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All-in-One Molecular Cloning as a New Gene Manipulation Method

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Abstract

Introduction: DNA cloning plays a crucial role in biotechnology laboratories and biotechnology-related fields, which facilitates the primary yet crucial step for further studies in molecular biology. Many laboratories worldwide still use restriction enzyme-based cloning methods to construct expression vectors owing to financial constraints, time-consuming, and the unavailability of appropriate vectors. In the present study, we introduced a novel method inspired by the restriction enzyme-based cloning method with some modifications named All-In-One (AIO) cloning.

Materials and Methods: The PCR product and vector for cloning were digested in one 0.2 ml tube with a total volume of 20 µl. Completely digested products were checked and inactivated by heat treatment. Digested genes and vectors were directly used for the ligation step in this 0.2 ml tube, without any purification step required. Finally, ligation products were transformed into competent *E. coli* DH5α by the heat shock method.

Results: More than eight different clones were generated by using AIO cloning, which all the necessary reactions were performed in one single 0.2 ml tube. This method was efficient in cloning a wide range of DNA fragments, from 200 to 1300 bps.

Conclusions: Collectively, AIO provided an alternative yet sufficient cloning protocol, reducing the loss of DNA components, and saving materials, labor and time, especially where research materials were not abundantly available.

Keywords: All-In-One, DNA Manipulation, Molecular Biology

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Introduction

DNA cloning builds the first step for molecular biology research. Many efforts have been conducted to improve and develop this step in the most convenient, efficient yet inexpensive way. Conventional cloning methods, known as Restriction Enzyme-based Cloning (REC) methods, depend on the enzymatic digestion of gene of interest and vector before ligation to obtain a clone.[1] REC is one of the most popular methods of choice today. Restriction enzymes derived from certain bacteria and archaea, cleave double-stranded DNA at specific sites. In REC, particular restriction enzymes are employed to cut DNA of interest into fragments containing precise 5' or 3' single strand to create ligating ends. Two pieces of DNA can then be fused together during a ligation reaction with DNA ligase. REC benefits from the abundance of accessible enzymes, plenty of which are relatively affordable. These enzymes target and cleave specific sequences ranging from 4 to 13 bp, generating blunt or sticky ends in DNA fragments.[2] Besides, most plasmids utilized for cloning and expression contain popular restriction sites. Any fragments of DNA with restriction sites on their ends can simply be inserted into a plasmid which has the same restriction sites in the correct orientation within its multiple cloning site. The short length of these specific sites can be incorporated into any DNA fragment using PCR amplification.

Last but not least, directional cloning can easily be done by using two different restriction enzymes. However, REC depends too much on enzyme activity and requires multiple purification steps prior to transformation. This fact could lead to unexpectedly low efficiency if one of these steps does not work well. Another obvious consequence is time-consuming when REC needs more time to finish compared to the other cloning methods. To overcome these limitations, various approaches have been introduced. Some restriction-free methods include the TA cloning,[3] ligation-independent cloning,[4] restriction-free cloning,[5] based on the generation of Manmade Cohesive Termini cloning (MCT cloning),[6] etc. However, some of them rely on impossible DNA end modifications,[4] or lacking directional cloning.[3] Homologous recombination is an alternative, in which commercial recombinase[7,8] or megaprimers[9] are utilized. Nevertheless, these approaches need expensive enzymes which leads to their limit of use.[7,8] Other methods employ *Dpn*I digestion before transformation[5,6,9] to eliminate methylated parental plasmids. However, the efficiency is not sufficient owing to the existence of resistant hemimethylated DNA (parental strand combined with PCR-generated strand) in the PCR products,[10] which can lead to a source of false-positive clones.

We characterized a method that was designed to be a saver, quicker and simpler cloning, named All-In-One (AIO). This method was established based on REC[1] with shortened steps, thereby reducing time, materials, costs, and lowering the loss of reaction components.[11] The basic REC method requires complicated purification steps for each component including PCR products, enzymatic digestions, etc which cause the loss of DNA products and is time-consuming. The AIO method was performed in only one 0.2 ml PCR tube for two crucial steps: enzymatic digestion and ligation.[12] This alteration resulted in the reduction of materials required, as well as the time in need for these steps. Furthermore, as all the residues after digestion remained in only one tube, this can extend the ability of swapping interested genes, which introduced a new insert into a plasmid that already carried a gene. It also requires no complications in primers design. In general, amplified product purification always yields good concentrations, but it is not the same for purification of enzymatically digested products. Purification step contributes to removing residues of the reaction and to capture clean DNA at a determined concentration. Gel purification may yield good recovery, but this method depends upon multiple steps and expensive materials. Currently, DNA purification kits are commercially available, which make simpler manipulations, but the yield of digested products is quite low, and column purification cannot eliminate all small DNA fragments. Therefore, a method without purification steps was about to be introduced.

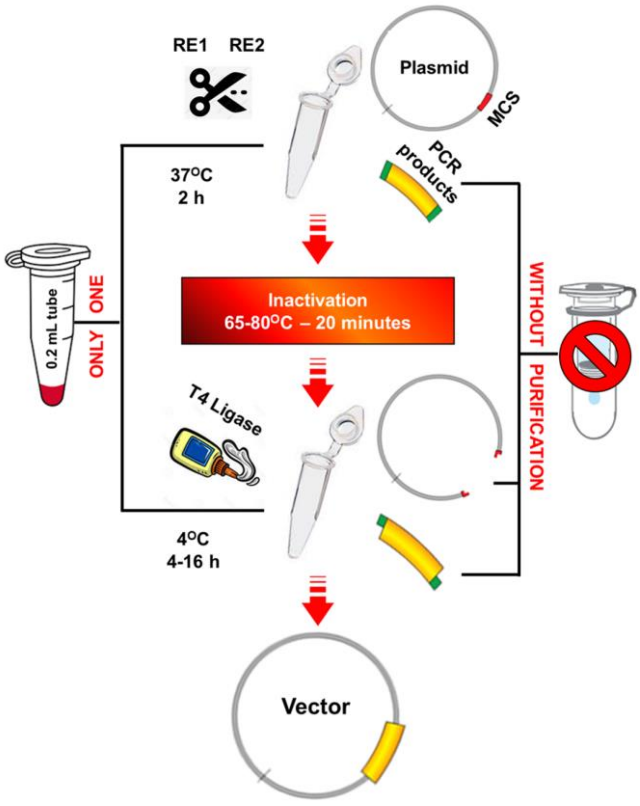

Figure 1. Schematic of AIO Method.

Materials and Methods

Isolation of PCR Products

PCR products in AIO were amplified by MyTaq™ Red Mix (Bioline) using the PCR profile: 95 °C for 5 min followed by 30 cycles at 95 °C for 30 s, 30 s at an annealing temperature based on primer sequence (55 °C is recommended for most pair of primers), 72 °C for a variable amount of time depending on the amplicon size (10 s/kb). A final 5-min extension at 72 °C was done to complete all amplifications. Three µl of PCR products were analyzed on a 1.5% agarose gel to confirm amplification efficiency. The PCR products were then digested without a purification step.

AIO Inserting a PCR Product into Vector

The construction of recombinant vectors using AIO cloning is described below. This method required four steps. The PCR products and vector for cloning were digested in one 0.2 ml tube with all necessary components including 200 ng plasmid, 300 ng gene, restriction enzymes of choice, restriction enzyme buffer, and molecular-grade water to the total volume of 20 µl. Completely digested products were confirmed via agarose gel electrophoresis using a 3 µl mixture and inactivated at 80 °C for 20 min. Thermal-treated products were directly used for ligation step as followed, 17 µl of the mixture in this 0.2 ml tube, supplemented with 2 µl ligase buffer and 1 µl DNA T4 ligase, and no purification step. Finally, ligation products were transformed into competent *E. coli* DH5α by using the heat shock method and spread onto selective-factor-containing medium. Colonies were screened by PCR with specific primer and plasmid primer.

Expression of Recombinant Protein, and Western Blot

The AIO-constructed recombinant plasmids were transformed into *E. coli* BL21 (DE3) by using the heat shock method. Transformants were selected by selective-factor-containing medium. Positive colonies were transferred into liquid medium and were then induced. Cells were collected, lysed for extraction, and further protein analysis including SDS-PAGE, and Western Blot were done.

Results

AIO Inserting a PCR Product into Vector

In this research, we constructed an *E. coli* expression vector named pET22b-*gfp*. The origin pET22b vector contains an AmpR cassette for positive selection in *E. coli* DH5α. To verify the efficiency of the AIO method, we inserted the *gfp* gene (0.7 kb) into pET22b. The *gfp* gene was initially amplified by PCR with specific primers. The PCR products were inserted into vector through AIO methods, and the ligation products were transformed into *E. coli* DH5α. The results showed that the AIO method generated many colonies, whereas controls (without vector) did not generate any colonies on the selective plate (Figure 2A). Transformants

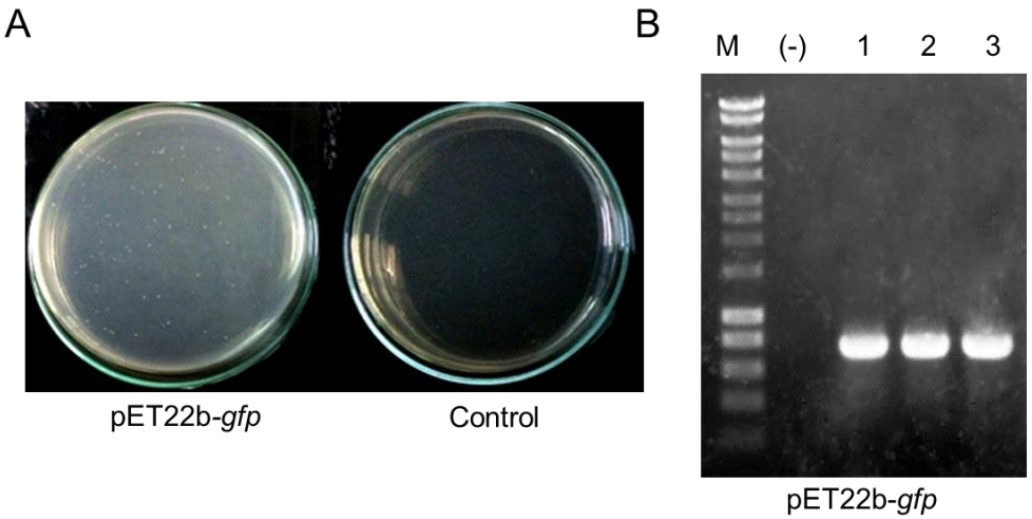

Figure 2. Cloning by AIO Method. **(A)** transformation into competent *E. coli* DH5α; **(B)** colony PCR of pET22b-*gfp*.

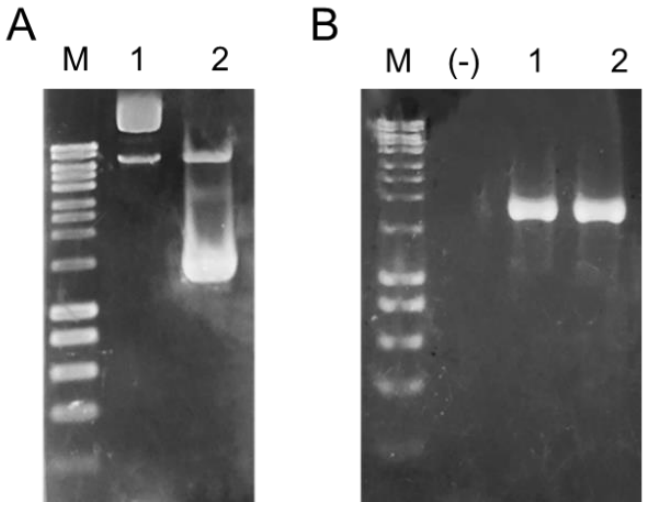

Figure 3. (A) isolated and digested gene, plasmid by restriction enzymes; **(B)** colony PCR of pET22b-*pep1-f18-agglutinin*.

were screened by PCR with specific primer and plasmid primer. Electrophoresis results showed that ligation without purifying digested product still gave recombinant plasmids (Figure 2B).

In addition, to verify the swapped ability of the AIO method, we swapped the *gfp* gene (0.7 kb) for *f18-agglutinin* gene (1.3 kb) from pET22b-*pep1-gfp* vector (Figure 3A, lane 1) to generate pET22b-*pep1-f18-agglutinin.* The pET22b-*pep1-gfp* vector and *f18-agglutinin* gene were digested with two restriction enzymes to remove the *gfp* gene (Figure 3A, lane 2). After ligation and transformation, transformants were screened by PCR colonies with a pET22b primer and a specific primer. Results showed that AIO was able to generate recombinant plasmids (Figure 3B).

The efficiency of the AIO cloning method was qualified by eight different genes with sizes ranging from 0.2 to 1.3 kb inserted into different vectors (Table 1).

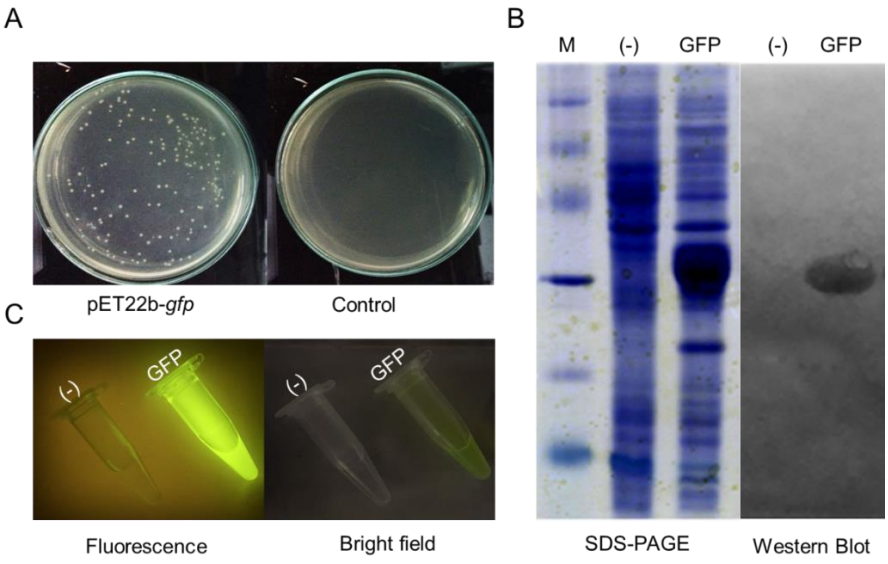

Figure 4. Expression Protein Cloned by AIO Method. **(A)** transformation recombinant plasmid into competent *E. coli* BL21(DE3); **(B)** expression of GFP protein; **(C)** fluorescence of GFP protein.

Expression of Recombinant Protein and Western Blot

Electrophoresis results showed that recombinant plasmids had been successfully transformed into expressing cells (Figure 4A). As shown in Figure 4, overexpression of GFP protein (Figure 4B) was observed. The expression of recombinant proteins was also confirmed by the Western Blot. The fluorescence activity of the GFP protein was also demonstrated (Figure 4C).

Discussion

At present, several novel cloning technologies have been developed to replace REC, such as Gateway system, TA, MCT, LIC, etc. But difficulties appeared gradually, including technical difficulties, multiple cloning procedures requirements, long primers, and so on. Also, in developing countries, access to molecular biology materials is sometimes a difficult issue. Therefore, REC still plays a critical role in recombinant plasmid construction. Here we characterized a method based on REC, designed to be a save, quick and simple cloning. Compared with REC, PCR products in AIO were directly involved in digestion without purification steps. This helped to reduce DNA loss as well as materials. However, PCR products included various components, which can affect digestion step. Optimizing PCR to obtain high concentrations of amplified products was necessary, which helped minimize the amount of volume added to digestion. According to normal methods, genes and plasmids were advisedly digested with 1000 ng DNA in each tube. In AIO, the genes and plasmids were cut together in one single tube with a smaller amount of DNA (about 400-500 ng). This actually reduced the amount of DNA involved in the reaction, as well as restriction enzymes, buffer, and related materials. Before ligation, digested products had to be confirmed and inactivated. Verifying after digestion was necessary to control the entire cloning process. After inactivation, digested products were used for ligation without purification steps. In AIO, no loss occurred, components in small quantities would be maximumly used.

Results showed that the target gene had been successfully obtained. Gene and plasmid were completely digested by restriction enzymes and followed by ligation in only one 0.2 ml tube. Digesting gene and plasmid in one tube did not affect the ability of digestion, as being observed in this study, it helped save the amount of DNA involved in the reaction, amount of enzyme, and buffer used. In addition, the AIO method was also successful in swapped cloning procedure. Based on REC but being cheaper and faster, AIO could completely replace REC for current research purposes. However, AIO still had some limitations. It was impossible to determine the amount of DNA involved in the ligation, the digestion and ligation efficiency, as well as self-ligation effect. The problems above could be explained as components of PCR reaction could have a negative impact on the next steps. The molar ratio between the gene and the plasmid was not reasonable, the enzymatic digests were not completely processed, and the DNA residues of the reactions could affect the ligation efficiency. Besides, the salt composition and concentration in restriction buffers could have effects on the ligation efficiency. Too much salt (from restriction and ligation buffers) might result in the decrease in ligase activity. In fact, this lazy-to-purify method did succeed with all the clones that had been tested, at one time and needed no repetition. However, the efficacy was quite low, as only 10-20% of screened colonies carried the targeted vector.

Further experiments should be conducted to improve and fulfill this method, such as investigating the amount and proportion of DNA involved in digestion process, increasing the number of gene copies to increase the ability to compete with small DNA fragments, and optimizing the amount of restriction enzyme. Compatible buffers for both restriction and ligation reaction (e.g. from NEB) would be an ideal solution if consumable costs were not an issue. Additionally, purification of PCR products could also be done by alcohol precipitation to remove impurities. Initially, PCR products purification was performed and gave higher cloning performance, without this step reduced efficiency but did not affect cloning results. Lastly, larger sizes of DNA fragments (>1300 bp) could be tested in the following stages of our study to clarify the dependence of cloning efficiency on insert size.

There are two methods similar to AIO including the Improved Restriction Digestion-Ligation (IRDL), and Pyrite cloning. In the IRDL method, plasmid must be pre-treated by inserting the *ccdB* gene. Although the efficiency was 100% successful, the recombinant plasmids no longer contained the *ccdB* gene and many restriction sites.[11] The next cloning step will be exactly the same as the REC, and the limitation of restriction sites will cause many difficulties. The difference between AIO and Pyrite cloning is the time for adding ligase enzyme.[12] In Pyrite cloning, ligase enzyme was added at the same time with digestion. Restriction enzymes in Pyrite have short-digested time, and help reduce the needed time. Digestion was performed at 37 °C, which can adversely affect the ATP required for ligation. Pyrite cloning cannot check whether the digestion has completely occurred; these restriction enzymes have a short-digested time (1.5-2 h), so the time problem for inactivation is unreasonable. Also, thermal cycling like this method is difficult in terms of facilities. In the AIO method, ligase enzyme was added after the end of digestion, and restriction enzymes were inactivated. The ligation mix was incubated at 16 °C for 4 h, or at 4 °C overnight. This condition made the most perfect for ligase enzyme activity.

Table 2 showed that the AIO experiment time was always the shortest (it was only 9 hours in the most favorable case). When compared with REC, the AIO method using Thermo's

restriction enzyme was faster, which required only 2 hours but reduced the loss of PCR products, as well as materials and chemicals. Compared with Pyrite cloning, the AIO (NEB) method using NEB's restriction enzyme greatly reduced the time (more than 6 hours), chemicals, and materials.

Conclusion

We set up a novel cloning method that shortened the purification step but still resulted in the success of obtaining target clones. This method was effective with a wide range of fragments (200-1300 bp) and could be combined with other methods to improve the cloning efficacy. This AIO cloning offered the benefit of simplicity, low cost, and time-saving. It could be expected to contribute a huge effort in cloning strategy, which is an important part of biological researches, especially for laboratories in developing countries where research materials are not abundantly available.

Authors' Contributions

QGM and HTV were responsible for conception and design of the study. QGM and HVVN contributed to acquisition of data, and drafting of the manuscript. HTV, TLT, HVVN, QGM participated in analysis and/or interpretation of data. Finally, HTV and TLT took responsibility for revising the manuscript for significant intellectual content.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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