APPLICATION OF MICROFLUIDIC SYSTEM FOR SCREENING OF ANTIBODY PRODUCING CHO CELLS

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APPLICATION OF MICROFLUIDIC SYSTEM FOR SCREENING OF ANTIBODY PRODUCING CHO CELLS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ซัลมา ฟัวดิยาห์ : การประยุกต์ใช้ระบบไมโครฟลูอิดิกส์เพื่อการคัดแยกโซเซลล์ผลิตแอนติบอดี (APPLICATION OF MICROFLUIDIC SYSTEM FOR SCREENING OF ANTIBODY PRODUCING CHO CELLS) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร. พันธุ์วงค์ คุณธนะวัฒน์, 83 หน้า.

โมโนโคลนอลแอนติบอดี/ไมโครฟลูดิก/รังไข่หนูแฮมสเตอร์จีน (CHO) เซลล์/การสร้างเซลล์ที่เสถียร/การแยกเซลล์เดียว

โมโนโคลนัลแอนติบอดี (mAbs) มีบทบาทสำคัญในอุตสาหกรรมชีวเภสัชภัณฑ์ เนื่องจากมีการนำไปใช้ในการบำบัดรักษาโรคหลากหลายชนิด เช่น มะเร็ง โรคภูมิคุ้มกันต่อต้านตนเอง และโรคติดเชื้อ ความต้องการ mAbs ในตลาดมีแนวโน้มจะสูงขึ้นเรื่อยๆ ขั้นตอนสำคัญขั้นตอนหนึ่งสำหรับการผลิตมอนอโคลนอลแอนติบอดี สำหรับการรักษาโรคคือการสร้างเซลล์ไลน์ที่สำหรับการแสดงออกของแอนติบอดีที่เสถียร โซเซลล์ (CHO cell) ถูกใช้เป็นเซลล์ตั้งต้นมากที่สุด ในการศึกษาวิจัยนี้ ผู้วิจัยได้ตรวจสอบผลของการใช้เวคเตอร์ที่แตกต่างกันสองชนิดที่เหมาะสม สำหรับระบบการแสดงออกของ CHO cell ที่แตกต่างกันสองระบบ นั่นคือ DHFR และ GS ต่อการแยก CHO เซลล์ที่เสถียร CHO เซลล์ที่ยีน GS ถูกน็อคเอาท์ ถูกใช้ทรานส์เฟกด้วยพลาสมิดสองชนิด ได้แก่ พลาสมิดสำหรับการแสดงออกของแอนติบอดี Adalimumab สำหรับระบบ GS (pWS_AdaliH7HC+L1LC) และพลาสมิดการแสดงออก GFP สำหรับระบบ DHFR (Cloned_EmGFP-เข้า-pCHO1.0) จากการทดลองพบว่าเซลล์ไลน์สามารถแสดงออก mAb และ GFP ได้พร้อมกัน รูปแบบการแสดงออกที่แตกต่างกันของ mAb และ GFP ซึ่งเป็นผลมาจากการรวมจีโนมแบบสุ่มของเวกเตอร์ทั้งสองสามารถสังเกตได้ ผลจากการศึกษานี้จะเป็นทรัพยากรที่ใช้สำหรับการศึกษาความต่างกันของการแสดงออกของยีน CHO ในอนาคต

การพัฒนาเซลล์ไลน์ที่เสถียรสำหรับการผลิต mAbs สามารถทำได้โดยใช้วิธีการทั่วไป เช่น วิธีลิมิตติงไดลูชั่น (limiting dilution) อย่างไรก็ตาม ข้อเสียของวิธีดังกล่าวคือ การดำเนินการต้องใช้เวลาและแรงงานมาก และมีโอกาสเกิดโมโนโคลนอลได้น้อยกว่า แม้ปัจจุบันจะมีเทคโนโลยีที่มีประสิทธิภาพสูง เช่น การคัดแยกเซลล์ด้วยเทคนิคทางฟลูออเรสเซนท์ (FACS) ที่สามารถใช้แทนวิธี limiting dilution ได้ แต่เทคนิคดังกล่าวไม่ใช่เทคโนโลยีที่เข้าถึงได้ง่ายสำหรับห้องปฏิบัติการทั่วไปมีต้นทุนสูง อาศัยทักษะในการใช้งาน ในงานวิจัยนี้ผู้วิจัยได้ศึกษาการนำอุปกรณ์ไมโครฟลูอิดิกเชิงหลุมระดับจุลภาค ที่ได้รับการดัดแปลงจากรายงานโดยกลุ่มของเราก่อนหน้านี้ (MBM) มาประยุกต์ใช้เพื่อแยก CHO เซลล์เดี่ยว เซลล์ในอาหารเหลวถูกโหลดเข้าไปในอุปกรณ์โดยไมโครปิเปต จากนั้นเซลล์ที่ถูกดักจับด้วยไฟฟ้าสถิตของหลุมจะสามารถสังเกตได้ภายใต้กล้องจุลทรรศน์แบบหัวกลับ เมื่อเปรียบเทียบกับ limiting dilution พบว่า อุปกรณ์ MBM ให้จำนวนเซลล์เดี่ยวที่เพิ่มขึ้น 4.7 เท่า ต่อการโหลดเซลล์หนึ่งรอบ และแสดงให้

เห็นว่าเวลาที่ใช้ในการปฏิบัติการทั้งหมดลดลง 1.91 เท่า วิธีการนี้มีต้นทุนต่ำ สามารถดำเนินการได้โดยง่าย มีประสิทธิภาพ และเป็นทางเลือกใหม่สำหรับการพัฒนาเซลล์ไลน์

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ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

SALMA FUADIYAH : APPLICATION OF A MICROFLUIDIC SYSTEM FOR SCREENING OF ANTIBODY PRODUCING CHO CELLS. THESIS ADVISOR : ASSIST. PROF. PANWONG KUNTANAWAT, Ph.D., 83 PP.

MONOCLONAL ANTIBODY/MICROFLUIDIC/CHINESSE HAMSTER OVARY (CHO) CELLS/STABLE CELL LINE GENERATION/SINGLE CELL ISOLATION

Monoclonal antibodies (mAbs) are dominant in the biopharmaceutical industry due to their function for curing diseases such as cancer, auto immune diseases, and infectious diseases. The demands for mAbs will always be high in the coming years. One of the key steps for the manufacturing of the therapeutic monoclonal antibody is to establish a stable cell line for expressing the antibody, of which the CHO cell is most commonly used. In this research study, we investigated the effect of using two different expression vectors suitable for two different CHO expression systems, i.e., DHFR and GS on the isolation of the stable CHO cell. GS knockout CHO cells were used and transfected with two plasmids, namely the Adalimumab antibody expression plasmid for the GS system (pWS_AdaliH7HC+L1LC) and the GFP expression plasmid for DHFR system (Cloned_EmGFP-into-pCHO1.0). It was found that cell lines could be expressed as mAb and GFP simultaneously. Different expression patterns of mAb and GFP, resulting from random genomic integration of the two vectors, could be observed. Stable cell lines expressing the dual plasmid system that were generated from this study will be valuable resources for the study of heterogeneity of the CHO cell gene expression in the future.

Furthermore, developing a stable cell line for mAbs production could be achieved using conventional methods such as limiting dilution. Unfortunately, the drawback of limiting dilution is time-intensive, laborious, and less probability of monoclonality. Moreover, high-throughput technology such as fluorescence activated cell sorting (FACS) could be used to replace limiting dilution. However, it is cost prohibitive, requires special skills and is less accessible to general laboratories. Here, in this research the implementation of the adapted version of a simple microwell-based microfluidic (MBM) device which was previously reported by our group was used to isolate single CHO cells. Cell suspension was loaded into the device by

simple micropipette, then the electrostatically trapped cells could be observed under an inverted microscope. Compared to the limiting dilution method, the MBM device offered a 4.7-folded increase in the number of single cells found per round of cell loading and demonstrated a 1.91-fold decrease in total working time. This approach could serve as affordable, simple and efficient alternative limiting dilution for cell line development.

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Salma Fuadiyah

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

EmGFP	=	Emerald Green Fluorescent Protein
ELISA	=	Enzyme-linked immunosorbent assay
CHO	=	Chinese hamster ovary
HRP	=	Horseradish peroxide
DNA	=	Deoxyribonucleic acid
GS-KO	=	Glutamine synthetase knockout
mAb	=	Monoclonal antibody
q_p	=	Cell-specific productivity
GS	=	Glutamine synthetase
DHFR	=	Dihydrofolate reductase
MSX	=	Methionine sulfoximine
MTX	=	Methotrexate
MBM	=	Microwell-based microfluidic

CHAPTER I
INTRODUCTION

1.1 Background and significance of the study

Monoclonal antibodies (mAbs) are a prominent player for drug discovery and drug development that can be used to cure several diseases such as cancer, cardiovascular, inflammatory, autoimmune diseases (Liu, 2014). Due to its pharmaceutical advantages, the market value of mAbs reached over a hundred billion dollars in 2018 and is expected to one of the most significant classes of biopharmaceuticals for the next couple of years (Urquhart, 2020). The increasing demand for mAbs products remains a challenge and drives innovation in the development of producing process. Optimizing protein expression to obtain stable high-producing clone is the purpose in the development of mAbs production (Tihanyi & Nyitray, 2021). Majority of producing mAbs are by molecular biology technique, which involves identifying and optimizing construction for a stable high-producing clone.

Chinese Hamster Ovary (CHO) cells have been considered as a suitable cell clone for mAbs production. Due to their adapted expression system, provide human-like post translational modification pattern with low immunogenic reactions, and secrete the highly complex molecules (Grilo & Mantalaris, 2019). Moreover, CHO cells based mAbs production could produce the highest antibody product titter around ~1 g/L in batch and 1–10 g/L in fed-batch processes (Kunert & Reinhart, 2016). To obtain large-scale production of mAbs using CHO cells could be achieved by modifying their expression system based via metabolic selection systems.

Nowadays, there were two main CHO cell based expression systems used in the biopharmaceutical industry which consist of Dihydrofolate reductase (DHFR) (Kaufman & Sharp, 1982) and Glutamine synthetase (GS) system (Cockett, Bebbington, & Yarranton, 1990). A selection reagent such as Methotrexate (MTX) acts as a selectable marker in DHFR system are beneficial to improve the productivity

(Ringold, Dieckmann, & Lee, 1981). Selection then occurs when hypoxanthine and thymidine are absence and adding MTX will inhibits DHFR enzyme activity (Ghaderi, Zhang, Hurtado-Ziola, & Varki, 2012). As an alternative, when using GS system as selectable marker, stable cell lines were selected through their ability growing in lack of glutamine and in combination with Methionine sulfoximine (MSX) to generate stable high-producing cell lines (Rita Costa, Elisa Rodrigues, Henriques, Azeredo, & Oliveira, 2010). Using two different selectable markers together for stable mAb-producing cell line have been reported (F Li, Kao, & Ryll, 2004). The double selection approach could lead to improve the productivity and generate high productive stable cell lines expressing mAb (Feng Li, Vijayasankaran, Shen, Kiss, & Amanullah, 2010).

Since mAbs production can be obtained by constructing the desired gene interest into a plasmid DNA, and then transfected into CHO cells, the cell populations usually have high diverse gene complements due to random integration and gene amplification system (S. M. Browne & M. Al-Rubeai, 2007). Consequently, presenting the few high expressing cells in many heterogeneous cell populations. Therefore, an efficient single cell isolation technique is crucial for obtaining a highly rare expressing cell clone. One of the conventional techniques is limiting dilution. This technique uses the dilution of cell concentration and pipetting in 96 well plates to obtain a single monoclonal cell. However, the limiting dilution technique has the main hindrance, such as the time-consuming, low probability of monoclonality, and is limited by the number of clones that can be screened (Ye, Wilhelm, Gentschev, & Szalay, 2021). The other technique is fluorescence activated cell sorting (FACS), which recognizes the single-cell characteristics by fluorescence imaging. This technique can reduce time consumption for screening steps and increase monoclonality (DeMaria et al., 2007). Although FACS is higher throughput than the limited dilution, it remains a disadvantage, such as requiring expensive reagents, skillful and cells could be damaged by the high voltage of the machine (Gross et al., 2015).

Currently, many techniques have been developed in this field, such as nanofluidic and microfluidic systems that could be used to screen the mAbs of CHO cells production. There is one platform of nanofluidic systems developed by

Berkeley Light (Chiou, Ohta, & Wu, 2005). Their fully integrated technology platform combines the use of nanofluidic on a temperature-controlled chip for obtaining a single cell and the high-resolution fluorescence imaging for downstream analytics automatically. In this technology, cell culture tasks are programmed through software, and it could process a thousand cells simultaneously (Le et al., 2018). Moreover, microfluidic systems have emerged as valuable tools for single-cell analysis. For example, microfluidic single cell cloning (SCC) device is used to obtain a single cell. This device holds great potential for functional high-throughput screening at a single cell level. Using this device, the single-cell efficiency could surpass the conventional method, such as the limiting dilution technique. However, the fabrication of this device is quite complicated and required high technology (Ye et al., 2021).

Here, we used the existing microwell-based microfluidic systems to implement and optimize single-cell isolation technology. Previously, this device was used to trap microalgal cells that has single cell efficiency up to ~30%. By successful trapping microalgae using the device, we continued to trap different cells such as CHO cells a mammalian cell lines. To optimize the single cell efficiency for CHO cells using this device, we optimize the initial cell concentration loading. We also demonstrate the microwell-based device that provides a simplicity and low cost for fabrication. We used GS knockout Chinese Hamster Ovary (CHO-K1) cells double deletion to demonstrate the usability of the device and compared the efficiency with the conventional method, i.e., limiting dilution. We hypothesized that the better single-cell clone isolation could be achieved by carefully optimized of their cell loading concentration and cell loading time and can be used in general laboratories due to its requirement of general equipment such as micropipette and inverted microscope.

1.2 Research objectives

The purpose of this study is

1.2.1 To investigate the effect of using double selection approach which together co-transfects two plasmids into GS-KO, CHO-S, and CHO-K1 cells for stable cell line development.

1.2.2 To optimize single cell isolation using existing microwell-based microfluidic device.

1.3 Research Hypotheses

When two plasmids with different selection systems co-transfected into GS-KO CHO-S and CHO-K1 cells, the expression pattern will vary and could lead for useful information about the nature of CHO cell expression system. In addition, microwell-based microfluidic device could be used to isolate CHO cell after optimizing the initial cell loading density and settle time.

1.4 References

Browne, S. M., & Al-Rubeai, M. (2007). Selection methods for high-producing mammalian cell lines. Trends Biotechnol, 25(9), 425-432. doi:10.1016/j.tibtech.2007.07.002.

Chiou, P. Y., Ohta, A. T., & Wu, M. C. (2005). Massively parallel manipulation of single cells and microparticles using optical images. Nature, 436(7049), 370-372. doi:10.1038/nature03831.

Cockett, M. I., Bebbington, C. R., & Yarranton, G. T. (1990). High Level Expression of Tissue Inhibitor of Metalloproteinases in Chinese Hamster Ovary Cells Using Glutamine Synthetase Gene Amplification. Bio/Technology, 8(7), 662-667. doi:10.1038/nbt0790-662.

DeMaria, C. T., Cairns, V., Schwarz, C., Zhang, J., Guerin, M., Zuena, E., . . . Karey, K. P. (2007). Accelerated clone selection for recombinant CHO CELLS using a FACS-based high-throughput screen. Biotechnol Prog, 23(2), 465-472. doi:10.1021/bp060298i.

Ghaderi, D., Zhang, M., Hurtado-Ziola, N., & Varki, A. (2012). Production platforms for biotherapeutic glycoproteins. Occurrence, impact, and challenges of non-human sialylation. Biotechnol Genet Eng Rev, 28, 147-175. doi:10.5661/bger-28-147.

Grilo, A. L., & Mantalaris, A. (2019). The Increasingly Human and Profitable Monoclonal Antibody Market. Trends Biotechnol, 37(1), 9-16. doi:10.1016/j.tibtech.2018.05.014.

Gross, A., Schoendube, J., Zimmermann, S., Steeb, M., Zengerle, R., & Koltay, P. (2015). Technologies for Single-Cell Isolation. International Journal of Molecular Sciences, 16(8), 16897-16919. doi:10.3390/ijms160816897.

Kaufman, R. J., & Sharp, P. A. (1982). Amplification and expression of sequences cotransfected with a modular dihydrofolate reductase complementary dna gene. J Mol Biol, 159(4), 601-621. doi:10.1016/0022-2836(82)90103-6.

Kunert, R., & Reinhart, D. (2016). Advances in recombinant antibody manufacturing. Appl Microbiol Biotechnol, 100(8), 3451-3461. doi:10.1007/s00253-016-7388-9.

Le, K., Tan, C., Gupta, S., Guhan, T., Barkhordarian, H., Lull, J., . . . Munro, T. (2018). A novel mammalian cell line development platform utilizing nanofluidics and optoelectro positioning technology. Biotechnol Prog, 34(6), 1438-1446. doi:10.1002/btpr.2690.

Li, F., Kao, E., & Ryll, T. (2004). Generic High Cell Mass Fedbatch Process for NS0 Cells. Poster Presentation, Cell Culture Engineering IX.

Li, F., Vijayasankaran, N., Shen, A. Y., Kiss, R., & Amanullah, A. (2010). Cell culture processes for monoclonal antibody production. mAbs, 2(5), 466-479. doi:10.4161/mabs.2.5.12720.

Liu, J. K. H. (2014). The history of monoclonal antibody development - Progress, remaining challenges and future innovations. Annals of medicine and surgery (2012), 3(4), 113-116. doi:10.1016/j.amsu.2014.09.001.

Ringold, G., Dieckmann, B., & Lee, F. (1981). Co-expression and amplification of dihydrofolate reductase cDNA and the Escherichia coli XGPRT gene in Chinese hamster ovary cells. J Mol Appl Genet, 1(3), 165-175.

Rita Costa, A., Elisa Rodrigues, M., Henriques, M., Azeredo, J., & Oliveira, R. (2010). Guidelines to cell engineering for monoclonal antibody production. Eur J Pharm Biopharm, 74(2), 127-138. doi:10.1016/j.ejpb.2009.10.002.

Tihanyi, B., & Nyitray, L. (2021). Recent advances in CHO cell line development for recombinant protein production. Drug Discovery Today: Technologies. doi:https://doi.org/10.1016/j.ddtec.2021.02.003.

Urquhart, L. (2020). Top companies and drugs by sales in 2019. Nat Rev Drug Discov, 19(4), 228. doi:10.1038/d41573-020-00047-7.

Ye, M., Wilhelm, M., Gentschev, I., & Szalay, A. (2021). A Modified Limiting Dilution Method for Monoclonal Stable Cell Line Selection Using a Real-Time Fluorescence Imaging System: A Practical Workflow and Advanced Applications. Methods Protoc, 4(1). doi:10.3390/mps4010016.

Yeh, C.-F., Lin, C.-H., Chang, H.-C., Tang, C.-Y., Lai, P.-T., & Hsu, C.-H. (2020). A Microfluidic Single-Cell Cloning (SCC) Device for the Generation of Monoclonal Cells. Cells, 9(6), 1482. doi:10.3390/cells9061.

CHAPTER II

LITERATURE REVIEWS

2. 1 Cell line development

The cell line development process is used to produce cell lines for biomanufacturing process especially in biopharmaceuticals for recombinant proteins such as monoclonal antibodies (mAbs). The process including screening and optimization of cell line to obtain the target productivity and quality. This process made the cell line establishment laborious, time-consuming, and challenging. Many researchers are creating advanced strategies and approaches to deal with those challenges, such as engineering host cells, enhanced the plasmid DNA, recombinant protein expression system, and the utilization of high-throughput tools for isolating single cell for potential cell line candidates (H. Le, N. Vishwanathan, N. M. Jacob, M. Gadgil, & W.-S. Hu, 2015).

The process of cell line establishment begins with transfection of plasmid DNA encoding for gene of interest, selectable marker, and gene regulatory elements into a host cell line which is usually mammalian cell lines (T. K. Kim & Eberwine, 2010). The transfection efficiency could be a fundamental part of cell line development. To achieve high transfection efficiency, there are some methods for transfection process. The transfection methods are generally classified into three groups including chemical, biological, and physical. These are the application of electroporation, calcium phosphate, cationic lipids, and cationic polymer (Geisse, 2009). To achieve high transfection efficiency, an appropriate transfection method should be considered.

Once production of transfected cell line has been done, cells are then subjected to screening process by selection pressure to achieve stable transfected cell pool with genomic integration of the expression cassette. The selection system that is widely used are comprising Dihydrofolate Reductase (DHFR) system and Glutamine synthetase (GS) system. Regarding DHFR system, selection pressure will be carried out by lacking thymidine and hypoxanthine in the medium and this resulting

in the survival of cells that expressing sufficient exogenous DHFR gene. To obtain high producing cells, the addition of methotrexate (MTX) in the medium will increase the copy numbers of the product genes and yielding high producing cells. Meanwhile for GS system lacking glutamine in the medium and addition of methionine sulfoximine (MSX) will yielding high producing cells (Huong Le et al., 2015). Moreover, the stable integration of transgenes and selective markers which introduced into host cell genome normally occurs in random integration site. The randomized way yielding heterogeneous mixture cells in a stably selected cell pool will affect in cell-specific productivity. These cells are named cell pool or bulk pools. To improve the probability of developing high-producing cells, stable cell pools can be enriched for cell populations using single-cell isolation approach.

As mentioned above that after transfection process, a heterogeneous cell pools are generated and this limiting insufficient of monoclonality. Monoclonality is very crucial for cell line development especially for monoclonal antibodies (mAbs) production. Based on the regulatory safety system, it is needed that biopharmaceuticals are to be used for treatment of disease in human have to be produced by monoclonal cell line that derived from single-cell (Barnes, Moy, & Dickson, 2006). Therefore, an approach to isolate single-cell is required and nowadays, there are some techniques that have been existed. Limiting dilution is a conventional technique for generating single cell by serial dilution. A cell pool is serially diluted from high cell density into low cell density and the cells then distributed into 96 well plates using micropipette. The final cell density of the diluted culture will contain less than one cell per well and this yielding some wells will empty, and some wells will have single cell. Subsequently, from single-cell it will be proliferated to form a monoclonal cell population (Susan M. Browne & Mohamed Al-Rubeai, 2007). However, the main hindrance of this technique is time-consuming, laborious, and low single-cell efficiency. Moreover, Fluorescent Activated Cell Sorting (FACS) is emerged as a high throughput technology for single-cell isolation and could overcome limiting dilution's drawbacks. The advantages using FACS allows the rapid screening of millions of cells in one round experiment. The cellular parameters for FACS to screening cells are cell size, viability, granularity, apoptosis, transfection efficiency, and cell surface protein expression (Carroll & Al-

Rubeai, 2004). FACS also allows sorting single-cell from millions of cells and then deposit a single-cell into a multi-well plate. Furthermore, an automated high-throughput microscopy technique has been implemented in the biopharmaceutical industry to confirm the purity of single-cell that deposited into micro-well plates (Evans et al., 2015). The utilization of a high-throughput technology of microscopic system with imaging system using SynenTec Bio Services or Solentim including the Cellavista/NyOne and Cell Metric provide an excellent approach for cell analysis after single-cell sorting and allow to take cell image by both brightfield and also fluorescent images.

2.2 Monoclonal antibodies

The immune system acts as a protection towards various infectious agents that can cause different forms of diseases. There are two major components in the immune system including the humoral (antibody-mediated) and cellular (cell-mediated) immune responses. The humoral immune system comprises B-lymphocytes which recognizes antigen and produces specific antibodies against them. Antibody has two important characteristics including their specificity to the antigen and their assurance to provide continual resistance to that particular of antigen (Ribatti, 2014). Considering their functions, researcher use them for the defense against diseases. The technique that could be produced antibody was developed using *in vitro* technique. This technique resulting the production of monoclonal antibodies for therapeutic application. Figure 2.3 represents the steps of monoclonal antibodies production.

Monoclonal antibodies are monovalent antibody which recognize and bind to the specific epitope and are produced from a single hybridoma cell to provides protection against disease. In the 1975 monoclonal antibody was generated for the first time and fully licensed in 1986, this made the development of monoclonal antibodies represent a novel way to target specific mutations and defects in protein structure and expression in a wide range of diseases and conditions. Currently, monoclonal antibodies are the fastest growing group of biotechnology derived molecules in clinical trials (Nelson, Dhimolea, & Reichert, 2010). Monoclonal antibodies are promising because in scale of the global value of antibody market

itself is almost $20 billion per year (Liu, 2014). Nowadays, about 30 monoclonal antibodies could be approved by the FDA for use in humans for treating various serious diseases and clinical conditions such as cancer, autoimmune diseases, chronic inflammatory, infectious disease (Jung, Jeong, & Chung, 2008).

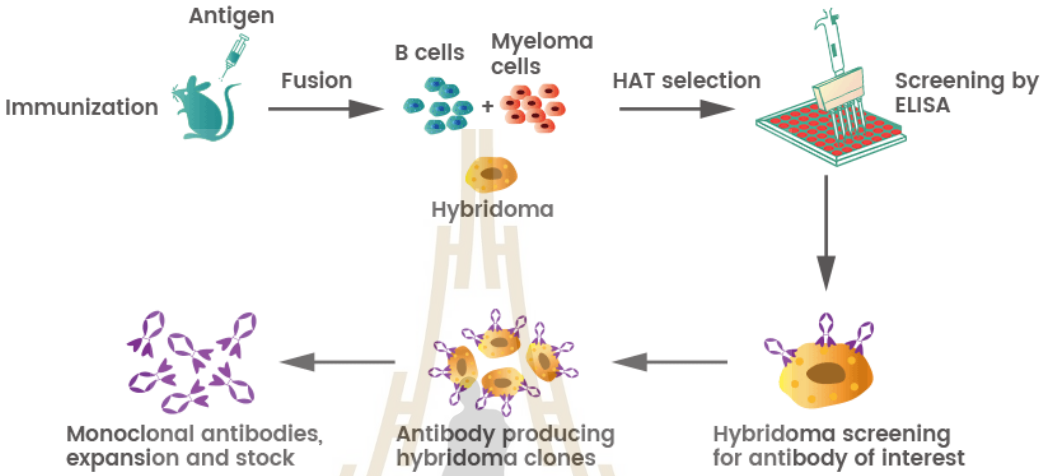

Figure 2.1 Steps involved in the production of mAbs (Saeed, Wang, Ling, & Wang, 2017).

The first generation of monoclonal antibodies were generated in mice in 1975 by hybridoma technique (Köhler & Milstein, 1975). The hybridoma technique involves immunizing a certain species toward a specific epitope on an antigen and obtaining the B-lymphocytes from the spleen of the animal. The fusing B-lymphocytes by chemical or virus induced methods with an immortal myeloma cell line lacking the hypoxanthine-guanine-phosphoribosyl transferase (HGPRT) gene and not contain another immunoglobulin producing cells. The hybridoma cells then cultured in selective medium which containing hypoxanthine-aminopterin-thymidine where only the hybridomas whose fussed with primary B-lymphocytes and myeloma cells, they will survive in the selective medium (Little, Kipriyanov, Le Gall, & Moldenhauer, 2000). The first culture of hybridomas contains a mixture of antibodies derived from many different primary B-lymphocyte clones where each secreting its own individual specific antibody into the culture medium (i.e., polyclonal of

antibodies). The hybridomas then separated by dilution for each individual clone into different culture wells. The cell culture medium then can be screened from hundreds of different wells for the specific antibody activity required and the interested B-lymphocytes grown from the positive wells and subsequently re-cloned and retested for activity assay (Feng Li et al., 2010). The positive results of hybridomas and monoclonal antibodies generated can then be stored in liquid nitrogen.

2.3 Adalimumab antibody

Adalimumab is recombinant immunoglobulin G1 (IgG1) anti-TNF monoclonal antibody and was the first fully humanized monoclonal antibody. It is high affinity and composed of human derived heavy and light chain variable regions and human IgG1: k constant regions and bind specifically to TNF-α. The structure of adalimumab has two antigen binding Fab domains linked to the Fc domain via a hinge. There are six complementarity determining regions of each Heavy:Light chain pair compose the antigen binding site on the Fab domain of the monoclonal antibody (Tracey, Klareskog, Sasso, Salfeld, & Tak, 2008). Adalimumab is engineered by phage display technology and produced in a CHO cell line.

Phage display technology provides the selection of a fully human antibody specific for a specific antigen. The generation of adalimumab is firstly used the anti-human TNF murine antibody MAK195 for the isolation of a human antibody which can recognize the same neutralizing epitope as MAK195. This antibody has a high affinity and low off rate for human TNF. VH and VL MAK195 are paired with human cognate repertoires. These phage antibody libraries, recombinant human TNF serves as the antigen for antigen binding selection. Generation of a fully human anti-TNF antibody is by combination of selected human VH and VL genes. Moreover, early human anti-TNF antibodies were optimized in a second phase which using natural process for antibody optimization. Adalimumab is produced in a CHO cell line and transfected with a plasmid vector containing the expression cassettes for adalimumab light and heavy chains (Radeke & Boehncke, 2007).

Adalimumab is designed as a fully human anti-TNF monoclonal antibody to have some characteristics such as high selectivity and affinity for TNF, suitability for long term chronic administration with a low degree of immunogenicity, low allergic reactions (Cooper, Fava, Gates, Cremer, & Townes, 1992). It is highly specific because it could not bind to or inhibit other forms of TNF, such as lymphotoxin. Adalimumab only binding to TNF, and once binding to TNF, it will neutralizes the biologic activities of cytokine by blocking its interaction with the TNF-RI/II cell surface receptors and modulating biologic responses that are induced or regulated by TNF (Jang et al., 2021). Additionally, adalimumab can binds to and neutralizes the cell membrane-associated form of TNF and may play a role in disease (Georgopoulos, Plows, & Kollias, 1996).

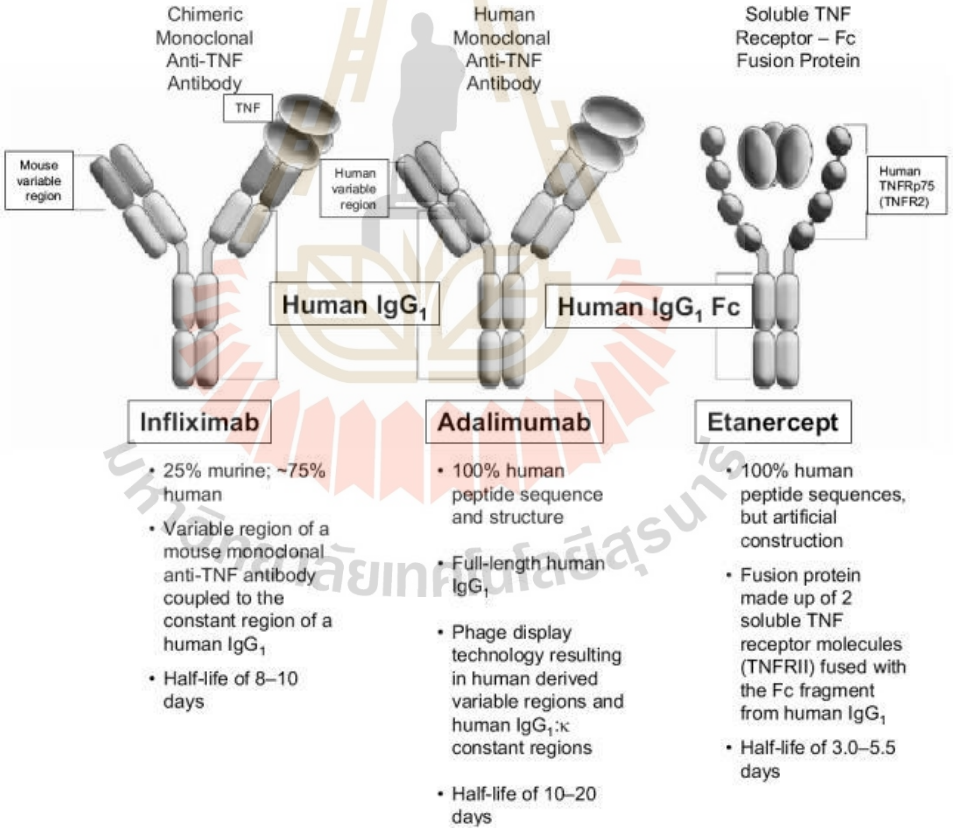

Figure 2.2 Adalimumab structure in comparison with other TNF antagonists. Copyright © 2005. Adapted from Anderson PJ. 2005.

2.4 Chinese Hamster Ovary (CHO) cells

Culture history of CHO cells were more than 50 years, since they were established from the ovary of a Chinese hamster and became “immortal” in the laboratory of Dr. Theodore Puck in the late 1950s (Puck, 1957). The first immortalized cells of CHO cells called CHO-ori which were becoming popular for the study of mammalian genetics and physiology due to two factors. First, they have large chromosomes and the diploid hamster genome contain of 11 pairs of easily identifiable and thus modifications can be observed under microscope. Second, the cells were grown fast and robust. Resulting induced or spontaneous mutagenesis (Puck, 1957).

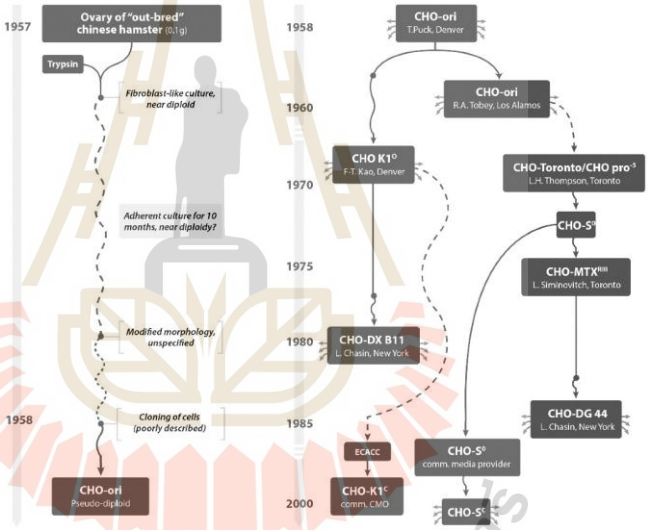

Figure 2.3 Origin, names, and constructed history of industrially relevant CHO cell lines (Wurm, 2013).

CHO cell populations in figure 5 demonstrated the number of labs handled and passed to many researchers in the field. Stippled lines demonstrate the uncertain handling and non-verifiably historic tracing of names and cells. Small arrows mean distribution of cells to numerous laboratories. The following names are still in use today such as CHO K1, DXB11, S, DG 44 which contain of high value therapeutic proteins (Wurm, 2013).

Chinese hamster ovary (CHO) cells are a cell line derived from the ovary of the Chinese hamster which usually used in biopharmaceutical and production of therapeutic proteins (Omasa, Onitsuka, & Kim, 2010). Growing demands for therapeutic proteins promotes the development of technologies for high quality and productivity in CHO expression systems (Omasa et al., 2010). CHO cells are also used as mammalian host for monoclonal antibodies (mAbs) due to their ability to perform post-translational modification (Orellana et al., 2015). Furthermore, CHO cells are able to produce high titers of human compatible therapeutic proteins and easily cultured in suspension (Wurm, 2004). CHO cells offer the benefits that they are easy for genetic manipulation, can be scale up in large scale suspension culture, and also can increase proteins with glycans which are similar even though not identical to those found on human glycoproteins.

Nowadays, products from mammalian cell cultivation are launched in market for more than 40 products and large scale (more than 20,000 L) cultivation is performed worldwide. These products made from CHO cells were sale worldwide over $140 billion per year (Walsh, 2014). Although these cells being used commonly, there is not yet a deep understanding of how to manipulate and control CHO cells to maximize productivity of product interest as host. The product titers are still low (5-10 g/L) which contributes to the high costs of biopharmaceuticals (Kelley, 2009).

2.5 CHO cell line expression systems

Mammalian cell line such as CHO cells are commonly used as a host for commercial production of therapeutic proteins, involving monoclonal antibodies (mAbs). Their expression system have been reported have the capability to perform correct (human-like) post-translational modifications (Werner, Noé, Kopp, & Schlüter, 1998). These made them as the most suitable expression system. DHFR-based methotrexate (MTX) selection and glutamine synthetase (GS)-based methionine sulphoximine are two of the most used in CHO expression systems (Rita Costa et al., 2010).

2.5.1 DHFR expression system

The DHFR (Dihydrofolate reductase) system is a commonly used as expression system in CHO cells and contribute to produce for high level-

recombinant protein expression (Chusainow et al., 2009). This system is based on the *dhfr* gene that coding for DHFR enzyme which can catalyzes the reduction of 5,6-dihydrofolate to 5,6,7,8-tetrahydrofolate which important for DNA synthesis as shown in figure 6. The transfected cells is resistance to geneticin and selection is performed by culturing the cells in medium without thymidine and hypoxanthine but containing geneticin (Wurm, 2004). Moreover, DHFR system allows a process of gene amplification which can increases the cell capacity of production by using methotrexate (MTX).

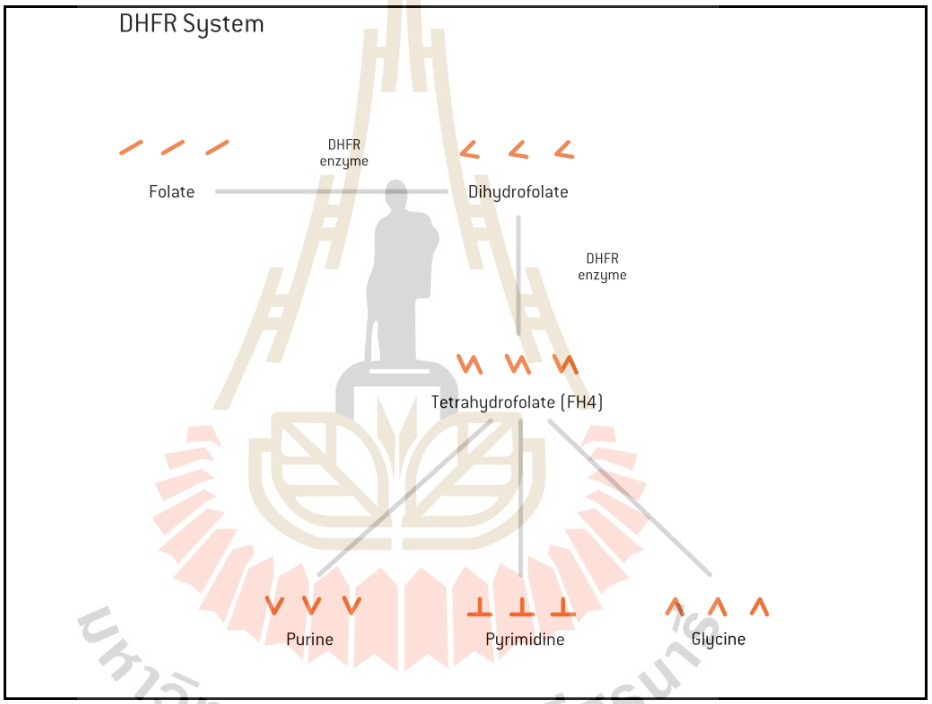

Figure 2.4 The DHFR expression system (Goeddel, 1990).

Methotrexate (MTX) is a folic acid antagonist which is actively transported into cells by the folate transporter. When MTX is presence in the cell, it will be converted to a high molecular weight polyglutamate metabolite by folylpolyglutamate synthetase, which binds to DHFR and inhibits its activity, but DHFR deficient CHO cells that have taken up an expression vector containing the *dhfr* gene could be developed resistance to MTX (Andersen & Reilly, 2004). The amplification unit is larger (100-3,000 kb) than the size of the *dhfr* gene, it makes

target gene that located in the same expression vector as the *dhfr* gene or adjacently resides in the host chromosomal DNA is co-amplified (Kaufman, 1990). Furthermore, high level resistance to MTX may result in cells synthesizing an MTX-resistant DHFR mutant or in cells with changed MTX-transport properties, gene amplification is usually obtained by selection for resistance to gradually increasing concentrations of MTX in multiple steps (Chusainow et al., 2009). The clonal variation in foreign protein expression is significant because clone can still acquire MTX resistance by mechanism other than DHFR-mediated gene amplification.

To obtain a high expression level of the target gene there are two strategies which commonly used. The first one is based on individual clones and the second one is parental cell pools. Both are isolated by the limiting dilution technique in 96-well culture plates. They are independently grown in medium with increasing concentrations of MTX. Even though the final clones are resistant to high level of MTX, they are derived clonally and become heterogenous with expression of the foreign protein (Cacciatore, Chasin, & Leonard, 2010).

As mention above that the DHFR system could be resulted in very high levels of amplification and expression, however there still remains limitation which is the amplification process could takes longer time and may require several months to isolate and characterize a stable amplified line (Kingston, Kaufman, Bebbington, & Rolfe, 2002).

2.5.2 GS-expression system

The Glutamine Synthetase (GS) expression system which using the glutamine metabolism in mammalian CHO cell lines are designed by Lonza. Glutamine formation in mammalian cells follows an enzymatic pathway of biosynthesis which from glutamate and ammonium using the GS system. Figure 7 A represents the GS expression system which has a GS (L-glutamate:ammonia ligase) that is a universal housekeeping enzyme that catalyzes the synthesis of glutamine from glutamate and ammonia using the hydrolysis of ATP to ADP and phosphate to drive the reaction (Purich, 1998). It is the only one of enzyme which can synthesize glutamine *de novo* (Lie-Venema, Hakvoort, van Hemert, Moorman, & Lamers, 1998). Figure 9 B shows the CHO GS gene was identified from a CHO cell line over expressing(P G Sanders & R H Wilson, 1984). It is consisting of six exons, and exons number 5 comprising the sequence critical for GS activity (Krajewski et al., 2008).

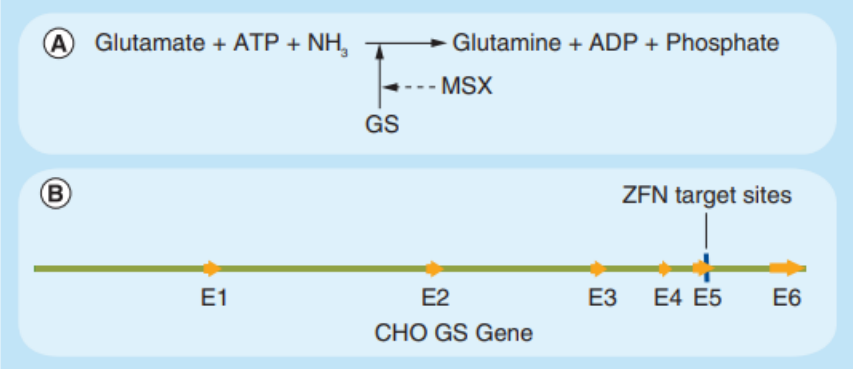

Figure 2.5 GS expression system (Goeddel, 1990).

GS-expression system by Lonza utilize a strong promoter which is the human CMV-MIE promoter (hCMV) to drive the gene of interest. The GS selectable marker is driven by a weaker promoter which is SV40E. Stringent selection in a glutamine-free medium with the addition of methionine sulphoximine (MSX), an irreversible inhibitor of GS, selects for rare integrations of the vector into transcriptionally active sites of the genome. Since the selection marker is on the same vector as the genes of interest, these are also likely to be expressed at high levels. The current vector utilizes the hCMV promoter to drive expression of both the heavy chain (HC) and the light chain (LC) with the LC placed in front of HC within the vector (Park & Shuler, 2003).

CHO cells required glutamine in culture medium because glutamine provides an essential amino acids and allowing the enzymatic pathway of biosynthesis (Cockett et al., 1990). The function of glutamine in cell culture are as an energy source, and as a nitrogen donor in the synthesis of amino acids and nucleotides. CHO cells can express GS to survive by the addition of methionine sulphoximine (MSX). Methionine sulphoximine is an inhibitor of the endogenous GS activity. It also allowing the selection of productive stable cell (Cockett et al., 1990). To obtain the selection of productive stable cell, there are 2 step selection strategies involving 25-50 μM MXS for enhancing stringency in the first round and second round using 100-1000 μM for amplification (Kingston et al., 2002). However, the survival of a large number of poor producers is still result in the selection for such CHO cell lines. To improve selection stringency for GS system, a CHO GS knockout host cell line was generated (Fan et al., 2012). The

SV40E promoter was used to improve selection stringency and eliminate the use of MSX. This can increase the higher producer in the absence of MSX.

Using the GS system rather than DHFR system could be more beneficial because in the GS system requires only a single round of selection for amplification to achieve higher expression levels. The time necessary to isolate stable amplified clones from the primary transfectants is less compared to DHFR system (Kingston et al., 2002).

2.6 Green Fluorescent Protein (GFP)

Green fluorescent protein (GFP) is broadly used as a reporter gene expression and could act as fusion tags to monitor protein localization within living cells. Until now, the utilization of GFP is still attracting immensely interest as the general method to create a visible fluorescence which can emit green color. In the beginning, GFP was isolated from jellyfish *Aequorea Victoria* which have 238 amino acids polypeptide that is highly fluorescent and stable in many assay conditions (Cubitt et al., 1995). Based on their sequences from many research that study on its expression in heterologous systems made it as an attractive reporter gene.

GFP has been used in application for monitoring the transfer and expression of genes in cells by fusion to the genes of desired. The advantage using GFP as a reporter gene is low toxicity and could not interrupt normal cellular activities and easy to detect its fluorescent by using fluorescence microscopy and flow cytometry (Cheng, Fu, Tsukamoto, & Hawley, 1996). In addition, a main benefit using GFP as a marker protein is the lack of requirement for exogenous substrates or cofactor to produce fluorescent molecule for detection (Chalfie, Tu, Euskirchen, Ward, & Prasher, 1994).

2.7 Microfluidics

Microfluidics is a study of fluids manipulation by using tiny channels that having dimensions at microscale. Microfluidics itself comes from the words of micro and fluid which means that using with very small volumes of fluids. Microfluidics controls the fluid in small volumes using a microsystem (Whitesides, 2006).

A microfluidic device is a tool that manipulates, analyze, and study about fluids and their behaviors. The microfluidic device can be fabricated with various materials and techniques. Materials that can be used to fabricate microfluidic

devices including silicon, glass, and polymer such as polydimethylsiloxane (PDMS), polymethylmethacrylate (PMMA), polystyrene (PS), and polycarbonate (PC). These materials have advantages and disadvantages for making a microfluidic device which depends on the purpose of fabrication in any research field. There are some techniques to fabricate microfluidic devices such as soft lithography, photolithography, wet etching, hot embossing, laser machine, conventional machining, and injection molding. In addition, the selection of a fabrication technique among them can be determined by the material and some factors such as cost and size of the device (Wu & Whitesides, 2001). Among these techniques for the fabrication of microfluidic, the most widely utilized is soft lithography. Soft lithography provides access to using a wide variety of materials, generally compatible with biological applications, and low in cost. Soft lithography forms micropatterns of self-assembled monolayers (SAM) by contact printing, and forms microstructures in materials by embossing (imprinting) and replica molding (Xia & Whitesides, 1998). Microfluidic devices have been established and provide promising platforms for lab on a chip application, such as high throughput analysis for single-cell isolation and cell culture.

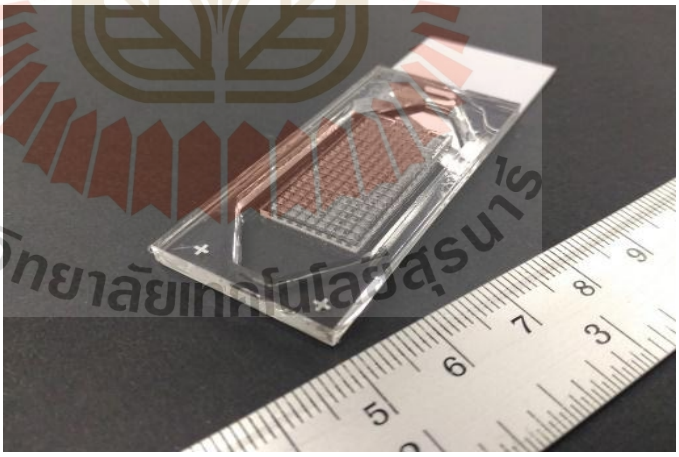

Figure 2.6 An electrostatic microwell-based biochip for phytoplanktonic cell trapping (Kuntanawat et al., 2014).

One example of an application for a microfluidic device is used to isolate and culture single-cells for different microalgae. Kuntanawat et al (2014) were

successfully fabricated a microwell-based-biochip device for trapping and culturing a single-cell microalga. They used an electrostatic force for trapping microalgae. The benefit of using this device is portability and simplicity (Kuntanawat et al., 2014).

2.8 Microwell-based microfluidic device

A microwell-based microfluidic is a combination of a microwell with microfluidic that is often used to isolate a single cell by using gravity force. Microfluidics provides smaller sample consumption, size, high throughput, and faster analysis due to their small size and can reduce the time handling. Subsequently, microwells are fabricated from an array of wells that function for separate single cells without external force and isolated by gravity force. The principle of the microwell mechanism is cells are trapped into a vessel is called well by gravity force (Charnley, Textor, Khademhosseini, & Lutolf, 2009). Microwell offers simplicity, reduces sample and reagent consumption and is also cost-effective for the development of single-cell analytical technique (Galler, Bräutigam, Große, Popp, & Neugebauer, 2014). The material to fabricate microwells is commonly similar to the microfluidic device. In 1950 microwell was patterned on PMMA (polymethyl methacrylate) with a loop array on the tray that has a 25 µL of volume capacity (Figueroa, Cooksey, Votaw, Horowitz, & Folch, 2010). Therefore, the fabrication techniques are more advanced since then and this made the microwell a versatile tool. The different shapes, sizes, usage, and throughput are evolved in microwell fabrication (Manzoor, Romita, & Hwang, 2021).

To fabricate a microwell device, some parameters are required to be optimized to obtain the maximum single-cell capture efficiency, especially the well geometry is crucial factor for determining microwell's application. The well geometry of microwell including size and shape. To obtain the optimal condition for microwell size, the height of the microwell should have an equal of cell's size to prevent the shear stress, cells washed out, and move to neighbor well. The width of microwell size also should be large enough and the ideal size is greater than 10 µm of the cell's size. If the width is larger than that it resulted in multiple cells trapped into one well (Rettig & Folch, 2005).

Microwell shape is important to be optimized when fabricating a microwell device. Some shapes are used to design for different research purposes. Microwell shapes including squares, circles, triangles, rectangles, or spindles are depicted in figure 2 (Ochsner et al., 2007). These shapes are widely used for trapping single cells. Furthermore, microwells also can be used to facilitate long-term cell culture by considering the aspect ratio of the diameter and depth of the device. Isolating individual cell and let cells proliferate can be done in one device by combining chip-based methods and microwell technology (Lindström et al., 2009).

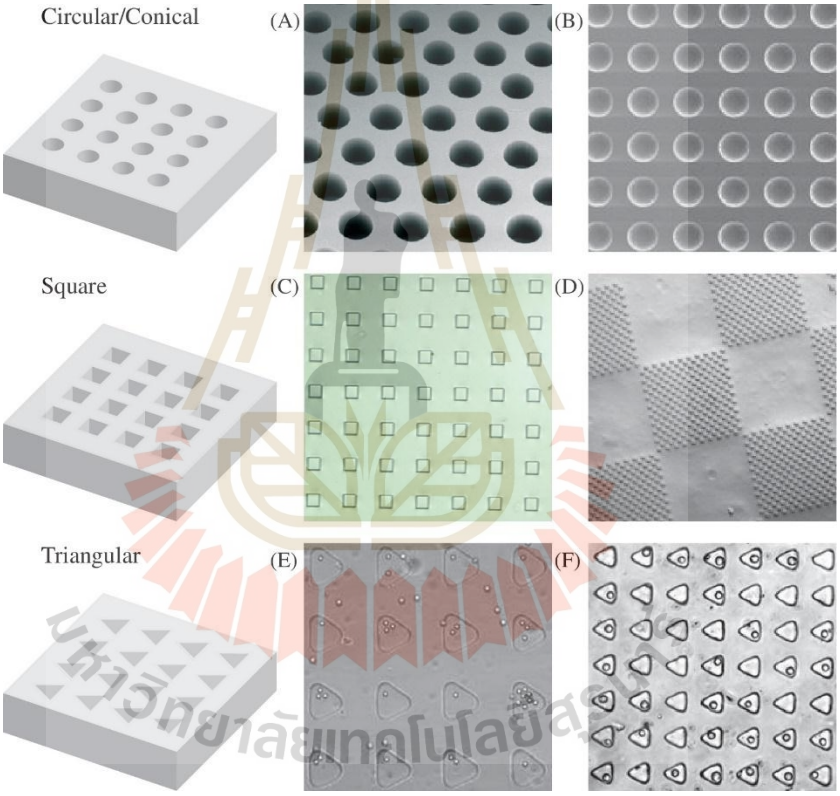

Figure 2.7 A various shape of microwell device and fabrication methods: A, Circular microwell shape fabricated by soft lithography. B, Conical microwell shape fabricated by ion etching. C,D Square microwells shape fabricated by soft lithography, E,F, triangle microwell shape fabricated by soft lithography (Manzoor et al., 2021).

2.9 Microwell fabrication techniques

A microengineering method is have been used to offer low cost and time saving for fabricating microwell devices that can be useful in cell-based drug screening applications. The microwell fabrication methods are laser, direct printing, and photolithography-based microwell.

The laser fabrication method is an alternative method to pattern a microwell using material of polymers such as PMMA and glass-based devices and cuts with CO_2 laser system (C.-H. Yeh, Lin, & Lin, 2009). The CO_2 laser could make thermal depolymerization and melt, and the melting in polymeric material proceeds in the conical formation (Manzoor et al., 2021). Laser systems use the integration of a galvo-scanning system and computer-aided design (CAD) software to cut the desired design on PMMA materials. This system can generate 2-D dimension patterns with different depths and widths in each layer. Using different patterns can be done by setting up the laser machine parameters (Wlodarczyk et al., 2018). The benefits of using this method for fabricating microwell devices are cost-effective, simplicity, and timesaving compared with other fabrication methods.

Moreover, direct printing can be used as an alternative technique for lithography. This technique offers a mask-free material patterning approach to make concave microstructures like microwells. Direct printing also can serve as an etching method by selectively removing the deposited materials through evaporation of the printed solvents ink. However, the limitation of this technique is less printing precision. The tiny size of microwells only affords within the sub-micrometer range (Manzoor et al., 2021).

Photolithography-based microwell is a common method for patterning microwell devices using different materials. The first microwell device was fabricated using this method was applied to fabricate poly (ethylene glycol) PEG-based microwells to create miniaturized cell culture wells with dimensions range from ten to hundreds of microns (Bernard, Lin, & Anseth, 2012). This method offers great control over shape, size, and dimension into microns. However, the lack benefit of using this method is the low production rate (Manzoor et al., 2021).

2.10 The principle of cell trapping using microwell device

Trapping a single cell using a microwell device is based on gravity forces and cell sedimentation where cells are seed and let to settle down into the device. By placing cells on an array of wells and let them be trapped by gravity force. There is no external force to trap an individual cell using a microwell device and this could be a benefit to prevent cell damage from external force, such as shear stress. This mechanism is also widely used over other cell trapping mechanisms due to being simple, easy to implement, less analyzing time, and cost-effective for production.

To obtain a high single-cell trap efficiency, cell seeding, and time settling are needed to be optimized. Cell seeding is a simple process where cells are introduced into the device with different initial cell seeding concentrations. Cell seeding concentration can be controlled by the initial cell seeding concentration and microwell geometry. Then, the cell will be settled, and this cell sedimentation could be improved by using the combination of wiping approach. After cells are introduced into the device, cells will be allowed to settle in a period then the remaining cells will be wiped out by pipetting out (Revzin, Sekine, Sin, Tompkins, & Toner, 2005). Settle times for cells are different and based on well and cell size, the deeper wells will need longer settle times (Cappello et al., 2016). In addition, the function of wiping out of cells remaining in the device could be beneficial for removing cells that stay on the top surface (L. Kang, M. J. Hancock, M. D. Brigham, & A. Khademhosseini, 2010).

2.11 Static microwell arrays

The static microwell is used for passive microstructure for single-cell trapping. This microstructure is based on the size cell between microwell. Using static microwell arrays is requiring no external tool and is straightforward and could minimizing external forces on the cell to reduce shear stress since gravity sediments let cells trapped into the wells. Cell seeding in static microwell is easy process and this technique is based on the cell sedimentation and further improved by combining with wiping method to obtain individual cell in one well. A suspension cell is loaded into the wells and subsequently wait for cell sediment from 5 minutes up to 2 hours. A round repetition is required to maximize percentage of single cell

found (Revzin et al., 2005). The duration of settling time is depends on the well and cell's size. The deeper wells need a longer settling times. This is one of the crucial factors for optimizing to achieve the maximum well filled with individual cell (Cappello et al., 2016). Wiping out the excess cell suspension and medium is used to remove the un-trapped cell in the wells. This process results in an balance amount of solution remaining in each well (Jackman, Duffy, Ostuni, Willmore, & Whitesides, 1998). The benefit by wiping out is that cells are directly placed in the wells, and they do not remain on the top surface of the well (Lifeng Kang, Matthew J. Hancock, Mark D. Brigham, & Ali Khademhosseini, 2010).

2.12 Single-cell isolation techniques

Single-cell isolation technique commonly achieved by using limiting dilution as conventional method or fluorescence-activated cell sorting (FACS) as a high-throughput method. Limiting dilution technique is a popular method for isolating single-cell because it only requires a pipette and tissue culture plates. The principle of this technique is using a serial dilution where cell suspension is serially diluted to an appropriate cell concentration and then pipetting into 96 well plates using a microchannel pipette. Moreover, the Poisson distribution is also involved with the single-cell events in well plates. The percentage of single-cell found using limiting dilution technique is restricted by Poisson distribution itself. Then, this technique is low-throughput, time-consuming, and laborious (Lin, Chang, & Hsu, 2016). Subsequently, a high-throughput such as fluorescence-activated cell sorting (FACS) is emerging to overcome the limitation of limiting dilution technique. Using FACS will allows the purity and accuracy of single-cell that placing in culture well at a time (Leong, Wang, Johnson, & Gao, 2008). However, the drawback of using FACS for isolating single-cell is because the high mechanical shear stress will affect to the death cells and the cost is high so that not many laboratories could afford it (Shapiro, Biezuner, & Linnarsson, 2013).

To overcome the above drawbacks, microwell-based microfluidic devices have been emerged and developed to isolate single-cell (Rettig & Folch, 2005). The well's diameter is crucial for obtaining high single-cell isolation. The well's diameter and

cell's diameter are usually equal due to obtaining high single-cell found. This results in less adequate space for the trapped cells to grow in the device. Larger microwells (90 – 650 μm in diameter) are required for cell culture assay. However, when the diameter well's size is increased, the single-cell found will be decreased due to Poison distribution similar to limiting dilution technique, ranging from 10-30% (Lindström et al., 2009). The advantage of using this technique is simplicity, which required low reagent and media consumption. Due to their efficiency for single-cell isolation, this technique could be useful for long-term cell culture experiments for cell heterogeneity studies and cell line development for monoclonal production (Lin et al., 2016).

2.13 Heterogeneity of single cells

Heterogeneity of single cells meaning that the diversity of single cell from each cells in many aspects such as molecular actions and signaling in a bulk pool populations (Raser & O'Shea, 2005). This can be conceived by variations in drug efficacy, cell cycle phase, cell division, phenotype, and gene expression (Teruel & Meyer, 2002). The diversity of single cells could lead many reasons why single cell responds differently to a particular stimulus. Single-cell analysis could answer those questions and assist in better understanding of the molecular machinery of a cell in a complex environment. Using single-cell analysis allows dynamic information of individual cells, such as differences in cell growth, drug response, and cell productivity. Moreover, the aim of single-cell biology is to gain information and better understanding at level of single-cell.

A common restriction for single-cell analysis is that cells require interaction and signaling between each other cells to function properly and without this condition, cells will change their normal function such as low viability. In a study of cell-to-cell communication showed that monitoring signaling between some single cells is attractive. Using bulk pool for study cell-to-cell communication is challenging and almost impossible due to their heterogeneity. In addition, an average response from bulk pool can hide data and could be misleading. To circumvent this issues, single cell technology is well suited to control single-cell and their behavior that

they offer. Thus, the benefits of single-cell analysis are crucial especially for the drug discovery such as monoclonal antibodies production (Lindström & Andersson-Svahn, 2011).

2.14 References

Andersen, D. C., & Reilly, D. E. (2004). Production technologies for monoclonal antibodies and their fragments. Curr Opin Biotechnol, 15(5), 456-462. doi:10.1016/j.copbio.2004.08.002.

Barnes, L. M., Moy, N., & Dickson, A. J. (2006). Phenotypic variation during cloning procedures: Analysis of the growth behavior of clonal cell lines. Biotechnology and Bioengineering, 94(3), 530-537. doi:https://doi.org/10.1002/bit.20856.

Bernard, A. B., Lin, C.-C., & Anseth, K. S. (2012). A microwell cell culture platform for the aggregation of pancreatic β-cells. Tissue engineering. Part C, Methods, 18(8), 583-592. doi:10.1089/ten.TEC.2011.0504.

Browne, S. M., & Al-Rubeai, M. (2007). Selection methods for high-producing mammalian cell lines. Trends Biotechnol, 25(9), 425-432. doi:10.1016/j.tibtech.2007.07.002.

Cacciatore, J. J., Chasin, L. A., & Leonard, E. F. (2010). Gene amplification and vector engineering to achieve rapid and high-level therapeutic protein production using the Dhfr-based CHO cell selection system. Biotechnol Adv, 28(6), 673-681. doi:10.1016/j.biotechadv.2010.04.003.

Cappello, V., Marchetti, L., Parlanti, P., Landi, S., Tonazzini, I., Cecchini, M., . . . Gemmi, M. (2016). Ultrastructural Characterization of the Lower Motor System in a Mouse Model of Krabbe Disease. Sci Rep, 6(1), 1. doi:10.1038/s41598-016-0001-8.

Carroll, S., & Al-Rubeai, M. (2004). The selection of high-producing cell lines using flow cytometry and cell sorting. Expert Opinion on Biological Therapy, 4(11), 1821-1829. doi:10.1517/14712598.4.11.1821.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., & Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. Science, 263(5148), 802-805. doi:10.1126/science.8303295.

Charnley, M., Textor, M., Khademhosseini, A., & Lutolf, M. P. (2009). Integration column: microwell arrays for mammalian cell culture. Integr Biol (Camb), 1(11-12), 625-634. doi:10.1039/b918172p.

Cheng, L., Fu, J., Tsukamoto, A., & Hawley, R. G. (1996). Use of green fluorescent protein variants to monitor gene transfer and expression in mammalian cells. Nat Biotechnol, 14(5), 606-609. doi:10.1038/nbt0596-606

Chusainow, J., Yang, Y. S., Yeo, J. H., Toh, P. C., Asvadi, P., Wong, N. S., & Yap, M. G. (2009). A study of monoclonal antibody-producing CHO cell lines: what makes a stable high producer? Biotechnol Bioeng, 102(4), 1182-1196. doi:10.1002/bit.22158.

Cockett, M. I., Bebbington, C. R., & Yarranton, G. T. (1990). High Level Expression of Tissue Inhibitor of Metalloproteinases in Chinese Hamster Ovary Cells Using Glutamine Synthetase Gene Amplification. Bio/Technology, 8(7), 662-667. doi:10.1038/nbt0790-662.

Cooper, W. O., Fava, R. A., Gates, C. A., Cremer, M. A., & Townes, A. S. (1992). Acceleration of onset of collagen-induced arthritis by intra-articular injection of tumour necrosis factor or transforming growth factor-beta. Clinical and experimental immunology, 89(2), 244-250. doi:10.1111/j.1365-2249.1992.tb06939.x.

Cubitt, A. B., Heim, R., Adams, S. R., Boyd, A. E., Gross, L. A., & Tsien, R. Y. (1995). Understanding, improving and using green fluorescent proteins. Trends Biochem Sci, 20(11), 448-455. doi:10.1016/s0968-0004(00)89099-4.

Fan, L., Kadura, I., Krebs, L. E., Hatfield, C. C., Shaw, M. M., & Frye, C. C. (2012). Improving the efficiency of CHO cell line generation using glutamine synthetase gene knockout cells. Biotechnol Bioeng, 109(4), 1007-1015. doi:10.1002/bit.24365.

Figueroa, X. A., Cooksey, G. A., Votaw, S. V., Horowitz, L. F., & Folch, A. (2010). Large-scale investigation of the olfactory receptor space using a microfluidic microwell array. Lab on a chip, 10(9), 1120-1127. doi:10.1039/b920585c.

Galler, K., Bräutigam, K., Große, C., Popp, J., & Neugebauer, U. (2014). Making a big thing of a small cell--recent advances in single cell analysis. Analyst, 139(6), 1237-1273. doi:10.1039/c3an01939j.

Geisse, S. (2009). Reflections on more than 10 years of TGE approaches. Protein Expression and Purification, 64(2), 99-107. doi:https://doi.org/10.1016/j.pep.2008.10.017.

Georgopoulos, S., Plows, D., & Kollias, G. (1996). Transmembrane TNF is sufficient to induce localized tissue toxicity and chronic inflammatory arthritis in transgenic mice. J Inflamm, 46(2), 86-97.

Goeddel DV. Systems for heterologous gene expression. Methods Enzymol. 1990;185:3-7. doi: 10.1016/0076-6879(90)85003-7. PMID: 2116575.

Jackman, R. J., Duffy, D. C., Ostuni, E., Willmore, N. D., & Whitesides, G. M. (1998). Fabricating large arrays of microwells with arbitrary dimensions and filling them using discontinuous dewetting. Anal Chem, 70(11), 2280-2287. doi:10.1021/ac971295a.

Jang, D.-i., Lee, A.-H., Shin, H.-Y., Song, H.-R., Park, J.-H., Kang, T.-B., . . . Yang, S.-H. (2021). The Role of Tumor Necrosis Factor Alpha (TNF-α) in Autoimmune Disease and Current TNF-α Inhibitors in Therapeutics. International Journal of Molecular Sciences, 22(5), 2719. Retrieved from https://www.mdpi.com/1422-0067/22/5/2719.

Jung, Y., Jeong, J. Y., & Chung, B. H. (2008). Recent advances in immobilization methods of antibodies on solid supports. Analyst, 133(6), 697-701. doi:10.1039/b800014.

Kang, L., Hancock, M. J., Brigham, M. D., & Khademhosseini, A. (2010). Cell confinement in patterned nanoliter droplets in a microwell array by wiping. Journal of Biomedical Materials Research Part A, 93A(2), 547-557. doi:https://doi.org/10.1002/jbm.a.32557.

Kelley, B. (2009). Industrialization of mAb production technology: the bioprocessing industry at a crossroads. mAbs, 1(5), 443-452. doi:10.4161/mabs.1.5.9448.

Kim, T. K., & Eberwine, J. H. (2010). Mammalian cell transfection: the present and the future. Analytical and Bioanalytical Chemistry, 397(8), 3173-3178. doi:10.1007/s00216-010-3821-6.

Kingston, R. E., Kaufman, R. J., Bebbington, C. R., & Rolfe, M. R. (2002). Amplification Using CHO Cell Expression Vectors. Current Protocols in Molecular Biology, 60(1), 16.23.11-16.23.13. doi:https://doi.org/10.1002/0471142727.mb1623s60.

Köhler, G., & Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, 256(5517), 495-497. doi:10.1038/256495a0.

Krajewski, W. W., Collins, R., Holmberg-Schiavone, L., Jones, T. A., Karlberg, T., & Mowbray, S. L. (2008). Crystal structures of mammalian glutamine synthetases illustrate substrate-induced conformational changes and provide opportunities for drug and herbicide design. J Mol Biol, 375(1), 217-228. doi:10.1016/j.jmb.2007.10.029.

Kuntanawat, P., Ruenin, J., Phatthanakun, R., Kunhorm, P., Surareungchai, W., Sukprasong, S., & Chomnawang, N. (2014). An electrostatic microwell–based biochip for phytoplanktonic cell trapping. Biomicrofluidics, 8(3), 034108. doi:10.1063/1.4882196.

Le, H., Vishwanathan, N., Jacob, N. M., Gadgil, M., & Hu, W. S. (2015). Cell line development for biomanufacturing processes: recent advances and an outlook. Biotechnol Lett, 37(8), 1553-1564. doi:10.1007/s10529-015-1843-z.

Leong, K. G., Wang, B. E., Johnson, L., & Gao, W. Q. (2008). Generation of a prostate from a single adult stem cell. Nature, 456(7223), 804-808. doi:10.1038/nature07427.

Lie-Venema, H., Hakvoort, T. B. M., van Hemert, F. J., Moorman, A. F. M., & Lamers, W. H. (1998). Regulation of the Spatiotemporal Pattern of Expression of the Glutamine Synthetase Gene. In K. Moldave (Ed.), Progress in Nucleic Acid Research and Molecular Biology (Vol. 61, pp. 243-308): Academic Press.

Lin, C.-H., Chang, H.-C., & Hsu, C.-H. (2016). A Microfluidic Platform for High-throughput Single-cell Isolation and Culture. Journal of visualized experiments : JoVE(112), 54105. doi:10.3791/54105.

Lindström, S., Eriksson, M., Vazin, T., Sandberg, J., Lundeberg, J., Frisén, J., & Andersson-Svahn, H. (2009). High-Density Microwell Chip for Culture and Analysis of Stem Cells. PLOS ONE, 4(9), e6997. doi:10.1371/journal.pone.0006997.

Little, M., Kipriyanov, S. M., Le Gall, F., & Moldenhauer, G. (2000). Of mice and men: hybridoma and recombinant antibodies. Immunol Today, 21(8), 364-370. doi:10.1016/s0167-5699(00)01668-6.

Manzoor, A. A., Romita, L., & Hwang, D. K. (2021). A review on microwell and microfluidic geometric array fabrication techniques and its potential applications in cellular studies. The Canadian Journal of Chemical Engineering, 99(1), 61-96. doi:https://doi.org/10.1002/cjce.23875.

Nelson, A. L., Dhimolea, E., & Reichert, J. M. (2010). Development trends for human monoclonal antibody therapeutics. Nat Rev Drug Discov, 9(10), 767-774. doi:10.1038/nrd3229.

Ochsner, M., Dusseiller, M. R., Grandin, H. M., Luna-Morris, S., Textor, M., Vogel, V., & Smith, M. L. (2007). Micro-well arrays for 3D shape control and high resolution analysis of single cells. Lab on a chip, 7(8), 1074-1077. doi:10.1039/b704449f.

Omasa, T., Onitsuka, M., & Kim, W. D. (2010). Cell engineering and cultivation of chinese hamster ovary (CHO) cells. Curr Pharm Biotechnol, 11(3), 233-240. doi:10.2174/138920110791111960.

Orellana, C. A., Marcellin, E., Schulz, B. L., Nouwens, A. S., Gray, P. P., & Nielsen, L. K. (2015). High-antibody-producing Chinese hamster ovary cells up-regulate intracellular protein transport and glutathione synthesis. J Proteome Res, 14(2), 609-618. doi:10.1021/pr501027c.

Park, T. H., & Shuler, M. L. (2003). Integration of cell culture and microfabrication technology. Biotechnol Prog, 19(2), 243-253. doi:10.1021/bp020143k.

Puck, T. T. (1957). The genetics of somatic mammalian cells. Adv Biol Med Phys, 5, 75-101. doi:10.1016/b978-1-4832-3111-2.50006-7.

Purich, D. L. (1998). Advances in the enzymology of glutamine synthesis. Adv Enzymol Relat Areas Mol Biol, 72, 9-42. doi:10.1002/9780470123188.ch2.

Radeke, H., & Boehncke, W. H. (2007). Biologics in General Medicine.

Raser, J. M., & O'Shea, E. K. (2005). Noise in gene expression: origins, consequences, and control. Science, 309(5743), 2010-2013. doi:10.1126/science.1105891.

Rettig, J. R., & Folch, A. (2005). Large-scale single-cell trapping and imaging using microwell arrays. Anal Chem, 77(17), 5628-5634. doi:10.1021/ac0505977.

Revzin, A., Sekine, K., Sin, A., Tompkins, R. G., & Toner, M. (2005). Development of a microfabricated cytometry platform for characterization and sorting of individual leukocytes. Lab on a chip, 5(1), 30-37. doi:10.1039/b405557h.

Ribatti, D. (2014). From the discovery of monoclonal antibodies to their therapeutic application: an historical reappraisal. Immunol Lett, 161(1), 96-99. doi:10.1016/j.imlet.2014.05.010.

Rita Costa, A., Elisa Rodrigues, M., Henriques, M., Azeredo, J., & Oliveira, R. (2010). Guidelines to cell engineering for monoclonal antibody production. Eur J Pharm Biopharm, 74(2), 127-138. doi:10.1016/j.ejpb.2009.10.002.

Saeed, A. F. U. H., Wang, R., Ling, S., & Wang, S. (2017). Antibody Engineering for Pursuing a Healthier Future. Frontiers in Microbiology, 8(495). doi:10.3389/fmicb.2017.00495.

Sanders, P. G., & Wilson, R. H. (1984). Amplification and cloning of the Chinese hamster glutamine synthetase gene. The EMBO Journal, 65-71.

Shapiro, E., Biezuner, T., & Linnarsson, S. (2013). Single-cell sequencing-based technologies will revolutionize whole-organism science. Nat Rev Genet, 14(9), 618-630. doi:10.1038/nrg3542.

Teruel, M. N., & Meyer, T. (2002). Parallel single-cell monitoring of receptor-triggered membrane translocation of a calcium-sensing protein module. Science, 295(5561), 1910-1912. doi:10.1126/science.1065028.

Tracey, D., Klareskog, L., Sasso, E. H., Salfeld, J. G., & Tak, P. P. (2008). Tumor necrosis factor antagonist mechanisms of action: a comprehensive review. Pharmacol Ther, 117(2), 244-279. doi:10.1016/j.pharmthera.2007.10.001.

Walsh, G. (2014). Biopharmaceutical benchmarks 2014. Nat Biotechnol, 32(10), 992-1000. doi:10.1038/nbt.3040.

Werner, R. G., Noé, W., Kopp, K., & Schlüter, M. (1998). Appropriate mammalian expression systems for biopharmaceuticals. Arzneimittelforschung, 48(8), 870-880.

Whitesides, G. M. (2006). The origins and the future of microfluidics. Nature, 442(7101), 368-373. doi:10.1038/nature05058.

Wlodarczyk, K. L., Carter, R. M., Jahanbakhsh, A., Lopes, A. A., Mackenzie, M. D., Maier, R. R. J., . . . Maroto-Valer, M. M. (2018). Rapid Laser Manufacturing of Microfluidic Devices from Glass Substrates. Micromachines, 9(8), 409. doi:10.3390/mi9080409.

Wu, M.-H., & Whitesides, G. M. (2001). Fabrication of arrays of two-dimensional micropatterns using microspheres as lenses for projection photolithography. Applied Physics Letters, 78, 2273-2275.

Wurm, F. M. (2004). Production of recombinant protein therapeutics in cultivated mammalian cells. Nature Biotechnology, 22(11), 1393-1398. doi:10.1038/nbt1026.

Wurm, F. M. (2013). CHO Quasispecies-Implications for Manufacturing Processes. Processes, 1(3), 296-311. Retrieved from https://www.mdpi.com/2227-9717/1/3/296.

CHAPTER III

DEVELOPMENT OF STABLE CELL LINE FOR PRODUCING MONOCLONAL ANTIBODY USING GS-KO CHO CELLS WITH GS AND DHFR SYSTEMS

3.1 Abstract

Chinese Hamster Ovary (CHO) cells are generally used as host cells for the production of biopharmaceutical drugs, particularly monoclonal antibodies (mAbs). The cell line development process is crucial for selecting stable and high producer cell lines among a large population of heterogeneous population of low-producing and non-producing cells, generated from random integration and gene amplification in transfected cells. For the glutamine synthetase (GS) system, the selection of top-producer cell lines is based on the expression of GS and its inhibitor, methionine sulfoximine (MSX). In addition, the dihydrofolate reductase (DHFR) system used methotrexate (MTX) for selection of stable high-producing cell lines. This study reports an attempt to develop a platform to produce Adalimumab antibody using GS-knockout CHO cell lines with a double selection approach. Two vectors comprising GS system for Adalimumab antibody production, and dihydrofolate reductase (DHFR) system, expressing green fluorescent protein (GFP), a marker for high-producing clones, were co-transfected into GS-KO CHO-S and CHO-K1 cells. Furthermore, transfected cells were selected by removing L-glutamine from the medium. Subsequently, 25 μM of MSX was added to improve selection stringency for the selection of high-producing clones. Moreover, selection by MTX addition was performed after single cell isolation to obtain stable high-producing clone and improve the monoclonality. The results showed that addition of MSX and MTX could significantly accelerate the production of mAbs titer and productivity. The double selection approach by transfecting two expression constructs has been successfully demonstrated for stable cell line establishment.

Keywords: monoclonal antibodies, Chinese hamster ovary (CHO) cells, glutamine synthetase (GS) system, dihydrofolate reductase (DHFR) system.

3.2 Introduction

Biopharmaceutical industry has established complex therapeutic drugs to face the medical needs of patients. The therapeutic drugs that mostly demanded is monoclonal antibodies (mAbs). mAbs are very popular in biopharmaceutical industry due to their higher usage of curing the uncurable diseases such as cancer and auto immune diseases. There were approximately 70 mAbs approved in 2020 with a global value of more than hundreds billion dollars (Ecker, Jones, & Levine, 2015).

mAbs were produced mostly by mammalian cell system, mainly due to their capability to produce active molecules with humanlike post-translational modifications (PTM) such as glycosylation, formation of disulphide bonds and proteolytic processing. These are the features that essential for the biological function of the final mAbs product (Fisher, Mayr, & Roth, 2016). The mammalian cell system that widely used in mAbs production is Chinese Hamster Ovary (CHO) cells

In CHO cell system, they have two systems that have become industrial standard. These systems are dihydrofolate reductase (DHFR) system with methotrexate (MTX) as inhibitor (Page & Sydenham, 1991) and glutamine synthetase (GS) system with methionine sulfoximine (MSX) as inhibitor (Cockett et al., 1990). In DHFR system, lacking *dhfr* gene will allow the selection process by growing the cell lines in the absence of hypoxanthine and thymidine in medium (Ringold et al., 1981). Increasing concentration of MTX will yielding higher productivity by amplification steps. Furthermore, in GS system allows the usage of glutamine synthetase enzyme to catalyze the ammonia and glutamate to generate glutamine. Selection step is carried out by growing cell lines into medium lack of glutamine. To increase the selection stringency, addition of MSX will yielding productive stable cell (Brown, Renner, Field, & Hassell, 1992). The MSX concentration for selection stringency is ranging from 25-50 μM MSX and to increase the amplification is 100–1000 μM (Cockett et al., 1990).

In this research, using two different selection systems that comprise GS and DHFR systems are reported. By using double selection system approach, the stable

mAb-producing cell line could accelerate the mAb titer and productivity. The information from this research will be fruitful for the development of stable mAbs cell line establishment.

3.3 Materials and methods

3.3.1 Host cell line and cell culture

GS-Knockout CHO-K1 and CHO-S cell lines were used as a host cell line for Adalimumab antibody expression. These cell lines were engineered by Witsanu Srila by knocking out the *gs* gene using CRISPR-cas technology. The cells were cultured in HyClone ActiPro medium (Cat: HAD3103701, Marlborough, MA, USA) and CD-CHO medium (#10743029, Waltham, Massachusetts, USA) supplemented with 8mM L-glutamine (# 25030-081) and 0.2% anti-clumping (# 0010057DG) into 50 mL TPP tube (# 1BS-010158-CK) containing 10 mL. The cells were cultured at 37°C with 7% CO_2, 200 RPM in shaker incubator. The passaging cells were performed regularly for maintaining the cell in exponential phase at cell density 3 x 10^5 cells/mL.

3.3.2 Expression vector in this study

In this study, we used two vectors that using one a mammalian vector Freedom™ pCHO_1.0 as a vector backbone (Invitrogen/Thermo Fisher, Waltham, Massachusetts, United States) that contain strong promoter elements for high expression level of one or two subunits. The pWS_AdaliH7HC+L1LC vector was used for expressing Adalimumab antibody under the control of CMV hybrid promoter and phosphoglycerate kinase (PGK) promoter driven glutamine synthetase (GS) gene. Subsequently, the Cloned_EmGFP_into_pCHO1.0 vector was used for expressing EmGFP gene that act as a reporter gene and PGK promoter driven dihydrofolate reductase (DHFR) genes

3.3.3 Transfection of cell line

Prior to transfection, the purified plasmid DNA was linearized with restriction enzyme *NdeI.* The cells were cultured at 2.0 x 10^5 viable cells/mL in 15 mL for 1 µg of pWS_AdaliH7HC+L1LC and 0.5 µg of Cloned_EmGFP_into_pCHO1.0 linearized plasmid transfection. Subsequently, the transfection was carried out via electroporation (SF Cell Line 4D-Nucleofector™ X Kit S) (#V4XC-2032, Lonza, Germany). After 48 hours transfection, the transfected cells were placed under

L-glutamine removal selection until their viability recovered to more than 90% by measuring the cell viability using Luna-II^{TM} automated cell counter (Logos Biosystems, Dongan-gu, Anyang-si, Gyeonggi-do, South Korea) with trypan blue exclusion method (Strober, 2015)

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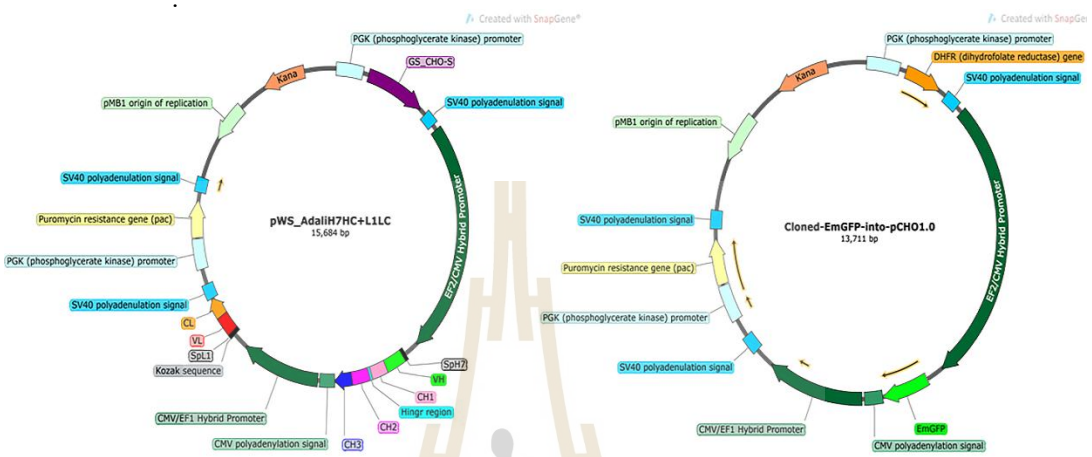


Figure 3.1 The map of expression vectors in this study comprised pWS_AdaliH7HC+L1LC expressing Adalimumab antibody using glutamine synthetase system and Cloned_EmGFP_into_pCHO1.0 expressing EmGFP gene using dihydrofolate reductase system.

3.3.4 Stable pool generation

Afterwards, the transfected stable pools were further subjected for selection in the absence of L-glutamine in the media and further selection stringency by adding 0 and 25 μM MSX (# 15985-39-4, Sigma-Aldrich, Germany) selection. To maintain the viability and cell density for recovering after transfection, changing selection media was performed for once in a week. The cells were resuspended by centrifuged at 1000 RPM for 5 minutes and subsequently discard the supernatant. Then, the cell pellet was resuspended with fresh selection media that contain 0.2% anti-clumping (# 0010057DG), lack L-glutamine, and 25 μM MSX (# 15985-39-4, Sigma-Aldrich, Germany).

3.3.5 Flow cytometry

Flow cytometry was used to check transfection efficiency and to monitor GFP expression of stable cell pool during the selection stringency. A total 2x

10^5 cells were washed in 0.4 mL of sterile PBS. Subsequently, cells were analyzed with 10,000 events were recorded each sample using Invitrogen Attune™ NxT Acoustic Focusing 3-Laser System Cytometer (Thermo Fisher Scientific# P3566, USA) and fluorescence intensity was measured using the BL-1 detector at 530/30.

A viable cell population was gated in accordance with FSC and SSC. Non-transfected cells were used to measure cell auto-fluorescence and used to set a biomarker gate to distinguish between GFP and non-GFP producing cells, so that 99% of cells were in the negative gate in a non-transfected cell sample.

3.3.6 Assess productivity

The indirect Enzyme-Linked Immunosorbent Assay (ELISA) was used to measured antibody titer and specific productivity of stable pools. For the indirect ELISA, 100 µL of Protein A diluted in phosphate-buffer saline (PBS) pH 7.4 was coated onto Maxisorp 96-well plates (Thermo Scientific #44-2404-21, USA) and the incubation was overnight at 4℃. The washing step was performed by 200 µL with 0.05% PBST thrice. Afterward, the wells were blocked with 2% of skim milk (# 1HMD-M530-500G) in PBST (PBS pH 7.4 containing 0.05% Tween 20) (# A4974.0500) in 200 µL and incubated for 1 hour. Then, the plates were washed three times in PBST by hands. Standard Humira and samples were diluted in PBS then added in duplicate to the antigen-coated wells in 100 µL. Subsequently, after incubation for 1 hour the plates were washed three times in PBST. The labelled 2^{nd} antibody Peroxidase AffiniPure F(ab')$_2$ fragment Goat-anti human IgG (H+L) HRP (# JIR-109-036-088, Jackson Immuno Research, West Grove, Pennsylvania, USA) diluted at 1:5000 in blocking buffer were coated into well plates for 100 µL and incubated for 1 hour. Then, washing step was carried out with PBST. Subsequently, 100 µL of ABTS substrate (# A1088.0005) that diluted in Citric acid buffer pH 4.0 with 0.05% H_2O_2 (# C412072) and incubated in dark condition for 30 minutes. Finally, the reaction was stopped with 50 µL of 1% SDS. The optical density (OD) was determined at 405 nm with Sunrise microplate reader (Tecan, Switzerland). Positive clones which showed OD value of at least two times higher than negative control were selected.

3.3.7 Ab titer and cell-specific productivity (q_p)

Cell culture and antibody secreting were assessed to determine high yield and productivity (q_p). The specific antibody productivity was measured in pg

cell^{-1} day $^{-1}$ (pcd). The viable cell density and Ab titer were evaluated and q_p was calculated as previously described (Meleady et al., 2011).

3.3.8 Single-cell isolation and screening process

After measuring the antibody product titer, the stable mini pools were subjected into single cell isolation using limiting dilution. The protocol for limiting dilution was begun with cell preparation and then serially diluted with PBS to the optimal cell concentration of 4.5 cells/mL (Underwood & Bean, 1988). Subsequently, the diluted cell suspension was loaded into 96 well plates (NEST Scientific 96 well cell culture plate, flat, non-treated, sterile) (# NES701011) with 200 µL using multichannel pipette (Gilson Pipetman, L Multichannel, Middleton, USA). Subsequently, the cells were incubated for 14 days to form colonies un-disturb at 37°C with 7% CO_2 static incubator. After cells were grown into colonies on 96-well plates, cells were scaled up to 24-well plates (NEST Scientific 24-well cell culture plate, flat, non-treated, sterile) (# NES702011) by transferring 200 µL cell suspension from 96-well plate onto 24-well plate that contain 800 µL of fresh media. Then incubated for 7 days until the confluency reach 90%. Afterwards, cell supernatants were collected for primary screening. Then, the cell suspensions were scale up to 6-well plate (NEST Scientific 6-well cell culture plate, flat, non-treated, sterile) (# NES703011) by seeding at cell density 1x10^5 cells/mL in 3 mL. Afterwards, cell supernatants were collected to assess secondary screening. After that the cell suspensions were scaled up to T25-flask (Thermo Fisher, CA, USA) with volume of 5 mL. The final scaled up was cell suspensions were scaled onto 50 mL TPP tube (# 1BS-010158-CK).

3.3.9 Selection of stable GFP clones

To obtain stable GFP clones, selection using complete CD CHO medium containing combination of Puromycin and MTX in 6 well plate was performed. The first round of selection was conducted by adding 20 µg/mL of Puromycin and 200 nM MTX. Subsequently, the cells were incubated for 7 days at 37°C, 7% CO_2, in static incubator. Sample cells for fluorescence microscope and flow cytometry to check the selected GFP cells. The confluency was measured under microscope. Media exchange was performed when confluency is <30% once per week. Afterwards, the second selection was conducted by increasing concentration of Puromycin and MTX at 30 µg/mL and 500 nM MTX, and 50 µg/mL and 1000 nM MTX. When the cells

recovered from selection condition, cells were subjected to culture in 100 nM MTX complete CD-CHO medium to maintain the selection phase. Selection is complete when viability and viable cell density exceeds 80% and 1 x 10^6 cells/mL respectively.

3.3.10 Fed-Batch culture

Fed-batch culture were assayed by seeding the cells at 3 x 10^5 cells/mL in 15 mL fresh medium of CD-CHO (#C10743029, Waltham, Massachusetts, USA) and 0.2% anti-clumping (#0010057DG) into 50 mL TPP tube (#1BS-010158-CK) with >90% viability. The cells were incubated for 14 days in 37°C, 7% CO_2, 200 RPM in shaker incubator. Sample cultures daily were collected at regular interval on day 0, 3, 5, 7, 10, 12, and 14 until the cell density, viability, and productivity were drop below <50%. After sampling, the cultures were fed with glucose (Fisher Scientific, #1IVG2-A24940-01, USA) by adding 4 g/L of glucose on day 3 and day 5, and then for day 7 the glucose was added at 6 g/L.

3.3.11 Fluorescence microscopy

Fluorescence and brightfield images were taken using a ZOE™ fluorescent cell imager (Bio-Rad Laboratories Ltd #145-0031, USA). Cells were placed on 96 well plate and observed under the microscope. ZOE™ fluorescent cell imager is an inverted microscope imaging system with brightfield and three fluorescent channels (emitting in blue, green, and red color), incorporating 20X lens. The cells were imaged using green channel with excitation 480/70 nm and emission 517/23 nm.

3.3.12 Confocal laser scanning microscopy

For IF staining, viable cell density 10^7 cells/mL were used. Subsequently, cells were centrifuged (100g, 5 min), washed with PBS two times and resuspended in PBS. Then, cells were fixed with 4% PFA (paraformaldehyde) for 30 minutes at room temperature. Cells were washed by centrifuged (100 g, 5 min). Cells were permeabilized with 0.1% (v/v) Triton X-100 (room temperature, 10 min). Cells were washed by centrifuged (100 g, 5 min). Cells were then blocked with 1% BSA-300 mM glycine-0.1% PBST for 20 minutes. Cells were washed by centrifuged (100 g, 5 min). Cells were incubated with 1:5000 dilution of goat F(ab')^2 anti-human IgG-Fc (DyLight 650) (Abcam #ab98593, UK) in PBS for 1 h. Cells were washed by centrifuged

(100 g, 5 min). Cells were counterstained with 300μM DAPI (4',6-diamidino-2-phenylindole) for 5 min. Cells were washed by centrifuged (100 g, 5 min). Cells were resuspended with gold mountant (Invitrogen#S36936, USA) on a glass slide and cover it. The last step was examined with confocal microscope (Nikon A1 R, Japan).

3.4 Results and discussion

3.4.1 Transfected cells

Using two different type of CHO cell lines comprising GS knockout CHO-K1-P1F3 and CHO-S-P2E5 that referred to double *gs* gene deletion were compared in this study. After 48 hours transfection process, the cells were analyzed using flow cytometry to determine the transfection efficiency. The result show that in Figure 3.2, both cells were had transfection efficiency about 45%. This indicate that one or two plasmids were successfully integrate into cell's genome. The frequency of integration of transgene DNA into chromosomal loci is very low and random event. To accelerate cell line generation and allow for protein production with a stable pool, highly efficient systems for chromosomal integration is required (Büssow, 2015). In this study, we used two different expression system that comprised Glutamine synthetase (GS) system to express Adalimumab antibody and Dihydrofolate reductase (DHFR) system to express GFP. In GS system, transfected cells were selected in a glutamine-free medium. After 48 hours of transfection process, cells were cultured in lack L-glutamine medium to achieve a stable transfected cell. Cells were allowed to proliferate until the viable cell density and viability increased.

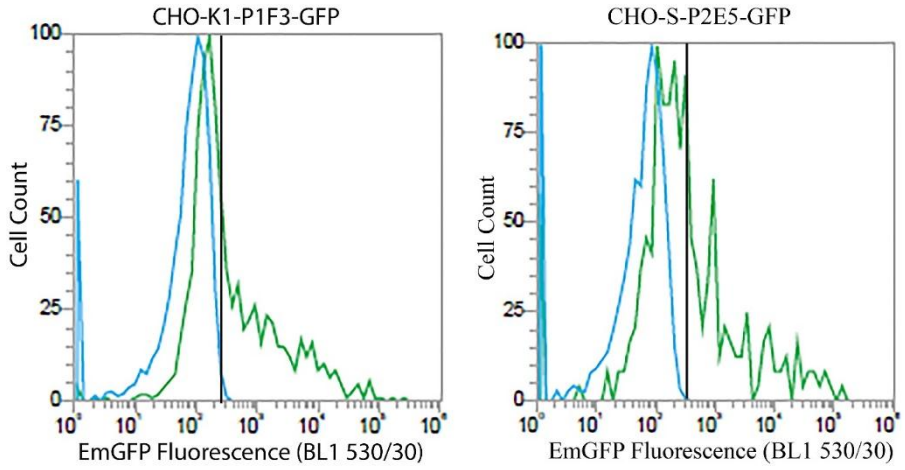


Figure 3.2 Transfection efficiency by flow cytometry analysis of pWS_ AdaliH7HC+ L1LC and Cloned_EmGFP_into_pCHO1.0 vectors in GS-KO- CHO-K1-P1F3 and GS-KO-CHO-S-P2E5 after 48 hours. Blue and green colors represent un-transfected and transfected cells respectively.

3.4.2 Generation of stable pool

Transfected cell lines stably expressing protein of interest are useful tools to facilitate functional studies, especially for developing monoclonal cell lines. After 48 hours of transfection, both transfected cells were cultured in medium without L-glutamine for selection of stable pools. As shown in figure 3.3 GS-KO CHO-K1-EmGFP could survive under selection condition. Viable cell density of GS-KO CHO-K1-EmGFP at day 3 was reached at 10^5 cells/mL and remain stable until day 24. Then, on day 27 and 30 the viable cell density was reached at 10^6 cells/mL. In contrast, GS-KO CHO-S-EmGFP could not survived after changing medium in L-glutamine free condition. This is due to perhaps the plasmid that carry GS system is less integrated into cell's genome. The integration of transgene DNA into host's genome is random event and because we used two plasmids that carry two different expression system, the probability of two plasmids integrate into cell's genome was rare. Recombinant DNA integrates randomly into the genome resulting in clones with variable transgene integration site and copy number in the nucleus (Hamaker & Lee, 2018).

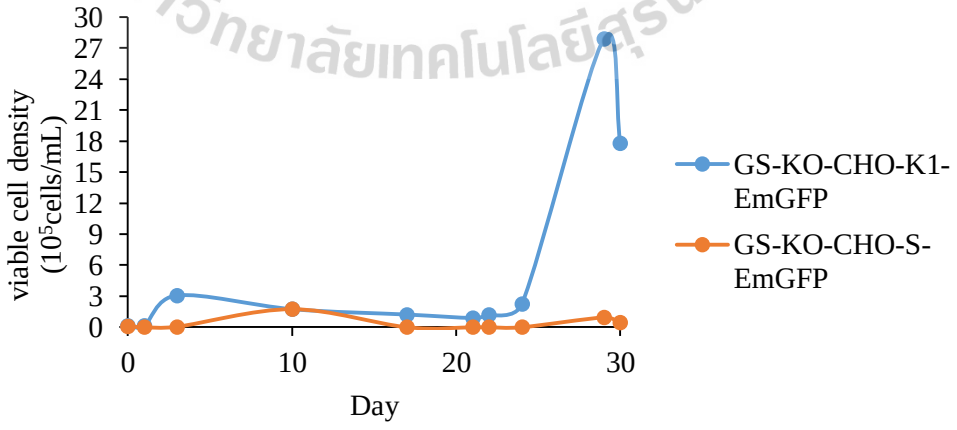


Figure 3.3 Viable cell density of transfected GS-KO CHO-K1-EmEGFP and GS-KO CHO-S-EmGFP cells in L-glutamine free media.

Furthermore, selection stringency was performed by adding 25 μM MSX into the medium lack L-glutamine. MSX is particularly applied during stable pool generation and adding MSX could generate high producing clone and eliminate cells that do not harbor the transgene, therefore stably transfected pools were generated (Fan et al., 2012). Due to GS-KO CHO-S-EmGFP cells could not survive under L-glutamine free media, these cells were eliminated for further experiment

Figure 3.4 showed that the viability of GS-KO CHO-K1-EmGFP with 0 μM MSX is fluctuated. In contrast, after adding 25 μM MSX cells were stable after day 7. Methionine sulfoximine (MSX) is a small molecule compound that act as selection reagent for cell clone generation with glutamine synthetase (GS) expression system (Fan et al., 2012). The range concentration of MSX that usually used is 25-50 μM for selection stringency (Feary, Racher, Young, & Smales, 2017). Previous study has shown that GS-knockout cells could accelerate cell line generation efficiency by removing background GS expression from the GS endogenous *gs* gene (Fan et al., 2012). The result showed that in the absence of MSX culture, the selection stringency was not sufficient for generating stable cell line and addition of MSX was provide sufficient selection stringency in the culture.

3.4.3 GFP mini pool of stable transfected cells

The GFP mini pool of stable transfected cells were measured after culturing cells for 30 days with 0 and 25 μM MSX supplement. In this study, we used flow cytometry to measure GFP intensity for both cell pools. The result showed that depict in figure 3.5, GFP mini pool that treated with 25 μM MSX could increase the GFP signal compared to GFP mini pool that treated without MSX. These results indicates that the MSX addition in the cell culture allows the selection of productive mAb clones. MSX allow select for high producing cell (Tian et al., 2020).

The histogram of MSX and without MSX treatment showed that there were two peaks which indicated there were two populations comprising cell pool that produce antibody and GFP cell pool. As expected, GFP cell pool is more dominant in the addition of MSX because the peak is quite separate from the negative control.

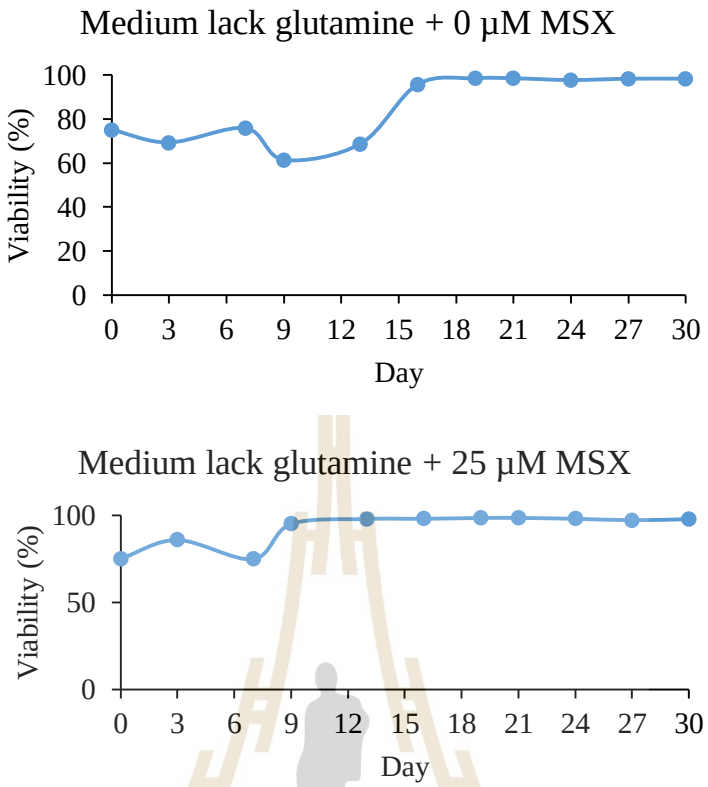


Figure 3.4 Viability of transfected GS-KO CHO-K1-EmEGFP cells in L-glutamine free media with 0 and 25 µM MSX.

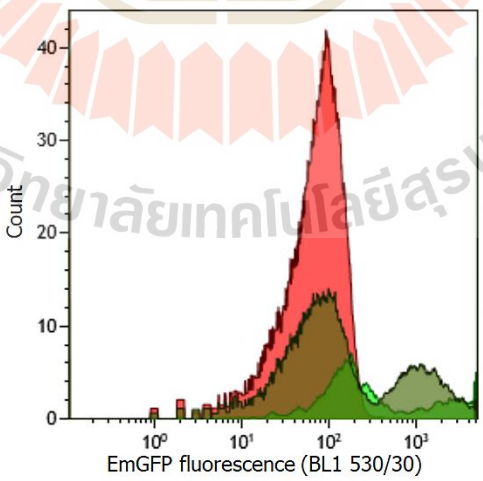


Figure 3.5 GFP expression level in mini pools of stable transfected cell line. The GFP cells were measured after adding 25µM MSX and 0 µM MSX using Flow cytometry. Red, dark green, and bright green colours represent negative control, with MSX, and without MSX, respectively.

3.4.4 Productivity of mini pool culture

The cell specific productivity rate (q_p) is one critical measure in the process of screening high producing clone in cell line generation (Bandyopadhyay, Khetan, Malmberg, Zhou, & Hu, 2017). As expected, in the absence of MSX, mini pools showed little mAb production. The mAb production was significantly increased in the presence of MSX. When the cells were selected from absence MSX media and subsequently cultured in higher MSX media at 25 μM, the protein titer was improved by up to more than 50% (Fan et al., 2012). Based on previous study suggest that increasing the level of MSX at seed train phase could improve production of q_p and overall process yield (Tian et al., 2020). Cell pool then were subjected for single cell isolation. Prior isolate single cell using limiting dilution, cells were subjected onto confocal microscopy to observe the population diversity in the culture.

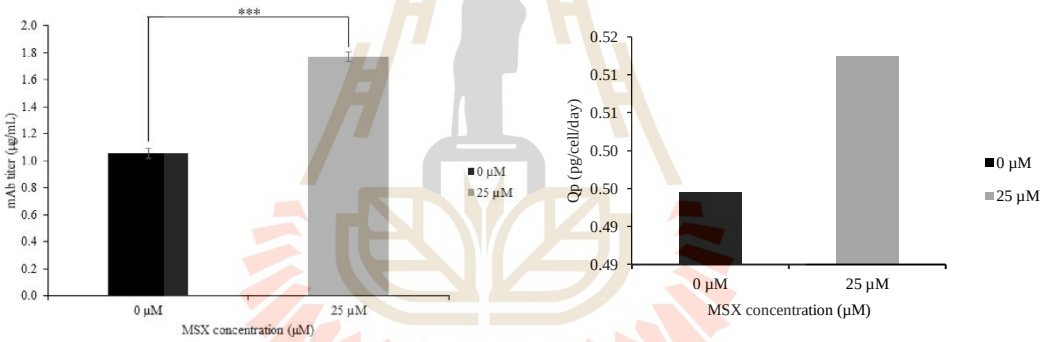


Figure 3.6 mAb concentration and specific productivity (Qp) of the culture supernatant in mini pool at 0 and 25 μM MSX. Error bars represent the standard deviation determined by triplicate experiments. Asterisks (*) indicate a significant difference at P<0.001 using Paired-T test.

3.4.5 Population in mini pool culture using confocal microscopy

Confocal microscopy was conducted to observe population mini pool culture of MSX treatment. In figure 3.7 showed that there were four population in mini-pool culture with MSX addition that consists of no Ab & no GFP, Ab & no GFP, no Ab & GFP, and Ab & GFP cells. This indicates that selection stringency is required to be increased because the GS-KO CHO-K1 cells were still grown in the medium

with 25 μM MSX. Removing background GS expression from the CHO endogenous GS gene could improve selection stringency (Fan et al., 2012).

Glutamine synthetase (GS) in CHO cells provide one way to catalyze the synthesis of glutamine as an essential nutrient (Wurm, 2004). Addition of GS inhibitor such as L-methionine sulfoximine (MSX) into cell culture irreversibly inhibits GS activity and will eventually cause cell death (P.G. Sanders & R.H. Wilson, 1984). GS-CHO selection system is particularly based on the balance of MSX and GS (Cockett et al., 1990). Then, two sources of GS comprised endogenous GS and GS expressed from transfected plasmids will determine the stringency and efficiency of MSX selection (Fan et al., 2012).

In this study, we used two different systems comprising GS and DHFR systems because these systems are widely used for biopharmaceutical company in development of stable cell line. However, the result for cell clone obtained using these systems are highly heterogenous due to random integration by the gene of interests and the gene amplification process (Lai, Yang, & Ng, 2013). The different intensity of GFP and antibody secreting from cells that depicted in Figure 3.7 represents that the heterogenous of random integration. Subsequently, single cell isolation using limiting dilution technique was performed to obtain a high rare producing cell.

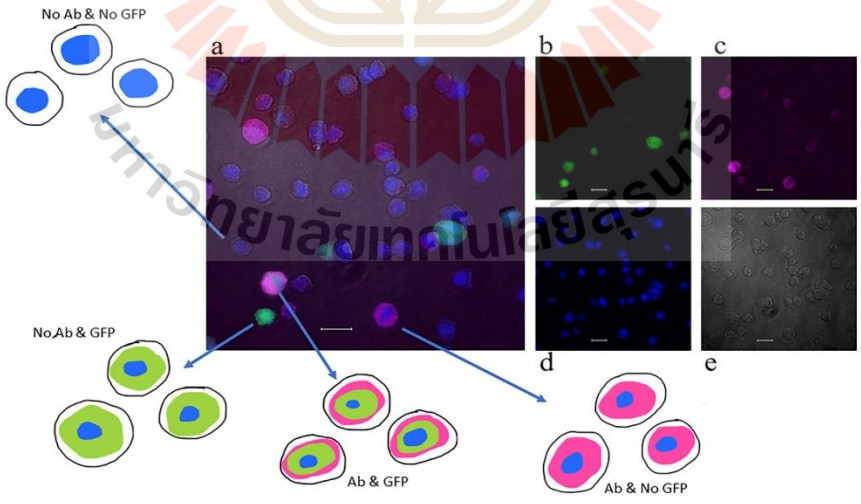


Figure 3.7 Confocal microscopic images of mini-pool culture with 25 μM MSX. DAPI was used for nucleus staining, goat F(ab') 2anti-human IgG-Fc (DyLight 650) (Abcam #ab98593, UK) was used for antibody staining. White scale bar represents 20 μm.

3.4.6 Growth and productivity of single cell clones derived from GFP mini pool

Single cell isolation was conducted using limiting dilution technique. Both cell pools that treated with and without MSX were subjected to limiting dilution technique. A single cell was obtained only from cell pool that treated with 0 μM MSX. There were 2 single cells survived from primary screening, named CHO-K1-F3-GFP-Ab14 and CHO-K1-F3-GFP-Ab15. This caused by media consumption for cell cultivation was using Hyclone ActiPro medium. This medium is specialized to be used for increasing viable cell density and protein production (Mayrhofer, Reinhart, Castan, & Kunert, 2020). In figure 3.8 depicted that clone CHO-K1-F3-GFP-Ab15 showed the highest mAb titer production. Subsequently, to confirm that these clones were derived from single cell, confocal microscopy was performed.

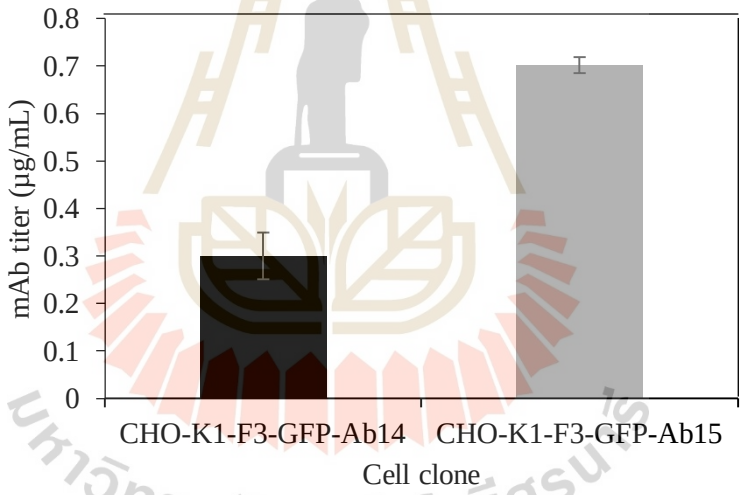


Figure 3.8 A primary screening from limiting dilution. mAb concentration of the culture supernatant of single clone at 0 MSX.

An individual cell of CHO-K1-F3-GFP-Ab14 and CHO-K1-F3-GFP-Ab15 were shown in figure 3.9. It showed that both cell clones have more than one population in each culture. It indicates that both cell clones were not derived from single cell. To circumvent this problem, limiting dilution was performed again to achieve single cell clone using CD-CHO medium. Due to mAb titer and many GFP

cells were found in CHO-K1-F3-GFP-Ab15, then this clone was selected to conduct single cell isolation.

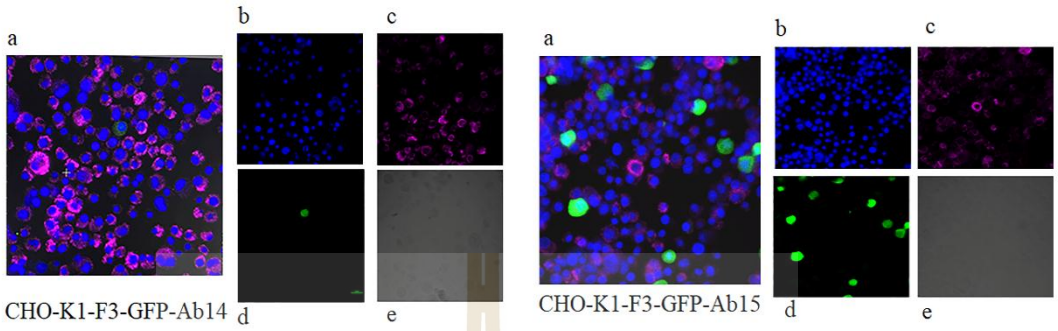


Figure 3.9 Confocal microscopic images of single clones culture with 0 μM MSX from limiting dilution technique. DAPI was used for nucleus staining, goat F(ab') 2anti-human IgG-Fc (DyLight 650) (Abcam #ab98593, UK) was used for antibody staining.

Single cell isolation was conducted using limiting dilution technique to the cloning density at 5 cells/mL and grown in static culture in 96 well plates for three weeks. There were 192 clones were obtained from limiting dilution technique of CHO-K1-F3-GFP-Ab15 cell clone. The 192 cell clones were expanded into 24 well plates, and primary screening were collected after 7 days. Subsequently, the highest cell clone that could produce mAb titer was reached 0.829 μg/mL from primary screening which depicted in figure 3.10. Afterwards, all cell clones were subjected to fluorescence microcopy and flow cytometry to confirm the single cell. The selected clones were then expanded into 6 well plates for secondary screening and further expanded into shaken culture tubes for fed-batch culture for 14 days. Clone scale up and screening process play a crucial role in identifying diverse and predictably productive high cell production in cell line development (Wang et al., 2018).

The results showed in figure 3.11 that among 192 clones there were only two clones that derived from single cell that confirmed from fluorescent microcopy and flow cytometry. The result from fluorescent microscopy showed that green field GFP cells were absence in these two cell clones. In addition, the

histogram result from flow cytometry showed that only one peak exists in for both cell clones and this indicated that cell clones were derived from single cell. However, the results from other cell clones more than hundreds of clones have two population that consists of Ab and GFP clones. Our results indicated that using limiting dilution technique for isolation a single cell is not effective due to their low probability of monoclonality.

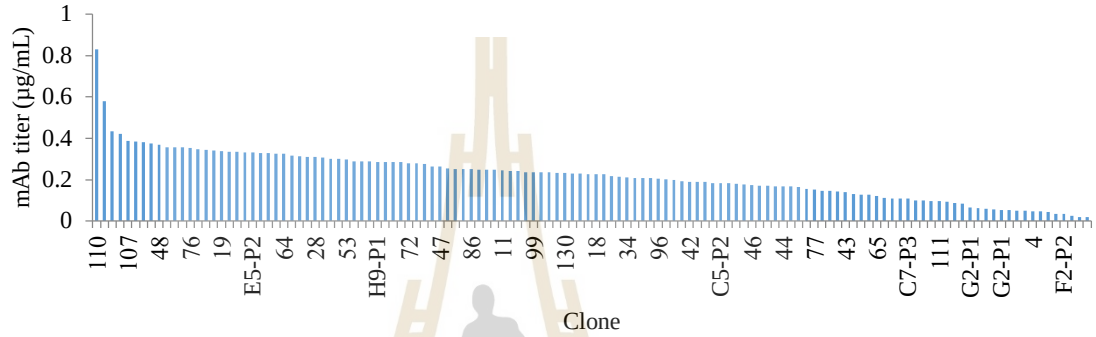


Figure 3.10 A primary screening from limiting dilution. mAb concentration of the culture supernatant of single cell clone derived from CHO-K1-F3-GFP-Ab15.

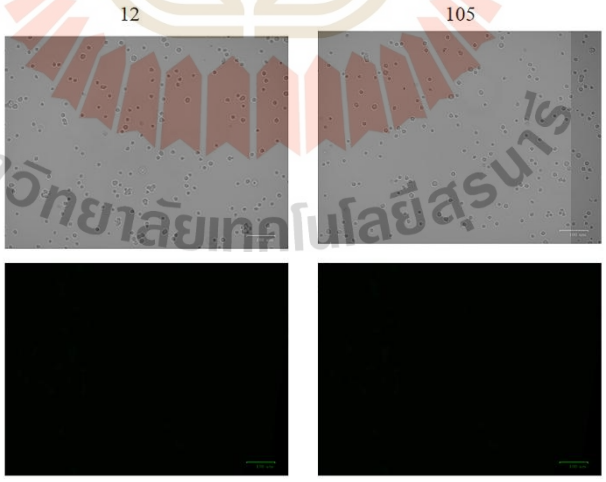


Figure 3.11 Fluorescence microscopic images of brightfield and green field in single cell CHO-K1-F3-Ab12 and CHO-K1-F3-Ab105 performed by a ZOE fluorescent cell imager (Bio-Rad Laboratories, Hercules, CA, USA). White and green scale bar represents 100 µm.

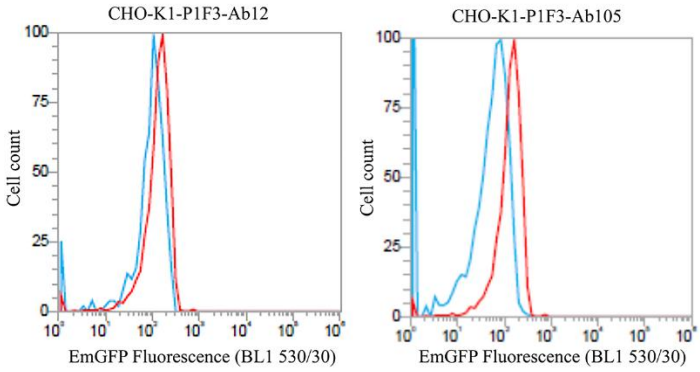


Figure 3.12 Ab expression level in single cell clone of CHO-K1-F3-Ab12 and CHO-K1-F3-Ab105. The Ab cells were measured using Flow cytometry. Red and blue colour represent negative control and sample, respectively.

Meanwhile, these two clones of CHO-K1-F3-Ab12 and CHO-K1-F3-Ab105 were then subjected into clone scale up and screening step. Secondary screening was performed for CHO-K1-F3-Ab12 and CHO-K1-F3-Ab105 in 6 well plates. The mAbs titer and q_p were measured and it shows that mAbs titer and q_p from CHO-K1-F3-Ab12 clone is higher than CHO-K1-F3-Ab105 clone. Furthermore, fed-batch culture was performed only for clone CHO-K1-F3-Ab12.

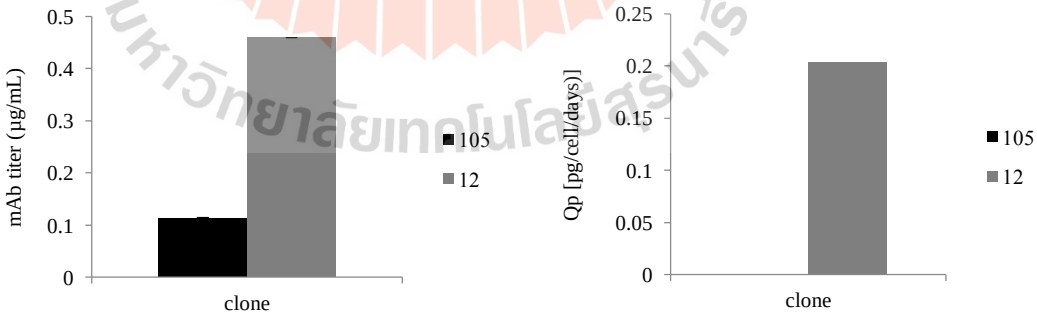


Figure 3.13 The secondary screening of mAb concentration and specific productivity (Qp) of the culture supernatant in single cell clone of CHO-K1-F3-Ab12 and CHO-K1-F3-Ab105.

3.4.7 Fed-batch culture from single cell clones

In each screening step during clone scale up, low producing clones were removed to decrease workload in the subsequent steps. The CHO-K1-F3-Ab12 cell is the top cell lines that was assigned for evaluation in fed-batch cultures. In figure 3.14, showed the maximum mAb titer production could reach 4.83 µg/mL and q_p 0.22 pg/cell/day. This data demonstrated that fed-batch culture was resulting in higher viable cell density and highest overall productivity. Fed-batch culture is particularly the ultimate production process, so prediction of productivity in that process is the most preferable for screening (Wang et al., 2018).

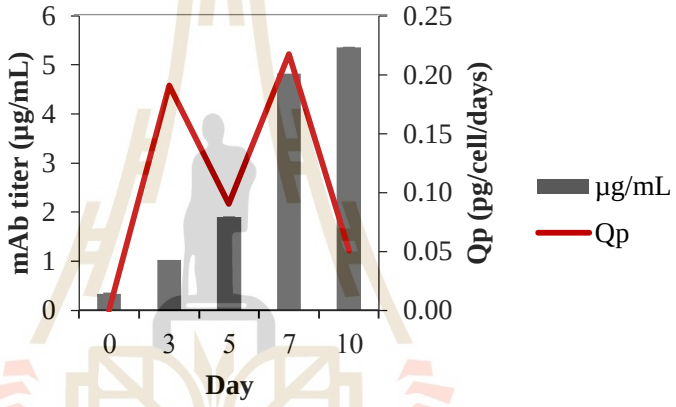


Figure 3.14 Fed-batch analysis of stable single CHO-K1-F3-Ab12 clone derived from CHO-K1-F3-GFP-Ab15. Cells supernatants were collected on day 0, 3, 5, 7, 10. Cells were fed with glucose (v/v) at day 3, 5 with 4 g/L, and at day 7 with 6 g/L. Viable cell density, viability, mAb titers were measured at the time points indicated in the graph and fed-batch cultures were terminated when the viability droped below 50%.

3.4.8 Growth and productivity of single GFP clones

As mentioned previously, there were two population from single cell isolation. GFP and Ab clones were existed that is shown in figure 3.15. There were two peaks from flow cytometry that indicates GFP, and Ab clones were presence. Hereinafter, MTX selection was performed to achieve uniformity of GFP clones. The addition of MTX (methotrexate) in this study is because GFP gene in the DHFR selection system. The dihydrofolate reductase (DHFR) system offered by the use of

dhfr $^{-}$ (deficient) CHO cells are based on DHFR enzyme that catalyze dihydrofolate to tetrahydrofolate. MTX is a selection drug that induces cell death by inhibiting DHFR enzyme. In *dhfr* $^{-}$ CHO cells, that taken up the expression vector containing *dhfr* and gene of interest (GFP) could develop resistance to MTX by amplifying *dhfr* (Urlaub & Chasin, 1980).

All 12 GFP candidates' clones were subjected into 24 well plate with 20 µg/mL Puromycin and 200 nM MTX in complete CD-CHO medium. After 3 days, cells were observed under fluorescence microscope to check the survival of cells. However, among 12 GFP clones there were 5 GFP clones that survived from selection phase that depicted in figure 3.17, cells were analyzed using flow cytometry and the results showed that GFP cells were separated from the negative control.

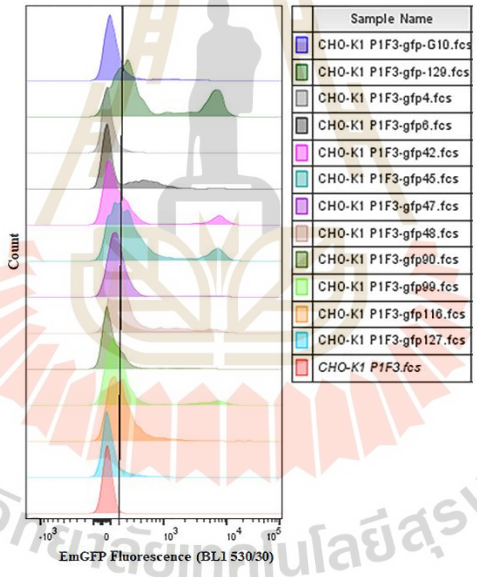


Figure 3.15 GFP and Ab clones derived from single cell isolation of CHO-K1-F3-GFP-Ab15. Flow cytometry was used to analyze GFP and Ab cells. There were 12 GFP and Ab candidate clones.

Further, the concentration of puromycin and MTX were increased to 30 and 50 µg/mL, 500 and 1000 nM respectively. However, increasing concentration of Puromycin and MTX were resulted in cell's stress. Cell size is decreased to less than 10 µm and grow slowly. To overcome this problem, cells from 20 µg/mL Puromycin

and 200 nM MTX were resuspended into 100 nM MTX in complete CD-CHO media. Adding MTX on media is used to maintain the selection stringency (Ng, 2012). After changing the condition, there were two top cells that recovered from selection phase. Cells were then grown in complete media without adding MTX.

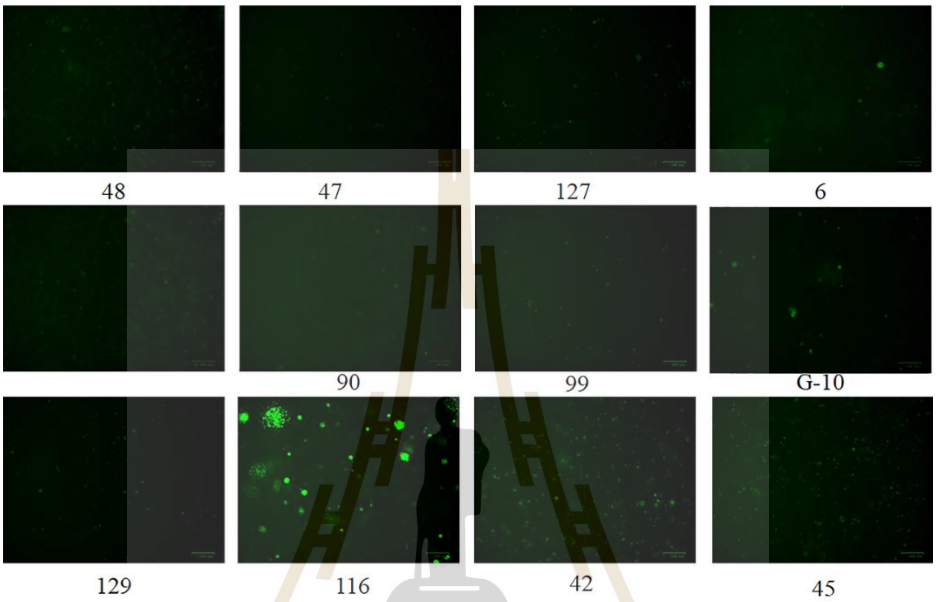


Figure 3.16 GFP candidates from single cell isolation using limiting dilution by fluorescence microscope.

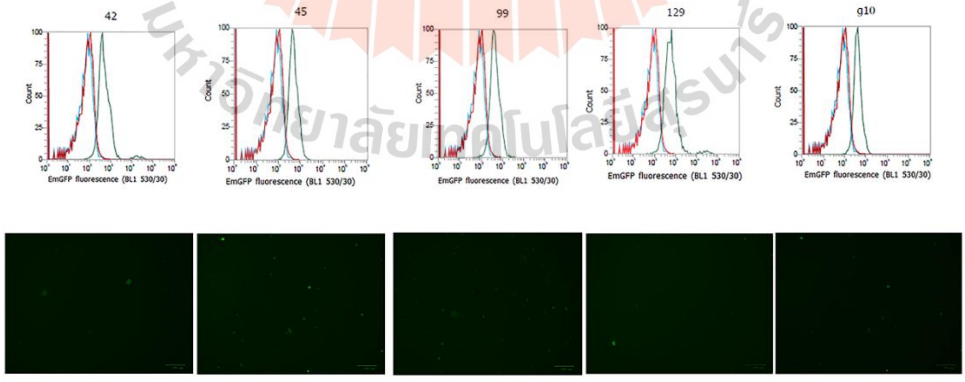


Figure 3.17 Flow cytometry for GFP cells when selected on 20 ug/mL Puromycin and 200 nM MTX.

The top GFP clones were CHO-K1-F3-GFP-Ab42 and CHO-K1-F3-GFP-Ab129. In figure 3.18 in green field of both clones by fluorescence microcopy. In addition, the results from flow cytometry showed that GFP clones were separated from negative control. These results indicated that selection with Puromycin and MTX were successful. Interestingly, these clones also have mAb titer that measured by indirect ELISA. These data suggest that both plasmids that comprises Ab with GS system and GFP with DHFR system were successfully integrated into cell's genome. As previous study reported that double selection approach which simultaneously transfects two plasmids into CHO cells for stable antibody cell line development, cells were selected with two different selective reagents such as MSX and MTX. Our results showed that the mAb titer of clones generated using this approach was higher than that of clones which transfected with one plasmid. Our system is similar with Li, F et all (2010) that used simultaneous transfection with two different expression system.

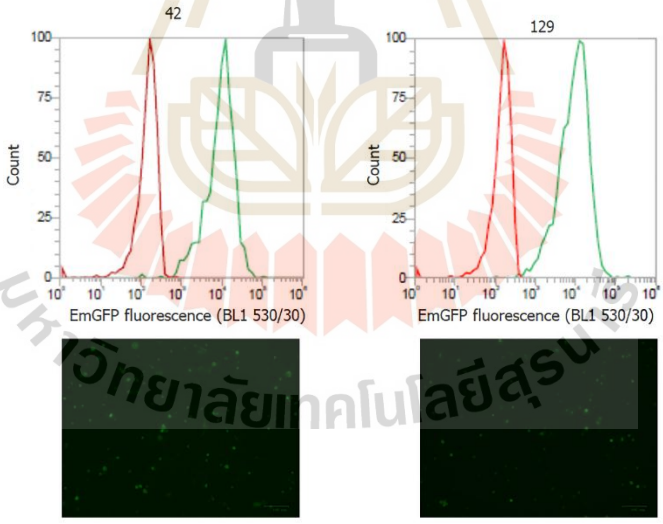


Figure 3.18 Analysis of CHO-K1-F3-GFP-Ab42 and CHO-K1-F3-GFP-Ab129 cells stably expressing EmGFP by fluorescence microscope and flow cytometry. Flow cytometry analyses of CHO-K1-F3-GFP-Ab42 and CHO-K1-F3-GFP-Ab129 (green peaks) compared to untransfected GS-KO-CHO-K1F3 cells (red peaks). Plots show the number of cells against green fluorescence

intensity. Note the higher levels of expression and the narrower peaks detected in cells expressing EmGFP.

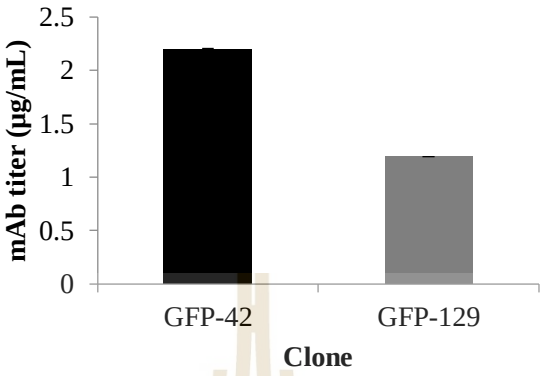


Figure 3.19 mAb titer from CHO-K1-F3-GFP-Ab42 and CHO-K1-F3-GFP-Ab129 cells.

3.4.9 Fed-batch culture from GFP-Ab single cell clones

Fed-batch culture for CHO-K1-F3-GFP-Ab42 and CHO-K1-F3-GFP Ab129 cells were perfomed for 12 days. The results were shown in figure 3.20 that the maximal density of viable cells reached in different day, but still in exponential phase. CHO-K1-F3-GFP-Ab42 clone had the maximal viable cell density on day 5 which 7.92 x 10^6 cells/mL and mAb titer reached 3.7 µg/mL. However, the mAb titer was continuously increased until day 12 that reached 7.2 µg/mL while viable cell density decreased. In contrast for clone CHO-K1-F3-GFP-Ab129 had maximal viable cell density and mAb titer on day 7 that could reach 10.3 x 10^6 cells/mL and 4.6 µg/mL respectively. Then, the mAb titer was increased to 5.9 µg/mL on day 10 then slowly decreased to 4.9 on the last day. The highest cell specific productivity (Qp) was achieved by clone CHO-K1-F3-GFP-Ab42 that reached 0.4 pg/cell/days. This data demonstrated that fed-batch culture was resulting in higher viable cell density and highest overall productivity. Fed-batch culture is particularly the ultimate production process, so prediction of productivity in that process is the most preferable for screening (Wang et al., 2018).

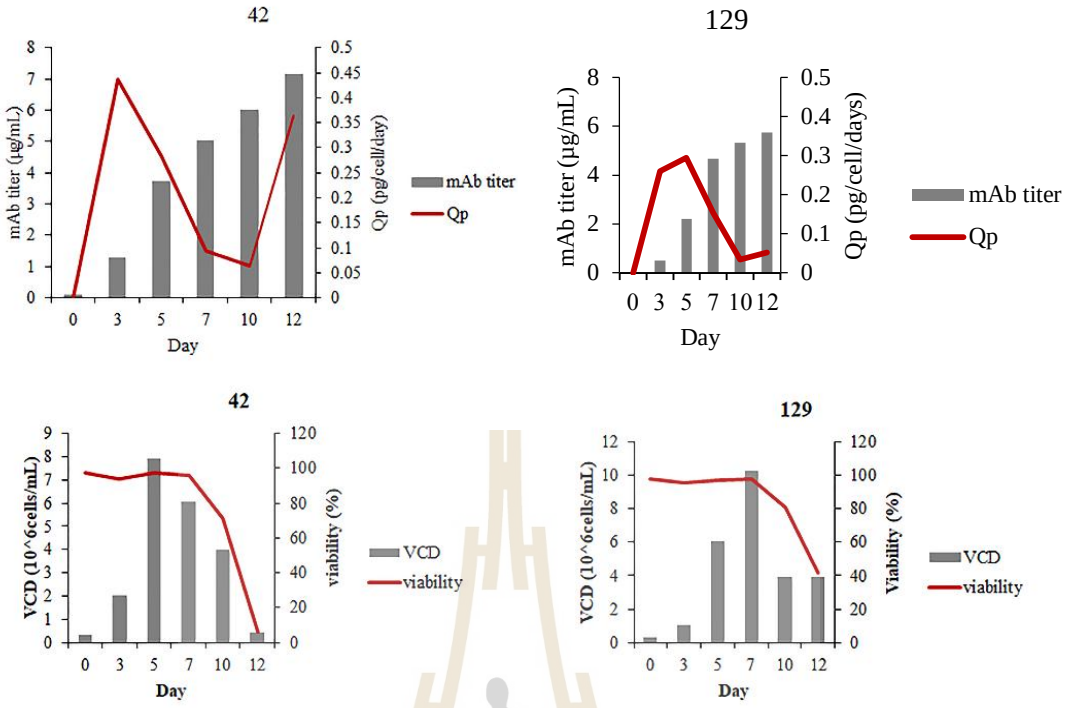


Figure 3.20 Fed-batch analysis of stable single CHO-K1-F3-GFP-Ab42 and CHO-K1-F3-GFP-Ab129 clones derived from CHO-K1-F3-GFP-Ab15 by limiting dilution. Cells supernatants were collected on day 0, 3, 5, 7, 10, 12. Cells were fed with glucose (v/v) at day 3, 5 with 4 g/L, and at day 7 with 6 g/L. Viable cell density, viability, mAb titers were measured at the time points indicated in the graph and fed-batch cultures were terminated when the viability droped below 50%.

To check the stability of GFP cells during fed-batch culture, fluorescence microscope and flow cytometer analysis were performed. The results depicted in figure 3.21 analysis by flow cytometry confirmed that approximately 99% and 95% of the cells expressed GFP for CHO-K1-F3-GFP-Ab42 and CHO-K1-F3-GFP-Ab129, respectively. GFP cells were confirmed by fluorescence microscope during fed batch culture. However, for CHO-K1-F3-GFP-Ab42 clone on day 12 showed that the GFP cells were reduced due to the cell death. These results indicated that GFP cells were stable under fed batch culture.

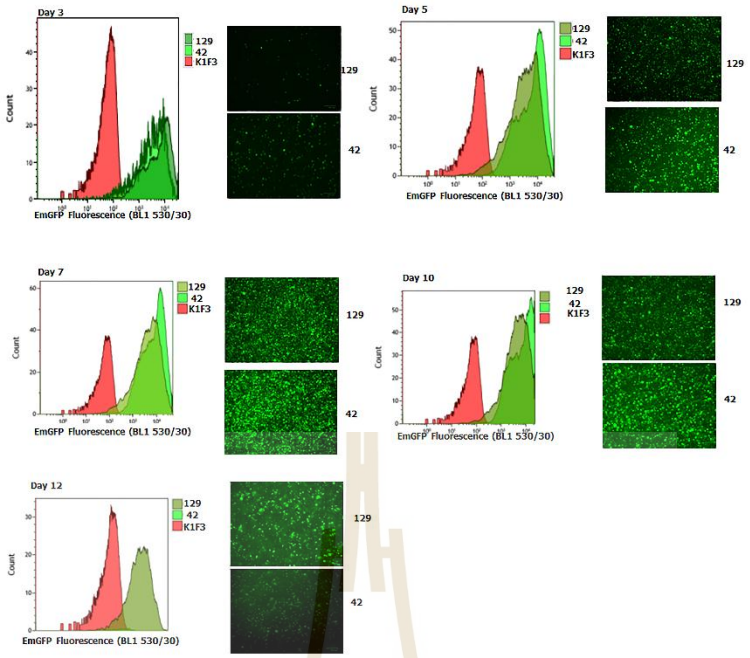


Figure 3.21 Flow cytometry and fluorescence microscopic analysis of fed-batch culture of stable single CHO-K1-F3-GFP-Ab42 and CHO-K1-F3-GFP- Ab129 clones derived from CHO-K1-F3-GFP-Ab15 by limiting dilution. Cells were collected on day 3, 5, 7, 10, 12. Cells were washed and dilute into sterile PBS for flow cytometry analysis. Cells were undiluted and placed on well plate for observed cells under fluorescence microscope.

To confirm that both clones were derived from single cell, confocal microcopy was performed. The confocal microscopic images of GFP and antibody producing clones from fed-batch culture on day 7 were depicted in Figure 3.22. Based on GFP fluorescence in confocal figures, GFP is mostly localized in both nucleus and cytoplasm. Further, the pink fluorescence in confocal figures represent the expression of Adalimumab antibody that is localized in cytoplasm of the cells but in some cells, they were localized in the outer cells. This indicated that the expression of antibody when localized in outer cells were ready to be secreted out. The un-uniformity of cell's size for both clones were due to the different cell's age. Thus, due to cells were collected from day 7 on fed-batch culture and it resulted in different cell's size. However, the majority of cells express both GFP and antibody that indicated cells were derived from single cell. Nevertheless, the intensity of

fluorescence shows variation. Thus might be due to heterogeneity of the clones that resulted from random integration of the transfected gene of interest into cell's genome (Würtele, Little, & Chartrand, 2003). This result also corresponding to previous study that demonstrate genetic heterogeneity may arise from each clone making up a pool which could have a significant fraction of total variation (Pilbrough, Munro, & Gray, 2009). It is hard to achieved homogenous level of protein expression among individual since the chromosomal surrounding employ strong influences on the promoter that result in transfection rate of the gene of interest (West & Fraser, 2005). Since we used two expression systems by the addition of MSX and MTX selection reagent for gene amplification process this might be results in large genomic rearrangements that lead to heterogeneity in protein expression level (Wurm, 2004). Previous study demonstrated that CHO cell exhibits rapid genetic changes from random mutations and genetic drift (Davies et al., 2013).

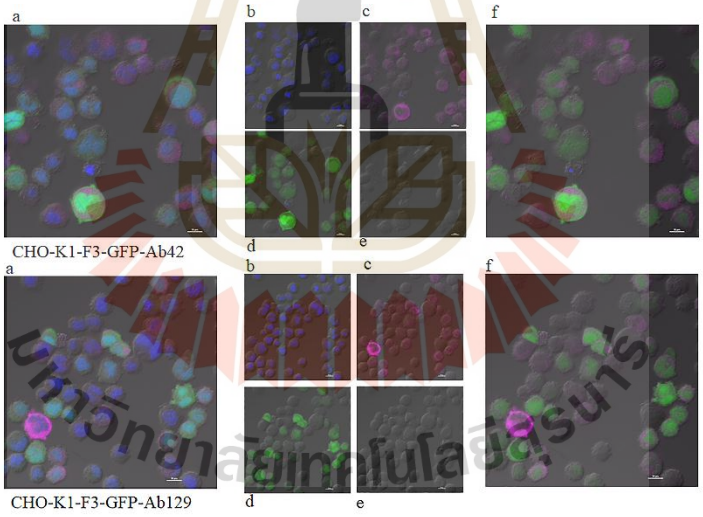


Figure 3.22 Confocal microscopic images of single clones culture from fed batch culture on day 7 of CHO-K1-F3-GFP-Ab242 and CHO-K1-F3-GFP-Ab129 from limiting dilution technique. DAPI was used for nucleus staining, goat F(ab') 2anti-human IgG – Fc (DyLight 650) (Abcam #ab98593, UK) was used for antibody staining. White scale bar represents 10 μm.

GFP gene in this study is used as a reporter gene that could detect the expression of antibody. GFP has widely used for monitoring protein localization in living cells, gene expression and intracellular protein targeting (Misteli & Spector, 1997). In addition, GFP fusion does not need the fixation or cell permeabilization when GFP is fused to Ab (Tsien, 1998). However, a main problem in the establishment of cell line using reporter is to verify the co-relationship between the expression level of the reporter and the antibody titer. The screening system that used intracellular reporter must be evaluated because antibody is generally secreted protein. Previous reports demonstrated that utilizing a GFP as an intracellular reporter proved the mutual relation between productivity of desired protein and fluorescence intensity (Y.-G. Kim et al., 2012). In contrast to our results that GFP fluorescence intensity was not related to the antibody intensity based on confocal microscopy. Cells have strong GFP intensity but low antibody intensity and this might be due to the heterogeneity of CHO cells.

To conclude, we have described the establishment of double selection approach system for stable cell line development. The GS and DHFR systems are used as an alternative approach to identify high producing and stable clones in the cell line development steps. Using the GS-CHO system is used to generate the highly producing clone by utilizing combination of MSX and GS-knockout cell line where the endogenous CHO *gs* gene is removed. Additionally, using DHFR-CHO system is used for expressing GFP gene that act as reporter gene for antibody titer. The results showed that we successfully established double selection system for expressing antibody and GFP cells simultaneously. Then, the long-term stability of this system to determine the association of Ab and GFP expression will be an interesting extension of this study.

3.5 Conclusions

The double selection approach was successfully developed for generating stable high producing cell line. Using this system demonstrated that GFP and Ab cells were established simultaneously. Furthermore, this system has a potential to identify hotspot region in CHO cell's genome for the application of site-specific integration (SSI) in the future.

3.6 References

Bandyopadhyay, A. A., Khetan, A., Malmberg, L. H., Zhou, W., & Hu, W. S. (2017). Advancement in bioprocess technology: parallels between microbial natural products and cell culture biologics. J Ind Microbiol Biotechnol, 44(4-5), 785-797. doi:10.1007/s10295-017-1913-4.

Brown, M. E., Renner, G., Field, R. P., & Hassell, T. (1992). Process development for the production of recombinant antibodies using the glutamine synthetase (GS) system. Cytotechnology, 9(1-3), 231-236. doi:10.1007/bf02521750.

Büssow, K. (2015). Stable mammalian producer cell lines for structural biology. Current Opinion in Structural Biology, 32, 81-90. doi:https://doi.org/10.1016/j.sbi.2015.03.002.

Cockett, M. I., Bebbington, C. R., & Yarranton, G. T. (1990). High Level Expression of Tissue Inhibitor of Metalloproteinases in Chinese Hamster Ovary Cells Using Glutamine Synthetase Gene Amplification. Bio/Technology, 8(7), 662-667. doi:10.1038/nbt0790-662.

Ecker, D. M., Jones, S. D., & Levine, H. L. (2015). The therapeutic monoclonal antibody market. mAbs, 7(1), 9-14. doi:10.4161/19420862.2015.989042.

Fan, L., Kadura, I., Krebs, L. E., Hatfield, C. C., Shaw, M. M., & Frye, C. C. (2012). Improving the efficiency of CHO cell line generation using glutamine synthetase gene knockout cells. Biotechnol Bioeng, 109(4), 1007-1015. doi:10.1002/bit.24365.

Feary, M., Racher, A. J., Young, R. J., & Smales, C. M. (2017). Methionine sulfoximine supplementation enhances productivity in GS-CHOK1SV cell lines through glutathione biosynthesis. Biotechnol Prog, 33(1), 17-25. doi:10.1002/btpr.2372.

Fisher, D. I., Mayr, L. M., & Roth, R. G. (2016). Expression Systems. In R. A. Bradshaw & P. D. Stahl (Eds.), Encyclopedia of Cell Biology (pp. 54-65). Waltham: Academic Press.

Hamaker, N. K., & Lee, K. H. (2018). Site-specific Integration Ushers in a New Era of Precise CHO Cell Line Engineering. Current opinion in chemical engineering, 22, 152-160. doi:10.1016/j.coche.2018.09.011.

Li, F., Vijayasankaran, N., Shen, A. Y., Kiss, R., & Amanullah, A. (2010). Cell culture processes for monoclonal antibody production. mAbs, 2(5), 466-479. doi:10.4161/mabs.2.5.12720.

Ng, S. K. (2012). Generation of high-expressing cells by methotrexate amplification of destabilized dihydrofolate reductase selection marker. Methods Mol Biol, 801, 161-172. doi:10.1007/978-1-61779-352-3_11.

Ringold, G., Dieckmann, B., & Lee, F. (1981). Co-expression and amplification of dihydrofolate reductase cDNA and the Escherichia coli XGPRT gene in Chinese hamster ovary cells. J Mol Appl Genet, 1(3), 165-175.

Sanders, P. G., & Wilson, R. H. (1984). Amplification and cloning of the Chinese hamster glutamine synthetase gene. The EMBO Journal, 65-71.

Strober, W. (2015). Trypan Blue Exclusion Test of Cell Viability. Current Protocols in Immunology, 111(1), A3.B.1-A3.B.3. doi:https://doi.org/10.1002/0471142735.ima03bs111.

Tian, J., He, Q., Oliveira, C., Qian, Y., Egan, S., Xu, J., . . . Li, Z. J. (2020). Increased MSX level improves biological productivity and production stability in multiple recombinant GS CHO cell lines. Engineering in life sciences, 20(3-4), 112-125. doi:10.1002/elsc.201900124.

Underwood, P. A., & Bean, P. A. (1988). Hazards of the limiting-dilution method of cloning hybridomas. J Immunol Methods, 107(1), 119-128. doi:10.1016/0022-1759(88)90017-8.

Wang, B., Albanetti, T., Miro-Quesada, G., Flack, L., Li, L., Klover, J., . . . Hawley-Nelson, P. (2018). High-throughput screening of antibody-expressing CHO clones using an automated shaken deep-well system. Biotechnology Progress, 34(6), 1460-1471. doi:https://doi.org/10.1002/btpr.2721.

Wurm, F. M. (2004). Production of recombinant protein therapeutics in cultivated mammalian cells. Nature Biotechnology, 22(11), 1393-1398. doi:10.1038/nbt1026.

CHAPTER IV

A MICROWELL-BASED MICROFLUIDIC DEVICE FOR ISOLATION OF SINGLE ANTIBODY PRODUCING CHO CELLS APPROACH

4.1 Abstract

Monoclonal antibodies (mAbs) play a crucial role in the biopharmaceuticals due to their ability to cure diseases such as cancer and auto immune diseases. The main hinderance for developing monoclonal cell lines is the single cell isolation technique. Single cell isolation technique that usually used is limiting dilution which time intensive, laborious, and low probability of monoclonality. Further, the high-throughput approaches such as fluorescence activated cell sorting (FACS) is expensive and less accessible to general laboratories. In this study, we report an approach for single cell isolation of Chinese hamster ovary (CHO) cells using an adapted version of a simple microwell-based microfluidic (MBM) device previously reported by our group. Using the electrostatic force, cell suspension was trapped into the device and can be observed under a microscope and transferred using a micropipette for a further clone development. Comparing to the traditional method, using our approach provided a 4.7-fold increase in a number of single cells isolated per round of cell loading and demonstrated a 1.91-fold decrease total performing time. In addition, the percentage of correct single cell assessment was improved significantly, especially in novice testers. Using MBM device could serve a simplicity, cost-effective alternative, efficient, and less skill intensive to the traditional single cell isolation for monoclonal cell lines development.

Keywords: monoclonal antibodies (mAbs), Chinese hamster ovary (CHO) cells, microwell device, microfluidic system, single cell isolation.

4.2 Introduction

Monoclonal antibodies (mAbs) are popular in biopharmaceuticals industry due their advantage to cure various of diseases including cancer, infectious diseases, and auto immune disease (Chung, 2017). mAbs are dominating the demand in pharmaceutical market. Total global sales of mAbs were reached over $200 billion and this will continue to rise in 2024 (Urquhart, 2020).

Generating mAbs could be achieved by traditional hybridoma technology and phage display technology. Using traditional hybridoma technology is dependent on animal immunization and screening of the immortalized (Lu et al., 2020). However, the limitation of this technique is that difficulty of producing a large titer on mAbs (Stapleton, Kennedy, & Tully, 2005). To overcome this problem, phage display is emerged by constructing a library in filamentous bacteria by fusing multiple genes into it (Smith, 1985). In comparation to hybridoma technique, phage display is more expensive and technically more complicated. Further, genetic engineering is represent to fix those limitations by generate mAbs based on molecular biology technology which involves identifying and optimizing construction for a stable high-producing clone (Dalton & Barton, 2014).

Chinese hamster ovary (CHO) cells are widely used as host system for mAbs production because of their adapted system and allow correct post-translational modification of the recombinant protein, proper folding, assembly (Wurm, 2004). Their production mAbs titer could reached around 1 g/L in batch and 1-10 g/L in fed-batch cultures (Kunert & Reinhart, 2016).

mAbs production can be achieved by constructing the plasmid that carry gene of interest and then subjected to transfection process into CHO cells. The plasmid with gene of interest will integrate into CHO genome by random amplification system and this yielding the transfected cells have high diversity of gene complement (S. M. Browne & M. Al-Rubeai, 2007). Due to their heterogenous cell population, it makes difficult to obtain a high expressing cell. Screening and selection process are needed in early stage of mAbs production. Therefore, an efficient single cell isolation method is a main for achieving a high-producing cell clone.

A conventional limiting dilution technique is widely used in single cell isolation process. This technique is based on Poisson distribution that allows obtaining single

cell from diluted cell suspensions by pipetting into 96 well plates. To obtain high number of single cells, diluting cell suspension at density ranging from 0.5 to 0.9 cells per well in well plates (Underwood & Bean, 1988). Unfortunately, due their time consuming, laborious, and less probability of monoclonality this technique is not sufficient for mAbs production. Then, a high-throughput method such as fluorescence activated cell sorting (FACS) could isolate individual cell characteristics based on granularity, size, and fluorescence from cell mixture (DeMaria et al., 2007). However, the main drawback of FACS is that they are expensive, skillful, and cells could be damaged due to the shear stress (Hong & Lukes, 2008).

Nowadays, microfluidic systems have been considered as a valuable device for isolating individual cell. A microfluidic for single cell cloning (SCC) device was fabricated for isolate and cultivate single cell into the device. Their device could be beneficial for high-throughput screening at a single cell level. The percentage of single cell efficiency using this device is significantly higher compared with limiting dilution method. Their device used dual well for single cell trapping into small microwell and then individual cell will be flipped into large microwell to let cell proliferate. Unfortunately, using dual well in one device could make the fabrication process more complicated, expensive, and disposable (C.-F. Yeh et al., 2020).

In this study, we used an approach for isolate an individual cell using the existing simple microwell-based microfluidic (MBM) device to overcome those problems. Our approach is based on Kuntanawat (2014) work that using electrostatic force for trapping single cells. To optimize the single cell efficiency for GS-KO CHO cells using this device, we optimize the initial cell loading density and settle times. We compared the single cell efficiency of our device towards limiting dilution.

4.3 Materials and Methods

4.3.1 Design and fabrication technique of MBM device

The simple microwell-based microfluidic device were modified and fabricated according to our previous study (Kuntanawat et al., 2014). The modification was conducted by removing upper cover, the inlet and outlet channel. CorelDraw software (CorelDraw Graphics Suite 2021, Ottawa, Ontario, Canada) was

used to draw the device design. There were three parts, including 200 arrays microwell, a side-open fluidic chamber, and a positive charge glass slide. In figure 27 is depicted the pattern of MBM device. There were 200 wells in microwell comprise 10 rows x 20 wells. Each well has dimension (width x length x depth) of 1000 x 1000 x 1000 µm, and 500 µm in spacing. Side open fluidic chamber and microwell were aligned together with positive glass slide (75 x 25 x 1 mm) (Superfrost® Plus J1800AMNZ, Saarbrückene, Germany), and attached using silicone mixture (Silicone SF 820 A and Silicone SF 820 B) and allowed to dry for 15 minutes.

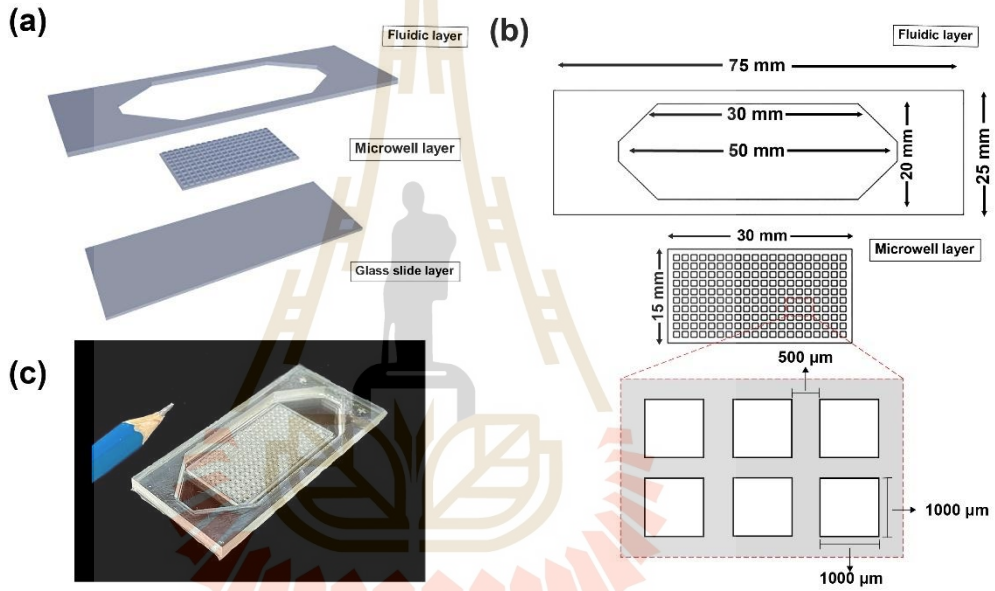


Figure 4.1 The fabricated a MBM device for single-cell isolation. a. The device is consist of 3 layers: a side-open fluidic chamber, microwell, positive glass slide. The side-open fluidic chamber and microwell were fabricated using laser technique. b. The details of MBM device, c. A finished microwell-based microfluidic device.

Poly (methyl methacrylate) or acrylic was used as a main material for microwell and side-open fluidic chamber. Laser cutting technique (HanMa Laser model HM-1310J, Guangdong, China) was used to fabricate the device.

4.3.2 Cell culture and harvesting

GS-KO CHO-K1 cell was used as a model for single cell isolation in this study. The culturing cells were similar with the step in previous section. Furthermore, the harvest cells were centrifuged (Biosan, LCM-3000, Riga, Latvia) at 1,000 RPM for 5 minutes and resuspended in sterile phosphate-buffer saline (PBS) at pH 7.4.

4.3.3 Protocol of MBM device for single cell isolation

Sterilization of the device by rinsed with 70% (v/v) ethanol were performed and then rinsed with sterile deionized water. The protocol for using the MBM device is depicted in figure 28. There were three steps, including preparation of cell suspension, cell loading into the device, and single cell measurement. At the beginning, cell preparation is performed. The maximum capacity by 3 mL of cell culture medium was introduced into the device. Then, cell suspension was prepared and diluted to cell concentration at $10x10^3$ cells/mL. Afterwards, 1 mL of cell medium inside the device was removed and add 1 mL of cell suspension in a side open fluidic chamber. Further, the device was allowed to settle for quite some time. Approximately 2.8 mL of mixed suspension was taken out from the device by pipetting out in the corner of open side up fluidic chamber. At last, the validation and quantification of single cell isolation through the device was conducted by inverted microscope (TS100F, Nikon, Melvile, NY, USA).

4.3.4 Optimization of the initial cell loading density for the MBM device

The optimization of initial cell loading density ranging at 10,000, 12,500, 15,000, and 17,500 cells/mL. Preparation of initial cell loading density was conducted by measuring the viable cell density using Luna-II™ automated cell counter (Logos Biosystems, Dongan-gu, Anyang-si, Gyeonggi-do, South Korea) with trypan blue exclusion method (Strober, 2015). Subsequently, 1 mL of cell suspension was introduced separately with the cell culture medium in the cell loading steps. The optimum initial cell loading density that provides the highest percentage of single cell trapped was used as an optimum.

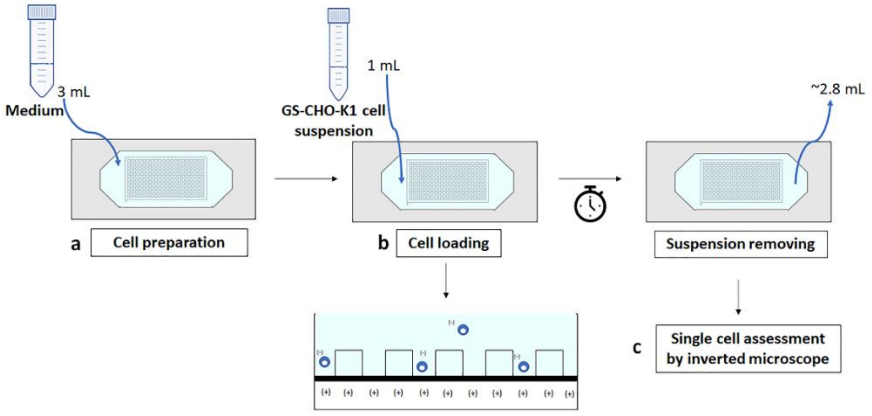


Figure 4.2 The steps of operation using microwell-based microfluidic device for single-cell isolation.

4.3.5 Optimization of settle times for the MBM device

The optimization of settle time was used to increase single cell trapped by the device. The range of settle time was 1, 3, 5, 7,9 minutes were conducted. To perform this, the optimized cell loading density was used earlier and the process for isolating single cell was begun with preparation of cell suspension. The cells were measured using Luna-II™ automated cell counter (Logos Biosystems, Dongan-gu, Anyang-si, Gyeonggi-do, South Korea) with trypan blue exclusion method (Strober, 2015). Further, 1 mL of cell suspension was introduced into the device. The settle time which offers the highest percentage of single cell found was used as an optimum settle time for the device.

4.3.6 Evaluation of single cell isolation by limiting dilution and the MBM device

In this study, there were ten testers which consists of experienced testers (n=5), and inexperience testers (n=5). They were asked to perform the single-cell isolation using two techniques. All testers had not been experienced with microwell-based microfluidic device before. While only experienced testers have familiar with limiting dilution technique. The introduction of both techniques, including the protocol and the validation of single-cell isolation, was performed either or both of tester groups. All testers were allowed to practice until they were felt comfortable to perform both techniques.

The protocol for limiting dilution was begun with cell preparation and then serially diluted with PBS to the optimal cell concentration of 4.5 cells/mL (Underwood & Bean, 1988). Subsequently, the diluted cell suspension was loaded into 96 well plates with 200 µL using multichannel pipette (Gilson Pipetman, L Multichannel, Middleton, USA) as much as 200 wells. Then, the single-cell assessment of whole plate was validated and quantified by inverted microscope (TS100F, Nikon, Melvile, NY, USA). For the microwell-based microfluidic device, the protocol was performed as aforementioned described previously.

To evaluate the efficiency of both techniques, the percentage of single-cell found and the time-spent in each process, including cell preparation, cell loading, and single-cell assessment, were recorded. All testers were allowed to rest between performing each technique and the sequence of both techniques for the testers was randomized.

4.3.7 Statistical analysis

All experimental data are reported as mean ± standard deviation using thrice replication. The statistical analysis of the data was conducted using one-way analysis of variance (ANOVA) with IBM SPSS Statistics (SPSS Inc, Version 21.0, Chicago, IL, USA). The significance difference between groups were performed using paired t-test and Duncan's new multiple range test. The level of the statistical significance was defined as $p<0.05$.

4.4 Results and discussion

4.4.1 Applied microwell device for single cell isolation

4.4.1.1 Optimization of initial cell density and settle time into microwell-based microfluidic device

The optimal cell density of initial cell loading is depicted in Figure 29. was ranging from 10,000, 12,500, 15,000, and 17,500 cells/mL. These initial cell densities were affected the number of single cells trapped in the well. The percentage of single cell found was varied with the range ~12.50-311%. The highest percentage of single cell found was at 15,000 cells/mL, it reached 31% and it is significant difference ($p< 0.05$) among other cell concentration.

Initial cell density for cell loading into a microwell-based microfluidic device is one of the critical parameters to obtain a single-cell. Among the optimization of initial cell density, the percentage of single cell found was accelerate through the increasing number of initial cell density until the optimal point. Furthermore, the reduction of its percentage was noticed when further increase to the highest amount of initial cell density. This phenomenon can be explained by the Poisson's distribution based on the relational distribution between initial cell concentration and single cell found event found (Summers, Wills, Brown, & Rees, 2015). With this distribution effect, the increase of initial cell density directly affects to increase the probability of single cell found in each well of the device. While the excessive number of initial cell density led to the decline of single cell found due to the increasing multiple cells found. Our finding was agreed with Kuntanawat and colleagues that the highest single cell found is related to the optimal initial cell density (Kuntanawat et al., 2014). In addition to the initial cell density, the function of time allowing suspension cells to be trapped on a device is another parameter that could influence the percentage of single cell found. This random event is occurred through the principle of an electrostatic microwell-based biochip that the cells could be trapped into the device via electrostatic interaction between the positive charge of the microscope glass slide and the negative charge cells surface (Kuntanawat et al., 2014). The investigation of the influencing time for cell trapping could provide the greater percentage of single cell found.

The optimal settle time incubation ranging from 1, 3, 5, 7, 10 minutes using initial cell loading density at 15,000 cells/mL were conducted. The results showed that the percentage of single cell found was ranging at 10-31%. Then, at 5 minutes reached 31% of the highest percentage single cell found. The settling time parameter is required to be optimized in order to obtain higher percentage of single cell found. Suspension cells were taken time to sediment into the well. The gravity force is affecting cells to fall inside microwell. Smaller settle time for 1- and 3-minutes results in 10% of single cell found using our device. Subsequently, the highest percentage of single cell found was at 5 minutes that could reach ~30% of single cell occupancy. However, the longer settle time for 7- and 10-minutes result in decreased single cell found. This is due to more microwells have multiple cells. Our

results are corresponding with Kobel and colleagues that for large and deep microwells when using longer settle time result in fewer single cell found in microwells due to multiple cells trapped inside the device (Kobel, Valero, Latt, Renaud, & Lutolf, 2010).

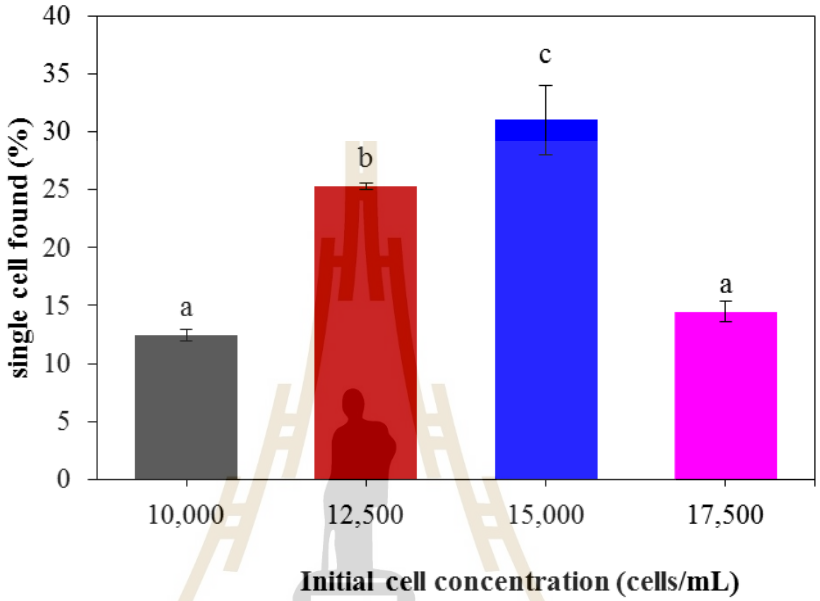


Figure 4.3 Percentage of single cell found using the different initial cell concentration ranging from 10,000 to 17,500 cells/mL. Experimental data are reported as mean±standard deviation (n=3). Means followed by the different letters within columns indicate a significant difference at $p<0.05$ using Duncan's multiple range test.

4.4.2 Efficiency of single cell isolation using limiting dilution and MBM device

The single cell isolation efficiency and time spent performing to complete all the tasks for ten persons were conducted in this study. There were 10 human testers which consists of 5 experience persons and 5 inexperience persons that asked to perform isolate single cell using limiting dilution and microwell-based microfluidic device methods. The results showed that using MBM device could provide higher single cell found compared to limiting dilution technique. It suggests

that using our device could improve the efficiency single cell isolation up to 24.3% over limiting dilution 5.15% (n=10) which depicted in figure 31. The mean of percentage of single cell found was significantly difference ($p<0.001$) between the device and limiting dilution.

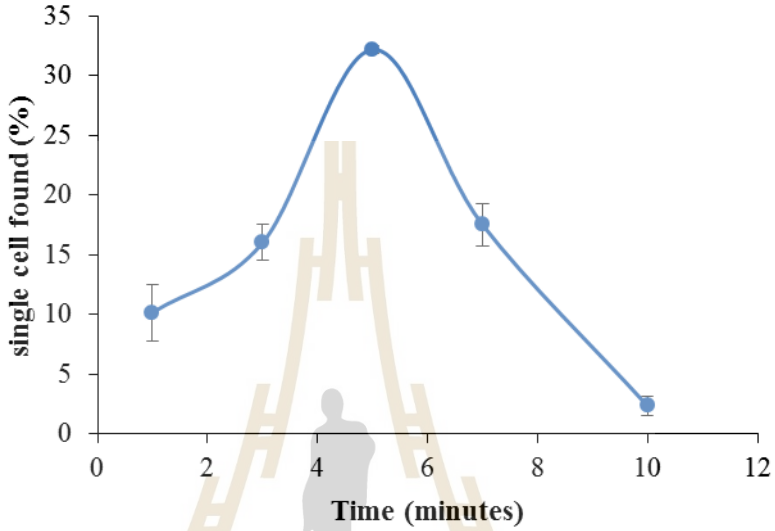


Figure 4.4 Percentage of single cell found using the different settle time incubation ranging from 1 to 10 minutes. Experimental data are reported as mean± standard deviation (n=3).

The improvement by 4.3-folded increase of single cell found was noticed in the MBM device. This finding could be elucidated by the ration between cell size and well's diameter. The increasing ratio in MBM device is resulted from the decreasing diameter of well, preventing the random event of multiple cells trapping against using 96 well plate. The results are agreed with Rettig and Folch (2005) that the decrease of size based lead to trap single cell at high efficiency (Rettig & Folch, 2005).

Yeh et al have been fabricated single cell cloning (SCC) device which used for single cell isolation and culture cells at same time with dual well system. Comparing the single cell found efficiency to their device, our device is lower 2.5-folded than theirs. This is due to diameter size of trapping well that they used is smaller compared to our device. Although our device has higher diameter size

compared to SCC device, it still could trap single cell and possible to grow the cell at same time without having two different wells. Additionally, using SCC device could not support the reusability because the design is closed and required to be punched for transferring cell clone into 96 well plate (C.-F. Yeh et al., 2020).

On the other hand, using our device could prompt to reusable because the device is open side up and easy to be sterilized again.

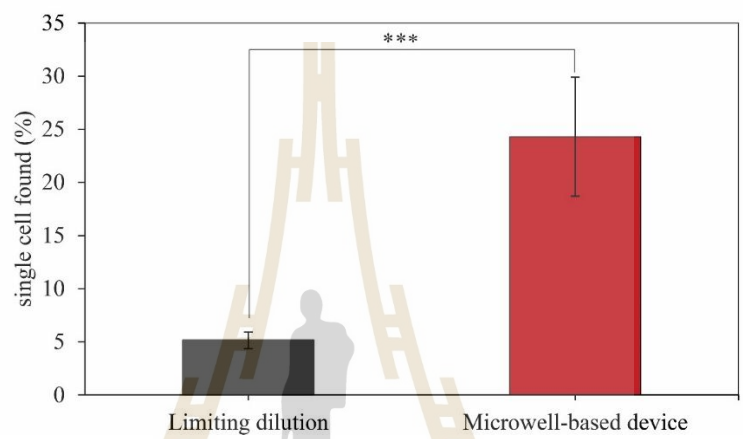


Figure 4.5 Comparison of the percentage of single-cell found using limiting dilution technique and microwell-based microfluidic device. Experimental data are reported as mean±standard deviation (n=10). Means followed by the asterisk indicate a significant difference at $p<0.001$ using paired t-test.

The result for comparing the time spent performing tasks of limiting dilution and MBM device was displayed in figure 32. Performing single cell isolation using limiting dilution was 48.14 minutes (n=10) and for the device was 25.15 minutes (n=10). The time was significantly different ($p<0.001$) between both methods.

The first task to performed is time spending process on cell dilution preparation. It found that MBM device could decrease time spending towards limiting dilution. In limiting dilution, all testers must to serially diluting cell suspension from high concentration into low concentration and therefore it takes time.

The second task is time spent for cell loading into both techniques. The results showed that time spending when using MBM device was higher compared to

limiting dilution. This is due to the waiting time incubation to let cell sediment into microwell. Hence, the actual time for microwell operation must be minus 5 minutes from the total time-spent, resulting the actual time of 2.3 minutes (7.3 minutes-5 minutes) which is shorter than the actual time of limiting dilution.

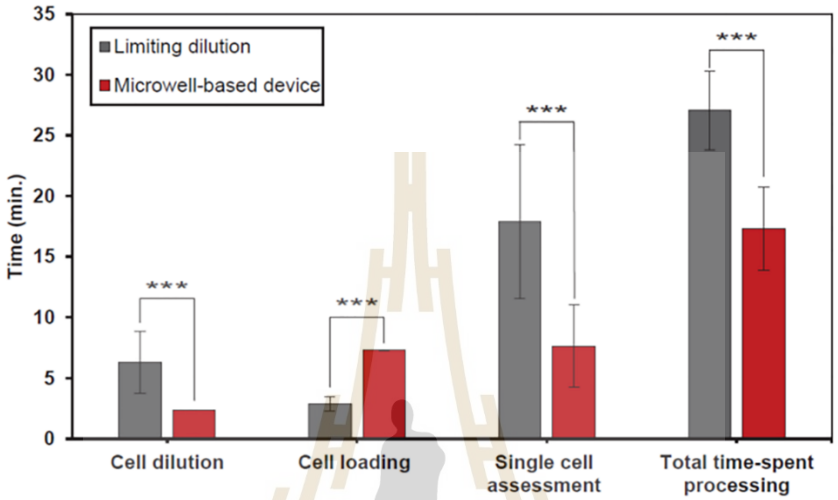


Figure 4.6 The time-spending using limiting dilution and a microwell-based microfluidic device. The data were based on each category, including preparation of cell dilution, cell loading, single-cell assessment, and total time-spent processing. Experimental data are reported as mean±standard deviation (n=10). Means followed by the asterisk within column indicate a significant difference at $p<0.001$ using paired t-test. Each column was analyzed separately.

The result of re-checking the single cell captured for both methods from experienced and inexperienced testers is displayed in figured 33. It was found that the percentage of single cell found correction when using our device and limiting dilution for experienced testers was 95.50% and 83.33% (n=5) and it was not significant difference ($p>0.05$), respectively. In contrast, the percentage of single cell found correction for inexperienced testers when using our device to isolate single cell, it was found that it reached 81.26% and limiting dilution was 61.43% which was significantly difference ($p<0.05$).

In this study, the efficiency of single cell measurement was assessed in order to validate the single cell found using both methods by observing single cells under inverted microscope directly. Then, it showed that MBM device could save time over limiting dilution. It suggests that the advantage using our device could reduce time handling and assurance for single cell found.

Reducing time-spent when using our device is because the smaller of well's diameter compared to well's diameter of 96 well plate. Hence, the smaller well's diameter of our device makes great easier observation for the cell and less time required to identify single cell. A single cell is crucial for monoclonal antibody production because of the regulatory of monoclonality assurance in order to sell to marketplace. Monoclonality assurance is used to qualify and assure that the cell line derives from a single progenitor (H. Le, N. Vishwanathan, N. M. Jacob, M. Gadgil, & W. S. Hu, 2015). Using single cell could be used to study the antibody responses to infection, autoimmune responses, and B cell developments (Guthmiller, Dugan, Neu, Lan, & Wilson, 2019).

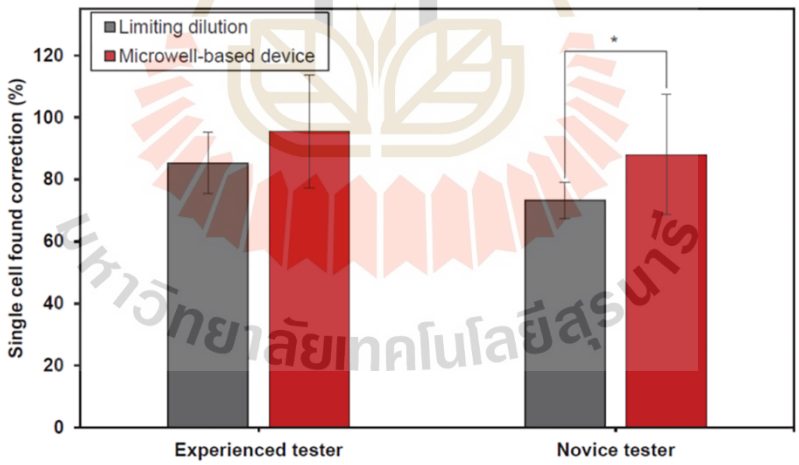


Figure 4.7 Comparison of the percentage of single-cell found correction using limiting dilution and microwell-based microfluidic device. The result obtained from experienced testers (n=5) and inexperienced testers (n=5), reported as mean±standard deviation. Mean followed by the asterisk within columns indicate a significant difference at $p<0.05$ using paired t-test. Each column was analyzed separately.

Based on human testers, they feel comfortable when they observe single CHO cell using our device. They could find single cell easily on the well of microwell-based microfluidic device because the diameter size of the well and cells are proportional. They also reflected that when observing single cell using well plate under inverted microscope were tough because cell size is smaller than the well plate, and they must screen 200 wells which was exhausting.

The total time-spent processing for single cell isolation by microwell-based microfluidic device showed the less-time consumption for 30 minutes significantly compared to limiting dilution method. This timesaving can be explained by reducing time-spent processing in each step of single cell isolation, including cell dilution, cell loading, and single cell measurement. For cell dilution, the serial dilution must prepare for getting the exact number of initial cell concentration following the Poisson's distribution. Limiting dilution technique prefers the initial cell concentration for 0.5-0.9 cells per aliquot to load into 96-well plate (Brunner, 2005). For this reason, many serial dilutions from the initial cell mixture, which usually has the concentration of 10^6 cells/mL, needs to be prepared. While, microwell-based microfluidic device needs the initial cell concentration for 15,000 cells/mL in presented study. This number is high enough to be diluted from the initial cell mixture only single dilution resulting in reduction of cell dilution. For cell loading, using limiting dilution requires pipetting one well to other well as much as 200 wells by multichannel pipette. However, using our device only need single pipetting and the cells are allowed to float freely and they will be trapped randomly by gravitation force. Then, for single cell assessment the well's diameter size strongly affect the time-spent for observing single cell.

Additionally, MBM device demonstrated the higher percentage of correct single cell identification in novice testers significantly than limiting dilution technique. This indicate that this device could be operate for lay people with less skill demanding. This finding could be reflexed by single step for cell dilution, shorter process, easy observation from smaller diameter, easy and simple for handling processing.

Table 4.1 Comparison of single-cell isolation using microwell based microfluidic device, limiting dilution, and FACS.

	Limiting Dilution	**Microwell-based microfluidic device**	**FACS (C.-F. Yeh et al., 2020)**
Time consumption	High	Low	Low
Reagent consumption	High	Low	High
Skill intensity	Moderate	Low	High

The conventional method for single cell isolation that has been exist is limiting dilution, this method is based on the use of serially dilution which dilute cells from high concentration to low concentration which then plated to produce single cell derived colonies. The cell dilution step can be performed using micropipette and replication of well plate, and this demanding high time and reagent consumption. Therefore, microwell-based microfluidic device is present to overcome the current method. Using our device could promote less handling time, low reagent consumption and low skill intensity. This is due to the size of our device is small and it requires small volume which correlate with reagent consumption. Additionally, a high throughput method such as fluorescence-activated cell sorting (FACS) can reduce time consumption for performing single cell cloning, but the disadvantages are it requires a lot of reagent consumption and also should have a skill to perform this technique. Based on Table 1, our device could be as an alternative approach for monoclonal cell generation and offers benefits such as cost effective over limiting dilution and FACS, moreover it also offers time effective same as FACS, and less skill required to perform single cell cloning isolation using our device in general biology laboratory.

4.5 Conclusions

Isolating a single CHO cell using microwell-based microfluidic device was successfully done by implement and optimize initial cell loading density and settle time. Using MBM device significantly improves the efficiency of single cell found comparing to limiting dilution. The MBM device provides simplicity, cost-effective,

and save time. In addition, the MBM device could be used for further clone development by isolation and cultivation simultaneously in the future.

4.6 References

Browne, S. M., & Al-Rubeai, M. (2007). Selection methods for high-producing mammalian cell lines. Trends Biotechnol, 25(9), 425-432. doi:10.1016/j.tibtech.2007.07.002.

Brunner, G. (2005). Limiting Dilution Analysis. In M. Assenmacher, H. K. Avraham, S. Avraham, S. Bala, J. Barnett, D. Basketter, Y. Ben-David, C. Berek, J. Blümel, A. P. Bolliger, B. Bolon, S. G. Bradley, K. M. Brundage, G. Brunner, P. J. Bugelski, S. W. Burchiel, L. A. Burns-Naas, J. L. Bussiere, S. B. Cameron, M. Carey, K. Cederbrant, A. W. Chow, M. D. Cohen, D. Colagiovanni, M. Contreras, J. B. Cornacoff, E. Corsini, R. Crevel, C. Cuff, C. J. Czuprynski, J. G. M. C. Damoiseaux, G. Daniels, A. D. Dayan, R. J. Dearman, S. V. M. Dodson, A. Ebringer, A. Engel, C. Esser, K. J. Fairley, R. Fernandez-Botran, R. Fernandez-Botran, D. K. Flaherty, W. Frings, S. C. Gad, D. E. Gardner, S. C. Gardner, J. Garssen, A. A. Gashev, J. Geffner, G. Geginat, D. Gemsa, F. Gerberick, D. Germolec, K. M. Gilbert, J. Giles-Komar, E. R. Gore, P. Griem, I. Hagelschuer, H. G. Haggerty, A. Hall, S. Encyclopedic Reference of Immunotoxicology. Springer Berlin Heidelberg. https://dol.org/10.1007/3-540-27806-0_887.

Hanneken, K. L. Hastings, A. H. Havelaar, E. Heisler, R. M. Helm, R. Henschler, T. Herrmann, D. J. Herzyk, R. R. Higgins, B. Hitzfeld, S. Holladay, M. Holsapple, R. V. House, L. Hughes, T. C. Jeong, V. J. Johnson, W. H. de Jong, R. de Jonge, A. Kamath, N. E. Kaminski, R. Kaminsky, M. Karol, M. L. Kashon, N. I. Kerkvliet, I. Kimber, D. M. Knight, A. C. Knulst, E. Koren, G. Kraal, A. Kretz-Rommel, C. F. Kuper, G. Ladics, M. Laiosa, K. S. Landreth, B. P. Lawrence, D. A. Lawrence, B.-C. Lee, W. Lee, L. Leino, H. Lemke, J. G. Lewis, J. Liebau, P.-L. Lollini, H. van Loveren, B. Luebke, M. I. Luster, R. G. Mage, C. C. Maier, M. U. Martin, T. Maurer, S. C. McKarns, B. J. Meade, B. Moser, S. Nagata, M. Nain, N. J. Neumann, D. L. Novicki, J. L. Olsen, J. Pauluhn, W. Pichler, R. Pieters, K. M. Pollard, K. T. Preissner, S. B. Pruett, N. R. Pumford, T. Rashid, H. V. Ratajczak, F. A. M. Redegeld, J. F. Regal, K. Resch, K. Rodgers, D. Roman, N. R. Rose, G. J.

Rosenthal, T. Sali, J. N. Samsom, H. F. J. Savelkoul, R. Schafer, M. Schatz, H. Schild, D. Shepherd, T. Shiohara, A. Silverstone, P. P. Simeonova, R. J. Smialowicz, R. J. Smialowicz, K. G. C. Smith, J. M. Soos, K. J. Stittelaar, F. Straube, C. E. W. Sulentic, B. Swart, K. Takumi, M. Tarkowski, J. W. C. Tervaert, P. T. Thomas, S. S. Tinkle, G. Treacy, K. Trouba, H. Tryphonas, M. Uguccioni, P. Ulrich, M. W. van der Heijden, H. Van Loveren, R. J. Vandebriel, K. Vleminckx, H.-W. Vohr, G. F. Weinbauer, I. B. Weinstein, H. U. Weltzien, A. Weston, K. L. White, C. Wilson, M. Wing, A. M. Wolf, P. Yaqoob, B. Yucesoy, D. C. Zawieja, J. T. Zelikoff, H. Zola, P. A. van Zwieten & H.-W. Vohr (Eds.), Encyclopedic Reference of Immunotoxicology. Berlin, Heidelberg: Springer Berlin Heidelberg. 385-387.

Chung, J. (2017). Special issue on therapeutic antibodies and biopharmaceuticals. Experimental & molecular medicine, 49(3), e304-e304. doi:10.1038/emm.2017.46.

Dalton, A. C., & Barton, W. A. (2014). Over-expression of secreted proteins from mammalian cell lines. Protein Sci, 23(5), 517-525. doi:10.1002/pro.2439.

DeMaria, C. T., Cairns, V., Schwarz, C., Zhang, J., Guerin, M., Zuena, E., . . . Karey, K. P. (2007). Accelerated clone selection for recombinant CHO CELLS using a FACS-based high-throughput screen. Biotechnol Prog, 23(2), 465-472. doi:10.1021/bp060298i.

Guthmiller, J. J., Dugan, H. L., Neu, K. E., Lan, L. Y.-L., & Wilson, P. C. (2019). An Efficient Method to Generate Monoclonal Antibodies from Human B Cells. In M. Steinitz (Ed.), Human Monoclonal Antibodies: Methods and Protocols. New York, NY: Springer New York. 109-145.

Hong, J., & Lukes, J. R. (2008). Flow Cytometer Lab-on-Chip Devices. In D. Li (Ed.), Encyclopedia of Microfluidics and Nanofluidics. Boston, MA: Springer US. 696-708.

Kobel, S., Valero, A., Latt, J., Renaud, P., & Lutolf, M. (2010). Optimization of microfluidic single cell trapping for long-term on-chip culture. Lab on a chip, 10(7), 857-863. doi:10.1039/B918055A.

Kunert, R., & Reinhart, D. (2016). Advances in recombinant antibody manufacturing. Appl Microbiol Biotechnol, 100(8), 3451-3461. doi:10.1007/s00253-016-7388-9.

Kuntanawat, P., Ruenin, J., Phatthanakun, R., Kunhorm, P., Surareungchai, W., Sukprasong, S., & Chomnawang, N. (2014). An electrostatic microwell–based biochip for phytoplanktonic cell trapping. Biomicrofluidics, 8(3), 034108. doi:10.1063/1.4882196.

Le, H., Vishwanathan, N., Jacob, N. M., Gadgil, M., & Hu, W.-S. (2015). Cell line development for biomanufacturing processes: recent advances and an outlook. Biotechnology Letters, 37(8), 1553-1564. doi:10.1007/s10529-015-1843-z.

Lu, R.-M., Hwang, Y.-C., Liu, I. J., Lee, C.-C., Tsai, H.-Z., Li, H.-J., & Wu, H.-C. (2020). Development of therapeutic antibodies for the treatment of diseases. Journal of Biomedical Science, 27(1), 1. doi:10.1186/s12929-019-0592-z.

Rettig, J. R., & Folch, A. (2005). Large-scale single-cell trapping and imaging using microwell arrays. Anal Chem, 77(17), 5628-5634. doi:10.1021/ac0505977.

Smith, G. P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science, 228(4705), 1315-1317. doi:10.1126/science.4001944.

Stapleton, S., Kennedy, R. O., & Tully, E. (2005). IMMUNOASSAYS | Production of Antibodies.

Strober, W. (2015). Trypan Blue Exclusion Test of Cell Viability. Current Protocols in Immunology, 111(1), A3.B.1-A3.B.3. doi:https://doi.org/10.1002/0471142735.ima03bs111.

Summers, H. D., Wills, J. W., Brown, M. R., & Rees, P. (2015). Poisson-event-based analysis of cell proliferation. Cytometry A, 87(5), 385-392. doi:10.1002/cyto.a.22620.

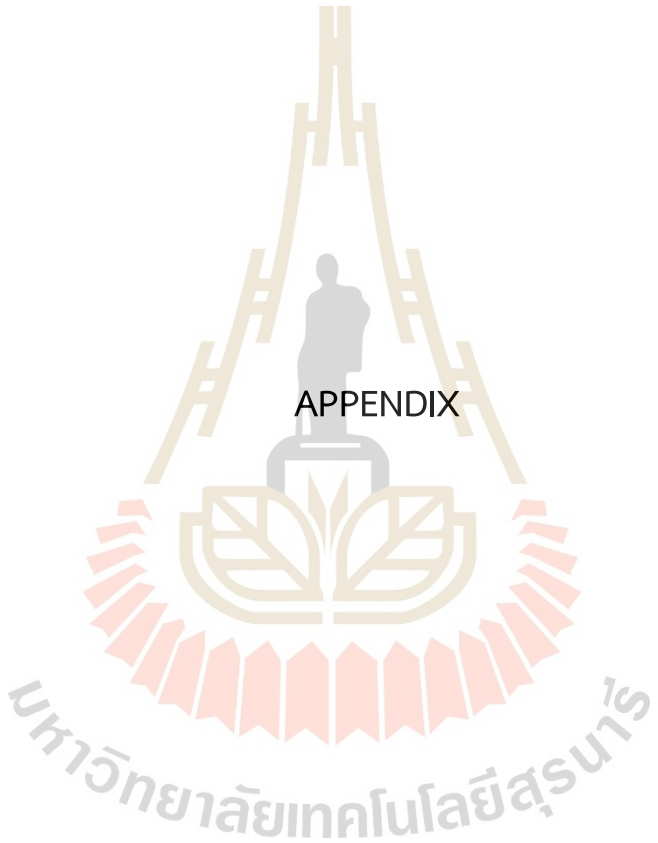
Underwood, P. A., & Bean, P. A. (1988). Hazards of the limiting-dilution method of cloning hybridomas. J Immunol Methods, 107(1), 119-128. doi:10.1016/0022-1759(88)90017-8.

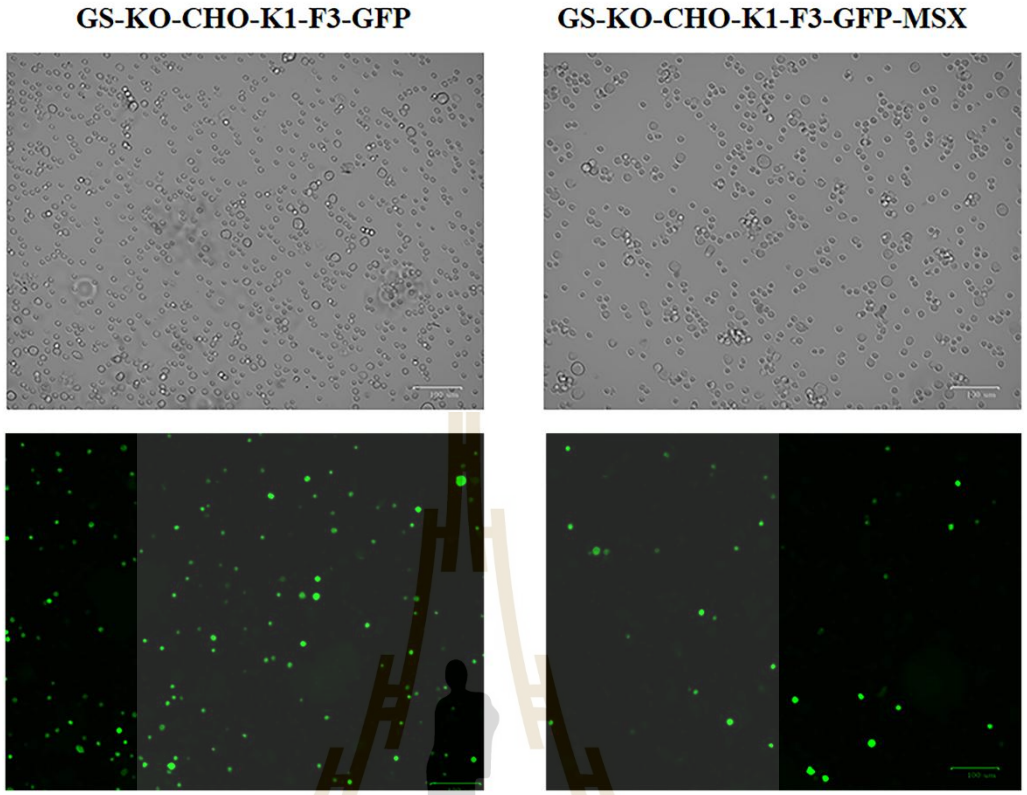
Urquhart, L. (2020). Top companies and drugs by sales in 2019. Nat Rev Drug Discov, 19(4), 228. doi:10.1038/d41573-020-00047-7.

Wurm, F. M. (2004). Production of recombinant protein therapeutics in cultivated mammalian cells. Nature Biotechnology, 22(11), 1393-1398. doi:10.1038/nbt1026.

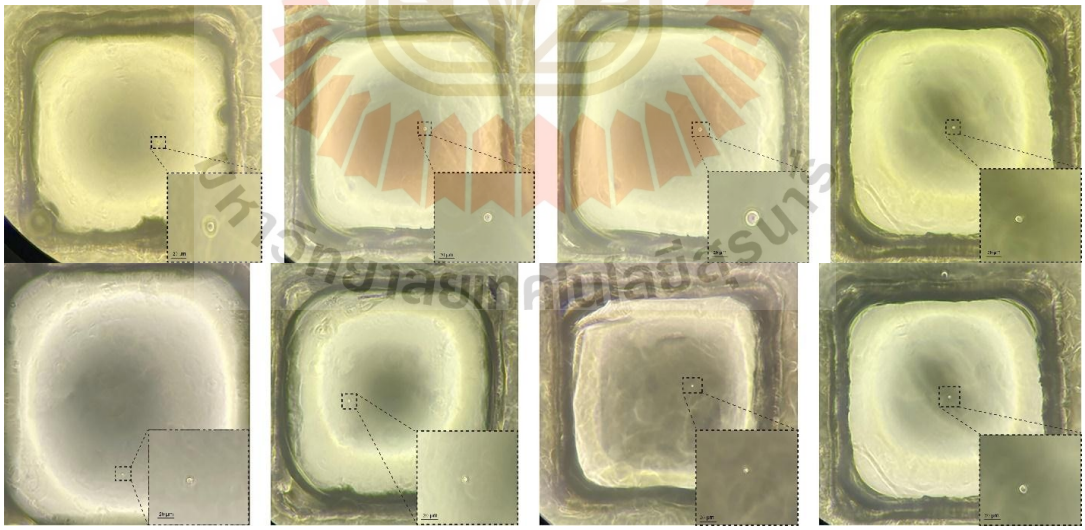
Yeh, C.-F., Lin, C.-H., Chang, H.-C., Tang, C.-Y., Lai, P.-T., & Hsu, C.-H. (2020). A Microfluidic Single-Cell Cloning (SCC) Device for the Generation of Monoclonal Cells. Cells, 9(6), 1482. doi:10.3390/cells9061482.

APPENDIX

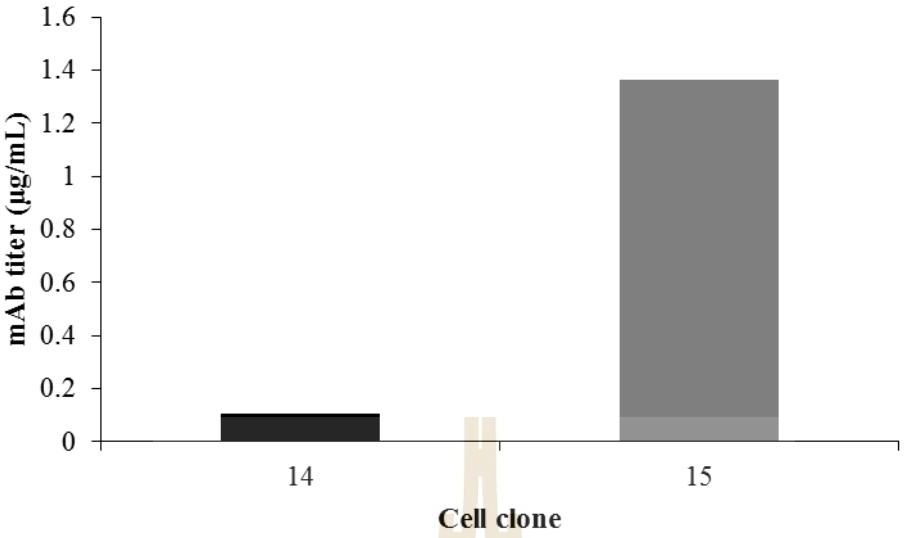




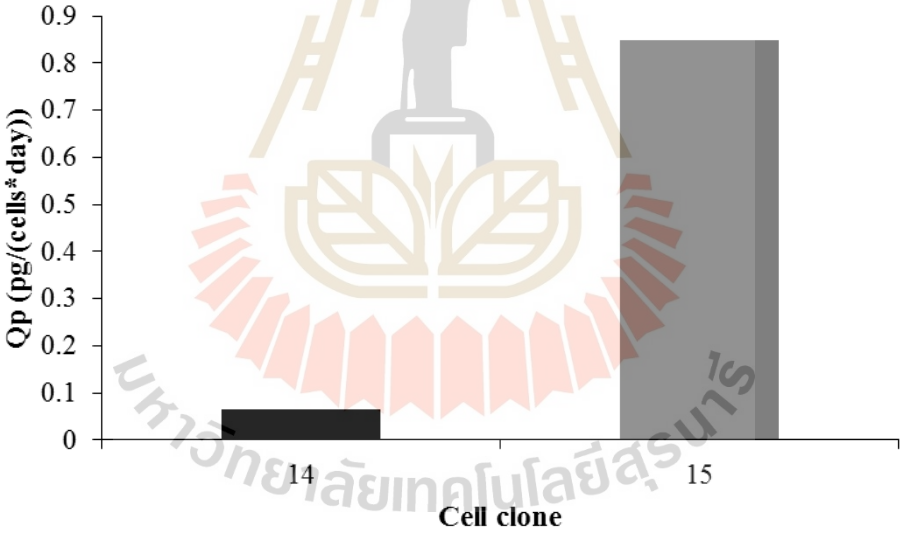
Stable GFP mini pools



Single CHO cell is trapped into microwell-based microfluidic device



Secondary screening to assess productivity



Productivity of cell clone

BIOGRAPHY

Miss Salma Fuadiyah was born on November 30, 1995, in Kudus, Indonesia. She had an internship program at BIOTEC, NSTDA, Thailand in 2017. She graduated with a bachelor's degree from Department of Biology, Faculty of Science and Mathematics, Diponegoro University in 2018. She had an enormous opportunity to study master's degree in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology. She received the OROG scholarship from Suranaree University of Technology to support her study. Her work and research interests in stable cell line development for producing monoclonal antibody and isolation of single cell using microfluidic system. She had presented some part of her research in Asian Federation of Biotechnology (AFOB) Virtual Conference 2021, entitled of the poster was Development of Stable Cell Line for Producing Monoclonal Antibody using GS-Knockout CHO Cells with Glutamine Synthetase System.