Multidisciplinary Surgical Research Annals
https://msrajournal.com/index.php/Journal/issue/view/15
Volume 3, Issue 3 (2025)

Online ISSN 3007-1941
Print ISSN 3007-1933



# DNA Restriction-Free Overlapping Sequence Cloning Techniques: A Modern Toolkit for Synthetic Biology and Genetic Engineering

https://doi.org/10.5281/zenodo.16977920

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## Article Details

**Keywords:** Restriction-free cloning, Gibson Assembly, Circular Polymerase Extension Cloning, In Vivo Cloning, Polymerase Incomplete Primer Extension, Sequence and Ligation Independent Cloning, Overlap Extension PCR Cloning

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## ABSTRACT

**Background**: Traditional restriction enzyme–based cloning techniques are limited by dependence on specific recognition sites, reduced flexibility, and introduction of unwanted sequences. To overcome these barriers, restriction-free overlapping sequence cloning methods have emerged as powerful alternatives. These strategies exploit homologous sequence overlaps and enzymatic reactions to enable scarless, seamless, and efficient DNA assembly. Over the past two decades, approaches such as Gibson Assembly, Circular Polymerase Extension Cloning (CPEC), In Vivo Cloning, Polymerase Incomplete Primer Extension (PIPE), Sequence and Ligation Independent Cloning (SLIC), and Overlap Extension PCR Cloning (OE-PCR) have reshaped synthetic biology and genetic engineering. **Methods**: A comprehensive literature search was performed for studies published between 2000 and 2025 in peer-reviewed journals. Inclusion criteria encompassed experimental studies, reviews, and mechanistic reports focusing on restriction-free, overlap-based cloning methods. Exclusion criteria removed articles not in English, lacking full-text availability, providing insufficient methodological details, or relying exclusively on computational predictions. Non–peer-reviewed publications, editorials, conference abstracts, and unrelated molecular biology studies were also excluded. **Results**: Analysis of selected literature demonstrated that overlap-based cloning strategies provide higher versatility and fidelity compared with conventional methods. Gibson Assembly and In Vivo Cloning showed the highest adoption due to efficiency and user-friendliness, while CPEC, PIPE, SLIC, and OE-PCR offered cost-effective and flexible alternatives for specific applications. These methods enable efficient construction of genetic circuits, gene synthesis, metabolic pathway engineering, and therapeutic constructs. Comparative assessments revealed trade-offs in error rates, fragment size limits, scalability, and cost across different techniques. Integration into high-throughput and automated systems has further enhanced their applicability in synthetic biology. **Conclusion**: Restriction-free overlapping sequence cloning has become a cornerstone of modern molecular biology, providing a robust toolkit for seamless DNA assembly. Its adoption facilitates rapid progress in synthetic biology, functional genomics, and therapeutic engineering. Future directions include integration with CRISPR-based editing, cell-free systems, and emerging DNA synthesis technologies, which together promise to expand the scope and efficiency of genetic engineering.

## INTRODUCTION

DNA cloning methods are fundamental tools in molecular biology, synthetic biology, and genetic engineering that enable precise DNA manipulation for various scientific and biotechnological applications (Bomfiglio et al., 2025). One of the most crucial underlying technologies for metabolic engineering and synthetic biology is DNA assembly. Much work has gone into creating better DNA assembly techniques with greater efficiency, fidelity, and modularity as well as quicker and easier protocols since the invention of the restriction digestion and ligation approach in the early 1970s (Chao et al., 2015). Rapid prototyping of the desired metabolic pathways or genetic circuits depends on techniques for assembling genetic components into reproducible and expressible DNA molecules in metabolic engineering and synthetic biology projects. Even though DNA can be chemically produced up to a certain length, enzymatic assembly techniques are still needed to create longer pieces (Kosuri and Church, 2014). Plasmid vectors with small or multiple-tags, and small RNAs are commonly used in biomedical studies. Current methods of plasmid construction require obtaining the gene of interest from the amplification of the DNA template or chemically synthesized oligonucleotides (Liang et al., 2011). Obtaining a gene fragment of interest from the DNA library is the most commonly used method (Fenderson et al., 2008). It relies on digestion of DNA by type II restriction enzymes, and cutting the plasmid DNA circles with the same restriction nuclease to create linear DNA molecules. Finally, the gene of interest and the linearized vector are separately purified, mixed together at a certain molar ratio, and ligated with T4 DNA ligase to form recombinant DNA circles (Berg et al., 2011). This process involves cumbersome subcloning procedures including designing primers, digestion, ligation and verification of recombinant plasmids (Xu et al., 2008).

Therefore, its efficiency is dependent on both the restriction enzyme and the DNA ligase. In addition, unwanted nucleotide sequences may be added to the insert, and result in undesirable changes in the final products (Liu et al., 2017). The chemical synthesis method is also called oligo overlap cloning, which can be used to add a short stretch of DNA to a plasmid, such as adding short tags (HA-tag or Flag) and cloning shRNAs (Liang et al., 2011). The DNA fragment of interest with restriction site is obtained through chemical synthesis, and the linearized vector with sticky ends is obtained by digesting the vector with restriction endonuclease (Miyagishi et al., 2004). Both methods share similar limitations. To overcome these limitations, several PCR-based cloning protocols have been proposed to skip the use of DNA ligases and restriction endonucleases. Similar to our method, these methods are based on homologous recombination. The PCR products are flanked by 15 to 60 bp sequences, which exactly match the ends of the linear vector (Jacobus et al., 2015). These include in vitro Gateway recombination cloning technology, (Hartley et al., 2000) seamless ligation cloning extract (SLiCE) (Okegawa et al., 2015) and RecET, etc. The SLiCE method from a commonly used laboratory E. coli strain, JM109 and DH5α, can efficiently function as an effective alternative to commercially available seamless DNA cloning kits (Okegawa et al., 2015). It also includes in vivo recombination

methods, such as relying on RecA recombination enzymes, Red/ET recombination systems, (Lovett et al., 2002) and DH5α competent cells (Motohashi et al., 2017). These methods require preparing the target gene and linearized vector, respectively. The target gene can also be inserted by the QuikChange site-directed mutagenesis method (Li et al., 2008). Similar to our one-step PCR method, the target fragment can be inserted at any amplifiable site of the vector. The difference is that when we designed the primers, we divided the target sequence into three parts, the target sequence at the 50 end of the forward primer, the target sequence at the 50 end of the reverse primer, and a 15 bp homology arm with over lapped region for target gene. In the QuikChange site directed mutagenesis method, the sequence of the target fragment was designed at the 50 end of the forward primer, while the 50 end of the reverse primer contained only a 15 bp homologous arm. Compared with the QuikChange site-directed mutagenesis method, our one-step PCR method reduces both the cost for primer synthesis and the formation of primer dimers. Although DNA inserts below 90 bp can be inserted using the SLiCE method, (Messerschmidt et al., 2016) it is still difficult in most plasmid construction methods to insert short DNA fragments less than 150 bp.22 DNA inserts below 150 bp can be directly synthesized, but it is cost prohibitive with limited productivity.

Also, the effects of synthesizing two short oligonucleotides below 150 bp still depend on the efficiency of annealing, endonuclease, and ligase, etc. The fidelity also significantly decreases when synthesizing oligonucleotides between 100 bp and 150 bp. Therefore, with these traditional methods it is especially inappropriate to construct plasmids with DNA fragments below 150 bp. In this study, we amplified a linear vector including the short target gene below 150 bp by one-step PCR. A DNA repair system in E. coli, homologous recombination, which can cyclize linearized vectors with homology arms, has been previously reported (Liu et al., 2017). Employing the characteristics of E. coli, we developed a method for constructing plasmids based on one-step PCR and homologous recombination. Previously reported methods of constructing plasmid using homologous recombination of E. coli are all for genes longer than 150 bp (Kostylev et al., 2015). Despite its popularity, this 40-year-old method's shortcomings limit our capacity to synthesize complex DNA molecules. Higher efficiency and fidelity in DNA assembly are becoming more and more necessary for complex DNA construct design including numerous genes and intergenic components, which is beyond the scope of conventional cloning techniques based on restriction digestion and ligation (Cobb *et al.*, 2013). Furthermore, choosing distinct restriction sites is very challenging due to the size of these DNA constructions. The modularity of DNA assembly, a defining feature of synthetic biology, is severely hindered by the fact that restriction sites, even if they could be chosen for a particular construct, would probably not be appropriate for another construct. In fact, many research initiatives strongly favor modularity. Combinatorial pathway library construction and screening, for instance, has been shown to be a successful method for pathway optimization. This method involves mixing and matching different genetic components with similar functions in order to find

specific combinations that enhance the metabolic flux or other characteristics (Kim *et al.*, 2013; Xu *et al.*, 2013; Yuan *et al.*, 2013). Similar to this, rapid prototyping in genetic circuit design necessitates the assembly of several prefabricated, defined components (Canton *et al.*, 2008; Purnick and Weiss, 2009). Thus, widely applicable and extremely effective DNA assembly techniques are preferred. This review systematically summarizes the major restriction-free overlapping sequence cloning (RFOSC) techniques currently used in synthetic biology and genetic engineering and examines their development, efficiency, practicality, and specific applications.

## 2. METHODOLOGY

To uncover, assess, and incorporate pertinent results from old and new developed research, the review employs a systematic methodology.

### 2.1. LITERATURE SEARCH STRATEGY

A comprehensive literature search was conducted using databases such as PubMed, Scopus, Web of Science, and Google Scholar. The search included studies published between 2000 and 2025, ensuring a broad coverage of both foundational and recent advancements in the field. Keywords used in the search included "DNA Restriction-Free Overlapping Sequence Cloning Techniques". Boolean operators (AND, OR) were applied to refine search queries and capture relevant studies. A total of 877 records were initially identified. After removing duplicates and applying inclusion/exclusion criteria, 110 studies were included in the final qualitative synthesis.

### 2.2. INCLUSION AND EXCLUSION CRITERIA

To guarantee their quality and relevance, studies were chosen using predetermined inclusion and exclusion criteria.

**• Inclusion Criteria**

Studies focusing on DNA cloning methodologies that utilize restriction-free, sequence-overlap-based strategies such as Gibson Assembly, Circular Polymerase Extension Cloning (CPEC), In Vivo Cloning, Polymerase Incomplete Primer Extension (PIPE), Sequence and Ligation Independent Cloning (SLIC), Overlap Extension PCR Cloning (OE-PCR), and other related techniques. Research articles, reviews, and experimental studies published in peer-reviewed journals that describe the mechanism, applications, or efficiency of restriction-free overlapping sequence cloning approaches. Papers published in English between 2000–2025 to capture both the origin and recent advancements of restriction-free cloning methods.

**• Exclusion Criteria**

Studies based solely on incorporation of overlap-based techniques without traditional restriction enzyme-dependent cloning methods were excluded. Publications not available in English or without full-text accessibility were excluded. Articles lacking sufficient methodological details or experimental validation (e.g., computational predictions only) were excluded. Conference abstracts, editorials, letters to the editor, and non–

peer-reviewed sources were not included. Studies focusing exclusively on vector design, bioinformatics tools, or unrelated molecular biology techniques without direct relevance to restriction-free cloning were excluded. Duplicate publications or studies with overlapping data were excluded to avoid redundancy.

Data Extraction and Synthesis limited Key themes, such as data were extracted systematically from the included studies regarding methodological principles, efficiency, fidelity, cost, and scalability of restriction-free overlapping sequence cloning techniques. The extracted information was synthesized around key themes, including: mechanistic principles of each cloning strategy (e.g., Gibson Assembly, SLIC, CPEC, etc). Where necessary, summaries of the retrieved data are provided to improve accessibility and clarity.

## 3. KEY RESTRICTION-FREE CLONING TECHNIQUES

Restriction-free cloning techniques provide seamless and efficient DNA assembly without relying on restriction enzymes or ligases. Methods such as Gibson Assembly, CPEC (Circular Polymerase Extension Cloning), Polymerase Incomplete Primer Extension (PIPE), Overlap Extension PCR Cloning (OE-PCR), Sequence and Ligation Independent Cloning (SLIC) and In Vivo cloning methods exploit overlapping homologous sequences and enzymatic reactions to generate precise constructs. These strategies reduce sequence limitations, enable multi-fragment cloning, and simplify synthetic biology workflows, making them vital tools in modern genetic engineering and molecular cloning research.

### 3.1 Gibson Assembly

A well-liked molecular cloning technique called the Gibson Assembly was created expressly to fuse many fragments in a certain order without being constrained by restriction enzyme sites. This technique involves assembling overlapping fragments, typically from PCR, and then merging them in an isothermal process using three enzymes: a DNA polymerase, a DNA ligase, and a $5'$ exonuclease (Avilan et al., 2023). Large DNA molecules can be effectively assembled using the Gibson one-step, isothermal assembly method (Gibson assembly), which involves in vitro recombination with a $5'$ -exonuclease, DNA polymerase, and DNA ligase. This reliable DNA assembly technique has been used extensively in recent years to build genes, genetic pathways, and even whole genomes with ease. Here, we extend this technique to clone large DNA segments with high GC contents, including Streptomyces gene clusters involved in antibiotic biosynthesis. The Gibson reaction system's low isothermal setting (50 °C) makes it easy for complementary overlaps with high GC contents to generate mismatched linker pairs, which mostly results in low assembly efficiencies because of vector self-ligation. Thus, we made the following two modifications to this traditional approach. The BAC vector is first modified by adding two universal terminal single-stranded DNA overhangs with high AT contents to its ends. Second, to accomplish the hierarchical assembly of big DNA molecules, two restriction enzyme sites are added to the corresponding sides of the planned overlaps. The enhanced Gibson assembly

technique makes it easier to quickly obtain big DNA fragments from Streptomyces that have high GC contents (Li et al., 2018).

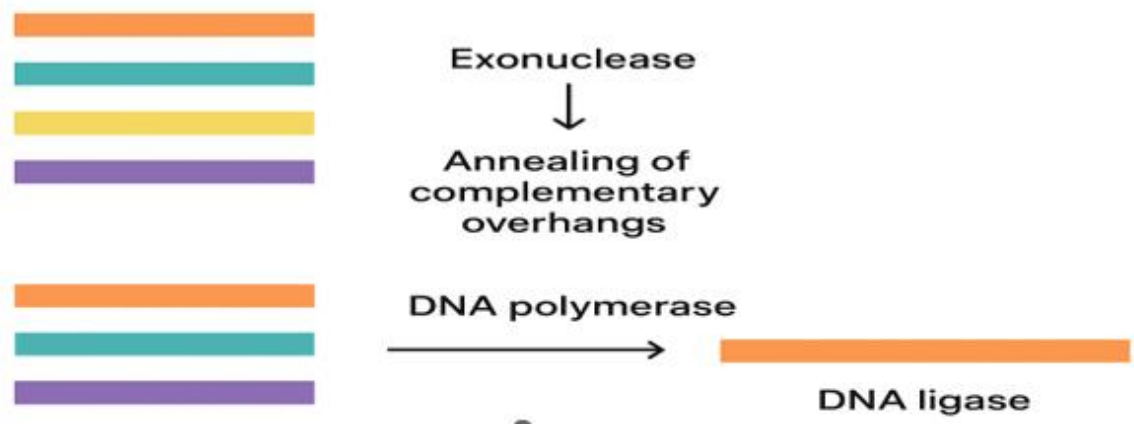


***FIGURE 1. MECHANISM OF GIBSON ASSEMBLY***

The Gibson Assembly method employs a one-pot, isothermal reaction for seamless DNA assembly. Exonuclease first creates single-stranded $3'$ overhangs, allowing fragments with complementary ends to anneal. DNA polymerase then fills in the gaps, and DNA ligase seals the nicks, resulting in a continuous, double-stranded DNA construct without the need for restriction enzymes.

**3.2 Circular Polymerase Extension Cloning (CPEC)**

High-throughput genomics and the emerging field of synthetic biology demand ever more convenient, economical, and efficient technologies to assemble and clone genes, gene libraries and synthetic pathways. The development of a novel and extremely simple cloning method, circular polymerase extension cloning (CPEC). Circular polymerase extension cloning (CPEC) represents a relatively cost-effective, high-efficiency cloning technique compared to the Gibson assembly and other protocols used in molecular cloning. It operates on the principle of polymerase overlap extension and is considered a robust alternative due to its omission of restriction digestion, ligation, and other procedural steps. CPEC enables the transformation of overlapping DNA fragments into a double-stranded circular form via the polymerase extension mechanism, thereby facilitating the integration of the insert into the target plasmid. During the CPEC reaction, linear double-stranded inserts and vectors are first separated through increasing temperature (denaturation). Subsequently, the resulting single-stranded products anneal through their overlapping regions and use each other as templates to construct the circular plasmid. Through the CPEC method, not only a single gene but also multi-fragment assembly could be obtained (Quan et al., 2014). This method uses a single polymerase to assemble and clone multiple inserts with any vector in a one-step reaction *in vitro*. No restriction digestion, ligation, or single-stranded homologous recombination is required (Quan et al., 2009). Polymerase extension is the basis of the

polymerase chain reaction (PCR) used for amplification of DNA sequences. The same principle is also used for gene assembly with overlapping oligonucleotides or gene fragments (Tian et al., 2004). However, to our knowledge, there has been no reported gene cloning method which solely relies on this mechanism. Here we report the development of a much-simplified sequence-independent cloning technology based entirely on the polymerase extension mechanism. This method extends overlapping regions between the insert and vector fragments to form a complete circular plasmid and is therefore named "Circular Polymerase Extension Cloning" (Quan et al., 2009).

### *3.3* Polymerase Incomplete Primer Extension (PIPE)

The speed and effectiveness of conventional restriction site and ligase-based cloning techniques have been greatly increased by notable advancements in molecular biology techniques. "Enzyme-free" techniques do not require modifying the ends of DNA fragments produced by the Polymerase Chain Reaction (PCR) or adding restricted sequences. Cloning and mutagenesis are further reduced to a relatively straightforward two-step technique with total design flexibility that is not achievable with previous strategies thanks to the Polymerase Incomplete Primer Extension (PIPE) technology. By converting competent cells with PCR products right after amplification, this approach accomplishes all significant cloning processes. Mixtures of partial extension products are produced by standard PCRs. Short, overlapping sequences are added to the ends of these incomplete extension mixtures using PCR and basic primer design guidelines. This enables complementary strands to anneal and create hybrid vector/insert combinations. Without undergoing any post-PCR enzymatic modifications, these hybrids are converted straight into recipient cells. Comparing this strategy to other accessible approaches, we have found it to be highly simple, quick, and efficient. This method has allowed us to clone thousands of genes simultaneously with little effort. Because it requires only a few basic processing steps, the approach is reliable and easily automated (Klock et al., 2009).

The finding that typical PCR reactions produce a population of partly single-stranded DNA fragments due to inadequate primer extension is exploited by the PIPE method. Each $3'$ -end varies in length according to primer extension; however, these PCR products have consistent $5'$ -ends with sequences determined by the amplification primers. Complementary $5'$ -ends are made using a straightforward primer design guideline to serve as annealing templates for joining PCR fragments throughout the cloning or mutant-generating processes. According to this primer design rule, each primer's $5'$ end should have 14–17 bases of sequence added. As long as this complementarity is maintained, the overlapping sequence can be made up of any sequence, but it must be reverse-complementary to the $5'$ end of an opposite strand primer where annealing is desired. Template DNA concentrations can be lowered to the point where background colonies are insignificant because the cloned product is the result of PCR amplifications. The PIPE method's ease of use, adaptability, and efficiency make it

perfect for rapidly and simply producing a large number of expression clones, including protein variations to improve biophysical properties (Klock et al., 2008).

**3.4 Overlap Extension PCR Cloning (OE-PCR)**

Extension of overlap PCR is a useful method that is frequently used to fuse two gene elements together, modify cloned genes, and clone huge complicated segments (Roland et al., 2020). First, basic DNA segments are amplified in distinct PCRs that have homologous ends. In the second round of PCR, these DNA segments can anneal to one another with the matching homologous regions, allowing DNA polymerase to expand them and produce spliced DNA molecules. The restriction digestion and ligation technique can then be used to insert the bigger DNA fragments that are produced into plasmids. Special GC-rich overlap sequences are made to mediate dependable fusion PCR in order to increase annealing efficiency (Cha-aim *et al.*, 2009). Several other methods have been developed as alternatives to PCR cloning (Sambrook et al., 2001), such as recombinase-dependent cloning (Court et al., 2002), PCR-mediated cloning (Zuo et al., 2009), and TA cloning ligation independent cloning (LIC) (Weeks et al., 2007). Any cloning method's practical usefulness depends more on its dependability than on its affordability, ease of use, or efficiency in ideal circumstances. In the end, the most dependable techniques are those that are the simplest to track and improve. Gel electrophoresis cannot track the terminal changes needed for TA cloning and LIC. Few customers optimize the in vitro recombination reactions since recombinases are often offered as proprietary parts of cloning kits. Extension of overlap This type of PCR-mediated cloning is not the original PCR cloning technique (Zuo et al., 2009). Nonetheless, it is comparatively simple, effective, and dependable. A simple, effective, and dependable method for cloning a desired insert into a desired plasmid without the need for T4 DNA ligase or restriction endonucleases. The genes for GFP (gfp), β-D-glucuronidase (gusA), and β-galactosidase (lacZ), as well as the complete luxABCDE operon, were PCR-amplified using chimeric primers that had plasmid sequence at the $5'$ ends and insert sequence at the $3'$ ends. These inserts were used with a circular plasmid template as mega-primers in a subsequent PCR (Bryksin et al., 2010).

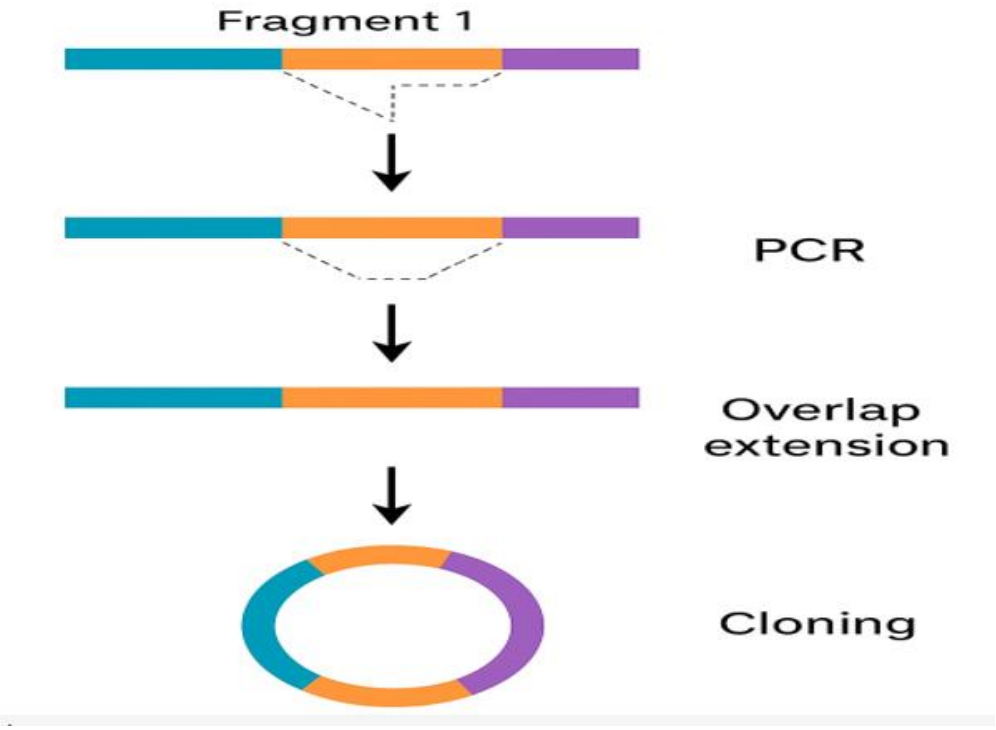


***FIGURE 2: MECHANISM OF OVERLAP EXTENSION PCR (OE-PCR) CLONING***

OE-PCR is a restriction-free cloning method that enables the seamless assembly of DNA fragments through complementary overlapping ends. Initially, two or more DNA fragments are amplified with primers designed to introduce overlapping regions. In the extension step, these overlapping sequences anneal, and DNA polymerase extends the strands, fusing the fragments into a single continuous sequence. The final product can then be amplified and inserted into a vector for cloning. This method eliminates the need for restriction sites and allows flexible, precise DNA assembly for genetic engineering and synthetic biology applications.

**SEQUENCE AND LIGATION INDEPENDENT CLONING (SLIC)**

Sequence-and ligation-independent cloning (SLIC) creates single-stranded DNA overhangs in insert and vector sequences by using the exonuclease T4 DNA polymerase. To create the desired recombinant DNA, these fragments are subsequently put together in vitro and converted into Escherichia coli. Additionally, imperfect PCR (iPCR) and mixed PCR can produce SLIC inserts (Li et al., 2012). Sequence-and-ligation-independent cloning (SLIC) is a straightforward, economical, time-efficient, and adaptable cloning technique. Two and a half minutes after combining any linearized vector, a PCR-prepared insert or inserts, and T4 DNA polymerase in a tube at room temperature, direct bacterial transformation can be used to do very effective and directed cloning (Jeong et al., 2012). Although sequence-and ligation-independent cloning (SLIC) gets beyond LIC's sequence restriction and enables the simultaneous assembly of several overlapping fragments, SLIC's cloning efficiency is

not very high when RecA is not present (Li et al., 2007). An example of one-step SLIC is provided by the vector must first be linearized using inverse PCR or restriction enzyme digestion. PCR is used to create an insert or inserts using primers homologous to each end of the linearized vector that have an extension of at least 15 bp. Second, to create 5′ overhangs, the vector and insert(s) are combined and treated with T4 DNA polymerase for 2.5 minutes at room temperature. A 1:2 to 1:4 molar vector-to-insert ratio is ideal for best outcomes. Third, competent Escherichia coli cells are immediately transformed using the annealed DNA complex after the reaction mixture has been on ice for 10 minutes to allow for single-strand annealing. Through highly efficient homologous recombination in vivo, the annealed complex transforms into seamless recombinant DNA. Refer to the accompanying material for a thorough explanation of the procedure (Jeong et al., 2012).

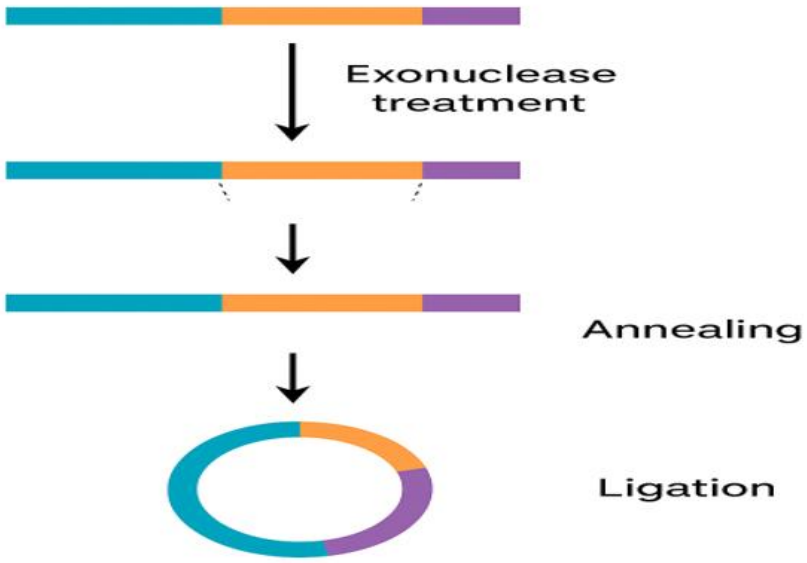


***FIGURE 3: MECHANISM OF SEQUENCE AND LIGATION INDEPENDENT CLONING (SLIC)***

SLIC is a versatile restriction-free cloning method that enables the seamless assembly of DNA fragments without the need for specific restriction sites or ligases. In this approach, exonuclease treatment generates single-stranded overhangs at the ends of DNA fragments. These complementary overhangs anneal through base pairing, forming recombinant molecules. The host cell's DNA repair machinery then seals the nicks, completing the circular plasmid. This strategy is efficient, cost-effective, and widely applied in molecular cloning and synthetic biology.

**3.6 In Vivo Cloning Methods**

Through recombination mechanisms, the DNA fragments can be assembled inside the cell using in vivo cloning techniques. The in vivo methods have not been extensively employed for cloning applications, despite their ease of usage and affordability. Since great efficiency can often take precedence above affordability, this could be the

result of their low efficiency. But the straightforward idea of in vivo cloning led to additional study to increase its effectiveness (Kostylev et al., 2015). All cloning processes (insertions, deletions, mutagenesis, and sub-cloning) may now be carried out using a single universal approach that solely uses a single-tube PCR thanks to the IVA (in vivo assembly) technology (García-Nafría et al., 2016). In vivo cloning of the PCR product by site-specific recombination (ICPS) is a molecular technique designed to make gene cloning easier and less expensive. Neither costly supplies nor the somewhat intricate PCR technique for generating DNA fragments with overlapping ends are needed for this plan. All of the conditions needed to produce the recombinant colony are present in the cell once the PCR-amplified target gene is directly transformed into the host. This in vivo cloning technique uses two plasmids in Escherichia coli BL21(DE3) to carry out gene exchange and negative screening procedures. The first plasmid (pLP-AmpR) features a landing pad (LP) flanked by attP sites, which includes a SpCas9-cleavage sequence (SCS) and two genes (λ int and λ gam). The Gam protein stops the host exonucleases V (RecBCD) and I (SbcB) from breaking down linear DNA (Datsenko and Wanner 2000; Mosberg et al. 2010). Following the transformation of the PCR product flanked by attB sites, the LP is substituted for this linear fragment by the Int and IHF proteins. Cas9 and a certain gRNA are inducibly co-expressed in the second plasmid (pScissors-CmR), which eliminates the non-recombinant plasmids. After an effective pre-selection by CRISPR-Cas9 and ampicillin screening, only colonies with the recombinant plasmid survive when using the ICPS, in contrast to laborious screening techniques (Aliakbari et al., 2024).

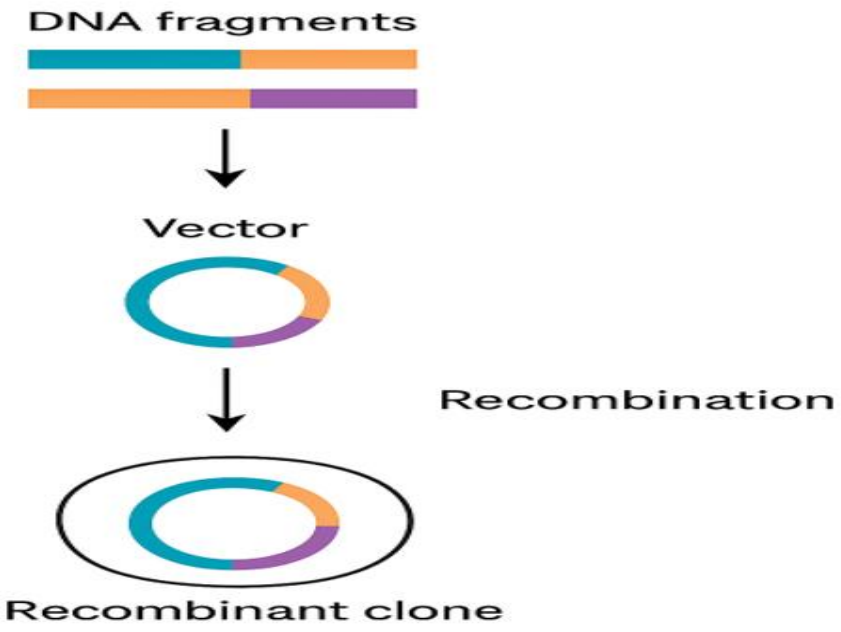


*FIGURE 4: MECHANISM OF IN VIVO CLONING METHODS*

In vivo cloning methods utilize the natural recombination machinery of host cells (commonly *E. coli* or yeast) to assemble DNA fragments directly inside the organism. DNA fragments with overlapping homologous ends are introduced along with a vector into the host. The cellular recombination system aligns and joins the fragments with the vector, creating a recombinant plasmid. The host cell then propagates the recombinant plasmid, resulting in stable recombinant clones. This strategy is efficient, avoids in vitro ligation, and is widely used for large or complex DNA assemblies in synthetic biology and molecular genetics.

**TABLE: COMPARATIVE SUMMARY OF RESTRICTION-FREE OVERLAPPING SEQUENCE CLONING TECHNIQUES**

| Method | Core Principle | Major Advantages | Limitations | Typical Applications |
|---|---|---|---|---|
| **Gibson Assembly** | Exonuclease creates single-stranded overhangs; fragments anneal via homologous sequences; polymerase and ligase fill gaps | Highly efficient, seamless, handles multiple fragments, commercial kits available | Relatively expensive, risk of errors in very large assemblies | Gene synthesis, pathway engineering, synthetic circuit construction |
| **Circular Polymerase Extension Cloning (CPEC)** | Linear DNA fragments with overlaps are extended by PCR cycles until circularized | No ligase or exonuclease required, cost-effective, high fidelity | Less efficient with >6 fragments, requires precise overlap design | Gene libraries, mutagenesis, synthetic pathway cloning |
| **In-Fusion Cloning** | 15 bp overlaps allow recombinase-based fusion of DNA fragments | Rapid, efficient, scarless, widely commercialized | Dependence on proprietary kits, moderate cost | High-throughput cloning, vector construction, protein expression |
| **Sequence and Ligation** | T4 DNA polymerase | Simple, inexpensive, | Requires host repair system, | Gene libraries, metabolic engineering |

| | | | | |
|---|---|---|---|---|
| **Independent Cloning (SLIC)** | generates single-stranded overlaps; fragments anneal and are repaired in vivo | multi-fragment cloning possible | higher background colonies | |
| **Polymerase Incomplete Primer Extension (PIPE)** | PCR with incomplete primer extension generates compatible ends for direct annealing | No ligase/enzymes needed post-PCR, inexpensive, easy setup | Limited to fewer fragments, optimization needed | Site-directed mutagenesis, small insertions/deletions |
| **Overlap Extension PCR Cloning (OE-PCR)** | PCR-amplified fragments with homologous ends fused via overlap extension | Flexible, low cost, no specialized reagents | Labor-intensive, less efficient with long/complex sequences | Gene fusion, domain swapping, small-scale construct design |

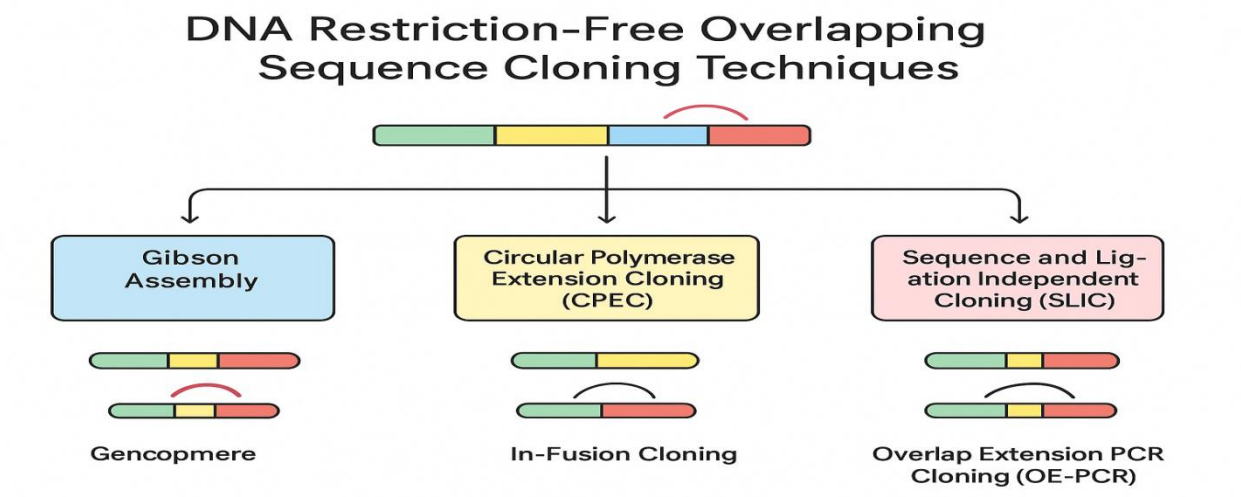


***FIGURE 5: OVERVIEW OF RESTRICTION-FREE OVERLAPPING SEQUENCE CLONING TECHNIQUES.***

Multiple DNA fragments containing homologous ends can be seamlessly assembled using methods such as Gibson Assembly, Circular Polymerase Extension Cloning (CPEC), In-Fusion Cloning, Sequence and Ligation

Independent Cloning (SLIC), and Overlap Extension PCR (OE-PCR). These approaches bypass the need for restriction enzymes and ligases, enabling scarless and efficient DNA assembly for synthetic biology and genetic engineering applications.

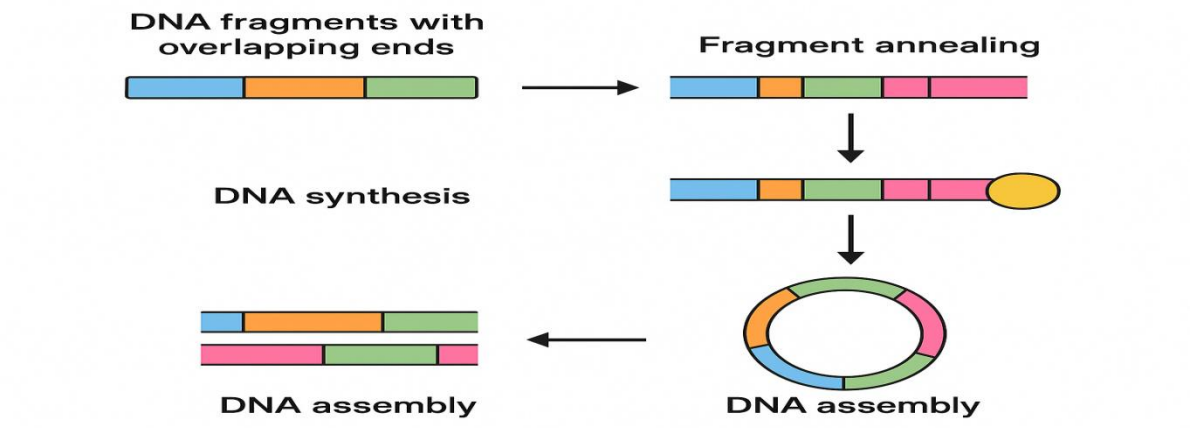


***FIGURE 6: SCHEMATIC REPRESENTATION OF RESTRICTION-FREE OVERLAPPING SEQUENCE CLONING WORKFLOW.***

DNA fragments with designed overlapping ends undergo fragment annealing, followed by DNA synthesis and extension. The process enables seamless assembly of multiple fragments into linear or circular constructs without the use of restriction enzymes or ligases, providing a versatile toolkit for synthetic biology and genetic engineering.

**4. CONCLUSION**

Restriction-free overlapping sequence cloning techniques have revolutionized the landscape of molecular cloning by eliminating the constraints of traditional restriction enzyme–dependent strategies. By harnessing sequence homology and enzymatic reactions, methods such as Gibson Assembly, In-Fusion Cloning, Circular Polymerase Extension Cloning (CPEC), Sequence and Ligation Independent Cloning (SLIC), Polymerase Incomplete Primer Extension (PIPE), and Overlap Extension PCR (OE-PCR) have enabled seamless, scarless, and efficient DNA assembly. These approaches provide unparalleled flexibility, allowing researchers to construct complex genetic circuits, engineer metabolic pathways, and design synthetic constructs with high precision.

Comparative analysis across the literature highlights that each method carries unique strengths and trade-offs in terms of cost, scalability, fragment size, error rates, and technical complexity. Gibson Assembly and In Vivo Cloning have become the most widely adopted due to their efficiency and robustness, while methods

like PIPE, SLIC, and OE-PCR offer simpler, cost-effective alternatives for resource-limited laboratories. Importantly, integration of these cloning strategies with high-throughput automation, CRISPR-based editing, and emerging cell-free expression systems is further accelerating advances in synthetic biology and genetic engineering.

Overall, restriction-free overlapping sequence cloning has established itself as a cornerstone methodology for modern molecular biology. Its continued refinement, coupled with advances in DNA synthesis technologies, promises to expand the boundaries of what can be achieved in genome engineering, therapeutic construct design, and industrial biotechnology. Future research should focus on improving fidelity, reducing costs, and streamlining automation, thereby making these powerful tools accessible to a broader scientific community.

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