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Advances in Biochemistry and Biotechnology

Review Article

Kammara R and Vinaytheerthan L. Adv Biochem Biotechnol 6: 10106.
DOI: 10.29011/2574-7258.010106

ATM: Adenine, Thymine Mediated Ligase Free Ligation

Rajagopal Kammara[1*], Lakshmanan Vinaytheerthan[2]

[1]AcSIR, and Department of Protein Chemistry and Technology (PCT), Central Food Technological Research Institute (CSIR-CFTRI), Mysore, India

[2]LV a Junior Research Fellow under DST-SERB project, India

[*]**Corresponding Author:** Rajagopal Kammara, AcSIR and Department of Protein Chemistry and Technology (PCT), Central Food Technological Research Institute (CSIR-CFTRI), Mysore, India.

Citation: Kammara R and Vinaytheerthan L (2021) ATM: Adenine, Thymine mediated ligase free ligation. Adv Biochem Biotechnol 6: 10106. DOI: 10.29011/2574-7258.010106

Received Date: 06 September, 2021; **Accepted Date:** 21 September, 2021; **Published Date:** 24 September, 2021

Abstract

We distinguish a simple method of efficient sequence dependent and natural concatemerization subsequent to the cloning of PCR products bypassing ligation. Tailed PCR primer pairs were employed to create complementary staggered termini with AT (Adenine, Thymine) bases only on the insert / PCR product. The introduced AT bases either in 5' or 3' termini were designed to allow effective intermolecular ligation to internalize the terminal restriction sites in a ligase free manner. The presence of extra nucleotide sequence in the termini does not restrict the subsequent steps of cloning. This ligase free ligation procedure is extremely efficient, and the subsequent cloning not restrained by the presence of an extra nucleotide in either of the termini. The avoidance of ligase based, chimera formation makes the procedure, rapid, reliable and efficient. The process also bypasses the multiple sequence incorporations at the termini to increase the efficiency of restriction digestion.

Introduction

PCR products containing terminal restriction endonuclease sites allow directional cloning to construct recombinant DNA molecules [1]. The entire process depends on the use of (un-phosphorylated) synthesized primers having desired restriction endonuclease site along with 3-4 extra 5' bases flanking the site. Cetus Corporation reported that incorporation of various restriction sites into the terminus of PCR amplified products is not only difficult to digest thoroughly, but also the resulted products found to be partially digested [2]. There are many causes behind their inefficient digestion; one of the most important reason is the breathing effect where the presence of 3-4 or fewer bases flanking the terminal restriction endonuclease recognition site. That does not allow the stable association of restriction endonuclease resulting partial digestion.

Jung, et al. 1990 [3], concluded that the addition of Proteinase K (de-blocking agent), Spermidine (stabilizer) and use of Klenow or T4 DNA polymerase (repairing agent) in the digestion reaction did not improve the cleavage efficiency subsequently the cloning efficiency. Overnight digestion created many other problems such as nuclease contamination and smearing of DNA bands, etc., Therefore overnight digestion did not result in better outcomes. Later, it was evidence that the concatemerization of PCR products, involving kinasing and ligation, are more efficient. There are two different ways of restriction site internalization. The natural and cost efficient way may be by adopting the method introduced by Scharf 1986 [4]. Here they have hypothesized that for efficient digestion of PCR products necessarily requires sufficient distance from the ends of the PCR products. The other way may be by observing the process of internalization, further leading to efficient digestion and cloning involving many steps and resources. The steps involved in the process were purification of the PCR product, kinasing, ligation hence it is laborious, cost efficient and time consuming. Nevertheless, the efficiency was not more than 50 %, and the method lacks reproducibility. Adoption of this procedure results in the conversion of the terminal restriction endonuclease cleavage sites into an internal site, which is an efficiently cleaved. To prove this hypothesis, the PCR product was amplified with phosphorylated primers subsequently, purified as a single band from the agarose gel. Purified and phosphorylated products were ligated resulting in internalization of terminal restriction endonuclease sites. The chimeras subsequently digested with the desired restriction endonucleases. Finally, the single band of digested products was purified from the agarose gel. From the above procedure, it may be seen that the procedure followed by complete digestion, and subsequent cloning was considered laborious, cost efficient and multi-step. To overcome incompetence in the existing process, we present a simple and novel method where kinase and ligase dependent concatemerization of amplified products

bypassed. Hence, it is a ligase free ligation. We state that it is a sequence dependent process, where the N-terminal AT sequence plays a significant role.

Jung, et al. (1990) [3], developed a method for efficient digestion of amplified products further leading to 1000 fold increase in cloning efficiency. Therefore, internalization of terminal restriction endonuclease sites can overcome the problem of incomplete digestion, which further results in decreasing the cloning efficiency. In the present manuscript, we introduce a new method of internalization of terminal restriction endonuclease sites just by the introduction of adenine, and thymine nucleotides at N-terminal or flanking regions of a restriction site. Following PCR parameters were followed to amplify Serine Threonine Kinase (STK) gene of Bifidobacterium catanulatum. PCR amplifications were carried out in a 50 µl reaction volume containing 100–250 ng of template DNA, 0.2 µM of each primer, 200 µM of dNTPs (New England Biolabs, Inc. MA, USA) buffer (10 mM Tris-HCl, pH 8.3 (at 25 ˚C), 10 mM KCl and 3 mM $MgCl_2$ and Taq DNA polymerase (New England Biolabs, Inc.MA, USA). After PCR amplification, the product was purified and concatemerized by bypassing kinasing as well as ligation to internalize the terminal restriction sites. The pre-requisite for successful internalization is the introduction of adenine and thymine nucleotide bases in the flanking regions of cloning sites that do not hybridize to the target DNA sequence.

Regardless of the type of cloning site, it is essential to incorporate minimum two bases adenine and thymine flanking the site. The presentation of these nucleotide bases at its N-termini helps in dimerization of the fragment and in internalization of the restriction endonuclease site. Internalization further helps in efficient digestion and leading to increasing in its cloning efficiency. To accomplish this, we used oligonucleotide primers with only two nucleotides adenine and thymine flanking the cloning site. This procedure aids in making only dimers in a single direction (head to head), after the introduction of repetitive adenine and thymine nucleotide bases did not succeed in making oligomerization of the PCR product. Therefore, we standardized the methodology just by an introduction of adenine and thymine nucleotide bases (ATATAT) only PCR amplifications were followed, and validated the amplification by agarose gel electrophoresis (Figure 1A). Purified PCR products (Figure 1B) incubated at room temperature irrespective of their concentration, pH and temperature. After 30 min to 2 days of incubation (at room temperature and 4.0 ˚C) (Figure 1C) they were subjected to restriction enzyme digestion. The formation of dimers is validated by running agarose gel electrophoresis (Figure 1D). After confirmation of dimer formation, they do not require kinasing/phosphorylation or ligation; one can go for digestion of the fragments. It was noticed that the addition of multiple AT nucleotides at the N-termini or C-termini of the fragment forms dimer only, but not trimer or oligomer (Figures 1B and G). Establishment of these results through southern blotting confirm that only the dimer formation observed, but not the trimer or oligomer (data not shown). This method rapidly increases the efficiency, reproducibility of restriction digestion (Figure 1D). Therefore, it is simple, cost effective method of cloning DNA fragments with flanking restriction endonuclease recognition sites.

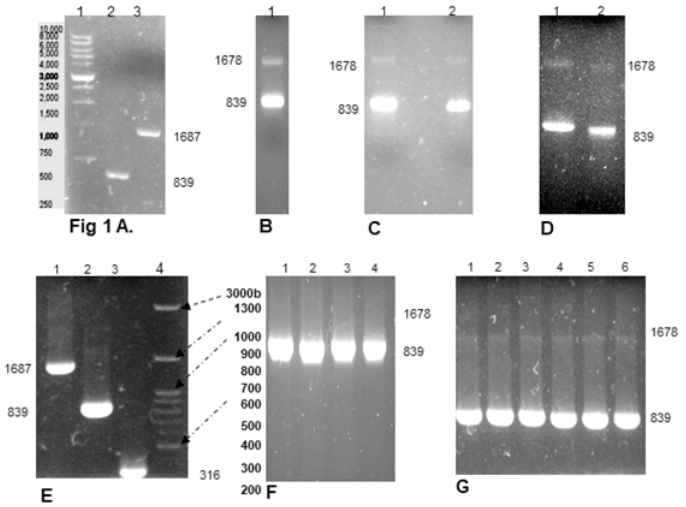

Figure 1: A, B, C, D, E, F, and G.

A: Lane 1. Molecular weight marker, Lane 2. Amplified PCR product 839 bp fragment, Lane 3. Dimerized state of 839 bp fragment as soon as after amplification and purification; **B:** 839 bp fragment showing dimerization after incubation at room temperature (contain terminal AT nucleotides); **C:** Purified terminal AT containing fragments subjected to high temperature incubation 37 and 42ºC for 15 min. **D:** Purified terminal AT containing fragments subjected to high temperature incubation at 42 and 50ºC for 15 min; **E:** Lane 1 Dimerized product, Lane 2. Dimerized product digested with Nco1 and Xho1 (head to tail orientation). **F:** Lane 1 Mol. wt marker, Lane 2 to 5. Amplification without terminal AT nucleotides. **G:** Amplification with different primers having combinations of AT, and TT etc.

The unidirectional dimerization of the fragments is independent of temperature, concentration (Figure 1F) and pH. It may be an intrinsic property of AT-rich region. To understand the stability of the dimers formed, the dimerized products were heat treated at 50-60 ˚C for 15-20 min, resulting in conversion to monomer from dimer (data not shown), and the dimers formed are transient and unstable in nature. To corroborate the importance of AT-rich terminal regions, experiments performed where AT-rich regions removed and observed that they do not form dimers (Figure 1E). This further confirms that concatemerization is by-passed to internalize terminal restriction sites to enhance restriction enzyme digestion, subsequently, improving the cloning efficiency.

The AT mediated dimerization is a molecular weight dependent process, does not work if the molecular weight of the fragment is above 800 bp (base pairs). The fragments of around 1387 and 2250 bp containing terminal AT-rich regions do not show dimerization, except the 800 bp product (Figure 1F). This further proves that the essential requirement is 800 bp fragment, higher molecular weight fragments do not form dimers. Formation of only dimers further emphasizes the importance of molecular weight of the fragment and its essentiality to dimerize. It may be because it is easy to find terminal regions of smaller fragments in comparison with larger fragments. Formation of trimers does not occur because it is hard to identify the termini of dimerized DNA fragments. Further to understand the importance of AT-rich regions, single, double and triple AT nucleotides were introduced into the primer, and they were further used for PCR amplification. The products purified and subjected to agarose gel electrophoresis and found that regardless of the number of terminal AT nucleotides, they form only the dimers but not trimers or oligomers (Figure 1G). It is noticed that the addition of multiple AT nucleotides at the N-termini or C-termini of the fragment forms dimer only, but not trimer or oligomer (Figure 1B and G).

We further confirm and state that to increase the efficiency of restriction digestion and ligation introduction of terminal AT nucleotides is most indispensable. This process does not require and bypasses internalization of terminal restriction sites by kinasing and ligation. The method does not depend on the terminal transferase activity of polymerases like the other reported methods [5]. Hence, the method is not specific for a single polymerase, and it can be subjected irrespective of polymerase. Therefore, the method is a very useful instrument for molecular scientists. Previous reports by Jong, et al. 1990 in ligation independent cloning of PCR products is considered to be again laborious and cost efficient. Method mainly depends on many modifying enzymes as well as DNA polymerase to generate long cohesive ends. The essential requirement of the method is an amplification of whole plasmid DNA; therefore, there are brighter chances for the introduction of mutations as the molecular weight of the plasmid increases. The method also mainly relies on the introduction of additional 12 nucleotide sequences lacking dCMP. In comparison, the present method is highly efficient, reproducible and cost ineffective.

Conclusion

We successfully developed a sequence dependent oligomerization of DNA fragments a method. The process bypasses the kinasing and ligation, in this process the incorporated AT-rich terminal ends helps in internalization. The process is molecular weight dependant and helps in efficient restriction enzyme digestion.

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