# IDENTIFICATION AND ENGINEERING OF HUMAN ANTIBODY AGAINST ACUTE MYELOID LEUKEMIA (AML)

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**A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology**

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# การคัดหา และวิศวกรรมแอนติบอดีที่จำเพาะต่อเซลล์มะเร็งเม็ดเลือดขาวชนิดเฉียบพลัน

นางสาวฐิติมา สัมพันธ์อภัย



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# IDENTIFICATION AND ENGINEERING OF HUMAN ANTIBODY AGAINST ACUTE MYELOID LEUKEMIA (AML)

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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มะเร็งเม็ดเลือดขาวไมอีลอยด์ชนิดเฉียบพลัน (AML) เป็นโรคมะเร็งเม็ดเลือดแบบเฉียบพลันที่พบได้มากที่สุด และยังเป็นหนึ่งในปัญหาหลักของประเทศไทยและทั่วโลก การรักษาแบบมุ่งเป้าเพื่อกำจัดเซลล์มะเร็งด้วยวิธีจำแบบจำเพาะ โดยใช้แอนติบอดีเป็นวิธีที่ได้รับความสนใจในปัจจุบัน องค์ความรู้ด้านชีวโมเลกุลสามารถนำมาใช้ในการพัฒนาเทคโนโลยีการแสดงแอนติบอดีบนผิวเฟจเพื่อใช้ในการผลิตแอนติบอดีสำหรับการรักษา และตรวจวิเคราะห์เพราะเทคโนโลยีนี้สามารถนำไปใช้ในการสร้างโมโนโคลนอลแอนติบอดีมนุษย์ที่จับแบบจำเพาะต่อโมเลกุลบนผิวของเซลล์มะเร็ง อีกทั้งยังสามารถปรับแต่งพันธุกรรมของแอนติบอดีให้มีคุณสมบัติเหมาะสมตามวัตถุประสงค์การใช้งานในวิทยานิพนธ์นี้แอนติบอดีได้ถูกคัดเลือกจากคลังที่สร้างจากเม็ดเลือดขาวของคนปกติ คือคลังย่าโม 1 โดยใช้ cell line AML ชนิด HL-60 เป็นเซลล์เป้าหมาย ผลการทำการคัดเลือกหาแอนติบอดีที่จำเพาะ แสดงว่าสามารถคัดเลือกแอนติบอดีทีสามารถจับกับเซลล์เป้าหมาย HL-60 ได้ โดยได้วิเคราะห์ความสามารถในการจับแบบจำเพาะของแอนติบอดีต่อเซลล์เป้าหมายด้วยวิธีโฟลไซโตเมตรี (Flow cytometry) และพบว่า แอนติบอดีโคลน y1HL63152 แสดงความสามารถในการจับได้ดีที่สุด และเป็นแอนติบอดีที่มีความจำเพาะเจาะจงสูงมาก คือจับจำเพาะกับเซลล์ AML ชนิด HL-60 ในระยะที่ยังไม่ได้พัฒนา (non-differentiate) เท่านั้น จึงได้เลือกใช้แอนติบอดีโคลนนี้ในการทดสอบคุณสมบัติทางชีววิทยาต่อเซลล์เป้าหมายต่อไป ผลของการย้อมเซลล์ด้วยแอนติบอดีและส่องใต้กล้องจุลทรรศน์คอนโฟคอลพบว่า แอนติบอดี y1HL63152 จับอยู่บนผิวของเซลล์ HL-60 และเมื่อทดสอบความสามารถในการเข้าไปในเซลล์ HL-60 พบว่า แอนติบอดี y1HL63152 สามารถเข้าไปในเซลล์ได้ นอกจากนั้นแล้วแอนติบอดีโคลน y1HL63152 และ anti-CD33 ยังได้ถูกดัดแปลงทางพันธุวิศวกรรมเป็นสองแบบ คือหนึ่งเชื่อมเข้ากับโปรตีนเรืองแสงฟลูออเรสเซนส์สีเขียวได้เป็นโมเลกุลแอนติบอดี scFv-GFP เพื่อความสะดวกในการตรวจวิเคราะห์การจับกันของแอนติบอดีและผิวเซลล์ด้วยวิธี flow cytometry นอกจากนั้น 152-GFP ได้ถูกนำไปตรวจสอบความสามารถในการจับกับเซลล์มะเร็งที่สกัดได้จากผู้ป่วย ซึ่งได้พบว่า แอนติบอดี 152-GFP สามารถจับกับเซลล์มะเร็งที่สกัดมาจากไขกระดูกของผู้ป่วยบางรายได้ แอนติบอดีโคลน y1HL63152 ได้ถูกดัดแปลงทางพันธุวิศวกรรมอีกแบบคือเชื่อมต่อกับสารพิษ และพบว่า แอนติบอดีที่เชื่อมต่อกับสารพิษสามารถกำจัดเซลล์มะเร็ง HL-60 ได้ที่ความเข้มข้นในช่วงไมโครโมลาร และเมื่อได้ทดสอบความสามารถในการกำจัดเซลล์ HL-60 โดยผสมกันระหว่าง 152-Sarcin กับ anti-CD33-Sarcin พบว่า สามารถเพิ่ม

ความสามารถในการกำจัดเซลล์ HL-60 เมื่อเทียบกับการใช้ 152-Sarcin อย่างเดียว โดยสรุปเทคโนโลยีเฟจสามารถนำมาใช้ในการคัดเลือกหาแอนติบอดีที่จำเพาะต่อเซลล์ AML ได้สำเร็จ และแอนติบอดีนี้ยังอาจนำเอาไปใช้ในการศึกษาโครงสร้าง และหน้าที่รวมถึงกลไกการเจริญเติบโตและพัฒนาเซลล์ AML อีกทั้งยังสามารถพัฒนาต่อยอดเพื่อใช้รักษาหลังจากที่มีการทดสอบทางคลินิกในอนาคต



สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2561 ลายมือชื่ออาจารย์ที่ปรึกษา_____

THITIMA SUMPHANAPAI : IDENTIFICATION AND ENGINEERING OF HUMAN ANTIBODY AGAINST ACUTE MYELOID LEUKEMIA (AML). THESIS ADVISOR : PROF. MONTAROP YAMABHAI, Ph.D., 101 PP.

RECOMBINANT ANTIBODY/ACUTE MYELOID LEUKEMIA/PHAGE DISPLAY ANTIBODY/ANTIBODY ENGINEERING

Acute myeloid leukemia (AML) is a cancer of blood and bone marrow that is rapidly fatal within months if untreated. It is one of the major health problems in Thailand and around the world. Targeted therapy using recombinant antibody is currently an attractive approach to treat cancer. Recent advances in molecular biology have led to the development of phage display antibody technology for the development of therapeutic and diagnostic antibodies. This method allows the generation of human monoclonal antibodies against specific molecules on the surface of cancer cells. It also allows the engineering of antibodies to suit specific purposes. In this thesis, the human scFv antibody fragments were isolated from a non-immunized phage display antibody library, using whole AML cell line (HL-60) as a target. The binding specificity and cross-reactivity of the isolated soluble scFv antibody fragments were evaluated by flow cytometry assay. The scFv antibody clone y1HL63152, which showed specific binding to HL-60 cells, was selected for further analysis. Immunofluorescent staining indicated that it could bind to the surface of the cell, and internalized. Moreover, the y1HL63152 and anti-CD33 scFv were further engineered into two different formats. Firstly, fusions to a fluorescent reporter, scFv-GFP, were created and used as convenient probes for the analysis of cell surface binding by flow cytometer. The results indicated that the target of y1HL63152 only expressed in the non-differentiated HL60 cell line and CD33 did not serve as a target for y1HL63152 scFv antibody. Most importantly, the y1HL63152 scFv could bind to certain samples of bone

marrow mononuclear cells derived leukemic patients. Secondly, fusions of scFv to ribotoxin, Sarcin, was generated and it showed that this immunotoxin could lead to a cytotoxic effect against AML-derived HL-60 cells at a micromolar concentration. In addition, the combining effect of y1HL63152scFv-Sarcin with anti-CD33-Sarcin was demonstrated. In conclusion, phage display technology was successfully used to isolated antibodies against AML cells. This antibody has the potential to be used as a reagent for the study of AML as well as further developed to be used in combination therapy for the treatment of certain types of leukemia after further clinical investigations.



School of Biotechnology

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Student's Signature _____

Advisor's Signature _____

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| Ab | Antibody |
| Abs | Absorbance |
| Ag | Antigen |
| Amp | Ampicillin |
| bp | Base pairs |
| BSA | Bovine serum albumin |
| cfu | Colony forming units |
| CDR | Complementary determining region |
| $CH_1$ | Constant heavy chain 1 |
| $CH_2$ | Constant heavy chain 2 |
| $CH_3$ | Constant heavy chain 3 |
| CL | Constant light chain |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotidyl triphosphates |
| *E. coli* | *Escherichia coli* |
| EDTA | Ethylenediaminetetra-acetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| Fc | Constant region of an antibody molecule |
| Fv | Variable binding region of an antibody |
| HRP | Horse radish peroxidase |

# LIST OF ABBREVIATIONS (Continued)

| | |
|---|---|
| IgG | Immunoglobulin class |
| IMAC | Immobilized metal affinity chromatography |
| Kan | Kanamycin |
| Log | Logarithmic |
| MW | Molecular weight |
| OD | Optical density |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffer saline |
| PBST | Phosphate buffer saline/Tween |
| PEG | Polyethylene glycol |
| pH | Log of the hydrogen ion concentration |
| RT | Room temperature |
| scFv | Single chain Fv antibody derivative |
| SDS | Sodium dodecyl sulphate |
| UV | Ultraviolet |
| µg | microgram |
| kDa | (kilo) Daltons |
| µl | microlitre |
| µM | micromoles |
| $^{o}C$ | degrees Celcius |
| g | grams |

# LIST OF ABBREVIATIONS (Continued)

| | |
|---|---|
| hr | hours |
| kg | kilogram |
| L | litre |
| m | metre |
| M | molar |
| mg | milligram |
| min | minute |
| ml | millilitre |
| nM | nanomolar |
| pg | picograms |
| rpm | revolutions per minute |
| sec | seconds |
| v/v | volume per unit volume |
| w/v | weight per unit volume |

# CHAPTER I

# INTRODUCTION

## 1.1 Significance of this study

Acute myeloid leukemia (AML) is a malignant disease of the bone marrow in which hematopoietic precursors are arrested in an early stage of development. The bone marrow is replaced by blast cells. Therefore, the most important complications are progressive anemia, leukopenia, and thrombocytopenia. To treat patients with AML, chemotherapy is the currently conventional method. However, it has been reported that in younger adults with AML, multiagent consolidation using mitoxantrone and amsacrine in combination with high dose cytarabine does not improve treatment outcome and confers additional toxicity (Schaich et al. 2013), while older patient or those in poor health may not be able to tolerate such intensive consolidation treatment because the risk of serious side effects, including treatment related death. In addition to chemotherapy, stem cell transplantation can be done to restore blood cell production after intensive induction by chemo to destroy all bone marrow cells. However, patients who treated by stem cell transplantation are also more likely to have serious complications, including an increased risk of death from treatment. Targeted immunotherapy is an attractive alternative approach because this method is possible to specifically eliminate only the cancer cells but not normal cells. Therefore, antibody directed against specific cell surface marker of leukemic cell and leukemic stem cell has been suggested as a magic bullet for the treatment of this disease.

Monoclonal antibodies have been demonstrated to be effective in cancer therapy for decades (Albanell and Baselga 1999). Many cell surface membranes can be used as site for targeted therapy. It has been reported that all of human genes, there are 23% classified as

membrane protein (Uhlén et al. 2015). So, membrane proteins involve in many roles for transducing signal into cells. Targeting cancer cells by engaging surface antigens that display cancer-restricted antigens seem promising. Many antibodies currently used as anti-cancer drug are humanized or fully human monoclonal antibody. Even though, they can induce immunogenicity but still lower risk than antibodies from other species. Phage display technology is a powerful tool in molecular biotechnology. A number of peptides or proteins including antibodies can be displayed on the surface of M13 or T7 bacteriophage. This technique can be used to generate therapeutic antibody without using mice or other non-human mammals in the process. The technique also does not induce immune responses to patients and can be manipulated easily *in vitro*.

Selection of antibodies against target of interest is mostly based on bio panning with purified antigen coating on solid phase (Zhang et al. 2017). However, this method has some weakness. Some purified proteins poor mimic of the conformation present on the cell surface. The conformation and natural orientation of the antigen can affect the binding of antibodies. Whole cell bio panning is an alternative method to overcome this problem. There are some bio panning methods applied cell bio panning. Cell sorting bio panning methods have been reported (Yuan et al. 2008) and (Muraoka et al. 2009). The selection of membrane protein by overexpressing the target of interest on host cell lines has been reported (Jones et al. 2016, Crepin et al. 2017) but the selection on native cancer cell has not been well explored. Here we described another method of viable whole cells bio panning using acute myeloid leukemia cancer cells. The lymphoid leukemia was negative selected for reducing undesired hematopoietic common antigen prior to panning with target of interest.

The single chain variable fragment (scFv) is a small antibody fragment that maintains binding ability of the antibody. It has been shown that several tags can be appended to antibody fragments by genetically fused to produce recombinant immunoconjugates depending on their purpose. It has been reported that green fluorescent protein (GFP) could

be produced in bacteria and preserved their property of chimeric fusion protein; therefore, it becomes a very popular reporter tag for protein imaging in intact cells and organisms (Tsien 1998, Tanenbaum et al. 2014). The scFv-GFP fusion protein has been done for decade. For example, anti-B-cell-activating factor of the TNF family (BAFF) scFv fused with GFP retained its fluorescence and high antigen-binding affinity which was useful for investigating the relation between BAFF and autoimmune diseases (Cao et al. 2008). The anti-LDL scFv fused GFP targeted against atherosclerosis could be used as a noninvasive diagnosis reagent of atherosclerotic vascular disease (Faulin Tdo et al. 2014). Using GFP fusion protein eliminates secondary antibody staining step so it is directly target to antigen of interest without amplifying false positive signal.

Immunotoxin has the potential to be widely applied in cancer therapy. The fusion of scFv and toxin has been reported in many publications for example anti-HER2 scFv fused with the A-chain of the ricin protein (RTA) showed strongly inhibit effect to ovarian cancer cells that overexpress HER2 but little effect on normal cells (Jiao et al. 2018). In AML, it has been reported that anti-CD123 conjugated with a truncated Pseudomonas Exotoxin A delivered cytotoxic component to cells and eliminated AML derived cell lines (Stein et al. 2010). Anti-MUC1 conjugated with Gelonin, a type I ribosome-inactivating toxin, showed highly cytotoxic to MUC1+ monocytic lineage AML cell lines (Guillaume et al. 2019). For immunotoxin fusion protein, scFv has been linked with toxins for cytotoxic purpose. Ribotoxin is a group of extracellular ribonucleases that target a conserved sequence of RNA in the ribosome. The α-sarcin is a cytotoxic protein that cleave a single phosphodiester bond in the sarcin–ricin loop of the large ribosomal subunit. The ribosome cannot be recognized to elongation factors so the protein synthesis is inhibited (Endo and Wool 1982). The conjugation of scFv specific to Glycoprotein A33 (GPA33), a colon cancer marker, with α-sarcin showed efficient cytotoxic effect in both *in vitro* and *in vivo* (Carreras-Sangrà et al. 2012, Tomé-Amat et al. 2015). In 2016, the deimmunized α-sarcin was introduced by Jones

and colleagues. They identified two T cell epitopes inside the sarcin gene. They eliminated those epitopes and got the variant clone that retain equal or better cytotoxicity and also lack of T cell activation compared with wild-type (Jones et al. 2016).

The aim of my thesis is to apply phage display antibody technology for the development of a novel target-based therapy against AML, using involve bio-panning of naïve phage display antibody library against AML cell lines. The scFv was engineered to link with GFP and sarcin for detection and cell cytotoxicity purposes. The recombinant antibodies were evaluated according to their specificity, localization and biological activity.

## 1.2 Research objectives

To explore a novel therapeutic approach based on antibody engineering in AML according to the detailed objectives as follows;

1.2.1 To identify human monoclonal antibody against specific cell surface antigens on AML cells by affinity selection (Bio-panning) of naïve (Yamo 1) phage display scFv antibody library

1.2.2 To engineer selected antibodies and evaluate their therapeutic and diagnostic potentials. The engineered antibodies for therapeutic purpose include toxin conjugated, whereas those for diagnostic purpose include green fluorescent protein (GFP) fusion

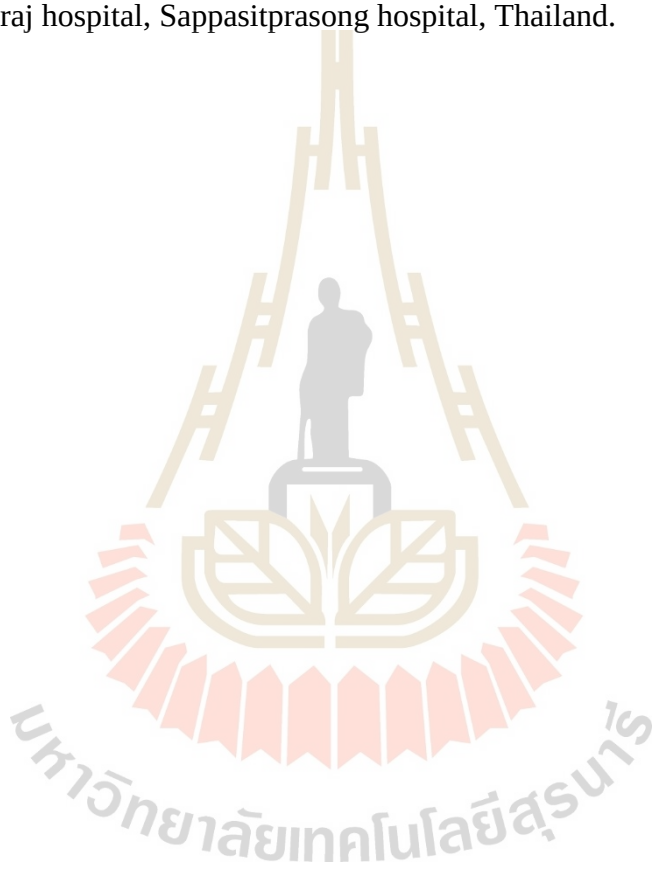
1.2.3 To investigate the biological effects of selected engineered antibodies on AML cells

## 1.3 Research hypothesis

This study hypothesized that the scFv specific to AML cells could be isolated from phage display scFv antibody technology. This scFv antibody could be used for detection and therapeutic purposes.

## 1.4 Scope and limitation of this study

Only single chain variable fragment (scFv) form of the antibody will be displayed on the pIII coat of bacteriophage M13. Biological activities of the recombinant antibodies will only be evaluated i*n vitro*. No experiment was done on animal or human. This work was done according to the guidelines and under the approval of the Ethical Committee for Human Research of Siriraj hospital, Sappasitprasong hospital, Thailand.



# CHAPTER II

# LITERATURE REVIEWS

## 2.1 Therapeutic antibody

Antibodies are proteins that can bind to specific molecules called antigens. Several types of antibodies have been developed in recent years. These developments have made antibodies essential components that useful for applying in medicine and biomedical research. Over the decades, monoclonal antibodies have become the most important class of therapeutic biologicals on the market. The market for monoclonal antibodies has grown exponentially. They accounted for a share of 43% in 2017 and was worth $94 billion from global market. Cancer and autoimmune diseases are approximately 78% of the total antibody sales in the US. Development of therapeutic antibodies was push ahead by recombinant DNA technologies. Adalimumab is the first fully human antibody that approved by U.S. Food and Drug Administration for treatment of rheumatoid arthritis in 2002.

### 2.1.1 Antibody fragments

The fully antibody consists of two identical heavy (H) and light (L) chains connected by disulfide bridges. The chains composed of constant (C) and variable (V) domains, combine to form one interaction site for the antigen. There are many formats of antibody fragment that display on surface of phage particle, e.g., scFv or fragment antigen-binding (Fab). The smallest antibody fragments are single domain (VH) antibodies (dAbs) or the variable domains of the naturally occurring camel heavy chain antibodies (VHH). The structure of these antibody fragments is shown in Fig. 2.1.

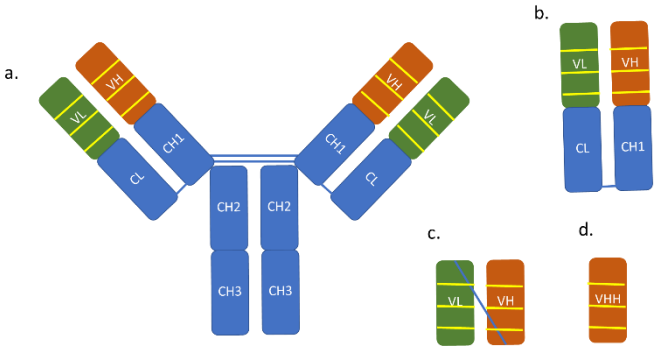



**Figure 2.1** Schematic of antibody structure in full length (a), Fab (b), scFv (c), and VHH (d) CH: Constant domain Heavy chain, CL: Constant domain Light chain, VH: Variable domain Heavy chain, VL: Variable domain Light chain

### 2.1.2 Types of monoclonal antibodies

There are different types of monoclonal antibodies that used in cancer treatment.

- Naked monoclonal antibodies which are antibodies that work by themselves.

These are the most common type of antibodies used to treat cancer. Most naked antibodies target to antigens on cancer cells. It can work in different ways. For example, Alemtuzumab (Campath) is used to treat some patients with chronic lymphocytic leukemia (CLL). It binds to the CD52 antigen, which is found on lymphocytes. Once attached, the antibody attracts immune cells to destroy these cells. It also is administered prior to transplant for depleting T-cell. These treatment results in low rates of Graft-versus-host disease (GvHD) and treatment-related mortality (TRM) (Poiré and van Besien 2011). Some naked antibodies boost the immune response by targeting immune system checkpoints. For example, Ipilimumab, an antibody blocking the receptor CTLA4, showed efficacy in treating malignant melanoma (Hodi et al. 2010). Nivolumab, antibodies blocking PD1, was associated with significant improvements in overall survival and progression-free survival of untreated patients who had metastatic melanoma (Robert et al. 2015). Other naked antibodies work by attaching and blocking antigens on cancer cells that help cancer cells grow or spread. For example, trastuzumab (Herceptin) is an antibody against the HER2

protein. Breast and stomach cancer cells sometimes have large amounts of this protein on their surface. When HER2 is activated, it helps these cells grow. Trastuzumab binds to these proteins and stops them from becoming active. The other mechanisms of naked antibody that leads to the activation of immune effector mechanisms are complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC). Binding of Herceptin also induces ADCC mechanism. Ofatumumab, an alternative anti-CD20 antibody to rituximab, showed enhance killing by CDC both *in vitro* and in preclinical xenograft models (Barth et al. 2012).

- Conjugated monoclonal antibodies which are an antibody that join to a chemotherapy drug or a radioactive particle. Once, antibodies circulate throughout the body. It targets on antigen specifically then delivers the toxic substance. Conjugated antibodies used to treat cancer include: Brentuximab vedotin (Adcetris), an antibody that targets the CD30 antigen. It conjugated to a chemo drug called MMAE. This drug is used to treat Hodgkin lymphoma. Ado-trastuzumab emtansine (Kadcyla), an antibody that targets the HER2 protein, attached to a chemo drug called DM1. It's used to treat some breast cancer patients.

- Bispecific monoclonal antibodies which contained two antibodies binding site. So, they can bind two different antigens. An example is blinatumomab (Blincyto), which is used to treat some types of acute lymphocytic leukemia (ALL) one-part targets to the CD19 protein. Another part binds to CD3. By binding to both of these proteins, this drug brings the cancer cells and immune cells together, which is thought to cause the immune system to attack the cancer cells.

### 2.1.3 Possible side effects of monoclonal antibodies

An antibody are proteins which can sometimes cause an allergic reaction. Possible side effects can include: Fever, chills, weakness, headache, nausea, vomiting, diarrhea, low blood pressure, and rashes. Monoclonal antibody leads to have lesser serious

side effects than chemotherapy drugs because their specificity to cancer cells. However, the side effect of monoclonal antibody also depends on other factors including what it does to the target. For example, Monoclonal anti-CD28 antibody such as TGN1412 was capable of activating T cells. Severe cytokine release syndrome (CRS) and faced multiple-organ failure were observed in clinical drug trial in 2006 (Attarwala 2010). Some monoclonal antibodies can have side effects that are related to the antigens they target. For example: Bevacizumab is a monoclonal antibody that targets VEGF and affects tumor blood vessel growth. It can cause side effects such as high blood pressure, bleeding, poor wound healing, blood clots, and kidney damage. Anti-CD20 monoclonal antibody is used to treated CLL patient even though it depletes normal B cells. But depleting mature and memory B cells by anti-CD20 treatment does not have a dramatic negative effect on preexisting Ab levels (DiLillo et al. 2008). The Conjugated antibodies can be more powerful than naked monoclonal antibodies, but they can also cause more side effects. The side effects depend on which type of substance they're attached to.

## 2.2 Acute myeloid leukemia

Acute myeloid leukemia (AML) is defined as a cancer stem cell with a hierarchy close to normal hematopoietic cell and characterized by clonal expansion and accumulation of myeloid blast. As described in 1994, Lapidot and colleagues have identified an AML-initiating cell by transplantation into severe combined immune-deficient (SCID) mice. These cells homed to bone marrow and proliferated myeloid like cell similar to that seen in the original patients after treating with cytokine. They found that the leukemia-initiating cells that could engraft SCID mice to produce large numbers of colony-forming progenitors were CD34+ CD38- (Lapidot et al. 1994). In 1997, Bonnet & Dick demonstrated that the cell capable of initiating human AML in the SCID leukemia-initiating cell possesses differentiate and proliferate capacities and the potential for self-renewal expected of a leukemic stem cell

(Bonnet and Dick 1997). Since AML is leukemic stem cell so the leukemic clone may be maintained by a rare population of stem cells. Survival of these cells after chemotherapy may lead to minimal residual disease (MRD) and trend to recurrence which is the major problem in the treatment of AML. Recently published demonstrate that AML relapse is associated with the addition of new mutations and clonal evolution by the chemotherapy that the patients receive to establish and maintain remissions (Ding et al. 2012). Moreover, it has been reported that patients who has a high percentage of CD34+ CD38- stem cells at diagnosis significantly correlated with a high MRD frequency, especially after the third course of chemotherapy. Also, it directly correlated with poor survival. It is hypothesized that a large CD34+ CD38- population at diagnosis reflects a higher percentage of chemotherapy-resistant cells that will lead to the outgrowth of MRD, thereby affecting clinical outcome (van Rhenen et al. 2005). Even if CD34+ CD38- are detected in most AML samples but it has been reported that the CD34+CD38+ fraction of myeloid leukemia samples contains leukemia-initiating cell capacity (Taussig et al. 2008). This group also has demonstrated that anti-CD38 antibodies have a profound inhibitory effect on the engraftment of both normal and leukemic repopulating cells. The estimated new case in United states on last five years are shown in table 2.1.

**Table 2.1** The estimated new case and death of all leukemia and AML in 2015-2019.

| Year | Estimated new case of leukemia | Estimated new case of AML | (%) | Estimated deaths of leukemia | Estimated deaths of AML | (%) | References |
|---|---|---|---|---|---|---|---|
| 2015 | 54,270 | 20,830 | 38.38 | 24,450 | 10,460 | 42.78 | Siegel et al. 2015 |
| 2016 | 60,140 | 19,950 | 33.17 | 24,400 | 10,430 | 42.74 | Siegel et al. 2016 |
| 2017 | 62,130 | 21,380 | 34.41 | 24,500 | 10,590 | 43.22 | Siegel et al. 2017 |
| 2018 | 60,300 | 19,520 | 32.37 | 24,370 | 10,670 | 43.78 | Siegel et al. 2018 |
| 2019 | 61,780 | 21,450 | 34.72 | 22,480 | 10,920 | 48.57 | Siegel et al. 2019 |

### 2.2.1 Diagnosis of AML

To diagnose patient with AML, physical examination, complete blood count and bone marrow biopsy to investigate cell morphology, immunophenotype, cytogenetics and molecular genetics are usually performed. Patients with abnormal complete blood count, contain at least 20% of leukemia blast cells in the bone marrow and have signs or symptoms of leukemia including weight loss, fatigue, fever, night sweats, and loss of appetites are considered to have AML.

### 2.2.2 AML classification

There are two main systems to classify AML into subtype, the French-American-British (FAB) classification and the newer World Health Organization (WHO) classification. For the French-American-British (FAB) classification, AML are divided into subtypes, M0 through M7, based on the type and the maturation of leukemia cells. FAB classification M0 to M7 is shown in table 2.2 below.

**Table 2.2** FAB classification.

| FAB subtype | Name |
|---|---|
| M0 | Undifferentiated acute myeloblastic leukemia |
| M1 | Acute myeloblastic leukemia with minimal maturation |
| M2 | Acute myeloblastic leukemia with maturation |
| M3 | Acute promyelocytic leukemia (APL) |
| M4 | Acute myelomonocytic leukemia |
| M4 eos | Acute myelomonocytic leukemia with eosinophilia |
| M5 | Acute monocytic leukemia |
| M6 | Acute erythroid leukemia |
| M7 | Acute megakaryoblastic leukemia |

However FAB classification does not cover many of the factors that are known to affect prognosis. The recently update World Health Organization (WHO) classification in 2016 categorizes AML focusing on their significant cytogenetics and molecular cytogenetic subgroup (Arber et al. 2016). The WHO classification divides AML in several groups as shown below (American cancer society accessed at https://www.cancer.org/cancer/acute-myeloid-leukemia/detection-diagnosis-staging/how-classified.html on March 26, 2019).

**AML with certain genetic abnormalities (gene or chromosome changes)**

- AML with a translocation between chromosomes 8 and 21 [t(8;21)]
- AML with a translocation or inversion in chromosome 16 [t(16;16) or inv(16)]
- APL with the PML-RARA fusion gene
- AML with a translocation between chromosomes 9 and 11 [t(9;11)]
- AML with a translocation between chromosomes 6 and 9 [t(6:9)]
- AML with a translocation or inversion in chromosome 3 [t(3;3) or inv(3)]
- AML (megakaryoblastic) with a translocation between chromosomes 1 and 22 [t(1:22)]
- AML with the BCR-ABL1 (BCR-ABL) fusion gene
- AML with mutated NPM1 gene
- AML with biallelic mutations of the CEBPA gene (that is, mutations in both copies of the gene)
- AML with mutated RUNX1 gene

**AML with myelodysplasia-related changes**

**AML related to previous chemotherapy or radiation**

**AML not otherwise specified** (This includes cases of AML that don't fall into one of the above groups, and is similar to the FAB classification.)

- AML with minimal differentiation (FAB M0)
- AML without maturation (FAB M1)

- AML with maturation (FAB M2)
- Acute myelomonocytic leukemia (FAB M4)
- Acute monoblastic/monocytic leukemia (FAB M5)
- Pure erythroid leukemia (FAB M6)
- Acute megakaryoblastic leukemia (FAB M7)
- Acute basophilic leukemia
- Acute panmyelosis with fibrosis

**Myeloid sarcoma (also known as granulocytic sarcoma or chloroma)**

**Myeloid proliferations related to Down syndrome**

### 2.2.3 Treatment of patient with AML

There are 2 phases of treatment patient with AML except Acute Promyelocytic Leukemia (APL). The first phase is remission induction which is to quickly get rid of leukemia cells as many as possible by using the combination chemo drug (Cytarabine (ara-C) and an anthracycline drug). In some case, the third drug might be added to improve the remission chance. The list of drugs that have been approved are shown in table 2.3. After remission induction treatment, leukemia cells are usually does not completely destroyed. The second phase, i.e., consolidation, need to be done to destroy any remaining leukemia cells. These step helps to prevent a relapse. The consolidation can be done in many ways including using standard chemotherapy, high dose of chemo drug or bone marrow transplantation.

For acute promyelocytic leukemia (APL), another type of AML which have rearrangement of chromosome relate with retinoic acid alpha receptor (RARα) on chromosome 17 and promyelocytic leukemia (PML) on chromosome 15, all trans retinoic acid and anthracycline base chemotherapy are used to treat APL patient.

**Table 2.3** The list of drugs that have been approved for AML patient.

| Drug | Description | Year |
|---|---|---|
| Gilteritinib | treatment of adult patients who have relapsed or refractory acute myeloid leukemia (AML) with a FLT3 mutation | November 28, 2018 |
| Venetoclax | combine with Azacitidine or Decitabine for the treatment of newly-diagnosed AML in adults | November 21, 2018 |
| Ivosidenib | adult patients with relapsed or refractory AML with a susceptible IDH1 mutation | July 20, 2018 |
| Gemtuzumab Ozogamicin | newly-diagnosed CD33-positive acute myeloid leukemia (AML) in adults and for treatment of relapsed or refractory CD33-positive AML in adults and in pediatric patients 2 years and older. | September 1, 2017 |
| Liposome-encapsulated combination of daunorubicin and cytarabine | of adults with newly-diagnosed therapy-related AML or AML with myelodysplasia-related changes | August 3, 2017 |
| Midostaurin | Adult patients with newly diagnosed acute myeloid leukemia (AML) who are FLT3 mutation-positive (FLT3+), as detected by an FDA-approved test, in combination with standard cytarabine and daunorubicin induction and cytarabine consolidation | April 28, 2017 |

## 2.3 Phage display technology

Since, the monoclonal antibody for new treatment of AML patient is still a need to develop. Phage display technology can be applied to be used as a tool to generate novel antibodies that recognized AML targets. Filamentous M13 phage can be inserted foreign gene to make fusion phage was described by George P. Smith in 1985 (Smith 1985). The DNA encoding of protein of interest is inserted into phagemid vector contains pIII gene. The phagemid harboring phage gene and inserted DNA is transduced into *Escherichia coli* bacteria. To complete phage particle, helper phage is super infected to bacteria. Assembled mature phage containing protein that display on the surface of minor coat protein phage is released from *E. coli*. Life cycle of filamentous phage is clearly described. Briefly, phage binds to bacterial host by F' pilus and imports single stand DNA into host. A replicative form double stands DNA is converted by host enzymes then single DNA are coated with pV protein dimers. The proteins pI and pXI combine with pIV to form a channel to facilitate phage secretion. pV is replaced by pVIII and then mature phage particles are assembled and released (Sioud 2019).

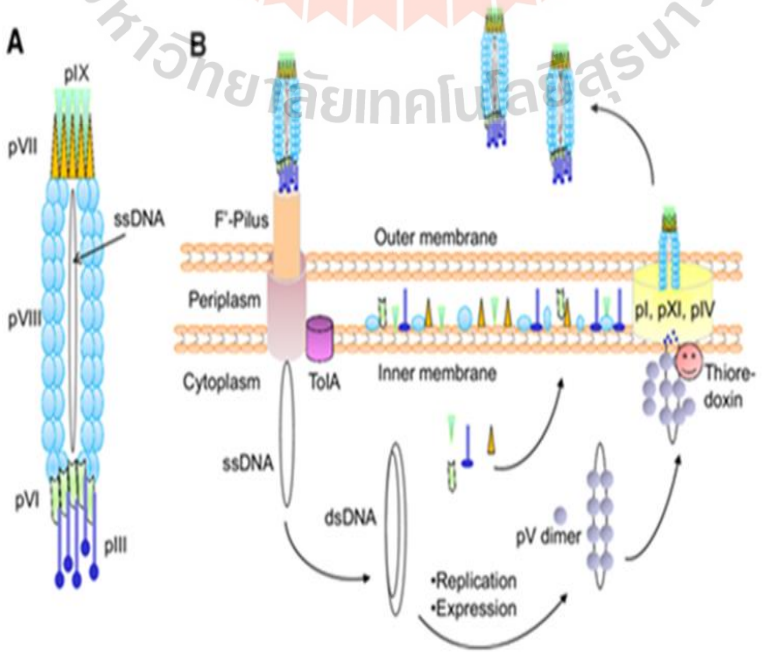



**Figure 2.2** Schematic of phage particle (a) and phage replication step (b) (Sioud 2019).

The simplicity, high efficiency, and low-cost lead to phage display technology has been widely applied to many technological challenges including therapeutic, diagnostic purposes. The outstanding property of phage display antibody is the linkage of genotype and phenotype that lead to accessible for downstream manipulation. The advantage over other techniques such as hybridoma technique is phage display can produce fully human monoclonal antibody so the limitation about immune response using species different monoclonal antibody is omitted.

**2.3.1 Phage display antibody library**

Phage display antibody library is act as a gold mine according to their variety of phage that display a collection of different antibody fragment on their surface. There are two main types of phage display antibody library, naïve or non-immunized and immunized library. Non immune libraries are constructed from healthy donor B cells. This library contains board antibody gene repertoire close to germ line. These antibodies have undergone *in vivo* selection for V gene rearrangement. It has been reported about constructing of naïve antibody library by (Pansri et al. 2009, Yuan et al. 2012, Schwimmer et al. 2013). It showed that the size was $1.5 \times 10^8$, $2.5 \times 10^8$ and $8.2 \times 10^{10}$ clones, respectively. The advantage of naïve antibody is containing a board diversity of antigen specific antibody but the limitation of the naïve is lower affinity than immunized antibody library. To overcome this limitation, antibody engineering including site direct mutagenesis (Yang et al. 1995), chain shuffling (Rangnoi et al. 2018) showed improvement of binding affinity by 420-fold and 7.5-fold from their parental antibody, respectively. In addition of fully natural naïve library, there are another synthetic and semi synthetic antibody library which is constructed from fully synthetic gene or using one antibody framework subsidized with synthetic randomized sequence at CDR position, respectively. A large Fab antibody library has been reported by (Griffiths et al. 1994, Lee et al. 2004) and other groups. Immunized libraries are made from B cells of immunized animal for example constructing immune library by immunizing mice

with CD123 recombinant protein (Stein et al. 2010) or patient donor who got diseases i.e. construction of phage antibody from patient with B cell lymphoma (Shen et al. 2007) and breast cancer (Rodriguez-Pinto et al. 2009, Ayat et al. 2013, Novinger et al. 2015). Immunized library is already affinity maturated from *in vivo* somatic hypermutation of donors. So, it is generally higher quality affinity than non-immunized library.

**2.3.2 Phage-derived antibodies in clinical use**

Despite, patient can still develop anti-human antibody immune response. But the human antibody can lower risk of immune response than using different specie monoclonal antibody. Antibodies in clinical use should be as human as possible. Phage display provides fully human antibody. So, it is a powerful tool for developing antibody for therapeutic purposes. The fist phage derived antibody that have been approved by FDA in 2002 is Adalimumab (Humira), an antibody target to Tumor necrosis factor-α (TNF). Later on, a number of phage derived antibodies have been approved i.e. Ranibizumab (Lucentis) an anti-Vascular endothelial growth factor A (VEGF-A), Belimumab (Benlysta) an anti- B-lymphocyte stimulator (BLyS), Raxibacumab (ABthrax) an anti-Protective antigen (PA) component of anthrax (Bacillus anthracis). Whereas, some are in clinical study such as Cixutumumab an anti-insulin-like growth factor-1 receptor (Weickhardt et al. 2012). Here is a list of phage derived antibody which was summarized in table 2.4. by (Nixon et al. 2014)

**Table 2.4** Summary of drug that have been studied in clinical trial and approved by US. FDA.

| Drug | Target | Disease | Approval year |
|---|---|---|---|
| Adalimumab | to Tumor necrosis factor-α (TNF) | Rheumatoid arthritis, juvenile idiopathic arthritis, psoriatic arthritis, ankylosing spondylitis, crohn's disease, ulcerative colitis, plaque psoriasis | 2002 |
| Ranibizumab | Vascular endothelial growth factor A (VEGF-A) | Neovascular (wet) age-related macular degeneration | 2006 |
| Belimumab | B-lymphocyte stimulator (BLyS) | Autoantibody-positive, systemic lupus | 2011 |
| Raxibacumab | Protective antigen (PA) component of anthrax (Bacillus anthracis) | Prophylaxis and treatment of anthrax | 2012 |
| Ramucirumab | Vascular endothelial growth factor receptor 2 (VEGFR2) | Cancer (NSCLC, breast, metastatic gastric adenocarcinoma) | 2014 |
| Necitumumab | Epidermal Growth Factor Receptor (EGFR) | Cancer (NSCLC) | Phase III |

**Table 2.4** (continued).

| Drug | Target | Disease | Approval year |
|---|---|---|---|
| Fresolimumab | TGF-β | Primary brain tumors, primary focal segmental glomerulosclerosis | Phase II |
| Mapatumumab | TNF-related apoptosis-inducing ligand receptor 4 (TRAIL-4) | Cancer (NSCLC, non-Hodgkin lymphoma, liver, cervical) | Phase II |
| Cixutumumab | anti-insulin-like growth factor-1 receptor | non-small cell lung cancer | Phase I/II |

### 2.3.3 Current advances in phage display research on AML

There have been many reports describing the application of phage display technology to identify antibodies or peptides for targeting AML cells as summarized in Table 2.5. The selection of specific scFv antibodies with biological functions against HL-60 and Kasumi-1 AML cell lines, using whole cells as affinity matrix have been previously reported. Surface molecules on AML cells for targeting by antibody are attractive target. Antibodies against FLT3, KIT, CD33, CD123, CD157, C-type lectin-like molecule-1 (CLL-1), and leukocyte antigen-related receptor protein tyrosine phosphatase (LAR PTP) have been identified by phage display technique. Furthermore, anti-CD33 VH was generated by human antibody domain (VH) library to further engineered to be CD33-targeting Chimeric antigen receptor (CAR) T cells. Not only antibody can be used for targeting AML cells but peptide also can be used for binding to leukemia cells, resulting in more effective treatment with less side effects. It has been reported that the selection of random phage display peptide libraries on Kasumi-1 AML cells and the result showed CPLDIDFYC peptide bound to Kasumi-1 AML cells and internalized upon receptor binding (Jager et al. 2007). In 2008, Galili and colleagues have screened peptide from a phage peptide display library that can induce differentiation of leukemia cells to make cells more sensitive to chemotherapy. (Galili et al. 2008). Peptide targeting Toll-like receptor 2 (TLR2) which highly express on AML was identified by screening of a phage display peptide library. Conjugation between TRL2 binding peptide and proapoptotic peptide reduced myeloid leukemia-induced animal death (Li et al. 2014). Antibodies can be engineered for more cytotoxic activity by fusing with potent immunotoxins and encapsulated with nanoparticles. It has been reported that anti-CD123 scFv antibody conjugated with Pseudomonas Exotoxin A and anti-CD64 conjugated with Pseudomonas Exotoxin A showed cytotoxic effect to AML cells. Moreover, the peptide that specifically bind to CLL-1 was coated with nanoparticles for targeted drug delivery (Zhang et al. 2012). Together these reports show that phage display technique is an efficient and flexible tool for generation of antibody against AML.

**Table 2.5** The application of phage display technology to identify antibodies or peptides for targeting AML cells.

| Phage Library | Bio-panning Target/Methods | Biological Function | Reference |
|---|---|---|---|
| Synthetic library | Unidentified epitope on HL-60 | Inhibited HL-60 cells proliferation. | Shadidi and Sioud 2001 |
| Tomlinson phage display library | Unidentified epitope on Kazumi-1 | Internalized into Kasumi-1 cells and induction apoptosis by fusing with truncated form of Pseudomonas Exotoxin A. | Fitting et al. 2015 |
| non-immunized phage antibody library in Fab format | FLT3 | Competed binding of FL ligand to FLT3 on cell surface and inhibited FLT3 stimulated proliferation of AML cells | Li et al. 2004 |
| large human Fab phage display library | FLT3 | Inhibited FLT3 ligand-induced receptor phosphorylation and cell proliferation in leukemia cells | Williams et al. 2005 |
| A large nonimmune phage antibody library | KIT | targeted KIT membrane and inhibited KIT-dependent signaling and cell growth. | Le Gall et al. 2015 |
| Immunized mice library | CD33 | The CD33 scFv antibody fragment was fused with Pseudomonas exotoxin A and it induced apoptosis in AML cells | Schwemmlein et al. 2006 |

**Table 2.5** (continued).

| Phage Library | Bio-panning Target/Methods | Biological Function | Reference |
|---|---|---|---|
| Immunized mice library | CD123 | The isolated scFv was either fused with ETA for inducing apoptosis or engineered to be bispecific scFv for mediating cell lysis by ADCC mechanism. | Stein et al. 2010 |
| Immunized mice library | CD123 | The isolated scFv bound specifically to CD123 | Moradi-Kalbolandi et al. 2016 |
| Phage Display at Alere TM | CD157 | CD157 could be validated as a strongly expressed target antigen in AML and could induced lysis of AML cell lines and primary AML cells | Krupka et al. 2017 |
| Semi-synthetic library | leukocyte antigen-related receptor protein tyrosine phosphatase (LAR PTP) activated leukocyte adhesion molecule (ALCAM) | The well-characterized cell surface antigens, LAR PTP and ALCAM were identified. | Geuijen et al. 2005 |

**Table 2.5** (continued).

| Phage Library | Bio-panning Target/Methods | Biological Function | Reference |
|---|---|---|---|
| Semi-synthetic phage library | CLL-1 | A novel C-type lectin that is broadly expressed on AML blasts were identified. | Bakker et al. 2004 |
| Immunized mice library | CD33 | The CD33 scFv antibody fragment was fused with Pseudomonas exotoxin A and it induced apoptosis in AML cells | Schwemmlein et al. 2006 |
| VHH library | TIM-3 | A functional anti-human TIM-3 specific nanobody was isolated. It suppressed the proliferation of TIM-3 expressing HL-60 cell. | Homayouni et al. 2016 |
| Human antibody domain (VH) library | Anti-CD33VH-CAR | The CAR33VH was efficient in tumor killing *in vitro* and *in vivo*. | Schneider et al. 2018 |

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Materials

A naive phage display scFv antibody library (Yamo I) was used (Pansri et al. 2009). M13KO7 helper phage was purchased from New England Biolabs, NEB (Massachusetts, United States). Immunotubes and bioassay plate were obtained from Nunc (Denmark). *Escherichia coli* TG1 was used for phage amplification and scFv antibody production which was obtained from MRC, Cambridge, UK. Bovine serum albumin (BSA), Doxorubicin, and IgG from human serum were obtained from Sigma (St. Louis, MO). Dimethyl sulfoxide (DMSO) and Isopropyl β-D-1-thiogalactopyranoside (IPTG) was obtained from Amresco (United States). HL-60 cells (CCL-240) and THP-1 cells were purchased from ATCC. OCIM-1 and Jurkat cells were a gift from Dr. Jenny Yeung, Cancer Institute UCL. Cell culture medium and supplements including Iscove's Modified Dulbecco's Medium (IMDM), RPMI 1640, Fetal Bovine Serum (FBS) and 1X penicillin streptomycin were purchased from Gibco. *Escherichia coli* strain BL21(DE3), K12, Shuffle B cell, DH5αF', Top 10, all restriction enzymes and Calf Intestinal Alkaline Phosphatase (CIP) were obtained from New England Biolabs, NEB (Massachusetts, USA). Ficoll Plague plus solution was obtained from GE health care. Gel purification kit was obtained from Promega (Madison, USA). Plasmid preparation kit was purchased from Qiagen (Hilden, Germany). pET21d+ vector was obtained from NEB (Massachusetts, USA). APC anti-human CD11b Antibody, Anti-CD45 APC (clone 2D1) and anti-CD33 PE antibodies (clone p67.6) were purchased from Bio Legend (San Diego, California). Mouse anti-Histidine dylight 488 was obtained from

Abcam. Rabbit anti-Myc was obtained from Sigma-Aldrich. HisPur Ni-NTA Resin, Donkey anti rabbit conjugated with Alexa flour 647 IgG1 (H+L) and Propidium Iodide (PI) was obtained from Thermo fisher. FITC Annexin V Apoptosis Detection Kit was provided from BD Pharmingen. Amicon® Ultra-4 was obtained from Merck. Vivaflow 200 was provided from Sartorius. The flow cytometer was purchased from Thermo Scientific (Attune™ NxT Flow Cytometer). The Muse flow cytometer and Muse cell count and viability kit were purchased from Merck.

## 3.2 Methods

### 3.2.1 Biopanning of phage display scFv library against HL-60 AML cell

The selection of phage display antibody library against HL-60 cells was done in immunotube (Nunc, Denmark). This experiment was done 3 rounds of selection. The phage library was pre-blocked with 1 mL of Phosphate buffer saline (PBS) supplemented with 4% (w/v) skimmed milk (4% MPBS) for 30 minutes at room temperature with rotating. The pre-blocked phage was incubated with $5x10^6$ Jurkat cells as a negative selection for 1 hour at 4°C with rotating. After incubation, cells were centrifuged at 350 g for 5 minutes, 4°C. Half of the supernatant containing phage was added to $5x10^6$ HL-60 cells and another half was added to $5x10^6$ Jurkat cells as a negative control. Phages were incubated for 2 hours at 4°C with rotating. Unspecific phages were removed by centrifugation at 350 g for 5 minutes, 4°C. The supernatant was discarded. Cells were washed 5 times with 5 mL of 0.1% (w/v) BSA in PBS. Bound phages were eluted with 500 µL of 50 mM Citric acid pH 2.5. Cells were incubated for 5 minutes and centrifuged at 350 g for 5 minutes, 4°C. the supernatant containing phage was collected in new clean tube. Eluted phage was neutralized with 500 µL of 1M Tris-HCl pH 7.4. The 1 mL of eluted phage was recovered by infecting to 5 mL of exponential growing *E. coli* TG1 ($O.D._{600}$ 0.5) and 4 mL of 2xYT media. Bacteria were incubated at 37°C for 30 minutes without shaking. Bacteria containing phage were plated on

to 2xYT containing 100 µg/mL ampicillin and 1% glucose bioassay plate. Plate was incubated at 37°C for overnight. The negative control was done in parallelly in every step of selection.

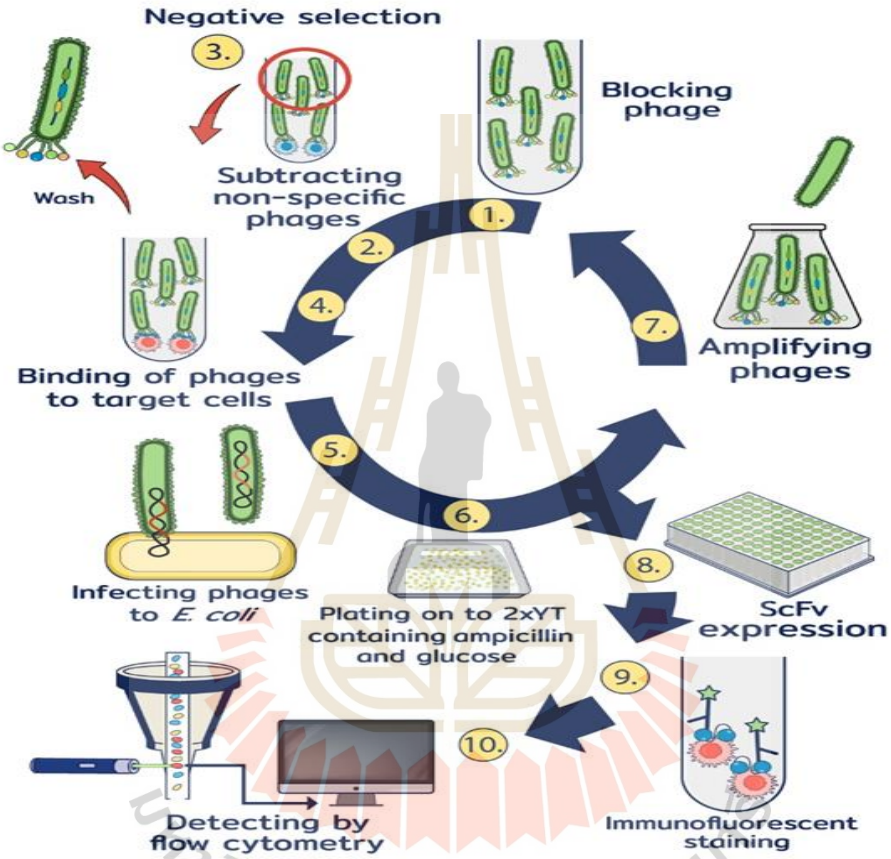



**Figure 3.1** Schematic representation of cell-based biopanning. (1) Phage library was pre-blocked in skimmed milk. (2) Pre-blocked phages were subtracted nonspecific phage on Jurkat cells. (3-4) Unbound phages were transferred to HL-60 target cells. Nonspecific phages were wash out. (5) Bound phages were eluted and used to infect *E. coli* TG1 bacteria. (6) Bacterial containing phagemid were plated on bioassay plate containing ampicillin and glucose. (7) Phages were amplified for the next round of biopanning. (8) After 3 round of selection, bacterial single colonies were randomly picked and scFv expression was induced on deep well plate. (9) Cells were stained with scFv and detected by flow cytometer (10).

### 3.2.2 Phage amplification

The bacterial cells which plated on bioassay plate were scraped and kept in 40% Glycerol storage at -80°C. Bacteria were inoculated into 100 ml 2xYT/A/G medium to obtain yield an $OD_{600nm}$ = 0.1. Cells were incubated 37°C with shaking at 250 rpm until $OD_{600nm}$ approximate 0.4-0.5. one hundred µl of helper phage M13KO17 (NEB) was added to infect bacterial containing phagemid. Superinfected bacteria were incubated at 37°C for 30 – 40 minutes without shaking. Cells were pelleted by centrifugation at 3,300 x g for 10 minutes and resuspend the pellet in 100 ml 2xYT containing 100 ug/mL ampicillin, 50 ug/mL kanamycin. Cells were incubated for overnight at 30°C, 250 rpm. Overnight cultured was centrifuged in 50 mL tubes at 3,300 x g for 20 minutes at 4°C then transferred 45 mL of supernatant from each tube to a clean 50 mL tube. Nine ml of 20% PEG 6000/2.5M NaCl was added into the supernatant and then leaved the tubes on ice for 1 hour to precipitate phage. Tubes were centrifuged at 3,300 x g for 10 minutes at 4°C. The supernatant was discarded and resuspended the pellet in 1.5 ml of sterile DI water. The supernatant containing phage was transferred to a new clean 1.5 mL tube and centrifuged at 13,000 rpm for 5 minutes to pellet any bacterial cells. The supernatant was transferred into 1.5 mL tubes. The 0.25ml of 20% PEG 6000/2.5M NaCl was added into the tube and leaved on ice for 1 hour. The tube was centrifuged at 13,000 rpm for 10 minutes to pellet phage and discarded supernatant. The phage pellet was resuspended in 500 µL of sterilized DI water. To pellet any remaining bacterial cells, tube was centrifuged at 13,000 rpm in a microcentrifuge for 2 minutes. The supernatant was transferred into a fresh tube and use for further round of selection or store at -20 °C. Ten µl of the phage was kept to obtain titer.

### 3.2.3 Production of polyclonal scFv antibody fragments

Bacteria in glycerol stock of each round bio-panning were inoculated to 5 mL 2xYT (16g of Bacto Tryptone, 10g Bacto Yeast Extract, 5g NaCl and adjust volume up to 1L) containing 100 µg/mL ampicillin and 1% (w/v) glucose in 1:100 ratio. Bacteria was

incubated at 37°C with 250 rpm shaking until OD. $_{600\ nm}$ reached 0.9. Cells were pelleted cells by centrifugation. The supernatant was discarded and resuspended the pellet in fresh 2xYT containing 100 µg/mL Ampicillin and 1mM IPTG. Bacterial cells were grown overnight at 30°C. The overnight cultured was centrifuged at 3,300 g for 30 minutes. The supernatant containing polyclonal scFv antibody was transferred to fresh tube and stored at 4°C until use.

**3.2.4 Production of monoclonal scFv antibody fragments**

After 3 rounds of selection, individual colony was randomly picked, grown in 5 ml of 2xYT medium containing 100 µg/ml ampicillin and 2% glucose and incubated for overnight, at 37 °C with 250 rpm shaking. One hundred µL of overnight cultured was inoculated to 5 mL 2xYT containing 100ug/mL ampicillin and 1% glucose and incubated 37°C with 250 rpm shaking until OD $_{600\ nm}$ approximate 0.9. Once the $OD_{600\ nm}$ reached 0.9, cells were pelleted by centrifugation. The supernatant was discarded and resuspended pellet in fresh 2xYT containing 100 µg/mL ampicillin and 1 mM IPTG. Bacterial cells were grown overnight at 30°C. The overnight culture was centrifuged at 3,300 g for 30 minutes. The supernatant containing scFv antibody was transferred to fresh tube and stored at 4°C until use.

**3.2.5 Flow cytometry analysis**

HL-60 cells were counted and washed with 1x PBS for 3 times by centrifugation at 330xg for 5 minutes, 4°C. The $2x10^5$ cells was used for one sample. The Fc receptor on cells surface were blocked with human IgG for 30 minutes at room temperature. Cells were washed once. The supernatant containing scFv of clone 1-180 were incubated with HL-60 cells and stained with rabbit anti-myc antibody in 1:1000 dilution on ice for 1 hour. After that, cells were washed with 1x PBS containing 0.1%BSA and then the donkey anti-rabbit conjugated with Alexa flour 647 was used as a secondary antibody for 30 minutes on ice in dark. Stained cells were washed and resuspended in PBS containing 0.1% BSA and 1mg/ml

of PI in 1:1000 dilution. Cells were analyzed by flow cytometer using PI negative exclude dead cells. The supernatant containing scFv of clone 181-276 were incubated with HL-60 for 1 hour on ice and then cells were washed and stained with anti-Histidine conjugated with dylight 488 in 1:1000 dilution for 1 hour on ice. Stained cells were washed and resuspended in PBS containing 0.1% BSA and 1mg/ml of PI in 1:1000 dilution. Cells were analyzed by flow cytometer using PI negative exclude dead cells.

**3.2.6 Immunofluorescent staining**

HL-60 cells were washed once with 1xPBS containing 0.1% BSA. The $2x10^6$ cells were used for one test. Cells were blocked with human IgG for 30 minutes at room temperature. Cells were centrifuged at 330xg for 5 minutes for washing. Cells were incubated with 0.2 µg/µl of purified scFv antibody in 100 µl of reaction for 1 hour on ice. Cells were washed and incubated with secondary antibody, mouse anti-Histidine dylight 488 in 1:1000 dilution, for 1 hour on ice at dark. Cell nucleus were stained with Hoechst 33342 for 30 minutes. Cells were washed and dropped on glass slide. The slow fade reagent was mixed with the stained cells. Glass slide was stand on table for 30 minutes to let the cells sink to the bottom of the glass slide. Stained cells were investigated under confocal microscope. In this experiment, scFv anti-aflatoxin, 3E3 was used as an isotype control. The exposure time was fixed to avoid positive bias.

**3.2.7 Recombinant protein engineering and expression**

**3.2.7.1 scFv cloning and expression**

The scFv gene of clone y1HL63152 in pMOD phagemid vector was double digested with *NcoI* (10U/µl, NEB, USA) and *NotI* (10U/µl, NEB, USA) restriction enzymes to generate compatible sticky end. The reaction was incubated at 37°C for 3 hours. The pET21d+ vector was also digested with compatible enzymes. Digested vector was heat inactivated at 65°C for 20 minutes and followed by adding 0.5 µl of Alkaline Phosphatase, Calf Intestinal Alkaline Phosphatase (CIP) (10U/µl) for further 1 hour at 37°C. The digested

vector and insert gene were purified by agarose gel electrophoresis and clean up by gel purification kit. (Promega, USA). The insert and vector were ligated by ligase enzyme in 3:1 ratio and incubated at 16°C for 20 hours and heat inactivated at 65°C for 20 minutes. The ligated insert was transformed by chemically prepared *E. coli* DH5αF' competent cells. Then transformed cells were spread on LB plate containing 100 μg/ml ampicillin and incubated at 37°C for overnight. The transformed cells were picked in 5 ml of LB containing ampicillin and incubated at 37°C for overnight. The overnight culture was centrifuged at 3,300 xg for 10 minutes. Plasmid was prepared by plasmid miniprep kit. The plasmid was cut for determining insert. The positive clones were sent for sequencing.

The recombinant plasmids were transformed into expression host (SHuffle B cell). The bacteria containing expression vectors were picked into 5 ml of LB containing 100 μg/ml of ampicillin and incubated at 30°C with shaking for overnight. The overnight cultured was inoculated to 200 ml of LB containing ampicillin in 1:100 dilution. Bacterial cells were incubated at 30 °C with shaking until OD 600 reach 0.6. The IPTG was added into the culture flask to final concentration 0.4 mM and incubated at 25°C with shaking for 20 hours. The overnight cultured was centrifuged at 3,300 g for 10 minutes and discarded supernatant. The cell pellet was incubated with lysis buffer (20 mM of Tris-HCl, 500 mM of NaCl, 20 mM of Imidazole, pH 7.4) containing 1 mg/ml of Lysozyme and 1X protease inhibitor cocktail for 30 minutes. Bacterial cells were disrupted by sonication for 5 minutes pulse 30 seconds and break 30 seconds. The cell lysate was centrifuged at 8,000 g for 30 minutes, 4°C. The clear supernatant was moved to the gravity flow chromatography containing 1 ml of the immobilized metal affinity chromatography (IMAC) resin. The column was incubated for 2 hours with rotating at 4°C after that the column was sit on stand and opened the cap and drained the flow through according to gravity. Column was washed 10 times of the resin by washing buffer (20 mM of Tris-HCl, 500 mM of NaCl, 60 mM of Imidazole, pH 7.4) and eluted by elution buffer (20 mM of Tris-HCl, 500 mM of NaCl, 250 mM of Imidazole, pH 7.4). Every fraction was collected for SDS-PAGE analysis.

### 3.2.7.2 scFv-GFP cloning and expression

The scFv gene from the phagemid vector were inserted into an EmGFP expression vector (pWS-Green: the vector that was made in our lab) between (10U/µl, NEB, USA) *NcoI* and (10U/µl, NEB, USA) *NotI* sites. The pWS-Green vector is based on pET21d+ vector under transcriptional controlled by T7 promotor. scFv gene is in N terminus and followed by EmGFP gene. FLAG tag and Histidine tag are in C terminus. The DNA of scFv fragments and pWS-Green vector was double digested with *NcoI HF* (10U/µl, NEB, USA) and *NotI HF* (10U/µl, NEB, USA) enzymes, to generate compatible sticky ends. The reactions were incubated at 37°C for 3 hours. The reactions were heat inactivated at 65°C for 20 minutes. After heat inactivate enzyme, the *NcoI/NotI* digested vector was dephosphorylated by adding 0.5 µl of CIP enzyme (10U/µl, NEB, USA) and incubated at 37°C for 1 hour. The inserts and vectors were gel purified followed by Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The scFv DNA was ligated into pWS-Green vectors at a 3:1 ratio. The ligation reaction was incubated at 16°C for overnight and heat inactivate enzyme at 65°C for 20 minutes. Then, the ligation reaction was transformed into 100 µl of *E. coli* DH5αF' competent cells. After that the transformed cells were spread on LB plates, containing 100 µg/ml ampicillin and incubated for overnight at 30°C. Three individual colonies were picked and analyzed by double digestion with *NcoI/NotI*. Moreover, the constructs were confirmed by DNA sequencing.

The recombinant vector was transformed into three expression hosts which are *E. coli* SHuffle® B cell (NEB), BL21, and K12. These expression hosts were induced expression of scFv-GFP fusion protein. Bacteria were inoculated into LB media containing 100 µg/ml ampicillin and incubated at 30°C for overnight with 250 rpm shaking. Overnight cultured was inoculated 1:100 to 200 ml of LB media containing 100 µg/ml of ampicillin incubated at 30°C until OD 600 reached 0.9 then IPTG in final concentration 0.4 mM was added and changed temperature to 25°C with 250 rpm for 20 hours. The bacterial

culture was pelleted by centrifugation at 4000 rpm for 30 minutes, 4°C. Cell pellet was homogenized by sonication. The supernatant was centrifuged at 6,000xg for 30 minutes, 4°C. Protein purification was done by immobilized metal affinity chromatography (IMAC) using Ni-NTA column. One ml His-Trap column (GE Healthcare, USA) was equilibrated with 10 ml buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 7.4). Then, the filtered supernatant was loaded into column. To remove loosely bound protein, the column was washed with the washing buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 7.4). The scFv was eluted from the column with high concentration of imidazole (20 mM Tris-HCl, 500 mM NaCl, 250 mM imidazole, pH 7.4). The antibody fraction was concentrated by Amicon® Ultra-4 (Merck KGaA, Germany) and exchanged to PBS buffer. Protein concentration was quantified by a Nanodrop ND2000 spectrophotome (Thermo Scientific, USA). To determine the efficiency of purification, the flow through fraction, wash fraction and elution fraction were compared by SDS polyacrylamide gel electrophoresis (SDS-PAGE). The binding ability was tested by flow cytometry.

#### 3.2.7.3 scFv-Sarcin cloning and expression

##### 3.2.7.3.1 scFv-SaWT in *Pichia pastoris*

The amino acid sequence of sarcin wild type was provided from published article (Lacadena et al. 2007). The gene was designed by flanking *SfiI* and *NotI* restriction enzyme at 5’ and 3’ terminal of the scFv gene, respectively. The scFv was linked with sarcin by glycine and serine linker (GGGS). The *XbaI* restriction site was added at 3’ terminal of the sarcin gene. These set of gene were synthesized by Genscript and cloned into pPICZαB vector at *SfiI* and *XbaI* sites. The scFv-sarcinWT was transformed into *E. coli* Top10 to amplify plasmid. The plasmid was linearized using 10,000 U of the *PmeI* restriction enzyme. The reaction was incubated at 37°C for 4 hours and separated the cut plasmid by running in 0.9% agarose electrophoresis. The agarose gel was cut at the expected size and purified by Promega kit.

To prepare *Pichia pastoris* competent cells, single colony of *Pichia* was picked up and inoculated into 5 mL of YPD and incubated at 30°C for overnight. The overnight cultured was inoculated to 50 mL of YPD media and incubated at 30°C for overnight. The optical density of cells was measured to reach OD600 1.3-1.5. Yeast cells were centrifuged at 1,500 g for 5 minutes at 4°C. The supernatant was discarded. Fifty mL of prechilled sterilized DI water was resuspended into cells. Yeast cells were centrifuged and discarded supernatant and then resuspended in 25 mL of prechilled sterile DI water after that followed by centrifugation and the supernatant was discarded. Cells were resuspended in 2 mL of 1 M sorbitol and centrifuged. And then, 100 µL of 1M sorbitol was added. The linearized plasmid was mixed with 100 µL of the prepared competent cells. The mixture was incubated on ice for 5 minutes and shot by electroporator. The electroporator was set at voltage 1500 V, time constant 5 millisecond, resistance 200-ohm, capacity 25 µF. After electroporated, the mixture was incubated at 30°C for 1 hour and spread on YPDS containing 300 µg/mL zeocin. The plates were incubated at 30°C for 3 days. The yeasts containing plasmid were investigated by colony directed PCR.

**3.2.7.3.2 Expression testing in *Pichia pastoris***

The selected clones were inoculated into 5 mL of YPD containing 300 µg/mL of Zeocin. Cells were incubated at 30°C with shaking for 16 hours. Five mL of overnight cultured were inoculated into 100 mL of BMGY and incubated at 30°C for 16 hours. Yeast cells were pelleted by centrifugation at 3,300 g for 10 minutes. The supernatant was discarded. Yeast cells were resuspended with 50 mL of BMMY and incubated at 25°C for 5 days. The absolute methanol was added to the culture at final concentration 0.05% in every 24 hours. Sample was collected in every 24 hours. Supernatant and cell lysate of the samples were analyzed by SDS-PAGE and Western blot.

The preparation of YPD, BMGY, and BMMY medium were done according to user manual from EasySelect™ Pichia Expression Kit (http://tools.thermofisher.Com/content/sfs/manuals/easyselect_man.pdf).

**3.2.7.3.3 Large scale production in *Pichia pastoris***

The best expressing clone was inoculated into 6 tubes of 5 mL of YPD containing 300 μg/mL of zeocin and incubated at 30°C with shaking for 16 hours. The 25 mL of overnight cultured were transferred into 350 mL of BMGY in 2L baffled flask. The culture was incubated at 30°C further 16 hours. Yeast cells were pelleted by centrifugation at 3,300 g for 10 minutes. Supernatant was discarded and cells were resuspended with 250 mL of BMMY. The culture was incubated at 25°C for 5 days. The absolute methanol was added into the culture at final concentration 0.05% in every 24 hours. After 5 days, cells were pelleted by centrifugation and keep the supernatant containing protein to fresh bottle. The supernatant was concentrated by Viva flow to final volume 50 mL. A one hundred mL of buffer A (50 mM of Sodium phosphate buffer + 0.1 M of NaCl, pH 7.5) was added to exchange buffer. Protein was concentrated and repeated exchange buffer again. The purification process was followed by previously published article (Carreras-Sangrà et al. 2012). The concentrated protein was loaded into the gravity flow chromatography containing 1 ml of the immobilized metal affinity chromatography (IMAC) resin. The column was washed with 20 mL of buffer A and washed again with 20 mL of buffer A plus 25 mM of Imidazole. The protein was eluted with 1 mL of buffer A containing 250 mM of Imidazole. The flow through, washed and eluted fraction were collected for SDS-PAGE and Western blot analysis.

**3.2.7.3.4 scFv-SaDI in *E. coli***

The information of Sarcin deimmunized (SaDI) gene was taken from published journal (Jones et al. 2016). The gene was designed by introducing *NotI* and *XhoI* restriction site at 5’ and 3’end respectively. This gene was synthesized by Genscript. The

SaDI gene was amplified by transforming into *E. coil* Top10 and plasmid was purified by Qiagen midi prep. The pET21d+ vector and SaDI gene were digested with *NotI* (10U/µl, NEB, USA) and *XhoI* (10U/µl, NEB, USA) restriction enzymes at 37°C for 3 hours. Digested vector and insert were purified by gel electrophoresis and clean up by Promega kit. Purified vector and insert were ligated with T4 ligase enzyme at 16°C for 16 hours and heat inactivated at 65°C for 20 minutes. Ligated vector was transformed into *E. coli* DH5αF' and spread on LB containing 100 µg/ml ampicillin. Plate was incubated for overnight at 37°C. Some single colonies were picked and grew in 5 ml of LB containing 100 µg/ml ampicillin. Tubes were incubated at 37°C for overnight. Bacterial cells containing plasmid were extracted using Qiagen mini prep. Plasmids were double digested with *NotI* and *XhoI* and run agarose gel electrophoresis for determining the insert. The plasmids containing insert were sent for sequencing.

ScFv genes from pMOD phagemid vector was digested with *NcoI* and *NotI* and ligated to the *NcoI* - *NotI* digested pET21d+ contain SaDI gene. Ligated vector was transformed into *E. coli* DH5αF' and plated onto LB containing 100 µg/ml of ampicillin. Plated cells were incubated at 37°C for overnight. Single colony was picked and cut check. Positive transformed cells were sent for sequencing. The production of scFv-SaDI was previously described.

### 3.2.8 Flow cytometry analysis of 152-GFP fusion antibody

HL-60 cells were blocked with human IgG for 30 minutes at room temperature. Cells were washed with 1xPBS containing 0.1% BSA. The 10 µg and 1µg of 152-GFP protein were incubated to cells in total volume 100 µl for 1 hour on ice. Cells were washed by centrifugation. Cells were suspended with 1xPBS containing 0.1% BSA. Debris were stained by 1mg/ml of PI in 1:1000 dilution for dead cells exclusion. Ten thousand events were acquired. Anti-aflatoxin conjugated GFP, 3E3-GFP, was used as isotype control.

### 3.2.9 Surface expression analysis

After written informed consent and approval by the Ethical Review Board, Sapphasitthiprasong hospital, Ubon Ratchathani province. Bone marrow samples from AML and non-AML patients were collected from patients at primary diagnosis. Patient characteristics were summarized in Table 8. Bone marrow mononuclear cells (BMMC) from AML patients and peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by density gradient centrifugation according to instruction protocol. Briefly, bone marrow or whole blood was diluted with 1XPBS in equal volume. Diluted blood was overlaid into tube containing Ficoll Plague plus solution. The tube was centrifuged at 1,800 rpm, 20°C for 30 minutes with break off. Buffy coat phase was transferred to new tube. Buffy coat containing mononuclear cells were washed with 10 ml of 1x PBS. The tube was centrifuged at 1,800 rpm for 10 minutes. Supernatant was discarded and resuspend cell pellet with 10 ml of 1XPBS. The tube was centrifuged again and discarded supernatant cells were cryoconserved at -80°C in RPMI media containing 10% dimethyl sulfoxide or immediately used.

Surface expression of the target to which y1HL63152 scFv antibody binds was assessed by flow cytometry using a 152-GFP fusion antibody. Patients and healthy donors were analyzed using the following antibodies: anti-CD45 and anti-CD33. A $CD45^{DIM}/SSC^{LOW}$ gate was used to limit the analysis to myeloid progenitor cells according to previous report (Krupka et al. 2014). Surface expression analysis was performed on an Attune NxT focusing flow cytometer. Median Fluorescent Intensity (MFI) values were determined using Attune NxT software. The MFI ratio was calculated by MFI of 152-GFP divided by MFI of 1A8-GFP which is an isotype control. The MFI ratio of each sample was plotted into graph and the average value of MFI in each group was determined. Statistical analysis was calculated by Dunn's multiple comparisons test.

HL-60 and mononuclear cells were blocked with human IgG for 30 minutes at room temperature and then cells were washed and discarded supernatant. Cells were stained in total volume 100 µl with 0.2 µg/µl of 152-GFP, 3 µl of APC anti-human CD45 Antibody (Biolegend, USA), and 3 µl of PE anti-human CD33. The stained cells were incubated on ice for 1 hour. After that, cells were washed and resuspended with 0.1%BSA in PBS. The intermediate CD45 positive population was gated. These cell population were used to determine the mean fluorescent intensity against 152-GFP fusion antibody. A ten thousand events of CD45 intermediate positive population were acquired.

**3.2.10 Internalization assay**

HL-60 cells were incubated with 152-GFP at 37°C 5% $CO_2$ for 2 hours. Cells were striped with 0.05% trypsin for 10 min and inactivated by complete media and washed 1 time with PBS. Cells were dropped on glass slide and the slow fade was added. Cells were observed under fluorescence microscope.

**3.3.11 HL-60 cells differentiation**

A $5x10^5$ cells/ml of HL-60 were cultured in IMDM medium supplemented with 5% FBS and 1.5% Dimethyl sulfoxide (DMSO) at 37°C, 5% $CO_2$ for 72 hours. Cells at 0 hour and 72 hours were collected to investigate the binding of 152-GFP and the expression of CD11b, a granulocyte marker. Cell were blocked with human IgG for 30 minutes at room temperature. Cells were washed by centrifugation 330 g for 5 minutes and discarded supernatant. Cells were incubated in total volume 100 µl with 0.2 µg/µl of 152-GFP and 3 µl of 0.2 µg/µl APC anti-human CD11b Antibody for 1 hour. Cells were washed and resuspended with 0.1% BSA in PBS and 1mg/ml of PI in 1:1000 dilution . Stained cells were determined by flow cytometer.

**3.2.12 Cell viability testing**

A 50 µl of $2x10^4$ cells of HL-60 was seed into 96 well plate in IMDM supplemented with 5% FBS and incubated at 37°C, 5% $CO_2$ for overnight. The proteins

including y1HL63152 scFv, CD33 scFv, 152-SaDI, CD33-SaDI, and SaDI protein were diluted in IMDM medium and seed into the cultured plated in total volume 100 µl and further incubated for 24 hours. HL-60 was treated for 24 hours. Cells were measured cell viability by the Muse cell count and viability kit according to manufacturer's instruction and detected by flow cytometer.

**3.2.13 Apoptosis assay**

Five milliliters of $5x10^5$ HL-60 cells/ml were cultured in IMDM supplemented with 20% FBS and treated with 1 µM and 10 µM of 152 scFv, 3E3 scFv, 152-SaWT, and Doxorubicin. Cells were incubated at 37°C, 5% $CO_2$ for 72 hours. The typhan blue exclusion test was conducted at 24, 48, and 72 hours of incubation. The apoptosis assay was done by staining the treated cells with Annexin V and PI followed by flow cytometry analysis.

**3.2.14 MTT cell proliferation assay**

A ten thousand cells per well of HL-60 was seed into 96 well plate in IMDM supplemented with 20% FBS and incubated at 37°C, 5% $CO_2$ for overnight. The proteins including y1HL63152 scFv, 3E3 scFv, 152-SaWT protein and Doxorubicin were diluted in media to be final concentration 1µM and 10 µM. These diluted proteins were seed into the cultured plated and further incubated for 24,48, and 72 hours. The MTT dye was added into the culture plates at final concentration 0.05 µg/ml. Plates was incubated for 4 hours and then the supernatant was discarded followed by adding 100 µl of DMSO to solubilize the formazan pellet. Plate was measured absorbance at 590 nm.

**3.2.15 Cells and culture method**

HL-60 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) medium supplemented with 20% fetal bovine serum (FBS) and 1X penicillin streptomycin. OCIM-1 cells were maintained in IMDM supplemented with 10% FBS and 1X penicillin streptomycin. THP-1 and Jurkat were maintained in RPMI 1640 (Gibco)

supplemented with 10% FBS and 1X penicillin streptomycin. All cell lines were cultured at 37°C, 5% CO2 and sub-cultured in every 2 days to remain cell density between $5x10^5$ to $1x10^6$ cell/ml.

### 3.2.16 SDS-PAGE and western blot preparation

The SDS-PAGE gels were made according to SureCast™ Handcast System user guide (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/surecast system_UG.pdf). Protein ladder and protein of interest were loaded in to the gel. The voltage was set up at 100 V. Gels were run until the dye front reaches the bottom of the gel. Gels were disassembled and proceed with Coomassie staining or Western blot procedures. To do Western blot, the PVDF membrane was activated by soaking with absolute methanol for 1 minute and then rinsed membrane with transfer buffer. The stack was prepared as follows: sponge, filter paper, gel, membrane, filter paper, and sponge, respectively. Proteins were transferred for 2 hours at 100 V. The membrane was blocked with 5% Skim milk in 1xPBS for 1 hour at room temperature or 4°C for overnight. The membrane was washed three times with 1xPBS and then incubated with rabbit anti-myc in 1:1000 dilution for 1 hour. The membrane was washed for 3 times with 1xPBS containing 0.05% Tween and 2 times with 1xPBS. The membrane was incubated with anti-rabbit conjugated with HRP in 1:1000 dilution for 1 hour. The membrane was washed for 3 times with 1xPBS containing 0.05% Tween and 2 times with 1xPBS. The chemiluminescent substrate was added to membrane and detected signal by exposing with X-ray film. In case of detection immunotoxin, His probe conjugated with HRP was used to probe the protein and detected chemiluminescent signal by Gel Doc machine.

# CHAPTER IV

# RESULTS

## 4.1 Generation of recombinant scFv antibody against HL-60 AML cells by phage display technology

### 4.1.1 Affinity selection of scFv antibody specific for HL-60 cells

To select the potentially scFv antibody fragment binding to AML cell, a naive phage display scFv antibody library named Yamo I (Pansri et al. 2009) was screened using viable HL-60 AML cells. The library is based on pMOD phagemid vector containing of scFv antibody fragment, which fused to phage pIII coat protein as shown in Fig. 4.1. *E. coli* cells strain TG1 containing the phagemids was infected with M13KO7 helper phage for the production of scFv-presenting phage particles suitable for panning. The scFv pIII fusion protein is transcriptional controlled under the lactose promoter. The recombinant protein is easily detected and purified a histidine and myc tag at the downstream of the scFv antibody fragment.

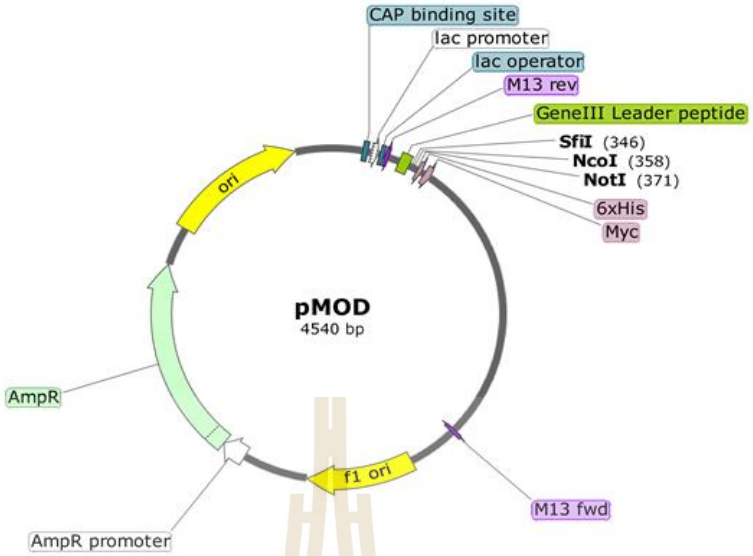



**Figure 4.1** Schematic of pMOD phagemid vector. The phagemid vector is transcriptional controlled under the lactose promoter and terminator. The scFv insert is joined between SfiI and NotI cloning site which is in-frame to the gene encoding the pIII phage coat protein (gIII). The myc, histidine tag and amber termination codon (TAG) are located in the downstream of the gene.

To do bio-panning, the library was negative selected with lymphoblastic leukemia cell line, Jurkat, followed by positive selection on intact HL-60 cells. Recovered phage of each round was amplified and used for further round of bio panning. After three rounds of selection, the enrichment factor was calculated based on the titer of input and recovered phage suspensions. The recovered phage ratio between HL-60 target cell and Jurkat was increased from 0.53 to 30.13 and 237.5-fold in the first, second and third round of biopanning, respectively (Table 4.1). The scFv library was strongly enriched for HL-60 specific clones by determining of polyclonal scFv antibody in each selection round. The Polyclonal scFv antibody in the first, second and third round of bio panning were induced and tested specific binding against HL-60 cells. The production of scFv antibody was investigated by Western blotting as shown in Fig. 4.2. It demonstrated that the scFv antibody from the first round did not have positive signal whereas scFv antibody from the second and the third round showed positive signal in the same molecular weight as isotype control.

The flow cytometry analysis indicated that scFv antibody from the third-round bio panning increased fluorescent intensity compared to scFv antibody from the first and the second that showed signal same as secondary antibody background (Fig. 4.3). These results indicated that scFv antibody from the third round might have some clone that specific to target cells. So, pooled scFv antibody was used for monoclonal scFv antibody production in further step.

**Table 4.1** Overview of screening of antibody against the target.

| Round | Target | Input | Output | Output/Input | HL-60/Jurkat |
|---|---|---|---|---|---|
| | HL-60 | $5x10^{13}$ | $2.6x10^{5}$ | $5.2x10^{-7}$ | |
| 1st round | Jurkat | $5x10^{13}$ | $4.9x10^{5}$ | $9.8x10^{-7}$ | 0.53 |
| | HL-60 | $3.75x10^{13}$ | $4.4x10^{7}$ | $1.17x10^{-6}$ | |
| 2nd round | Jurkat | $3.75x10^{13}$ | $1.46x10^{7}$ | $3.83x10^{-8}$ | 30.13 |
| | HL-60 | $5x10^{13}$ | $1.9x10^{7}$ | $3.8x10^{-7}$ | |
| 3rd round | Jurkat | $5x10^{13}$ | $8x10^{4}$ | $1.6x10^{-9}$ | 237.5 |

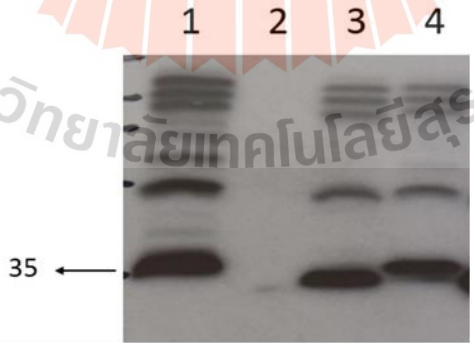



**Figure 4.2** Western blot result of soluble scFv antibodies. Western blot result of soluble scfv antibodies from bacterial supernatant of isotype control (lane 1), polyclonal scfv antibody from the first, second, and third round (lane 2, 3, and 4, respectively).

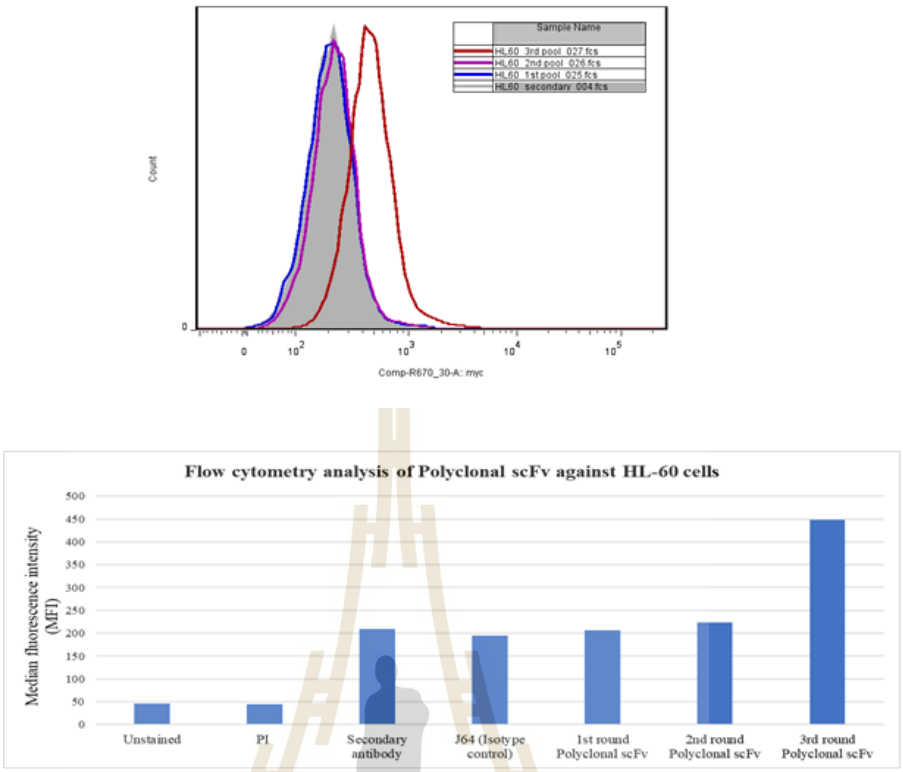


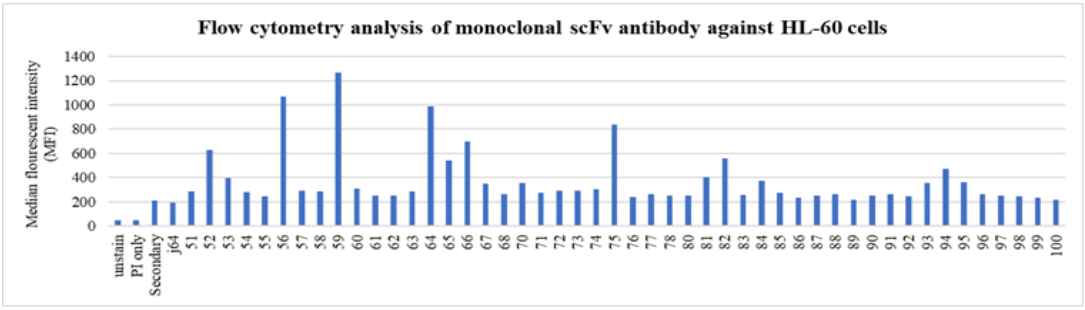

**Figure 4.3** Flow cytometry analysis of polyclonal scFv antibodies. (a) Overlay histogram of polyclonal scFv antibody from the first second and third round bio-panning in blue pink and red curve, respectively. (b) Median fluorescent intensity (MFI) of each antibody were plot into the graph.

### 4.1.2 Screening of bound soluble scFv antibodies

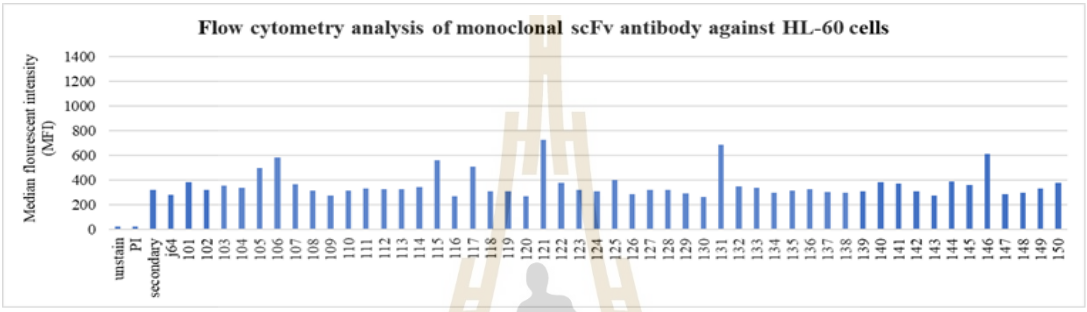
Individual binders were identified by picking single colonies of bacteria containing phagemid vector from the third round and inducing monoclonal scFv antibody expression in deep well plate. The specificity of clones isolated by cell-based bio panning on HL-60 was tested by flow cytometry. In this experiment, the supernatant containing scFv of clone 1-180 were incubated with HL-60 cells and stained with rabbit anti-myc antibody in 1:1000 dilution. After that, the donkey anti-rabbit conjugated with Alexa flour 647 was used as a secondary antibody. The supernatant containing scFv of clone 181-276 were incubated with HL-60 and stained with anti-Histidine conjugated with dylight 488 in 1:1000 dilution. To classify the positive clone, the clone that showed two time higher than secondary antibody control was used as a threshold value. The result showed that there were 30 out of 276 clones

(10.86%) performed positive binding signal as shown in Fig. 4.4a-e. These clones were induced expression in 5 ml culture and checked specificity to target cells again. The result showed that clones y1HL63152, y1HL63214, y1HL63222, y1HL63228 and y1HL63255 still showed high signal (Fig. 4.4f). These 5 clones were sent for sequencing. There was a stop codon and out of frame inside the clone y1HL63214 gene and the DNA sequence of clones y1HL63152 and y1HL63222 were identical. The others were a complete scFv antibody fragment gene. So, clones y1HL63152, y1HL63228, and y1HL63255 were further checked cross binding with OCIM-1 (another AML cell line), Jurkat (a non-AML cell line), Peripheral blood mononuclear cell (PBMC, a representative of normal blood cells), and SHSY5Y (a neuroblastoma cell line, representative of non-blood cancer cells). The result revealed that clones y1HL63152 and y1HL63255 bound specifically to HL-60. Clone y1HL63228 bound to HL-60, OCIM-1, and Jurkat but not bound to PBMC and SHSY5Y (Fig. 4.5).

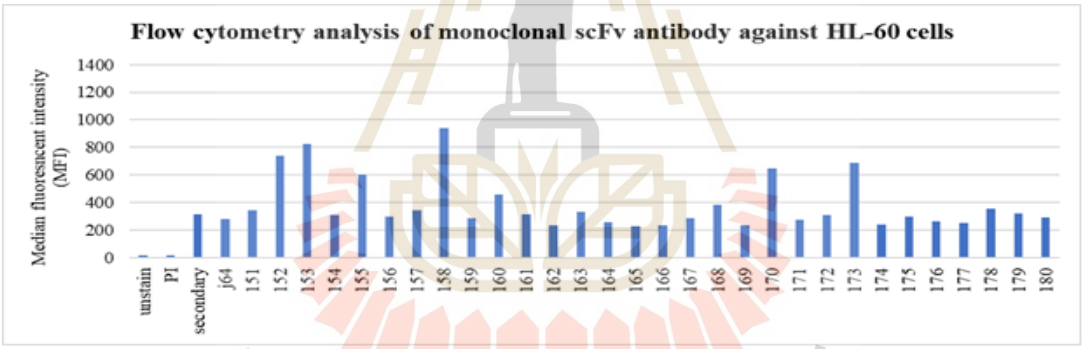
**a.**




**b.**




**c.**




**d.**

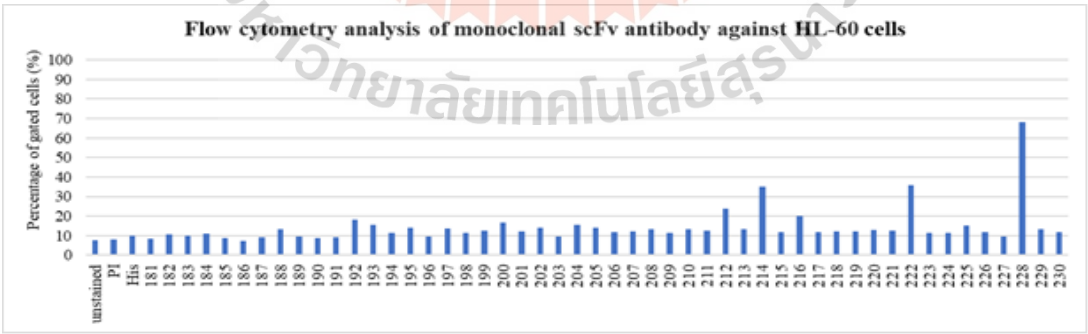
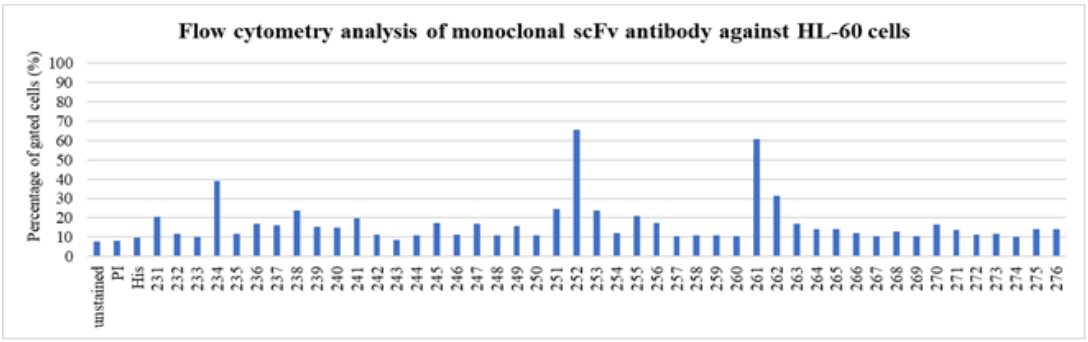





**Figure 4.4** Screening of soluble scFv antibody against HL-60 cells. Clone 1-180 (a-c) were plotted according to their median fluorescent intensity (y axis), clone 181-276 (d-e) were plotted according to their percentage of gated cells (y axis). The comparison graph of scFv binding to HL-60 in 200 µl (orange bar) and 5 ml (blue bar) were plotted (f).

**e.**




**f.**

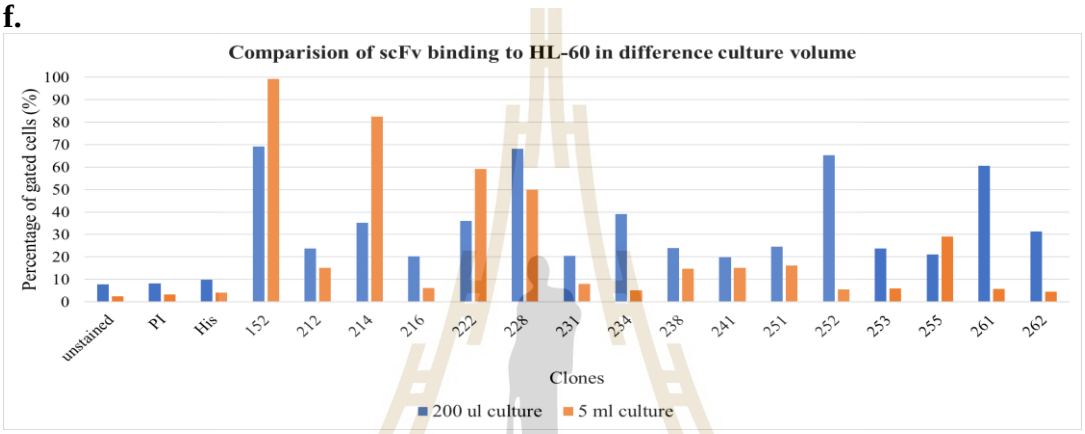



**Figure 4.4** (continued).

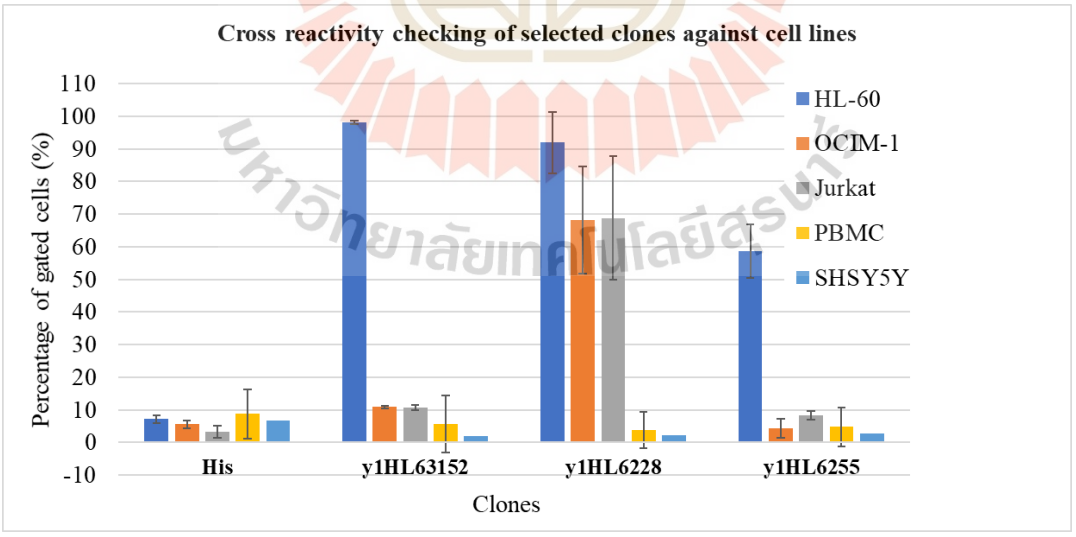



**Figure 4.5** Cross reactivity of soluble scFv antibody against difference cell lines.

### 4.1.3 Sequence analysis of the selected scFv antibodies

Five positive clones (y1HL63152, y1HL63214, y1HL63222, y1HL63228, y1HL63255) were sent for sequencing. Clone y1HL63214 contained stop codon and out of frame inside the gene. Clone y1HL63152, y1HL63222, y1HL63228, y1HL63255 showed complete scFv gene. However, clone y1HL63152, y1HL63222 showed DNA sequence identical. So, finally, there were 3 clones showed specific binding to target cells and consist of complete DNA sequence. The antibodies were analyzed by IMGT/V-QUEST website. Variable heavy chain and light chain of clone y1HL63152 is in IGHV5-51*01and IGLV2-14*01 family, respectively. The percentage identity to germ line of variable heavy chain and light chain is 99.31% (286/288 nucleotides) and 99.31% (286/288 nucleotides), respectively. Variable heavy chain and light chain of y1HL63228 is in IGHV3-48*03 F and IGLV6-57*02 F family, respectively. The percentage identity to germ line of variable heavy chain and light chain is 99.31% (286/288 nucleotides) and 98.97% (288/291 nucleotides), respectively. Variable heavy chain and light chain of y1HL63255 is in IGHV6-1*01 F and IGLV1-40*01 F family, respectively. The percentage identity to germ line of variable heavy chain and light chain is 98.32% (292/297 nucleotides) and 97.92% (282/288 nucleotides), respectively. Amino acid sequence alignment of 3 clones is shown in Fig. 4.6. The amino acid of frame work and CDR were categorized in table 4.2. Homology modeling of the three-dimension (3D) structure of scFv clone y1HL63152 was generated by the SWISS-MODEL website (Schwede et al. 2003). Models were visualized by PyMOL program.

Together these result, clone y1HL63152 showed specific binding to HL-60 target cells and higher signal than clone y1HL63255. So, clone y1HL63152 was selected for further analysis.

**a.**

```
                                    CDR1                                CDR2
152     MAQVQLVQSGAEVKKPGESLKISCKGSGYSFT--SYWIGWVRQMPGKGLEWMGIIYPG-D       57
228     MAQVQLVQSGGGLVQPGGSLRLSCAASGFTFS--SYEMNWVRQAPGKGLEWVSYISSS-G       57
255     MAQVQLQQSGPGLAKPSQTLSLTCAISGDSVSNNSAAWNWIRQSPSRGLEWLGRTYYRSK       60
        ****** ***  : :*. :* ::*  ** :.:  *   .*:** *.:****:.

                                                        CDR3
152     SDTRYSPSFQGQVTISADKSISTAYLQWSSLKASDTAMYYCARH--QGSWYSDAFDIWGQ       115
228     STIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGGHDDFWY---FDYWGQ       114
255     WYNDYAVSVKSRITINPDTPKNQFSLQLNSVTPEDTAVYYCAREN----IHLDAFDIWGQ       116
            *: *.:.:.**. *.  .   ** .*:  .***:*****       :   ** ***

                                                CDR1
152     GTTVTVSSGGGGSGGGGSGGGGSQSALTQPASVSGSPGQSITISCTGTSSDVGGYNYVSW       175
228     GTLVTVSSGGGGSGGGGSGGGGSNFMLTQPHSVSESPGKTVTISCTGSSGSI-ASNYVQW       173
255     GTMVTVSSGGGGSGGGGSGGGGSQAVLTQPSSVSGAPGQRVTISCTGSSSNIGAGYDVHW       176
        ** ********************:  **** *** :**: :******:*..: .   * *

                    CDR2                                        CDR3
152     YQQHPGKAPKLMIYDVSNRPSGVSNRFSGSK--SGNTASLTISGLQAEDEADYYCSSYTS       233
228     YQQRPGNAPTTVIYEDNQRPSGVPDRFSGSIDSSSNSASLTISGLRTEDEADYYCQSYDS       233
255     YQQLPGTAPKLLIYGNSNRPSGVPDRFSGSR--SGTSASLAITGLQAEDEADYHCQSYDS       234
        *** **.**. :**  .:***** :*****   *..:***:*:**::******:*.** *

152     S--STLVFGGGTKVTVLGAAAHHHHHHGAAGPEQKLISEEDLNGTA*     277
228     S--NLVVFGGGTKLTVLRAAAHHHHHHGAAGPEQKLISEEDLNGTA*     277
255     SLSGSGVFGTGTQLTVLRAAAHHHHHHGAAGPEQKLISEEDLNGTA*     280
*  .  *** **::*** ****************************
```

**b.**

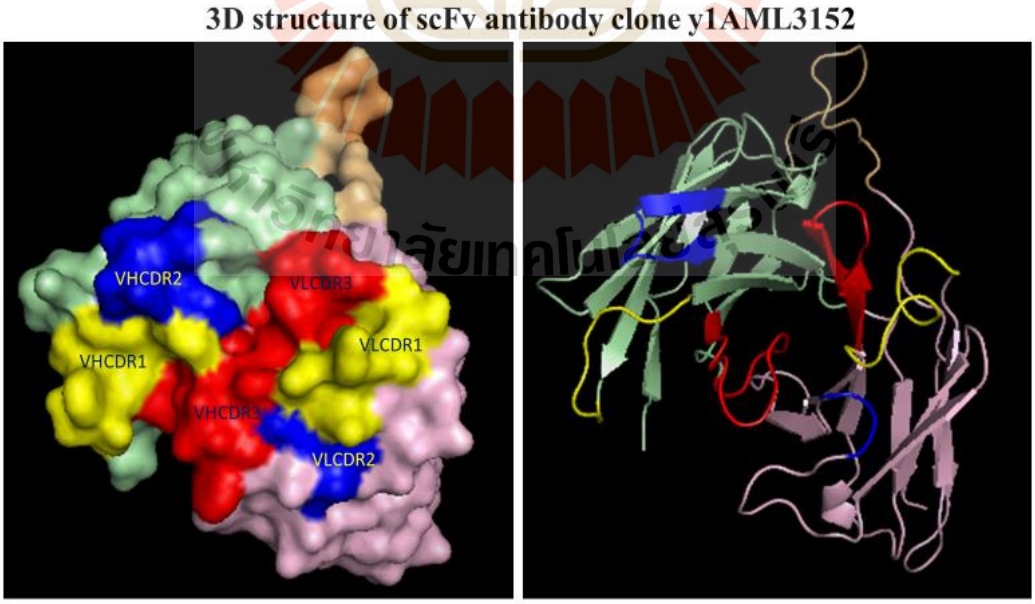



**Figure 4.6** Amino acid sequence analysisof the selected scFv antibodies. The sequence alignment of the selected clone (a) and 3D structure modeling of the y1HL63152 antibody (b).

**Table 4.2** Sequence analysis of the selected clones.

| Clone | FR1 | CDR1 | FR2 | CDR2 | FR3 | CDR3 | AA change | Germline | Family |
|---|---|---|---|---|---|---|---|---|---|
| 152 | QVQLVQSGAEVKKPGESLKISCKGS | GYSFTSYW | IGWVRQMPGKGLEWMGI | IYPGDSDT | RYSPSFQGQVTISADKSISTAYLQWSSLKASDTAMYYC | ARHQGSWYSDAFDI | 1 | IGHV5-51*01 | VH5 |
| 228 | QVQLVQSGGGLVQPGGSLRLSCAAS | GFTFSSYE | MNWVRQAPGKGLEWVSY | ISSSGSTI | YYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYC | ARGGHDDFWYFDY | 2 | IGHV3-48*03 | VH3 |
| 255 | QVQLQQSGPGLAKPSQTLSLTCAIS | GDSVSNNSAA | WNWIRQSPSRGLEWLGR | TYYRSKWYN | DYAVSVKSRITINPDTPKNQFSLQLNSVTPEDTAVYYC | ARENIHLDAFDI | 3 | IGHV6-1*01 | VH6 |
| 152 | QSALTQPASVSGSPGQSITISCTGT | SSDVGGYNY | VSWYQQHPGKAPKLMIY | DVS | NRPSGVSNRFSGSKSGNTASLTISGLQAEDEADYYC | SSYTSSSTLV | 1 | IGLV2-14*01 | VL2 |
| 228 | NFMLTQPHSVSESPGKTVTISCTGS | SGSIASNY | VQWYQQRPGNAPTTVIY | EDN | QRPSGVPDRFSGSIDSSSNSASLTISGLRTEDEADYYC | QSYDSSNLVV | 2 | IGLV6-57*02 | VL6 |

## 4.2 Characterization of selected scFv antibody from pET expression system

The scFv genes of clone y1HL63152 and anti-CD33 scFv antibody which was taken from US patent (US7557189B2) were cut with *NcoI* and *NotI* restriction enzymes, ligated into pET21d+ and then transformed to *E. coli* DH5αF' competent cells. A three clones of the transformed bacteria were picked and then plasmids were extracted. These plasmids were cut to investigate the insert. An agarose gel analysis showed that the ligated plasmid contained insert with the expected size at approximately 800 bp. (figure 10 a-b). The recombinant vectors were transformed to *E. coli* strain Shuffle B cell and induced scFv expression. The recombinant proteins were purified by IMAC. The molecular weight of y1HL63152 and anti-CD33 scFv antibody was approximately 27 KDa (Fig. 4.7 c-d).

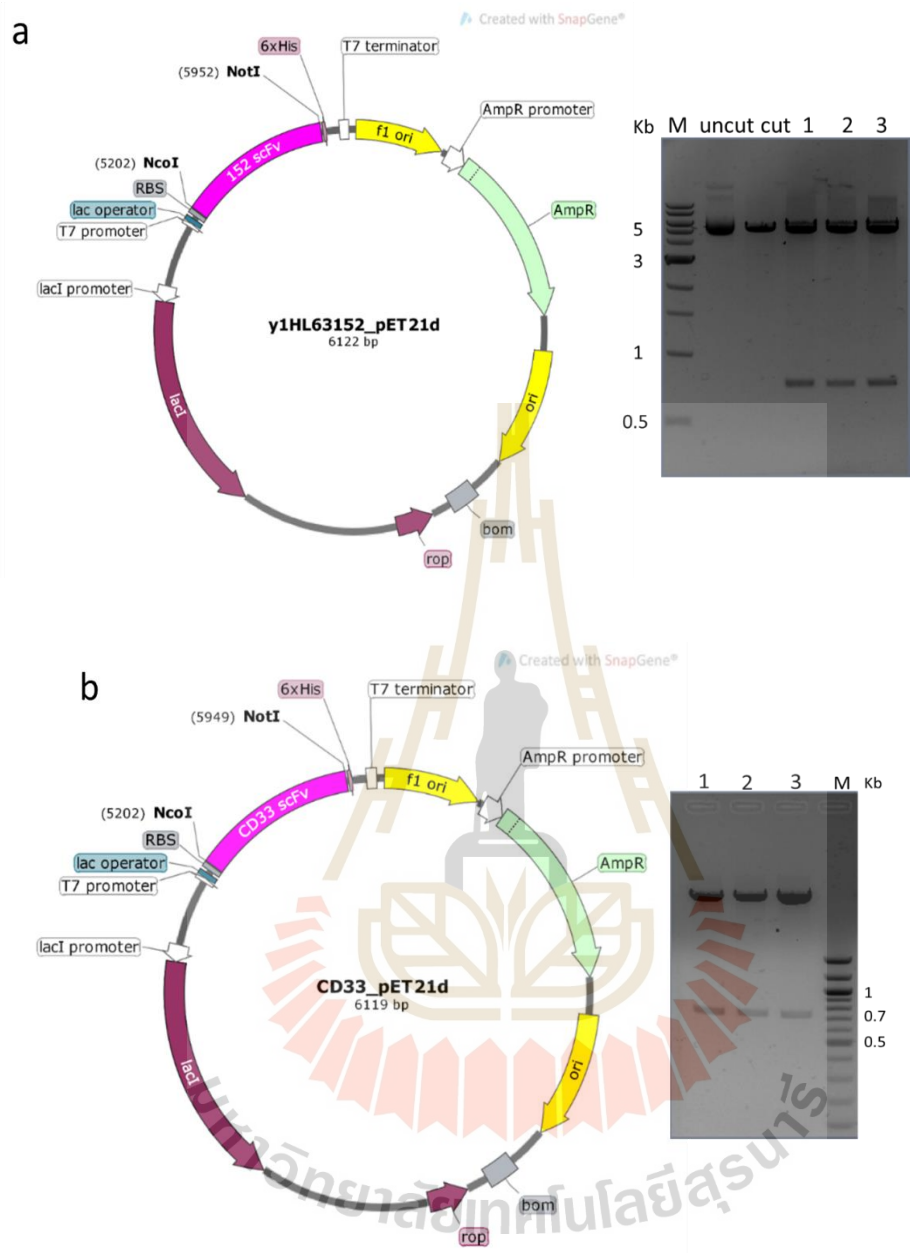
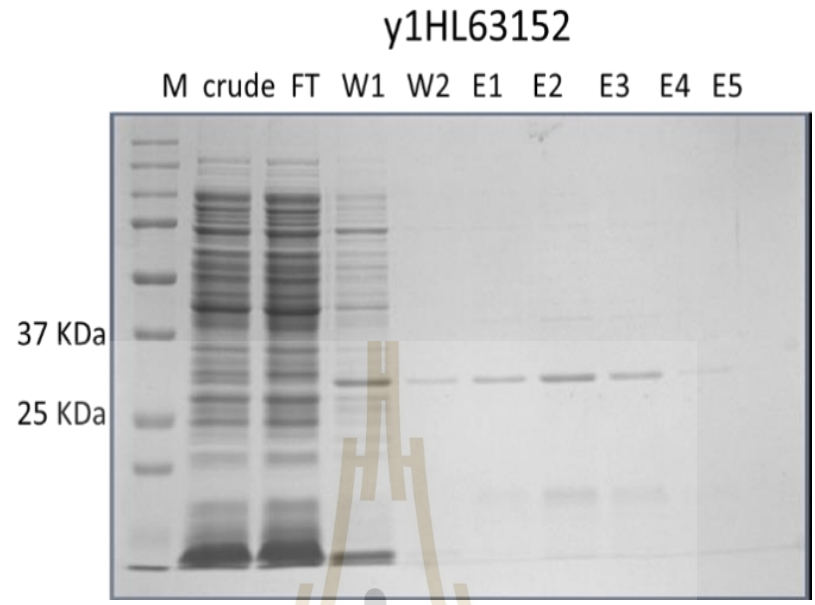



**Figure 4.7** Diagram of plasmid map of scFv expression vector and SDS PAGE analysis. The scFv gene of y1HL63152 (a) and anti-CD33 (b) were sub-cloned into pET21d+ vector between *NcoI* and *NotI* restriction sites. Agarose gel electrophoresis showed the subcloned vector contained scFv insert. 1-3 indicated an individual transformed clone. SDS-PAGE of purified scFv of y1HL63152 (c) and CD33 (d), FT: flow through, W1: wash, E1-E5: elute fraction.

**c.**




**d.**

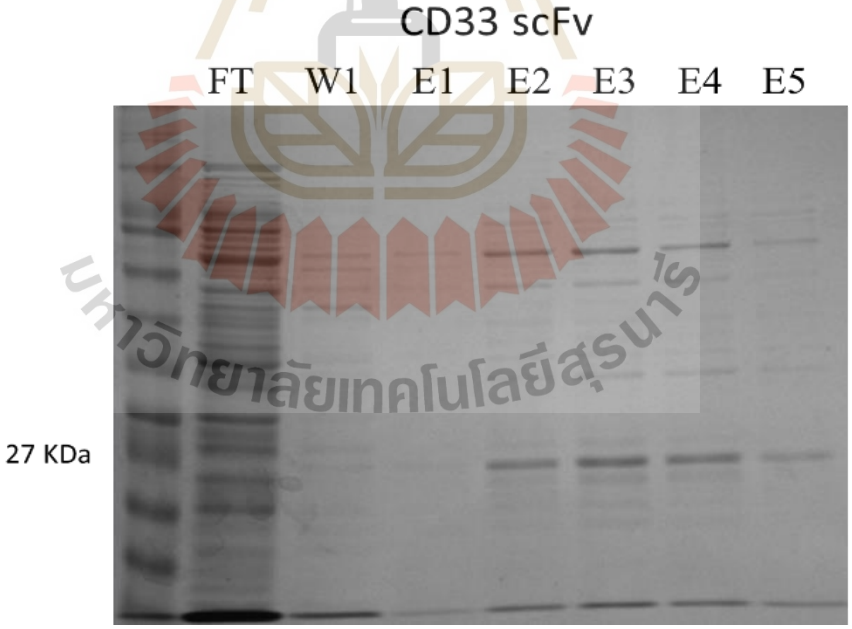



**Figure 4.7** (continued).

### 4.2.1 Binding test of scFv antibody against HL-60 cells

The scFv antibodies in final concentration 0.1 µg/µl were tested against HL-60 cells. The result showed that the average value of y1HL63152, anti-CD33, and 3E3 scFv antibody were 83.66±5.57, 96.45±2.69, and 9.98±9.33, respectively. These experiments were done for three times independently. The histograms of flow cytometry analysis are shown in Fig. 4.8.

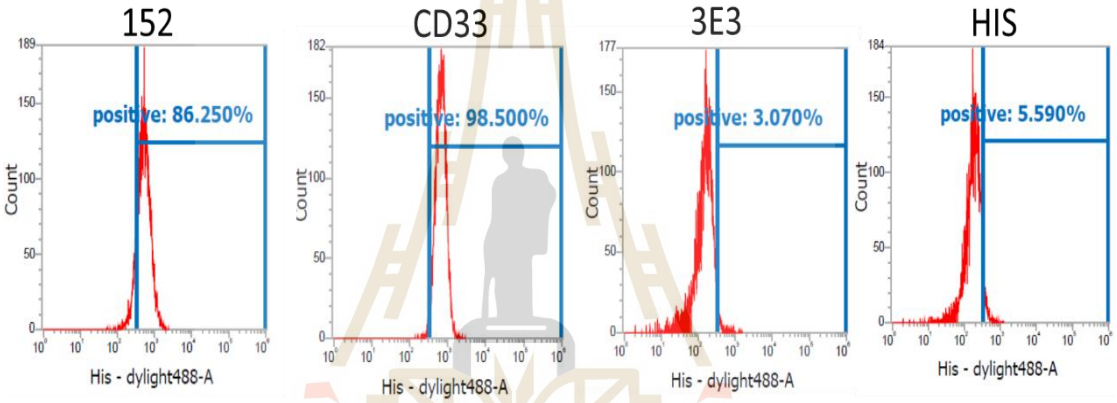



**Figure 4.8** Flow cytometry analysis of y1HL63152 scFv, anti-CD33 scFv, and 3E3 scFv against HL-60. The purified scFv antibodies were incubated with HL-60. Cells were washed and incubated with secondary antibody conjugated with fluorescent dye (anti-His-dylight 488). The fluorescent signal was detected by flow cytometer. The result showed the percentage of positive cells against each scFv antibody. Blue gate was set by using secondary antibody as a signal background.

To compare the binding ability of the scFv antibody, purified scFv antibodies were diluted in 100-fold serial dilution from 0.0001- 20 μM, incubated into HL-60 cells, and analyzed by flow cytometer. The fluorescent intensity was plotted into curve. The result demonstrated that the logEC50 of y1HL63152 and anti-CD33 scFv antibody was 0.3259 and 0.1672, respectively. It showed that the EC50 of clone y1HL63152 was 1.94 times higher than anti-CD33 scFv antibody (Fig. 4.9).

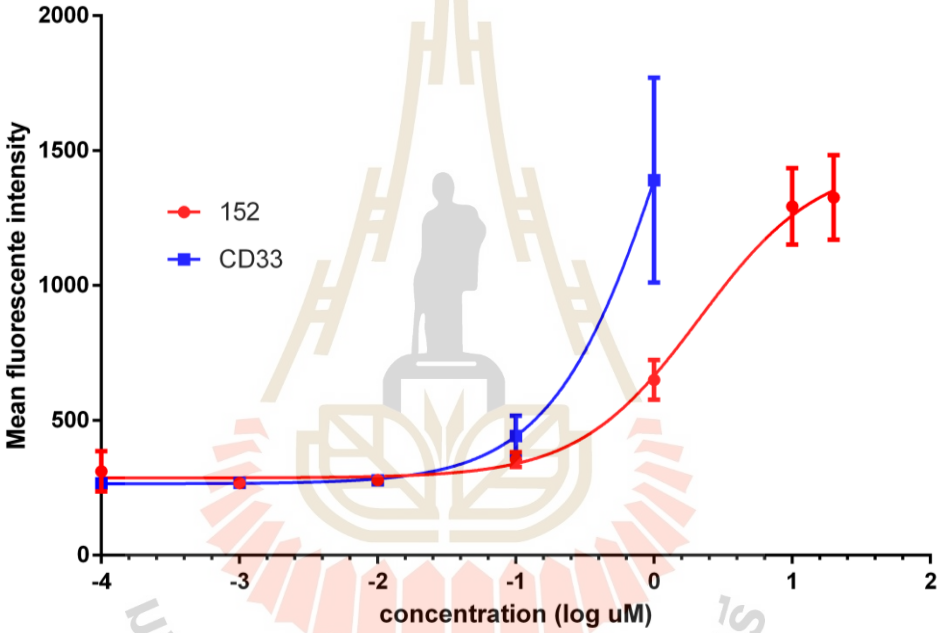



**Figure 4.9** The EC50 determination of y1HL63152 and CD33 scFv antibody. Varies concentration of scFv antibodies were tested with HL-60 and analyzed by flow cytometer. The mean fluorescent intensity was plot in Y axis and protein concentration in log scale was plot in X axis. The signal of y1HL63152152 and anti-CD33 scFv antibody was shown as a red and blue line, respectively.

### 4.2.2 Cross reactivity analysis

To check cross reactivity of scFv antibodies, 0.1 µg/µl of purified antibodies were incubated with AML cell lines including HL-60, OCIM-1, and THP-1 and non-AML cell line which is Jurkat cells. The result showed that y1HL63152 scFv antibody bound specifically to HL-60. Whereas, CD33 scFv bound to HL-60, OCIM-1 and THP-1 which are express CD33 on their surface but not bound to Jurkat. The 3E3 scFv antibody is antibody against aflatoxin so it did not bind to any cells. The histogram of flow cytometry analysis is shown in Fig. 4.10

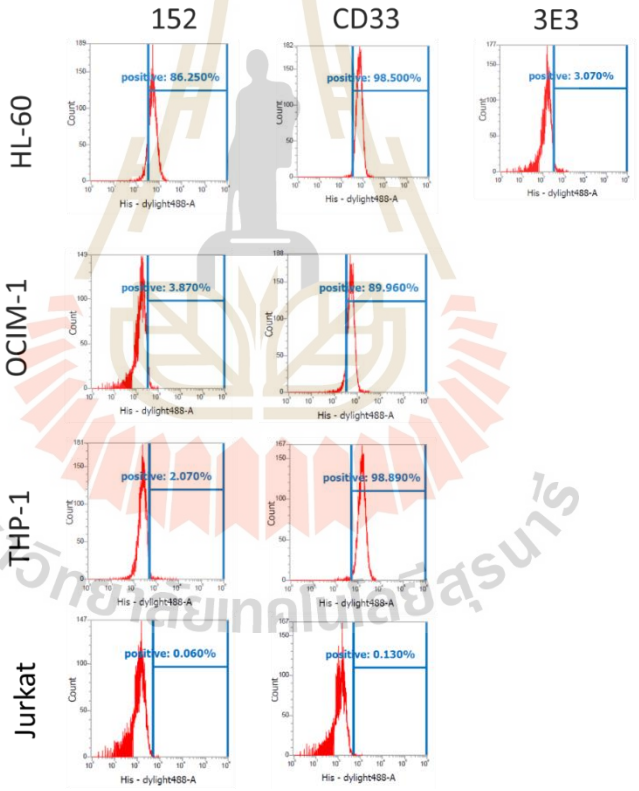



**Figure 4.10** Cross reactivity checking of y1HL63152 scFv antibody against different cell lines. Purified scFv antibodies were tested the binding to HL-60, OCIM-1, and THP-1 AML cell lines, and Jurkat, a non-AML cell line. Row indicates antibody use and column indicates cells type. Blue gate indicates the percentage of positive cells. Blue gate was set by using secondary antibody as a signal background.

### 4.2.3 Immunofluorescent staining of scFv antibody on the HL-60 cells

To investigate the localization of scFv antibody on HL-60, 0.2 µg/µl of y1HL63152 scFv antibody was stained to HL-60 target cells on ice. Nuclei were stained by Hoechst. Stained cells were visualized by confocal microscope. The result showed that scFv located on cells surface membrane (Fig. 4.11).

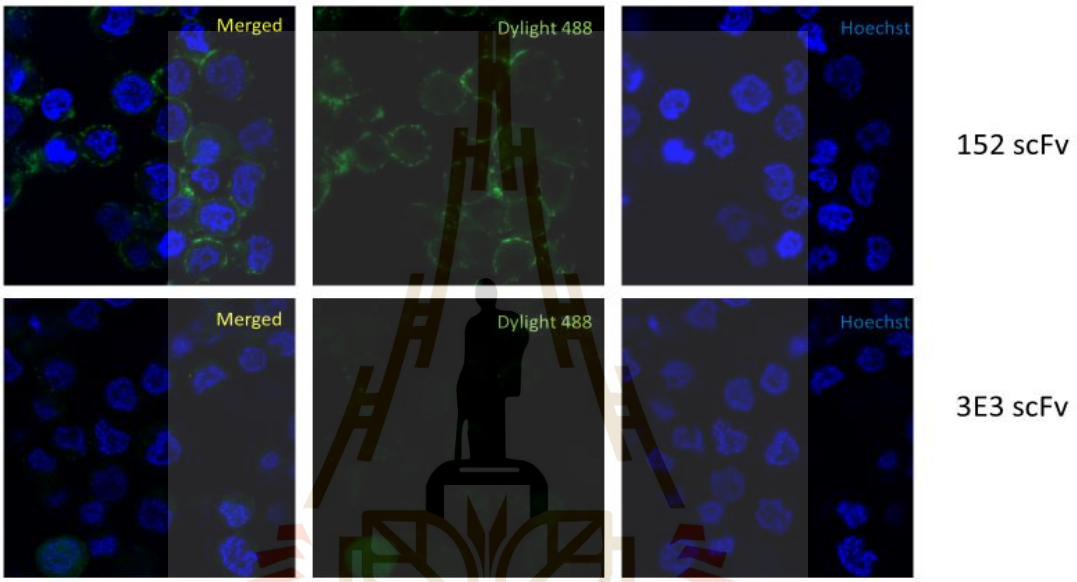



**Figure 4.11** The fluorescent staining of y1HL63152 scFv antibody to HL-60. The HL-60 cells were stained with scFv antibody and visualized under confocal microscope. The green signal was from anti-His dylight 488, a secondary antibody. The blue signal was from DNA DNA staining by Hoechst 33342. The exposure time and laser intensity were fixed in order to compare the signal between scFv and isotype control.

## 4.3 Engineering of anti-AML y1HL63152 scFv antibody

### 4.3.1 scFv-GFP

In order to produce scFv-GFP fusion protein to be used as one step detection, the scFv gene of y1HL63152 was sub-cloned into the in-house generated GFP expression vector, pWS-Green vector, between NcoI and NotI restriction sites. The construct used pET21d+ as a backbone. The backbone consists of a bacterial origin of replication (ori) and the ampicillin resistance gene (AmpR) for plasmid amplification and selection in *E. coli*. These constructs were regulated under T7 promotor. Histidine tag is encoded at C terminal of the construct. The scFv gene was located at *NcoI* and *NotI* sites and followed by EmGFP gene. The scFv-GFP construct is illustrated in figure 15. The agarose gel analysis after digestion with *NcoI* and *NotI* enzymes showed the desired scFv insert gene at 800 bp (Fig. 4.12).

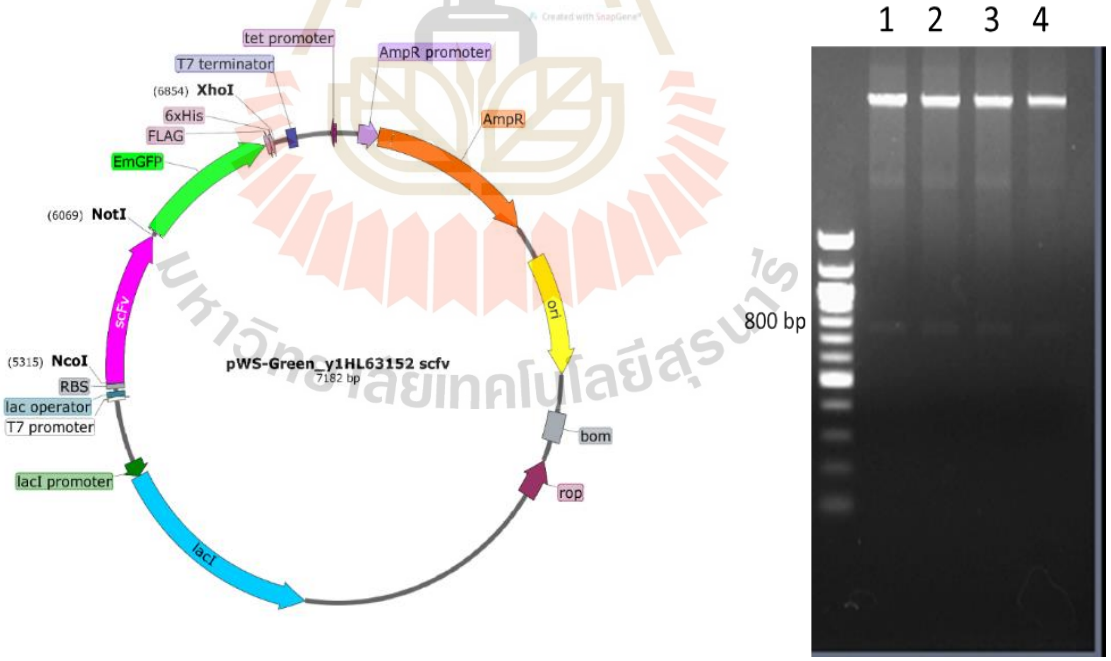



**Figure 4.12** Diagram of plasmid map of scFv-GFP expression vector. scFv gene was inserted into pWS-Green at NcoI and NotI site. Agarose gel electrophoresis showed the subcloned vector contained scFv insert. 1-4 indicated the individual clone that were cut check.

For 152-GFP, the expression was done in 3 bacteria host strains including Shuffle B cell, K12, and BL21(DE3) to investigate expression level and binding ability of scFv-GFP fusion protein which were expressed in different hosts. The result showed that all of host strains expressed protein in the same manner. The fluorescent signal and SDS-PAGE of purified protein in each host strain were shown in Fig. 4.13. The expected size of y1HL63152 scFv conjugated with GFP is 55.13 KDa.

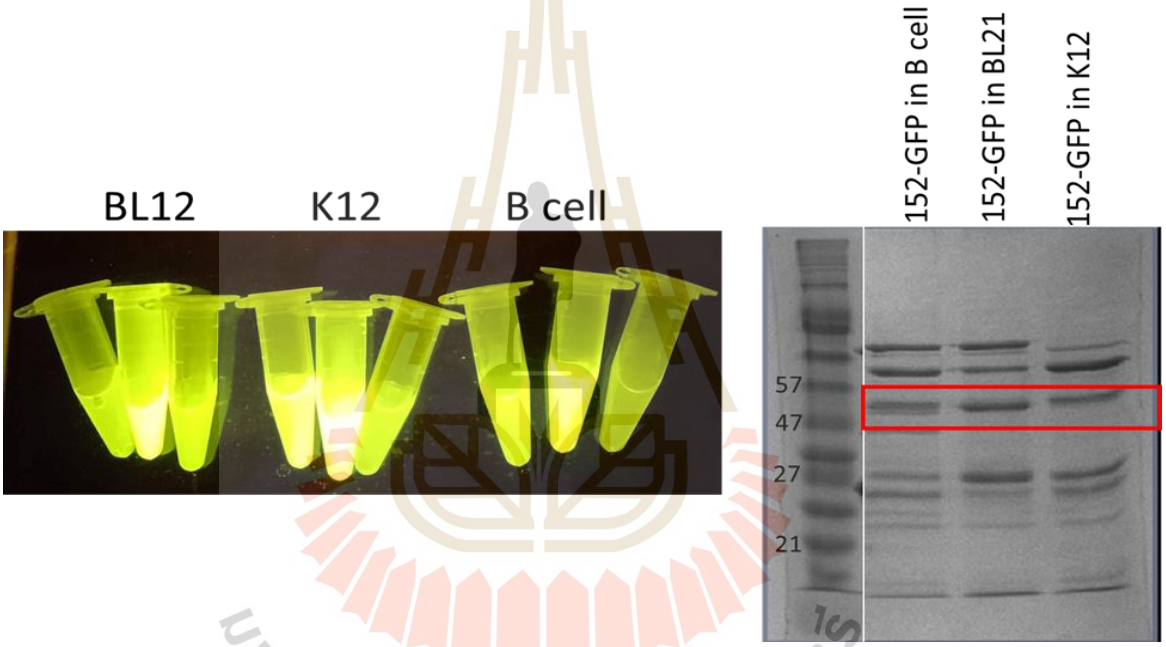



**Figure 4.13** Purification of 152-GFP in different expression hosts. Clone 152-GFP was expressed in different expression hosts including E. coli strain BL21(DE3), K12, and Shuffle B cells. The fusion proteins were purified by Ni-NTA column. The purified proteins were analyzed by SDS-PAGE (right) and observe fluorescent signal under Blue light LED epi illuminator (the first, second, and third tube in each host strain represent purified protein in elute 1-3 fraction) (left).

### 4.3.2 scFv-Sarcin

#### 4.3.2.1 Yeast expression of scFv-SaWT

To test cell cytotoxicity of scFv-toxin fusion protein, the scFv clone y1HL63152 linked with α-sarcin wild type (SaWT) was synthesized and cloned to pPICZαB vector at *NcoI* and *XbaI* sites. The size of the scFv-SaWT gene insert was 1,231 bp. The plasmid was amplified by transforming to *E. coli* Top10. To determine the insert, *NcoI* and *XbaI* were used to digest with the plasmid. Since there were two *NcoI* sites in the plasmid. So, the agarose gel analysis showed 2 bands at 1,254 and 860 base pair. To linearize the plasmid, *PmeI* was used. The cut band showed upper band than uncut plasmid. The agarose gel analysis result is shown in Fig. 4.14.

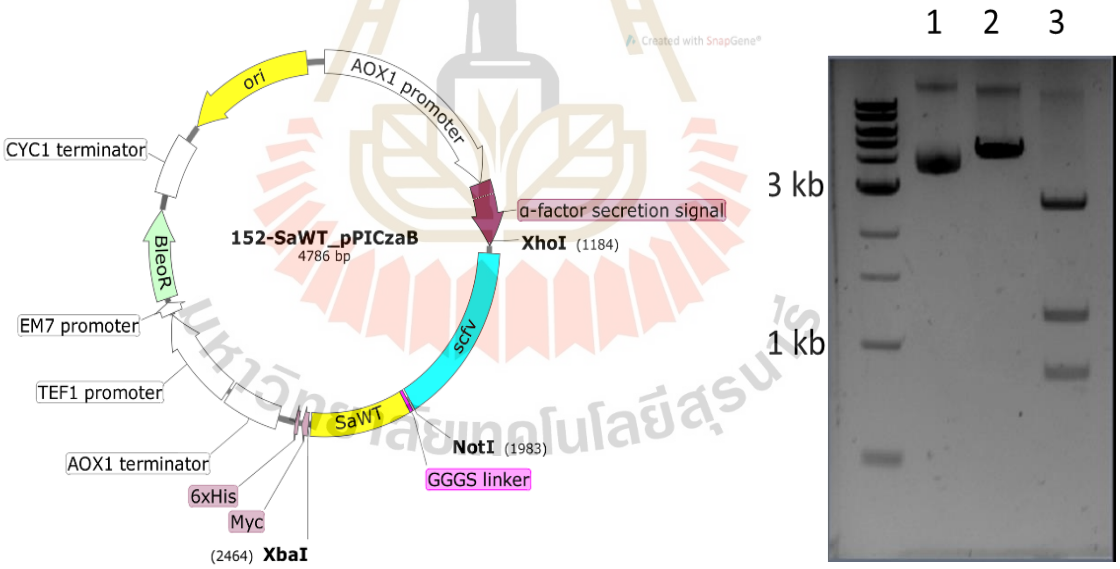



**Figure 4.14** Diagram of plasmid map of scFv-SaWT expression vector. Agarose gel electrophoresis showed the subcloned vector contained scFv insert. Lane 1: uncut vector, Lane 2: PmeI linearized vector, Lane 3: NcoI and XhoI digested vector.

The immunotoxin was expressed in *Pichia pastoris* system under Aox promotor induced by methanol. The plasmids containing recombinant 152-SaWT gene were electroporated to yeast cells and plated onto YPD agar supplemented with Zeocin. Plates were incubated for 3 days. Colonies were colony directed PCR by using AOX primer set. The result of agarose gel electrophoresis demonstrated that clone 2 and 5 showed bands at the expected size (Fig. 4.15a). The actual size was 1,786 base pair. So, these 2 clones were expressed in small scale. The small-scale expression was done in 100 mL culture. Supernatants and cell lysates were collected in every 24 hours. Supernatant and cell lysate of day 2 to 5 were analyzed by SDS-PAGE. The result showed that clone 5 secreted protein to supernatant and increased protein concentration from day 2 to day 5 (Fig. 4.15b). Clone 5 was up-scaled to express in 250 mL culture for 4 flasks. After 5 days of induction, 1 liter of culture was purified using His affinity chromatography. Flow through, washed and eluted fraction were collected and analyzed by SDS-PAGE. The size of 152-SaWT was 47.91 KDa. All of the elution was in the expected size (Fig. 4.15c). The elute fraction 2 and 3 showed bigger band so these fractions were combined and exchanged buffer to PBS. The protein concentration was measured by spectrometer. The yield of the combined fraction after exchanging buffer was 0.735 mg/l.

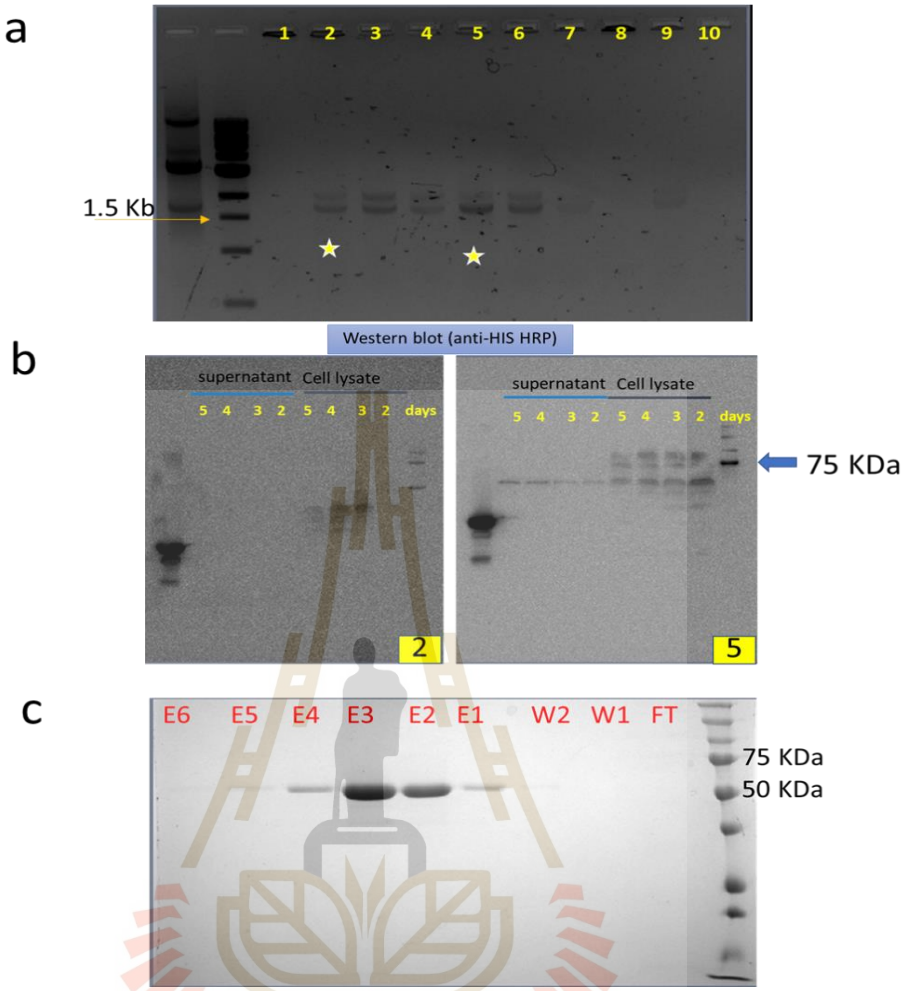



**Figure 4.15** Expression testing of 152-SaWT. 152-SaWT immunotoxin gene was sub-cloned into pPICZαB vector. This vector was linearized and transformed to *Pichia pastoris* competent cells. The transformed cells (clone number 1-10) were checked insert by colony directed PCR (a). The star indicated that clone was selected for expression testing. The yeast cells containing insert were expressed in small scale and checked expression by Western blot using anti-Histidine HRP as a detection probe. The chemiluminescent was captured (b). Clone that well expressed was expressed in larger scale (1 liters). The expressed protein was determined by SDS-PAGE (c).

#### 4.3.2.2 Bacteria expression of scFv-SaDI

Since the purification process of scFv-SaWT fusion protein was complicated so the fusion protein was changed to bacterial expression system. The newer version of sarcin toxin was reported and it has been reported that it could reduce immunogenicity (Jones et al. 2016). So, the deimmunized sarcin (SaDI) gene was used for cell cytotoxicity experiment. The SaDI gene was constructed by inserting SaDI gene at *NotI* and *XhoI* sites of the pET21d+ vector as shown in Fig. 4.16a. To determine the insert, plasmid was amplified by transforming to *E. coli* DH5αF'. Plasmid was extracted and double digested with *NotI* and *XhoI* for checking the insert. The size of SaDI gene was 485 bp. The agarose gel analysis showed the corresponded size of the insert. The SaDI-pET21d+ vector was further digested with *NcoI* and *NotI* enzymes and inserted the scFv gene of clone y1HL63152 and CD33 at *NcoI* and *NotI* sites. The agarose gel analysis showed the approximately 750 and 747 bp, respectively. The agarose gel analysis is shown in Fig. 4.16b-c.

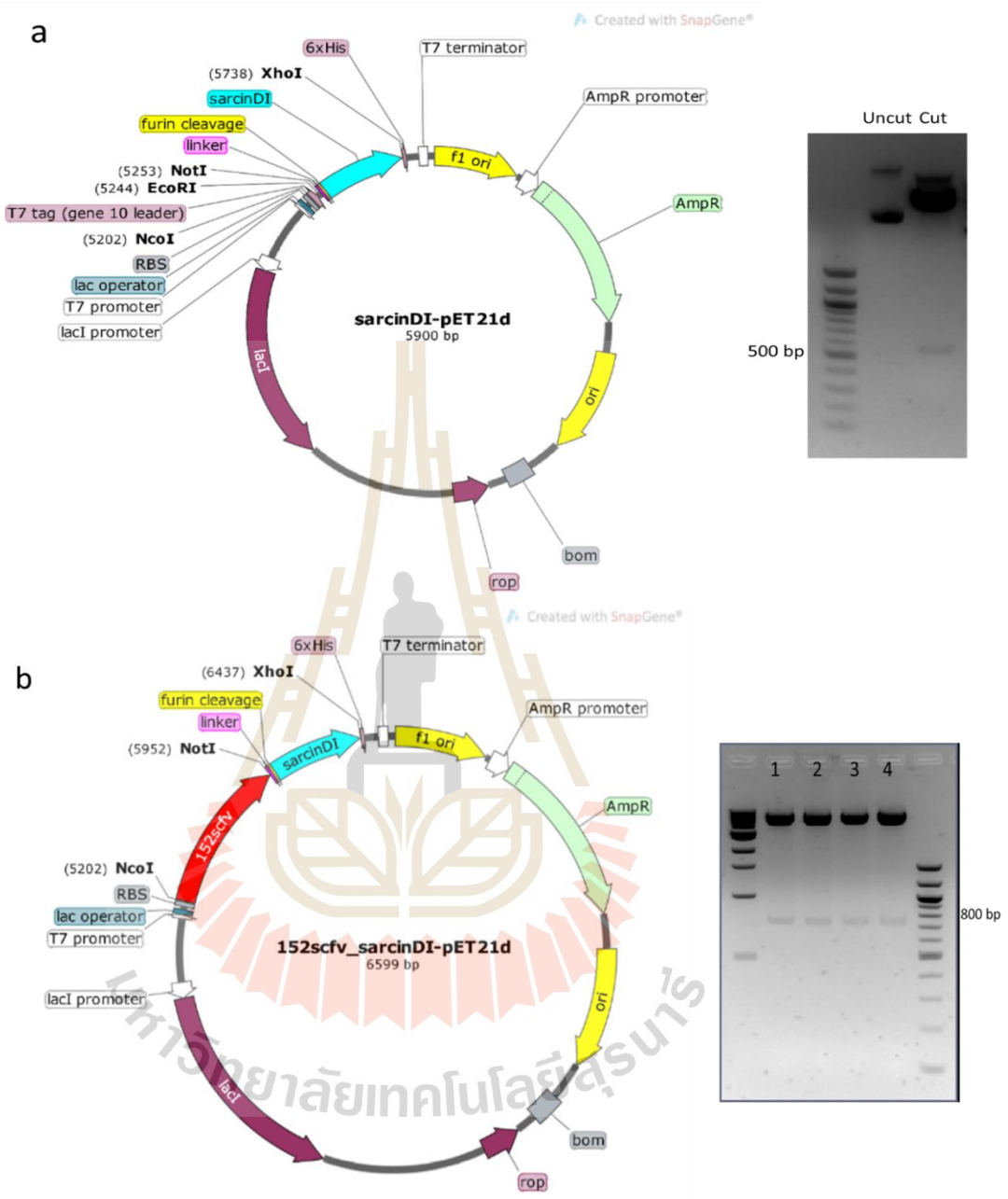



**Figure 4.16** Diagram of plasmid map of scFv-SaDI expression vector. SarcinDI-pET21d+ vector was double digested with NotI and XhoI restriction enzymes (a), 152_SaDI-pET21d+: four transformed clones (1-4) were double digested with NcoI and NotI restriction enzymes (b) and CD33_SaDI-pET21d+: three transformed clones (1-3) were double digested with NcoI and NotI restriction enzymes (c). Agarose gel electrophoresis showed the subcloned vector contained scFv insert.

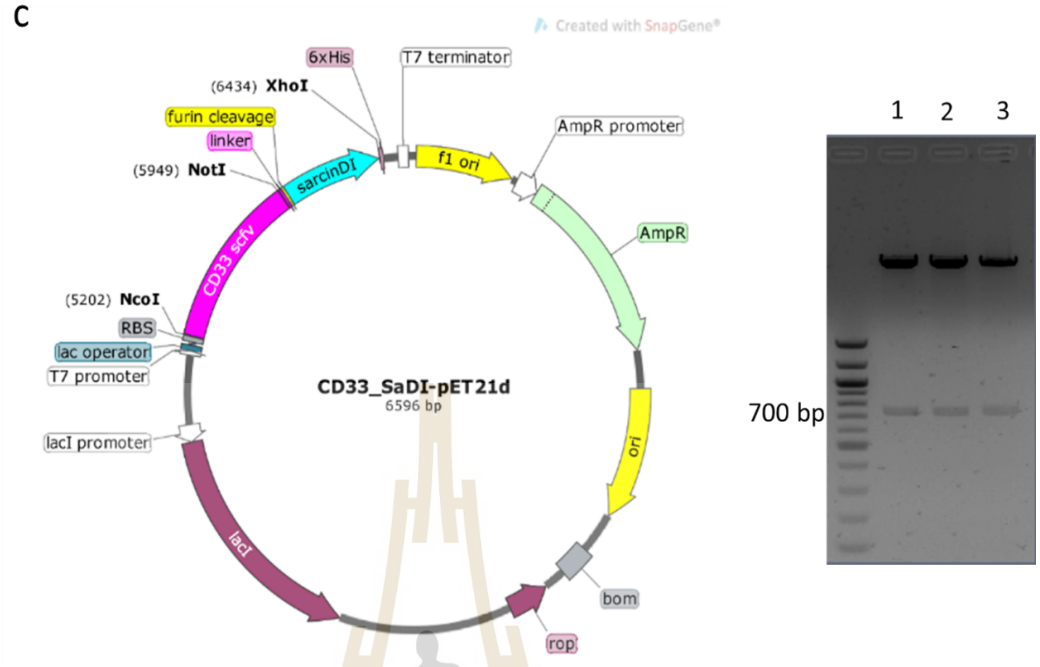



**Figure 4.16** Diagram of plasmid map of scFv-SaDI expression vector. SarcinDI-pET21d+ vector was double digested with NotI and XhoI restriction enzymes (a), 152_SaDI-pET21d+: four transformed clones (1-4) were double digested with NcoI and NotI restriction enzymes (b) and CD33_SaDI-pET21d+: three transformed clones (1-3) were double digested with NcoI and NotI restriction enzymes (c). Agarose gel electrophoresis showed the subcloned vector contained scFv insert (continued).

The scFv antibodies were expressed in *E. coli* system under lac promotor induced by IPTG. The recombinant proteins were expressed in bacterial cells so cell lysate was purified by IMAC using His affinity chromatography. The expected sized of y1HL63152 scFv antibody, CD33 scFv antibody, sarcin immunotoxin, and the fusion of scFv and sarcin of clone y1HL63152 and CD33 were 27.21 KDa, 27.98 KDa, 19.91 KDa, 44.97 KDa, and 45.75 KDa, respectively. The purified proteins were investigated by SDS-PAGE as shown in Fig. 4.17.

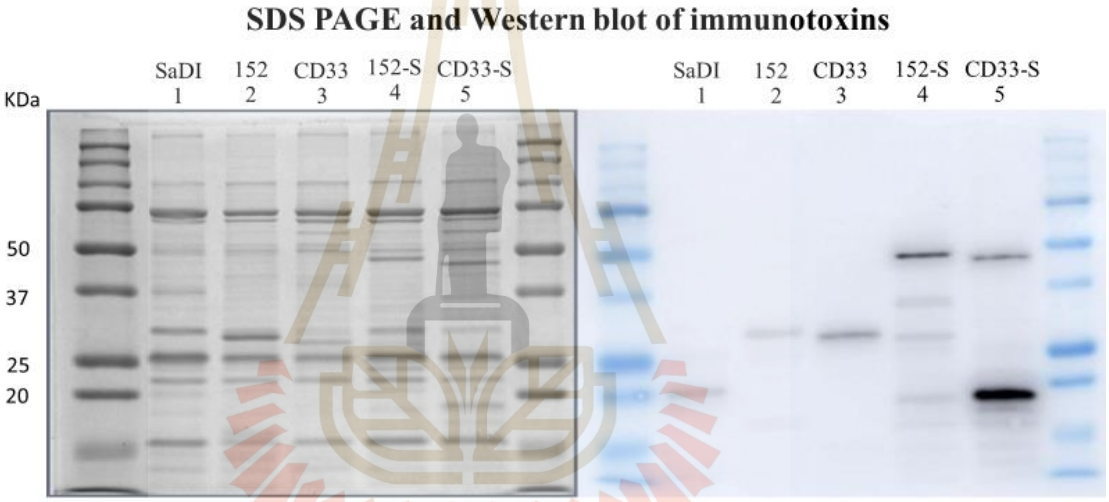



**Figure 4.17** SDS-PAGE and western blot analysis of purified proteins. Recombinant proteins were expressed in bacteria system and then cells were lysed. The crude lysates were purified through Ni-NTA column. Proteins were eluted. The elute proteins were analyzed by the Coomassie stained SDS-PAGE (left gel) and western blot (right gel). The purified proteins were detected by His probe HRP and the chemiluminescent signal was detected. Lane 1: clone SaDI, 2: clone y1HL63152, 3: clone CD33, 4: clone 152-SaDI, 5: clone CD33-SaDI.

## 4.4 Biological activities of engineered antibody

### 4.4.1 Binding specification of y1AML3152 against cell lines

#### 4.4.1.1 Binding test of scFv- GFP antibody against HL-60

The GFP fusion antibody was expressed in 3 difference hosts. These antibodies were determined binding activity against HL-60 by flow cytometry. The result showed that antibodies which were produced in difference hosts did not affect binding activity. Fusion proteins which was produced in B cell, BL21, and K12 were positive to target 87.91±8.82, 85.82±9.19, and 89.35±10.56, respectively. All of antibodies bound to target cells as shown in Fig. 4.18. These experiments were done for 3 times independently.

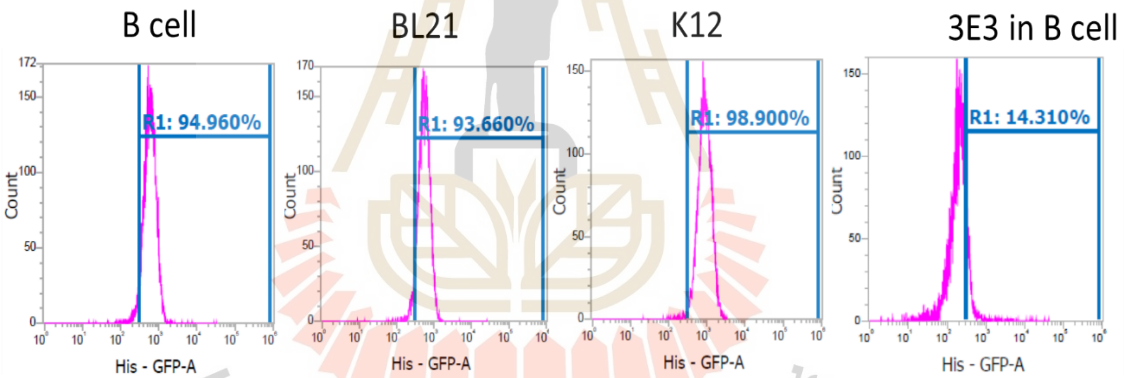
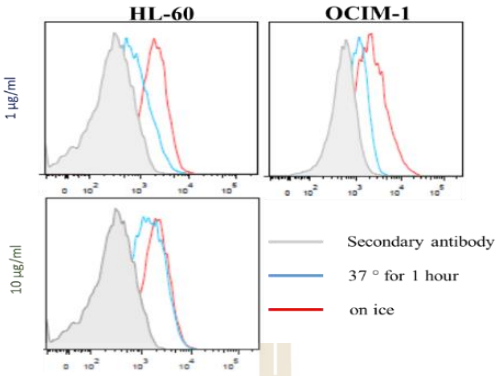



**Figure 4.18** Flow cytometry analysis of 152-GFP against HL-60. The 152-GFP from different expression hosts were incubated to cells and analyzed by flow cytometer. The result showed the percentage of positive cells (blue gate) against each scFv antibody. Blue gate indicates the percentage of positive cells. Blue gate was set by using secondary antibody as a signal background. 3E3-GFP, anti-aflatoxin scFv was used as an isotype control.

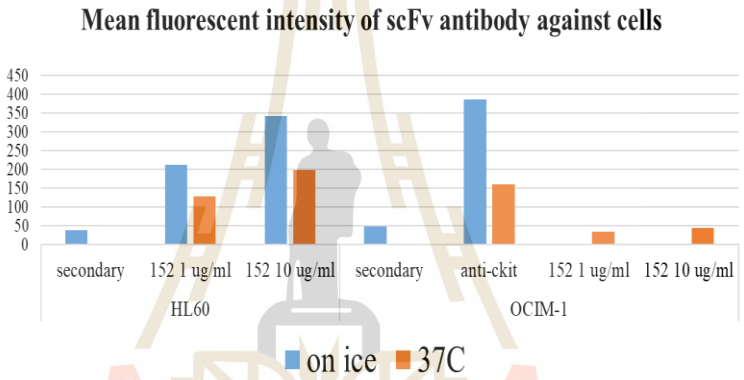
#### 4.4.1.2 Internalization of scFv-GFP fusion antibody

To investigate the ability of y1HL63152 scFv antibody could internalize into cells, two independent methods were set up. The HL-60 cells were incubated with y1HL63152 scFv antibody in 1 and 10 µg/ml final concentration. Cells were incubated on ice and 37°C for one hour and then cells were stained with secondary antibody. The fluorescent signal of the stained cells was detected by flow cytometer. The result showed that the mean fluorescent intensity (MFI) of the cells which were incubated at 37°C with 1µg/ml y1HL63152 scFv antibody was lower than the cells which were incubated on ice. However, the MFI of cells which were incubated with 10 µg/ml of y1HL63152 scFv antibody at 37°C and on ice showed similar level. This similarity MFI might because of the saturation of antibody usage. In this experiment, the OCIM-1 cells which were incubated with anti-c-kit scFv antibody at 37°C was used as a positive control for internalization (Fig. 4.19a-b). The second method was investigated by fluorescent microscope. HL-60 cells were incubated with 152-GFP fusion antibody at 37°C for 1 hour and then the stained cells were observed under fluorescent microscope. The result showed that the green spots were observed inside the cell as compared with bright field as shown in Fig. 4.19c.

**a.**




**b.**




**c.**

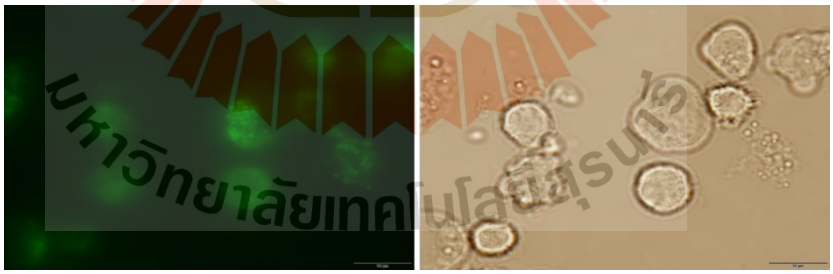


**Figure 4.19** Internalization assays. HL-60 cells were incubated with y1HL63152 scFv antibody at 37°C and then detected the fluorescent signal by flow cytometry analysis. The overlay histogram (a) and MFI of fluorescent signal compared between incubate on ice and at 37°C (b) of 152-GFP against HL-60. OCIM-1 cells were incubated with anti-c kit on ice and at 37°C was used as a positive control for internalization. The 152-GFP fusion protein was incubated at 37°C. Cells were visualized by fluorescent field (left panel) and bright field (right panel)(c).

### 4.4.2 Binding of 152-GFP against DMSO differentiated HL-60

To investigate the binding ability of y1HL63152 scFv antibody when cells were differentiated by DMSO, HL-60 cells were treated with DMSO to induce cell differentiation to granulocyte. Uninduced HL-60 and 72 hours-induced HL-60 were incubated stained with 152-GFP and CD11b. The fluorescent signal was determined by flow cytometer. The result showed that the 152-GFP fluorescent signal of DMSO treated cells at 0 hour and 72 hours was decreased from 93.94% to 24.55%. Whereas, the anti-CD11b-APC fluorescent signal of DMSO treated cells at 0 hour and 72 hours was increased from 9.68% to 98.08% (Fig. 4.20). These results indicated that the y1HL63152 scFv antibody target to receptor that highly express on immature HL-60 leukemia cells.

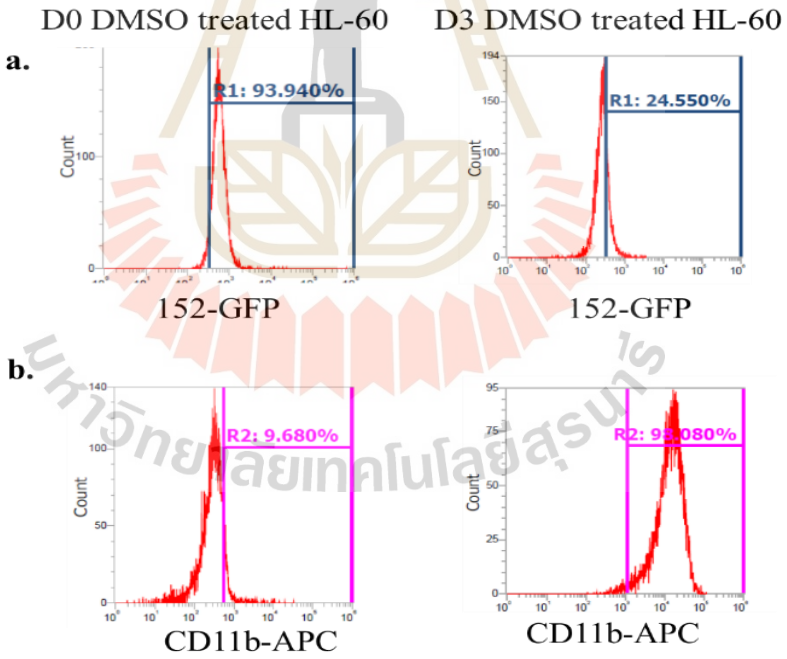



**Figure 4.20** Flow cytometry analysis of 152-GFP to DMSO treated HL-60. The HL-60 cells were treated with DMSO for 3 days and then cells were stained with 152-GFP and anti-CD11b APC. Panel (a) indicated 152-GFP staining of DMSO treated HL-60 at day 0 (left) and day 3 (right) and panel (b) indicated CD11b expression of DMSO treated HL-60 at day 0 (left) and day 3 (right).

### 4.4.3 Binding of 152-GFP against patient samples

To determine the binding ability of 152-GFP in patient derived sample, the 152-GFP fusion protein was used to stain with AML and non-AML patient bone marrow. Since, the bone marrow from healthy donor is difficult to get access. So, PBMC from healthy donors were substituted. The fluorescent signal was detected by flow cytometer. To detect blast cells, anti-CD45 APC was used to distinguish from other cells. Blast cells were intermediate positive to anti-CD45. This population was gated and used for further detection. The example for gating was shown in Fig. 4.21a-c. The MFI ratio was defined by MFI of the 152-GFP divided by isotype control. The result showed that the average value of MFI ratio from AML patient sample, healthy donor and non-AML sample was 1.48±0.78, 0.70±1.43, and 0.23±1.57, respectively. Even through, the average value of the MFI ratio from AML showed highest. But the actual MFI ratio value from healthy donor and non-AML showed similar to AML patient sample. The MFI ratio of AML patient sample was not significant different from healthy donors ($p$ = 0.3968) and non-AML patient sample ($p$ = 0.3571). The graph of MFI ratio in different groups was shown in Fig. 4.21d. The patient characteristics which were reported by the hospital and MFI ratio of samples against 152-GFP was list in table 4.3.

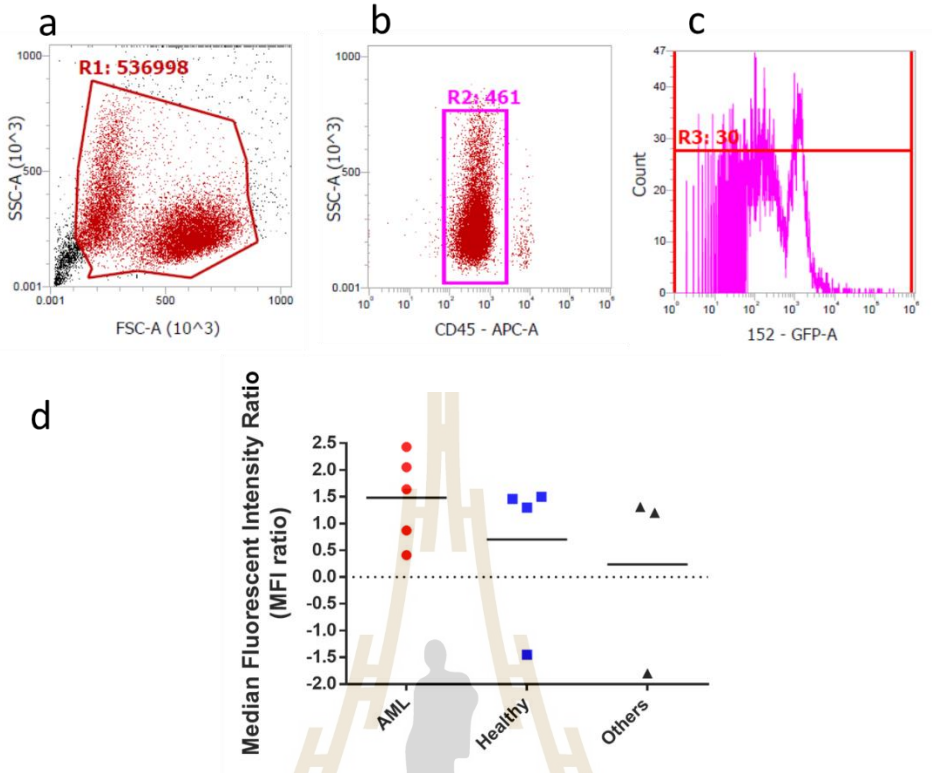



**Figure 4.21** Flow cytometry analysis of 152-GFP to AML patient derived samples. Cells were gated by FSC-A and SSC-A (a). The gated cells were subsequence gated based on CD45-APC and SSC-A (b). Cell population from gate R2 were further acquired against 152-GFP (c). MFI ratio of AML bone marrow samples, healthy donor PBMC, and non-AML leukemia bone marrow were plotted (d).

**Table 4.3** Patient characteristics.

| Patient number | Gender | Age | Interpretation | MFI ratio | FAB |
|---|---|---|---|---|---|
| 1 | Female | 20 | CD13 +, CD15+, CD33+, CD64+, CD117+, HLA-DR-, MPO+ | 2.05 | M3 |
| 2 | Female | 61 | CD13+, CD33-, CD34+, CD56+, CD117+, HLA-DR+, MPO+ | 0.41 | M1 |
| 3 | Female | 46 | CD13+, CD15+, CD33+, CD64+, CD117+, HLA-DR-, MPO+ | 0.87 | M3 |
| 4 | Female | 19 | CD11b+, CD13+, CD33+, CD34-, CD64+, CD117-, HLA-DR+, MPO+ | 1.64 | M5 |
| 5 | Male | 34 | CD45-, CD10+, CD19+, CD34-, HLA-DR+, TdT+ | 1.3 | Precursor B Acute Lymphoblastic Leukemia (ALL) |
| 6 | Female | 53 | CD56-, CD64-, CD117- | 1.2 | No evident of acute leukemia |
| 7 | Female | 35 | Not determined | -1.8 | Non-AML |
| 8 | Female | 56 | CD13(+). CD33(+). CD34(+). CD117(+), h HLA-DR (+), MPO (+) | 2.43 | M1 |

### 4.4.4 Cytotoxicity of scFv immunotoxins

#### 4.4.4.1 Binding test of scFv-sarcin immunotoxins against HL-60

To determine specific binding of recombinant immunotoxin, flow cytometry analysis was done 3 times independently. The result showed that the positive percentage of gated cells were 95.47±4.5, 96.89±1.25, 98.87±0.94, 96.57±2.3, 3.11±0.23 for y1HL63152-scFv, 152-SaDI, CD33-scFv, CD33-SaDI, and SaDI, respectively (Fig. 4.22).

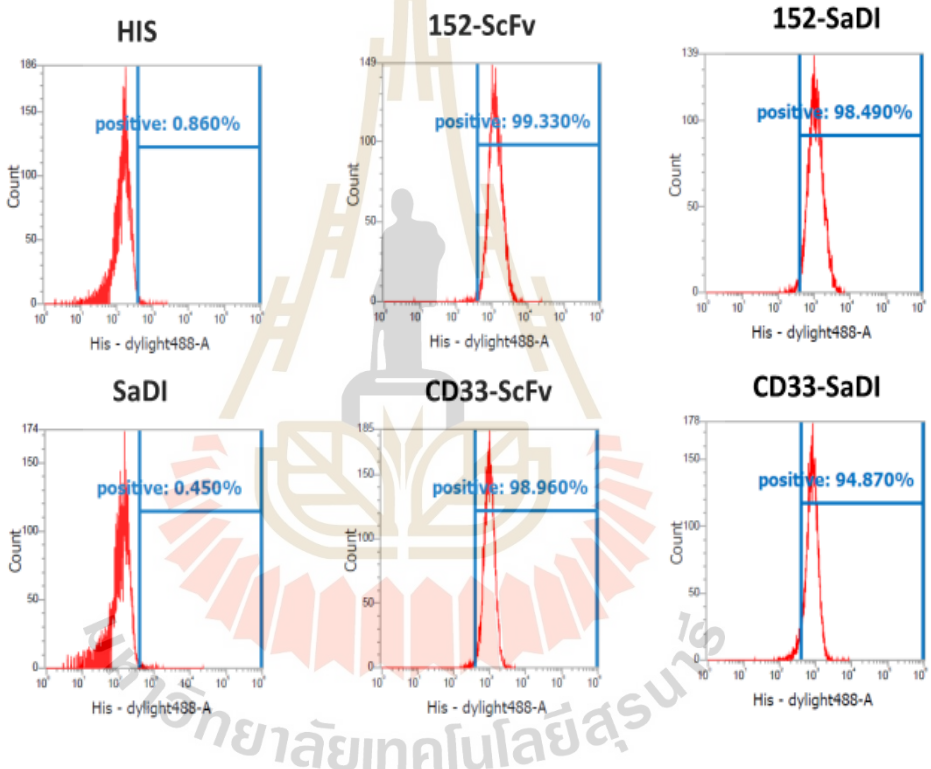



**Figure 4.22** Flow cytometry analysis of recombinant immunotoxins against HL-60. Cells were incubated with recombinant immunotoxin and stained with secondary antibody, anti-His dylight 488. The fluorescent signal was detected by flow cytometer. The result showed the percentage of positive cells (blue gate) against each scFv antibody. Blue gate was set by using secondary antibody as a signal background.

#### 4.4.4.2 Cell cytotoxicity of scFv fusion to wildtype sarcin

To test cell cytotoxicity after treating with 152-SaWT, the typhan blue exclusion test and apoptosis assay were conducted. For cells viability, the dead cells were stained with trypan blue dye. The live cells were counted. The percentage of living cells were calculated and then plotted into graph as shown in Fig. 4.23a. The result showed that the percentage of cell viability of the cells treated with 10 µM of 152-SaWT decreased from 93.67 %, 80.67 %, and 57.08 % at 24, 48, and 72 hours of incubation, respectively. Whereas, the percentage cell viability of untreated cells, cells treated with 1 µM and 10 µM of 152 scFv and 1 µM and 10 µM of 3E3 scFv, an isotype control was between 97 % to 87 % during 72 hours of incubation. The percentage viability of cells treated with 1 µM of 152-SaWT was also between 92 % and 90% during 72 hours of incubation. Cells treated with 10 µM of Doxorubicin were used as positive control. It showed that the percentage of cell viability decreased from 90.32 %, 66.67 %, and 29.25 % at 24, 48, and 72 hours for incubation, respectively. For apoptosis assay, the 72 hours treated cells were stained with Annexin V and PI. The stained cells were analyzed by flow cytometer. The result showed that cells treated with 10 µM 152-SaWT showed 61.8 % of Annexin $^{-}$ / PI $^{-}$ cells, 5.95% of Annexin $^{+}$ / PI$^{-}$ cells, 25.7% of Annexin$^{+}$/ PI$^{+}$ cells, and 6.62% of Annexin$^{-}$and PI$^{+}$ cells (Fig. 4.23b).

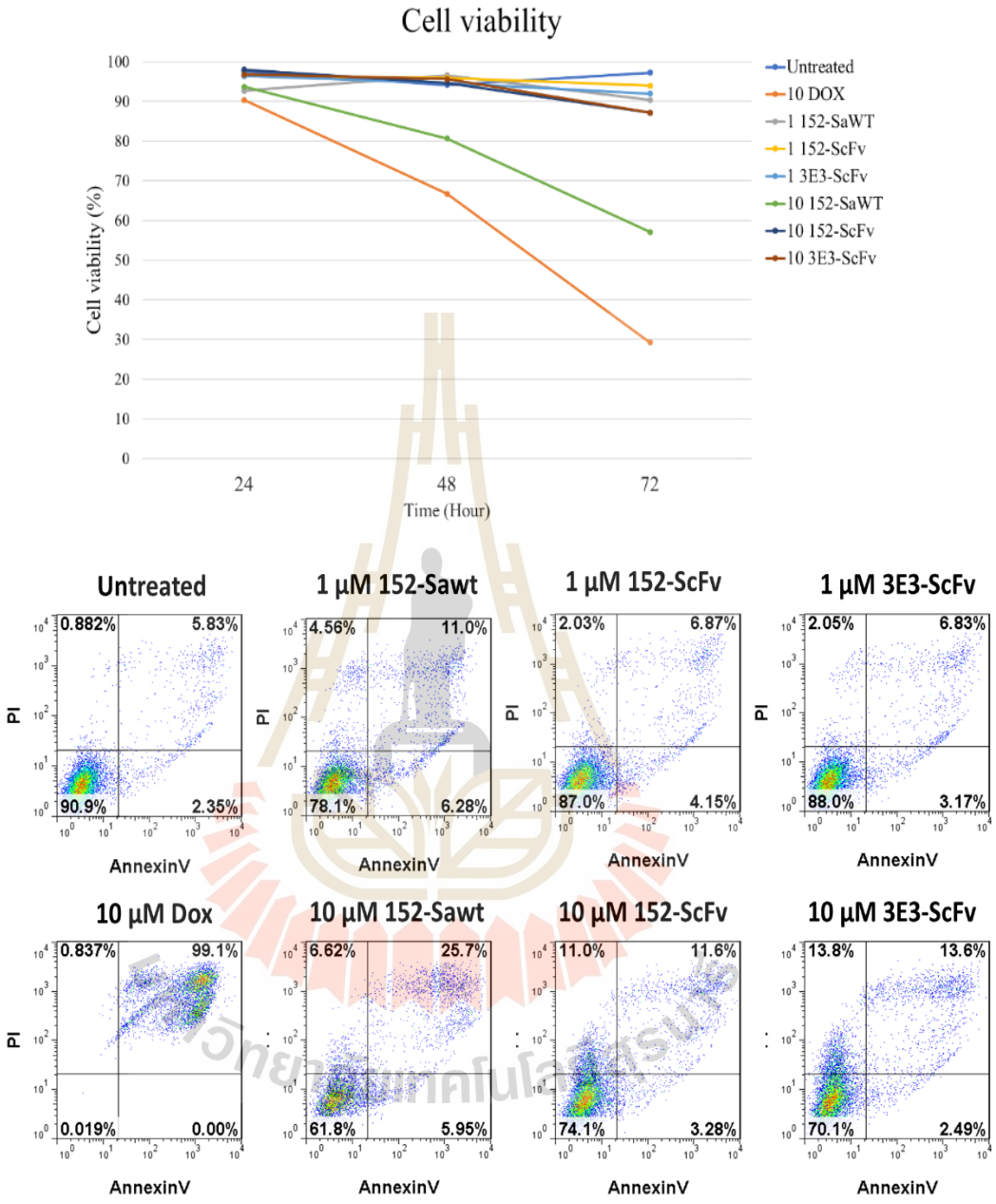



**Figure 4.23** Cell cytotoxicity of HL-60 to recombinant proteins. (a) Cell viability testing. The percentage of viable cell (Y axis) in each protein and in each incubation time (X axis). (b) Apoptosis assay, treated cells were stained with AnexinV (X axis) and PI (Y axis).

The cells treated with 152-SaWT were further tested with MTT assay by incubating cells with recombinant proteins and Doxorubicin for 72 hours and observed cells mitochondrial activity. The MTT was added and measured formazan formation by spectrophotometer. The result showed that the absorbance of cell treated with 152-SaWT decreased lower than y1HL63152 scFv and 3E3 scFv as shown in Fig. 4.24.

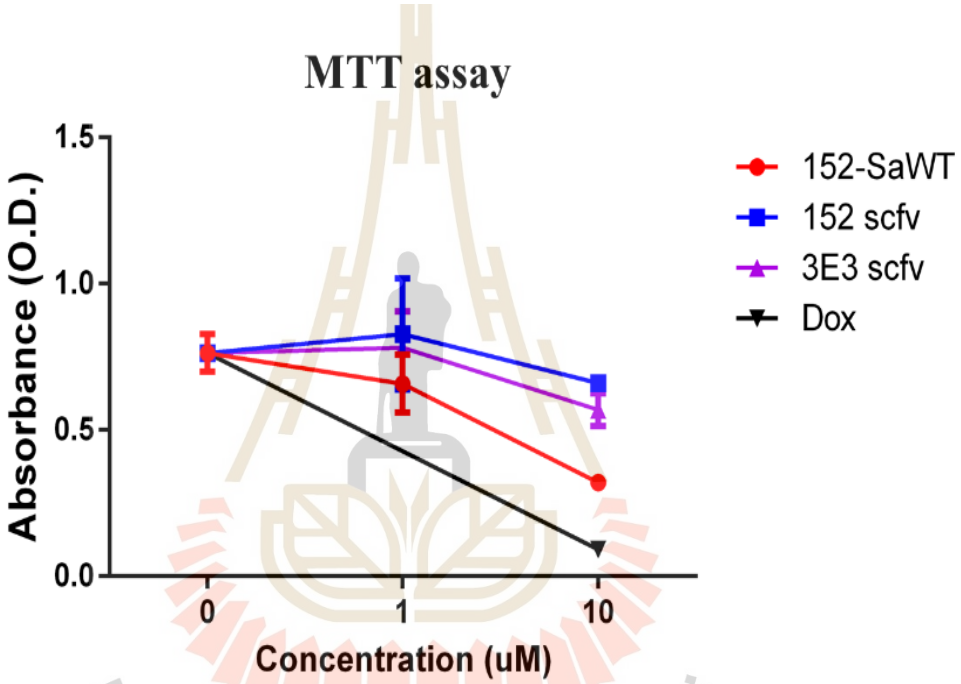



**Figure 4.24** Cell cytotoxicity of HL-60 treated with recombinant proteins. Cells were treated with proteins and doxorubicin for 72 hours. The absorbance of colorimetric signal of cell treated with proteins (Y axis) in vary concentrations (X axis) were plotted.

### 4.4.4.3 Cell cytotoxicity of scFv fusion to deimmunized Sarcin

HL-60 cells were treated with two-fold serial dilution from 0 to 10 µM of y1HL63152 scFv antibodies, anti-CD33 scFv, 152-SaDI fusion protein, anti-CD33-SaDI, and SaDI protein. Treated cells were incubated for 24 hours and the cell viability were determined by Muse cell count and viability kit. The percentage of viable cells of anti-CD33-SaDI fusion protein at 0, 0.625, 1.25, 2.5, 5, and 10 µM was 94.9 %, 94.9%, 93.9%, 80.9%, 41.8%, and 13.1%, respectively. It showed the lowest viable cells and decreased in dose dependent manner. The percentage of viable cells of 152-SaDI fusion protein at 0, 0.625, 1.25, 2.5, 5, and 10 µM was 94.3%, 96.8%, 95.2%, 92.8%, 64.3%, and 27.8%, respectively. In contrast, the percentage of viable cells of cells treated with SaDI, 152 scFv, and anti-CD33 scFv were not less than 80% (Fig. 4.25).

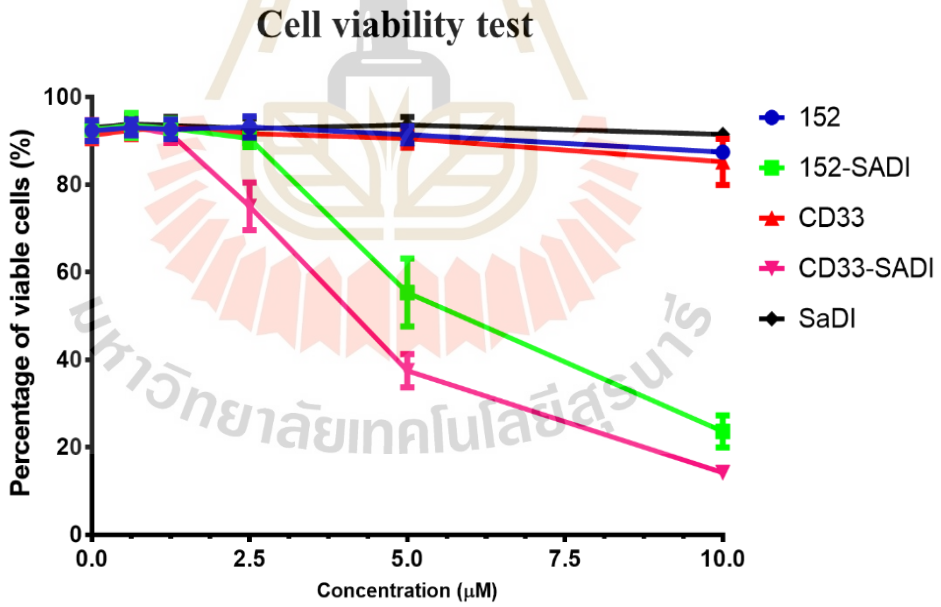



**Figure 4.25** Cell viability of HL-60 treated with immunotoxins. The treated cells were incubated for 24 hours. Cell viability was determined. The percentage of viable cells of recombinant proteins (Y axis) in vary concentrations (X axis) was plotted.

#### 4.4.4.4 Cell cytotoxicity of anti-AML immunotoxin combinations

To investigate cell cytotoxicity of the combination treatment of 152-SaDI and CD33-SaDI, HL-60 cells were treated with the 2-fold serial dilution of 152-SaDI, CD33-SaDI, and the combination in 50:50 ratio of 152-SaDI and CD33-SaDI for 24 hours and cell viability was detected by Muse cell count and viability kit. The percentage of viable cells of 152-SaDI, CD33-SaDI, and mixed of proteins in term of total protein was 44.3 %, 26.55 %, and 35.65%, respectively (Fig. 4.26a). To investigate the additive effect of each immunotoxin, the percentage of viable cell was plotted base on equimolar amount of protein. The result presented the additive effect of combination treatment of immunotoxin in equal equimolar amounts compare to single immunotoxin treatment (Fig. 4.26b).

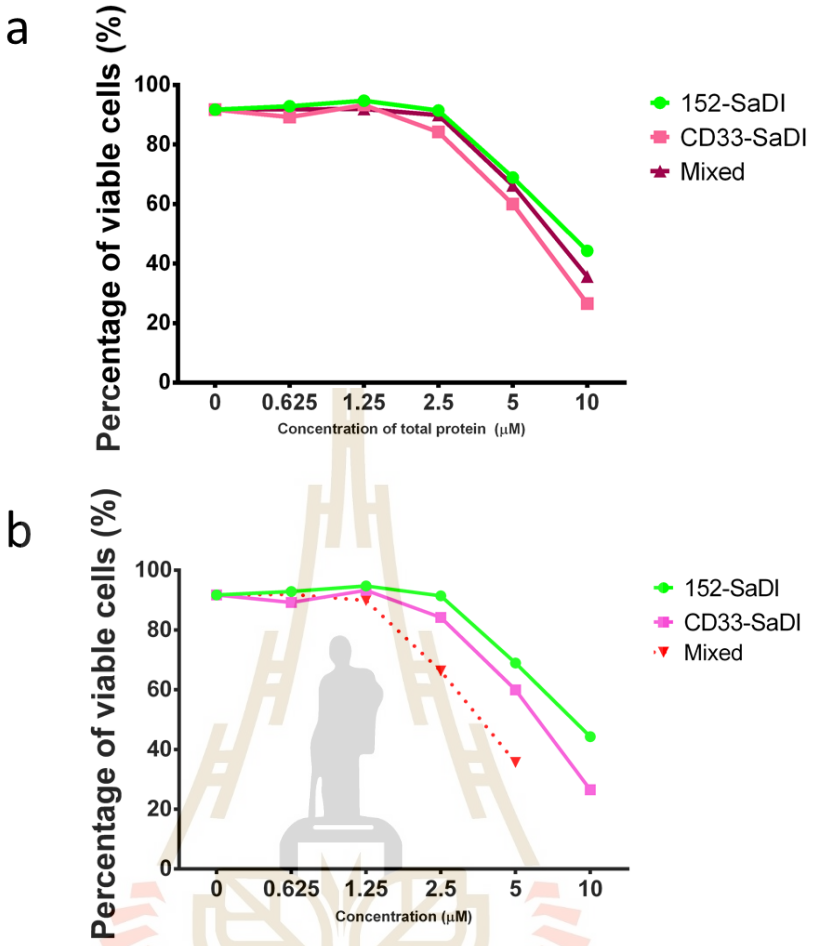



**Figure 4.26** Cell viability of HL-60 treated with immunotoxins. a ) The percentage of viable cells of recombinant proteins (Y axis) in vary concentrations of total protein in 2-fold serial dilution (X axis). Cells were treated with 152-SaDI (green), CD33-SaDI (pink). Cells were treated with total protein of the combination in 50:50 ratio of 152-SaDI and CD33-SaDI was defined as mixed (red). b) The percentage of viable cells of recombinant proteins (Y axis) in vary concentrations of equimolar amounts in 2-fold serial dilution (X axis). Cells were treated with 152-SaDI (green), CD33-SaDI (pink). Cells were treated with equimolar amounts of the combination in 50:50 ratio of 152-SaDI and CD33-SaDI (red dot line).

# CHAPTER V

# DISCUSSION AND CONCLUSION

Phage display technology is widely used to generate fully human antibody. *In vitro* selection can be used to identify antibody to nearly all type of antigen. Targeting membrane proteins is a powerful mechanism of therapeutic antibodies. Identifying specific antibody against membrane proteins is promising method to get novel therapeutic antibodies. Purified antigens coated on solid surface has limitation. Membrane proteins preparation loss conformation stability and orientation. Cells based bio-panning has been studied by using overexpressed cell lines and activated cells to archive membrane proteins of interest. (Eisenhardt et al. 2007, Stein et al. 2010) However, selection of membrane proteins on native blood cancer cell lines and subtraction with closely related cancer cell line has not been reported. In this study, HL-60 was used as a target. It was isolated from patient with acute myeloblastic leukemia with maturation, FAB-M2 (Dalton et al. 1988). Because it is in the early stage of development of leukemia cells and has been reported that this subtype was the most commonly found in patients (Wakui et al. 2008). The subtractive cells line, Jurkat, are an immortalized line of human T lymphocyte which is hematopoietic cell but different linage from HL-60. Subtraction of non-specific phage in each biopanning would increase ratio of specific and non-specific phage. Temperature control for biopanning process is an important concern depending on intention. For therapeutic purposes, antibody drug conjugates need to endocytose into cells, cells panning process has to be done at 37°C (Fitting et al. 2015, Mandrup et al. 2017). In this study, we carried out at 4°C to preserve cell surface antigens. The screening process of individual clone in this study, phage-based assay was skipped out. The scFV antibodies were directly produced in 96 deep well plate and binding activity is

tested by flow cytometry. This method can overcome the problem of time consuming, phage sticky cause high background and scFv-unproducible phage. Furthermore, this small-scale expression could narrow down the screening clone and confirmed binding by expressing scFv in 5 ml culture. All three clones were neither bind to non-related blood cancer cell nor normal blood cells. y1HL63152 and y1HL63255 showed specifically bind to HL-60 target cells. It indicated that subtraction step might worked well. The scFv antibody clone y1HL63152 was used for further experiment.

The recombinant proteins for bacteria expression in this work based on pET21d+ vector. Because of this vector contained compatible restriction sites suitable for molecular cloning and the pET system is strongly controlled under T7 promoter. Bacterial host that used to produce the recombinant proteins was Shuffle B cells. This bacterial strain was engineered to enhance the stability of disulfide bond formation under oxidative condition inside the host cells (Lobstein et al. 2012). So, the recombinant proteins were extracted from cell cytoplasm. The purified proteins from cell lysate contained non-specific proteins as shown in SDS-PAGE (figure 16). However, the recombinant proteins were in the correct size. More optimization is necessary to obtained purer scFv for bioactivity study. This can be done by double purification process with different affinity column.

*Pichia pastoris* is a methylotrophic yeast. It has capability to metabolize methanol as its sole carbon source. Protein was secreted to culture supernatant. The purified protein was very pure without any contaminate protein. These purified proteins were used for further experiments. However, the purification process was complicated so the expression of immunotoxin via yeast system was discontinued. The expression of immunotoxin in yeast system might be done in the future if the concentrator and dialysis set are set up.

The recombinant antibody in various formats were characterized their binding ability, specificity, and their localization on the target cells. From EC50 determination, the affinity binding of y1HL63152 scFv antibody was 1.44 higher than CD33. Since CD33 scFv was a

humanized antibody which was originated from mouse hybridoma. So, their affinity was high. The 1.44-fold lower of y1HL63152 scFv antibody was not typical for scFv isolated from naïve phage display scFv antibody library. To improve the binding affinity of clone y1HL63152, affinity maturation can be done. The target antigen of y1HL63152 scFv antibody is still unknown. To identify the target antigen, immuno-precipitation, followed by mass spectrometry will be done once the full-length monoclonal antibody is successfully constructed. The density of the antigen that expressed on HL-60 cell surface is unclear. So, the density of target antigen of y1HL63152 on HL-60 cells will be quantified by the Fluorescent Bead Assays. The comparison of binding ability between y1HL63152 and anti-CD33 scFv suggested that the target antigen of y1HL63152 scFv antibody might express on HL-60 lower than CD33 receptor. The result of cross reactivity checking, the scFv clone y1HL63152 bound specifically to HL-60 cells. CD33 scFv bound to HL-60, OCIM-1, and THP-1 which are overexpressed CD33 on their surface. Thus, it suggested that y1HL63152 scFv antibody did not bind to CD33 receptor on HL-60. The immunofluorescent staining showed that it located on the surface of the cells. This result corresponded to selection method that the bio-panning was done at 4°C to get phage that bind on cell surface of intact cells. For 152-GFP, the GFP protein is a molecule that preferred folding in oxidative condition. Difference expression hosts were tried. The binding ability of 152-GFP in difference hosts was tested. The result showed that the binding ability of 152-GFP was not different in each expression host. The fluorescent signal of flow cytometry analysis was comparable to scFv antibody. Therefor, 152-GFP could be used for one step detection. Moreover, 152-GFP was used to detected patient derived mononuclear cells. It suggested that the y1HL63152 scFv antibody could positivity for some AML patient samples and could demonstrate with substantial inter-patient heterogeneity in expression level. Furthermore, the 152-GFP also bound to ALL patient's sample. It might be because of the antigen that target to y1HL63152 also express on ALL patient sample. Although the result showed that

152-GFP also bound to healthy donor PBMC. But this antibody is still interesting to explore. Although, it binds to normal cells but it might be still use to treat cancer patient. Rituximab (anti-CD20) is a good example. It is used to treated CLL patient even though it depletes normal B cells. Antibody directed enzyme prodrug therapy which it is selectively released drug at tumor specific site could be worked out this limitation (Schellmann et al. 2010). However, the assay still has not completed and more patients should be evaluated. Since, the patient samples who were observed are not cover all AML FAB subtypes due to the limitation of sample access. Even thought, the scFv-GFP fusion protein is convenient for detection, it still has some limitation. The monovalent of scFv antibody might cause lower sensitivity than bivalent from monoclonal antibody and GFP is not sufficiency bright. So, using monoclonal antibody which chemically conjugate with brighter fluorophore could be the better way for diagnosis. It also more powerful to distinguish between high, medium, and dim fluorescent signal in sample that various expression the target of interest.

The 152-GFP was used to detected differentiated HL-60 which co-staining with a granulocyte marker, CD11b. The result suggested that the 152 scFv antibody bound to the epitope that overexpressed on immature HL-60. Furthermore, 152-GFP was incubated to HL-60 at 37°C for investigating cell internalization. It showed that the scFv-GFP fusion protein was endocytosed into cells. So, it suggested that scFv-GFP clone y1HL63152 could be internalized into cells. This property might be useful for drug delivery.

For the immunotoxin, 152-SaDI and CD33-SaDI bound to HL-60. Whereas, SaDI alone did not bind to the target cells. Therefore, it could make sure that SaDI might not toxic to the cells. Overall, these experiments showed that the immunotoxin was able to bind specifically to HL-60, confirming the functionality of its scFv moiety. However, isotype control, irrelevant scFv conjugated with SaDI, could be used to prove specificity. The cytotoxicity of CD33-SaDI to HL-60 was higher than 152-SaDI. It might be because of HL-60 express CD33 higher than antigen that specific for y1HL63152. This result corresponded

to the binding ability results that previously mentioned. The combination treatment between 152-SaDI and CD33-SaDI to HL-60 showed that it was not enhanced cytotoxicity compare to cells treated with CD33-SaDI alone. However, the cytotoxicity was higher than 152-SaDI alone. Furthermore, the purified proteins were not pure as shown in figure 20. So, the amount of expected proteins was not exactly equally used. This experiment has to be repeat again with the purer proteins. The combination treatment by using same cytotoxic agent and same mechanism of action might not improve cytotoxicity even though they target into different antigen. The combination treatment in different mechanism of action, for example, the combination treatment between scFv-SaDI, which inhibits protein synthesis of leukemia cells and doxorubicin which is known as chemotherapy medication used to treat cancer including leukemia, might enhance cell cytotoxicity. Furthermore, Jurkat or other irreverent cell line, can be used to treat in the same condition as a negative control.

In the conclusion, the scFv that specifically bound to HL-60 AML cells was isolated. The characteristics, specificity, binding ability, and biological activities of scFv, scFv-GFP, and immunotoxin were investigated. Together these data, it showed that clone y1HL63152 is an interesting antibody which can be applied for diagnostic and therapeutic purposes.

To further development of clone y1HL63152, the monoclonal antibody IgG format will be constructed, and the epitope that target to this antibody will be identified. The IgG conjugated to a brighter fluorophore will be made for detection purpose in clinical diagnostic. This IgG conjugated with fluorophore might be use to bind relapse sample or monitor disease. This investigation may be done in mouse model. For the immunotoxin, the localization of the toxin after internalization will be investigated by co-staining with Endoplasmic Reticulum and Golgi tracker dye and detect by confocal microscopy.

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# APPENDIX

# APPENDIX

## A.1 Patent application

Thai Patent Application No. 1901004141

Yamabhai M., Sumphanapai, T.

Title: A recombinant single chain variable fragment antibody which bind specifically to acute myeloid leukemia cells

## A.2 DNA sequence of selected clones

**y1HL63152**

GGCCCAGCCGGCCATGGCCCAGGTGCAGCTGGTGCAGTCTGGGGCAGAGGTGA

AAAAGCCCGGGGAGTCTCTGAAGATCTCCTGTAAGGGTTCTGGATACAGCTTT

ACCAGCTACTGGATCGGCTGGGTGCGCCAGATGCCCGGGAAAGGCCTGGAGTG

GATGGGGATCATCTATCCTGGTGACTCTGATACCAGATACAGCCCGTCCTTCCA

AGGCCAGGTCACCATCTCAGCCGACAAGTCCATCAGCACCGCCTACCTGCAGT

GGAGCAGCCTGAAGGCCTCGGACACCGCCATGTATTACTGTGCGAGACATCAG

GGCAGCTGGTATTCGGATGCTTTTGATATCTGGGGCCAAGGGACCACGGTCAC

TGTCTCCTCAGGTGGTGGTGGTAGCGGCGGCGGCGGCTCTGGTGGTGGTGGAT

CCCAGTCTGCCCTGACTCAGCCTGCCTCCGTGTCTGGGTCTCCTGGACAGTCGA

TCACCATCTCCTGCACTGGAACCAGCAGTGACGTTGGTGGTTATAACTATGTCT

CCTGGTACCAACAACACCCAGGCAAAGCCCCCAAACTCATGATTTATGATGTC

AGTAATCGGCCCTCAGGGGTTTCTAATCGCTTCTCTGGCTCCAAGTCTGGCAAC

ACGGCCTCCCTGACCATCTCTGGGCTCCAGGCTGAGGACGAGGCTGATTATTAC

TGCAGCTCATATACAAGCAGCAGCACTCTCGTGTTCGGCGGAGGGACCAAGGT

CACCGTCCTAGGTGCGGCCGCACATCATCATCACCATCACGGGGCCGCAGGGC

CCGAACAGAAACTGATCTCTGAAGAAGACCTGAACGGTACCGCATAG

**y1HL63288**

ATGGCCCAGGTGCAGCTGGTGCAGTCTGGGGGAGGCTTGGTACAGCCTGGAGG

GTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTCAGTAGTTATGAAAT

GAACTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTTTCATACATTA

GTAGTAGTGGTAGTACCATATACTACGCAGACTCTGTGAAGGGCCGATTCACC

ATCTCCAGAGACAACGCCAAGAACTCACTGTATCTGCAAATGAACAGCCTGAG

AGCCGAGGACACGGCTGTTTATTACTGTGCGAGAGGGGGCCATGACGATTTTT

GGTACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAGGTGGTG

GTGGTAGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCAATTTTATGCTGACTC

AGCCCCACTCTGTGTCGGAGTCTCCGGGGAAGACGGTAACCATCTCCTGCACC

GGCAGCAGTGGCAGCATTGCCAGCAACTATGTGCAGTGGTACCAGCAGCGCCC

GGGCAATGCCCCCACCACTGTGATCTATGAGGATAACCAAAGACCCTCTGGGG

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**y1HL63255**

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GTTTTACGTGCGGCCGCACATCATCATCACCATCACGGGGCCGCAGGGCCCGA

ACAGAAACTGATCTCTGAAGAAGACCTGAACGGTACCGCATAG

# BIOGRAPHY

Miss Thitima Sumphanapai was born on March 1, 1989 in Uthaitani, Thailand. She graduated with the Bachelor Degree of Science, Department of Medical Technology, Faculty of Allied Health Sciences, Naresuan University, Thailand in 2010 with the first-class Honor. She did the practical work in Medical Technology Department of clinical pathology, Uthaitani hospital, Thailand in 2010. In 2011-2019, she got financial support by The Royal Golden Jubilee Ph.D program to study Doctoral Degree enrollment in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology. During her study, she received scholarship names "The Royal golden Jubilee PhD programme (One-year PhD placement in the UK co-funded by Newton fund, 2015) to experience on her research for 1 year at Prof. Kerry Chester's Laboratory for Antibody Engineering, University College London Cancer Institute. London, United Kingdom. She had presented research work in the 5th RGJ congress and seminar series 118, 1st -2nd May, 2017. At Suranaree University of Technology, Thailand (Poster presentation) in title "Selection of scFv antibody specifically bound to AML cell line by the human naïve phage display scFv antibody library". And also presented in the 14th Asian Congress on Biotechnology, 1st -4nd July, 2019. At Fullon Hotel, Tamsui fisherman's Wharf, Taiwan (Poster presentation) in title "Targeting Membrane Protein for Antibody Discovery by Whole Cell Bio-Panning of Recombinant Single Chain Fragment Variable (ScFv) Antibody from Phage Display Antibody Library"