APPLICATION OF PHAGE DISPLAY TECHNOLOGY FOR THE STUDY OF CHOLANGIOCARCINOMA ASSOCIATED WITH CONSUMPTION OF FLUKE INFECTED FOOD

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การใช้เทคโนโลยีฟาจในการศึกษาโรคมะเร็งท่อน้ำดีที่สัมพันธ์กับการบริโภคอาหารที่ปนเปื้อนด้วยพยาธิใบไม้ตับ

นางสาวนันทนิจ จารุเศรณีย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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โรคมะเร็งท่อน้ำดีเป็นหนึ่งในปัญหาสุขภาพที่สำคัญในภาคตะวันออกเฉียงเหนือของประเทศไทย ซึ่งเป็นแหล่งที่มีผู้ป่วยโรคนี้มากที่สุดในโลก การตรวจหามะเร็งชนิดนี้ในระยะต้นนั้นทำได้ยากมาก ถึงทำไม่ได้เลย และยังไม่มีวิธีการรักษาที่มีประสิทธิภาพ วัตถุประสงค์ของโครงการวิจัยนี้ คือการพยายามใช้เทคโนโลยีฟาจในการแก้ปัญหานี้ โดยการนำมาประยุกต์ใช้ในการค้นหาเปปไทด์และแอนติบอดีที่สามารถจับอย่างจำเพาะกับเซลล์ KKU-100 ซึ่งเป็นสายพันธุ์ของเซลล์ที่เตรียมได้จากมะเร็งชนิดนี้ ผลจากกระบวนการคัดหาเส้นเปปไทด์จากคลังของฟาจที่แสดงเปปไทด์หลากหลายชนิดต่างๆ พบเปปไทด์ที่มีโครงสร้างเป็นวงกลม ๒ เส้น ที่สามารถจับกับเซลล์ KKU-100 ได้อย่างจำเพาะเจาะจงโดยมีลำดับกรดอะมิโนคือ ซิสเตอีน ทรีโอนีน ทรีโอนีน โพรลีน โพรลีน ไทโรซีน เวลีน ไซโตซีน (CTTPPYVC) และ ซิสเตอีน ทรีโอนีน ซีรีน โพรลีน โพรลีน ไทโรซีน เวลีน ไซโตซีน (CTSPPYVC) โดยทดสอบยืนยันความสามารถในการจับ ด้วยวิธีอีไลซ่า และการย้อมเซลล์ นอกจากนั้นแล้วเส้นเปปไทด์สังเคราะห์ CTTPPYVC ยังสามารถยับยั้งการเจริญของเซลล์เมื่อทำการทดสอบในจานทดลองด้วย ในขณะเดียวกันยังได้นำเทคโนโลยีฟาจไปใช้คัดหาแอนติ บอดีส่วน scFv ที่สามารถจับกับเซลล์ KKU-100 ได้เป็นจำนวนมาก แอนติบอดีส่วน scFv เหล่านี้ เมื่อผลิตในรูปของ scFv เดี่ยวและ scFv ที่เชื่อมติดกับ เอนไซม์อัลคาไลฟอสฟาเทส (scFv-AP) แล้ว พบว่ายังสามารถจับกับเซลล์เมื่อทดสอบด้วยวิธีการอีไลซ่า การตกตะกอนด้วยอิมมูนและการย้อมเซลล์ แต่ยังไม่พบแอนติบอดีที่สามารถใช้ในการทดสอบโดยวิธีเวสเทิร์นบลอตหรือแอนติบอดีที่มีผลต่อการเจริญของเซลล์ นอกจากนั้นแล้วยังได้ทำการทดสอบความสามารถในการจับของแอนติบอดีเหล่านี้กับเซลล์อื่นๆ เช่น H69, COS-1 และ HepB3 รวมทั้งยังได้นำแอนติบอดีส่วน scFv โคลน YM12 และ YM13 ไปทดสอบผลต่อโครงสร้างของเซลล์ด้วยเทคนิค Fourier Transform Infrared (FTIR) microspectroscpy ซึ่งผลจากการศึกษาพบว่าแอนติบอดีส่วน scFv ทั้งสองชนิด สามารถทำให้โครงสร้างของคาร์โบไฮเดรตและโปรตีนของเซลล์ KKU-100 เกิดการเปลี่ยนแปลง แต่ไม่พบการเปลี่ยนแปลงที่โครงสร้างของไขมัน ผลจากการศึกษาดังกล่าวมาทั้งหมด แสดงให้เห็นว่าเทคโนโลยีฟาจนั้นมีประสิทธิภาพในการใช้ค้นหาเปปไทด์และแอนติบอดีส่วน scFv ที่จำเพาะกับเซลล์ KKU-100 ได้ ซึ่งทั้งเปปไทด์และแอนติบอดี

ส่วน scFv ที่คัดเลือกมาได้นี้ สามารถนำไปใช้ในการศึกษากลไกการเกิดมะเร็งท่อน้ำดีได้ ส่วนการนำไปประยุกต์ใช้ในระดับคลีนิคนั้นยังต้องทำการวิจัยและพัฒนาต่อไป

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2552

ลายมือชื่อนักศึกษา นันทกานิจ จารุเศรษฐนัย
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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

NANTHANIT JARUSERANEE : APPLICATION OF PHAGE DISPLAY TECHNOLOGY FOR THE STUDY OF CHOLANGIOCARCINOMA ASSOCIATED WITH CONSUMPTION OF FLUKE INFECTED FOOD. THESIS ADVISOR : ASSOC. PROF. MONTAROP YAMABHAI, Ph.D., 155 PP.

PHAGE DISPLAY TECHNOLOGY/CHOLANGIOCARCINOMA/ANTIBODY/ PEPTIDE/CELL SURFACE MARKER

Cholangiocarcinoma is one of the most serious health problems in Northeastern region of Thailand, where the prevalence of this form of cancer is the highest in the world. Unfortunately, early detection of this cancer is difficult to impossible and there is still no effective treatment. To overcome this limitation, phage display technology was used to identify peptide ligands and antibody fragments bound selectively to the surface of the cholangiocarcinoma cell line, KKU-100. Affinity selection of various phage-displayed combinatorial peptide libraries yielded two cyclic peptides: CTTPPYVC and CTSPPYVC, bound specifically to KKU-100, as shown by ELISA and cell staining assays The synthetic form of one peptide, CTTPPYVC, could inhibit cell growth *in vitro*. In parallel, a number of human single-chain fragments of variable regions (scFv) antibody fragment were isolated from phage-display libraries bound selectively to the KKU-100 cell surface. These antibody fragments, prepared as soluble scFv or scFv-alkaline phosphatase (scFv-AP) fusions, were evaluated in cell-based ELISA, cell-staining, and immunoprecipitation experiments. None of antibody tested so far yielded a signal by western blot analysis or showed any effects on cell

growth. Cross-reactivity of these antibodies to other cell lines, namely H69, HepG2, COS-1 and HepB3 cells, was also investigated. Moreover, Fourier Transform Infrared (FTIR) microspectroscopy analysis was carried out to observe the effect of two scFv antibodies on KKU-100 cells. The results showed that the YM12 and YM13 scFvs could alter the structure of carbohydrate and protein, but not lipid. Thus, phage display was successfully used to isolate specific peptides and antibodies against KKU-100 cells. These peptides and scFv antibodies have the potential to be used as reagents for the study of cholangiocarcinogenesis, although their clinical applications requires further investigation.

School of Biotechnology

Academic Year 2009

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ABBREVIATIONS

AP	=	Alkaline phosphatase
ABTS	=	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
BCIP	=	5-Bromo-4-Chloro-3-Indolyl Phosphate
CB	=	Carbenicillin
CCA	=	Cholangiocarcinoma
ELISA	=	Enzyme linked immunosorbance assay
HRP	=	Horse radish peroxidase
IMAC	=	Immobilized metal affinity chromatography
IPTG	=	Isopropyl β-D-1-thiogalactopyranoside
LB	=	Luria-Bertani
L.F.	=	Lactoferrin
MPBS	=	Skim Milk in PBS
NBT	=	Nitro Blue Tetrazolium
PBS	=	Phosphate buffer saline
S.A.	=	Streptavidin
scFv	=	Single-chain variable fragment
S.V.	=	Sullivan library
YM	=	YAMO library

CHAPTER I

INTRODUCTION

In the northeastern of Thailand, people have daily habit of eating raw fish (figure 1) and salted-fermented fresh water fish. One problem from consuming these food products is increasing a chance of being infested with liver fluke if human or other mammals ingest raw fish containing the infective cysts of the fluke (metacercariae) in muscle and connective tissue. There has been strong evident suggesting the association of liver fluke infection and exposure to nitrosamine-contaminated food with a type of cancer called cholangiocarcinoma (CCA) (Sripa et al., 2007). This disease is a fatal, malignant neoplasm with no curative treatment, neither by chemotherapy nor radiation (de Groen PC, 1999). Besides it shows high resistance to anticancer drug. There is no effective treatment for CCA at present, probably due to it enhanced resistance to apoptosis (Wongkham et al., 2009). Many patients are presented with unresectable or at metastatic stage. Early detection of cholangiocarcinoma is difficult and not clinically available at present. Cholangiocarcinoma-associated soluble antigen for early detection of cancer development has been reported only in an experimental study. This antigen is a 200-kDa glycoprotein and the level of the antigen in serum and bile was raised markedly in animals with progressive tumors, compared with controls (Watanapa and Watanapa, 2002).

Figure 1. Preparation of a Meal of *Koi-Pla* Using Uncooked Cyprinoid Fishes. (A) Fluke-infected fish are plentiful in the local rivers such as the Chi River in Khon Kaen province, Thailand. (B) Local people catch the fish in nets and prepare the fish-based meals with local herbs, spices, and condiments. (C) The finished dish of *koi-pla* accompanied by rice and vegetables. This dish is a dietary staple of many northeastern Thai villagers and is a common source of infection with *O. viverrin* (Sripa et al., 2007).

So far, there has been no report on the study of surface molecules of cholangiocarcinoma by using phage display technology. However, the cell line of patient living in Northeast Thailand, with an egg-proven, *Opisthorchis*-associated cholangiocarcinoma has been developed and desiginated KKU-100 (Sripa et al., 2005). This cholangiocarcinoma cell line that will be used in this study was derived from poorly differentiated tubular adenocarcinoma, the common histologic cholangiocarcinoma reported in Thailand. It is the first cell line derived from porta hepatic. Tumor at this site is lethal and was found around two-third of all cholangiocarcinomas (Sinawat P, 1991). Since most intrahepatic cholangiocarcinoma

are rapidly develop and extremely invasive without any clinical symptom, the identification of novel tumor markers and development of effective therapeutic strategies are urgently needed (Jinawath et al., 2006). Novel methods for early detection and the development of effective treatments are an eminent priority in medicine. For this reason, isolation of tumor-specific ligands is a growing area of research nowadays and phage display is a powerful technique for the isolation of peptides and antibody that bind to particular target with high affinity and specificity (Brown, 2009).

In this study, surface markers expressed by KKU-100 will be investigated for studying tumor biology, cellular and molecular carcinogenesis, and identification of biomarkers for early diagnosis. Better understanding of the disease mechanisms and early diagnosis might help to reduce severity of this disease or better design of prevention program. In addition, both peptides and antibodies specific to surface molecule on this cancer cell can be used as an efficient tool to study the disease mechanism. Finally, peptides and antibodies that demonstrate inhibitory effect on cell growth could be potential drugs lead for future treatment.

Research objectives

The purpose of this thesis is to use phage display technology to study cell surface and cancer associated antigen of cholangiocarcinoma cell line (KKU100)**.** It can be divided into 3 main following parts.

1. To select the specific peptide ligand that can interact specifically with KKU-100 surface molecules.
2. To select scFv from phage display library that can interact specifically with KKU-100 surface molecules.

3. To characterize the biochemical and biological properties of specific antibody/ peptide *in vitro.*

Research hypothesis

Specific molecules are expressed on cholangiocarcinoma cell surface and these molecules can interact specifically with peptides or antibodies that are displayed on phage coat protein. Both peptides and antibodies have potential to be developed into useful diagnostic and therapeutic reagents. In addition, these peptides and antibodies can be used as molecular tools to study molecular biology of cholangiocarcinoma.

Scope and limitation

Both phage display of peptide and antibody libraries will be used in this study. The surface molecules on KKU-100 cell will be focused.

CHAPTER II

REVIEW AND LITERATURE

2.1 Cholangiocarcinoma

Cholangiocarcinoma (CCA) can be described as the malignancy of bile ducts, an organ that drains bile from the liver into the small intestine. Risk factors for cholangiocarcinoma include primary sclerosing cholangitis (an inflammatory disease of the bile ducts), congenital liver malformations, infection with the parasitic liver flukes *Opisthorchis viverrini* (found in Thailand, Laos, and Malaysia) or *Clonorchis sinensis* (found in Japan, Korea, and Vietnam) (Sripa et al., 2007; Sripa et al., 2005), and exposure to a chemical in aircraft, rubber, and wood finishing industries or medical imaging (thorium dioxide). The etiology of most bile duct cancers remains undetermined. The possible mechanism of carcinogenesis include chronic irritation, nitric oxide formation, intrinsic nitrosation, and activation of drug-metabolizing enzymes (Watanapa, 1996b; Watanapa and Watanapa, 2002). The incidence of association between infection with *Opisthorchis viverrini* and cholangiocarcinoma had been reported for many years and the carcinogenesis induced by the fluke has been actively investigated. However the actual causal relationship between the parasite and the cancer is still poorly understood (Watanapa and Watanapa, 2002). In Thailand, CCA is associated with liver flukes *Opisthochis vivierrini.* It is a eukaryote desiginated as a class I carcinogen by International Agency for research on Cancer (Sripa et al., 2007). Liver fluke-associated cholangiocarcinoma is still a serious health problem in developing countries, especially in the Northeast of Thailand, which has the

highest incidence of cholangiocarcinoma reported in the world (Vatanasapt V and Chartbanchachai W, 1990).

There have been only a few reports describing *Opisthorchis* –related cholangiocarcinoma from Thai people, KKU-100 may probably be the first porta hepatic-derived and egg-proven Opisthorchis-associated cholangiocarcinoma cell line. It is the second cell line developed in the area endemic for opisthorchiasis and cholangiocarcinoma (Sripa et al., 2007; Sripa et al., 2005). Clinical studies and experimental models have uncovered that *O. viverrini* infection plays a crucial role in the promotion of cholangiocarcinoma (Jinawath et al., 2006). Cumulative data suggested that the pathogenesis of this bile duct cancer in Thailand is different from that observed in Western countries with different etiologies (Vatanasapt V, 1999), even though long term survival after surgical treatment of liver fluke-associated cancer is similar to that reported in patients without liver fluke infestation (Watanapa, 1996a). The gene expression profile of tissue from patient with *O. viverrini* and non- *O. viverrini* infected by using DNA microarray technique revealed the different of gene expression profile (Jinawath et al., 2006). The biology of cholangiocarcinoma is need to be studied in order to understand the mechanism of the disease in both liver fluke and non-liver fluke associated in order to find effective treatment and prevention.

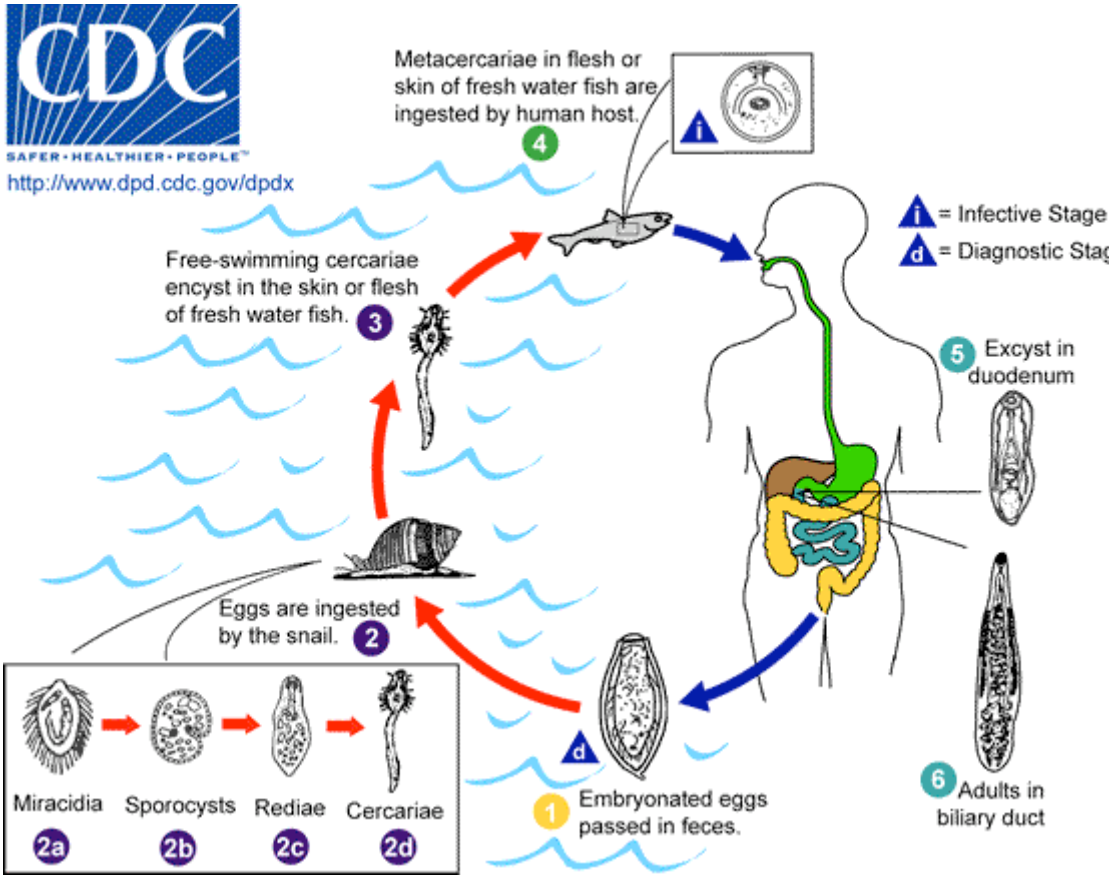

Figure 2. Liver fluke life cycle

There are several risk factors invloved in cholangiocarcinoma pathogenesis including amplification of epidermal growth factor receptor, interleukin-6 signaling pathway, inducible nitric oxide, erb2 and cyclooxygenase-2 (Malhi and Gores, 2006). When epithelial cells surrounded by chronic inflammatory environment, they are constantly stimulated to participate in inflammation by generating chemokines and cytokines (Moss and Blaser, 2005). Cholangiocarcinoma cells constitutively secrete IL-6 (Isomoto et al., 2005). Proto-oncogene c-erbB2 (Her-2/neu) is activated in patients with cholangiocarcinoma (Malhi and Gores, 2006). Therefore, CA 19-9, CA 125 and CEA, the tumor associated markers, had been widely used in

cholangiocarcinoma studies (Malhi and Gores, 2006). However, CEA and CA-125 showed low sensitivity and are not specific only to cholangiocarcinoma as they also elevate in patients with other gut derived malignancies (Chen et al., 2002).

Galectin-3 (Gal-3), a β-galactoside-binding lectin, is a multifunctional protein that implicated in a variety of biological functions including cell adhesion, differentiation, proliferation, apoptosis, cancer progression and metastasis. Gal-3 is frequently expressed in CCA tissue, interfering with Gal-3 action either by using RNAi-mediated knockdown of Gal-3 or specific Gal-3 inhibitors can either be developed as a specific gene targeting therapy to treat CCA, or used in combination with chemotherapeutic agent to enhance apoptosis and chemosensitivity in CCA. However, low Galectin-3 expression was found in poorly differentiated cholangiocarcinoma (Wongkham et al., 2009).

Most reports of cholangiocarcinoma cell line focused on extrahepatic type, only a few from intrahapatic. KKU-100 was established from liver of the tumor mass and hepathology indicated a poorly differentiated tubular adenocarcinoma and it was established from intrahepatic duct. Moreover biliary cytology revealed cluster of tumor cells with *O. vivernii* eggs. Tumor suppressor gene p53 is a common genetic alteration in various cancer and up to 78.5% of cholangiocarcinoma showed over-expressed, but KKU-100 does not express p53 (Sripa et al., 2005).

2.2 Phage display technology

Phage such as M13 have been widely used since the 1970s as ‘genetic warehouses’ for cloning and sequencing of DNA fragments (Smother 2002). In 1985, George Smith reported using phage to display foreign protein on its surface without

losing infectivity (Smith, 1985). The M13 viral particle carries five copies of pIII, at one of its ends, and 2700 copies of protein VIII, which covers the filamenteous virus along its length of approximately 1 μm. (figure 3) (Kriplani and and Kay, 2005). Foreign protein is typical expressed as N-terminal pIII or pVIII fusion. The key advantage of phage display is the direct linkage between DNA (genotype) and display protein (phenotype). Screening a phage-displayed peptide library is accomplished through an affinity-selection process referred to as biopanning (Krumpe and Mori, 2007).

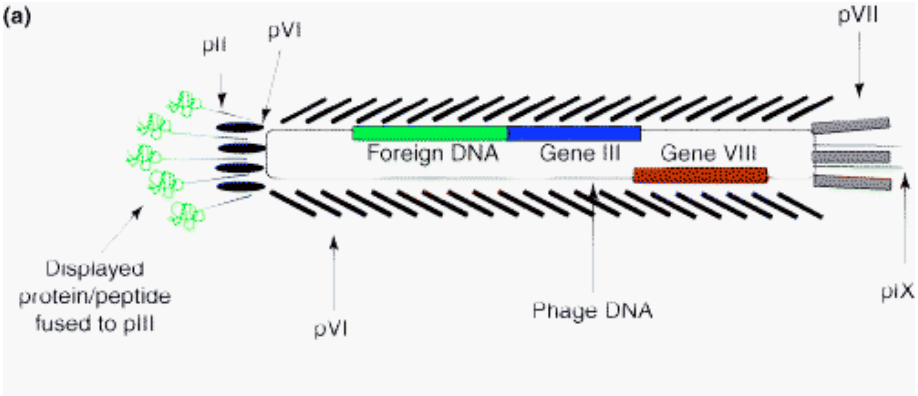

Figure 3. A schematic of M13 used in phage display library. Phage DNA modified to display protein or peptide as fusion to PIII phage coat protein. Five phage coat proteins were labeled (Kriplani and and Kay, 2005).

Phagemid system was developed for large protein display such as scFv. This system requires infection with helper phage which providing most wild type phage proteins to form recombinant phage particles. Phage life cycle with phagmid vector was shown in figure 4.

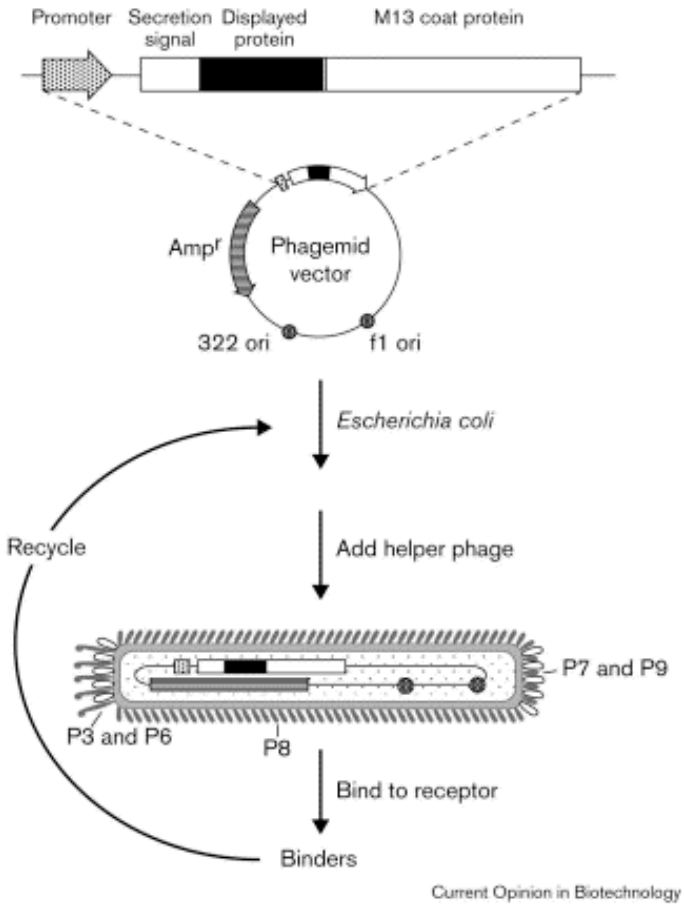

Figure 4. Schematic of phage life cycle with phagemid vector (Sidhu, 2000)

2.2.1 Phage display peptide library

Phage display peptide libraries are created under standard molecular cloning technique by insertion randomized oligonucleotide fragments in frame with phage coat protein gene. Linear libraries can be constructed by elimination of nucleotide encode cysteines, on the other hand, cyclic libraries can be constructed with even number of cystein coding sequences (Aina et al., 2007) Phage display peptides can be affinity selected with a certain target molecule from complex mixture of billions of display peptides in library (Krumpe and Mori, 2007).

2.2.2 Phage display human scFv Library

Two sources of human antibody genes were used to construct phage display scFv library. Phage display library constructed from non-immunized antibody genes is a naïve library. Alternatively, the antibody genes from immunization with certain antigen were used to construct the immunized library.

YAMO library, phage display human scFv, was based on the naïve human re-arranged V-genes and assembled through the use of a gene repertoire derived from 140 non-immunized donors (Pansri et al., 2009). It was constructed in pMOD1 phagemid with 1.5 x 10^8 diversity (Figure 5).

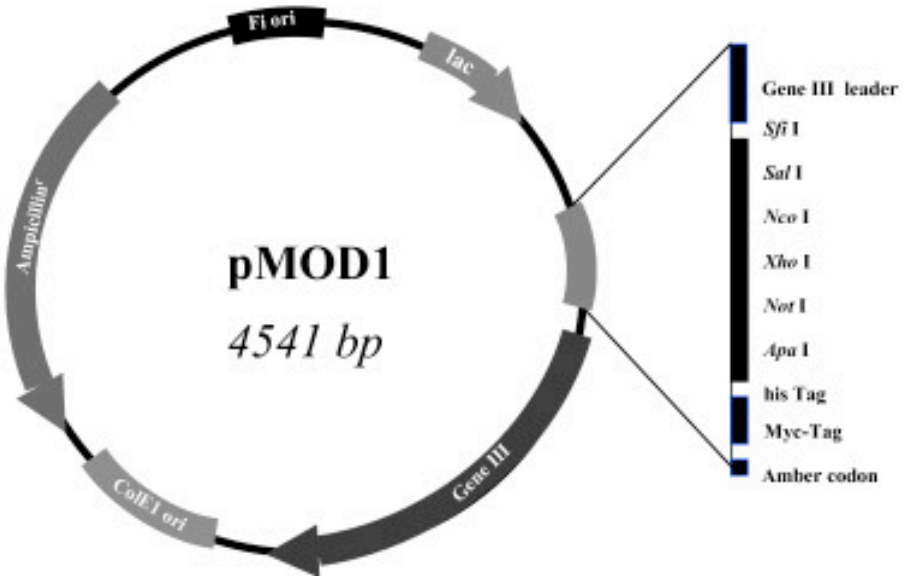

Figure 5. pMOD1 phagemid

Tomlinson I library is human synthetic scFv library. It was constructed with phagemid pIT2 (HIS myc tag) (Figure 6) based on a single human framework for V_H (V3-23/DP-47 and J_H4b) and V_κ (O12/O2/DPK9 and J_κ1). The CDR3 of heavy chain

was designed to be as short as possible, but still able to bind to antigen. Side chain diversity was incorporated at antigen binding site sequences.

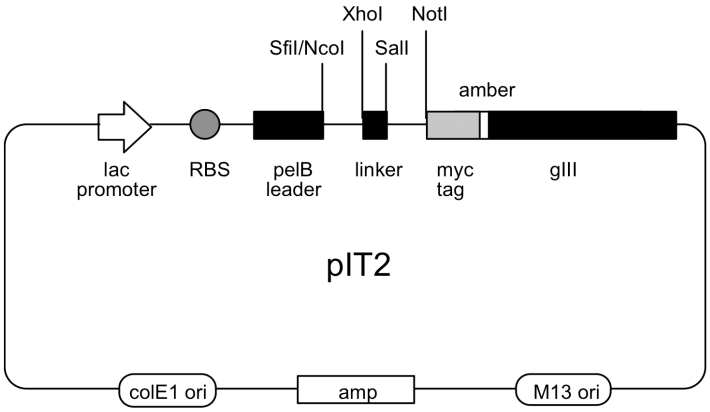

Figure 6. pIT2 phagemid vector

Sullivan library, human scFv naïve library, was constructed from 100 donors donated B lymphocyte in AP-III$_6$ phagemid (Figure 7) under alkaline phosphatase promoter (*phoA*). Ninety percent of this library are in VL-linker-VH orientation and 10% are in the reverse for kappa light chain with 2 x 10^9 diversity (unpublished information, Phage antibody methods Sullivan lab, 2007).

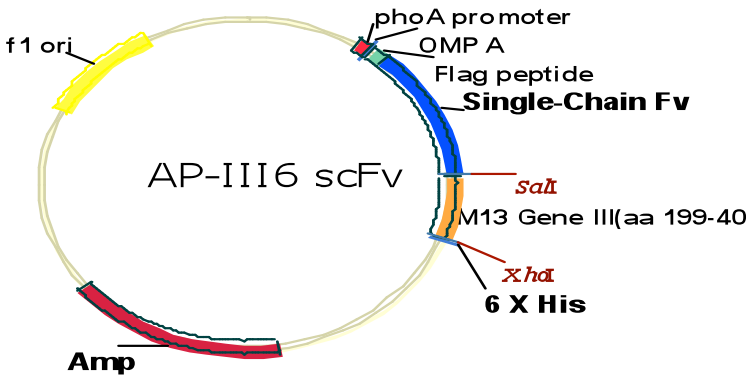

Figure 7 AP-III$_6$ phagemid vector

2.3 Application of phage display peptide and scFv in cancer study

The study of surface molecules on cancer cells not only help to extend the understanding of cancer mechanism but also develop a novel marker for early diagnostic, especially for cholangiocarcinoma, of which early diagnosis is essential. The surface of cancer cell expresses certain molecules that are different from normal cells. Some of them involve in cell division, invasion and metastatic (Jia et al.), or escape from macrophage elimination (Intasai et al., 2006). Many of them involve in cell cycle and growth control, for example, epidermal growth factor receptor (EGFR), a 170-kDa transmembrane glycoprotein, is a cell surface receptor that overexpressed on many solid tumors (Heitner et al., 2001). The intercellular adhesion molecule (ICAM-1) is expressed selectively at low levels on endothelial cells but is strongly upregulated in dysfunctional endothelial cells associated with inflammation, cancer, and atherogenesis (Belizaire et al., 2003). In addition to being used as diagnostic tools, specific molecules on surface of cancer cells also have potential roles in developing specific ligands for drug delivery in targeting therapy of cancer (Du et al., 2006). Moreover, it can also lead to the identification of drug target for the development of novel, efficient therapeutic agents. Human solid tumor cell lines are important sources for studying of tumor biology, including tumor cell growth, differentiation, metastasis, molecular pathogenesis and drug susceptibility. Phage display is one of the techniques that can be used to investigate cell surface molecules. Selection of phage-displayed peptide/antibody libraries on intact cells in culture (cell based biopanning) has been proven to be successful for isolating cell-binding peptides/antibodies (Oyama et al., 2006). One advantage of cell based biopanning is the selected phage typically show cell-specificity, another advantage is this technique requires no prior knowledge of

cell surface biomarker, allowing for isolation of targeting peptides/antibodies for cell types of which little is known about the cellular receptor profile (Loo and Mather, 2008; Oyama et al., 2003). Moreover, the panning occurs on cells, so the ligands isolated can bind the cellular receptors in their native context (Oyama et al., 2006). There are a number of previous reports (Table 1) demonstrating the successful identification of cells markers by using phage display technique, either phage display of peptide or antibody libraries. Most of peptide and antibody selection generated cell specific markers. Some of them showed the ability to reduce cell invasion and migration *in vitro* (Hu et al., 2006). This peptide could be developed to use as a therapeutic agent. In addition, a clinically applicable anti-tumor huMabs (human monoclonal antibodies) based on scFv obtained from a semi-synthetic phage antibody display library had also been reported (Gerwin A. Huls, 1999).

Table 1. Cell specific marker identified by phage display technology.

Library	Cell	Subtractor cells	Identified	Reference
Combinatorial peptides 12-mer and 7-mer	Hepatocellular cell line (BEL-7402)	Normal liver cell line HL-7702	Specific marker	(Du et al., 2006)
12-mer	Hig liver-metastatic gastric cancer cell (XGC9811-L)	XCG9811	Specific marker, suppress motility and invasion	(Hu et al., 2006)
7-mer	Hepatocellular carcinoma (HCC) (HCCLM3)	MHCC97L	Specific marker	(Jia et al.)

Table 1. (Continued)

Library	Cell	Subtractor cells	Identified	Reference
12-mer	Irradiated human pancreatic adenocarcinoma cell line (Capan-2-cell)	Nonirradiated Capan-2-cell	Specific marker	(Huang et al., 2005)
16-mer	COS-7 cell transfected with murine ICAM-1 (mICAM-1)	Non-transfected COS-7 cells	Specific marker	(Belizaire et al., 2003)
20-mer	Lung tumor cell lines(NCI-H1299, NCI-H2009 and A549)	—	Specific marker and internalizing	(Oyama et al., 2006; Oyama et al., 2003)
	Neuroblastoma cell line (WAC2)	—	Specific marker and internalizing	(Zhang et al., 2001)
	Tissue culture cell line and primary cell		Cell surface biomarker	(McGuire et al., 2009)
Antibody Naïve scFv and human semi-synthetic scFv	Breast carcinoma (Erb2-expressing breast carcinoma SKBR3)	MCF10A and 184B5 cell line human breast non-tumorigenic epithelial cell	Specific marker	(Mazuet et al., 2006)
scFv	Leukocyte and platelets receptors.	Cell lines expressing activated or non-activated conformation of cell surface receptors	High specific scFv antibody to various conformation states of cell surface receptors of integrin family	(Eisenhardt et al., 2007)

Phage display peptide is one of the most widely used in drug discovery (Krumpe and Mori, 2007). Most peptides which were selected by phage display peptide biopaning are cyclic peptides studied *in vitro* (Table 2) and *in vivo* (Table 3). *In vitro* biopanning of phage display library is performed on cells. *In vivo* biopanning

is mainly performed on mice and only one example of screening on human patients is available (Balestrieri and Napoli, 2007). Advantage of using peptide in human for *in vivo* tumor imaging and diagnosis is high kidney uptake observed with radiolabeled peptides and phage display peptides. Many of peptides selected via phage display had been radio or optically labeled for imaging of a range of tumors *in vivo* for the last five years, as shown in table 4. This indicates a rapid, economical and efficacious of phage display technology in development of agents for molecular imaging and diagnosis of cancer (Deutscher, 2010).

Rapidly internalization of scFv selected via phage display to breast tumor cell was previously described. This scFv could have a potential role for the delivery of cytotoxic agents target to breast cancers. This study demonstrated that direct selection of phage display antibody library on tumor cells could effectively lead to the identification and functional characterization of relevant tumor markers (Goenaga et al., 2007). The identification of more than 90 internalizing phage monoclonal antibodies targeting prostate cancer was reported and the cell type-specific internalizing human antibody can be readily identified from naïve phage antibody display library (Liu et al., 2007).

Table 2. Cyclic peptides selected by phage display peptide *in vitro* biopanning.

Target	Peptide sequences	Reference
In vitro B-cell lymphoma	CTLPHLKMC	(Choi et al., 2008)
Cervical carcinoma	C(R/Q)L/RT(G/N)XXG(A/V)GC	(Robinson et al., 2005)
CRIP-1 (Cys-rich intestinal protein)	CLKDNHRSC	(Hao et al., 2008)
Glucose-regulated protein 78	CTVALPGGYVRVC	(Kim et al., 2006)
HSP90	CVPELGHEC	(Vidal et al., 2004)
HUVEC (human colon CA)	CPHSKPCLC	(Zhao et al., 2007)
HUVEC/gastric cancer	CTKNSYLMC	(Liang et al., 2006)
Integrin $\alpha_4\beta_1$	CPLDIDFYC	(Jager et al., 2007)
Integrin $\alpha_v\beta_3/\alpha_5\beta_1$	*CQQSNRGDRKRC*	(Witt et al., 2009)
Interleukin-11 receptor α	CGRRAGGSC	(Zurita et al., 2004a)
Lectin	CNTPLTSRC, CKPHASSMC, CSRILTAAC, CSPIYKDTC, CNNPRAINC	(Yu et al., 2009)
Lymphatic (breast)	<u>CGNKRTRGC</u>	(Laakkonen et al., 2004)
Lymphatic (leukemia cells)	CAYHRLRRC	(Nishimura et al., 2008)
MMP-2/MMP-9	CTTHWGFTLC	(Chen et al., 2005)
Pancreatic islets	CVSNPRWKC, CHVLWSTRC	(Yao et al., 2005)
Plasma	CGLIIQKNEC, CNAGESSKNC	(Pilch et al., 2006)
Prostate specific antigen (PSA)	CVFXXXYXXC, CXFXXXYXYLMC CVXYCXXXXCYVC, CVXYCXXXXCWXC	(Wu et al., 2000)
Protein kinase CK2	<u>CWMSPRHLGTC</u>	(Perea et al., 2008)

Underline indicated peptides used in tumor reduction study. Italic indicated peptides used in biodistribution studies.

Table 3. Cyclic peptides selected by phage display peptide *in vivo* biopanning.

Target	Peptide sequences	Reference
In vivo Aminopeptidase A (APA) (tumour endothelium, perycites, stromal cells)	CPRECESIC	(MarchiÚ et al., 2004)
Aminopeptidase N (APN)/CD13 (proteasenormal/breast tumour endothelium, perycites)	CNGRC, CNGRCVSGCAGRC, CVCNGRMEC	(Curnis et al., 2000) (Pasqualini et al., 2000)
Aminopeptidase P (APP) (breast tumour endothelium)	CPGPEGAGC	(Burg et al., 1999)
Bladder cancer	CXNXDXR(X)/(R)C	(Lee et al., 2007)
Cardiac endothelium	CRSTRANPC, CPKTRRVPC	(Zhang et al., 2005)
Clotted plasma proteins and tumor tissue	CGLIIQKNEC CNAGESSKNC	(Pilch et al., 2006)
Coated superparamagnetic fluorescent nanoparticle targeted VCAM-1-expressing endothelium and atherosclerotic lesions	CVHSPNKKC	(Kelly et al., 2005)
Ephrin receptor (EphA2, EphA4 and EphB)	*CVSNPRWKC,* *CHVLWSTRC*	(Yao et al., 2005), (Koolpe et al., 2005; Koolpe et al., 2002)
IL11-rcytokine receptor (prostate tumour endothelium)	CGRRAGGSC	(Zurita et al., 2004b)
Kallikrein-9 substrateprotease substrate (tumour endothelium)	CSRPRRSEC	(Hoffman et al., 2003)
MMP2 (matrix metalloproteases) (normal/tumor endothelium)	CTTHWGFTLC	(Hajitou et al., 2006)
αv Integrins (normal/breast tumour endothelium)	CDCRGDCFC	(Dickerson et al., 2004)

Italic indicated peptides used in biodistribution studies.

Table 4. Peptide imaging molecules selected from phage display biopanning.

Target	Peptide sequences	Reference	Labeling agents
HUVEC (gastric cancer)	CGNSNPKSC	(Hui et al., 2008)	(99)Tc(m)O(4) (-)
Integrin $\alpha_v\beta_3/\alpha_5\beta_1$	CDCRGDCFC	(Cai et al., 2006)	quantum dots (QDs)
Invasive colon cancer	CPIEDRPMC	(Kelly et al., 2004)	FITC
αvβ3, αvβ5	(RGD-4C) CDCRGDCFC	(Liu, 2006)	(99m)Tc
Colon cancer cells	CPIEDRPMC	(Kelly and Jones, 2003)	FITC
Lymphatic (rhabdomyosarcoma)	CMGNKRSAKR PC	(Witt et al., 2009)	Biotinylated peptide and avidin-Alexa488
Linear peptide (*in vitro*)			
Neuroblastoma	VPWMEPAYQR FL	(Askoxylakis et al., 2005)	(131)I-labeled
Linear peptide (*in vivo*)			
β3	GRDSPK	(Achilefu et al., 2005; Bloch et al., 2006)	carbocyanine molecular probe (Cypate)

An example of therapeutic peptides and antibodies that are in the market or at late stage of clinical trails are shown in table 5 and 6, respectively. Top 5 therapeutic antibodies are Rituxan, Remicade, Herceptin, Humira and Avastin. There are currently 22 monoclonal antibodies (mAbs) approved by the United States Food and Drug Adminstration (FDA) for clinical use and hundreds are in clinical trials for treatment of various diseases including cancers, immune disorders and infections. (Dimitrov and Marks, 2009).

Table 5. Some peptide therapies in late-stage clinical trials.

Company	Therapy/indiction	Manufacture	Status
Advanced Viral Research (Hallandale,FL)/Beijing Unistone Pharmaceutical (Beijing,China)	Reticulose/HIV infection		Phase III
Allelix Biopharmaceuticals	ALX-0600/short bowel syndrome	Recombinant	Phase III
Aphton (Woodland,CA) Pasteur Mérieux (Lyon, France)	Gastrimmune/Pancreatic cancer	Synthetic	Phase III
Connectics (Palo Alto,CA)/Medeva (London)/Suntory (Osaka, Japan)	ConXn/scleroderma, hardening of skin and internalorgans	Recombinant	Phase III
COR Therapeutics (South San Francisco, CA)/Schering-Plough(Madison, NJ)	Integrilin/unstable angina	Synthetic	PLA/NDA
Diatide/Nycomed Amersham(Little Chalfont, UK)	Neotect(P829)/imaging non-small cell lung cancer		PLA/NDA
Diatide (Londonderry, NH)	Neotect(P829)/imaging metastasis of malignant melanoma	Synthetic	Phase III
Discovery Laboratories (Doylestown, PA)	Surfaxin/acutent respiratory distress syndrome		Phase II/III
Immune Response (Carlsbad, CA)	Combination vaccine/ rheumatoid arthritis		Phase III
Magainin (Plymouth Meeting, PA)	Locilex cream 1% (formally Cytolex)/infections in diabetic foot uclers		FDA non approved
Peptide Technologies (Louisville, KY/Allergan (San Diego, CA))	GMDP/ psoriasis		Phase III
SciClone Pharmaceuticals (San Mateo, CA)	Zadaxin/HepatitisB	Synthetic	Phase III

Source:Therapeutic pepetide revisited, Peter W, Latham, 1999, Nature Biotechnology, volume 17, p756.

Table 6. Therapeutic antibodies approved by the FDA for cancer treatment.

Therapy	Type	Isotype/ conjugate	Target	Indiction	Year of FDA approval
Monoclonal antibodies Rituximab (Rituxan)	Chimeric	IgG1	CD20	Low-grad B-cell NHL	1997
Trastuzumab (Herceptin)	Humanized	IgG1	HER2/ neu	Metastatic breast cancer	1998
Alemtuzumab (Campath)	Humanized	IgG1	CD52	CLL	2001
Bevacizumab (Avastin)	Humanized	IgG1	VEGF	Metastatic CRC	2004
Cetuximab (Erbitux)	Chimeric	IgG1	EGF recepto r	Metastatic CRC	2004
Immunoconjugates Gemtuzumab ozogamicin (Mylotarg)	Humanized	IgG4/calic heamicin	CD33	AML	2000
Ibritumomab tiuxetan (Zevalin)	Murine	IgG1/90Y	CD20	Relapsed or refractory NHL	2002
Tositumomab and ^{131}I tositumomab (Bexxar)	Murine	IgG2a/131 I	CD20	NHL refractory to Rituximab and relapsed following chemothera py	2003

AML, acute myeloid leukaemia; CLL, chronic lymphocytic leukemia; CRC, colorectal cancer; EGF, epidermal growth factor; NHL, non-Hodgkin's lymphoma; VEGF, vascular endothelial growth factor (Schrama et al., 2006).

CHAPTER III

MATERIAL AND METHOD

3.1 Materials

3.1.1 Phage display peptide library

Phage displayed random peptide libraries

1. SUT 12: 12 mer (12 NN G/T)
2. ANL 6: 12 mer (12 NNK) complexity 1.6 X 10^9
3. ANL10: fixed cysteine constrained XCxxxxxxCX complexity 3.6X 10^{10}
4. ANL12 displayed 8NNK (8-mer) complexity 13 X 10^9

3.1.2 Phage display human scFv library

Phage display human scFv libraries from 3 different sources of scFv genes were used.

1. Tomlinson I, a semi-synthetic human scFv library (MRC, Cambridge, UK).
2. Sullivan library a kind gift from Dr. Brian Kay, University of Illinois at Chicago, USA.
3. YAMO library (Pansri et al., 2009).

3.1.3 Cell lines

Cell line HepB3, COS-1 and HepG2 were grown in DMEM (Invitrogen), H69 were grown in RPMI-1640 medium (invitrogen) containing 10% fetal bovine

serum, 25 mM HEPES (Invitrogen) and 1X Antibiotic-antimycotic (Invitrogen) and incubated at $37^{O}C$ in 5% CO_2 incubator. Cholangiocarcinoma cell line KKU-100 was grown in HamF12 media (Invitrogen) containing 10% fetal bovine serum, 25 mM HEPES and 1XAntibiotic-antimycotic.

3.1.4 Instruments

Autoclave:	Hiclave HA-3000MIV, Hirayama, Japan
Balance:	Precisa 205A, Precisa Instruments, Switzerland
	Precisa 3000C, Precisa Instruments, Switzerland
Centrifuge machine:	Sorvall RC5C plus, Kendro laboratory Products, USA
Deep freezer -70 ^{o}C:	Heto, Ultra Freeze, Denmark.
	Eppendrof centrifuge 5810 R, Eppendrof, US
ELISA reader:	Sunrise, TECAN, Austria
Electroporator :	Eppendrof 2510, Eppendrof, USA
FTIR microscope:	Bruker infrared microscope and Bruker IFS55 FTIR spectrometer
Freezer -20 ^{o}C:	Heto, HLLF 370, Denmark.
	MyBio LFT420, DAIREI, Denmark
Gel Document set:	White/Ultraviolet Transilluminator GDS7500, UVP, USA
	Digital Graphic Printer UP-D890, Sony, Japan.
Gel dryer:	Drygel sr. SLAB GEL Dryer model SE1160, Hoefer Scientific Instruments, USA
Gel electrophoresis:	Mini Protean® 3 cell, BioRad, USA

Apparatus:

Heat Box:	HB1, Wealtee Corp., USA
Incubator shaker:	C24 Incubator shaker, New Brunswick Scientific, USA
Incubator:	Memmert, BE 500, WTB Binder BD115, Shel-Lab 2020 Low Temperature Incubator, Sheidon, USA
Laminar hood:	Holten LaminAir HBB 2448, Denmark. BH2000 Series ClassII Biological Safety Cabinets, BHA120 & BHA180, Clyde-Apac,
Membrane transfer:	Semi Phor, Hoefer Scientific instruments, USA
Machines:	
Automate electrophoresis:	Experion system, BioRad, USA
Microcentrifuge:	Mini spin plus, Eppendrof, USA Eppendorf 54154, Eppendorf, Germany
PCR machine:	DNA Engine PTC 200 peltier Thermal cycler, MJ Research, USA
pH meter:	Ultra Basic pH meter UB-10, Denver Instruments, Germany
Protein purification system:	KingFisher mL, Thermo Fisher Scientific, USA
Rotator:	Certomat TCC, B. Braun Biotech International, Germany Rotator AG, Fine PCR, Korea

Shaker:	Innova 2300 platform shaker, New Brunswick Scientific, UK Certomat TC2, B. Braun Biotech International, Germany
Stirrer:	Variomag Electronicrührer Poly 15, Germany Magnetic stirrer MSH300, USA Hot plate stirrer Labtech, Korea
Thermomixer:	Thermomixer compact, Eppendrof, USA

3.2 Method

3.2.1 Biopanning with phage display peptide library

Before selection, cells were incubated in serum free media for at least 2 hours. Pre-incubation of library with non-specific cell lines was applied before each round of selection with KKU-100 cells. Phage library pool with 100 equivalents of individual clone were incubated with HepB3, COS-1 and HepG2 cell on ice for 1 hour for each cell line and unbound phage in supernatant was used in selection with KKU-100 cells. After library was incubated with KKU-100 cells on ice for 1 hour, cells were washed with PBST05 and PBS for 10 times with 2 minutes incubation of each wash. The bound phage was eluted with 400 μl of 0.25% trypsin solution, 400 μl of 0.1M Glycine-HCl pH 2.0 and neutralized with 400 μl of 2 M Tris base. The eluted phages were pooled together and amplified by infected into log phase TG1 and grown at 37^{o}C 200 rpm for 4-6 hr. The amplified phage particles were concentrated by PEG precipitation and resuspended in PBS solution before next round of selection. The selection with pre-incubation with HepB3, COS-1 and HepG2 was done for 3-5 rounds to eliminate non-specific binding. After five rounds of selection, individual

phage clone was grown and cell based ELISA was also performed to confirm the specific binding. Cross reactivity binding with other cell lines was done by cell based ELISA. The O.D. value in the graph is the O.D. of phage clone minus cell background (without phage added).

3.2.1.1 Cell based phage display peptide ELISA

Cells were trypsinized and splited to grow in a 96-well plate until reach 70-90% confluence. Cells were washed with warm PBS 3 times and fixed with freshly prepared 4% PFA in PBS. Then, cells were washed with PBS 3 times and blocked with 4% MPBS for 1 hr. Phage supernatant of individual clone 75 μl and PBS 25 μl were added to each well cells were incubated for 2 hr. Cells were washed with 5 times PBST01 and 5 times with PBS, incubated for 2 minutes each wash. Anti M13 HRP 1:5000 was added and ABTS substrate was also added. The plate was incubated until color developed and the absorbance values were determined at 405 nm.

3.2.1.2 Cell based free peptide ELISA

The ELISA protocol as described in 3.2.1.1 was used. Biotinylated free peptide 0.4 μg/μl was added in stead of phage supernatant. After incubation for 1 hr., cells were washed with PBST05 5 times and PBS 5 times. Streptavidin HRP or AP conjugated 1:500 or 1: 5000 were added separately. After washing step, ABTS with H_2O_2 and pNPP, appropriate substrate for HRP and AP was applied, respectively. The absorbance at 405 nm. was determined.

3.2.1.3 Peptide binding efficiency test

The efficiency in binding test was carried out as same as the selection protocol as described in 3.2.1.1. Cells were grown in a 25 cm^3 flask to reach 70-90% confluence. Cells were washed with warm PBS 3 times (5ml/time). Each

individual phage clone supernatant 50 µl (10^{12} PFU/ml), 10^9 PFU in 500 µl of 4% MPBS were placed on cells instead of phage display peptide library. After washing and elution step, number of eluted phage was determined by serial dilutions. After incubated the plated at 37^{O}C overnight, infected bacteria which shown in blue plaques were counted. The PFU and binding efficiency were calculated. Binding efficiency define by the ratio of output phage versus input phage.

3.2.1.4 Phage peptide sequence analysis

Each phage clone was grown by infection into *E.coli*TG1 and plasmid preparation with Promega kit was performed. The quantity and purity of DNA was determined by Nano drop spectrophotometer. DNA sequencing was done by the UIC Research Resources Center (RRC DNA Sequencing center).

3.2.1.5 Free peptide synthesis

Free peptides were synthesized by UIC RRC. In order to do further detection, these free peptide were synthesized with biotin labeled at N terminal.

3.2.1.6 Cell staining with free peptide

KKU-100 cells and other control cells, H69, HepG2, HepB3 and COS-1 were cultured on LabTekII chamber slide (Nunc™) in CO_2 incubator. The cells were washed with warm PBS before 4% PFA or 4% formaldehyde fixation at room temperature for 20 min. After 3 times washing with PBS, cells were blocked with 4% MPBS or SuperBlock™ or 3% BSA for 1 h. Then free peptide solution between 100 ng/ml to 2 µg/ml were added to cells and incubated for 1 h. After intensive washing step with PBST05 10 times and PBS 10 times with 2 min incubation during each wash, cells were incubated with streptavidin AlexaFluor 488 dilution 1:X000 for 30 min to 1 h. Cells were removed remaining fluorescence residual with PBS and

VectaMount™ with DAPI was applied to slide and covered with coverslip (Fisher Scientific). The slide were stored at -20^{O}C or 4^{O}C before photograph under Axivert 200 or LSM 700 confocal microscope (Carl Ziess, Germany) or fluorescence microscope (Olympus). All images were analyzed using Axiovision program.

3.2.2 Biopanning with phage display scFv library

3.2.2.1 Biopanning with YAMO phage display human scFv library

The selection was done as described in previous report (Pansri et al., 2009). KKU-100 cells were incubated in serum free media at least for 2 hours before selection. Phage library pool with 100 equivalents of individual clone was used in selection with KKU-100 cell. After library was incubated with KKU-100 cells at 37 ^{O}C for 2 hours, cells were washed with PBST05 for 10 times and PBS 10 times with 2 minutes incubation each wash. The bound phage was eluted with 300 μl trypsin solution (10 mg/ml). After that 300 μl of 0.1M Glycine-HCL pH2.0 was added and neutralized with 300 μl of neutralization solution. Ten-fold serial dilution of eluted phage was done and spread on 2XYT containing amplicillin and 1% glucose to separate individual phage clone. Single colony was grown to rescue phage display scFv. An overnight culture was done by inoculated single colony in 200 μl of 2XYT containing amplicillin and 1% glucose and incubated at 200 rpm 37^{O}C for overnight. Next day, 1:100 dilution of an overnight culture was inoculated to freshly prepared 200 μl of 2XYT containing amplicillin and 1% glucose in 96 well plate and grown until cell reach log phase. Then super infection with helper phage was done at 37^{O}C for 1 hr. Bacteria cells were separated and resuspend in 200 μl of 2XYT containing amplicillin, kanamycin and 0.1% glucose. The culture was incubated at 30^{O} at least 20

hr to produce phage display scFv. Phage supernatant was used in cell based ELISA to confirm the specific binding. Cross reactivity binding with other cell lines was done by cell based ELISA. The O.D. value in the graph is the O.D. of phage clone minus cell background (without phage added).

3.2.2.2 Biopanning with Sullivan phage display human scFv library

The same selection protocol as described in 3.2.2.1 was performed with modification. Before selection cell were incubated in serum free media at least for 2 hours. Library pre incubation with non-specific cell lines was applied before each round of selection. Phage library pool 100 equivalents were incubated with HepB3, COS-1 and HepG2 cell on ice for 1 hour for each cell line and unbound phage in supernatant was used in selection with KKU-100 cell. After library was incubated with KKU-100 cells on ice for 1 hour, cells were washed with PBST05 for 10 times and PBS 10 times with 2 minutes incubation each wash. The bound phage was eluted by 0.25% trypsin solution, acid elution (0.1M Glycine-HCl pH 2.0) and neutralized with 2 M Tris base. The eluated phage was amplified by infected into log phase TG1 cell. A single colony of individual clone was separate by spreading on LB containing carbenicillin and the plated was incubated at 37^{O}C overnight. Next day all colonies was scraped and pooled together then amplified by growing TG1 cell harboring phagemid with in LB containing carbenicillin at 250 rpm 37^{O}C for 3 h. After cell growing reach log phase, helper phage infection was done. This infection was incubated at 150 rpm at 37^{O}C for 2 hr. Then all culture was growing in LB containing carbenicillin and kanamycin at 30 ^{O}C 250 rpm at least 20 hr. Phage particles were precipitate by 0.2 volume of 24% PEG/2.5 M NaCl for 1 hour and resuspended in PBS for next round of selection. In next round of selection, 100 μl of amplified phage

was used. After 5 rounds of selection, individual phage clone was separated by serial dilution before infection into *E.coli* TG1. Infected *E.coli* were spread on LB containing carbenicillin agar plate and incubated at 37 ^{O}C overnight. Each colony represent individual scFv clone was randomly selected and grown in deep well plated filled with 100 μl of LB media containing carbenicillin at 37^{O}C 250 rpm. for 3-4 hr. until cell reach log phase, then infected with helper phage at 150 rpm 37^{O}C for 1 hr. and the media was removed and resuspended cell in LB contain carbenicillin and kanamycin at 30^{O}C 250 rpm at least 20 hr. Phage supernatant was used in phage cell based ELISA.

3.2.2.3 Cloning scFv gene into expression vector pKP300ΔIII

After DNA analysis of scFv sequence, scFv clones with different DNA sequence were cloned into alkaline phosphatase expression vector (pKP300ΔIII). Both phagemids containing scFv genes and expression vector were digested with two restriction enzymes, *HindIII* and *SalI*. Restriction digested scFv insert and vector were ligated together to generate vector to express scFv with FLAG tag at N terminal and enzyme alkaline phosphatase and 6His tag fusion at C terminal as shown in figure 7. This reaction was first incubated at 37^{O}C for 1 h., and heat inactivated at 65^{O}C for 20 min. Then *SalI* digestion was continued at 37^{O}C for 1 h. and heat inactivated at 65^{O}C for 20 min. For vector, CIP was added into reaction and continue reaction at 37^{O}C for 1 h. before heat inactivation at 65^{O}C for 20 min (detail in appendix).

After complete digestion, vector and insert were separated in 1.2% agarose gel and the complete digested scFv (~ 800 bp.) and vector (~5 kb.) fragment were excised from gel. Gel purification was done and DNA concentration was

determined by NanoDrop spectrophotometer. The ligation reaction was set up with insert : vector ratio was 3:1. Vector should be at least 50 ng for each ligation reaction.

The ligation reaction was incubated at 16^{O}C for 16 h. and heat inactivated at 65^{O}C for 20 min. Ligation reaction was desalted on membrane to reduce salt from reaction before electroporated into *E.coli* TG1 competent cell. Recovered *E.coli* cell contained plasmid was selected by spread *E.coli* on LB + CB plate with Coli roller plating beads. The plates were incubated at 37^{O}C for overnight. Next day single colony was picked to confirm scFv insert by colony PCR technique. Single colony was picked and dropped into sterile water 10 µl and the same colony was replicate by dipping into LB+CB plate. This plate was grown at 37^{O}C for stock of *E.coli.* Bacterial cell was lysed by heating at 95^{O}C for 10 min and 5 µl of solution was used as DNA sample for PCR amplification for scFv insert following the reaction below. After that PCR amplification product was separated by 1.2% agarose gel and the positive clone was selected to grow and glycerol were made for next step.

Each positive clone was selected to grow in LB media with carbenicillin at 37^{O}C, 250 rpm for overnight and plasmid purification using Promega plasmid preparation kit. The concentration of plasmid was measure by NanoDrop. The AP positive plasmid was deleted AP gene by enzyme *AscI* digestion and re-ligated vector with scFv fragment together to generate plasmid for soluble scFv expression.

Digestion reaction was done with DNA at 500 ng in volume 20 µl (This will make DNA concentration is 25 ng/ µl) and after finished reaction it was diluted with water to 1:20 (This will decrease DNA concentration to 1.25 ng/µl) to prevent digested AP fragment re-ligation. Only 4 µl was taken to do ligation. The ligation reaction was carried out at 25^{O}C for 3 h. and dialysis was done by spot

ligation reaction on membrane floating on deionized water for 1 hr before electroporated into *E.coli* TG1. The positive clones on LB contained carbenicillin plate after incubated at 37^{O}C for overnight was picked and confirmed complete removing AP fragment by PCR as described above. The positive clones were picked to grow and store in glycerol stock for scFv expression and purification.

3.2.2.4 Production of soluble scFv with AP (scFv_AP) and soluble scFv

Plasmid for scFv_AP and scFv expression were constructed and transformed into TG1 host cell. Cells harboring constructed was grown in low phosphate media with carbenicillin at 30^{O}C 250 rpm at least 20 h. Then cell was collected by centrifugation and lysed with 1X bugbuster (Novagen) contain benzonase (Novagen). This reaction was incubated on ice for at least 15 minutes. Clear cell lysate was separated from cell debris and used in cell based ELISA to confirm the binding affinity. Purify soluble scFv_AP and soluble scFv by 6XHis Tag was carried out using clear cell lysate.

3.2.2.5 Soluble scFv purification by IMAC

Clear cell lysate supernatant was mixed with NovaHisMag™ beads in the ratio 1 ml of supernatant with 20 μl of magnetic bead slurries and the protein purification program by KingfishermL protein purification system was applied. The purification program is set as shown below

Step1	Incubation	30 min.
Step2	Washing	2 min. (3 times)
Step3	Elution	30 min.

The elution was done with final volume 100 μl of elution buffer. Immidazole remaining in elution buffer was eliminated with ZebraDesalt™ column.

Purified soluble scFv was determined quatitatively and quanlitatively by Experion automated gel electrophoresis (detail in appendix).

3.2.2.6 Soluble scFv cell based ELISA

The same protocol of phage ELISA was done. Crude cell extract from clear cell lysate of individual clone 20 µl and PBS 80 µl was added instead of phage supernatant. The washing step was done as previous described. And anti FLAG M2 conjugated with HRP (Sigma) or AP were used to detect FLAG tag in soluble scFv. After washing step, appropriate substrate, ABTS or pNPP were added to develop color. For soluble scFv with AP fusion in cell based ELISA, the ELISA protocol as previously described was done. Crude cell extract containg soluble scFv with AP fusion were used. After washing step, Sigma fast pNPP substrate was added to detect remained bound AP fusion protein.

3.2.2.7 Soluble scFv in western blot assay

The cells were grown in cell culture dish until reach >70% confluence, they were washed with pre-warm PBS and 1X Cytobuster (Novagen) cell lysis buffer was applied. After incubated for 5 min, all cells were scraped and spun at 10,000 rpm to obtain clear cell lysate and stored in -20^{O}C. Novex Tris-Glycine SDS Sample Buffer (2X) (Invitrogen) was equally mixed with cell lysate sample. SDS poly acrylamide gel electrophoresis was carried out and separated proteins were transferred into PVDF membrane at 30V for o/n. Next day, transferred membrane was blocked with 4% MPBS. After washing step, clear cell lysate and/or purified form of soluble scFv protein was incubated for 1 h. Then BCIP/NBT substrate for AP was applied after washed with PBST and PBS. For soluble scFv with FLAG tag, anti FLAG tag HRP/AP was applied first then ECL chemiluminescence kit (Amersham Pharmacia) or

BCIP/NBT (Sigma) appropriate substrate was poured on top of membrane. After developed color, the membrane was washed to reduce any non-significant background binding. For chemiluminescence substrate, the membrane was washed then read with StromImager scanning to obtain signal.

3.2.2.8 Soluble scFv in pull down assay

The cells were grown on cell culture dish until reach 70-90% confluence. After 3 times washed with warm PBS, 1X Cytobuster (Novagen) was added to lyse cell. All cells were scraped from dish and any unsoluble remaining was removed by centrifugation. Clear cell lysate supernatant were stored at -20^{O}C until used. Purified soluble scFv was incubated with clear cell lysate at least 4 hr. at 4^{O}C. Then KingfishermL automated protein purification was used to re-purified soluble scFv and binding components by immobilized metal ion affinity chromatography (IMAC). All pull down proteins was separated by SDS gel electrophoresis and stained with coomassie blue.

3.2.2.9 Soluble scFv in cell staining test

The cells were grown on LabTekII Chamber slide (Nunc) until reach 70% confluence. After pre-incubated in serum free media for at least 2 hr, cells were washed with warm PBS and blocked with 4%MPBS/SuperBlock™ for 1h. The cells were washed and purified soluble scFv was added. The pNPP substrate for AP was used to developed color and applied VectaMount™ before cover with coverslip. Anti FLAG M2 biotin (Sigma) 1:5000 was applied to detect FLAG tag and Streptavidin AlexaFlur488 1:1000 was added. VectaMount™fluorescence mounting medium was put before coverslip and nail polish was sealed. The slides was sealed with nail polish to prevent drying and stored at -20^{O}C before photograph under

Axivert 200 confocal microscope (Carl Ziess, Germany). All images were analyzed using Axiovision program.

3.2.2.10 Soluble scFv in FTIR microscopy analysis

Cells were grown to reach 80% confluence. After trypsinization cells were resuspend in PBS buffer. Soluble and supernatant of scFv were added to 10^5 cells in each suspension and incubated at $4^{O}C$ for 1 hr. Cells were wash with PBS 3 times and changed to resuspend in 0.85% NaCl. Cells in each condition were spot on slide, infrared transparent substrate. The samples were then desiccated for 24 hr and washed with distilled water twice. The slide was dry again before observe under FTIR microscope. Spectra were collected and analyzed with OPUS 65 and Unscrambler program.

CHAPTER IV

RESULT

4.1 KKU-100 biopanning optimization

4.1.1 Biopanning with phage display peptide libraries

Since the method for cholangiocarcinoma cell based selection has not been established. The first step is to established biopanning method for both phage display peptide and scFv libraries against KKU-100 cell line. The biopanning method for KKU-100 was optimized according to successful selection protocol such as gastric cancer cell line (Hu et al., 2006), large lung carcinoma cell line (Hu et al., 2006; Lipes et al., 2008; Oyama et al., 2006; Oyama et al., 2003) and hepatocarcinoma (Du et al., 2006).

Various selection procedures were performed as listed in table 7. These included library pre-incubation with COS-7 (experiment 1) and selection with fixed cells instead of live cell (experiment 2). No specific peptide could be selected from experiment 1 and 2 when selection was done with ~10^5 cells in 6 well plate format (Figure 8 and 9). Therefore, numbers of target cells were increased in experiment 3, 4 and 5 for library ANL10, ANL12 respectively. Unfortunately, neither higher cell number nor different phage display peptide library yielded any positive result after 3 rounds of selection (Figure 10 and 11). The next step, the selection experiment was further modified following McGuire et al., 2009, as described in experiment 6. Finally, after five rounds of selection with library pre-incubation (substraction) with

cell lines, e.g. HepB3, COS-1 and HepG2 the affinity selection experiment was successful. Thus the result indicated that experiment 6 which library pre-incubation with other cell lines and using CX6C library is the most efficient method for the selection of phage display peptide library against KKU100 cells.

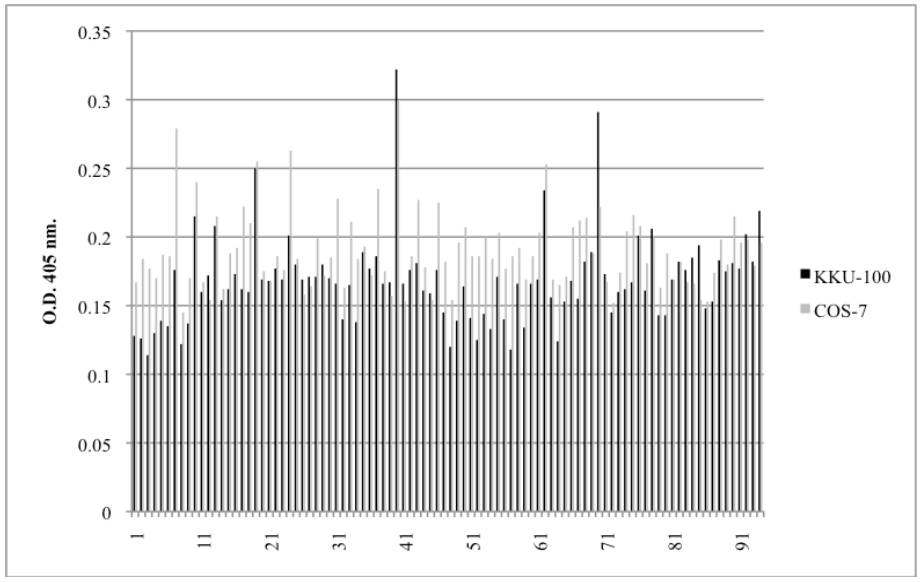

Figure 8. Phage cell based ELISA of KKU-100 selected clones from 3 round of selections with SUT12 library using experiment 1.

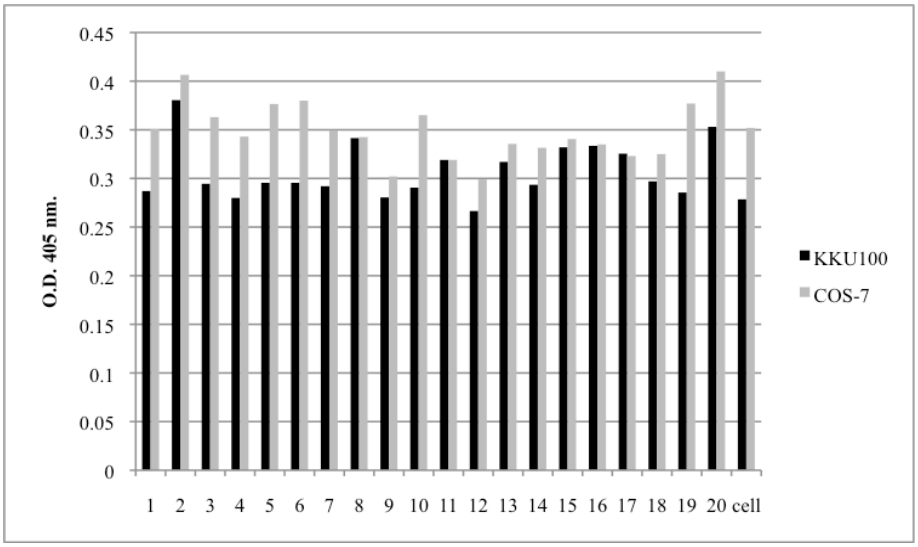

Figure 9. Phage cell based ELISA of KKU-100 selected clones from 3 round of selections with SUT12 library using experiment 2.

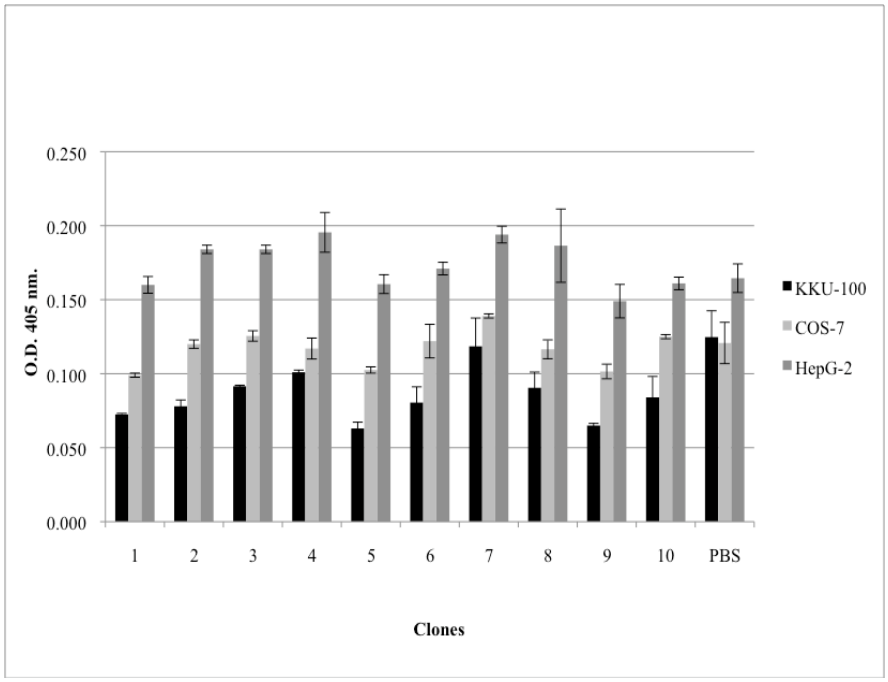

Figure 10. Phage cell based ELISA of KKU-100 selected clones from 3 round of selections withSUT12 library using experiment 3.

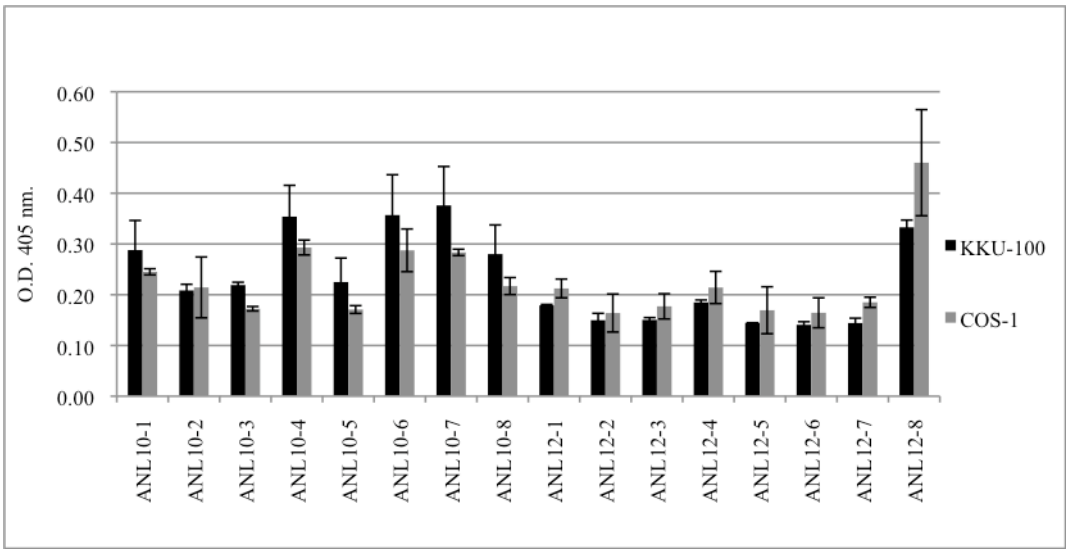

Figure 11. Phage cell based ELISA of KKU-100 selected clones from 3 round of selections with ANL10 and ANL12 library using experiment 4 and 5 respectively.

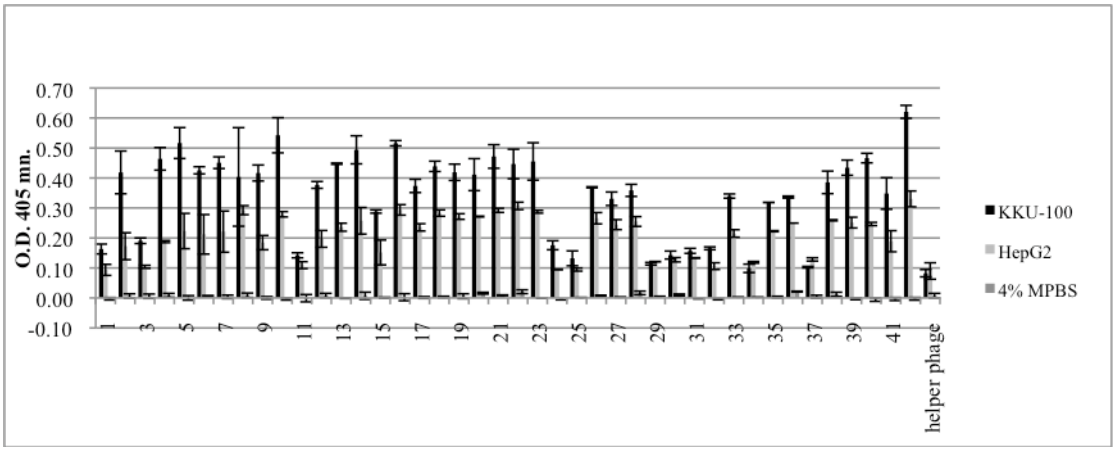

Figure 12. Phage cell based ELISA of KKU-100 selected clones from 5 round of library pre-incubations and selections with ANL6 and ANL10 library using experiment 6.

Table 7. Various trial experiments for biopanning of phage display peptide against KKU-100 cells.

Experiment*	Library‡	Cell number	Lived/ Fixed cell	Library pre-incubation	Temperature (^{O}C)	Round of selection	Blocking buffer	Washing buffer	Elution buffer	Specific peptide
1	SUT12	~ 10^5	Lived	COS-7	37^{O}C	3	3% MPBS	PBS with 0.05% Tween 20 And PBS	Glycine HCl pH 2.0	0/94
2	SUT12	~ 10^5	Fixed	-	25^{O}C	3	3% MPBS			0/20
3	SUT12	5 x 10^5	Lived	-	37^{O}C	3	3% MPBS			0/10
4	ANL10	5 x 10^5	Lived	H69	37^{O}C	3	4% MPBS			0/8
5	ANL12	5 x 10^5	Lived	H69	37^{O}C	3	4% MPBS			0/8
6	ANL6 : ANL10 (1:1)	5 x 10^5	Lived	HepB3, COS-1 and HepG2	4^{O}C	5	4% MPBS		Trypsin 0.25 % and Glycine HCl pH 2.0	42/42

* Detail in appendix .

‡Phage display peptide library description SUT12 displayed $NN(G/T)_{12}$, ANL 6 displayed 12NNK codons (12-mer), ANL 10 displayed XCXXXXXXCX (fixed cysteine constrained), ANL12 displayed 8NNK codons (8- mer).

4.1.2 Biopanning with phage display scFv libraries

Since number of cells had an effect on successful selection with KKU-100 cells, biopanning with phage display scFv was done with ~5 x 10^5 cells. Successful selection experiments are shown in table 8. Optimization experiment indicated that, different libraries require different methods.

Table 8. Successful methods for KKU-100 biopanning with phage display scFv.

Experiment*	Phage display scFv library	Library preincubation (substraction)	Temperature (^{O}C)	Round of selection	Blocking buffer	Washing buffer	Elution buffer	% positive clones
1	YAMO	-	37^{O} C	1	3% MPBS	PBS with 0.05% Tween 20 And PBS	Trypsin 0.25 % and Glycine HCl pH 2.0	2.08% (2/96)
2	Tomlinson I	-		1				2.08% (2/96)
3	Sullivan's lab	-		1	0.5 % casein			2.08% (2.96)
4	Sullivan's lab	COS-1		1	4% MPBS			0.52% (1/192)
5	YAMO	-		2				7.29 % (14/192)
6	Sullivan's lab	-		2				5.57% (16/287)
7	Sullivan's lab	H69		2				15.79% (15/95)
8	Sullivan's lab	4 COS-1		2				9.90% (19/192)
9	Sullivan's lab	HepB3, COS-1 and HepG2	4^{O} C	5				72.63 % (69/95)

* Detail in appendix

4.2 Selection and characterization of KKU-100 specific peptide.

Following experiment 6, the number of recovery phage was increased from ~10^3 to ~10^5 when selection round was increased from 3 to 5 (Figure 7). Most of selected clone showed specific binding to KKU100 when compare with control wild type phage (helper phage), and exhibited low cross reactivity with HepG2 cell (Figure 12).

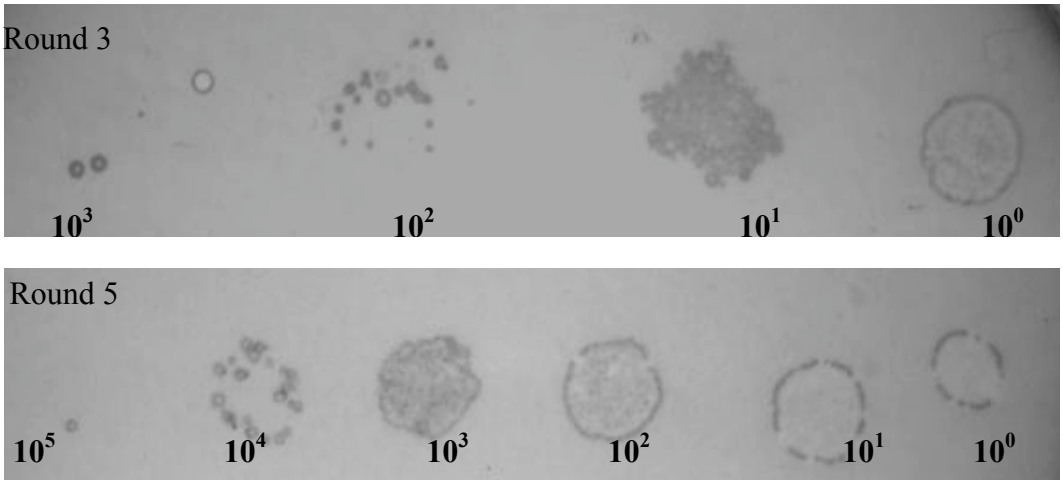

Figure 13. Phage titer after three and five rounds of selection with ANL6 and ANL10 library using experiment 6.

4.2.1 Peptide sequence analysis

Twenty phage clones that showed relatively high positive signals in ELISA were selected for DNA sequencing to determine amino acid sequences. Only two types of ligand sequences had been selected (Table 9). And the frequency of CTSPPYVC peptide is higher than another peptide CTTPPYVC. These are novel peptides that show specific to cancer cell line.

Table 9. Frequency of peptide sequence from 20 selected phage clones.

Clone*	**Peptide sequence**	**Frequency**	**%**
P 1, 4,24,25,49	CTTPPYVC	5	25
P2,4,5,6,7,9,10,13,14,43,47,48,50,57,58,	CTSPPYVC	15	75

*These are all selected clones that showed selectivity to KKU100 cell signal higher than two fold as judged from ELISA when compared with those from HepG2 and COS-1 cells (Figure 14).

Specific binding of phage displaying CTTPPYVC and CTSPPYVC was confirmed by cell-based phage ELISA. The two phages could bind specifically to KKU-100 showed low cross-reactivity with other cell lines e.g. HepG2 and COS-1 cells (Figure 14).

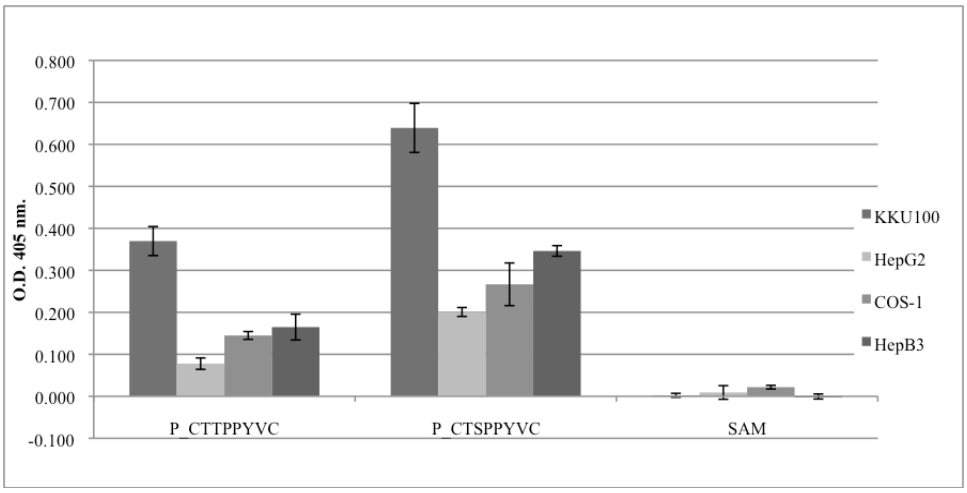

Figure 14. Cell based ELISA showed the specific binding of phage particle display two types of peptide, CTTPPYVC and CTSPPYVC, when compare with wild type phage (SAM phage). These phages showed ELISA signal with KKU100 cell line 2 times higher than HepG2, COS1 and HepB3.

4.2.2 Phage display peptide binding efficiency test

Binding efficiency of the two selected phage displayed peptides was carried out to confirm their specificity to KKU-100 cells. The results indicated that efficiency of binding of the two phages to KKU100 was at least 1,000 times higher than those to HepG2 cell, which were used in library pre-incubation (Figure 15).

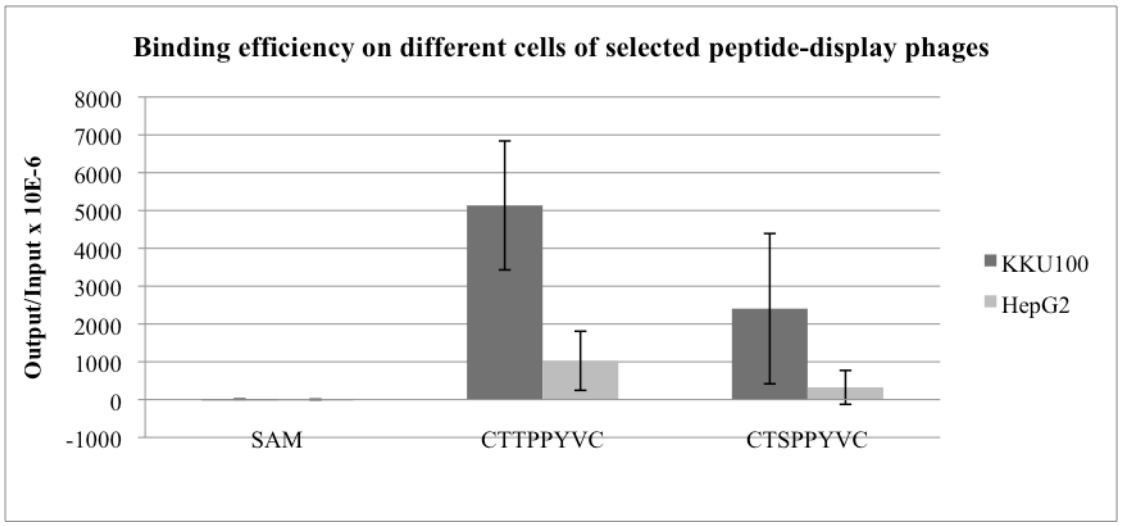

Figure 15. Specific binding efficiency of peptide attached with gene III in phage context. Mean values from two or more independent experiments are shown.

4.2.3 Binding of free biotinylated peptide in cell based ELISA

Two peptides, CTTPPYVC and CTSPPYVC, were synthesized with N-terminal biotin labeled by UIC RRC (UIC Research Resources center, IL, USA) and confirmed the binding ability of free peptide without gene III by cell-based ELISA. Biotinylated peptides were detected by streptavidin HRP conjugated (1:500) (Figure 16) or streptavidin alkaline phosphatase conjugated (1:1,000) (Figure 17). Free peptide showed capability in binding to KKU-100 when compared with control peptide. This revealed that peptides could function without gene III. However, they also bind to other cells.

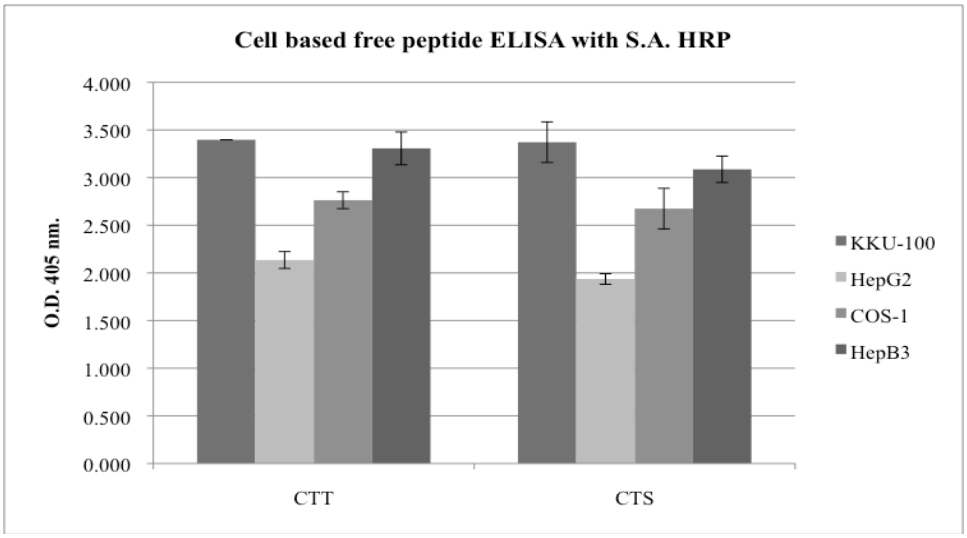

Figure 16. Cell-based ELISA with free peptide N-terminal biotin labeled. These peptides were detected with streptavidin HRP conjugated and ABTS with H_2O_2 substrate.

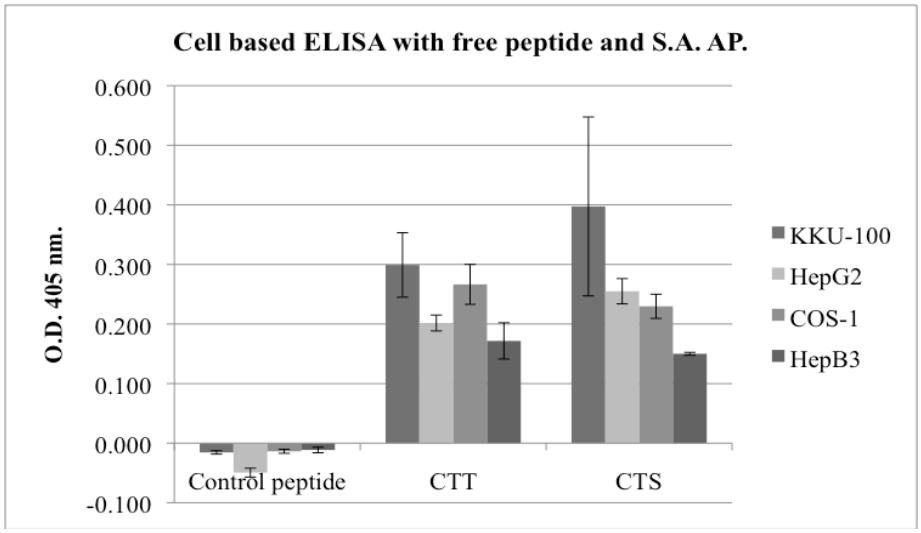

Figure 17. Cell-based ELISA with N-terminal biotin labeled peptide. These peptide were detected by streptavidin alkaline phosphatase conjugated and pNPP substrate.

4.2.4 Peptide mutational analysis

To analyze the peptides in more detail, three more peptides were synthesized ATTPPYVA, ATTPPYVCS and CAAAAAAC. Cell-based ELISA showed that two of synthesized peptide did not function due to lack of cysteine residue at both ends (Figure 18) indicating that cyclization is required for binding. Surprisingly this peptide still function when other amino acids between cysteine residues were substituted by alanine (Figure 18).

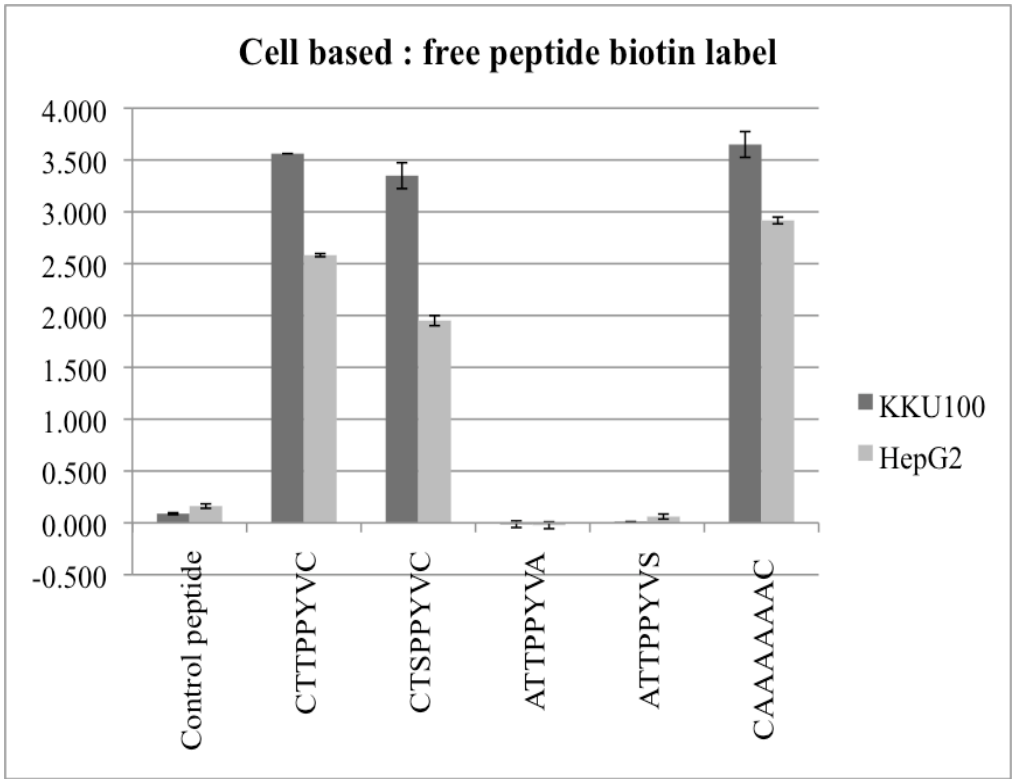

Figure 18. Cell based ELISA result with synthetic biotin label free peptide. Streptavidin HRP conjugated and ABTS with H_2O_2 substrate were applied.

4.2.5 Free peptide in cell staining assay

Immunocytochemistry is a technique that can reveal a position or pattern of ligand in the cell, since free peptides could function without gene III, they were explored in cell staining. Cells were fixed with 4% formaldehyde and blocked with 4% MPBS. Free peptides with biotin labeled were incubated with cells, and washed with PBS + 0.05% Tween 20 and PBS. Alexa Flour! 488 conjugated strepavidin was added to stain biotinylated peptide. In addition, DAPI mounting was applied to stain DNA. The staining pattern showed that these two peptides bind to molecules expressed all over the cells (Figure 19 and 20) and could react with other cell lines. In addition staining pattern of ATTPPYVA, ATTPPYVCS and CAAAAAAC peptides were also explored to confirm ELISA result. As shown in Figure 21, CTSPPYVS, CTTPPYVC and CAAAAAAC could bind to KKU-100. This is confirmed that the binding of CX6C peptide function depending on two cysteine residues.

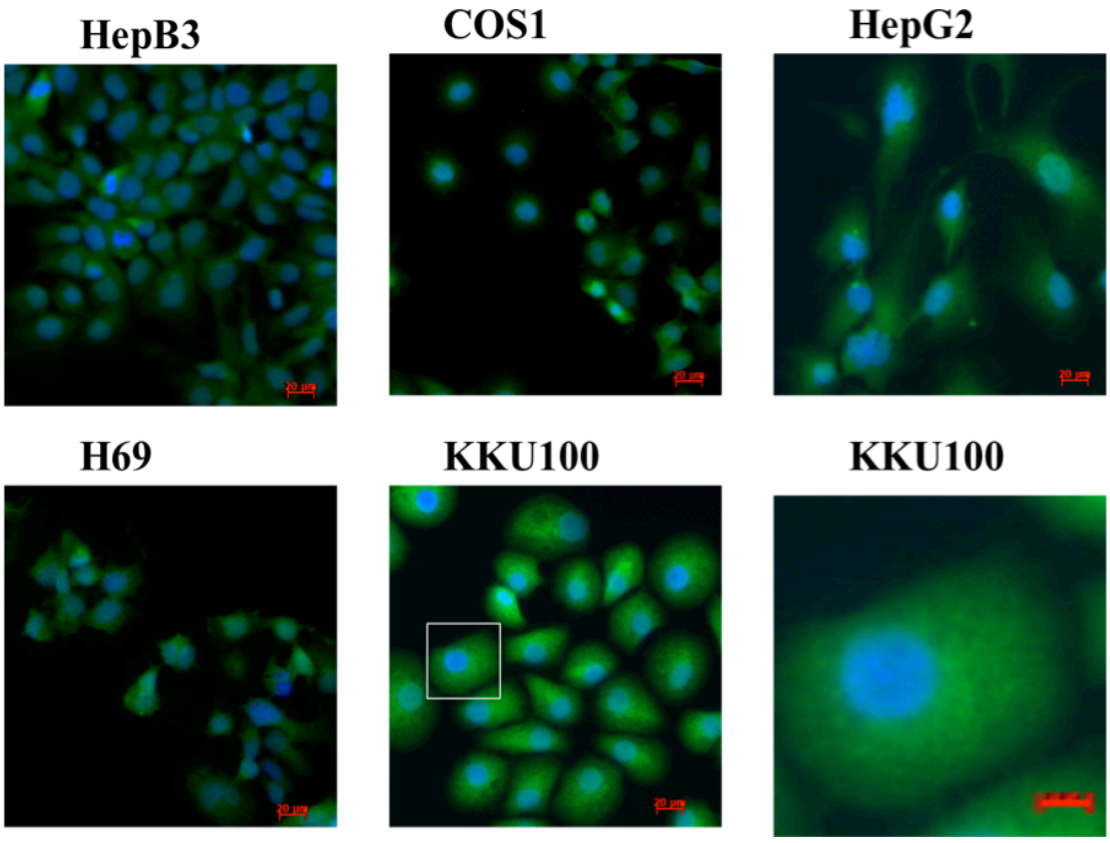

Figure 19. Staining pattern of CTSPPYVC free peptide. Cells were grown in slide chamber and fixed with 4% formaldehyde. After cells were blocked with 4% MPBS, free peptide 4 μg/ml was incubated with cells. Biotinylated peptide was detected by Alexa Flour! 488 conjugated streptavidin, nucleus was stained with DAPI (as shown in green and blue fluorescence respectively)

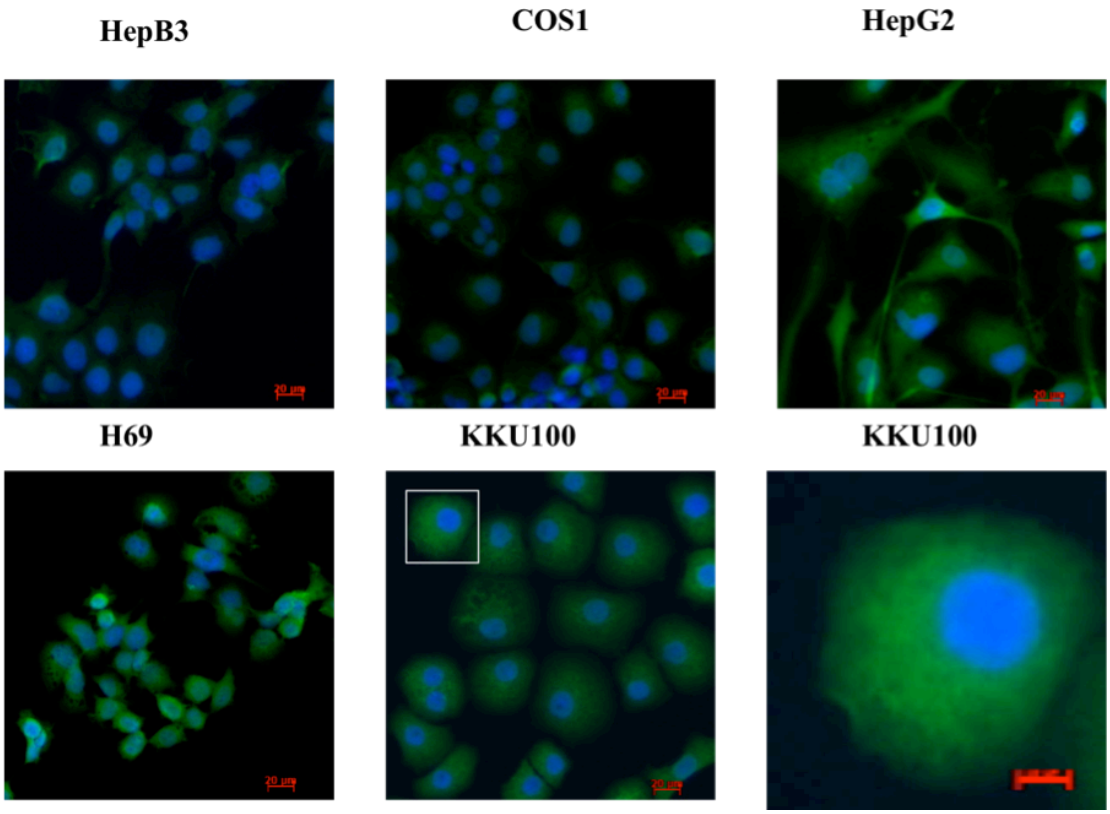

Figure 20. Staining pattern of CTTPPYVC free peptide. Cells were grown in slide chamber and fixed with 4% formaldehyde. After cells were blocked with 4%MPBS, free peptide 4 μg/ml was incubated with cells. Biotinylated peptide was detected by Alexa Flour! 488 conjugated streptavidin, nucleus was stained with DAPI (as shown in green and blue fluorescence respectively)

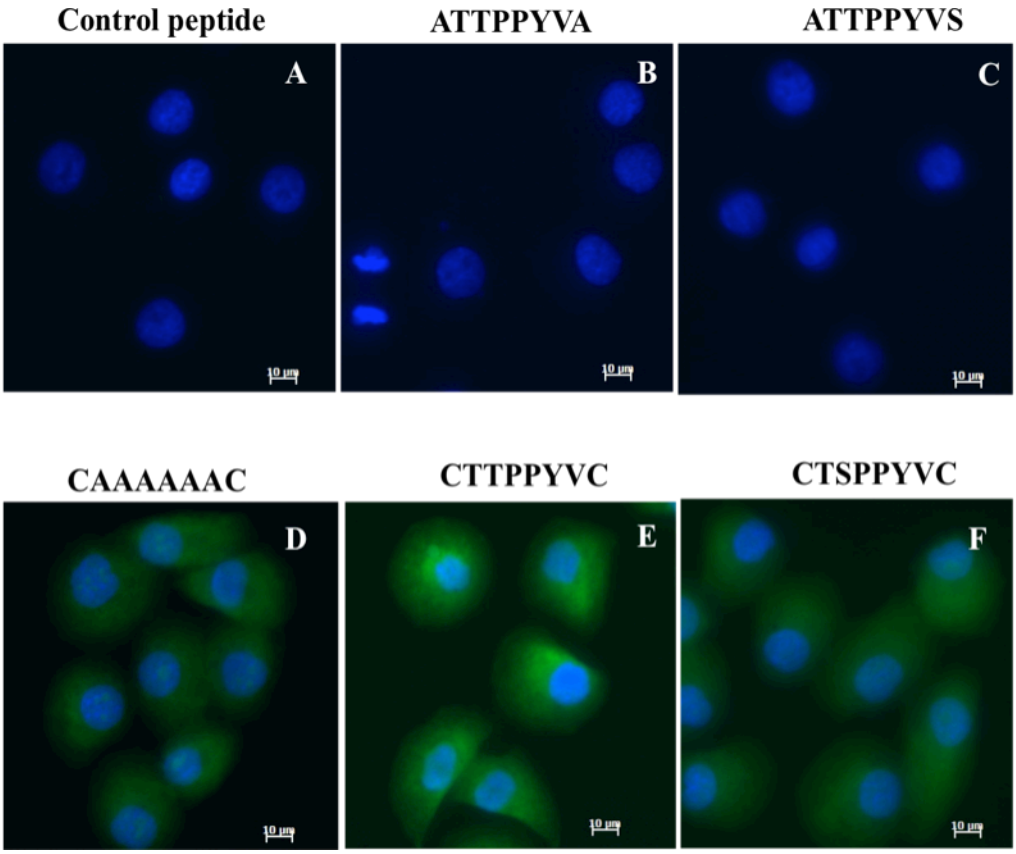

Figure 21. Staining pattern of KKU100 with 6 different peptides, control peptide, ATTPPYA, ATTPPYVS, CAAAAAAC, CTTPPYVC and CTSPPYVC. Cells were grown in slide chamber, fixed with 4% paraformaldehyde and blocked with 4% MPBS. Free peptide 4 μg/ml was incubated with cells. Biotinylated peptide was detected by Alexa Flour! 488 conjugated streptavidin, nucleus was stained with DAPI (as shown in green and blue fluorescence respectively)

Since previous staining result showed that the peptide cross react to other cells, modification of cell staining protocol was done to improve the staining patten. When using commercial SuperBlock , cell-staining signal of KKU-100 and

H69 cells were changed. CTSPPYVC peptide showed higher signal to KKU-100 than H69 cells (Figure 22). The binding pattern was found when 3% BSA was used as blocking solution, moreover, clear specific signal could be obtained with CarlZiess LSM700 microscope. It showed higher specific binding to KKU-100 when compared with HepG2 (Figure 23).

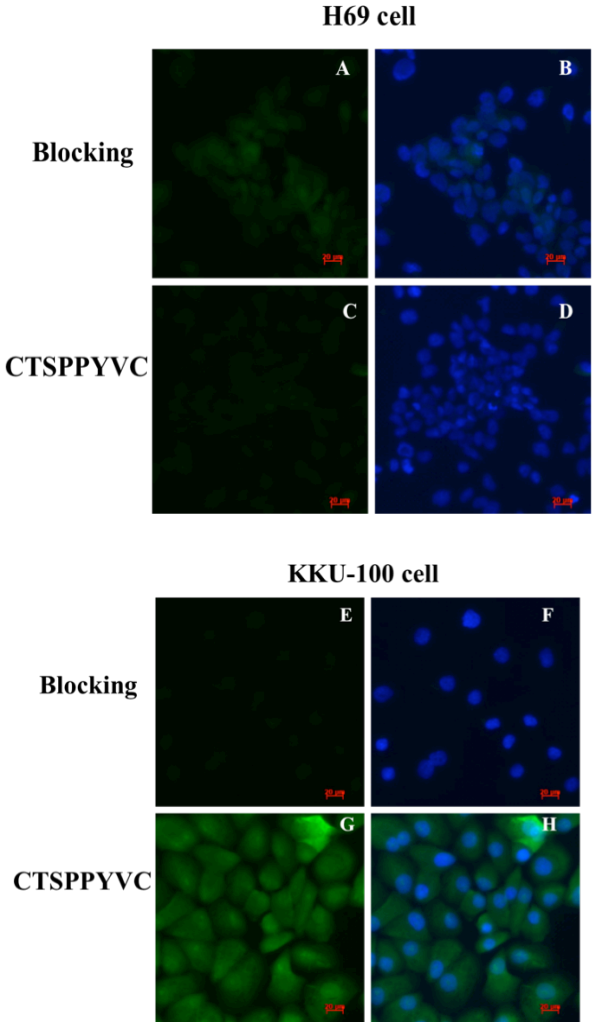

Figure 22. CTSPPYVC free peptide staining pattern different from H69, control cell. Cells were grown in slide chamber, fixed with 4% paraformaldehyde and blocked with SuperBlock . Free peptide 4 μg/ml was incubated with cell. Biotinylated peptide was detected by Alexa Flour! 488, nucleus was stained with DAPI (as shown in green fluorescence)

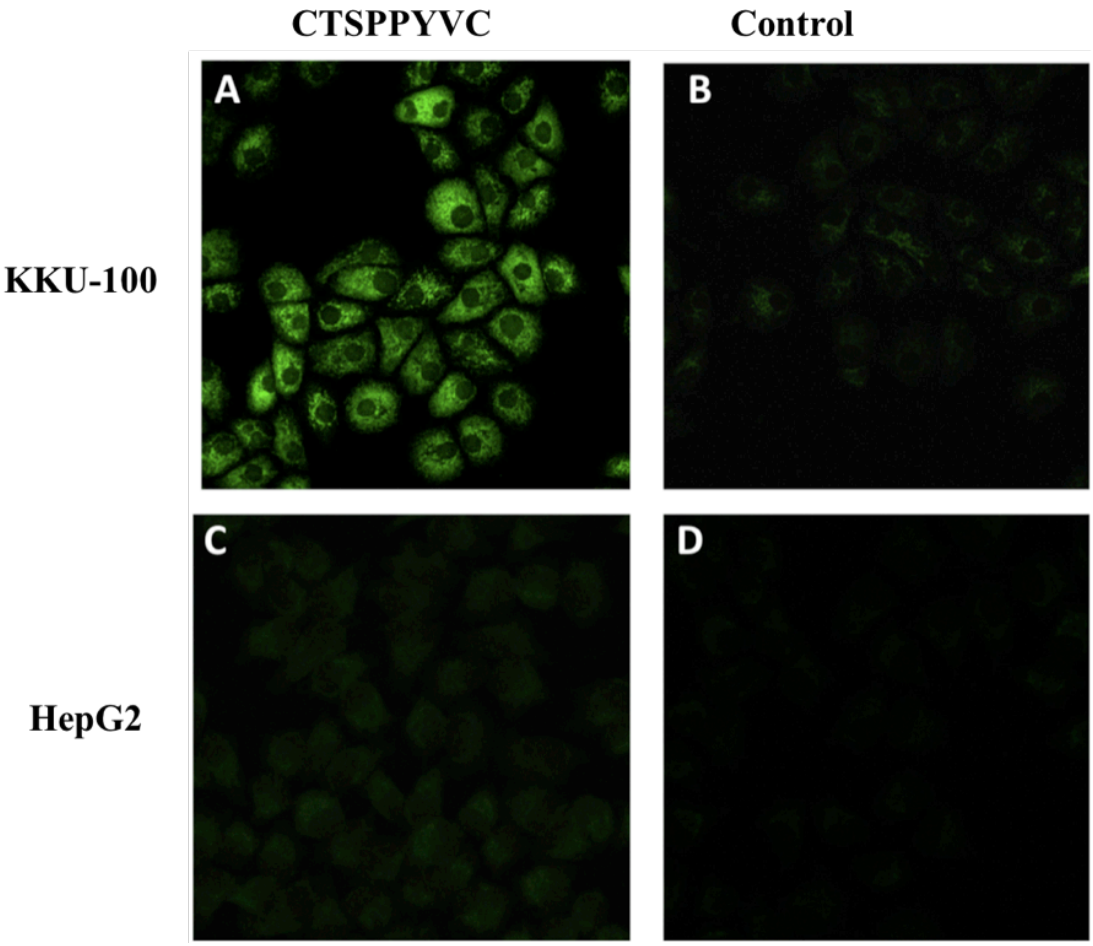

Figure 23. Peptide binding was analyzed by CarlZiess LSM700 microscope**.** Cells were grown in slide chamber, fixed with 4% paraformaldehyde and blocked with 3%BSA. After blocking with 3% BSA, free peptide 4 μg/ml was incubated with cell. Biotinylated peptide was detected by Alexa Flour! 488 conjugated streptavidin (as shown in green fluorescence)

In conclusion specific peptides CTSPPYVC and CTTPPYVC against KKU-100 cells were selected using experiment 6. Cyclization is an important role in binding to ligand as indicated by ELISA and cell staining.

4.3 Selection and characterization of phage display human scFv antibody against KKU-100 cell

4.3.1 Affinity selection of phage display human scFv

To identify cell surface targets for cholangiocarcinoma, three phage display human scFv libraries, YAMO library, Tomlinson I library and Sullivan's lab library were used in selection with cholangiocarcinoma cell line, KKU-100. Biopanning with lived KKU-100 cell was performed as described in Table 8. The cell based ELISA was performed to test the binding ability of selected clone. From biopanning with YAMO library, only one round selection with PBS with 0.05% Tween20 and PBS as washing buffer revealed specific scFv to KKU-100 cell (Figure 24). Selection with Tomlinson I library as described in experiment 2 obtained only one positive clone (Figure 25). Two positive clones were selected when biopanning with Sullivan library using experiment 3 was done (Figure 26), as YAMO library.

Repeated round of selection increased number of positive clones as shown in Figure 27 and 28 but also reduced the variety of scFv (*BstNI* finger print analysis: appendix).

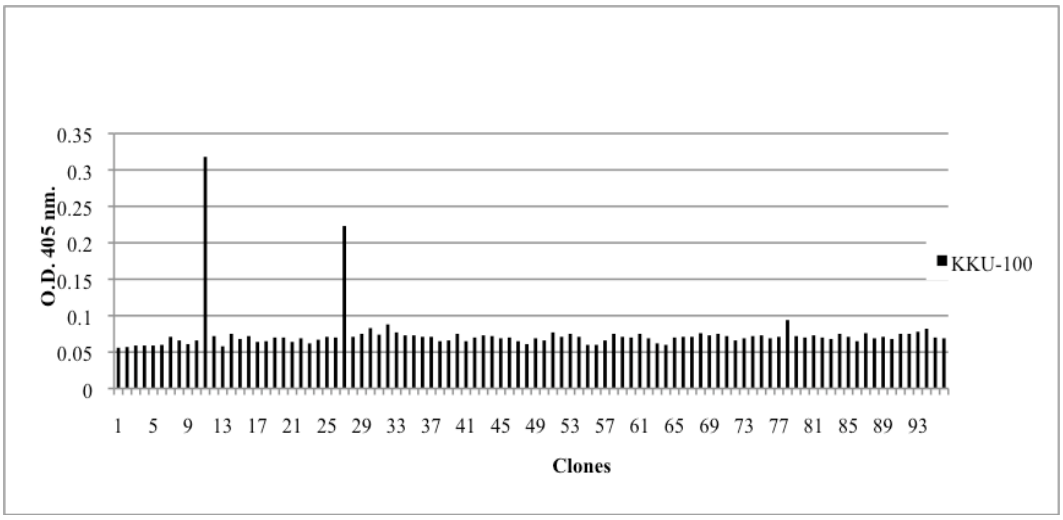

Figure 24. Cell based phage ELISA showed positive clones selected using experiment 1 : Direct selection using YAMO library at 37^{O}C.

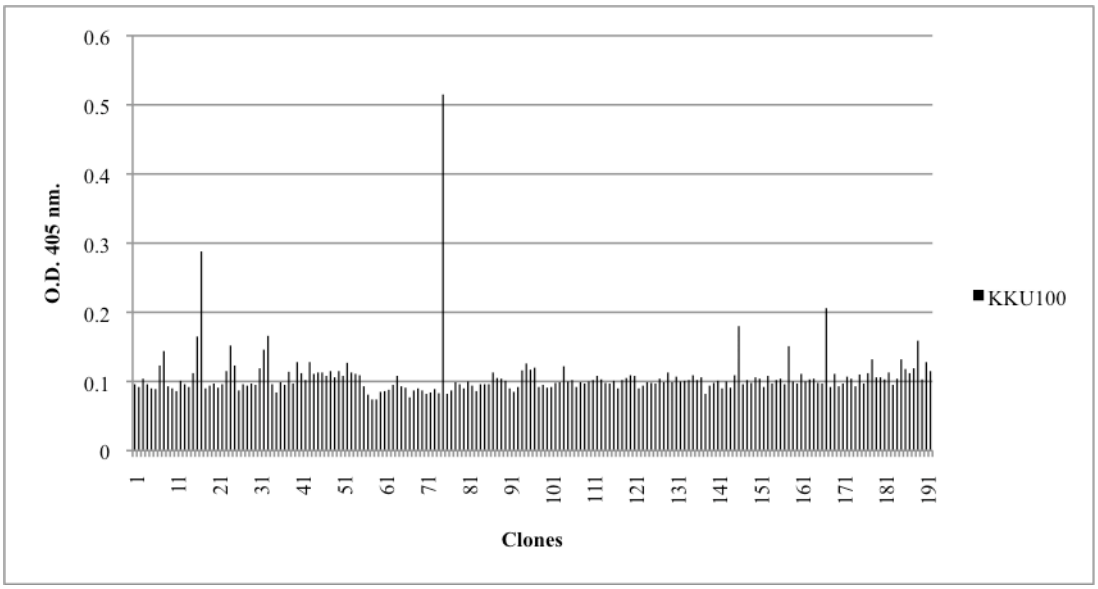

Figure 25. Cell based phage ELISA showed positive clones selected using experiment 2 : Direct selection with KKU100 using Tomlinson I library.

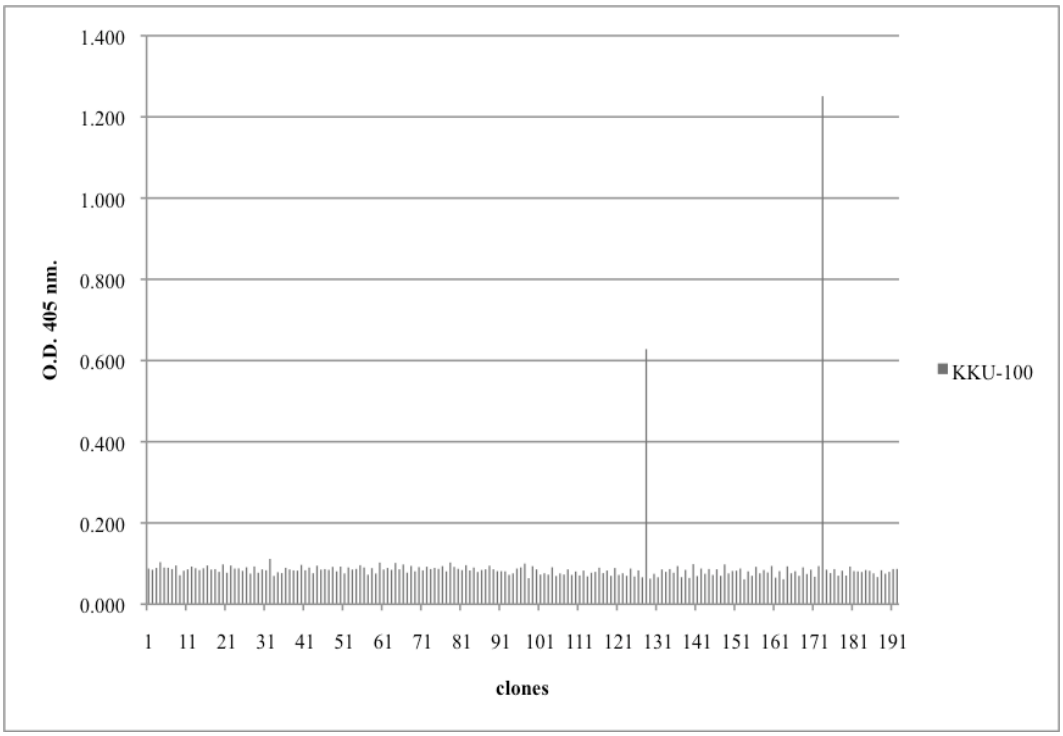

Figure 26. Cell based phage ELISA showed positive clones selected using experiment 3 : Direct selection using Sullivan library one round of selection.

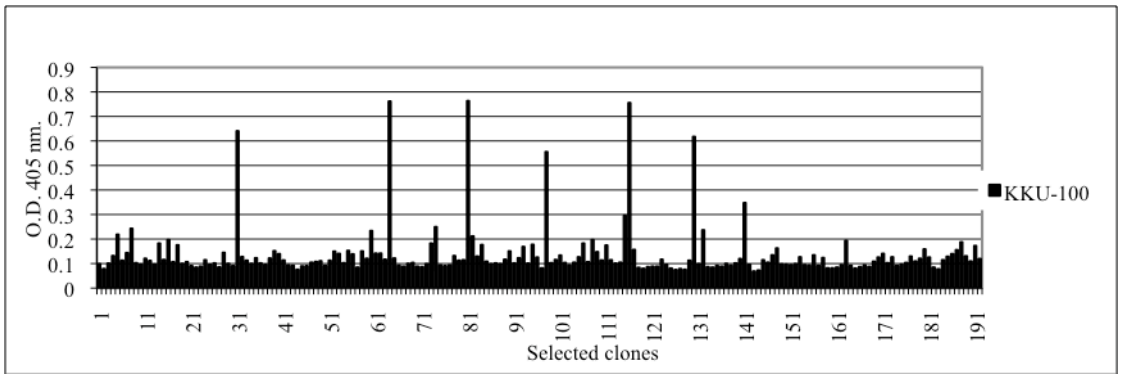

Figure 27. Cell based phage ELISA showed positive clones selected using experiment 5 : Direct selection using YAMO library at 37^{O}C two rounds of selection.

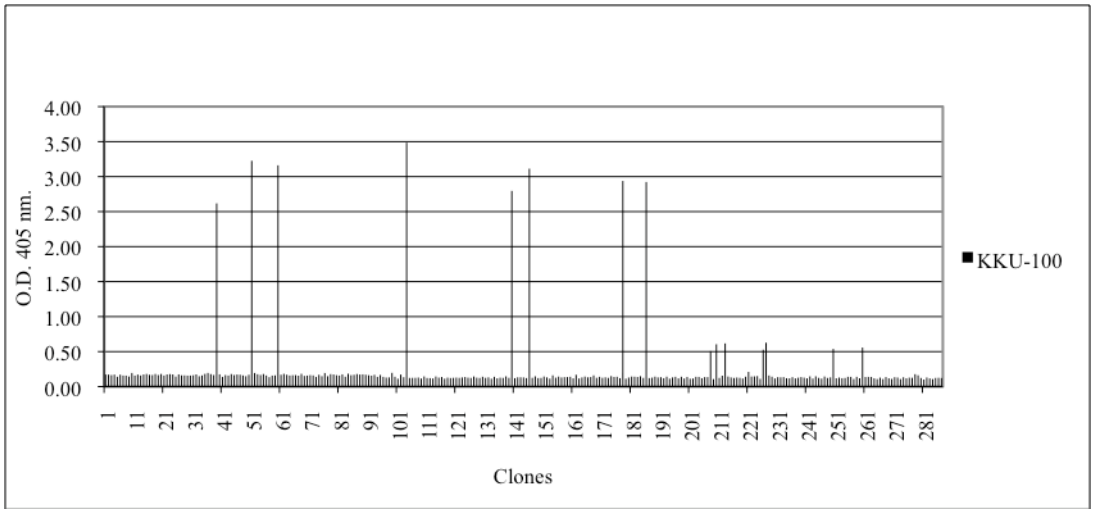

Figure 28. Cell based phage ELISA showed positive clones selected using experiment 6 : Direct selection using Sullivan library two rounds of selection.

Various selection experiments were modified to gain variety of specific scFv to KKU-100 cell (Table 8). The optimization of selection procedure included substraction or library pre-incubation with other cell lines such as HepG2, HepB3 and COS-1 cell to reduce scFv specific to common targets that expressed in other cell lines. After that unbound phage scFv was incubated to select specific scFv to target cell line, KKU-100 cell. The pre-incubation procedure was applied with success (Figure 29 to 32). In fact COS-1 library pre-incubation for only 1 round of selection (experiment 4) did not generate higher positive clones (Figure 29) than selection with KKU-100 alone (Figure 26). Two rounds of selection in cooperation with H69 cells library-depletion immediately after the 1st round of selection as described in experiment 7 generated more positive clones (Figure 30) when compare with biopanning without pre-incubation (Figure 28) as same as library pre-incubation with

COS-1 cells for 4 hr (experiment 8) (Figure 31). When selection was repeated, selected scFv showed higher phage ELISA signal, this indicated higher specific binders were selected as shown in both YAMO and Sullivan's lab library. Moreover 5 round of selections with library pre-incubation (experiment 9) increased the frequency of positive clones (72 %) (Figure 32) when compare with the first round and second round (2% and 15-19% respectively).

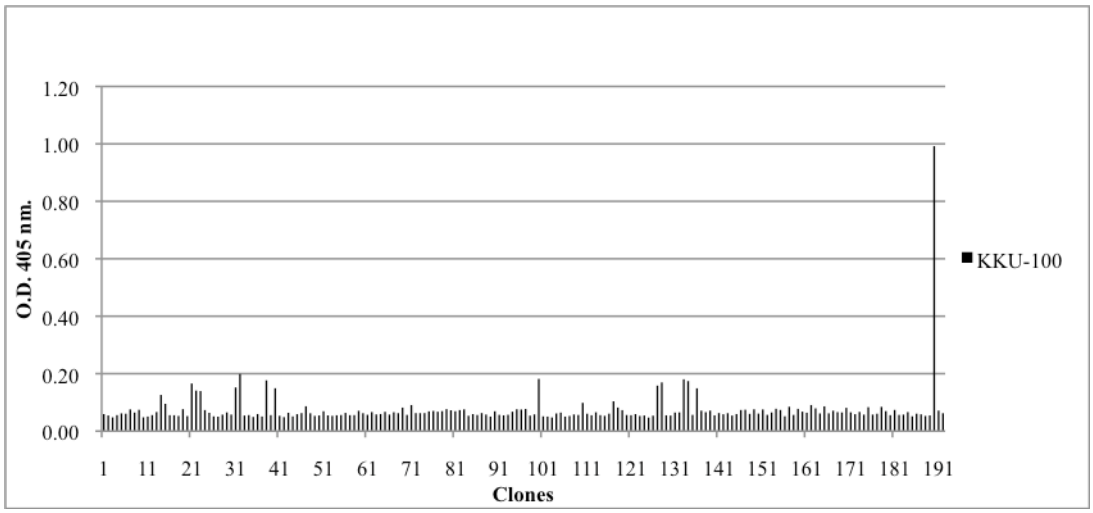

Figure 29. Cell based phage ELISA showed positive clones selected using experiment 4 : Library pre-incubation with COS-1 cell before selection with KKU-100.

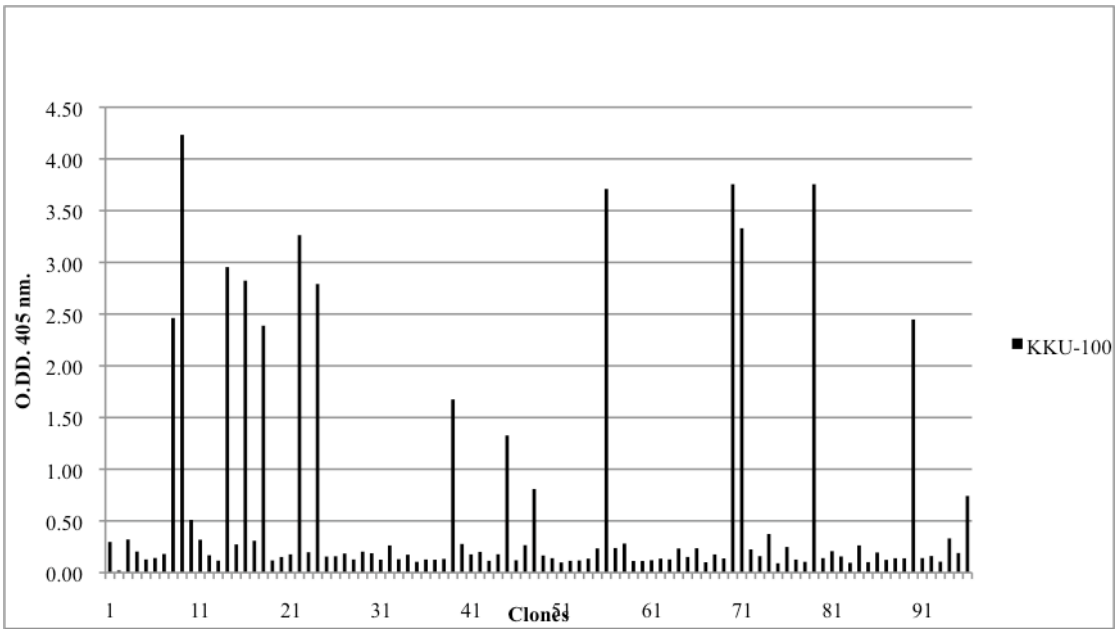

Figure 30. Cell based phage ELISA showed positive clones selected using experiment 7 : Sullivan library selection by direct selection with KKU100 and subtraction with H69 cell after finished first round of selection.

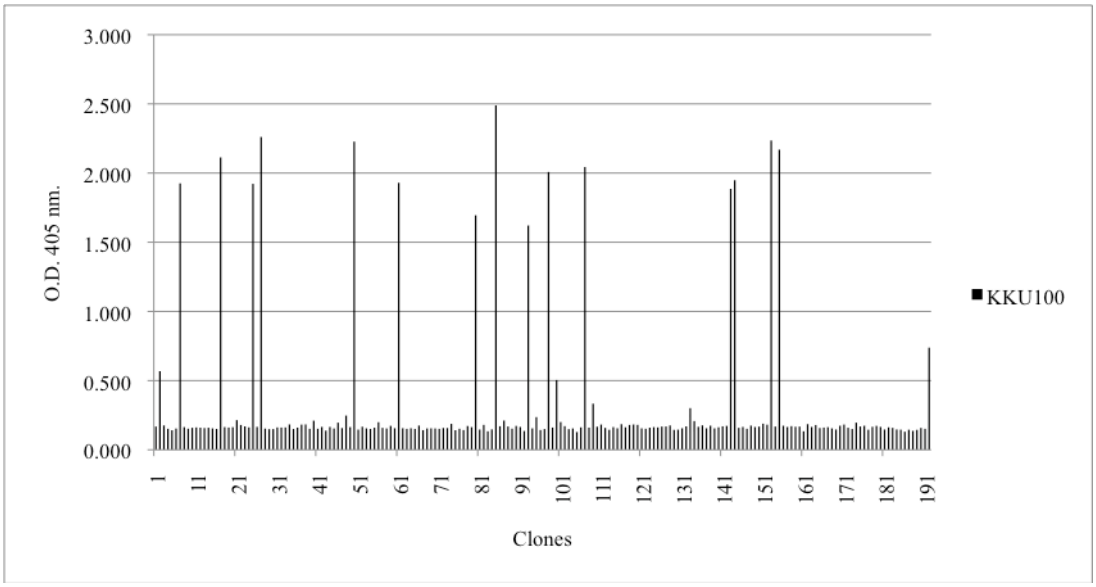

Figure 31. Cell based phage ELISA showed positive clones selected using experiment 8 : Library pre-subtraction with COS-1 cell 4 times before the first round of selection with KKU100 and the second round of selection is directly select with KKU100 cell.

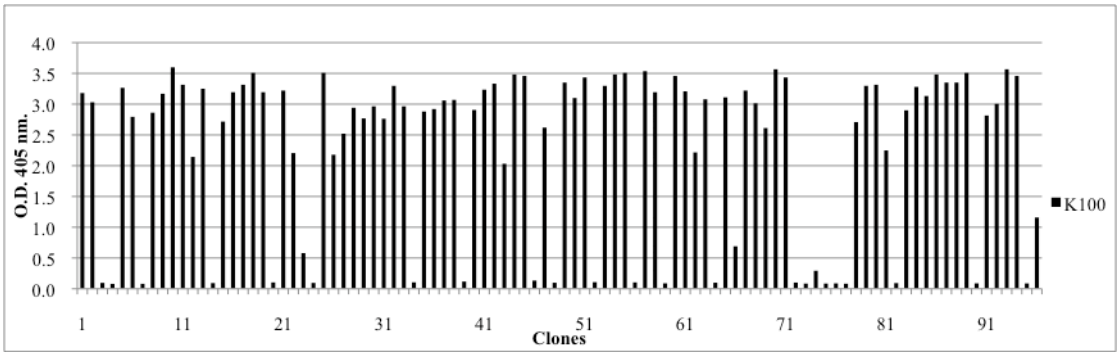

Figure 32. Cell based phage ELISA showed positive clones selected using experiment 9 : Selection with pre library substraction with HepB3, COS1 and HepG2 before selection with KKU100 cell.

4.3.2 Specificity of phage displayed human scFv

The binding to KKU-100 of phage display scFv from primary ELISA was confirmed and cross reactivity was also tested with other cell lines, HepG2, HepB3 and COS-1 cells. Phage displayed scFv clone YM12 showed specific binding to only KKU100 with low cross-reactivity with HepG2 and COS-7 (ELISA signal 2 times higher) (Figure33). Only selected clones from Tomlinson I showed specifically bind to KKU-100 but cross-reacted with HepG2 and COS-1 as shown in Figure 34 the results were the same as clones from the Sullivan library (Figure 35 and 36). Not only binding to other cell lines, non-related target proteins were also tested against zebra fish protein target; ZF621 and lactoferin protein (Figure 36 and 37). Phage ELISA signals indicated that selected phages scFv showed specific binding to KKU-100 but not to unrelated target proteins.

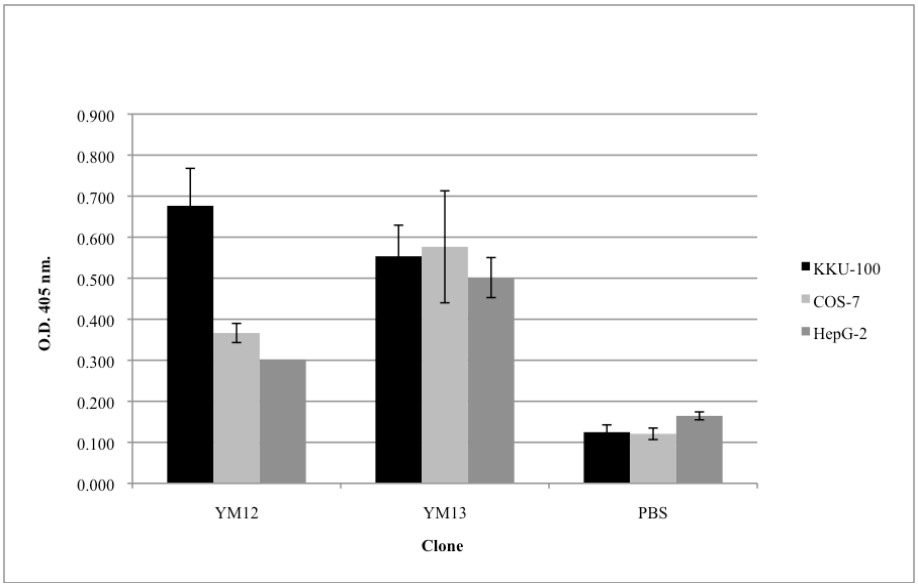

Figure 33. Cell based ELISA confirmation of specific scFv to KKU-100 cells.

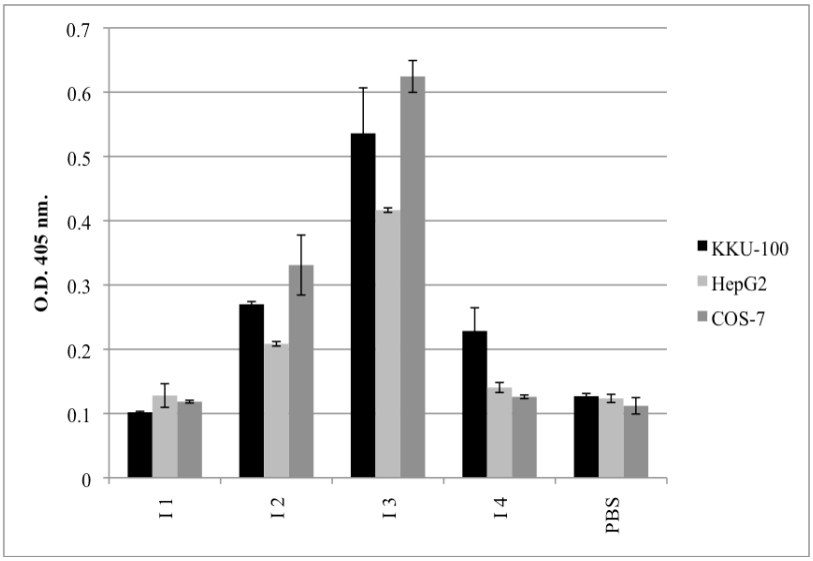

Figure 34. Cell based ELISA confirmation of selected Tomlinson I scFv with KKU-100 cells.

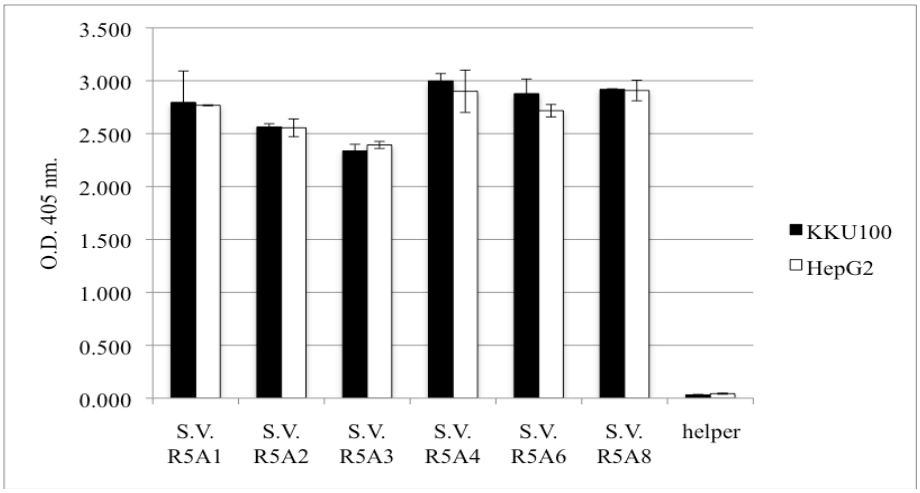

Figure 35. Cell based ELISA confirmation of selected scFv from 5 round of selection with KKU-100 cells.

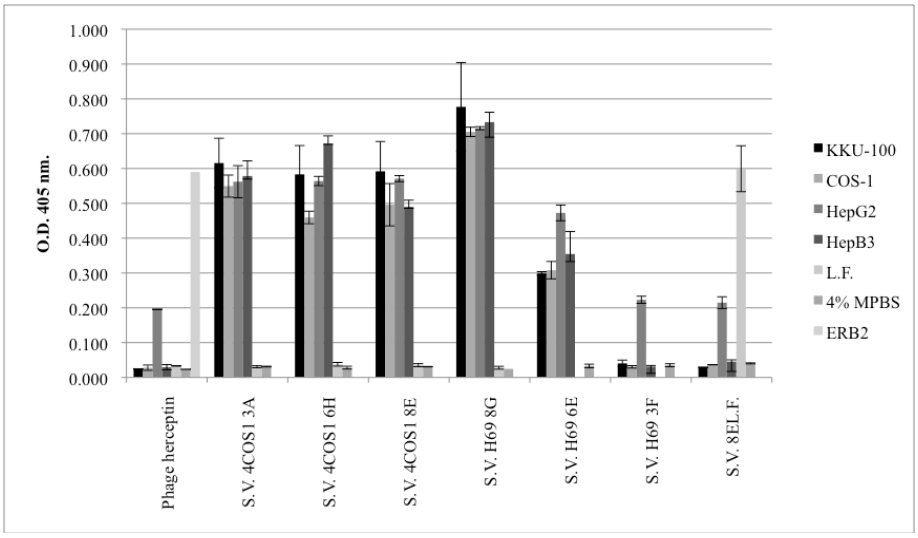

Figure 36. Cell based ELISA confirmation of KKU-100 positive clones when compare phage display Herceptin as a control.

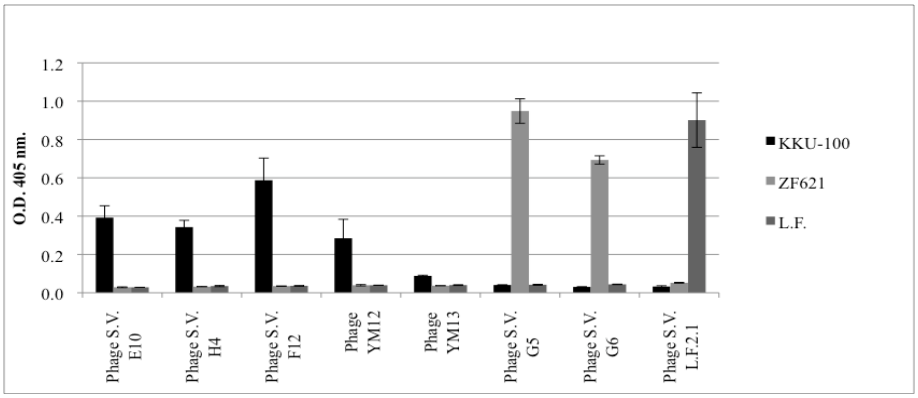

Figure 37. Confirmation of phage specific to KKU-100 cells and other targets proteins as control.

4.3.3 Production of soluble scFv and scFv with enzyme alkaline phosphatase fusion (scFv_AP)

Phage scFv from YAMO library contain an amber stop codon in phagemid. This allows soluble scFv production from non-suspressor host *E.coli* such as strain HB2151 and/or XL1blue. The 2-phage scFv clones that were selected from YAMO library were transfected into *E.coli* HB2151 and XL1blue and the expression under Lac promoter with IPTG induction was observed (Figure 38). However, the expression of soluble scFv from this promoter was quite low (Table 10). To overcome induction and expression problems, these 2 scFv were subjected to express under *phoA* promoter, using pKP300ΔIII vector.

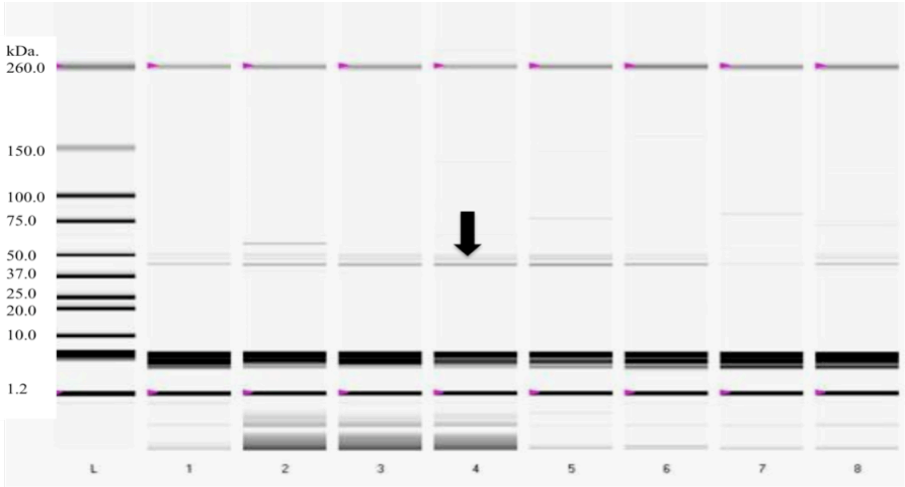

Figure 38. Gel image of purified soluble scFv by infection phage into *E. coli* HB2151 Lane1=HB2151, 2=clone YM3, 3= clone YM5, 4=clone YM6, 5= clone YM9, 6=clone YM12, 7= clone YM13, 8= clone YM15 (arrows marked purified protein).

Tabel 10. Purification profile from gel image after infected into *E. coli* HB2151

scFv	Culture volume (ml)	Soluble scFv		
		Concentration (ng/µl)	Total protein (µg)	Purity (%)
YM3	10	55.3	0.553	37.6
YM5	10	57.7	0.577	46
YM6	10	80	0.805	51.5
YM9	10	70	0.708	44.1
YM12	10	37.4	0.374	39.5
YM13	10	9.7	0.097	27.4
YM15	10	35.2	0.352	36.4

Next, The DNA fragments encoding individual single chain variable regions were subcloned into pKP300ΔIII expression vector to generate scFv_AP expression. After *AscI* digestion to remove gene encoding AP, recombinant vector with scFv fragment was re-ligated to generate recombinant plasmid for soluble scFv expression (appendix). Production of recombinant scFv_AP and scFv in *E.coli* strain TG1 was controled under *phoA* promoter. To produce scFv_AP and scFv, recombinant plasmids were transformed into *E.coli*TG1 and grown in LowPhos media containing carbenicillin at 30^{O}C 250 rpm for 20-22 h. Soluble scFv_AP and scFv were purified from total cell lysate by IMAC purification system with HisMag™ beads (Novagen). Protein quantity and purity was determined by Pro260 automate gel electrophoresis system (BioRad). Recombinant scFv_AP and scFv were expressed shown as approximately 80 and 37 kDa respectively (Figure 39 and 40).

Small-scale protein purification was done with 10 ml culture. Purification profile of both soluble scFv_AP and scFv were shown in Table 11 and gel images were shown in Figure 39 and 40. All scFv_AP and scFv were successfully express under *phoA* promoter with different level of expression. Soluble scFv_AP clone S.V. R5A1 showed highest expression level. Whereas, R5A6 showed the lowest expression level. When consider soluble scFv expression, R1E10 and R1S.V. H4 showed the highest and the lowest expression level respectively. The promising clones were selected to do large scale production.

Table 11. Small-scale protein purification profile under *phoA* promoter.

Clone	scFv_AP				scFv			
	Culture volume (ml)	**Conc. (ng/µl)**	**Purity (%)**	**Total protein (µg)**	**Culture volume (ml)**	**Conc. (ng/ µl)**	**Purity (%)**	**Total protein (µg)**
S.V. R5A1	10	48.1	85.4	4.810	10	122.6	98.2	12.2
S.V. R5A2	10	-	-	-	10	-	-	-
R5A4	10	13	70.4	1.3	10	20.7	100	2.07
R5A6	10	4.3	69.7	0.43	10	31.7	100	3.17
S.V. R5A8	10	34.7	71.5	3.47	10	14.2	100	1.42
R1YM12	10	8.4	84.5	0.84	10	62.4	91.7	6.24
R1YM13	10	32.1	77	3.2	10	28.8	95.1	2.88
S.V. E10	10	19.2	71.2	1.92	10	342.9	97.1	34.2
S.V. H4	10	11.2	52.2	1.12	10	5.3	54.7	0.53

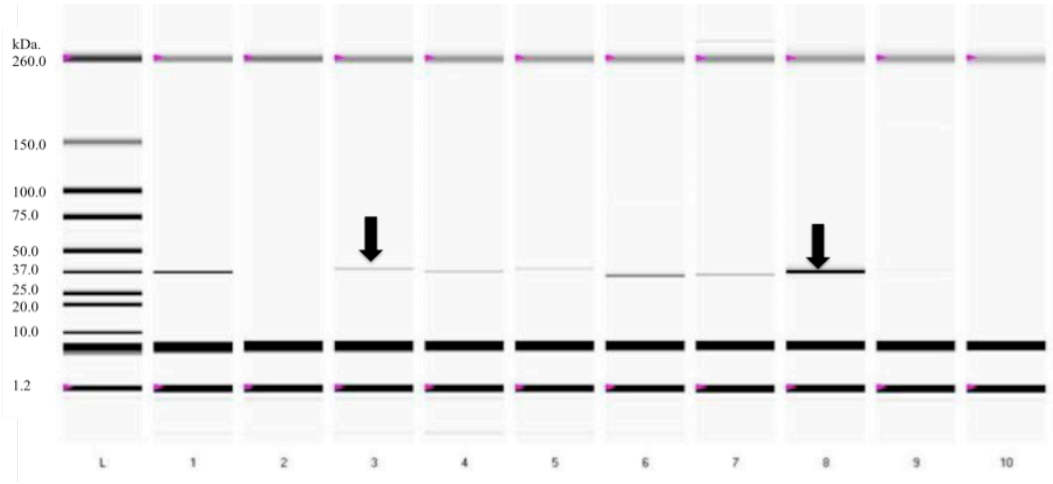

Figure 39. Gel image from small scale purification of soluble scFv from different clones after scFv genes were subcloned into pKP300deltaIII vector with AP gene drop out and expressed in *E.coli* TG1 , L=marker, 1=S.V. R5A1, 2=S.V. R5A2, 3=R5A4, 4=R5A6, 5=S.V. R5A8, 6=YM12, 7=YM13, 8=E10, 9=S.V. H4 (arrows marked purified protein).

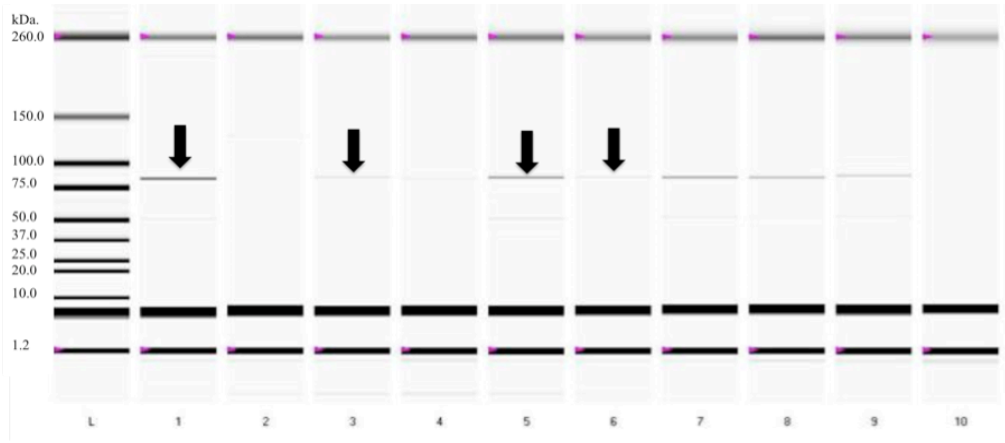

Figure 40. Gel image from small scale purification of soluble scFv_ AP from different clones after scFv genes were subcloned into pKP300deltaIII vector and expressed in *E.coli* TG1, L=marker, 1=S.V. R5A1, 2=S.V. R5A2, 3=R5A4, 4=R5A6, 5=S.V. R5A8, 6=YM12, 7=YM13, 8=S.V. E10, 9=S.V. H4 (arrows marked purified protein).

Large-scale purification was done in flask culture system in volume 50-200 ml. Gel image analysis of clone S.V. E10 and S.V. H4 purified proteins was shown in Figure 41. Different expression level was observed in both scFv_AP and scFv as shown in Table12. Clone E10 in scFv_AP and scFv format had the highest purification yield (0.67 mg from 50 ml of culture with 85% purity). Soluble scFv purification indicated that clone E10 had the highest purification yield (1.66 mg from 50 ml of culture with 98.1% purity). The lowest purification yield obtained from clone S.V. H4 in both scFv_AP and scFv. The yield of purified scFv with AP varied from 0.08 to 0.67 mg with 87.8 to 99.7% purity and purified scFv varied from 0.03 to 1.66 mg of with 60 to 85% purity per 50 ml of bacteria culture. Thus these clones were selected for the next assay.

Table 12. Total protein of purified soluble scFv_AP in large-scale production under *phoA* promoter.

scFv_AP	Culture volume (ml)	Total protein (mg)	Purity (%)
1. YM12_AP*	50	0.474	64.8
2. YM13_AP	50	0.10	60.6
3. S.V. E10_AP**	50	2.37	85
4. S.V. H4_AP	50	0.08	59.4
5. S.V. R5A8_AP	50	0.67	72.4

*Estimated from 100 ml bacteria culture

** Estimated from 150 ml of bacteria culture

Table 13. Total protein of purified soluble scFv in large-scale production under *phoA* promoter.

scFv	Culture volume (ml)	Total protein (mg)	Purity (%)
1. YM12*	50	1.43	94
2. YM13*	50	1.51	99.7
3. S.V. E10	50	1.66	98.1
4. S.V. H4	50	0.03	90.6
5. S.V. R5A1*	50	0.96	87.8
6. S.V. R5A8	50	0.37	98

*Estimated from 100 ml bacteria culture

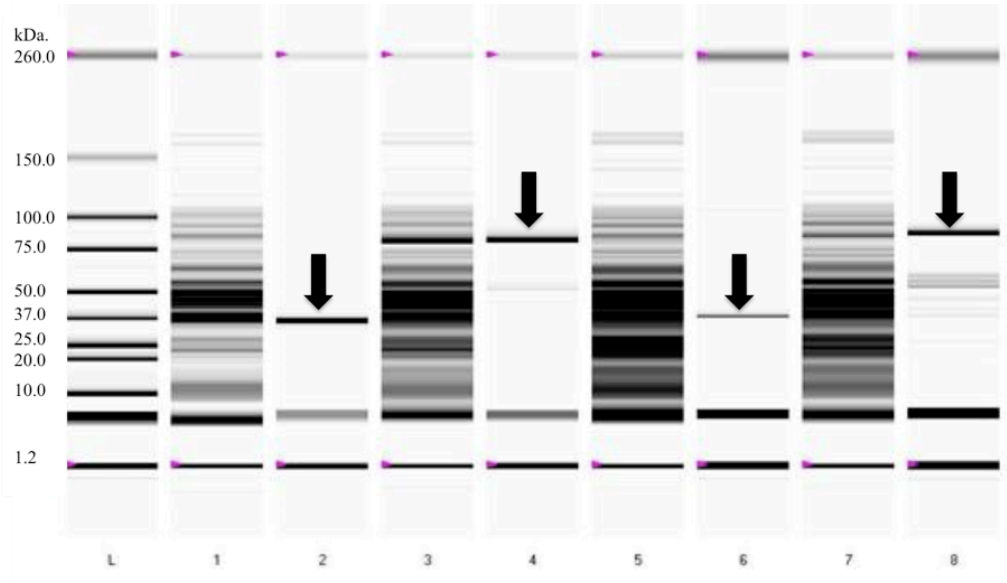

Figure 41. Gel image represent large scale purification from 50 ml culture represent two scFv clones in both soluble scFv and soluble scFv with AP fusion form.

L= Marker,

1=Clone S.V. E10 clear cell lysate flow through,

2= clone E 10 scFv purified,

3=clone S.V. E10 scFv fused AP clear cell lysate flow through,

4=clone S.V. E10 scFv fused AP purified,

5= clone S.V. H4clear cell lysate flow through,

6=clone S.V. H4 scFv purified,

7= clone S.V. H4 scFv fused AP clear cell lysate flow through,

8= clone S.V. H4 scFv fused AP purified.

4.3.4 scFv_AP and scFv binding confirmation by cell based ELISA

The function of soluble scFv and scFv_AP in binding to KKU-100 cells were examined by cell based ELISA. Without purification, total protein in clear cell lysate was used in cell based ELISA and the result showed that all selected scFv_AP and scFv could function without gene III except S.V. R5A2_AP (Figure 42, 43 and 44). After that modified ELISA experiment was done by washing with TBS+0.05% Tween 20 and TBS with 2 min. incubation during each wash, the result showed that clone S.V. R5A1_AP, YM12_AP and S.V. R5A8 and YM12 scFv showed KKU-100 specific binding (Figure 45 and 46). The signal of scFv_AP could be directly detected with substrate for enzyme alkaline phosphatase (pNPP). Soluble scFv with FLAG and 6His tag was detected with anti FLAG antibody conjugated with HRP and/or AP and anti His antibody conjugated HRP.

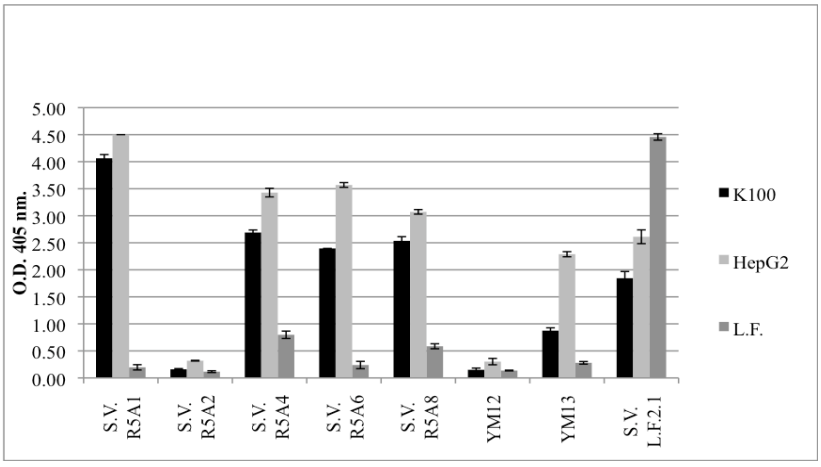

Figure 42. Cell based ELISA using scFv_ AP fusion using clear cell lysate

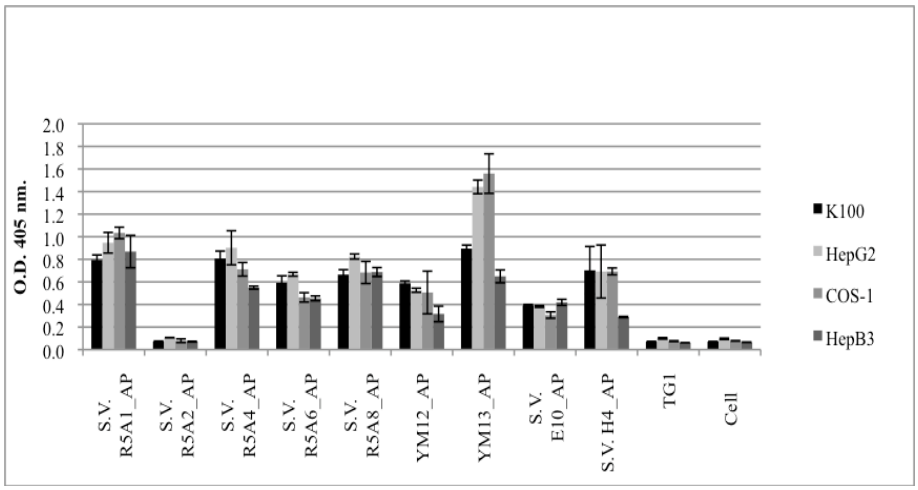

Figure 43. Cell based ELISA with scFv_AP using clear cell lysate.

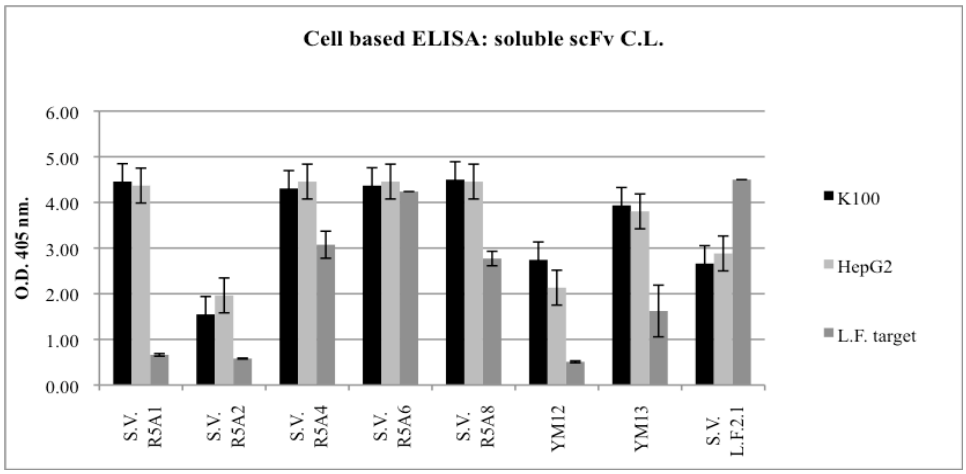

Figure 44. Cell based ELISA with soluble form of scFv, 20 µl of clear cell lysated were used in ELISA test, anti FLAG-HRP antibody was used to detected scFv.

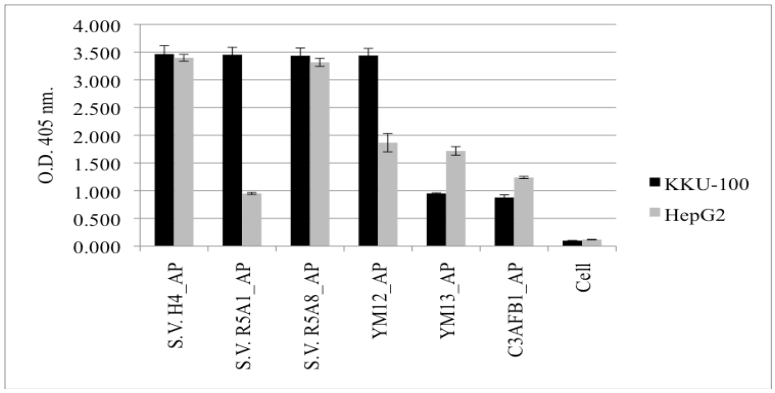

Figure 45. Cell based ELISA with scFv_AP in clear cell lysate. Modified ELISA experiment was done by changed washing step from 3 times PBS +0.05% Tween 20 and 3 times with PBS without incubation to TBS + 0.05% Tween 20 and TBS washing buffer with 2 min. incubation during each wash.

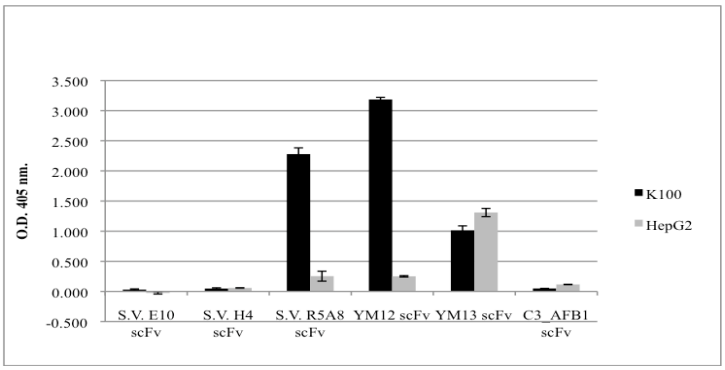

Figure 46. Cell based ELISA with soluble scFv in clear cell lysate. Modified ELISA experiment was done by changing washing step from 3 times with PBS +0.05% Tween 20 and 3 times with PBS without incubation to TBS + 0.05% Tween 20 and TBS washing buffer with 2 min. incubation during each wash.

The binding ability of scFv_AP and scFv after purification was also confirmed. Promising clones which showed higher yield in small-scale purification were selected. Soluble scFv_AP clone YM13 and S.V. R5A8 showed specific binding to KKU-100 when compared with maltose binding protein (MBP) (Figure 47). Soluble scFv after purification could bind to KKU-100 but not to lactoferrin protein (L.F.) (Figure 48).

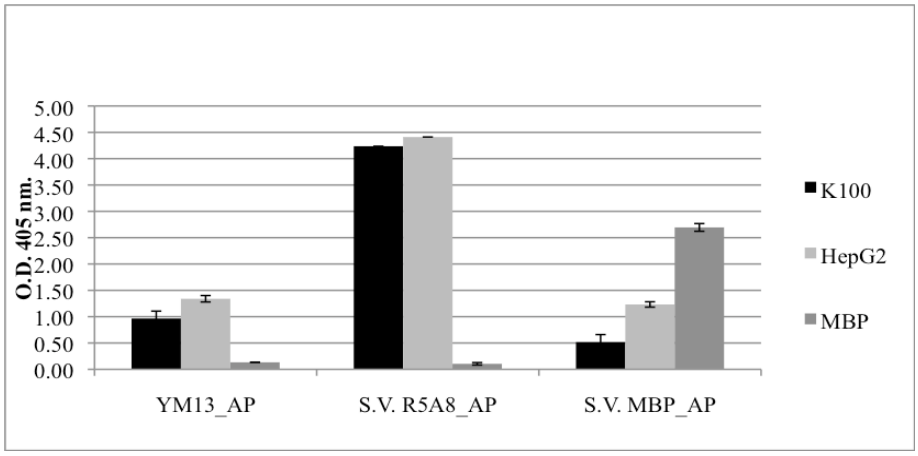

Figure 47. Cell based ELISA from scFv fused with AP in purified format.

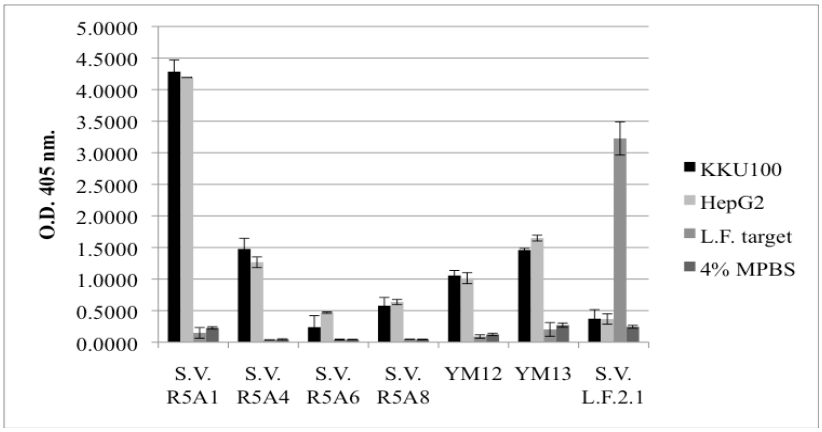

Figure 48. Cell based ELISA from purified scFv.

4.3.5 Soluble scFv in immunoblotting

When using purified scFv_AP or scFv as probes, the result showed that none of all selected scFv recognized their cognate antigen by western blot assay (Figure 49, 50).

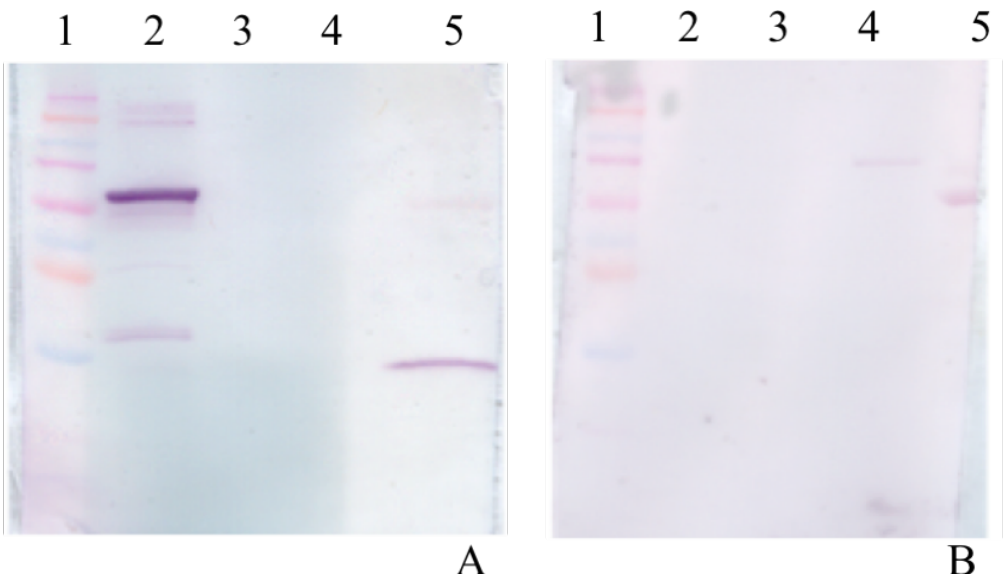

Figure 49. Western blot analysis of soluble scFv_AP. Lane1= marker, 2= maltose binding protein, 3= zebra fish protein, 4= lactoferrin protein, 5= KKU-100 clear cell lysate. A and B were probed with control scFv_AP specific to maltose binding protein and scFv_AP clone S.V. E10, respectively.

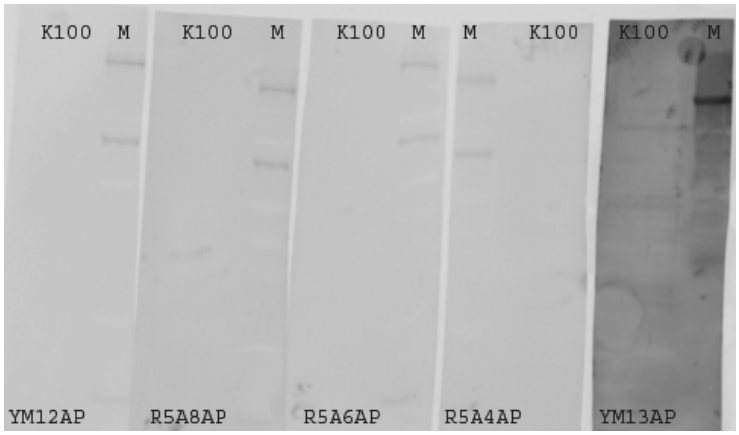

Figure 50. Different scFv_AP probe YM12_AP, S.V. S.V. R5A8_AP, S.V. R5A6_AP, S.V. R5A4_AP and YM13_AP ECF substrate for AP were used to detect.

4.3.6 Immunoprecipitation assay

The scFv_AP or scFv were also used in immunoprecipitation assay. KKU-100 and H69 clear cell lysate were incubated with purified soluble scFv at $4^{O}C$ for overnight. It was re-purified by IMAC. Purified scFv and their cognate antigen were separated by SDS-PAGE (Figure 51). Some proteins were co-purified with scFv as indicated by arrows in Figure 51. Immunoprecipitation experiment was also done with biotinylated cell surface protein. Using KingFisher ml automate protein purification system to purify scFv after incubated with clear cell lysate at $4^{O}C$ for overnight, the purified protein and its cognated were separated by SDS-PAGE. Biotinylated proteins were detected by streptavidin conjugated HRP and ECL substrate. The signal was detected by Stromimager. As shown in Figure 52, none specific bands become clearly visible but distinct signal appear from scFv clone S.V. E10 compare with control scFv G5.

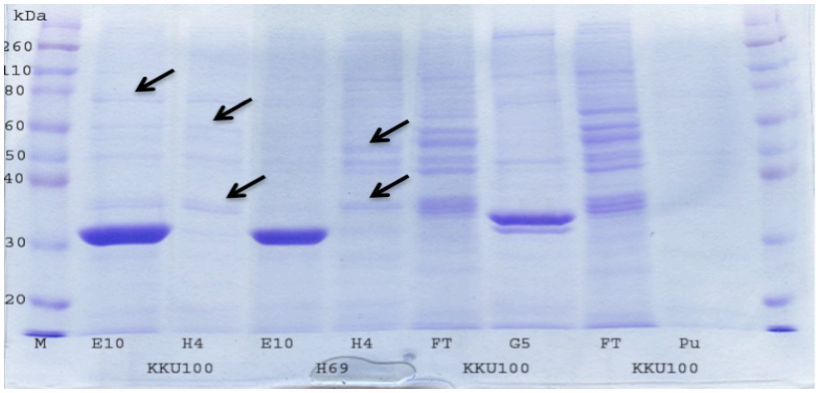

Figure 51. Gel image of protein profile after immunoprecipitation experiment. Two purified scFv, S.V. E10 and S.V. H4 were incubated with KKU-100 and H69 clear cell lysate. Then these scFv and their cognate were re-purified with HisMag beads.

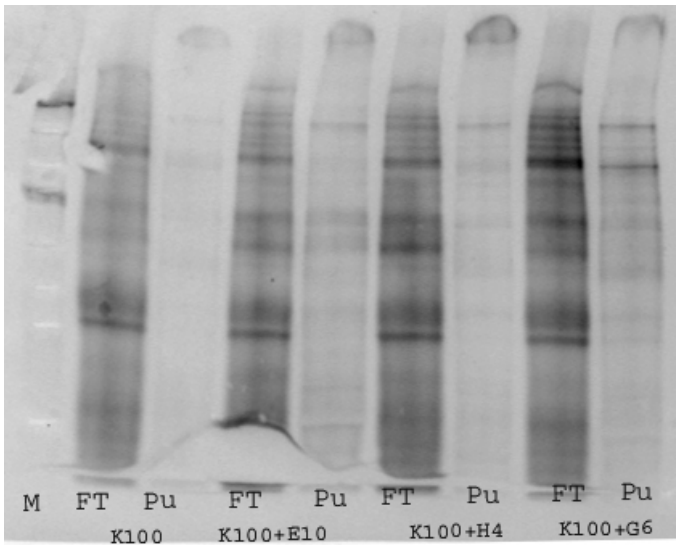

Figure 52. Western blot of cell surface biotin marked protein using S.A AP to detect biotinylated protein and ECF substrate.

4.3.7 Effect of soluble scFv_AP and scFv on cell growth

Since soluble scFv_AP and scFv clone S.V. E10 and S.V. H4 could be expressed and purified. The primary screening of biological effect of these scFvs was carried out. These two clones were incubated with KKU-100 for 48 hr. Viable cells were detected by MTT assay. Increased scFv_AP and scFv concentration did not affect cell growth compared to control elution buffer (Figure 53).

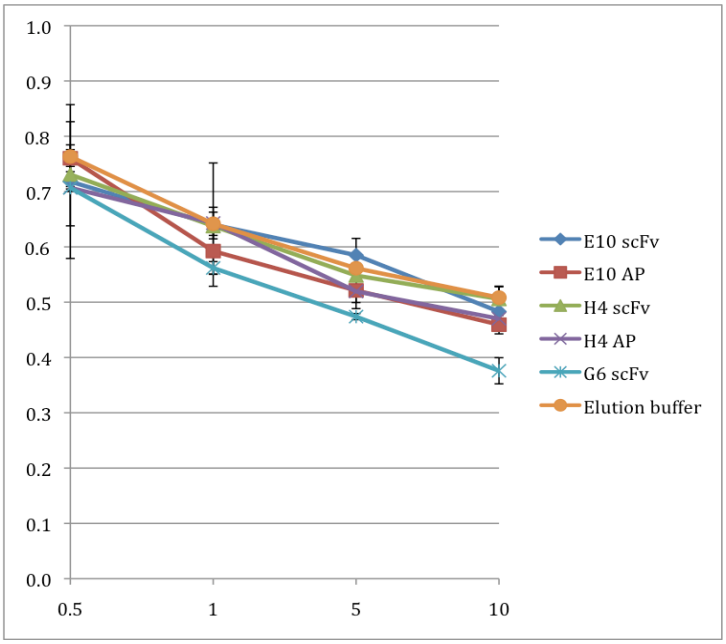

Figure 53. The effect of scFv and scFv_AP on cell growth.

4.3.8 Soluble scFv_AP and scFv in cell staining assay

Several well cultures were expressed soluble scFv_AP and scFv. The binding pattern of scFv_AP and scFv express culture were investigated by cell staining. Soluble scFv_AP clone S.V. R5A8 and YM13 were used to stain KKU-100

and HepG2 cells. As shown in Figure 54, scFv clone YM13_AP showed stronger binding signal to KKU-100 when compare to HepG2.

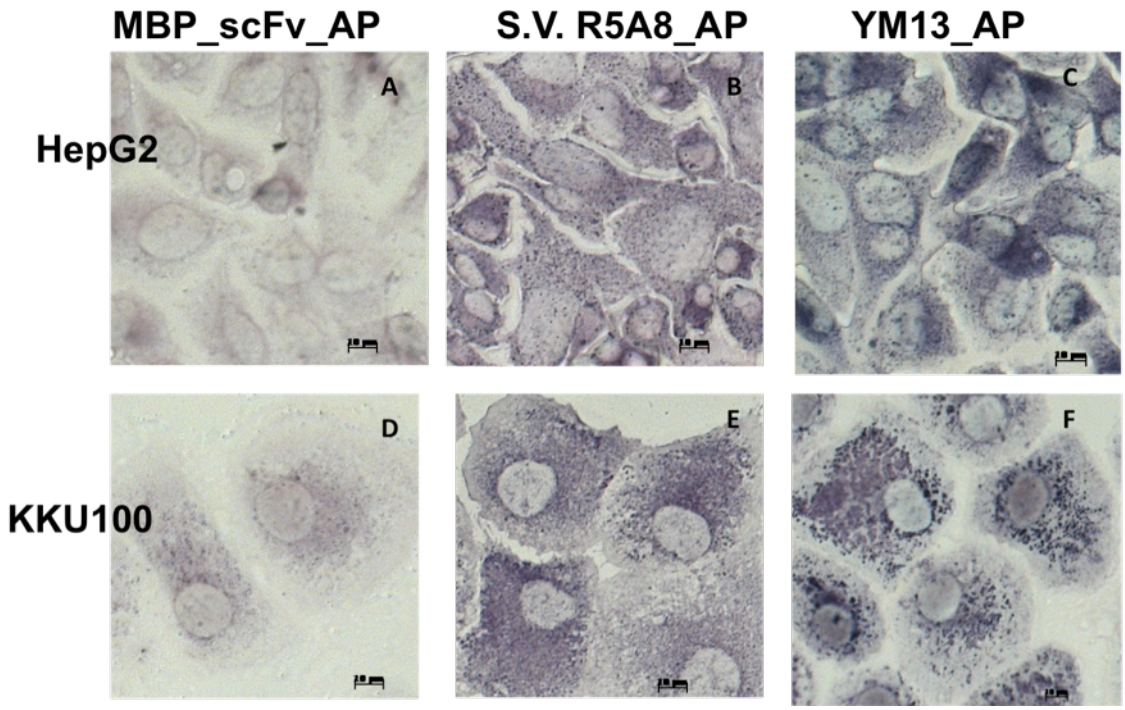

Figure 54. HepG2 cell (A,B,and C) and KKU100 (D, E, and F) were fixed with 4% PFA and permeabilized with cold methanol and stained by scFv AP fusion format. (A and D: S.V. MBP_scFv_AP, B and E: S.V. R5A8_AP, C and F : YM13_AP)

Soluble scFv clone YM12, YM13 and S.V. R5A1 staining pattern were shown in Figure 55, 56, 57 and 58. In conclusion, the staining result suggested that all three soluble scFv exhibited specific KKU-100 staining.

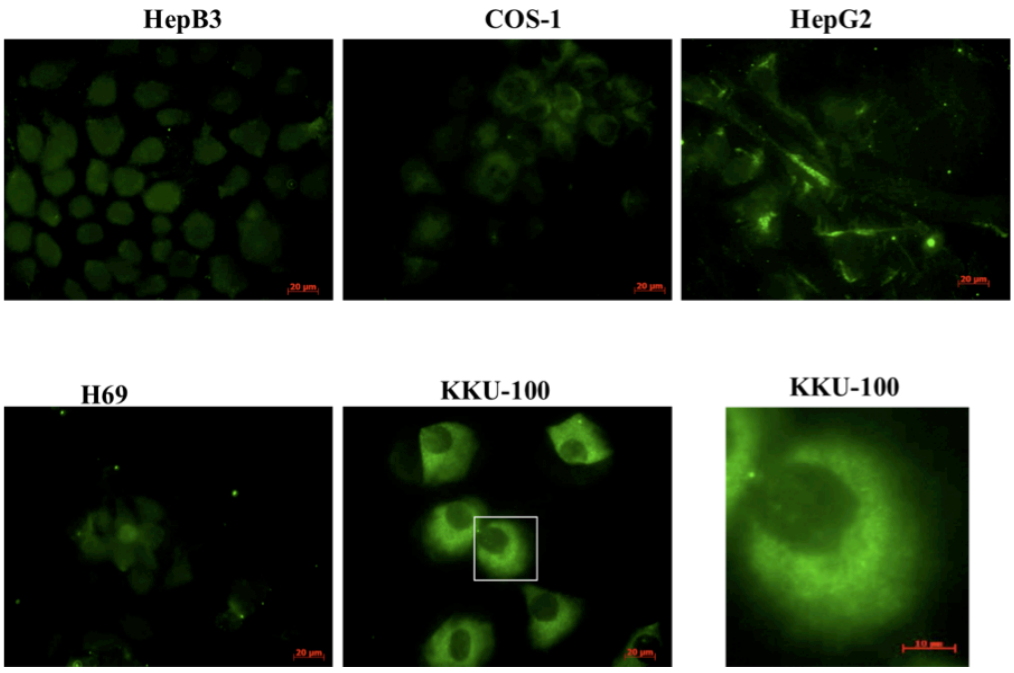

Figure 55. scFv YM12 staining pattern. HepB3, COS-1, HepG2, H69 and KKU-100 cells were fixed with 4% paraformaldehyde and incubated with purified scFv. Streptavidin conjugated AlexaFluor488 was used to detect scFv that labeled anti FLAG M2 biotin (which shown in green fluorescence signal).

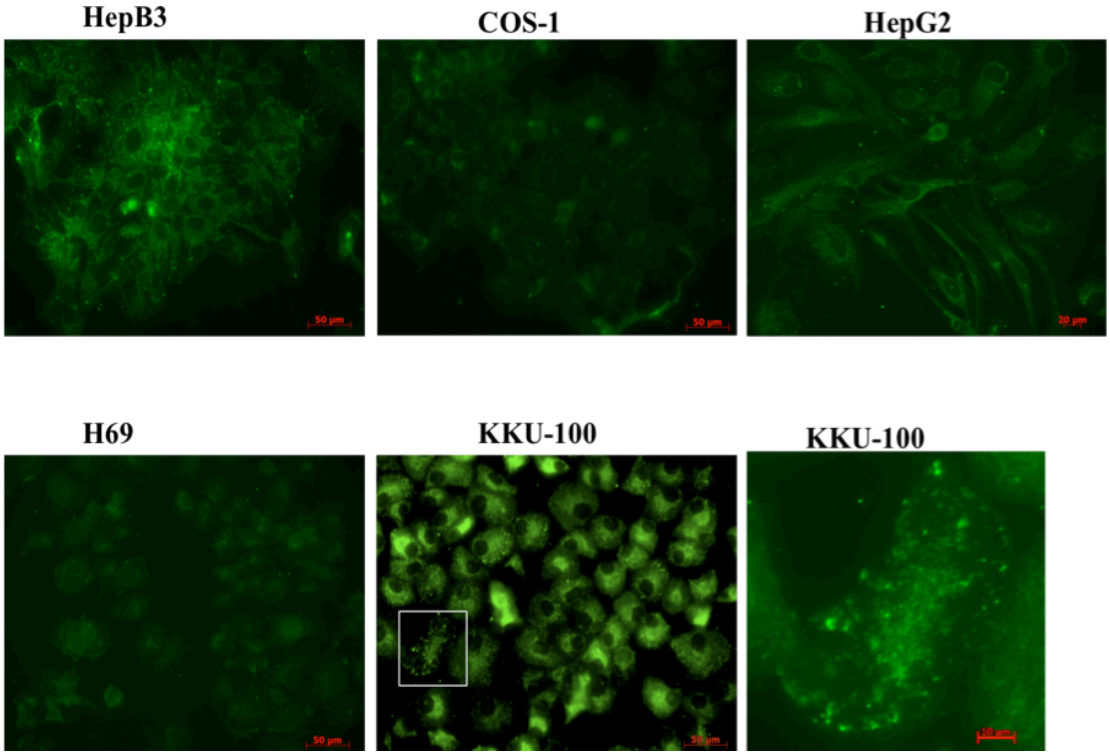

Figure 56. Cell staining pattern of purified scFv YM13 in formaldehyde fixation. HepB3, COS-1, HepG2, H69 and KKU-100 cells were fixed with 4% formaldehyde and incubated with purified scFv. Streptavidin conjugated AlexaFluor488 was used to detect scFv that labeled anti FLAG M2 biotin (which shown in green fluorescence signal).

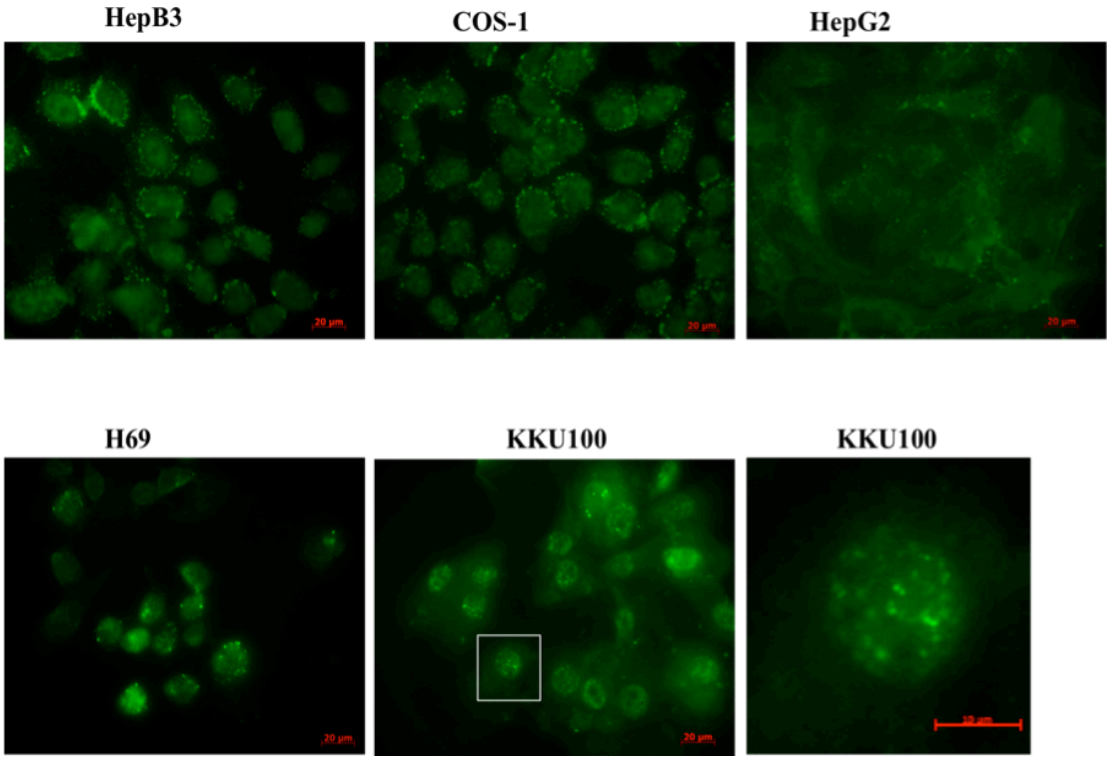

Figure 57. Cell staining pattern of purified YM13 with paraformaldehyde fixation. HepB3, COS-1, HepG2, H69 and KKU-100 cells were fixed with 4% paraformaldehyde and incubated with purified scFv. Streptavidin conjugated AlexaFluor488 was used to detect scFv that labeled anti FLAG M2 biotin (which shown in green fluorescence signal).

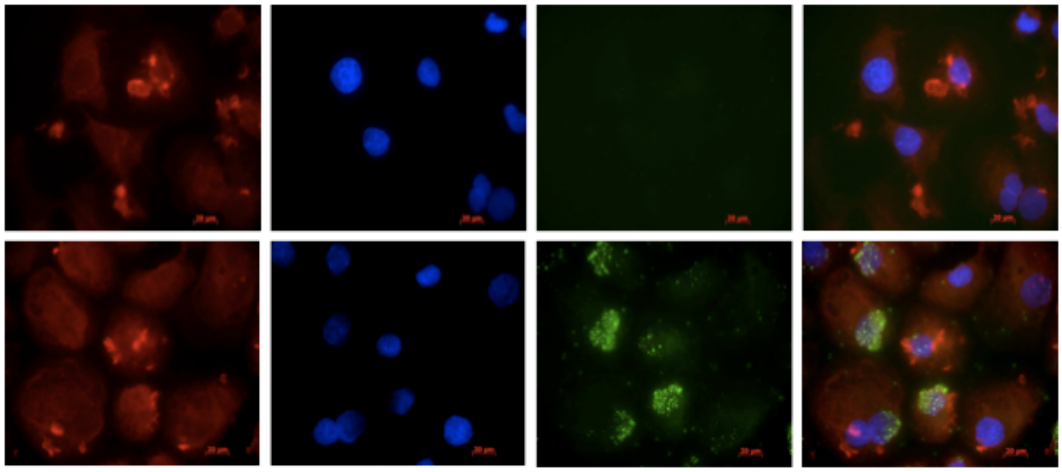

Figure 58. Cell staining pattern of purified scFv clone S.V. R5A1. HepG2 (upper panel) and KKU-100 (lower panel) cells were fixed with 4% paraformaldehyde and incubated with purified scFv. Streptavidin conjugated AlexaFluor488 was used to detect scFv that labeled anti FLAG M2 biotin (which shown in green fluorescence signal). ConcanavalinA TexasRed conjugated and DAPI mounting were used to stain lectin and DNA respectively.

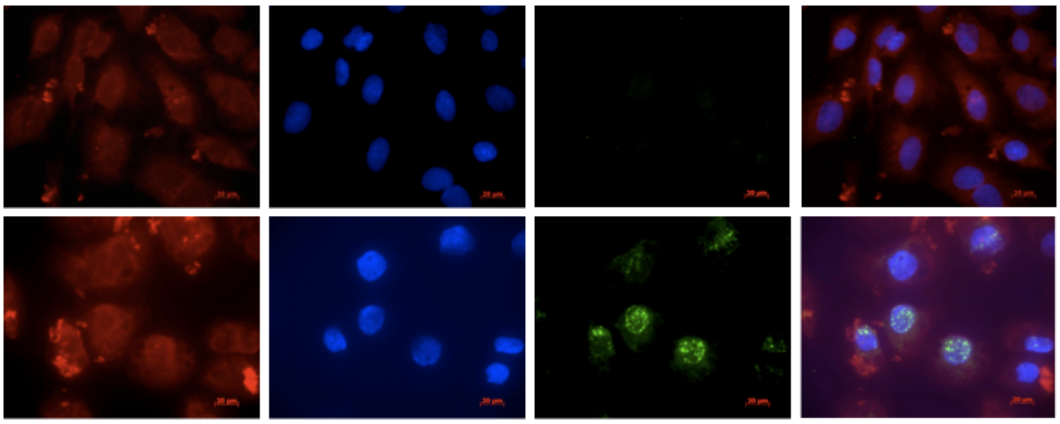

Figure 59. Cell staining pattern of purified scFv clone YM12. HepG2 (upper panel) and KKU-100 (lower panel) cells were fixed with 4% paraformaldehyde and incubated with purified scFv. Streptavidin conjugated AlexaFluor488 was used to detect scFv that labeled anti FLAG M2 biotin (which shown in green fluorescence signal). ConcanavalinA TexasRed conjugated and DAPI mounting were used to stain lectin and DNA respectively.

4.3.9 Binding of soluble scFv with KKU-100 cells analysis by FTIR spectromicroscopy

To predict the target of scFv, FTIR spectromicroscopy was used for study molecular structure of cellular constituents such as proteins, lipids and carbohydrate. The result showed the most significance differences spectra in absorptions from C-O-C stretching vibration of polysaccharides (~966 cm^{-1}) obtained when cells incubated with YM13 scFv as indicate by P5 and P2 in Figure 60. It is indicated YM13 might involve in binding with carbohydrate part of cell.

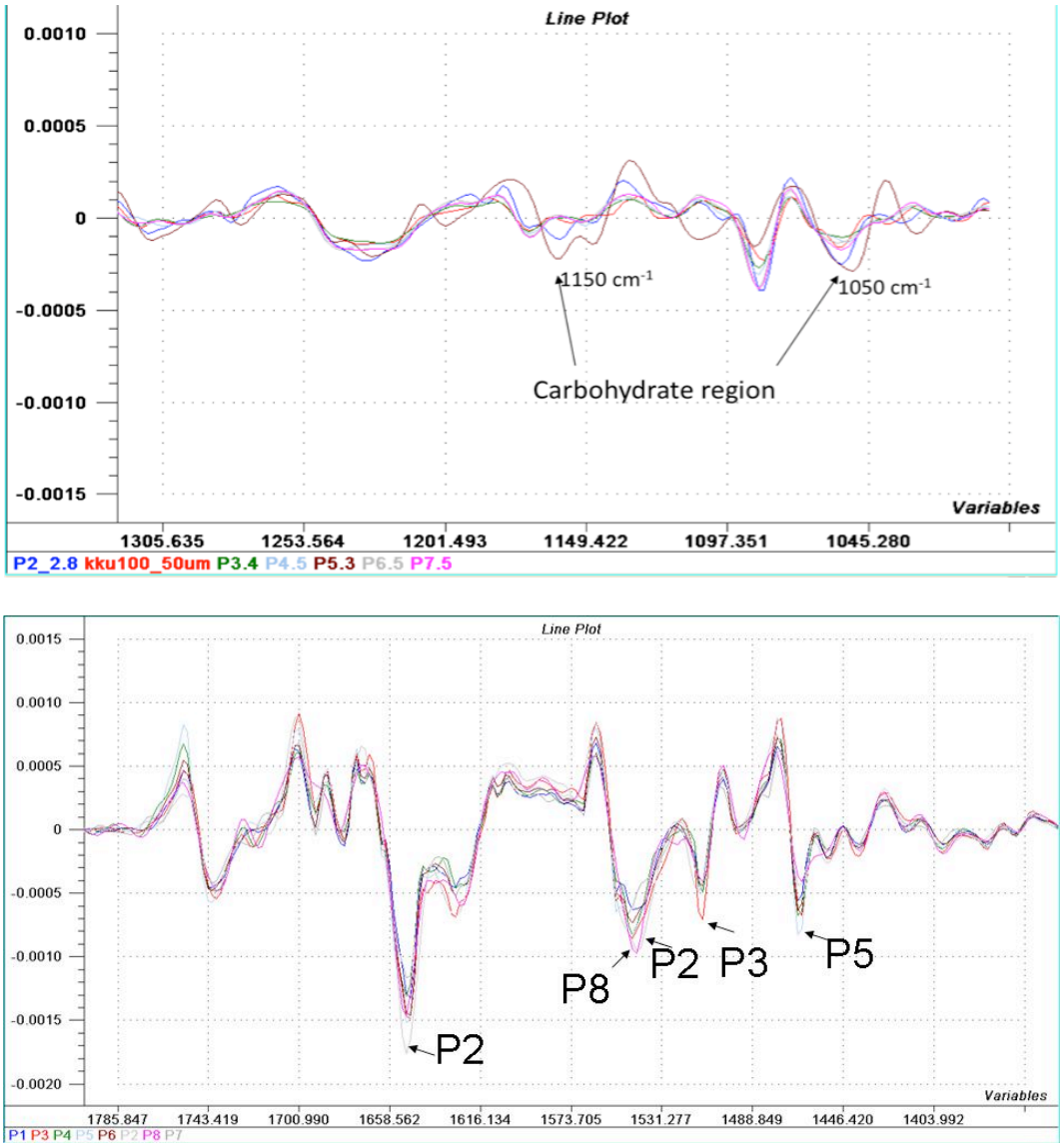

Figure 60. FTIR spectra result. All spectra were and phosphodiester backbone and carbohydrate shown. Cells were incubated with supernatant (P2 to P4) and purified scFv (P5 to P8) and spot on slide. After cell spot were dried, slide was examined under infared microscope. P1=KKU-100 , P2=+ S.YM13 scFv, P3=+ S.pK300 vector, P4= + S.YM12 scFv, P5= + P.YM13 scFv, P6=+ P.YM12 scFv, P7=+ P.L.F.2.1 scFv.

4.3.10 Sugar and scFv inhibition assay

Since the previous result showed that sugar might be the target of scFv YM13. Cell based ELISA was set up with scFv_AP in clear cell lysate with addition of 0.5 M NAG and glucose as inhibitor to test the binding ability of scFv_AP .If scFv clone YM12_AP and YM13_AP share some ligands with these sugar, ELISA signal with sugar inhibitor will decrease. The ELISA signal of scFv_AP clone YM12 and YM12 in the presence of sugar was decreased. The result could be implied that two scFv_AP might bind to the sugar target (Figure 61).

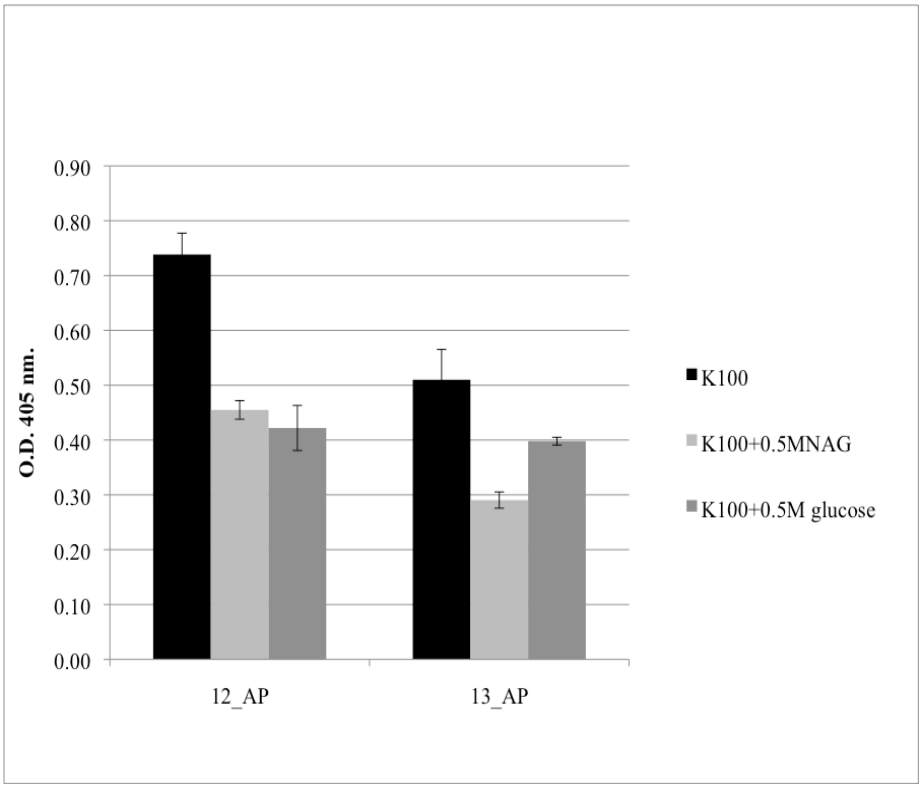

Figure 61. Cell ELISA of YM12_AP and YM13_AP using clear cell lysate with 0.5M NAG and 0.5 M glucose as inhibitor.

CHAPTER V

DISCUSSION AND CONCLUSION

Both peptide and antibody were studied in this thesis because both have their advantages. Antibody seems to have high affinity than short peptide. However using antibody as targeting molecule still has a limitation. Its size is relatively large and has difficulty infiltrating the entire tumor mass and the Fc region of antibody binds to the reticuloendothelial system, result in high uptake of conjugated toxic drug into bone marrow, liver and spleen, leading to high toxicity. In contrast peptides or small molecules are alternative and possibly more effective cancer targeting agents. The use of antibody fragment or peptides could overcome these problems due to its smaller size, more stable and easy to synthesis. In addition peptide based therapeutic is considered as promising drug in clinical because of their ease of derivatization and low cost of manufactory. Peptides with N- and C- terminally blocked, cyclized, and/or contain D-amino acid and unnatural amino acids are generally very stable to proteolysis (Aina et al., 2007). However the efficacy of peptide therapeutic is limited by relative low affinity, proteolytic degradation, immunogenicity potential and rapid renal filtration (Krumpe and Mori, 2007). Therefore specific ligand for KKU-100 from both peptide and antibody libraries were selected in this study.

5.1 Selection of specific peptide to KKU-100 cells

Thus, various factors needed to be concerned in selection of phage display library

against cell surface target. These are number of phage used, stringency of required at least ~ 5 x 10^5 cells (in 25 cm^3) for successful selection. None of specific peptide was selected when using ~10^5 cells (6-well plate format).

KKU-100 cells biopanning with phage display peptide library were done for 3 rounds, cell based ELISA result did not reveal any specific peptide to KKU-100 (Figure 8). After 5 rounds of library pre incubation/selection, selected peptides are derived from CX_6C library, none of them are from (NNK)12 library. The similar phenomenon was shown on selection with glucose-manose binding lectin. Selection of phage display peptide library with lectin described the majority of clones are from C-X_7C library demonstrated higher specificity than X12 library (Yu et al., 2009). This is proved that cyclic peptide library providing higher specific binding ligand than linear peptide. The two KKU-100 specific peptides, CTTPPYVC and CTSPPYVC, composed of rich proline and/or threonine residues. Proline (P) residues, which appear in many of the convergent sequences from the screening of lectin target, may serve an important structural role by spatially orienting key side chains (Yu et al., 2009).

From phage display peptide binding efficiency test confirmed the specificity of these two peptides. The phage binding efficiency was calculated by dividing the number of recover phage (out put) by the input number. Thus, CTTPPYVC and CTSPPYVC phages showed higher binding efficiencies to KKU-100 cells than HepG2 cells. CTTPPYVC and CTSPPYVC are novel peptides specific to KKU-100 cells. These peptides were selected using ligand in native context. Thus this format of selection enables to generate novel ligand from unknown cell surface receptors (Aina et al., 2007). It is also the first report of peptide specific to cholangiocarcinoma.

5.2 Application of free peptides in cell staining

Free peptides cell based ELISA indicated the CTTPPYVC and CTSPPYVC capability in binding to KKU-100 cells without gene III. Most peptides selected from cell based selection with protocol described by McGuire and colleagues are active outside context of phage, retaining their cell specific and affinity (McGuire et al., 2009).When free peptide was synthesized with alanine residue at both ends and one end, ELISA and cell staining result showed that these peptides lost their binding ability. It is implied that both cysteine residues with cyclization play an important role in binding, even all amino acid inside this cyclic had been changed to alanine residue, the CAAAAAAC peptide still function. CTSPPYVC showed specific binding to KKU-100 in staining experiments. Its pattern is seems likely bind to ligand that expressed all over cells except nucleus. Nevertheless, KKU-100 peptide staining pattern is different from wheat germ agglutinin (WGA) plasma membrane staining pattern. Somehow the staining pattern is different depending on staining condition such as fixation and blocking solution (Figure 22 and 23). The result of cell staining using paraformaldehyde and 3% BSA as blocking agent generated the best signal of peptide staining with KKU-100 cells. Further analysis of peptide staining needs to be investigated to improve staining pattern and signal. Since CTTPPYVC and CTSPPYVC were selected with live cells, *in vivo* application is beneficial. The improvement of molecular targeting of these two peptides could be valuable for imaging diagnosis in the future. Example of peptides in molecular imaging was described in Table 4. In past decade, there have been great advances in molecular imaging study. Cancer cell surface targeting ligand will play an essential role as imaging probe in clinical cancer detection and staging in future (Aina et al., 2007).

5.3 Selection of specific scFv to KKU-100 cells

The cell based selection of phage display peptide and human scFv for cholangiocarcinoma cell line had not been established. Various selection experiments had been optimized from previous report that had been done with different cell types and targets including tumor cells or tumor associated cells (Gao et al., 2003; Gerwin A. Huls, 1999; Oyama et al., 2003; Rasmussen et al., 2002), cancer cell lines (Eisenhardt et al., 2007) and freshly removal tumor (Roovers et al., 2001) or transfected cell line (Heitner et al., 2001). In order to obtain specific ligand to target molecule under native context, panning on live cells were done. The selection condition was done at different temperature 37^{O}C and 4 ^{O}C. Selection at 37^{O}C incubation, internalization process is occurred, on the other hand, reduce internalization occurs at 4^{O}C (Eisenhardt et al., 2007).

Phage display scFv specific to KKU100 cell line were selected since the first round selection by using experiment 1, with YAMO library. Most of previous cell-based selection reports indicated that 3 to 5 were done to obtain specific ligand (Gur et al., 2009; McGuire et al., 2009). The cell based ELISA for confirmation of positive scFv clones demonstrated that non-specific scFv to KKU-100 was selected from Tomlinson I and Sullivan library. This might be influence from different source of scFv genes in each library. The higher complexity of library seems to be better choice for selecting scFv against complex target even naïve library were used in biopanning. Liu and co-workers reported that naïve phage antibody library is highly complex to use in selection with complex target such as whole cell target. More than 90 internalizing phage monoclonal antibodies targeting prostate cancer and cell type-

specific internalizing human antibody can be readily identified from naïve phage antibody display library (Liu et al., 2007).

Roovers and colleagues revealed that selection exhibit antibodies cross-react with normal tissue. Even though the selection protocol with different patients section was done to avoid possible selection of antibodies directed to antigens unique for one, or a subset of patients (e.g. human leucocyte antigen (HLA) molecule. Immuno-histochemistry test showed that 14 of 17 clones after 5 rounds of selection did not show positive signal. The use of selection strategy without library depletion or substraction does not avoid selecting antibodies that coss-react with normal or common target. The selection for tumor-specific epitopes required depletion/ substraction step (Roovers et al., 2001).

Applying library pre-incubation before and/or after selection with KKU100 target seems to be no consequence on decreasing selected scFv display on phage that bind to common target on pre-incubation cell. All selected scFv phage from library pre-incubation procedures could react with HepG-2 cells and COS-1 cells.

Library pre-incubation and 2-5 rounds of selection as mentioned before increased number of positive scFv and might not limit variety of scFv. *BstNI* digestion pattern of selected clones from 5 rounds of selection is still diverse. The cell based ELISA signal of positive scFv is increased when more than single round of selection was applied, indicated that scFv with higher affinity was selected. Since the selected pool of scFv after five selection rounds was still diverse and only a limited number of the selected clones were tested, it is expected that many more specificity may be identified by additional screening.

5.4 Production of soluble scFv and their applications

To ensure that these selected scFvs can function without gene III context, soluble scFv were expressed. Most promising clones were selected to produce soluble scFv. Phage scFv from YAMO library were constructed into pMOD1 vector that has an amber stop codon and the production of scFv is control by lac promoter in non-suppressor host *E.coli* (Pansri et al. , 2009). Since the expression of scFv from this promoter was quite low, these 2 scFv were subjected to express under another promoter. Vector for scFv expression named pKP300ΔIII allows protein expression under *phoA* promoter. Two scFv, YM12 and YM13, were successfully expressed and purified using this vector. Others clone such as S.V. E10, S.V. H4, S.V. R5A1 and S.V. R5A8 were also done with success. The quantity and quality of purified scFv_AP and scFv analysis from Pro260 was shown in Table 12. It is indicated that scFv_AP and scFv were expressed and purified with approximate size 80 and 30 kDa, respectively. The quantity of each scFv_AP clone is varied between 0.08 to 2.37 mg per 50 ml of bacteria culture. While each scFv quantity is 0.03 to 1.66 mg. Purity of purified scFv (87.8-99.7 %) is higher than scFv_AP (59.4-85%). Since, many scFv failed to express in *E. coli* at accepTable levels or misfolding forming in inclusion bodies. Typical yield for scFv in *E.coli* are 0.1 to 0.2 mg/l of culture whereas in *Drosophila* expression system are 1-2 mg/l of culture and range from 0.5 to 5 mg/l of culture (Lipes et al., 2008).

Immunoblotting using purified scFv/scFv with AP fusion as a probe, the result showed that none of all selected scFv recognized their cognate antigen by this method, meaning that they might recognized non-linear epitope that are sensitive to denaturation of the antigen. The immunoblot result was shown negative under

reducing condition, indicating that these scFv bound epitopes that are altered by breaking of disulfide bonds. The same phenomena was noted by other selected antibodies such as HUVEC (human umbilical vein endothelial cells) antigen and EpCAM (epithelial cell adhesion molecules) (Mutuberria et al., 2004; Roovers et al., 2001). By the way, these scFv selected from native context unmodified cell surface. This scFv might be useful *in vivo* application, targeting.

KKU-100 cell line did not give a positive ELISA signal when incubated with Herceptin displayed on phage (Figure 36) indicating low ErbB2 expression. The staining pattern of scFv YM12 and YM13 have a unique different pattern from HepB3, COS-1, HepG2 and H69 (Figure 55, 56 and 57). The finding suggested that the scFv from YAMO library bound to ligand that was more / over-expressed in KKU-100 than HepG2 and COS-1 cells. Further quantitative expression studies are needed to deeply validate these antigens as tumor markers.

Whether or not the exact epitope recognized by selected scFv is composed of carbohydrate structure has not been assessed. The binding of scFv in cell based ELISA was interfered by both 0.25 mM N-acetlyglucosamine and glucose. Moreover different pattern of cells after incubated with scFv from FTIR microspectroscopy indicated these scFv binding invlove with both protein and carbohydrate.

All selected clones from YAMO library derived VH gene from VH1 VH3 and. VL originated from both κ and λ isotypes (Vκ3 and Vλ1, 2,3 and 5 families). Sullivan library in addition of VH1 and VH3, selected clones derived from VH6 family were found and VL from κ and λ isotypes only Vκ1 and Vλ1 families. Human monoclonal antibodies to antigen associated with cholangiocarcinoma cell line were generated from poorly diffentiated adenocarcinoma. This format of scFv with approximately 35

kDa might be useful in immunotherapy or immunodiagnosis due to smaller size, less accumulate in kidney.

In conclusion, we have proven that phage display is a powerful mean to selected peptide and scFv specific to cholangiocarcinoma cell line, KKU-100 that could be developed into molecular targeting agents for cholangiocarcinoma.

REFERENCES

Achilefu, S., Bloch, S., Markiewicz, M. A., Zhong, T., Ye, Y., Dorshow, R. B., Chance, B., and Liang, K. (2005): Synergistic effects of light-emitting probes and peptides for targeting and monitoring integrin expression. **Proc Natl Acad Sci U S A** 102, 7976-81.

Aina, O. H., Liu, R., Sutcliffe, J. L., Marik, J., Pan, C. X., and Lam, K. S. (2007): From combinatorial chemistry to cancer-targeting peptides. **Mol Pharm** 4, 631-51.

Arap, W., Pasqualini, R., and Ruoslahti, E. (1998): Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. **Science** 279, 377-80.

Askoxylakis, V., Zitzmann, S., Mier, W., Graham, K., Kramer, S., von Wegner, F., Fink, R. H., Schwab, M., Eisenhut, M., and Haberkorn, U. (2005): Preclinical evaluation of the breast cancer cell-binding peptide, p160. **Clin Cancer Res** 11, 6705-12.

Balestrieri, M. L., and Napoli, C. (2007): Novel challenges in exploring peptide ligands and corresponding tissue-specific endothelial receptors. **Eur J Cancer** 43, 1242-50.

Belizaire, A.-K., Tchistiakova, L., St-Pierre, Y., and Alakhov, V. (2003): Identification of a murine ICAM-1-specific peptide by subtractive phage library selection on cells. **Biochemical and Biophysical Research Communications** 309, 625-630.

Bloch, S., Xu, B., Ye, Y., Liang, K., Nikiforovich, G. V., and Achilefu, S. (2006): Targeting

Beta-3 integrin using a linear hexapeptide labeled with a near-infrared fluorescent molecular probe. **Mol Pharm** 3, 539-49.

Brown, K. C. (2009): Peptidic Tumor Targeting Agents: The Road from Phage Display Peptide Selections to Clinical Applications. **Curr Pharm Des**.

Burg, M. A., Pasqualini, R., Arap, W., Ruoslahti, E., and Stallcup, W. B. (1999): NG2 proteoglycan-binding peptides target tumor neovasculature. **Cancer Res** 59, 2869-74.

Cai, W., Shin, D. W., Chen, K., Gheysens, O., Cao, Q., Wang, S. X., Gambhir, S. S., and Chen, X. (2006): Peptide-labeled near-infrared quantum dots for imaging tumor vasculature in living subjects. **Nano Lett** 6, 669-76.

Chen, C. Y., Shiesh, S. C., Tsao, H. C., and Lin, X. Z. (2002): The assessment of biliary CA 125, CA 19-9 and CEA in diagnosing cholangiocarcinoma--the influence of sampling time and hepatolithiasis. **Hepatogastroenterology** 49, 616-20.

Chen, J., Tung, C. H., Allport, J. R., Chen, S., Weissleder, R., and Huang, P. L. (2005): Near-infrared fluorescent imaging of matrix metalloproteinase activity after myocardial infarction. **Circulation** 111, 1800-5.

Choi, J. H., Lee, W. K., Han, S. H., Ha, S., Ahn, S. M., Kang, J. S., Choi, Y. J., and Yun, C. H. (2008): Identification and characterization of nonapeptide targeting a human B cell lymphoma, Raji. **Int Immunopharmacol** 8, 852-8.

Curnis, F., Sacchi, A., Borgna, L., Magni, F., Gasparri, A., and Corti, A. (2000): Enhancement of tumor necrosis factor alpha antitumor immunotherapeutic properties by targeted delivery to aminopeptidase N (CD13). **Nat Biotechnol** 18,1185-90.

de Groen PC, G. G., LaRusso NF, Gunderson LL, Nagorney DM. (1999): Biliary tract cancers. **New England Journal of Medicine** 341, 1368-1378.

Deutscher, S. L. (2010): Phage display in molecular imaging and diagnosis of cancer. **Chemical reviews**.

Dickerson, E. B., Akhtar, N., Steinberg, H., Wang, Z. Y., Lindstrom, M. J., Padilla, M. L., Auerbach, R., and Helfand, S. C. (2004): Enhancement of the antiangiogenic activity of interleukin-12 by peptide targeted delivery of the cytokine to alphavbeta3 integrin. **Mol Cancer Res** 2, 663-73.

Dimitrov, D. S., and Marks, J. D. (2009): Therapeutic antibodies: current state and future trends--is a paradigm change coming soon? **Methods Mol Biol** 525, 1-27, xiii.

Du, B., Qian, M., Zhou, Z., Wang, P., Wang, L., Zhang, X., Wu, M., Zhang, P., and Mei, B. (2006): In vitro panning of a targeting peptide to hepatocarcinoma from a phage display peptide library. **Biochemical and Biophysical Research Communications** 342, 956-962.

Eisenhardt, S. U., Schwarz, M., Bassler, N., and Peter, K. (2007): Subtractive single-chain antibody (scFv) phage-display: tailoring phage-display for high specificity against function-specific conformations of cell membrane molecules. **Nat Protoc** 2, 3063-73.

Gao, C., Mao, S., Ronca, F., Zhuang, S., Quaranta, V., Wirsching, P., and Janda, K. D. (2003): De novo identification of tumor-specific internalizing human antibody-receptor pairs by phage-display methods. **Journal of Immunological Methods** 274, 185-197.

Gerwin A. Huls, I. A. F. M. H., Maria E. Cuomo, Jacob C. Koningsberger, Luus

Wiegman, Edwin Boel, Anne-Renee van der Vuurst de Vries, Sabine A.J. Loyson, Wijnand Helfrich, Gerard P. van Berge Henegouwen, Marja van Meijer, John de Kruif, Ton Logtenberg (1999): A recombinant, fully human monoclonal antibody with antitumor activity constructed from phage-displayed antibody fragments. **Nature Biotechnology** 17, 276-281.

Goenaga, A. L., Zhou, Y., Legay, C., Bougherara, H., Huang, L., Liu, B., Drummond, D. C., Kirpotin, D. B., Auclair, C., Marks, J. D., and Poul, M. A. (2007): Identification and characterization of tumor antigens by using antibody phage display and intrabody strategies. **Molecular Immunology** 44, 3777-3788.

Gur, D., Liu, S., Shukla, A., Pero, S. C., Wicha, M. S., and Krag, D. N. (2009): Identification of single chain antibodies to breast cancer stem cells using phage display. **Biotechnol Prog** 25, 1780-7.

Hajitou, A., Pasqualini, R., and Arap, W. (2006): Vascular Targeting: Recent Advances and Therapeutic Perspectives. **Trends in Cardiovascular Medicine** 16, 80-88.

Hao, J., Serohijos, A. W., Newton, G., Tassone, G., Wang, Z., Sgroi, D. C., Dokholyan, N. V., and Basilion, J. P. (2008): Identification and rational redesign of peptide ligands to CRIP1, a novel biomarker for cancers. **PLoS Comput Biol** 4, e1000138.

Heitner, T., Moor, A., Garrison, J. L., Marks, C., Hasan, T., and Marks, J. D. (2001): Selection of cell binding and internalizing epidermal growth factor receptor antibodies from a phage display library. **Journal of Immunological Methods** 248, 17-30.

Hoffman, J. A., Giraudo, E., Singh, M., Zhang, L., Inoue, M., Porkka, K., Hanahan, D and Ruoslahti, E. (2003): Progressive vascular changes in a transgenic mouse

mouse model of squamous cell carcinoma. **Cancer Cell** 4, 383-91.

Huang, C., Liu, X. Y., Rehemtulla, A., and Lawrence, T. S. (2005): Identification of peptides that bind to irradiated pancreatic tumor cells. **International Journal of Radiation Oncology*Biology*Physics** 62, 1497-1503.

Hui, X., Han, Y., Liang, S., Liu, Z., Liu, J., Hong, L., Zhao, L., He, L., Cao, S., Chen, B., Yan, K., Jin, B., Chai, N., Wang, J., Wu, K., and Fan, D. (2008): Specific targeting of the vasculature of gastric cancer by a new tumor-homing peptide CGNSNPKSC. **J Control Release** 131, 86-93.

Hu, S., Guo, X., Xie, H., Du, Y., Pan, Y., Shi, Y., Wang, J., Hong, L., Han, S., and Zhang, D. (2006): Phage display selection of peptides that inhibit metastasis ability of gastric cancer cells with high liver-metastatic potential. **Biochemical and Biophysical Research Communications** 341, 964-972.

Intasai, N., Mai, S., Kasinrerk, W., and Tayapiwatana, C. (2006): Binding of multivalent CD147 phage induces apoptosis of U937 cells. **International Immunology** 18, 1159.

Isomoto, H., Kobayashi, S., Werneburg, N. W., Bronk, S. F., Guicciardi, M. E., Frank, D. A., and Gores, G. J. (2005): Interleukin 6 upregulates myeloid cell leukemia-1 expression through a STAT3 pathway in cholangiocarcinoma cells. **Hepatology** 42, 1329-38.

Jager, S., Jahnke, A., Wilmes, T., Adebahr, S., Vogtle, F. N., Delima-Hahn, E., Pfeifer, D., Berg, T., Lubbert, M., and Trepel, M. (2007): Leukemia-targeting ligands isolated from phage-display peptide libraries. **Leukemia** 21, 411-20.

Jia, W.-D., Sun, H.-C., Zhang, J.-B., Xu, Y., Qian, Y.-B., Pang, J.-Z., Wang, L., Qin, L.-X., Liu, Y.-K., and Tang, Z.-Y. A novel peptide that selectively binds highly

metastatic hepatocellular carcinoma cell surface is related to invasion and metastasis. **Cancer Letters** In Press, Corrected Proof.

Jinawath, N., Chamgramol, Y., Furukawa, Y., Obama, K., Tsunoda, T., Sripa, B., Pairojkul, C., and Nakamura, Y. (2006): Comparison of gene expression profiles between Opisthorchis viverrini and non-Opisthorchis viverrini associated human intrahepatic cholangiocarcinoma. **Hepatology** 44, 1025-38.

Joyce, J. A., Laakkonen, P., Bernasconi, M., Bergers, G., Ruoslahti, E., and Hanahan, D. (2003): Stage-specific vascular markers revealed by phage display in a mouse model of pancreatic islet tumorigenesis. **Cancer Cell** 4, 393-403.

Kelly, K. A., Allport, J. R., Tsourkas, A., Shinde-Patil, V. R., Josephson, L., and Weissleder, R. (2005): Detection of vascular adhesion molecule-1 expression using a novel multimodal nanoparticle. **Circ Res** 96, 327-36.

Kelly, K. A., and Jones, D. A. (2003): Isolation of a colon tumor specific binding peptide using phage display selection. **Neoplasia** 5, 437-44.

Kelly, K., Alencar, H., Funovics, M., Mahmood, U., and Weissleder, R. (2004): Detection of invasive colon cancer using a novel, targeted, library-derived fluorescent peptide. **Cancer Research** 64, 6247-6251.

Kim, Y., Lillo, A. M., Steiniger, S. C., Liu, Y., Ballatore, C., Anichini, A., Mortarini, R., Kaufmann, G. F., Zhou, B., Felding-Habermann, B., and Janda, K. D. (2006): Targeting heat shock proteins on cancer cells: selection, characterization, and cell-penetrating properties of a peptidic GRP78 ligand. **Biochemistry** 45, 9434-44.

Kolonin, M. G., Saha, P. K., Chan, L., Pasqualini, R., and Arap, W. (2004): Reversal of obesity by targeted ablation of adipose tissue. **Nat Med** 10, 625-32.

Kolonin, M. G., Sun, J., Do, K. A., Vidal, C. I., Ji, Y., Baggerly, K. A., Pasqualini, R., and Arap, W. (2006): Synchronous selection of homing peptides for multiple tissues by in vivo phage display. **FASEB J** 20, 979-81.

Koolpe, M., Burgess, R., Dail, M., and Pasquale, E. B. (2005): EphB receptor-binding peptides identified by phage display enable design of an antagonist with ephrin-like affinity. **J Biol Chem** 280, 17301-11.

Koolpe, M., Dail, M., and Pasquale, E. B. (2002): An ephrin mimetic peptide that selectively targets the EphA2 receptor. **J Biol Chem** 277, 46974-9.

Kriplani, U., and and Kay, B. K. (2005): Selecting peptides for use in nanoscale materials using phage-displayed combinatorial peptide libraries. **Current Opinion in Biotechnology** 16 470-475.

Krumpe, L. R., and Mori, T. (2007): Potential of phage-displayed peptide library technology to identify functional targeting peptides. **Expert Opin Drug Discov** 2, 525.

Laakkonen, P., Akerman, M. E., Biliran, H., Yang, M., Ferrer, F., Karpanen, T., Hoffman, R. M., and Ruoslahti, E. (2004): Antitumor activity of a homing peptide that targets tumor lymphatics and tumor cells. **Proc Natl Acad Sci U S A** 101, 9381-6.

Laakkonen, P., Porkka, K., Hoffman, J. A., and Ruoslahti, E. (2002): A tumor-homing peptide with a targeting specificity related to lymphatic vessels. **Nat Med** 8, 751-5.

Lee, L., Buckley, C., Blades, M. C., Panayi, G., George, A. J., and Pitzalis, C. (2002): Identification of synovium-specific homing peptides by in vivo phage display selection. **Arthritis Rheum** 46, 2109-20.

Lee, S. M., Lee, E. J., Hong, H. Y., Kwon, M. K., Kwon, T. H., Choi, J. Y., Park, R. W., Kwon, T. G., Yoo, E. S., Yoon, G. S., Kim, I. S., Ruoslahti, E., and Lee, B. H. (2007): Targeting bladder tumor cells in vivo and in the urine with a peptide identified by phage display. **Mol Cancer Res** 5, 11-9.

Liang, S., Lin, T., Ding, J., Pan, Y., Dang, D., Guo, C., Zhi, M., Zhao, P., Sun, L., Hong, L., Shi, Y., Yao, L., Liu, J., Wu, K., and Fan, D. (2006): Screening and identification of vascular-endothelial-cell-specific binding peptide in gastric cancer. **J Mol Med** 84, 764-73.

Lipes, B. D., Chen, Y. H., Ma, H., Staats, H. F., Kenan, D. J., and Gunn, M. D. (2008): An entirely cell-based system to generate single-chain antibodies against cell surface receptors. **J Mol Biol** 379, 261-72.

Liu, B., Conrad, F., Roth, A., Drummond, D. C., Simko, J. P., and Marks, J. D. (2007): Recombinant full-length human IgG1s targeting hormone-refractory prostate cancer. **J Mol Med** 85, 1113-23.

Liu, S. (2006): Radiolabeled multimeric cyclic RGD peptides as integrin alphavbeta3 targeted radiotracers for tumor imaging. **Mol Pharm** 3, 472-87.

Loo, D. T., and Mather, J. P. (2008): Antibody-based identification of cell surface antigens: targets for cancer therapy. **Curr Opin Pharmacol** 8, 627-31.

Malhi, H., and Gores, G. J. (2006): Cholangiocarcinoma: modern advances in understanding a deadly old disease. **J Hepatol** 45, 856-67.

MarchiÚ, S., Lahdenranta, J., Schlingemann, R. O., Valdembri, D., Wesseling, P., Arap, M. A., Hajitou, A., Ozawa, M. G., Trepel, M., Giordano, R. J., Nanus, D. M., Dijkman, H. B. P. M., Oosterwijk, E., Sidman, R. L., Cooper, M. D.,

Bussolino, F., Pasqualini, R., and Arap, W. (2004): Aminopeptidase A is a functional target in angiogenic blood vessels. **Cancer Cell** 5, 151-162.

Mazuet, C., Lerouge, D., Poul, M.-A., and Blin, N. (2006): Breast carcinoma specific antibody selection combining phage display and immunomagnetic cell sorting. **Biochemical and Biophysical Research Communications** 348, 550-559.

McGuire, M. J., Li, S., and Brown, K. C. (2009): Biopanning of phage displayed peptide libraries for the isolation of cell-specific ligands. **Methods Mol Biol** 504, 291-321.

Michon, I. N., Penning, L. C., Molenaar, T. J., van Berkel, T. J., Biessen, E. A., and Kuiper, J. (2002): The effect of TGF-beta receptor binding peptides on smooth muscle cells. **Biochem Biophys Res Commun** 293, 1279-86.

Moss, S. F., and Blaser, M. J. (2005): Mechanisms of disease: Inflammation and the origins of cancer. **Nat Clin Pract Oncol** 2, 90-7; quiz 1 p following 113.

Mutuberria, R., Satijn, S., Huijbers, A., van der Linden, E., Lichtenbeld, H., Chames, P., Arends, J.-W., and Hoogenboom, H. R. (2004): Isolation of human antibodies to tumor-associated endothelial cell markers by in vitro human endothelial cell selection with phage display libraries. **Journal of Immunological Methods** 287, 31-47.

Nie, Y., He, F., Li, Z., Wu, K., Cao, Y., Chen, B., and Fan, D. (2002): Identification of tumor associated single-chain Fv by panning and screening antibody phage library using tumor cells. **World Journal of Gastroenterology** 8, 619-623.

Nishimura, S., Takahashi, S., Kamikatahira, H., Kuroki, Y., Jaalouk, D. E., O'Brien, S. , Koivunen, E., Arap, W., Pasqualini, R., Nakayama, H., and Kuniyasu, A. (2008): Combinatorial targeting of the macropinocytotic pathway in leukemia

and lymphoma cells. **J Biol Chem** 283, 11752-62.

Oyama, T., Rombel, I. T., Samli, K. N., Zhou, X., and Brown, K. C. (2006): Isolation of multiple cell-binding ligands from different phage displayed-peptide libraries. **Biosensors and Bioelectronics** 21, 1867-1875.

Oyama, T., Sykes, K. F., Samli, K. N., Minna, J. D., Johnston, S. A., and Brown, K. C. (2003): Isolation of lung tumor specific peptides from a random peptide library: generation of diagnostic and cell-targeting reagents. **Cancer Letters** 202, 219-230.

Pakkala, M., Jylhäsalmi. A., Wu, P., Leinonen, J., Stenman, U., Santa, H., Vepsäläinen, J., Peräkylä, M., and Närvänen, A. (2004): Conformational and biochemical analysis of the cyclic peptides which modulate serine protease activity. **Journal of peptide science : an official publication of the European Peptide Society** 10, 439-447.

Pansri, P., Jaruseranee, N., Rangnoi, K., Kristensen, P., and Yamabhai, M. (2009): A compact phage display human scFv library for selection of antibodies to a wide variety of antigens. **BMC Biotechnol** 9, 6.

Pasqualini, R., Koivunen, E., Kain, R., Lahdenranta, J., Sakamoto, M., Stryhn, A., Ashmun, R. A., Shapiro, L. H., Arap, W., and Ruoslahti, E. (2000): Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. **Cancer Res** 60, 722-7.

Perea, S. E., Reyes, O., Baladron, I., Perera, Y., Farina, H., Gil, J., Rodriguez, A., Bacardi, D., Marcelo, J. L., Cosme, K., Cruz, M., Valenzuela, C., Lopez-Saura, P. A., Puchades, Y., Serrano, J. M., Mendoza, O., Castellanos, L., Sanchez, A., Betancourt, L., Besada, V., Silva, R., Lopez, E., Falcon, V., Hernandez, I.,

Solares, M., Santana, A., Diaz, A., Ramos, T., Lopez, C., Ariosa, J., Gonzalez, L. J., Garay, H., Gomez, D., Gomez, R., Alonso, D. F., Sigman, H., Herrera, L., and Acevedo, B. (2008): CIGB-300, a novel proapoptotic peptide that impairs the CK2 phosphorylation and exhibits anticancer properties both in vitro and in vivo. **Mol Cell Biochem** 316, 163-7.

Pilch, J., Brown, D. M., Komatsu, M., Jarvinen, T. A., Yang, M., Peters, D., Hoffman, R. M., and Ruoslahti, E. (2006): Peptides selected for binding to clotted plasma accumulate in tumor stroma and wounds. **Proc Natl Acad Sci U S A** 103, 2800-4.

Rasmussen, U. B., Schreiber, V., Schultz, H., Mischler, F., and Schughart, K. (2002): Tumor cell-targeting by phage-displayed peptides. **Cancer Gene Ther** 9, 606-12.

Robinson, P., Stuber, D., Deryckere, F., Tedbury, P., Lagrange, M., and Orfanoudakis, G. (2005): Identification using phage display of peptides promoting targeting and internalization into HPV-transformed cell lines. **J Mol Recognit** 18, 175-82.

Roovers, R. C., van der Linden, E., de Bruine, A. P., Arends, J.-W., and Hoogenboom, H. R. (2001): Identification of colon tumour-associated antigens by phage antibody selections on primary colorectal carcinoma. **European Journal of Cancer** 37, 542-549.

Schrama, D., Reisfeld, R., and Becker, J. (2006): Antibody targeted drugs as cancer therapeutics. **Nat Rev Drug Discov** 5, 147-59.

Sidhu, S. S. (2000): Phage display in pharmaceutical biotechnology. **Curr Opin Biotechnol** 11, 610-6.

Sinawat P, H. V. (1991): A histopathologic study of 61cases of peripheral intrahepatic cholangiocarcinoma. **Journal of the Medical Association of Thailand** 74, 448-453.

Smith, G. (1985): Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. **Science** 228, 1315.

Sripa, B., Kaewkes, S., Sithithaworn, P., Mairiang, E., Laha, T., Smout, M., Pairojkul, C., Bhudhisawasdi, V., Tesana, S., Thinkamrop, B., Bethony, J. M., Loukas, A., and Brindley, P. J. (2007): Liver fluke induces cholangiocarcinoma. **PLoS Med** 4, e201.

Sripa, B., Leungwattanawanit, S., Nitta, T., Wongkham, C., Bhudhisawasdi, V., Puapairoj, A., Sripa, C., and Miwa, M. (2005): Establishment and characterization of an opisthorchiasis-associated cholangiocarcinoma cell line (KKU-100). **World Journal of Gastroenterology** 11, 3392-3397.

Vatanasapt V, S. B., Sithithaworn P, Mairiang P. (1999): Liver flukes and liver cancer. **Cancer Surveys** 33, 314-343.

Vatanasapt V, U. T., Mairiang EO, Pairojkul C,, and Chartbanchachai W, H.-E. M. (1990): Northeast Thailand: A region with a high incidence of cholangiocarcinoma. **Lancet** 335, 116-117.

Vidal, C. I., Mintz, P. J., Lu, K., Ellis, L. M., Manenti, L., Giavazzi, R., Gershenson, D. M., Broaddus, R., Liu, J., Arap, W., and Pasqualini, R. (2004): An HSP90-mimic peptide revealed by fingerprinting the pool of antibodies from ovarian cancer patients. **Oncogene** 23, 8859-67.

Watanapa, P. (1996a): Cholangiocarcinoma in patients with opisthorchiasis. **Br J Surg** 83, 1062-64.

Watanapa, P. (1996b): Cholangiocarcinoma in patients with opisthorchiasis. **British Journal of Surgery** 83, 1062-64.

Watanapa, P., and Watanapa, W. (2002): Liver fluke-associated cholangiocarcinoma. **British Journal of Surgery** 89, 962-970.

Witt, H., Hajdin, K., Iljin, K., Greiner, O., Niggli, F. K., Schafer, B. W., and Bernasconi, M. (2009): Identification of a rhabdomyosarcoma targeting peptide by phage display with sequence similarities to the tumour lymphatic-homing peptide LyP-1. **Int J Cancer** 124, 2026-32.

Wongkham, S., Junking, M., Wongkham, C., Sripa, B., Chur-In, S., and Araki, N. (2009): Suppression of galectin-3 expression enhances apoptosis and chemosensitivity in liver fluke-associated cholangiocarcinoma. **Cancer Sci** 100, 2077-84.

Wu, P., Leinonen, J., Koivunen, E., Lankinen, H., and Stenman, U. H. (2000): Identification of novel prostate-specific antigen-binding peptides modulating its enzyme activity. **Eur J Biochem** 267, 6212-20.

Yao, V. J., Ozawa, M. G., Trepel, M., Arap, W., McDonald, D. M., and Pasqualini, R. (2005): Targeting pancreatic islets with phage display assisted by laser pressure catapult microdissection. **Am J Pathol** 166, 625-36.

Yu, L., Yu, P. S., Yee Yen Mui, E., McKelvie, J. C., Pham, T. P., Yap, Y. W., Wong, W. Q., Wu, J., Deng, W., and Orner, B. P. (2009): Phage display screening against a set of targets to establish peptide-based sugar mimetics and molecular docking to predict binding site. **Bioorg Med Chem** 17, 4825-32.

Zhang, J., Spring, H., and Schwab, M. (2001): Neuroblastoma tumor cell-binding peptides identified through random peptide phage display. **Cancer Letters** 171, 153-164.

Zhang, L., Hoffman, J. A., and Ruoslahti, E. (2005): Molecular profiling of heart endothelial cells. **Circulation** 112, 1601-11.

Zhao, X., Hu, J., Huang, R., and Yang, L. (2007): Identification of one vasculature specific phage-displayed peptide in human colon cancer. **J Exp Clin Cancer Res** 26, 509-14.

Zurita, A. J., Troncoso, P., Cardo-Vila, M., Logothetis, C. J., Pasqualini, R., and Arap, W. (2004a): Combinatorial screenings in patients: the interleukin-11 receptor alpha as a candidate target in the progression of human prostate cancer. **Cancer Res** 64, 435-9.

Zurita, A. J., Troncoso, P., Cardo-Vila, M., Logothetis, C. J., Pasqualini, R., and W., a. A. (2004b): Combinatorial screenings in patients: the interleukin-11 receptor alpha as a candidate target in the progression of human prostate cancer. **Cancer Research** 64 435-439.

APPENDICES

APPENDIX I

ADDITIONAL METHODS

1. Mycoplasma detection and Elimination

Mycoplasma is one major problem in animal cell culture due to this might effect the protein expression in cell culture. There are various methods to detect Mycoplasma contamination. Detection by PCR method is based on the amplification of Mycoplasma 16s DNA contaminated in culture. Another method is direct DNA staining, only nuclear DNA will be stained in uncontaminated culture while contaminated culture, extra nuclear DNA will be also appear with the nuclear DNA staining.

1.1 Mycoplasma detection by PCR based method

The **LookOut™ Mycoplasma PCR Detection Kit** from Sigma company (Catalog Number **MP0035)** utilizes the polymerase chain reaction (PCR), which is established as the method of choice for highest sensitivity in the detection of *Mycoplasma* and *Acholeplasma* contamination in cell cultures and other cell culture derived biologicals. Detection requires less than 5 mycoplasma genomes per microliter of sample. The primer set is specific to the highly conserved 16S rRNA coding region in the mycoplasma genome. The cell culture supernatant can be used directly to detect Mycoplasma DNA by preparation following the protocol from **LookOut™ Mycoplasma PCR Detection Kit.** Cell lines should be pre-cultured in the absence of Mycoplasma active antibiotics for several days to maximize test sensitivity. Samples

should be derived from cultures that are at 90-100 % confluence. Culture supernatant was transferred from the test culture to a sterile amplification tube and boiled or incubated the sample supernatant at 95 ^{o} C for 5 minutes. Then it was briefly centrifuged (5 seconds) the sample supernatant to pellet cellular debris before adding to the PCR mixture and the templates are stable at 2–8^{O}C for at least 1 week. PCR reactions were set up.

	Sample	**Positive**	**Negative**
DNA Polymerase/ Rehydration Buffer	23 µl	25 µl	23 µl
Sample Volume	2 µl	-	-
DNA-free Water	-	-	2 µl
Taq polymerase	2 units	2 units	2 units

The amplification was done using the PCR machine with this program.

1 cycle	94 ^{O}C	for 2 minutes
40 cycles	94 ^{O}C	for 30 seconds
	55 ^{O}C	for 30 seconds
	72 ^{O}C	for 40 seconds
Cool down to 4-8 ^{O}C		

After the amplification was completed, 1.2% agarose gel electrophoresis was done. Mycoplasma positive samples show bands in the range of 260 ±8 bp.

1.2 DAPI staining method

DNA staining can be used to detect Mycoplasma contamination in animal cell culture. This method is over 95% effective. Mycoplasma DNA will be stained and

appeared as an extra nuclear DNA staining when compare with non- contaminated cell which only nuclear DNA is stained. Cells were grown in chamber slide to reach 70% confluence and were fixed with Carnoy's fixative for 30 min. Then cell were washed with PBS and Fluorescence mounting medium with DAPI and cover slip was applied. Slides were observed under fluorescence microscope.

1.3 Mycoplasma Elimination protocol

The elimination protocol was carried out according to Mycoplasma Elimination kit from Sigma company. Contaminated cell were cultured in media without antibiotic for Mycoplasma until cell reach 80 to 90% confluence. Mycoplasma Elimination Initial Treatment from Sigma company (Product Code M4569) 250 µl was added to prewarmed medium (37 °C) 2.5 ml of Ham F12 with 5% fetal bovine serum. The cell were split and passaged as thinly as possible given the nature of the cell line. Cell suspension 2.5 ml was added to medium/elimination reagent mixture as described previous. Try to avoid cell aggregates, by using single cell suspension during treatment thus it can be confirmed by observation under microscope. If there are some aggregated cell trypsinization or trituration can be applied as necessary. This culture will be grown until 80-90% confluent. Cell can be sub-cultured at usual rate and the contents of one vial (250 µl) of Mycoplasma Elimination Final will also added to cell suspension during sub culture. Finallly Treatment (Product Code M4444) was added to 4.5 ml of passaged cells in Ham F-12 with 10% Fetal bovine serum.

The last step of treatment was done through two additional passages. At this time cultures are Mycoplasma free.

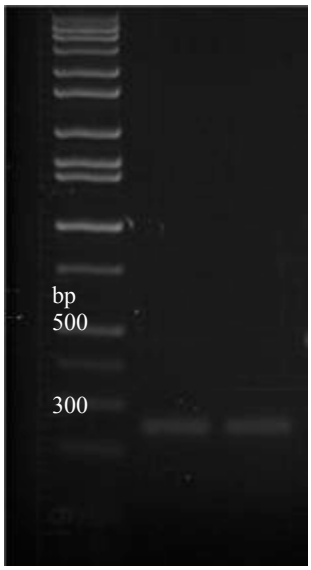

Figure 62. Positive bands of Mycoplasma 16s DNA amplification from PCR Mycoplasma detection kit . Lane 1= marker,Lane 2and 3 =KKU-100 .16s DNA Mycoplasma product were amplified the PCR product size is in the range of 260 ±8 bp.

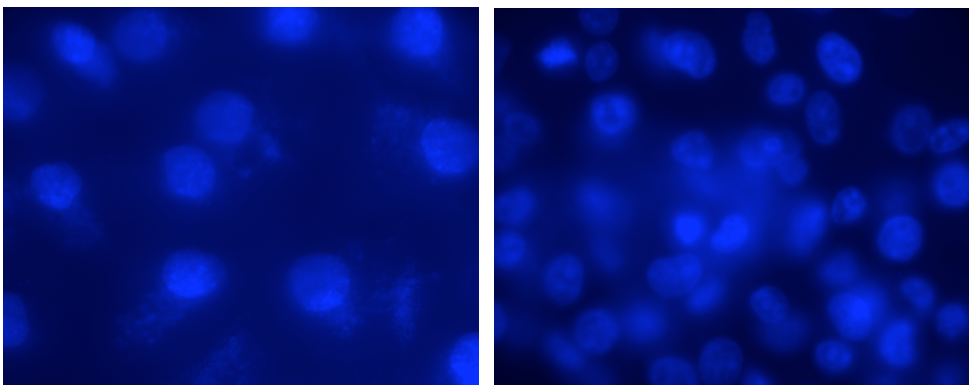

Figure 63. DAPI staining KKU-100 ; Mycoplasma contaminated Mycoplasma DNA were stained in cytoplasm and COS-1 cell ; non contaminated cell only nuclear DNA were stained.

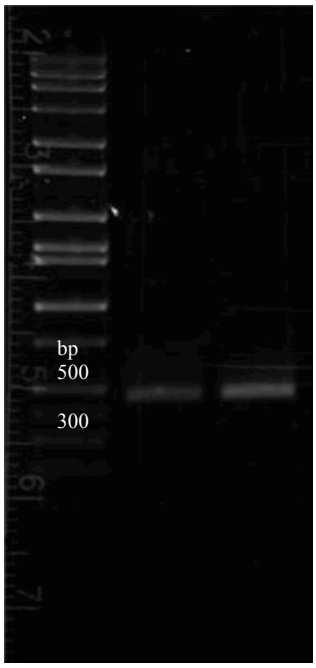

Figure 64. PCR Mycoplasma detection after elimination process. Lane1=marker, 2 and 3= KKU-100 no PCR product from Mycoplasma contamination in culture.

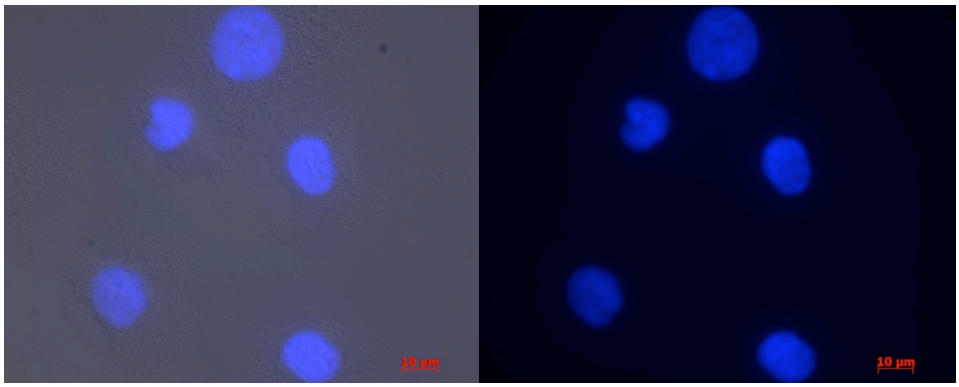

Figure 65. KKU-100 cell with DAPI staining after Mycoplasma elimination only nuclear DNA were stained.

2. Optimization of Biopanning with phage display peptide

Experiment 1 : Live KKU-100 selection with SUT12 library with COS-7 preincubation

1. COS-7 cells were grown in 6-wells plate to 80 % confluence.
2. Cell was washed with 1 ml of warm PBS 2 times
3. Phage display peptide SUT12; 50 µl (~10^{13} pfu/ml) with 1450 µl 3% BSA in PBS was added to cell.
4. Cell and Phage library were incubate at 37^{O}C for 1 hr.
5. Supernatant was transferred to 2nd COS-7 cells, 5 times preincubation was done.
6. After that unbound phage supernatant was incubated with KKU-100 cells.
7. Cell and Phage library were incubate at 37^{O}C for 1 hr.
8. Then follow by acid elution by adding 750 µl of 0.1M glycine HCl pH 2.0 and neutralize with 750 µl NU solution.
9. In order to break cell, PBS with 1% TritonX 750 µl was added.
10. All eluted phage 2,250 µl was incubated with 1:100 *E.coli*K12f' 3 ml and shaken at 200 rpm 37^{O}C for 8 hr.
11. Amplified library was rescued by centrifugation at 10,000 rpm 5 minutes and supernatant was ready for second round of selection or stored at 4^{O}C.
12. Phage supernatant 750 µl was equally mixed with 3%BSA and continue selection with KKU-100 cells.
13. The selection procedure was described in 7 to 10 and 3rd round was done.
14. After 3rd round, individual phage clones was separated by ten fold serial dilution of phage particle was carried out and spot on 0.8% top agar contained 1:100

E.coli cells, 50 µl of 0.1M IPTG and 50 µl of 2% X-gal. The plate was incubated at 37^{O}C for overnight.

15. Next day, number of blue plaque from phage infection indicate number of phage particle multiply dilution.

16. The appropriate titer of phage was selected to separate each phage clone. A certain mount of phage particle was mixed with 0.8% top agar containing 1:100 *E.coli* cells, 50 µl of 0.1M IPTG and 50 µl of 2% X-gal. Then it was mixed thoroughly and pour on top of LB agar. The plate was incubated at 37^{O}C for overnight.

17. Single plaque was picked and co-inoculated with 1:100 *E.coli* cells and incubated with agitation at 200 rpm 37^{O}C for 8 hr.

18. Phage supernatant were rescued by centrifugation to separate cell pellet and ready for phage ELISA.

Experiment 2 : Fixed KKU-100 selection with SUT12 library

1. KKU-100 cells were grown in 6-wells plate to 80 % confluence.

2. Cells were washed with 1 ml of warm PBS 2 times and fixed with freshly prepare 4% paraformaldehyde in PBS for 20 minutes

3. Cells were washed with 1 ml of warm PBS 2 times.

4. Phage display peptide SUT12; 50 µl (~10^{13} pfu/ml) with 1450µl 3% BSA in PBS was added to cell.

5. Cell and Phage library were incubate at room temperature for 1 hr.

6. Cells were washed with 1 ml of warm PBS 10 times

7. Then follow by acid elution by adding 750 µl of 0.1M glycine HCl pH 2.0 and neutralize with 750 µl NU solution.

8. Eluted phage 1,500µl was incubated with 1:100 *E.coli*K12f' 3 ml and shaken at 200 rpm 37^{O}C for 8 hr.

9. Amplified library was rescued by centrifugation at 10,000 rpm 5 minutes and supernatant was ready for second round of selection or stored at 4^{O}C.

10. Phage supernatant 750 µl was equally mixed with 3%BSA and continue selection with KKU-100 cells.

11. The selection procedure was repeated for 2nd as described in 7 to 10 and 3rd round was done without amplification of 2nd round eluted phage.

12. After 3rd round, individual phage clones was separated and produced as described in protocol 1.

Experiment 3 : Live ~ 5 x 10^{5} KKU-100 cells selection with SUT12 library

1. KKU-100 cells were grown in 25 cm^{3} to 80 % confluence.

2. Cells were washed with 1 ml of warm PBS 2 times. Cells were washed with 1 ml of warm PBS 2 times.

3. Phage display peptide SUT12; 50 µl (~10 ^{13} pfu/ml) with 450µl 3% BSA in PBS was added to KKU-100 cell.

4. Cells and Phage library were incubate at 37^{O}C for 2 hr.

5. Cells were washed with 3 ml of PBS 10 times and 3 ml PBST 10 times, 3 minutes incubation during each wash.

6. Then follow by acid elution by adding 350 µl of 0.1M glycine HCl pH 2.0 and neutralize with 350 µl NU solution.

7. Eluted phage 700µl was incubated with 1:100 *E.coli*K12f' 3 ml and shaken at 200 rpm 37^{O}C for 8 hr.

8. Amplified library was rescued by centrifugation at 10,000 rpm 5 minutes. 2.5 M Nacl PEG was added to supernatant and incubated at $4^{O}C$ for overnight.

9. After centrifuged to separate precipitate phage, 1XPBS 200 µl was added to resuspend concentrate amplified library.

10. Amplified phage 200 µl was mixed with 3% BSA 500 µl and continue selection with KKU-100 cells.

11. The selection procedure was repeated for 2^{nd} and 3^{rd} as described in 5 to 11.

12. After 3^{rd} round, individual phage clones was separated and produced as described in protocol 1.

Experiment 4 : Live ~ 5 x 10^5 KKU-100 cells selection with ANL10 (CX_6C) library

The selection protocol was done as described in protocol 4 and 100 µl of ANL10 (10^{13} pfu/ml) and 400 µl of 4% MPBS was added to cell instead of SUT 12 library. Eluted phage from the 1^{st} round of selection was incubated with H69 for 1 hr and unbound phage supernatant was amplified and continued further rounds of selection.

Experiment 5 : Live ~ 5 x 10^5 KKU-100 cells selection with ANL12 (8NNK) library

The selection protocol was done as described in protocol 5 and 100 µl of ANL12 (10^{13} pfu/ml) and 400 µl of 4% MPBS was added to cell instead of SUT 12 library.

Experiment 6 : Live Protocol 3 : Live ~ 5 x 10^5 KKU-100 cells selection with ANL6 (12NNK) : ANL10 (CX_6C) library and library pre-incubation with HepB3, COS1 and HepG2 before selection with KKU100 cell at 4^OC

1. Cells were washed with warm PBS 1 time and serum free media were added and incubated at 37°C incubator at least 2 hr. before selection or substraction.

2. First depletion the library with HepB3 cell,

200 µl of ANL6 (10^{13}pfu/ml) and 100 µl of ANL10 (10^{13} pfu/ml) and 200 µl of 4% MPBS was added to cell. The library was incubated with cell on ice for 1hr.

3. All supernatant with unbound phage was transferred to second depleted cell COS-1 and incubate on ice for another 1 hr.

4. Then all supernatant was also transferred to third cell HepG2, incubated on ice for 1 hr.

5. All supernatant of subtracted library was transferred to KKU-100 cell for selection and incubated on ice for 1hr.

6. The cell was washed with PBST (0.5% tween 20) 5 times and PBS 5 times; 5ml and 2 min incubation per time

7. Trypsin (100µg/ml) 400 µl was added to eluted phage and incubated for 10 minutes. Then follow by acid elution by added 500 µl of 0.1M glycine HCl pH 2.0 and neutralize with 2M Tris base 30 µl.

8. Last elution, log phase TG1 cell 500µl was added and incubated at 37 °C for 15 minutes.

9. All eluted phage were pooled together to amplify and perform further 4 rounds of library pre-incubation and selection.

10. The amplification and phage rescue was carried out as describe in protocol 3.

3. Optimization of biopanning with phage display human scFv

Experiment 1 : YAMO library selection by direct selection at 37^{O}C, one selection round

1. The cells were grown in 25 cm^3 flask to 80 % confluence.
2. Cell was washed with 5ml of warm PBS 1 time
3. Phage scFv library; 5 µl of YAMO library (10^{13} pfu/ml) with 450µl 3% MPBS was added to cell.
4. Cell and Phage library were incubate at 37^{O}C for 2 hr.
5. The cell was washed with PBST (0.5% Tween 20) 10 times and PBS 10 times; 5ml and 3 min. incubation per time with rotation during washing.
6. Trypsin (10mg/ml) 300 µl was added to eluted phage and incubated for 10 minutes. Then follow by acid elution by added 300 µl of 0.1M glycine HCl pH 2.0 and neutralize with 300 µl NU solution.
7. Eluted phage 250 µl was incubated with 1.5 ml log phase TG1 cell and the infection was done at 37 ^{O}C for 30 min.
8. The serial dilution was performed and plated on 2XYT agar plate with 1% Glucose and Ampillicin (100 µg/ml) to separate individual phage clone. Plates were incubated at 37 ^{O}C overnight.
9. Next day single colony was selected to grow and produce phage particles.

Experiment 2 : Tomlinson I library selection by direct selection with KKU100 at 37^{O}C, one selection round.

The same selection protocol as described in protocol 1 was done

but phage displayed human scFv TomlinsonI library 5 µl in 450 µl of 4%MPBS was used.

Experiment 3: Sullivan library selection by direct selection one selection round at 37^{O}C.

1. The cells were grown in 25 cm^3 flask to 80 % confluence.
2. Cell was washed with 5ml of warm PBS 1 time
3. Phage scFv library; 5 µl of Sullivan library (10^{13} pfu/ml) in 450 µl of 0.5% casein was mixed by vortex and added to cell.
4. Cell and Phage library were incubate at 37^{O}C for 2 hr.
5. The cell was washed with PBST (0.5% Tween 20) 10 times and PBS 10 times; 5ml and 3 min. incubation per time with rotation during washing.
6. 0.1M Glycine HCl pH 2.0 500 µl was added to elute bound phage and incubate for 15 min. Then 30 µl of 2M Tris base was also added to neutralize the pH of solution.
7. Eluted phage 10 µl and its serial dilution was incubated with 200 µl of log phase TG1 cell and the infection was done at 37 ^{O}C for 15 min.
8. Then LB media 200 µl was added and incubated at the same temperature for 30-45 min. Infected *E. coli* TG1 was plated on LB agar plate contained Carbenicillin (100 µg/ml) to separate individual phage clone. Plates were incubated at 37 ^{O}C overnight.
9. Next day single colony was selected to grow and produce phage particles.

Experiment 4: Sullivan library selection by library pre-substraction with COS-1 cell before selection with KKU100 at 37^{O}C, one selection round.

1. The cells were grown in 25 cm^3 flask to 80 % confluence.
2. Cell was washed with warm PBS 1 time
3. First depletion the library with COS-1 cell, scFv library; 5 µl of Sullivan library (10^{13} pfu/ml) with 450µl 4% MPBS was added to cell. The library was incubated with cell at 37^{O}C for 1hr.
4. All supernatant of subtracted library was transferred to KKU-100 cell for selection and incubated at 37^{O}C for 2hr.
5. The cell was washed with warm PBST (0.5% Tween 20) 5 times and PBS 5 times; 5ml and 2 min incubation per time
6. Then follow by acid elution by added 500 µl of 0.1M glycine HCl pH 2.0 at RT for 15 min. and neutralize with 2M Tris base 30 µl.
7. Eluted phage 30 µl and its serial dilution was incubated with 750 µl of log phase TG1 cell and the infection was done at 37 ^{O}C for 30 min.
8. Then LB media 200 µl was added and incubated at the same temperature for 30-45 min. Infected *E. coli* TG1 was plated on LB agar plate contained Carbenicillin (100 µg/ml) to separate individual phage clone. Plates were incubated at 37 ^{O}C overnight.

Experiment 5 : YAMO library selection by direct selection at 37^{O}C, two selection rounds.

The same selection protocol was done after first round of selection the all colony from first round of selection was scraped from the plate and

amplified for next round of selection.

1. Media 2XYT +1% glucose + Ampillicin 1 ml was added to plated that contained all colony from first round of selection and loosen *E. coli* with spreader.

2. Scraped cell 5 µl was added to 5 ml of 2XYT+1% glucose +Amplicillin and incubated at 37^{O}C 200 rpm. for 2-3 hr. until the culture become turbid.

3. Helper phage 0.33 µl was added and incubated at 37^{O}C for 30 min.

4. The culture was spin at 4000 rpm for 20 min and all supernatant was removed.

5. Cell pellet was resuspended in 5 ml of 2XYT + 0.1% glucose + Amplicillin and incubate at 25^{O}C 200 rpm at least 20 hr.

6. Next day, The culture was spin to collect phage supernatant and 1 ml of 20% PEG/2.5M NaCl was added to precipitated phage particles.

7. This supernatant was mixed by invert tube 4-5 times and incubated at 4^{O}C for 1 hr.

8. Then this tube was spin at 4000 rpm. 4^{O}C for 20 min and remove all supernatant. And re-spin for 5 min. was done to remove all remaining supernatant.

9. The phage precipitate pellet was resuspended in 100 µl of 1XPBS and phage particle can be store at 4^{O}C for several weeks.

10. For round two of selection 100 µl of amplified from round one was mixed with 500 µl of 2% MBPS to perform the selection.

11. The same selection protocol as described in round one of selection was done.

Experiment 6: Sullivan library selection by direct selection, two selection rounds at 37^{O}C.

The same selection protocol was done after first round of selection All colony from first round of selection was scraped from the plate and amplified for

next round of selection.

Experiment 7 : Sullivan library selection by direct selection with KKU100 and subtraction with H69 cell after finished first round of selection, 2 selection rounds at 37^{O}C.

1. The cells were grown in 25 cm^3 flask to 80 % confluence.

2. Cell was washed with warm PBS 1 time and serum free media were added and incubated at 37^{o}C incubator at least 2 hr. before selection or substraction.

3. scFv library; 5 µl of Sullivan library (10^{13} pfu/ml) with 450µl 4% casein was added to cell and incubated at 37^{o}C for 2 hr.

4. The cell was washed with PBST (0.5% tween 20) 5 times and PBS 5 times; 5ml and 2 min incubation per time

5. Then follow by acid elution by added 500 µl of 0.1M glycine HCl pH 2.0 at RT 15 min. and neutralize with 2M Tris base 30 µl.

6. Eluted phage was gently mixed and added to H69 cell to do substraction for 15 min.

7. All unbound phage supernatant and detached cell was collect to new tube and spin to separate cell and unbound phage.

8. Unbound phage supernatant 30 µl and its serial dilution was incubated with 750 µl of log phase TG1 cell and the infection was done at 37 ^{O}C for 30 min.

9. Then LB media 200 µl was added and incubated at the same temperature for 30-45 min. Infected *E. coli* TG1 was plated on LB agar plate contained carbenicillin (100 µg/ml) to separate individual phage clone. Plates were incubated at 37 ^{O}C overnight.

Experiment 8 : Sullivan library selection by library pre-subtraction with COS-1 cell 4 times before the first round of selection with KKU100 and the second round of selection is directly select with KKU100 cell, 2 selection rounds.

1. The cells were grown in 25 cm^3 flask to 80 % confluence.
2. Cell was washed with warm PBS 1 time
3. First depletion the library with COS-1 cell, scFv library; 5 µl of Sullivan library (10^{13} pfu/ml) with 450µl 4% MPBS was added to cell.The library was incubated with cell at $37^{O}C$ for 1hr.
4. All supernatant of subtracted library was transferred to another COS-1 cell to continue substraction at $37^{O}C$ for 1hr. The same substraction was performed for other two more times.
5. All supernatant of subtracted library was transferred to KKU-100 cell for selection and incubated at $37^{O}C$ for 2hr.
6. The cell was washed with warm PBST (0.5% tween 20) 5 times and PBS 5 times; 5ml and 2 min incubation per time
7. Then follow by acid elution by added 500 µl of 0.1M glycine HCl pH 2.0 at RT for 15 min. and neutralize with 2M Tris base 30 µl.
8. Eluted phage 30 µl and its serial dilution was incubated with 750 µl of log phase TG1 cell and the infection was done at 37 ^{O}C for 30 min.
9. Then LB media 200 µl was added and incubated at the same temperature for 30-45 min. Infected *E. coli* TG1 was plated on LB agar plate contained Carbenicillin (100 µg/ml) to separate individual phage clone. Plates were incubated at 37 ^{O}C overnight.

Experiment 9 : Sullivan library selection by pre-incubation library with HepB3, COS1 and HepG2 before selection with KKU100 cell, 5 round of selections at $4^{O}C$

1. The cells were grown in 25 cm^3 flask to 80 % confluence.

2. Cell was washed with warm PBS 1 time and serum free media were added and incubated at 37^{o}C incubator at least 2 hr. before selection or substraction.

3. First depletion the library with HepB3 cell,

scFv library; 5 µl of Sullivan library (10^{13} pfu/ml) with 450µl 4% MPBS was added to cell.

The library was incubated with cell on ice for 1hr.

4. All supernatant with unbound phage was transferred to second depleted cell COS-1 and incubate on ice for another 1 hr.

5. Then all supernatant was also transferred to third cell HepG2, incubated on ice for 1 hr.

6. All supernatant of subtracted library was transferred to KKU-100 cell for selection and incubated on ice for 1hr.

7. The cell was washed with PBST (0.5% tween 20) 5 times and PBS 5 times; 5ml and 2 min incubation per time

8. Trypsin (100µg/ml) 400 µl was added to eluted phage and incubated for 10 minutes. Then follow by acid elution by added 500 µl of 0.1M glycine HCl pH 2.0 and neutralize with 2M Tris base 30 µl.

9. Last elution, log phase TG1 cell 500µl was added and incubated at 37 ^{o}C for 15 minutes.

10. All eluted phage were pooled together to amplify and perform further 4 rounds of substraction and selection.

4. Cloning and expression

Alkaline phosphatase (AP) is an enzyme whose function is dephosphorylation target molecule. AP has been use widely in molecular biology. For instance, AP has been used to eliminate PO^{4-} from DNA or RNA to prevent self ligation in DNA cloning application. And DNA or RNA tag protocol also uses AP to remove old PO^{4-} before tagged with new radiolabeled PO^{4-}. This enzyme can use with colorimetric substrate such as p-Nitrophenyl phosphate (pNPP), which phosphate group had been removed and generated p-nitrophenol product with yellow color, highly absorb at 405 nm. More over Nitro Blue Tetrazolium (NBT) is used with enzyme alkaline phosphatase substrate 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP) in immunohistological staining procedures. It generates an insoluble NBT diformazan end product that is blue color.

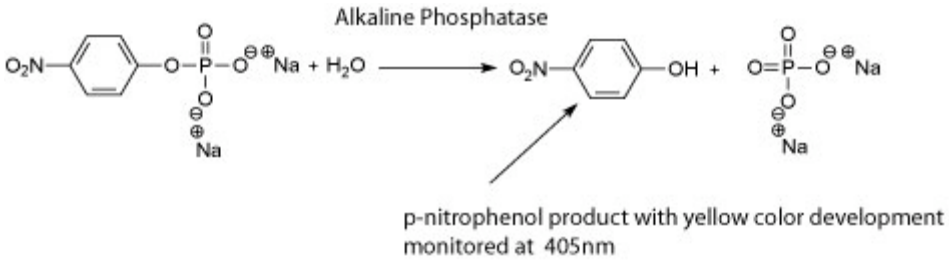

Figure 66. Generating p-nitrophenol yellow color product, alkaline phosphatase digest substrate diagram. (From Sigma pNPP data sheet)

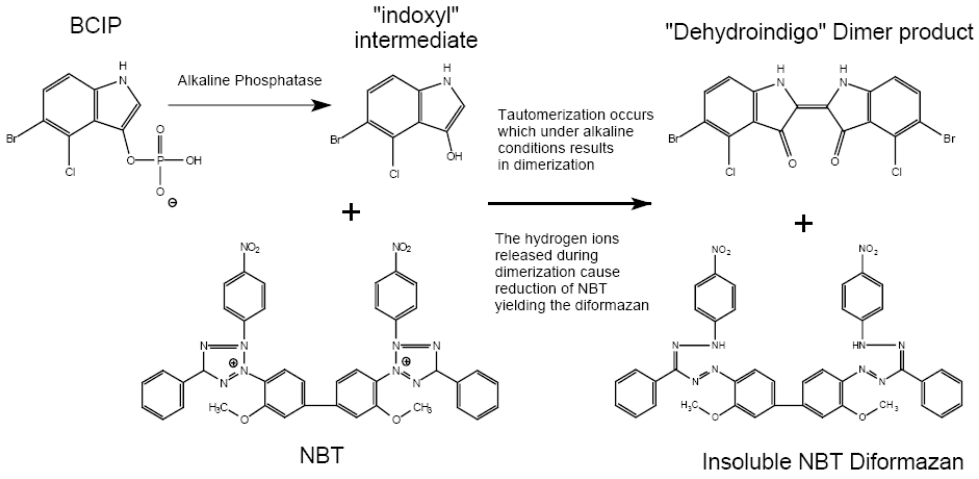

Figure 67. Dephosphorylated substrate, BCIP, generate insoluble blue color with NBT (From sigma BCIP/NBT product sheet)

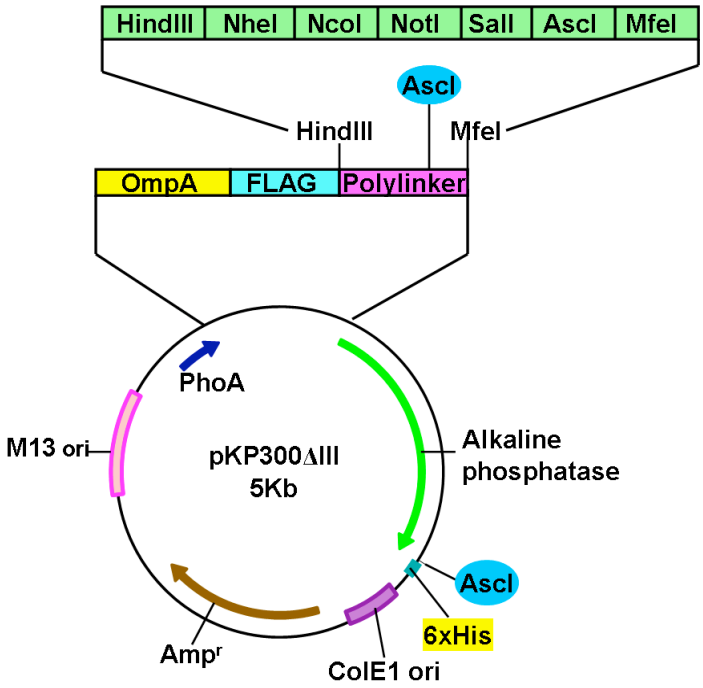

Figure 68. **Vector** pKP300deltaIII, vector for expression protein with enzyme alkaline phosphatase fusion.

Vector pKP300deltaIII was a kind gift from Dr. Brian Kay and Ms. Kritika Pershad from University of Illinois at Chicago, IL. USA. This vector contains *PhoA* promoter which control protein expression depending on phosphate level.

1. Oligonucleotide encoding C-X6-C peptide cloning into pKP300deltaIII

1.1 Oligonucleotide design

a. Oligonucleotide had been designed to be synthesized with restriction enzyme flanking : *HindIII* and *MfeI.*

For example

Chitin binding peptide (E G K G V E A V G D G R)

Oligonucleotide sequence

5’ GAGGGGAAGGGTGTGGAGGCGGTGGGGGATGGGAGG 3’

1) Edited sequence with restriction enzyme site at both ends

5’ aagcttGAGGGGAAGGGTGTGGAGGCGGTGGGGGATGGGAGGcaattg 3’

```
          Hind3          BpuE1        Mfe1
           |              |           |
           AAGCTTGAGGGGAAGGGTGTGGAGGCGGTGGGGGATGGGAGGCAATTG
         1 ---------+---------+---------+---------+-------- 48
           TTCGAACTCCCCTTCCCACACCTCCGCCACCCCCTACCCTCCGTTAAC
frame 0   > K   L   E   G   K   G   V   E   A   V   G   D   G   R   Q   L
```

agcttGAGGGGAAGGGTGTGGAGGCGGTGGGGGATGGGAGGc

aCTCCCCTTCCCACACCTCCGCCACCCCCTACCCTCCgttaa

2) There are sequences of oligonucleotide will be synthesized

Chitin binding peptide up (CBPup)

5’ AGC TTG AGG GGA AGG GTG TGG AGG CGG TGG GGG ATG GGA GGC 3’ (Tm = 77.2 °C)

Chitin binding peptide dn (CBPdn)

5’ AAT TGC CTC CCA TCC CCC ACC GCC TCC ACA CCC TTC CCC TCA 3’ (Tm = 75.2 °C)

1. 2 Oligonucleotide annealing

a. Equal amount of two complementary oligonucleotide encode interested short peptide was mixed (5 μl of 100 mM each).

b. After well mixing and brief spining to collect all mixture down, This reaction was performed to slow anneal complementary oligonucleotide together by PCR machine. The temperature had been being decrease from 95^{O}C and incubate for 5 minutes, 5^{O}C every 5 minute until reach final temperature at 25^{O}C .

c. At this step, complete annealing oligonucleotide can be stored at 4^{O}C for longer storage at -20^{O}C.

d. The concentration of oligonucleotide was determined by Nanodrop spectrophotometer. (At this step 10 fold dilution was performed to 1:1000). The amount of oligonucleotide will be used in ligation was calculated.

1. 3 Preparation of vector pKP300deltaIII

a. From overnight culture of *E.coli* harbored plasmid, Plasmid preparation using QIA Minipred was done with final sterile water elution.

b. The concentration of prepared vector was determined by nanodrop spectrophotometer.

c. Plasmid vector was digested by *HindIII* and *MfeI.*

(Plasmid DNA 1 μg will require 5 units of enzyme. For 3 hours incubation)

i. *HindIII* digestion

1. Plasmid DNA (~ 100 ng/ μl)	10	μl
2. 10X NEB buffer2	2	μl

3. H_2O	7.5	µl
4. *HindIII* (20 units/µl)	0.5	µl
Total	20	µl

The reaction was incubated at 37 ^{O}C for 3 hours and heated inactivation at 65^{O}C for 20 minutes.

ii. *MfeI* digestion

1. Plasmid DNA (from reaction I)	20	µl
2. 10X NEB buffer4	3	µl
3. H_2O	6	µl
4. *MfeI* (10 units/µl)	1	µl
Total	30	µl

The reaction was incubated at 37 ^{O}C for 3 hours and heated inactivation at 65^{O}C for 20 minutes.

After that digested vector was prepared from gel purification process. Digested vector was run in 1.2% agarose gel with 70V for 2-3 hr. then the correct size of vector DNA was excised from gel and purified by Promega gel purification kit with final elution DNA with sterile water.

1.4 Ligation ratio calculation

Vector: Insert

1:3

(~0.1-1 µg of vector per ligation reaction)

pKP300deltaIII	size 5000 bps.
Insert	size 30bps.

Vector/Insert = 5000/30 = 166.6 times

If we use 100 ng of Vector , It means 1 molecule vector

If we use 100 ng of Insert , It can be assume 166.67 molecules of insert.

So If we use insert 166.67 molecules It will be 100 ng of insert

Then If we need 3 molecules, It will be 1.8 ng. of insert.

Finally vector 0.1 μg will require insert ~2 ng. per one ligation reaction.

1.5 Ligation reaction

Vector concentration after gel purification is 20 ng/μl

Insert 1:100 dilution concentration 20 ng/μl, So 1:1000 dilution is ~2 ng/μl

1. Insert DNA (2 ng/μl)	1	μl
2. Vector (20ng/μl)	5	μl
3. 10X T4 DNA ligase buffer	1	μl
4. H_2O	3.9	μl
5. Ligase enzyme (400units/μl)	0.2	μl
Total	10	μl

The ligation reaction was done at $16^{O}C$ for 16hr. Then heat inactivated at $65^{O}C$ for 20 min. and all reaction in 10 μl was transferred to do heat shock transformation. Only 10-20 μl of culture can be spread on LB+Amp plate and incubated at $37^{O}C$ for o/n. Next day, single colony was picked and grown in LB+Amp with 250 rpm. at $37^{O}C$ for 3-4 hr. And the plasmid from each colony was confirmed the positive clone by restriction digestion pattern. The positive clone containing insert oligonucleotide will not be digested by *SalI* but only ligated vector will be digested.

1.6 *SalI* restriction digestion pattern

1.	Plasmid DNA	2.5	µl
2.	10X buffer3	1	µl
3.	100X BSA	0.1	µl
4.	H_2O	6.3	µl
5.	*SalI* (units/µl)	0.1	µl
		Total 10	µl

The reaction was incubated at 37 ^{O}C for 3 hours and heated inactivation at 65^{O}C for 20 minutes. And restriction digestion pattern was observed by running 1.2% agarose gel at 95 V for 2-3 hr.

2. Subcloned scFv fragment from AP-III$_6$ vector into pKP300ΔIII vector

Restriction digestion reaction

1) *HindIII* digestion reaction

1.	Phagemid or vector	1	µg (~ 10 µl)
2.	10X NEB buffer2	2	µl
3.	H_2O	6.5	µl
4.	Enzyme *HindIII* (20 u/ µl)	1.5	µl
		Total 20	µl

2) *SalI* digestion reaction

1.	Phagemid or vector (from *HindIII* reaction)	20	µl
2.	10X NEB buffer3	5	µl
3.	100X BSA	0.5	µl
4.	H_2O	23	µl

5. Enzyme *SalI* (20 u/ µl)		1.5	µl
	Total	50	µl

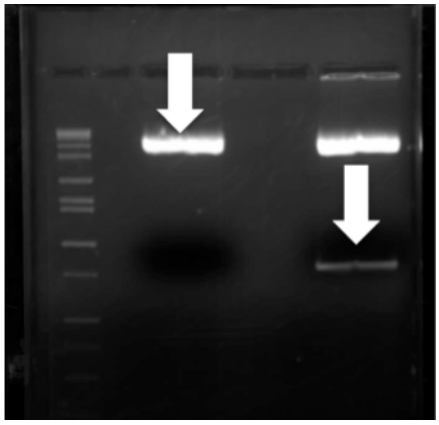

Figure 69. Agarose gel electrophoresis image of pKP300ΔIII vector and phagemid with scFv insert after *HindIII* and *SalI* restriction digestion.

3) Ligation reaction

1. DNA insert scFv fragment (~22 ng)		5	µl
2. DNA vector pKP300deltaIII fragment (~50 ng)		5	µl
3. 10X NEB Ligase buffer		2	µl
4. H_2O		7.6	µl
5. NEB Ligase enzyme (400u/ µl)		0.4	µl
	Total	20	µl

4) PCR Amplification reaction (for positive scFv with AP fusion)

1. DNA		5	µl
2. 5X GoTaq Buffer		4	µl

3.	25 mM $MgCl_2$	1.6	µl
4.	1mM dNTP	4	µl
5.	Forward primer : OmpAup primer 20 µM	0.2	µl
6.	Reverse primer : APstRv primer 20 µM	0.2	µl
7.	Taq polymerase (20 u/ µl)	0.2	µl
8.	H_2O	8.6	µl
	Total	20	µl

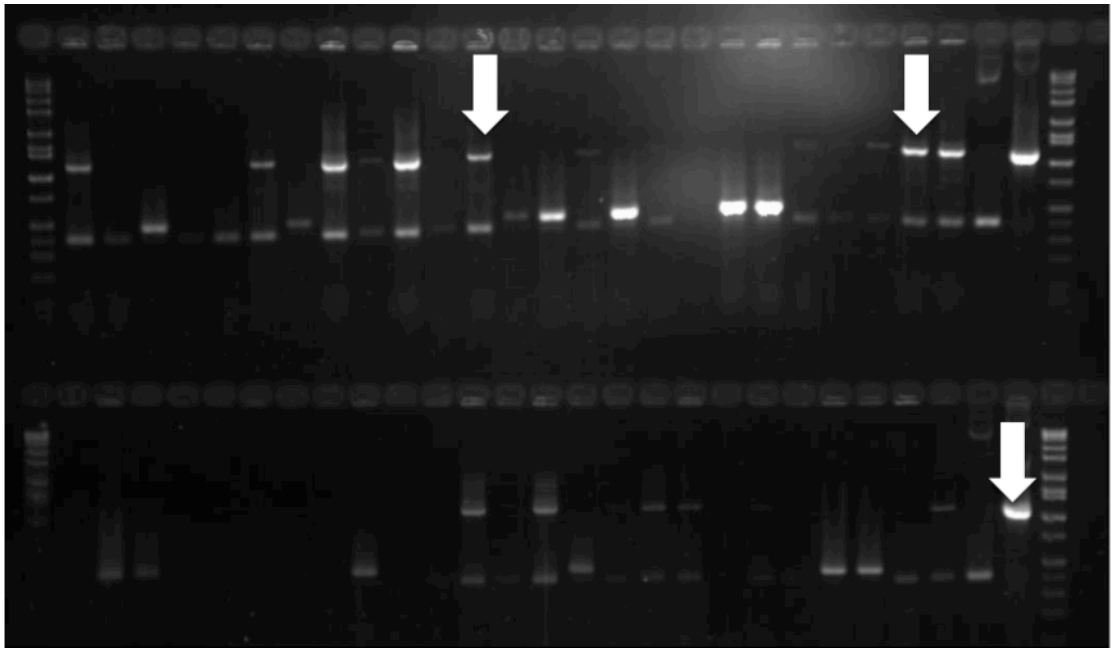

Figure 70. Agarose gel elctrophoresis of PCR product of scFv with AP fusion.

PCR reaction was programmed as shown below.

1 cycle,	$95^{O}C$	1	min.
30 cycles,	$94^{O}C$	30	sec.
	$57^{O}C$	45	sec.
	$72^{O}C$	1.2	min

1 cycle,	$72^{O}C$	7	min.
1 cycle,	$4^{O}C$		forever

5) *AscI* digestion reaction

1. DNA from plasmid (500 ng)		10	µl
2. 10X NEB buffer4		2	µl
3. *AscI* enzyme (10 u/ µl)		1	µl
4. H_2O		7	µl
	Total	20	µl

6) Ligation reaction after *AscI* digestion

1. DNA (1.25 ng/ µl)		4	µl
2. 10X Ligase buffer		1	µl
3. NEB Ligase enzyme (40 u/ µl)		0.4	µl
4. H_2O		4.6	µl
	Total	10	µl

7) PCR Amplification reaction (positive scFv without AP fusion)

1. DNA		5	µl
2. 5X GoTaq Buffer		4	µl
3. 25 mM $MgCl_2$		1.6	µl
4. 1mM dNTP		4	µl
5. Forward primer : OmpAup primer 20 µM		0.2	µl
6. Reverse reaction : AmpstRv primer 20 µM		0.2	µl
7. Taq polymerase (20 u/ µl)		0.2	µl
8. H_2O		8.6	µl
	Total	20 µl	

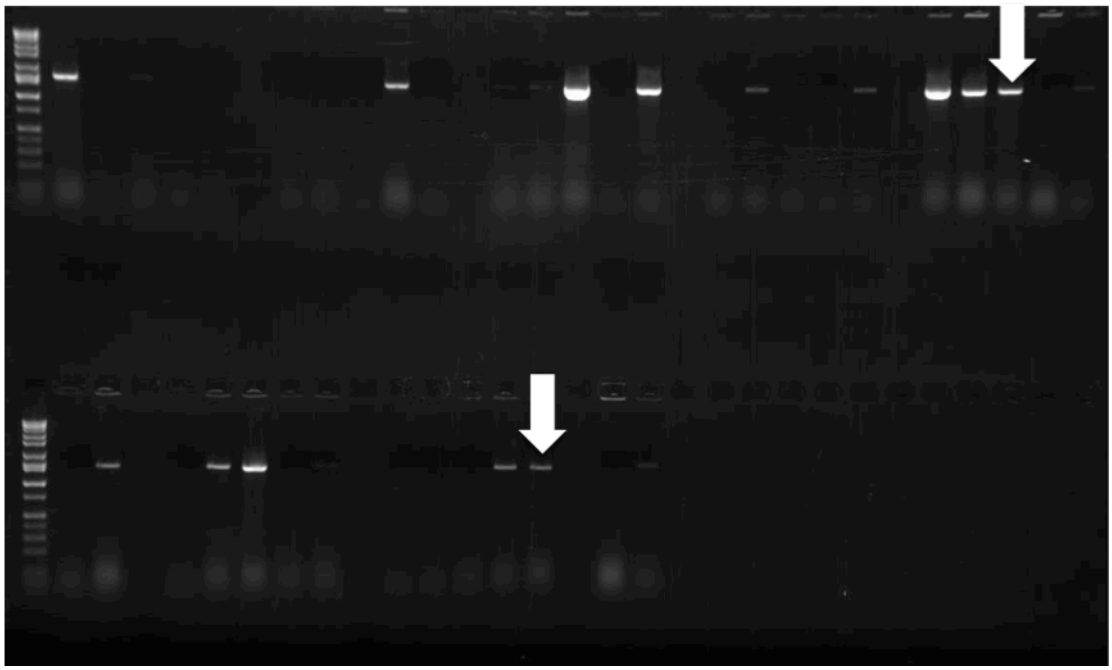

Figure 71. Agarose gel elctrophoresis of PCR product of scFv without AP fusion.

8) PCR amplification of scFv insert from YAMO library.

In order to produce scFv in soluble form and scFv with enzyme alkaline phosphatase fusion, scFv insert had been amplified by PCR with AccuPrime™ *Pfx* DNA polymerase (invitrogen) and subcloning into pKP300ΔIII expression vector.

PCR condition

1. Plasmid DNA template (~100 ng/µl) 1 µl
2. Pfx DNA polymerase enzyme (2.5 u/µl) 1 µl
3. Forward primer (100 µM) 0.15 µl
4. Reverse primer (100 µM) 0.15 µl

5. 10X Pfx Buffer		5	µl
6. H_2O		42.7	µl
	Total	50	µl

PCR reaction was programmed as shown below.

1 cycle,	$95^{O}C$	2	min.
30 cycles,	$95^{O}C$	15	sec.
	$64^{O}C$	45	sec.
	$68^{O}C$	50	sec.
1 cycle,	$68^{O}C$	5	min.
1 cycle,	$4^{O}C$	forever	

Figure 72. Agarose gel electrophoresis image of PCR product of scFv from phage display scFv clone YM12 and YM13.

The insert PCR product size is around 750 bp. After *NcoI* and *NotI* digestion, Vector and insert were ligated together at 16 ^{O}C for o/n. Ligated vector were transformed in to TG1 competent cell. The positive clones were selected by resistant to antibiotic cb. Single colony was grown and checked for insertion of scFv by PCR. The positive insert clone was grown in low phosphate media at $30^{O}C$ o/n and soluble

scFv with AP fusion was obtained from clear cell lysate extract. The soluble scFv can be generate by *AscI* digestion the alkaline phosphatase fusion expression plasmid to cleave out enzyme alkaline phosphatase and re-ligated the same plasmid together

5. Protein purification and analysis protocols

5.1. Protein purification with KingFisher®mL

Combine the beads (20-25 µl) with 1ml BugBuster lysate in well 1 of the KF strips1-5, the next three wells of each strip will be 1 ml PBST washes, the fifth well of each strip will be the elution step where the protein will be eluted from the beads with 100 µl of Elution buffer containing 500 mM Imidazole.

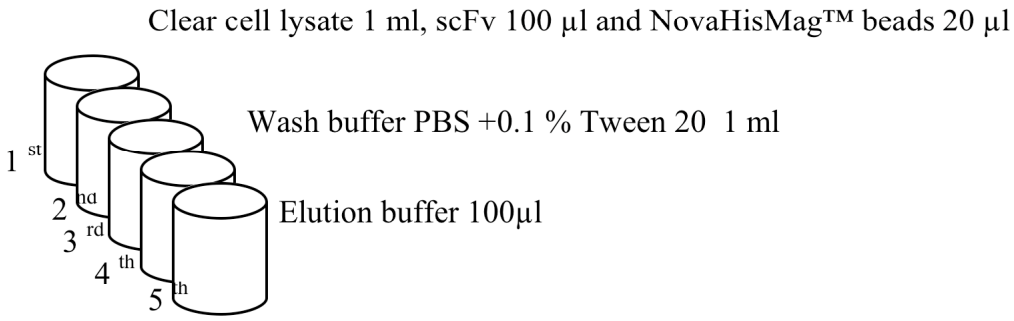

Figure 73. KingFisher®mL purification diagram

5.2 Imidazole elimination with Zeba™ desalting columns (Thermo Scientific)

1. Twist off the column's bottom closure and loosen cap and place column in a collection tube.

2. Centrifuge column at 1,000 x *g* for 2 minutes to remove storage solution. Resis will appear compacted after centrifugation.
3. Place column in a new collection tube, remove cap and slowly apply sample to the center of compact resin bed.
4. Centrifuge column at 1,000 x *g* for 2 minutes to collect desalted sample. Discard column after use.

5.3 Protein quantification using BioRad Experion Pro260 kit

5.3.1 Equilibrating kit reagents

Remove Pro260 stain gel, sample buffer and ladder from $4^{O}C$ storage and equilibrate to room temperature. Protect kit component from light at all times. Vortex tubes briefly centrifuge tubes to collect solutions.

5.3.2 Preparing the gel-stain and sample buffer

Add 20 µl Pro260 stain (blue cap) to 520 µl Pro260 gel (green cap) in the gel tube provided. Vortex the gel-stain solution. Transfer the gel-stain solution (GS) and 520 µl gel (G) to separate spin filters and centrifuge at 10,000 x g for 5 min. For separations under reducing conditions, add 1 µl ß-mercaptoethanol to 30 µl sample buffer (yellow cap) for each chip to be run.

5.3.3 Preparing the samples and Pro260 ladder

Add 2 µl sample buffer to 4 µl sample and vortex.

Add 2 µl sample buffer to 4 µl Pro260 ladder (red cap) and vortex.

Heat the samples and ladder at 95-$100^{O}C$ for 3-5 min. Briefly centrifuge the samples.

Add 84 µl deionized water (0.2 µm filtered) to samples and the ladder and vortex.

5.3.4 Priming the chip

Remove a Pro260 chip from its packaging and place it on the chip platform in the Experion priming station.

Add 12 µl gel-stain solution into the well labeled GS (gel priming well). Close the lid of the priming station, set the pressure to B, and set the time to 3. Press the start button. After priming is finished, remove the primed chip from the priming station and visually inspect the microchannels for trapped air bubbles or incomplete priming.

5.3.5 Loading the samples and Pro260 ladder into the chip

Remove the remaining gel-stain from the top right well labeled GS (gel priming well)

Pipet 12 µl filtered gel-stain solution into all 4 wells labeled GS.

Pipet 12 µl filtered gel into the well labeled G.

Pipet 6 µl diluted protein ladder into the well labeled L.

Pipet 6 µl diluted sample into each sample well. Do not leave any sample well empty.

Run the chip in the Experion electrophoresis station within 5 min of loading.

5.3.6 Run the Pro260 analysis

Power the electrophoresis station on and launch the Experion software. Place the loaded chip onto the electrophoresis station chip platform and close lid.

Selected New run and then Experion Pro260.

Click the start button in the software to begin the run. Select the number of samples to run.

After the run is complete, remove and discard the used chip.

5.3.7 Cleaning the chip after a run

Place a cleaning chip filled with 800 ! l water (0.2 ! m filtered) into the electrophoresis station.

Close the lid for 60 second to clean the electroades, and then open it for 60 seconds to allow the electrodes to completely dry.

Remove the cleaning chip.

APPENDIX II

ADDITIONAL DATA

1. Phage tittering after three selection rounds with ANL10 and ANL12 library

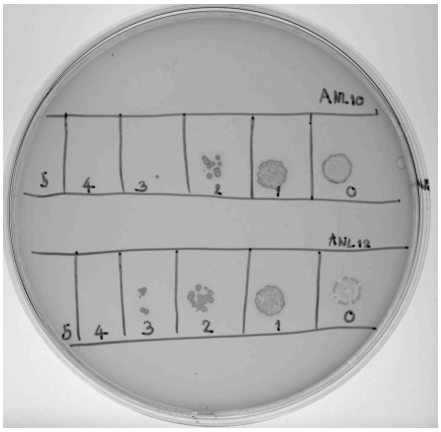

Figure 74. Blue plaque of infected *E.coli* TG1 by phage display peptide ANL10 and ANL12 library. After 3 rounds of biopanning with KKU-100 cells, serial dilution eluted phages were done and spot on LB contained X-gal and IPTG top agar. The plate was incubated at 37^{O}C, next day each blue plaque represent each phage clone.

2. Phage display peptide selectivity index

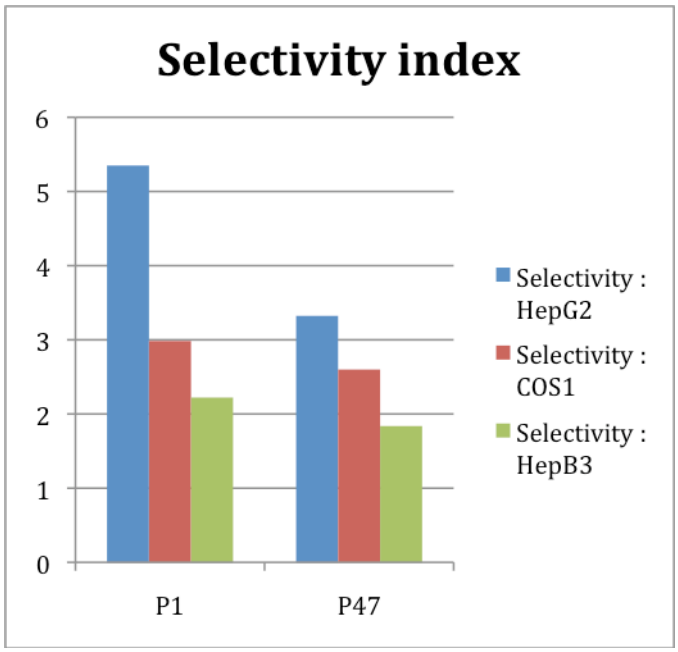

Figure 75. Selectivity index of phage clone P1(CTTPPYVC) and P47 (CTSPPYVC). The selectivity index was calculated from O.D. of cell based phgae ELISA. Selectivity index = (O.D. selected phage from target cell – O.D. control target cell)/ (O.D. selected phage non-target cell- O.D. control non-target cell).

3. Phage tittering from phage peptide selectivity experiment

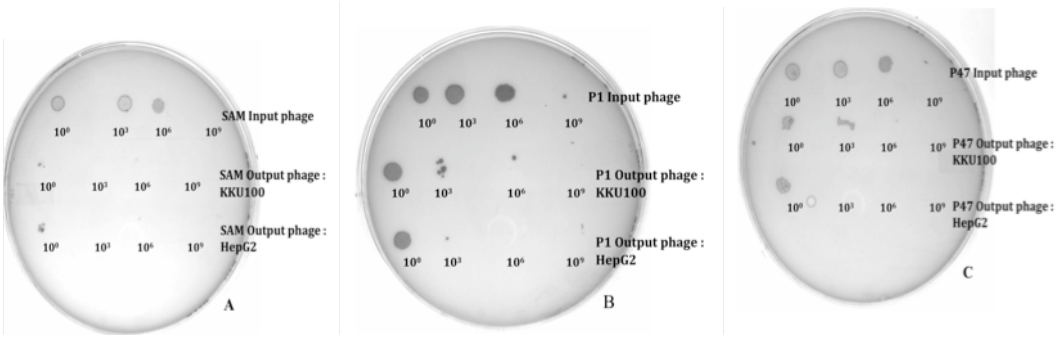

Figure 76. Phage tittering after incubation with KKU-100 cell and HepG2 cells. A,B and C showed recovery phage from SAM as a control phage, phage display peptide clone P1 (CTTPPYVC) and P4 (CTSPPYVC).

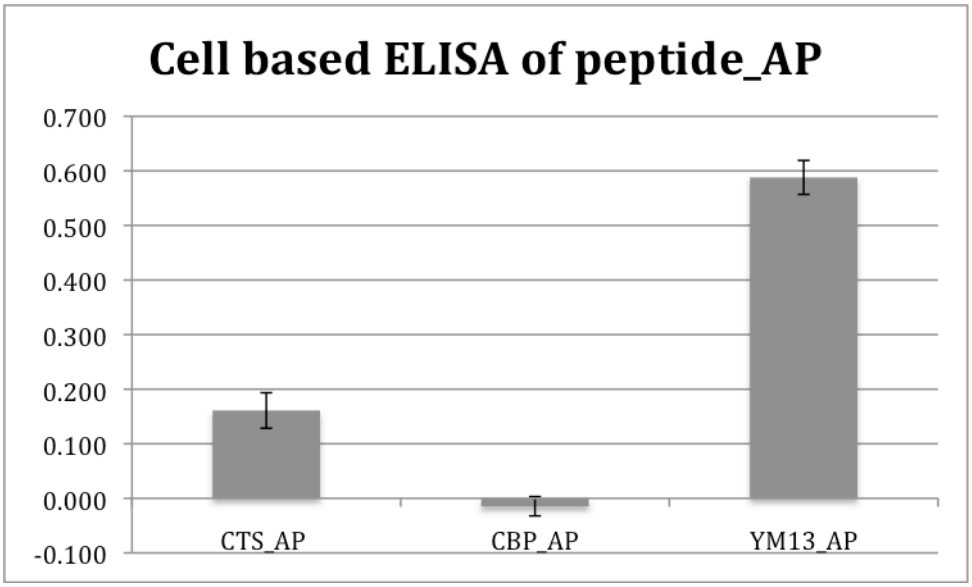

Figure 77. The signal of cell based ELISA with peptide fused with AP compare to scFv YM13 fused with AP.

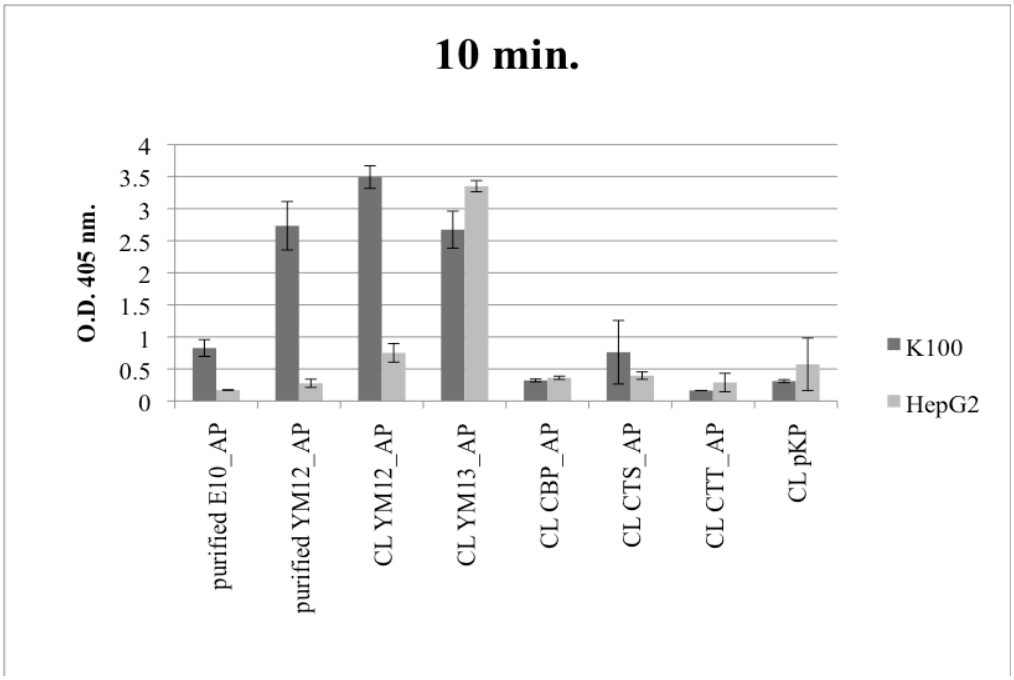

Figure 78. Cell based ELISA binding confimation of CTSPPYVC peptide with AP fusion. The signal was measured at 10 min.

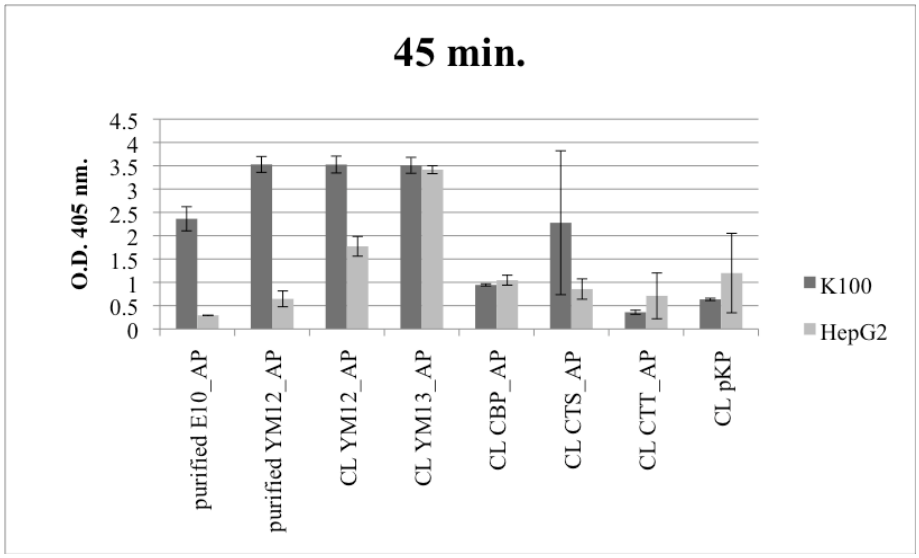

Figure 79. Cell based ELISA binding confimation of CTSPPYVC peptide with AP fusion. The signal was measured at 45 min.

4. DNA fingerprinting analysis with *Bst*NI digestion

1. Plasmid	5	! l (from 100 ng/! l)
2. Buffer	1	! l
3. 100X BSA	0.1	! l
4. H_2O	3.6	! l
*5. BstN*I	0.2	! l
	Total 10	! l

60°C, 1 hr.

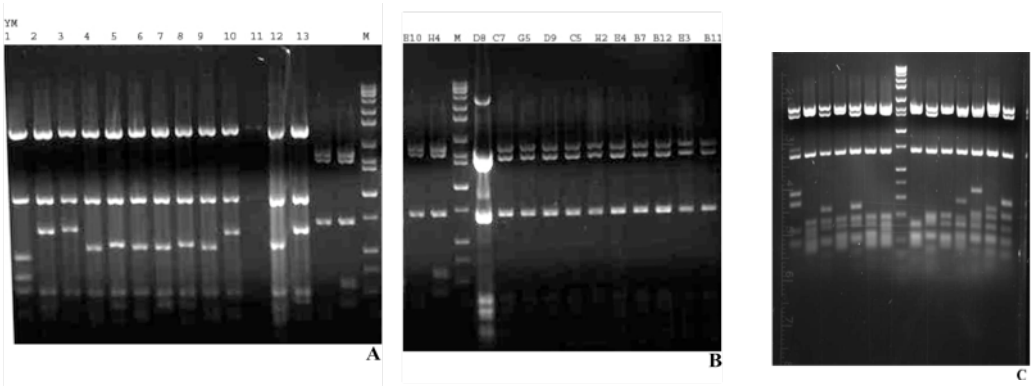

Figure 80. *BstNI* restriction digestion pattern of selected clone from phage display human scFv YAMO library (A) and Sullivan library after round 2 of selection direct selection and library pre incubation of COS-1 cell 4 hr. after sselection with KKU-100 cells directly substract with H69 cells (B and C respectively).

*BstN*I digestion profile of all the specific clone from different way of selection, showed the selection method and the diversity of clones. From the direct selection with KKU-100 and 2 rounds of selection, all of these clone seem to be the same clone. In the other hand, library depletion with COS-1 before selection or substraction with H69 cell after round 1 direct selection with KKU-100 seem to select more diversity of selected clones. All of these clones showed crossreactivity with COS-1 and H69 cell in different level.

5. Quanlitative and quatitative analysis of purified scFv_AP and scFv

Table 14. Large-scale scFv_AP protein purification profile.

scFv_AP	Culture volume (ml)	Concentration of purified protein (ng/ ! l)	Total protein (mg)	Purity (%)	Total volume of purified protein (! l)
1. YM12_AP	100	948	0.948	64.8	1000
2. YM13_AP	50	216.1	0.10	60.6	500
3. S.V. E10_AP	150	4740	7.11	85	1500
4. S.V. H4_AP	50	176.2	0.08	59.4	500
5. S.V. R5A8_AP	50	1344.6	0.67	72.4	500

Table 15. Large-scale scFv protein purification profile.

scFv	Culture volume (ml)	Concentration of purified protein (ng/ ! l)	Total protein (mg)	Purity (%)	Total volume of purified protein (! l)
1. YM12	100	2869.5	2.86	94	1000
2. YM13	100	3020	3.02	99.7	1000
3. S.V. E10	50	3323	1.66	98.1	500
4. S.V. H4	50	60.7	0.03	90.6	500
5. S.V.R5A1	100	1923.5	1.92	87.8	1000
6. S.V. R5A8	50	785.8	0.37	98	500

Table 16. Light chain complementary determination region of selected scFv.

LC	FR1	CDR1	FR2	CDR2	FR3	CDR3	Germ line	Germline different	Family
YM1	TQSPATLSVSPGERATLSC	RASQSVSS	NLAWYQQKPGQAPRLLIH	GASTRAT	GIPARFSGSGSGTEFTLTISSLQSEDFAVYYC	QQYNNWPPYTFGQGTELEIK	IGKV3-15*01	5	KV3
YM3	QAVLTQPSSLSASPGASASLTC	TLPSGFTVSSYRIY	WYQQKPGSPPQFLLR	YKSESEKHQAS	GVPSRFSGSKDTSANAGILYISGLQSEDEADYYC	AVWHNKAWVFGGGTKLTVL	IGLV5-45*02	16	LV5
YM5	VLTQPPSTSGTPGQRVTISC	SGSRSNIGSNTVN	WYQQLPGKAPKLLIY	ANNQRPS	GVPDRFSGSKSGTSASLAISGLRSEDEADYYC	AARGDRLSGHVFGTGTKVTVL	IGLV1-47*01	11	LV1
YM7	QSALTQPASASGSPGQSVTISC	TGTSGDIGYYNYVS	WYQQYPGKVPKLIIY	EVNKRPS	GVPARFSGSKSGNTASLTVSGLQAADEAQYYC	TSYALGDNLIVFGGGTKLTVL	IGLV2-8*01	15	LV2
YM8	VLTQPPSTSGTPGQRVTISC	SGSRSNIG	SNTVNWYQQLPGKAPKLLIY	ANNQRPS	GVPDRFSGSKSGTSASLAISGLRSEDEADYYC	AARGDRLSGHVFGTGTKVTVL	IGLV1-47*01	11	LV1
YM10	EIVLTQSPGTLSLSPGERATLSC	RASQSVSSSYLA	WYQQKPGQAPRLLIY	GASSRAT	GIPDRFSGGGSGTDFTLTISRLEPEDFAVYYC	QQYGSSPPRTFGQGTKLEIK	IGKV3-20*01	1	KV3
YM11	LTQPPSVSNGLGQTATLTC	TGTSNIVAKHGAA	WLQQHQGHPPKVLSY	RNNDRPS	GVSERFSATKSGDTASLTITGLQPEDEADYFC	SAWDSSLSGYVFGTGTKVTV	IGLV10-54*02	14	LV1
YM12	VLTQPPSASGTPGQRVTISC	SGSSSNIGSNTVN	WYQQLPGTAPKLLIY	SNNQRPS	GVPDRFSGSKSGTSASLAISGLQSEDEADYYC	AAWDDSLNGYVFGTGTQLTV	IGLV1-44*01	0	LV1
YM13	SYVLTQPPSVSLTLGQTARITC	EGNSIGRKHVH	WYQQKPGQAPVLVIY	RDIQRPS	GIPERFSGSNSGNTATLTITGVQVGDDSDYYC	QVWDRDSRTIVFGGGTQLTVL	IGLV3-9*01	16	LV3
S.V.E10	QAVLTQPPSASGTPGQRVTISC	SGSSSNIESNAVN	WYQQLPGTAPKLLIY	SNNQRPS	GVPDRFSGSKSGTSASLAISGLQSEDEADYYC	AAWDDSLNGRVFGGG	IGLV1-44*01	3	LV1
S.V.H4	DIQMTQSPSSLSASVGDRVTITC	RASQSIRSYLN	WYQQKPGKAPKLLIY	TASNLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQFYSLSPTFGGGTKVEIK	IGKV1D-39*01	4	KV1
S.V.R5A1	ELTQPPSASGTPGQRVTISC	SGSSSNIGSNYVY	WYQQLPGTAPKLLIY	RNNQRPS	GVPDRFSGSKSGTSASLAISGLRSEDEADYYC	AAWDDSLRRVFGGGTKLTVLGEGKSSG	IGLV1-47*01	3	VL1

BIOGRAPHY

Nanthanit Jaruseranee was born on January 2nd, 1980 in Lampang, Thailand. She graduated with Honors in Bachelor of Sciences (B.S.) in Biology major from Biology Department, Faculty of Sciences, Chiang Mai University, in 2002. Then she got a Master of Sciences (M.S.) from the same University in 2004. In this year she joined PhD. program in school of Biotechnology, Institute of Agricultural at Suranaree University of Technology. Her research is focused on applying phage display technology to select marker for cholangiocarcinoma. She conducted her research with Dr. Montarop and her lab members. Moreover she had a chance to do her research with Professor Dr. Brian K. Kay in UIC (University of Illinois at Chicago) for one year (July 2008 - June2009) before she graduated.

Publications

Yamabhai, M., Emrat, S., Sukasem, S., Pesatcha, P., **Jaruseranee, N**., Buranabanyat, B**.**: Secretion of recombinant *Bacillus* hydrolytic enzymes using *Escherichia coli* expression systems. J Biotechnol 2008, 133: 50-57.

Pansri, P., **Jaruseranee, N**., Rangnoi, K., Kristensen, P., and Yamabhai, M. (2009): A compact phage display human scFv library for selection of antibodies to a wide variety of antigens. BMC Biotechnol 9, 6

Table 16. (Continued).

LC	FR1	CDR1	FR2	CDR2	FR3	CDR3	Germ line	Germline different	Family
S.V. R5A6	QSVLTQPPSASGTPG QRVTISC	SGSSSNIGSNYV Y	WYQQLPGTA PKLLIY	RNNQRP S	GVPDRFSGSKSGTSAS LAISGLRSEDEADYYC	AAWDDSLSGWVFGGGTK VTVLGESKSSG	IGLV1 - 47*01	0	VL1
S.V. R5A6	QSVLTQPPSASGTPG QRVTISC	SGSSSNIGSNYV Y	WYQQLPGTA PKLLIY	RNNQRP S	GVPDRFSGSKSGTSAS LAISGLRSEDEADYYC	AAWDDSLSGWVFGGGTK VTVLGESKSSG	IGLV1 - 47*01	0	VL1
S.V. R5A8	ELTQPPSASGTPGQR VAISC	SGSNSNIGSNYV Y	WYQQLPGTA PKLLIY	RNDQRPS	GVPDRFSGSKSGTSAS LAISGPRSEDEAHYYC	AVWDDSLRGWVFGGGTK LTVL	IGLV1 - 47*01	9	VL1
YM12 scFv	VLTQPPSASGTPGQR VTISC	SGSSSNIGSNTV N	WYQQLPGTA PKLLIY	SNNQRPS	GVPDRFSGSKSGTSAS LAISGLQSEDEADYYC	AAWDDSLNGYVFGTGTQ LTV	IGLV1 - 44*01	0	VL1
YM13 scFv	SYVLTQPPSVSLTLG QTARITC	EGNSIGRKHVH	WYQQKPGQA PVLVIY	RDIQRPS	GIPERFSGSNSGNTAT LTITGVQVGDDSDYYC	QVWDRDSRTIVFGGGTQ LTVL	IGLV3 -9*01	16	LV3
YM13 scFv	SYVLTQPPSVSLTLG QTARITC	EGNSIGRKHVH	WYQQKPGQA PVLVIY	RDIQRPS	GIPERFSGSNSGNTAT LTITGVQVGDDSDYYC	QVWDRDSRTIVFGGGTQ LTVL	IGLV3 -9*01	16	LV3

Table 17. Heavy chain complementary determination region of selected scFv.

HC	FR1	CDR1	FR2	CDR2	FR3	CDR3	Germ line	Germline different	Family
YM1	VQLVQSGGGLVKPGG SXRLSCAASGFTFS	SYSMN	WVRQAPGKG LERVS	SISSSSS YIYYADS VKG	RFTISRDNAKNSLYLQ MNSLRAEDAAVYYCAR	EVYDYYDSSGYGASD IW	IGHV3-21*01	5	VH3
YM3	QVQLVQSGGGVVQPG RSLRLSCVASGFSFR	NYGMH	WVRQAPGKG LEWVA	IVWSDGS TKYYADS VKG	RFTISRDNSRNTLYLQ MDSLRAEDTAVYYCAR	GSQSGMITGFDNW	IGHV3-33*01	11	VH3
YM4	QVQLVQSGAEVKEPG ASVKVSCKASGYTLN	DYYMH	WVRQAPGQG PEWMG	WLNPNSG AAGYSQK FQG	RVTLTRDTSISTAYME VNRLTSADTAIYYCAR	GRAVVSSYKRGNDYW	IGHV1-2*02	16	VH1
YM5	VQLVQSGAEVKKLGA SVKVSCKASGYTFT	SYYMH	WVRQAPGQG LEWMG	IINPSGG STSYAQK FQG	RVTMTRDTSTSTAYME LSGLRSEDTAVYYCAR	GAMFDYW	IGHV1-46*01	4	VH1
YM8	GQLVQSGAEVKKPGA SVEVSCKASGYTFT	SYYMH	GVRQAPGQG LEWMG	IINPSGG STSYAQK FQG	RVTMTRDTSTSTAYME LSGLRSEDTAVYYCAR	GAMFDYW	IGHV1-46*01	6	VH1
YM1	VQLVQSGGGLVKPGG SXRLSCAASGFTFS	SYSMN	WVRQAPGKG LERVS	SISSSSS YIYYADS VKG	RFTISRDNAKNSLYLQ MNSLRAEDAAVYYCAR	EVYDYYDSSGYGASD IW	IGHV3-21*01	5	VH3
YM3	QVQLVQSGGGVVQPG RSLRLSCVASGFSFR	NYGMH	WVRQAPGKG LEWVA	IVWSDGS TKYYADS VKG	RFTISRDNSRNTLYLQ MDSLRAEDTAVYYCAR	GSQSGMITGFDNW	IGHV3-33*01	11	VH3
YM4	QVQLVQSGAEVKEPG ASVKVSCKASGYTLN	DYYMH	WVRQAPGQG PEWMG	WLNPNSG AAGYSQK FQG	RVTLTRDTSISTAYME VNRLTSADTAIYYCAR	GRAVVSSYKRGNDYW	IGHV1-2*02	16	VH1
YM5	VQLVQSGAEVKKLGA SVKVSCKASGYTFT	SYYMH	WVRQAPGQG LEWMG	IINPSGG STSYAQK FQG	RVTMTRDTSTSTAYME LSGLRSEDTAVYYCAR	GAMFDYW	IGHV1-46*01	4	VH1
YM8	GQLVQSGAEVKKPGA SVEVSCKASGYTFT	SYYMH	GVRQAPGQG LEWMG	IINPSGG STSYAQK FQG	RVTMTRDTSTSTAYME LSGLRSEDTAVYYCAR	GAMFDYW	IGHV1-46*01	6	VH1
YM11	VQLV*SGGGLVQPGG SLRISCAASGFSFS	SYEMN	WVRQAPGKG LEWVS	YNSRSGN IKYYADS VKG	RFTIPRDNAKNSLYLQ MNSLRAKDTAVYHCAR	GIGIVDYW	IGHV3-48*03	11	VH3
YM12	VQLLQSAGGLVQXGG XLRLSCAASGFTFS	SYAMS	WVRQAPGKG LEWVS	AISGSGG STYYADS VKD	RFTISRDNSKDTLYLQ MNSLRAEDTAVYYCAK	DRGKYPGDGMGVW	IGHV3-23*01	6	VH3

Table 17. (Continued).

HC	FR1	CDR1	FR2	CDR2	FR3	CDR3	Germ line	Germline different	Family
YM13	QVQLVQSGAEVKKPG SSVKVSCKASGGTFT	RYAFS	WVRQAPGQG LEWMG	RIIPMFD KTNYAQK FQG	RVTITADKSTSTAYME LSSLRSEDTAVYYCAT	GPRWGLAAFDIW	IGHV1-69*09	9	VH1
S.V. H4	QVQLQQSGPGLVKPS QTLSLTCAISGDSVS	RKSAAWN	WIRQSPSRG LEWLG	RTYYRSK WYNDYAV SVKS	RITINPDTSKNQFSLQ LNSVTPEDTAVYYCAR	DYYYGMDVW	IGHV6-1*01	2	VH6
S.V. R5A1	EVQLVESGGGLVQPG GSLRLSCAASGFTFS	SYAMS	WVRQAPGKG LEWVS	AISGSGG STYYADS VKG	RFTISRDNAKNSLYLQ MNSLRAEDTAVYYCAR	DRYLSYW	IGHV3-23*04	2	VH3
S.V. R5A6	QVQLQSGAEVKKPGA SVKVSCKASGYTFT	GYYMH	WVRQAPGQG LEWMG	RINPNSG GTNYAQK FQG	RVTMTRDTSISTAYME LSRLRSDDTAVYYCAR	VPALWFRDDLYGMDV W	IGHV1-2*02	2	VH1
S.V. R5A8	QVQLQQSGPGLVKPS Q TLSLTCAISGDSVS	SNSAAWN	WIRQSPSR GLEWLG	RTYYRSK WYNDYAV SVKS	RITINPDTSKNQFSLQ LNSVTPEDTAVYYCAR	GPRFQHW	IGHV6-1*01	0	VH6
YM12 scFv	VQLLQSAGGLVQPGG SLRLSCAASGFTFS	SYAMS	WVRQAPGKG LEWVS	AISGSGG STYYADS VKD	RFTISRDNSKDTLYLQ MNSLRAEDTAVYYCAK	DRGKYPGDGMGVW	IGHV3-23*01	4	VH3
YM13 scFv	QVQLVQSGAEVKKPG SSVKVSCKASGGTFT	RYAFS	WVRQAPGQG LEWMG	RIIPMFD KTNYAQK FQG	RVTITADKSTSTAYME LSSLRSEDTAVYYCAT	GPRWGLAAFDIW	IGHV1-69*09	9	VH1