

**DEVELOPMENT OF A SINGLE CHAIN VARIABLE FRAGMENT
(ScFv) FOR DIRECT DETECTION OF TROPHOBLAST CELL
SURFACE ANTIGEN 2 ON CIRCULATING TUMOR CELLS**




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OF THE REQUIREMENTS FOR THE DEGREE OF
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ABSTRACT

Trophoblast cell surface antigen 2 (Trop2) overexpression has been reported in various human cancers. A single chain variable fragment (ScFv) is a common type of antibody fragments that can be developed and modified to use for many purposes. Circulating tumor cells (CTCs) play a role in tumor progression and become the interesting biomarker for detection among cancer patients because it can be simplified for monitoring progression of patients. This study aimed to develop an easy usage of ScFv against Trop2 to detect Trop2-expressing cancer cells. The ScFv against Trop2 with mCherry labelling (Trop2mCherry ScFv) was constructed by phage display technique and expressed by bacterial system (*Escherichia coli*). After production process, it was tested for the binding efficiency on many types of cancer cell lines to screen for Trop2 expression by immunofluorescence analysis (IFA) and confocal microscopy analysis, in comparison to the staining by commercial anti-Trop2 antibody. For application, situation of CTCs was performed by mixing the Trop2-expressing cancer cell with normal blood and stained by this ScFv and then detected under fluorescence microscope. The results showed KKU-213, as the highest, and KKU-055, as the lowest Trop2-expressing cells. The staining of Trop2mCherry ScFv was significantly reduced in Trop2-knockdown KKU-213 cells when compared with un-transfected cells. Overexpression of Trop2 were performed in KKU-055 cells when observed by confocal microscope analysis. The results showed the co-localization between Trop2-GFP tag and binding of antibody had no difference between commercial antibody and Trop2mCherry ScFv. When the situation of CTCs was performed by KKU-213 cells mixing together with blood from healthy donor, only KKU-213 cells were stained by Trop2mCherry ScFv. Therefore, Trop2mCherry ScFv could stain Trop2 expressing cells compatible to commercial antibody. Moreover Trop2mCherry ScFv may detect Trop2-expressing cancer cells in blood circulation. In summary, Trop2mCherry can be produced for detection of Trop2 expression on surface of cancer cells and should be developed for simplified utilization.

KEY WORDS: CIRCULATING TUMOR CELLS / MCHERRY / SINGLE CHAIN VARIABLE FRAGMENT / TROPHOBLAST CELL SURFACE ANTIGEN 2

89 pages

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LIST OF ABBREVIATIONS

Abbreviation	Term
%	Percent
μg	Microgram
μl	Microliter
μm	Micrometer
μM	Micro molar
°C	Degree Celsius
a.a	Amino acid
Ab	Antibody
BCA	Breast cancer
bp	Base pair
CaCl ₂	Calcium chloride
CCA	Cholangiocarcinoma
CO ₂	Carbon dioxide
C _H	Constant (heavy) chain
C _L	Constant (light) chain
CTCs	Circulating tumor cells
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DW	Distilled Water
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial mesenchymal transition
EOC	Epithelial ovarian cancer
FACS	Fluorescence-activated cell sorting
Fab	Antigen binding fragment

LIST OF ABBREVIATIONS (cont.)

Abbreviation	Term
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FCM	Flow cytometry
FITC	Fluorescein isothiocyanate
Fv	Variable fragment
HAMA	Human anti-murine antibody
IFA	Immunofluorescence assay
IgG	Immunoglobulin G
IHC	Immunohistochemistry
kDa	Kilo Dalton
L	Liter
LB	Luria-Bertani
mAbs	Monoclonal antibodies
mg	Milligram
mL	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
ng	Nanogram
nm	Nanometer
NSCLC	Non-small cell lung cancer
OD	Optical density
PBS	Phosphate Buffered Saline
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
Ref	Reference
rpm	Revolutions per minute

LIST OF ABBREVIATIONS (cont.)

Abbreviation	Term
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
ScFv	Single chain variable fragment
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEMED	Tetramethylethylenediamine
Trop2	Trophoblast cell surface antigen 2
V _H	Variable heavy chain
V _L	Variable light chain
WB	Western blot

CHAPTER I

INTRODUCTION

Trophoblast cell surface antigen 2 (Trop2), also known as tumor-associated calcium signal transducer 2 (TACSTD2) or gastrointestinal antigen 733-1 (GA733-1) or epithelial glycoprotein 1 (EGP-1), is a transmembrane type-1 glycoprotein (1, 2), and firstly discovered on the surface of trophoblast cells since 1981 (3). Trop2 is coded by *tacstd2* on chromosome 1p32. Trop2 functions as an intracellular calcium signal and also involves in many pathways, including mitogen-activated protein kinases (MAPK) signaling (4), and Insulin-like growth factor-1 receptor (IGF-1R) signaling in lung cancer (5)

Trop2 overexpression correlates with poor prognosis in various cancer types such as breast cancer (BCA) (6, 7), ovarian cancer (8, 9), cervical cancer (10), non-small cell lung cancer (NSCLC) (11), pancreatic cancer (12), and cholangiocarcinoma (CCA) (13, 14). Moreover, the expression level in normal tissues was found only low or no expressions (15, 16). The investigation of Trop2 mRNA level by using real-time PCR has shown that CCA cell lines including KKU-213 and KKU-214 had the highest Trop2 expression when compared with other cell lines (14). Several reports have demonstrated that Trop2 can promote cancer progression by proliferation, invasion, metastasis, and survival (4, 15).

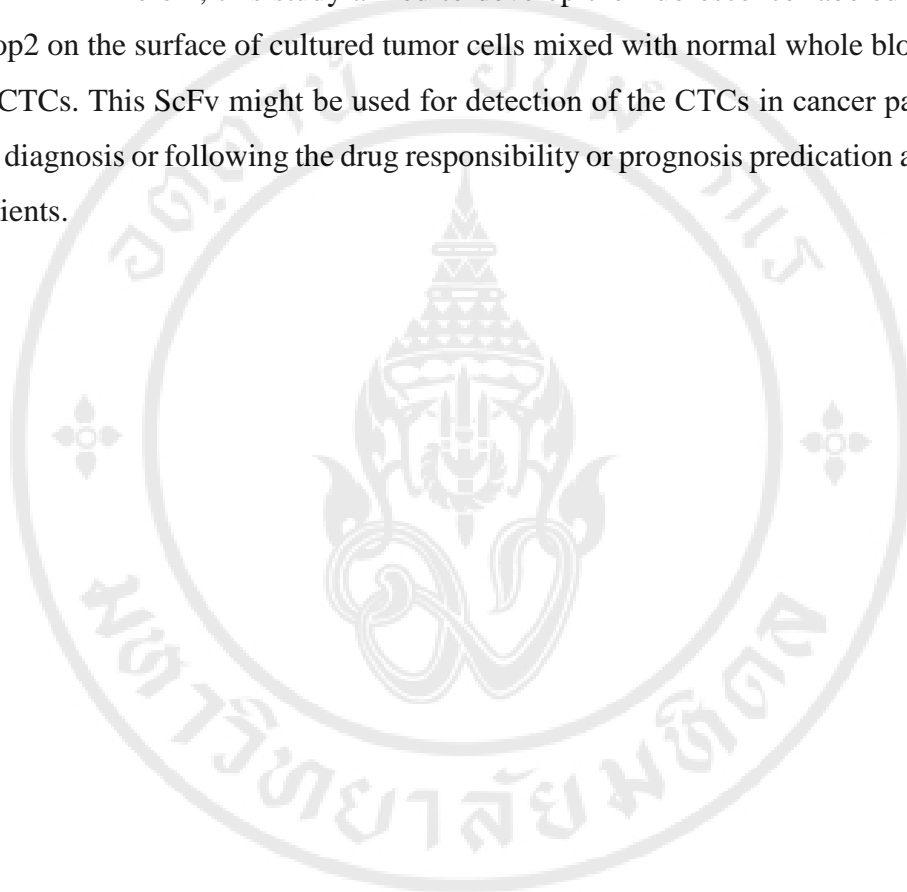
Nowadays, we have several technologies for generating antibodies for molecule detection. Firstly, since 1975, the monoclonal antibodies (mAbs) were produced by hybridoma technology (17, 18). These mAbs has been commonly used in many fields such as diagnostic, medical research and also therapeutic treatment (19). But it still has some limitations about time consuming, requirement of the tissue culture facility, and especially adverse effect in clinical applications (20). This may cause by the mAbs are usually mouse protein that can activate the immune response in the human, called human anti-mouse antibody response (21). Furthermore, the mAbs produced from small animals such as mouse, have usually high affinity, but cannot be improved

or modulated (22). The researchers attempt to reduce these problems so they try to produce many types of antibodies by many techniques. They produced the chimeric human-mouse mAb by the recombinant DNA techniques (23). The antibody fragments, including antigen binding fragment (Fab), variable fragment (Fv), and single chain variable fragment (ScFv) have been improved (24-26). Interestingly, the ScFv has been successfully improved by phage display technology (27, 28). The ScFv can be constructed by PCR amplification of human immunoglobulin gene from B-lymphocytes isolated from peripheral blood, spleen, or bone marrow. The large libraries that contained the diverse range of antibodies (V_H and V_L gene) were created *in vitro* (20, 28). All of gene libraries were cloned into a phagemid vector to perform recombinant phagemid and further transformed into bacteria such as *Escherichia coli* (*E. coli*) (29). Finally, the ScFv can be displayed on phage surfaces, each phage represents individual B-lymphocyte clone. So the antibody display phage library can be constructed similar to a pool of B lymphocytes in human and used to generate the ScFv against specific epitope of each antigen (22). Therefore, ScFv has a good potential for using in immunodetection and also medical application (20).

The sample collection for cancer detection is the important step that we should concern about their limitations. The limitation of tissue biopsies, the old sampling technique, that are invasive, unrepeatable, and especially it cannot be performed for monitoring the responsibility during treatment. The liquid biopsy is the new diagnostic tool that has high potential and rapidly growing not only for diagnostic application but also for improvement in therapeutics approach. This technique offers a non-invasive, reproducible, and high specificity because it requires only the blood sample (30). The circulating tumor cells (CTCs) from both primary tumor and secondary tumor and can be entered into the blood circulation by either directly shedding via surrounding vessels or through the lymphatic system (31). Although CTCs are the rare population in the blood sample; approximately 1-10 CTCs per mL of whole blood (32), it can be enriched and isolated from the whole blood sample by several techniques. The most popular technique is the CellSearch (CELLSEARCH® Circulating Tumor Cell Kit), the only approved for CTCs diagnostic technology from American Food and Drug Administration (FDA). By CellSearch technique, CTCs population can be isolated using epithelial cell surface markers for positive selection of CTCs such as Epithelial cell

adhesion molecule (EpCAM) and cytokeratin (CK) (33). However, it has some limitation about evident that some CTCs subpopulation without these cell surface markers such as cancer stem cells, and especially epithelial cells undergoing epithelial to mesenchymal transition (EMT) (34). So, it might be lost from the detection (35).

Herein, this study aimed to develop the fluorescence-labeled ScFv against Trop2 on the surface of cultured tumor cells mixed with normal whole blood, as mimic of CTCs. This ScFv might be used for detection of the CTCs in cancer patients' blood for diagnosis or following the drug responsibility or prognosis predication among cancer patients.



CHAPTER II

OBJECTIVES

2.1 Research questions

Can the Trop2mCherry ScFv detect Trop2-expressing CTCs?

2.2 Hypothesis

The Trop2mCherry ScFv can be produced to use as a detector for Trop2-expressing CTCs.

2.3 Research objectives

1. To produce Trop2mCherry ScFv for directly binding to Trop2 molecule on cancer cell surface.
2. To investigate the efficiency of Trop2mCherry ScFv for detection of Trop2 molecule that express on the surface of experimental mimic CTCs.

CHAPTER III

LITERATURE REVIEW

3.1 Trophoblast cell surface antigen 2 (Trop2)

3.1.1 Introduction of Trop2

Trop2 is a member of Trop family. Lipinski et al. identified the Trop family which composed of Trop1, Trop2, Trop3, and Trop4 by mAbs that produced from hybridoma cells, the fusion between the spleen cells of mice that were immunized by the BeWo choriocarcinoma cell lines and NS-1 myeloma cells (3). And the results were further confirmed by Alberti et al (36). They transfected genomic DNA of BeWo, choriocarcinoma cell lines into mouse L cells, so it can express Trop2 molecule on their surface. The transfected L cells were selected by fluorescence-activated cell sorting (FACS) that they used Trop2 mAbs labeled with fluorescein isothiocyanate (FITC) as a selective marker. They found that the mAbs can specifically recognize to Trop2 molecule and also proved the Trop2 structure by immunoprecipitation. These findings demonstrated that the molecular weight of Trop2 molecule was approximately 38 kDa (36).

3.1.2 Structure of Trop2

Trop2 is encoded by the *tacstd2* gene that located on chromosome 1p32. This gene is formed by the retroposition of the *tacstd1* gene that can produce 35 kDa of Trop2 molecule, consists of 323 amino acids, and can be divided into four domains (37).

The primary structure of Trop2 composed of a hydrophobic leader peptide that contains 26 amino acids (amino acid number 1-26) followed by the largest part of the extracellular (EC) domain with 248 amino acids (amino acid number 27-274). The extracellular domain composed of cysteine-rich domain (CRD), thyroglobulin type-1 domain (TY), and cysteine-poor domain (CPD). The glycosylation sites also located on the extracellular domain that represented on the amino acid number 33, 120, 168, and

208. The next domain is the 23 amino acids of the transmembrane (TM) domain (amino acid number 275-297) and the last domain is the short intracellular (IC) domain of 26 amino acids (amino acid number 298-323) (38).

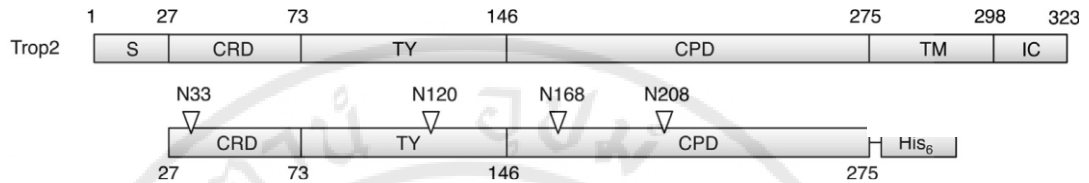


Figure 3.1 Schematic representation of domain organization of the Trop2 (38)

The tertiary structure model of Trop2 was constructed by Pavsic et al (39). Trop2 is a monomeric cell surface that can form the dimerization. The one of subunit can form with adjacent subunit as shown in Figure 3.2. The CRD domain represented in green color, TY domain represented in pink color, and CTD domain represented in yellow color and the other Trop2 subunit represented in gray color. The TM represented in orange and IC represented in blue color and contained the serine phosphorylation site (Ser303) represented in red color (39).

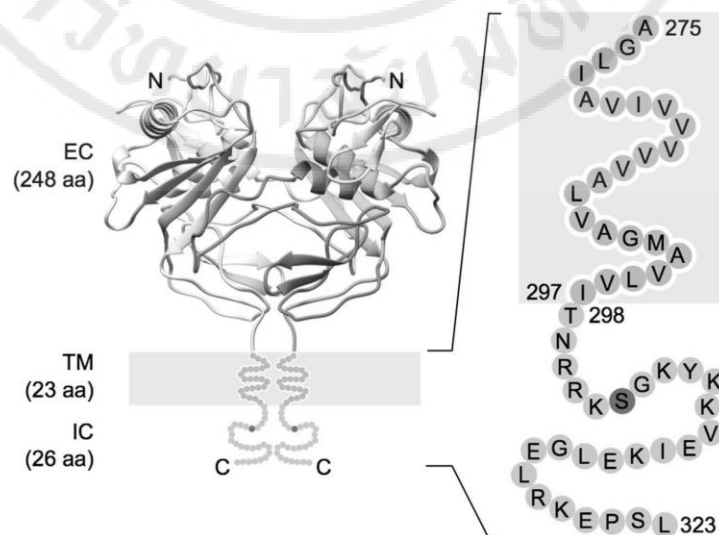


Figure 3.2 The domain structure of Trop2 (39)

3.1.3 Functions of Trop2

Many publications reported that Trop2 could interacted with many molecules and involved in many pathways (40). The important role of Trop2 is transduction an intracellular calcium signaling. Ripani et al. investigated the Trop2 signaling by specific mAb that could cross-link with Trop2 and the results showed increasing of calcium concentration in the cell, interpreted that the mobilization of calcium was coming from internal storage (41).

The cytoplasmic tail of Trop2 contains the phosphatidylinositol 4, 5-bisphosphate (PIP₂) - binding domain is bound to PIP₂, when the phosphorylation site (Ser303) is phosphorylated by protein kinase C (PKC). The PIP₂ is cleaved by phospholipase C (PLC) leading to increase the inositol 1, 4, 5 – triphosphate (IP₃) in the cytoplasm as well as diacylglycerol (DAG) in the plasma membrane. IP₃ can enhance Ca²⁺ release from the smooth endoplasmic reticulum in the cell. Both high level of free Ca²⁺ and DAG can increase the PKC. Therefore, once the PKC more increased it could more phosphorylation of Trop2 and also activation of the Raf and NF-κB pathway. Moreover, the free Ca²⁺ could activate MAPK pathway and cell cycle that could further increase the level of phosphorylated-extracellular signal-regulated kinases 1/2 (ERK1/2). Trop2 also increases the level of cyclin D1 and cyclin E. All of them, ERK1/2, cyclin D1, and cyclin E, are involved in cell cycle progression. For downstream signaling of ERK1/2, it could induce the expression of activator protein 1 (AP-1) transcription factor. The AP-1 is the important factor that involves in tumor progression including, proliferation, apoptosis, angiogenesis, invasion, and metastasis (4, 40).

Lin et al. reported that the insulin-like growth factor 1 (IGF-1) might be the one ligand of Trop2 (5). They conclude that it might has two possible mechanisms. The first one is the Trop2 might be bound with IGF-1 and allow to activate its downstream mediators. Another one possible mechanism is that the Trop2 might form a complex directly with IGF-1 led to prevent the IGF-1/IGF-1R signaling (5).

3.1.4 Trop2 overexpression

Generally, Trop2 is low or no expressed on normal cells. Trop2 is necessary in early development. On the other hand, Trop2 is overexpressed in various cancer types (15, 16). Many researchers found that the overexpression of Trop2 is associated with poor prognosis and important in cancer progression including proliferation, invasion, metastasis, and survival rate (15).

3.1.4.1 Breast cancer (BCA)

BCA is the most commonly diagnosed cancer and the leading cause of cancer death among women people in the world (42). So, many researchers still try to find the possible way that can reduce these incidents. Kluger et al. reported the first time of Trop2 expression in BCA cell lines (6). They found the overexpression of Trop2 in two BCA cell lines including BT-474, and BT-20 by immunohistochemistry (6). Furthermore, Huang et al. studied about the expression of the cell surface markers on both BCA cell lines and tissues (7). The result demonstrated that Trop2 overexpression was significantly high in ER-negative/HER2-positive BCA when compared with ER-positive/HER2-negative BCA (7).

3.1.4.2 Ovarian cancer

In year 2016, Ning et al. tried to investigate the expression of Trop2 in epithelial ovarian cancer (EOC) that is the advanced stage of ovarian cancer (8). The results demonstrated that both level of Trop2 mRNA and protein were upregulated in EOC cell lines. They use the quantitative polymerase chain reaction (qPCR) and FACS technique to investigate the Trop2 expression. They also studied in EOC tissue samples and the results showed that the overexpression of Trop2 in EOC tissue samples was 60% and associated with poor overall survival (8). Bin et al. studied about the overexpression of Trop2 and its mechanism (9). They investigated the Trop2 expression by immunofluorescence assay (IFA) in SKOV-3, HO8910, and A2780 ovarian cancer cell lines. The A2780 cell lines were highly expressed. Therefore, they tried to knockdown Trop2 by siRNA in A2780 cell lines and observed the cell migration and invasion by wound healing and cell invasion assays, respectively. The results showed that the ability of cell migration, cell invasion, and proliferation were inhibited when Trop2 was downregulated. So, they concluded that Trop2 might has potential as a novel target for ovarian cancer treatment (9).

3.1.4.3 Cervical cancer

The cervical cancer is the fourth for both incidence and mortality rate in the world (42). Ting et al. found the consistent result of the Trop2 expression between tissue samples and four types of cervical cancer cell lines (10). For tissue samples that showed the high expression level of Trop2 was significantly associated with decreased overall survival. For cervical cancer cell lines that they also tried to knockdown the Trop2 expression, the proliferation and invasion were inhibited. All of results can be concluded that the overexpression in cervical cancer lead to the aggressive cancer and poor survival outcome (10).

3.1.4.4 Non-small cell lung cancer (NSCLC)

The one type of lung cancer that highly aggressive vascular cancer, NSCLC. Xiaobin et al. reported that the Trop2 expression was significantly up-regulated in both tumor tissues and human NSCLC cell lines including A549, NCI-H520, NCIH441, NCI-H226, and HBE by using RT-PCR assay and western blot. Interestingly, they found that Trop2 can promote angiogenesis in NSCLC through ERK1/2 signaling pathway. So, they concluded that Trop2 was a critical angiogenesis regulator of NSCLC as well as proliferation and migration ability (11).

3.1.4.5 Pancreatic cancer

Trop2 overexpression was also reported in pancreatic cancer. Fong et al. found high Trop2 expression in pancreatic cancer tissue samples by IHC (12). Mao et al. also investigated Trop2 expression, they found the high level Trop2 expression in pancreatic cancer tissues when compared with non-cancerous tissues (43)

3.1.4.6 Prostate cancer

Prostate cancer is the most common and high incident rate in men. Although, prostate cancer is high cure rate in earlier stage but for advance stage is aggressive cancer and low survival outcome. Trerotola et al. studied in invasive prostate cancer, they found that Trop2 is high expression in metastatic cancer. The result demonstrated that Trop2 plays a role during cancer progression through $\alpha 5\beta 1$ integrin signaling pathway. Surprisingly, Trop2-expressing exosome can be isolated from PC3 cell lines (human prostate cancer cell line) and it has ability to promote cell migration (44).

3.1.4.7 Cholangiocarcinoma (CCA)

CCA is the second most common of hepatic malignancy. It is rare but is the aggressive cancer because there is no specific diagnostic in earlier stage lead to patients become advanced-stage disease as well as no effective treatment for these patients (45). Moreover, the prevalence rate of CCA are increasing worldwide especially in Thailand and also Southeast Asia (46). Shanglei et al. tried to investigate the Trop2 expression in hilar CCA, the one type of CCA, among Chinese patients (13). They also found that the high Trop2 expression was correlated with poor overall survival rate and microvessel density. The microvessel density widely used to determine the degree of angiogenesis in the cancer. Therefore, they suggested that Trop2 may promote cancer invasion and metastasis through angiogenesis (13). In Thailand, Sawanyawisuth et al. investigated Trop2 expression in six types of CCA cell lines (14). The highest Trop2 expression was KKU-213 and KKU-214 and the lowest expression was KKU-055 and KKU-100. KKU-213 cell lines were further suppressed Trop2 expression and observed the ability of cell proliferation and cell migration. Interestingly, the controversy result was found from this report because the result demonstrated that the ability of cell proliferation and cell migration was enhanced when Trop2 was suppressed (14).

The most of reports showed in the same way that Trop2 could activate several pathways that contribute to its cancer progression and acts as an oncogene. The elevated Trop2 expression is significantly correlated with cell migration, cell invasion, poor overall survival, and poor prognosis. Therefore, Trop2 might be used to the effective prognostic factor for various cancer types.

The summary of Trop2 expression in various cancer types including BCA, ovarian cancer, cervical cancer, NSCLC, pancreatic cancer, prostate cancer, and CCA was concluded and shown in Table 3.1.

Table 3.1 The summary of Trop2 expression in various cancer types

Cancer types	Samples	Testing methods	%Positive* (positive cases/ total cases)	Expression level	Ref.
BCA	Cell lines: BT-474, BT-20	IFA	-	High expression	(6)
	Cell lines: BT474 MDA-MB-231	qPCR	-	High expression	(7)
	Cell lines: SKBR3 MDA-MB-435 MDA-MB-468			Low expression	
Ovarian cancer	Tissue	IHC	90.6 % (119/128)	Low-high expression	(8)
	Cell lines: SKOV3	IFA	-	weak	(9)
	Cell lines: A2780			High expression	
Cervical cancer	Tissue	IHC	88.7 % (94/106)	Low-high expression	(10)
	Cell lines: CaSki, Siha	WB and IFA	-	High expression	
	Cell lines: HeLa, C33A			Low expression	
NSCLC	Tissue	qPCR	55 % (58/107)	High expression	(11)
	Cell lines: A549 NCI-H520 NCIH441 NCI-H226	RT-PCR and WB	-	High expression	

Table 3.1 The summary of Trop2 expression in various cancer types (cont.)

Cancer types	Samples	Testing methods	%Positive* (positive cases/ total cases)	Expression level	Ref.
Pancreatic cancer	Tissue	IHC	55 % (109/197)	High expression	(12)
	Tissue	qPCR, and IHC	60% (144/189)	High expression	(43)
	Cell lines: BxPC3, PL45	FCM, IFA	-	High expression	
Prostate cancer	Tissue	IHC	45% (67/148)	High expression	(44)
CCA	Tissue	IHC	61.4 % (43/70)	High expression	(13)
	Cell lines: KKU-055 KKU-100	IFA, Real time PCR	-	Low expression	(14)
	Cell lines: KKU-213 KKU-214			High expression	

* **% Positive** means the percentage of cases that express Trop2 when compare with total cases.

Note: **IFA:** Immunofluorescence assay; **FCM:** Flow cytometry; **IHC:** Immunohistochemistry; **qPCR:** quantitative polymerase chain reaction; **RT-PCR:** Reverse transcription polymerase chain reaction; **WB:** Western blot

3.2 Antibody production

The one application of antibodies that widely used to detect in immunodetection. Many types of antibodies are selected to use in different experiment depend on the objective. Especially, the mAbs are the most powerful in many methods. Since 1975, George Köhler and Cesar Milstein developed the mAbs by the hybridoma technology (17).

For hybridoma technology as shown in Figure 3.3, in brief, the first step starts with the mouse that was immunized with interesting antigen. After the immunization, the spleen of mouse is removed and isolated the splenocytes. The B-lymphocytes, one of cell populations in splenocytes, are fused with immortalized myeloma cells become hybridoma cells that continuously produce mAbs specific to antigens of interest. The hybridoma cells are cultured in selective medium and only clone that can produce mAbs specific to antigens of interest is selected and further expansion (47).

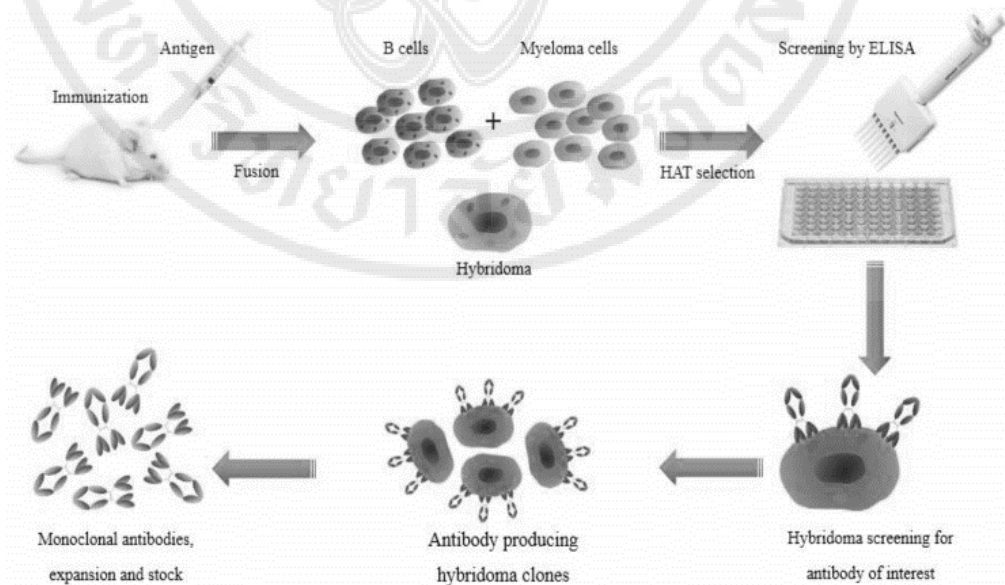


Figure 3.3 The procedure of mAbs production by hybridoma technology (47)

The mAbs have been chosen to use not only in the laboratory but also in clinical medicine (48). Nevertheless, the mAbs that produced from hybridoma cells are usefulness in many fields, still have some limitations. The human anti-murine antibody response is the adverse effects from human immune responses against murine protein due to the mAbs are generated by the mouse (21). Furthermore, the hybridoma technology has the long step so it requires the time as well as tissue culture facility, it also can't be improved or modulated for further development (22). So, the scientists try to develop the many techniques that further reduce the limitation of these mAbs. The antibody engineering technology has been developed to solve this problem which is *in vitro* technique such as phage display technology. The phage display technology was discovered by George P. Smith since 1985, by this work he had won the Nobel Prize in Chemistry, in 2018. He successfully constructed the fusion phage that fusion between the interested protein and gene III of filamentous phage. The exogenous proteins, interested protein, can be displayed on the outside of filamentous phage (49). The bacteria especially *E. coli* were used for cloning and expression of recombinant antibodies but it was difficult because the expression of full-length antibodies in bacteria can be disturbed by protein of bacteria in their cytoplasm. The antibody fragments were designed for solving problem (50).

The full-length antibodies, molecular weight around 150 kDa, consist of four polypeptide chains, two identical heavy chains and two identical light chains (47). For each heavy chain contains one of variable (V_H) domain and three or four constant (C_H) domains depends on type of antibody such as IgG contains three C_H while each light chain contains one of variable (V_L) domain and one constant (C_L) domain. Two regions are classified, antigen binding fragment (Fab) and fragment crystallizable (Fc) region. Fab region contains the antigen-binding site (paratope), the site of binding with specific epitope, is the forming of V_H and V_L domains. Fc region contains only two C_H , to generate an immune cell activity. Hinge region is a linker between Fab and Fc region. Antibody structure was shown in Figure 3.4 (22).

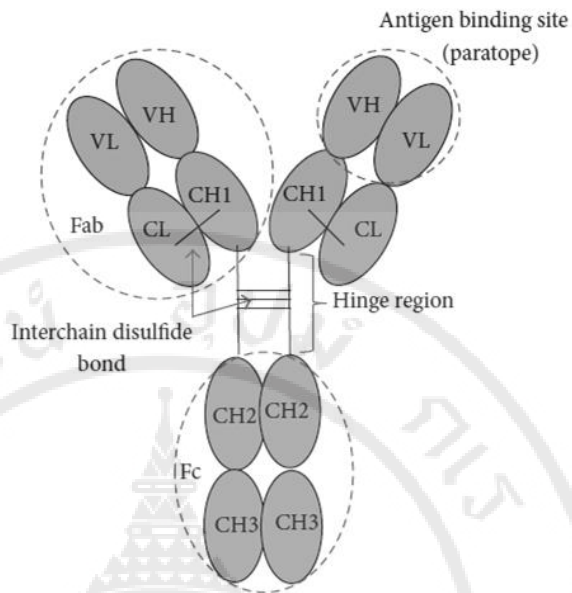


Figure 3.4 The structure of full-length antibody (23)

The engineering of various antibody fragments that are smaller than full-length antibodies and still contain an antigen-binding site are developed including single chain variable-Fc fragment (ScFv-Fc), (Fab')₂ fragment, minibody, Fab (monovalent), diabody, ScFv, and peptide (47) as shown in Figure 3.5.

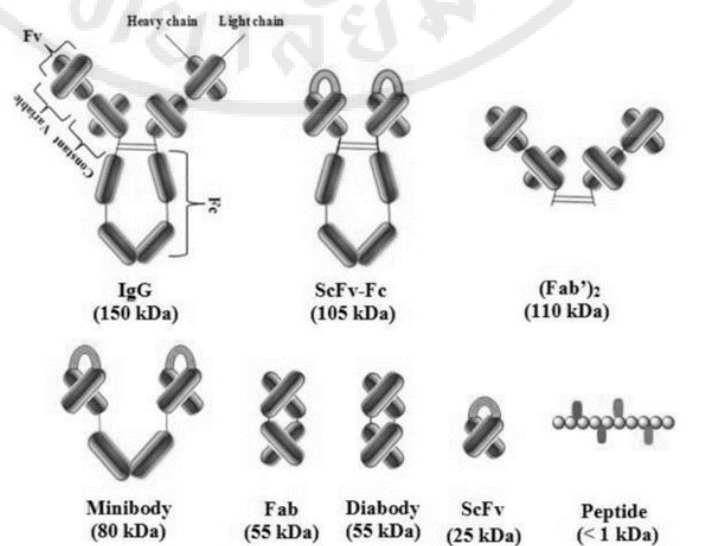


Figure 3.5 The format of antibody fragments (47)

Proteolytic enzyme digestion of IgG can produce two types of antibody fragments. (Fab')₂ fragment, two Fab with hinge region, is produced by digestion with pepsin enzyme. Fab (monovalent) is digested by papain enzyme (51). Genetic engineering technique is applied to produce the variable fragments including ScFv (V_H and V_L domains are linked together by flexible peptide), diabody (complementary domains of ScFv), minibody (ScFv fusion with one C_H), and ScFv-Fc (ScFv fusion with Fc region) (47).

These fragments are better than full-length antibodies for production, modification and some usages (50, 52). The advantage about immunotherapy such as tissue penetration, blood clearance, short retention times as well as a reduction of immunogenicity (50). Moreover, they are easier for transformation into bacterial and well display on a filamentous phage (50). So, they are good choice for both diagnostic and immunotherapy (50). The most popular antibody fragment is the ScFv because it has high affinity, high specificity, highly solubility. Moreover, it is a primarily monomeric fragment that can be further applied to another antibody fragments as well as be easy to modify their function such as carrying toxins to kill the cancer cells, carrying cytokines to activate the immune cells, and containing the fluorescence for immunodetection (52).

3.2.1 Single chain variable fragment (ScFv)

The ScFv is interesting for using in many purposes. It has been successfully improved by phage display technology (27). The structure of ScFv consists of V_H and V_L are linked together by short peptide and has molecular weight approximately 25 kDa (20, 47).

3.2.1.1 Generation of human ScFv by phage display technology

Phage display technology is mostly useful for specific gene expression and study of protein-protein or protein-peptide interaction. This technique is easy for screening of peptide or antibody fragments especially a ScFv. Phage display system is based on various bacteriophage. Bacteriophage was classified into four types. T4 Phage is distinguished for cDNA expression and display of peptides or proteins on phage capsid surface at high copy numbers. T7 Phage can carry large protein fragments

at low level and has a high stability under various extreme conditions such as high or low pH, high salt concentration. Lambda phage can display multiple copies of peptides or the large protein domains but it has some evident that foreign protein may interfere with the assembly of the Lambda phage tail. Filamentous bacteriophage is commonly used for phage display. It contains a genome of single-stranded DNA and has four family including Ff, M13, fd, and f1. M13 bacteriophage is the most common use. Their genome contains five coat protein, major coat proteins (pVIII) and minor coat proteins (pVII, pIX, pVI and pIII). Most common of antibody fragments or proteins are display on pIII and pVIII (53).

In order to construct the phage display libraries, the messenger ribonucleic acids (mRNA) were extracted from many sources of B-lymphocytes of human such as peripheral blood, spleen, or bone marrow as well as hybridoma cells (22). The mRNA should be reversed into complementary deoxyribonucleic acids (cDNA), act as template of antibody repertoires gene. Next important step is PCR assembly, the most popular technique. The amplified sequences of V_H and V_L gene are linked by polynucleotide linker coding, a triplicate of four glycines and one serine (G4S)₃. The large libraries that contain the diverse range of antibodies, V_H -linker- V_L gene, or human ScFv gene repertoires were created *in vitro*. The human ScFv gene are cloned into phagemid (plasmid contains a replication of bacteria for propagation of the plasmid in *E. coli* and a phage origin for replication and packaging of single-stranded DNA) to perform recombinant phagemid. The *E. coli*, the most popular organisms for the phage display, are used for transformation of recombinant phagemid. The successful transformed *E. coli* are grown and co-transfected with helper phage that are essential for the replication and assembly of phagemid particles. After co-transfected with helper phage, the human ScFv are displayed on phage surface (20, 22, 47).

The procedures for the generation of human ScFv phage display library was shown in Figure 3.6.

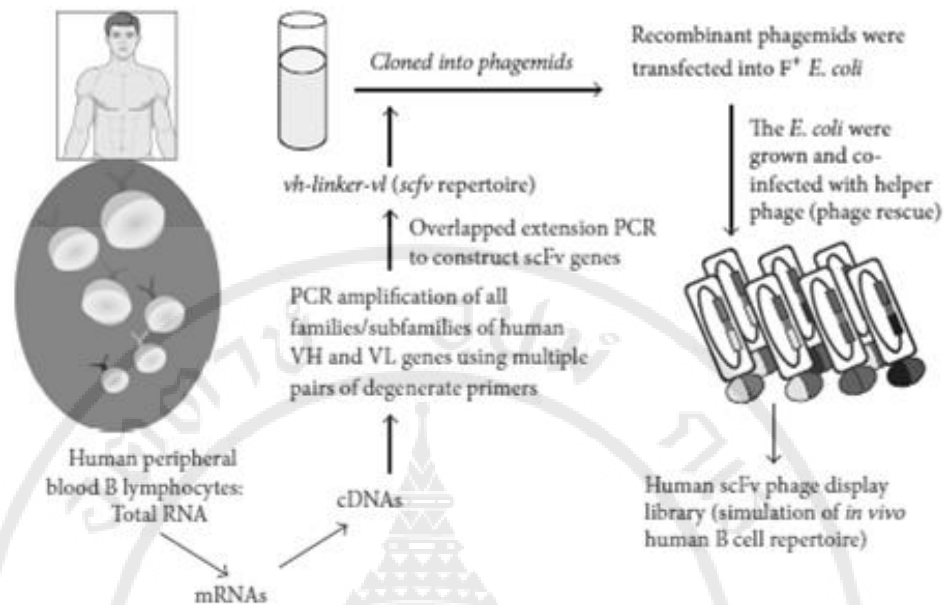


Figure 3.6 The procedures for generation of human ScFv phage display library (23)

3.2.1.2 The screening of ScFv by phage biopanning

The main objective of phage display is the high affinity selection because the fusion human ScFv that display on phage surface can specifically bind to target antigen while the other phages can't bind to the antigen (20). Therefore, the phage display is the useful technique for screening the ScFv. After the human ScFv phage display library are generated, the target antigen that already coated on the solid phase such as immunotubes, microtiter plates or solid particles can be bound by the only phages that display their specific ScFv. For the unbound phages will be washed out. The specifically bound phages are eluted and further amplified by the *E. coli*. After amplification, each clone is screened with target antigen again, the cycle can be repeated around 3-5 cycles. The DNA sequencing might be performed for confirming the primary structure of each clone after finish in final cycle for determination of structural and functional property. The numerous screening cycles are important in the phage biopanning in order to make sure that the specifically bound phages are unique bind to target antigen. The clone that is successful from screening step can further express by bacterial expression system especially *E. coli* (20, 22, 47). The summarized of procedures of screening by phage biopanning was shown in Figure 3.7.

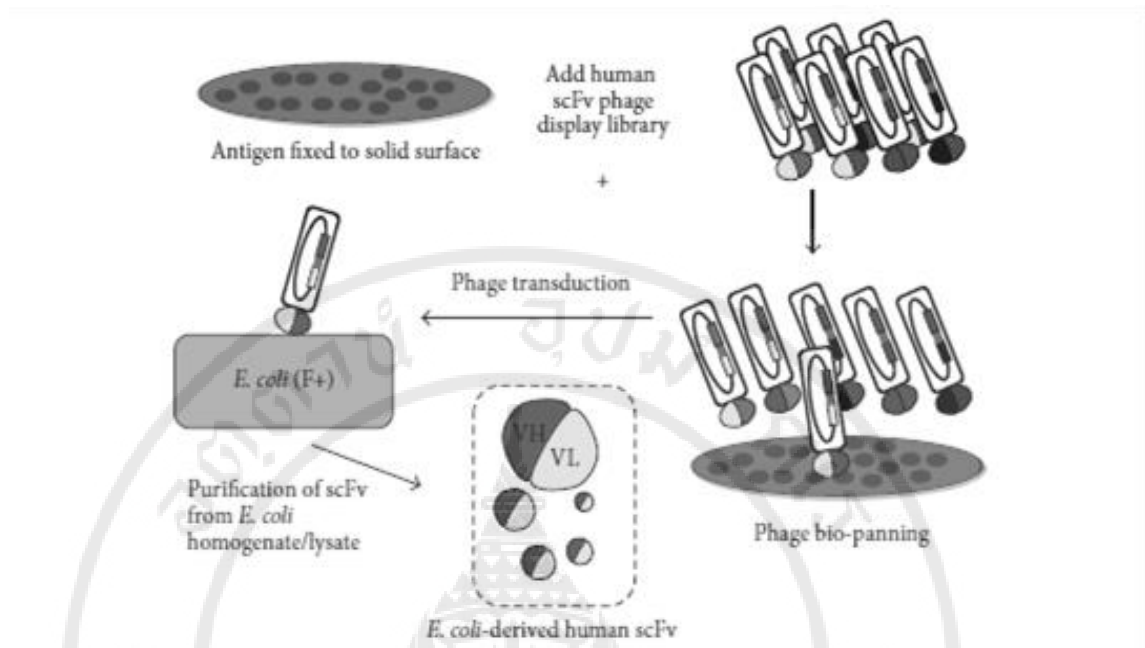


Figure 3.7 The screening of human ScFv by phage biopanning (23)

3.2.1.3 Application of ScFv

The ScFv that produce by phage display technology are powerful for several applications including medical treatment, diagnosis, and research field. Because they have many advantages such as inexpensive, easy to modify, high throughput, high stability, and easy to perform (20). Examples of ScFv application were described below.

- Medical treatment

The ScFv are smaller than the whole antibody, so they can penetrate rapidly into tumor mass or other tissue (54). The ScFv can fuse with toxin or drug and form to immunotoxin or therapeutic drug delivery, respectively. Therefore, the ScFv can specifically deliver their agents to target tissue (55). The rapidly clearance from blood is the one of property of ScFv, so when they are fused with toxin, the toxicity might be less harmful to healthy tissue (56).

- Laboratory diagnostic

The one advantage of ScFv is they can be easily modified, which means that the ScFv can be fused with another agent such as a fluorescent proteins as well as color-generating enzyme (20). Remko et al., they successfully designed the ScFv-GFP fusion proteins, called fluobodies. They used the phage display technique for

the production of fluobodies (57). These fluobodies can be used for both flow cytometry and immunofluorescent assay (57). Martin et al. also successfully produced the ScFv-alkaline phosphatase fusion molecules for using in enzyme-linked immunosorbent assay (ELISA), western blot, and immunohistochemistry (58). Therefore, it can be used for direct detection instead of the expensive conjugated-enzyme antibody (58). Moreover, the ScFv can be fused with various fluorescent proteins as well. Interestingly, the fluorophor-linked immunosorbent assay (FLISA), this assay is similar to ELISA, has been developed by Peter et al. The ScFv were fused with fluorescent proteins instead of a color-generating enzyme. This assay is faster and easier than conventional ELISA (59).

Therefore, the antibody fragments especially ScFv offer to the potential for both medical treatment and laboratory diagnostic. However, the production of ScFv should be well suitable to get the well functional and antibody properties (20).

3.3 Antibody fragment for Trop2 targeting

As mention previously, Trop2 may be used as a maker for diagnosis, prognosis, or targeted tumor therapeutic in cancer patients. Therefore, many researchers take an interest in the development of antibody fragment against Trop2 molecule on cancer cell surface to respond for their purpose (60).

3.3.1 Human anti-Trop2 antibody IgG

Ikedo et al. tried to develop the new human anti-Trop2 antibody IgG by immunization of prostate cancer cell in Balb/c mice, they called Pr1E11. They found that Pr1E11 had high specificity to Trop2 molecule and they also investigated the binding site of Pr1E11, they found that Pr1E11 bind to CRD, one part of extracellular domain, of Trop2 molecule. Next, they expected that Pr1E11 might be use as a naked antibody *in vivo*, they tried to investigate the internalization activities of Pr1E11. The result demonstrated that Pr1E11 had low internalized into cancer cell. They mention that the high internalization activity antibody might reduce the efficacy of naked antibody. Therefore, Pr1E11 might be used as a naked antibody for therapeutic purpose (61). They further studied on antibody effector function especially antibody-dependent

cellular cytotoxicity (ADCC), because ADCC is the one mechanism for anti-tumor effect. The result demonstrated that Pr1E11 had high anti-tumor activity and ADCC activity *in vivo* (62).

Wang et al. tried to construct the whole antibody molecule specifically to Trop2 molecule by PCR amplification to generate the sequence of each chain which is light chain and heavy chain and then combined together within recombinant expression vector. Interestingly, they tried to express by eukaryotic expression system, Chinese hamster ovary cell line with dihydrofolate reductase defect (CHO/dhfr- cell line), and hypothesized that their anti-Trop2 antibody IgG could bind to Trop2 molecule specifically on cancer cell surface and inhibit cell proliferation in pancreatic cancer cells (63). They got the good result, the binding activity was determined by flow cytometry and IFA, the result indicated that anti-Trop2 antibody IgG can bind only cell that express Trop2 molecule on their surface. Moreover, anti-Trop2 antibody IgG has an effect to inhibit proliferation rate of pancreatic cancer cells, *in vitro* (63).

3.3.2 Human Antigen binding fragment (Fab) antibody

Lin et al. tried to produce the human Fab antibody against Trop2 molecule by phage display technology and investigated the inhibitory effect of tumor progression on BCA both *in vitro* and *in vivo*. They determined the binding activity of Trop2 Fab first, the result demonstrated that Trop2 Fab bind to only Trop2-expressing cancer cells. *In vitro*, Trop2 Fab could inhibit cancer cell proliferation and migration of Trop2-expressing cancer cells as well as induce cancer cell apoptosis. *In vivo*, at only the high concentration of Trop2 Fab could inhibit cancer growth (60).

Although, human antibody fragment against Trop2 was constructed and developed in many methods and used mainly in therapeutic purpose, another purpose still interesting.

3.4 Circulating tumor cells (CTCs)

3.4.1 Characteristics of CTCs

The CTCs have been first discovered in cancer patients since 1869 (64). The CTCs are the rare population in the blood circulation, approximately 1-10 CTCs per mL of whole blood (32), but they still have a potential to use as a real-time marker of prognosis in the cancer patients (31). The CTCs can be entered into blood circulation from both primary and secondary tumor site as well as they can be entered into the blood circulation by either directly shedding into surrounding vessels or through the lymphatic system (31, 65). In the circulation, CTCs can circulate both single cell and cluster cells. The cluster form of CTCs has increased metastatic potential and higher clearance rate from the blood circulation when compared with single CTCs (66).

3.4.2 Detection of CTCs

The rare population of CTCs can be enriched and isolated from the whole blood sample by several techniques. The most popular technique is the CellSearch[®], the only approved for CTCs diagnostic technology from Food and Drug Administration of the United State of America (US-FDA). The CellSearch[®] technique can isolate CTCs population by using epithelial cell surface markers for positive selection of CTCs such as EpCAM and cytokeratin (CK) (33). This technique still has some limitation about evident that some CTCs subpopulation isn't express these cell surface markers such as cancer stem cells, and especially epithelial cells undergoing epithelial to mesenchymal transition (EMT) (34). So, it might be lost from the detection (35). The other techniques have been developed to reduce the limitation such as microfluidic, the microfluidic has many platforms including immunoaffinity chromatography, magnetic activated micro-sorting, and size-based cell separation resulting in high throughput, high CTCs recovery, high purity, and high cell viability (67).

3.4.3 Application of CTCs

The one advantage of CTCs to use as a biomarker for detection in cancer patients that is non-invasive because it is only drawing the blood sample from the patients (30). For the monitoring of patients that following the drug responsibility that

they should be followed on the time during the drug treatment. The liquid biopsy becomes the technique that can use as a new diagnostic tool which is high potential and rapidly growing not only diagnostic application but also improvement in therapeutics cancer patients (30, 68). Many few year ago, the CTCs were used as a parameter for diagnosis, prognosis, and therapeutics responsibility (30).

3.4.3.1 Diagnosis

The problem of CTCs when used as a parameter of diagnosis is the early detection it might be misdiagnosed due to the very small amount of CTCs that entered into blood circulation of early stage of cancer (30). On the other hand, some researchers tried to use CTCs for distinguishing the malignant and benign lesions (69).

3.4.3.2 Prognosis

The prognosis role of CTCs is very useful in clinical application. The many results have been confirmed that the number of CTCs in blood sample of cancer patients are associated with poor prognosis as well as metastatic lesions (30).

3.4.3.3 Therapeutics

The CTCs are also used as predictive of therapeutics responsibility (70). The CTCs that found in the blood sample can be used to monitor the effectiveness of treatments. For example, the patients with poor reduction of CTCs level should be changed or optimized the treatment. In the same way, The CTCs level is significantly decreased after treatment when compared with base line (64). Moreover, the molecules that expressed on CTCs are very helpful to use as prognosis marker especially immune checkpoint such as programmed death-1 (PD-1) and programmed death-ligand-1 (PD-L1). Kallergi et al. evaluated the expression of PD-1 and PD-L1 on the isolated CTCs from blood of non-small cell lung cancer (NSCLC) patients (71). They found that the high number of PD-1 positive CTCs before treatment (front-line chemotherapy) is associated with poor survival outcome (71).

CHAPTER IV

MATERIALS AND METHODS

4.1 Materials

4.1.1 Plasmid

4.1.1.1 Trop2mCherry ScFv recombinant plasmid

The recombinant plasmid that contains the sequence of anti-Trop2 ScFv labelled as clone number 31 and conjugated with mCherry was kindly contributed from Associate Professor Peti Thuwajit, Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University. The procedure to produce this plasmid was summarized in Appendix B.

4.1.1.2 TACSTD2 (GFP-tagged) - Human tumor-associated calcium signal transducer 2

TACSTD2 (GFP-tagged) Human-tumor-associated Calcium Signal Transducer 2 (RG202519, OriGene Technologies, Rockville, USA), the commercial plasmid that contains the sequence of *human Trop2* gene (NM_002353) combined with *gfp* gene, a green fluorescence, and constructed on pCMV6-AC-GFP vectors was kindly contributed from Professor Byung-Heon Lee, Department of Biochemistry and Cell Biology School of Medicine, Kyungpook National University, Daegu, Republic of Korea. This plasmid contains ampicillin resistant gene for *E.coli* selection and neomycin resistant gene for cell lines selection. This plasmid was used for transfection with cancer cell line which has no or low Trop2 expression. The map of this plasmid was shown in Figure 4.1.

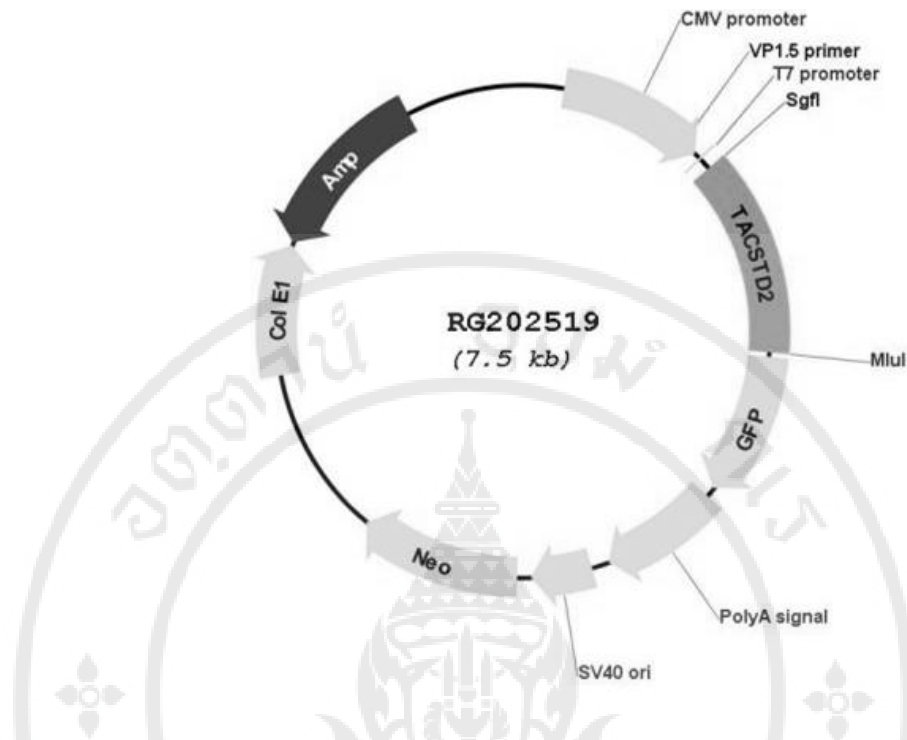


Figure 4.1 Map of TACSTD2 (GFP-tagged) plasmid

4.1.1.3 Human *TACSTD2* FlexiTube siRNA

Human *TACSTD2* FlexiTube[®] siRNA (SI00036596, Qiagen, Maryland, USA) a siRNA targeted to *human TACSTD2* gene. To knockdown Trop2 targeting on cancer cell lines.

4.1.2 Bacterial stains

4.1.2.1 *E. coli* DH5 α

DH5 α , one strain of *E. coli*, the high transformation efficiency is the one of properties that make it suited for cloning (72). This stain was used to amplify stock of all plasmid used in this experiment. The cells were kept in 50% glycerol at -80°C. The selective antibiotic is 100 μ g/mL of ampicillin (Merck, USA).

4.1.2.2 *E. coli* Rosetta

The *E. coli* Rosetta is the derivatives of *E. coli* BL21 that is designed to enhance the expression of eukaryotic proteins because it contains rare tRNA supplementation (73). Cells were kindly given by Dr. Chunya Puttikhunt, Division of Dengue Hemorrhagic Fever Research, Faculty of Medicine Siriraj Hospital, Mahidol

University. Therefore, this strain provides for universal translation and suitable for production of proteins. This strain was used for production of recombinant ScFv. The selective antibiotic is 100 µg/mL of ampicillin (Merck) and 100 µg/mL of chloramphenicol (Merck).

4.1.3 Protein purification and concentration equipment

4.1.3.1 HisTrap high performance (HP) columns

The HisTrap[®] HP columns (GE Healthcare Life Sciences, Marlborough, MA, USA) contain Ni Sepharose, which has high binding capacity. It were designed for simple, high-resolution purification of histidine-tagged proteins by immobilized metal ion affinity chromatography (IMAC).

4.1.3.2 Vivaspin 6 mL column

Vivaspin[®] 6 (GE Healthcare Life Sciences), a protein concentrator spin column, was used for recovery the concentration of ScFv by ultracentrifugation. Polyethersulfone is the type of membrane and the body of tube consist of polycarbonate. The all materials are the reducing protein absorption.

4.1.4 Antibodies and counter staining

4.1.4.1 Mouse anti-human Trop2

Human anti-TROP-2 antibody (MAB650, R&D system, Minneapolis, MN, USA) was a monoclonal mouse IgG_{2A} antibody against human Trop2 molecule. It was be used as positive control for comparing with Trop2mCherry ScFv.

4.1.4.2 Goat anti-mouse IgG antibody

Cy[™]3-conjugated Affini Pure[®] Goat Anti-Mouse IgG, (115-165-071, Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA) was used as a secondary antibody for primary antibody detection.

4.1.4.3 Hoechst 33258 staining

Hoechst 33258 (Invitrogen, Thermo Fisher Scientific Corporation, Waltham, MA, USA) which is the blue fluorescent dyes, nucleic acid staining, which emits blue fluorescence when it bound to double stand DNA.

4.1.4.4 CellTracker™ Green CMFDA

CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate C2929, Invitrogen), a green fluorescence probe that can pass through cell membrane and has less toxicity will be used for staining of viable cells. For stock, at concentration 10 mM in dimethylsulfoxide (DMSO) was kept in -20°C with light protection.

4.1.5 Cancer cell lines

4.1.5.1 BCA cell lines

MCF-7 and MDA-MB-231 cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco®, Thermo Fisher Scientific Corporation, Waltham, MA, USA) with heat-inactivated of 10% fetal bovine serum (FBS, Gibco®). 100 Units/mL of Penicillin and 100 µg/mL of Streptomycin (Gibco®) was added for prevention of microbial contamination.

4.1.5.2 CCA cell lines

KKU-213 cell lines and KKU-055 were grown in DMEM with 10% FBS. 100 Units/mL of Penicillin and 100 µg/mL of Streptomycin (Gibco®), maintained in 5% CO₂ at 37°C.

4.1.5.3 Ovarian cancer cell lines

Kuramochi cell lines were grown in Roswell Park Memorial Institute (RPMI) 1640 (Gibco®) with 10% FBS. 100 Units/mL of Penicillin and 100 µg/mL of Streptomycin (Gibco®), maintained in 5% CO₂ at 37°C.

4.1.5.4 Ethical approval

Procedures of human blood collection were approved by ethical committee on Human Research Protection Unit, Faculty of Medicine Siriraj Hospital, Mahidol University. Certification number 841/2019.

4.1.6 Chemical reagents

All chemical reagents used in this study is a molecular biology and culture grade.

4.2 Experimental Design

The experiment started from amplification of ScFv recombinant plasmid and transformation into expression strain of *E. coli* Rosetta. The transformed *E. coli* were induced for ScFv-fusion proteins expression. The purification and concentration of ScFv was performed in order to obtain the best productivity of ScFv. The favorable ScFv would be used to detect the target molecules, Trop2, which express on the surface of cancer cell lines by immunofluorescence assay (IFA) for determination of binding affinity. For the application of these ScFv, the Trop2mCherry ScFv could bind to Trop2 molecules on the cancer cells that circulated in patients. If the Trop2mCherry ScFv can bind to Trop2 molecules very well, it may apply to use with other technique such as microfluidic technique. The diagram of experimental design was shown in Figure 4.2.

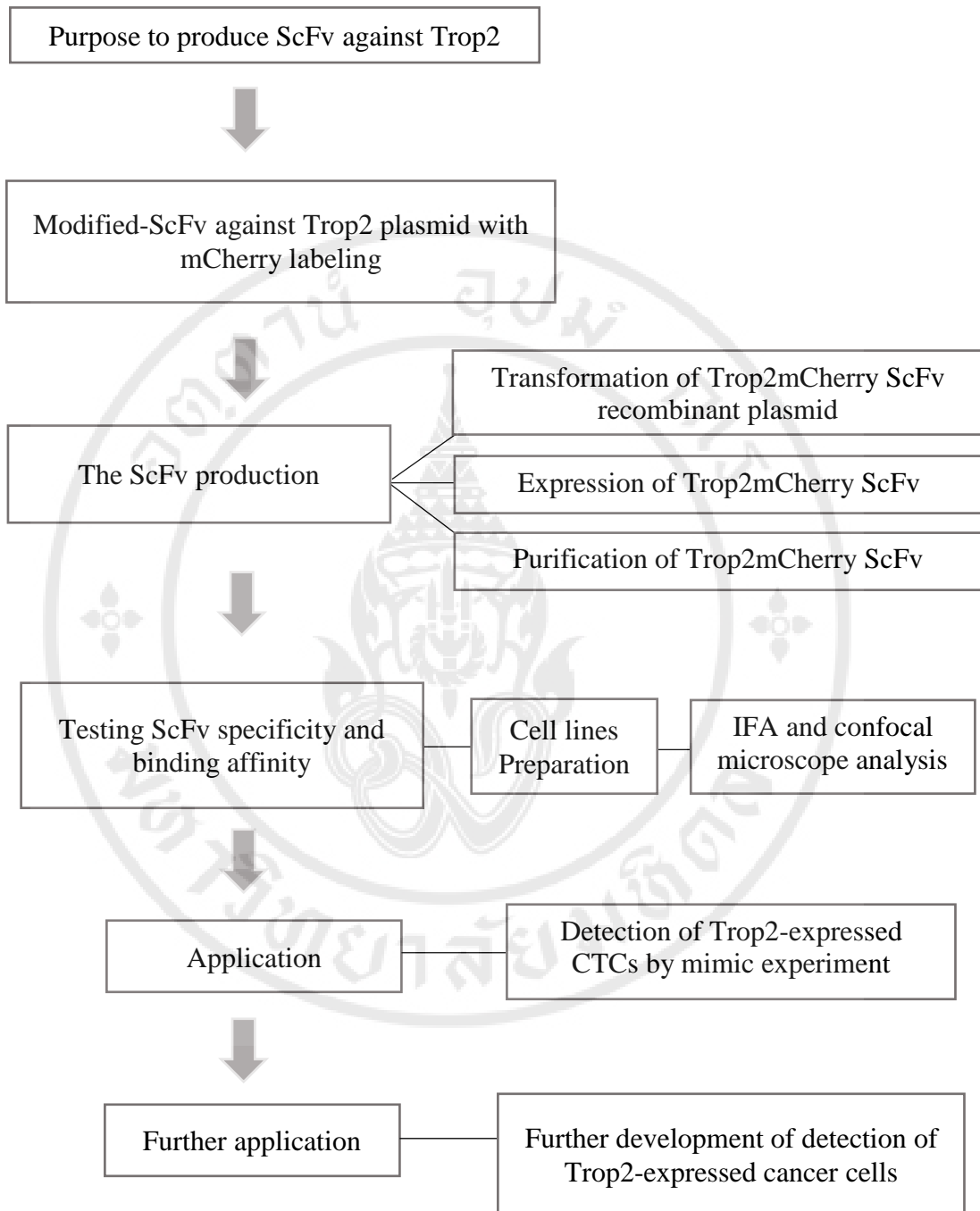


Figure 4.2 Diagram of experimental design

4.3 Methods

4.3.1 Transformation

The clone of recombinant plasmid (clone number 31) that contains the sequence of anti-Trop2 together with mCherry was transformed using the two types of *E. coli* including, *E. coli* DH5 α for amplification and *E. coli* Rosetta for expression.

To prepare competent cells using CaCl₂ method, *E. coli* stock was incubated at 37°C around 2 hours until reach 0.25 of optical density (OD) at 600 nm. Then aliquot them into 1.5 mL tube (1 mL/tube). Let them on ice for 30 minutes and then centrifuge at 4,000 rpm, 4°C for 2 minutes, the supernatant was discarded. The cell pellets were gently resuspended with 150 μ L of 100 mM CaCl₂ and then centrifuge at 4,000 rpm, 4°C for 2 minutes, the supernatant was discarded. The cell pellets were gently resuspended with 100 μ L of 100 mM CaCl₂. After complete the competent cells preparation, the desire plasmid was applied and incubated on ice for 30 minutes. The heat shock method was performed immediately, 42°C for exactly 1 minute and then let them on ice. 900 μ L of Luria-Bertani (LB) broth was added and incubated at 37°C, 1 hour. The transformed cells were spread on LB agar with suitable antibiotics (100 μ g/mL of ampicillin for *E. coli* DH5 α , 100 μ g/mL of ampicillin and 34 μ g/mL of chloramphenicol for *E. coli* Rosetta). After incubated at 37°C overnight, colonies on agar were collected to grow further in LB broth at 37°C overnight and extracted plasmid following protocol of AccuPrep[®] Plasmid Mini Extraction Kit (Bioneer Corporation, Korea). Finally, the plasmid was observed the size by agarose gel electrophoresis.

4.3.2 Protein expression

The protein expression on a large scale using the bacterial system was selected to perform in this study. The autoinduction technique is the technique for inducing of protein expression to produce a lot of ScFv. The pre-cultured *E. coli*, transformed-*E. coli* Rosetta with Trop2mCherry ScFv recombinant plasmid, was grown in LB broth plus ampicillin and chloramphenicol at shaking 250 rpm, 37°C overnight. The expression medium that consists of fresh LB broth base, sugar mix solution, and the pre-cultured *E. coli* were incubated at shaking 250 rpm, 37°C until OD at 600 nm is approximately 0.6. The temperature was changed from 37°C to 16°C for overnight.

4.3.3 Protein purification

The induced *E. coli* cells were harvested by centrifugation. The cell pellets were collected after centrifugation at 8,000g, 4°C for 5 minutes, and resuspended in cold 40 mM imidazole. The cell suspension were lysed by sonication. After that, the only cell lysate that contains the soluble Trop2mChery ScFv were separated by high speed centrifugation (10,000g, 4°C for 30 minutes). HisTrap® HP columns, the Ni sepharose high performance affinity resin that contains the highly cross-linked agarose beads to bind with chelating group (in this case is histidine) was selected to use for protein purification. After crude extracts were separated, all of them were loaded into column and eluted by two concentrations of imidazole (250 mM and 500 mM imidazole, respectively). The concentration was measured following protocol Bradford protein assay (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad, USA). The purity of ScFv would be further evaluated by SDS-PAGE analysis.

4.3.4 Increasing of protein concentration

To recover the Trop2mChery ScFv, the concentration of each fraction was tried to be concentrate by Vivaspin® 6. Trop2mChery ScFv fraction was mix with PBS and then was loaded to vivaspin column and centrifuge at 4,000g for 15 minutes. The remaining volume was observe after centrifugation. If the remaining volume is close to 1 mL it was collected to new tube. If the remaining volume is too high, it will be gently pipette mixed and re-centrifuged until the remaining volume is close to 1 mL. After concentration, Trop2mChery ScFv were preserved in PBS (10 mM phosphate buffer, 137 mM NaCl) and 25% glycerol. Protein was kept at -20°C and avoided to freeze-thaw.

4.3.5 Cancer cell line preparation

4.3.5.1 Cell culture procedure

All of cancer cell lines, except Kuromochi cell line, were grown in DMEM with 10% FBS plus 100 Units/mL of Penicillin and 100 µg/mL of Streptomycin (Gibco®), maintained in 5% CO₂ at 37°C and lets them grow until they reach optimal confluence. After that, they were detached by 0.25% trypsin/EDTA solution (Gibco®) and determined the total number of cells. The calculated volume of cells will be used for each experiment.

4.3.5.2 Endogenous Trop2 knockdown

The cancer cell lines that has high Trop2 expression which is KKU-213 were knocked down by ten ng of Human TACSTD2 FlexiTube siRNA (Qiagen®). The RNAiMAX® (Invitrogen) was used as a transfection reagent.

4.3.5.3 Transfection of TACSTD2 (GFP-tagged)-Human tumor-associated calcium signal transducer 2 plasmid to cancer cell lines

The cancer cell lines that has no or low Trop2 expression which is KKU-055 were transfected with two ul of TACSTD2 (GFP-tagged) plasmid using Lipofectamine™ 3000 Reagent (Invitrogen). After transfection, the transfected cells were selected by geneticin (Gibco®, Thermo Fisher Scientific Corporation, Waltham, MA, USA) to create stable cell lines expressed Trop2GFP protein. Therefore, these transfected cells will be expressed Trop2 protein with green fluorescence on their cell membrane. The bright GFP expression cells were selected by fluorescence-activated cell sorting, FACS (BD FACSAria™ III, BD Biosciences, CA, USA).

4.3.6 Immunofluorescence assay and confocal microscope analysis

The cancer cell lines were seeded in 96-well plate for IFA and 24-well plate with coverslip for confocal microscope analysis and then grown for 16-18 hours at 37°C, 5% CO₂. The medium will be removed after completed the time and washed with 1x phosphate-buffered saline (PBS). Four percent paraformaldehyde was used as a fixative for 10 minutes at RT. Five percent of FBS was used as a blocking solution for 15 minutes at RT. For positive control, mouse anti-human Trop2 (R&D system) was used as a primary antibody (concentration 0.001 mg/mL) and incubated at 37°C for 4 hours. Goat anti-mouse IgG antibody conjugated Cy3 (Jackson Immuno Research Laboratories) was used as a secondary antibody (concentration 0.00075 mg/mL) and Hoechst 33258 (Invitrogen) was used as a counter stain DNA (concentration 0.02 mg/mL), both of them were incubated at RT for 1 hour. For negative control, no adding of primary antibody, just incubated with blocking solution. For Trop2mCherry ScFv staining, purified-Trop2mCherry ScFv (concentration 0.20 mg/mL) was incubated at 37°C for 4 hours and Hoechst 33258 (Invitrogen) was used as a counter stain DNA, incubated at RT for 1 hour. The observation of fluorescence signal will be examined by the fluorescence

microscope (Olympus, IX71, Tokyo, Japan) and confocal microscope (LSM800, Carl Zeiss, Jena, Germany).

4.3.7 Detection of CTCs

To determine the sensitivity and specificity of Trop2mCherry when use as a detector in blood circulation, both CCA cell lines were used as a model for determination.

4.3.7.1 Cancer cell preparation

KKU-213 and KKU-055 were used as a positive and negative control cells, respectively. Both of them were detached by 0.25% trypsin/EDTA solution and counted them for 1,000,000 cells and then washed with 1x PBS. CellTracker™ Green CMFDA was diluted with Opti-MEM™ (Invitrogen) to final concentration 5 μ M and warmed at 37°C for 30 minutes. And then each of CCA suspension cells were incubated with 5 μ M pre-warmed CellTracker™ Green CMFDA at 37°C for 30 minutes. After incubation, washing by 1X PBS was performed.

4.3.7.2 Blood preparation

Whole blood in EDTA preservation was collected from healthy donor. Blood was separated into 1 mL/tube.

4.3.7.3 Cancer cells mixing in blood

The number of cells, according to 500, 1,000, 5,000, 10,000, 50,000, were calculated and added to 1 mL/tube of blood. Red blood cells (RBCs) were lysed by 1x RBC lysis buffer. And then, washing by 1x PBS was performed until the lysate was clear.

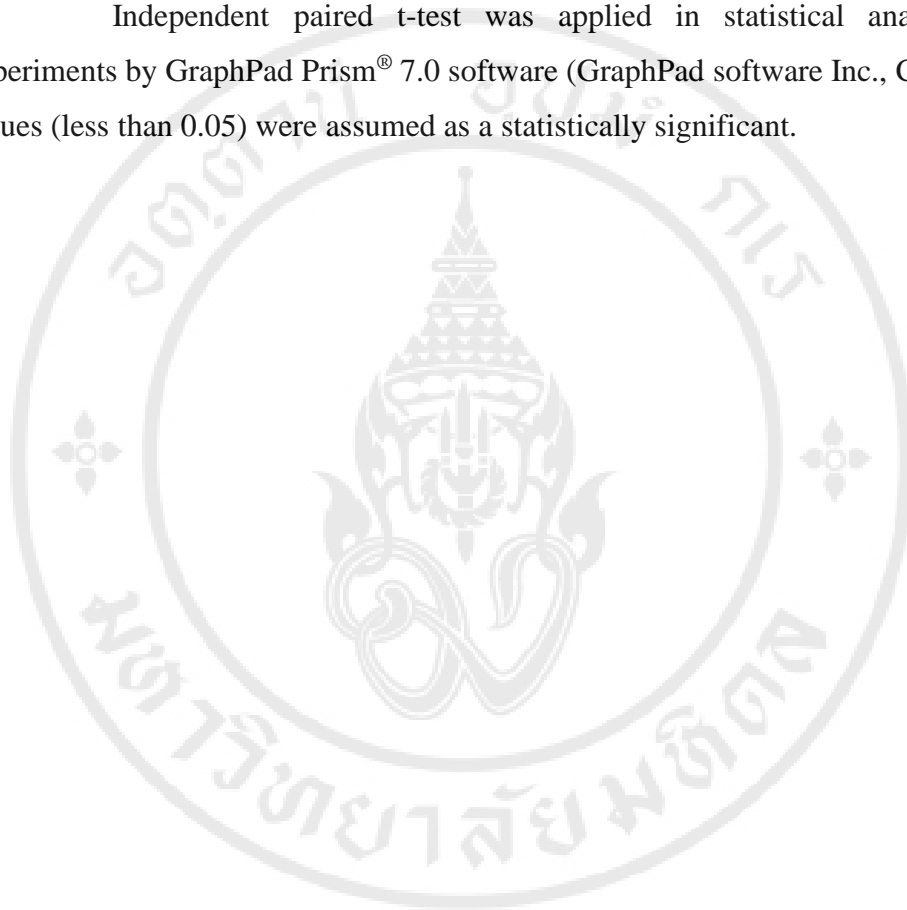
4.3.7.4 Staining of Cancer cells mixing

When cancer cells mixing in blood was prepared already, antibody was added to stain cancer cells. For positive control, mouse anti-human Trop2 (R&D system) was used as a primary antibody (concentration 0.001 mg/mL) and incubated at 4°C for 1 hour. Goat anti-mouse IgG antibody conjugated Cy3 was used as a secondary antibody (concentration 0.0015 mg/mL) and incubated at RT for 30 minutes. For negative control, no adding of primary antibody, just incubated with blocking solution. For Trop2mCherry ScFv staining, purified-Trop2mCherry ScFv (concentration 0.20 mg/mL), was incubated at 4°C for 1 hour. And then, washing by 1x

PBS was performed. The final volume of these suspension was 100 μ L. All of conditions were observed under fluorescence microscope.

4.3.8 Statistical analysis

Independent paired t-test was applied in statistical analysis of all experiments by GraphPad Prism[®] 7.0 software (GraphPad software Inc., CA, USA). *P*-values (less than 0.05) were assumed as a statistically significant.



CHAPTER V

RESULTS

5.1 Production of Trop2mCherry ScFv

The plasmid that contained sequence of Trop2 ScFv fusion with mCherry was further transformed into competent *E.coli* Rosetta. The pink-colonies with ampicillin resistance were selected for ScFv expression by autoinduction system. The induction time was around 20 hours at 16°C and cell pellets were collected by high-speed centrifuge. Sonication was performed for break out cell wall of *E.coli* and released ScFv protein. The supernatant was further purified by HisTrap[®] HP columns (GE Healthcare Life Sciences). Total yield was approximately 1 mg/L from total 4 L. After column, only pink fraction (5 fractions) was selected for further concentration by Vivaspin[®] 6 (GE Healthcare Life Sciences). Total 5 mL of 5 pink-fractions was pooled and concentrated until the remaining volume on concentrator around 1 mL, the concentration up to 5 fold from original. The measurement of concentration by Bradford protein assay, pooled fraction before concentrated was 0.06 mg/mL and after was 0.20 mg/mL. For sampling, crude extract, flow-through after column, pink-fraction, and pink-fraction concentrated was collected for measurement of protein concentration by Bradford assay and purity determination by SDS-PAGE analysis. The result of SDS-PAGE analysis was shown in Figure 5.1.

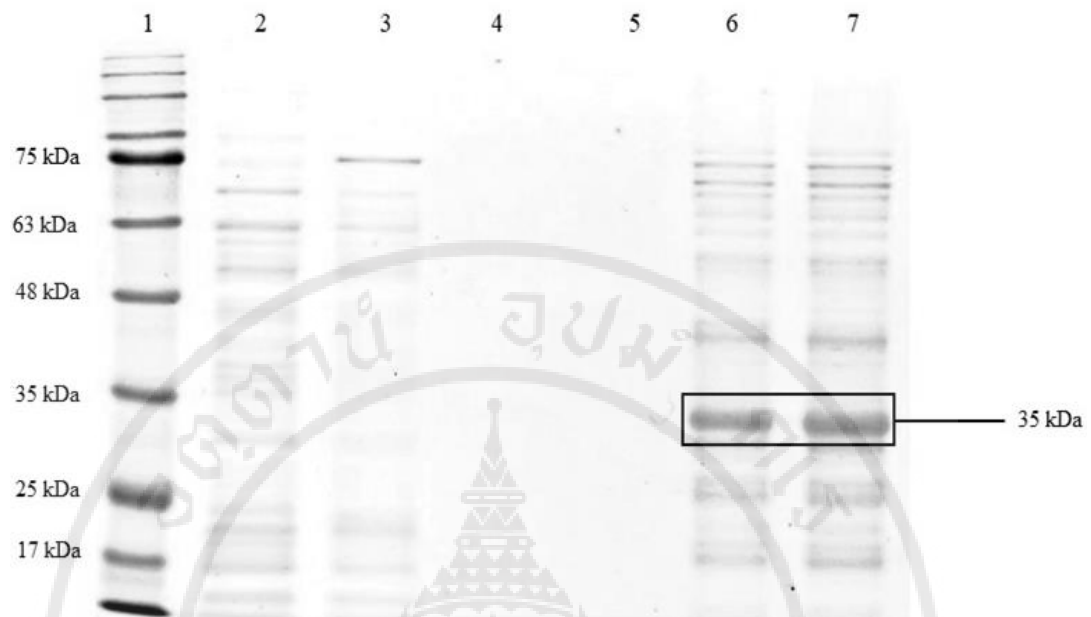


Figure 5.1 SDS-PAGE analysis of Trop2mCherry ScFv expression in *E.coli*.

(**Lane 1:** protein ladder, **Lane 2:** Crude extract, **Lane 3:** Flow-through after column in 40 mM imidazole, **Lane 4:** Flow-through after column in 250 mM imidazole, **Lane 5:** Flow-through after column in 500 mM imidazole, **Lane 6:** Pooled-pink fraction after 250 mM imidazole elution, **Lane 7:** Pooled-pink fraction after concentration)

5.2 Screening for Trop2 staining positive cells

Trop2mCherry ScFv was produced and kept in glycerol stock, in -20°C , for few months. To determine the specificity of Trop2mCherry ScFv, several types of cancer cell lines were used as a model and be comparative with specific mouse anti-human Trop2 (R&D system). IFA was performed, and results were shown in Figure 5.2-5.4.

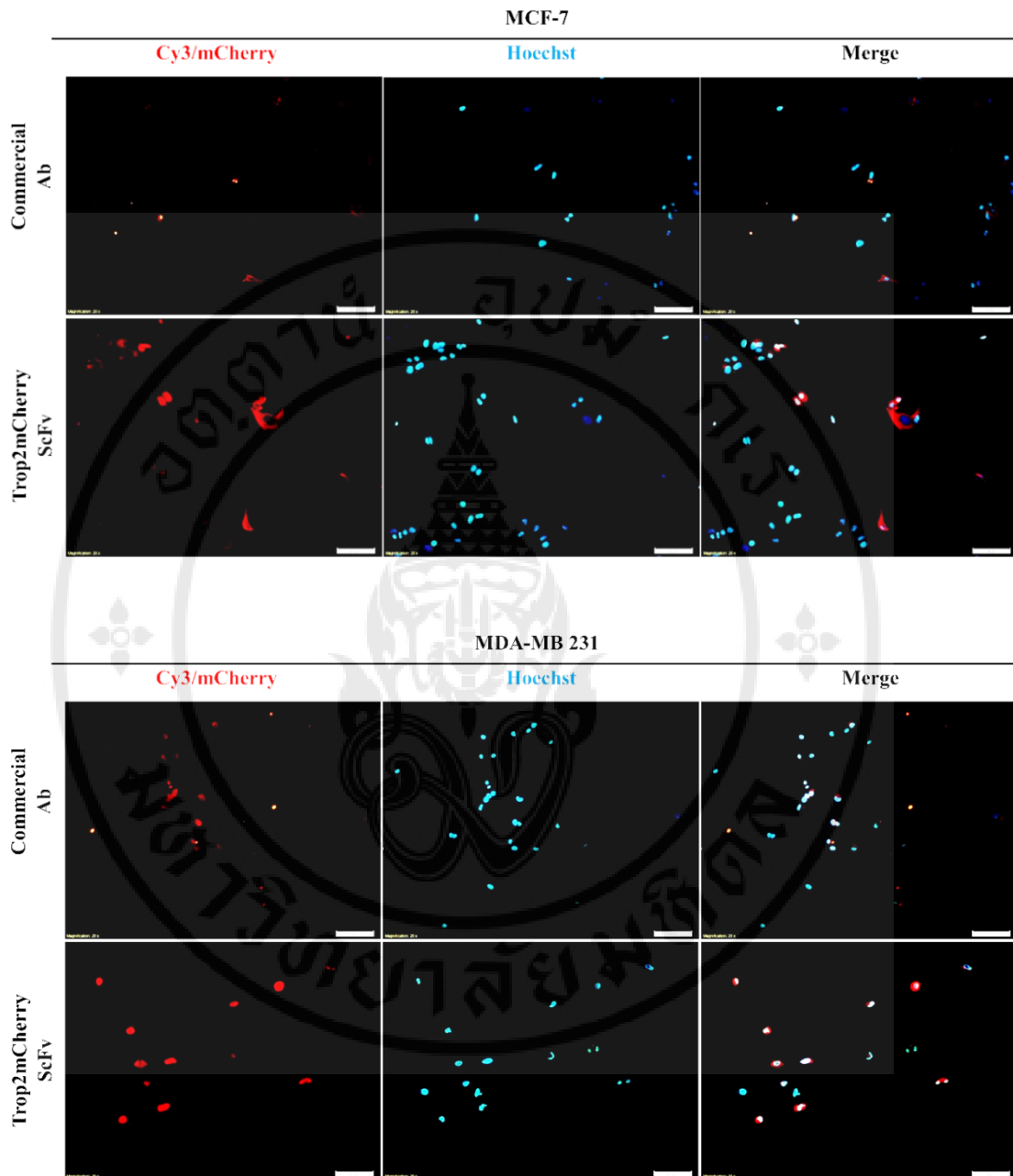


Figure 5.2 IFA results of Trop2 expression in BCA cell lines.

Commercial Ab staining, mouse anti-human Trop2 was used as a positive control and detected by goat anti-mouse IgG antibody conjugated Cy3 (Red). Signal of mCherry (Red) was tested. Hoechst 33258 (Blue) was used for nuclear staining. Original magnification was 200x. (Scale bar = 50 μ m)

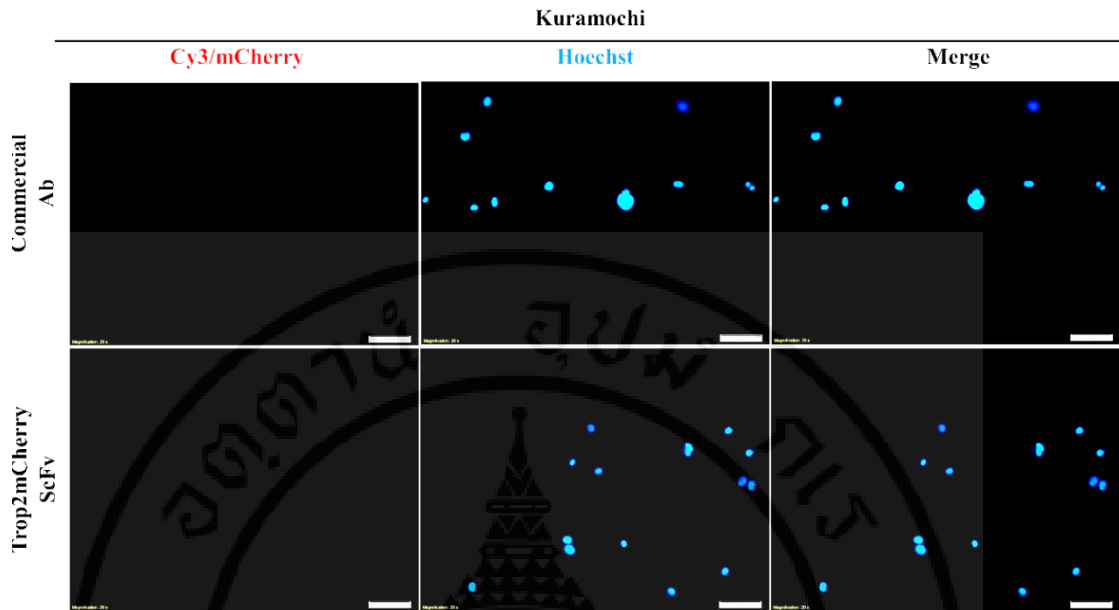


Figure 5.3 IFA results of Trop2 expression in ovarian cancer cell lines.

Commercial Ab staining, mouse anti-human Trop2 was used as a positive control and detected by goat anti-mouse IgG antibody conjugated Cy3 (Red). Signal of mCherry (Red) was tested. Hoechst 33258 (Blue) was used for nuclear staining. Original magnification was 200x. (Scale bar = 50 μ m)

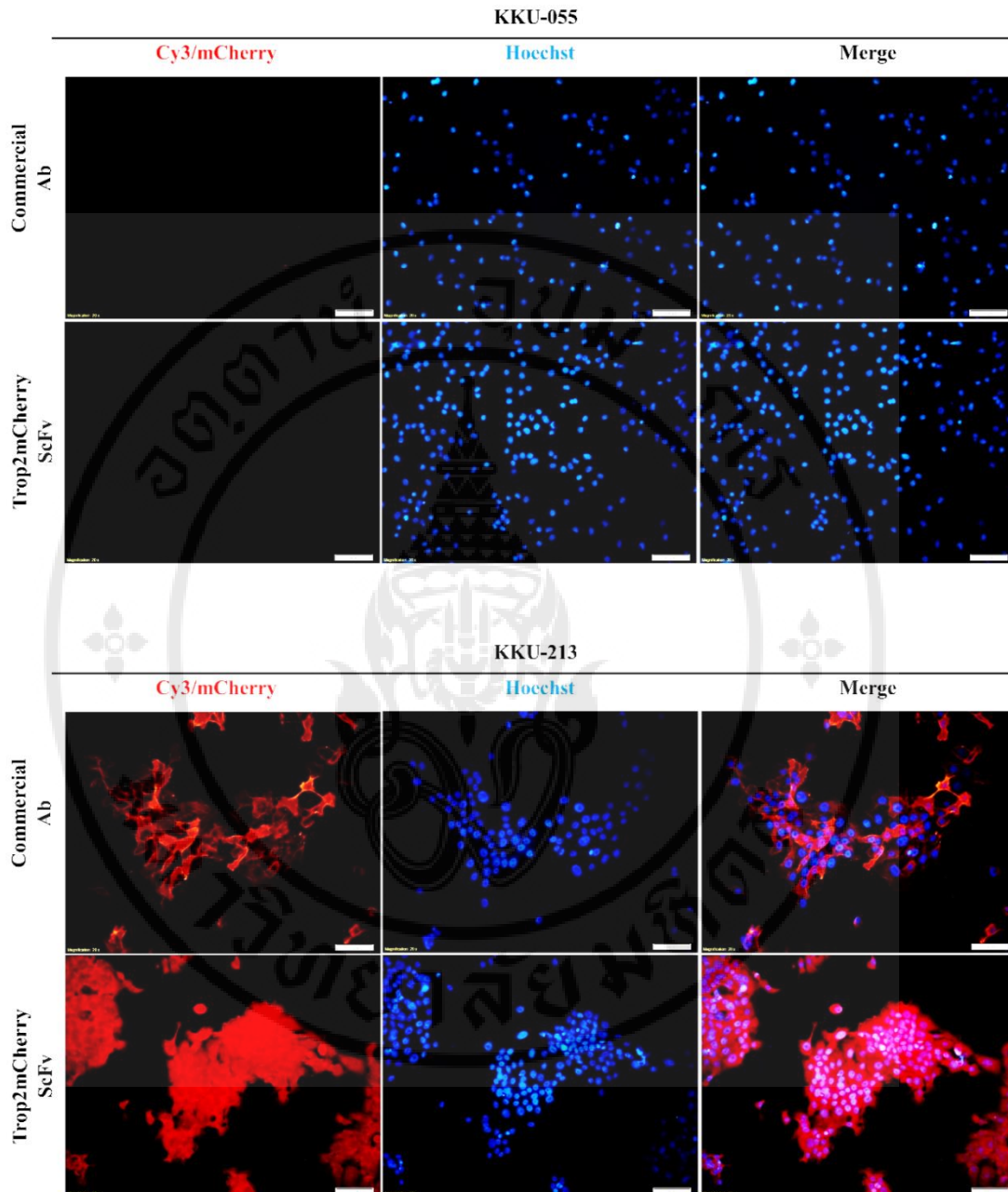


Figure 5.4 IFA results of Trop2 expression in CCA cell lines.

Commercial Ab staining, mouse anti-human Trop2 was used as a positive control and detected by goat anti-mouse IgG antibody conjugated Cy3 (Red). Signal of mCherry (Red) was tested. Hoechst 33258 (Blue) was used for nuclear staining. Original magnification was 200x. (Scale bar = 50 μ m)

From all of IFA results, Trop2 staining positive cells were counted to compare between staining of commercial antibody and Trop2mCherry ScFv. The percentage of Trop2 staining positive cells was shown in Figure 5.5

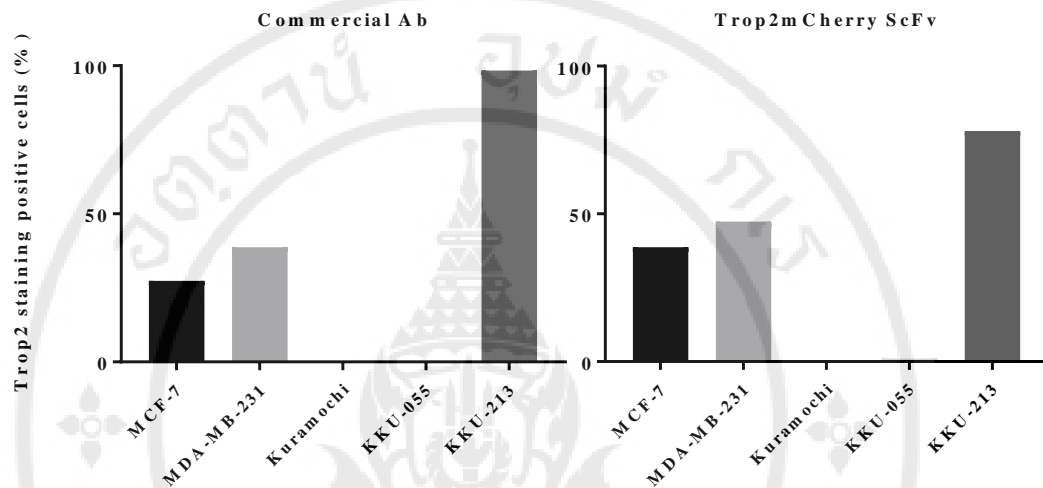


Figure 5.5 Percentage of Trop2 positive staining cells among various types of cancer cell lines.

Staining between commercial antibody and Trop2mCherry ScFv was compared. The percentage was counted from three images.

The highest Trop2 expression from both commercial staining and Trop2mCherry ScFv staining, K KU-213, was selected for next experiments as a positive control cell lines. The low Trop2 expression, K KU-055, was selected for next experiments as a negative control cell lines.

5.3 Testing of Trop2-unrelated mCherry ScFv

The other clone of ScFv fusion mCherry that has no specificity to Trop2 molecule was compared with Trop2mCherry ScFv staining in KKU-213 (positive control cells) and KKU-055 (negative control cells) as shown in Figure 5.6 and Figure 5.7, respectively.

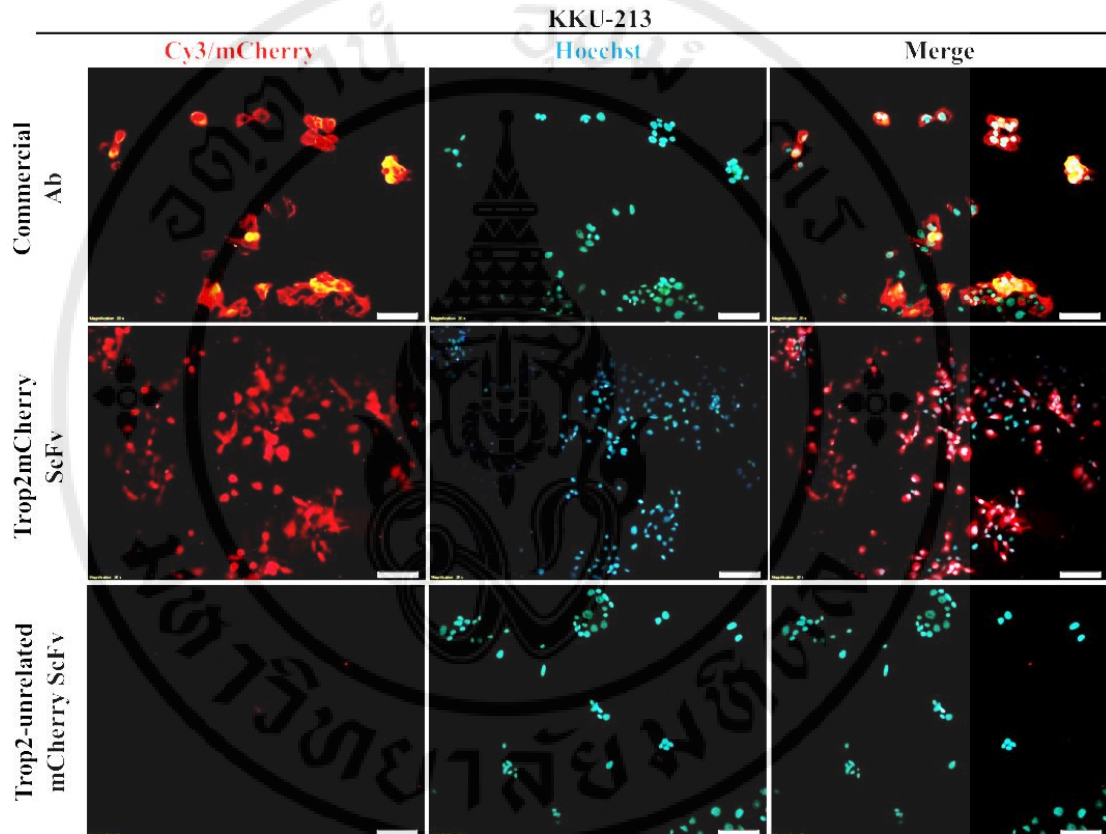


Figure 5.6 IFA results of Trop2-unrelated mCherry ScFv staining on KKU-213 cell lines.

Commercial Ab staining, mouse anti-human Trop2 was used as a positive control and detected by goat anti-mouse IgG antibody conjugated Cy3 (Red). Signal of mCherry (Red). Hoechst 33258 (Blue) was used for nuclear staining. Original magnification 200x. (Scale bar = 50 μ m)

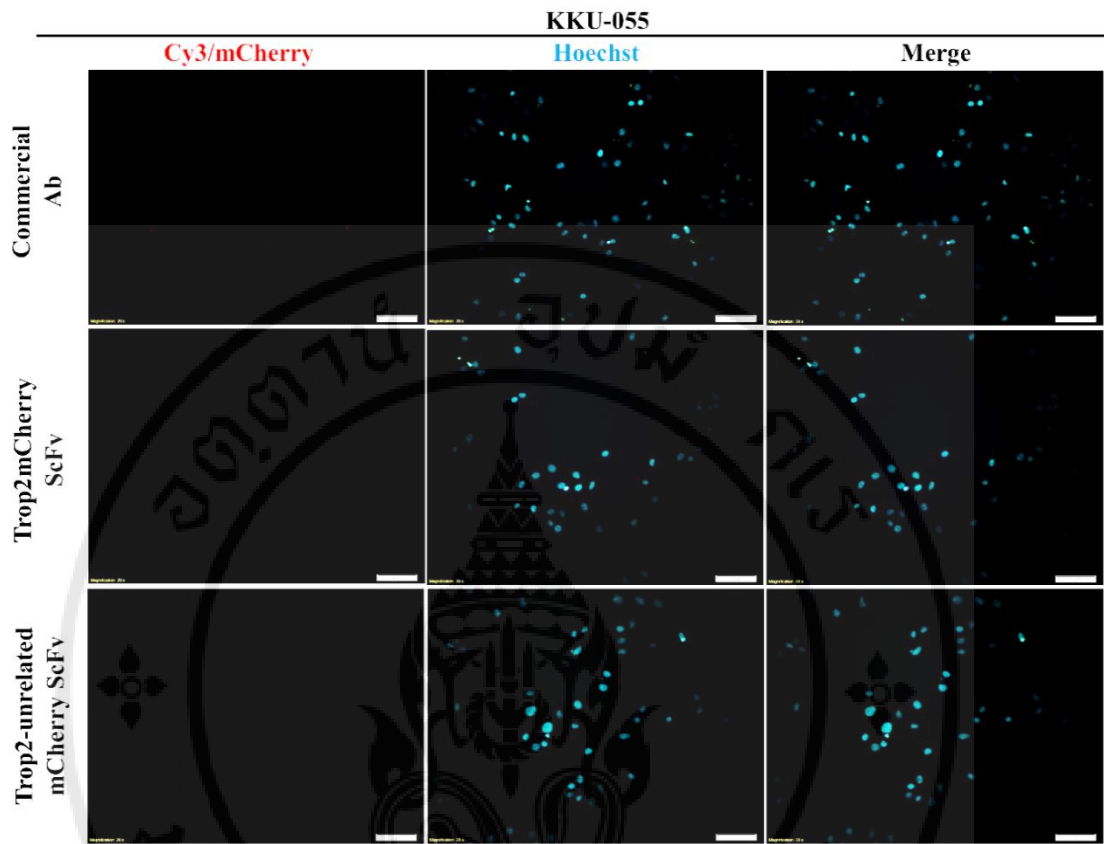
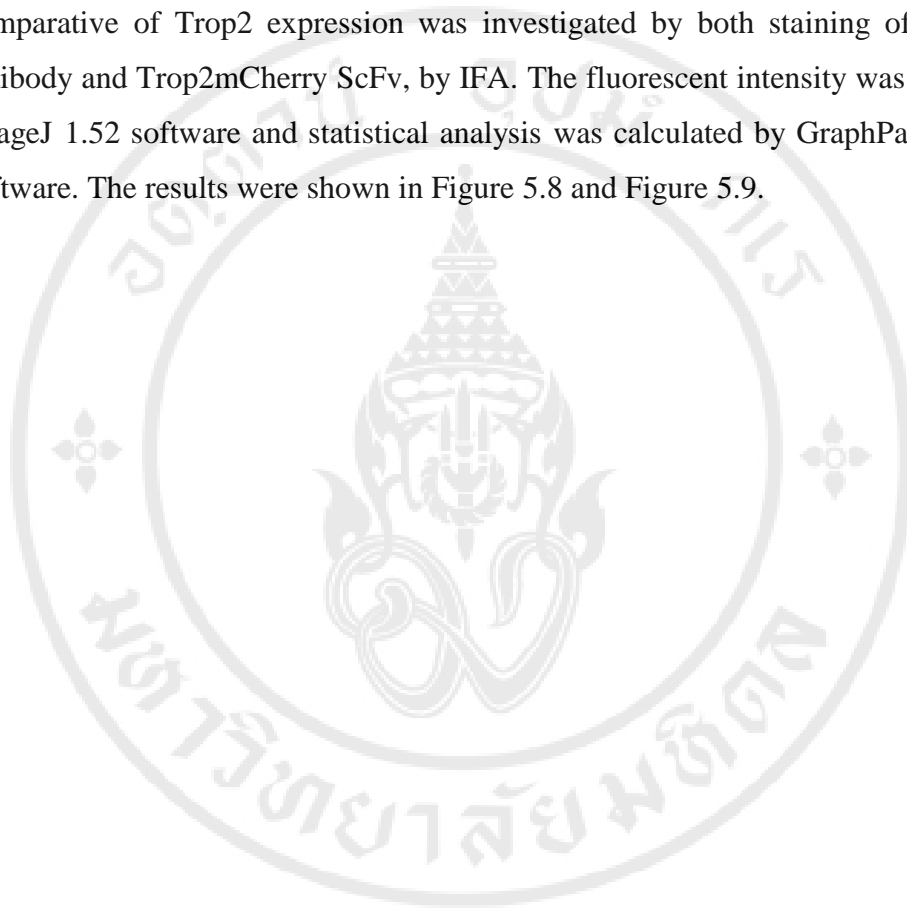


Figure 5.7 IFA results of Trop2-unrelated mCherry ScFv staining on KKU-055 cell lines.

Commercial Ab staining, mouse anti-human Trop2 was used as a positive control and detected by goat anti-mouse IgG antibody conjugated Cy3 (Red). Signal of mCherry (Red). Hoechst 33258 (Blue) was used for nuclear staining. Original magnification 200x. (Scale bar = 50 μ m)

5.4 Determination of Trop2mCherry ScFv with endogenous Trop2 knockdown in KKU-213 cell lines

To knock down Trop2 expression in KKU-213 cell lines. The Trop2-siRNA (Qiagen[®]) was used and RNAiMAX[®] was used as a transfection medium. The comparative of Trop2 expression was investigated by both staining of commercial antibody and Trop2mCherry ScFv, by IFA. The fluorescent intensity was measured by ImageJ 1.52 software and statistical analysis was calculated by GraphPad Prism[®] 7.0 software. The results were shown in Figure 5.8 and Figure 5.9.



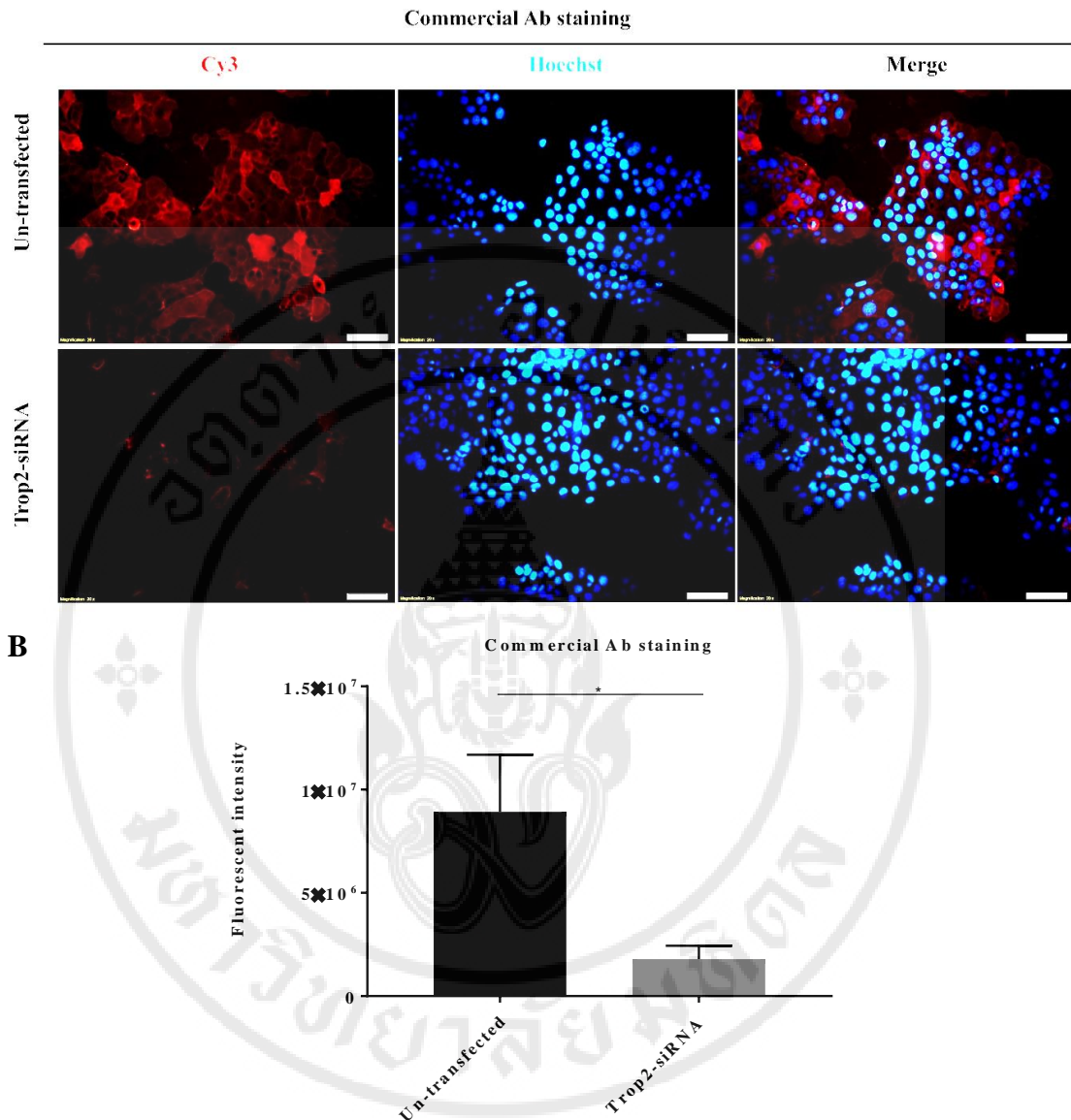


Figure 5.8 Downregulation of endogenous Trop2 was determined by commercial antibody staining.

(A) IFA results, comparative staining of Trop2 expression between KKU-213 un-transfected cell lines and KKU-213 Trop2 knock down by commercial Ab, mouse anti-human Trop2 was used as a positive control and detected by goat anti-mouse IgG antibody conjugated Cy3 (Red). Hoechst 33258 (Blue) was used for nuclear staining. Original magnification 200x. (Scale bar = 50 μ m). (B) Fluorescence intensity of Trop2 staining. The experiment was repeated three times. The results was shown as mean \pm SD. * $p < 0.05$

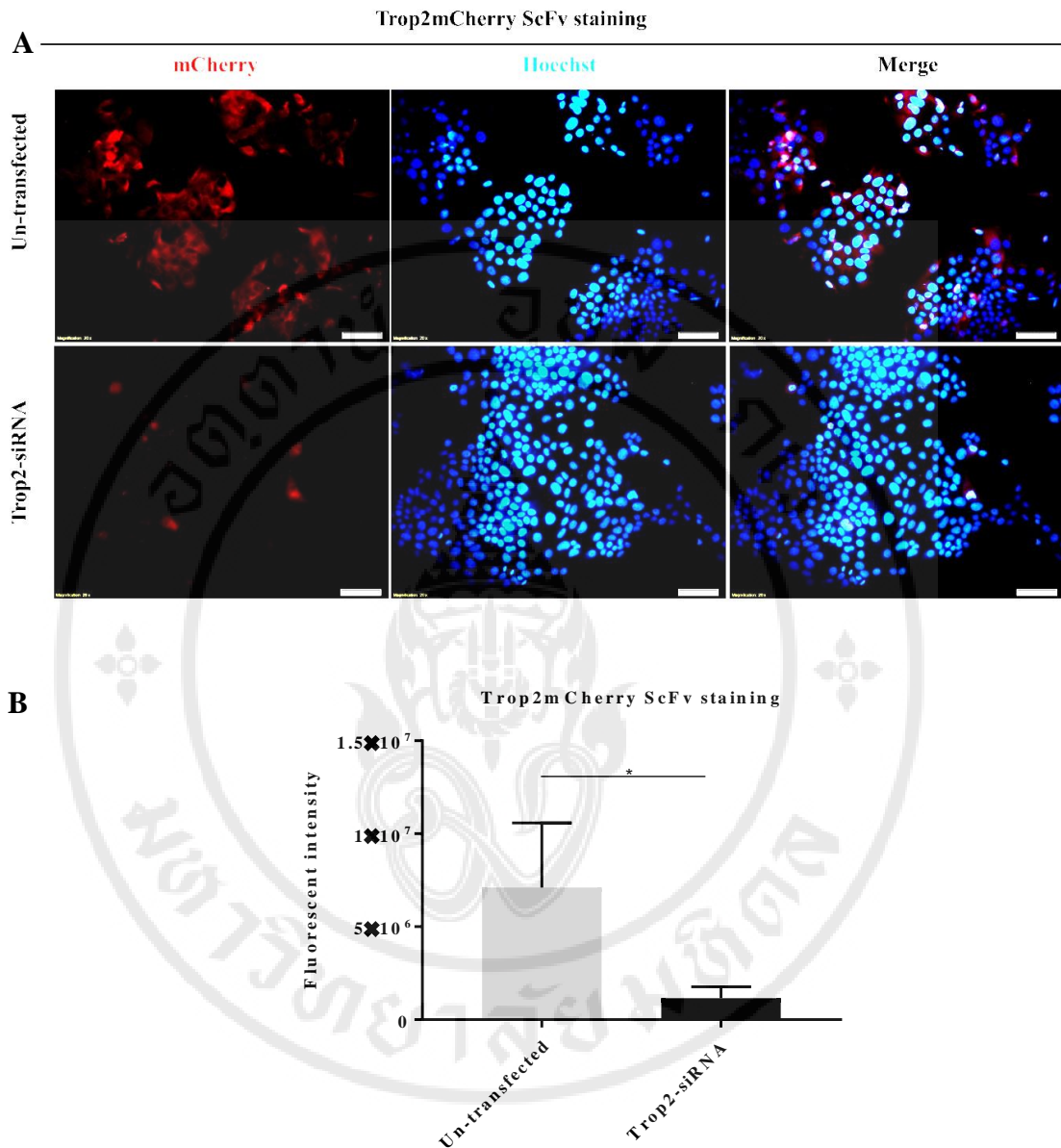


Figure 5.9 Downregulation of endogenous Trop2 was determined by Trop2mCherry ScFv staining.

(A) IFA results, comparative staining of Trop2 expression between KKU-213 un-transfected cell lines and KKU-213 Trop2 knock down by Trop2mCherry ScFv (Red). Hoechst 33258 (Blue) was used for nuclear staining. Original magnification 200x. (Scale bar = 50 μ m). (B) Fluorescence intensity of Trop2 staining. The experiment was repeated three times. The results was shown as mean \pm SD. *p < 0.05

5.5 Determination of Trop2 membranous pattern

To determine the membranous pattern of Trop2 molecules on membrane of cancer cells. Confocal microscope analysis was performed.

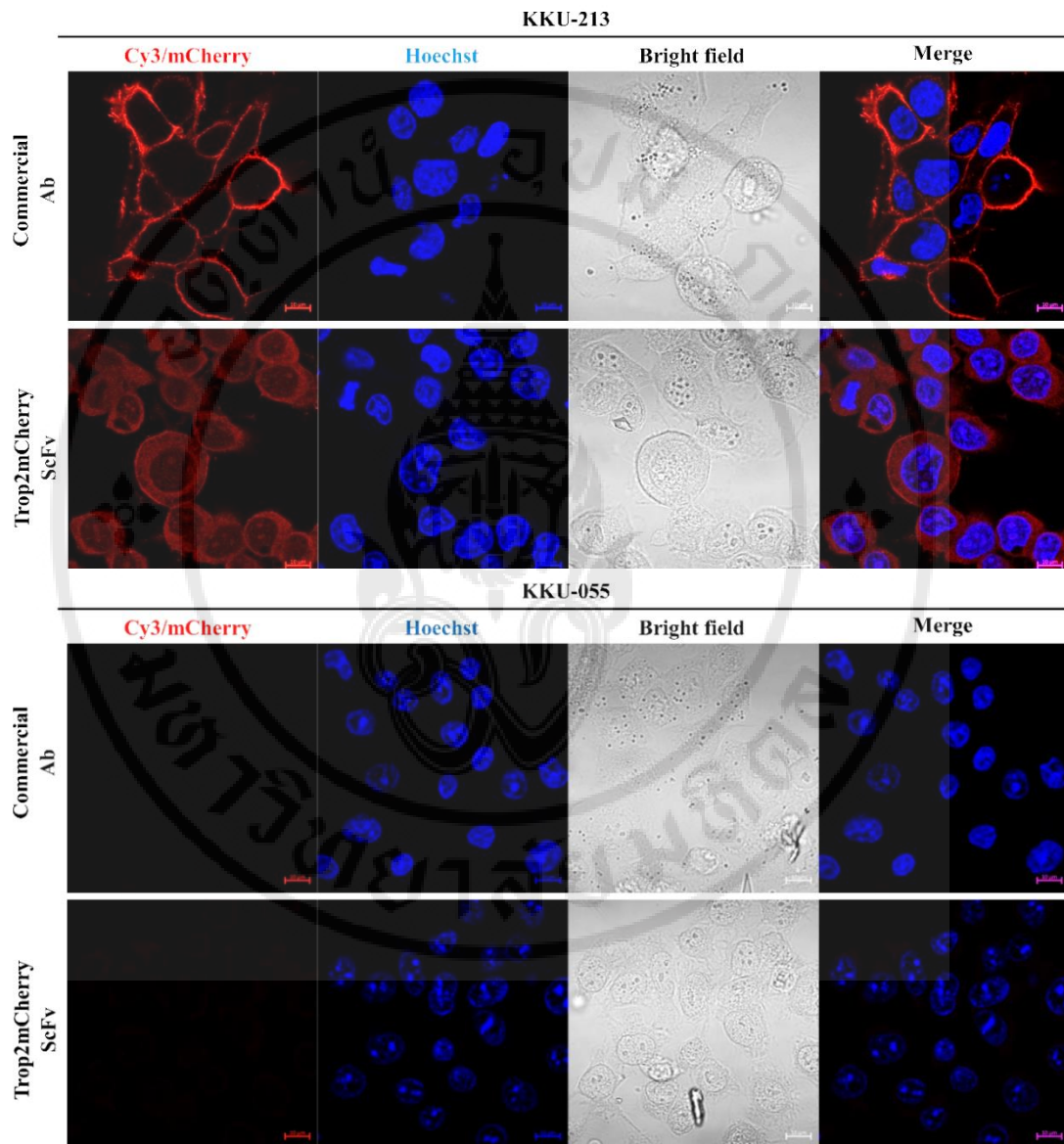


Figure 5.10 Confocal microscope analysis of Trop2 expression on KKU-213 and KKU-055 cell lines.

Commercial Ab staining, mouse anti-human Trop2 was used as a positive control and detected by goat anti-mouse IgG antibody conjugated Cy3 (Red). Signal of mCherry (Red). Hoechst 33258 (Blue) was used for nuclear staining. Magnification was 63x. (Scale bar = 10 μ m)

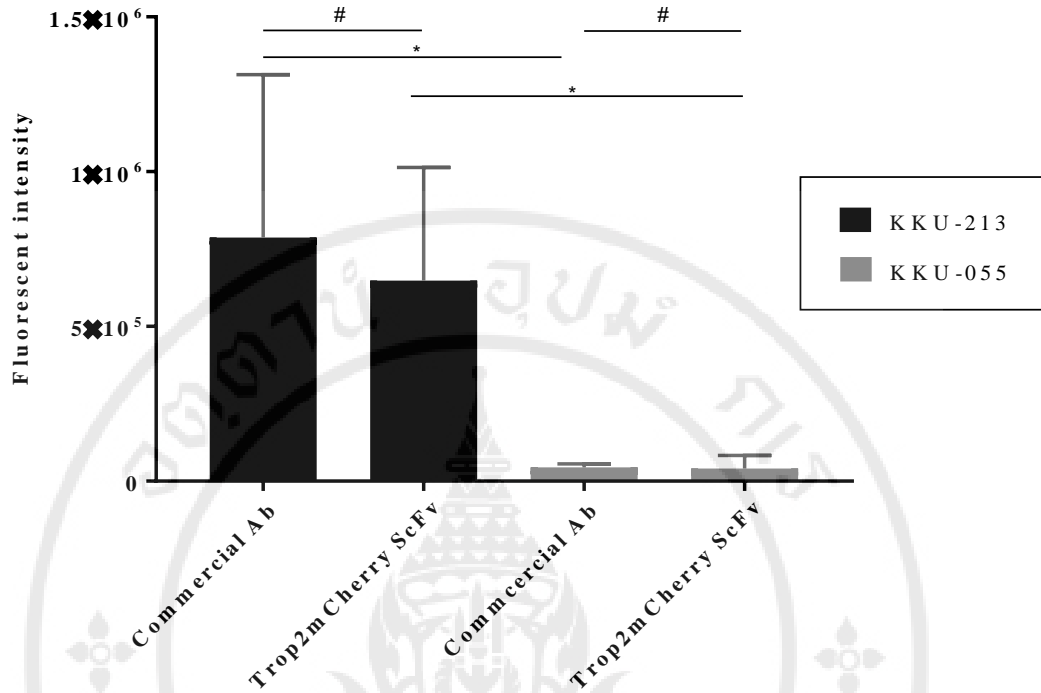


Figure 5.11 Fluorescence intensity of Trop2 staining observed by confocal microscope.

KKU-213 and K KU-055 cell lines were stained by commercial antibody and Trop2mCherry ScFv. The fluorescent intensity was measured from randomly 50 cells per condition. The results were shown as mean ± SD. *p < 0.05, # no significant.

5.6 Determination of Trop2 overexpression by confocal microscope analysis

To determine the efficiency of Trop2mCherry ScFv on Trop2-overexpressed cell lines, KKU-055 cell lines that showed Trop2 negative staining was further transfected with TACSTD2 (GFP-tagged) plasmid (OriGene Technologies) and determined the co-localization by confocal microscope analysis.

KKU-055 cell lines were transfected with TACSTD2 (GFP-tagged) plasmid (OriGene Technologies). To select the bright GFP expression, the transfected cells were sorted by FACS (BD FACSAria™ III, BD Biosciences). After cell sorting, Trop2 GFP tag showed the percent expression around 80%.

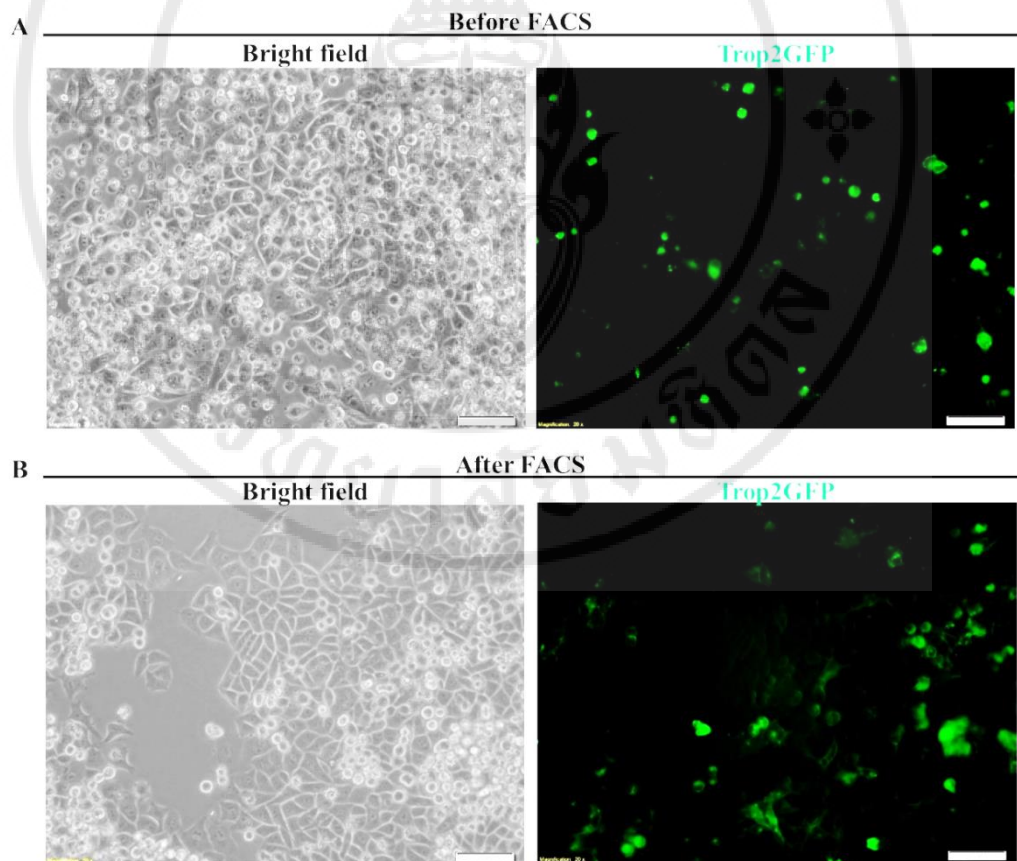


Figure 5.12 Trop2 overexpression on KKU-055 cell lines.

(A) Bright field (Left) and Trop2-GFP tag (Right) expression observed under fluorescent microscope. (B) Bright field (Left) and Trop2-GFP tag (Right) after FACS. Original magnification 200x. (Scale bar = 50 μ m).

After selection of the bright Trop2-GFP tag expression, these cells were stained by commercial antibody and Trop2mCherry ScFv and observed under confocal microscope.

To evaluate the co-localization between two channels (Green channel: Trop2-GFP tag, Red channel: a signal from Trop2 staining which is Cy3 and mCherry). The ImageJ Co-localization Color map plugin was used as a software for calculation. The normalized mean deviation product (nMDP) values were calculated, a correlation between pairs of individual pixels (green and red channels), and represented as a color map. The picture of color map showed the nMDP values from each pixel, ranging from -1 to 1. The index of correlation which is the fraction of positively correlated or co-localized between individual pixels in the images.

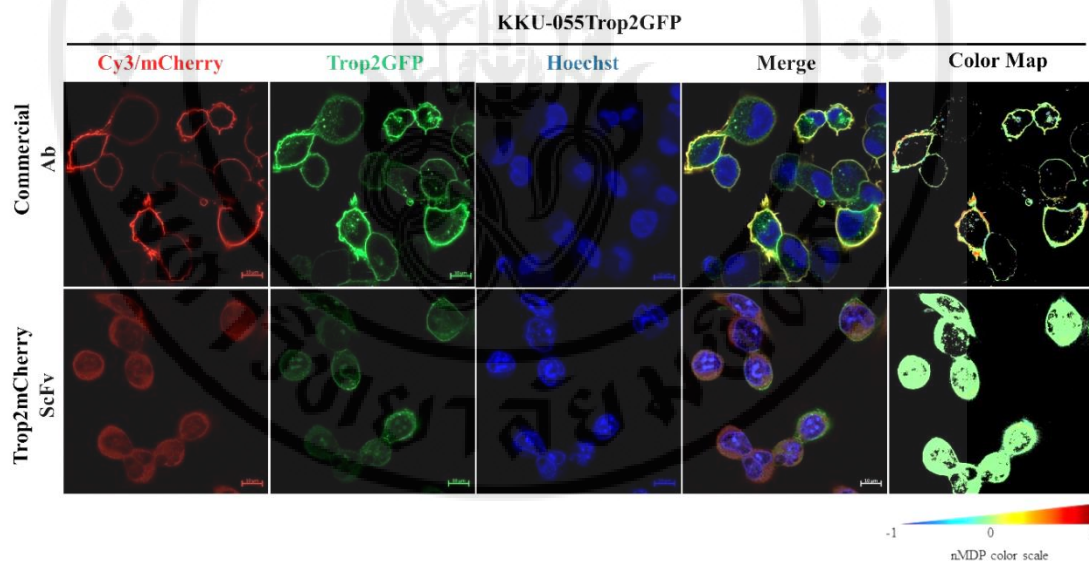


Figure 5.13 Confocal microscope analysis of Trop2 overexpression on KKKU-055 cell lines.

Commercial Ab staining, mouse anti-human Trop2 was used as a positive control and detected by goat anti-mouse IgG antibody conjugated Cy3 (Red). Signal of mCherry (Red). Hoechst 33258 (Blue) was used for nuclear staining. Color map represented co-localization, calculated from ImageJ software, determined by normalized mean deviation product (nMDP) values. Magnification 63x. (Scale bar = 10 μm)

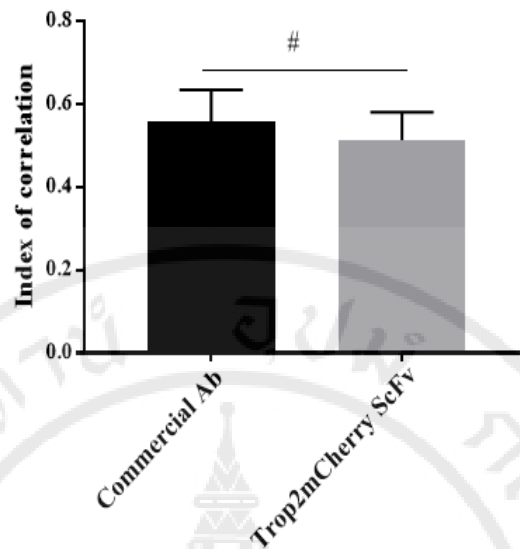


Figure 5.14 Index of correlation values of positively co-localization.

Between Trop2-GFP tag and antibody detection (commercial antibody and Trop2mCherry ScFv). The calculation from ImageJ Co-localization Color map plugin. The results was shown as mean \pm SD. The experiment was repeated three times. The results was shown as mean \pm SD. #: no significant

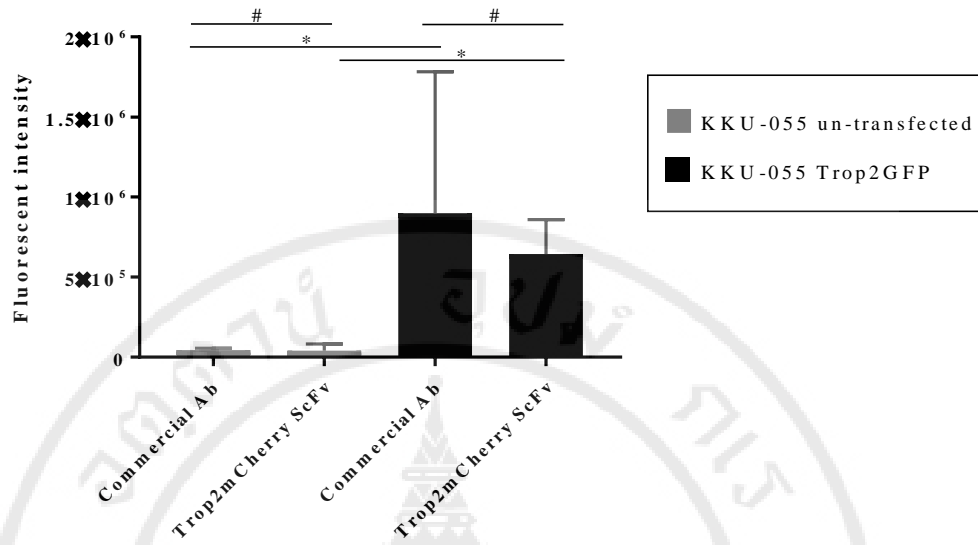


Figure 5.15 Fluorescence intensity of Trop2 staining for determination of Trop2 overexpression.

KKU-055-untransfected and KKU-055Trop2GFP (transfected) cell lines were stained by commercial antibody and Trop2mCherry ScFv. The fluorescent intensity was measured from randomly 50 cells per condition. The results were shown as mean ± SD. *p < 0.05, # no significant.

5.7 Efficiency of Trop2mCherry ScFv for using as a detector of circulating tumor cells (CTCs)

Situation likes CTCs from the patients was performed by mixing between whole blood from healthy donors and cancer cells. After mixing, 1x RBC buffer was added to lyse RBC, the biggest population among type of blood cells. 4% paraformaldehyde was added and stained by both commercial antibody and Trop2mCherry ScFv. Determination of Trop2-expressing cancer cells staining was observed by fluorescence microscope. The only blood cells were used as a negative control staining. KKU-213 and KKU-055 cell lines were used as a positive control cells and negative control cells, respectively.

Because of CTCs are the small population among type of blood cells. To investigation of the lowest number of detectable cancer cells were counted. The lowest number of Trop2 positive KKU-213 cell lines were counted by this technique, 20 cells, in total WBC 120,000 cells. The results of staining by this technique, in blood alone, cancer cells alone, cancer cells mixing in blood, were shown below.



Figure 5.16 Testing of staining in blood alone.

Commercial Ab staining, mouse anti-human Trop2 was used as a positive control and detected by goat anti-mouse IgG antibody conjugated Cy3 (Red). Signal of mCherry was tested (Red). Original magnification was 200x. (Scale bar = 50 μ m)

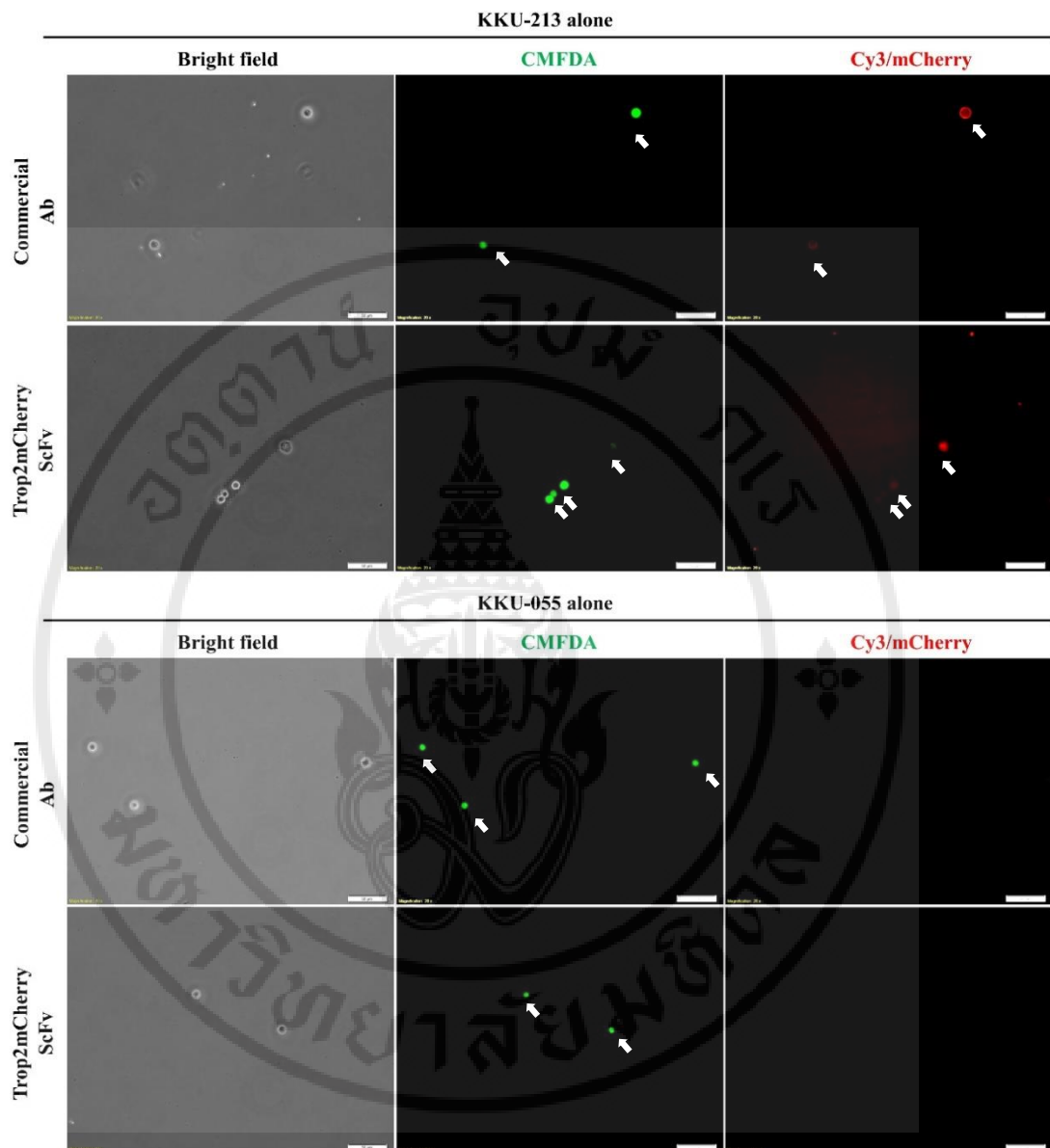


Figure 5.17 Testing of staining in cancer cells alone.

Commercial Ab staining, mouse anti-human Trop2 was used as a positive control and detected by goat anti-mouse IgG antibody conjugated Cy3 (Red). Signal of mCherry was tested (Red). CMFDA was stained in cancer cells (Green). White arrow point to cancer cells. Original magnification was 200x. (Scale bar = 50 μ m)

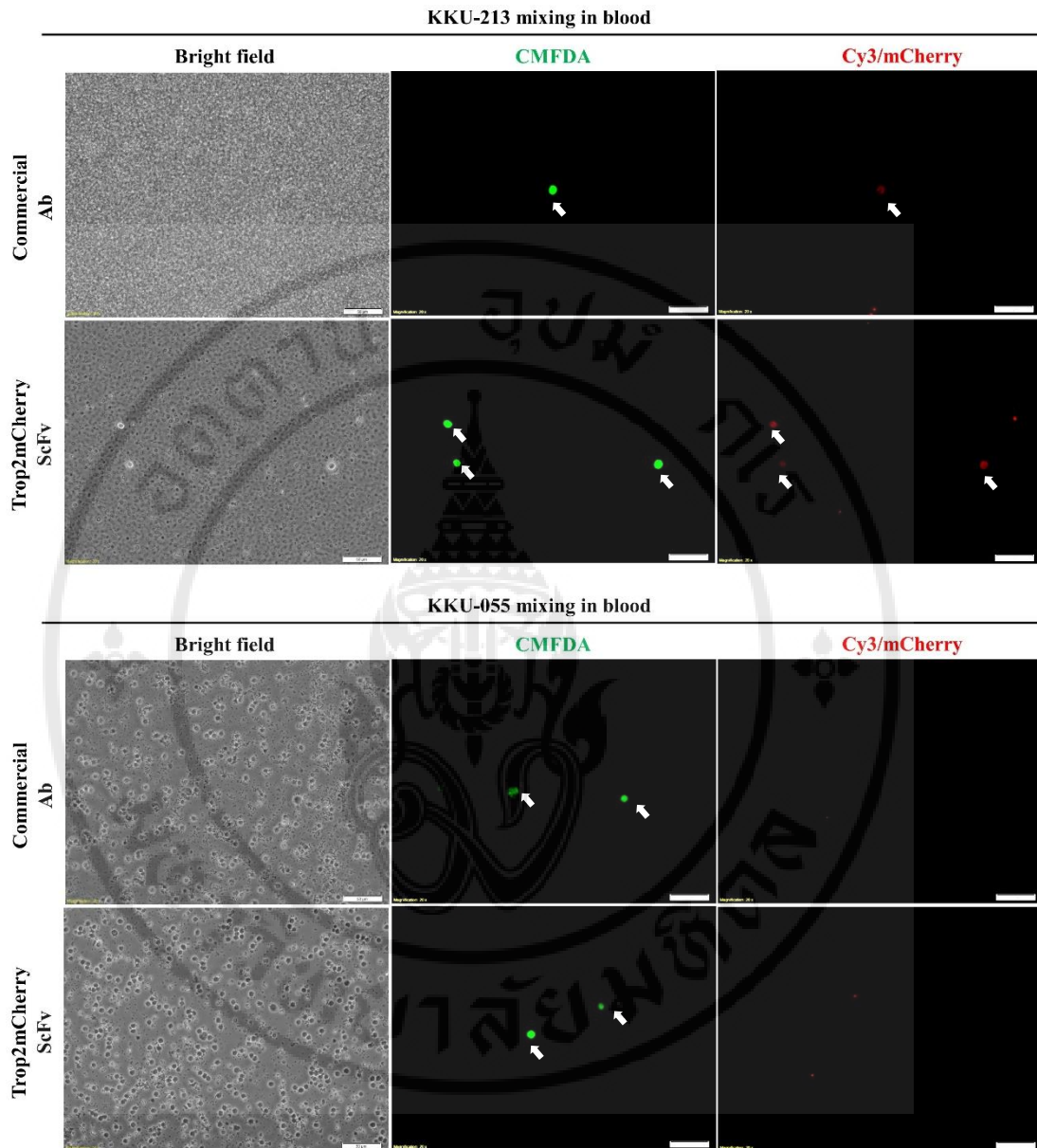


Figure 5.18 Testing of staining in cancer cells mixing.

Commercial Ab staining, mouse anti-human Trop2 was used as a positive control and detected by goat anti-mouse IgG antibody conjugated Cy3 (Red). Signal of mCherry was tested (Red). CMFDA was stained in cancer cells (Green). White arrow point to cancer cells. Original magnification was 200x. (Scale bar = 50 μ m)

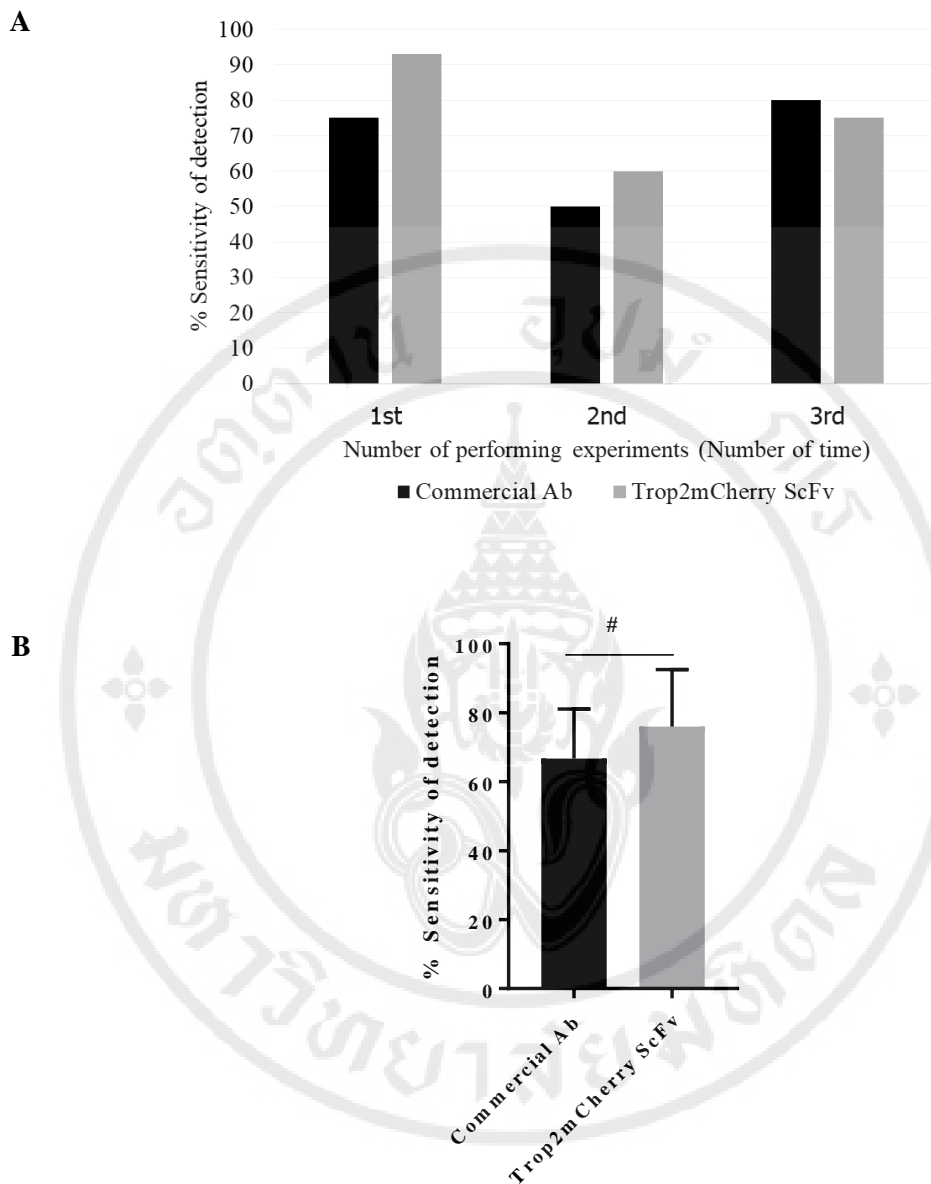


Figure 5.19 The sensitivity of detection.

(A) Determination by the number of cells that presented green signal from CMFDA and red signal from staining, from three different experiments. (B) Average of sensitivity, to compare between commercial antibody and Trop2mCherry ScFv. The results were shown as mean \pm SD. # no significant.

CHAPTER VI

DISCUSSIONS

Cancer is the most common cause of death worldwide, estimated around 9.6 million deaths in 2018 (42). Several things have been developed and improved for better cancer management, in recent years. Antibodies are well developing in biomedical research field (19). Phage display technique is a suitable system for antibody fragments production especially ScFv (19). ScFv is a monovalent antibody fragment, consists of V_H and V_L domain linked together with flexible peptide (20). Because of easy to modify, ScFv can be fused with other proteins such as hapten, cytotoxic proteins, drugs, as well as fluorescent protein (20). Trop2 overexpression was reported in various tumors and also associated with poor prognosis (40). Trop2 is a monomeric cell surface, consists of four domains including leader peptide domain, extracellular domain, transmembrane domain, and intracellular domain (74). Several antibodies were constructed for Trop2 targeting, mainly in therapeutic purposes (75). IMMU-132, Sacituzumab govitecan, an antibody drug conjugate (ADC) targeting Trop-2 conjugated with antineoplastic drug (SN-38), is going on study in clinical trial (76). It has some adverse effects reported (75). Many scientists have been trying to develop both mAbs and antibody fragments that specific to Trop2 molecules for more optional treatment (40). For example, novel specific mAbs of Trop2 were produced and investigated the anti-cancer ability (62). Human Fab fragments against Trop2 were produced and studied the inhibitory effect of tumor progression both *in vivo* and *in vitro* (60). In addition, the follow up of treatment is the important one in cancer management (77). CTCs are the one biomarker of liquid biopsy that have been studying a lot for cancer detection. CTCs can be detected in blood circulation of cancer patients (78). Moreover, Trop2 can also be expressed on CTCs. Therefore, the monitoring of Trop2-expressing CTCs during treatment may be used as a predictor in clinical practice (77).

The present study aimed to produce a novel ScFv fused with mCherry fluorescence protein against human Trop2 on the surface of cultured cancer cells mixed with normal whole blood, as mimic of CTCs. This ScFv may be used for detection of the CTCs in cancer patients' blood for diagnosis or following the drug responsibility or prognosis predication among cancer patients.

6.1 Production of Trop2mCherry ScFv

Human ScFv phage display library was constructed first. By overlapped extension PCR of *vh* and *vl* sequences that came from peripheral blood mononuclear cells (PBMCs) of healthy donors. Then phage biopanning was performed by 3-5 rounds of selection with antigen of interest (22). In this study, the sequence of Trop2mCherry ScFv was constructed in pET23b+ plasmid that contained 590 bp of Trop2 ScFv sequence, 709 bp of mCherry sequence, 1332 bp of total Trop2mCherry ScFv sequence.

The results of SDS-PAGE, under reducing conditions, (Figure 5.1) showed several protein bands in all fractions. It means that Trop2mCherry ScFv contained impurity. For pooled-pink fraction after concentration, showed the one remarkable band at around 35 kDa and also showed a bit tightly band when compared with pooled-pink fraction before concentration. These results correlated with the concentration measurement by Bradford protein assay (Bio-Rad), before concentrated was 0.06 mg/mL and after was 0.20 mg/mL. After concentration, the Trop2mCherry ScFv protein was preserved in PBS (10 mM phosphate buffer, 137 mM NaCl) and 25% glycerol for proteins stabilization.

The mCherry, monomeric red fluorescent protein, is the one of mFruit family derived from monomeric red fluorescent protein 1 (79). A mCherry is most widely used for fusion with another protein because it has several advantage including monomeric structure, high brightness, fast maturation, high photo stability, and pH resistance (80). mCherry has maximum excitation wavelength at 587 nm (540-590 nm) and maximum emission wavelength at 610 nm (550-650 nm) (80).

6.2 Screening of Trop2 expression in cancer cell lines

Trop2 is overexpressed in various cancer types (6), however, not all types of cancer cell lines express Trop2 molecule on their cell surface. In the same way, the clinical tissue from cancer patients showed the variation of Trop2 expression depending on many factors such as stage of cancer, tumor aggressiveness, and also types of cancer (15, 81).

In this study, the screening of Trop2 expression was performed in three types of cancer (five cancer cell lines) including BCA (MCF-7, MBA-MB-231), ovarian cancer (Kuramochi), and CCA (KKU-055 and KKU-213). To determine the Trop2 expression, the screening was performed by IFA (Figure 5.2-5.4). The staining of commercial antibody and Trop2mCherry ScFv was performed in parallel to determine the correlation of detection by both antibodies. The percentage of positive Trop2 staining was determined (Figure 5.5). Kuramochi was only one ovarian cell line that tested in this experiment but it was no expression of Trop2 showed in this cell line. For BCA cell lines, 27%, and 38% of Trop2 positive on MCF-7 cell lines staining by commercial antibody and Trop2mCherry ScFv, respectively. MDA-MB-231 showed 38% and 47%. For ovarian cancer cell lines showed no Trop2 staining by both commercial antibody and Trop2mCherry ScFv. For CCA cell lines, KKU-213 showed the highest Trop2 staining, nearly one hundred percent, similar to that previously reported (14). On the other hand, KKU-055 showed lowest Trop2 staining. KKU-055 is a moderately differentiated CCA cell lines, showed low Trop2 expression both mRNA level and protein level when determined by real-time PCR and IHC, respectively, that correlated to our results (14). Therefore, KKU-055 cell lines were further used as a negative control cell lines and KKU-213 were used as a positive control cell lines for further experiments.

6.3 Determination of unrelated ScFv

To determine the binding effect of mCherry, the Trop2-unrelated mCherry ScFv was stained with KKU-213 and KKU-055 cell lines. The Trop2-unrelated mCherry was also constructed by phage display technique and performed protein expression similar with Trop2mCherry ScFv but this ScFv couldn't bind to the Trop2

molecules. So, it could be used as another mCherry ScFv. The results demonstrated that Trop2-unrelated mCherry couldn't stain even KKU-213 and KKU-055 cell lines. Therefore, mCherry folding protein mightn't affect the Trop2 binding activity (Figure 5.6 and Figure 5.7).

6.4 Determination of Trop2 knockdown

To further confirmed that Trop2mCherry ScFv bind specifically to Trop2 molecules. KKU-213 cell lines were transfected with Trop2-siRNA (Qiagen®) to knockdown endogenous Trop2 from their membrane. After knockdown, Trop2 expression was determined by IFA with both commercial antibody and Trop2mCherry ScFv. Twenty hours after transfection with siRNA, IFA results of both commercial antibody and Trop2mCherry ScFv demonstrated that Trop2 expression was significantly reduced when compared with un-transfected cell lines (Figure 5.8 and Figure 5.9). This evidence confirmed the binding ability of Trop2mCherry ScFv to cellular Trop2.

6.5 Determination of membranous pattern of Trop2

Trop2 is a transmembrane protein that has extracellular domain appear out of cells surface (38). Trop2 is synthesized in the endoplasmic reticulum and transported to Golgi apparatus for glycosylation and then transported to cell membrane (81). So, the detection of Trop2 might be occurred for two different site, one localized on cell membrane and one in the cytoplasm (during maturation) (74).

Confocal microscope analysis was further performed for determination of Trop2 membranous patterns to determine the membranous patterns of Trop2. From results of confocal microscope analysis showed clearly Trop2 membranous pattern on KKU-213 cell lines when staining with commercial antibody and a bit cytoplasmic pattern. On the other hand, the staining of Trop2mCherry ScFv was almost pattern showed the cytoplasmic patterns and surround with membranous patterns (Figure 5.10).

The process of IFA in this experiment was not include the step of detergent application, therefore only permeable substance such as Hoechst 33258 could penetrate

to intracellular part. Both commercial antibody and ScFv were macromolecule that had less ability for penetration, however, during process of fixation, cell membrane might have some destruction and allow these molecules to bind to cytosolic or nuclear targets. And because ScFv had a smaller size than intact antibody, it might have more penetration ability than mAbs (50). Moreover, commercial antibody also needed secondary antibody to bind and showed the signal. So, it might be the reason that the staining with Trop2mCherry ScFv showed more cytoplasmic pattern than commercial antibody. Another reason, it might be came from the impurity of Trop2mCherry ScFv production. It might be presented the non-specific binding. However, the impurity of Trop2mCherry hadn't effect to the binding activities because Trop2mCherry ScFv showed the corrected staining that could be observed on positive (KKU-213) and negative (KKU-055) Trop2-expressing cell lines.

The comparison of fluorescent intensity, the results showed no significantly between commercial antibody and Trop2mCherry ScFv staining on both KKU-213 and KKU-055 cell lines (Figure 5.11).

6.6 Establishment of Trop2 overexpression

To determine whether the Trop2mCherry ScFv can bind to Trop2 molecule that overexpressed on KKU-055 cell lines, cells were transfected with TACSTD2 (GFP-tagged) plasmid (OriGene Technologies) to construct the expression model and to more selection of positive Trop2 expression was performed by FACS (Figure 5.12). Confocal microscope analysis was performed for determination of co-localization between Trop2 tag with GFP (green signal) and binding of Trop2mCherry ScFv (red signal). After performing, the images were analyzed by ImageJ Co-localization Color map plugin software. The nMDP values, a correlation between pairs of individual pixels (green and red channels), were used for determination (82). This values represented in color map ranging from -1 to 1. The high co-localization showed in hot color, for low co-localization showed in cold color (82). The index of correlation which is the fraction of positively correlated or co-localized between individual pixels in images was used for statistical analysis (82).

The statistical analysis of index of correlation values showed no significantly between commercial antibody and Trop2mCherry ScFv with KKU-055Trop2GFP (Figure 5.14).

To determine the different between KKU-055 un-transfected and KKU-055Trop2GFP, the fluorescent intensity was used for statistical analysis (Figure 5.15). The statistical analysis showed no significantly between antibody staining. For different between two cell lines showed highly significant. It might be concluded that Trop2mCherry ScFv could bind to Trop2 molecules not different from commercial antibody. The results from this experiment could support the specificity of Trop2mCherry ScFv to bind to Trop2 molecule.

6.7 Determination of Trop2mCherry ScFv efficiency for CTCs detection

CTCs are the one biomarker of liquid biopsy technique that important for both early diagnosis and prognosis of cancer patients (83). The major challenge of CTCs utilization is that the very rare CTCs population, approximately 1-10 CTCs per mL of whole blood in patients with metastatic disease (32). It means that one mL of whole blood it consists of a million white blood cells, a billion red blood cells, and a just 10 CTCs (32). Therefore, the sensitivity and specificity of technique should be high.

To evaluate the efficiency of Trop2mCherry ScFv for CTCs detection among blood cells. KKU-213 (Trop2-expressing cancer cells) and KKU-055 (Trop2 negative cancer cells) cell lines were mixed in whole blood of healthy donors. The ratio variation of cancer cells in 1 mL of whole blood was determined. For this technique, the lowest Trop2-expressing cancer cells that could be detected by Trop2mCherry ScFv was 20 cells. To determine sensitivity of detection, the number of cancer cells that presented green signal from CMFDA compared with red signal from staining by individual comparison were evaluated. The sensitivity of detection from commercial antibody showed 67%, Trop2mCherry ScFv showed 76%. The better sensitivity of detection from Trop2mCherry ScFv than commercial antibody, it might be came from the smaller size of Trop2mCherry ScFv that it might be easy to bind in the cell suspension. However, it

mightn't be good as much as possible but it had be promising for further apply with other techniques.

The other techniques have been introduced such as microfluidic. The microfluidic has many platforms including immunoaffinity chromatography, magnetic activated micro-sorting, and size-based cell separation resulting in high throughput, high CTCs recovery, high purity, and high cell viability (67). By principle, CTCs are captured by mAbs that are coated on the wall of flow-channel and then will be released out for further step (83). To increase efficiency of microfluidic technique, the nanomaterials were used. The nanoparticles are coated with mAbs instead for increase the binding surface area (84). CTCs analysis is developing for more reliable and currently there is no suitable technique (83). Therefore, this ScFv, it might be a candidate one for applied to use in CTCs analysis.

CHAPTER VII

CONCLUSIONS

The results of current study could be concluded as the following:

1. Trop2mCherry ScFv was successfully produced by bacteria expression system in our laboratory, it could be used as an in house production.
2. Trop2mCherry ScFv could bind specifically to Trop2 molecules on cancer cells surface and it could be used as a direct fluorescent staining.
3. Trop2mCherry ScFv statistically has no efficiency difference from the commercial Trop2 antibody.
4. Trop2mCherry ScFv was demonstrated to be used in CTCs detection.
5. For further modification, Trop2mCherry ScFv could be adapted for another purposed such as therapeutic cancer.

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APPENDICES

APPENDIX A

GENERAL REAGENTS

100 mM CaCl₂

CaCl ₂ (MW 147.02g)	4.41 g
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Dissolve in distilled water 300 mL and sterilized by autoclaving, store at RT.

1X PBS, pH 7.4

NaCl (MW 58.4 g/mol)	8 g
KCl (MW 74.551 g/mol)	0.2 g
Na ₂ HPO ₄ (MW 141.96 g/mol)	1.44 g
KH ₂ PO ₄ (MW 136.086 g/mol)	0.24 g

Dissolve in sterile water 900 mL and adjust pH to 7.4.

Sterilized by autoclaving and store at 4°C.

MEDIA FOR BACTERIA CULTURE

LB Medium

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

Dissolve in distilled water to 1 L and sterilized by autoclaving and store at RT.

LB agar

Tryptone	3 g
Yeast extract	1.5 g
NaCl	3 g

Dissolve in distilled water to 300 mL and microwaved until dissolved.

Agar	4.5 g
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Sterilized by autoclaving and store at RT.

LB broth base for protein expression

Tryptone	40 g
Yeast extract	10 g
NaCl	10 g
Na ₂ HPO ₄	12 g
KH ₂ PO ₄	6 g

Dissolve in distilled water to 2 L and sterilized by autoclaving and store at RT.

Sugar mix solution

Glucose	12.5 g
Lactose	50 g
Glycerol	150 mL

Dissolve in distilled water 850 mL and sterilized by autoclaving, store at RT.

100 mg/mL Ampicillin

Ampicillin Sodium equivalent to ampicillin	1 g
Sterile distilled water	10 mL

Aliquot for 1 mL/Eppendorf tube for working solution and store at -20°C.

100 mg/mL Chloramphenicol

Chloramphenicol	1 g
Absolute ethanol	10 mL

Aliquot for 1 mL/Eppendorf tube for working solution and store at -20°C.

REAGENTS FOR AGAROSE ELECTROPHORESIS**5X Tris-Borate-EDTA (TBE) buffer**

Tris-base	27 g
Boric acid	13.75 g
EDTA	0.93 g

Dissolve in distilled water 400 mL and adjust pH to 8.0.

Adjust final volume to 500 mL with distilled water and store at RT.

Dilute with distilled water to 1X before use.

0.8% agarose for gel electrophoresis

Agarose	0.8 g
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Dissolve in 1X TBE buffer 100 mL and melting by microwave before pour in the tray.

REAGENTS FOR PROTEIN EXPRESSION**5x Binding buffer (Phosphate buffer)**

NaH ₂ PO ₄ ·H ₂ O (MW 137.99 g/mol)	13.8 g
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NaCl (MW 58.44 g/mol)	146.1 g
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Dissolve in distilled water 1 L and store at RT.

1x Binding buffer (Phosphate buffer)

5x Binding buffer (Phosphate buffer)	100 mL
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Add distilled water 400 mL and store at RT. Filter (0.22 μm) before use.

1.0 M imidazole

Imidazole (MW 68.08 g/mol)	13.6 g
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Dissolve in distilled water 200 mL and store at 4°C with light protection.

For several concentrations of imidazole**40 mM imidazole**

1 M imidazole	20 mL
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1x Binding buffer	480 mL
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250 mM imidazole (Elution buffer)

1 M imidazole	62.5 mL
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1x Binding buffer	187.5 mL
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500 mM imidazole (Elution buffer)

1 M imidazole	125 mL
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1x Binding buffer	125 mL
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Store at 4°C with light protection. Filter (0.22 μm) before use.

REAGENTS FOR SDS-PAGE**1.5 M Tris-HCl pH 8.8 (Stock buffer for separating gels)**

Tris-Base (MW 121.14 g/mol) 181.71 g

Dissolve in distilled water 800 mL and adjust pH to 8.8.

Adjust final volume to 1,000 mL with distilled water and store at 4°C.

1.0 M Tris-HCl pH 6.8 (Stock buffer for stacking gels)

Tris-Base (MW 121.14 g/mol) 121.14 g

Dissolve in distilled water 400 mL and adjust pH to 6.8.

Adjust final volume to 500 mL with distilled water and store at 4°C.

30% Acrylamide/ 0.8 w/v bis

Acrylamide 90 g

N,N-bis-methylene-acrylamide 2.4 g

Dissolve in distilled water 300 mL and store at 4°C with light protection.

10% Sodium Dodecyl Sulfate (SDS)

SDS 10 g

Dissolve in distilled water 100 mL and store at RT.

10% Ammonium persulfate (APS)

APS 1 g

Dissolve in distilled water 10 mL. Aliquot and store at -20°C with light protection.

5X running buffer

Tris-base (MW 121.14 g/mol) 15 g

Glycine (MW 75.07 g/mol) 72 g

SDS (MW 288.372 g/mol) 5 g

Dissolve in distilled water 800 mL and adjust pH to 8.3.

Adjust final volume to 1,000 mL with distilled water and store at RT.

Dilute to 1X with distilled water before use.

2X Sample Buffer

Glycerol	2 mL
1.0 M Tris-HCl pH 6.8	2.5 mL
0.05% w/v Bromophenol blue	0.2 mL
10% SDS	4 mL
Add β -Mercaptoethanol	1 mL

Add distilled water 0.5 mL. Aliquot and store at -20°C.

Preparing for 12% separating gel (1 gel)

Distilled water	2.65 mL
1.5 M Tris-HCl pH 8.8	2.0 mL
30% Acrylamide/ 0.8 w/v bis	3.19 mL
10% SDS	80 μ l
10% APS	80 μ l
TEMED	4 μ l

Preparing for 5% stacking gel (1 gel)

Distilled water	1.35 mL
1.0 M Tris-HCl pH 6.8	250 μ l
30% Acrylamide/ 0.8 w/v bis	340 μ l
10% SDS	20 μ l
10% APS	20 μ l
TEMED	2 μ l

Staining buffer (Commasie Blue solution)

Commasie Blue	0.06 g
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Add 10% v/v acetic acid 1 L, and store at RT.

Destain solution

Absolute methanol	400 mL
-------------------	--------

Add distilled water 600 mL, and store at RT.

REAGENTS FOR CELLS CULTURE**DMEM media, pH 7.4**

DMEM, powder, high glucose, pyruvate media (Gibco®)	1 pack
NaHCO ₃	3.7 g

Dissolve in sterile water 890 mL and adjust pH to 7.2.

Sterilized by using filter 0.2 µm (pH will increase 0.2 approximately after filtration).

Add FBS (Gibco®)	100 mL
------------------	--------

Add Penicillin-Streptomycin 10,000 U/mL (Gibco®)	10 mL
--	-------

Store at 4°C.

RPMI 1640 media, pH 7.4

RPMI 1640 medium (Gibco®)	1 pack
NaHCO ₃	2 g

Dissolve in sterile water 890 mL and adjust pH to 7.2.

Sterilized by using filter 0.2 µm (pH will increase 0.2 approximately after filtration).

Add FBS (Gibco®)	100 mL
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Add Penicillin-Streptomycin 10,000 U/mL (Gibco®)	10 mL
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Store at 4°C.

APPENDIX B

INFORMATION OF TROP2MCHERRY PLASMID

The recombinant plasmid was developed by phage display technique using bacteriophage library that was constructed by Professor Wanpen Chaicumpa, Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University (22). In brief, mRNA was extracted from B-lymphocytes of volunteer donors and converted to cDNA that used as a template for antibody repertoires gene. The ScFv fragments were randomly assembled V_H link with V_L using overlap extension PCR.

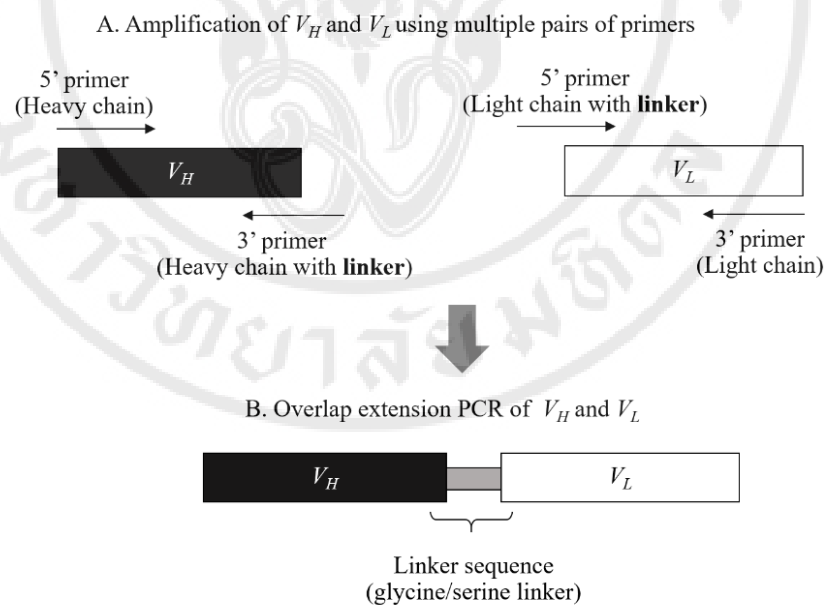


Figure B.1 Assembly strategy of ScFv by overlap extension PCR

The ScFv gene repertoires were further cloned into phagemid (pCANTAB 5E), amplified, and used in phage biopanning procedure. The genetic map of pCANTAB 5E was shown in Figure B.2.

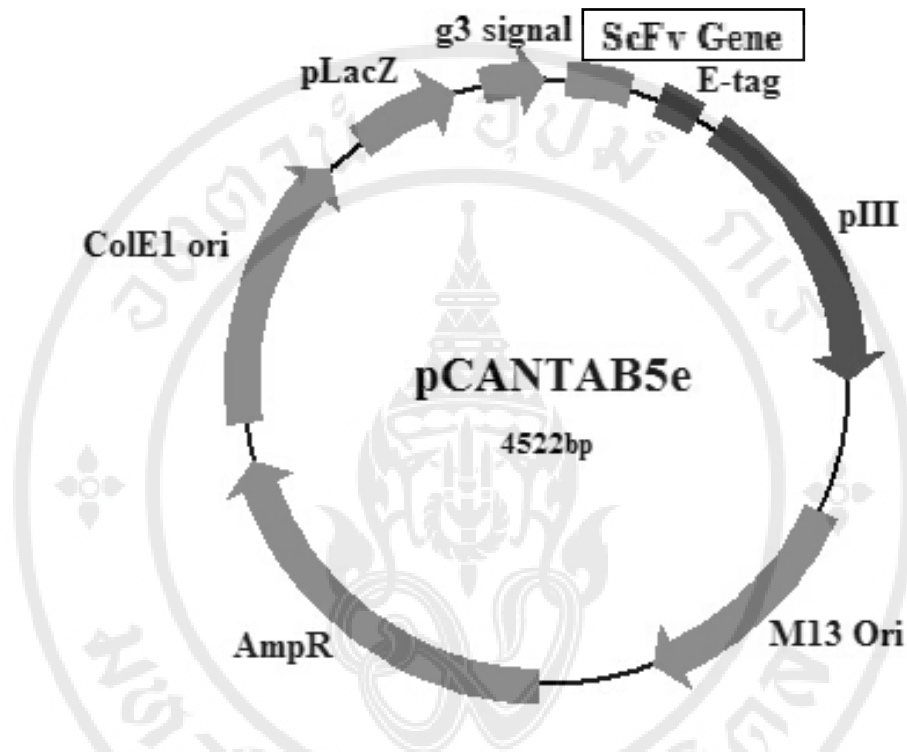


Figure B.2 Map of pCANTAB 5E

By principle, the individual ScFv molecule was displayed on the phage surface because the ScFv gene was fused with phage genome. The phage display library would be selected by binding with antigen of interest, in this case is some part of Trop2 extracellular domain, and only clone that can bind specifically with that specific peptide would be eluted and further amplified.

For phage biopanning procedure, a short peptide corresponded to linear structure, extracellular domain of Trop 2 labelled with biotin was synthesized (Synpeptide, Shanghai, China). This Trop2 peptide primary structure was Biotin-Lysine-Glycine- Glutamic Acid-Serine-Leucine-Phenylalanine-Glutamine-Glycine-Arginine-Glycine-Glycine-Leucine-Aspartic Acid-Leucine-Arginine-Valine-Arginine-Glycine-Glutamic Acid-Proline (Biotin-KGESLFQGRGGLDLRVRGEP), be adhered on streptavidin-coated plate and then performed phage biopanning. After that phages were transformed into *E. coli*. Crude extractions from *E. coli* were tested the binding with Trop2 peptide compared with bovine serum albumin (BSA) and the clones that had high affinity with more than double signal of BSA would be selected for sequencing. The selected clones were performed the DNA sequencing and only clone with complete ScFv sequence (composed of both V_H and V_L sequence) would be selected.

One of the clones of ScFv named clone No. 31 has been chosen for further used in this project. Only sequence of ScFv against Trop2 contains 590 bp (Table B.1) would be transferred to pET-23b+ plasmid, an expression vector with T7 promoter, by amplification and recombination with specific primers and restriction endonucleases (Table B.2). After that, the sequence of mCherry contains 709 bp, a red fluorescent protein, has been transferred by amplification and recombination with specific primers and restriction endonucleases (Table B.2) to this plasmid at 3' position of *Trop2 ScFv* gene for production of fluorescence fusion protein. Next to sequence of His-Taq protein at C-terminal, a string of six histidine residues, that already modified in pET-23b+ it will help for protein purification. Therefore, Trop2mCherry ScFv contained DNA sequences 1332 bp, 443 amino acids (Table B.1). This plasmid contains ampicillin resistance gene as a selective marker. The map of this plasmid was shown in Figure B.3.

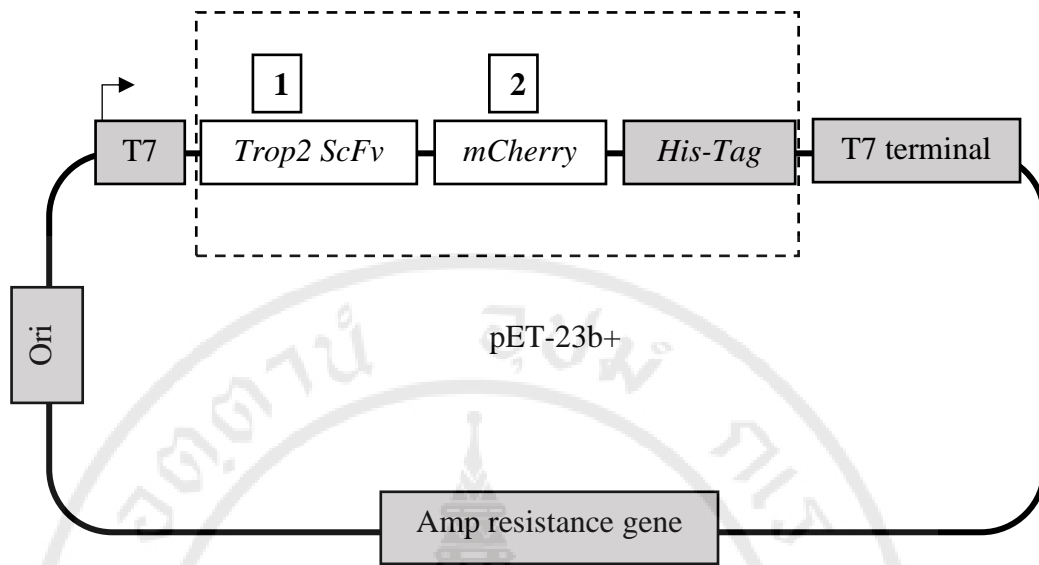


Figure B.3 Map of plasmid construction (pET-23b+) contained Trop2mCherry ScFv sequences.

1: sequences of Trop2 ScFv. **2:** sequence of mCherry. **Dashed lines square:** total sequence of Trop2mCherry ScFv.

Table B.1 The synthesized DNA sequence and amino acid sequence of Trop2mCherry ScFv

<p>DNA sequence 1: <i>Trop2 ScFv</i></p>	<p>ATGGGGTGGATGAGCCCTACCAGTGGCAATACAGACTATG CACAGAAGTTCAGGGCAGAGTCACGATTACCGCGGACA AATCCACGAGCACAGCCTACATGGAGCTGAGCAGCCTGA GATCTGACGACACGGCCGTGTATTACTGTGGCTACGAACC GCGGGGGGAGCTACGTGGATTGACTACTGGGGTCAGGG AACCACGGTACCGTCTCCTCAGGTGGCGGTGGCTCGGGCG GTGGTGGGTCGGGTGGCGGCCGGATCTAAAATTGTGTTGA CGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAG AGCCACCCTCTCGTGCAGGGCCAGTCAAAGTATTTCGTGGT ACTGACTTAGCCTGGCACCAGCAGAAACCTGGCCAGCCTC CCAGGCTCCTCATTTATGACACATCCATCAGGGCCACTGG CATCCAGACAGGTTTCAGTGGCAGTGGGTCTGGGACAGAC TTCACTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTG CAGTGTATTACTGTCAGCAGTATGGTATCTCACCGTGGAC GTTCGGCCAAGGGACCAAGCTGGAAATCAAAGCGGCCGC A</p>
<p>DNA sequence 2: <i>mCherry</i></p>	<p>ATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATC AAGGAGTTCATGCGCTTCAAGGTGCACATGGAGGGCTCCG TGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGG GCCGCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGG TGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCT GTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAG CACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCC CCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGG ACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCA GGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCAC CAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACC ATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGG ACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGC TGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGACCA CCTACAAGGCCAAGAAGCCCCTGCAGCTGCCCGGCGCCTA CAACGTCAACATCAAGTTGGACATCACCTCCCACAACGAG GACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGC CGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGCTCG AGCACCACCACCACCACACTGA</p>
<p>Amino acid sequence (Fully molecule)</p>	<p>MGWMSPTSGNTDYAQKFQGRVTITADKSTSTAYMELSSLRS DDTAVYYCGYEPRGGATWIDYWGQGTTPVSPQVAVARAVV GRVAAGSKIVLTQSPGTLSPGERATLSCRASQSIRGTDLA WHQQKPGQPRLLIYDTSIRATGIPDRFSGSGSDFTLTISRL EPEDFAVYYCQYGISPWTFGQGTKLEIKAAAMVSKGEEDN MAIIEFMRFKVMHEGSVNGHEFEIEGEGEGRPYEGTQTAKL KVTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFP EGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNF PSDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLD GGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTI VEQYERAEGRHSTGGMDELYKLEHHHHHH</p>

Table B.2 Specific primer used for PCR amplification and product size

Gene name	Direction	Primer sequence (5'-->3')	Size (bp)
Gene 1: Trop2 ScFv	Forward	ATA-AGG-ATC-CGC-TAG-TTC-CTC- GTG-GAT-CTA-TGG-GGT-GGA-TGA- GCC-CTA-CC	600 bp
	Reverse	CTT-TCG-GGC-TTT-GTT-AGC-AGC	
Gene 2: mCherry	Forward	TTT-GCG-GCC-GCA-ATG-GTG-AGC- AAG-GGC-GAG-GA	700 bp
	Reverse	TAT-CTC-GAG-CTT-GTA-CAG-CTC- GTC-CAT-GC	
Gene A-B: Trop2mCherry	Forward	ATA-AGG-ATC-CGC-TAG-TTC-CTC- GTG-GAT-CTA-TGG-GGT-GGA-TGA- GCC-CTA-CC	1300 bp
	Reverse	CTT-TCG-GGC-TTT-GTT-AGC-AGC	

PCR reaction was performed by several reagents including Platinum Taq DNA polymerase, 500U (Invitrogen) that also contains 10X buffer and 50 mM MgCl₂, dNTP set (Invitrogen). The reagents for each reaction were shown in Table B.3 and PCR cycle was shown in Table B.4. All PCR reactions were performed by PCR machine (5 Prime G gradient thermal cycler, Techne[®], USA) and observe the PCR product by Agarose gel electrophoresis with 0.8% agarose gel. All primers for using in PCR reaction were synthesized by BioDesign (Pathumthani, Thailand). The determination of each sequence gene and plasmid was shown in Figure B.4.

Table B.3 PCR reagents for PCR amplification

Reagents	Final concentration	Volume (uL)
10X buffer	1X	2.5
50 mM MgCl ₂	2.5 mM	1.25
10 μM Forward Primer	0.5 μM	1.25
10 μM Reverse Primer	0.5 μM	1.25
5 mM dNTP	0.2 mM	1
DW	-	15.55
Template	-	2
Total volume		25

Table B.4 PCR cycle

Step	Temperature	Time	Cycles
Pre-denature	95 °C	5 min	-
Denature	95 °C	30 sec	35 cycles
Annealing	55 °C	45 sec	
Extension	72 °C	1 min	

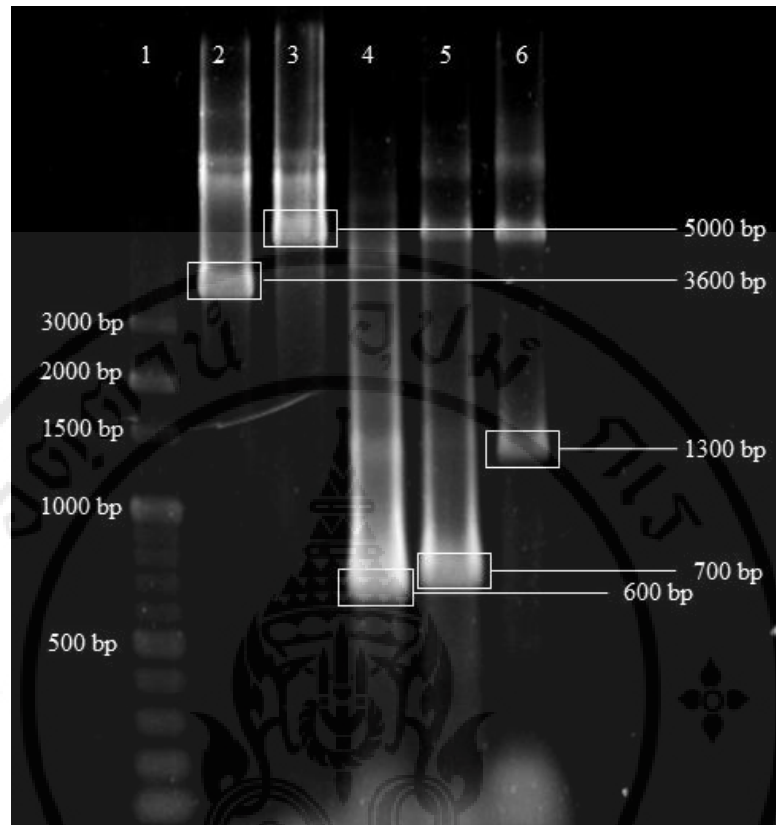


Figure B.4 Determination of each gene segment.

Lane 1: 100 bp DNA marker, **Lane 2:** pET23b+ vector, **Lane 3:** Trop2mCherry ScFv plasmid, **Lane 4:** Sequence of Trop2 ScFv gene, **Lane 5:** Sequence of mCherry gene, **Lane 6:** Sequence of Trop2 mCherry ScFv gene

APPENDIX C

CERTIFICATE OF HUMAN APPROVAL

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Siriraj Institutional Review Board

Certificate of Approval

COA no. Si 841/2019

Protocol Title(English) : Development of Single Chain Variable Fragment for Direct Detection of Trophoblast Cell Surface Antigen 2 on Circulating Tumor Cells

Protocol Title(Thai) : การผลิตแอนติบอดีชนิดขนาดเล็กที่มีความจำเพาะต่อโปรตีนทรอป 2 ที่พบบนเซลล์มะเร็งในกระแสเลือด

SIRB Protocol No. : 727/2562(IRB3)

Principal Investigator/Affiliation : Assoc. Prof.Peti Thuwajit, M.D., Ph.D. / Department of Immunology
Faculty of Medicine Siriraj Hospital, Mahidol University

Research site : Faculty of Medicine Siriraj Hospital

Duration of research : 1 year 6 months

Approval date : December 13, 2019

Expired date : December 14, 2020

This is to certify that Siriraj Institutional Review Board is in full compliance with international guidelines for human research protection such as the Declaration of Helsinki, the Belmont Report, CIOMS Guidelines and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

(Prof. Chairat Shayaku, M.D.)
Chairperson

18 DEC 2019

date

(Prof. Dr. Prasit Watanapa, M.D., Ph.D.)
Dean of Faculty of Medicine Siriraj Hospital

19 DEC 2019

date

Approval includes :

1. SIRB submission form, date October 11, 2019
2. Participant information sheet, date December 9, 2019
3. Informed consent form, date December 9, 2019
4. Case record form
5. Curriculum vitae

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PUBLICATION/PRESENTATION

Poster presentation:

Yuti P., Thuwajit C., Thuwajit P. Development of single chain variable fragment for direct detection of trophoblast cell surface antigen 2 expressing cancer cells. . Seminar “Kumamoto-Mahidol (Siriraj)-Khon Kaen” at SiMR Building, Mahidol University, Bangkok, Thailand. Dec 2-3, 2019.

PROCEEDING

Short oral presentation for M.Sc. Student in Biomedical Science, Development of single chain variable fragment for direct detection of trophoblast cell surface antigen 2. Graduate Research Forum 2018, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. May 16, 2019.