# Molecular Cloning

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**Submission date:** 14-Oct-2020 09:15AM (UTC+0700)
**Submission ID:** 1414557871
**File name:** Molecular_Cloning.pdf (13.85M)
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Lombok-Indonesia. December, 1th-2nd 2016

# ICST 2016

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(Revised Edition-1)


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**Proceedings**
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**Theme** : The Emerging Innovation On Science And Technology

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# PREFACE

Bismillaahirrahmaanirrahiim
Assalaamu'alaikum warahmatullaahi wabarakaatuh.

Praise always we pray to God Almighty for giving us the abundance of grace, guidance and inayah, so that we all can met in the "1stInternational Conference on Science and Technology (ICST) 2016". ICST is a conference where researchers can share and publish their scientific papers about science and technology. The theme of this conference is "Emerging Innovation on Science and Technology for Sustainable Development".

This conference was done for two days, from 1st to 2nd December 2016, and took place in the Green Campus of the University of Mataram.

We received more than one hundred papers from various universities and research institutions in Indonesia and from overseas, but not all of the papers were published in this proceeding. The paper has been selected and grouped based on the similarity of the research field, which then are presented and discussed. Presentation of the papers will be held in eight parallel classes.

At this moment, the organizing committee would like to expressour gratitude to all of you who have participated this conference, especially to the all keynote speakers, presenters who have submitted posters or orally presented papers and also to the participants. Our special gratitude also goes to the Rector of the University of Mataram who has been highly supporting this conference. Last but not least, the organizing committee would like to thank to all of you who have supported this conference.

Wassalamu'alaikum warohmatullahi wabarakatuh.

Chairman of 1st ICST 2016

Dr. Satrijo Saloko

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# Molecular Cloning of Immunoglobulin Genes Repertoire from a Variety of Animals Using a Robotic System

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**Abstract**

Cloning and expression of immunoglobulin light chain (Lc) and heavy chain (Hc) genes from a single cell of hybridoma and immunized mouse have been previously reported (Ali *et al*., 2006; Sabrina *et al*., 2010). Since the cloning relies on a manually single cell isolation and a reverse transcription polymerase chain reaction (RT-PCR), however, the recovery ratio of coupled Lc and Hc genes obtained from the single cell was very low (11.3%). Here, a cloning of the immunoglobulin genes (Ig-genes) from a variety of animals (mouse, rat, and Guinea pig) cells was performed by the use of a robotic system. A single plasma cell of immunized animals was isolated using a fluorescent-activated cell sorting (FACS) and subsequently continued with cDNA synthesis in free-hand handling procedures. The results showed that the recovery ratio of amplified Lc and Hc genes from a variety of animals cells was increased up to 60-90%. This method is useful not only to obtained larger antibody-encoding gene from animal repertoires but also by-pass some steps which laborious, time consuming, and prone to contamination.
*Keywords: Immunoglobulin genes, light chain, heavy chain, RT-PCR, cDNA synthesis.*

## 1. Introduction

Production of a huge diversity of lymphocytes expressing immunoglobulin is the most important tasks of an adaptive immune system in response to large numbers of pathogenic antigens. Based on the "one-cell-one-antibody" clonal selection theory, each developing B-cell of pathogen-infected animals is committed to produce only one antibody structure (Gherardi and Milstein, 1992). During B-cell proliferation, there are multiple rounds of immunoglobulin gene recombination and conversion which then create vast diversity of antibody library. Therefore, the immunized animals can be a major contributor towards generation of the primary immunoglobulin light chain (Lc) and heavy chain (Hc) repertoires for monoclonal antibody generation.

A molecular cloning of immunoglobulin-encoding genes is a powerful tool to immortalize the diversity of immune repertoires and allows for construction of wide varieties of antibody library, and provides re-construction to produce antibody with any desired specificity. Once these genes have been cloned, the antibody fragments can be further modified and expressed in many expression platforms. The cloning which is continued by sequencing forms the basis of antibody modelling, experimental structure determination or X-ray crystallography at a high resolution (Ostermier *et al*., 1995). In addition, the cloning of immunoglobulin genes (Ig-genes) can facilitates antibody engineering to generate another antibody variant which has better quality (Jackson *et al*., 1995; Sturbe and Chen, 2004; Rajval *et al*., 2005) or a modified utility (Maynard *et al*., 2002).

A variety of recombinant DNA technologies have been applied to get the immune repertoire of antibody. The construction of combinatorial libraries by the reverse transcription-

polymerase chain reaction (RT-PCR) to generate cDNAs from pooled B-cells or plasma cells provides a wealth of immunoglobulin sequences (Orlandi, 1989). However, the technique cannot be used to provide the original pairs of heavy (h) and light (L) chains produced in single B cell (Gherardi and Milstein, 1992).

We previously observed that the immunoglobulin G genes (Lc and Hc) were separately amplified from a single hybridoma cell using cDNA-specific primers with 5' homotags and a homotag-specific primer (Ali *et al*., 2006). Furthermore, cloning and expression of Lc and Hc genes from a single plasma cell of immunized mouse were also reported (Sabrina *et al*., 2010). However, since the cloning relies on manually cell isolation and RT-PCR, the recovery ratio of coupled Lc and Hc genes from single cell was very low (11.3%). The other drawback is cross-contaminations due to the manual input of all required materials.

Thus, in this present study, cloning of the Ig-genes from a variety of animal cells was performed by the use of a robotic system. A single plasma cell of immunized animals was isolated using a fluorescent-activated cell sorting (FACS) and subsequently continued by cDNA synthesis in free-hand handling procedures. The results of this study indicated that the recovery ratio of amplified Lc and Hc genes from a variety of animals including mouse, rat, and Guinea pigs cells was increased up to 60-90% comparing to previous method. Cross-contaminations which usually occur during cDNA synthesis in manually input procedure were successfully reduced. This method is not only useful to obtained larger antibody-encoding gene from animal repertoires but also could skip or by-pass some procedures which expensive, laborious, time consuming, and prone to contamination.

## 2. Materials and Methods

### 2.1 Immunization of animals and preparation of plasma cells

Plasma cells of an immunized animal were prepared according to a modified protocol of Kurosawa *et al*. (2012). Briefly, Hartley guinea pigs, 6-weeks female ICR mice, and Wistar rats were purchased from Japan SLC, Inc. and used as animal experiments with approval of the Committee on Animal Experimentation at the University of Toyama. Starting with Guinea pigs, immunization was performed four times intramuscularly at the tail with 200 $\mu$l of 50:50 water in oil TiterMax Gold adjuvant emulsion containing 100 $\mu$g of *Plasmodium falciparum* lactate dehydrogenase (PfLDH) antigen. Then, mice were immunized three times (at interval of 3 weeks) subcutaneously in the hind foodpad with a 50 $\mu$l of 50:50 water in oil TiterMax Gold adjuvant emulsion (Sigma-Aldrich) containing 25 $\mu$g of the PfLDH. Lastly, immunization of rats was conducted four time intramuscularly at the tail with a 200 $\mu$l of 50:50 water in oil TiterMax Gold adjuvant emulsion containing 100 $\mu$g of the PfLDH. A week after the final booster, the popliteal lymph nodes were surgically removed and used for the isolation of plasma cells using FACS based on Kurosawa *et al*. (2012). The obtained of cells were stored at -80$^{o}$C until further used.

### 2.2 Set up of Robotic System

A robotic system was set up and arranged as shown in the Fig. 1. To start the cloning of immunoglobulin gene using robotic system, the equipments were turn on including the control computer. Installation of twenty-four neodymium magnetic rods (magfine, ϕ1.5 mm x 20 mm 2910 Gauss) was performed on the head whose pitch matches with 96-well plates. By using a special designed software program, the non-contact magnetic power transmission can be adapted to achieve optimal output.

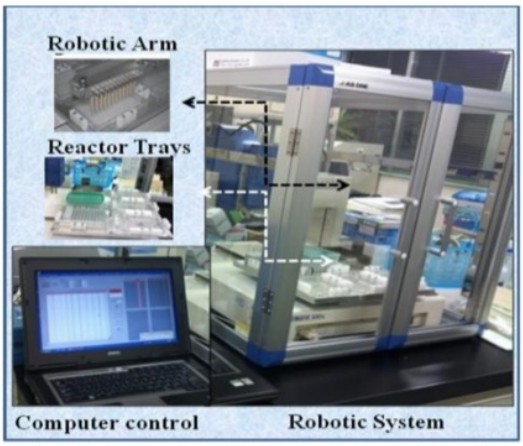


**Figure 1. A robotic system for Ig-genes amplification**

### 2.3 Fabrication of reactor trays

Reactor trays were fabricated according to Yoshioka *et al*. (2011) with small modification. Omni tray (Nunc), thin glass cover plate (Matsunami, No. 1, 73 mm x 118 mm, 0.1 mm thickness), and hydrophobic parafilm were used to produce the trays. Firstly, the parafilm was pressed with an aluminum stamp which has a small protrusion on the surface against a silicone plate. The film has a 24 x 8 grid of positive relief features which consist of two cup-like arcs with 3 mm diameter. Each arc is separated from one another by gaps of approximately 2.5 mm in width to allow magnetic beads to move into the cup and 1 mm in width on the bottom side to allow magnetic beads to move through the grids. Secondly, the surface of film was bound to the inner surface part of the thin plate (Nunc) after pressed against the weight load for 3-5 min at 37°C. Finally, plastic on the surface of layer was removed and used for sample preparation. Droplets of cells or magnetic bead were self-centered which facilitated parallel sample processing by robotic system.

### 2.4 Synthesis of 3'-end homopolymer-tailed cDNA and amplification of Ig-genes

mRNA isolation, 3'-end homopolymer-tailed cDNA synthesis, and Ig-genes amplification from single plasma cells were prepared as previously reported (Kurosawa *et al*., 2011) by a droplet-based solid-phase cDNA synthesis. Primers used in this study were listed elsewhere (Kurosawa *et al*., 2012). The recovery ratio of coupled Lc and Hc genes obtained from the single cell was counted according to agarose gel electrophoresis results.

## 3. Results and Discussion

Single cell-based RT-PCR is a expensive, laborious, time consuming, easily contaminated, and a sensitive technique for the immortalization of Ig-genes from large numbers of single cells (Ali *et al*., 2006). As an alternative solution, a robotic system with an automatic non-contact magnetic power transmission instrument has been developed (Yoshioka *et al*., 2011). The robotic system consists of four main units: computer control, reactor trays, robotic arm, and temperature controller. Every step of robot activity can be programmed and controlled from the computer. The reactor tray is made of a reusable plastic tray and hydrophobic parafilm layer. The robotic arm consisted of 12-chanel parallel magnetic rods which mixes and transports nucleic acid-bound magnetic beads into the reactor trays.

Temperature of the robotic system was controlled and measured by the temperature controller.

During operation, the robotic system mixes nucleic acid and magnetic beads, isolates and extract mRNA, performs reverse transcription, and a homopolymer-tailing reaction (Fig. 2). Touching of robotic arm to the opposite site of thin glass surface of reactor tray allows the transportation of nucleic acid-bound magnetic beads to the next step reaction. Therefore, mRNA from single cell bound to the oligo-dT magnetic beads, can be cleaned from cell lysate by washing buffer, and transported into the reverse transcription mixtures in the next reaction drop for cDNA synthesis.

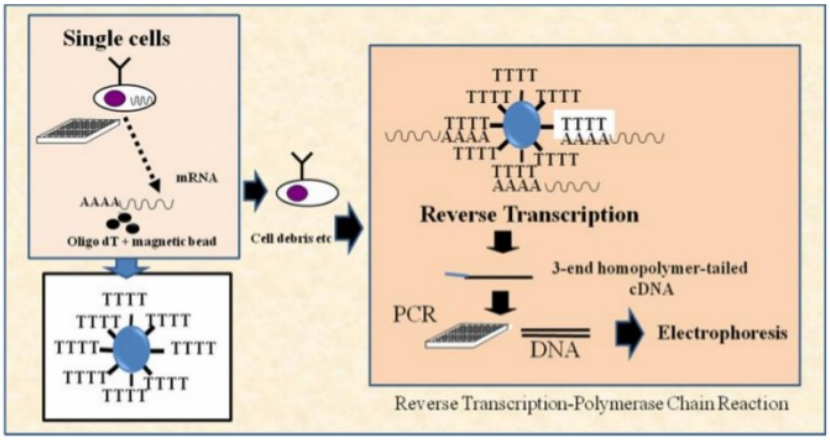


**Figure 2. Cloning of Ig-genes repertoires from single plasma cell using a robotic system**

The results showed that amplification of Lc and Hc were successfully amplified with an overall success rate of 60-90%, Figure 3. The recovery ratio observed in this present study was higher than a recovery ratio of Ig-genes amplification performed by Ali *et al*. (2006) and Sabrina *et al*. (2010) which used manually input. The higher recovery ratio obtained in this study might be due to a better quality of mRNA and cDNA product which were obtained using the robotic system. It seemed that the robotic system made better separation of nucleic acid-bound magnetic beads were separated from residual contaminants, such as cells debris, PCR mixtures (dNTPs, enzyme, primers, salt, etc.). And the pure mRNA and cDNA allowed more efficient downstream processes to generate Lc and Hc of Ig-genes.

In addition, since the processes of mRNA extraction and 3'-end homopolymer-tailed cDNA synthesis were proceed in a closed space (the opposite site of thin glass surface of reactor tray), cross-contamination from laboratory environment could be avoided. The use of the system protected the sensitive RT-PCR mixtures against all sources of contamination.

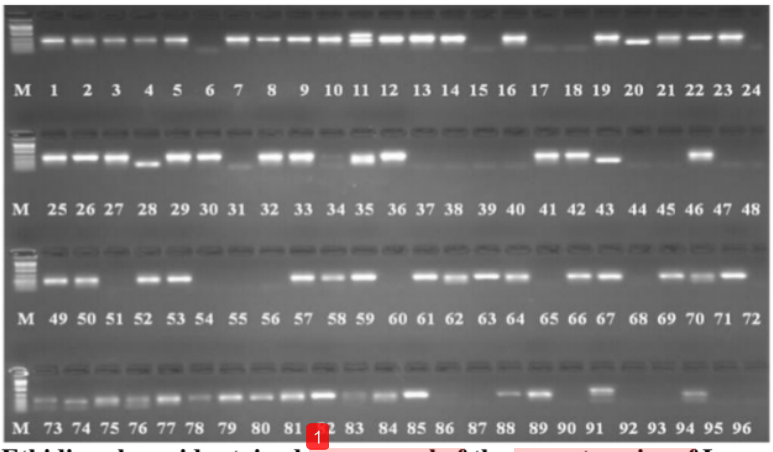


**Figure 3. Ethidium bromide stained agarose gel of the cognate pairs of Lc and Hc genes amplified from single plasma cells. Each lane contains 5 μl of a 25 μl Lc or Hc PCR product. Odd number = Lc, and even = Hc**

Because the screening for Ig-genes repertoire using this system is performed free of hand, millions of lymphocyte cells can be assayed during each round of reaction, which requires only a few hours instead of the many days, weeks, and months required for the conventional screening. This method is useful not only to obtained larger antibody-encoding gene from animal repertoires but also could skip several steps which are laborious, time consuming, and prone to contamination.

## 4. Conclusion

The recovery ratio of amplified Lc and Hc genes from a variety of animals including mouse, rat, and marmot cells was increased up to 60-90%. This method is useful not only to obtained larger antibody-encoding gene from animal repertoires but also by-pass some steps which laborious, time consuming, and prone to contamination.

## Acknowledgments

Thanks to 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 robotic equipment and RACE-PCR. 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 Research, Technology, and Higher Education, Indonesia).

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