

GENE CLONING: A FREQUENTLY USED TECHNOLOGY IN A MOLECULAR BIOLOGY LABORATORY - ALTERNATIVE APPROACHES, ADVANTAGES AND LIMITATIONS

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ABSTRACT

Gene synthesis is a convenient tool that is widely used to make genes for a variety of purposes. All current protocols essentially take inside-out approaches to assemble complete genes using DNA oligonucleotides or intermediate fragments. Molecular manipulations, including DNA cloning are basic tools used on a routine basis in all life-science disciplines. The cloning and engineering of genes are widely used techniques to study DNA and protein function. Conventional methods to insert genes into vectors are based on DNA cleavage by restriction endonucleases and then ligation by DNA ligase. However, this DNA engineering method is time-consuming and relatively inefficient. Over the last decade, the emergency of new methodologies has facilitated and expanded the applications for DNA cloning. Thus, this review will introduce the gene cloning as an important molecular biology tool currently used in biomedical research laboratories and will explore the alternative cloning approaches recently developed, their applications, advantages and limitations.

Keywords: Ligation-dependant cloning (LDC); TA-Cloning; Ligation independent-cloning (LIC); TA-Cloning; RF-Cloning

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I. INTRODUCTION

Gene cloning and vector construction are widely applied techniques in DNA and protein research and are the most frequently used technologies in a molecular biology laboratory. In fact, to study a particular gene, the first step is usually to clone and express it (An *et al.*, 2007). There are many approaches available for DNA cloning. The classical technique generally involves cleaving a destination plasmid and a target insert sequence with restriction enzymes, and then stitching them together with DNA ligase. This approach is enormously convenient and straight forward when the appropriate restriction sites are well positioned in the sequences being manipulated, but becomes problematic when these restriction sites are not present. Additionally, most of the eukaryotic genes are interrupted by intervening sequences (introns), which make the gene of interest very large. Manipulation of the large genomic DNA is tedious and problematic due to size capacity of cloning vectors and multiple restriction endonucleases which make it difficult to find appropriate enzymes for subcloning. To circumvent these difficulties, numerous alternative cloning approaches have been developed (Sambrook and Russell, 2001) including TA cloning (Marchuk *et al.*, 1991), ligation independent cloning (LIC) (Weeks *et al.*, 2007; Yang *et al.*, 2010; Thieme *et al.*,

2011), recombinase-dependent cloning (Court *et al.*, 2002; Cheo *et al.*, 2004), and PCR-mediated cloning (Shuldiner *et al.*, 1990, 1991; Zuo and Rabie, 2009; Bond and Naus, 2012). This review will introduce the different molecular cloning approaches currently used in biomedical research laboratories and will explore both advantages and limitations of its technique.

II. CLASSICAL LIGATION DEPENDENT CLONING AND LIMITATIONS

The classical Ligation Dependent Cloning (LDC) approach, which utilizes restriction enzyme digestion followed by a ligation step of the vector and DNA insert, has been used for several decades. Traditionally, molecular cloning joins insert and vector by T4 DNA ligase after restriction digestion to excise insert from a donor vector or from a PCR product with restriction enzyme recognition sites added to the ends (Sambrook and Russell, 2001). Although this is a widely used method, it involves multiple steps and is time consuming. This multi-step process also makes it difficult or complicated for troubleshooting (Li *et al.*, 2011).

With a rising number of sophisticated functional and structural studies involving proteins and to overcome the difficulties encountered in the original cloning method, there is a need for development of efficient and robust techniques, which can be adapted for high-throughput platforms (Erijman *et al.*, 2011). The practical utility of any cloning method is predicated upon its reliability, rather than its convenience, price, or efficiency under optimum conditions. The methods that are easiest to monitor and optimize ultimately prove the most reliable.

III. EMERGENCY OF NEW ALTERNATIVE CLONING APPROACHES

Many alternative cloning methods have been developed over the last two decades. These methods include TA cloning (Marchuk *et al.*, 1991), ligation independent cloning with T4 DNA polymerase (Haun *et al.*, 1992), GATEWAY recombinational cloning (Walhout *et al.*, 2000), and more recent sequence- and ligation-independent cloning kits, such as CloneEZ (GenScript USA Inc., Piscataway, NJ, USA), one step cloning (Zuo and Rabie, 2009), and overlap extension PCR cloning (Bryksin and Matsumura, 2010; Li *et al.*, 2011).

III.1. Direct Cloning of PCR products using TA Cloning System

The polymerase chain reaction (PCR) is recognized as a basic tool in molecular biology and biotechnology. Therefore, PCR-based gene probing, cloning, and then expression are widely used for gene manipulations. Most of these approaches require ligation of the targets genes, or DNA fragments, with various specialized vectors for subsequent applications. For this purpose, it is well known that TA cloning systems are the most effective methods for direct cloning of PCR products, because the method can avoid laborious and time-consuming steps of restriction enzyme digestion and ligation (Lim *et al.*, 2010). This approach is based on the principle that non-proofreading polymerases such as Taq and Tth attach a nucleotide adenosine into the 3'-end in a template-independent manner. Thus, protrusion of a thymine at the terminal ends of the cloning vector can ligate PCR products. This TA cloning system was realized by some reports (Marchuk *et al.*, 1991; Mead *et al.*, 1991), and further improved by the incorporation of recognition sites by XcmI or Eam1105I into the vector as cloning sites, resulting in exclusion of the T-tailing step (Kovalic *et al.*, 1991).

Additional attempts to inactivate the reporter proteins by using restriction sites could provide a method of simple selection owing to substitution of the intact open reading frame (ORF), encoding functional reporter, with PCR products in the recombinant plasmids (Park and Zeng, 2008) or destruction of α -complementation for the functional expression of reporter enzyme (Holton and Graham, 1991). For this purpose, two reporters, green fluorescent protein (GFP) and β -galactosidase, are generally used as selection reporters for gene cloning (Kwon *et al.*, 1998).

The blue-white screening system based on β -galactosidase activity for the selection of recombinants requires 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) as a substrate and isopropyl- β -D-thiogalactopyranoside (IPTG) as an inducer for increased fidelity; therefore, it has a relatively high cost. Additionally, this system is also functional in a *lacZ*-mutation or -deficient host. The other reporter, GFP, shows autofluorescence without any substrates and is available for a range of host strains of any genotypes (Inouye *et al.*, 1997). However, the time-consuming maturation of the fluorophore and the need to expose the clones to toxic UV to excite the reporter protein are issues that must be solved to enable GFP to be applicable for more general use (Lim *et al.*, 2010).

III.2. Ligation-Independent Cloning Approach

In recent years, a variety of new alternative methodologies for DNA cloning were reported based on Ligation-Independent Cloning (LIC) principles. These methodologies are gradually replacing the traditional LDC approach (Graslund *et al.*, 2008). The principle of the LIC strategy is based on regions of homology present in the primers used for amplification of the PCR product and the ends of a linearized cloning vector. Vector and insert are treated with an exonuclease such as T4 DNA polymerase or exonuclease

III (Yang *et al.*, 1993), leading to formation of complementary single-stranded DNA overhangs that are able to anneal with each other. Annealed vector-insert complexes can be transformed directly in *Escherichia (E.) coli* cells without ligation (Li and Evans, 1997).

Several of the LIC procedures are based on recombination between the insert and the destination vector including the Gateway-system (Invitrogen, Carlsbad, CA) that utilizes site specific recombination. Other recombination-based technologies such as the In-Fusion™ system (Benoit *et al.*, 2006) (Clontech, Mountain View, CA) and the Sequence and Ligation-Independent Cloning (SLIC) (Li and Elledge, 2007) rely on homologous recombination. Other LIC procedures use complementary single strand overhangs to combine the vector and the insert (Gileadi *et al.*, 2008).

A commercial LIC system based on similar principles is available from Novagen. A different LIC approach for DNA cloning and mutagenesis is based on whole plasmid amplification of the insert and the plasmid and designated as Restriction Free (RF) cloning (van den Ent and Lowe, 2006; Unger *et al.*, 2010).

LIC presents many advantages. LIC is simple to perform and can be done using common reagents found in any molecular biology laboratory, and therefore does not require the purchase of a kit, but is nevertheless very efficient (Thieme *et al.*, 2011). It does not require cleavage of insert DNA with restriction enzymes, and therefore can be used to clone libraries of unknown sequences. Additionally, LIC is highly efficient, in part because empty vector cannot relegate without insert, but also because annealing of long single-stranded DNA ends (of more than 12 nucleotides) allows direct transformation in *E. coli* cells without the need for an *in vitro* ligation step (Aslanidis *et al.*, 1994; Thieme *et al.*, 2011).

III.3. “Quick Assemble”: One-step DNA Fragment Assembly and Circularization for Gene Cloning

Recombinant DNA projects generally involve the ligation of two or more long fragments of DNA, but this process is sometimes not possible with conventional cloning techniques, or unwanted sequences need to be inserted first (Shevchuk *et al.*, 2004). Several alternative approaches have been developed, but they usually require the use of a single-stranded DNA template, which must be pre-prepared from double-stranded DNA. One method not requiring single-stranded DNA is DNA shuffling, in which pools of selected mutant genes are randomly fragmented and reassembled by *in vitro* homologous recombination, followed by the polymerase chain reaction (PCR) (Stemmer, 1994). Some other ligase-free cloning methods need to use either topoisomerase I (Heyman *et al.*, 1999) or uracil DNA glycosylase (Buchman *et al.*, 1993). Both of them need special vectors. Another method is overlap extension PCR. For example, Shuldiner *et al.* (1990, 1991) developed a PCR-induced (ligase-free) subcloning method. However, the PCR protocols are still limited by existing techniques of DNA purification to remove excess primers through an Utrafree-MC unit. The exact steps contain (i) PCR amplification from genomic DNA or cDNA, (ii) Filtration to remove excess primers, (iii) Two sets of PCR reactions with 2 sets primers, “ac” and “bd”, (iv) Combine 2 sets of PCR products and cyclization. (v) Concentrating the mixture. It is labor-intensive.

Consequently, Zuo and Rabie (2010) described a modified PCR method called “Quick Assemble” that simultaneously and precisely anneals and ligates DNA fragments, thereby allowing recombinant products to be created in fewer steps than conventional restriction enzyme cloning. It is a one-step procedure based on *Taq* polymerase for the precise assembly of DNA fragments into circular constructs as long as 6 kb. The only prior step needed is the amplification of the gene to be cloned and the linear vector

backbone, and the whole process up to assembly and circularization lasts only 2 days, compared with the conventional method's 2 weeks. Furthermore, the final DNA construct is used to transform *E. coli* directly without any further treatment (Zuo and Rabie, 2010).

By circumventing the need for DNA ligase, "Quick Assemble" method offers an improvement over the combination of long PCR and overlap extension PCR, and is expected to facilitate various kinds of complex genetic engineering projects that require precise in-frame assembly of multiple fragments, such as multiple site-directed mutagenesis and whole-DNA library gene shuffling, as well as the construction of new plasmids with any promoter, resistance gene marker, restriction site, or any DNA tag. In the future, DNA shuffling might become easier and quicker with this method to offer new opportunities in genetic engineering (Zuo and Rabie, 2010). Additionally, this method would enable researchers to replace promoters or modify resistance to antibiotics by using different or multiple resistance gene markers such as ampicillin, kanamycin, neomycin, chloramphenicol, gentamycin, tetracycline, erythromycin, streptomycin, vancomycin, and spectinomycin (Wang *et al.*, 2009).

III.4. Fast Cloning

Fast Cloning is a highly simplified, reliable, and efficient PCR-based cloning technique to insert any DNA fragment into a plasmid vector or into a gene (cDNA) in a vector at any desired position. With this method, the vector and insert are PCR amplified separately, with only 18 cycles, using a high fidelity DNA polymerase. The amplified insert has the ends with ~16-base overlapping with the ends of the amplified vector. After DpnI digestion of the mixture of the amplified vector and insert to eliminate the

DNA templates used in PCR reactions, the mixture is directly transformed into competent *E. coli* cells to obtain the desired clones (Li *et al.*, 2011).

This technique has many advantages over other cloning methods. First, it does not need gel purification of the PCR product or linearized vector. Second, there is no need of any cloning kit or specialized enzyme for cloning. Furthermore, with reduced number of PCR cycles, it also decreases the chance of random mutations. In addition, this method is highly effective and reproducible. Finally, since this cloning method is also sequence independent, Li and collaborators (2011) demonstrated that it can be used for chimera construction, insertion, and multiple mutations spanning a stretch of DNA up to 120 bp.

Because the PCR amplification of vector can be controlled by primers to exact positions, Fast Cloning method is truly sequence-independent. Thus, one can put an insert to any position and in any frame. This feature, although with only a small modification of standard cloning protocol, makes it easy to construct cDNAs for fusion proteins or chimeras. Furthermore, a minor variation of this technique can be applied for insertion of a short DNA fragment directly from two relative long primers for PCR amplification of a cDNA along with its vector (Li *et al.*, 2011).

The Fast Cloning technique provides a very simple, effective, reliable, and versatile tool for molecular cloning, chimera construction, insertion of any DNA sequences of interest and also for multiple mutations in a short stretch of a cDNA (Li *et al.*, 2011).

III.5. Quick and Clean Cloning QC Cloning

The principle of the quick and clean (QC) cloning strategy is based on homology between sequences present in both the vector and the PCR product. However, in contrast to other ligation independent strategies, the cloning vector has homology with

only one of the two primers used for amplification of the insert. The other side of the linearized cloning vector has homology with a sequence present in the insert, but nested and non-overlapping with the gene-specific primer used for amplification. Since only specific products contain this sequence, but none of the non-specific products, only specific products can be cloned (Thieme *et al.*, 2011).

Cloning is performed using a one-step reaction that only requires incubation for 10 minutes at room temperature in the presence of T4 DNA polymerase to generate single-stranded extensions at the ends of the vector and insert. The reaction mix is then directly transformed into *E. coli* where the annealed vector-insert complex is repaired and ligated. Thieme *et al.* (2011) have tested this method, which they call quick and clean cloning (QC cloning), for cloning of the variable regions of immunoglobulins expressed in non-Hodgkin lymphoma tumor samples. This method can also be applied to identify the flanking sequence of DNA elements such as T-DNA or transposon insertions, or be used for cloning of any PCR product with high specificity.

III.6. Restriction-Free Cloning

Restriction-free cloning (RF-cloning) is a PCR based technology that expands on the QuikChange mutagenesis process originally popularized by Stratagene in the mid-1990s, and allows the insertion of essentially any sequence into any plasmid at any location (Bond and Naus, 2012). In RF cloning, the gene of interest is PCR-amplified using two primers, each containing a target-specific sequence and a 5' extension that overlaps the insertion sites in the destination vector. Following purification, the double-stranded PCR product is used as a set of mega-primers for the second reaction. In this step, each of the DNA strands anneals to the destination vector at a pre-designed

position and is extended in a linear-amplification reaction (Unger *et al.*, 2010; Erijman *et al.*, 2011).

The RF cloning procedure has several major advantages, which makes it a highly attractive alternative to other LIC procedures. From one hand, it can be performed using any destination vector and the sequence of interest may be inserted at any desired position within the vector. From the other hand, this method allows precise and seamless insertion of the DNA insert without any additional unnecessary sequences. Additionally, the reaction is fast, highly efficient and is suitable for high-throughput cloning (Unger *et al.*, 2010). While RF-cloning is a powerful tool for the design of custom plasmids when restriction sites are not conveniently situated, manually designing the requisite primers can be tedious and error prone (Bond and Naus, 2012).

Recently, the applications of the RF cloning were expanded for diverse molecular manipulations including simultaneous cloning at distinct positions and multi-component assembly (Unger *et al.*, 2010; Erijman *et al.*, 2011). In fact, it is a powerful technique for constructing custom plasmids, but designing the necessary hybrid primers has been a manual task up until now.

IV. Limitations of Cloning Techniques

Conventional methods to insert genes into vectors are based on DNA cleavage by restriction endonucleases and then ligation by DNA ligase. However, this DNA engineering method is time-consuming and relatively inefficient. To more efficiently clone DNA molecules, several ligation-independent cloning methods have been developed as described above. Nevertheless, each of these techniques has its own limitations. For example, "TA cloning" is a popular method of cloning without the use of restriction enzymes; instead, PCR products are amplified with only *Taq* DNA polymerase

and other polymerases. These polymerases lack 5'-3' proofreading activity and add an adenosine triphosphate residue to the 3' ends of the double-stranded PCR products. Such PCR amplified products can thus be cloned in a linearized vector that has complementary 3' thymidine triphosphate overhangs (Zuo and Rabie, 2010; Li *et al.*, 2011). The limitations of this method are low fidelity of Taq DNA polymerase causing unwanted mutations and requirement of subcloning into the final target vector with restriction digestion and ligation (Li *et al.*, 2011). Additionally, another major problem is that the gene has a 50% chance of getting cloned in the reverse direction (Zuo and Rabie, 2010). The early ligation independent cloning uses the 3'-exonuclease activity of T4 DNA polymerase to create 15-base 5' overhangs in the ends of insert and complementary 5' overhangs in the ends of vector. This technique requires specific sequences to create 15-base overhangs (Li *et al.*, 2011).

Gateway recombinational cloning uses site-specific recombination to transfer cDNAs between donor and destination vectors, which requires additional specific sequences for recombination. The latest ligation-independent cloning, such as Clone EZ and In-Fusion cloning kits, uses some DNA polymerase to generate sticky ends in the vector and insert without specific sequence requirement, except for restriction sites to linearize the vector. However, the new ligation independent cloning still requires purification of the digested vector and PCR-amplified insert, and the purchase of purification and cloning kits (Li *et al.*, 2011).

Similarly, overlap extension PCR cloning also requires purification of the first round PCR products (vector and insert) and an additional round overlap extension PCR, which usually generates multiple bands, for producing linked vector and insert. One-step "quick assemble" cloning does not need purification of PCR products. However, it includes two sequential 35-cycle PCRs with a total number of 70 cycles. The first-round

PCRs are used to amplify insert and linear vector. The second-round PCR is essentially the overlap extension PCR to assemble vector and insert into a single linear PCR product. Another ligation independent cloning technique, using nick DNA endonuclease to create long single-strand 5' overhangs in the vector and PCR-amplified insert (Yang *et al.*, 2010), requires specific sequences for nick DNA endonuclease and purification of the PCR product (Li *et al.*, 2011).

One limitation for cloning of PCR products containing unknown flanking sequences is that a substantial fraction of the products can be non-specific. One source of non-specific products consists of sequences amplified with the adaptor primer only. Other non-specific products can be produced by non-specific annealing of one or both primers during amplification. Finally primer-dimers are a source of non-specific products that can occur during any PCR amplification (Thieme *et al.*, 2011). Due to the requirement for specific sequences on both sides of the insert, ligation-independent cloning should not lead to cloning of the nonspecific products that result from amplification from a single primer. However, all other non-specific products can theoretically still be cloned (Thieme *et al.*, 2011).

V. CONCLUSION

DNA cloning and protein engineering are basic methodologies employed in all life-science disciplines and are essential for many biological and biochemical applications. Initial approaches for DNA manipulation, including cloning, were developed decades ago. In most molecular biology laboratories, plasmid construction remains a costly and time-consuming exercise. With so much genome sequence data available, more recombinant vectors are being produced than ever before, often sets of related constructs containing different sequences representing a panel of particularly

interesting genes. It is therefore highly desirable to develop tools that alleviate the plasmid construction bottleneck. Although several commercially available vectors allow efficient high-throughput cloning, the selection of vectors is limited, and many rely on steps with which many researchers have no first-hand experience. Only simple, versatile technologies that employ familiar procedures are likely to be widely tested and adopted by the research community. The number of vectors and DNA-cloning technologies is continually growing, thus, the correct vector and the convenient time-, and cost-effective cloning method must be carefully chosen to match the purpose of the research.

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