The Cloning Enzymes

Introduction

Enzymes that modify nucleic acids provide the foundation for many molecular biology techniques. These enzymes are used to synthesize, degrade, join or remove portions of nucleic acids in a controlled and generally defined manner. Specific features of the *in vivo* functions of these enzymes have been exploited *in vitro* to provide many of the protocols currently used in nucleic acid manipulations.

The enzymes highlighted in this second Enzyme Resource Guide, *Cloning Enzymes*, are those important in nucleic acid cloning procedures. Figure 1 summarizes the activities of the cloning enzymes: ligases, kinases and phosphatases, and RecA Protein. Table 1 provides a list of the common applications of the six enzymes included in this guide.

Ligases are used to join nucleic acid segments, primarily when cloning a DNA fragment into vector DNA (Figure 1, Panels A-C).

Phosphatases remove the 5′-phosphate from nucleic acid strands. This serves several useful functions, including (1) preventing vector religation, which reduces the number of background colonies and (2) producing substrate to which a kinase can attach a new, often radiolabeled phosphate (Figure 1, Panel D).

Kinases add new phosphate groups to a nucleic acid, usually as a means to label a nucleic acid fragment or synthetic oligonucleotide. Under certain conditions, kinases can exchange an existing phosphate group from a nucleic acid fragment with the γ-phosphate of a nucleotide triphosphate (Figure 1, Panels E-H).

RecA Protein and Agar*ACE*® Enzyme[a] are included in this guide because of their roles in protection in certain cloning procedures or facilitating nucleic acid purification. The *E. coli* RecA Protein has a remarkable ability to facilitate the pairing of homologous DNA sequences (Figure 1, Panel I). Agar*ACE*® Enzyme is a novel, patented agarose-lysing enzyme produced and extensively tested for the harvest of DNA from agarose gels.

Table 1. Applications of Cloning Enzymes.

Application	T4 DNA Ligase	T4 RNA Ligase	T4 Polynucleotide Kinase	Calf Intestinal Alkaline Phosphatase	RecA Protein	Agar*ACE*® Enzyme
Cloning (DNA) Ligation						
Blunt-ended	✓					
Cohesive-ended	✓					
Single-stranded		✓				
Nicks	✓					
Cloning (5′-RACE)						
RNA Ligation		✓				
Genomic Cloning Enrichment					✓	
Phosphorylation						
5′			✓			
Dephosphorylation						
3′			✓			
5′				✓		
Purification from Agarose Gel						✓
Labeling DNA and RNA						
5′			✓			
3′-addition		✓				
Sequencing						
DNA			✓			
RNA			✓			
Sequence-Specific Cleavage of DNA					✓	

Cloning Tips

- Addition of acetylated BSA to restriction enzyme digests will ensure the highest possible enzyme activity.
- Three types of ends can result from restriction enzyme digestion of double-stranded DNA: a 5′ ssDNA overhang, a 3′ ssDNA overhang (a cohesive or 'sticky' end) or no overhang (a blunt end).
- DNA ends that have complementary overhanging sequences can be ligated directly to each other. While complementary cohesive ends are usually the result of digestion with the same restriction enzyme, they may also be generated by different enzymes. Blunt ends are compatible with any other blunt end.
- Ligation of complementary cohesive ends is much more efficient than ligation of blunt ends. However, it is often desirable to convert a cohesive end to a blunt end since a blunt-ended fragment can be ligated to any other blunt-ended fragment. T4 DNA Polymerase can be used to blunt-end both 5′- and 3′- overhangs.
- Dephosphorylation prevents the religation of cohesive ends. Even if the vector has been digested to generate noncompatible ends, dephosphorylation may be desirable. Double digests are rarely 100% efficient, and the number of background colonies resulting from singly-cut vector can be significant.
- While insert and vector can be purified directly from the restriction and dephosphorylation reactions using the Wizard® DNA Clean-Up System[b] (Cat.# A7280), an agarose gel purification allows size selection of the fragments to be ligated. Simply separate the fragments by agarose gel electrophoresis and isolate the proper fragments using the Wizard® PCR Preps DNA Purification System[c] (Cat.# A7170) or Agar*ACE*® Agarose-Digesting Enzyme (Cat.# M1741).
- Calf Intestinal Alkaline Phosphatase is a very efficient enyzme. It is not necessary to use more than 0.01u CIAP/pmol ends.
- Removal of the alkaline phosphatase by phenol:chloroform extraction or using the Wizard® DNA Clean-Up System is recommended.
- A formula for converting µg DNA to pmol can be found on pages 6 and 20.
- Resuspend the purified DNA in water or buffer without EDTA. EDTA will chelate the magnesium required by T4 DNA Ligase.

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Cloning Protocol

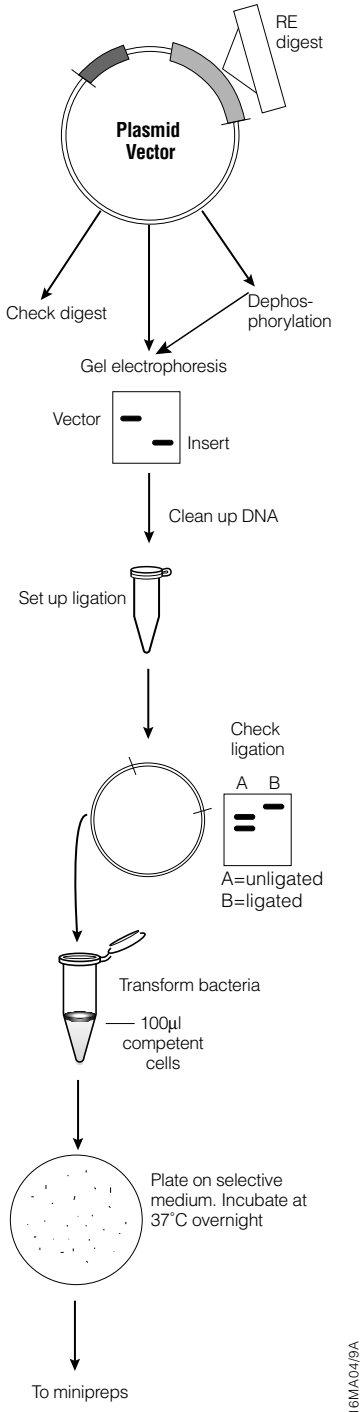

Figure 2. Cloning protocol.

This protocol provides essential information on the cloning of DNA fragments into plasmid or phage vectors. The protocol provides 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. The flow diagram and complementary written protocol and tips are designed to walk the user through a typical cloning procedure.

Digestion of Vector and Insert DNA

1. Vector (1µg/µl) 5µl
   Appropriate restriction enzyme 10X buffer 5µl
   Acetylated BSA (1µg/µl) 5µl
   Appropriate restriction enzyme(s) 4-5u/µg DNA 20-25u
   Deionized water to a final volume of 50µl

   Note: This reaction can be scaled up or down depending on the amount of DNA needed for ligation.
2. Incubate at the appropriate temperature for approximately 2 hours.
3. Run a small sample (5µl) on a 1% agarose gel to check for complete digestion. If the DNA fragment is to be blunt-ended or dephosphorylated, the restriction enzyme should be heat-inactivated or purified if possible after digestion. If desired, the DNA can be concentrated by ethanol precipitation.

Converting a 5′- or 3′-Overhang to a Blunt-End Terminus using T4 DNA Polymerase.

T4 DNA Polymerase is active in most restriction enzyme buffers and can be used immediately after the restriction enzyme digestion without prior purification of the DNA. If desired, a T4 DNA Polymerase buffer consisting of 33mM Tris-acetate (pH 7.9), 66mM potassium acetate, 10mM magnesium acetate, 0.5mM DTT may be used.

1. In a reaction volume of 10–100µl add 100µM each dNTP, 0.1mg/ml acetylated BSA and 5 units of T4 DNA Polymerase per microgram DNA.
2. Incubate at 37°C for 5 minutes.
3. Stop the reaction by heating to 75°C for 10 minutes or by adding EDTA to a final concentration of 25mM.
4. The blunt-ended DNA should be either ethanol-precipitated or gel-purified prior to dephosphorylation and ligation to remove unincorporated nucleotides.

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Vector Dephosphorylation Reaction

1. Dilute sufficient Calf Intestinal Alkaline Phosphatase (CIAP) for immediate use in CIAP 1X Reaction Buffer to a final concentration of 0.01u/µl. Each pmol of DNA ends will require 0.01u CIAP (added in two aliquots of 0.005u/pmol ends).

   Example: 1µg of a 1kb DNA fragment equals approximately 1.5pmol DNA or 3pmol ends.

2. Purify the DNA to be dephosphorylated by ethanol precipitation and resuspend the pellet in 40µl of 10mM Tris-HCl (pH 8.0) or water.

   Set up the following reaction:

DNA (up to 10pmol of 5′ ends)	40µl
CIAP 10X Reaction Buffer,	5µl
Diluted CIAP (0.01u/µl, add 0.005u/pmol ends)	up to 5µl
Deionized water to a final volume of	50µl

   Note: CIAP may be added directly to digested DNA. Add 5µl 10X CIAP reaction buffer, 0.005u CIAP/pmol ends and water to a final volume of 50µl.

3. For 5′-protruding ends, incubate at 37°C for 30 minutes. For 5′-recessed or blunt ends, incubate at 37°C for 15 minutes and then at 56°C for 15 minutes.

4. Add another aliquot of diluted CIAP (equivalent to the amount used in Step 2) and repeat incubation.

5. Add 300µl CIAP stop buffer (10mM Tris-HCl [pH 7.5], 1mM EDTA, 200mM NaCl, 0.5% SDS). Phenol/chloroform extract and ethanol precipitate by adding 0.5 volume 7.5M ammonium acetate (pH 5.5), and 2 volumes of 100% ethanol to the final aqueous phase. Alternatively, the Wizard® DNA Clean-Up System (Cat.# A7280) or gel purification may be used to purify the DNA prior to ligation.

Ligation of Vector and Insert DNA

1. Using a 1:1 insert:vector ratio (this example uses a 3.0kb vector and a 0.5kb insert), set up the following ligation reaction. For optimal ligation efficiency the insert:vector ratio may need to be optimized. Ratios 1:1, 1:3 or 3:1 of insert:vector are most common. Typical ligation reactions use 10–200ng of vector DNA.

Vector DNA (3.0kb)	100ng
Insert DNA (0.5kb)	17ng
T4 DNA Ligase 10X Buffer	1µl
T4 DNA Ligase (Weiss units)	1u
Deionized water to a final volume of	10µl

   Perform ligation reactions according to the following guidelines for incubation temperature and time:

4°C	overnight
or 15°C	4–6 hours
or 20–22°C	1–3 hours

2. Following ligation use the DNA to transform competent cells from an appropriate bacterial strain. Further information on bacterial transformation may be found in Promega's *Protocols and Applications Guide,* Third Edition (p. 45) and in Promega Technical Bulletin #TB095. See also pages 14 and 35–36 of this guide.

Further information on cloning in plasmid vectors can be found in Promega's *Protocols and Applications Guide,* Third Edition, Chapter 3, and in Promega Product Information Sheets #PIM180, #PIM182, #PIM421, and Technical Bulletin #TB095.

Cloning Tips (continued)

- Many strategies have been reported to increase the efficiency of blunt-end ligations. These include increasing the amount of ligase up to 50-fold, increasing the time of ligation, and the addition of PEG or hexamine chloride (1). Generally, if good quality DNA and reagents are used, standard conditions will suffice for blunt-end ligations.
- T4 DNA Ligase can be inactivated by incubation at 70°C for 10 minutes. This should be done if the ligation is to be further manipulated before bacterial transformation.
- ATP is required for ligation. ATP is labile; repeated freeze/thaws will destroy the ATP in the ligase buffer. To maintain the ATP concentration, store the 10X ligase buffer frozen in small aliquots.
- Generally, an insert:vector molar ratio of 1:1 will work for most ligations. However, the optimal insert:vector ratio may range from 1:3 to 3:1 or even higher. If a ligation is unsuccessful, try optimizing the this ratio.
- Confirm the concentration of insert and vector by gel electrophoresis or spectrophotometry before setting up ligations.
- Controls are essential for interpreting the results of a cloning experiment. Controls should be included with every ligation. A 'vector only' control will indicate the number of colonies due to vector religation. An 'insert only' control will indicate any background resulting from contamination of the insert with either intact plasmid or vector sequences from the source of insert.
- A ligation control can be performed by ligating a DNA size marker. The marker should be phosphorylated and have compatible ends. Compare the ligation reaction to unligated DNA on an agarose gel. A shift of the marker bands toward the top of the gel indicates successful ligation.
- There is considerable latitude in the temperature and time needed for a successful ligation. Blunt-end ligations benefit from lower temperatures (4–16°C) and longer incubation times (4 hours to overnight). Cohesive-end ligations are generally performed at higher temperatures (15–20°C) and with shorter incubation times (3–16 hours).
- Experienced users may find a 30-minute incubation at 20°C sufficient for ligation of cohesive ends.

Reference

1. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, **1,**1.70.

Notes