Gene Cloning

Gene cloning or recombinant DNA technology is the joining of two or more segments of DNA to generate a single DNA molecule capable of autonomous replication within a given host (1). Ligase enzymes catalyze the joining or ligation of DNA or RNA segments, where phosphodiester bonds are formed between adjacent 3′-OH and 5′-phosphate termini of DNA and RNA. The most widely used of such enzymes is T4 DNA Ligase. This enzyme can join DNA fragments having cohesive or blunt ends. It requires ATP as the energy yielding cofactor and Mg^{2+} ions for activity.

A good general vector for DNA cloning should contain basic elements, such as: an origin of replication enabling replication in bacteria; a multiple cloning site (MCS) with an array of unique restriction enzyme sites suitable for cloning of a double-stranded DNA insert and; selectable markers such as antibiotic resistance genes (tet^r and amp^r) (2).

Additional elements of cloning vectors include a phage-derived *f1* origin of replication for the production of single-stranded DNA and a MCS containing a DNA segment from the *E. coli lacZ* operon that encodes the amino-terminal fragment of β-galactosidase. This *lacZ* fragment can be induced by isopropylthio-β-D-galactosidase (IPTG), and complements a defective form of β-galactosidase encoded by the *E. coli* host, allowing blue/white colony or plaque selection of clones (2). In addition, some plasmids contain bacteriophage promoters adjacent to the MCS, so that foreign DNA cloned within this site can be transcribed *in vitro* (2).

Depending on the size of the DNA to be cloned, DNA fragments are inserted into plasmid (~0.1–10kb), lambda phage (~0–23kb), or cosmid (~35–50kb) vectors. Common cloning vectors such as the pGEM® series[d], pBR322, pUC/M13, pSP series[d], pET series[e], and others can easily replicate DNA inserts of 0–10kb. The multiplication rates of large recombinant plasmids slow as the plasmids get larger, with those that have lost large pieces of their foreign DNA eventually predominating. Larger DNA inserts, up to 15kb, are more easily cloned in lambda vectors, without affecting the normal packaging of the lambda chromosome into functional virus particles (4). Still larger DNA inserts, up to 50kb, can be cloned into cosmid vectors, constructed from the two ends of the lambda chromosome (3). Very large DNA fragments can be cloned into P1, BAC and YAC vectors.

The insert sequences can derive from practically any organism. They may be isolated directly from the genome, from mRNA by reverse transcription, from previously cloned DNA segments (subcloning) or from synthetic DNA sequences (1).

The following discussion focuses on DNA cloning in *E. coli* hosts, using common plasmid vectors and T4 DNA Ligase. For a discussion on cloning in other bacterial hosts, yeast, plants, *Drosophila*, and viruses, please refer to reference 4. Figure 2 provides essential information on the cloning of DNA fragments into plasmid or phage vectors, including basic directions on 1) digestion of vector and insert DNA; 2) conversion of 5′- or 3′-overhangs to blunt ends; 3) vector dephosphorylation and; 4) ligation of vector and insert DNA.

Preparation of Vector and Insert

There are four major steps in DNA cloning: 1) preparation of vector and insert; 2) ligation of vector and insert; 3) transformation into a host; 4) screening of selected clones.

In preparing the vector and insert DNA for ligation, it is best to digest purified plasmid with two different restriction enzymes having recognition sites within the MCS of the vector. The digestions should generate compatible ends for the cohesive-end ligation of vector and insert in the desired orientation (forced cloning). Cohesive-end ligation combined with blunt-end ligation at one of the ends is the second easiest type of ligation.

When the foreign DNA does not contain the same restriction sites as the vector MCS, it can be digested with appropriate restriction enzymes and blunt-ended for ligation to the vector. Both Klenow (DNA Polymerase I Large Fragment) and T4 DNA Polymerase can be used to fill 5′-protruding ends with dNTPs because both enzymes have 5′→3′ polymerase activity. T4 DNA Polymerase can be used to polish 3′-protruding ends in the presence of dNTPs due to its robust 3′→5′ exonuclease activity. Protocol information for these two enzymes is described in references 5 and 6.

When blunt-end ligation is not desired, it is sometimes possible to generate partial filling of 5′-overhangs with Klenow in the presence of selected dNTPs (5); this alters the overhangs and can make compatible overhangs or cohesive ends. An alternative strategy is to ligate linkers containing the appropriate restriction enzyme sites to the blunt-ended DNA insert.

For cloning PCR products, restriction enzyme sites can be added at the 5′- and 3′-end during amplification to ensure forced cloning. The restriction enzyme site is designed approximately four nucleotides from the 5′-end of the PCR primers to ensure proper digestion; some restriction enzymes do not digest DNA when the recognition site is too close to the end of the DNA fragment (5). Since nonproofreading (no 3′→5′ exonuclease activity) polymerases add an extra nucleotide, usually an adenine, to the 3′-end of the PCR product, a practical approach for cloning PCR products is to use a vector containing single T-overhangs. The pGEM®-T or pGEM®-T Easy Vector Systems[d,f] (Cat.# A3600 and A1360, respectively), posess a 3′-terminal thymidine, which can pair with the adenine overhang of the PCR product facilitating ligation.

References

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8. *Wizard® PCR Preps DNA Purification System Technical Bulletin* #TB118, Promega Corporation.
9. *T4 DNA Ligase* Promega Product Information Sheet #9PIM180, Promega Corporation.
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When doing blunt-end ligation or single restriction enzyme site ligation, it is advisable to dephosphorylate the vector with Calf Intestinal Alkaline Phosphatase (CIAP). Removing 5′-phosphate groups from the vector prevents self-ligation, thus reducing background levels observed after transformation into the host cells. Exceptions are linear vectors having unique 5′-ends, or nucleotide cloning where the insert:vector molar ratio is >10:1. CIAP is recommended at 0.01 units per picomole of DNA ends:

$$\frac{\mu g\ DNA}{(kb\ size\ of\ DNA)} \times 3.04 = pmol\ of\ ends\ (for\ linear\ dsDNA)$$

For example, 5µg of linearized DNA (6kb) contains 2.5pmol of ends. For detailed protocol information see references 5 and 6.

In a situation where the vector is dephosphorylated, the insert must be phosphorylated at the 5′-ends to ensure ligation. DNA fragments derived from restriction enzyme digestions will be phosphorylated. PCR DNA inserts and synthetic double-stranded DNA inserts can be phosphorylated with T4 Polynucleotide Kinase and ATP. See references 5 and 6 for protocol information.

Another important step to ensure successful ligation is the purification of vector and insert DNA. To reduce background transformation of uncut vector, it is desirable to gel-purify the linearized vector from uncut vector. The linearized vector DNA can be gel-purified once it has been treated with restriction enzymes, modifying enzymes and dNTPs, which may inhibit ligation. If the vector DNA has been cut to near completion (>99%) it may not be necessary to gel-purify it, although a few background transformants may result. The Wizard® DNA Clean-Up System (Cat.# A7280) or Agar*ACE*® Enzyme (Cat.# M1741) are appropriate methods of purification when DNA has not been eluted from agarose gels (6,7).

The DNA insert of interest may need to be gel-purified to remove donor vector or additional DNA fragments that have been digested from a donor. DNA inserts generated by PCR may need to be gel-purified if contaminant DNA fragments are present. When a single PCR fragment of the expected size is generated, gel purification is not necessary. PCR products can be purified using the Wizard® PCR Preps DNA Purification System (Cat.# A7170, A7181), which allows purification from agarose gels or directly from the PCR reaction (8).

Ligation

The efficiency of ligation between vector and insert is best represented by the yield of transformable products. Conditions such as insert:vector molar ratios, amount of total DNA, insert size, units of ligase, ATP and Mg^{2+} concentrations, and appropriate incubation time and temperature should also be considered to ensure successful ligation reactions.

When cloning a DNA fragment into a plasmid vector, the recommended molar ratio of insert:vector DNA is 3:1–1:3. To convert molar ratios to mass ratios the following formula can be applied:

> (ng of vector x size of insert in kb/size of vector in kb) x (molar ratio of insert:vector) = ng of insert. For example, to calculate the amount of 0.5kb DNA insert to use in a ligation reaction containing 100ng of a 5kb vector, in a 3:1 insert:vector molar ratio is 30ng of insert DNA (5,9).

Generally, the recommended amount of total DNA in a ligation reaction varies from 1–10ng per microliter of reaction in a final volume of 10–20µl (10–200ng total DNA mass) (1,9,10). More difficult ligations, such as blunt-end and greater than two fragment ligations, may require more DNA (1). However, some blunt-end ligations have optimal total DNA concentrations as low as 2–3ng per microliter of reaction (10).

There is a consensus that T4 DNA Ligase performs best at 0.1u/µl of reaction (1,9–12). The buffer components for optimal T4 DNA Ligase activity should be at a final concentration of 30–50mM Tris-HCl (pH 7.6–7.8), 10mM $MgCl_2$, 1–10mM DTT and 10µM–1mM ATP (Table 15). The integrity of the ATP is essential (9,12). ATP forms a gradient upon thawing in the 10X T4 DNA Ligase Buffer. The 10X buffer should be vortexed to completely incorporate ATP into solution. Also, the DTT in this buffer tends to precipitate upon freezing; vortexing is important to thoroughly mix the DTT into solution. The 10X T4 DNA Ligase Buffer should be stored frozen at –20°C in aliquots to maintain the stability of the ATP molecule.

In general, optimal ligation occurs at a balance between the optimal temperature for T4 DNA Ligase activity (25°C) and the optimal joining temperature of the termini (a few degrees below the melting temperature or T_m) (11,12). There is considerable latitude concerning optimal incubation times and temperatures for blunt- and cohesive-end ligations. Reference 11 outlines some commonly used conditions.

In the presence of up to 5% (w/v) high-quality PEG 8000, cohesive-end ligations can be accomplished in 1–2 hours at room temperature, or overnight at 4°C. Under the latter condition, ligation efficiency is improved by approximately three- to six-fold, as determined by the number of transformants (6,11). Blunt-end ligation efficiencies can also be improved with 5% (w/v) PEG 8000, HMG (high mobility group) 14 DNA binding protein and T4 RNA Ligase (11). Macromolecules such as PEG 8000 greatly stimulate ligation by increasing macromolecular crowding and aggregation of DNA molecules (2,11).

References (continued)

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HMG 14 DNA binding protein associates with active chromatin through electrostatic interactions with the phosphate backbone of DNA, stabilizing the DNA duplex and promoting intermolecular ligation of linear DNA. A 50% increase in transformants was observed in blunt-end ligations using T4 DNA Ligase with 50 moles of HMG 14 per mole DNA. Intramolecular interactions (recircularization) under these conditions were inhibited (11).

Low concentrations of T4 RNA Ligase stimulate T4 DNA Ligase in the ligation of blunt ends as much as 20-fold, approaching the efficiency of cohesive-end ligation. The joining of cohesive ends increases slightly in the presence of T4 RNA Ligase (11).

Another important component in a ligation reaction is the use of nuclease-free water. Nucleases are active in the presence of Mg^{2+} in the T4 DNA Ligase buffer. Moreover, it is important to isolate plasmid DNA from bacterial strains that are *end*A–. If this is not possible, the plasmid DNA preparation should be phenol:chloroform extracted, as it may contain nuclease. Alternatively, if a Wizard® DNA Purification System is used for DNA purification, nuclease can be removed with an additional 40% isopropanol/4.2M guanidine-HCl wash (13,14).

In addition to the need for appropriate reaction conditions, it is critical to perform negative control reactions during the ligations. Each control reaction should lack a single DNA component; the excluded DNA component should be replaced with an equal volume of water. In a successful cloning reaction the number of transformed colonies, when all the reaction components are present, should be greater than the number of colonies obtained for any of the control reactions.

An additional reaction without T4 DNA Ligase provides information on the number of colonies that result from background uncut vector.

Transformation and Selection

Several *Escherichia coli* strains can be used for transformation with Promega cloning vectors. However, an appropriate host should be compatible with the method of selection. Genotype information on commonly used strains is presented in chapter 24 of reference 5, in reference 11 and Tables 13 and 14 of this guide. *E. coli* JM109 is a preferred host because it is *rec*A–, which reduces undesirable recombination between the insert and host chromosomal DNA (5). *E. coli* cells can be made competent for transformation by different methods (5,15).

Generally, 10ng of ligated DNA is sufficient to transform 100–200µl of competent cells. The transformation efficiency of uncut, supercoiled plasmid in high efficiency competent cells is about 1×10^7–1×10^8cfu/µg DNA. For each batch of competent cells it is advisable to perform a transformation control with 0.1ng of uncut plasmid, and to calculate the transformation efficiency according to the following formula:

$$\frac{\text{cfu on control plate}}{\text{ng of uncut vector}} \times \frac{10^3\text{ng}}{\mu\text{g}} \times \text{final dilution} = \frac{\text{cfu}}{\mu\text{g DNA}}$$

(cfu = colony forming units; 5)

For example:

One hundred microliters of competent cells is transformed with 1ng of supercoiled plasmid DNA. Ten microliters of the transformation reaction (0.1ng total DNA) is added to 990µl SOC medium (1:100 dilution). A 100µl aliquot is plated and 100 colonies are counted. The transformation efficiency =

$$\frac{100\text{cfu}}{0.1\text{ng of supercoiled plasmid}} \times \frac{10^3\text{ng}}{\mu\text{g}} \times 10^3 = \frac{1 \times 10^7\text{ cfu}}{\mu\text{g DNA}}$$

When the transformation efficiency of uncut supercoiled plasmid is approximately 1×10^9cfu/µg of DNA, the transformation efficiency of recombinant clones is usually about 1×10^5cfu/µg of DNA for blunt-end ligation. This means that 1–1,000 colonies could be obtained from 10ng of ligated DNA. However, the results will also depend on what percentage of the DNA is successfully ligated. The transfection efficiency of recombinant clones is about 1×10^6cfu/µg of DNA for cohesive-end ligation. This means that 10–1,000 colonies could be obtained from 1ng of ligated DNA. However, these results also depend on the percentage of DNA that is successfully ligated.

A common method of screening transformed plasmids in *E. coli* is accomplished by plating transformed cells on media containing ampicillin, tetracycline or other antibiotics and IPTG and X-Gal. Antibiotic selection is based on the presence of a selectable antibiotic marker in the cloning vector. Successful ligation can be determined by a change in colony color when a vector designed for blue/white color selection is used. A colony color change results from insertion of a DNA fragment into the *lac*Z region of the plasmid, generally causing a white colony upon induction with IPTG and color detection with X-Gal (5,6).

Candidate colonies containing recombinant plasmids are identified and single clones are then grown in liquid media containing antibiotics. Recombinant plasmids are verified by purifying plasmid DNA and digesting with selected restriction enzymes, by hybridization to a specific nucleic acid probe, by sequencing or by PCR.