

A New Overview on the Old Topic: The Theoretical Analysis of “Combinatorial Strategy” for DNA Recombination

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Abstract To clone genes of interest into suitable vectors is the first step to investigate their functions *in vitro* and *in vivo*. At the genome era, the sequences of more and more genes were decoded and available gradually. Therefore, it is critical to develop high efficient strategies for cloning genes of interest into different vectors to facilitate the functional analyses of them. In our previous studies, we created “Combinatorial strategy” for DNA recombination. Here, I theoretically analyzed the procedure of DNA recombination, the mechanism of this strategy, and further gave suggestions and predictions for various ligation-dependent molecular cloning experiments.

Keywords: *combinatorial strategy, DNA recombination, clone sites, CIP, T4 DNA ligase, transformation, Top10*

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1. Introduction

The ligation-dependent DNA recombination technique was first developed at 1972 and 1973 [1,2]. From then on, the functions of genes of interest could be investigated intentionally *in vitro* and *in vivo* by cloning those of interest into appropriate vectors. In addition, the establishment of site-directed mutagenesis technique enabled the scientists to change the DNA sequences at specific locations [3]. These two techniques made the gene engineering and protein engineering from concepts into reality. During the past decade, the advent of the era of genomics unveiled the miracles of life codes unprecedentedly [4,5,6]. A main task of current biomedical research is to investigate the interactions of different proteins within the complicated networks, and the relationships of their functions. Thus far, more and more gene sequences of different organisms were gradually available, therefore, it is obvious that to efficiently clone appropriate vectors with genes of interest is the pivotal step for successful experimental investigations. At present, although several different strategies for molecular cloning were established, there were still no generalized methods to clone different vectors accurately and quantitatively with high efficiencies. In this review, based on our previous reports [7,8], I theoretically analyzed our new method, “Combinatorial strategy”, for efficient cloning different vectors.

2. Analysis of the Procedure of DNA Recombination

2.1. What Happened in the Ligation Reaction System?

In a typical 20µl ligation system, about 100 ng insert and 200 ng vector DNA were mixed together with 1 µl T4 DNA ligase. Let us suppose that the sizes of the insert and vector are about 1.5kb and 5kb, respectively. The average molar weight of A, G, C, T is about 660 g. Therefore, 100 ng inserts contained about 3×10^{10} insert DNA molecules, and 200 ng vectors contained about 2×10^{10} vector DNA molecules. So, how did these billions of insert and vector DNA molecules react with each other?

2.2. Main Procedure of DNA Recombination

Generally to speak, the main procedure of DNA recombination includes five steps. The first step is to choose and create compatible clone sites between the vectors and inserts. This is the starting point and critical for the successful cloning. The basic rule is to choose high efficient clone sites, such as EcoR I, BamH I, EcoR V, etc. both over-hang and blunt end sites, and to avoid using sites of low efficiencies. The second step is to digest and purify the vector and insert DNA, and the value of the purities (A260/280) of all the DNAs must be above 1.80. At the ligation step, high concentration T4 DNA ligase

should be used. And competent cells, such as DH5 α , Top10 cells, could be used for transformation. Finally, 5 to 10 clones might be selected for identification by restriction digestion and confirmed by sequencing.

2.3. Approaches to Create Compatible Clone Sites

During the molecular cloning, the ideal circumstance was to find matching clone sites between the inserts and vectors among the multiple clone sites. Unfortunately, this is not always realistic in many molecular cloning experiments. In these cases, it was necessary to create the compatible clone sites between vectors and inserts. The mostly used method is to design PCR primers containing proper clone sites for the inserts. This is an easy and simple way to incorporate clone sites into the inserts, because both the 5' and 3' end clone sites could be designed and created in a single PCR reaction. The main disadvantage of this method is that the extra 3-4 bases at the terminal restriction sites are insufficient for stable association with and cut by certain restriction endonucleases [9,10]. In addition, because the PCR products of this method are linear DNA with incorporated clone sites, after restriction endonuclease digestion and agarose gel purification, theoretically, it could not guarantee that 100% of the digested DNA molecules were with correct cutting ends. Therefore, this method is only suitable for the small size, high efficient cloning, such as pET, pcDNA vectors, but might not for the large size, low efficient cloning, such as lentiviral vector cloning. Making blunt ends for the inserts and vectors is another method for molecular cloning. This method could neglect the differences between different over-hang clone sites. But in real experiments, this method is also of low efficiency due to two reasons. One is the low efficiency of blunt end ligation compared with cohesive ends. The other is the inefficiency of creation of correct blunt ends. The functions of Klenow fragment and T4 DNA polymerase are to make blunt ends by filling-in the 5'-overhangs, and removing the 3'-overhangs. At the same time, both of them could result in recessed ends due to their 3' to 5' exonuclease activity [11]. As a result, this method also could not guarantee that 100% of the products were with correct ends. Therefore, it is only suitable for high efficient cloning, but not for low efficient cloning. Site directed mutagenesis is a powerful tool to change the DNA sequences specifically [3]. It could be used to insert clone sites for molecular cloning [12]. The working format of the Stratagene SDM contained three steps. The first is to synthesize the mutated DNA strands by PCR. Then the methylated and hemimethylated parental DNA was digested by Dpn I enzyme. Finally, the mutated plasmids were transformed into the competent cells for multiplication. After growing bacteria and miniprep, the modified clone sites were created within the circular double-stranded plasmid DNA. Because the mutated products by SDM are circular double-stranded plasmid DNA, after digestion and purification, the linearized DNA is theoretically 100% with the correct-cutting ends. As a result, the maximal ligation could be achieved with the vectors, and the efficiencies of transformation could be improved radically. Therefore, this method is not only

suitable for regular cloning, but also for low efficient cloning [7,8].

2.4. The Function of T4 DNA Ligase

The function of DNA ligase is to catalyze the formation of 3', 5'-phosphodiester bond between the juxtaposed 5'-phosphate and 3'-hydroxyl groups [13]. *In vitro* experiments using plasmid or synthetic oligonucleotide substrates revealed that T4 DNA ligase exhibited a relaxed specificity, 3' and 5' A-A or T-T mismatches, 5' G-T mismatches or 3' C-A, C-T, T-G, T-T, T-C, A-C, G-G or G-T mismatches [14,15,16]. Therefore, ligation could take place when there were mismatches at or close to the ligation junction by T4 DNA ligase. That is to say, between different clone sites, phosphodiester bond could be formed. This is the reason why there are lots of clones with empty vectors in molecular cloning experiments.

2.5. Procedure of Regular Ligation

There are three steps during regular ligation. One is the Inter-molecular reaction, at this step, to form non-covalently bonded, linear vector-insert hybrids, and this reaction requires high DNA concentrations. The other is the intra-molecular reaction, at that step, to form non-covalently bonded, circular molecules. That reaction works efficiently with low DNA concentrations. Finally, the annealing between inter and intra molecules brings the 5'-phosphate and 3'-hydroxyl residues of the vectors and inserts into close alignment, which allows T4 DNA ligase to catalyze the formation of 3', 5'-phosphodiester bond [17] (Figure 1). Because the ligation could take place between inter and intra molecules. At the same time, T4 DNA ligase could catalyze the ligation between different clone sites. Therefore, at least, there are three kinds of ligation can happen, which are the vector self-ligation, the insert self-ligation, and the vector-insert ligation. After transformation, all the circular DNAs could be efficiently transformed into the competent cells [2]. Because the vectors contain antibiotic resistance genes, the clones with vectors and recombinants can survive after antibiotic selection. Whereas, the clones only with circular inserts cannot survive the selection. Therefore, there are many background clones with empty circularized vectors (Figure 2).

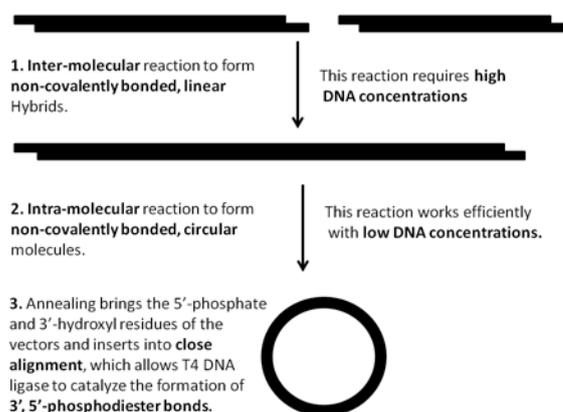


Figure 1. The procedure of regular ligation (modified from Sambrook J, Russell DW. 2001. *Molecular Cloning: A Laboratory Manual*)

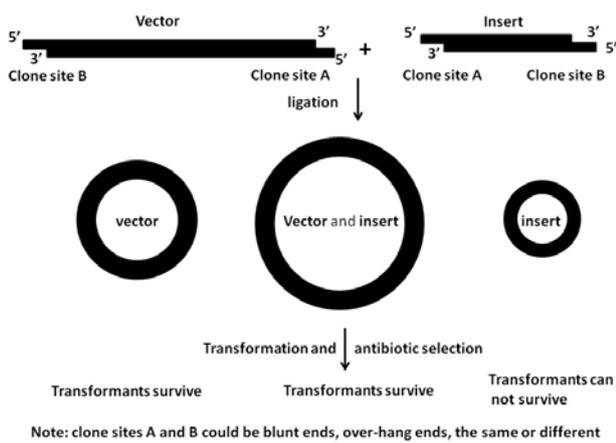
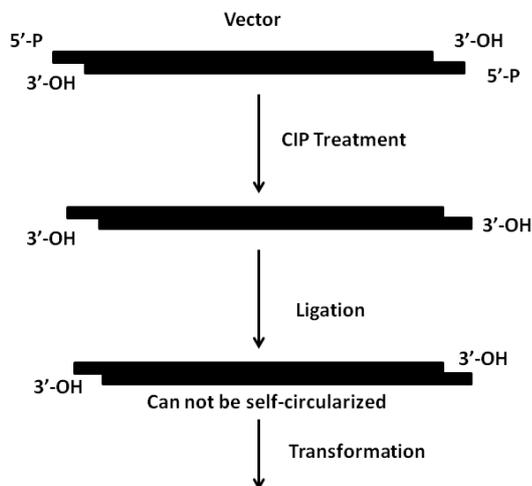


Figure 2. Types of ligation and transformation

3. Working Mechanism of “Combinatorial Strategy”

3.1. The Function of CIP Treatment of the Vector after Enzyme Digestion

The function of calf intestinal phosphatase (CIP) is to remove the 5'-phosphate group from the vector DNA [17]. After CIP treatment, the vectors could not be self-ligated. Because the transformation efficiencies of linear DNA are much lower than circularized DNA [2], the backgrounds with empty-vectors are decreased radically (Figure 3). Almost all the clones are with recombinant vectors [7,8].



Because the transformation efficiencies of linear DNA are very low, the backgrounds with empty-vectors are decreased radically.

Figure 3. Function of CIP treatment (modified from Sambrook J, Russell DW. 2001. *Molecular Cloning: A Laboratory Manual*)

3.2. Choosing Proper Competent Cells for Transformation

After CIP treatment, the ligation efficiencies between the vectors and inserts are decreased significantly [17]. To increase the transformation efficiencies, more efficient competent cells should be used. DH5 α and Top10 competent cells are commonly used for transformation, because the transformation efficiency of Top10 cells (1×10^9 cfu/ μ g super coil DNA, Life Technologies) is much higher than DH5 α (1×10^6 cfu/ μ g super coil DNA,

Life Technologies), Top10 cells are recommended as transformation hosts for CIP-treated ligation, in order to obtain sufficient clones for identification, particularly for CIP-treated, blunt-end cloning [7,8].

3.3. Theoretical Analysis of Combinatorial Strategy

From the above analysis, we could conclude that, first of all, digesting the inserts from circular vectors could guarantee that 100% of the linearized inserts were with correct cutting ends. Secondly, the vectors treated with CIP could efficiently protect the vector-self-ligation, and decrease the background clones with empty-vectors. Finally, the clones with circular inserts could not survive the selection. And the transformation efficiencies of the linear vectors were very low. Therefore, most of the clones should be with the recombinant vectors. We could predict that, with different clone sites, nearly 100% of the clones should be the positive clones. With the same overhang, and blunt clone sites, about 50% of the clones should be the positive clones [7,8] (Figure 4).

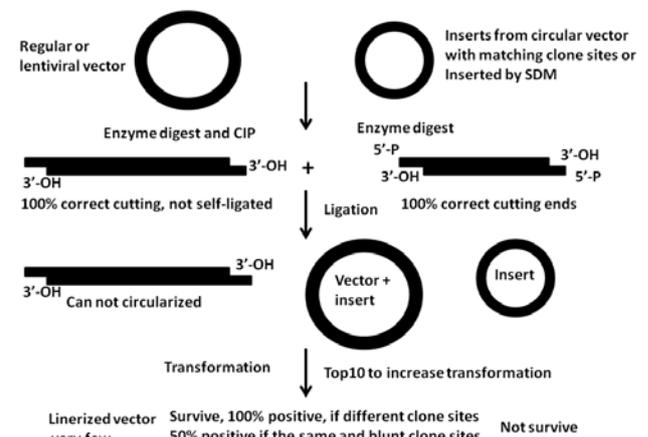


Figure 4. Working mechanism of “Combinatorial Strategy”

4. Suggestions and Predictions for Molecular Cloning with CIP-Treated Vectors

Our previous reports demonstrated that, with our “Combinatorial strategy”, different plasmid vectors could be efficiently cloned with various clone sites [7,8]. Based on these studies, we could make the following suggestions and predictions for molecular cloning (Table 1). These suggestions could significantly facilitate the construction of vectors with genes of interest, and further speed up the biomedical research radically.

Competing Interests

The author declares that there is no competing interest.

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Table 1. Suggestions and predictions for molecular cloning with CIP-treated vectors

Clone sites	Sizes (kb)	Methods for clone sites	Transformation host	No. of colonies	Positive clones
Blunt sites	Small (vector<5, insert<1.5)	Existed	Top10	Dozens or more	About 50%
	large (vector>5, insert>1.5)	Existed	Top10	A few to dozens	About 50%
Different over-hang sites	Small (vector<5, insert<1.5)	SDM	Top10	Hundreds or more	Nearly 100%
	Large (vector>5, insert>1.5)	SDM	Top10	Dozens to hundreds	Nearly 100%
One over-hang site	Small (vector<5, insert<1.5)	SDM	Top10	Hundreds or more	About 50%
	Large (vector>5, insert>1.5)	SDM	Top10	Dozens to hundreds	About 50%

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