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On the "All or Half" Law of Recombinant DNA

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Abstract Plasmid vectors are one of the most important tools for the investigation of the functions of genes of interest. Efficient cloning of various vectors, according to different purposes, is critical for biomedical research. Previously, we reported a new method, designated as "Combinatorial Strategy", for cloning different vectors with various clone sites. We demonstrated that it is a quantitative law for recombinant DNA with our method, which is when two different clone sites are used, almost 100% transformants are positive clones, on the other hand, when one over-hang clone site, or different blunt clone sites are used, about 50% of the transformants are positive clones. We named this quantitative law as the "All or Half" law of recombinant DNA. Here, we summarized the mechanisms of recombinant DNA, provided a general protocol and suggested the predicted results for plasmid vector cloning. This is a revolutionary breakthrough of recombinant DNA technology.

Keywords: *recombinant DNA, Combinatorial Strategy, "All or Half" law, Calf Intestinal Phosphatase, site-directed mutagenesis, Top10, clone sites*

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1. Introduction

The advent of the ligation-dependent DNA recombination technique at 1970s [1,2] opened an efficient way for researchers to investigate the functions of genes of interest intentionally by cloning those genes into appropriate vectors. Ever since then, investigators made great efforts to improve the efficiencies for cloning different vectors, such as directional cloning method, adding synthetic linkers or adaptors to the termini of the linearized plasmid and/or fragment of foreign DNA, amplifying the fragment of foreign DNA by PCR using primers that contained the desired restriction sites to one or both termini, and so forth [3]. However, even after these optimizations, there are still many troubles in plasmid vector cloning. For example, after transformation, there are usually many transformants contained empty circularized vectors, rather than circularized recombinant vectors with correct inserts. This phenomenon increased the task to screen correct clones from the transformation plates. Therefore, the cloning efficiencies are often low.

In our previous study, we created a new method, called "Combinatorial Strategy", to clone various plasmid vectors with different clone sites [4,5,6]. With this method, according to the different types of clone sites employed, the cloning efficiencies can be radically improved to almost 100% or about 50%. More importantly, the improvement of the cloning efficiencies is not caused by the techniques of different individual investigators. Rather, it is due to the inherent characteristics of the ligation-dependent DNA recombination processes. Because, if the clone sites at both ends of the linearized vectors and inserts are different, the matched clone sites are always ligated by T4 DNA ligase with much higher efficiencies [7,8,9]. Hence, the cloning efficiencies can be almost 100%. Whereas, if the same overhang clone sites or blunt clone sites are adopted, the vectors and inserts only have two choices, either in correct orientation or in opposite orientation. Therefore, in these circumstances, the cloning efficiencies are about 50% [4,5,6]. In this paper, we briefly summarized this quantitative feature of plasmid vector cloning, and designated it as the "All or Half" law of recombinant DNA.

2. The Mechanisms of Recombinant DNA

In order to quantitatively clone DNA vectors, the following mechanisms of recombinant DNA technology should be considered. 1) Digestion of inserts from circular vectors can make sure that, theoretically, 100% of the linearized, purified inserts are with correct cutting-ends. 2) The removal of 5'-phosphate group from the digested vectors with calf intestinal phosphatase (CIP) treatment can radically decrease the self-circularization of vectors. 3) Since all the linearized inserts are with correct-cutting ends, the maximal ligation can be achieved between the inserts and the vectors. 4) The CIP treatment of digested vectors can drastically decrease the ligation efficiencies between the vectors and inserts, therefore, Top10 competent cells, or other competent cells with equivalent efficiencies, or more efficient suitable competent cells, are used for transformation. This is very important for large-size, CIP-treated, low-efficiency ligations, such as

lentiviral vector cloning. 5) Because the transformation efficiencies of linear DNA are much lower than circular DNA, almost all the transformants are with recombinant vectors after antibiotic selection [2,4,5].

Based on the above analysis, we simplified the recombinant DNA technique according to the following three steps. 1) Using originally existed clone sites from circular vectors to prepare the inserts, and, if no appropriate clone sites are available between vectors and inserts, performing site-directed mutagenesis (SDM) to create compatible clone sites, to achieve maximal correct-digestion of the inserts. 2) Different vectors were digested with various restriction endonucleases matched with the inserts, and then dephosphorylated with CIP. 3) Top10 competent cells, or equivalent efficiency cells, or more efficient suitable competent cells, were used for transformation to obtain sufficient transformation colonies for identification [4,5].

Our reported results demonstrated that, when blunt clone sites (such as Swa I, Pme I, EcoR V, etc.), or a unique overhang clone site (such as Xba I or BamH Ietc.) was adopted for ligation, the percentages of positive clones were about 50%. Whereas, when different clone sites, including one blunt clone site (for example, EcoR V) and another overhang clone site (for example, Pst I), and two different overhang clone sites (such as Not I and Xho I, etc.), the percentages of positive clones were nearly 100%. We found that, even though the number of transformation colonies obtained was different, when different vectors, inserts, and clone sites were adopted, the percentages of 50% and 100% for positive clones are consistent [4,5]. Our reported data demonstrated that those of 50% and 100% are general quantitative feature for recombinant DNA by Combinatorial Strategy. We designated this feature as the "All or Half" law of recombinant DNA. Using this strategy, almost all the vectors can be successfully cloned through “one ligation, one transformation, 2 to 3 minipreps”. Therefore, dozens of plasmid vectors can be successfully cloned in a week efficiently.

General Protocol Procedures (all the enzymes used are from NEW ENGLAND BioLabs, the equivalent products from other commercial companies could be adopted as well).

Day 1: Design the clone sites and growing bacteria for miniprep

1. Using online software (such as NEBcutter V2.0, etc.) to analyze the sequences of the vectors and inserts to determine the clone sites between vectors and inserts, including blunt clone sites, one unique overhang clone site, two different overhang clone sites, or one blunt clone site and another overhang clone site. If there are no suitable clone sites existed for the vectors and inserts, site-directed mutagenesis (such as Stratagene Kit) will be adopted to create matched clone sites for the inserts/vectors (not included in this protocol).
   CRITICAL: According to the product instructions, choosing efficient clone sites for cloning experiments, such as BamH I, EcoR V, Pst I, etc. (for example, NEW ENGLAND BioLabs, information about ligation and recutting).
2. It is recommended to seed the proper bacteria with suitable plasmids at around 5:00 pm in 3-5 ml LB broth with appropriate antibiotics overnight in a 37°C shaker, at 225 rpm, in order that the bacteria will have grown for about 16 hours before miniprep the next day.
   CRITICIAL STEP The plasmid DNAs should be freshly made for efficient cloning. The plasmids can be stored at -20°C freezer for several weeks. The long-stored plasmid DNAs, such as more than half years were not recommended to use for cloning.

Day 2: Miniprep, restriction digestion, CIP treatment, purification and ligation

1. Miniprep plasmid DNAs with QIAprep Spin Miniprep Kit using 3-5 ml bacteria exactly according to the manufacturer’s protocol in 50 µl EB buffer, the concentrations of the plasmid DNAs should be around 300-500ng/µl, and the purities of DNA (A260/280) must be ≥1.80 [4,5].
   CRITICAL STEP The purities of the plasmid DNAs are critical for the subsequent restriction digestion and ligation, therefore, the values of A260/280 MUST be ≥1.80.
2. Digest the plasmid vectors and inserts with suitable restriction endonuclease according to the instructions of manufacturer’s guides. It is recommended to perform the digestion as follows: in a 100 µl reaction system, 20-30µg plasmid DNAs of vectors or inserts were digested with 5 µl of restriction endonuclease(s) totally, with 10 µl appropriate 10 X buffer for 1-3 hours.
   CRITICAL STEP: It is important to let the total volume of the restriction enzymes less than 1/10 of the whole volume of the digestion system to avoid star reactions of the digestion.
   OPTIONAL: It is also very convenient to let the digestion overnight, if there are other experiments scheduled.
3. For the vectors, after digestion, directly add 3µl (10U/µl) Calf Intestinal Phosphatase (CIP, about 1U CIP for 1µg DNA), 1µl NEBuffer 2, 3, 4, or NEBuffer for EcoR I, and 6µl ddH_2O, mix thoroughly, and at 37°C water bath for 1 hour to remove the 5’-phosphate group from the linearized vector DNA.
   IMPORTANT: Because CIP is not work well in NEBuffer 1, if the restriction digestions are performed in NEBuffer 1, it is necessary to purify the vector DNA with QIAGEN Gel Extraction Kit. Then new reactions for CIP treatment are set up according to about 1U CIP for 1µg DNA (NEW ENGLAND BioLabs). In this case, in order to obtain sufficient DNA for ligation, more vector DNAs should be digested due to the loss of DNA during purification.
4. The digested inserts and CIP treated vectors are purified by 1% Agarose Gel Electrophoresis running at 80V for 1.5 hours and recovered by QIAGEN Gel Extraction Kit.
   CRITICAL: After CIP treatment, the vectors must be purified through Agarose Gel Electrophoresis or Gel Extraction Kit to remove the CIP enzyme thoroughly. If not, remnant CIP enzyme will

dephosphorylate the inserts during ligation step, and decrease the efficiencies of ligation.

IMPORTANT: To obtain better separation of the DNAs, the Agarose Gel Electrophoresis should be run at lower voltages with longer time, such as 80V and 1.5 hours [3].

5. In 20µl ligation systems, at first, appropriate volume of ddH_2O, about 200ng vector and 100ng insert (up to 17µl totally with the vector and insert), are pooled together for each ligation reaction, mixed thoroughly, and warmed at 45°C for 5 min to melt any cohesive termini that had re-annealed during fragment preparation, then chilled on ice for 2 min [3,4,5]. Then, 2µl 10 X T4 DNA ligase buffer, and 1µl (2000U/µl) T4 DNA ligase are added into the PCR tube (NEW ENGLAND BioLabs), mixed thoroughly. Finally, the 20µl reaction mixtures are incubated in a PCR System at 16°C for 16 hours followed by inactivation at 65°C for 10 min, and then set at 4°C until transformation [4,5].

IMPORTANT: The warm and chill step is important for efficient ligation between the vectors and inserts.

TIPs: The ratios of inserts to vectors can be varied from 1 to 1 up to 22 to 1 [5].

Day 3: Transformation

1. 1-2µl (about 30-60ng) volumes of the ligation products are used to transform 50µl of Top10 competent cells according to the manufacturer's instructions (Invitrogen, Life Technologies). Briefly, the ligation reaction tubes are centrifuged briefly and placed on ice. One 50µl vial of One Shot® Top10 cells is thawed on ice for 30 min for each ligation/transformation. 1-2µl of each ligation product are added directly into the vial of TOP10 competent cells and mixed by tapping gently for 7-10 times rather than pipetting up and down. The vials are incubated on ice for 30 min. Then, Top10 cells are incubated for exactly 30 seconds in a 42°C water bath without shaking. The vials are then removed from the 42°C bath and placed on ice immediately for 2 min. 250µl of pre-warmed S.O.C medium (Invitrogen, Life Technologies) are added to each vial, and the vials are shook at 37°C for about 1 hour at 225 rpm in a shaking incubator. In order to obtain more colonies, all the transformation cells are used to spread plates. After the plates were dried, they were inverted and incubated at 37°C overnight [4,5].

   CRITICAL: Mix the ligation product with TOP10 cells by tapping gently for 7-10 times rather than pipetting up and down. Do not use more than 2µl ligation products for transformation. For low-efficiency ligation, adding more ligation products into competent cells will decrease the transformation efficiencies [4,5].

Day 4: Growing bacteria

1. To check the transformation plates the following day at morning, and different numbers of colonies will be in the plates due to different clone sites and sizes of vectors and inserts. For each transformation, 2 to 3 colonies are chosen and seeded in 3 ml LB broth with appropriate antibiotics, shaking at 37°C incubator at 225 rpm overnight.

Day 5: Miniprep and identification by restriction digestion

1. Miniprep plasmid DNAs with QIAprep Spin Miniprep Kit using 1ml bacteria exactly according to the manufacturer's protocol in 50 µl EB buffer, the concentrations of the plasmid DNAs should be around 100ng/µl, and the purities of DNA (A260/280) must be ≥1.80 [4,5].
2. According to the sequences of different vectors and inserts, suitable restriction enzymes are chosen to digest the selected vectors. If blunt clone sites and one unique overhang clone site are adopted, the restriction enzymes used must both identify the existence of the inserts and the correct orientation of the inserts, such as Not I restriction endonuclease [4]. If necessary, some of those positive clones are further confirmed by DNA sequencing.

   IMPORTANT: To make the identification step simple and easy, check the sequences of the vectors and inserts to find suitable restriction sites both for the recombination and orientation of inserts. These restriction sites do not need to be the adopted clone sites, because the adopted clone sites can merely identify the recombination but not the orientation [4].

3. Predicted Results

Based on different clone sites employed, and different sizes of inserts and vectors, different numbers of colonies can be obtained after transformation [4,5]. The predicted results are as the following. If different clone sites are used, nearly 100% of the colonies are positive clones. On the other hand, if different blunt-end clone sites, or a unique overhang clone site, are adopted, approximately 50% of the colonies are positive clones. This is the "All or Half" law of recombinant DNA with our "Combinatorial Strategy".

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Conflict of the Interest

The authors declared that there is no conflict interest for this paper.

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