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Application of Molecular Evolution Techniques in the Field of Modern Biology

Junqin Guo

College of Biology Science and Medical Engineering, Donghua University, Shanghai, China
211140113@mail.dhu.edu.cn

Abstract. Molecular evolution is gaining momentum in the field of modern biology, especially in molecular biology and protein and enzyme engineering. Among the masses of techniques, error-prone PCR, DNA shuffling and saturation mutagenesis have stood out from the crowd and become three of the most commonly adapted research techniques in molecular evolution. The outcomes of molecular evolution have been widely applied to biological and medical industries, including the development of engineered enzymes, improving existing proteins' or peptides' properties, therapeutic protein modification, protein structural and functional studies, etc. However, these three techniques occupy distinct advantageous niches and concomitantly, they are constrained by their respective limitations. For the purpose of solving the limitations, a multi-technology combination is suggested and the the rapid proliferation of cross-disciplinary research and technological innovations, molecular evolution combined with computer science and bioinformatics have developed several groundbreaking research paradigms and technical solutions. Among these techniques, multiple sequence alignment is one of the technologies most closely associated with molecular evolution, especially with saturation mutagenesis. Current flourishing applications of molecular evolution and continuous technological refinements provide compelling evidence that molecular evolution research will remain a pivotal component in modern biological sciences.

Keywords: Molecular Evolution, Error-prone PCR, DNA Shuffling, Saturation Mutagenesis

1. Introduction

Molecular evolution techniques have emerged since the end of the 20th century. Based on the central dogma, The substitution of bases in DNA sequence can finally reflect the substitution of amino acid in polypeptide sequence after transcription and translation. As the primary structure of the protein, the mutation of the sequence of protein may modify the function of the protein in aspects such as stability and reactivity, etc. which is the general theory of molecular evolution.

With the development of technology, in the field of protein engineering, scientists have developed three different techniques to achieve Molecular evolution which are Error-prone PCR, DNA shuffling, and saturation mutagenesis.

In 1989, Lenug proposed an inchoate error-prone PCR (epPCR) technique that utilizes the characteristic lack of 3' to 5' exonuclease activity in Taq DNA polymerase [1]. EpPCR does not

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require structural studies, the key point of whether the evolution is successful or not mainly depends on fortune because the mutation randomly occurs in DNA fragments when applying PCR. With the accumulation of mutation in target fragments, molecular evolution based on error-prone PCR will become possible. In order to increase the mutation rate to achieve evolutionary goals faster, researchers have developed several methods to optimize epPCR including applying additional metal ions like manganese and magnesium and utilizing lopsided dNTP concentration which can promote the possibility of DNA polymerase's error frequency [2, 3].

In 1994, Stemmer presented an innovative technique known as DNA shuffling which concentrates on molecular evolution research [4, 5]. Stemmer claimed that DNA shuffling is an advanced technique compared to epPCR because of the higher mutation rate the DNA shuffling has and DNA shuffling can avoid the accumulation of neutral mutation which may enhance the immunogenicity of the target protein. The principle of DNA shuffling is quite different compared to epPCR, DNA shuffling requires the homology between fragments that participate in DNA shuffling. After digested by DNase I or broken by ultrasonic, fragmented parental DNA fragments will be assembled by primerless PCR. After amplified by DNA polymerase, homologous DNA fragments from diverse sources will be assembled into chimeric constructs. These chimera DNA fragments may exhibit different properties compared to their parents.

Both PCR and DNA shuffling are random mutageneses, they do not pay attention to a specific point on the target sequence, they are much simpler to operate but for performing more elaborate molecular evolution manipulations, saturation mutagenesis (SM) will be more appropriate. In 1979, S. Gillam and M. Smith proposed a specific point mutation approach which set a precedent for the subsequent research on saturation mutagenesis [6]. In 1985, wells et al. developed cassette mutagenesis which systematically leverages degenerate codons as the main measure of saturation mutagenesis [7]. Three years later, Reidhaar et al. modified this technique and designed a standardized degenerate codon NNK which includes all 20 amino acids' codons and only one termination codon [8]. Since then, employing degenerate codons has become a popular issue of SM and is widely used in the field of molecular evolution. SM requires foundational research of the structure of target protein and rational design. All of these evolutionary strategies have a comprehensive application in modern biological research.

Recently, all of these three biotechnology have wildly applied to various life science research fields including optimization of protein properties, protein interactions, functional studies of protein domains, etc. Employing these technologies separately may cause several shortcomings which include a lack of evolutionary targeting, an insufficient amount of introduced variants, restriction to the parental fragments, high demand for structural understanding, etc. However, these shortcomings do not prevent these techniques from becoming mainstream in the direction of molecular evolution in modern biological research.

2. Applications of error-prone PCR

Owing to its straightforward operational principle and broad applicability, epPCR has become one of the most widely utilized techniques in structure-independent molecular evolution approaches.

In 2021, Gallo et al. applied epPCR to the haemagglutinin protein of peste des petits ruminants virus to investigate its receptor-binding function [9]. After analyzing the receptor binding domain, they build a mutation library and research the effect of point mutations on ligand-receptor binding. The research team has detected two major types in the library, functional mutants and non-functional mutants. For the functional mutants, the activity of haemagglutinin is not significantly affected. These mutants can be summarized and generalized as conservative mutations like I498V, D530E

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which amino acids have similar properties between the pre-mutant and post-mutant, and neutral mutations like R556Q and H575Y which do not affect the key structure of the protein. Additionally among the functional mutants, there still are individuals who do not have amino acid substitution. On the other hand, non-functional mutants have exposed massive information about the function of haemagglutinin. About 38% of individuals exhibit a nonsense mutation. These mutants shorten the original protein and dramatically affect the function of the protein, all of these have exhibited a loss of function. Other individuals exhibit significant variations of the function, for example, mutant H536L breaks the hydrogen bond between Y529, mutant I538N causes the structure to collapse to the hydrophobic core which decreases the expression of the protein and lack of glycosylation, mutant N468I, V515F, Y529, etc., these mutants causes structure alteration and misfolding and finally leads to degradation. This research pays attention to structure investigation, through negative evolution, disclosing the critical residue in the protein of interest and will have an essential contribution to protein science.

EpPCR can also be applied to improve the function and properties of proteins. In 2020, Simons et al. applied epPCR to evolve antibodies targeted to four immune-oncology proteins [10]. The research team employed Mutazyme II from Agilent Technology as their polymerase and the results of the evolution exhibit a uniform distribution of mutants among the sequence of single-chain variable fragments (scFv) which includes complementarity-determining regions and framework regions. After the mutagenesis, there are three amino acid mutants are induced into the antibodies on average. The research team paid attention to the affinity of the antibodies and the yeast display exhibits that scFv's affinity against PD-1, CTLA-4, and OX40 has promoted 8.3-fold, 5.8-fold, 8.3-fold respectively, but scFv against PD-L1 do not exhibit a significant improve. Meanwhile, in vitro experiments display that epPCR-evolved antibodies have exhibited a 2-fold improvement of blocking activity against PD-1 and CTLA-4. Another research done by Liu et al. in 2023 focuses on improving pullulanase (PulB)'s catalytic performance by epPCR [11]. After two rounds of mutagenesis, they build the error-prone library and screen the property of the library. They have achieved four individuals who exhibit improved activity. Among these mutants, G250P, T252S, G253S, and N255K have improved their activity 1.9-fold and the value of km, kcat and their ratio have improved for 22.7%, 28.7%, and 68.4% respectively. Moreover, the thermal stability of these mutants has significantly improved, these mutants' half-life at 60°C has improved by 87.5% compared to their parents. Furthermore, epPCR does not have a restriction on the length of DNA fragments and it can be applied to evolve polypeptides or even oligonucleotides. Zhan modified a mini-split intein Npu DnaE (NE) by epPCR [12]. NE is a naturally occurring split intein from cyanobacterial whose length is only 143 amino acids. Zhan's research exhibited the feasibility of applying epPCR to polypeptide. The sequencing results revealed that the mutant library was built successfully and laid the foundation of molecular evolution aiming to inteins. In the same research group, zhan's study is following up by others and continued. Researchers have developed a modified mini intein named Ter DnaE3 (TE3) which is homologous to NE by PCR. Modified TE3 has exhibited a significant improvement in splice activity and has fewer side effects. Another research reported by Lee et al. in 2023 has paid attention to the initiation site of translation in mRNA which only contains 36 base pairs [13]. They have developed an advanced epPCR protocol which is aimed to short DNA fragments. According to their reports, employing multiple dilutions to the templates amplifying the fragments with low cycles, and repeating the amplification program will be helpful to induce substitution to the short DNA fragments. Lee et al. claimed that they have successfully induced 1.2 mutations in the parents on average.

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Error-prone PCR has emerged as one of the most widely adopted techniques in molecular evolution due to its operational simplicity and versatility. However, this method exhibits inherent limitations: completely random mutagenesis patterns, lack of target specificity, low mutational density per round and dependence on iterative PCR amplification cycles that render the procedure labor-intensive despite its straightforward principle. These characteristics currently position epPCR primarily as a foundational tool for preliminary molecular evolution studies, particularly for proteins lacking structural characterization.

3. Applications of DNA shuffling

DNA shuffling is a next-generation molecular evolution technique, it has a higher mutation rate than epPCR but also has limitations on parental selection. Generally, parental chains applied in DNA shuffling require at least 70% identity to ensure the feasibility of DNA shuffling. Fortunately, several groups of researchers have developed the original DNA shuffling protocol and improved its operability. Ma et al. reviewed these improved techniques in their textbook and summed by the author in the table 1 below [14]. These improved techniques have widely increased DNA shuffling's applicability, but due to economic aspects and convenience of operation, original DNA shuffling and DNA family shuffling are more widely applied in molecular evolution.

Table 1. The process of improvement of DNA shuffling

Developers	Technique	Improvement
W. Stemmer et al.	DNA family shuffling	Higher recombination efficiency and mutation possibility
Zhao et al.	staggered extension process	Omitting the DNase I digestion process and better hybridization effect
Lutz et al.	SCRATCHY	Combine with incremental truncation for the creation of a hybrid enzyme and do not depend on sequence homology.
J.A. Bittker et al.	An unnamed nonhomologous random recombination method	Do not require homology as well as employ controllable length of parental chains

In 2022, Yao et al. pay attention to AprE which is an alkaline serine protease from Bacillus subtitles [15]. The research team collected four different AprE from different species of B.subtilis which are AprE3-5, AprEJS2, AprESJ4, and AprE176. After applying a DNA family shuffling program, they acquired a modified aprE enzyme which is named AprEF5M4 by the team. According to their test, AprEF5M4 exhibits higher thermal stability and catalyze activity. AprEF5M4 can tolerate at 55°C and its activity still remains at 21.26% while the parental AprE is devitalized. According to the sequencing results, AprEF5M4 is chimera which is mainly recombined with AprEJS2 and AprE176, some regions of AprEF5M4 are contributed from AprE3-5 and AprESJ4. Another molecular evolution research done by Nunez et al. in 2024 focuses on an outer membrane protein of E.coli named OmpA applying ordinary DNA shuffling [16]. The main purpose of their study on OmpA is to evolve mutants with lower interfacial tension (IFT) and analyze the relationship between structure and function. The result exhibits that among the 25 clones they screened, clones 6 and 12 have exhibited a significant decrease in the value of IFT. The analysis of the structure by AlphaFokd2 revealed that clone 12 contains an explicit α helix which provides stability to the whole structure.

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Besides the limitations on homologous, fragment length is another key aspect of DNA shuffling. Also in the research group which study about inteins mentioned previously, inchoate research on employing DNA family shuffling to three homologous inteins is exploited by the author and followed up by others in the group. The selected parental fragments were two naturally occurring intein Npu DnaE and Ter DnaE3 and an artificial intein Cfa DnaE. All of these fragments are shorter than 500 nt, and the result of BLASTn indicated that the homologous between the three fragments is between 71% to 79%. The research shows that these fragments are not sufficient to form a chimera by DNA family shuffling but appear to acquire point mutations and the amount of the mutations in each individual ranges from 0 to 6. Researches suggest that DNA family shuffling of short DNA fragments should utilize parental strands with both high sequence homology (identity should be higher than 90% at least) and extended continuous regions of similarity to ensure effective chimeric formation. Failure to meet these criteria may compromise recombination efficiency.

Moreover, an interdisciplinary study published in 2024 by Schweiggert et al. has combined DNA shuffling with computer science which will bring convenience to modern life science research [17]. The research team devised a Python-based program named Shuffle Analyzer which aims to analyze the sequencing results after DNA shuffling. The computational algorithm probabilistically assigns parental origins to DNA segments in chimeric sequences by comparing user-submitted parental templates with post-shuffling recombinants. It can also identify the mutation sites on the chimera segments that do not belong to the parental segments and are induced by DNA shuffling. All these computational results will be exhibited in a GUI frame with colored figures which will help the user to understand the analytical results.

DNA shuffling is a more advanced technique compared to epPCR, but its strict restrictive requirements limit its range of applications. Although researchers have developed several improved techniques based on DNA shuffling, these techniques are still not widely utilized in the field of modern biology. However, this cannot prevent DNA shuffling from becoming a great and ingenious invention.

4. Applications of saturation mutagenesis

Differing from epPCR and DNA shuffling, saturation mutagenesis requires an understanding of protein structure. Molecular evolution employing SM usually focuses on specific sites in the protein like the active center of the enzyme, the critical domain of protein. While PCR and DNA shuffling represent more contemporary approaches in directed evolution, SM stands as an earlier-generation technique with distinct methodological characteristics.

Early saturation mutations based on degenerate codon technology utilize “NNN” as a degenerate codon which includes all 64 types of codons. In 1992, Sherman et al, concentrated on two positions in the reaction center of Plasminogen Activator Inhibitor-1(PAI-1). Two positions they chose were marked as P1 and P1’ respectively and after screening the library, researchers considered that only basic amino acid in site P1 can generate the inhibition while site P1’ is not sensitive to the replacement beside proline. According to the sequencing of evolved PAI-1, P1Lys-P1’Ala exhibits 40-fold inhibitory activity compared to the parental enzyme. Meanwhile, another mutant P1Lys-P1’Trp exhibits 6.5-fold inhibitory activity.

For modern molecular evolution studies, researchers prefer to utilize more concise combinations of codons. Except for the degenerate codon “NNK” which is mentioned previously in the introduction, researchers have also developed another degenerate codon “NNS”. Both NNK and NNS contain 32 different codons and fully cover all 20 common amino acids that construct proteins. In 2020, Raluca et al. applied NNK as a degenerate codon to modify Phenylalanine Ammonia

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Lyases (PAL). Researchers focus on the critical residue I460 of PAL for the purpose of enhancing the activity to catalyze non-natural substrates. They have screened several mutants which exhibit significantly improved activity to p-MeO-Phe which is a non-natural substrate to PAL. According to the screening results, I460T and I460S exhibited a high transformation rate to the p-MeO-Phe ammonia elimination reaction. Notably, variant I460T still maintains a high catalytic activity to L-Phe which is the natural substrate of PAL while mutant I460V which is reported by the research team previously exhibited a decreased activity to L-Phe. Moreover, variant I460S manifests instability while variant I460T has a similar stability to the wild type. Finally, the research team employs molecular docking to simulate the conformation of the activity center of PAL. Both I460T and I460V have demonstrated an advantageous conformation to p-MeO-Phe while wild-type PAL has steric hindrance that is unable to catalyze p-MeO-Phe. Another research done by Helen et al. in 2022 pays attention to the spleen tyrosine kinase (Syk) family [18]. Human Syk is unable to be expressed in E.coli which will pose an obstacle to the research. In order to improve the stability of Syk, researchers utilize a multiple sequence alignment based on 183 different modern Syk family kinases and design an ancestral kinase named AncSZ which is an artificial common ancestor of Syk and ZAP-70 which is a control kinase of Syk. After that, they applied NNS as a degenerate codon to modify AncSZ to promote expression efficiency and improve the properties of enzyme and molecular biological study. They have selected several positions as their saturation mutagenesis site and the screening result demonstrated that mutant L616R has a significantly improved soluble expression in E.coli 75 times. Meanwhile, mutant M426E which is a critical residue for regulation has promoted the activity 2.5 times while mutant L616R does not display a promotion in activity. A similar study was done by Julita et al. in 2023 also applied NNS as a degenerate codon. The main purpose of their study is to understand the relationship between the structure as well as the critical residue of α-synuclein and the aggregation principle of α-synuclein. They have a bold research program and construct a library that contains saturation mutations at every amino acid site in the whole segment. After being screened by fluorescence-activated cell sorting, the research team has identified two essential positions among the α-synuclein. Mutations on the 65th to 78th residue have prime importance for the aggregation of α-synuclein. Mutants on this residue will inhibit the formation of aggregation. On the opposite, the local structure of the N-terminal will inhibit the aggression by electrostatic interaction. Mutants in this region like E13K which is mentioned in the article will increase the aggregation tendency. Julita et al.'s study has set a precedent for saturation mutagenesis-based molecular evolution research without structural biology foundations. However, the enormous workload may hinder the effective generalization of this approach to other molecular evolution studies, especially for large proteins or proteins that are difficult to purify or express.

Nevertheless, utilizing a degenerate codon is not the only option for SM. A study done by Vitaly et al. constructed a primer set that contains different primer pairs for the molecular evolution of β-Lactamase TEM-1 [19]. Researchers focus on M182 residue which has already been reported as a frequently-used variant of TEM-1. Some previous studies have reported M182T as an advanced variant and have already been applied to treatment. The research team is aimed at the regulation mechanism of this site and designing a primer set to substitute methionine with every other 19 kinds of amino acids. Their research results indicate that M182X (X refers to any amino acid) does not display a significant difference in the catalytic parameter, while M182T, M182S, M182A, and M182C exhibit improved thermal stability. In contrast, M182L and M182D display reduced thermal stability. Additionally, researchers also measured the thermoreactivation ability of each mutant and demonstrated that M182E, M182I, and M182T exhibit improved thermoreactivation ability compared to the wild type. Structural research reveals that M182 interacts with V159–R65–E177;

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mutants M182E and M182T maintain the interaction through electrostatic or hydrophobic forces and stabilize the loop structure. In contrast, mutants M182L and M182G disrupt the interaction and render the loop more flexible, which decreases thermal stability and thermoreactivation ability.

Saturation mutagenesis represents a structure-guided approach in molecular evolution. Compared to DNA shuffling and epPCR, this technique offers more predictable and reproducible evolutionary outcomes. However, it requires substantially more preparatory work to establish experimental frameworks. While exceptions exist - such as the whole-sequence saturation mutagenesis reported by Julita et al. - the associated labor intensity and economic costs significantly exceed those of DNA shuffling or epPCR. In contemporary biological research, saturation mutagenesis is increasingly integrated with bioinformatic tools like multiple sequence alignment, and such interdisciplinary synergies are expected to advance molecular evolution studies.

5. Conclusion

In summary, epPCR, DNA shuffling and SM are three of the most popular techniques in the field of contemporary molecular biology. For the early research, epPCR and DNA shuffling do not require deep understanding of the structure and mechanism of the target protein. EpPCR is an experimental techniques that are easy to operate, it is an excellent choice for elementary researches of molecular evolution. On the premise that conditions permit, DNA shuffling demonstrates superior operational efficiency compared to error-prone PCR (epPCR), offering both shorter experimental cycles and higher mutagenic diversity, making it the more effective choice for molecular evolution applications. Furthermore, for the advanced applications, saturation mutagenesis remains one of the most prominent molecular evolution techniques in modern molecular biology, even four decades after its introduction, owing to its exceptional outcome reliability and comprehensive coverage of mutational possibilities.

Notably. rather than employing one of the above three techniques alone for molecular evolution, modern molecular evolution researchers are more inclined to combine multiple evolutionary methods. For instance, an initial mutagenic library can be constructed and screened using epPCR due to its random mutagenesis and broad applicability. This primary library then serves as the foundation for subsequent DNA shuffling. Or, applying a sequence alignment to the sequences in the random library and followed by focused saturation mutagenesis at key divergent positions. Such an integrated approach effectively addresses the limitations of applying these techniques alone and will be a potential research thought for molecular evolution.

Also, interdisciplinary applications are extremely prominent in recent years. With the integration of computational technologies and bioinformatics, molecular evolution techniques have experienced a renaissance in recent years. This interdisciplinary synergy has enabled several amazing next-generation technologies possible. For example, more precise identification of evolutionary targets through combined multiple sequence alignment and molecular evolution analysis, visualization of evolutionary outcomes via computational protein structure and function simulations, and algorithm-guided designs of iterative evolution pathways. These technological advances have enhanced the efficiency and rationality of achieving desired evolutionary outcomes. However, as much of the research in this field remains in its nascent stages and the author's expertise in computer science is limited, this paper does not provide a comprehensive review of this aspect.

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