

Mammalian

by Muhamad Ali

Submission date: 05-Oct-2019 08:57AM (UTC+0700)

Submission ID: 1186404419

File name: Mammalian.pdf (1.22M)

Word count: 5947

Character count: 32435

ORIGINAL ARTICLE

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

Muhamad ALI

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

Corresponding author: Muhamad Ali, Laboratory of Microbiology and Biotechnology, Faculty of Animal Sciences, Mataram University, Jl. Majapahit No. 62 Mataram, 83125, Indonesia. E-mail: ali.molbiotech@gmail.com

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 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 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.

(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 Biotech 2017;29:33-40. DOI: 10.23736/S1120-4826.16.02120-0)

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 specificity³ 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 deadly 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 MAGrahder 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[®] mRNA DIRECT[™] 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[™] III Reverse Transcriptase, Invitrogen), TdT buffer, TdT reaction, TE buffer, and 1 x Primer-STAR GC Buffer-0.1% TritonX-100 were dispensed automatically using MAGrahder 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 MAGrahder 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.

4

TABLE I.—List of primers used in this study.

Name	Sequence (5'-3')	Application
dC13 S	CGGTACCGCGGGCCCGGGATCCCCCCCCCCCCDN	5'-RACE PCR 1st PCR
P0 for IgG	ACCYTGCATTGAACTCCTTGCC	5'-RACE PCR 1st PCR
P0 for IgK	ACTGCCATCAATCTTCCACTTGACA	5'-RACE PCR 1st PCR
IgG	ACCYTGCATTGAACTCCTTGCC	5'-RACE PCR 1st PCR
IgK	ACTGCCATCAATCTTCCACTTGACA	5'-RACE PCR 1st PCR
IgGV AS2	CTGCCGACGACGGGAAGGTGCGTAC	5'-RACE PCR 2nd PCR
IgKV AS2	GGGTGGTGCCATCCACCTCCCAGGTGAC	5'-RACE PCR 2nd PCR
Igλ AS2	CTGGACAGGGATCCAGAGTTCCA	5'-RACE PCR 2nd PCR
Nhe-Eco47	CGCTAGCGCTACCGACTCAGATCC	5'-RACE PCR 2nd PCR
BgIT7P	AGAGATAGATCTATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAAT	Cell-free expression construct
BgIT7T	GTGTGTGGGCCCATTGGTATATCTCTTCTTAAAGTTAAACAAAATTATTCTAGAGGGAAACCG	Cell-free expression construct
Mini.CMV.S	AGAGAAACCGTCTATCAGGGCGATGGC	TS-jPCR
Mini.CMV.AS	AGAGACCCTTTGACGTTGGAGTGCCACG	TS-jPCR

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 (40) dT) 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 *DpnI* 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 BgIT7SD-S and BgIT7SD-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.

7

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 used²¹ are listed in Table I. The reaction was performed 10 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 at 4 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

DNA transfection of the mammalian expression constructs produced by TS-jPCR was³⁰ formed 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.

29

Bacterial cell-free expression

For the cell-free expression, the cell-free expression construct produced¹⁹ 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 instruction²⁸ manual of PURExpress *In Vitro* Protein Synthesis Kits (New England Biolabs, Ips-

wich, MA, USA). Briefly, 3 µl each of TS-jPCR 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, 1.2 µl of disulfide enhancer-2, and nuclease-free dH₂O). 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 previously¹³ 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 MEM 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

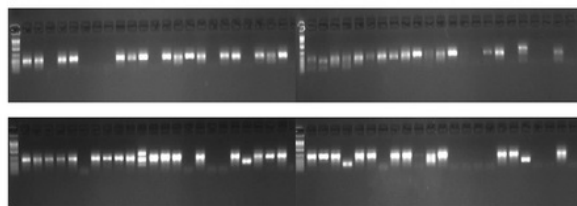


Figure 1.—Results of 5'-RACE PCR for amplification of the cognate pair of VL (upper) and VH genes (below).

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 an 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 MAGrahder 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

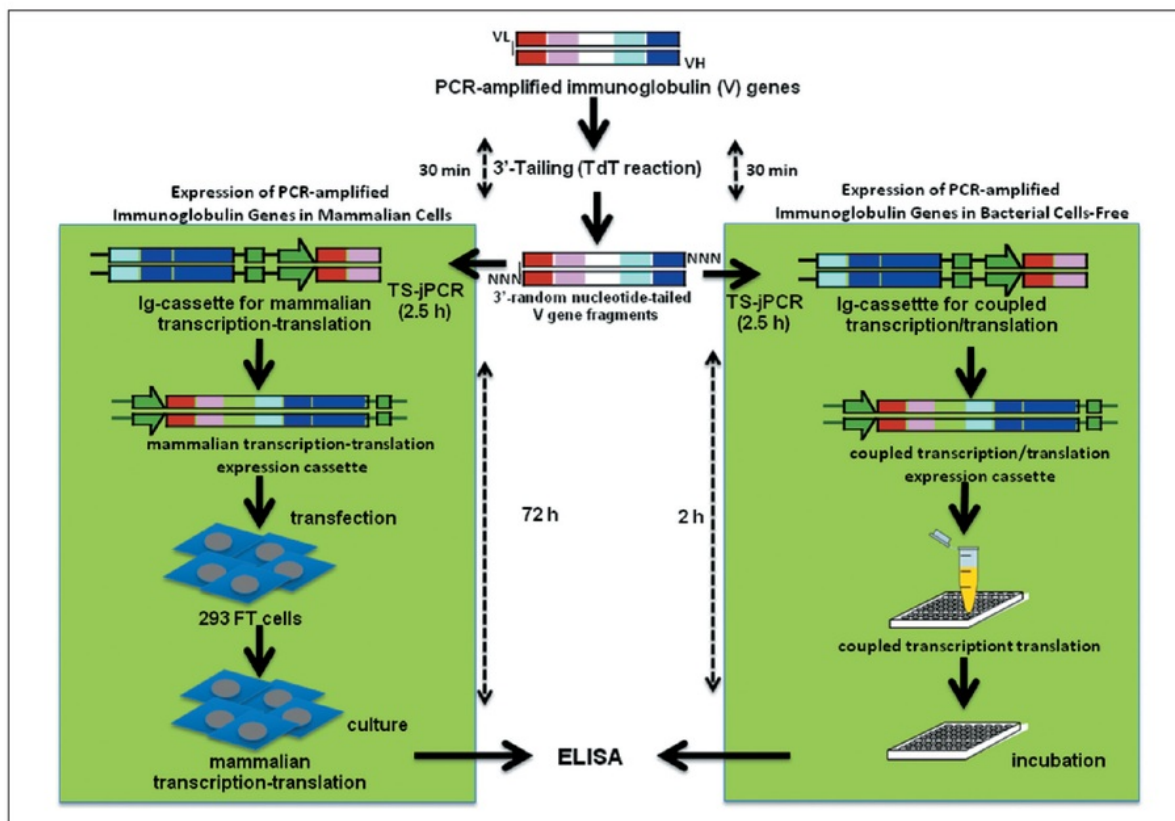


Figure 2.—Schematic illustration of antibody generation using mammalian cells and cell-free expression system. The time requirements in the cell-free expression is very fast (5 h) over the mammalian cell-based expression which needs 75 h.

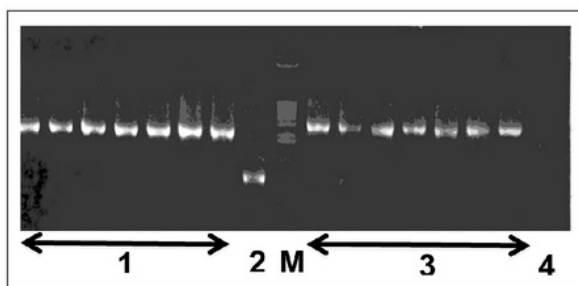


Figure 3.—TS-jPCR products analysis of linear PCR-amplified immunoglobulin variable genes with Ig-cassette. The results of TS-jPCR the Lc (1) and Hc genes (3) are partly shown. The PCR product (3 µl) was analyzed on a 1.0% agarose gel. Corresponding lane numbers for the (lane 1) indicate fragments jointed 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).

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 activity 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

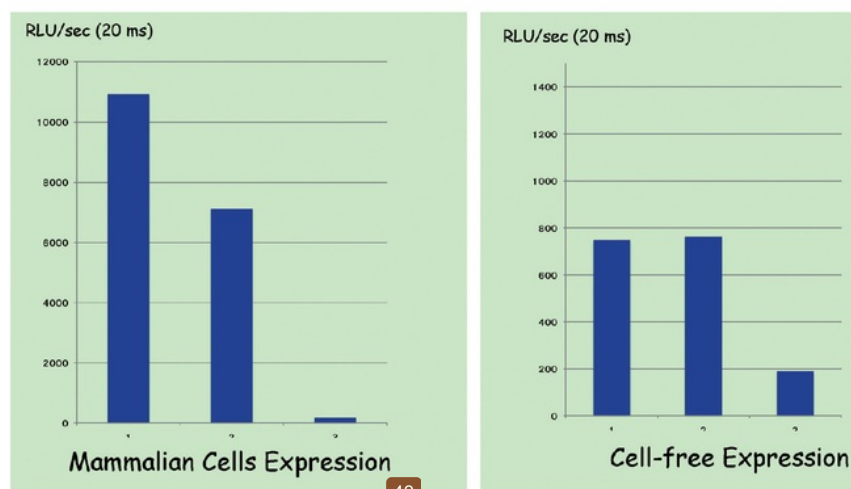


Figure 4.—ELISA analysis of 3 (three) Fab fragments synthesized in mammalian cell expression and cell-free expression. The specific activity of recombinant antibodies is expressed as relative light units (RLU/s).

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 (1) 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 (26) per 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 (1) 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 (36) amount of protein produced and then reduce the antigen-binding activity of the antibody.

(35) 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 (5) 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 (2) 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 (5) responding Fab fragments. The scFv lacks the whole constant region of light and heavy chain, making the antibody fragment unable to mediate effector function (2) 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 (34) 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.

References

- Berger M, Shankar V, Vafai A. Therapeutic applications of monoclonal antibodies. *Am J Med Sci* 2002;324:14-30.
- Krauss J. Recombinant antibodies for the diagnosis and treatment of cancer. *Mol Biotechnol* 2003;25:1-17.
- Fang J, Qian JJ, Yi S, Harding TC, Tu GH, VanRoey M, Jooss K. Stable antibody expression at therapeutic level using the 2A peptide. *Nat Biotech* 2015;23:584-90.
- Nelson PN, Reynolds GM, Waldron EE, Ward E, Giannopoulos K, Murray PG. Monoclonal antibodies. *J Clin Pathol* 2006;53:111-7.
- Aston R, Cowden WB, Ada GL. Antibody mediated enhancement of hormone activity. *Mol Immunol* 1989;26:435-46.
- Ali M. High-throughput monoclonal antibody production using cell-free protein synthesis system. Dissertation. Nagoya University, Japan, 2006.
- Houdebaine LM. Transgenic animal bioreactors. *Transgenic Res* 2000;9:305-20.
- Schwartz JR. Advances in *Escherichia coli* production of therapeutic proteins. *Curr Opin Biotechnol* 2001;12:195-01.
- Chu L, Robinson DK. Industrial choices for protein production by large-scale cell culture. *Curr Opin Biotechnol* 2001;12:180-87.

10. Ali M, Hitomi K, Nakano H. Generation of monoclonal antibodies using simplified single-cell RT-PCR and cell-free protein synthesis. *J Biosci Bioeng* 2006;101:284-6.
11. Sabrina Y, Ali M, Nakano H. In vitro generation of anti-hepatitis B monoclonal antibodies from a single plasma cell using single-cell RT-PCR and cell-free protein synthesis. *J Biosci Bioeng* 2010;109:75-82.
12. Yoshioka M, Kurosawa N, Isobe M. Target-selective joint polymerase chain reaction: A robust and rapid method for high-throughput production of recombinant monoclonal antibodies from single cells. *BMC Biotechnol* 2011;11:75.
13. Kurosawa N, Yoshioka M, Fujimoto R, Yamagishi F, Isobe M. Rapid production of antigen-specific monoclonal antibodies from a variety of animals. *BMC Biology* 2012; 10: 80.
14. Agüena M and Spira B. RT-PCR of long prokaryotic operon transcripts without DNase treatment. *J Microbiol Methods* 2003;55:419-23.
15. Lowe JB, Marth JD. A genetic approach to mammalian glycan function. *Annu Rev Biochem* 2003;72:643-91.
16. Butler M. Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals. *Appl Microbiol Biotechnol* 2005;68:283-91.
17. Katzen F, Chang G, Kudlicki W. The past, present and future of cell-free protein synthesis. *Trends in Biotechnol* 2005;23:150-6.
18. Ali M, Suzuki H, Fukuba H, Jiang X, Nakano H, Yamane T. Improvements in the Cell-Free Production of Functional Antibodies Using Cell Extract from Protease-Deficient *Escherichia coli* Mutant. *J Biosci and Bioeng* 2005;99:181-6.
19. Simmons LC, Reilly D, Klimowski L, Raju TS, Meng G, Sims P, *et al.* Expression of full-length immunoglobulins in *Escherichia coli*: rapid and efficient production of glycosylated antibodies. *J Immunol Methods* 2002;263:133.
20. Hudson PJ, Souriau C. Engineered antibodies. *Nat Med* 2003;9:129.

Funding.—This work was supported by grants from Hokuriku Innovation Cluster for Health Science (the Ministry of Education, Culture, Sport, Science, and Technology, Japan) and Directorate General of Higher Education (the Ministry of Education and Culture, Indonesia).

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

Acknowledgments.—The author would like to thank Professor Masaharu ISOBE, Dr. Nobuyuki KUROSAWA and Dr. Yoshioka MEGUMI (Laboratory of Molecular and Cellular Biology, Graduate School of Innovative Life Sciences, Toyama University) for their advice and support on MAGRAhd, RACE-PCR, TS-jPCR, transfection, and mammalian cells expression. Also to Professor Hideo NAKANO and Dr. Takaaki KOJIMA (Laboratory of Molecular Biotechnology, Graduate School of Bioagricultural Sciences, Nagoya University) for their helpful discussion on the advance cell-free expression system.

Manuscript accepted: April 22, 2016. - Manuscript received: April 14, 2016.

Mammalian

ORIGINALITY REPORT

17%

SIMILARITY INDEX

9%

INTERNET SOURCES

15%

PUBLICATIONS

5%

STUDENT PAPERS

PRIMARY SOURCES

- | | | |
|---|---|--|
| <div style="background-color: red; color: white; width: 40px; height: 40px; display: flex; align-items: center; justify-content: center; margin-bottom: 10px;">1</div> | <div style="color: red;">Muhamad Ali, Hirotatsu Suzuki, Takako Fukuba, Xiuping Jiang, Hideo Nakano, Tsuneo Yamane. "Improvements in the cell-free production of functional antibodies using cell extract from protease-deficient Escherichia coli mutant", Journal of Bioscience and Bioengineering, 2005</div> <div style="color: gray; font-size: small;">Publication</div> | <div style="font-size: 2em; color: gray;">2%</div> |
| <div style="background-color: purple; color: white; width: 40px; height: 40px; display: flex; align-items: center; justify-content: center; margin-bottom: 10px;">2</div> | <div style="color: purple;">www.isisn.org</div> <div style="color: gray; font-size: small;">Internet Source</div> | <div style="font-size: 2em; color: gray;">2%</div> |
| <div style="background-color: purple; color: white; width: 40px; height: 40px; display: flex; align-items: center; justify-content: center; margin-bottom: 10px;">3</div> | <div style="color: purple;">In-Seok Oh. "Cell-free production of functional antibody fragments", Bioprocess and Biosystems Engineering, 08/23/2009</div> <div style="color: gray; font-size: small;">Publication</div> | <div style="font-size: 2em; color: gray;">1%</div> |
| <div style="background-color: teal; color: white; width: 40px; height: 40px; display: flex; align-items: center; justify-content: center; margin-bottom: 10px;">4</div> | <div style="color: teal;">toyama.repo.nii.ac.jp</div> <div style="color: gray; font-size: small;">Internet Source</div> | <div style="font-size: 2em; color: gray;">1%</div> |
| <div style="background-color: green; color: white; width: 40px; height: 40px; display: flex; align-items: center; justify-content: center; margin-bottom: 10px;">5</div> | <div style="color: green;">Jostock, T.. "Rapid generation of functional human IgG antibodies derived from Fab-on-phage display libraries", Journal of Immunological Methods, 200406</div> <div style="color: gray; font-size: small;">Publication</div> | <div style="font-size: 2em; color: gray;">1%</div> |

6

Muhamad Ali, Kiyotaka Hitomi, Hideo Nakano.
"Generation of monoclonal antibodies using
simplified single-cell reverse transcription-
polymerase chain reaction and cell-free protein
synthesis", Journal of Bioscience and
Bioengineering, 2006

Publication

1 %

7

www.biomedcentral.com

Internet Source

1 %

8

www.jove.com

Internet Source

<1 %

9

Julian K-C. Ma, Pascal M. W. Drake, Paul
Christou. "The production of recombinant
pharmaceutical proteins in plants", Nature
Reviews Genetics, 2003

Publication

<1 %

10

P. KENGNE. "Molecular differentiation of three
closely related members of the mosquito
species complex, Anopheles moucheti, by
mitochondrial and ribosomal DNA
polymorphism", Medical and Veterinary
Entomology, 6/2007

Publication

<1 %

11

Michael Hust. "Selection Strategies II: Antibody
Phage Display", Handbook of Therapeutic
Antibodies, 01/01/2007

Publication

<1 %

12

Submitted to National University of Singapore

Student Paper

<1 %

13

www.tandfonline.com

Internet Source

<1 %

14

Yunita Sabrina, Muhamad Ali, Hideo Nakano.
"In vitro generation of anti-hepatitis B
monoclonal antibodies from a single plasma cell
using single-cell RT-PCR and cell-free protein
synthesis", Journal of Bioscience and
Bioengineering, 2010

Publication

<1 %

15

repositorium.sdum.uminho.pt

Internet Source

<1 %

16

Sulaiman, Ngongu Depamede, Asri Niken,
Indah Julisaniah Nur, F Suryadi Bambang, and
Kisworo Djoko. "Isolation and partial purification
of lysozyme from saliva of Bali cattle (*Bos
sondaicus*) using an aqueous mixture of
polyethylene glycol (PEG) with sodium sulfate",
AFRICAN JOURNAL OF BIOTECHNOLOGY,
2012.

Publication

<1 %

17

microbialcellfactories.biomedcentral.com

Internet Source

<1 %

18

Submitted to Mahidol University

Student Paper

<1 %

- | | | |
|----|--|------|
| 19 | Jonghyeon Shin, Vincent Noireaux. " An Cell-Free Expression Toolbox: Application to Synthetic Gene Circuits and Artificial Cells ", ACS Synthetic Biology, 2012
Publication | <1 % |
| 20 | www.nature.com
Internet Source | <1 % |
| 21 | Ana Silva. "Molecular Basis of Cold Resistance: the role of Frost and Muc68E", Repositório Aberto da Universidade do Porto, 2014.
Publication | <1 % |
| 22 | "Cell-Free Protein Synthesis", Wiley, 2007
Publication | <1 % |
| 23 | www.physiology.org
Internet Source | <1 % |
| 24 | Aaron Winters, Karyn McFadden, John Bergen, Julius Landas et al. "Rapid single B cell antibody discovery using nanopens and structured light", mAbs, 2019
Publication | <1 % |
| 25 | publish.kne-publishing.com
Internet Source | <1 % |
| 26 | Jermutus, L.. "Recent advances in producing and selecting functional proteins by using cell-free translation", Current Opinion in | <1 % |

- 27 Lavinia Liguori. "A Bacterial Cell-Free Expression System to Produce Membrane Proteins and Proteoliposomes: From cDNA to Functional Assay", Current Protocols in Protein Science, 11/2008

Publication

- 28 Philip G. Penketh. "1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine (KS119): a Cytotoxic Prodrug with Two Stable Conformations Differing in Biological and Physical Properties : Small Molecule Conformation and Activity", Chemical Biology & Drug Design, 10/2011

Publication

- 29 Eingereicht an Universitaet Dortmund Hochschulrechenzentrum am 2012-12-11

Student Paper

- 30 Shun Matsuzawa, Yuka Wakata, Fumiya Ebi, Masaharu Isobe, Nobuyuki Kurosawa. "Development and validation of monoclonal antibodies against N6-methyladenosine for the detection of RNA modifications", PLOS ONE, 2019

Publication

- | | | |
|---|---|----------------|
| <div style="background-color: #8B4513; color: white; display: inline-block; width: 40px; height: 40px; text-align: center; line-height: 40px;">31</div> | <p>Biljana Bogicevic, Hélène Berthoud, Reto Portmann, Leo Meile, Stefan Irmler. "CysK from <i>Lactobacillus casei</i> encodes a protein with O-acetylserine sulfhydrylase and cysteine desulfurization activity", <i>Applied Microbiology and Biotechnology</i>, 2011</p> <p>Publication</p> | <p><1 %</p> |
| <hr/> | | |
| <div style="background-color: #005596; color: white; display: inline-block; width: 40px; height: 40px; text-align: center; line-height: 40px;">32</div> | <p>Tomohiro Makino, Ryuichi Nakamura, Maki Terakawa, Satoshi Muneoka et al. "Analysis of peripheral B cells and autoantibodies against the anti-nicotinic acetylcholine receptor derived from patients with myasthenia gravis using single-cell manipulation tools", <i>PLOS ONE</i>, 2017</p> <p>Publication</p> | <p><1 %</p> |
| <hr/> | | |
| <div style="background-color: #800080; color: white; display: inline-block; width: 40px; height: 40px; text-align: center; line-height: 40px;">33</div> | <p>Submitted to University of Leeds</p> <p>Student Paper</p> | <p><1 %</p> |
| <hr/> | | |
| <div style="background-color: #6B8E23; color: white; display: inline-block; width: 40px; height: 40px; text-align: center; line-height: 40px;">34</div> | <p>Submitted to University College London</p> <p>Student Paper</p> | <p><1 %</p> |
| <hr/> | | |
| <div style="background-color: #005596; color: white; display: inline-block; width: 40px; height: 40px; text-align: center; line-height: 40px;">35</div> | <p>elib.uni-stuttgart.de</p> <p>Internet Source</p> | <p><1 %</p> |
| <hr/> | | |
| <div style="background-color: #0070C0; color: white; display: inline-block; width: 40px; height: 40px; text-align: center; line-height: 40px;">36</div> | <p>"Human Monoclonal Antibodies", Springer Nature, 2019</p> <p>Publication</p> | <p><1 %</p> |
| <hr/> | | |
| <div style="background-color: #FF0000; color: white; display: inline-block; width: 40px; height: 40px; text-align: center; line-height: 40px;">37</div> | <p>Hong, S.H.. "A phagemid system enabling easy estimation of the combinatorial antibody library</p> | <p><1 %</p> |

38

jcb.rupress.org

Internet Source

<1 %

39

edoc.ub.uni-muenchen.de

Internet Source

<1 %

40

Submitted to iGroup

Student Paper

<1 %

41

Submitted to University of Queensland

Student Paper

<1 %

42

Nobuyuki Kurosawa, Yuka Wakata, Tomonao Inobe, Haruki Kitamura et al. "Novel method for the high-throughput production of phosphorylation site-specific monoclonal antibodies", Scientific Reports, 2016

Publication

<1 %

43

Submitted to Indiana University

Student Paper

<1 %

44

Submitted to University of Birmingham

Student Paper

<1 %

45

M. Sato. "Production and Characterization of Transgenic Mice Systemically Expressing Endo-Galactosidase C", Glycobiology, 10/27/2007

Publication

<1 %

46

Shun Matsuzawa, Masaharu Isobe, Nobuyuki

Kurosawa. "Guinea pig immunoglobulin VH and VL naïve repertoire analysis", PLOS ONE, 2018

Publication

<1%

47

Submitted to Queen's University of Belfast

Student Paper

<1%

Exclude quotes Off

Exclude matches Off

Exclude bibliography On