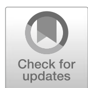
Chapter 5
Overview of Gene Cloning Strategies



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5.1 Introduction

The production of first rDNA molecules using restriction enzymes was carried out in early 1970s, reinforcing focus on molecular genetics. Thereafter, RDT is one of the fields that has extensively ramped up in its utilization and complexity, yielding increasingly potent methods for DNA manipulation. It has multidisciplinary applications in agriculture, hormones, vaccines, therapeutic agents, antimicrobial peptides, and recombinant diagnostic probes [1]. Discovery of bacterial enzymes that can cleave DNA molecules at specific positions led to a significant development in molecular cloning techniques. Molecular cloning is the most crucial and popular set of techniques to bring together DNA molecules forming recombinants and hybrids DNA constructs capable of performing a plethora of functions. By definition, molecular cloning is a process in which recombinant DNA molecules of interest are assembled in vitro and replicated into a host organism. This process constitutes two elements: isolation and amplification of a specific DNA fragment to be replicated and a vector for propagation.

By means of restriction enzymes the DNA fragments of interest are isolated from the source, copied, and amplified using PCR. Upon isolation, clones can be used to generate numerous copies of the DNA for analysis, and/or to express the proteins

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for the study or employment of its function. Moreover, site-directed mutations of the clones allow them to detect the function of target protein, by altering the quantity and quality of protein.

However, the choice of restriction enzymes is more critical for designing and cloning thereby increasing the efficiency by generating complementary “sticky ends” [2]. In sticky ends the DNA has a single stranded overhang on either 3′ or 5′ ends. The sticky ends must be converted into blunt ends by either by removing the overhangs or by inserting complementary base pairing.

In RDT, a gene of interest is obtained by splicing it from source, copying, or assembling it using oligonucleotides and inserting it into a suitable vector. The DNA becomes an integral part of the new vector by phosphodiester bonds and is replicated by the host. The vectors can be prokaryotic (plasmid, bacteriophages, cosmid vectors) or eukaryotic (yeast or mammalian artificial chromosomes). Generally, plasmids (replicates independently of chromosomal DNA) are introduced as vectors into the bacterial host. Important feature in plasmid is the presence of a short segment of DNA which contains multiple restriction sites called multiple cloning sites, also known as a polylinker.

Basically, molecular cloning includes four fundamental steps:

1. Isolation of insert or target DNA fragments
2. Ligating the insert into suitable vector plasmids
3. Transformation of recombinant plasmids into host for multiplication
4. Identify the correct host cell carrying the recombinant molecule

Nowadays, different molecular cloning strategies have been developed for different purposes. It is important to note that for a single cloning project, a combination of several methods may actually yield the best results. The choice of most appropriate cloning strategy would depend on various factors like the efficiency of cloning, availability of infrastructure and reagents, the efficiency of each method and the time available with the researcher. In this chapter, we will be walking you through different cloning strategies in detail.

5.2 PCR Cloning

PCR cloning is a versatile technique and has been widely used for biological engineering. It allows DNA fragments to be inserted into the backbone of the vector even when it is in minimal quantity [3]. In this method, a PCR generated DNA fragment is directly ligated into the vector. One of the most commonly used and the simplest form of PCR cloning techniques is the TA cloning. The method requires designing of appropriate primers and optimization of the PCR conditions. During amplification of a template, Taq polymerase favors addition of an adenosine molecule at 3′ ends. These A sites can be directly ligated with T-tailed vectors, hence also known as TA cloning. This method utilizes the advantage of hybridizing adenine and thymine on different fragments in presence of ligase. The TA cloning method

can be easily modified and is especially useful for restriction digestion free insertion of fragments in vector DNA. It is a convenient and less laborious method, and can be performed even with a limited quantity of the starting material [4]. This method also helps in avoiding the restriction digestion of the insert and the vector. However, one of the major disadvantages is that this method cannot be used for directional cloning. The DNA fragment used for cloning should be less than 5 kb. As this method relies on Taq DNA polymerase which does not have a proofreading activity, the error rate may also be high. In order to reduce the errors, high-fidelity enzymes can be used for amplification. These enzymes do not produce the Adenosine overhangs. These blunt ended DNA fragments are ligated with linearized vectors (which are also blunt ended). This may result in lower cloning efficiency. Several commercially available vectors are available which have improved the efficiency of cloning of blunt ended and sticky ended DNA fragments. Figure 5.1 gives the details of PCR cloning.

Long-term storage and presence of endonucleases may cause degradation of nucleotide overhangs of PCR products, thereby reducing the efficiency. Hence fresh and purified PCR products in TA cloning are recommended. Using terminal transferase, the T vector is prepared allowing T and A annealing and ligation. The unidirectional in TA cloning can be achieved by manipulating the phosphorylation status of the DNA molecules ensuring cloning of insert in right the orientation.

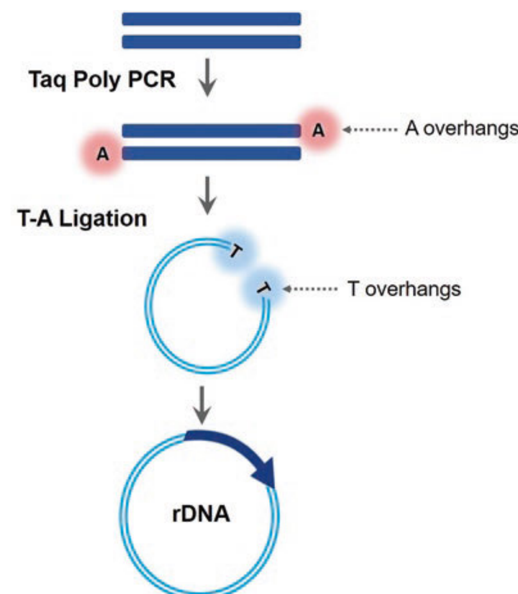

Fig. 5.1 TA Cloning method: Illustration of TA-mediated cloning. The PCR catalyzed by Taq polymerase add an A overhang to the products. Vectors with T overhang match with the PCR product and are ligated to obtain recombinant plasmid

5.3 Restriction and Ligation-Based Cloning

Special bacterial enzymes that cut DNA at specific sites (known as restriction sites) are called restriction enzymes. Depending on the ends generated after digestion, the restriction enzyme is termed as sticky end cutters or blunt end cutters. As the name suggests a blunt end cutter generates blunt ends while sticky end cutters create 3′ or 5′ overhang. Endonuclease restriction enzymes make a cut within the DNA while exonuclease restriction enzymes chop the DNA from an end. The restriction enzymes have been covered in detail in Chap. 5.

Restriction enzyme-based cloning is one of the most popular cloning strategies, with very versatile applications. In this technique, both the DNA fragment to be cloned and the vector are treated with appropriate restriction enzymes. After digestion, the insert and the vector are ligated with DNA ligase, which will result in a circular vector which contains the insert. This cloned vector can be maintained in different biological systems, like the bacteria *E. coli*.

Restriction enzymes like the *Eco*RI recognize conserved short inverted repeats (also known as palindromes) generating reproducible set of fragments called as restriction fragments. Upon digestion with the same enzyme, the same complementary tails are generated in insert and vector DNA. At room temperature, these compatible ends readily base-pair with each other. This base pairing of sticky ends permits DNA from widely differing species to be ligated, forming chimeric molecules.

Other restriction enzymes, such as *Alu*I (5′-AG/CT-3′) and *Sma*I (5′-CCC/GGG-3′), generated fragments with blunt ends. In these ends, all the complementary nucleotides are base paired. Blunt end cloning involves ligation of both strands of DNA into the vector with no overhanging bases at the termini. The phosphodiester bond of nearby 5′ phosphate and 3′ hydroxyl groups of cut DNA in presence of T4 ligase (isolated from phage T4) is sufficient to produce clones. T4 DNA ligase work in sequential steps of (a) enzyme adenylation: addition of adenosine monophosphate (AMP) molecule from ATP or NAD+ into ligase, (b) transfer of AMP: the AMP molecule is transferred at the 5′ phosphate at the site of nick, (c) nucleophilic attack of 3′-OH on 5′ phosphate to form phosphodiester bond. Blunt end cloning preparation is easy because it avoids enzymatic digestion and subsequent purification needed in sticky end cloning.

T4 DNA ligase is a ligating enzyme that can join any two cohesive or blunt DNA ends by forming phosphodiester bonds between adjacent nucleotides (Fig. 5.2). This enzyme will not join single stranded DNA. Blunt end ligation, on the other hand, demands a higher DNA concentration than sticky end ligation [3].

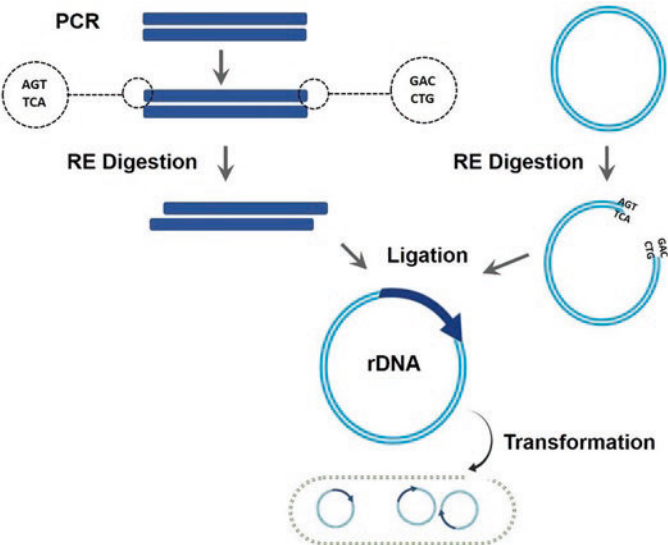

Fig. 5.2 The insert DNA is PCR amplified using primers with restriction site linkers. The vector is digested with the same enzyme to generate compatible ends. Next the insert and vector are ligated together using the matching ends and introduced in host cells

5.4 Ligation-Independent Cloning

The success of restriction digestion and ligation cloning method depends on the presence of appropriate restriction site selection and efficient ligation between the DNA molecules [5]. In case the DNA molecule does not have specific restriction enzymes the method would fail to produce recombinant plasmids. Therefore, to overcome this lacuna, Ligation-Independent Cloning (LIC) was developed which is highly efficient, simple, and faster. In this technique short sequences of DNA (12 nucleotides long) are added to the clone fragments that are homologous to the vector. Complementary and compatible ends are generated by 3′ → 5′ exonuclease activity of T4 DNA polymerase. The resulting molecules are mixed together to form non covalent association and annealed. T4 DNA Polymerase's polymerization and exonuclease activity are balanced by exonuclease processing to the first complementary C residue by addition of dGTPs. Thus the vector has four nicks on each strand which are repaired by host (*E. coli)* during transformation (Fig. 5.3). In this technique the resultant recombinant plasmid is "scarless" as it does not contain any unwanted sequences or new restriction enzyme sites [3]. Ligation-independent cloning. LIC includes polymerase incomplete primer extension (PIPE) cloning which is a two-step process. Hybrid vectors are formed when complementary strands anneal overlapping sequences which are introduced at the ends of incomplete extensions. These hybrids are directly transformed into the host without any enzymatic manipulations [6], sequence and ligation-independent cloning (SLIC)

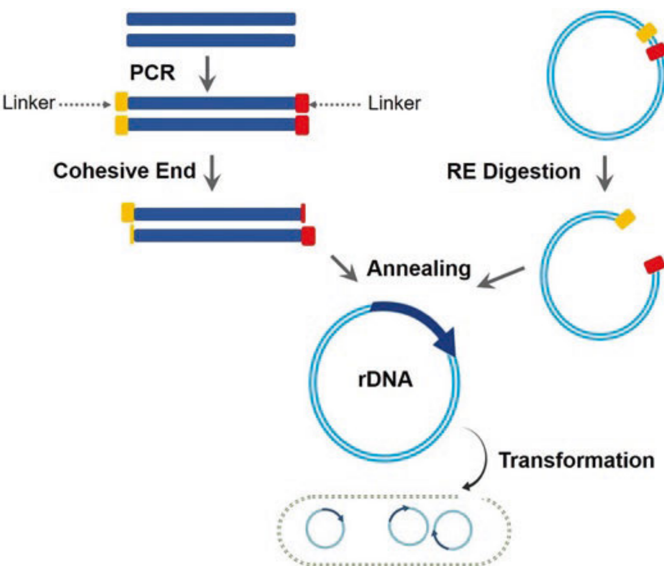

Fig. 5.3 Ligation-Independent Cloning (LIC) Workflow: First the insert DNA is PCR amplified using LIC primers. The PCR product and vector are treated with 3′ to 5′ exonuclease to create cohesive overhangs. The nicks in the complementary overlap are sealed by annealing to obtain rDNA which can be multiplied by transforming in competent host cells

uses in vitro homologous recombination and single strand annealing to assemble multiple fragments of DNA in a single reaction [7], and overlap extension cloning (OEC) employs chimeric primers to the insert creating overlapping regions with the vector. By mixing the insert and vector, hybrids are generated. The inserts are then extended using Phusion DNA polymerase, using vectors as template until polymerase reaches 5′ end forming a new plasmid. The parent plasmid is degraded by DpnI restriction enzymes leaving behind newly synthesized plasmid which can now be transformed into the host [8].

LIC is based on the exonuclease activity of T4 polymerase generating complementary overhangs without dependency on presence of specific restriction sites [9]. The extended length of compatible ends holds the insert and vector together, the nick between the nick is sealed by the DNA repair machinery of the host in which the rDNA is transformed. As background recombinants are not formed, thereby reducing the need to screen for recombinants [10].

Advantages

- It is highly efficient, allowing direct transformation into the host without any in vitro ligation.
- It does not cleave the insert sequence using restriction enzymes.
- It can be used to clone a library of unknown sequences.

Disadvantages

- Every fragment needs to be sequenced when used in different vectors.
- Cloned fragments cannot be recombined as in other techniques (Gateway).

5.5 Recombinational Cloning

Traditional methods of cloning allow handling only a few genes of interest at a time [11]. Recombinational cloning gives flexibility to insert multiple genes using site-specific recombination into multiple expressions and cloning systems simultaneously. These enzymes are capable of swapping and shifting single DNA sequences into multiple expression systems or multiple DNA inserts into a single expression vector at the same time, with widely available open reading frame (ORF) collections [12].

Gateway cloning system (Invitrogen) and Creator system (BD Clontech) is the most widely used system in this category [12]. It is based on a site-specific recombination system used by phage to integrate DNA in bacterial host cells. This technique relies on two proprietary enzyme mixes BP and LR clonase to transfer DNA fragments across recombination sites. Initially appropriate sequence of interest is cloned into a holding vector ("Entry" for Gateway) using traditional cloning methods. Once the new clone is created, it can be easily transferred to a variety of "destination" or "acceptor" vectors that include sequences which can be identified by recombinase (Fig. 5.4). This in vitro version of integration and excision reactions are made directional by developing att1 and att 2 recombinational sites.

Advantages

- Robust and high cloning efficiency.
- It allows to maintain the desired reading frame and orientation.

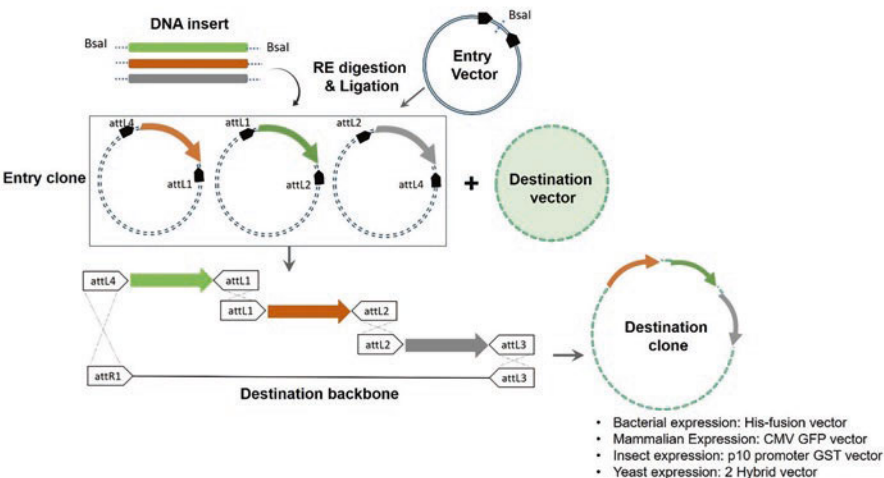

Fig. 5.4 Recombinational cloning workflow: In the first step Insert DNA sequences are cloned into entry vectors by traditional cloning methods or LIC. Using the flanking BsaI restriction sites, multiple fragments can be sub cloned as desired. The site-specific recombination between the flanking att sequences allows rearrangement and transfer into various destination vectors to create the final expression vector

- Single entry clone genes can be easily subcloned into a variety of destination vectors [13].

Disadvantage

- It is difficult to switch to another recombination system (lack convenient restriction endonuclease sites and start and stop codons are removed).
- Recombination enzymes are very expensive where the vector sets are defined by the supplier and often require proprietary enzymes to be used [14].

5.6 Mating-Assisted Genetically Integrated Cloning (MAGIC)

A highly engineered process of in vivo cloning "Mating-Assisted Genetically Integrated Cloning" or MAGIC was developed by Li and Elledge enabling rapid assembly of recombinant DNA molecules. During bacterial mating, it uses site-specific DNA cleavage and homologous recombination. The transfer of DNA fragments is aided by specifically made "donor" and "recipient" vectors (e.g., insert with specific recipient expression vectors and donors). The recombination events do not need any DNA preparation or in vitro manipulations. Recombination events are genetically chosen which results in the efficient positioning of the target gene under the control of new regulatory elements [7]. As this new method involves mixing of bacterial strains, it brings about a high-throughput recombinant DNA production that is seamless, saves time and effort.

Bacterial mating can efficiently transfer large DNA fragments (>100 kb). The donor strain must have conditional origin of replication and is nonfunctional in the recipient strain Two genetic systems initiate homologous recombination when the two strains are in the same cell. The first is by I-SceI site-specific endonuclease activity over donor and the recipient. The second is the lambda recombinase system which brings about homologous recombination between 50 bp. Combining all these features allows efficient and enhanced transfer of fragments from the donor vector onto the recipient [15].

Advantages

- The technique permits bacteria to mix inserts and vectors, allowing constructs to be generated without restriction enzyme digestion, gel purification, or the use of recombination-promoting enzymes [15].
- MAGIC can generate multiple constructs at the same time by using different recipient strains.

Disadvantage

- During the transfer of genes, there may be gain or loss of some nucleotides.

5.7 Summary

In this chapter, we provided an outline of various molecular cloning techniques and described fundamental working mechanisms for each. It also provides an overview of the progress made in development of specialized cloning techniques to improve efficiency by exploiting the properties of enzymes. Molecular cloning has evolved from cloning a few sequences of single DNA fragments to assembly of multiple fragments onto a single stretch of DNA. Additionally, these methods have been advantageous for making processes simple, seamless, and ability to bring about high-throughput production of recombinant DNA in a short stretch of time. In future, these technologies will emerge into processes that are able to insert or build sequences adjacent to each other to synthesize large DNA molecules. It will provide molecular biologist tools to explore, alter, and harness DNA, further broadening the horizon of science.

Self Assesment

Q1. What are the different types of DNA cloning methods?

A1. There is the classical traditional cloning method, PCR-based cloning and restriction enzyme-based cloning along with advanced methods such as recombination-based cloning and Mating-Assisted Genetically Integrated Cloning. Each method is based on the principle of inserting target DNA into a vector, but uses different mechanisms and approaches to achieve the insertion. The advances in the method make it affordable, efficient, and time saver.

Q2. Which of the DNA cloning methods are available commercially?

A2. Various types of DNA cloning kits are available based on Traditional and advanced DNA cloning methods. Few commercial products are TOPO TA cloning kit, In-Fusion, Gibson assembly GeneArt, Gateway Echo cloning, and Creator.

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