# HIGH-THROUGHPUT IN VITRO

*by* Muhamad Ali

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**Mataram, 10th December 2018**

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**Preface of the Proceeding of**
**The 3rd International Conference on Science and Technology 2018**

Bismillaahirrahmaanirrahiim
Assalaamu'alaikumwarahmatullaahwabarakaatuh.
Peace be upon us.

Praise always we pray to God Almighty for giving us the abundance of grace, guidance and inayah, so that we all could meet in Lombok, a beautiful island "the Island of Thousand Mosques" in West Nusa Tenggara Province. Our Lombok island known to its many natural and cultural diversity where you can enjoy cuisines, beaches, waterfalls, mountain, traditional villages and handicraft of many ethnics in this Island.

On Behalf the Committee, I would like to thank you all attendee of the "3rd International Conference on Science and Technology (ICST) 2018" on December 10th 2019 and shared impressive ideas, knowledge and experiences through the article to build network for possible future collaboration, therefore the proceedings can be realized.

This proceeding published 43 articles from 107 presenters that came from various universities and research institutions in Indonesia and from overseas. Research papers already reviewed on the basis of a full length manuscript that accepted based on quality, originality and relevance.

At this moment, the organizing committee would like to express our gratitude to the all keynote speakers and presenters who have submitted for article and also to all participants to share their acknowledged works, your effort and contribution to the conference are absolutely valuable. Our special gratitude also goes to the Rector of the University of Mataram and Head of Institute for Research and Community Services) University of Mataram, West Nusa Tenggara, Lombok, Indonesia, who have been highly supporting the conference.

Last but not least, I would like to thank the organizing committee as well as all other supporters and participants, as without their effort, commitment and hard work, the publication of this proceeding will be hardly achieved. Critics and suggestions on the improvement of this proceeding will be highly appreciated. Hopefully the next ICST can be better prepared based on this recommendation.

Wassalamu'alaikum warohmatullahi wabarakatuh.

Chairman of 3rd ICST 2018

Dr.rer.nat. Lalu Rudyat Telly Savalas, M.Si.

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# HIGH-THROUGHPUT *IN VITRO* CONSTRUCTION OF PROTEIN LIBRARY: A POTENTIAL TECHNOLOGY FOR CONTAINING AN EMERGING PANDEMIC AVIAN INFLUENZA

**Muhamad Ali[1] and Yunita Sabrina[2*]**

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**Abstract**. A highly pathogenic avian influenza A virus (subtype H5N1) that circulates in wild and domesticated poultry the most serious human disease for decades. This virus is sporadically transmited from poultry to human and limited probable human-to-human transmission. Since the avian virus is RNA virus which reassort quickly, transmission to human is very fast and difficult to combat. Therefore, identifying a ultra-high-troughput technology to halt a pandemic is very urgent. *In vitro* construction of protein library using cell-free technology was found to be successful in rapid screening and generation of vaccine and monoclonal antibodies. This technology can by pass most of the biological process and allows itself to robotic automation for ultra-high-throughput screening and generation of suitable recombinant functional protein to combat highly spread and pathogenic H5N1 viruses.

**Keywords:** Avian influenza, H5N1, Protein library, cell-free protein synthesis system

## 1. Introduction

Influenza virus infection is a common disease infecting a variety of animals, birds, and humans. The infection is caused by single-stranded negative sense RNA viruses under family *Orthomyxoviridae*. The influenza viruses are divided into three categories; type A, B, and C based on their nucleoproteins and matrix proteins. Type A is those viruses which are generally infecting swine, horses, domesticated and wild birds as well as human. Meanwhile, type B and C are viruses which are predominantly infect humans [1]. Influenza A viruses are commonly known to circulate in wild aquatic birds population and ocassionally transmitted to other hosts, including domestic poultry, pigs, horses, and humans [2].

Viral proteins in the influenza virus are grouped into three which are: the internal proteins, the surface proteins, and nonstructural proteins. The surface proteins are the only proteins which could induce neutralizing antibody and therefore a protective immune response in infected animals, bird, and humans. Based on the surface proteins, influenza A viruses divide into two subtype, hemagglutinin (HA) and neuraminidase (NA). Both the subtypes have high differences in sequences and antigens. HA surface protein consists of 16 subtypes (H1-H16), and NA surface protein consists

of 9 subtypes (N1-N9) [3]. Out of HA subtype recovered from poultry, the H5 and H7 subtypes are the most associated with t high-pathogenicity phenotype [4].

Highly pathogenic avian influenza A (subtype H5N1), is causing widespread outbreaks among wild and domestic poultry in Africa, Southeast Asia, and the Middle East, with sporadic transmission from the poultry to human and human-to-human. The influenza virus has been reported to infect humans from several countries including Indonesia, Bangladesh, Cambodia, China, and Egypt. According to World Health Organization, Indonesia leads the number of total human influenza (H5N1) cases followed by Vietnam, Thailand, and China [5]. The spread of the virus in wild and domestic poultry represent the most serious human disease for decades.

A key format in the global strategy to co rol influenza pandemic is the development of an effective vaccine. Therefore, numerous vaccines have been developed and experimentally displayed to be eff cious for the disease prevention. These include the commercially fowlfox recombinant vaccine, baculovirus expressed proteins, DNA vaccines, and plant based vaccines.

In therapeutic opportunity point of view, monoclonal antibody which specifically inhibits antigen activity of highly pathogenic avian influenza was identified and characterized [6 ,9,10]. Moreover, an antibody (65C6) was reported by Hu *et al*. [11] to recognize a neutralization epitope in the globular head of HA, a conserved epitope among all divergent H5N1 influenza stains. These research results suggest that the antibody might be an effective therapeutic candidate for a wide range of influenza viruses type A.

However, the number of pathogenic avian influenza outbreaks were increasing even though various vaccines and antibodies were available [12]. Evolution and antigenic drift accumulation particularly in hemagglutinin where antibodies induced from prior infection is less competent in protecting against the mutated virus appear to be reasons of this lack of control [12]. Immunity against the new subtype does not exist making it capable of spreading worldwide [13] Therefore, the vaccines or antibodies have been evaluated every year to display how simila they are to the recent wild-type virus. As the results, the vaccines or antibodies have ben frequently reformulated with more recent isolates to optimize protection against the circulating influenza viruses.

To obtain a novel vaccines and antibodies from the reformulation, construction of wide protein libraries is absolutely necessary. The protein libraries should be constructed in high-throughput format. In this article, we offer cell-free protein synthesis technology for generation of protein libraries which are needed for containing pandemic avian influenza.

## 2. *In vivo* and *In vitro* Construction of Protein Library

Since the virus RNA polymerase complex of H5N1 virus lacks of proof reading capability, the genetic changes of H5N1 virus (antigenic shift and antigenic drift) occur at a high rate. Antigenic shift or gene reassortment mainly causes the virus become pandemic, whereas antigenic drift which caused by minor change (point mutations) in the gene encoding the viral surface antigen commonly causes annual epidemics.

To meet the wide variety of vaccines or antibodies needed because of the fast antigenic shift and antigenic drift, the high-throughput construction of the protein libraries for screening a suitable recombinant protein is absolutely important. Although conventional recombinant protein production in large scale using bacterial living cells (cells expression system) is widely used now for high-throughput generation, however, it is indispensable for rapidly testing affinity and specificity.

In this review, we compare the conventional library construction using bacterial system and more high-throughput version using cell-free system. As shown in Figure 1, the conventional *in vivo* version seems a long and detour way than *in vitro* format. The conventional protein library construction in *E. coli* system takes many steps and extensive time (50 h). Moreover, the system need time consuming steps, expensive cost, and laborious work. The basic overhead cost comes primarily from extensive use of restriction and ligation enzyme, vector, competent cells of host, plasmid, media, and protein purification apparatus. The PCR colony, sequencing, and protein purification are the most extremely labor intensive among the procedures needed.

The bacterial construct may not always be a final format of the antigen or antibody, and bacterial expression may become a detour. Because living cells are: (a) very complex, diverse interacting gene products as well as subcellular components, (b) very crowded (protein concentrations

within the cell); (c) being surrounded by a plasma membrane, obscuring the reaction environment for manipulation.

A significant, attractive, and powerful option in the high-throughput construction of protein libraries for generation of recombinant protein is the use of cell-free protein synthesis system in which the recombinant proteins are expressed in an *in vitro* coupled transcription-translation reaction. The application of this method requires shorter time (6 h) than *in vivo* one. Figure 1 shows the Schematic diagram of the construction of a protein library using in vivo and in vitro system.

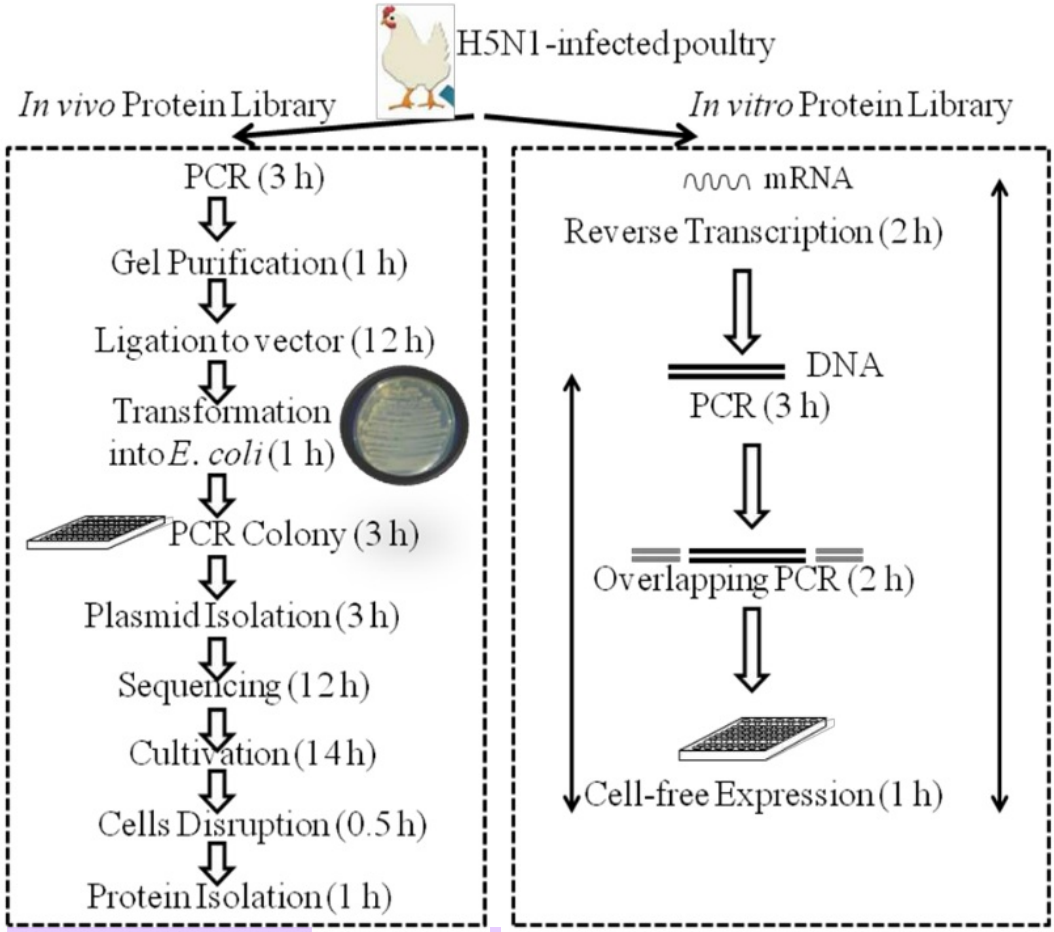


**Figure 1**. Schematic diagram of the contruction of a protein library. The *iv vitro* system can construct a protein library within 6-8 h (6 h for antigen indicated by shorter arrow and 8 h for monoclonal antibodies indicated by longer arrow) while the conventional method (expression in *E. coli*) takes 50 h.

### 3. Merits of *In vitro* Protein Library Construction

The cell-free protein synthesis systems have various advantages over *in vivo* systems, for example, much higher throughput because it is fast process and can be performed in a multi-well plate [14], a PCR product can be used as template directly without cloning [15,16], and reaction conditions can be easily modified to provide an optimum environment for recombinant proteins expression in their native conformations.

Protein synthesized in the cell-free systems display the same accuracy as in *in vivo* expression [17]. Ali *et al*. [18] reported that 20 $\mu$g of the 6D9 Fab fragment could be purified from 1 ml of cell-free reaction, which was comparative to the production using *in vivo* hybridoma cells. Moreover, Ali *et al*. [19] and Ali [20] indicated that the cell-free system could be used effectively to produce fully functional antibody with an excellent antigen-binding capacity.

The systems can by passess most of the biological processes and allows itself to robotic automation for high-throughput expression of genetic information [21]. Because of these merits, it was used as a powerful tool for protein folding studies [22], functional monoclonal antibodies production [23] (Sabrina *et al*., 2010), antibody engineering [24], and vaccine production [25].

After finding PCR products to be used as templates for protein synthesis in the cell-free expression system [15], functional mapping of genomes become one of the most important applications of the cell-free protein synthesis technology [24]. This can be performed in a multi-well format for cell-free systems and robotic automation of the process. A similar approach can be applied to high-throughput, cloning-independent screening of a large diversity mutant and engineering protein [26].

One of the greatest impacts of cell-free protein synthesis system could be in the production of membrane protein. The overexpression of membrane proteins by *in vivo* frequently cause cell toxicity, largely owing to their hydrophobicity, misfolding, protein aggregation, and low yields. In some cases, the overexpression of integral membrane proteins (ion channel proteins, transporters, and receptors) may disrupt integrity of the cell membrane and to cell lysis. By contrast, nearly all of these obstacles can be overcome by cell-free protein synthesis system [27].

Another exciting feature of the cell-free systems is the extension of the genetic code by using unnatural amino acids. Several unnatural amino acids can be fused into peptides with *in vivo* system. However, the types of amino acid and the sites of incorporation are limited by the numbers of the naturally occurring aminoacyl transferase. In addition, the use of artificial aminoacyl tRNA and in vitro translation has also been developed [28].

The cell-free protein synthesis system has been successfully used for production of functional antibodies with antigen-binding activities and two single chain monoclonal antibodies against lysozyme [29]. Jiang *et al*. [30] reported that the Fab fragment of a catalytic antibody 6D9 has been successfully synthesized in the *E. coli* cell-free protein synthesis system. More recently, Sabrina *et al* (2010) [25] reported *in vitro* generation of anti-hepatitis B monoclonal antibodies from a single plasma cell using single-cell RT-PCR and cell-free protein synthesis.

The use of cell-free expression system for virion formation of infectious poliovirus [31], hepatitis B capsid and HIV-1 capsid [32] has been reported. Several cytotoxic proteins, which are difficult or impossible to express in cellular environments can be readily produced in vitro. More recently, Yang *et al*., [25] reported that the idiotype (Id)-granulocyte-macrophage colony-stimulating factor (GM-CSF) fusion protein, which a potential vaccine for immunotherapy of B-cell lymphoma, were rapidly expressed in a high amount in an *E. coli* cell-free system.

Specific antibodies for hemagglutinin of the influenza virus have been isolated through a combinatorial expression library which were generated using mRNA obtained from an immunized donor mouse [40]. This finding indicated that the adaptation of these procedures to the generation of libraries from human peripheral blood lymphocytes may provide an effective way of assessing human immune responses to a variety of pathogenic agents.

Since the vast and growing amount of genetic information are available today, the use of traditional cell-based expression systems (bacterial fermentation and mammalian cell culture) to rapidly screen protein activities or interactions using a multiplexed approach is becoming increasingly difficult. These methods require extensive time and labor for cloning, transformation, fermentation, and downstream processing and not easily adaptable to multiplexed format. What is exciting is that the cell-free expression system offers tremendous flexibility for fast and parallel analysis of the products.

The cell-free expression system has taken considerable interest in the protein truncation assay as a method to detect frame-shift or nonsense mutations in marker genes. In brief, PCR amplified gene or specific RT-PCR products are added to transcription and translation reactions, thereafter truncated products are detected using ELISA or SDS-PAGE in a high-throughput format. At the end, the use of cell-free expression is used to study of synthesis and assembly of macromolecules protein complexes.

Among them, *de novo* synthesis of infectious viral particles by the cell-free protein synthesis system has become considerable attention during the past years. *In vitro* production of virus has enabled the development of multiple antiviral agents, aside from renewed concerns about biological weapons and new threats posed by genetic engineering [33].

## 4. Conclusion

The use of *in vitro* cell-free protein synthesis system was found to be successful in rapid screening and generation of antigens and monoclonal antibodies. This method can bypass conventional technology which is time consuming, labor-intensive multistep process. Since this method is rapid, simple, and entirely performed *in vitro*, it is promising for vaccine and antibody generation of highly infectious diseases. Since construction of protein library for containing an emerging pandemic avian influenza need high-throughput technology, the use of *in vitro* cell-free protein synthesis system is very significant, attractive and powerful method.

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