Chapter 19

DNA Cloning and Sequencing

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Abstract

The plasmid DNA is cleaved with an enzyme and joined in vitro to foreign DNA; the resulting recombinant plasmids are then used to transform bacteria. The plasmid vectors must be carefully chosen and processed to minimize the effort required to identify and characterize recombinants. This chapter provides guidelines for preparation of DNA fragment for cloning, transformation into chemically competent host, and selection of positive clones. The write-up will also describe basic methods used in the cloning of PCR amplified rRNA gene into appropriate vector and followed by sequencing.

19.1 Introduction

In principle, cloning in plasmid vectors is very straightforward. The easiest fragment to clone carries a noncomplementary protruding termini generated by digestion with two different restriction enzymes. Since most of the present-day vectors contain poly-linker that has multiple cloning sites, it is almost always possible to find restriction sites that are compatible with the termini of the foreign DNA fragment. The fragment of foreign DNA is then inserted into the vector by a process known as directional cloning.

Fragment of foreign DNA carrying identical termini (either blunt-ended or protruding) must be cloned in a linearized plasmid vector bearing compatible ends. During the ligation reaction, the foreign DNA and the plasmid DNA should have the capacity to circularize and to form tandem oligomers. It is therefore necessary to carefully adjust the concentrations of the two types of DNA in the ligation reaction to optimize the number of correct ligation products. In addition, removal of the 5′-phosphate groups with alkaline phosphatase will help to suppress self-ligation and circularization of the plasmid DNA.

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19.2 Materials

1. Glassware, instruments, and other materials: Electrophoresis tank (horizontal/submarine), UV transilluminator, sterile microfuge tubes, micropipettes, sterile micropipette tips, shaker incubator, sterile 50 ml polypropylene centrifuge, ice-box, microfuge, vortex mixture, water bath, etc.
2. Chemicals:
   (a) Agarose
   (b) 50× TAE buffer
   (c) Ethidium bromide (10 mg/ml)
   (d) Restriction endonucleases (*Eco*RI, *Hind*III, etc.)
   (e) λ DNA digested with *Hind*III
   (f) Isolated plasmids (with and without inserts of foreign DNA)
   (g) Calf Alkaline Phosphatase (CIP)
   (h) T4 DNA ligase and 10× ligase buffer
   (i) Buffer saturated phenol
   (j) Chloroform
   (k) 3 M Sodium acetate (pH 7)
   (l) 3 M Sodium acetate (pH 5.2)
   (m) 10 M Ammonium acetate
   (n) LB broth
   (o) LB agar plates with and without ampicillin
   (p) *E. coli* DH5α Competent cells
   (q) IPTG (200 mg/ml)
   (r) X-gal (20 mg/ml in dimethylformamide)
   (s) Ptz57R/T vector (MBI Fermentas) or PCR™ II vector (Invitrogen)
   (t) Calcium chloride—0.2 M (prepare 1 M stock solution of $CaCl_2$ and store 10 ml aliquots frozen at −20 °C). Just before use, dilute an aliquot to 100 ml with sterile water and by filter through 0.45 μm filter, and then chill on ice for use.
   (u) BDT v3.1 Reaction Mix (Applied Biosystems #4337455) [1]
   (v) 5× Sequencing buffer (Applied Biosystems #4336697)
   (w) Hi-Di formamide (Applied Biosystems)
   (x) 0.5 M EDTA pH 8.0
   (y) 125 mM EDTA
   (z) TE buffer

19.3 Methods

19.3.1. Ligation of Vector and Foreign DNA Fragment

Ligation of a segment of foreign DNA to a linearized plasmid vector involves the formation of new bonds between phosphate residues located at the 5′-hdroxyl moieties. Ligation of one end of DNA to another can be regarded as a bimolecular reaction whose velocity under standard conditions is determined solely by the concentration of same DNA molecule (intramolecular ligation) or on different molecules (intermolecular ligation). Low concentration of DNA in the ligation reaction may lead to intramolecular ligation, whereas high DNA concentration may result in the formation of dimers and/or larger oligomers of the plasmid.

19.3.2. Preparation of Vector DNA and Foreign DNA Fragment for Cloning

1. Restriction digestion of plasmid DNA (prepared by mini prep.) and foreign DNA with the desired endonucleases.
2. Check 5 μl of the above plasmid for completion of digestion in 0.8 % agarose gel. Use undigested plasmid and marker DNA for comparison.
3. Mix following components in the order mentioned below in a microfuge tube on ice:

To linearize the plasmid:

Plasmid DNA (1–2 μg)	5 μl
10× RE buffer	2 μl
Restriction enzyme 1 (e.g., *Eco*RI)	2 μl
Restriction enzyme 2 (e.g., *Hind*III)	2 μl
Deionized distilled water	to 20 μl

4. Mix the contents by gentle tapping and pulse spin in a microfuge to bring down all the liquid to the bottom of the tube and incubate at 37 °C for 1 h.
5. Heat at 65 °C for 10 min to stop the reaction. Chill on ice. Add 5 μl of loading dye, mix, and pulse spin.
   Load the digested products on 1 % TAE/agarose gel, with λ DNA digested with *Hind* III as marker.

19.3.3. Preparation of Phosphatase-Treated Vector

1. Restriction digested plasmid will be now proceeding for phosphatase treatment as follows:

Digested plasmid	15 µl
10× CIP buffer	5 µl
H_2O	49 µl
Calf alkaline phosphatase (1 U)	1 µl

2. Incubate at 37 °C for 30 min.
3. Add another 1 µl of CIP and continue the incubation for 30 min.
4. After 1 h of CIP treatment, add 1 µl of 0.5 M EDTA (pH 8.0) to get a final concentration of 5 mM.
5. Incubate at 75 °C for 10 min to inactivate the CIP.
6. Cool the reaction to room temperature and extract once with equal volume of phenol and once with equal volume of phenol–chloroform mixture.
7. To the aqueous phase, add 0.1 volume of 3 M sodium acetate (pH 7.0), and 2 volume of ethanol, mix well, and precipitate the linear dephosphorylated vector at −20 °C for 30 min.
8. Recover the DNA by centrifugation at 4 °C in a microfuge.
9. Wash the pellet with 70 % ethanol, air dry, and dissolve in 10 µl of distilled water.
10. Run an aliquot of both the vector and the insert DNA to estimate the concentration of DNA in the gel before setting up the ligation.

19.3.4. Ligation

1. Set up ligation reaction in total volume of 10 µl as follows (for a foreign DNA fragment that has length equal to vector DNA) (Table 19.1).
2. If the foreign DNA is smaller than vector, reduce the concentration of foreign DNA accordingly to bring it equal to molar concentration of the vector.
3. Include necessary control like ligation without foreign DNA fragment (Vector re-circularization control), ligation vector only that was not treated with phosphatase (Ligation control), etc.
4. In each case, adjust the volume to 10 µl with phosphatase (Ligation control), etc.
5. In each case, adjust the volume to 10 µl with H_2O.
6. Incubate the reaction for 4–16 h at 16 °C.
7. Use 2 µl of the ligation mixture for transformation of bacteria. Store the remaining ligation reaction at −20 °C for further use.

Table 19.1
Ligation reaction mixture of 10 l volume

Ingredients	Volume	Final concentration
Vector DNA (100 ng/μl)with dephosphorylated 5′termini	2 μl	200 ng
Foreign DNA fragment (100 ng/μl) with compatible phosphorylated termini	2 μl	200 ng (equimolar to vector)
10× ligase buffer with 10 mM ATP	1 μl	1×
T4 DNA ligase	1 μl	0.1 Weiss Unit
Deionized distilled water	To 10 μl	0.1 Weiss Unit

19.3.5. Transformation

When the bacteria are treated with ice-cold solution of $CaCl_2$ and then briefly heated, they could be transfected with plasmid DNA. Apparently the treatment induces a transient state of "competence" in the recipient bacteria, during which they are able to take up DNAs derived from a variety of sources. Most commonly used methods yield transformants at a frequency of 10^7–10^8 transformants/μg of supercoiled plasmid. Competent cells of the *E. coli* strains such as JM 107, XL 1-Blue, DH5α, SURE, and NM522 can be used for transformation.

19.3.6. Competent Cell Preparation

Day 1: Selection of *E. coli* DH5α on LB Agar Plates

1. Streak *E. coli* DH5α culture either from glycerol stock or from any viable culture stored at 4 °C, with platinum loop on LB agar plates.
2. Incubate the LB plate at 37 °C overnight (O/N) and isolate a single colony.

Day 2: Preparation for O/N Culture

1. Inoculate 5 ml of LB broth with a single colony from LB agar plate.
2. Let it be grown for O/N in a shaking incubator (150 rpm) at 37 °C.
3. Keep the following in the cold room for next day use: 50 ml centrifuge tubes, microfuge tubes, 10 and 5 ml glass pipettes, $CaCl_2$ solution.

Day 3: Preparation of Competent Cells

1. Inoculate 100 ml LB medium in 1 liter flasks with O/N culture (make 1 % inoculums).

2. Let the flask grow at 37 °C shaking for 2–3 h. Measure OD at A_{550} after every 30 min to find out when A_{550} is between 0.4 and 0.5 (i.e., when the cells are in early log phase).
3. Chill the flaks in ice for 5–10 min. Transfer the culture to pre-chilled 50 ml centrifuge tubes.
4. Centrifuge at 5,000 rpm/5 min at 4 °C.
5. Decant the supernatant and suspend the pellet gently with the help of a pre-chilled glass pipette in 20 ml of 200 mM $CaCl_2$ and incubate in ice water for 20 min. Centrifuge at 5,000 rpm/5 min at 4 °C.
6. Decant the supernatant and suspend the pellet gently in 4 ml 80 mM $CaCl_2$ solution.
7. Aliquot the cells in microfuge tubes either in 200 µl or its multiples, and immediately freeze in liquid nitrogen and store the cells at −70 °C.
8. If not used immediately, the competent cells may be stored on ice O/N.

19.3.7. Transformation

1. Thaw a 200 µl aliquot of competent cells on ice.
2. Add 2 µl of ligation mix containing approximately 50–100 ng of DNA to the competent cells.
3. The volume of DNA/ligation mix to be added to a 200 µl aliquot of competent cells should not exceed 10 µl.
4. Mix the contents of tube by swirling gently. Incubate on ice for 30 min.
5. Heat shock at 42 °C for exactly 120 s. (Do not shake the tubes.)
6. Rapidly transfer the tubes to ice bath and allow the cells to chill for 1–2 min.
7. Add four volumes (0.8 ml) of LB medium and keep in a water bath at 37 °C for 1 h to allow the bacteria to recover and express the antibiotic resistance marker encoded by the plasmid.
8. Centrifuge at 5,000 rpm for 5 min to settle down the cells. Remove medium and resuspend the cells in the remaining medium and plate on the LB ampicillin plates with IPTG and X-gal.
9. Include following control:
   (a) No DNA control
   (b) Transformation efficiency control
   (c) Incubate the plates at 37 °C O/N

19.3.8. Selection of Positive Clones

There are four following methods that are commonly used to identify bacterial colonies that contain recombinant plasmids:

19.3.8.1. Restriction Analysis

In restriction analysis, a number of independently transformed bacterial colonies are picked and grown in small cultures. Plasmid DNA isolated from each culture is analyzed by digestion with restriction enzyme and gel electrophoresis.

19.3.8.2. Insertional Inactivation

It can only be used with order vectors (Pbr322) that carry two or more antibiotic resistance genes and an appropriate distribution of restriction enzyme cleavage sites. The foreign DNA is cloned in the plasmid in such a way that it disrupts the reading frame of one of the antibiotic resistant genes. Recombinant bacteria are screened by growing identical colonies separately on more than one antibiotic plate. If the bacteria become sensitive to the antibiotic, whose gene was disrupted by the insertion of the foreign DNA, and remains resistant to the others, it indicates that the bacteria contain the plasmid having foreign DNA.

19.3.8.3. Screening by Colony Hybridization

In this method the bacterial colonies are transferred onto a nitrocellulose paper and lysed to release and denature the DNA that is immobilized on the nitrocellulose paper. This nitrocellulose paper is then hybridized with radioactive labeled DNA fragments that were used for cloning. The autoradiogram is aligned with the original bacterial plate, from where the colonies were transferred, to identify the bacteria carrying foreign DNA.

19.3.8.4. α-Complementation

Many of the vectors in current (e.g., the pUC Series) carry a short segment of *E. coli* DNA that contains the regulatory sequences and the coding information for the first 146 amino acids of the β-galactosidase gene (*lac* Z). Embedded in this coding region is polycloning or multiple cloning site that does not disrupt the reading frame but results in the harmless interpolation of a small number of amino acids to the amino-terminal fragment of β-galactosidase. Vectors of this type are used in host cells that code for the carboxy-terminal portion of the β-galactosidase. Although neither the host-encoded nor the plasmid-encoded fragments are themselves active, they can associate to form enzymatically active protein. This type of complementation, in which deletion mutants of the operator-proximal segment of the *lac*-Z gene are complemented by β-galactosidase—negative mutants that have the operator-proximal region intact, is called α-complementation. The lac^+ bacteria that result from α-complementation are easily recognized because they form blue colonies in the presence of chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glactose (X-gal). However insertion of a fragment of foreign DNA into the polycloning site of the plasmid almost invariably results in the production of an amino-terminal fragment that is not capable of α-complementation.

Bacteria carrying recombinant plasmid therefore form white colonies. The development of this simple color test has greatly simplified the identification of recombinants constructed in plasmid vectors of this type. It is easily possible to screen many thousands of colonies visually and to recognize colonies that carry putative recombinant plasmids. The structure of these plasmids is then verified by restriction analysis of mini preparation of plasmid DNA.

19.3.9. Experimental Procedure

1. To a premade LB agar plate containing ampicillin, add 40 µl of stock solution of X-gal (20 mg/ml) and 4 µl of solution of IPTG (200 mg/ml).
2. Using a sterile glass spreader, spread the solution over the entire surface of the plate.
3. Incubate the plate at 37 °C until the fluid has disappeared.

   (**Note**: It may take up to 2–3 h, if the plate is freshly made.)
4. Inoculate the plate with 200 µl of transformation mixture and spread the solution over the entire surface of the entire plate using a sterile glass spreader.
5. Incubate the inoculated plate in an inverted position for 12–16 h at 37 °C.
6. Store the plate at 4 °C for several hours. This allows the blue color to develop fully.
7. Pick few blue and few white colonies for analysis by restriction endonuclease digestion.

   Note: Colonies that contain active β-galactosidase are pale blue in the center and dense blue at the periphery. White colonies occasionally show a faint blue spot in the center, but these are colorless at the periphery.

19.3.10. Cloning of PCR Product Using T/A Over Hang

Cloning of PCR product into appropriate vector followed by sequencing allows the product identification and characterization. The basic methods used in cloning of PCR product include:

TA cloning: Since PCR product generated by *Taq* polymerase is appended with a single extraneous d*A* at 3′ ends, the easiest way of cloning is by using a plasmid tailed with d*T*.

Blunt end cloning: The blunt end PCR product generated by *Pwo* or *Pfu* polymerase can be cloned into a plasmid restricted with blunt end generating enzymes.

Directional cohesive end cloning: In this case PCR product is first restricted with appropriate restriction enzymes followed by ligating them onto plasmid linearized by same restriction enzymes.

Table 19.2
Components of ligation reaction mixture

Ingredients	Volume (μl)
Plasmid vector pTZ57R/T vector (0.165 μg)	1
PCR fragment (approx 0.495 μg)	2
10× ligation buffer	1
PEG 4000 solution	1
Deionized water	5
T4 DNA Ligase, 5 U	1

19.3.11. Ligation

The following protocol describes cloning of PCR amplified product using TA vector:

1. PCR products are first purified to remove enzymes, unused primers, dNTPs, etc. For this the PCR product is first run on low melting agarose gel, followed by extraction and purification (many commercial gel extraction kits are available).

   Note: The efficiency of ligation is known to be dependent on the purity of the PCR fragments and if a single homogenous band of desired size is observed on the gel after purification of PCR product, it can be directly used in the ligation reaction. tpb 6pt

2. Dissolve the purified PCR fragment in 10–20 μl of TE buffer, determine the DNA concentration using nanodrop UV spectrophotometer, or alternatively load 2 μl of DNA into agarose gel electrophoresis and compare with the known amount of DNA markers.
3. Calculate the amount of PCR fragment required for ligation (in equimolar concentration) using the formula-

   $$X ng\ of\ PCR\ product\ to\ ligate = (Y bp\ of\ PCR\ product)\text{-}(50\ ng\ of\ vector)/(size\ in\ bp\ of\ the\ vector)$$

   Three time "*X*"ng would be used for a 1:3 molar ratio.
4. Ligation Reaction Mixture: Ligation reaction mixture has been listed in Table 19.2.
5. Incubate at 22 °C for 1 h.
6. For maximum yield, the reaction time can be extended overnight.

19.3.12. Transformation and Selection of the Clones

1. Follow the procedure mentioned in the above sections.
2. Plasmid isolation may be done following the protocol of this manual.

19.3.13. Release of the Insert

To release the insert:

Plasmid DNA (1–2 µg)	−5 µl
10× RE buffer	−2 µl
Restriction enzyme 1 (e.g., *Eco*RI)	−2 µl
Restriction enzyme 2 (e.g., *Hind*III)	−2 µl
Deionized distilled water	−20 µl

19.3.14. Sequencing of PCR Product

Sequencing may be done following protocol of "Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing kit" [1].

19.3.15. Removal of Excess of Salts and Enzymes from PCR Products

The PCR amplified products can be subjected for the removal of excess salts as follows:

1. To the template DNA, add enough MQ water to make the volume to 100 µl. Add 10 µl of 3 M sodium acetate pH 5.5 and 250 µl of chilled absolute ethanol.
2. Mix the contents well and incubate on ice for 20–30 min.

   Note: Incubation at lower temperatures and for longer periods may cause precipitation of salts and hence is not recommended.
3. Spin at 12,000×*g* for 20 min and remove the supernatant.
4. Wash the pellet by adding 500 µl of 70 % ethanol at room temperature and centrifuge at 12,000×*g* for 5 min.
5. Aspirate or decant the supernatant and repeat the 70 % ethanol wash step once more.
6. Air dry the pellet and resuspend in a suitable volume of water.
7. Check an aliquot on gel and quantification.

19.3.16. Exo–SAP Digestion of PCR Product

1. Make a master mix of *Exonuclease* I and *Shrimp Alkaline Phosphatase* (SAP) for 10 µl of PCR product as per Table 19.3.
2. Add 1 µl of the mastermix to 10 µl of PCR product and set up the following incubation protocol in a thermal cycler:

Hold: 37 °C 120 min
Hold: 85 °C 15 min
Hold: 4 °C

Both *Exo*I and SAP are active in 1× Taq Buffer and can be easily denatured by heating to 85 °C for 15 min.

19.3.17. Sequencing PCR

1. Make a master mix of your sequencing reaction based on the following volumes given in Table 19.4.

Table 19.3
Mastermix of Exo–SAP digestion

Components	Units/Rxn.	1×	100×
*Exo*I (20 U/μl)	0.5	0.025	2.5
SAP (1 U/μl)	0.5	0.5	50
PCR Buffer 10×	1×	0.1	10
MilliQ		0.375	37.5
Total volume		1	100

Table 19.4
Ingredients of master mix of sequencing reaction

Reaction	1/4×	1/8×
BDT	1	0.5
5× Seq Buffer	1.5	1.75
Primer 3.2 μM	3.2 pmol	3.2 pmol (1 μl)
Template	–	–
Water	To 10 μl	To 10 μl

2. Thaw out your primer first and add the correct amount of this and water to a tube.
3. Thaw out the 5× buffer, mix well, and add the correct amount to the tube.
4. Remove an aliquot of BDT and thaw on ice. Mix well and spin down the tube.
5. Add the correct amount to the reaction mix.
6. Mix the master mix well by inversion and spin down. The master mix is now ready to be aliquoted into strip tubes, a plate, or single tubes.
7. Add the purified template (up to 6.75 μl), typically 1–5 μl for 300–1,500 bp products depending on concentration, based on 3–10 ng for 200–500 bp, 5–20 ng for 500–1,000 bp, or 10–40 ng for 1,000–2,000 bp.
8. Seal the plate with PCR film, or tubes as per normal. Mix the reaction by vortexing for 3 s. Flick the product back down to the bottom of the wells.
9. Place the plate/tubes in a PCR machine.

The reaction is as follows:

96 °C 10 s
50 °C 10 s
60 °C variable[a]
Repeat for 25 cycles
Hold at 15 °C

[a]This is the extension step and so alter the time to be the same as what you would use for a PCR, i.e., ≤1,000 bp/min

10. The primer temp can be altered for difficult templates.
11. Once the sequencing reaction is finished, the samples can be stored at −20 °C for few days or else continue for the cleanup step.

19.3.18. PCR Product Cleanup

After sequencing PCR, the amplified products should be carried out for the removal of the excess salts, primers, and enzymes as follows:

1. Transfer the reaction product into a 1.5 ml tube.
2. Make a master mix I of 10 μl Milli-Q and 2 μl of 125 mM EDTA per reaction.
3. Add 12 μl of master mix I to each reaction containing 10 μl of reaction.
4. Ensure the contents are mixed.
5. Make master mix II of 2 μl of 3 M NaOAc pH 4.6 and 50 μl of ethanol per reaction.
6. Add 52 μl of master mix II to each reaction.
7. Mix the contents well and incubate at room temperature for 15 min.
8. Spin at a speed of 12,000×*g* for 20 min at room temperature.
9. Decant the supernatant.
10. Add 250 μl of 70 % and spin at 12,000×*g* for 10 min at room temperature.
11. Decant the supernatant.

Add 12–15 μl of Hi-Di formamide, transfer to sample tubes cover with septa, denature, snap chill, and proceed for electrophoresis.

19.3.19. Sequence Analysis

The resulting ITS sequences were analyzed for homologies to sequences deposited in the GenBank and EMBL databases.

Reference

1. Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit Protocol.