Original Article

An Improved Homologous Recombination Method for Rapid Cloning of the Antibody Heavy Chain Gene and Its Comparison with the Homologous Recombination and Traditional Cloning Methods

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Abstract

Background: The homologous recombination (HR) is one of the conventional cloning methods for the production of recombinant DNA. It is a quick method for *in vivo* DNA cloning without using the high cost restriction enzymes. A few modifications in the cloning procedure can increase the efficiency of this method.
Materials and Methods: In this study, effect of heating on the rate of the IgG1 heavy chain gene cloning was investigated in the HR method and then it was compared with HR method without heating and traditional cloning method. For doing this comparison, three cloning methods including HR, HR with the heat treatment, and traditional cloning were used to clone the human IgG1 heavy chain into the pcDNA3.1(+) plasmid.
Results: The results showed that adding heat in the HR method converts insert and vector from the double strand DNA to the single strand DNA. This allows them to copulate with each other better and faster than the two other methods. The heat addition also helps the cell enzyme system for a faster and easier recombination and moreover it increases the cloning efficiency of the HR method in case of *in vitro* processing.
Conclusion: The results showed that giving heat in the HR method increases cloning rate 7.5% and this increase reaches 10% in comparison with the traditional method.
Keywords: Homologous recombination, Gene cloning, Heat, pcDNA3.1, IgG1

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Introduction

In recent years, because of stability and specification of antibodies to join their antigens[1], tumor treatment using immunotherapy became important[2] and so more studies have been carried out on the production of humanized and chimeric antibodies[3]. More antibodies, i.e. Rituximab (Rituxan)[4-5], Infleximab[6-8], Alemtuzumab[7], Panitumumab[5], Trastuzumab[5,9], Infleximab[10], Cetuximab (Eribtux)[5,11], and Herceptin[5,12], which are used and adapted for disease treatment, have been produced by the United states Food and Drug Administration (FDA). Most of the recombinant antibodies have been developed using a traditional cloning method which uses the restriction enzymes. Traditional cloning method is time

consuming and expensive. This method also has some limitations of using enzymes which are not in the insert sequence. Furthermore, there is a possibility of breaking large sequence of DNA using this method[13] as this is an *in vitro* method.

Homologous recombination is one of the cloning methods for the production of recombinant DNA[14, 16]. Bateson[17] realized that because of linked genes on a chromosome there is an exception with one of the principles of inheritance which was originally introduced by Gregor Mendel. Morgan[18] suggested a physical exchange between the homologous chromosomes to cause a recombination in genome. Creighton and McClintock[19] found that this exchange occurs in the meiosis. Later, some studies showed that this exchange has an essential role in repairing damaged DNA, replication fork, and the telomere conservation[13,20,21]. Nowadays, it is used as a method in genetic engineering studies and named the homologous recombination (HR) method. In this method, the RecA protein is needed to join the single strand DNA and search a homolog molecule for it and exchanges homolog sequence[13,21]. The Rec BCD enzyme is needed in cells for producing the single strand DNA at 3´ end of fragment if the double strand DNA is used in the HR method. This single strand DNA is used in the RecA protein as well[22]. The HR is an *in vivo* method and safe to clone large sequence of DNA[13]. The HR has many applications and needs few equipment and materials[23,24]. It is used for chromosome recombination, conditional knockout mutation[25], and DNA cloning[16]. To do DNA cloning, the double-stranded DNA (PCR product) and plasmid DNA are transferred to cells by the electroporation or the chemical methods[25].

The linear plasmid and the DNA fragment, which have two homolog ends with each other, are co-transformed into the *E. coli* RecA positive strains and then the DNA recombination is done[25].

The HR depends on a few numbers of intracellular enzymes and changing the extracellular factors can greatly increase the efficiency of this method.

Yu et al. used the HR with the RecA-negative bacteria and defected λ phage with heat dependent repressors for the Exo, Beta, and Gama genes. They showed that increasing culture temperature improves the recombination efficiency[14]. Heyer compared the HR intermediary proteins in two eukaryotic cells (*i.e.* Human and yeast)[20].

Li and Elledge used a new method based on the HR method which is called the SLIC and assembled several pieces of DNA in a single reaction. They used the Rec A positive bacteria[15]. Filippo et al. studied catalytic effects of two recombinases; i.e. the RAD51 and the Dmc1, on the HR method improvement in the eukaryotic cells[26]. Gao et al. also developed a new method (i.e. SIRT) based on the HR method. They could repeat a target gene in a specific site in drosophila and other organisms[27]. Sharan et al. removed a DNA fragment from the genome using the HR method[23]. Lopez and Blázquez evaluated effects of ciprofloxacin and 10 other antibiotics on the HR method in the *E. coli* cells[28]. Lee et al. improved the HR method for epitope tagging and gene deletion by electroporation DNA fragment, and making DNA fragments *in vivo*[29].

The production of recombinant antibodies as therapeutic proteins is great importance. Because of the antibodies large size of heavy chain, using a reliable and rapid method for its cloning is essential. The HR method is a good method for cloning this sequence. Making some changes in the HR method can make this method even more efficient. Based on the knowledge of the authors and the reviewed literature, none of the recent studies has investigated the effect of adding heat as a factor outside the cell on the homologous recombination method so far.

In this study, the effect of heat on the DNA cloning before entering the gene into the cells was investigated in the homologous recombination method, and the results were compared with the traditional method.

Methods

Effect of heating on IgG1 HR cloning efficiency was investigated and compared with the traditional cloning method. For doing this, IgG1 heavy chain gene was synthesized (Takapouzist Company) and then the study was performed in three parts, namely, HR cloning method without giving heat (Method I), HR cloning method with giving heat up to 95ºC (Method II), and traditional cloning method (Method III).

Insert preparation and plasmid

Preparation of insert and plasmid for the HR methods

A pair of primers with 40 nucleotides for the heavy chain gene of immunoglobulin G1 (1400 base pair) was synthesized (Takapouzist Company). There were 20 nucleotides in the 5` end in each primer which had similar end with the plasmid sequence. Sequences of the used primers were as follows:

Forward:

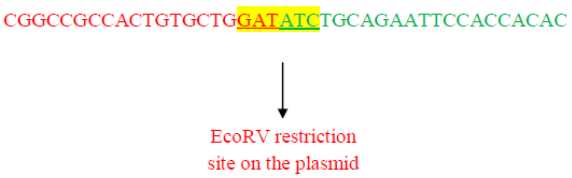
5`CGGCCGCCACTGTGCTGGATATGGGCGTGC CCAGACCCCA -3`

Reverse:

5`GTGTGGTGGAATTCTGCAGATTGCCGGGGC TCAGGCTCAGG-3`

PCR reaction was done for heavy chain gene. Components of PCR reaction: 20ng DNA, 0.2 mM dNTP, 1 unit Pfu DNA polymerase, 3µL 10x Pfu (Fermentas Company) Buffer with MgSO4, 200 Mm Tris-HCl (pH 8.8 at 25°C), 100 mM KCl, 100 mM (NH4)2SO4, 1% Triton X-100, 1 mg/ml BSA, 25 mM MgSO4), 20pmol each of R and F primers, H_2O up to final volume of 30 µL.

pcDNA3.1+ (Neo) plasmid (Pasteur Institute of Iran) was digested by the *EcoRV* restriction enzyme (Takara Company) and dephosphorylated[23]. In order to increase transformation efficiency, the PCR product and the linear plasmid were purified first by electrophoresing on low melting temperature agarose and then using agarose gel extraction kit (QIAGEN).



Preparation of insert and plasmid for the traditional method

A pair of primers with the *BamHI* and the *HindIII* restriction sites for the heavy chain gene of the IgG1 (1400 base pair) was synthesized (Takapouzist Company). Used primers Sequences were as follows:

Forward 5`- TTTTGGATCCCTTGCCGGGGCT-3`

Reverse 5`- TTTTAAGCTTATGGGGGTGCCC-3`

PCR reaction was done for heavy chain gene. Components of PCR reaction are like before.

PCR product of heavy chain gene of the IgG1 and the pcDNA3.1(+) (Neo) plasmid were digested using the *BamH1* and the *HindIII* restriction enzymes in order to sub-clone the gene into the plasmid. To increase the transformation efficiency, the PCR product and the linear plasmid were purified using an agarose gel extraction kit (QIAGN).

Preparation of competent cell

A colony of HB101 (Rec A+) strain (Pasteur Institute of Iran) was cultured in 3 ml LB liquid medium. It was incubated in a shaking incubator for 16 hours at 37°C. Overnight culture was sub-cultured until (OD 600=0.6). Competent cell was prepared using the Hanahan method[30].

Homologous recombination

Because of similarity between the two ends of PCR products with linear plasmid sequence in EcoRV restriction enzyme site, the HR is done after transforming them into RecA+ cells. For doing this, different amounts of insert and vector (1:1, 2:1, and 3:1) were mixed together[30]. At this stage, only prepared samples for studying the heat addition were employed. These samples were heated for 5 minutes at 95°C, and then they were severely shaken and immediately transferred into ice cubes. 50 µL of the competent cells were transferred into each micro-tube containing a mixture of insert and vector. They were then kept on ice for 30 minutes and after that they were heat shocked for 90 seconds at 42°C. They were then kept on ice for 2 minutes; afterwards 100 µL of LB without antibiotics was added to each micro tube. Micro tubes then incubated for 90 to 120 minutes in a shaker incubator. Then the cells were distributed on agar plate with 100 µg/ml Ampicillin and finally they were incubated at 37°C for an overnight[31]. The above procedure was repeated three times for each method.

The Traditional method

Different amounts of insert and vector (1:1, 2:1, and 3:1) were mixed with a T4 ligase enzyme (Fermentas Company) (1unit) for one night at 22ºC. Then they were transferred into the HB101 competent cell. This procedure also was repeated three times.

Screening of recombinant plasmid

The PCR reactions were done on bacterial colonies by universal primers and the gene-specific primers[31]. PCR products of the recombinant plasmids (1400 nucleotides) were digested by the *Hinf I* restriction

enzyme (Takara Company)[31]. It has only one restriction site for the IgG1 heavy chain gene at position 1120.

Results

Preparation of insert and plasmid for the HR method

To prepare insert and vector for doing cloning in both HR methods, PCR reaction was done using specific homologous recombination primers for the IgG1 heavy chain gene. A 1440 bp fragment was seen after electrophoresis on 1.5% agarose gel (Figure 1a). The pcDNA3.1/Neo was digested using the *EcoRV* and compared with undigested plasmid by electrophoresis on 0.8% agarose gel after two hours (Figure 1b).

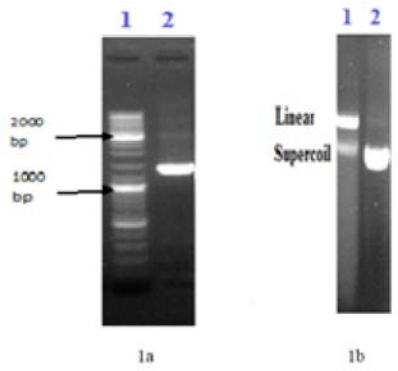


Figure 1. a: Homologous specific PCR from heavy chain gene. 5µl PCR product was loaded and 1440 bp band was seen. b: The digested pcDNA3.1 (+) with the EcoRV for 2 hours at 37℃. Lane 1: 5µl digested plasmid, Lane 2: 5µl undigested plasmid.

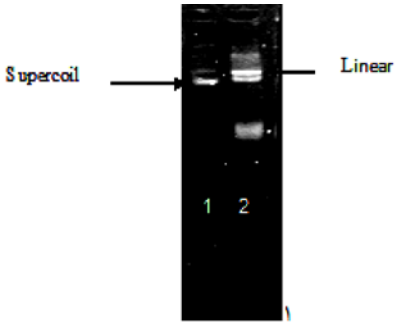


Figure 2. The digested pcDNA3.1(+) with the *HindIII* and the *BamHI* for 2 hours at 37°C. (Lane 1: 5µl undigested plasmid, lane 2: 5µl digested plasmid).

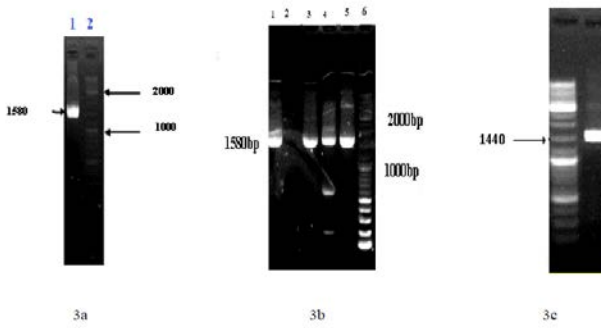


Figure 3. a: The PcDNA3.1(+) universal colony PCR was performed to screen recombinant colony in the HR method (lane 1: 5µl of the PCR product was loud and only one colony had 1580 bp band, lane 2: 2µl ladder). b: PcDNA3.1(+) universal colony PCR was performed to screen recombinant colony in the improved HR method and 4 colonies had band (lane 1, 3, 4 and 5: 1580 bp band, lane 6: ladder). c: specific colony PCR was done from the positive colonies, (lane 1: 2µl ladder, lane 2 and 3: 5µl of positive PCR colony product with 1400 bp).

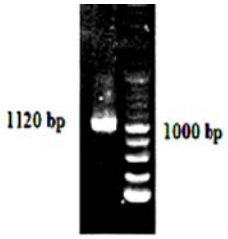


Figure 4. Specific PCR product was cut with the *HinfI* and1120 bp band was seen after loading in 1.5% agarose gel.

Preparation of insert and plasmid for the traditional method

The heavy chain genes of the IgG1 and the pcDNA3.1(+) (Neo) were digested using *BamH1* and *HindIII* restriction enzymes and then electrophoresed on an agarose gel (Figure 2). Because the restriction sites were close to the gene sequence, no differences were seen between the not digested and digested PCR products on agarose gel in the electrophoresis process.

Screening of recombinant plasmid

Bacterial colonies were screened, using PCR reaction which has universal primers of the pcDNA3.1+. Since the universal primers are 90 nucleotides away from multiple cloning sites, 1580bp band should be seen in the PCR product in each method. To check this, 40 colonies were individually extracted in three methods. After cloning PCR with universal primers in the samples in Method I only one colony, on average, with 1580bp band was seen in all the experiments (Figure 3a). 1580bp band was observed in 4 colonies in the samples in Method II (Figure 3b). No colony with

1580bp band was seen in the samples in Method III. Then specific colony PCR was done on positive colonies with 1580bp band in Methods I and II (Figure 3c).

The PCR products (1400bp) of recombinant plasmids were digested with the *HinfI* restriction enzyme, which has only one restriction site for the IgG gene sequence, and then 1120bp band was seen (Figure 4).

Discussion

Homologous recombination (HR) is a quick method for *in vivo* DNA cloning without using the high cost restriction enzymes[21]. This method is useful for cloning of DNA large sequence, because using *in vitro* methods for such cloning may break DNA[13].

Some modifications in HR method have been implemented to increase cloning efficiency in the past. Li and Elledge used the negative recA bacterial cells and the T4 DNA ligase enzyme. They also used SLIC (sequence and ligation-independent cloning) and incomplete PCR methods, they showed that changes can increase the efficiency of this method[15]. Yu et al. also used defected λ phage with heat dependent repressors for the Exo, the Beta, and the Gama genes in the negative recA bacterial cells[14]. They could clone genes with using phage recombinases[14]. Lee et al. made two modifications to increase cloning efficiency of the HR method[24], namely electroporation DNA fragment and making DNA fragment by a nuclease *in vivo.*

It should be mentioned that so far the effect of heat on the HR efficiency has not been studied yet. The heat treatment of the mixed insert and vector prior to transforming them into the *E. coli* competent cells improves the cloning efficiency considerably compared to the samples which their mixed insert and vector were not given heat. Therefore in this case, there are more bacteria containing the recombinant plasmid. This proves that the heat treatment increases the efficiency of the homologous recombination method and makes the cloning procedure faster. This fact is important in cloning of the immunoglobulin heavy chain to produce the recombinant therapeutic antibodies.

Conclusion

In this study, for the first time, the effect of heating on the homologous recombination efficiency was investigated. The heating process was implemented in the HR method for cloning heavy chain gene of antibody and then the results were compared with the traditional and the original HR cloning methods. The results showed that the HR method is an efficient method for cloning heavy chain gene, and using the heat treatment in that increases the DNA cloning performance significantly. The heat converted the insert and the vector from the double strand DNA to the single strand DNA and allowed them to pair with each other better. This helps the cell enzyme system for faster and easier recombination. The results also showed that giving heat in the HR method increases the cloning rate 7.5% and this increase reaches 10% compared with the traditional method.

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