COVER LETTER

April 14, 2016

Dear Professor Roberto Gambari
Chief Editor of the Journal Minerva Biotecnologica
Via Spallanzani 9 - 00161 ROMA - ITALY

My name is, Muhamad Ali, graduated from Laboratory of Molecular Biotechnology Nagoya University Japan in 2006 and followed Postdoctoral Program at Laboratory of Cellular and Molecular Biotechnology Toyama University Japan in 2012. Now, I am researcher at Laboratory of Microbiology and Biotechnology Mataram University Indonesia with research focusses on the monoclonal antibody engineering. I would like to submit the attached research paper to the Journal Minerva Biotecnologica.

Tittle of the research paper is "**Comparison between Mammalian Cell and Bacterial Cell-free System for High-throughput Expression of Linear PCR-amplified Immunoglobulin Genes**". Cell-free expression system is the most rapid and robust method for screening of immunoglobulin gene library as shown in the most recent article entitled "Ultra-high-throughput screening of an in vitro-synthesized horseradish peroxidase displayed on microbeads using cell sorter. (Zhu B, Mizoguchi T, Kojima T, Nakano H. in PLoS One. 2015). A mammalian cell-based expression have been developed for the same purpose (Rapid production of antigen-specific monoclonal antibodies from a variety of animals. Kurosawa N, Yoshioka M, Fujimoto R, Yamagishi F, Isobe M. in BMC Biol. 2012). However, the use of both methods to express the same linear Polymerase Chain Reaction (PCR)-amplified immunoglobulin genes (Ig-genes) for generation of a functional monoclonal antibody fragment (Fab) has not been compared yet.

This manuscript is the first report describes the comparison of mammalian cell and bacterial cell-free system for high-throughput expression of linear PCR-amplified immunoglobulin genes, which strongly necessary to reduce the burden of a novel antibody discovery.

I deeply hope that this article is suitable to publish in the Journal of Minerva Biotecnologica. I looking forward your responses.

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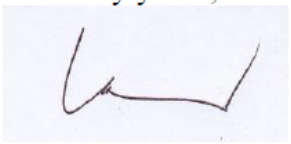

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Faculty of Animal Sciences, Mataram University, Indonesia
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AUTHOR STATEMENT FORM

Dear editorial board of the journal Minerva Biotecnologica

I, undersigned

Name : Muhamad Ali

Position : author of manuscript entitled "Comparison between Mammalian Cell and Bacterial Cell-free System for High-throughput Expression of Linear PCR-amplified Immunoglobulin Genes"

Declare that there are no competing interests with any financial organization regarding the material discussed in the manuscript.

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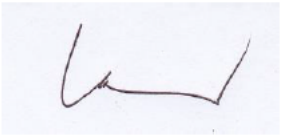

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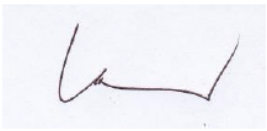
MUHAMAD ALI	

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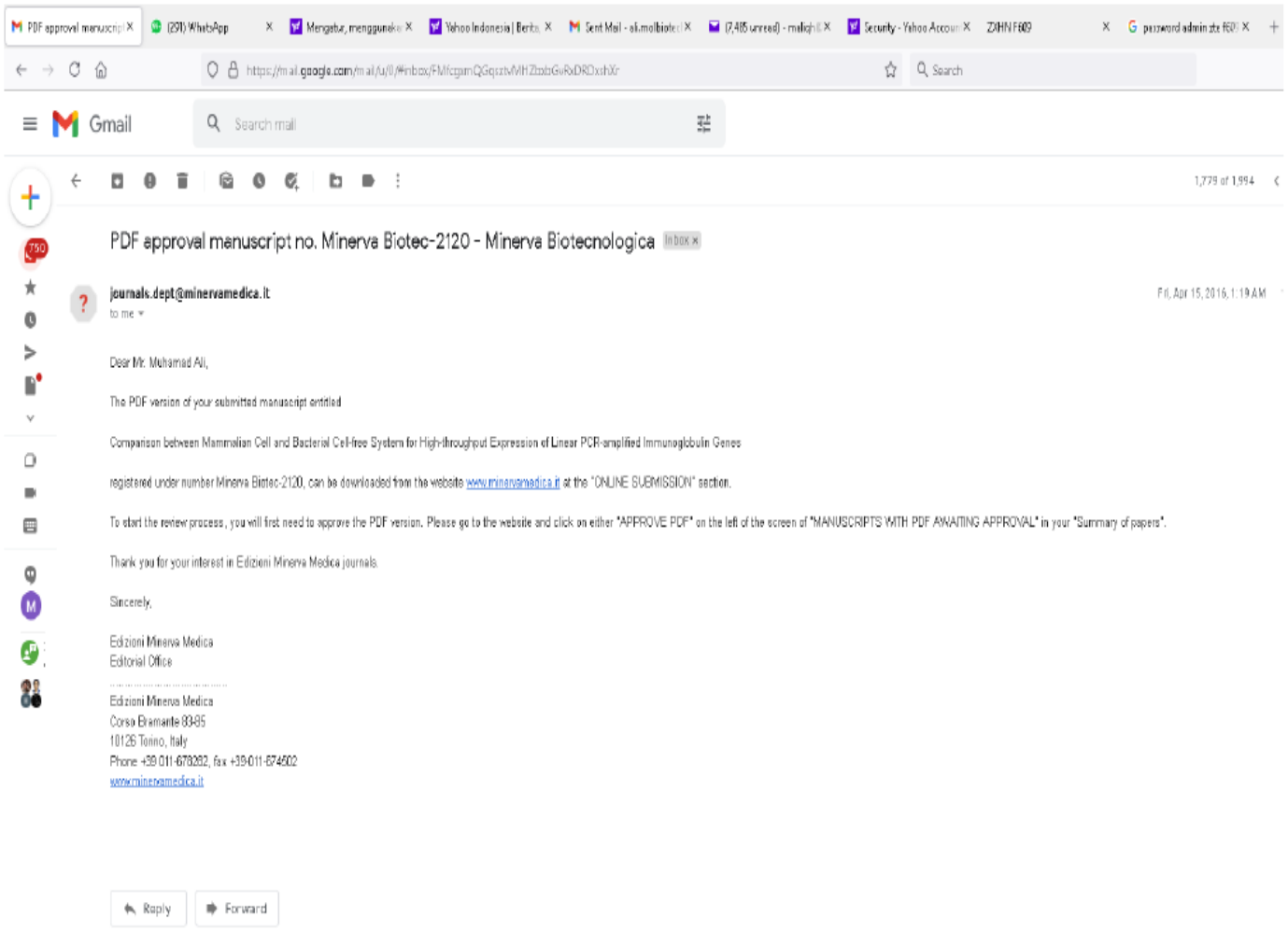

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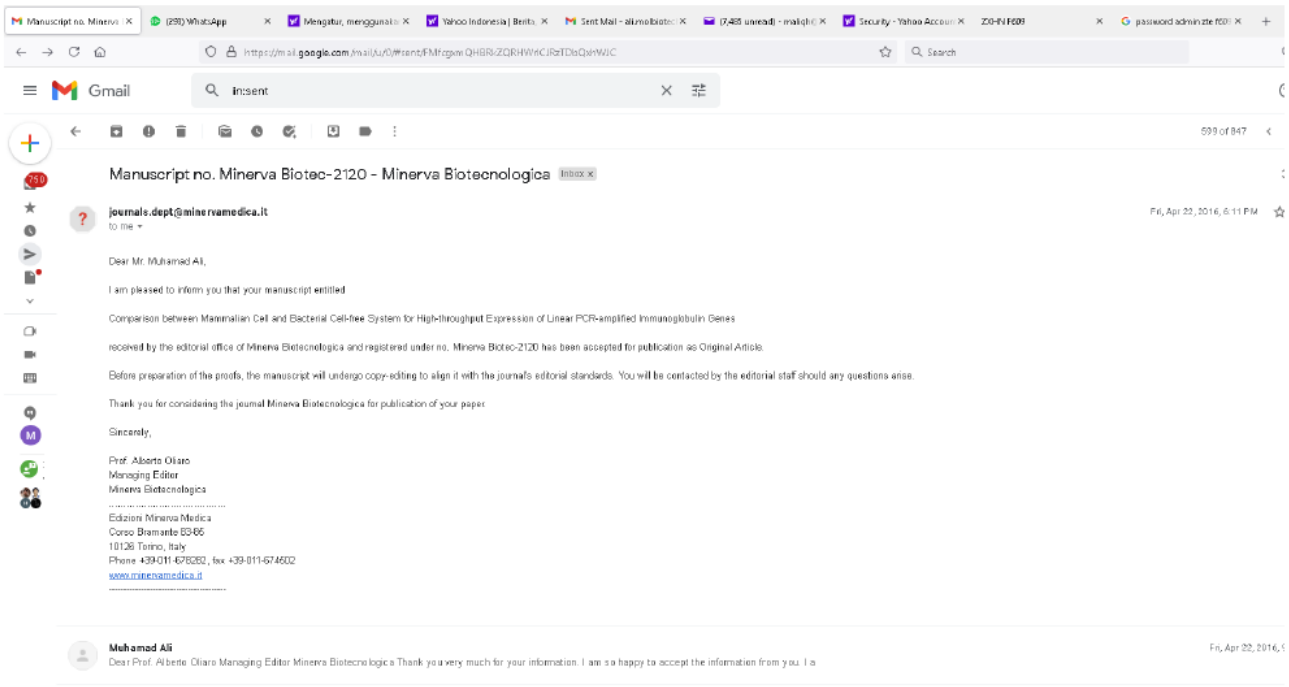

Muhamad Ali <ali.molbiotech@gmail.com> Apr 22, 2016, 9:42 PM

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Dear Prof. Alberto Oliaro
Managing Editor
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Thank you very much for your information. I am so happy to accept the information from you.
I am waiting further information or questions from your staff.

Sincerely yours,
Muhamad Ali, Ph.D
Lab. Microbiology and Biotechnology
Faculty of Animal Sciences
Mataram University, Indonesia, 83125.

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ORIGINAL ARTICLE

Comparison between mammalian cell and bacterial cell-free system for high-throughput expression of linear PCR-amplified immunoglobulin genes

SHORT TITLE: LINEAR PCR-AMPLIFIED IMMUNOGLOBULIN GENES

Muhamad ALI

Laboratory of Microbiology and Biotechnology, Faculty of Animal Sciences, Mataram University

Mataram, Indonesia

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ABSTRACT

BACKGROUND: Monoclonal antibodies have become the most crucial and fastest growing group of protein therapeutics dedicated by modern biotechnology. These molecules represent a powerful reagent not only as a weapon to fight against lethal pathogens, but also as tools for many molecular immunology investigations. Therefore, the development of a high-throughput procedure to generate the antibodies in quite an amount is highly required. Two rapid methods, mammalian cells-based expression and bacterial cell-free transcription/translation system, have been developed for the rapid generation of a functional monoclonal antibody. However, the use of both methods to express the same linear polymerase chain reaction (PCR)-amplified immunoglobulin genes (Ig-genes) for generation of a functional monoclonal antibody fragment (Fab) has not been compared yet.

METHODS: Ig-genes were amplified from single plasma cell of an immunized mouse using MAGrahd Reactor. The PCR-amplified Ig-genes were then treated with TdT for random nucleotide tailing in the 3'-end and continued with target-selective joint PCR (TS-jPCR) to produce linear Ig-expression constructs. DNA

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transfection of the constructs was performed using the FuGENE HD transfection reagent into 293FT cells. At three days after transfection, the culture supernatants were analyzed for the secretion of Fab fragment. In the cell-free expression, the linear Ig-expression constructs were directly used as templates for Fab generation in *E. coli* cell-free expression system.

RESULTS: Mammalian cell and a bacterial cell-free system could be used to express a linear PCR-amplified Ig-genes to generate functional antibody fragment. The antigen binding activity of the mammalian cell-generated Fab fragment was 20-fold higher than those of the cell free-generated Fab fragment. However, the use of the cell-free system is faster, enabling the screening of antigen binding by ELISA within two~~2~~ hours.

CONCLUSIONS: This report describes the first comparison of mammalian cell and bacterial cell-free system for high-throughput expression of linear PCR-amplified immunoglobulin genes. Bacterial cell-free system entirely bypasses tedious *in vivo* process required for antibody production, which dramatically reduced the burden of a novel antibody discovery.

(Cite this article as: Ali M. Comparison between mammalian cell and bacterial cell-free system for high-throughput expression of linear PCR-amplified immunoglobulin genes. Minerva Biotec 2016;)

Key words: Antibodies, monoclonal - Genes, immunoglobulin ~~Linear PCR-amplified Ig-genes, *cell free expression system, mammalian 293-FT cell, single plasma cell, Fab*~~ Plasma cells.

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Therapeutic use of monoclonal antibody (mAb) has become a major part of treatments for various important human diseases including oncology, autoimmune diseases, cardiovascular, transplantation, viral infection, and infectious diseases.[1-3] This is due to the antibodies having several advantages, such as being reproducible, having low antigenicity, high specificity, and potentially inexhaustible in supply.[4] Also, it has been used in a wide array of applications, including as rapid diagnostic materials for diseases in clinical laboratories and used as research tools for many molecular immunology investigations. The emergence of

several molecular techniques, such as epitope mapping and molecular modeling, facilitate the utilization of the mAb~~monoclonal antibody~~ for antigenic profiling and macromolecular surfaces visualization to identify previously unknown cell molecules.[5, 6]

One of the most serious issues still pending for widespread application of antibodies is a labor-efficient, cost-effective, simple, rapid and efficient preparation of various antibodies. The use of microbial fermentation, insect, and conventional mammalian cell cultures, and transgenic animals have some drawbacks in term of cost, time-consuming, scalability, product safety, and authenticity.[7-9] Therefore, a method that enables rapid production of antibody molecules will give a significant contribution to deal with pandemic situations related to viruses and other deathly pathogens.

An antibody fragment with an antigen-binding activity has been rapidly generated using single-cell RT-PCR-linked cell-free expression (SICREX) system, which enables the high-throughput screening and generation of monoclonal antibodies from single plasma cells.[10,11] Functional light chain (Lc) and immunoglobulin heavy chain (Hc) genes were successfully amplified using the RT-PCR method and then actively expressed in the cell-free expression system, indicating that the functional fragments synthesized *in vitro* possess an affinity for their cognate antigen.

More recently, a higher affinity of mAb~~monoclonal antibody~~ fragment was generated by expression of a linear PCR-amplified immunoglobulin gene (Ig-gene) in mammalian 297FT cells.[12] A robotic magnetic head handling instrument

(MAGrahder) and a target-selective joint polymerase chain reaction (TS-jPCR) have been developed to facilitate the effective molecular cloning of the Ig-variable gene from a single cell for the expression. The use of the MAGrahder and TS-jPCR allow efficient amplification of Ig-gene from single B or plasma cell.

In this research, a high-throughput expression of linear PCR-amplified Ig-genes coding for anti-green fluorescence protein (anti-GFP) mAb~~monoclonal antibody~~ using mammalian cell-based expression and cell-free expression were compared. Results indicated that the antigen binding activity of the Fab produced by the mammalian cell system was 20-fold higher than those of bacterial cell-free expression system. However, the use of the cell-free system is faster, enables to screen antigen binding activity by ELISA within 2 hours. These systems entirely bypassed tedious *in vivo* process required for antibody production, which dramatically reduced the burden of a novel antibody discovery.

Materials and methods

Arrangement of MAGrahd Reactor Tray and cDNA synthesis

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Superhydrophobic layer, which has a 24 x 8 grid of positive relief features, was made by pressing the layer with an aluminum stamp that contained the positive relief features. The layer was bound to the inner surface part of the thin glass plate (Nunc) by incubation for 5 min at 37°C. A single plasma cell of an immunized mouse, which was isolated as previously described~~, previously~~[12], was

dropped on the center of the each 24 grids in the second row after oligo-dT magnetic bead (Dynabeads^{R} mRNA DIRECT^{TM} Kit, Invitrogen, Carlsbad, CA; USA) dropping on the center of each 24 grids in the first row. The reverse transcription (RT) buffer, RT reaction (SuperScript^{Tm} III Reverse Transcriptase, Invitrogen), TdT buffer, TdT reaction, TE buffer, and 1 x PrimerSTAR GC Buffer-0.1% TritonX-100 were dispensed automatically using MAGrahd Reactor Tray onto 24 grids of the 3rd, 4th, 5th, 6th, 7th, and 8th row respectively. For cDNA synthesis, the oligo-dT magnetic bead was removed from 1st row to 2nd, 3rd, and 4th rows by touching neodymium permanent magnet rods to the opposite side of the thin glass surface of the MAGrahd reactor tray above the drops. After 20 min, the reagent was removed to the 5th and 6th rows for a homopolymer-tailing reaction. Finally, the reagent was then removed into the 7th and 8th rows and used as templates for the Ig-gene (VL and VH) amplification.

5'-RACE-PCR

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5'-RACE-PCR was used for Ig-gene amplification with 3'-end homopolymer-tailed cDNA as templates. The first round of PCR was performed with dC13 forward primer, mixture of reverse primers (P0) specific for the respective IgG and IgK constant region, PrimeStar DNA polymerase, 1x PrimeStar GC buffer with the BIO-RAD MyCycler (35 cycles with denaturation at 95^{o}-C for 30 s, annealing and strand elongation at 68^{o}-C for 90 s and a final extension at 72^{o}-C for 180 s. In the 2nd round of PCR, the PCR products were diluted 10x with water and used (1 µl) as a template with a forward primer (Nhe-Eco47) and a respective nested reverse

primer (IgGV AS2, IgKV AS2 or IgλV AS2). Primers used in this study are listed in Table 1.

Synthesis of 3'-end random nucleotide tailing

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The amplified Ig variable genes (VL and VH) were then treated with a terminal deoxynucleotidyl transferase (TdT) for 3'-end random nucleotide tailing. The TdT reaction (1x PrimeStar GC buffer containing 0.2 mM of each dNTP and 2 units of TdT) was added with 1.0 µl 5'-RACE-PCR products and incubated at 37°C for 30 min after pre-incubation at 37°C for 2 min. TdT reaction was then heat inactivated at 94°C for 5 min.[12]

Construction of Ig-cassette

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The Ig-cassettes (IgG and Ig-K) for mammalian cell-based expression were amplified from pJON-mIgG and pJON-mIgK plasmids and purified using S-400 spin column after *Dpn*I treatment. For bacterial cell-free-based expression, Ig-cassettes (IgGT7P and IgKT7P) were amplified from pJONmIgGT7P and pJONmIgKT7P, which was constructed from pJON-mIgG and pJON-mIgK. Fragments of T7 promoter, Shine-Dalgarno (SD) sequence, and T7 terminator were synthesized by primer-self amplification using *Bgl*T7SD-S and *Bgl*T7SD-AS primers. Then, these fragments were ligated with the linearized pJON-mIgG and pJON-mIgK plasmids to generate pJONmIgGT7P and pJONmIgKT7P. Primers used for the amplification of these cassettes are available in Table 1.

Target selective-joint polymerase chain reaction ~~(TS-jPCR)~~

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Target selective-joint polymerase chain reaction (TS-jPCR) was conducted by joining the 3'-random nucleotide-tailed variable gene fragment and an Ig-cassette as previously described.[10] The TS-jPCR reaction consists of 10 ng of the 5'-RACE-PCR product (VL and VH), 0.25 mM dNTPs Mix, 2.0 U PrimeStar DNA Polymerase, 0.2 pM mini CMV primer (S and AS), and 1x PrimeStar GC buffer. Sequences of primers used are listed in Table ~~1~~I. The reaction was performed 2 min at 94°C, 5 cycles of 30 s at 94°C, 4 min at 70°C, 30 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C. The 3'-random nucleotide variable gene fragments were joined to their respective Ig-cassette to build linear expression construct.

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Transfection and mammalian cell expression

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DNA transfection of the mammalian expression constructs produced by TS-jPCR was performed using the FuGENE HD transfection reagent ~~(Roche, http://www.roche.com/research_and_development.htm)~~ into 293FT cells and grown in 96-well culture dishes for mammalian cells expression. At three days after transfection, the culture supernatants were analyzed for the secretion of recombinant antibodies.

Bacterial cell-free expression

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For the cell-free expression, the cell-free expression construct produced by TS-jPCR have directly used as templates for Fab expression in *E. coli* cell-free

expression system. The cell-free reactions were carried out as described in the instruction manual of PURExpress *In Vitro* Protein Synthesis Kits (New England~~,~~ Biolabs, Ipswich, MA, USA). Briefly, 3 µl each of TS-jPR products was mixed with 25 µl of a cell-free mixture (10 µl of solution A, 7.5 µl of solution B, 1.0 µl of disulfide enhancer-1, 1.0 µl of disulfide enhancer-2, and nuclease-free dH_2O). At the same time, the reaction mixture without TS-jPCR products was used as a control reaction. The reactions were incubated at 37^{o}~~-~~C for 2 h and analyzed for activity measurement.

Activity measurement

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The activity of the Fab fragment synthesized in the mammalian and cell-free expression system was examined using an ELISA against a green fluorescent protein (GFP) as described previously [13] with minor modification. Briefly, high-binding-capacity ELISA plates (Corning) were coated with 50 µl of 5 ng/µl GFP protein in NaHCO3 Buffer for overnight at 4^{o}C. About 200 µl of DMEM containing 10% FBS was used for blocking at room temperature for one hour~~1 h~~. After washing the ELISA plates 3x with 1x PBS, 50 µl of these samples (supernatant of mammalian cell culture or cell-free reaction mixtures) were transferred to the ELISA plates and incubated at room temperature for two~~2 h~~hours. Each sample was tested in duplicate. Anti-mouse IgG-AP was used as the second antibody after washing 3x with 1x PBS. Then, Fab fragment was detected with alkaline-phosphatase-conjugated anti-mouse IgG (Sigma) at a concentration of 0.8 µg/m~~l~~L after washing with Tris-NaCl buffer. The developed chemiluminescence was

quantified using a Tecan GENios microplate reader (TECAN, Crailsheim, Germany). The magnitude of the light emission was expressed as relative light units (RLU).

Results

Amplification of Ig variable genes

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The MAGrahder instrument used in this research performs mRNA extraction, reverse transcription (cDNA synthesis), and homopolymer-tailing reaction (3'-end homopolymer-tailed cDNA). The instrument has magnetic rods which transported and mixed nucleic acid-bound magnetic beads in the reactor tray (super-hydrophobic layer) with externally applied magnetic force. Since the scale of reaction in the reactor tray is small (3.0 µl), it can be inversely placed on the opposite side of the thin glass surface of the reactor tray. Therefore, by touching the neodymium permanent magnetic rods to the opposite side of the glass surface above the drop, nucleic acid-bound magnetic beads could be transferred to the next reaction. The products of 3'-end homopolymer-tailed cDNA synthesis were then used as templates to amplify the variable region of the immunoglobulin heavy chain (VH) and the variable region of the immunoglobulin light chain (VL) genes by 5'-RACE PCR.

Results of Ig-gene amplification are shown in Figure 1. Based on the figure, there are major clear bands with the appropriate size to light and heavy chain of the Ig-gene. Thus, the 5'-RACE PCR resulted in the successful

amplification of the cognate pair of VL and VH genes with an overall success rate of 85%. The amplification success rate of these genes using this method was higher than the previous method.[10,11] The use of FACS for single plasma cell isolation in this method gave a higher success rate comparing to the limiting dilution technique which used in the previous method. Moreover, the success rate was increased by the use of MAGrahd reactor tray and TS-jPCR.

TS-jPCR products

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After 3'-end random nucleotide tailing of the 5'-RACE PCR products, they were joined to the respective Ig-cassette using TS-jPCR. The procedures used in this research are shown in Figure 2. The construct contains homology sequence on its ends with the end of the 5'-RACE PCR products as shown by the same color in the figure. Thus, the PCR amplified V gene fragment can be selectively assembled with a linear Ig-expression construct.

Figure 3 shows the joining of the 5'-RACE PCR products with the Ig-cassette produced a single major band corresponding to the expected size of IgG- and IgK-expression construct. These results indicate that the linear PCR-amplified Ig variable genes were successfully hybridized with the Ig-fragment after TS-jPCR.

mAb~~Monoclonal Antibody~~ generation

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The activity of the Fab fragment synthesized in the mammalian cell and cell-free expression system was examined using an ELISA against a green fluorescent protein (GFP). Figure 4 shows the ELISA results of antibody affinity generated by

expression in mammalian cell and bacterial cell-free expression system. As seen in the figure, the ELISA signal of the Fab fragment generated by both methods produces an active Fab fragment. However, the antigen binding activity of the Fab generated by mammalian cells is 20x higher than those of the Fab synthesized by the *in vitro* cell-free expression system.

Discussion

As shown in Figure 1, 5'-RACE PCR resulted in the successful amplification of the cognate pair of Ig-genes (VL and VH) with an overall success rate of 85%. The use of FACS for plasma cell isolation in this method was to make sure the single cell isolation for Ig-gene amplification was better than the limiting dilution technique used in the previous method. By using the MAGRAhder, mRNA could be isolated by oligo-dT-bounding magnetic bead from DNA or other RNA and transcribed into cDNA. Therefore, the amplification of false-positive products from contamination as the most serious drawback of RT-PCR in amplification of mRNA from single cell[10,14] could be avoided. Results of this experiment indicated that the use of MAGRAhder, 5'-RACE-PCR, and TS-jPCR facilitated effective molecular cloning of Ig-encoding genes from a single plasma cell.

To express the amplified Ig-genes in a mammalian cell or cell-free system, the VL or VH was joint to Ig-cassettes by PCR to build a linear Ig-gene expression construct. The procedures used after the amplification are illustrated in Figure 2. Firstly, the PCR products were treated with TdT for random nucleotide tailing in the 3'-end. TS-jPCR was performed by mixing the TdT reaction products with Ig-

cassettes to produce linear Ig-expression constructs. Since the Ig-cassette shares homology sequences with the tailed VL and VH gene, overlapping hybridization between these fragments would generate the intact linear Ig-gene expression construct. Ig-cassettes for mammalian cell expression contain all crucial sequences for gene expression in the cell, such as the CMV promoter, the Ig chain constant region, and the poly (A) signal.[10-12] Instead of the CMV promoter, Ig-cassettes for bacterial cell-free expression contains T7 promoter and T7 terminator which are recognized by T7 RNA polymerase used in the cell-free kit. As shown in Figure 3, the joining of the tailed variable gene fragment with the Ig-cassette produced a single major band corresponding to the expected size of IgG- and IgK-expression construct. These results indicate that the linear PCR-amplified Ig variable genes were successfully hybridized with the Ig-fragment after TS-jPCR. Secondly, the generated Ig-expression constructs were transfected into 293FT cells for mammalian cell expression or directly mixed with *E. coli* cell-free expression reagent for cell-free expression system. Lastly, the supernatant of 3 days 293FT cell culture or 2 h cell-free reaction was examine using ELISA for detection of Fab fragment activity.

During 3 days cultivation, the transfected 293FT cells produced and excreted a functional Fab antibody fragment into their supernatant. As occurred in the mammalian cells, the Ig expression construct of Fab is also simultaneously transcribed and translated in the *E. coli* cell-free expression system. Fab fragment generated in the mammalian cell has a higher antigen-binding activity than the Fab fragment generated in the *E. coli* cell-free expression system (Figure 4). It is

because the eukaryotic-based expression system provides a better environment for protein synthesis particularly for protein folding and post-translationally modification.[15,16] Since the *E. coli* cell-free system lack in compartments with oxidizing redox potential, such as periplasm or lumen of endoplasmic reticulum in eukaryotic, the formation of disulfide bonds for proper protein folding might be prevented.[17] Therefore, one of the biggest problems in the biotechnological application of the *E. coli* cell-free system is that proteins are frequently expressed as insoluble aggregates folding intermediates, known as inclusion bodies, which then reduce their activity.

Also, the expression of recombinant protein in bacterial cell-free expression systems has been limited partly because the proteins produced are susceptible to endogenous proteases from the *E. coli* extract.[18] These proteases, which are retained in the S30 extract during its preparation, degrade the expressed proteins, which would eventually reduce the total amount of protein produced and then reduce the antigen-binding activity of the antibody.

In the time requirements point of view, however, the cell-free protein synthesis system is very fast for rapid screening of antigen binding by ELISA within 2 h comparing to the expression time in the mammalian cell-based expression which needed 72 h as shown in Figure 2. Therefore, the cell-free method entirely bypasses tedious *in vivo* process required for antibody production. Thus, this method could dramatically reduce the burden of a novel antibody discovery.

Generation of antibody derivative in this research, Fab fragment, has some

potential advantages over the full-size immunoglobulins produced by the hybridoma. According to Simmons ~~et al~~*et al*. (2002),[19] ~~,~~ although complete IgG molecules can successfully express in *E. coli* and exhibit increased plasma half-lives compared to antibody fragments (Fab and ScFV), these proteins are still deficient in effector functions, mainly due to a lack of glycosylation. Since smaller antibody fragments such as antigen-binding fragment (Fab) and single chain variable fragment (ScFv) enable rapid screening and selection of high-affinity molecule, the potential range of applications for these molecules are rapidly expanding. Light chain (Lc) and heavy chain (Hc) of Fab are covalently linked by a disulfide bridge, which gives native structure, proper folding, a higher binding affinity, and longer term storage.

However, the scFv often forms unpredictable multimers, can be digested by proteases, and some scFvs show a reduced affinity of up to one order of magnitude compared to the corresponding Fab fragments. The scFv lacks the whole constant region of light and heavy chain, making the antibody fragment unable to mediate effector functions and leading to relatively short plasma half-lives.[20] ~~.~~ Moreover, construction of Fab in the recombinant plasmid is simpler and faster than the construction of scFv.

Conclusions

In conclusion, the expression of linear PCR-amplified immunoglobulin gene using mammalian cell gave a higher quantity and quality of recombinant mAb~~monoclonal antibody~~ fragment than the use of the *E. coli* cell-free expression

system. However, the expression of these genes in the *E. coli* cell-free system is the fastest (2 h), allowing the system for automation to reduce the burden of antibody discovery and engineering.

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~~*Competing interests*~~

~~*The author declare there are no competing interests.*~~

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Figure 1.—Results of 5′-RACE PCR for amplification of the cognate pair of VL (upper) and VH genes (below).

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Figure 2.—Schematic illustration of antibody generation using mammalian cells and cell-free expression system. The time requirements in the cell-free expression is very fast (5 h) over the mammalian cell-based expression which needs 75 h.

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Figure 3.—TS-jPCR products analysis of linear PCR-amplified immunoglobulin variable genes with Ig-cassette. The results of TS-jPCR the Lc (1) and Hc genes (3) are partly shown. The PCR product (3 µl) was analyzed on a 1.0% agarose gel. Corresponding lane numbers for the (lane 1) indicate fragments jointed with Ig-cassette. The lane 2 is linear PCR-amplified immunoglobulin cassette without TS-jPCR, and 4 control without template was also performed. The λ-DNA marker digested by EcoT141 was in the middle (M).

Figure 4.—ELISA analysis of 3 (three) Fab fragments synthesized in mammalian cell expression and cell-free expression. The specific activity of recombinant antibodies is expressed as relative light units (RLU/s).

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Table 14. List of primers used in this study.

Name	Sequence (5'–3')	Application
dC13 S	CGGTACCGCGGGCCCGGGATCCCCC CCCCCCCCDN	5'-RACE PCR 1st PCR
P0 for IgG	ACCYTGCATTTGAACTCCTTGCC	5'-RACE PCR 1st PCR
P0 for IgK	ACTGCCATCAATCTTCCACTTGACA	5'-RACE PCR 1st PCR
IgG	ACCYTGCATTTGAACTCCTTGCC	5'-RACE PCR 1st PCR
IgK	ACTGCCATCAATCTTCCACTTGACA	5'-RACE PCR 1st PCR
IgGV AS2	CTGCCGGACGGACGGGAAGGTGCGTAC	5'-RACE PCR 2nd PCR
IgKV AS2	GGGTGGTGCCATCCACCTCCCAGGTGAC	5'-RACE PCR 2nd PCR
Igλ AS2	CTGGACAGGGATCCAGAGTTCCA	5'-RACE PCR 2nd PCR
Nhe-Eco47	CGCTAGCGCTACCGGACTCAGATCC	5'-RACE PCR 2nd PCR
*Bgl*T7P	AGAGATAGATCTATTAATACGACTCACTAT AGGGAGACCACAACGGTTTCCCTCTAGAA ATAAT	Cell-free expression construct
*Bgl*T7T	GTGTGTGGGCCCATTGGTATATCTCCTTC TTAAAGTTAAACAAAATTATTTCTAGAGGG AAACCG	Cell-free expression construct
Mini.CMV.S	AGAGAAACCGTCTATCAGGGCGATGGC	TS-jPCR
Mini.CMV.AS	AGAGACCCTTTGACGTTGGAGTCCACG	TS-jPCR

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ORIGINAL ARTICLE

Comparison between mammalian cell and bacterial cell-free system for high-throughput expression of linear PCR-amplified immunoglobulin genes

SHORT TITLE: LINEAR PCR-AMPLIFIED IMMUNOGLOBULIN GENES

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ABSTRACT

BACKGROUND: Monoclonal antibodies have become the most crucial and fastest growing group of protein therapeutics dedicated by modern biotechnology. These molecules represent a powerful reagent not only as a weapon to fight against lethal pathogens, but also as tools for many molecular immunology investigations. Therefore, the development of a high-throughput procedure to generate the antibodies in quite an amount is highly required. Two rapid methods, mammalian cells-based expression and bacterial cell-free transcription/translation system, have been developed for the rapid generation of a functional monoclonal antibody. However, the use of both methods to express the same linear polymerase chain reaction (PCR)-amplified immunoglobulin genes (Ig-genes) for generation of a functional monoclonal antibody fragment (Fab) has not been compared yet.

METHODS: Ig-genes were amplified from single plasma cell of an immunized mouse using MAGrahd Reactor. The PCR-amplified Ig-genes were then treated with TdT for random nucleotide tailing in the 3'-end and continued with target-selective joint PCR (TS-jPCR) to produce linear Ig-expression constructs. DNA transfection of the constructs was performed using the FuGENE HD transfection reagent into 293FT cells. At three days after transfection, the culture supernatants

were analyzed for the secretion of Fab fragment. In the cell-free expression, the linear Ig-expression constructs were directly used as templates for Fab generation in *E. coli* cell-free expression system.

RESULTS: Mammalian cell and a bacterial cell-free system could be used to express a linear PCR-amplified Ig-genes to generate functional antibody fragment. The antigen binding activity of the mammalian cell-generated Fab fragment was 20-fold higher than those of the cell free-generated Fab fragment. However, the use of the cell-free system is faster, enabling the screening of antigen binding by ELISA within two hours.

CONCLUSIONS: This report describes the first comparison of mammalian cell and bacterial cell-free system for high-throughput expression of linear PCR-amplified immunoglobulin genes. Bacterial cell-free system entirely bypasses tedious *in vivo* process required for antibody production, which dramatically reduced the burden of a novel antibody discovery.

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Key words*:* Antibodies, monoclonal - Genes, immunoglobulin - Plasma cells.

Therapeutic use of monoclonal antibody (mAb) has become a major part of treatments for various important human diseases including oncology, autoimmune diseases, cardiovascular, transplantation, viral infection, and infectious diseases.[1-3] This is due to the antibodies having several advantages, such as being reproducible, having low antigenicity, high specificity, and potentially inexhaustible in supply.[4] Also, it has been used in a wide array of applications, including as rapid diagnostic materials for diseases in clinical laboratories and used as research tools for many molecular immunology investigations. The emergence of several molecular techniques, such as epitope mapping and molecular modeling, facilitate the utilization of the mAb for antigenic profiling and macromolecular surfaces

visualization to identify previously unknown cell molecules.[5, 6]

One of the most serious issues still pending for widespread application of antibodies is a labor-efficient, cost-effective, simple, rapid and efficient preparation of various antibodies. The use of microbial fermentation, insect, and conventional mammalian cell cultures, and transgenic animals have some drawbacks in term of cost, time-consuming, scalability, product safety, and authenticity.[7-9] Therefore, a method that enables rapid production of antibody molecules will give a significant contribution to deal with pandemic situations related to viruses and other deathly pathogens.

An antibody fragment with an antigen-binding activity has been rapidly generated using single-cell RT-PCR-linked cell-free expression (SICREX) system, which enables the high-throughput screening and generation of monoclonal antibodies from single plasma cells.[10,—11] Functional light chain (Lc) and immunoglobulin heavy chain (Hc) genes were successfully amplified using the RT-PCR method and then actively expressed in the cell-free expression system, indicating that the functional fragments synthesized *in vitro* possess an affinity for their cognate antigen.

More recently, a higher affinity of mAb fragment was generated by expression of a linear PCR-amplified immunoglobulin gene (Ig-gene) in mammalian 297FT cells.[12] A robotic magnetic head handling instrument (MAGrahder) and a target-selective joint polymerase chain reaction (TS-jPCR) have been developed to facilitate the effective molecular cloning of the Ig-variable

gene from a single cell for the expression. The use of the MAGrahder and TS-jPCR allow efficient amplification of Ig-gene from single B or plasma cell.

In this research, a high-throughput expression of linear PCR-amplified Ig-genes coding for anti-green fluorescence protein (anti-GFP) mAb using mammalian cell-based expression and cell-free expression were compared. Results indicated that the antigen binding activity of the Fab produced by the mammalian cell system was 20-fold higher than those of bacterial cell-free expression system. However, the use of the cell-free system is faster, enables to screen antigen binding activity by ELISA within 2 hours. These systems entirely bypassed tedious *in vivo* process required for antibody production, which dramatically reduced the burden of a novel antibody discovery.

Materials and methods

Arrangement of MAGrahd Reactor Tray and cDNA synthesis

Superhydrophobic layer, which has a 24 x 8 grid of positive relief features, was made by pressing the layer with an aluminum stamp that contained the positive relief features. The layer was bound to the inner surface part of the thin glass plate (Nunc) by incubation for 5 min at 37°-C. A single plasma cell of an immunized mouse, which was isolated as previously described,[12] was dropped on the center of the each 24 grids in the second row after oligo-dT magnetic bead (Dynabeads^{R} mRNA DIRECT^{TM} Kit, Invitrogen, Carlsbad, CA; USA) dropping on the center of each 24 grids in the first row. The reverse transcription (RT) buffer,

RT reaction (SuperScript^{Tm} III Reverse Transcriptase, Invitrogen), TdT buffer, TdT reaction, TE buffer, and 1 x PrimerSTAR GC Buffer-0.1% TritonX-100 were dispensed automatically using MAGrahd Reactor Tray onto 24 grids of the 3rd, 4th, 5th, 6th, 7th, and 8th row respectively. For cDNA synthesis, the oligo-dT magnetic bead was removed from 1st row to 2nd, 3rd, and 4th rows by touching neodymium permanent magnet rods to the opposite side of the thin glass surface of the MAGrahd reactor tray above the drops. After 20 min, the reagent was removed to the 5th and 6th rows for a homopolymer-tailing reaction. Finally, the reagent was then removed into the 7th and 8th rows and used as templates for the Ig-gene (VL and VH) amplification.

5'-RACE-PCR

5'-RACE-PCR was used for Ig-gene amplification with 3'-end homopolymer-tailed cDNA as templates. The first round of PCR was performed with dC13 forward primer, mixture of reverse primers (P0) specific for the respective IgG and IgK constant region, PrimeStar DNA polymerase, 1x PrimeStar GC buffer with the BIO-RAD MyCycler (35 cycles with denaturation at 95°-C for 30 s, annealing and strand elongation at 68°-C for 90 s and a final extension at 72°-C for 180 s. In the 2nd round of PCR, the PCR products were diluted 10x with water and used (1 µl) as a template with a forward primer (Nhe-Eco47) and a respective nested reverse primer (IgGV AS2, IgKV AS2 or IgλV AS2). Primers used in this study are listed in Table I.

Synthesis of 3'-end random nucleotide tailing

The amplified Ig variable genes (VL and VH) were then treated with a terminal deoxynucleotidyl transferase (TdT) for 3'-end random nucleotide tailing. The TdT reaction (1x PrimeStar GC buffer containing 0.2 mM of each dNTP and 2 units of TdT) was added with 1.0 µl 5'-RACE-PCR products and incubated at 37°-C for 30 min after preincubation at 37°-C for 2 min. TdT reaction was then heat inactivated at 94°-C for 5 min.[12]

Construction of Ig-cassette

The Ig-cassettes (IgG and Ig-K) for mammalian cell-based expression were amplified from pJON-mIgG and pJON-mIgK plasmids and purified using S-400 spin column after *Dpn*I treatment. For bacterial cell-free-based expression, Ig-cassettes (IgGT7P and IgKT7P) were amplified from pJONmIgGT7P and pJONmIgKT7P, which was constructed from pJON-mIgG and pJON-mIgK. Fragments of T7 promoter, Shine-Dalgarno (SD) sequence, and T7 terminator were synthesized by primer-self amplification using *Bgl*T7SD-S and *Bgl*T7SD-AS primers. Then, these fragments were ligated with the linearized pJON-mIgG and pJON-mIgK plasmids to generate pJONmIgGT7P and pJONmIgKT7P. Primers used for the amplification of these cassettes are available in Table I.

Target selective-joint polymerase chain reaction

Target selective-joint polymerase chain reaction (TS-jPCR) was conducted by joining the 3'-random nucleotide-tailed variable gene fragment and an Ig-

cassette as previously described.[10] The TS-jPCR reaction consists of 10 ng of the 5'-RACE-PCR product (VL and VH), 0.25 mM dNTPs Mix, 2.0 U PrimeStar DNA Polymerase, 0.2 pM mini CMV primer (S and AS), and 1x PrimeStar GC buffer. Sequences of primers used are listed in Table I. The reaction was performed 2 min at 94°C, 5 cycles of 30 s at 94°C, 4 min at 70°C, 30 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C. The 3'-random nucleotide variable gene fragments were joined to their respective Ig-cassette to build linear expression construct.

Transfection and mammalian cell expression

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DNA transfection of the mammalian expression constructs produced by TS-jPCR was performed using the FuGENE HD transfection reagent into 293FT cells and grown in 96-well culture dishes for mammalian cells expression. At three days after transfection, the culture supernatants were analyzed for the secretion of recombinant antibodies.

Bacterial cell-free expression

For the cell-free expression, the cell-free expression construct produced by TS-jPCR have directly used as templates for Fab expression in *E. coli* cell-free expression system. The cell-free reactions were carried out as described in the instruction manual of PURExpress *In Vitro* Protein Synthesis Kits (New England Biolabs, Ipswich, MA, USA). Briefly, 3 µl each of TS-jPR products was mixed with 25 µl of a cell-free mixture (10 µl of solution A, 7.5 µl of solution B, 1.0 µl of

disulfide enhancer-1, 1.0 µl of disulfide enhancer-2, and nuclease-free dH_2O). At the same time, the reaction mixture without TS-jPCR products was used as a control reaction. The reactions were incubated at 37°-C for 2 h and analyzed for activity measurement.

Activity measurement

The activity of the Fab fragment synthesized in the mammalian and cell-free expression system was examined using an ELISA against a green fluorescent protein (GFP) as described previously[13] with minor modification. Briefly, high-binding-capacity ELISA plates (Corning) were coated with 50 µl of 5 ng/µl GFP protein in NaHCO3 Buffer for overnight at 4°C. About 200 µl of DMEM containing 10% FBS was used for blocking at room temperature for one hour. After washing the ELISA plates 3x with 1x PBS, 50 µl of these samples (supernatant of mammalian cell culture or cell-free reaction mixtures) were transferred to the ELISA plates and incubated at room temperature for two hours. Each sample was tested in duplicate. Anti-mouse IgG-AP was used as the second antibody after washing 3x with 1x PBS. Then, Fab fragment was detected with alkaline-phosphatase-conjugated anti-mouse IgG (Sigma) at a concentration of 0.8 µg/mL after washing with Tris-NaCl buffer. The developed chemiluminescence was quantified using a Tecan GENios microplate reader (TECAN, Crailsheim, Germany). The magnitude of the light emission was expressed as relative light units (RLU).

Results

Amplification of Ig variable genes

The MAGrahder instrument used in this research performs mRNA extraction, reverse transcription (cDNA synthesis), and homopolymer-tailing reaction (3'-end homopolymer-tailed cDNA). The instrument has magnetic rods which transported and mixed nucleic acid-bound magnetic beads in the reactor tray (super-hydrophobic layer) with externally applied magnetic force. Since the scale of reaction in the reactor tray is small (3.0 µl), it can be inversely placed on the opposite side of the thin glass surface of the reactor tray. Therefore, by touching the neodymium permanent magnetic rods to the opposite side of the glass surface above the drop, nucleic acid-bound magnetic beads could be transferred to the next reaction. The products of 3'-end homopolymer-tailed cDNA synthesis were then used as templates to amplify the variable region of the immunoglobulin heavy chain (VH) and the variable region of the immunoglobulin light chain (VL) genes by 5'-RACE PCR.

Results of Ig-gene amplification are shown in Figure 1. Based on the figure, there are major clear bands with the appropriate size to light and heavy chain of the Ig-gene. Thus, the 5'-RACE PCR resulted in the successful amplification of the cognate pair of VL and VH genes with an overall success rate of 85%. The amplification success rate of these genes using this method was higher than the previous method.[10,11] The use of FACS for single plasma cell isolation in this method gave a higher success rate comparing to the limiting dilution technique

which used in the previous method. Moreover, the success rate was increased by the use of MAGrahd reactor tray and TS-jPCR.

TS-jPCR products

After 3'-end random nucleotide tailing of the 5'-RACE PCR products, they were joined to the respective Ig-cassette using TS-jPCR. The procedures used in this research are shown in Figure 2. The construct contains homology sequence on its ends with the end of the 5'-RACE PCR products as shown by the same color in the figure. Thus, the PCR amplified V gene fragment can be selectively assembled with a linear Ig-expression construct.

Figure 3 shows the joining of the 5'-RACE PCR products with the Ig-cassette produced a single major band corresponding to the expected size of IgG- and IgK-expression construct. These results indicate that the linear PCR-amplified Ig variable genes were successfully hybridized with the Ig-fragment after TS-jPCR.

mAb generation

The activity of the Fab fragment synthesized in the mammalian cell and cell-free expression system was examined using an ELISA against a green fluorescent protein (GFP). Figure 4 shows the ELISA results of antibody affinity generated by expression in mammalian cell and bacterial cell-free expression system. As seen in the figure, the ELISA signal of the Fab fragment generated by both methods produces an active Fab fragment. However, the antigen binding activity of the Fab generated by mammalian cells is 20x higher than those of the Fab synthesized by

the *in vitro* cell-free expression system.

Discussion

As shown in Figure 1, 5'-RACE PCR resulted in the successful amplification of the cognate pair of Ig-genes (VL and VH) with an overall success rate of 85%. The use of FACS for plasma cell isolation in this method was to make sure the single cell isolation for Ig-gene amplification was better than the limiting dilution technique used in the previous method. By using the MAGRAhder, mRNA could be isolated by oligo-dT-bounding magnetic bead from DNA or other RNA and transcribed into cDNA. Therefore, the amplification of false-positive products from contamination as the most serious drawback of RT-PCR in amplification of mRNA from single cell [10,14] could be avoided. Results of this experiment indicated that the use of MAGRAhder, 5'-RACE-PCR, and TS-jPCR facilitated effective molecular cloning of Ig-encoding genes from a single plasma cell.

To express the amplified Ig-genes in a mammalian cell or cell-free system, the VL or VH was joint to Ig-cassettes by PCR to build a linear Ig-gene expression construct. The procedures used after the amplification are illustrated in Figure 2. Firstly, the PCR products were treated with TdT for random nucleotide tailing in the 3'-end. TS-jPCR was performed by mixing the TdT reaction products with Ig-cassettes to produce linear Ig-expression constructs. Since the Ig-cassette shares homology sequences with the tailed VL and VH gene, overlapping hybridization between these fragments would generate the intact linear Ig-gene expression construct. Ig-cassettes for mammalian cell expression contain all crucial

sequences for gene expression in the cell, such as the CMV promoter, the Ig chain constant region, and the poly (A) signal.[10-12] Instead of the CMV promoter, Ig-cassettes for bacterial cell-free expression contains T7 promoter and T7 terminator which are recognized by T7 RNA polymerase used in the cell-free kit. As shown in Figure 3, the joining of the tailed variable gene fragment with the Ig-cassette produced a single major band corresponding to the expected size of IgG- and IgK-expression construct. These results indicate that the linear PCR-amplified Ig variable genes were successfully hybridized with the Ig-fragment after TS-jPCR. Secondly, the generated Ig-expression constructs were transfected into 293FT cells for mammalian cell expression or directly mixed with *E. coli* cell-free expression reagent for cell-free expression system. Lastly, the supernatant of 3 days 293FT cell culture or 2 h cell-free reaction was examine using ELISA for detection of Fab fragment activity.

During 3 days cultivation, the transfected 293FT cells produced and excreted a functional Fab antibody fragment into their supernatant. As occurred in the mammalian cells, the Ig expression construct of Fab is also simultaneously transcribed and translated in the *E. coli* cell-free expression system. Fab fragment generated in the mammalian cell has a higher antigen-binding activity than the Fab fragment generated in the *E. coli* cell-free expression system (Figure 4). It is because the eukaryotic-based expression system provides a better environment for protein synthesis particularly for protein folding and post-translationally modification.[15,16] Since the *E. coli* cell-free system lack in compartments with oxidizing redox potential, such as periplasm or lumen of endoplasmic reticulum in

eukaryotic, the formation of disulfide bonds for proper protein folding might be prevented.[17] Therefore, one of the biggest problems in the biotechnological application of the *E. coli* cell-free system is that proteins are frequently expressed as insoluble aggregates folding intermediates, known as inclusion bodies, which then reduce their activity.

Also, the expression of recombinant protein in bacterial cell-free expression systems has been limited partly because the proteins produced are susceptible to endogenous proteases from the *E. coli* extract.[18] These proteases, which are retained in the S30 extract during its preparation, degrade the expressed proteins, which would eventually reduce the total amount of protein produced and then reduce the antigen-binding activity of the antibody.

In the time requirements point of view, however, the cell-free protein synthesis system is very fast for rapid screening of antigen binding by ELISA within 2 h comparing to the expression time in the mammalian cell-based expression which needed 72 h as shown in Figure 2. Therefore, the cell-free method entirely bypasses tedious *in vivo* process required for antibody production. Thus, this method could dramatically reduce the burden of a novel antibody discovery.

Generation of antibody derivative in this research, Fab fragment, has some potential advantages over the full-size immunoglobulins produced by the hybridoma. According to Simmons *et al*. (2002),[19] although complete IgG molecules can successfully express in *E. coli* and exhibit increased plasma half-lives compared to antibody fragments (Fab and ScFV), these proteins are still

deficient in effector functions, mainly due to a lack of glycosylation. Since smaller antibody fragments such as antigen-binding fragment (Fab) and single chain variable fragment (ScFv) enable rapid screening and selection of high-affinity molecule, the potential range of applications for these molecules are rapidly expanding. Light chain (Lc) and heavy chain (Hc) of Fab are covalently linked by a disulfide bridge, which gives native structure, proper folding, a higher binding affinity, and longer term storage.

However, the scFv often forms unpredictable multimers, can be digested by proteases, and some scFvs show a reduced affinity of up to one order of magnitude compared to the corresponding Fab fragments. The scFv lacks the whole constant region of light and heavy chain, making the antibody fragment unable to mediate effector functions and leading to relatively short plasma half-lives.[20] Moreover, construction of Fab in the recombinant plasmid is simpler and faster than the construction of scFv.

Conclusions

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In conclusion, the expression of linear PCR-amplified immunoglobulin gene using mammalian cell gave a higher quantity and quality of recombinant mAb fragment than the use of the *E. coli* cell-free expression system. However, the expression of these genes in the *E. coli* cell-free system is the fastest (2 h), allowing the system for automation to reduce the burden of antibody discovery and engineering.

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Conflicts of interest.—The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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Figure 1.—Results of 5′-RACE PCR for amplification of the cognate pair of VL (upper) and VH genes (below).

Figure 2.—Schematic illustration of antibody generation using mammalian cells and cell-free expression system. The time requirements in the cell-free expression is very fast (5 h) over the mammalian cell-based expression which needs 75 h.

Figure 3.—TS-jPCR products analysis of linear PCR-amplified immunoglobulin variable genes with Ig-cassette. The results of TS-jPCR the Lc (1) and Hc genes (3) are partly shown. The PCR product (3 μl) was analyzed on a 1.0% agarose gel. Corresponding lane numbers for the (lane 1) indicate fragments jointed with Ig-cassette. The lane 2 is linear PCR-amplified immunoglobulin cassette without TS-jPCR, and 4 control without template was also performed. The λ-DNA marker digested by EcoT141 was in the middle (M).

Figure 4.—ELISA analysis of 3 (three) Fab fragments synthesized in mammalian cell expression and cell-free expression. The specific activity of recombinant antibodies is expressed as relative light units (RLU/s).

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