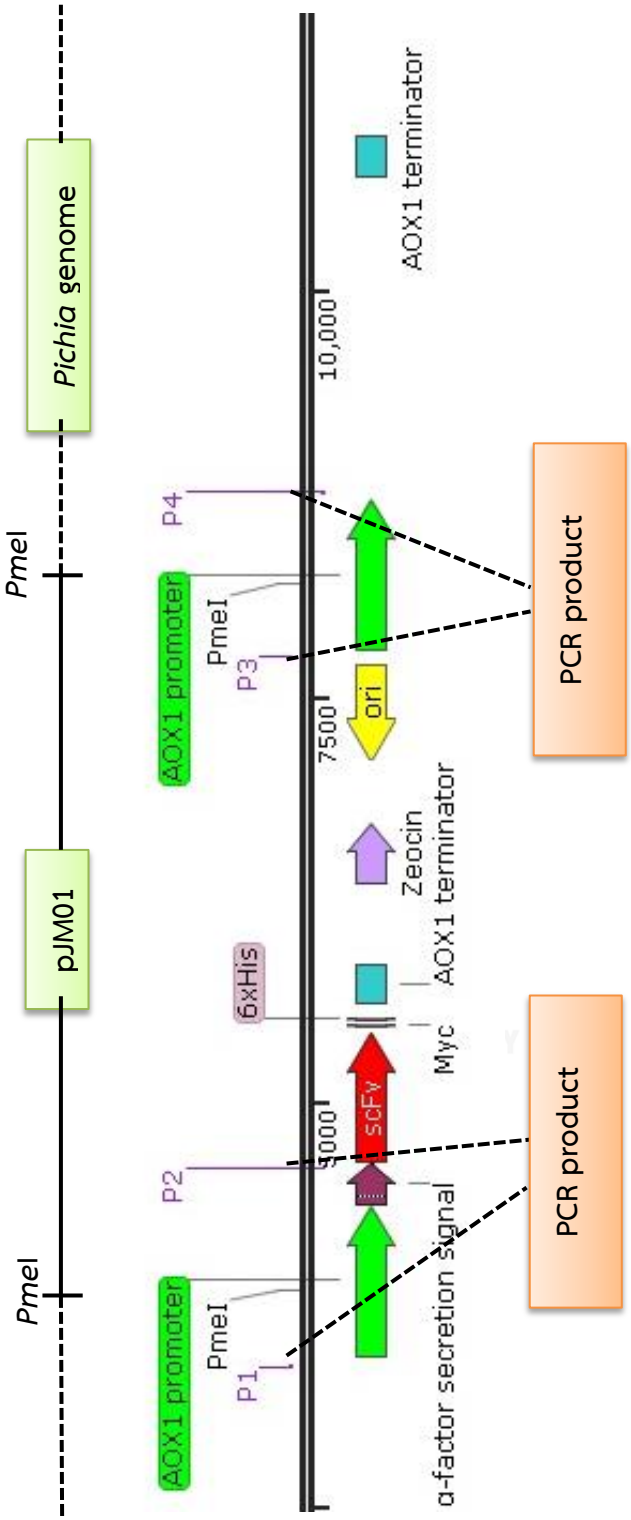

Figure 3.6 The schematic representation of confirmation of scFv integrated at AOX1promoter

3.2.9 Yeast Cultivation and Recombinant scFv Expression

The *Zeo*^{R} transformants yeast, which was confirmed as above and revealed the totally accuracy of nucleotide sequence, were selected to screen for high recombinant scFv expression. Fresh single colony of those was inoculated in 20 mL YPG medium and incubated with shaking at 200 rpm at 30°C for overnight. Five percent of the overnight culture was inoculated in 30 mL of YPG medium and incubated with shaking at 250 rpm at 30°C for an OD_{600} reach to 1.0 (approximately 3 hours) which used as a starter culture. After an OD_{600} reach to 1.0, ten percent of the starter culture was inoculated in 50 mL YPG medium in 250 mL Erlenmeyer flask, and incubated with shaking at 250 rpm at 30°C for 24 hours (cell production phase). After 24 hours in cell production phase, cells were collected by centrifugation at 2,000×g for 5 minutes at 20°C followed by decanting the supernatant. The cells were re-suspended in 50 mL of MMH induction medium in 250 mL baffled flask and incubated with shaking at 250 rpm at 30°C. Five milliliters of culture samples were taken at 0, 12, 24, 48, and 72 hours and 5 mL of MMH medium were added. In an induction phase, one-hundred percent of absolute methanol was added to a final concentration of 0.5% methanol every 24 hours to maintain an induction. The culture samples were centrifuged at 5,000×g at 4°C for 5 minutes, the supernatants were transferred to a new sterile tube and adjusted the pH value to ~7 with a sterilized 2.5 M NaOH. The supernatants were aliquoted and stored at +4°C until assay and cell pellet was washed twice with TE buffer (pH 8.0) and kept in -20°C for further assay. The procedure of this protocol is show in Figure 3.2.

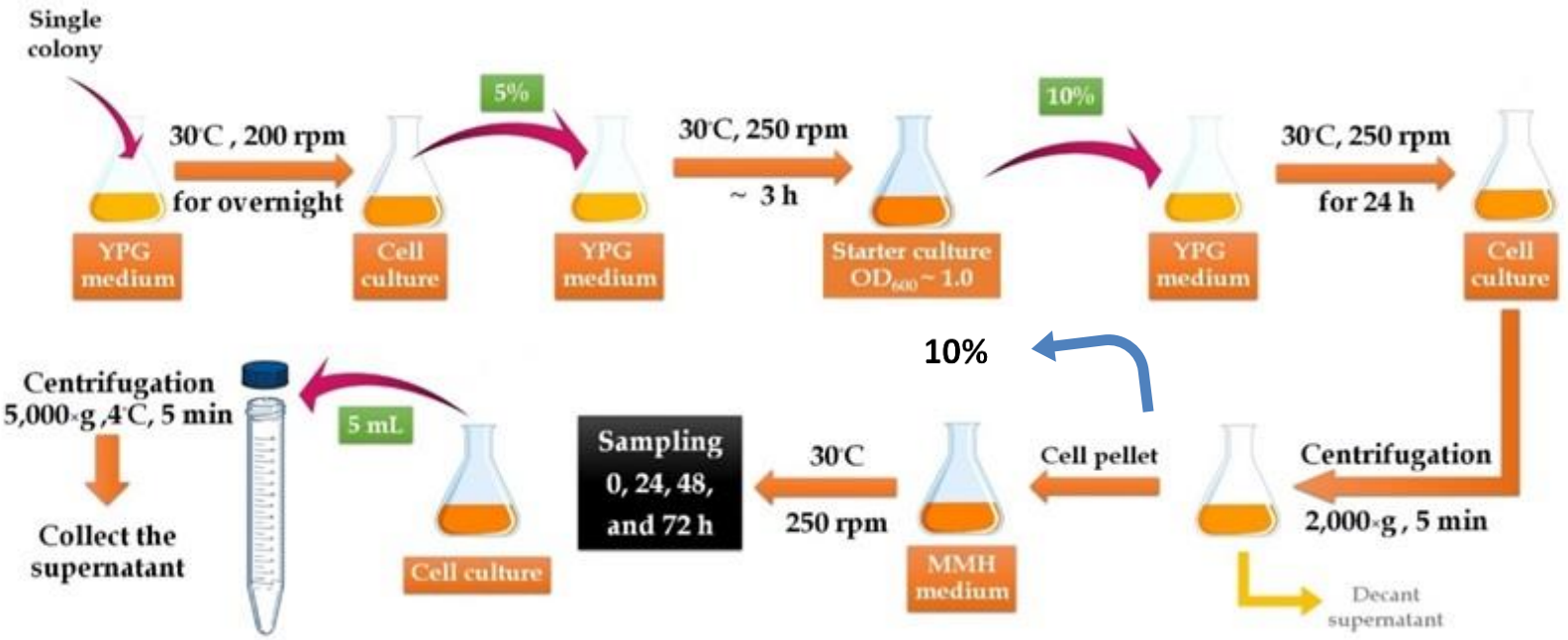

Figure 3.7 The cultivation and expression of the recombinant scFv by two step cultivation

3.2.10 Characterization of Recombinant scFv

3.2.10.1 Intracellular Expression [95]

The frozen cell pellets were re-suspended in 850 µl of sterilized water and mixed well by vortex. After that, 50 µl of these was taken to determine the cells concentration by spectrophotometry. Another was added immediately with 150 µl of 1.85 M NaOH and kept on ice for 10 minutes. Later, 150 µl of 55% (w/v) TCA was applied and kept on ice again. After incubation for 20 minutes, the mixture was centrifuged at 14000 rpm for 15 minutes at 4°C and the excess of supernatant was removed out. Afterward, high urea –DTT (HU-DTT) buffer was added according to the OD value. Normally, 50 µl of HU-DTT buffer was employed for one OD and then mixed well until the pellet re-suspend. Next, the samples were heated-up for 15 minutes at 65°C followed by centrifugation at 14000 rpm for 2 minutes at room temperature. Finally, these samples were loaded in further SDS-PAGE.

3.2.10.2 Secreted Expression

The proteins in supernatant were concentrated by precipitation with trichloro acetic acid (TCA). The protein concentration was measured by Qubit® 3.0 Fluorometer (Life Technologies). Equal amounts of supernatant and 100% TCA were mixed to final concentration of 20% and incubated on ice for 10 minutes. After centrifugation (13000 rpm, 4°C for 5 minutes), the pellets were washed twice with the same volume of cold acetone followed by air-dried and then re-suspended in 2x SDS sample buffer. Finally, these samples were loaded in further SDS-PAGE.

3.2.10.3 SDS-PAGE

The samples form 3.2.10.1 and 3.2.10.2 were analyzed by electrophoresis on 12% sodium dodecyl sulfate (SDS) polyacrylamide gel under denaturing conditions according to standard protocol. The condition was run at 200 V for 90 min. Then, the gel was either stained with a Coomassie brilliant blue R250 (Bio-Rad) or stained with silver stain (SilverQuest™ Silver Staining kit, Life technologies).

3.2.10.4 Chemiluminescent Western Blot Analysis

The protein gel was transferred to a PVDF membrane for Western blot analysis using a semi-dry transfer cell (Bio-Rad Laboratories, USA) for 1 hour at 10 V in transfer buffer (25 mM Tris base, 192 mM glycine, and 20% (w/v) methanol). The membrane was thereafter blocked in 5% non-fat dry milk (Bio-Rad) in 1xTris buffered saline (Sigma) for 1 hour with shaking. Then, the membrane was washed in 1xTris buffered saline (Sigma) with 0.1% Tween 20 at room temperature with shaking. The recombinant scFv on membrane was detected with mouse anti-c-myc monoclonal antibody (Clone 9E10) (Invitrogen) as primary antibody at 1:2000 at room temperature for 1 hour, alternatively 4°C for overnight, followed by goat anti mouse IgG-HRP (Invitrogen) in 5% non-fat dry milk in 1xTris buffered saline containing 0.1% Tween 20 as secondary antibody at 1:4000 at room temperature for 1 hour. Afterward, the ECL chemiluminescent reagent was used as substrate. The membrane was exposed and observed specific signal band by ChemiStage CC-16 mini (Scitech).

The clones which were shown the highest expression were selected and stored in 50 % sterilized glycerol at -70°C for further large scale expression.

3.2.11 Large-Scale Cultivation of O5 Strain

The O5, *Zeo*^{R} transformant yeast, was selected as the best candidate for large scale expression. The large-scale expression was scaled-up and the procedure of expression was carried out as previously described in 3.2.9 method section.

3.2.12 Determination of Transcriptional Level Using Real-time Quantitative PCR (RT-qPCR)

Total RNA was isolated from O5 cells, which were induced with methanol for 72 hours in period using E.Z.N.A.® Yeast RNA kit (Omega) following the procedure recommended by the manufacturer. After DNase I digestion, the concentration of total RNA was eluted and determined by Nanodrop sprectrophotometer (Optizen NanoQ). Then, the equal amount of RNA was reversed transcribed using the Tetro cDNA synthesis kit (Bioline™). After that, cDNA template was amplified in 25µl

containing 150 mM of the respective primers and 5 µl of 5x HOT FIREPol® Evagreen® qPCR mix plus (Solis Biodye). All real-time RT-qPCR reactions were run in triplicate using the following program: 95°C for 15 min, 40 cycles of 95°C for 15s, 55°C for 20s and 72°C for 20s. The TJ04F and TJ05R primers were used for scFv amplification. In this condition, a melting curve was applied to ensure that only a specific amplification product was obtained. The relative amounts of mRNA were calculated from the comparative quantification cycle (Cq) values using the actin gene as control.

3.2.13 Purification of Recombinant scFv

Large scale culture was used to prepare recombinant scFv antibody as described in 3.2.11.1. The yeast cells were removed by centrifugation at 10,000 g for 5 minutes and the cell-free medium further clarified through a 0.2 µM filter. The purification of recombinant scFv antibody under native conditions was carried out by immobilized metal affinity chromatography (IMAC) using a Ni-NTA agarose (Invitrogen™) according to the manufacturer's instructions. Briefly, the 50% Ni-NTA slurry was added to the supernatant at the ratio of 1:4 (v/v) and mixed gently by stirrer overnight at 4^0C. Then, the supernatant-agarose mixture was carefully loaded into an empty column with the bottom cap still attached. After removing the bottom cap, flow—through was collected and detected by UV 280 nm. All the following eluted fractions were also detected by UV 280 nm. The column was further washed twice with Native wash buffer. Subsequently, the protein was eluted with Native Elution buffer. The elution fractions were collected and analyzed by UV 280 nm, SDS-PAGE and Western blotting, respectively. The protein concentration after dialysis against PBS buffer was measured by Pierce™ BCA protein assay kit (Thermo scientific) using the protein standard bovine serum albumin (BSA).

3.2.14 Determination of Recombinant scFv Binding Activity

3.2.14.1 Indirect Enzyme-Linked Immunosorbent Assay (indirect ELISA)

After purification, the binding activity of the purified recombinant scFv antibody was determined by indirect ELISA. Initially, the dialyzed of 1µg/ml of BSA-

norfloxacin antigen was used to coat the 96 well plates (100 µL per well) at 4°C for overnight. The plate was washed three times with 300 µL per well of washing buffer (PBST) following by blocking with 300 µL per well of blocking buffer (5% skim milk in PBS buffer) and incubated at 37°C for 1 hour. The plate was washed again. Subsequently, the purified recombinant scFv was added into the reaction well (50 µL per well) and incubated at 37°C for 2 hours. Then, 50 µL of mouse anti-c-myc antibody at the dilution ratio of 1:2,000 was added. After incubation at 37°C for 2 hours and subsequent by washing, a secondary goat anti-mouse IgG conjugate with horseradish peroxidase (HRP) was added at the dilution ratio of 1:5,000 (100 µL per well). The plate was incubated at 37°C for 1 hour, subsequent by washing and adding the TMB substrate solution (100 µL per well). After incubation in the dark at room temperature for approximately 20 minutes, the reaction was stopped by adding 100 µL per well of 1 M H_2SO_4. The plate was measured the optical density at 450 nm using microplate reader.

3.2.14.2 Surface Plasmon Resonance (SPR)

The SPR assay was performed and measured the binding activity of purified recombinant scFv antibody on an Autolab SPRINGLE system. Briefly, instrument calibration was firstly done after gold disk and cuvette installation, and baseline adjustment was performed according to manufacturer's suggestion provided in the SPR user manual. Washing of gold disk were performed after the baseline adjustment at the start of the measurement; and after each ligand immobilization and association processes. A total of 130 µl of PBS buffer were introduced during the washing cycles. In every step within the SPR protocol, mixing of samples during measurement is efficiently carried out by a programmed intermittent introduction of air flow into the sample using a piston pump. Afterward, the gold sensor surface was initially saturated with purified recombinant scFv antibody by static mixing over the sensor surface for overnight at 4°C. After this point, the sensor surface was washed with 10 mM PBS buffer (pH 8.0) and drained before introducing the antigen solutions. Binding interactions between the antigen and antibody were allowed to proceed for a minimum period of 16 minutes. 5 similar measurements on the same disk were

carried out by changing the position of the sampling spot (Figure3.8). All SPR experiments were performed at Scientific and Technological Research Equipment Centre (STREC), Chulalongkorn University, Thailand.

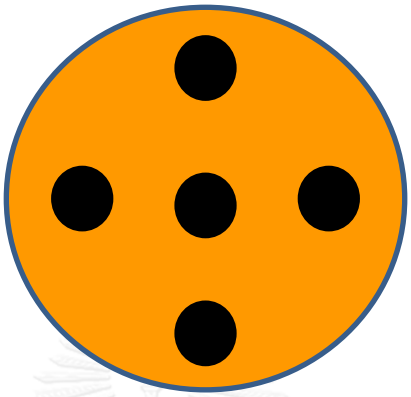

Figure 3.8 Gold-coated SRP sensor disk showing 5 different sampling spots on the disk which allow 5 individual sets of experiments to be carried out.

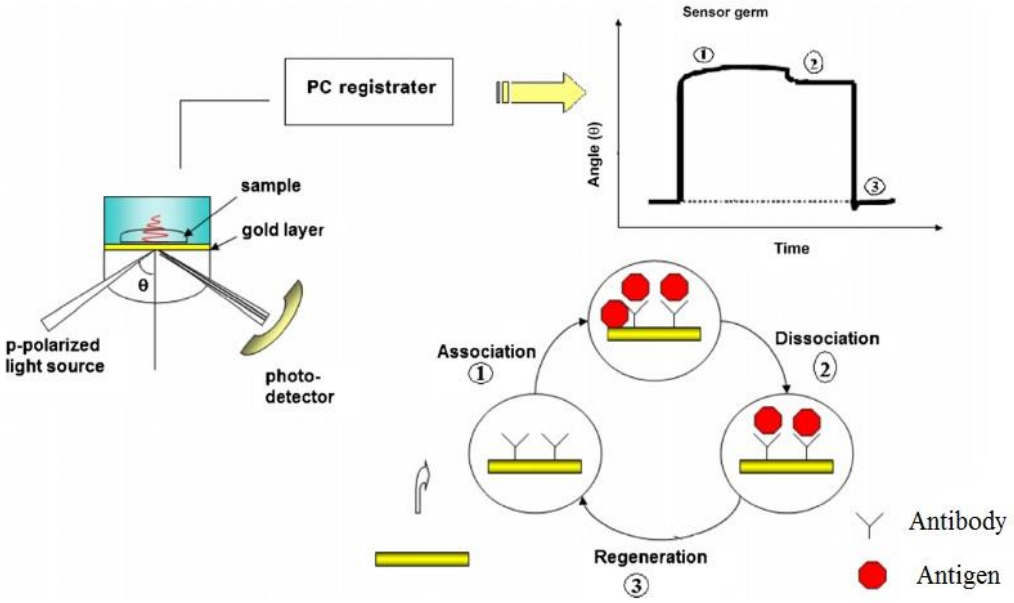

Figure 3.9 Schematic representation of the principle of SPR [96]

CHAPTER IV

RESULTS AND DISSCUSSIONS

4.1 Characterization of Nor155

In this study, the antinorfloxacin-producing hybridoma clone 155 (Nor155) was selected as the model for construction and production of single-chain variable fragment (scFv) antibody by methylotrophic yeast *Pichia patoris* GS115. The Nor155 was kindly provided by the Institute of Biotechnology and Genetic Engineering (IBGE), Chulalongkorn University since it poses good antibody properties for further development of an ELISA for norfloxacin detection. It revealed the best several characterizations over the other antinorfloxacin-producing hybridoma clones. As reported [97], the limit of detection (LOD) and the half maximal inhibitory concentration (IC_{50}) were 1.01 ng/mL and 0.42 µg/mL, respectively. Moreover, the cross-reactivity was ranged from 47% - 2100% with other FQs which is suitable for a generic test. It isotype was identified to be IgG1 and the light chain was a kappa type (Appendix C). Therefore, Nor155 was used as the source of genetic information for construction of scFv antibody.

4.2 RNA Extraction and 1st Strand cDNA Synthesis

The total RNA of monoclonal antibody Nor155 was isolated by using NucleoSpin® RNA II kit. The concentration and purity of the extracted RNA were estimated by spectrophotometry. The absorbance at 260 nm indicated the concentration of 2.19±0.07 µg/µL which the absorbance ratio of 260/280 nm estimated the purity of 2.03±0.09. It has been suggested that this absorbance ratio of a pure nucleic acid should be between 1.8 and 2.0. Since, the obtained value was slightly above the suggested value, the obtained extract was pure enough for futher study. The RNA integrity was assessed prior to cDNA synthesis. The quality of extracted RNA was observed via 1% formaldehyde agarose gel electrophoresis followed by ethidium bromide staining.

The figure 4.1 showed a major 2 sharp bands of the extracted RNA. As prediction, the first one was 28S rRNA with 3,354 nucleotides and the other one was 18S rRNA with 1753 nucleotides, respectively. From these results, the extracted RNA revealed high potential genetic information source for further using as template in 1st strand cDNA synthesis.

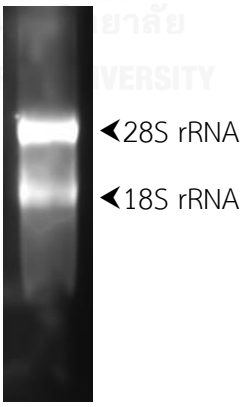

Figure 4.1 Total RNA from Nor155 were separated on 1% formaldehyde agarose gel electrophoresis

For synthesis of 1st strand cDNA coding for the V_H and V_L genes, an approximately 1 µg of isolated RNA was used as the template in the reaction. In this experiment, the oligo $(dT)_{18}$ primers was selected as the suitable primers to synthesize 1st strand cDNA with a reverse transcripase enzyme. This primer only hybridized to the poly-adenylated (A) tail, which found on the 3'-end of most eukaryotic mRNA. After completion of the reaction, the cDNA reaction mixture was examined by running through 1% agarose gel electrophoresis (Figure 4.2) and was then kept at -20°C for V_H and V_L amplification.

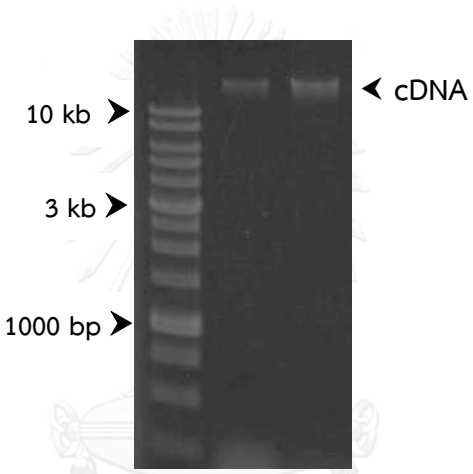

Figure 4.2 The ethidium bromide stained 1.0% agarose gel of cDNA revealed a major band over 10 kb of DNA marker. Lane M: 1 Kb plus DNA ladder (Invitrogen). The expected band was indicated by an arrow.

4.3 Amplification of V_H and V_L Genes

The V_H and V_L genes were amplified separately by PCR using the 1^{st} strand cDNA template. For V_H gene amplification, the PCR reaction was performed by using VHFwMH1 paired with VHRwIgG1 primers. After separation the DNA fragment through 1% agarose gel electrophoresis and subsequent ethidium bromide staining, the sharp band of V_H DNA fragment was observed at 402 bp (Figure 4.3A, lane 1-3). The agarose gel band containing the desired DNA fragment was carefully cut and purified. After elution with sterilized water, the purified V_H DNA fragment was examined by 1% agarose gel electrophoresis (Figure 4.3B, lane 1).

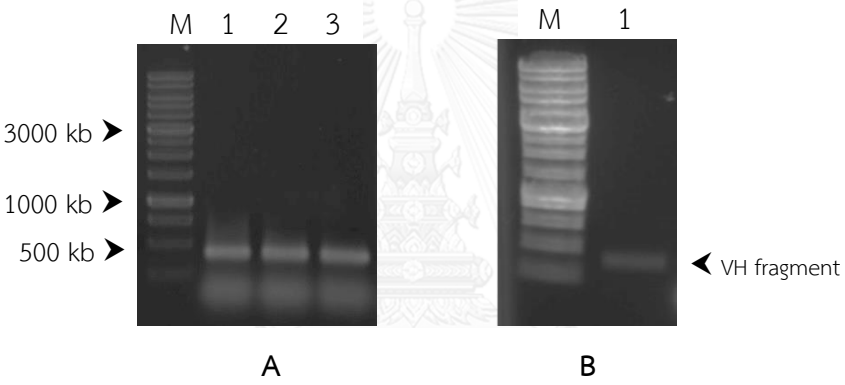

Figure 4.3 PCR product of V_H amplification. A) lane 1-3: amplified VH. B) lane 1: purified VH fragment. Lane M: 1 Kb plus DNA ladder (Invitrogen). The expected band was indicated by an arrow.

In the case of V_L gene amplification, the PCR reaction was carried out with VLFwMk and VLRwKc primers. The lengths of VL DNA fragment were approximately 363 bp (Figure 4.4A, lane 1-4). These sharp bands were also cut and purified. After elution with sterilized water, the purified VL DNA fragment was examined via 1% agarose gel electrophoresis (Figure 4.4B, lane 1-2).

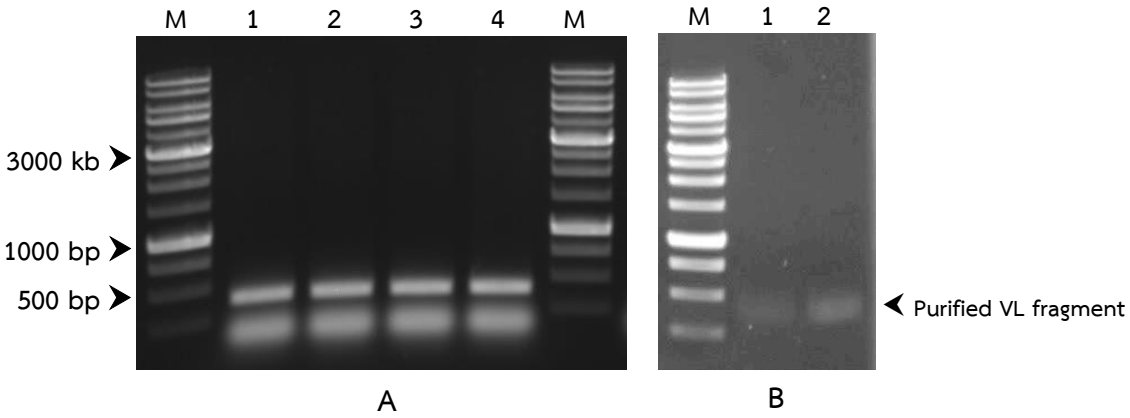

Figure 4.4 PCR product of V_L amplification. A) lane 1-4: amplified VL. B) Lane 1-2: purified VL fragment. Lane M: 1 Kb plus DNA ladder (Invitrogen). The expected band was indicated by an arrow.

The purified V_H and V_L DNA fragments were individual cloned into pGEM® T-easy vector by using T4 DNA ligase enzyme, then transformed to freshly competent *E. coli* TOP10F'. After 16 hours cultivation in LB plates containing ampicillin, IPTG and x-gal, the blue and white recombinant colonies were appeared. Over 50 white colonies of each VH and VL fragments plasmids were randomly picked up and cultured in LB/amp liquid medium at 37°C on shaker, overnight. Ten white colonies of VH and VL transformants were extracted by using alkaline lysis solution and the obtained plasmids were run through 1% agarose gel electrophoresis as shown in Figure 4.5A. The obtained DNA plasmid had the size of approximately 3.4 kb.

Figure 4.5B showed the linear DNA fragment after using *Eco*RI digestion. Lane 1 and lane 2 indicated that VHnor155-5 and VHnor155-9 clone were completely digested and the DNA fragment of V_H was released with the size of about 400 bp. Lane 3 represented pGEM® T-easy vector which extracted from blue colony on LB/amp plate that no insert DNA fragment (negative control).

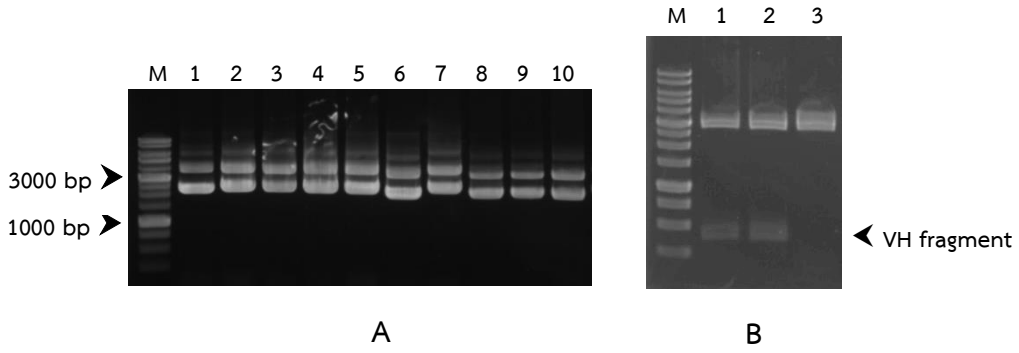

Figure 4.5 The 1.0% agarose gel electrophoresis of the recombinant pGEM-VH plasmid A) lane 1-10: the obtained plasmid of 10 white colonies B) lane 1-3: the plasmid from Figure 4.5A were digested with *Eco*RI. Lane M: 1 Kb plus DNA ladder (Invitrogen)

Figure 4.6 illustrated the inserted DNA fragment of V_L after digestion the recombinant pGEM_VL plasmid with *Eco*RI. Lane 1 to land 6 was VLnor155-12, VLnor155-13, VLnor155-14, VLnor155-18, VLnor155-19 and VLnor155-21, respectively. The released DNA fragment of V_L was observed at the size of approximately 360 bp, which found in VLnor155-12, VLnor155-13, VLnor155-18 and VLnor155-21 clone.

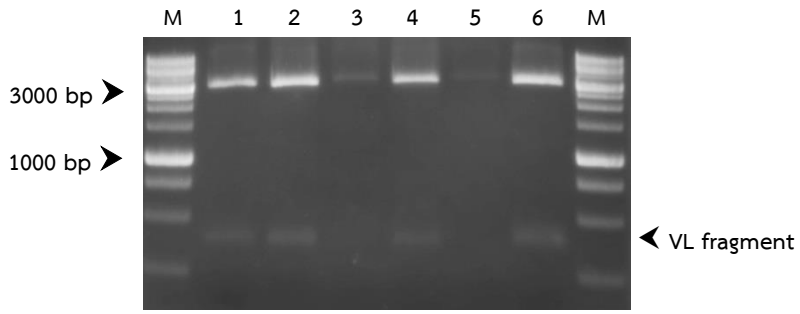

Figure 4.6 The 1.0% agarose gel electrophoresis of the recombinant pGEM-VL plasmid digested with *Eco*RI. Lane 1-6: the obtained plasmid of 6 white colonies were digested with *Eco*RI and revealed the expected size as indicated by arrow. Lane M: 1 Kb plus DNA ladder (Invitrogen)

After confirmation with *Eco*RI digestion, the VHnor155-9 and VLnor155-12 clones were sent to analyze the nucleotide sequence by Macrogen, Korea. By using T7 primers or SP6 primers for sequencing, the nucleotide sequences of VHnor155-9 showed 402 nucleotides and their deduced 134 amino acid sequence was shown in Figure 4.7. In addition, the nucleotide sequences of VLnor155-12 clones revealed 363 nucleotides and their 121 amino acid sequence was shown in Figure 4.8. These sequences have been submited to the NCBI GenBank with accession no. AJG06889.1 and KR261578.1, respectively.

```
  1 GAATTCGAAGTAGAG CTGGAGGAGTCTGGG GGAGGCTTAGTGAAG CCTGGAGGGTCCCTG AAACTCTCCTGTGCA
  1  E  F  E  V  E   L  E  E  S  G   G  G  L  V  K   P  G  G  S  L   K  L  S  C  A
 76 GCCTCTGGATTCAGT TTCAGTGACTATTAC ATGTATTGGGTTCGC CAGACTCCGGAAAAG AGGCTGGAGTGGGTC
 26  A  S  G  F  S   F  S  D  Y  Y   M  Y  W  V  R   Q  T  P  E  K   R  L  E  W  V
151 GCAACCATTAGTGAA GGTGCCAGTTATATC TATTATTCGGACAGT GTAAAGGGACGCTTC ACCATCTCCAGAGAC
 51  A  T  I  S  E   G  A  S  Y  I   Y  Y  S  D  S   V  K  G  R  F   T  I  S  R  D
226 AATGCCAGGAACACC CTGTACCTGCAAATG AGCAGTCTGAAGTCT GAGGACACAGCCATT TATTACTGTACAAGA
 76  N  A  R  N  T   L  Y  L  Q  M   S  S  L  K  S   E  D  T  A  I   Y  Y  C  T  R
301 GCCTATAATAACTAC GTGTCGAAATATTTC GATGTCTGGGGCGCA GGGACCACGGTCACC GTCTCCTCAGCCAAA
101  A  Y  N  N  Y   V  S  K  Y  F   D  V  W  G  A   G  T  T  V  T   V  S  S  A  K
376 ACGACACCCCCATCT GTCTATGAATTC
126  T  T  P  P  S   V  Y  E  F
```

Figure 4.7 The coding sequence (CDS) and amino acid sequence of V_H gene of Nor155 (accession no. AJG06889.1)


```
  1 GGGGAGCTCGACATT GTGATGACACAGTCT ACAGCAATCCTGTCT GCATCTCCAGGGGAG AAGGTCACAATGACT
  1  G  E  L  D  I   V  M  T  Q  S   T  A  I  L  S   A  S  P  G  E   K  V  T  M  T
 76 TGCAGGGCCAGTTCA AGGGTAAATTACATA CACTGGTTCCAGCAG AAGGCAGGATCCTCC CCCAAACCCTGGATT
 26  C  R  A  S  S   R  V  N  Y  I   H  W  F  Q  Q   K  A  G  S  S   P  K  P  W  I
151 TATGCCACATCCAAC CTGGCTTCTGGAGTC CCTGATCGCTTCAGT GGCAGAGGGTCTGGG ACCTCTTACTCTCTC
 51  Y  A  T  S  N   L  A  S  G  V   P  D  R  F  S   G  R  G  S  G   T  S  Y  S  L
226 ACAATCAGCAGAGTG GAGGCTGAAGATGCT GCCACTTATTACTGC CAGCAGTGGAGTAAT AACCCGTGGACGTTC
 76  T  I  S  R  V   E  A  E  D  A   A  T  Y  Y  C   Q  Q  W  S  N   N  P  W  T  F
301 GGTGGAGGCACCAAG CTGGAAATCAAACGG GCTGATGCTGCACCA ACTGTATCCGAGCTC CCC
101  G  G  G  T  K   L  E  I  K  R   A  D  A  A  P   T  V  S  E  L   P
```

Figure 4.8 The coding sequence (CDS) and amino acid sequence of V_L gene of Nor155 (accession no. KR261578.1)

These obtained sequences were aligned by searching against the Genbank database for sequence homology analysis. Through the BLASTp program, the result showed the similarity of either VHnor155-9 sequence or VLnor155-12 with others. Over 50 different homologous sequences, each first-five of these sequences were selected and demonstrated. Each NCBI GenBank accession number is a sequence identified by BLASTp program as a highly homologous protein sequence to the query putative VHnor155-9 sequence or VLnor155-12 sequence. The comparative analysis of the amino acid sequence with the five known sequences in database was shown and summarized in the Table 4.1 and Table 4.2.

In Table 4.1, it was found that the amino acid sequence of VHnor155-9 was closely related to the Ig heavy chain precursor V region (MAK33) with 80% identity followed by chain H, structure of a *Plasmodium vivax* apical membrane antigen 1-Fab F8.12.19 complex, immunoglobulin mu heavy chain variable region, chain H, crystal structure of Bmj4 P24 capsid protein in complex with A10f9 Fab and immunoglobulin heavy chain variable region with amino acid sequence homology 81%, 85%, 80% and 85% identity, respectively. In addition, the VHnor155-9 sequence was found to be immunoglobulin IgG.

Table 4.1 Comparative analysis of amino acid sequence of VHnor155-9 with sequences in the GenBank database using BLASTp program.

No.	Accession	Definition	Amino acid	Max score	Total score	Query cover	Ident (%)
1	B26471	Ig heavy chain precursor V region (MAK33) - mouse	152	214	214	98%	80%
2	2J4W_H	Chain H, structure of A *Plasmodium vivax* apical membrane antigen 1- Fab F8.12.19 complex	225	213	213	98%	81%
3	AGS48040.1	Immunoglobulin mu heavy chain variable region [*Mus musculus*]	117	208	208	89%	85%
4	3VRL_H	Chain H, crystal structure of Bmj4 P24 capsid protein in complex with A10f9 Fab	224	211	211	98%	80%
5	AAO19048.1	Immunoglobulin heavy chain variable region [*Mus musculus*]	119	207	207	90%	85%

Table 4.2 showed the result of amino acid sequence comparison between VLnor155-12 and the most identical sequence in the GenBank database with BLASTp program. The result indicated that VLnor155-12 sequence was found to be immunoglobulin kappa light chain. Moreover, VLnor155-12 sequence was most closely related to anti-KSHV gH immunoglobulin light chain variable region from *Mus musculus* with percentage of identity 87%.

Table 4.2 Comparative analysis of amino acid sequence of VLnor155-12 with sequences in the GenBank database using BLASTp program.

No.	Accession	Definition	Amino acid	Max score	Total score	Query cover	Ident (%)
1	AEC11639.1	Anti-KSHV gH immunoglobulin light chain variable region [*Mus musculus*]	142	197	197	97%	87%
2	ABC86053.1	Immunoglobulin kappa light chain [*Mus musculus*]	151	196	196	97%	87%
3	AAD01881.1	Ig light chain [*Mus musculus*]	120	194	194	95%	88%
4	ABC86098.1	Immunoglobulin kappa light chain [*Mus musculus*]	151	194	194	97%	79%
5	ABC86065.1	Immunoglobulin kappa light chain [*Mus musculus*]	151	192	192	97%	86%

4.4 Construction of scFv Antibody Gene

4.4.1 Construction of V_H and V_L Genes with Flexible Polypeptide Linkers

The V_H fragment from VHnor155-9 and the V_L fragment from VLnor155-12 were used to construct the V_H-linker and linker-V_L fragment by adding two units of the flexible linker [$(Gly_4Ser)_2$].

In the case of V_H fragment, two units of Gly_4Ser were added behind the V_H gene, but these two units were specifically added in front of the V_L gene. Then PCR was performed with N1 to N4 primers and the PCR product was observed via 1% agarose gel electrophoresis. The lengths of DNA fragments of VH-linker and linker-VL were estimated to be 426 bp (lane 1-4 in Figure 4.7A) and 380 bp (lane 1-7 in Figure 4.7B), respectively. Subsequently the agarose gel bands containing the VH-linker and linker-VL DNA fragments were carefully cut and purified. The purified fragments were served as the template for further cloning.

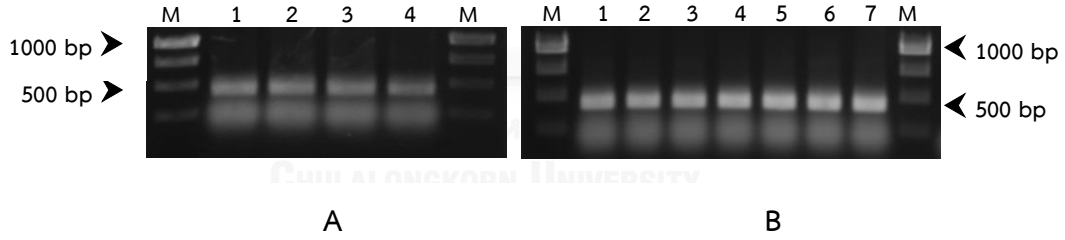

Figure 4.9 The 1.0% agarose gel stained with ethidium bromide. A) lane 1-4: PCR products of VH-linker amplification. B) lane 1-7: PCR products of linker-VL amplification. Lane M: 1 Kb plus DNA ladder (Invitrogen)

The purified VH-linker and linker-VL fragments were individually ligated to pGEM® T-easy vector by using T4 DNA ligase enzyme and were transformed to fresh competent *E. coli* TOP10F'. By blue and white screening method, over 50 white colonies of each fragment which were grown on LB agar plate supplemented with ampicillin antibiotic were randomly picked up for screening. At first, the plasmid of the four white colonies of each fragment were extracted by using alkaline lysis solution and the obtained plasmids were run through 1% agarose gel electrophoresis. The obtained DNA plasmid was shown in Figure 4.10A with the size of approximately 3.4 kb.

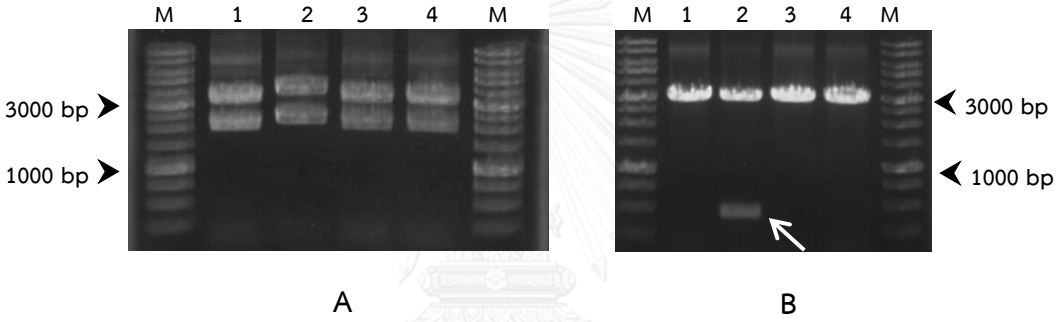

Figure 4.10 The 1.0% agarose gel electrophoresis of the VH-linker with digested by *Eco*RI. A) lane 1-4: the obtained plasmid of 4 white colonies of VH-linker B) four white colonies were digested by *Eco*RI and revealed the expected size as indicated by arrow. Lane 1-4: VH-linker#1 - VH-linker#4. Lane M: 1 Kb plus DNA ladder (Invitrogen)

After digestion with *Eco*RI, the DNA fragment was analyzed by gel electrophoresis as shown in the Figure 4.10B. The result revealed that the VH-linker#2 was completely digested and the DNA fragment was released with the size of approximately 426 bp (Figure 4.10B, lane 2). On the contrary, no band was observed in lane 1, 3 and 4 which corresponded to the VH-linker#1, VH-linker#3 and VH-linker#4, respectively. This indicated that there were no DNA fragments inserted into these vectors. Therefore, only VH-linker#2 was sent to confirm the accuracy of sequence. The obtained nucleotide sequences of VH-linker#2 was comprised of 426 nucleotides and their 142 amino acid residues (Figure 4.11).

```
  1 GAATTCGAAGTAGAG CTGGAGGAGTCTGGG GGAGGCTTAGTGAAG CCTGGAGGGTCCCTG AAACTCTCCTGTGCA
  1  E  F  E  V  E   L  E  E  S  G   G  G  L  V  K   P  G  G  S  L   K  L  S  C  A
 76 GCCTCTGGATTCAGT TTCAGTGACTATTAC ATGTATTGGGTTCGC CAGACTCCGGAAAAG AGGCTGGAGTGGGTC
 26  A  S  G  F  S   F  S  D  Y  Y   M  Y  W  V  R   Q  T  P  E  K   R  L  E  W  V
151 GCAACCATTAGTGAA GGTGCCAGTTATATC TATTATTCGGACAGT GTAAAGGGACGCTTC ACCATCTCCAGAGAC
 51  A  T  I  S  E   G  A  S  Y  I   Y  Y  S  D  S   V  K  G  R  F   T  I  S  R  D
226 AATGCCAGGAACACC CTGTACCTGCAAATG AGCAGTCTGAAGTCT GAGGACACAGCCATT TATTACTGTACAAGA
 76  N  A  R  N  T   L  Y  L  Q  M   S  S  L  K  S   E  D  T  A  I   Y  Y  C  T  R
301 GCCTATAATAACTAC GTGTCGAAATATTTC GATGTCTGGGGCGCA GGGACCACGGTCACC GTCTCCTCAGCCAAA
101  A  Y  N  N  Y   V  S  K  Y  F   D  V  W  G  A   G  T  T  V  T   V  S  S  A  K
376 ACGACACCCCCATCT GTCTATGGTGGAGGC GGTTCAGGCGGAGGT GGATCC
126  T  T  P  P  S   V  Y  G  G  G   G  S  G  G  G   G  S
```

Figure 4.11 The nucleotide sequence and putative amino acid sequence of VH-linker. The amino acid sequence was shown in blue with one-letter code under the nucleotide sequence. The linker was labeled in bolded-red color. The one-underline and double-underline represented the primers annealing sites. The restriction enzymes for cloning were labeled with bold letter.

As shown in Figure 4.12B, DNA fragment of linker-VL was released after *Eco*RI digestion. This result indicated that linker-VL#2 and linker-VL#3 were completely digested and the DNA fragment was released with the size of approximately 400 bp (Figure 4.12B, lane 2 and 3). On the contrary, no band was observed in lane 1 and 4 which corresponded to the linker-VL#1 and linker-VL#4, respectively. This indicated that there were no DNA fragments inserted into these vectors. Therefore, only linker-VL#2 and linker-VL#3 were sent to confirm the accuracy of sequence. The obtained nucleotide sequence of linker-VL#2 was comprised of 392 nucleotides and their 130 amino acid residues (Figure 4.13).

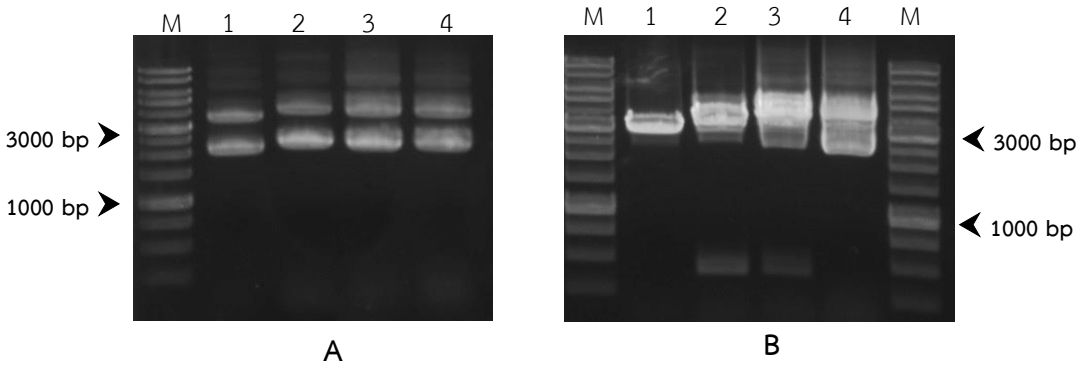

Figure 4.12 Screening and detection of the recombinant plasmid digested with *Eco*RI on 1.0% agarose gel electrophoresis. A) lane 1-4: the obtained plasmid of 4 white colonies B) Digested with *Eco*RI and revealed the expected size as indicated by arrow. Lane M: 1 Kb plus DNA ladder (Invitrogen)

```
  1 GGCGGAGGTAGATCT GGCGGTGGCGGATCG GACATTGTGATGACA CTGTCTACAGCAATC CTGTCTGCATCTCCA
  1  G  G  G  R  S  G  G  G  G  S  D  I  V  M  T  L  S  T  A  I  L  S  A  S  P
 76 GGGGAGAAGGTCACA ATGACTTGCAGGGCC AGTTCAAGGGTAAAT TACATACACTGGTTC CAGCAGAAGGCAGGA
 26  G  E  K  V  T  M  T  C  R  A  S  S  R  V  N  Y  I  H  W  F  Q  Q  K  A  G
151 TCCTCCCCCAAACCC TGGATTTATGCCACA TCCAACCTGGCTTCT GGAGTCCCTGATCGC TTCAGTGGCAGAGGG
 51  S  S  P  K  P  W  I  Y  A  T  S  N  L  A  S  G  V  P  D  R  F  S  G  R  G
226 TCTGGGACCTCTTAC TCTCTCACAATCAGC AGAGTGGAGGCTGAA GATGCTGCCACTTAT TACTGCCAGCAGTGG
 76  S  G  T  S  Y  S  L  T  I  S  R  V  E  A  E  D  A  A  T  Y  Y  C  Q  Q  W
301 AGTAATAACCCGTGG ACGTTCGGTGGAGGC ACCAAGCTGGAAATC AAACGGGCTGATGCT GCACCAACTGTATCC
101  S  N  N  P  W  T  F  G  G  G  T  K  L  E  I  K  R  A  D  A  A  P  T  V  S
376 GAGCTCCCGGTACCT CG
126  E  L  P  V  P
```

Figure 4.13 The nucleotide sequence and putative amino acid sequence of linker-VL. The amino acid sequence was shown in blue with one-letter code under the nucleotide sequence. The linker was labeled in bolded-red letter. The one-underline and double-underline represented the primers annealing sites. The restriction enzymes for cloning were shown in bold letter.

4.4.2 Construction of Recombinant scFv Antibody Expression Plasmid

To obtain the recombinant scFv antibody expression plasmid, the VH-linker fragment was double cleaved by *Eco*RI and *Bam*HI restriction enzymes which were specifically cleaved at the 5'end and 3'end of the VH-linker, respectively. While the *Bgl*II and *Kpn*I restriction sites were employed to digest the linker-VL fragment at the 5' and 3'of the fragment. These specific-digested fragments were ligated into the multiple cloning site regions of the *P. pastoris* between the *AOX1* promoter and the α-factor signal sequence of pPICZαA vector using *Eco*RI/*Kpn*I restriction enzymes to generate the pPICZαA-scFv plasmid. The resulting pPICZαA-scFv plasmid was named as pJM01 plasmid with the estimated of 4354 bp (Figure 4.14). The plasmid was then transformed into *E. coli* TOP10F'. The pJM01 plasmid contained the zeocin resistance gene (Zeo^{R}) for the selection in *E. coli*. Plasmids carrying the insert were selected on LB low salt plates containing zeocin.

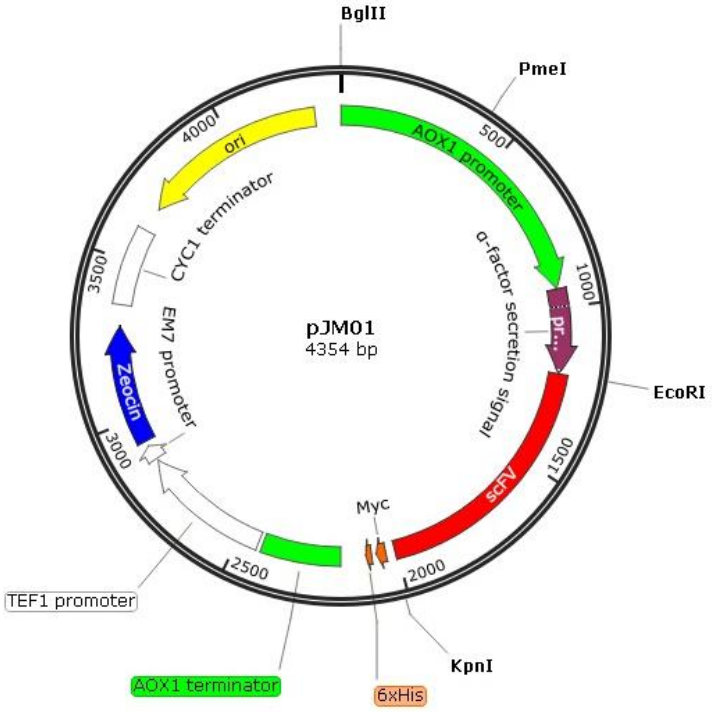

Figure4.14 Plasmid structure of pJM01.

The structure of the expression plasmid pJM01 (pPICZαA-scFv) comprised of

- 5'AOX1; alcohol oxidase 1 promoter
- scFv gene
- AOX1 TT; transcriptional terminator from *P. pastoris AOX1* gene
- TEF1 promoter; transcriptional elongation factor 1 promoter from *S. cerevisiae*
- EM7 promoter; synthetic prokaryotic promoter
- Zeocin resistance gene
- CYC1 TT; transcriptional terminator from *S. cerevisiae CYC1* gene
- PUC ori; PUC origin of replication

Twenty colonies of Zeo^R transformant were randomly selected on LB low salt plates containing zeocin. The pJM01 plasmid was isolated and examined the size on 1% agarose gel electrophoresis. The migration patterns of the obtained plasmids were shown in Figure 4.15. Among of 20 colonies, these were five Zeo^R transformants (Figure 4.15, lane 2, 4 and 7-9) that their bands were higher than the band of pPICZαA (lane 1). Therefore, these transformants were analyzed by cleaving with *Eco*RI and *Kpn*I restriction enzymes (Figure 4.16), respectively.

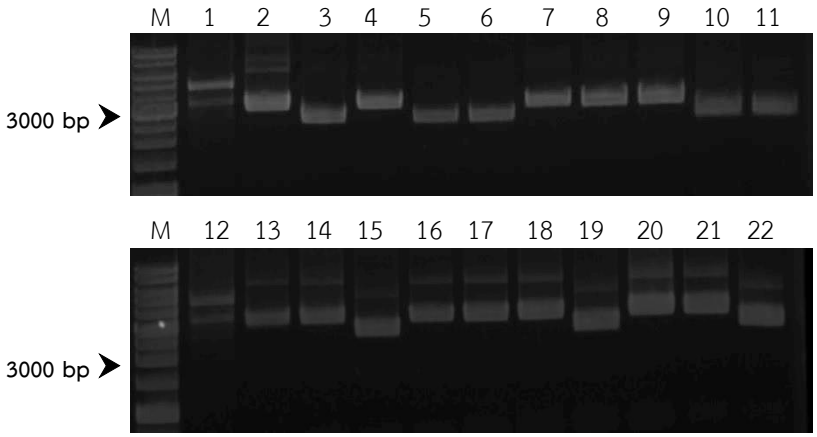

Figure 4.15 The 1.0% agarose gel electrophoresis of the extracted plasmid from twenty Zeo^R transformants. Lane 1 and12: pPICZαA as control. Lane 2-11: Zeo^R transformants clone no.1–no.10. Lane 13-22: Zeo^R transformants clone no.11–no.20. Lane M: 1 Kb plus DNA ladder (Invitrogen)

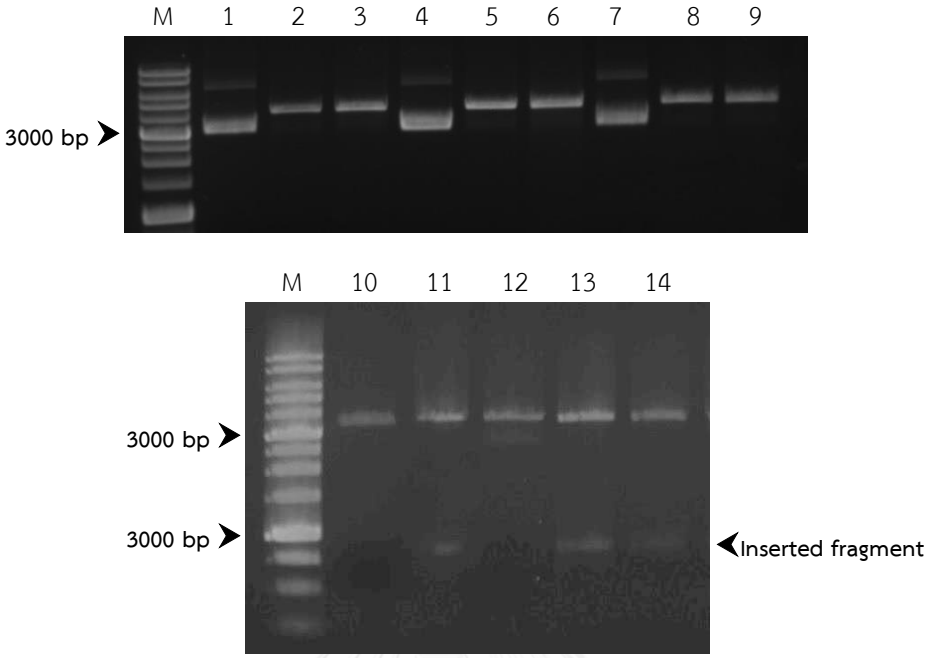

Figure 4.16 The 1.0% agarose gel electrophoresis of the plasmid of three Zeo^{R} transformants (no.1, no.2 and no.3) with *Eco*RI and *Kpn*I digestion.

Lane 1: intact form of Zeo^{R} transformants no.1

Lane 2: plasmid of Zeo^{R} transformants no.1 digested by *Eco*RI

Lane 3: plasmid of Zeo^{R} transformants no.1 digested by *Kpn*I

Lane 4: intact form of Zeo^{R} transformants no.2

Lane 5: plasmid of Zeo^{R} transformants no.2 digested by *Eco*RI

Lane 6: plasmid of Zeo^{R} transformants no.2 digested by *Kpn*I

Lane 7: intact form of Zeo^{R} transformants no.3

Lane 8: plasmid of Zeo^{R} transformants no.3 digested by *Eco*RI

Lane 9: plasmid of Zeo^{R} transformants no.3 digested by *Kpn*I

Lane10: plasmid of Zeo^{R} transformants no.1 digested by *Eco*RI

Lane11: plasmid of Zeo^{R} transformants no.1 digested by *Eco*RI and followed by *Kpn*I

Lane12: plasmid of Zeo^{R} transformants no.2 digested by *Eco*RI

Lane13: plasmid of Zeo^{R} transformants no.2 digested by *Eco*RI and followed by *Kpn*I

Lane14: plasmid of Zeo^{R} transformants no.3 digested by *Eco*RI and followed by *Kpn*I

Lane M: 1 Kb plus DNA ladder (Invitrogen)

4.5 Verification of pJM01 Plasmid

To improve and facilitate the expression of the antibody fragment, V_H-linker and linker-V_L DNA fragments were inserted into the open reading frame of the *P. pastoris* expression vector pPICZαA under the regulation of *AOX*1 promoter using the *Eco*RI (bp 1203-1213) and *Kpn*I (1241-1246) restriction sites. At the c-terminal, the constructs were fused to a *c-myc* epitope tag and a hexahistidine-tag. Colonies were screened for insertion using PCR analysis and restriction cleavage reactions. The plasmid of five Zeo^R transformants was extracted. The presence of scFv gene in the obtained plasmids was tested by PCR technique with N1 and N4 primers listed in 3.1.5. These primers were designed for up-stream amplification of V_H and down-stream amplification of V_L gene. The result reveals the 800 bp (Figure 4.17A) of the PCR product according to the right size of the predicted restriction map of the linearized pJM01 plasmid. This information indicated that all of the five positive transformants contained a scFv antibody gene inserted into the pPICZαA expression plasmid.

The scFv antibody gene was designed by cloning in frame with staring codon (ATG) of α-factor secretion signal in the pPICZαA vector and was possible translated to be amino acid. Therefore, the accuracy of the nucleotide sequence of scFv antibody gene was analyzed. First, the plasmid of the Zeo^R transformant No.1 was selected to be digested with several restriction enzymes. In accordance with the expected map, *Eco*RI, *Kpn*I, and *Pme*I were used. These enzymes have only one position of enzymatic digestion meaning that after completely digestion and observed by gel electrophoresis, the digested plasmid should show the linearized plasmid. Figure 4.17B showed the result bands corresponding to the correct size of 4354 bp plasmid (lane 2, 3, and 5), while lane 4 represented the use of *Eco*RI and *Kpn*I in the plasmid digestion at the same time. It showed the 800 bp of scFv gene when compared with the DNA marker. Moreover, we also confirmed the plasmid of clone No.1 by PCR with primers as described in the table 3.1.5. Figure 4.17C exhibited the positive bands of approximately 1461 bp of the PCR product (lane 2) by using TJ01F/TJ01R primers, approximately 800 bp of the PCR product (lane 4) by using

N1/N4 primers and approximately 1350 bp by using 5'AOX1/3'AOX1 primers (Figure 4.17D). In case of the control, these primers were used with pPICZαA vector showing approximately 700 bp (Figure 4.17C, lane 1), 589 bp (Fig.3D, lane1) and no band could be observed by using TJ01F/TJ01R, 5'AOX1/3'AOX1 and N1/N4 primers (Figure 4.17C, lane 3), respectively. The schematic of PCR analysis was clearly shown in Figure 3.2.

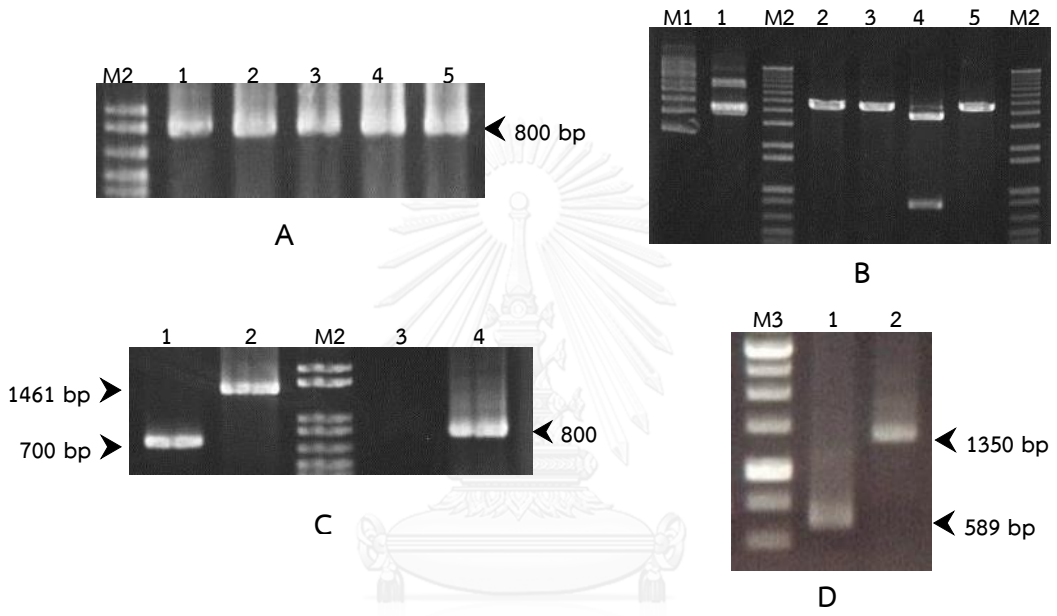

Figure 4.17 Electrophoretic analysis. A) PCR product of five Zeo^R transformants. Lane 1-5: positive transformants No.1 – No. 5, Lane M2: the 1 Kb plus DNA ladder (Invitrogen™). B) Restriction enzyme digestion. Lane 1: intact pJM01, lane 2-5: pJM01 plasmid of transformants No.1 digested with *Eco*RI, *Kpn*I, *Eco*RI/*Kpn*I, and *Pme*I, respectively. Lane M1: supercoiled plasmid ONESTEP Ladder (Wako Nippon GENE). C) PCR product. Lane1: pPICZαA, lane 2: Zeo^R transformants No.1. Lane 3: pPICZαA. Lane 4: Zeo^R transformants No.1, respectively. D) PCR product by using 5'AOX1/3'AOX1. Lane 1: pPICZαA vector, lane2: Zeo^R transformants No.1.

The pJM01 plasmid of Zeo^{R} transformant No.1 was sequenced with TJ03F, TJ04F, TJ05F and TJ06R primers. The result showed the nucleotide sequence of scFv antibody gene with, 800 nucleotides. The translation of this sequence was predicted to encode 266 amino acids with a molecular weight of 32.67 kDa including a flexible amino acid linker of $(Gly_4Ser)_3$ as shown in Fig. 4.18. The gene sequence of scFv was submitted to the NCBI database under the following accession number AJG06891.1 (Appendix E).

```
   1 GAATTCGAAGTAGAG CTGGAGGAGTCTGGG GGAGGCTTAGTGAAG CCTGGAGGGTCCCTG AAACTCTCCTGTGCA
   1  E  F  E  V  E   L  E  E  S  G   G  G  L  V  K   P  G  G  S  L   K  L  S  C  A
  76 GCCTCTGGATTCAGT TTCAGTGACTATTAC ATGTATTGGGTTCGC CAGACTCCGGAAAAG AGGCTGGAGTGGGTC
  26  A  S  G  F  S   F  S  D  Y  Y   M  Y  W  V  R   Q  T  P  E  K   R  L  E  W  V
 151 GCAACCATTAGTGAA GGTGCCAGTTATATC TATTATTCGGACAGT GTAAAGGGACGCTTC ACCATCTCCAGAGAC
  51  A  T  I  S  E   G  A  S  Y  I   Y  Y  S  D  S   V  K  G  R  F   T  I  S  R  D
 226 AATGCCAGGAACACC CTGTACCTGCAAATG AGCAGTCTGAAGTCT GAGGACACAGCCATT TATTACTGTACAAGA
  76  N  A  R  N  T   L  Y  L  Q  M   S  S  L  K  S   E  D  T  A  I   Y  Y  C  T  R
 301 GCCTATAATAACTAC GTGTCGAAATATTTC GATGTCTGGGGCGCA GGGACCACGGTCACC GTCTCCTCAGCCAAA
 101  A  Y  N  N  Y   V  S  K  Y  F   D  V  W  G  A   G  T  T  V  T   V  S  S  A  K
 376 ACGACACCCCCATCT GTCTATGGTGGAGGC GGTTCAGGCGGAGGT GGATCTGGCGGTGGC GGATCGGACATTGTG
 126  T  T  P  P  S   V  Y  G  G  G   G  S  G  G  G   G  S  G  G  G   G  S  D  I  V
 451 ATGACACTGTCTACA GCAATCCTGTCTGCA TCTCCAGGGGAGAAG GTCACAATGACTTGC AGGGCCAGTTCAAGG
 151  M  T  L  S  T   A  I  L  S  A   S  P  G  E  K   V  T  M  T  C   R  A  S  S  R
 526 GTAAATTACATACAC TGGTTCCAGCAGAAG GCAGGATCCTCCCCC AAACCCTGGATTTAT GCCACATCCAACCTG
 176  V  N  Y  I  H   W  F  Q  Q  K   A  G  S  S  P   K  P  W  I  Y   A  T  S  N  L
 601 GCTTCTGGAGTCCCT GATCGCTTCAGTGGC AGAGGGTCTGGGACC TCTTACTCTCTCACA ATCAGCAGAGTGGAG
 201  A  S  G  V  P   D  R  F  S  G   R  G  S  G  T   S  Y  S  L  T   I  S  R  V  E
 676 GCTGAAGATGCTGCC ACTTATTACTGCCAG CAGTGGAGTAATAAC CCGTGGACGTTCGGT GGAGGCACCAAGCTG
 226  A  E  D  A  A   T  Y  Y  C  Q   Q  W  S  N  N   P  W  T  F  G   G  G  T  K  L
 751 GAAATCAAACGGGCT GATGCTGCACCAACT GTATCCGAGCTCCCG GTACC
 251  E  I  K  R  A   D  A  A  P  T   V  S  E  L  P   V
```

Figure 4.18 The nucleotide sequence and putative amino acid sequence of the scFv antibody fragment. The amino acid sequence was shown in blue with one-letter codes under the nucleotide sequence. The $(G_4S)_3$ linker between V_H and V_L was labeled in bolded underline. The restriction enzymes for cloning were shaded in bold. (Accession number AJG06891.1)

4.6 Identification of Complementarity Determining Region (CDRs) and Framework Sequences (FR)

The CDR and framework sequences were identified by comparison with IgG1 and light chain sequences database from GenBANK (NCBI) or published data.

Complementarity determining regions (CDRs) are part of the variable chains in immunoglobulins (antibodies), where these molecules bind to their specific antigens. As the most variable parts of the molecules, CDRs are crucial to the diversity of antigen specificities. Within the variable domain, CDR I, CDR II, and CDR III are found in the variable region of a polypeptide chain, and CDRIII is the most variable. The complementarity determining regions (CDRs) I, II, and III of the variable region of both heavy chain (V_H) and light chain (V_L) were identified. The predicted of CDRI, CDR II and CDR III of V_H domain was located at position 29-37, 52-60, and 99-112 in the primary protein sequences, while the prediction of CDRI, CDR II and CDR III of V_L domain was specified at position 170-180, 195-202, and 235-243 as depicted in Figure 4.19, respectively.

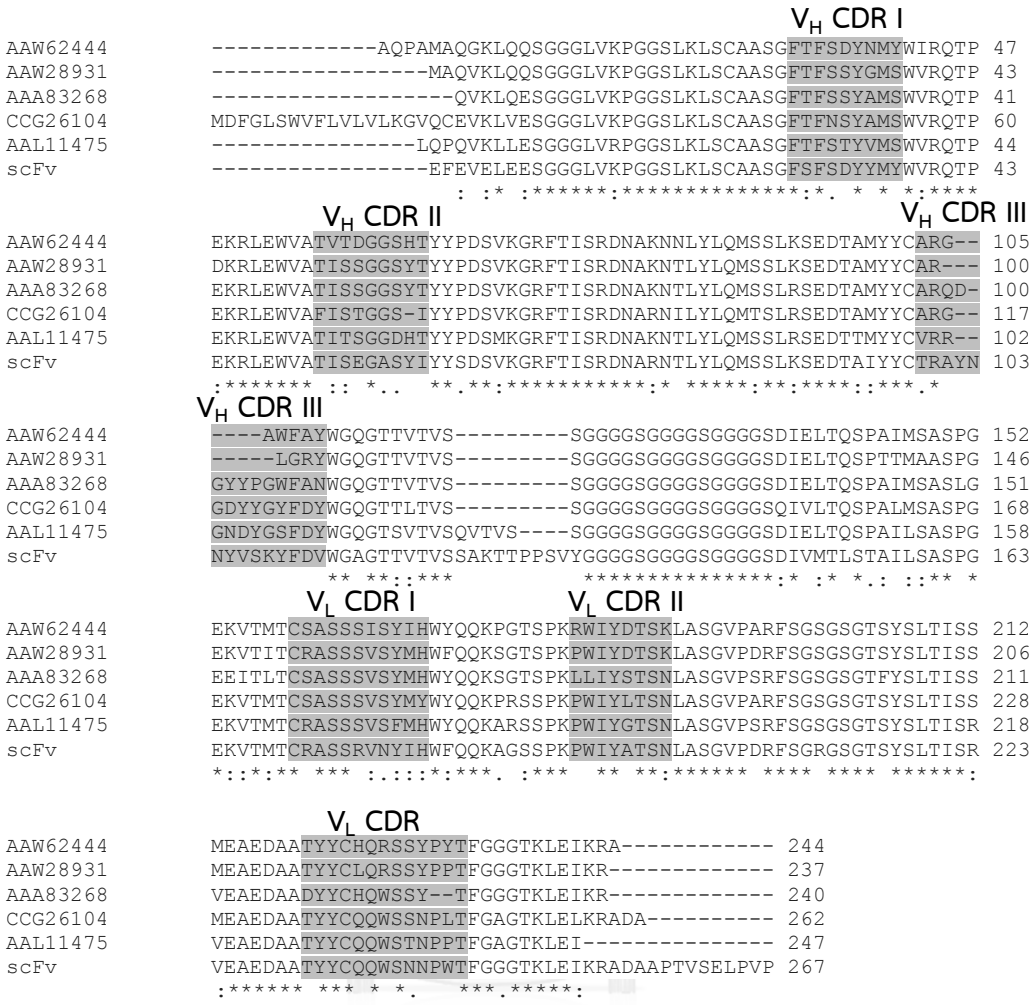

Figure 4.19 Primary sequence alignment of scFv (Nor155) antibody and those of five known scFv sequences with accession number were taken from GenBank. Predicted CDR indicates the complementary-determining regions (CDRs) and framework regions that exist between the CDRs. Asterisk (*) indicates the amino acid that is the same. Hyphen (-) indicates the amino acid that is missed compared with others.

The deduced amino acid sequence of the scFv (Nor155) antibody gene was aligned by searching against GenBank database. Through the BLASTp program, the result showed the similarity of scFv sequence with over 50 different scFv antibodies homologous sequences (data not shown). Each NCBI GenBank accession number exhibited a high degree of similarity to the corresponding sequences from closely related species. Comparative analysis of the amino acid sequence with the five known scFv sequences was shown and summarized in the Table 4.3. It was found that the scFv antibody was most closely related to the single chain antibody 12G9, (78% identity) followed by anti-porcine sialoadhesin scFv antibody, (75% identity) and both of scFv B4 anti-pectinase antibody, and anti-isoketal-adduct single chain variable fragment (74% identity), and scFv antibody from *Mus musculus* (71% identity), respectively.

Table 4.3 Comparative analysis of amino acid sequence of scFv with sequences in the GenBank database using BLASTp program.

Accession	Definition	Amino acid	Max score	Total score	Query cover (%)	Ident (%)
AAA83268	sFv antibody, partial [*Mus musculus*]	240	326	326	94	71
AAL11475	single chain antibody 12G9, partial	247	325	325	93	78
CCG26104	anti-porcine sialoadhesin scFv antibody, partial	262	324	324	96	75
AAW62444	ScFv B4 anti-pectinase antibody, partial	244	318	318	94	74
AAW28931	anti-isoketal-adduct single chain variable fragment, partial	237	315	315	94	74

4.7 Transformation

As in *S. cerevisiae*, linear vector DNAs can generate stable transformants of *P. pastoris* via homologous recombinant between sequences shared by the vector and host genome. The first and simplest way of integration is to digest the vector at a unique site with a restriction enzyme within either the marker gene (*HIS4*) or the *AOX1* promoter sequences, and then to transform the linearized vector into the appropriate auxotrophic mutant [53, 58, 60]. Methods employed for DNA-mediated transformation of *P. pastoris* are similar to those for *S. cerevisiae* and lead to similar result. *P. pastoris* can be transformed by spheroplast fusion, or by whole-cell methods such as electroporation, or DNA co-precipitation with lithium chloride, calcium chloride, or polyethylene glycol. Among of these methods, electroporation is a simple and fast method for transforming *P. pastoris* as well as commercial kit [53, 60, 61]. For these reasons, electroporation and commercial available kit were applied for transforming *P. pastoris*.

In this study, pJM01 plasmid had one *Pme*I restriction site (414 bp) in the *AOX1* promoter, while the auxotrophic mutant (*P. pastoris* GS115) had also one *Pme*I restriction site at 5' *AOX1* promoter region. For this reason, the *Pme*I-linearized pJM01 plasmid was introduced into *Pichia* genome by both electroporation and commercial available kit. The recombinants which were well grown on YPD plates containing Zeocin after 2-4 days incubation were selected.

4.7.1 Transformation by Electroporation and Screening of Zeo^R Transformants by Colony PCR

By electroporation, over one hundred Zeo^R transformants colonies were found on YPD/zeocin agar plates. Only twenty colonies of well grown were randomly picked up. To confirm whether or not the *P. pastoris* transformants contained the scFv gene, firstly, colony PCR assay was performed for primary screening. Nine of twenty Zeo^R transformants were selected to prepare its templates by using lyticase enzyme and incubated at -80°C for 10 min. The digestion of yeast cell wall by a lyticase enzyme was performed at -80°C to break spheroblast cell. Cell debris was

removed by centrifugation so that the DNA in the supernatant could be used as the template for PCR assay. The PCR products were then selected and observed by 1% agarose gel electrophoresis.

The products obtained from the colony PCR assay using N1 and N4 primers were shown in Figure 4.20. Size of the product was found to be about 800 bp. This size corresponded to the scFv gene. This result showed that only Zeo^R transformants no.1, no. 4 and no. 5 (lane 1, lane 4 and lane 5, respectively) contained the scFv gene in their genomes comparing with pJM01 plasmid (lane 10) as a positive control and pPICZαA (lane 11) as negative control. This result indicated that these clones had possible ability for using in further experiments. Therefore, they re-cultured and kept as stock cultures.

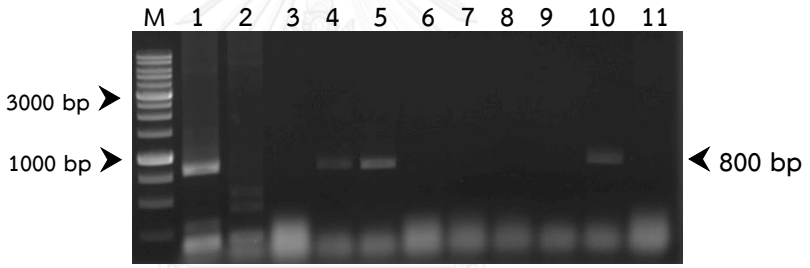

Figure 4.20 PCR products of colony PCR assay. Lane 1-9: Zeo^R transformants no.1-9. Lane 10: pJM01 as positive control. Lane 11: pPICZαA as negative control. Lane M: 1 kb DNA marker

In addition, the colony PCR assay was performed again with three Zeo^R transformants, which showed the expected product size from previous study. By using 5'AOX1 and 3'AOX1 primers in PCR reaction, the PCR product could be found on 1% ethidium bromide stained agarose gels with the expected product sizes of about 1351 bp and 2105 bp, while the PCR product of the negative control could be found on this gels with the expected product size of about 589 bp and 2105 bp, respectively.

However, 1% ethidium bromide stained agarose gels demonstrated that PCR products were not corresponded to the expected product size as mentioned above

(Figure 4.21). The result revealed that the PCR product of pJM01 plasmid (lane 1) was found with the size of about 1351 bp only, while the PCR product of pPICZαA, the negative control, (lane 2) yield only one product with the size of about 589 bp. Moreover, Zeo^{R} transformants no.1 and no.4 showed the product size less than 750 bp, while Zeo^{R} transformants no.5 was not amplified by this primer when comparing with 1 kb DNA marker (Figure 4.21). This result may imply that either these primers or colony PCR assay were not suitable to use in this experiment.

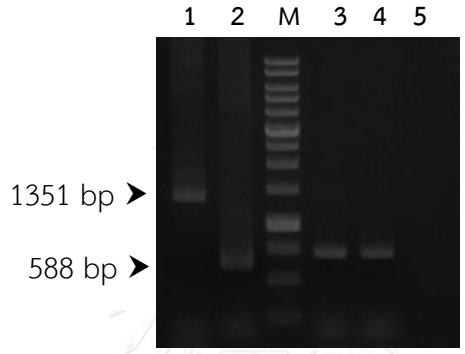

Figure 4.21 PCR products of colony PCR assay. Lane 1: pJM01 plasmid as positive control. Lane 2: pPICZαA as negative control. Lane 3-5: Zeo^{R} transformants no.1, no.4 and no.5, respectively. Lane M: 1 kb DNA marker

4.7.2 Transformation by Electroporation and Screening of Zeo^{R} Transformants by PCR

The genomic DNA of the five clones out of twenty Zeo^{R} transformants were extracted. The quality and quantity of the extracted genomic DNA were determined by using 1.0% agarose gel electrophoresis and compared with the 1 kb DNA marker. The concentration of the extracted genomic DNA was approximately 45-200 ng/µL and the high molecular weight DNA band over 10 kb DNA marker was obtained (Figure 4.22). The extracted genomic DNA was adjusted to approximately 20-30 ng/µL for use in PCR amplification.

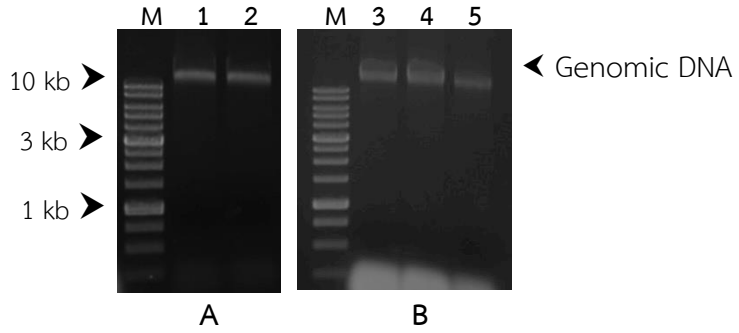

Figure 4.22 The 1.0% ethidium bromide stained agarose gel showing the quality of total DNA extracted from each Zeo^{R} transformant (lanes 1-5). Lane M: 1 kb DNA marker

PCR amplification was performed to investigate the scFv gene by using N1 and N4 primers. Figure 4.23 A and B showed the expected band of PCR products which was successfully amplified and revealed the expected product size of about 800 bp. Figure 4.23A represented PCR products of Zeo^{R} transformants no.1-3 (lane 1-3), while the PCR products of Zeo^{R} transformants no.4-5 was shown in Figure 4.23B (lane 4-5). The pJM01 plasmid and pPICZαA (empty vector) were used as positive and negative control (lane6 and 7), respectively. This result indicated that Zeo^{R} transformants no. 1, no.2 and no.5 showed the expected product band corresponding to pJM01 plasmid.

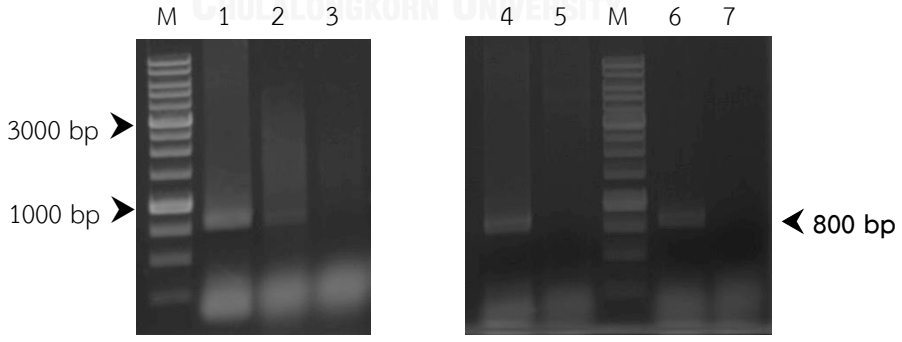

Figure 4.23 The 1.0% ethidium bromide stained agarose gels showing the PCR products. Lane1-5 represents PCR products of Zeo^{R} transformants no.1-5. Lane 6: pJM01 plasmid as positive control. Lane 7: pPICZαA as negative control. Lane M: 1 kb DNA marker.

According to the results of colony PCR assay and PCR assay for screening the obtained Zeo^{R} transformants, the results could be summarized that the Zeo^{R} transformant no.1 from those two method assays was selected as the best candidate, which had the size of the expected one. So, the Zeo^{R} transformant no.1 was called as T1.

4.7.3 Transformation by Frozen-EZ Yeast Transformation II™ kit and Screening of Zeo^{R} Transformants by PCR

Chemical solution kit, another transformation technique, was used for introduce *Pme*I-linearized pJM01 into *Pichia* genome. This kit has been wildly used and has several advantages over electroporation such as easy, rapid and convenient. For these reasons, Frozen-EZ Yeast Transformation II™ kit was selected for using in this study. By using this kit, only eight Zeo^{R} transformants were found on YPD/zeocin agar plate. They were named staring from O1 to O8 according to their Zeo^{R} transformants numbers, respectively. The genomic DNA of O1-O8 was extracted by using LiOAc-SDS lysis following the Looke and co-worker's protocol [98] comparing with T1, αA (empty vector) and GS115 as host. The DNA concentration was measured by Nanodrop Lite spectrophotometer (Thermo scientific) and was shown in Table 4.4. The quality and quantity of extracted genomic DNA were determined by using 1.0% agarose gel electrophoresis and comparing with the 1 kb DNA marker (Figure 4.24).

Table 4.4 Genomic DNA concentration of Zeo^{R} transformants O1-O8, T1, αA5 and GS115

Strains	Concentration (ng/μL)	A260/A280
O1	414.10	1.90
O2	471.90	1.90
O3	481.50	1.92
O4	621.10	1.94
O5	501.30	1.89
O6	538.50	1.91
O7	628.20	1.92
O8	673.50	1.94
T1	593.10	1.92
[a]αA	627.30	1.92
[b]GS115	751.60	1.94

a: GS115 carrying pPICZαA (*Pichia* expression vector)

b: *un-transformed P. pastoris* GS115 as control

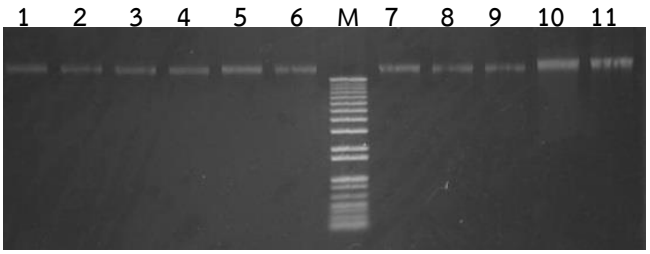

Figure 4.24 The 1% ethidium bromide stained agarose gels showing the genomic DNA. Lane1-8: O1-O8. Lane 9: T1. Lane 10: pPICZαA. Lane 11: GS115. Lane M: Lane M: 1 kb DNA marker.

The extracted genomic DNA was adjusted to approximately 20-30 ng/µL for use in PCR amplification. By using N1 and N4 primers, the PCR products revealed the product size of about 800 bp. Figure 4.25 Illustrated the PCR products on agarose gel after staining with ethidium bromide. This result indicated that the PCR product of O1 to O8 showed strong bands at about 800 bp (lane 1-8) when comparing with T1, αA and pJM01 (lane 9, 10 and 11), respectively.

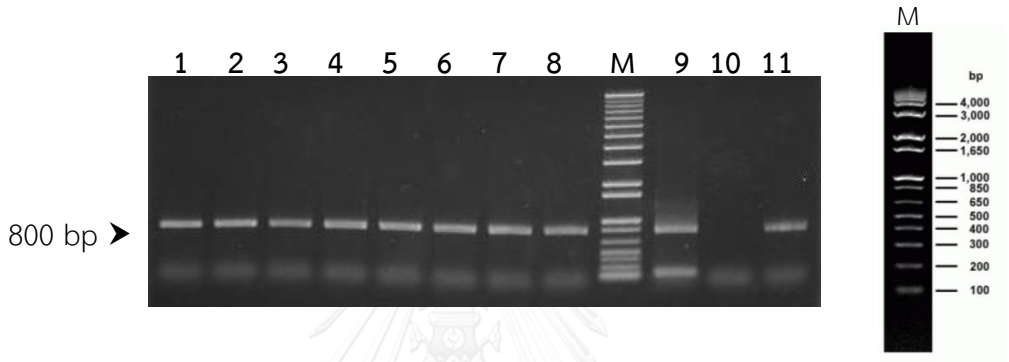

Figure 4.25 The 1.0% ethidium bromide stained agarose gels showing the PCR products by using N1 and N4 primers. Lane1-8: PCR products of O1-O8. Lane 9: PCR product of T1. Lane 10: pPICZαA. Lane 11: pJM01. Lane M: 1 Kb plus DNA ladder (Invitrogen)

Therefore, O1 – O8 and T1 were selected as candidate for recombinant scFv expression.

4.9 Confirmation of pJM01 Integrated into *Pichia* Genome

4.9.1 Southern Blot Analysis

There are several methods to investigate the integration of pJM01 plasmid into chromosomal DNA of *P. pastoris* GS115. In this study, Southern blotting analysis, sequencing analysis and PCR analysis were carried out. Following these methods, nine Zeo^R transformnts, O1-O8 and T1 as previously selected, αA and host *P. pastoris* GS115 (as negative control) were firstly tested. In Southern blotting analysis, the chromosomal DNA of the entire transformants was extracted and the DNA concentration was measured by Nanodrop Lite spectrophotometer (Thermo scientific) as shown in Table 4.4.

The quality and quantity of the extracted choromosomal DNA were determined via 1.0% agarose gel electrophoresis and compared with the 1 kb DNA marker. Afterward, *Kpn*I restriction enzymes were used for digestion of 2.5 µg chromosomal DNA for 2 hours. After that, *Kpn*I-digestion mixture was observed via 0.8% agarose gel electrophoresis and staining with ethidium bromide (Figure4.26). In this study, *AOX1* promoter was used as the probe for hybridization in Southern blotting assay. PCR was performed by using AOX1-F and AOX1-R primers and genomic DNA of *P. pastoris* GS115 was served as template. The PCR product was observed by running through 1% agarose gel. PCR assay revealed that the PCR product showed the expected band of *AOX1* promoter fragment with at 1000 bp (Figure 4.27). This sharp band was purified and used as probe for hybridization.

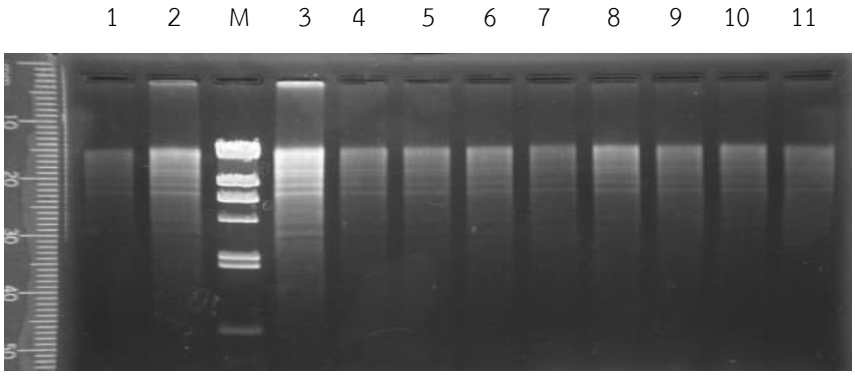

Figure 4.26 The 0.8% ethidium bromide stained agarose gel showed the quality of *Kpn*I digestion. Lane 1: *Pichia* untransformed control. Lane 2: transformed with pPICZαA. Lane 3: Zeo^R transformnts T1. Lane 4-11: Zeo^R transformnts O1-O8. Lane M: λDNA/*Hin*dIII marker

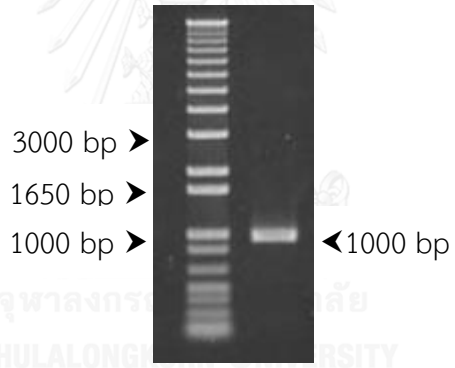

Figure 4.27 The 1.0% ethidium bromide stained agarose gel of *AOX1* promoter amplification.

The 0.8% ethidium bromide stained agarose gel was captured under UV light. Then, this gel was transferred to Hybron-N^+ membrane by capillary action. As predicted, the use of *Kpn*I-digestion of the entire chromosomal DNA and *AOX1* promoter as probe showed a 2.4 kb and 4.0 kb band products of Zeo^R transformants, while the use of *Eco*RI-digestion, the 2.1 kb and 7.9 kb band products were observed. Moreover, the *Pichia* chromosome harboring pPICzαA vector showed a 5.7 kb band and 2.2 kb band when *Eco*RI and *Kpn*I restriction enzyme were used, respectively.

As shown in Figure 4.28, the entire chromosomal DNA showed a 2.4 bp band and 4.0 kb band when digested with *Kpn*I, except for untransformed GS115 (negative control) which showed a 2.4 kb band (lane 1). While transformed GS115 with pPICZαA revealed a 1.7 kb band and 4.0 kb band. This result related to the expected map (Figure3.4) which was predicted before the experiment was done. Moreover, a strong band of 23.1 kb was found, indicating that *Kpn*I digestion was not complete. The bands pattern in Figure 4.28 demonstrated that the transformants contained the scFv gene in the chromosomal DNA.

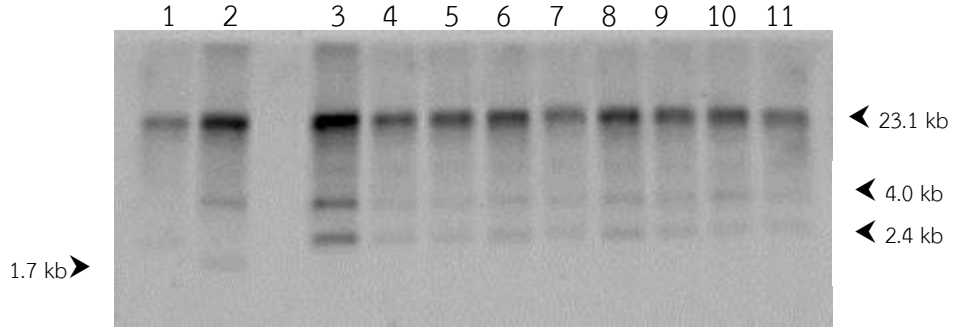

Figure 4.28 Southern blot analysis of chromosomal DNA from the nine Zeo^R transformants clones. The DNA was digested with *Kpn*I and hybridized with *AOX1* promoter as probe. Strains shown are following; Lane 1: untransformed control. Lane: 2 transformed with pPICZαA. Lane 3: T1. Lane 4 - lane 11: O1- O8.

4.9.2 Sequencing analysis

Because accuracy of the nucleotide sequence of scFv affects the accuracy of the translated amino acid of scFv antibody. In this study, linearized pJM01 expression cassette was inserted into the chromosomal of *Pichia* at *Pme*I site of *AOX1* promoter to generate genetically stable transformants. Therefore, the accurate sequence of pJM01 after insertion should be improved.

By using TJ03F, TJ04F, TJ05F and TJ06R primers (Table 3.1.5, Methodology section) for nucleotide sequencing, it was found that the sequence of a region was corresponded to 1161 nucleotides from α-factor secretion signal to stop codon (TGA) at c-terminus which was carried the scFv expression cassette (Figure 4.29). The coding sequence (CDS) of scFv expression cassette consisted of 800 nucleotides encoding a polypeptide of 266 amino acids with a predicted molecular weight of 42.5 kDa (intracellular expression including α-factor secretion signal) and 32.50 kDa (secreted).

```
   1 ATGAGATTTCCTTCA ATTTTTACTGCTGTT TTATTCGCAGCATCC TCCGCATTAGCTGCT CCAGTCAACACTACA
   1  M  R  F  P  S  I  F  T  A  V  L  F  A  A  S  S  A  L  A  A  P  V  N  T  T
  76 ACAGAAGATGAAACG GCACAAATTCCGGCT GAAGCTGTCATCGGT TACTCAGATTTAGAA GGGGATTTCGATGTT
  26  T  E  D  E  T  A  Q  I  P  A  E  A  V  I  G  Y  S  D  L  E  G  D  F  D  V
 151 GCTGTTTTGCCATTT TCCAACAGCACAAAT AACGGGTTATTGTTT ATAAATACTACTATT GCCAGCATTGCTGCT
  51  A  V  L  P  F  S  N  S  T  N  N  G  L  L  F  I  N  T  T  I  A  S  I  A  A
 226 AAAGAAGAAGGGGTA TCTCTCGAGAAAAGA GAGGCTGAAGCTGAA TTCGAAGTAGAGCTG GAGGAGTCTGGGGGA
  76  K  E  E  G  V  S  L  E  K  R  E  A  E  A  E  F  E  V  E  L  E  E  S  G  G
 301 GGCTTAGTGAAGCCT GGAGGGTCCCTGAAA CTCTCCTGTGCAGCC TCTGGATTCAGTTTC AGTGACTATTACATG
 101  G  L  V  K  P  G  G  S  L  K  L  S  C  A  A  S  G  F  S  F  S  D  Y  Y  M
 376 TATTGGGTTCGCCAG ACTCCGGAAAAGAGG CTGGAGTGGGTCGCA ACCATTAGTGAAGGT GCCAGTTATATCTAT
 126  Y  W  V  R  Q  T  P  E  K  R  L  E  W  V  A  T  I  S  E  G  A  S  Y  I  Y
 451 TATTCGGACAGTGTA AAGGGACGCTTCACC ATCTCCAGAGACAAT GCCAGGAACACCCTG TACCTGCAAATGAGC
 151  Y  S  D  S  V  K  G  R  F  T  I  S  R  D  N  A  R  N  T  L  Y  L  Q  M  S
 526 AGTCTGAAGTCTGAG GACACAGCCATTTAT TACTGTACAAGAGCC TATAATAACTACGTG TCGAAATATTTCGAT
 176  S  L  K  S  E  D  T  A  I  Y  Y  C  T  R  A  Y  N  N  Y  V  S  K  Y  F  D
 601 GTCTGGGGCGCAGGG ACCACGGTCACCGTC TCCTCAGCCAAAACG ACACCCCCATCTGTC TATGGTGGAGGCGGT
 201  V  W  G  A  G  T  T  V  T  V  S  S  A  K  T  T  P  P  S  V  Y  G  G  G  G
 676 TCAGGCGGAGGTGGA TCTGGCGGTGGCGGA TCGGACATTGTGATG ACACTGTCTACAGCA ATCCTGTCTGCATCT
 226  S  G  G  G  G  S  G  G  G  G  S  D  I  V  M  T  L  S  T  A  I  L  S  A  S
 751 CCAGGGGAGAAGGTC ACAATGACTTGCAGG GCCAGTTCAAGGGTA AATTACATACACTGG TTCCAGCAGAAGGCA
 251  P  G  E  K  V  T  M  T  C  R  A  S  S  R  V  N  Y  I  H  W  F  Q  Q  K  A
 826 GGATCCTCCCCCAAA CCCTGGATTTATGCC ACATCCAACCTGGCT TCTGGAGTCCCTGAT CGCTTCAGTGGCAGA
 276  G  S  S  P  K  P  W  I  Y  A  T  S  N  L  A  S  G  V  P  D  R  F  S  G  R
 901 GGGTCTGGGACCTCT TACTCTCTCACAATC AGCAGAGTGGAGGCT GAAGATGCTGCCACT TATTACTGCCAGCAG
 301  G  S  G  T  S  Y  S  L  T  I  S  R  V  E  A  E  D  A  A  T  Y  Y  C  Q  Q
 976 TGGAGTAATAACCCG TGGACGTTCGGTGGA GGCACCAAGCTGGAA ATCAAACGGGCTGAT GCTGCACCAACTGTA
 326  W  S  N  N  P  W  T  F  G  G  G  T  K  L  E  I  K  R  A  D  A  A  P  T  V
1051 TCCGAGCTCCCGGTA CCTCGAGCCGCGGCG GCCGCCAGCTTTCTA GAACAAAAACTCATC TCAGAAGAGGATCTG
 351  S  E  L  P  V  P  R  A  A  A  A  A  S  F  L  E  Q  K  L  I  S  E  E  D  L
1126 AATAGCGCCGTCGAC CATCATCATCATCAT CATTGA
 376  N  S  A  V  D  H  H  H  H  H  H  *
```

Figure 4.29 The coding sequence (CDS) and amino acid sequence of pJM01 plasmid from **α**-factor secretion signal to stop codon (TGA) at c-terminus. Underline: **α**-factor secretion signal nucleotide region. Non-labeled letter: CDS of scFv nucleotide. Double underline: Myc (human c-Myc oncogene) epitope tag. Bolded-italic: 6xHis affinity tag. Asterisk (*): stop codon, respectively.

4.9.3 Verification of pJM01 insertion at *AOX1* promoter

In *P. pastoris*, alcohol oxidase (*AOX*) is the first enzyme in the methanol utilization pathway and is encoded by two genes, *AOX1* and *AOX2*. The DNA and predicted amino acid sequences of the protein-coding portions of the genes are closely homologous at 97% homologous, whereas flanking sequences share no homology. The *AOX1* gene is responsible for the vast majority of *AOX1* activity in the cell and expression of the *AOX1* gene is tightly regulated and induced by methanol to very high level [99].

Therefore, the location that linearized pJM01 expression cassette could be integrated exactly at *AOX1* promoter must be investigated. The P1, P2, P3 and P4 primers were specifically designed corresponding to the nucleotide of *AOX1* promoter and pJM01 regions. The P1 primer can bind at downstream of AOX1 promoter, while the P4 primer anneals at CDS of *AOX1* gene. Moreover, the P2 and P3 primers were synthesized which was specifically annealed at α-factor secretion signal nucleotide region and at ori of pJM01 region. The sequence of these primers was confirmed that it could anneal to *AOX1* only (Figure3.6). By using P1&P2 primers, the PCR product could be found at 1231 bp and by using P3 and P4 primer, the 1021 bp band was amplied and detected on 1% agarose gel electrophoresis. The result indicated that linearized pJM01 expression cassette was integrated at *AOX1* promoter because the Zeo^R transformant O1 –O8 and T1 yield the PCR products at about 1231 bp band and 1021 bp band which related to the prediction map as shown in Figure 3.6.

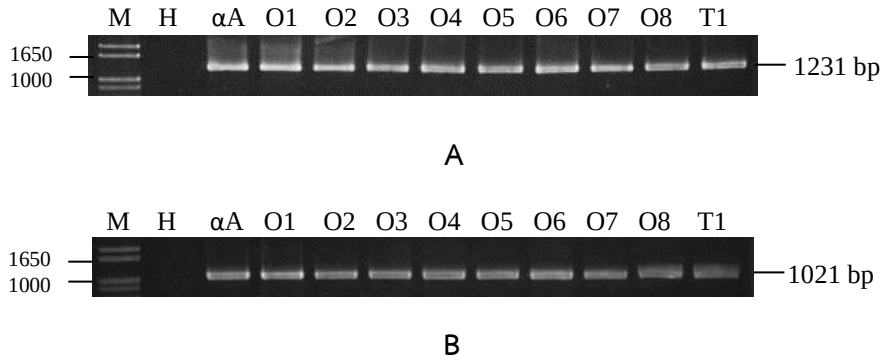

Figure 4.30 The 1.0% ethidium bromide stained agarose gel showed the PCR products. A) Using P1 and P2 primers. B) Using P3 and P4 primers.

4.10 Selection of Zeo^R Transformants with Recombinant scFv Expression

In this study, nine Zeo^R Transformants were subjected to a second screening for recombinant scFv expression, which was performed in shaker flask cultivation. The first step was cell production phase, which was cultured in the YPG medium for overnight. After harvesting, 10% inoculum of this cell culture was transferred to MMH medium for recombinant scFv expression by inducing with methanol. The optical density at 600 nm by spectrophotometry was used to observe the different cell growth. The results revealed that the optical density (OD) at 600 nm of all Zeo^R Transformants cell culture at 0 h were quite similar ranking from 0.28 to 0.36. After 24 h induction with methanol, the OD_{600} of O2, O5 and O8 showed higher cell concentration than the others with 3.74±0.45, 5.15±0.40 and 3.92±0.54, respectively. Moreover, among these clones, O2, O5 and O8 exhibited higher specific growth rate (SRG) at 24 h than the others with 0.14, 0.20 and 0.15 μ, respectively. In addition, SRG at 48 h of these clones still exhibited higher than others with 0.12, 0.14 and 0.13 μ of O2, O5 and O8, while the SGR at 72 h revealed that SGR of all clones were similarity. The summarized data was shown in Table 4.1 and Figure 4.1.

Table 4.5 The optical density at 600 nm of the cell culture in the induction phase (MMH medium) and specific growth rate at 24, 48 and 72 hours

Strains	OD_{600} of the cell culture in MMH medium*				Specific growth rate: SGR (μ)		
	0 h	24 h	48 h	72 h	24 h	48 h	72 h
O1	0.36±0.00	3.21±0.41	6.41±0.49	6.51±0.49	0.12	0.13	0.09
O2	0.34±0.03	3.74±0.45	6.24±0.00	6.88±0.00	0.14	0.12	0.09
O3	0.34±0.00	3.06±0.75	5.54±0.00	7.53±0.27	0.11	0.11	0.10
O4	0.34±0.00	3.37±0.35	5.98±0.50	7.33±0.18	0.13	0.12	0.10
O5	0.36±0.00	5.15±0.40	7.18±0.42	7.91±0.75	0.20	0.14	0.10
O6	0.36±0.00	2.79±0.98	6.06±0.31	8.42±0.31	0.10	0.12	0.11
O7	0.28±0.00	2.40±0.00	5.30±0.00	7.60±0.84	0.09	0.10	0.10
O8	0.32±0.03	3.92±0.54	6.38±0.00	7.00±0.00	0.15	0.13	0.09
T1	0.34±0.00	3.53±0.04	6.32±0.28	7.21±0.56	0.13	0.12	0.10
[a]αA	0.26±0.00	2.57±0.01	6.53±0.72	8.42±0.31	0.10	0.13	0.11
[b]lacZ	0.30±0.00	2.04±0.00	4.92±0.00	8.55±0.49	0.07	0.10	0.11
[c]albumin	0.32±0.00	0.64±0.00	2.30±0.00	4.11±0.00	0.01	0.04	0.05

*Data is shown in mean ± standard deviation a: pPICZαA (*Pichia* expression vector), b: intracellular clone as control, c: secretion clone as control

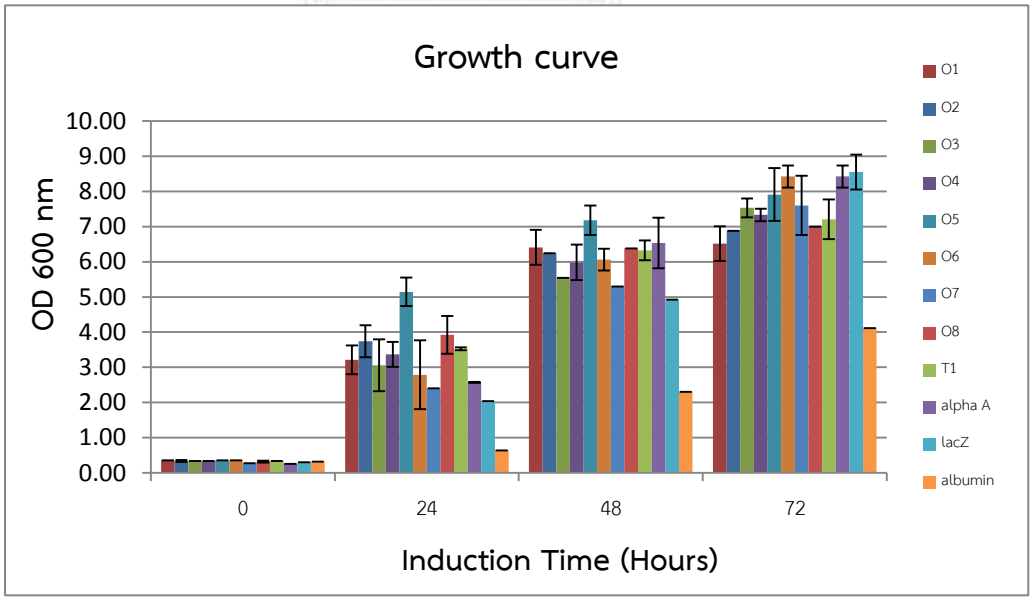

Figure 4.31 Growth curve of all transformants during cultivation in MMH medium with methanol induction for 72 h.

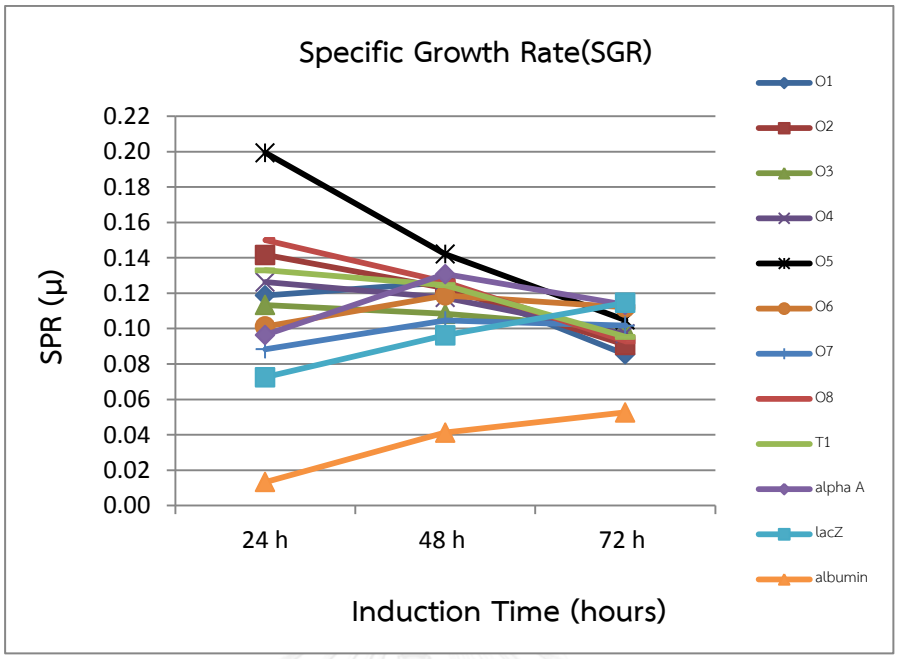

Figure 4.32 The specific growth rate (SGR) of all transgenic strains

Subsequently, the ability of the recombinants to produce protein of insert was investigated. The αA clone was employed as negative control for comparison of the amount of total protein production with positive clone and their proteins were measured using Qubit® 3.0 Fluorometer (Life Technologies). The result illustrated that the total protein concentration of all clones were not significant different in 24 h induction with methanol (ranging form 51.60 μg/mL in O1 to 60.40 μg/mL in O7, respectively). After 72 h with methanol induction, the result revealed that the total protein concentrations were significantly different among these clones. It was found that O6 showed the highest total protein concentration at about 143.50 μg/mL followed by O3, O5 and O7 at 140.50, 138.00 and 136.50 μg/mL, respectively. In this study, however, the total protein concentration of MMH medium was assumed to equal to the total protein concentration at 0 h induction with 35.97 μg/mL. Therefore, the increasing the protein concentration after 72 h induction was implied that each clone could produce the proteins on the recombinant scFv antibody. The

investigation of recombinant scFv secretion was done by SDS-PAGE and Western blotting analysis in the next experiments. The summarized statistic data of total protein concentrations were illustrated in Table 4.1 and Figure 4.1.

Table4.6 The total protein concentrations (μg/mL) of nine Zeo^{R} Transformants and others at 24, 48 and 72 hours

Strains	0 h	24 h	48 h	72 h
O1	35.97±0.55	51.60±0.16	84.25±6.15	102.00±4.07
O2		53.65±5.30	81.10±7.64	87.15±0.07
O3		51.65±0.21	83.10±14.57	140.50±14.85
O4		53.90±0.28	67.65±0.49	95.77±3.36
O5		56.35±2.90	88.27±8.18	138.00±14.14
O6		51.65±1.48	82.65±9.26	143.50±7.78
O7		60.40±2.83	89.15±8.56	136.50±7.78
O8		53.55±2.05	81.30±2.12	92.25±1.20
T1		52.00±0.42	75.35±1.48	97.00±5.76
[a]αA		55.20±0.02	75.60±0.42	102.00±1.41
[b]lacZ		57.45±0.07	88.55±0.07	141.50±10.61
[c]albumin		58.15±0.07	60.45±0.07	82.50±0.14

Data is shown in mean ± standard deviation

a: pPICZαA (*Pichia* expression vector),

b: intracellular clone as control

c: secretion clone as control

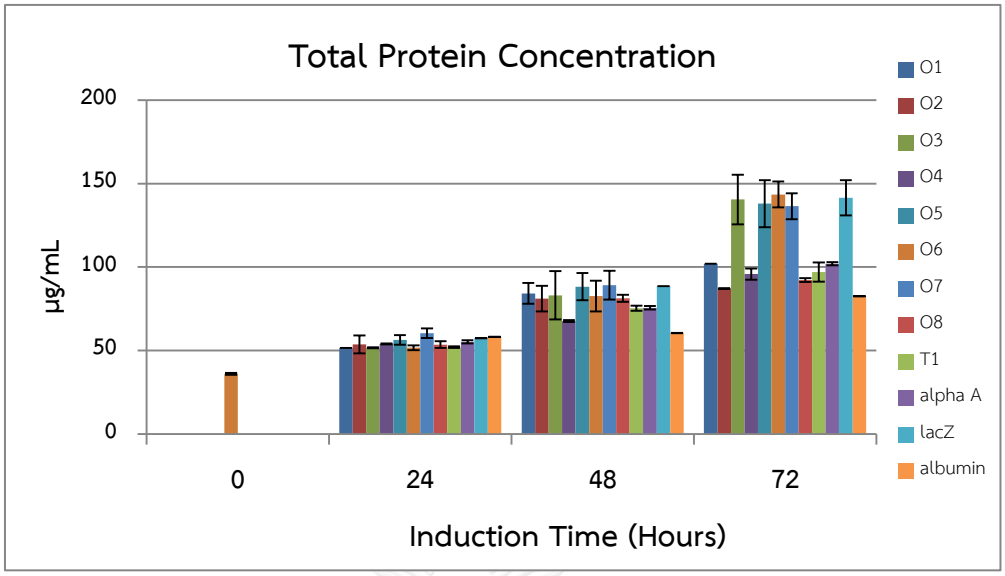

Figure 4.33 Total protein concentrations assay of all transformants during cultivation in MMH medium with methanol induction for 72 h.

4.11 Characterization of Recombinant scFv Expressed

4.11.1 Intracellular Expression

When expressing recombinant proteins in *P. pastoris*, heterologous proteins can either be expressed intracellular or secreted into the medium [54, 58]. To consider whether intracellular expression or extracellular secretion, in this study, intracellular expression was first examined by breaking the cells with lyticase enzyme and its intracellular protein was equally subjected onto 12% SDS-PAGE and detected the recombinant scFv by Western blot analysis. The GS115/pPICZ/lacZ (Mut^+) was used as the positive control because it can expressed ß-galactosidase intracellular. Moreover, *Pichia* expression vector, pPICZαA, was applied as the control for intracellular background. As shown in Figure 4.1, using mouse anti-c-myc antibody at the concentration of 1:2000 to detect the expressed recombinant scFv, it was found that four major bands were detected on the membrane. The upper band might be a glycosylated scFv with the size of approximately 55 kDa. This observation suggested that the lower band could be a recombinant scFv fused with α-factor secretion signal of *Pichia* expression vector which was predicted the MW of about 42.5 kDa.

However, the two other bands were also found on the membrane. These could be a recombinant scFv secretion form with the MW of about 32.67 kDa and a de-natured recombinant scFv.

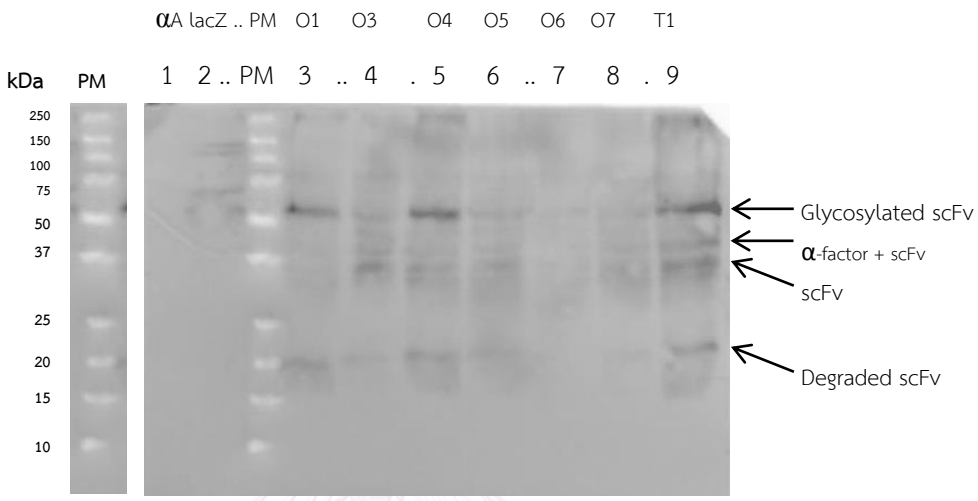

Figure 4.34 Western blot analysis of the recombinant scFv antibody intracellular expression by using mouse anti-c-myc antibody as a primary antibody and GAM-HRP as secondary antibody. Lane 1: intracellular protein of pPICZαA. Lane2: intracellular protein of GS115/pPICZ/lacZ. Lane 3-9: intracellular protein of O1, O3, O4, O5 O6, O7 and T1, respectively. Lane PM: protein marker.

In addition, the mouse anti-His (c-term) conjugated with HRP was also employed to detect recombinant scFv antibody on the membrane. By using the concentration of this antibody at 1:2500, the results revealed that two bands were detected on the membrane. As predicted, the upper band corresponded to recombinant scFv antibody fused with α-factor secretion signal of *Pichia* expression vector which was predicted the MW of about 42.5 kDa. While, the lower band could be claimed as a recombinant scFv with was predicted the MW of 32.67 kDa comparing with the protein marker, respectively (Figure4.1).

The use of mouse anti-His (c-term) conjugated with HRP antibody showed the major advantages than the use of mouse anti-c-myc antibody. At first, it can reduce the process of detection. Because its antibody conjugated with HRP enzyme, it does not require another enzyme for reaction. In the cases of using mouse anti-c-myc

antibody for detection, it does not conjugate with any enzymes, so another antibody which is labeled with an enzyme for detection is required. For these reasons, mouse anti-His (c-term) conjugated with HRP antibody is preferred. Unfortunately, the use this antibody to detect recombinant scFv antibody in this experiment showed that it was cross-react with protein marker. Therefore, this antibody was not suitable for further study.

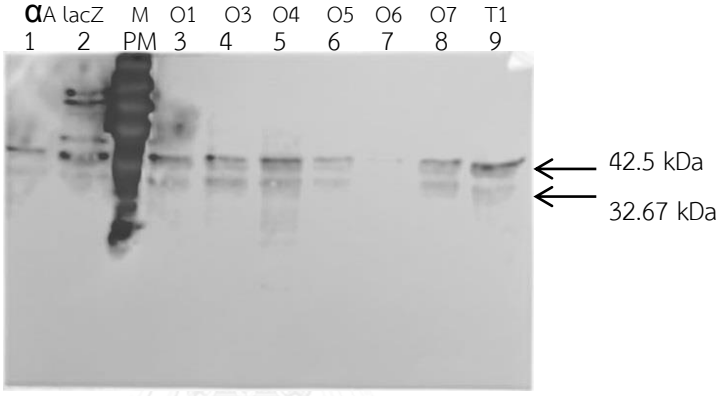

Figure 4.35 Western blot analysis of the recombinant scFv antibody intracellular expression by using mouse anti-his HRP (c-term) antibody conjugated with HRP. Lane 1: intracellular protein of pPICZαA as background control. Lane2: intracellular protein of GS115/pPICZ/lacZ as intracellular expressed. Lane 3-9: intracellular protein of O1, O3, O4, O5 O6, O7 and T1, respectively. Lane PM: protein marker.

4.11.2 Extracellular Expression (Secreted)

It has been known that *P. pastoris* secrets a very low level of native protein. Therefore, the majority of the proteins found in the medium should be the secreted recombinant proteinsthus. [52, 53, 61, 100].

The selected clones were cultured to check for their recombinant scFv antibody synthesis. The cell-free supernatant were clarified to remove cell debris. The equal amounts of clarified supernatant were concentrated by precipitation with trichloro acetic acid (TCA) and 15 µL of these samples were subjected onto 12% SDS-PAGE. After staining with SimplyBlue™ SafeStain (invitrogen), the SDS-PAGE gel analysis showed that the recombinant scFv antibody of O3 at 48 h induction (lane 2), O5 at 24 and 48 h induction (lane 3 and 4) and O6 at 48 h induction (lane 6) were found as a major band of approximately 50 kDa as compared with the protein markers. However, several minor contamination proteins of the yeast origin were also found on the gel.

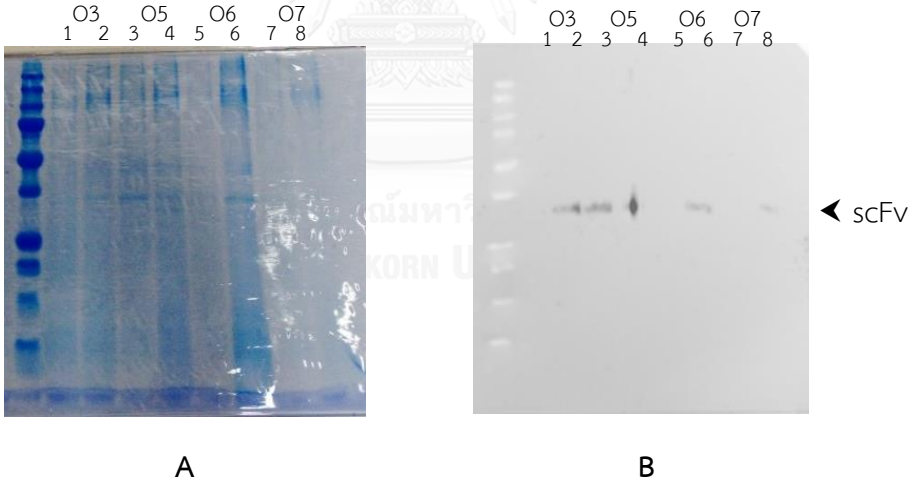

Figure 4.36 A) SDS-PAGE analysis of recombinant scFv antibody which produced by O3, O5, O6 and O7 transfromants. Lane. B) Western blotting of recombinant scFv antibody detected with mouse anti-c-myc antibody followed by GAM-HRP. Lane1-2: supernatant of O3 at 24 and 48 h induction. Lane3-4: supernatant of O5 at 24 and 48 h. Lane5-6: supernatant of O6 at 24 and 48 h. Lane7-8: supernatant of O7 at 24 and 48 h. lane PM: protein marker.

The obtained SDS-PAGE gel was transferred to the PVDF membrane and assayed the recombinant scFv antibody expressed by Western blotting. The results showed that a strong specific band appeared on the membrane with corresponding to the predicted recombinant scFv MW of about 32.67 kDa by using mouse anti-c-myc antibody at concentation of 1:2000. As shown in Figure 4.36, the specific bands of recombinant scFv antibody which exprssed by O5 were found on the membrane at 24 h and 48 h induction (lane 3 and lane 4, respectively). While, the specific bands of the recombinant scFv antibody which exprssed by O3, O6 and O7 were found at 48 h induction (lane2, 6 and 8) using mouse anti-c-myc antibody and GAM-HRP antibody for detection. This result indicated that the O5 gave highest amount of the recombinant scFv antibody than other clones at 24 h methanol induction.

In addition, the recombinant scFv antibody produced by O2 and O8 strains was also observed. The 15 µL of equal amounts of supernatant (designated as S) and 15 µL of equal amounts of precipitated supernatant (designated as PS) were subjected onto 12% SDS-PAGE. After that, a SDS-PAGE gel was transferred to PVDF membrane. The recombinant scFv antibodies were checked by Western blotting. As shown in Figure 4.37, several specific bands were found on the membrane. The result indicated that the precipitated supernatant at 48 h of O5 clone showed a specific intensive band which was found on the membrane (lane 4) than the precipitated supernatant of others (lane 2 for O2 and lane 6 for O8). From this result, it implied that this strong band corresponded to a large amounts of the expressed recombinant scFv antibody. In the case of supernatant (without precipitatation), the supernatant of O5 revealed a thin specific band (lane 3), while the antibody of other clones were not detected (lane 1 and lane 5).

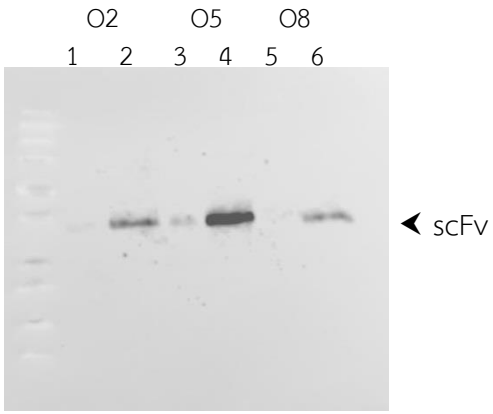

Figure 4.37 Western blotting of recombinant scFv antibody detected with mouse anti-c-myc antibody followed by GAM-HRP. Lane1-2: supernatant (S) and precipitated supernatant (PS) of O2 at 48 h induction. Lane3-4: S and PS of O5 at 48 h induction. Lane5-6: S and PS of O8 at 48 h induction. Lane PM: protein marker.

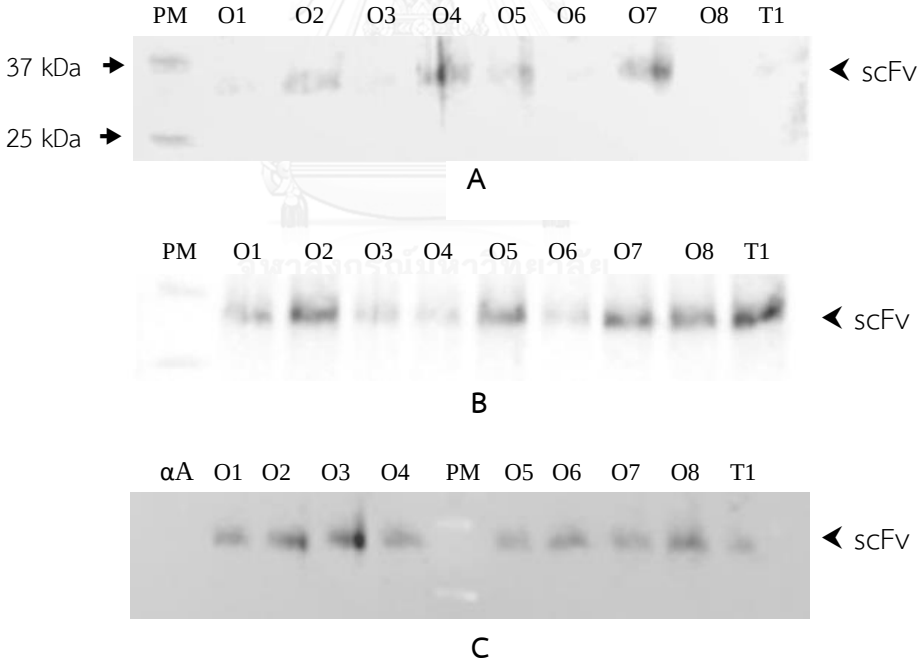

Figure 4.38 Western blotting of recombinant scFv antibody detected with mouse anti-c-myc antibody followed by GAM-HRP. A) 24 h induction with methanol B). 48 h induction with methanol. C) 72 h induction with methanol. Lane PM: protein marker.

4.12 Production of Recombinant scFv Antibody

The transgenic O5 strain which gave the highest level of recombinant scFv antibody expression was selected and analyzed in time course experiments. Cultivation of O5 strain to obtain high amounts of recombinant scFv antibody was performed. During 72h of O5 cultivation, several characteristics such as optical density at 600 nm (OD_{600}), biomass accumulation (dry cell weight (DCW), g/L), pH and total proteins concentration (µg/mL) were measured. The results revealed that the cell growth in MMH medium measured by OD_{600} was dramatically increased from 0.13 to 9.63. This result related to the dry cell weight. It was found that the dry cell weight of O5 rose gradually from 2.33 g/L to 28.67 g/L. While, the pH was decreased from 5.29 to 2.55. However, the total proteins which were produced and secreted into the medium were slightly increased from 38.25 µg/mL to 135.76 µg/mL, respectively. The statistic results were summarized in Table 4.7 and Figure 4.39.

Table4.7 Characterization of O5 during cultivation in MMH medium for 72 h

Induction Time (hours)	OD 600	Biomass accumulation (DCW, g/L)	pH	Total protein concentration (µg/mL)
0	0.13±0.02	2.33±0.0001	5.29±0.03	38.25±2.66
24	4.10±0.34	10.67±0.0004	4.89±0.11	65.77±0.86
48	6.47±0.27	20.00±0.0004	3.12±0.11	85.92±4.68
72	9.63±0.24	28.67±0.0003	2.55±0.04	135.67±1.15

The data is shown in mean ± standard deviation

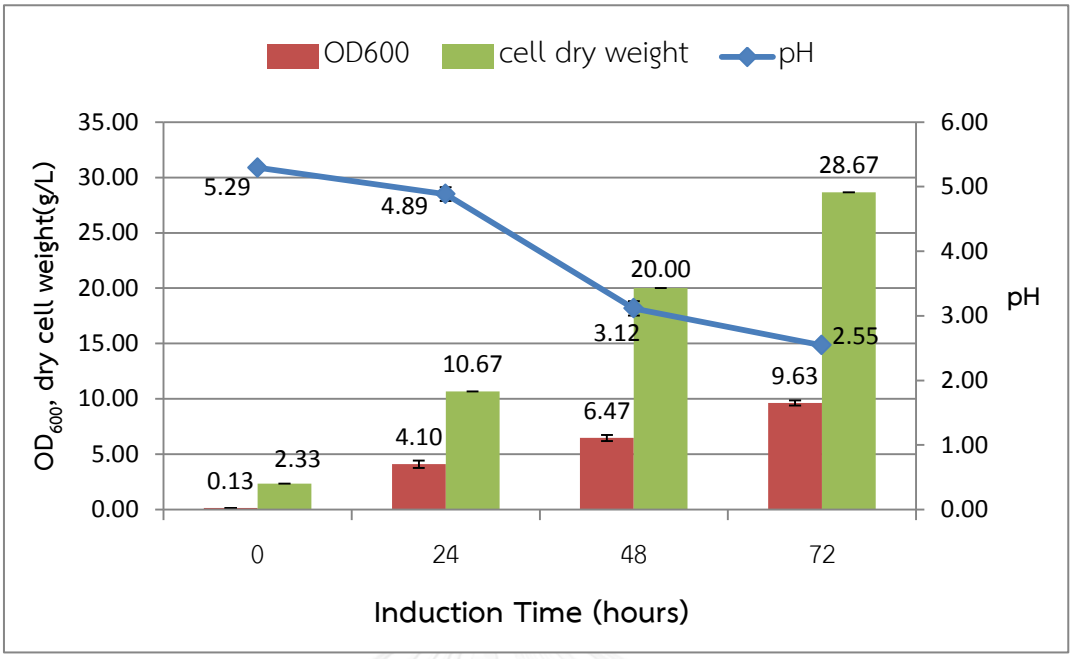

Figure 4.39 Cultivation of O5 strain in MMH medium with methanol induction for 72 h. Cultivation was performed in shake flasks at 30°C. Aliquots (5 mL) of the yeast cultivation were used to determine biomass (dry cell weight, g/L), OD600 and pH.

After 72 h induction with methanol, The O5 cell-free culture supernatants were clarified using 0.2 µm filter to eliminate small particles. The 15 mL of the clarified supernatants that related to 0, 24, 48 and 72 h of induction were concentrated using amicon 15kD cut-off followed by adjustment of the pH to pH 7.0 with 2.5N NaOH. A ten microliter of these samples were loaded onto 12% SDS-PAGE and followed by silver staining for observation of the recombinant scFv expression at each time point. The silver stained gel revealed that several bands were detected in the gel. However, this observation suggested that the size of the major tightly band appeared on the gel was about to 35 kDa which cloud be corresponded to a form of the recombinant scFv antibody (Figure 4.40).

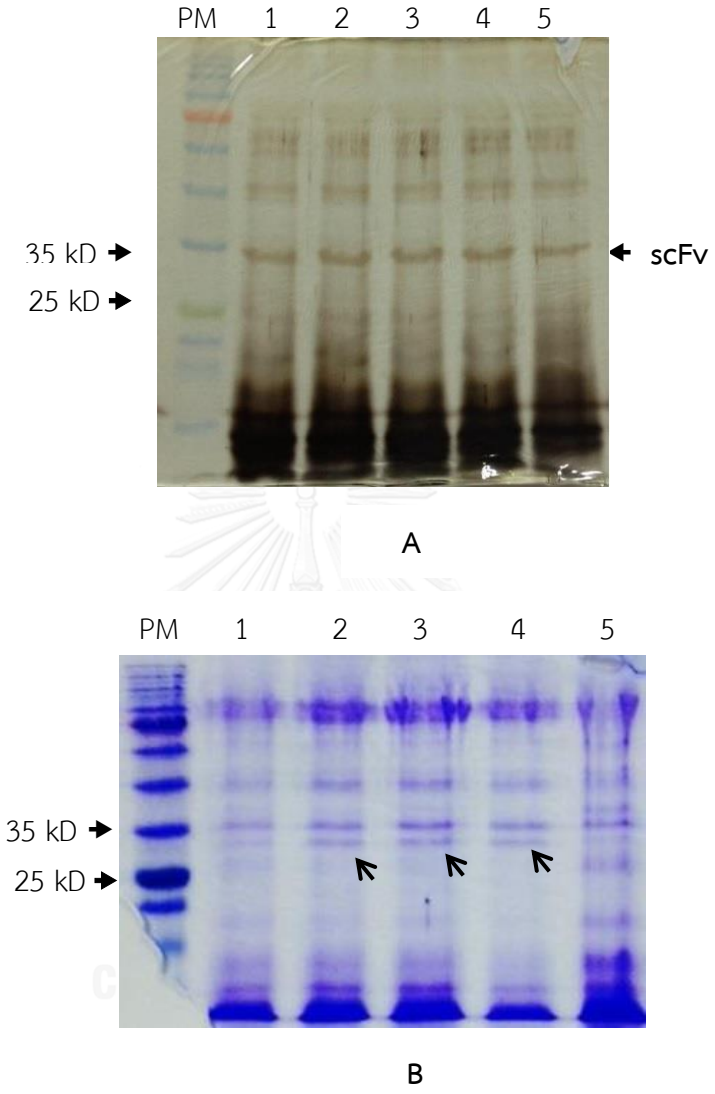

Figure 4.40 SDS-PAGE analysis of the scFv expressed by O5 strain during cultivation in MMH medium with methanol induction for 72 h.

A) Silver stained gel; Lane 1: 24 h. Lane 2: 48 h. Lane 3-5: 72 h.

B) Coomassie blue; Lane 1: 6 h. Lane 2: 24 h. Lane 3: 48 h. Lane 4: 72 h. Lane 5; αA at 72h (control)

Lane PM: protein marker.

4.13 Transcriptional Level of scFv Gene During O5 Strain Cultivation

The expression level of scFv transcription of O5 at each time point was analyzed by RT-qPCR to determine the different level of recombinant scFv antibody expression. After induction with methanol for 72 h, the cDNA was prepared from the total RNAs which was extracted and measured the concentration using Nanodrop spectrophotometry. The Table 4.8 showed the RNA concentration of O5. The result revealed that the extracted RNA concentration ranged from 722.4 ng/μL to 1084.4 ng/μL. Subsequently, equal amounts of RNA were used to synthesize cDNA which was used as the template for RT-qPCR analysis using specific primers, TJ04F and TJ05R (Table 3.1.5).

In this experiment, the actin gene, housekeeping gene was used as a reference gene and RT-qPCR was run in triplicate to calculate the statistical confidence in different gene expressions. After 40 cycles of RT-qPCR with EvaGreen® qPCR mixer, the normalized expression of scFv gene at each time point was examined by normalization the quantification cycle (Cq) values with using actin gene. For calculation the relative normalization expression, the transcription levels of αA strain (empty vector) were set as control to normalize the data. The mean Cq, Cq standard deviation, normalized expression, relative normalized expression and regulation were summarized in the Table 4.9.

As depicted in Table 4.9, the normalized expression of scFv mRNA transcripted in transgenic O5 at 0.5 h, 24 h, 48 h and 72 h was 1.03, 4.21, 1.23 and 0.66 times higher than the expression of actin gene at the same time point. Moreover, the relative normalized expression of scFv mRNA transcripted in transgenic O5 at different time point of induction was 167.77, 688.32, 200.74 and 107.42 times at 0.5 h, 24 h, 48 h and 72 h respectively. In this analysis, GS115 transformed with the empty vector, pPICZαA, was used as the control. This result revealed that the highest expression of transgenic O5 was found at 24 h of methanol induction (Figure 4.41). This suggested that the mRNA expression of scFv gene at 0.5h, 24h, 48h and 72h induction with methanol showed the up regulated expression comparing with the regulation threshold ($P<0.05$)

Table 4.8 The RNA concentration (ng/µL) measured by Nanodrop

Strain	Induction (hours)	Concentration (ng/µL)	A260 (10mm)	A260/A280
αA*	72	1084.4	27.110	2.17
O5	0	722.4	18.059	2.20
	24	919.8	22.996	2.15
	48	958.8	23.969	2.17
	72	951.2	23.780	2.20
O5Gly**	24	276.4	6.911	1.93
GS115***	24	317.5	7.937	2.10

*GS115 transformed with the empty vector, pPICZαA

**O5 cultivation in YPG medium (cells production phase)

*** Un-transformed GS115 strain

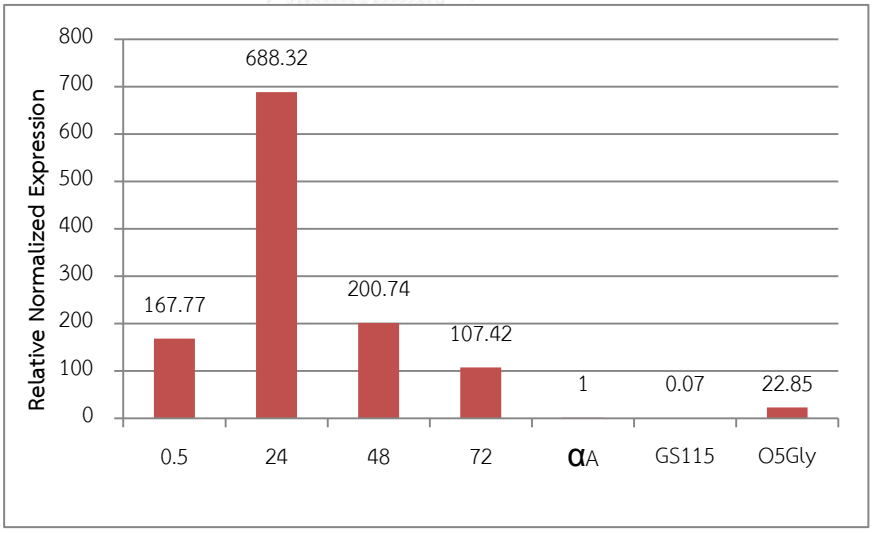

Figure 4.41 Expression profiles of scFv gene transcript of transgenic O5 at different time point compared to αA: GS115 transformed with the empty vector, pPICZαA. GS115: Un-transformed GS115 strain and O5Gly: O5 cultivation in YPG medium (cells production phase)

For an accurate assessment of gene expression by real-time PCR, the PCR efficiency and the PCR specificity of gene must be taken into consider. Real-time PCR efficiency was calculated from the slope, obtained from the curve plotted in log scale between the cDNA and the quantification cycle (Cq). The correlation coefficients, melting temperature and real-time PCR efficiencies were represented in Table 4.9.

The specificity of the product amplified by EvaGreen® qPCR mixer was monitored by analyzing the dissociation curve of each amplicon. The dissociation curve showed a single peak at the expected melting temperature, indicating that scFv gene was specifically amplified and there was no non-specific amplification or primer-dimer (Figure 4.42).

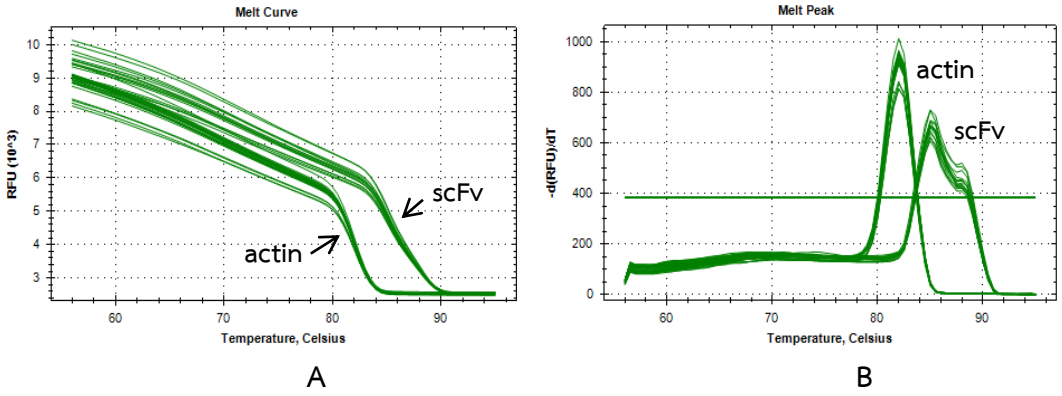

Figure 4.42 A) Melting curve of expected scFv gene and actin gene. B) Melting peak of expected scFv gene and actin gene as indicated in arrow.

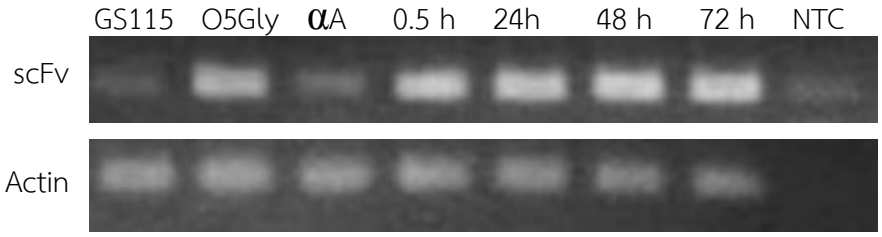

Figure 4.43 Expression of scFv gene of O5 at each time point. A) RT-qPCR amplified of scFv gene. B) RT-qPCR amplified of the actin gene as control. GS115: untransformed GS115 strain. O5Gly: O5 in YPG medium (production phase). αA: GS115 transformed with the empty vector, pPICZαA. NTC: no template control.

Table 4.9 Summarization of various values which were calculated and performed by RT-qPCR

Target	Samples	Mean Cq	Cq Std. Dev.	Normalized Expression	Relative Normalized Expression	Regulation	Compared to Regulation Threshold	P-Value	Exceeds P-Value Threshold
Actin	0.5 h	19.20	0.050	-	-	-	No change	0.97	Yes
Actin	24 h	18.38	0.045	-	-	-	No change	0.97	Yes
Actin	48 h	19.92	0.076	-	-	-	No change	0.98	Yes
Actin	72 h	20.66	0.048	-	-	-	No change	0.97	Yes
Actin	αA	20.96	0.135	-	-	-	No change	N/A	No
Actin	GS115	17.47	0.047	-	-	-	No change	0.97	Yes
Actin	O5Gly	16.78	0.034	-	-	-	No change	0.96	Yes
scFv	0.5 h	19.17	0.040	1.03	167.77	167.77	Up regulated	0.00	No
scFv	24h	16.30	0.029	4.21	688.32	688.32	Up regulated	0.00	No
scFv	48h	19.62	0.030	1.23	200.74	200.74	Up regulated	0.00	No
scFv	72h	21.27	0.019	0.66	107.42	107.42	Up regulated	0.00	No
scFv	αA	28.31	0.307	0.01	1.00	1.00	No change	N/A	No
scFv	GS115	28.58	0.204	0.00	0.07	-13.55	Down regulated	0.00	No
scFv	O5Gly	19.62	0.222	0.14	22.85	22.85	Up regulated	0.00	No

4.14 Purification of Recombinant scFv Antibody Fragment

Probond™ Purification System (Invitrogen) was used to purify recombinant scFv antibody under native condition. The recombinant scFv produced by O5 contained six histidine tag at c-terminal allowing purification by IMAC. The Ni-NTA agarose resin was incubated with 100 mL of neutralized supernatant at 4°C for overnight with rotating stirrer. After the non-specifically bound contaminants were washed away with binding buffer containing 20 mM imidazole, the recombinant scFv was eluted by stepwise gradient of imidazole at the flow rate of 1 mL per min. The eluted fractions were collected and analyzed by measurement the protein quantification at A 280 nm (Figure4.44), by SDS-PAGE and confirmed by Western blot analysis.

For SDS-PAGE, each eluted fraction was dialyzed against 10mM PBS buffer, pH 8.0. An aliquot sample was analyzed for the identity and purity on a 12% SDS-PAGE gel and confirmed by Western blotting using mouse anti-c-myc antibody followed by GAM-HRP antibody.

The results of Western blotting revealed that the recombinant scFv antibody was first eluted by 150 mM imidazole and followed by 200 mM imidazole which was present as a major band appeared on the blot after detection with specific antibody (Figure4.45; lane 6 and lane, Figure4.46; lane2, 3, 6 and 7). Its molecular weight was estimated to be approximately 35 kDa, which is consistent with the predicted molecular weight of recombinant scFv, 32.67 kDa.

To estimate the yield of the purified recombinant scFv antibody, BCA protein assay kit was used for protein quantification. After removing the excess imidazole contaminated by dialysis, the concentration of obtained purified recombinant scFv antibody was deduced from a standard curve constructed using bovine serum albumin (BSA) with a known concentration (Appendix F). It was found that the concentration of purified recombinant scFv was 7.42 µg/mL.

Table 4.10 The protein concentration of eluted fraction measured by A 280 nm

Fraction(s)	**A280 nm**	**Total protein concentration (mg/mL)***
Flow through	1.31	1.31
Wash I	1.65	1.65
Wash II	0.14	0.14
Wash III	0.09	0.09
100 mM imidazole in Elution buffer	0.12	0.12
150 mM imidazole in Elution buffer	2.56	2.56
200 mM imidazole in Elution buffer	1.52	1.52
250 mM imidazole in Elution buffer	0.21	0.21
500 mM imidazole in Elution buffer	0.20	0.20
500 mM imidazole in Elution buffer	0.27	0.27

*total protein concentration were determined by 1 optical density unit at A280 = 1 mg/mL [34]

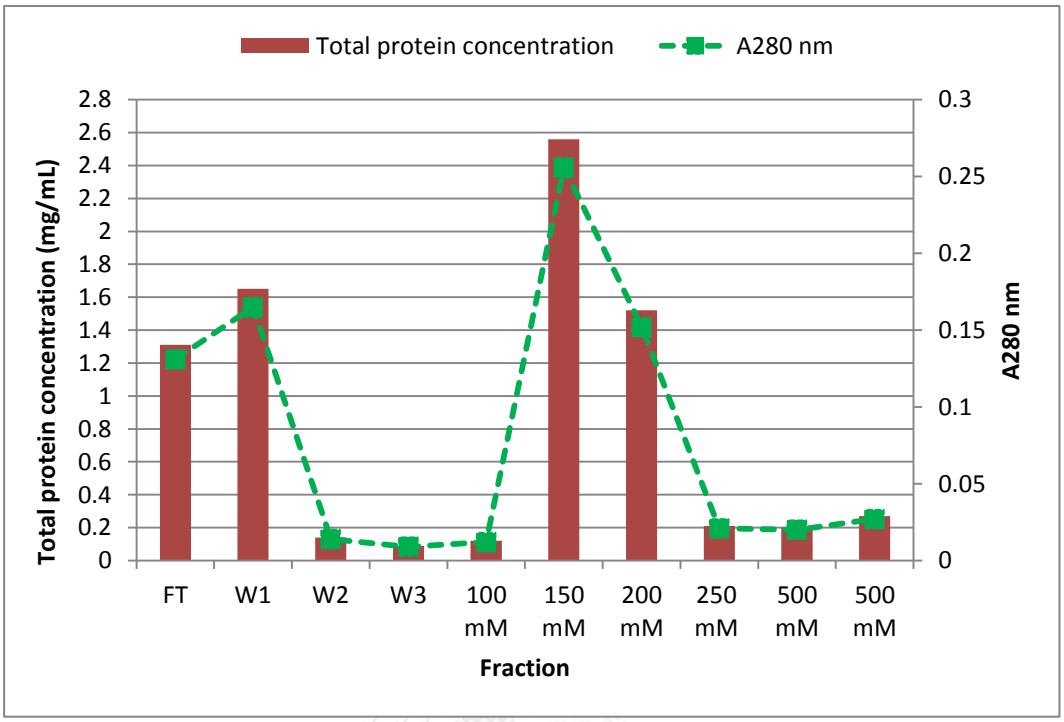

Figure 4.44 The protein concentration (mg/mL) of each eluted fractions determined by A 280 nm. F: flow-through, W: wash. 100mM, 150mM, 200mM, 250mM and 500mM related to the concentration of imidazole in elution buffer.

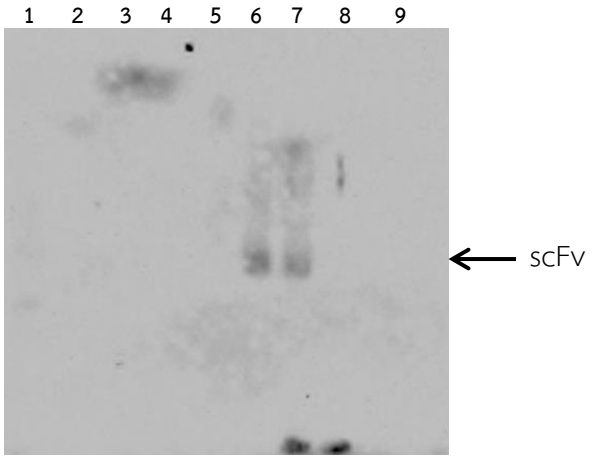

Figure 4.45 Western blotting of the purified recombinant scFv antibody expressed in *P. pastoris* O5 after Ni-NTA affinity column using mouse anti- c-myc antibody and GAM-HRP for detection.

Lane 1: Flow through fraction

Lane 2: Wash I fraction

Lane 3: Wash II fraction

Lane 4: Wash III fraction

Lane 5: 100 mM imidazole elution fraction

Lane 6: 150 mM imidazole elution fraction

Lane 7: 200 mM imidazole elution fraction

Lane 8: 250 mM imidazole elution fraction

Lane 9: 500 mM imidazole elution fraction

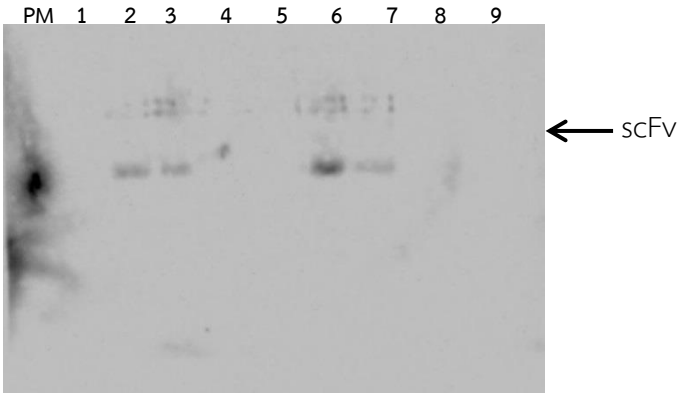

Figure 4.46 Western blotting of the purified recombinant scFv antibody expressed in *P. pastoris* O5 after Ni-NTA affinity column using mouse anti- c-myc antibody and GAM-HRP for detection.

Lane 1, 5: 100 mM imidazole elution fraction

Lane 2, 6: 150 mM imidazole elution fraction

Lane 3, 7: 200 mM imidazole elution fraction

Lane 4, 8: 250 mM imidazole elution fraction

Lane 5, 9: 500 mM imidazole elution fraction

Lane PM: Protein marker

4.15 Determination of Recombinant scFv Binding Activity

4.15.1 Determination of Recombinant scFv Binding Activity by Indirect ELISA

To examine functionality of the purified recombinant scFv antibody, its binding activity was assayed by an indirect ELISA and compared to their parental mAb Nor155.

Since the recombinant scFv was expressed as fusion protein, theyr contained the c-myc tag and hexa-histidine tag at the C-terminus for detection and purification. Therefore, to detect the binding ability of purified recombinant scFv antibody to its antigen (norfloxacin), the mouse anti-c-myc antibody as primary antibody and HRP-conjugated goat anti-mouse antibody were employed in the detection.

In the indirect binding assay, the scFv reacted to its antigen (norfloxacin) in a concentration-dependent manner (Table 4.11). The result showed that there was significant difference between the detection results by the purified recombinant scFv antibody and by their parental mAb nor155. The result demonstrated that the affinity of purified recombinant scFv antibody was lower than that of its parental mAb Nor155. This was probably due to the structure and folding of recombinant scFv antibody, which interferes slightly the formation of the antigen binding sites. Further investigations are necessary to clarify the binding properties of the recombinant scFv. However, these results indicated that the scFv still remain function in term of antigen binding ability. Moreover, it was also indicated that this recombinant scFv was successfully expressed by *P. pastoris*.

Table 4.11 the binding activities of recombinant scFv antibody and their parental mAb Nor155 assayed by Indirect ELISA

Concentration (ng/mL)	Monoclonal antibody Nor155			Purified recombinant scFv		
	OD 450		Average	OD 450		Average
	No.1	No.2		No.1	No.2	
7420.00	4.0778	4.1845	4.1312±0.075	0.4948	0.4966	0.4957±0.001
742.00	4.2010	4.0847	4.1429±0.082	0.4542	0.4515	0.4529±0.002
371.00	4.0124	4.1242	4.0683±0.079	0.4338	0.4394	0.4366±0.001
185.50	3.9973	3.3455	3.6714±0.461	0.3737	0.3940	0.3821±0.012
92.75	3.3466	3.1795	3.2631±0.118	0.3514	0.3714	0.3614±0.014
46.40	3.1135	3.1393	3.1264±0.018	0.2455	0.2602	0.2529±0.010
23.20	2.8137	2.797	2.8054±0.012	0.2499	0.2227	0.2363±0.019
0	0.1663	0.165	0.1657±0.001	0.0953	0.0765	0.0859±0.013

The data is shown in mean ± standard deviation

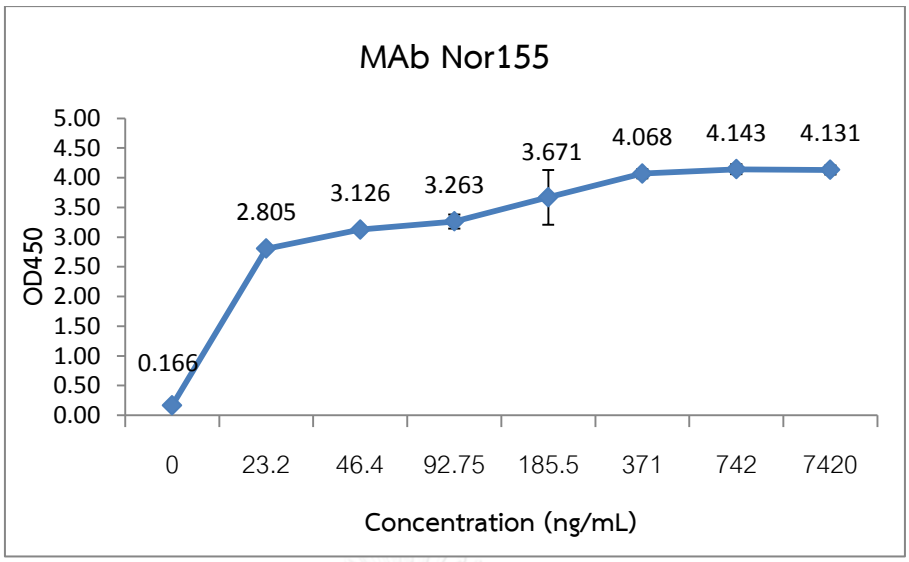

Figure 4.47 Indirect ELISA showed the binding of the mAb Nor155 to their antigen (norfloxacin) at different concentration (0 – 7420 ng/mL). Remarked that, the concentration at 0 ng/mL means that PBS buffer was instead of the antibody in the ELISA conditions as control. The concentration of coated antigen was 1 µg/mL.

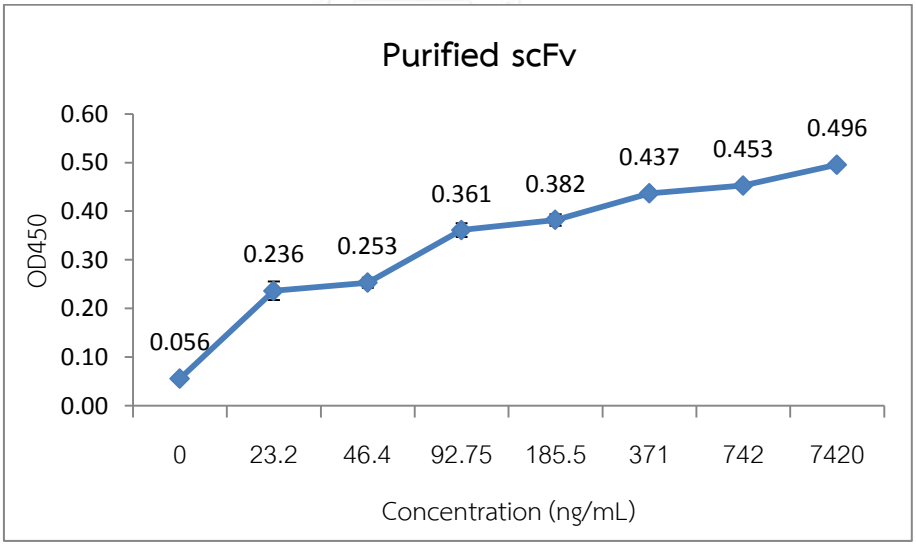

Figure 4.48 Indirect ELISA showed the binding of the purified recombinant scFv antibody to their antigen (norfloxacin) at different concentration (0 – 7420 ng/mL). Remarked that, the concentration at 0 ng/mL means that PBS buffer was instead of the antibody in the ELISA conditions as control. The concentration of coated antigen was 1 µg/mL.

4.15.2 Determination of Recombinant scFv Binding Activity by SPR

The affinity of the purified recombinant scFv was confirmed by surface plasmon resonance (SPR) on the Autolab. Since norfloxacin in the reaction buffer binds to the purified recombinant scFv as ligand, the accumulation of protein on the surface results in an increase in the refractive index. This change in refractive index is transformed into the sensorgram plotted as angle shift (mDegree) versus time, which is a continuous, real-time monitoring of the association and dissociation of the interacting molecules. The sensorgram provides the information in real-time on specificity of binding and affinity. The values of SPR angle shift was recorded for 40 min after the final wash (baseline constant).

As depicted in Figure 4.48 and Figure 4.49, a sensorgram showed the binding interaction between the purified recombinant scFv antibody, which was immobilized on the gold surface and the injected norfloxacin (as an antigen) in the flow cell. It was found that both scFv and parental mAb showed strong binding to norfloxacin which revealed the average angle shift of 579.85±133.27 mDegree (Table 4.12). While the mAb Nor155 showed the specific-dependent binding to norfloxacin with the angle shift of 505.1±28.28 mDegree. This result indicated that there was no significant different between the detection result by the purified recombinant scFv antibody and by their parental mAb Nor 155.

Moreover, the binding activity of the purified recombinant scFv antibody to other FQs such as ciprofloxacin and enrofloxacin was also examined. The results illustrated that the binding ability of scFv to enrofloxacin was lower than that to norfloxacin. In addition, their binding activity to ciprofloxacin showed the lowest value of the angle shift at only 36.68±19.01 mDegree.

The results of norfloxacin detection suggested that this recombinant scFv antibody could be used to screen the norfloxacin residues in real sample detection.

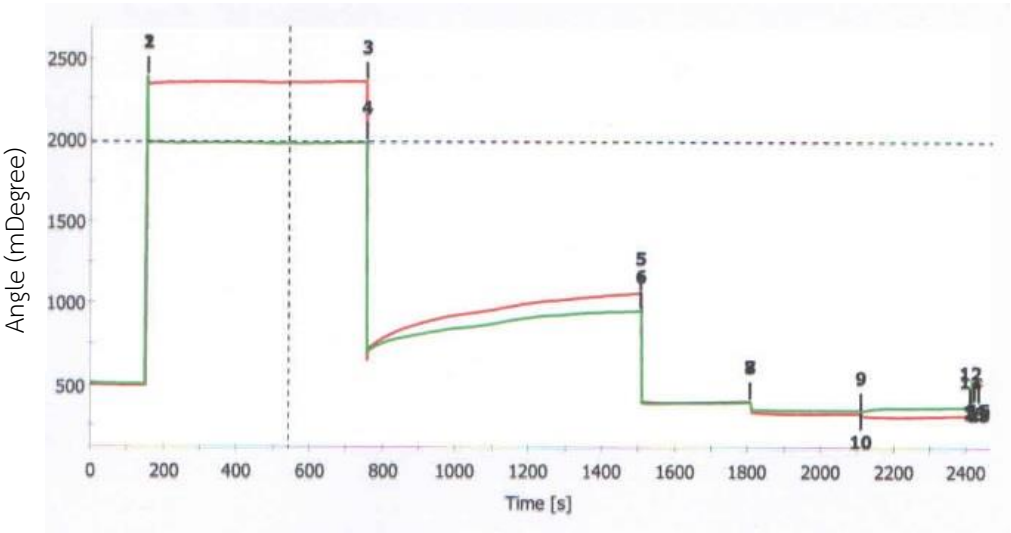

Figure 4.49 SPR sensorgram illustrated the binding interaction after the injection of norfloxacin at 1 mg/mL over the immobilized purified recombinant antibody at 7 µg/mL in channel 1 and channel 2. The numbers showed in sensorgram represented following: number 1 and 2; association phase. Number 3 and 4; Dissociation phase. Number 5 and 6; regeneration phase. Number 7 to 12: base line.

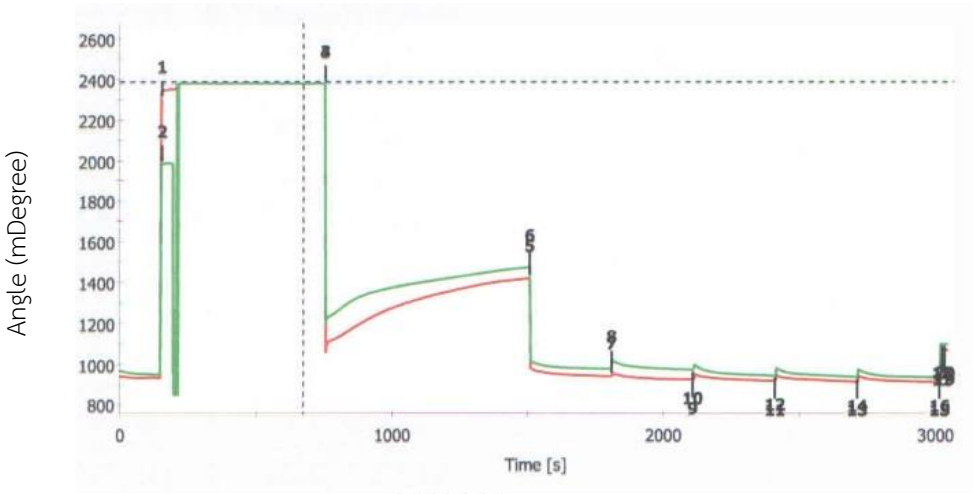

Figure 4.50 SPR sensorgram illustrated the binding interaction after the injection of norfloxacin at 1 mg/mL over the immobilized MAb Nor155 at 7 µg/mL in channel 1 and channel 2. The numbers showed in sensorgram represented following: number 1 and 2; association phase. Number 3 and 4; Dissociation phase. Number 5 and 6; regeneration phase. Number 7 to 12: base line.

Table 4.12 Summarization of binding activity of purified recombinant scFv antibody to their antigen (norfloxacin) and other FQs (enrofloxacin and ciprofloxacin)

Antibody* **(7µg/mL)**	**Antigen**** **(1mg/mL)**	**Channel**	**Angle shift (mDegree)**			**Angle shift (mDegree) Average ± SD**
			Rep.1	**Rep.2**	**Rep.3**	
Purified scFv	Norfloxacin	Ch.1	560.5	544.3	682.9	579.85±133.27[a]
		Ch.2	442.1	461.0	788.3	
	Enrofloxacin	Ch.1	421.7	422.1	-	394.08±50.77[b]
		Ch.2	414.4	318.1	-	
	Ciprofloxacin	Ch.1	53.9	59.3	40.0	36.68±19.01[c]
		Ch.2	9.0	21.8	36.1	
MAb Nor155	Norfloxacin	Ch.1	485.1	-	-	505.1±28.28[a]
		Ch.2	525.1	-	-	

* Immobilization on bare gold surface

** Injection into flow cell

a, b and c : statistical analysis (Appendix H)

CHAPTER V
CONCLUSIONS

In this study, the construction, expression, purification and characterization of a recombinant scFv antibody against norfloxacin have been successfully carried out.

The variable regions of the heavy and light chain (402 bp and 363 bp, respectively) amplified from the mouse antinorfloxacin-producing hybridoma cells (Nor155) were assembled with the $(GGGGS)_3$ linker DNA fragment and cloned into pPICZαA to construct pPICZαA-scFv, namely pJM01 (4354 bp) plasmid.

The expression cassette for pJM01 plasmid was driven by *AOX1* promoter contains *Zeocin* resistance gene.

In pJM01 plasmid, the scFv gene was inserted in-frame between N-terminal α-factor secretion signal sequence and C-terminal hexahistidine tag of pPICZαA vector.

The scFv has 800 nucleotides encoding 266 amino acids including a flexible amino acid linker of $(Gly_4Ser)_3$, c-myc tag and His_6 tag polypeptide with a predicted molecular weight of 32.67 kDa.

Amino acid numbering and complementarity determining regions (CDRs) of the V_H and V_L domains were determined. The gene segment families of V_H and V_L were identified by search for similarities and confirmed by the alignment with sequences of GenBank database. Accordingly, the variable regions of the heavy chain were determined to be IgV_H, while those of the light chain were IgV_L_kappa type.

The electroporation and commercial solution kit were used to transfer pJM01 to the *Pichia* chromosome. Transformants were confirmed by Southern blot analysis, PCR and sequencing analysis.

Transgenic O5 strain was selected as the best produced scFv. The mRNA transcription level of O5 strain was observed by RT-qPCR and showed 688 times highly relative normalized expression at 24 h induction with methanol compared to control.

The expression of the scFv was analyzed by SDS-PAGE as well as by Western blot analysis using mouse anti-c-myc antibody and GAM-HRP antibody. A predominant protein at 32.67 kDa, corresponding to the expected molecule size of scFv was observed.

The recombinant scFv proteins were purified by immobilized metal affinity chromatography (IMAC) from the soluble fraction of flask cultures using the hexahistidine tail presented at the C-terminus of the scFv. The purified 32.67 kDa scfv was confirmed by Western blot analysis. The purified proteins were used for measurement of the affinity and specificity against norfloxacin in the subsequent experiments.

Indirect ELISA was used for exploring the sensitivity and specificity of this scFv antibody. The result indicated that the purified scFv possessed desirable affinity and specificity to norfloxacin and still remained functionality in term of antigen binding. In addition, it was also indicated that this functionalized scFv antibody was successfully expressed by *P. pastoris*.

To confirm functionality of recombinant scFv, its binding activity was assayed by SPR and compared to their parental MAb Nor155 activity. The recombinant scFv showed strong binding to norfloxacin with $P>0.05$.

Taken together, use of recombinant scFv antibody form of monoclonal antibody offers an excellent alternative to the whole antibodies for determining their binding characteristics. The scFv could be used for the future development of sensitive and affordable immunoassays for detecting FQs in biological matrices.

Suggestions

1. To produce the large amounts of the functionalized scFv antibody by *P. pastoris*, there are several parameters should be further studied e.g. methanol concentration, inoculum density and cultivation strategy.

2. To improve high specific binding and affinity of scFv antibody, protein engineering should be performed.

3. The major advantages of *AOX1* promoter driven expression are the strength and the tight regulation. However, this requires a two-step cultivation procedure, which increases the process time and handling efforts. For nontoxic proteins, which do not hamper cell growth, a strong constitutive expression system can help to minimize cultivation efforts and lead to a higher space/time yield. Despite the advantages of tightly regulated *AOX1* promoter driven expression, the induction with methanol (a toxic and flammable compound) was considered problematic for safety reasons, especially for large-scale production. Furthermore, the strong expression from *AOX1* promoter is not always the most favorable condition for heterologous protein. To close this gap, *HpTEF1* promoter form *H. polymorpha* is suggested. Because of *HpTEF1* promoter is the constitutive expression, no induction step is needed which shortens and simplifies the process time for protein production.

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APPENDICES

APPENDIX A

Culture Media

1. Low salt LB medium

- 1% Tryptone
- 0.5% Yeast extract
- 0.5% Sodium chloride (NaCl)
- 2% Agar (Solid medium)

Adjust the pH value to 7.5 with 1 N NaOH and sterilized by autoclave at 121°C, 15 $lb.in^{-2}$ for 20 minutes. In case of Low salt LB with Zeocin™, cool the solution to ~ 50°C and add Zeocin™ to a final concentration of 25 $\mu g.mL^{-1}$ and store at +4°C in the dark.

2. Luria-Bertani (LB) medium

- 1% Tryptone
- 0.5% Yeast extract
- 1% Sodium chloride (NaCl)
- 2% Agar (Solid medium)

Adjust the pH value to 7.5 with 1 M NaOH and sterilized by autoclave at 121°C, 15 $lb.in^{-2}$ for 20 minutes. Store at 4°C.

3. Minimal Methanol Histidine (MMH) medium

- 1.34% YNB (Yeast nitrogen base w/o amino acid w/ ammonium sulfate) *
- 4×10^{-5}% Biotin *
- 0.004% Histidine *
- 0.5% Methanol

* Biotin, Histidine and YNB were dissolved in sterilized double distilled water and sterilized by filtration (pore size 0.22μm, mixed cellulose esters membrane). Store at 4°C.

Stock solution preparation

- 10×YNB; 13.4% YNB w/o amino acid w/ ammonium sulfate, 100 mL
  - YNB W/o amino acid W ammonium sulfate powder 13.4 g
  - Dissolved with sterilized double distilled water and adjust the volume to 100 mL using volumetric flask. Sterilized by filtration (mixed cellulose esters membrane, pore size 0.22 µm) and stored at +4°C.
- 500×Biotin; 0.02% Biotin, 50 mL
  - Biotin powder 10 mg
  - Dissolved with sterilized double distilled water and adjust the volume to 50 mL using volumetric flask. Sterilized by filtration (mixed cellulose esters membrane, pore size 0.22 µm) and stored at +4°C.
- 100×Histidine; 0.4% Histidine, 100 mL
  - Histidine powder 0.4 g
  - Dissolved with sterilized double distilled water and adjust the volume to 100 mL using volumetric flask. Sterilized by filtration (mixed cellulose esters membrane, pore size 0.22 µm) and stored at +4°C.
  -

- Compositions of MMH medium from stock solution above, for 1 L
  - 10×YNB 100 mL
  - 500×Biotin 2 mL
  - 100×Histidine 10 mL
  - Absolute methanol 5 mL
  - Sterilized double distilled water 883 mL (Autoclaved)

    Aseptically mix the solutions above in a biohazard cabinet.

4. Yeast Peptone Dextrose (YPD) medium

- 1% Yeast extract
- 2% Peptone
- 2% Dextrose (Glucose)

- 2% Agar (Solid medium)

Sterilized by autoclave at 121°C, 15 lb.in^{-2} for 20 minutes. In case of YPD with Zeocin™, add Zeocin™ to a final concentration of 100 µg.mL^{-1} and store at +4°C in the dark.

5. Yeast Peptone Glycerol (YPG) medium

- 1% Yeast extract
- 2% Peptone
- 1% Glycerol

Sterilized by autoclave at 121°C, 15 lb.in^{-2} for 20 minutes. Store at +4°C

APPENDIX B

Chemical Solutions Preparation

1. 1% Agarose for agarose gel electrophoresis

- Agarose (molecular grade) 1 g
- 10×TAE buffer solution 10 mL
- Double distilled water 90 mL

Melt the agarose solution by microwave oven until the gel completely melted. Cool the solution to +50°C and pour the gel to the gel boxes, inserts the combs and wait for the gel has been set.

2. 0.1 M $CaCl_2$ for fresh competent *E. coli* preparation

- $CaCl_2.H_2O$ powder (MW = 147.02 g.mole^{-1}) 14.702 g
- Dissolved in double distilled water and adjust the volume to 100 mL using volumetric flask. The solution was sterilized by autoclave and stored at +4°C.

3. 1 M DTT, Yeast competent cells preparation

- DTT powder (MW = 154.25 g.mole^{-1}) 0.2314 g
- Dissolved in sterilized double distilled water and adjust the volume to 1.5 mL and sterilize by filtration (pore size 0.22 µm). Store at 0°C.

4. 0.25% Glutaraldehyde (Fixing solution for dot-blot analysis)

- Glutaraldehyde 50% (w/v) in water 0.5 mL
- Double distilled water 99.5 mL

5. 1 M HEPES buffer (pH 8.0), Yeast competent cells preparation

- HEPES free acid powder (MW = 238.30 g.mole^{-1}) 2.383 g
- Dissolved in sterilized double distilled water and adjust the pH value to 8.0 with 5 M NaOH. Adjust the volume to 10 mL and sterilize by filtration (pore size 0.22 µm). Store at +4°C.

6. Lysis by alkali for plasmid extraction

Solution I

- 50 mM glucose (Glucose monohydrate; MW = 198.17 g.mole^{-1})
- 25 mM Tris·Cl (pH 8.0), (Tris base; MW = 121.1 g.mole^{-1})
- 10 mM EDTA (pH 8.0), (EDTA; MW = 292.25 g.mole^{-1})

Solution I was prepared in batches of approximately 100 mL, autoclaved for 15 minutes and stored at +4°C.

Stock solution preparation

- 1 M Glucose, 100 mL
  - Glucose monohydrate powder 19.817 g
  - Adjust the volume to 100 mL by sterilized double distilled water using volumetric flask. Stored at +4°C.
- 0.5 M Tris·Cl (pH 8.0), 100 mL
  - Tris·base powder 6.055 g
  - Dissolved with sterilized double distilled water and adjust the pH to 8.0 with 1 M HCl. Adjust the volume to 100 mL by double distilled water using volumetric flask. Stored at +4°C.
- 0.5 EDTA (pH 8.0), 100 mL
  - EDTA powder 14.6125 g
  - Dissolved with sterilized double distilled water and adjust the pH to 8.0 with 5 M NaOH. Adjust the volume to 100 mL by double distilled water using volumetric flask. Stored at +4°C.
- Compositions of Solution I from stock solution above
  - 1 M Glucose 5 mL
  - 0.5 M Tris•Cl (pH 8.0) 5 mL
  - 0.5 EDTA (pH 8.0) 2 mL
  - Adjust the volume with double distilled water to 100 mL

Solution II (freshly prepared before use)

- 0.2 N NaOH (NaOH; MW = 40 g.mole^{-1})
- 1% SDS (SDS; MW = 288.38 g.mole^{-1})

Stock solution preparation

- 2 N NaOH, 100 mL
  - NaOH pellet 4 g
  - Dissolved with sterilized double distilled water and adjust the volume to 100 mL using volumetric flask and stored at +4°C.
- 10% SDS, 100 mL
  - SDS powder 10.00 g
  - Dissolved with sterilized warm double distilled water and adjust the volume to 100 mL and stored at +4°C.
- Compositions of Solution II from stock solution above
  - 2 N NaOH 1 mL
  - 10% SDS 1 mL
  - Sterilized double distilled water 8 mL

Solution III

- 5 M Potassium acetate (MW = 98.14 g.mole^{-1}) 60 mL
- Glacial acetic acid 11.5 mL
- Double distilled water 28.5 mL

Stock solution preparation

- 5 M Potassium acetate, 100 mL
  - Potassium acetate powder 49.07 g
  - Dissolved with double distilled water and adjust the volume to 100 mL using volumetric flask. Sterilized by autoclave and stored at +4°C.

- Compositions of Solution III from stock solutions above
  - 5 M Potassium acetate (Sterilized) 60 mL
  - Glacial acetic acid 11.5 mL
  - Sterilized double distilled water 28.5 mL

7. 0.01 M Phosphate Buffer Saline (PBS), pH 7.4

- 200 mM Phosphate buffer, pH 7.4 1 L
- Sodium chloride (NaCl) 175.2 g
- Double distilled water 18 L

Stock solution preparation

- Stock solutions, 200 mM each for 1 L
  - Stock A: $NaH_2PO_4.H_2O$ (MW = 137.99 g.mole^{-1}) 27.6 g
  - Stock B: $Na_2HPO_4.12H_2O$ (MW = 358.135 g.mole^{-1}) 71.63 g
  - Each $NaH_2PO_4.H_2O$ and $Na_2HPO_4.12H_2O$ powder was separately dissolved in double distilled water and adjusts the volume to 1 L using volumetric flask. Stored at +4°C.
- 200 mM Sodium phosphate buffer (pH 7.4), 1 L
  - Stock A (774 mL) and B (226 mL) were mixed together and adjust the pH value to 7.4 by titration with 5 M HCl. Store at room temperature.

8. 0.05% PBS-Tween 20 or PBST (Washing buffer for dot-blot analysis and ELISA)

- Tween 20 0.5 mL
- 0.01 M Phosphate buffer saline (PBS), pH 7.4 1000 mL

9. 200 mM Potassium citrate buffer for ELISA, pH 4.0

- Stock A: 200 mM Citric acid monohydrate (MW= 210.14 g.mole^{-1})
- Stock B: 200 mM Potassium citrate (MW= 324.41 g.mole^{-1})

Each citric acid and potassium citrate powder was dissolved in double distilled water and adjusts the volume to 250 mL using volumetric flask. Stock A (200 mM citric acid) was titrated with stock B (200mM potassium citrate) until the pH value equal to 4.0. The solution was stored at +4°C in the dark.

10. 5% Skim milk in PBS buffer (Blocking buffer for dot-blot analysis and ELISA)

- Skim milk powder 5 g
- 0.01 M Phosphate buffer saline (PBS), pH 7.4 100 mL

11. 1 M D-Sorbitol

- D-Sorbital (D-Sorbital; MW = 182.18 g.mole^{-1}) 18.2 g
- Dissolved in sterilized double distilled water and adjust the volume to 100 mL using volumetric flask. Sterilized by filtration (0.22µm mixed cellulose esters membrane) and store at +4°C in the dark.

12. TMB substrate (for indirect competitive ELISA)

- TMB powder (3,3',5,5'-tetramethylbenzidine) 2.5 mg
- 200 mM potassium citrate buffer (pH 4.0) 10 mL
- 30% H_2O_2 solution 3.5 µL

TMB powder was completely dissolved in 200 mM potassium citrate buffer follow by adding 30% H_2O_2 solution.

13. Tris-EDTA (TE) buffer pH 8.0

- 10 mM Tris·Cl (pH 8.0); Stock solution 0.5 M Tris·Cl (pH 8.0) from Solution I
- 1 mM EDTA (pH 8.0); Stock solution 0.5 M EDTA (pH 8.0) from Solution I
  - Compositions of TE buffer (pH 8.0) from stock solutions above
    - 0.5 M Tris•Cl (pH 8.0) 20 mL
    - 0.5 EDTA (pH 8.0) 2 mL
    - Adjust the volume with double distilled water to 1000 mL

Sterilize by autoclave and store at +4°C.

APPENDIX C

Characterization of Norfloxacin-Producing Hybridoma Clones

Table 1 The IC_{50} of various monoclonal antibodies against norfloxacin (μg/mL)

Types	**Nor#7**	**Nor#119**	**Nor#132**	**Nor#155**	**Nor#49**
	1:16	1:50	1:2	1:8	1:2
	IgG2a	IgG2b	IgG1	IgG1	IgG1
Norfloxacin	0.62	0.08	4.92	0.42	0.91
Nalidixic acid	0.57	0.67	0.60	0.45	6.00
Enrofloxacin	1.01	0.10	6.31	0.47	7.34
Flumequin	0.77	<0.01	0.78	0.02	0.53
Cinoxacin	<0.01	>10	0.04	0.44	>10
Ofloxacin	8.88	0.35	>10	0.50	0.96
Oxolinic acid	0.58	<0.01	0.51	0.90	1.00
Pipemedic acid	0.84	6.25	4.59	0.7	>10
Enoxacin	0.84	0.57	5.76	0.33	5.35
Ciprofloxacin	5.14	0.67	6.32	0.48	6.25

[97]

Table 2 The %cross-reactivity of various monoclonal antibodies against norfloxacin

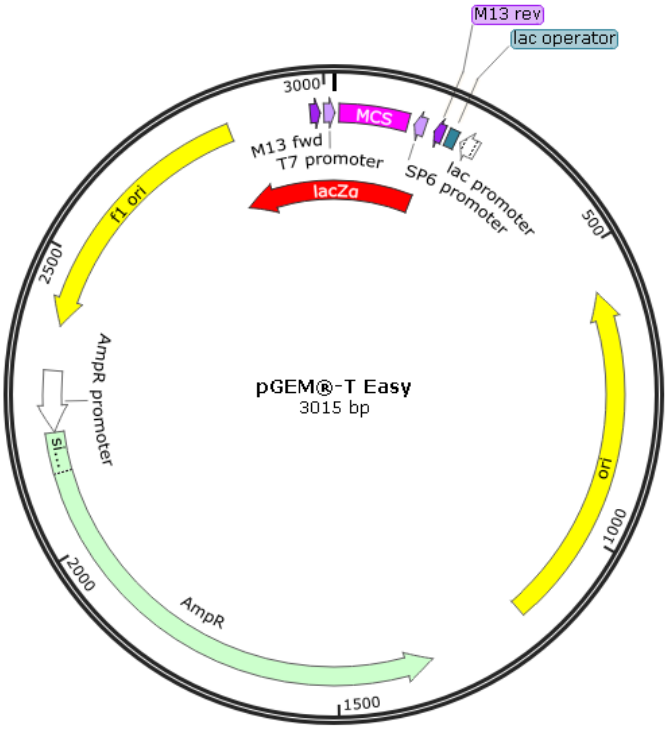
Types	Nor#7	Nor#119	Nor#132	Nor#155	Nor#49
	1:16	1:50	1:2	1:8	1:2
	IgG2a	IgG2b	IgG1	IgG1	IgG1
Norfloxacin	100	100	100	100	100
Nalidixic acid	109	12	820	93	15
Enrofloxacin	61	80	78	89	12
Flumequin	81	>800	631	2100	172
Cinoxacin	>6200	<0.8	12300	95	<9
Ofloxacin	7	23	<49	84	95
Oxolinic acid	107	1333	965	47	91
Pipemedic acid	74	1	107	60	<9
Enoxacin	74	14	85	127	17
Ciprofloxacin	12	12	78	88	15

[97]

APPENDIX D

Vectors

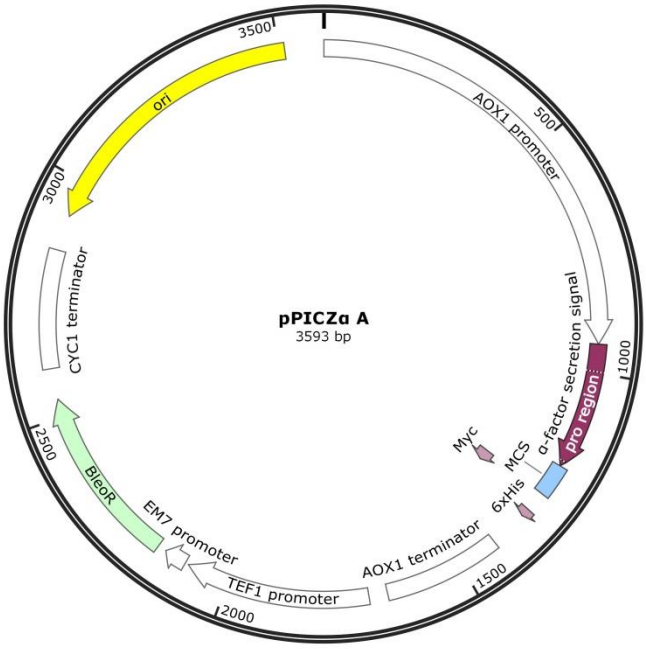
1. pGEM-T easy vector



Feature Name	Location	Size (bp)	Type
MCS	10...128	119	misc_feature
SP6 promoter	140...158	19	promoter
M13 rev	176...192	17	primer_bind
lac operator	200...216	17	protein_bind
lac promoter	224...254	31	promoter
ori	578...1166	589	rep_origin
Amp^R	1337...2197	861	CDS
AmpR promoter	2198...2302	105	promoter
f1 ori	2380...2835	456	rep_origin
lacZ-alpha	2812...180	384	CDS
M13 fwd	2976...2992	17	primer_bind
T7 promoter	2999...2	19	promoter

2. pPICZαA vector

2. pPICZαA vector



Feature Name	Location	Size (bp)	Type
AOX1 promoter	2...940	939	promoter
alpha-factor secretion signal	941...1207	267	CDS
MCS	1208...1276	69	misc_feature
Myc	1275...1304	30	CDS
6xHis	1320...1337	18	CDS
AOX1 terminator	1417...1663	247	terminator
TEF1 promoter	1703...2089	387	promoter
EM7 promoter	2097...2144	48	promoter
$Bleo^R$	2163...2537	375	CDS
CYC1 terminator	2603...2850	248	terminator
ori	2925...3513	589	rep_origin

Appendix E

Sequences Submission

1. Immunoglobulin heavy chain variable region, partial [*Mus musculus*]

GenBank: AJG06889.1

LOCUS AJG06889 134 aa linear ROD 24-MAR-2015

DEFINITION immunoglobulin heavy chain variable region, partial [*Mus musculus*].

ACCESSION AJG06889

VERSION AJG06889.1 GI:752273089

DBSOURCE accession KJ623260.1

KEYWORDS .

SOURCE *Mus musculus* (house mouse)

ORGANISM *Mus musculus*
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia; Sciurognathi; Muroidea; Muridae; Murinae; Mus; Mus.

REFERENCE 1 (residues 1 to 134)

AUTHORS Mala,J., Puthong,S., Komolpis,K., Sooksai,S. and Palaga,T.

TITLE Monoclonal antibody against enrofloxacin, VHnor155-9

JOURNAL Unpublished

REFERENCE 2 (residues 1 to 134)

AUTHORS Mala,J.

TITLE Direct Submission

JOURNAL Submitted (25-MAR-2014) The Institute of Biotechnology and Genetics Engineering, Chulalongkorn University, Institute Building 3, 5th Floor, Phayatai, Phatumwan, Bangkok, Bangkok 10330, Thailand

FEATURES Location/Qualifiers

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/db_xref="taxon:10090"
/clone="Nor155-9"
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      121 vssakttpps vyef
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```

2. *Mus musculus* clone nor155-12 anti-norfloxacin MAb light chain variable region mRNA, partial cds

GenBank: KR261578.1

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LOCUS       KR261578                363 bp    mRNA    linear   ROD 09-SEP-
2015
DEFINITION  Mus musculus clone nor155-12 anti-norfloxacin MAb light chain
            variable region mRNA, partial cds.
ACCESSION   KR261578
VERSION     KR261578.1  GI:925216692
KEYWORDS    .
SOURCE      Mus musculus (house mouse)
  ORGANISM  Mus musculus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia;
            Sciurognathi; Muroidea; Muridae; Murinae; Mus; Mus.
REFERENCE   1  (bases 1 to 363)
  AUTHORS   Mala,J., Sooksai,S. and Komolpis,K.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-APR-2015) Biotechnology, The Institute of
            Biotechnology and Genetics Engineering, Chulalongkorn
           University,Institute Building 3, 5th Floor, Phayatai, Phatumwan,
           Bangkok 10330, Thailand
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /clone="nor155-12"
                     /country="Thailand"
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ORIGIN

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      121 aaggcaggat cctcccccaa accctggatt tatgccacat ccaacctggc ttctggagtc
      181 cctgatcgct tcagtggcag agggtctggg acctcttact ctctcacaat cagcagagtg
      241 gaggctgaag atgctgccac ttattactgc cagcagtgga gtaataaccc gtggacgttc
      301 ggtggaggca ccaagctgga aatcaaacgg gctgatgctg caccaactgt atccgagctc
      361 ccc
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//

3. Anti-norfloxacin scFv antibody, partial [synthetic construct]

GenBank: AJG06891.1

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LOCUS       AJG06891                263 aa            linear   SYN 04-MAY-
2015
DEFINITION  anti-norfloxacin scFv antibody, partial [synthetic construct].
ACCESSION   AJG06891
VERSION     AJG06891.1  GI:752273093
DBSOURCE    accession KP170507.1
KEYWORDS    .
SOURCE      synthetic construct
  ORGANISM  synthetic construct
            other sequences; artificial sequences.
REFERENCE   1  (residues 1 to 263)
  AUTHORS   Mala,J., Sooksai,S., Puthong,S., Komolpis,K., Palaka,T. and
            Yoshinobu,K.
  TITLE     Cloning and expression of scFv fragment of monoclonal antibody
            against norfloxacin in Pichia pastoris
  JOURNAL   Unpublished
REFERENCE   2  (residues 1 to 263)
  AUTHORS   Mala,J., Sooksai,S., Puthong,S., Komolpis,K., Palaka,T. and
            Yoshinobu,K.
  TITLE     Direct Submission
  JOURNAL   Submitted (15-NOV-2014) Biotechnology, The Institute of
            Biotechnology and Genetics Engineering, Chulalongkorn
           University,Institute Building 3, 5th Floor, Phayatai, Phatumwan,
            Bangkok, Bangkok 10330, Thailand
FEATURES             Location/Qualifiers
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                     /clone="mAb#nor115"
                     /PCR_primers="fwd_name: fw-scfvnor115, fwd_seq:
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                     rev_seq: cgaggtaccgggagctcggatacagttggt"
                     /note="from Mus musculus"
     Protein         <1..>263
                     /product="anti-norfloxacin scFv antibody"
                     /name="single chain variable fragment"
     Region          4..120
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                     domain; cd04981"
                     /db_xref="CDD:143182"
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                     /note="intrachain domain interface"
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                     /note="L1 hypervariable region"
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                     /note="antigen binding site"
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                     /note="heterodimer interface [polypeptide binding]"
                     /db_xref="CDD:143182"
     Site            73..78
                     /site_type="other"
                     /note="L2 hypervariable region"
                     /db_xref="CDD:143182"
     Site            order(99,108..110)
                     /site_type="other"
                     /note="L3 hypervariable region"
                     /db_xref="CDD:143182"
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                     /note="Immunoglobulin domain; cl11960"
                     /db_xref="CDD:276006"
     Region          156..250
                     /region_name="IG_like"
                     /note="Immunoglobulin like; smart00410"
                     /db_xref="CDD:214653"
     CDS             1..263
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      121 sakttppsvy ggggsggggs ggggsdivmt lstailsasp gekvtmtcra ssrvnyihwf
      181 qqkagsspkp wiyatsnlas gvpdrfsgrg sgtsysltis rveaedaaty ycqqwsnnpw
      241 tfgggtklei kradaaptvs elp
//
```

APPENDIX F

Determination of Purified Recombinant scFv Antibody Concentration

1. Preparation of diluted albumin (BSA) standards

Use Table 1 as a guide to prepare a set of protein standard. Dilute the contents of one Albumin Standard (BSA) ampule into several clean vials, preferably using the same diluent as the sample(s). Each 1 ml ampule of 2 mg/ml Albumin Standard is sufficient to prepare a set of diluted standards for either working range suggested in Table 1. There will be sufficient volume for three replications of each diluted standard.

Table 1. Preparation of Diluted Albumin (BSA) Standards

Dilution Scheme for Standard Microplate Procedure (Working Range =5-250 µg/mL)			
Well	PBS (µl)	Volume and Source of BSA ((µl)	Final BSA Concentration (µg/ml)
A	700	100 of stock	250
B	400	400 of well A dilution	125
C	450	300 of well B dilution	50
D	400	400 of well C dilution	25
E	400	100 of well D dilution	5
F	400	0	0=Blank

2. Preparation of the BCA working reagent (WR)

Prepare WR by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A : B). For the above example, combine 50 ml of reagent A with 1 ml of Reagent B.

3. Microplate procedure (Sample to WR ratio =1:8)

- Pipette 25 µl of each standard and unknown sample replicate into a microplate well.
- Add 200 µl of the WR to each tube and mix well.
- Cover plate and incubate at 60°C for 30 minutes
- Cool all tubes to RT
- Measure the absorbance at or near 562 nm on a plate reader

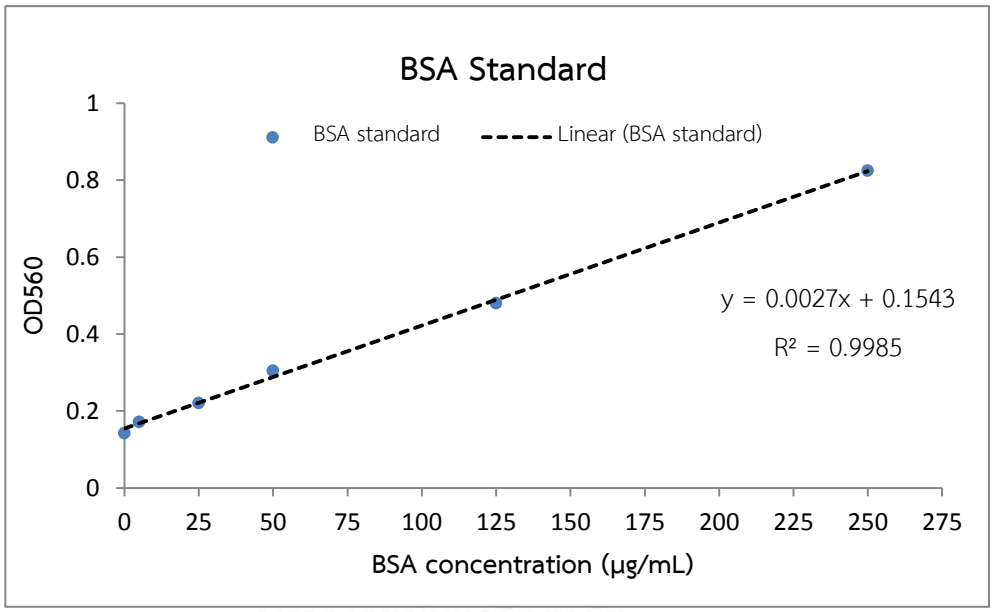

Figure F-1: Standard graph of standard BSA for calculation of the purified recombinant scFv antibody concentration

Standard equation to calculate the MIP concentration;

Y = 0.0027(X) + 0.1543

Description; Y = the optical density at 560 nm

X = the BSA concentration (µg/mL)

APPENDIX G

RT-qPCR Results

1. Plate View

		1	2	3	4	5	6	7	8	9	10	11	12
A	Content Sample Target			Unkn-1 GS115 scFv	Unkn-3 alp-A scFv	Unkn-5 O5Gly scFv	Unkn-7 0.5 h scFv	Unkn-9 24h scFv	Unkn-11 48h scFv	Unkn-13 72h scFv	NTC-15 scFv		
B	Content Sample Target			Unkn-1 GS115 scFv	Unkn-3 alp-A scFv	Unkn-5 O5Gly scFv	Unkn-7 0.5 h scFv	Unkn-9 24h scFv	Unkn-11 48h scFv	Unkn-13 72h scFv	NTC-15 scFv		
C	Content Sample			Unkn-1 GS115 scFv	Unkn-3 alp-A scFv	Unkn-5 O5Gly scFv	Unkn-7 0.5 h scFv	Unkn-9 24h scFv	Unkn-11 48h scFv	Unkn-13 72h scFv	NTC-15 scFv		
D	Content Sample												
E	Content Sample												
F	Content Sample Target			Unkn-2 GS115 actin	Unkn-4 alp-A actin	Unkn-6 O5Gly actin	Unkn-8 0.5 h actin	Unkn-10 24h actin	Unkn-12 48h actin	Unkn-14 72h actin	NTC-16 actin		
G	Content Sample Target			Unkn-2 GS115 actin	Unkn-4 alp-A actin	Unkn-6 O5Gly actin	Unkn-8 0.5 h actin	Unkn-10 24h actin	Unkn-12 48h actin	Unkn-14 72h actin	NTC-16 actin		
H	Content Sample Target			Unkn-2 GS115 actin	Unkn-4 alp-A actin	Unkn-6 O5Gly actin	Unkn-8 0.5 h actin	Unkn-10 24h actin	Unkn-12 48h actin	Unkn-14 72h actin	NTC-16 actin		

Note:

GS115: untransformed GS115 strain

Alp-A: GS115 transformed with the empty vector, pPICZαA

O5Gly: transgenic O5 strain grew in YPG medium (Production phase)

0.5h, 24h, 48h and 72h: transgenic O5 strain grew in MMH medium (Induction phase)

2. End point Results

Well	Fluor	Content	Sample	End RFU
A03	SYBR	Unkn-01	GS115	1340.18
A04	SYBR	Unkn-03	alp-A	1502.29
A05	SYBR	Unkn-05	O5Gly	4091.82
A06	SYBR	Unkn-07	0.5 h	4364.23
A07	SYBR	Unkn-09	24h	4540.16
A08	SYBR	Unkn-11	48h	4110.57
A09	SYBR	Unkn-13	72h	4263.20
A10	SYBR	NTC-15		2109.90
B03	SYBR	Unkn-01	GS115	1452.41
B04	SYBR	Unkn-03	alp-A	1586.00
B05	SYBR	Unkn-05	O5Gly	4416.14
B06	SYBR	Unkn-07	0.5 h	4378.10
B07	SYBR	Unkn-09	24h	4848.65
B08	SYBR	Unkn-11	48h	4450.91
B09	SYBR	Unkn-13	72h	4501.67
B10	SYBR	NTC-15		1795.13
C03	SYBR	Unkn-01	GS115	1403.57
C04	SYBR	Unkn-03	alp-A	1686.27
C05	SYBR	Unkn-05	O5Gly	4293.35
C06	SYBR	Unkn-07	0.5 h	4631.69
C07	SYBR	Unkn-09	24h	4862.73
C08	SYBR	Unkn-11	48h	4155.48
C09	SYBR	Unkn-13	72h	4406.13
C10	SYBR	NTC-15		579.66
F03	SYBR	Unkn-02	GS115	3200.25
F04	SYBR	Unkn-04	alp-A	3666.19
F05	SYBR	Unkn-06	O5Gly	3781.99
F06	SYBR	Unkn-08	0.5 h	3702.81
F07	SYBR	Unkn-10	24h	3719.91
F08	SYBR	Unkn-12	48h	3715.04

F09	SYBR	Unkn-14	72h	3869.60
F10	SYBR	NTC-16		2489.29
G03	SYBR	Unkn-02	GS115	3262.75
G04	SYBR	Unkn-04	alp-A	3559.83
G05	SYBR	Unkn-06	O5Gly	3654.37
G06	SYBR	Unkn-08	0.5 h	3365.90
G07	SYBR	Unkn-10	24h	3776.97
G08	SYBR	Unkn-12	48h	3793.19
G09	SYBR	Unkn-14	72h	3736.38
G10	SYBR	NTC-16		909.45
H03	SYBR	Unkn-02	GS115	3344.34
H04	SYBR	Unkn-04	alp-A	3795.08
H05	SYBR	Unkn-06	O5Gly	3768.58
H06	SYBR	Unkn-08	0.5 h	3713.12
H07	SYBR	Unkn-10	24h	3999.46
H08	SYBR	Unkn-12	48h	3867.78
H09	SYBR	Unkn-14	72h	3832.32
H10	SYBR	NTC-16		253.47

Note:

GS115: untransformed GS115 strain

Alp-A: GS115 transformed with the empty vector, pPICZαA

O5Gly: transgenic O5 strain grew in YPG medium (Production phase)

0.5h, 24h, 48h and 72h: transgenic O5 strain grew in MMH medium (Induction phase)

3. Melt Curve Peak Results

Well	Fluor	Target	Content	Sample	Melt Temperature	Peak Height	Begin Temperature	End Temperature
A03	SYBR	scFv	Unkn-01	GS115	None	None	None	None
A04	SYBR	scFv	Unkn-03	alp-A	None	None	None	None
A05	SYBR	scFv	Unkn-05	O5Gly	85.00	623.54	80.50	93.00
A06	SYBR	scFv	Unkn-07	0.5 h	85.00	647.99	79.50	93.00
A07	SYBR	scFv	Unkn-09	24h	85.00	682.46	81.00	94.00
A08	SYBR	scFv	Unkn-11	48h	85.00	607.93	79.00	92.50
A09	SYBR	scFv	Unkn-13	72h	85.00	638.63	79.50	92.00
A10	SYBR	scFv	NTC-15		None	None	None	None
B03	SYBR	scFv	Unkn-01	GS115	None	None	None	None
B04	SYBR	scFv	Unkn-03	alp-A	None	None	None	None
B05	SYBR	scFv	Unkn-05	O5Gly	85.00	669.16	80.00	93.00
B06	SYBR	scFv	Unkn-07	0.5 h	85.00	666.86	77.00	92.00
B07	SYBR	scFv	Unkn-09	24h	85.00	728.65	80.00	94.00
B08	SYBR	scFv	Unkn-11	48h	85.00	667.43	79.00	92.00
B09	SYBR	scFv	Unkn-13	72h	85.00	688.76	80.00	92.00
B10	SYBR	scFv	NTC-15		None	None	None	None
C03	SYBR	scFv	Unkn-01	GS115	None	None	None	None
C04	SYBR	scFv	Unkn-03	alp-A	None	None	None	None
C05	SYBR	scFv	Unkn-05	O5Gly	85.00	659.00	79.00	94.50
C06	SYBR	scFv	Unkn-07	0.5 h	85.00	684.02	79.00	92.00
C07	SYBR	scFv	Unkn-09	24h	85.00	729.19	77.50	92.00
C08	SYBR	scFv	Unkn-11	48h	85.00	617.97	78.00	93.00
C09	SYBR	scFv	Unkn-13	72h	85.00	667.46	77.50	94.00
C10	SYBR	scFv	NTC-15		None	None	None	None
F03	SYBR	Actin	Unkn-02	GS115	82.00	813.80	76.50	89.00
F04	SYBR	Actin	Unkn-04	alp-A	82.00	923.60	76.50	89.50
F05	SYBR	Actin	Unkn-06	O5Gly	82.00	949.18	77.50	94.50
F06	SYBR	Actin	Unkn-08	0.5 h	82.00	923.44	77.00	90.50
F07	SYBR	Actin	Unkn-10	24h	82.00	938.27	77.50	88.50
F08	SYBR	Actin	Unkn-12	48h	82.00	914.49	76.50	87.50
F09	SYBR	Actin	Unkn-14	72h	82.00	959.94	76.50	87.00
F10	SYBR	Actin	NTC-16		82.00	823.88	77.00	87.00
G03	SYBR	Actin	Unkn-02	GS115	82.00	813.56	77.00	88.00
G04	SYBR	Actin	Unkn-04	alp-A	82.00	909.88	76.00	87.00
G05	SYBR	Actin	Unkn-06	O5Gly	82.00	920.30	77.00	92.50

G06	SYBR	Actin	Unkn-08	0.5 h	82.00	842.36	77.00	90.50
G07	SYBR	Actin	Unkn-10	24h	82.00	955.21	76.00	88.50
G08	SYBR	Actin	Unkn-12	48h	82.00	937.75	77.00	95.00
G09	SYBR	Actin	Unkn-14	72h	82.00	929.24	77.00	87.50
G10	SYBR	Actin	NTC-16		82.00	433.88	78.00	88.00
H03	SYBR	Actin	Unkn-02	GS115	82.00	832.71	76.50	89.00
H04	SYBR	Actin	Unkn-04	alp-A	82.00	933.55	76.50	91.50
H05	SYBR	Actin	Unkn-06	O5Gly	82.00	939.13	77.00	91.00
H06	SYBR	Actin	Unkn-08	0.5 h	82.00	915.65	77.00	91.00
H07	SYBR	Actin	Unkn-10	24h	82.00	1012.07	76.50	92.50
H08	SYBR	Actin	Unkn-12	48h	82.00	965.22	76.00	91.50
H09	SYBR	Actin	Unkn-14	72h	82.00	950.59	76.00	87.00
H10	SYBR	Actin	NTC-16		None	None	None	None

Note:

GS115: untransformed GS115 strain

Alp-A: GS115 transformed with the empty vector, pPICZαA

O5Gly: transgenic O5 strain grew in YPG medium (Production phase)

0.5h, 24h, 48h and 72h: transgenic O5 strain grew in MMH medium (Induction phase)

4. Quantification Cq cycles

Well	Fluor	Target	Content	Sample	Cq	Cq Mean	Cq Std. Dev
A03	SYBR	scFv	Unkn-01	GS115	28.57	28.58	0.204
A04	SYBR	scFv	Unkn-03	alp-A	28.66	28.31	0.307
A05	SYBR	scFv	Unkn-05	O5Gly	19.43	19.62	0.222
A06	SYBR	scFv	Unkn-07	0.5 h	19.14	19.17	0.040
A07	SYBR	scFv	Unkn-09	24h	16.31	16.30	0.029
A08	SYBR	scFv	Unkn-11	48h	19.59	19.62	0.030
A09	SYBR	scFv	Unkn-13	72h	21.25	21.27	0.019
A10	SYBR	scFv	NTC-15		25.95	26.52	1.519
B03	SYBR	scFv	Unkn-01	GS115	28.38	28.58	0.204
B04	SYBR	scFv	Unkn-03	alp-A	28.22	28.31	0.307
B05	SYBR	scFv	Unkn-05	O5Gly	19.58	19.62	0.222
B06	SYBR	scFv	Unkn-07	0.5 h	19.15	19.17	0.040
B07	SYBR	scFv	Unkn-09	24h	16.27	16.30	0.029
B08	SYBR	scFv	Unkn-11	48h	19.65	19.62	0.030
B09	SYBR	scFv	Unkn-13	72h	21.27	21.27	0.019
B10	SYBR	scFv	NTC-15		28.24	26.52	1.519
C03	SYBR	scFv	Unkn-01	GS115	28.79	28.58	0.204
C04	SYBR	scFv	Unkn-03	alp-A	28.07	28.31	0.307
C05	SYBR	scFv	Unkn-05	O5Gly	19.86	19.62	0.222
C06	SYBR	scFv	Unkn-07	0.5 h	19.21	19.17	0.040
C07	SYBR	scFv	Unkn-09	24h	16.33	16.30	0.029
C08	SYBR	scFv	Unkn-11	48h	19.63	19.62	0.030
C09	SYBR	scFv	Unkn-13	72h	21.28	21.27	0.019
C10	SYBR	scFv	NTC-15		25.36	26.52	1.519
F03	SYBR	Actin	Unkn-02	GS115	17.51	17.47	0.047
F04	SYBR	Actin	Unkn-04	alp-A	21.07	20.96	0.135
F05	SYBR	Actin	Unkn-06	O5Gly	16.76	16.78	0.034
F06	SYBR	Actin	Unkn-08	0.5 h	19.15	19.20	0.050
F07	SYBR	Actin	Unkn-10	24h	18.41	18.38	0.045
F08	SYBR	Actin	Unkn-12	48h	19.95	19.92	0.076

F09	SYBR	Actin	Unkn-14	72h	20.63	20.66	0.048
F10	SYBR	Actin	NTC-16		32.30	35.63	3.528
G03	SYBR	Actin	Unkn-02	GS115	17.49	17.47	0.047
G04	SYBR	Actin	Unkn-04	alp-A	21.00	20.96	0.135
G05	SYBR	Actin	Unkn-06	O5Gly	16.82	16.78	0.034
G06	SYBR	Actin	Unkn-08	0.5 h	19.25	19.20	0.050
G07	SYBR	Actin	Unkn-10	24h	18.41	18.38	0.045
G08	SYBR	Actin	Unkn-12	48h	19.97	19.92	0.076
G09	SYBR	Actin	Unkn-14	72h	20.64	20.66	0.048
G10	SYBR	Actin	NTC-16		35.27	35.63	3.528
H03	SYBR	Actin	Unkn-02	GS115	17.42	17.47	0.047
H04	SYBR	Actin	Unkn-04	alp-A	20.81	20.96	0.135
H05	SYBR	Actin	Unkn-06	O5Gly	16.77	16.78	0.034
H06	SYBR	Actin	Unkn-08	0.5 h	19.21	19.20	0.050
H07	SYBR	Actin	Unkn-10	24h	18.33	18.38	0.045
H08	SYBR	Actin	Unkn-12	48h	19.83	19.92	0.076
H09	SYBR	Actin	Unkn-14	72h	20.72	20.66	0.048
H10	SYBR	Actin	NTC-16		39.33	35.63	3.528

Note:

GS115: untransformed GS115 strain

Alp-A: GS115 transformed with the empty vector, pPICZαA

O5Gly: transgenic O5 strain grew in YPG medium (Production phase)

0.5h, 24h, 48h and 72h: transgenic O5 strain grew in MMH medium (Induction phase)

APPENDIX H

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) software (15.0) was used for statistical analysis in this study. One-Way Analysis of Variance (ANOVA) was used to determine the difference means between/within groups of samples and Tukey HSD multiple comparisons was used to determine the difference means in homogeneous subsets between groups of samples. The null hypothesis will be rejected when the *p* value ≤0.05 (significant level = 0.05, $\alpha_{0.05}$) also means that the mean of data between groups is significantly different.

Hypothesis

H_0 = Null hypothesis, the data between two groups is not difference at significant level = 0.05.

H_1 = Alternative hypothesis, the data between two groups is difference at significant level = 0.05.

So; If the *p* value > $\alpha_{0.05}$ (Alpha = 0.05) ; Accept the null hypothesis (H_0),
There is sufficient evidence to accept the null hypothesis.

If the *p* value ≤ $\alpha_{0.05}$ (Alpha = 0.05) ; Reject the null hypothesis (H_0),
There is sufficient evidence to reject the null hypothesis.

Statistical Analysis of SPR

1. The interaction between purified scFv and their antigens (norfloxacin, enrofloxacin and ciprofloxacin)

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
Antibody	1.00	Purified scFv	18
Antigen	1.00	Norfloxacin	6
	2.00	Enrofloxacin	6
	3.00	Ciprofloxacin	6

Tests of Between-Subjects Effects

Dependent Variable: Angle Shift

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	917614.014[(a)]	2	458807.007	69.041	.000
Intercept	2046938.889	1	2046938.889	308.021	.000
Antibody	.000	0	.	.	.
Antigen	917614.014	2	458807.007	69.041	.000
Antibody * Antigen	.000	0	.	.	.
Error	99681.817	15	6645.454		
Total	3064234.720	18			
Corrected Total	1017295.831	17			

[a]R Squared = .902 (Adjusted R Squared = .889)

Homogeneous Subsets

Angle Shift

	Antigen	N	Subset		
			1	2	3
Tukey HSD[a,b]	Ciprofloxacin	6	36.6833		
	Enrofloxacin	6		394.0833	
	Norfloxacin	6			580.9000
	Sig.		1.000	1.000	1.000
Duncan[a,b]	Ciprofloxacin	6	36.6833		
	Enrofloxacin	6		394.0833	
	Norfloxacin	6			580.9000
	Sig.		1.000	1.000	1.000
Scheffe[a,b]	Ciprofloxacin	6	36.6833		
	Enrofloxacin	6		394.0833	
	Norfloxacin	6			580.9000
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Based on Type III Sum of Squares

The error term is Mean Square (Error) = 6645.454.

[a]Uses Harmonic Mean Sample Size = 6.000.

[b]Alpha = .05.

2. Comparison of binding activity between purified recombinant scFv antibody and their parental MAb Nor155

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
Antibody	1.00	Purified scFv	6
	2.00	MAb Nor155	6
Antigen	1.00	Norfloxacin	12

Tests of Between-Subjects Effects

Dependent Variable: Angle Shift

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	17236.920[(a)]	1	17236.920	1.863	.202
Intercept	3538188.000	1	3538188.000	382.338	.000
Antibody	17236.920	1	17236.920	1.863	.202
Antigen	.000	0	.	.	.
Antibody * Antigen	.000	0	.	.	.
Error	92540.820	10	9254.082		
Total	3647965.740	12			
Corrected Total	109777.740	11			

[a] R Squared = .157 (Adjusted R Squared = .073)

VITA

Mr. Jirawat Mala was born on July 5, 1983 in Surat Thani, Thailand. He graduated with the Bachelor degree of Science in Biology, Faculty of Science, Srinakharinwirot University, Prasarnmit in 2006. He continued his study in the Program of Biotechnology, Faculty of Science, Chulalongkorn University and graduated with the Degree of Master of Science in 2010. Since then, he has been working as a lecturer at the Program in Community Health, Faculty of Science, Suratthani Rajabhat University, Surat Thani, Thailand. In 2011, he began his Doctoral study in the same program as his Master Degree.

Award:

In 2015, he awarded the student best paper awards from Seoul International Conference on Applied Science and Engineering (SICASE 2015) under entitle “Construction and Sequencing Analysis of scFv Antibody Fragment Derived from Monoclonal Antibody against Norfloxacin (Nor155)”, Seoul, Korea.