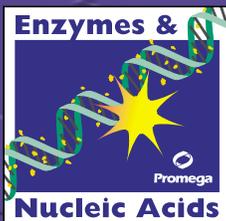


2

Enzyme Resource Guide

# Cloning Enzymes

from Promega





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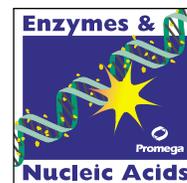
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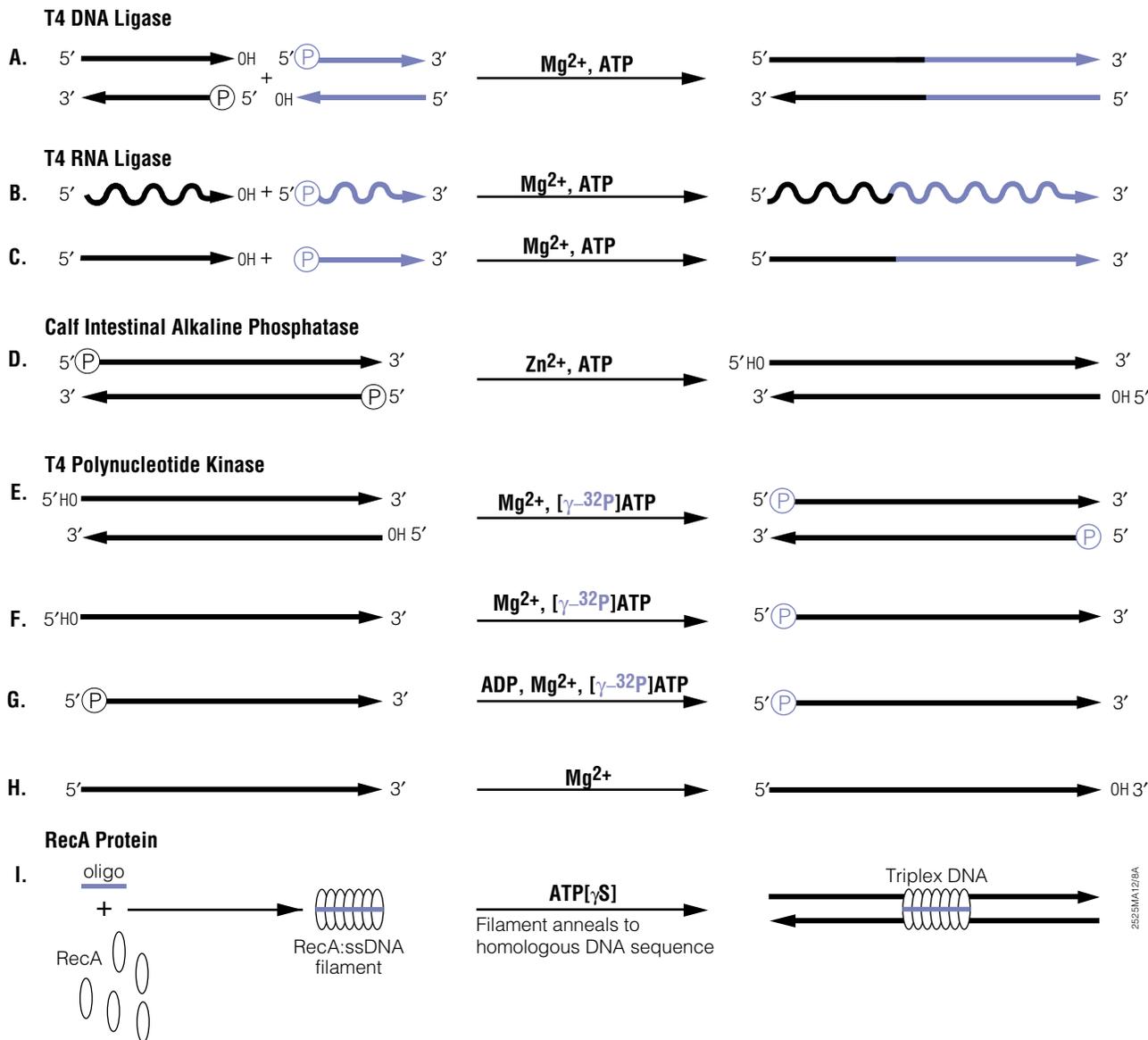
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**Figure 1. Activities of enzymes commonly used in cloning applications.** Straight lines indicate DNA molecules, wavy lines indicate RNA molecules and arrowheads indicate the 3'-end of a nucleic acid molecule. **Panel A:** T4 DNA Ligase catalyzes the joining of two DNA strands between the 5'-phosphate and 3'-hydroxyl groups. T4 DNA Ligase can catalyze the joining of RNA to either a DNA or RNA strand in a duplex molecule but will not join single-stranded nucleic acids. **Panels B and C:** T4 RNA Ligase catalyzes the joining of single-stranded nucleic acids, including RNA/DNA hybrids. T4 RNA Ligase catalyzes the joining of the 5'-phosphate of single-stranded RNA (donor) to the 3'-hydroxyl of single-stranded RNA (acceptor; Panel B). Single-stranded DNA may also serve as a donor but is a poor acceptor (Panel C). **Panel D:** Calf Intestinal Alkaline Phosphatase catalyzes the hydrolysis of 5'-phosphate groups from DNA, RNA and ribo- and deoxyribonucleoside triphosphates. **Panels E-H:** T4 Polynucleotide Kinase has several activities including catalysis of the transfer of a  $\gamma$ -phosphate from a nucleotide triphosphate to the 5'-hydroxyl terminus of single- and double-stranded mono- and polynucleotides (DNA and RNA) by the *forward reaction* (Panels E and F). If excess ADP is present in the reaction the enzyme can catalyze the exchange of phosphates between the  $\gamma$ -phosphate of a NTP and the 5'-phosphate terminus of a DNA molecule by the *exchange reaction* (Panel G). T4 Polynucleotide Kinase also possesses 3'-phosphatase activity (Panel H). **Panel I:** RecA Protein, isolated from *E. coli*, facilitates the pairing of homologous sequences. In the presence of a nonhydrolyzable ATP analog, RecA Protein binds to ssDNA to form a RecA:ssDNA filament. This RecA-coated oligonucleotide can anneal with homologous duplex DNA to form a stable DNA-protein triplex.



## 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  $\gamma$ -phosphate of a nucleotide triphosphate (Figure 1, Panels E-H).

RecA Protein and AgarACE® Enzyme<sup>(a)</sup> 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). AgarACE® 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	AgarACE® Enzyme
<b>Cloning (DNA)</b>						
<b>Ligation</b>						
Blunt-ended	✓					
Cohesive-ended	✓					
Single-stranded		✓				
Nicks	✓					
<b>Cloning (5'-RACE)</b>						
RNA Ligation		✓				
<b>Genomic Cloning Enrichment</b>					✓	
<b>Phosphorylation</b>						
5'			✓			
<b>Dephosphorylation</b>						
3'			✓			
5'				✓		
<b>Purification from Agarose Gel</b>						✓
<b>Labeling DNA and RNA</b>						
5'			✓			
3'-addition		✓				
<b>Sequencing</b>						
DNA			✓			
RNA			✓			
<b>Sequence-Specific Cleavage of DNA</b>					✓	



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## Cloning Protocol

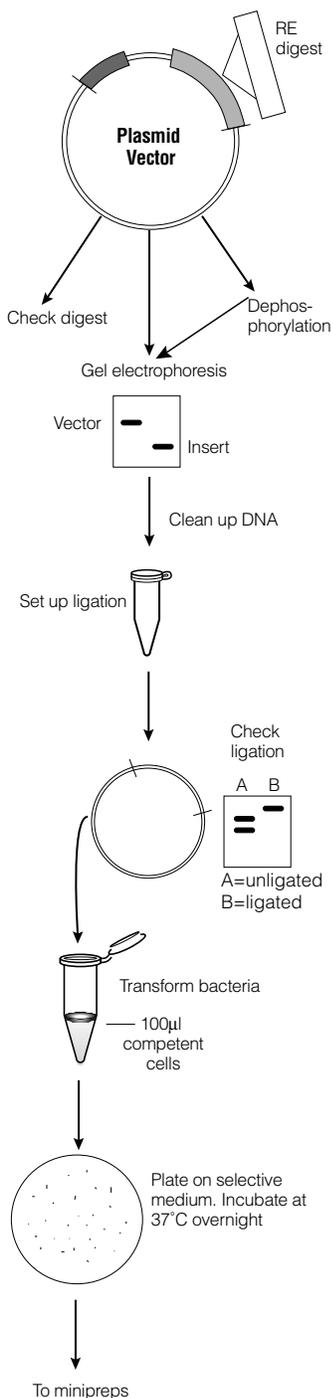


Figure 2. Cloning protocol.

### 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<sup>®</sup> (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<sup>®</sup> (Cat.# A7170) or AgarACE® Agarose-Digesting Enzyme (Cat.# M1741).
  - ◆ Calf Intestinal Alkaline Phosphatase is a very efficient enzyme. It is not necessary to use more than 0.01 u 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.
- (continued)

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.

(continued)

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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)	<u>up to 5μl</u>

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)	<u>1u</u>

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.



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## 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<sup>2+</sup> 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<sup>r</sup> and amp<sup>r</sup>) (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<sup>(d)</sup>, pBR322, pUC/M13, pSP series<sup>(d)</sup>, pET series<sup>(e)</sup>, 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<sup>(d,t)</sup> (Cat.# A3600 and A1360, respectively), possess a 3'-terminal thymidine, which can pair with the adenine overhang of the PCR product facilitating ligation.

### References

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5. *Protocols and Applications Guide*, Third Edition (1996) Promega Corporation.
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7. *Wizard® DNA Clean-Up System Technical Bulletin* #TB141, Promega Corporation.
8. *Wizard® PCR Preps DNA Purification System Technical Bulletin* #TB118, 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:

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

For example, 5 $\mu$ g 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<sup>®</sup> DNA Clean-Up System (Cat.# A7280) or AgarACE<sup>®</sup> 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<sup>®</sup> 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<sup>2+</sup> 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:

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

Generally, the recommended amount of total DNA in a ligation reaction varies from 1–10ng per microliter of reaction in a final volume of 10–20 $\mu$ l (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/ $\mu$ l 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<sub>2</sub>, 1–10mM DTT and 10 $\mu$ M–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<sub>m</sub>) (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).

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14. Wizard<sup>®</sup> Plus Maxipreps DNA Purification System<sup>(b)</sup> Technical Bulletin #TB139, Promega Corporation.
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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<sup>2+</sup> in the T4 DNA Ligase buffer. Moreover, it is important to isolate plasmid DNA from bacterial strains that are *endA*<sup>-</sup>. 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 *recA*<sup>-</sup>, 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 x 10<sup>7</sup>–1 x 10<sup>8</sup>cfu/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 x 10<sup>9</sup>cfu/µg of DNA, the transformation efficiency of recombinant clones is usually about 1 x 10<sup>5</sup>cfu/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 x 10<sup>6</sup>cfu/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 *lacZ* region of the plasmid, generally causing a white colony upon induction with IPTG and color detection with X-Gal (5,6).

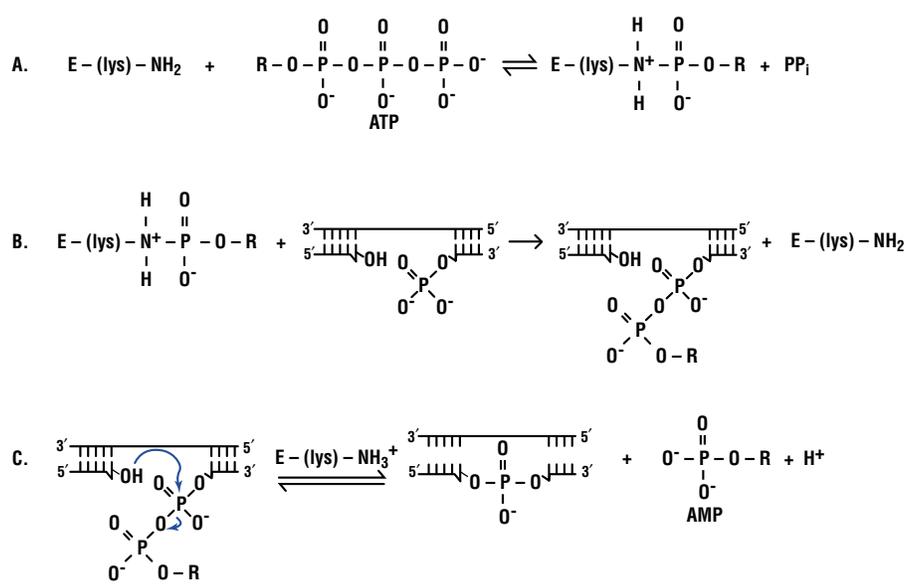
Candidate colonies containing recombinant plasmids are identified and single clones are then grown in liquid media containing antibiotics. Recombinant plasmids are verified by purifying plasmid DNA and digesting with selected restriction enzymes, by hybridization to a specific nucleic acid probe, by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

## Ligases

### Introduction

DNA Ligases are primarily responsible for joining the gaps that form in DNA during replication (i.e., the joining of "Okazaki" fragments formed by discontinuous or lagging strand replication; 1), DNA repair, and recombination. The best known RNA ligase is bacteriophage T4 RNA ligase. This enzyme does not appear to have any role in nucleic acid metabolism in bacteriophage T4 infected *E. coli*, but instead appears to be required for the attachment of the bacteriophage's tail fibers to its base plate during bacteriophage assembly (2). However, its activity as a ligase has been used effectively in various molecular biology applications.

Both DNA and RNA ligases catalyze the formation of a phosphodiester bond between adjacent nucleotides with the concomitant hydrolysis of ATP to AMP and inorganic pyrophosphate. Some DNA ligases (such as *E. coli* DNA ligase) use nicotinamide adenine dinucleotide (NAD) instead of ATP as a cofactor and release AMP and nicotinamide mononucleotide (NMN) as a result of phosphodiester bond formation. In general, DNA ligases will only form this covalent linkage in a duplex molecule, for example at a nick in double-stranded (dsDNA) or when joining cohesive- or blunt-ended dsDNAs (Figure 1, Panel A) (3). RNA ligase, on the other hand, has a preference for single-stranded RNA or DNA (Figure 1, Panels B and C) (4). The ligation mechanism is essentially identical for both DNA and RNA ligases, and occurs in three stages (Figure 3). First is the formation of an enzyme-nucleotide intermediate through transfer of an adenylyl group (AMP) from either ATP or NAD to the epsilon-amine group of a lysine residue in the enzyme. This results in the release of pyrophosphate when ATP is the cofactor and NMN when NAD is used. Second, the adenylyl group is transferred from the enzyme to the 5'-phosphate of the DNA (DNA ligases) or donor polynucleotide (RNA ligases), thereby activating it. Third, a phosphodiester bond is formed by nucleophilic attack of the 3'-hydroxyl group of the DNA (DNA ligases) or acceptor polynucleotide (RNA ligases) on the activated 5'-phosphate, with concomitant release of AMP.



**Figure 3. Ligation mechanism.** The three step reaction schematic for ATP-dependent DNA ligases is shown. Differences for NAD-dependent ligases and RNA ligases are noted in this legend. **Panel A:** Transfer of an adenylyl group from ATP (or NAD, such as in the case of *E. coli* DNA ligase) to the epsilon-amine group of a lysine residue in the enzyme with the concomitant release of pyrophosphate (or NMN when NAD is the adenylyl donor). **Panel B:** The adenylyl group is transferred from the enzyme to the 5'-phosphate of the DNA. In the case of RNA ligases, it is transferred to the donor polynucleotide. **Panel C:** Nucleophilic attack by the 3'-hydroxyl group on the activated 5'-phosphate group of the DNA (or acceptor polynucleotide in the case of RNA ligases) forms the phosphodiester bond, with simultaneous release of AMP. For Panels A-C, E = enzyme, lys = lysine residue.

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Bacteriophage T4 DNA ligase is the ligase most commonly used in the construction of recombinant DNA molecules for molecular biology applications. It is able to ligate DNA fragments having either complementary cohesive or blunt ends, and has an absolute requirement for ATP as a cofactor; it cannot use NAD. *E. coli* DNA ligase (which, like most prokaryotic DNA ligases uses NAD as a cofactor instead of ATP) can sometimes be used in place of T4 DNA ligase for ligation of single-strand breaks or joining of DNA molecules with cohesive termini. However, unlike T4 DNA ligase, the *E. coli* enzyme does not show blunt-ended ligation activity, except under conditions of molecular crowding with PEG 8000 (5). For this reason T4 DNA ligase has become more widely used in DNA manipulations (Table 1). For intermolecular ligations it is important that at least one of the DNA molecules possesses a 5'-phosphate at either end of the dsDNA in order to form a phosphodiester bond. T4 DNA ligase is also used to join adjacent single-stranded DNA (ssDNA) molecules that have been polymerized on a template from primers annealed at separate sites, such as during site-directed mutagenesis.

The various applications that use bacteriophage T4 RNA ligase are indicated in Table 1. This enzyme has been used for many years to label RNA molecules at their 3'-end with a radiolabeled nucleoside 3',5'-bisphosphate as a complementary approach to labeling the 5'-end using T4 polynucleotide kinase (6). More recently, this enzyme has proved useful in the cloning of full-length cDNAs by circular and 5'-rapid amplification of cDNA ends (RACE) (7,8). The former method involves circularization of first-strand cDNA followed by inverse PCR, whereas the latter involves ligation of an oligonucleotide linker onto the 3'-end of the first-strand cDNA synthesis product, followed by amplification with a primer complementary to this linker and a gene-specific primer.



## T4 DNA Ligase

E.C 6.5.1.1

### Description

*In vivo*, T4 DNA Ligase (Cat.# M1801, M1794) catalyzes the sealing of single-stranded nicks in double-stranded DNA molecules (1,2). It is commonly used for the joining of two strands of DNA between the 5'-phosphate and the 3'-hydroxyl groups of adjacent nucleotides in either a cohesive-ended or blunt-ended configuration (3; Figure 1, Panel A). The enzyme has also been shown to catalyze the joining of RNA to either a DNA or RNA strand in a duplex molecule, but will not join single-stranded nucleic acids (3).

### Applications

- Joining blunt-ended double-stranded DNA.
- Joining cohesive-ended double-stranded DNA.
- Sealing nicks on a DNA or RNA strand annealed to a DNA or RNA (4) complementary strand.

### T4 DNA Ligase is a component of the following Promega systems:

- Erase-a-Base® System Plus Vectors (Cat.# E5850)
- Erase-a-Base® System (Cat.# E5750)
- GeneEditor™ *in vitro* Site-Directed Mutagenesis System<sup>(g)</sup> (Cat.# Q9280)
- Altered Sites® II *in vitro* Mutagenesis Systems<sup>(h)</sup> (Cat.# Q6210, Q6491, Q6090, Q6501, Q6080, Q6511)
- Altered Sites® II Mammalian *in vitro* Mutagenesis Systems<sup>(h)</sup> (Cat.# Q5590, Q6600)
- pGEM®-T Vector Systems<sup>(d,f)</sup> (Cat.# A3600, A3610)
- pGEM®-T Easy Vector Systems<sup>(d,f)</sup> (Cat.# A1360, A1380)
- pTARGET™ Mammalian Expression Vector System<sup>(f,h)</sup> (Cat.# A1410)
- PinPoint™ Xa-1 T-Vector Systems<sup>(f,i)</sup> (Cat.# V2610, V2850)
- Universal RiboClone® cDNA Synthesis System<sup>(i)</sup> (Cat.# C4360)

### Enzyme Properties

**Requirements:** Mg<sup>2+</sup>, ATP, DTT (or other reducing agent such as β-mercaptoethanol) (1,2).

**Cofactor Concentration:** 10mM Mg<sup>2+</sup>, 10μM-1mM ATP (1), 10mM DTT (1).

**Optimal Substrate:** *In vivo*, single-stranded nicks in double-stranded DNA molecules (2). *In vitro*, two double-stranded DNAs containing either blunt ("flush") or cohesive ("sticky") ends (1,2,5). In all cases one DNA strand must have a 3'-OH and the other must have a 5'-phosphate.

**Typical Working Concentration:** 0.1–1.0 units T4 DNA Ligase per 10μl reaction for cohesive-end ligations or nick sealing reactions. Up to ten-fold more ligase may be required for blunt-ended ligations (1).

**Optimal pH:** The optimum pH range of T4 DNA Ligase has been reported to be 7.0–7.8 (2) or 7.2–7.6 (1).

**K<sub>m</sub>:** 5 × 10<sup>-5</sup>–5 × 10<sup>-8</sup>M for blunt-ended DNA (1,3,6), 6 × 10<sup>-7</sup>M for cohesive ends; 1.5 × 10<sup>-9</sup>M for nicks (2); 1.4 × 10<sup>-9</sup>M for ATP (1).

**Stimulators:** Polyethylene Glycol (PEG) 6000 or 8000 at 5% (w/v) (or other macromolecules such as Ficoll®, albumin, or glycogen) can stimulate ligation of blunt-ended DNAs over 1,000-fold, as judged by shifts in electrophoretic mobility. Cohesive-ended DNA ligation is stimulated by PEG to a far lesser extent. T4 RNA Ligase greatly stimulates blunt-end ligations (6).

Monovalent cations at low concentration (~20mM) can give slight stimulation (~30%), but at high concentrations they will inhibit ligation (1,7). Polyamines such as spermidine have been reported to both stimulate (1) and inhibit (7) ligation reactions at low (<1mM) concentrations. At higher concentrations polyamines inhibit ligation. HMG 14 has been shown to stimulate blunt-ended ligation by as much as 50% when present at a 50:1 molar ratio of HMG 14:DNA (1).

**Alternative Cofactors and Substrates:** Double-stranded DNA with blunt ends, single-stranded nicks in DNA/RNA hybrids or RNA/RNA hybrids. In all cases, one DNA or RNA strand must have a 3'-OH and the other must have a 5'-phosphate. Mn<sup>2+</sup> can substitute for Mg<sup>2+</sup> with reduced efficiency (1).

**Inhibitors:** dATP is a competitive inhibitor of ATP in ligation reactions (2). Monovalent cations inhibit ligations with almost no activity seen at greater than 200mM (1,7).



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There are conflicting reports concerning polyamines such as spermidine, which are reported to both stimulate (1) and inhibit (7) ligation reactions at low (<1mM) concentrations. At higher concentrations they inhibit ligation (1,7). Ethidium bromide inhibits ligations with an ID<sub>50</sub> of approximately 4.3μM (1). Anions are reported to inhibit blunt-end ligations at concentrations greater than 25mM for phosphates and greater than 50mM for salts in general (1). Hexaminecobalt chloride and Cibacron blue F3GA act as inhibitors (1). Excess ATP has been reported to inhibit blunt-ended ligations at high concentration (5mM) (5).

**K<sub>i</sub>:** 3.5 x 10<sup>-5</sup>M for dATP (2).

**Temperature Stability:** T4 DNA Ligase is inactivated at temperatures above 37°C.

**Inactivation:** Incubate at 70°C for 10 minutes or add EDTA to 25mM concentration.

**Genetic Locus:** Bacteriophage T4 gene 30 (1).

### Promega Product Information

**Source:** Purified from an *E. coli* strain expressing a recombinant clone.

**Molecular Weight:** 68kDa.

**Typical Working Conditions:** A typical ligation of cohesive-ended DNA contains 10–200ng of vector DNA and sufficient insert DNA to make a 1:1, 1:3 or 3:1 insert:vector molar ratio. The DNA is incubated in buffer containing 30mM Tris-HCl (pH 7.8), 10mM MgCl<sub>2</sub>, 10mM DTT and 1mM ATP in a final volume of 10μl. 0.1–1 unit of T4 DNA Ligase is added. For cohesive-end ligations, incubate at 20–25°C for approximately 3 hours or at 4–8°C overnight. For blunt-end ligations incubate at 15–20°C overnight.

**Storage Conditions:** Store at –20°C. T4 DNA Ligase is supplied in storage buffer containing 10mM Tris-HCl (pH 7.4), 50mM KCl, 1mM DTT, 0.1mM EDTA and 50% glycerol.

**Unit Definition:** 0.01 Weiss unit of T4 DNA Ligase is the amount of enzyme required to catalyze the ligation of greater than 95% of the *Hind* III fragments of 1μg of Lambda DNA at 16°C in 20 minutes. (One Weiss unit is the amount of enzyme that catalyzes the conversion of 1nmol of <sup>32</sup>PPI into a charcoal-adsorbable form in 20 minutes at 37°C in an ATP-PPi exchange form.)

**Purity:** The purity is ≥90% as judged by SDS-polyacrylamide gels with Coomassie® blue staining.

### Activity Assays

**Lambda Packaging Efficiency:** A control insert is ligated to 1μg of EMBL3 Vector Arms with 2.5 units of T4 DNA Ligase. The ligated DNA is packaged using Packagene® Extract (Cat.# K3151). The packaged phage is diluted 1:10,000 and used to infect bacterial strain LE392. After an overnight incubation at 37°C, the phage titer and the packaging efficiency are measured. The minimum packaging efficiency must be 5 x 10<sup>6</sup>pfu/μg DNA.

### Contaminant Assays

**Endonuclease Assay:** 1μg of pGEM®-5Zf(+) Vector is incubated with 5 units of T4 DNA Ligase in T4 DNA Ligase 1X Buffer (Tables 12,15) for 16 hours at 37°C. Following incubation the DNA is visualized on an ethidium bromide-stained agarose gel to verify the absence of visible nicking or cutting.

### Single-Stranded and Double-Stranded DNase Assay:

To test for DNase activity, 50ng of radiolabeled single-stranded or double-stranded DNA is incubated with 20 units of T4 DNA Ligase in 1X Buffer (Tables 12,15) for 16 hours at 37°C. Minimum passing specification is <2% release of single-stranded and <1% release of double-stranded radiolabeled nucleotides as monitored by scintillation counting of TCA-soluble material.

**RNase Assay:** To test for RNase activity, 50ng of radiolabeled RNA is incubated with 20 units of T4 DNA Ligase in 1X Buffer (Tables 12,15) for 5 hours at 37°C. Minimum passing specification is <3% release of radiolabeled nucleotides as monitored by scintillation counting of TCA-soluble material.

**Blue/White Cloning Assay:** This assay is performed to demonstrate that T4 DNA Ligase is free from contaminating activities, which can affect the efficiency and integrity of plasmid cloning. Any exonuclease or polymerase activity that alters the termini of linearized plasmids during ligation will result in a proportion of white-colored colonies above background levels.

A pGEM® series Vector is linearized with three different restriction enzymes in separate reactions to generate three different types of termini: *Eco*R I for 5'-overhangs, *Kpn* I for 3'-overhangs, and *Hinc* II for blunt ends. Linearized plasmids are purified, and ligations are performed using 12 units of T4 DNA Ligase in overnight incubations at 4°C. Competent JM109 cells are transformed with ligated plasmids and plated on X-Gal/IPTG/Amp plates. A minimum of 750 colonies is counted. White colonies result from transformation with ligated plasmids that have damaged ends. These white colonies represent the number of false positives expected in a typical cloning experiment. Enzymes that generate overhangs must produce fewer than 2% white colonies and blunt-cutting enzymes must produce less than 5% white colonies. The minimum transformation efficiency must be 1 x 10<sup>5</sup>cfu/μg DNA. Figure 4 includes a diagram of the blue/white color selection protocol.

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## T4 RNA Ligase

E.C. 6.5.1.3

### Description

T4 RNA Ligase (Cat.# M1051) catalyzes the ATP-dependent formation of either an intra- or intermolecular 3',5'-phosphodiester bond between a donor poly- or oligonucleotide containing a 5'-phosphate group and an acceptor poly- or oligonucleotide containing a 3'-hydroxyl group (1,2). The minimal length of a polyribonucleotide for circularization is 8 bases (3). For an intermolecular reaction the minimum size for an acceptor is a trinucleotide, whereas the donor can be as small as a nucleoside 3',5'-bisphosphate (4-6). This enzyme is used to circularize RNA molecules or join them to other RNAs. DNAs can also be used in intra- and intermolecular reactions, although with less efficiency than RNA (7-9).

T4 RNA Ligase is used to specifically label the 3'-end of RNA molecules with 5'-<sup>32</sup>P-radiolabeled nucleoside 3',5'-bisphosphate (e.g., [5'-<sup>32</sup>P]pCp) (10,11). The resulting 3'-end labeled RNAs can be used for enzymatic or chemical sequencing studies or for studies of ribonuclease activity and RNA/protein interactions (12,13). T4 RNA Ligase can also be used in 5'-RACE (Rapid Amplification of cDNA Ends) to ligate a specific oligodeoxyribonucleotide to the 3'-end of the first strand of cDNA synthesis. The oligodeoxyribonucleotide serves as a primer binding site for the upstream primer in 5'-RACE (14). Alternatively, the cDNA may be circularized in an intramolecular reaction for use in circular RACE (cRACE) (15).

T4 RNA Ligase has also been used for the site-specific incorporation of unnatural amino acids into protein (16,17). This involves using T4 RNA Ligase to ligate a CA dinucleotide modified with an unnatural amino acid onto the 3'-terminus of a 3'-CA deficient amber suppressor tRNA. Using this method, it is possible to introduce a variety of labels at specific sites in a protein (18,19).

Another useful application of T4 RNA Ligase involves the blunt-end ligation of double-stranded DNA. Although T4 RNA Ligase cannot by itself catalyze this reaction, it can stimulate the activity of T4 DNA Ligase in joining blunt ends by as much as 20-fold (20).

### Applications

- Labeling the 3'-end of RNA with cytidine 3',5'-bisphosphate (10,11).
- Cloning of full-length cDNAs/5'-RACE (14,15).
- Intra- and intermolecular ligation of single-stranded DNA, RNA and oligonucleotides (2,7-9,21).
- Incorporation of unnatural amino acids and labels into proteins (16-19).
- Stimulation of blunt-ended ligation efficiency (20).

### Enzyme Properties

**Requirements:** Both Mg<sup>2+</sup> and ATP are required for enzyme activity.

**Cofactor Concentration:** A concentration of 5-10mM Mg<sup>2+</sup> is optimal for activity. Concentrations above 10mM are inhibitory (9,22). Although a final ATP concentration of 1mM is used in the unit activity assay (circularization of 5'-[<sup>32</sup>P]-rA<sub>14-20</sub>) of T4 RNA Ligase, 100μM ATP is sufficient for ligation reactions (1,9,22).

**Optimal Substrate:** For intramolecular circularization reactions, the optimal substrate is a polynucleotide with a 5'-phosphate and a 3'-hydroxyl group. For intermolecular reactions, the donor should possess a 5'-phosphate and the acceptor a 3'-hydroxyl. To limit the reaction to a single ligation product, the acceptor should possess a hydroxyl group at both the 5'- and 3'-termini, whereas the donor should possess a 5'- and 3'-phosphate. For a single nucleotide donor, the reaction can be driven towards high yields of product by incubating with a molar excess of donor over acceptor (7,10). In contrast, the use of oligonucleotide donors requires an excess of acceptor to donor. A molar ratio of 5:1 is usually optimal (7,8).

**Typical Working Concentration:** 100 units per milliliter of reaction (10).

**Optimal pH:** pH 7.5-8.2 (Tris-HCl, 25°C) (1).

**K<sub>m</sub>:** For ATP, 12μM (22).

**Isoelectric Point:** pI = 6.1.

**Stimulators:** PEG 8000 increases the ligation efficiency of single-stranded DNA 30-fold at a concentration of 25%, whereas the addition of dimethyl sulfoxide (DMSO) to a final concentration of 10-20% increases the yield of RNA ligations 2- to 3-fold (8,11).

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**Alternative Cofactors and Substrates:** T4 RNA Ligase can utilize a wide variety of modified nucleoside 3',5'-bisphosphates as donors in ligation reactions. Examples include 5-bromodeoxyuridine, 2'-O-methylcytidine, and 1-methylguanosine (2).

**Inhibitors:** The nucleoside 2',5'-bisphosphate, 2',5'-ADP inhibits T4 RNA Ligase activity in the presence of magnesium (23).

**Inactivation:** 65°C for 15 minutes or 100°C for 2 minutes (1,9,22).

**Genetic Locus:** Bacteriophage T4 gene 63 (24).

### Promega Product Information

**Source:** Purified from an *E. coli* strain expressing a recombinant clone.

**Molecular Weight:** T4 RNA Ligase is a monomeric enzyme with a molecular weight of 43.5kDa (24).

**Typical Working Conditions:** For ligation of single-stranded nucleic acids to each other use the T4 RNA Ligase 10X Buffer supplied with the enzyme, diluted 1:10 (50mM Tris (pH 7.8), 10mM MgCl<sub>2</sub>, 5mM DTT and 1mM ATP) and BSA at a final concentration of 10µg/ml. Incubate at 17–25°C for 10–16 hours (8,14,15,21). Labeling the 3'-end of RNA molecules with cytidine 3',5'-[5'-<sup>32</sup>P] bisphosphate can be carried out in the same buffer, but reactions are incubated at 5°C for 6–24 hours and DMSO is added to 10% final concentration (10,11).

**Storage Conditions:** Store at –20°C. T4 RNA Ligase is supplied in storage buffer containing 10mM Tris (pH 7.5), 50mM KCl, 0.1mM EDTA, 1mM DTT, 50% glycerol and 0.1% Tween® 20.

**Unit Definition:** One unit is defined as the amount of enzyme required to catalyze the formation of 1 nanomole of 5'-[<sup>32</sup>P]-rA<sub>14-20</sub> into a phosphatase-resistant form in 30 minutes at 37°C at a 5'-terminal concentration of 10µM. The reaction conditions are specified below under Functional Assay.

**Purity:** T4 RNA Ligase is determined to be >90% homogeneous, as judged by SDS-polyacrylamide gels with Coomassie® blue staining.

### Activity Assay

**Functional Assay:** The RNA substrate (5'-[<sup>32</sup>P]-rA<sub>14-20</sub>, 10µM of 5'-termini) is ligated in the presence of T4 RNA Ligase 1X Buffer (Table 15) and T4 RNA Ligase for 15 minutes at 37°C. After ligation, the reaction is terminated by heating at 100°C for 2 minutes. The ligated substrate is then treated with 10 units of Calf Intestinal Alkaline Phosphatase (Cat.# M1821) for 10 minutes at 37°C. The amount of phosphatase-resistant substrate is monitored by scintillation counting of the TCA-precipitable material.

### Contaminant Assays

**DNase Assay:** To test for the absence of DNase activity, 50ng of radiolabeled DNA is incubated with 20 units of T4 RNA Ligase in T4 RNA Ligase 1X Buffer (Tables 12, 15) for 3 hours at 37°C. The minimum passing specification is <1% release of radiolabeled nucleotides as monitored by scintillation counting of TCA-soluble material.

**RNase Assay:** To test for the absence of RNase activity, 50ng of radiolabeled RNA is incubated with 20 units of T4 RNA Ligase in T4 RNA Ligase 1X Buffer (Tables 12, 15) for 3 hours at 37°C. The minimum passing specification is <1% release of radiolabeled nucleotides as monitored by scintillation counting of TCA-soluble material.

**Endonuclease Assay:** To test for endonuclease activity, 1µg of lambda or pGEM<sup>®(d)</sup> DNA is incubated with 20 units of T4 RNA Ligase in T4 RNA Ligase 1X Buffer (Tables 12, 15) for 3 hours at 37°C. Following incubation, the DNA is visualized on an ethidium bromide-stained agarose gel to verify the absence of visible nicking or cutting.

### References (continued)

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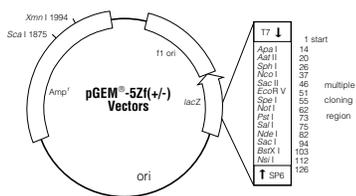


## Blue/White Color Selection

### Blue/White Color Selection Tips

Cells used for blue/white color selection have a deletion of the *lacZ* gene ( $\Delta lac$  in genotype). This mutation results in the production of nonfunctional  $\beta$ -galactosidase. However, if the deleted portion of  $\beta$ -galactosidase is supplied by a *lacZ*-containing plasmid,  $\beta$ -galactosidase activity can be restored. This process is known as  $\alpha$ -complementation. Cells that contain an intact *lacZ* gene (e.g., HB101) cannot be used for blue/white color selection.

- ◆ To determine the transformation efficiency of your cells, see Promega's *Protocols and Applications Guide*, Third Edition, page 46, or Technical Bulletin #TB095.
- ◆ LB medium can be used in place of SOC medium if desired. In our experience the use of SOC medium results in maximum transformation efficiencies.
- ◆ Perform a mock transformation of competent cells to which no DNA is added. No colonies should result. Presence of colonies may be the result of inactive antibiotic or contaminated cells.
- ◆ Light blue colonies? These colonies may contain inserts. The causes and cures for light blue colonies are discussed in *Promega Notes* 41.
- ◆ It is possible for an insert to ligate into a vector in-frame and not disrupt the *lacZ* gene. This is more likely to happen with short inserts. In such a case the dephosphorylated vector-only control will contain few colonies but the insert-plus-vector plate will contain many blue colonies.
- ◆ An alternative to preparing plates containing X-Gal and IPTG is to spread 20 $\mu$ l of 50mg/ml X-Gal and 100 $\mu$ l of 0.1M IPTG onto previously prepared LB/antibiotic plates. Allow these components to absorb for at least 30 minutes (or until the plate surface appears dry) at 37°C prior to plating cells.
- ◆ When cloning PCR products, the desired PCR product should be purified away from nonspecific products, especially primer-dimers. Primer-dimers ligate efficiently and will generate many white colonies that appear to contain no insert.
- ◆ Only cells that overexpress the *lac* repressor (*lacI<sup>s</sup>* in genotype) need to be plated on IPTG-containing plates. IPTG inactivates the *lac* repressor allowing the *lac* promoter to function. Overexpression of the *lac* repressor is desirable because it allows control of expression of plasmids containing *lac* or *tac* promoters.
- ◆ In JM109 cells, the *lacZ* gene harboring a deletion of the  $\alpha$ -peptide, which is required for blue/white color selection, is located on the F' episome. JM109 cells used to prepare competent cells should be maintained on M9 minimal medium to prevent loss of the F' episome. White colonies that do not contain inserts are generally the result of loss of the F' episome.



**Standard Cloning Experiment**  
 1. Linearize plasmid and purify  
 2. Ligate with insert

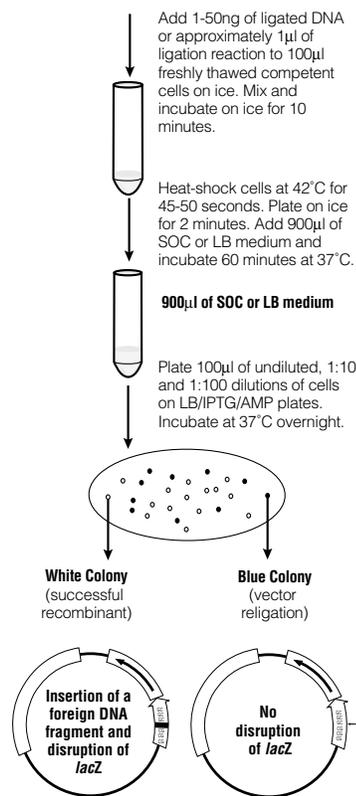


Figure 4. Blue/white color selection protocol.

2617NA04/9A

1. Pre-chill sterile 17 x 100mm polypropylene culture tubes (e.g., Falcon 2059) on ice, one per transformation.
  2. Remove frozen competent cells from -70°C and place on ice for 5 minutes or until just thawed. Competent cells will quickly lose their competency if warmed above 4°C.
  3. Gently mix the cells by flicking the tube and transfer 100 $\mu$ l of the thawed competent cells to each of the pre-chilled culture tubes.
  4. Add 1–50ng of ligated DNA (in a volume not greater than 10 $\mu$ l) to competent cells. Transformation efficiency will decrease with greater amounts of DNA. Move the pipette tip through the cells while dispensing. Quickly flick the tube several times. Do not pipet or vortex to mix.
  5. Immediately return the tubes to ice for 10 minutes.
  6. Heat-shock the cells for 45–50 seconds in a water bath at exactly 42°C. DO NOT SHAKE.
  7. Immediately place the tubes on ice for 2 minutes.
  8. Add 900 $\mu$ l of cold (4°C) SOC or LB medium to each transformation reaction then incubate for 60 minutes at 37°C with shaking (approximately 225rpm).
  9. For each transformation reaction, we recommend diluting the cells 1:10 and 1:100 in medium, and plating 100 $\mu$ l of the undiluted, 1:10 and 1:100 dilutions on antibiotic plates. The 1:100 dilution may be omitted for transformations from ligation reactions. Plate the transformed cells on LB plates containing 100 $\mu$ g/ml ampicillin, 0.1mM IPTG and 40 $\mu$ g/ml X-Gal.
- Most plasmids used for routine subcloning contain the gene for  $\beta$ -lactamase (*Amp<sup>r</sup>*) and should be selected for using liquid and solid medium containing 100 $\mu$ g/ml ampicillin. Other common selectable markers include tetracycline (use at 12.5 $\mu$ g/ml on plates, 10 $\mu$ g/ml in liquid culture), chloramphenicol (use at 20 $\mu$ g/ml) and kanamycin (use at 30 $\mu$ g/ml). Antibiotics are heat sensitive; do not add to culture medium above 55°C.
10. Incubate the plates at 37°C for 12–14 hours (overnight).
  11. Colonies that contain recombinant plasmid (disruption of *lacZ*  $\alpha$ -peptide) will appear white while colonies that contain nonrecombinant plasmid (intact *lacZ*  $\alpha$ -peptide) will appear blue.

Further information on bacterial transformation and blue/white color selection can be found in Promega's *Protocols and Applications Guide*, Third Edition (pp. 46, 51, 383), and in Promega Technical Bulletin #TB095.



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## Kinases and Phosphatases

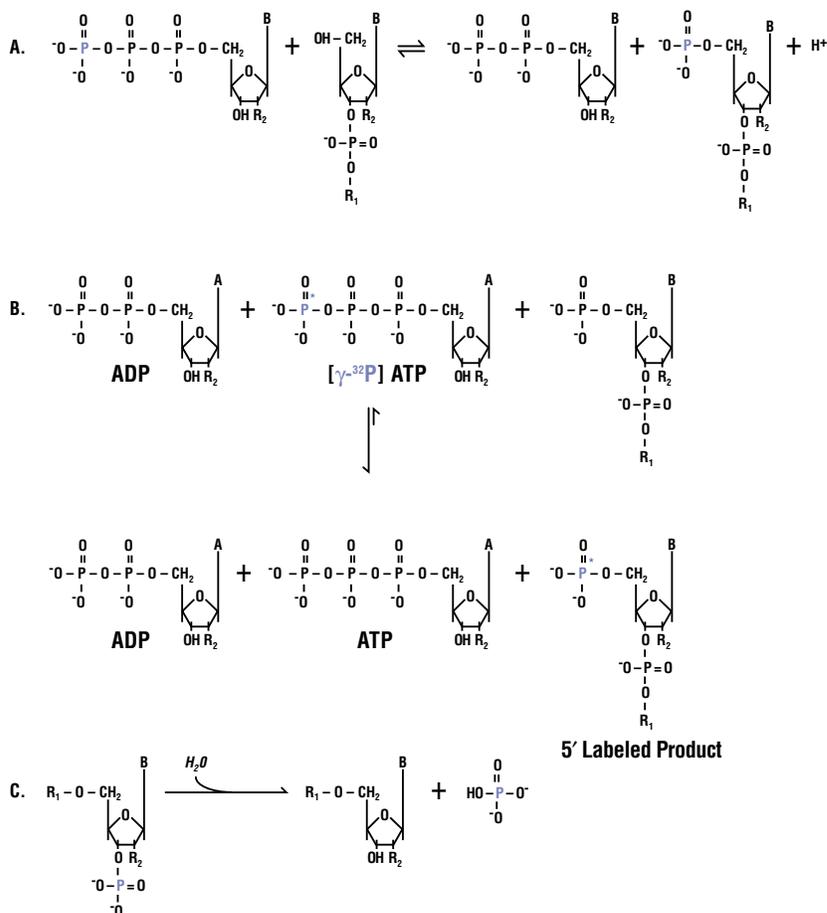
### Introduction

Kinases and phosphatases are common reagents in modern day molecular biology laboratories. Although there are a variety of sources for these enzymes, the most common are calf intestinal alkaline phosphatase (CIAP) and T4 polynucleotide kinase (T4 PNK). Their most frequent use is to modify the phosphorylation state of the 5'-ends of DNA molecules (Figure 1, Panels D-H).

CIAP is most commonly used to remove 5'-phosphates from vector DNA to prevent self-ligation during cloning. Only one strand of a DNA duplex must be joined prior to bacterial transformation; the other will remain nicked until it is repaired inside the bacteria. While the vector DNA is dephosphorylated, the insert DNA should not be dephosphorylated as 5'-phosphates are required for a successful ligation reaction. CIAP is also used to end-label DNA fragments by removing 5'-phosphates, making the DNA fragments better T4 PNK substrates.

Synthetic DNA, usually in the form of custom-made oligonucleotides, is devoid of 5'-phosphates and is therefore a less than ideal template for ligation reactions. T4 PNK is routinely used to transfer a  $\gamma$ -phosphate from a nucleotide triphosphate (usually ATP) to the 5'-end of oligonucleotides to facilitate ligation (Figure 5, Panel A). For blotting, gel-shift or sequencing procedures, [ $\gamma$ - $^{32}\text{P}$ ]ATP is used as the phosphate donor, resulting in a radiolabeled species. The 5'-end of a DNA molecule generated by restriction endonuclease cleavage can also be labeled, even though a phosphate already exists at that position. This can be achieved either by making use of the exchange activity of T4 PNK to exchange the existing phosphate with a radiolabeled phosphate from the phosphate donor (Figure 5, Panel B), or by first treating the DNA with CIAP to remove the existing phosphates, then adding the radiolabeled phosphate with PNK via the forward reaction, which will result in a high specific activity (Figure 5, Panel A). Finally, T4 PNK has a 3'-phosphatase activity that can be used to remove phosphate groups from the 3'-terminus of DNA and RNA (Figure 5, Panel C).

Although both enzymes are most commonly used for cloning purposes, they have other activities and are also used for other types of studies. These other activities will be listed in further detail in the following section. The robustness and versatility of CIAP and T4 PNK have made them staples in today's molecular biology applications.



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**Figure 5. Multiple activities associated with T4 polynucleotide kinase. Panel A:** The transfer of a phosphate from a nucleoside 5'-triphosphate to the 5'-OH group of an acceptor molecule by T4 polynucleotide kinase, via the forward reaction.  $\text{R}_1 = \text{H}$ , a nucleoside, a nucleotide or a polynucleotide;  $\text{R}_2 = \text{H}$  or  $\text{OH}$ . **Panel B:** With excess ADP an exchange reaction can occur.  $\text{A} = \text{adenine}$ ;  $\text{R}_1 = \text{H}$ , a nucleoside, a nucleotide or a polynucleotide;  $\text{R}_2 = \text{H}$  or  $\text{OH}$ ;  $\ast = ^{32}\text{P}$ . **Panel C:** The hydrolysis of a 3'-phosphoryl group by the 3'-phosphatase activity of T4 polynucleotide kinase.  $\text{R}_1 = \text{H}$ ,  $\text{PO}_4^{2-}$ , a nucleotide or a polynucleotide. For Panels A–C,  $\text{B} = \text{adenine, guanine, cytosine, thymidine or uracil}$ .



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## T4 Polynucleotide Kinase

E.C. 2.7.1.78

### Description

T4 Polynucleotide Kinase (polynucleotide 5'-hydroxyl-kinase or ATP:5'-dephospho-polynucleotide 5' phosphatase)

T4 Polynucleotide Kinase (T4 PNK; Cat.# M4101, M4103) is a tetramer composed of identical subunits and has multiple activities. The 5'-kinase activity of T4 PNK catalyzes the transfer of the  $\gamma$ -phosphate from NTP to the 5'-OH terminus of mono- or polynucleotides (Figure 5, Panel A;1). The reaction is reversible and in the presence of a nucleotide diphosphate such as ADP the enzyme has 5'-phosphatase activity (2). Dephosphorylation and subsequent rephosphorylation allow the enzyme to transfer phosphates between ATP and a 5'-phosphate group on an acceptor molecule in an exchange reaction (Figure 5, Panel B; 3). T4 PNK also has 3'-phosphatase activity (Figure 5, Panel C;4). For a review of T4 PNK see references 3, 5 and 6.

T4 PNK can be used to phosphorylate RNA, DNA and synthetic oligonucleotides prior to subsequent manipulations such as ligation. Radioactive phosphate can be used as a label for DNA sequencing (7,8), gel shift analysis (9), footprinting (10), primer extension (11), and restriction mapping (12,13). Labeling the 5'-ends of DNA and RNA may be done using a dephosphorylated template (5'-OH) using the forward or 5'-kinase reaction (Figure 5, Panel A). Alternatively, labeling of 5'-ends can be achieved without removal of the existing 5'-phosphate using the exchange reaction (Figure 5, Panel B; 2, 14, 15). The reaction conditions for the forward and exchange reactions are not the same. The forward reaction generally results in better incorporation. T4 PNK can also be used to remove 3'-phosphates from DNA and RNA (Figure 5, Panel C; 4).

### Applications

- 5' end-labeling of ss- and dsDNA and RNA (1,14,16).
- Phosphorylation of insert DNA prior to ligation.
- Phosphorylation of oligonucleotides.
- Removal of 3'-phosphates (4).

### T4 Polynucleotide Kinase is a component of the following Promega systems:

- *fmoI*<sup>®</sup> DNA Cycle Sequencing Systems<sup>(k)</sup> (Cat.# Q4100, Q4110)
- OmniBase<sup>®</sup> DNA Cycle Sequencing Systems<sup>(k)</sup> (Cat.# Q6800, Q4550)
- TaqTrack<sup>®</sup> Sequencing Systems (Cat.# Q5530)
- DNA 5' End-Labeling System (Cat.# U2010)
- Universal RiboClone<sup>®</sup> cDNA Synthesis System (Cat.# C4360)
- Gel Shift Assay Systems (Cat.# E3050, E3300)

<sup>k</sup> OmniBase<sup>®</sup> products are available in selected countries. Please inquire for additional information.

- Primer Extension System - AMV Reverse Transcriptase (Cat.# E3030)
- Core Footprinting System (Cat.# E3730)
- 5' End-Labeling Application Pack (Cat.# M7700)

### Enzyme Properties

**Requirements:** 5'-kinase (forward), exchange and 3'-phosphatase reactions require  $Mg^{2+}$  (1,2,17). A sulfhydryl compound such as DTT is essential for activity (2).

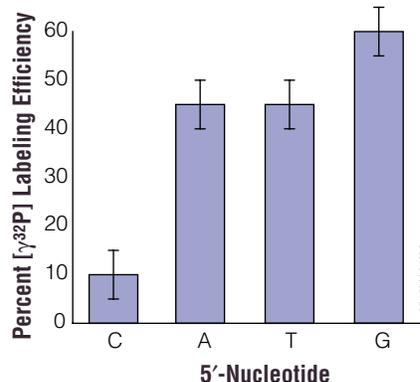
**Cofactor Concentration:** For the 5'-kinase reaction, 10mM  $Mg^{2+}$  optimal at pH 7.6 (1). For the 3' phosphatase reaction, 8mM  $Mg^{2+}$  optimal (17).

### Optimal Substrate

**5'-Kinase Activity:** ss- and dsDNA, ss- and dsRNA, synthetic oligonucleotides and nucleoside 3'-monophosphates (Figure 5, Panel A; 3). 5'-OH groups on ssDNA overhangs in dsDNA are phosphorylated more efficiently than 5'-OH groups on blunt or 5'-recessed ends (3). With increased concentration of ATP or enzyme, blunt- and 5'-recessed ends can be completely phosphorylated (3,5). Phosphorylation of 5'-OH groups located at nicks in dsDNA is 10–30X slower than for ssDNA (14,18). The reaction does not differ significantly for substrates 0.15–50kb in length (5). T4 PNK can also phosphorylate a variety of modified nucleotides and non-nucleotide substrates (6). Nucleosides (adenosine), nucleoside 2'-phosphates, 3'-termini, or 5'-termini bearing phosphomonoesters are not substrates (19).

**Exchange Reaction:** ss- and dsDNA, (6; Figure 5, Panel B). 5'-overhangs of dsDNA and single-stranded oligonucleotides are more efficient substrates than 5'-recessed or blunt ends (20). 5'-recessed and blunt ends are labeled 15–25% as efficiently as 5'-overhangs (20). 5'-phosphate groups at nicks are the most difficult to exchange (30X less efficient than 5'-overhangs [14]). Some tRNA species can act as substrates for phosphate exchange (6).

**5'-Phosphatase Activity:** DNA, RNA, oligonucleotides, 3',5'-bisphosphates (6).



**Figure 6. T4 polynucleotide kinase labeling efficiency.** Labeling of oligonucleotides by T4 polynucleotide kinase is dependent, in part, on the identity of the 5'-nucleotide, with 5'-cytidine having the lowest labeling efficiency (33).

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**3'-Phosphatase Activity:** Deoxynucleotide 3'-monophosphates, ss- and dsDNA (17; Figure 5, Panel C). Extracts of T4-infected *E. coli* have no phosphatase activity on 3'-phosphoryl groups of RNA and ribomononucleotides (17). However, the purified enzyme is reported to have weak 3'-phosphatase activity on RNA (4).

**Phosphate Donor:** ATP, CTP, UTP, GTP, dATP, and dTTP function equally well (3).

**Typical Working Concentration:** 10–20 units of T4 PNK are typically used in a 50µl reaction volume for 1–50pmol of 5' DNA ends, for both the forward and exchange reactions.

**Optimal pH**

**5'-Kinase Activity:** pH 7.4–8.0 in Tris-HCl buffer for DNA substrates (1). Maximal activity at pH 7.6 (19). For ss-oligonucleotide substrates an optimal pH of 9.5 in glycine-NaOH buffer has been reported (2).

**Exchange Reaction:** pH 6.2–6.6 in imidazole-HCl buffer (14). At pH 7.6 the exchange reaction is 20% as efficient as at pH 6.2 (6).

**5'-Phosphatase Activity:** pH 6.2–6.5 in imidazole-HCl buffer (2,14).

**3'-Phosphatase Activity:** pH 5.6–6.4 depending on buffer (5,18), pH 6.0 in imidazole-HCl buffer optimal (5).

**K<sub>m</sub>**

**5'-Kinase activity:** 4–260µM for ATP (pH 8.0 at 37°C) depending on DNA substrate (14, 23). The K<sub>m</sub> for DNA ranges from 7.6µM for large DNA fragments and between 1.8–143.0µM for nucleoside 3'-monophosphates and oligonucleotides depending on the 5' base and the length of the oligonucleotide (23). For the forward reaction the K<sub>m</sub> for ATP is 4µM. For the reverse reaction the K<sub>m</sub> for ADP is 200µM (14).

**5'-Phosphatase Activity:** ADP, 200µM for dsDNA 5'-overhangs at pH 7.4 (14).

**Stimulators**

**5'-Kinase Activity:** Spermidine (1.7mM optimal) can increase the rate of the reaction three-fold (24). Spermidine promotes tetramer formation (25). Salts such as NaCl, KCl and CsCl (125mM optimal) can increase activity up to 5X (24). LiCl and NH<sub>4</sub>Cl give similar stimulation (24). This effect is the same for 5'-overhangs on dsDNA and ssDNA, oligo and mononucleotides (24). Conversely, KCl decreases phosphorylation of 5'-recessed ends and at nicks (18). A sulfhydryl compound such as DTT (5mM DTT optimal) is essential for activity (19). 10mM 2-mercaptoethanol and 10mM glutathione result in 80% and 70%, respectively, of the activity observed with DTT (19). In the absence of a sulfhydryl compound only 2% of the optimal activity is observed. Several anions (at 125µM) are stimulatory, with Cl<sup>-</sup>, Br<sup>-</sup> > F<sup>-</sup>, NO<sub>3</sub><sup>-</sup>, SO<sub>4</sub><sup>2-</sup> (24). PEG 8000 at 4–6% improves the efficiency of labeling 5'- and 3'-overhangs, blunt ends and at nicks (26).

**Exchange Reaction:** In the presence of ADP, the phosphorylation reaction can be reversed and T4 PNK can remove a 5'-phosphate to yield a 5'-OH terminated molecule and ATP (2,3). PEG 8000 improves the efficiency of the exchange reaction (26). Spermidine has no significant effect on the exchange reaction (6).

**3'-Phosphatase Activity:** 2-mercaptoethanol, glutathione and DTT maintain activity during long (≥10 minutes) reactions (17). No effect seen for reactions <10 minutes. 0.3M NaCl promotes the 3'-phosphatase activity over the 5'-phosphatase activity (27).

**Alternative Cofactors and Substrates**

**5'-Kinase Activity:** Mn<sup>2+</sup> can partially fulfill the requirement for the divalent cation. 3.3mM is optimal and results in 50% the maximal activity with Mg<sup>2+</sup> (19).

**3'-Phosphatase Activity:** Co<sup>2+</sup> ions can replace Mg<sup>2+</sup> ions. At 0.8mM, the activity is 140% the optimal activity with Mg<sup>2+</sup>. At 0.08mM, 70% activity is observed (17).

**Inhibitors**

**5'-Kinase Activity:** Phosphate (P<sub>i</sub>) and pyrophosphate (PP<sub>i</sub>) anions are inhibitors of T4 PNK (24,28). Ammonium ions are strong inhibitors of T4 PNK (22). DNA should not be dissolved in or precipitated from buffers containing ammonium salts prior to treatment with kinase. Sulfate containing polymers (e.g., agar, dextran sulfate and heparin) are inhibitors of T4 PNK (29). However, addition of cationic compounds such as spermine or polylysine can counteract the inhibition (6). Nonsulfate polysaccharides have no effect on T4 PNK.

**Exchange Reaction:** Phosphate ions; 50mM potassium phosphate results in 60% inhibition (2).

**K<sub>i</sub>:** 29mM (P<sub>i</sub>), 2mM (PP<sub>i</sub>) (24).

**Temperature Stability:** T4 PNK is active over a range of temperatures. Different temperature optima have been reported; 37°C (5), 30–35°C (23). At 0°C the rate of the 5'-kinase reaction is reduced to 7% the rate at 37°C. At 0°C the exchange reaction is reduced to 1.2% the rate at 37°C (25).

**Inactivation:** 68–70°C for 10 minutes (11,30); 78°C for 1 minute (6). Reactions can also be terminated by addition of 20mM EDTA.

**Genetic Locus:** Bacteriophage T4 *pseT*.

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### Promega Product Information

**Source:** Purified from an *E. coli* strain expressing a recombinant clone.

**Molecular Weight:** T4 PNK is a tetramer composed of identical subunits. The relative mobility of the monomers as measured by SDS-PAGE is 33kDa (31), by centrifugation; 33.2kDa (25). From the sequence of T4 PNK, the monomer consists of 301 amino acids with a predicted molecular weight of 34kDa (32). The molecular weight of the tetramer as estimated by gel filtration is 140kDa (32), by centrifugation, 147.3kDa (25). Monomers and dimers are not enzymatically active (6).

**Typical Working Conditions:** ATP at  $\geq 2$ -5 fold molar excess over DNA ends results in  $\geq 95\%$  phosphorylation of 5'-overhangs (6). Increasing concentration of ATP to 100-fold molar excess will allow complete labeling of blunt ends or recessed 5'-ends (6,18). Protocols for using T4 PNK are available from Promega (21,22).

**Storage Conditions:** Store at  $-20^{\circ}\text{C}$ . T4 Polynucleotide Kinase is supplied in 20mM Tris-HCl (pH 7.5), 25mM KCl, 2mM DTT, 0.1mM EDTA, 0.1 $\mu\text{M}$  ATP and 50% (v/v) glycerol.

**Unit Definition:** One unit is defined as the amount of T4 PNK required to catalyze the transfer of 1 nanomole of phosphate to the 5'-OH end of a polynucleotide from [ $\gamma$ - $^{32}\text{P}$ ] ATP in 30 minutes at  $37^{\circ}\text{C}$ . The reaction conditions are: 40mM Tris-HCl (pH 7.5), 10mM  $\text{MgCl}_2$ , 5mM DTT, 0.1mM [ $\gamma$ - $^{32}\text{P}$ ]ATP, 0.5mM 5'-OH polynucleotide end concentration.

**Purity:** The purity is  $>90\%$  as judged by SDS-polyacrylamide gels with Coomassie® blue staining.

### Activity Assays

**End Labeling:** To test for activity, 5pmol of dephosphorylated primer is incubated with 8 units of T4 PNK for 1 hour at  $37^{\circ}\text{C}$  in 1X Kinase Buffer (Tables 12, 15) containing [ $\gamma$ - $^{32}\text{P}$ ]ATP. Following incubation, the amount of [ $\gamma$ - $^{32}\text{P}$ ]ATP converted to TCA-insoluble material is determined. The minimal passing specification is  $>20\%$  incorporation.

**Sequencing Assay:** This enzyme has been tested and qualified for performance in the TaqTrack® Sequencing Systems.

### Contaminant Assays

**Endonuclease Assay:** To test for endonuclease activity, 1 $\mu\text{g}$  of supercoiled plasmid DNA is incubated with 25 units of T4 PNK for 5 hours at  $37^{\circ}\text{C}$  in 1X Kinase Buffer (Tables 12, 15). Following incubation, the supercoiled DNA is visualized on an ethidium bromide-stained agarose gel to verify the absence of visible nicking or cutting.

**DNase and RNase Assay:** To test for nuclease activity, 50ng of radiolabeled DNA or radiolabeled RNA is incubated with 25 units of T4 Polynucleotide Kinase in 1X Kinase Buffer (Tables 12, 15) for 3 hours at  $37^{\circ}\text{C}$ , and the release of radiolabeled nucleotides is monitored by scintillation counting of TCA-soluble material. Minimum passing specification is  $<3\%$  release for both DNase and RNase.





## Calf Intestinal Alkaline Phosphatase

E.C. 3.1.3.1

### Description

Calf Intestinal Alkaline Phosphatase (CIAP; Cat.# M1821 and M2825) is a phosphomonoesterase that catalyzes the hydrolysis of 5'- and 3'-phosphates of DNA, RNA and nucleotides. CIAP is primarily used to dephosphorylate vector DNA prior to cloning and for removal of 5'-phosphates from RNA and DNA before labeling with [ $\gamma$ - $^{32}$ P]NTP and T4 polynucleotide kinase. CIAP has also been widely utilized to dephosphorylate proteins. In contrast to acid phosphatases, CIAP can be utilized for *in vitro* dephosphorylation of proteins under conditions that do not denature the substrate protein (1). CIAP effectively dephosphorylates proteins containing phosphoserine, phosphothreonine and phosphotyrosine, although it can show preferential dephosphorylation of phosphotyrosine under certain conditions (2).

### Applications

- Dephosphorylation of 5'-phosphorylated ends of cloning vectors.
- Dephosphorylation of 5'-phosphorylated ends of DNA or RNA for subsequent labeling with  $^{32}$ P using [ $\gamma$ - $^{32}$ P]NTP and T4 Polynucleotide Kinase.
- Dephosphorylation of proteins.

**CIAP is a component of the following Promega systems:**

- Core Footprinting System (Cat.# E3730)
- DNA 5' End-Labeling System (Cat.# U2010, M7700)
- Subcloning Application Pack (Cat.# M7670)

### Enzyme Properties

**Requirements:** Zn $^{2+}$ , Mg $^{2+}$  (3).

**Optimal Substrate:** 5'- and 3'-phosphates of RNA, DNA and nucleotides.

**Typical Working Concentration:** 0.01unit/pmol DNA ends.

**Optimal pH:** 9.1–10.5. (CIAP is stable at pH 7.5–9.5, but is rapidly inactivated at acidic pH [3]).

**K<sub>m</sub>:** 3.6 $\mu$ M and 1.5mM at pH 8.0 and 10.0, respectively, for p-nitrophenylphosphate (4).

**Inhibitors:** P<sub>i</sub>, Inositol hexaphosphate (5,6).

**K<sub>i</sub>:** 16.5 $\mu$ M at pH 9.2 for P<sub>i</sub> (6).

**Inactivation:** 65°C for 45 minutes or 75°C for 10 minutes.

### Promega Product Information

**Source:** Calf intestinal mucosa.

**Molecular Weight:** 68kDa.

**Typical Working Conditions:** 50mM Tris-HCl (pH 9.3 at 25°C), 1mM MgCl $_2$ , 0.1mM ZnCl $_2$  and 1mM spermidine.

**Storage Conditions:** Store at –20°C. CIAP is supplied in storage buffer containing 10mM Tris-HCl (pH 8.0), 1mM MgCl $_2$ , 0.1mM ZnCl $_2$ , 50mM KCl and 50% (v/v) glycerol.

**Unit Definition:** One unit is defined as the amount of enzyme required to catalyze the hydrolysis of 1 $\mu$ mol of 4-nitrophenyl phosphate per minute at 37°C in 1M diethanolamine, 10.9mM 4-nitrophenyl phosphate, 0.5mM MgCl $_2$  (pH 9.8).

### Contaminant Assays

**Endonuclease Assay:** 1 $\mu$ g of supercoiled plasmid DNA is incubated with 5 units of CIAP in 1X Reaction Buffer (Tables 12, 15) for one hour at 37°C. Following incubation, the supercoiled DNA is visualized on an ethidium bromide-stained agarose gel to verify the absence of visible nicking or cutting.

**Exonuclease Assay:** To test for DNase and RNase activity, 50ng of radiolabeled DNA or radiolabeled RNA is incubated with 5 units of Calf Intestinal Alkaline Phosphatase in 1X Reaction Buffer (Tables 12, 15) for one hour at 37°C, and the release of radiolabeled nucleotides is monitored by scintillation counting of TCA-soluble material. Minimum passing specification is  $\leq$ 3% release for both DNase and RNase.

**Blue/White Cloning Assay:** Five micrograms of pGEM<sup>®</sup>-3Zf(+) Vector<sup>(d)</sup> is digested with representative restriction enzymes (leaving 5'-termini, 3'-termini or blunt ends). The termini are treated with 5 units of Calf Intestinal Alkaline Phosphatase for 1 hour at 37°C, kinased and ligated. The religated plasmid is then transformed into JM109 cells that are plated on X-Gal/IPTG/Amp plates. A minimum of 200–400 colonies are counted. White colonies result from transformation with ligated plasmids with damaged ends. These white colonies represent the number of false positives expected in a typical cloning experiment. Enzymes that generate overhangs, such as *EcoR* I, *Hind* III or *Kpn* I, must produce fewer than 2% white colonies, and blunt-cutting enzymes, such as *Hinc* II, must produce fewer than 5% white colonies (1). Transformation efficiency must be  $\geq 1 \times 10^5$  cfu/ $\mu$ g DNA.

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### 5' End-Labeling Tips

- ◆ The forward reaction is much more efficient than the exchange reaction for labeling of phosphorylated ends. Promega Technical Bulletin #TB519 contains a protocol for labeling phosphorylated DNA via the exchange reaction.
- ◆ The template used should be gel-purified. Contamination by short oligonucleotides or tRNA will lower the efficiency of labeling. Consider the use of glycogen instead of tRNA for efficient precipitation of oligonucleotides.
- ◆ Oligonucleotides are synthesized with a 5'-hydroxyl group. They can be labeled directly in the forward reaction without prior dephosphorylation.
- ◆ Ammonium ions are strong inhibitors of T4 Polynucleotide Kinase; therefore, DNA should not be dissolved in, or precipitated from, buffers containing ammonium salts prior to treatment with T4 PNK.
- ◆ The final ATP concentration in the forward reaction should be at least 1 μM.
- ◆ Equal concentrations of ATP and 5' ends result in about 50% labeling of oligonucleotides. To obtain higher specific activity, the ATP:oligonucleotide ratio should be increased to 10:1. Only 10% of the label will be transferred but virtually every oligonucleotide molecule will be labeled (1).
- ◆ 0.5 pmol of linear DNA equals 1 pmol of 5' ends. To convert μg DNA to pmol:

**For dsDNA:**

$$\mu\text{g} \times 10^6 \text{ pg}/1\mu\text{g} \times \text{pmol}/660\text{pg} \times 1/\text{N} = \text{pmol}$$

where N is the number of nucleotide pairs and 660pg/pmol is the conversion factor for a nucleotide pair.

**For ssDNA:**

$$\mu\text{g} \times 10^6 \text{ pg}/1\mu\text{g} \times \text{pmol}/330\text{pg} \times 1/\text{N} = \text{pmol}$$

where N is the number of nucleotides and 330pg/pmol is the conversion factor for a single nucleotide.

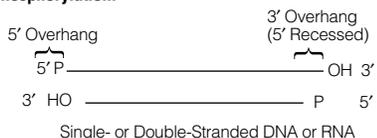
- ◆ Single-stranded DNA and double-stranded DNA containing 5'-overhangs are labeled more efficiently than 3'-overhangs (5'-recessed) or blunt ends. Labeling of blunt or recessed ends can be improved by increasing the concentration of ATP in the reaction or by denaturing the template with heat or NaOH prior to labeling (1,2). Alternative protocols involving the addition of PEG 8000 to the reaction may also be used to increase the labeling efficiency of these templates (1,2).

### References

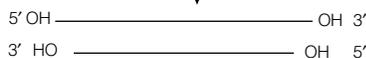
1. *Methods in Molecular Biology*, Vol. 16 (1993) Burrell, M.M. ed., Humana Press, Totowa, New Jersey, 343.
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## 5' End-Labeling Protocol

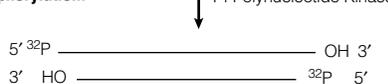
### Dephosphorylation:



↓ CIAP



### Phosphorylation:



2618MA04/9A

Figure 7. 5' end-labeling protocol.

### Dephosphorylation of 5'-Protruding Ends Using Calf Intestinal Alkaline Phosphatase (CIAP):

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

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

2. Purify the DNA to be dephosphorylated by ethanol precipitation and resuspend the pellet in 40 μl of 10 mM Tris-HCl (pH 8.0). Set up the following reaction:

DNA (up to 10 pmol of 5'-ends)	40 μl
CIAP 10X Reaction Buffer,	5 μl
Diluted CIAP (0.01 u/μl)	<u>up to 5 μl</u>
Deionized water to a final volume of	50 μl

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

3. Incubate at 37°C for 30 minutes.
4. Add another aliquot of diluted CIAP (equivalent to the amount used in Step 2) and continue incubation at 37°C for 30 minutes.
5. Add 300 μl CIAP stop buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 200 mM NaCl, 0.5% SDS). Phenol:chloroform extract and ethanol precipitate by adding 5 M NaCl to a final concentration of 0.2 M and 2 volumes of 100% ethanol to the aqueous phase.

### Dephosphorylation of 5'-Recessed or Blunt Ends:

When 5'-recessed or blunt ends are used as the substrate prepare CIAP by diluting the required amount in 1X Reaction Buffer (Table 15) to a final concentration of 0.01 u/μl. Each pmol of DNA ends will require 0.01 u CIAP (added in two aliquots of 0.005 μl/pmol ends). Add the first aliquot of CIAP and incubate at 37°C for 15 minutes and then at 56°C for 15 minutes. Add a second aliquot of CIAP and repeat the incubations at both temperatures. The higher temperature ensures accessibility of the recessed ends.

### 5' End-Labeling Protocol, Forward Reaction, Using T4 Polynucleotide Kinase

1. Assemble the following reaction in a sterile microcentrifuge tube:

5' ends of DNA (dephosphorylated)	1–50 pmol
Kinase 10X Buffer	5 μl
[γ- <sup>32</sup> P]ATP (at 3,000 Ci/mmol, 10 mCi/ml, 50 pmol total)	15 μl
T4 Polynucleotide Kinase	<u>10–20 u</u>
Deionized water to a final volume of	50 μl

2. Incubate at 37°C for 10 minutes.
3. Stop the reaction by adding 2 μl of 0.5 M EDTA. Depending on the application for which the labeled DNA is to be used, subsequent purification may not be necessary.
4. If desired, the labeled DNA can be separated from unincorporated labeled nucleotides by chromatography, by spin columns or by ethanol precipitation. However, it is generally not necessary to purify phosphorylated DNA to be used as hybridization probes or as primers for sequencing. But if the measured incorporation is low (below 30%) or if background problems are experienced, removal of unincorporated nucleotides may be desired. If the labeled DNA fragment is greater than 200 bp, the Wizard® DNA Clean-Up System<sup>(b)</sup> (Cat.# A7280) may also be used. See Promega's *Protocols and Applications Guide*, Third Edition (pp. 128–129), for more information.

Further information on the removal and addition of 5'-phosphates can be found in Promega's *Protocols and Applications Guide*, Third Edition (pp. 44, 125) and in Promega Technical Bulletins #TB096 and #TB519 and Product Information Sheet #9PIM182.



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## RecA Protein and AgarACE® Enzyme

### Introduction

RecA Protein and AgarACE® Enzyme are included in this Cloning Enzyme Guide because of their applications for nucleic acid purification or protection in cloning procedures. The RecA protein has the amazing capacity to facilitate the pairing of DNA molecules (for recent reviews see references 1 and 2). Agarases, such as AgarACE® Enzyme, have become a popular tool to liberate DNA from the commonly used electrophoretic separation matrix, agarose.

### RecA Protein

RecA forms a helical filament on single-stranded DNA, with a stoichiometry of one RecA monomer per 3 bases of ssDNA. RecA protein binds ATP in addition to DNA, and ATP binding is required for nucleoprotein filament assembly, homology searching, and DNA strand exchange (Figure 1, Panel I). ATP hydrolysis is not required for any of these processes, but it is required for disassembly of the nucleoprotein filament (i.e., disassociation of the RecA protein) upon completion of DNA strand exchange, bypass of structural barriers in DNA during replication, and 4-strand exchange reactions. RecA is also involved in the SOS response of bacteria. As cellular DNA damage levels increase to the point where replication is impeded, RecA protein can assemble a nucleoprotein filament on ssDNA that accumulates as a result of interrupted replication. The nucleoprotein filament facilitates autocatalytic cleavage of the LexA repressor and other proteins, such as the bacteriophage  $\lambda$  cI repressor. This co-protease activity of RecA results in the derepression of over 20 genes regulated by LexA and the induction of bacteriophage  $\lambda$  lysogens (1,2). One of the more useful *in vitro* properties of RecA is its ability to locate and pair a ssDNA sequence to its homologous dsDNA sequence in the presence of ATP[ $\gamma$ S] (Figure 1, Panel I). This behavior has been exploited to enrich for specific DNA sequences (3–5), and to protect specific DNA sites from methylation or endonuclease cleavage (RecA-assisted restriction endonuclease, RARE [6], Achilles' heel cleavage [7,8; Figure 9]).

### AgarACE® Enzyme

Agarose gels are widely used in the electrophoretic separation of polynucleotides. A highly heterogeneous polysaccharide, agarose is an alternating copolymer of 3-linked  $\beta$ -D-galactopyranose and 4-linked 3,6-anhydro- $\alpha$ -L-galactopyranose. Agarase enzymes are secreted by a wide variety of organisms as a means of degrading plants containing agaroses, and are classified as  $\alpha$  or  $\beta$  agarases, depending on whether they cleave  $\alpha$  or  $\beta$  intersaccharide linkages. The majority of reported agarases are of the  $\beta$  type, and most degrade agarose into mixtures of short polysaccharides (i.e., small sugars).

Although a large number of organisms secrete enzyme mixtures that completely hydrolyze unmelted agarose, all reported individual enzymes (and all tested mixtures of individual agarases) require melting of the agarose prior to enzyme treatment in order to obtain complete hydrolysis. As a hot agarose solution cools, it reaches a loose gel state in which agarose chains form double helices joined in loosely associated bundles or fibrils, called the gel I state. As the gel cools further, the bundles further associate with each other to a point where the bundles of agarose chains form an extended network and a hardened agarose gel is formed, called the gel II state, used for running electrophoresis gels (9). Agarases will attack agarose in either the gel I or gel II state, but will not attain complete hydrolysis of the most tightly bundled helices, presumably due to steric considerations.

For this reason, commercially available agarases require that the agarose be melted completely prior to enzyme treatment. For low melting agaroses, this means treatment at 70°C; for regular agarose, it requires 95–100°C. Since the enzymes are typically inactivated at these high temperatures, the agarose must be rapidly equilibrated to lower temperatures prior to enzyme treatment, and care must be taken to ensure that the gel I state agarose does not begin to form. AgarACE® Enzyme, isolated from a *Flavobacterium* sp. from the Dry Tortugas (10), is somewhat more thermostable than other known agarases, thus many of the typical difficulties of this equilibration step are avoided (11). AgarACE® Enzyme also exhibits unusual activity in high concentrations of KI and NaI, which allows protocols to be developed for easy recovery of polynucleotides from regular agarose gels (12). AgarACE® Enzyme also has a broad pH optimum, which allows its use in all common electrophoresis buffers without adjustment of unit addition or presoaking of gels to attain optimum activity. It also exhibits fast reaction rates, so Promega's standard unit definition is based on 15-minute hydrolysis times, as opposed to the industry standard 1 hour.

Since the polynucleotides are typically recovered in the electrophoresis buffer, some downstream applications can be negatively affected by either the high pH or the buffering of the solution. In rare cases, some inhibition has been seen due to residual oligosaccharide, and even more rarely, from the enzyme itself. In the first 2 cases (pH and buffering), soaking the gel prior to hydrolysis generally eliminates the problem. In the first three cases, ethanol precipitation after hydrolysis generally alleviates any problems, and high recoveries are typically obtained due to the fact that AgarACE® Enzyme also precipitates and acts as a carrier.

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## RecA Protein

### Description

The *E. coli* RecA protein plays a central role in homologous recombination, post-replication repair and the SOS response to DNA damage (for reviews see references 1–6). The purified RecA Protein (Cat.# M1691) binds cooperatively and stoichiometrically to single-stranded DNA (ssDNA) and is a prototype for DNA strand-exchange proteins. The active species in strand exchange is a nucleoprotein filament containing one RecA Protein monomer (38kDa) per 3 bases of ssDNA. ATP hydrolysis is not required for filament formation or paranemic pairing to duplex DNA, but ATP hydrolysis is required for unidirectional DNA strand exchange, dissociation of the protein, bypass of structural barriers in DNA during replication and 4-strand exchange reactions.

One of the more useful *in vitro* properties of RecA is its ability to locate and pair a ssDNA sequence to its homologous dsDNA sequence in the presence of ATP[ $\gamma$ S]. This behavior has been exploited for enrichment of specific DNA sequences (7–9), and to protect specific DNA sites from methylation or endonuclease cleavage (RecA-assisted restriction endonuclease, RARE [10], Achilles' heel cleavage [11, 12; Figure 9]).

### Applications

- Enrichment method for genomic cloning (7–9).
- Sequence-specific cleavage of large fragments of DNA (RecA-assisted restriction endonuclease [RARE10]; Achilles' heel cleavage [11,12]).
- D-loop mutagenesis (13).
- Coating of DNA with RecA Protein to enhance contrast of electron micrographs (14,15).

### Enzyme Properties

**Requirements:** ATP, MgCl<sub>2</sub>.

**Cofactor Concentration:** 10mM MgCl<sub>2</sub> in the presence of ATP (6).

**Optimal Substrate:** ssDNA.

**Typical Working Concentration:** Varies with the application, but at enzyme concentrations above 0.2 $\mu$ M, ATP hydrolysis becomes linear (6).

**Optimal pH:** Above pH 7, binding to ssDNA is much faster than binding to dsDNA. Binding to dsDNA, pH 6.0 (25°C) (16); DNA-independent ATP hydrolysis, pH 6.0 (25°C) (17), gives a  $k_{cat}$  of 0.1 min<sup>-1</sup> and the  $k_{cat}$  drops to 0.015 min<sup>-1</sup> at pH 7.5; ssDNA-dependent ATP hydrolysis, no dependence on pH between 5.5 and 9.0 (17).

**K<sub>m</sub>:** ATP range from 20 $\mu$ M for ssDNA to 100 $\mu$ M for dsDNA based on initial velocity measurement (18).

**k<sub>cat</sub>:** When RecA is bound to ssDNA, it hydrolyzes ATP with a  $k_{cat}$  of 28–30 min<sup>-1</sup> at

37°C (17). When RecA is bound to dsDNA, it hydrolyzes ATP with a  $k_{cat}$  of 20–22 min<sup>-1</sup> at 37°C (16).

**RecA Filament Assembly:** On ssDNA circles over 8,000 nucleotides long, filament formation is completed in less than 2 minutes at 37°C. Assuming a single nucleation event per circle gives a rate of filament extension at 1,100 RecA monomers per minute (19). Extension is polar and proceeds from 5'→3'; therefore, there is a much greater likelihood that the 3'-ends of linear ssDNA will be coated with RecA monomers than the 5'-ends (20).

**RecA Filament Disassembly:** RecA also displays a polar filament disassembly behavior on linear ssDNA, such that monomers come off at the end opposite to that where filament extension occurs; therefore, both processes proceed 5'→3' (21). Using dATP instead of ATP can prevent filament disassembly.

**Alternative Cofactors and Substrates:** dATP, UTP and PTP (purine ribonucleoside triphosphate) serve as cofactors in RecA-mediated reactions (17,22,23). ITP, CTP, dCTP and GTP are hydrolyzed with a nearly normal  $V_{max}$  but do not support DNA strand exchange. Reactions with dATP increase hydrolytic rates 20% over ATP (22,24,25).

**Inhibitors:** ADP, ATP[ $\gamma$ S], <25mM NaCl to prevent destabilization of RecA triplexes (26).

**K<sub>i</sub>:** For ATP[ $\gamma$ S], 0.6 $\mu$ M (27).

**Temperature Stability:** RecA Protein is stable at temperatures of 25–45°C as demonstrated by a linear Arrhenius plot (28).

**Inactivation:** 65°C for 15 minutes (29).

**Genetic Locus:** *E. coli* recA.

### Promega Product Information

**Source:** Purified from an *E. coli* strain expressing a recombinant clone.

**Molecular Weight:** 37,842 daltons; 352 amino acids.

**Typical Working Conditions:** RecA Protein reaction conditions vary with the application (see references 7–15), but in general there should be one RecA Protein for every three nucleotides in the ssDNA used for strand invasion protocols (e.g., 160ng oligonucleotide per 6.25 $\mu$ g RecA Protein [26]).

**Storage Conditions:** Store at –20°C. RecA Protein is supplied in storage buffer containing 20mM Tris-HCl (pH 7.5 at 25°C), 0.1mM EDTA, 1mM DTT and 50% glycerol.

**Concentration:** RecA concentration is determined by A<sub>280</sub> using an extinction coefficient of 0.516 at 1mg/ml (30).

**Purity:** The purity is ≥95% as judged by SDS-polyacrylamide gel electrophoresis with Coomassie® blue staining.



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**Activity Assays**

**ATPase Activity:** RecA Protein is a ssDNA-dependent ATPase. The rate of ATPase activity is measured as turnover number or apparent  $k_{cat}$ . One monomer of RecA protein binds per 3 nucleotides of ssDNA to form an active RecA:ssDNA complex. ATPase activity is determined by monitoring conversion of NADH to NAD in a coupled reaction. The rate of ATPase activity is calculated from the change in absorbance (31). An apparent  $k_{cat}$  per minute is calculated from the moles of ATP to ADP per mole of activated RecA:ssDNA.

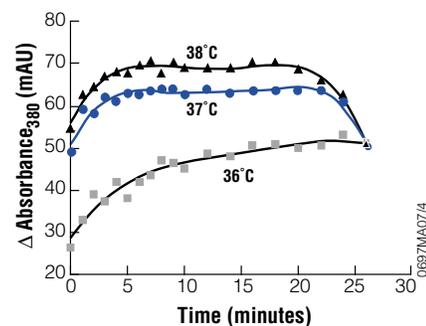
**DNA Strand Exchange:** Strand exchange is the ability of RecA Protein to exchange closed circular ssDNA with one strand of linearized dsDNA. The resulting products are open circular dsDNA and linear ssDNA. After 60 minutes of incubation with 15 $\mu$ g of RecA at 37°C

$\geq$ 50% of the linear dsDNA is converted to open circular DNA (28).

**Contaminant Assays**

**Endonuclease Assay:** To test for endonuclease activity, 1 $\mu$ g of Type 1 supercoiled plasmid DNA, lambda DNA and pGEM® marker DNA(+) is incubated with 15 $\mu$ g of RecA Protein in restriction enzyme buffer A for 16 hours at 37°C. Following incubation the DNA is visualized on an ethidium bromide-stained agarose gel to verify the absence of visible nicking or cutting.

**Exonuclease Assay:** To test for DNase activity, 50ng of radiolabeled DNA is incubated with 5 $\mu$ g of RecA Protein in restriction enzyme buffer A for four hours at 37°C, and the release of radiolabeled nucleotides is monitored by scintillation counting of TCA-soluble material. Minimum passing specification is <1% release of substrate.



**Figure 8. ATPase activity as a function of temperature.** ssDNA-dependent ATPase activity was determined spectrophotometrically by monitoring conversion of NADH to NAD in a coupled reaction (31). RecA Protein was slightly in excess of one monomer per 3 bases of M13mp18 ssDNA. Moles of ATP converted to ADP were calculated from the absorbance change of NADH, and the  $k_{cat}$  was calculated by dividing the rate of ATP conversion by the amount of activated RecA:ssDNA complex. Since RecA is in excess of ssDNA, the amount of activated RecA:ssDNA complex is equal to the known amount of ssDNA sites in the reaction (31). As seen in the above figure, there is a strong temperature-dependence of the ATPase activity. The measured  $k_{cat}$  for 36°C, 37°C, and 38°C was 24, 31, and 34 min<sup>-1</sup>, respectively.

**Table 2. Materials Needed for RecA Protein Protection and Cleavage Protocols.**

- Oligonucleotide: Prepared by user to be specific for the intended protected site. Diluted to 160ng/ml.
- RecA: 1-3mg/ml.
- SAM (1.6mM S-adenosyl methionine): Prepared immediately before use from a 32mM stock by dilution with ice-cold 5mM sulfuric acid.
- ATP[ $\gamma$ S]: Aliquots of a 10mM solution (in water) are stored at -70°C.
- 80mM magnesium acetate.
- Restriction Enzyme Buffer H (Promega).
- Methylase: In theory, any restriction enzyme/methylase pair could be used. In these protocols, 35u/ $\mu$ l of *EcoR* I methylase was used (Figure 9).
- Restriction Enzyme: In these protocols, 12u/ $\mu$ l *EcoR* I was used (Figure 9).
- Buffer A: 250mM Tris-acetate (pH 7.5 at 25°C), 1mM magnesium acetate.
- Buffer B: 166mM Tris-acetate (pH 7.5 at 25°C), 37mM magnesium acetate, 100mM DTT.
- 250mM potassium acetate.

**Table 3. Troubleshooting RecA Protein Cleavage and Protection Protocols.**

Symptoms	Possible Causes	Comments
RecA cleavage or protection: extra bands on a gel that look like restriction digest partials.	Nonspecific protection. RecA in excess of oligonucleotide. RecA favors binding to single-stranded DNA; however, it does have a low affinity for double-stranded DNA.	Try a number of different concentrations of oligonucleotide, holding all other reaction components constant.  Add 160ng of oligo(dT) immediately after the addition of ATP[ $\gamma$ S], which has been described to improve the process (11).
RecA cleavage: uncut DNA.	Incomplete protection of the targeted site.	Increase the concentration of RecA and oligonucleotide.
RecA protection: unexpected products that are comigrating with the products of an unprotected restriction digest.		Change the reaction buffer to decrease nonspecific cleavage by raising the pH from 7.5 to 7.85 (32).  Adjust the oligonucleotide size and/or sequence to target the oligonucleotide more specifically.
Low transformation efficiencies of RecA cleavage products.	Methylation is incompatible with restriction systems of the host.	Check host genotype for incompatible methylation-induced restriction system. Change host if necessary.

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## RecA Protein Cleavage and Protection Protocols

### RecA Protein Cleavage and Protection Reaction Tips

- RecA concentration.** 6.25mg RecA in a 10ml reaction works well. To maximize the specificity and efficiency of RecA protections, it may be necessary to manipulate the oligonucleotide:RecA ratio; we recommend holding the RecA concentration constant and titrating the oligonucleotide
- Oligonucleotide concentration.** The molar stoichiometry (moles of nucleotides to moles of RecA protein) of binding of the oligonucleotide to RecA is 3:1. This ratio is independent of oligonucleotide size and corresponds to 160ng of oligonucleotide per 6.25µg RecA. We recommend using a titration series of 40–280ng in 40ng increments to determine the optimal concentration of oligonucleotide to use with the RecA. If nonspecific protection is a problem, then 160ng of oligo(dT) can be added to the reaction after the addition of ATP[γS].
- Oligonucleotide design.** An oligonucleotide of 30–36 bases in length is recommended for both RecA cleavage and RecA protection in solution. Locate the protected site in the middle of the oligonucleotide. M. Koob recommends oligonucleotides designed with the restriction site ten nucleotides from the 3'-end (personal communication).
- Buffer.** The buffers shown in Figure 9 and Table 2 work well with either *EcoR* I methylase or restriction enzyme. If restriction enzymes or methylases other than these are used, it may be necessary to adjust the salt concentration to improve the activity of the enzyme. Acetate salts appear to be less destabilizing to the RecA triplex than chloride salts, and therefore we suggest using potassium acetate rather than potassium or sodium chloride. While Promega's MULTI-CORE™ Buffer contains potassium acetate, we do not recommend its use for these applications.
- Cloning the products of RecA cleavage.** Because the products of a RecA cleavage reaction are methylated, low transformation frequencies may arise from incompatibilities with the host's restriction/modification system. This will not be a problem when *EcoR* I methylase is used to protect the site, but could be with other restriction enzyme/methylase pairs. If transformation efficiencies are low, compare the genotype of your host to the known methylation-induced restriction systems to determine if this is the cause.

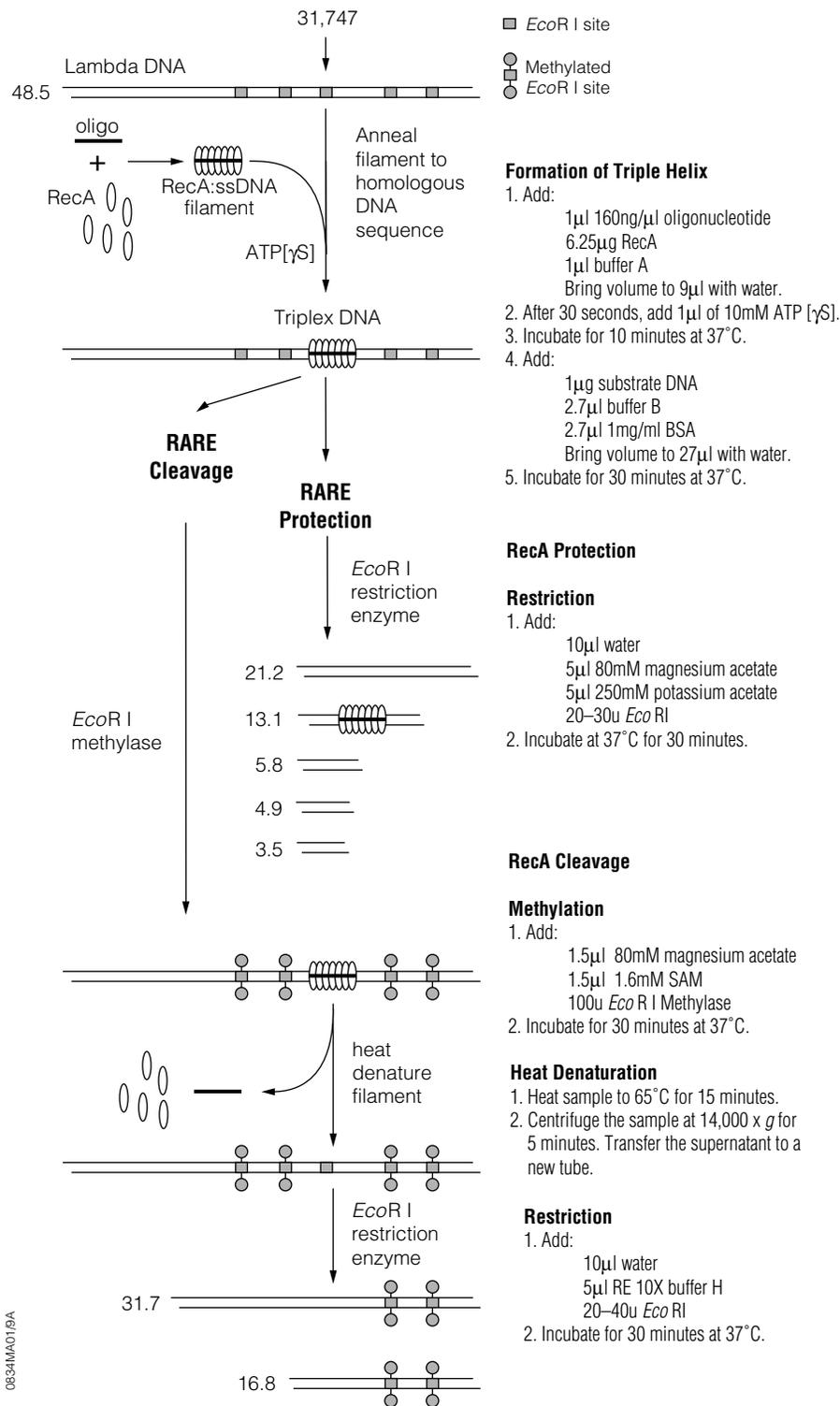


Figure 9. RecA cleavage and protection protocols.



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## AgarACE® Enzyme

### Description

AgarACE® Enzyme (Cat.# M1741, M1743) is a novel, patented agarolytic enzyme produced and extensively tested for the harvest of DNA and RNA from agarose gels (1,2). The harvested polynucleotide is suitable for a variety of downstream applications (page 29).

### Applications

- Harvest of DNA and RNA from low-melting agarose gels.
- Harvest of DNA from regular-melting agarose gels (3).

### Enzyme Properties

**Requirements:** Completely melted agarose, thorough mixing after enzyme addition, incubation temperature 42–47°C.

**Optimal Substrate:** Any agar or agarose, typically 1% (w/v) in electrophoresis buffers (TAE/TBE), melted completely.

**Typical Working Concentration:** One unit per 200mg of 1% gel.

**Optimal pH:** Approximately 6.75; however, AgarACE® Enzyme exhibits ≥80% activity at pH 6.0–8.3. The unit activity is determined at pH 8.3, where the enzyme is least active, so under all conditions that researchers are likely to use, the activity will be at or greater than the expected activity (Figure 15).

**Inhibitors:** 0.3% SDS inhibits 35% in 15 minutes, urea, formaldehyde and guanidine-HCl at ≥0.2M inhibit completely. **Note:** The latter 3 inhibitors can be soaked out of the gel prior to digestion (Table 5, Figure 16).

**Half-Life:** Approximately 15 minutes at 57°C, about 10 minutes at 60°C and >6 minutes at 65°C.

**Temperature Stability:** No significant loss of activity after approximately 90 days at 4°C or 3 days at 20–23°C.

**Inactivation:** 70°C for 15 minutes.

### Promega Product Information

**Source:** *Flavobacterium* strain NR19.

**Molecular Weight:** 42kDa.

**Typical Working Conditions:** One unit per 200mg melted 1% agarose in 1X TAE (pH 8.3) at 42–47°C.

**Storage Conditions:** Store at –20°C. AgarACE® Enzyme is supplied in storage buffer containing 20mM Tris (pH 7.3).

**Unit Definition:** One unit is defined as the amount of AgarACE® Enzyme required to completely degrade 200µl of 1% SeaPlaque® agarose in 1X TBE (pH 8.3) in 15 minutes at 42–47°C (Figure 11). AgarACE® Enzyme degradation of agarose is measured by monitoring sugar release (4).

**Purity:** >90% as determined by visual inspection of a Coomassie® blue-stained SDS-polyacrylamide gel.

### Activity Assays

**Functional Assay (DNA Recovery):** >95% recovery from 2µg of a DNA ladder ranging from 0.4 to 3.0kb in length.

### Contaminant Assays

**Endonuclease Assay:** To test for endonuclease activity, 1µg of supercoiled plasmid DNA is incubated with 5 units of AgarACE® Enzyme for 4 hours at 42°C. Following incubation, the supercoiled DNA is visualized on an ethidium bromide-stained agarose gel. There must be no visible nicking or cutting of the DNA.

**Exonuclease Assay:** To test for DNase and RNase activity, 50ng of radiolabeled DNA or RNA is incubated with 5 units of AgarACE® Enzyme for 4 hours at 42°C in 1X TAE buffer (pH 8.3) containing 6.6mM Mg<sup>2+</sup>. The release of radiolabeled nucleotides is monitored by scintillation counting of TCA-soluble material. Minimum passing specification is <1% release for DNase and <3% release for RNase.

**Single-Stranded DNase:** 5 units of AgarACE® Enzyme are tested with 50ng denatured substrate DNA in 1X TAE buffer with 6.6mM Mg<sup>2+</sup> for 4 hours at 42°C. Minimum passing specification is <5% release.

**Blue/White Cloning:** pGEM®-3Zf(+) Vector<sup>(d)</sup> is digested with *Hinc* II or *Eco*R I, separated on a 1% gel and the agarose digested using AgarACE® Enzyme. The harvested DNA is then religated and transformed into JM109 cells, which are then plated on X-Gal/IPTG/Amp plates. A minimum of 400 colonies is counted. White colonies result from transformation with ligated plasmids with damaged ends. These white colonies represent the number of false positives expected in a typical cloning experiment. Enzymes that generate overhangs, such as *Eco*R I, must produce fewer than 2% white colonies, and blunt-cutting enzymes, such as *Hinc* II, must produce fewer than 5% white colonies (5). Transformation efficiency must be ≥1 x 10<sup>5</sup> cfu/µg DNA.

### Additional Quality Control Assays

**T-Vector Cloning:** At least 100 colonies must be obtained, ≥60% of which are white, when JM109 cells are transformed with the pGEM®-T Vector<sup>(d,f)</sup> containing an insert purified using AgarACE® Enzyme. 80% of the white colonies generated must contain inserts.

**Labeling Efficiency:** Using the Prime-a-Gene® Labeling System, a labeling efficiency of ≥1 x 10<sup>9</sup>cpm/µg DNA must be obtained.

### References

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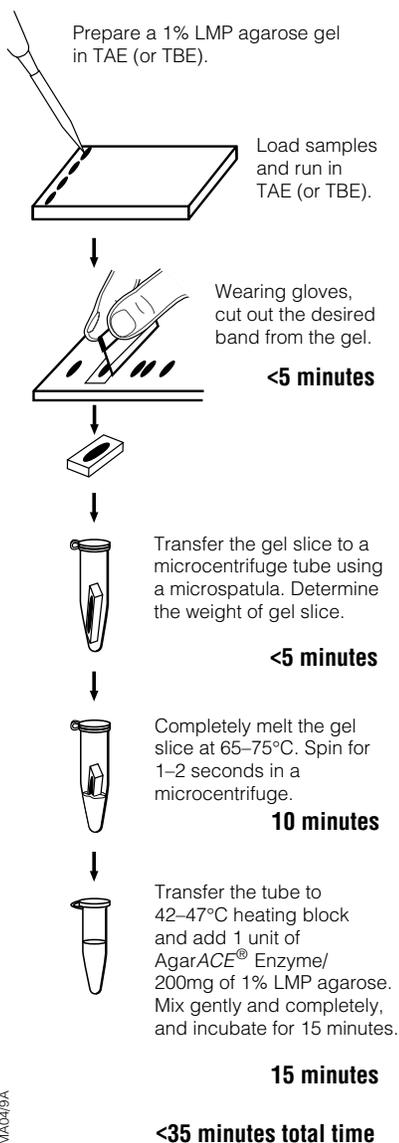
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## DNA Recovery Protocol



26 19/MA04/9A

Figure 10. DNA recovery protocol.

### Brief Protocol

1. Prepare a 1% low melting point agarose gel in TAE or TBE.
2. Load samples and DNA size standard(s). Run gel until bands are well separated.
3. Wearing gloves, refer to the DNA size standards to locate and cut out the desired band.
4. Transfer the gel to pre-weighed microcentrifuge tube using a clean spatula. Determine the weight of the gel slice. 1mg of gel equals approximately 1µl of volume.
5. Completely melt the gel slice at 65–75°C. Centrifuge for 1–2 seconds in a microcentrifuge to collect melted gel to bottom of tube.
6. Transfer the tube to a 42–47°C heating block or water bath and add 1 unit of AgarACE® Enzyme/200µl (200mg) of melted gel. Mix gently and completely, and incubate for 15 minutes.

The activity of AgarACE® Enzyme is linear with respect to agarose concentration. That is, 1 unit of AgarACE® Enzyme will digest 200µl of 1% agarose in 15 minutes, whereas 2 units of enzyme are required to digest an equivalent volume of 2% agarose in 15 minutes. Likewise, 200µl of 1% agarose will be completely digested in 1 hour using 0.25 units of AgarACE® Enzyme (Table 4 and Figure 13). Check for complete digestion by gently pipetting the reaction. The reaction should have the viscosity of water.

7. The DNA can be used in many applications without further purification. If desired, the DNA can also be ethanol precipitated before use. Some downstream applications require ethanol precipitation of the DNA (Table 8). Promega Technical Bulletin #TB228 contains an application compatibility guide for determination of what further purification, if any, is needed.

For a more detailed protocol on the use of AgarACE® Enzyme refer to Promega's *Protocols and Applications Guide*, Third Edition (pp. 84–86). Further information on downstream applications using AgarACE® Enzyme-purified DNA can be found in *Promega Notes* 53, 54 and 61, Table 8 and Figures 17–20.

### DNA Recovery Tips

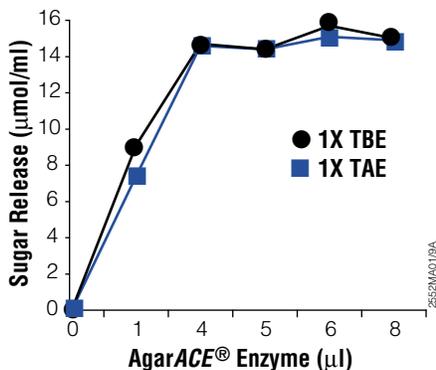
- ◆ TBE can be used instead of TAE for the gel running buffer. However, fewer downstream applications can be performed directly from a TBE gel digest. (See Table 8 and #TB228 for more information.)
- ◆ To visualize the DNA, ethidium bromide may be added to the gel directly (0.5µg/ml) or the gel can be stained after electrophoresis. To stain the gel after electrophoresis, soak in a solution of 0.5µg/ml ethidium bromide for 30 minutes at room temperature. Ethidium bromide added to the gel is lost during electrophoresis. As such, small fragments may not be visible. This can be avoided by adding ethidium bromide to the gel running buffer or staining the gel after electrophoresis. **Caution:** Ethidium bromide is a carcinogen. Wear gloves and use appropriate precautions when working with solutions and gels that contain ethidium bromide.
- ◆ It is important that the gel slice is completely melted. It will take approximately 10–15 minutes for 200µl of 1% LMP agarose to melt completely in a 1.5ml polypropylene microcentrifuge tube. If the gel slice is over 300mg it should be broken up using a spatula or razor blade prior to melting. Unless the sample contains large (>10kb) shearable DNA, monitor melting by gently pipetting the sample. **If the agarose is completely melted, it should pipet like water.**
- ◆ Use of a thermal cycler is convenient for isolation of DNA from many samples. Simply load the samples and program the melting and digestion temperatures into the unit. **Note:** Add the AgarACE® Enzyme after the temperature has been reduced to 42–47°C to avoid denaturation of the enzyme.
- ◆ AgarACE® Enzyme performs equally well in TAE (pH 7.3, 7.8 or 8.3) and TBE (pH 8.3) and performs adequately in 20mM phosphate and MOPS buffers across the pH range of 6.5–8.5. Note that the buffer selection may affect subsequent applications in which the isolated nucleic acid will be used (Table 8; #TB228).
- ◆ SDS (>0.3% w/v) and formaldehyde will inactivate AgarACE® Enzyme. (See Table 5 and Technical Bulletin #TB228 for more information.)
- ◆ In some cases, components of the running buffer may interfere with downstream applications. In many cases, these effects can be reduced by soaking the gel slice in water before digestion with AgarACE® Enzyme (Figure 16).



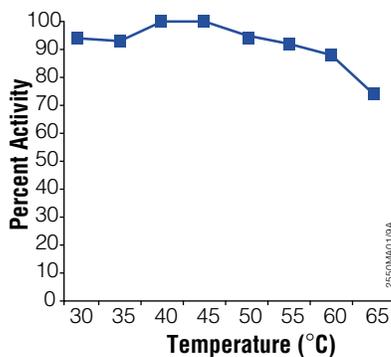
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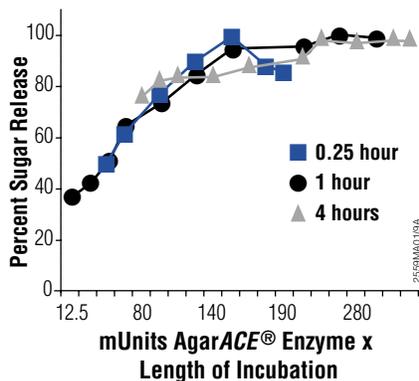
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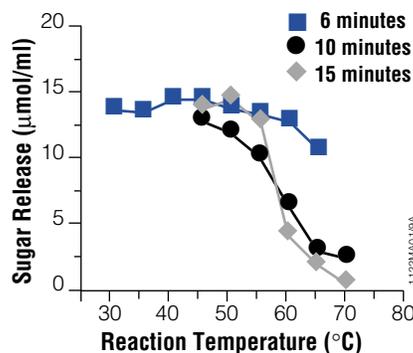
**Figure 11. Determination of AgarACE® Enzyme activity.** Melted agarose is incubated with a titration of enzyme. The point at which the maximal amount of sugar is released through agarose hydrolysis is measured. The same amount of enzyme is required for either TAE or TBE gels.



**Figure 12. Temperature profile for AgarACE® Enzyme.** The activity of AgarACE® Enzyme, added to a reaction at the indicated temperatures and allowed to act for 15 minutes, is shown. Note that although the temperature profile is fairly broad, 100% agarose hydrolysis (required for good DNA recovery) is only obtained at 42–47°C.



**Figure 13. AgarACE® Enzyme performance at longer digestion times.** In this experiment, various amounts of AgarACE® Enzyme were tested at incubation times of 15 minutes, 1 hour, and 4 hours. The graph shows that the enzyme titration maximizes at nearly the same value of enzyme x time for all three incubation times. This means that if the use of less enzyme is desired, the incubation time can be increased proportionately (up to a 10-fold enzyme dilution/4 hour incubation) (see Table 4 for precise values; 3).



**Figure 14. AgarACE® Enzyme survival at various temperatures.** AgarACE® Enzyme is relatively stable for a few minutes at temperatures of 65–70°C, which allows the hydrolysis protocol to be performed without a lower temperature equilibration step. By the time a sample is taken from a block and the AgarACE® Enzyme is added, the temperature is usually at 60–65°C, and is dropping (4).

**Table 4. Digestion Time Versus Units of Enzyme.**

Digestion Time (at 42–47°C, unless noted)	Units of AgarACE® Enzyme
15 minutes	1.0
1 hour	0.25
4 hours	0.1
16 hours (overnight)*	0.025

\*Perform overnight digestions at 37°C in an oven.

**AgarACE® Enzyme Activity Assays:**

Agarase activity assays are generally based on the fact that hydrolysis of the agarase polymer liberates free reducing sugar, which is then measured colorimetrically. Two types of assays are commonly used; one in which the amount of enzyme is varied and the maximal sugar release attained is measured (Figure 11). The other type of assay is a fixed enzyme/ fixed time format, where the amount of sugar released is compared to that obtained for a known standard condition (Figures 12–14).

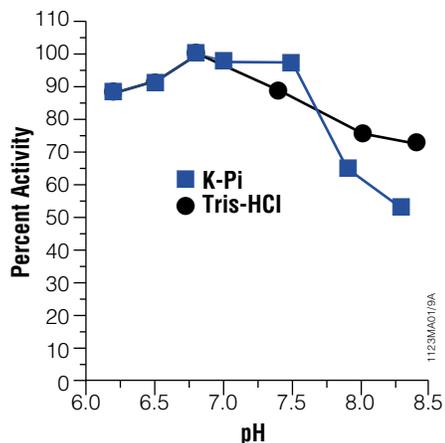
**Table 5. Effects of Buffer Additives on AgarACE® Enzyme (4).**

Additive	Effect on AgarACE® Enzyme
Ethidium Bromide (0.5–5mg/ml)	None detected
Glycerol (0–50%)	None detected
DTT (0–10mM)	None detected
NaCl (0–200mM)	None detected
NaCl (200–500mM)	Causes variation in the digestion rate, not recommended
EDTA (0–10mM)	None detected
SDS (<0.1% w/v)	None detected
SDS (>0.3% w/v)	Inactivates enzyme
Formaldehyde	Inactivates enzyme

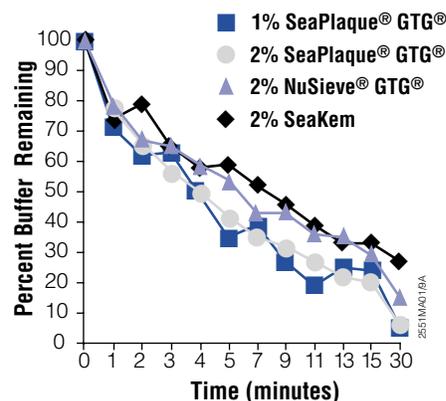


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**Figure 15. The effect of pH on AgarACE® Enzyme activity.** Although the pH optimum for AgarACE® is 6.75, the pH activity profile is fairly broad. The enzyme is assayed for quality control purposes under the low activity condition (pH 8.3), so that any pH deviations on the acidic side will result in higher activity than that indicated on the product label (4).



**Figure 16. Buffer removal by gel soaking.** About 70–80% of the 1X TBE buffer in slices of several types of gels can be removed by a 15-minute soak in 10 volumes of water. Other experiments (data not shown) demonstrate that DNA fragments as short as 200bp are largely retained in the gel under these conditions.

**Ethanol Precipitation of DNA**

- Hydrolyze the agarose completely as described in the standard procedure.
- For sodium acetate precipitation, add 0.1 volume of 3M NaOAc (pH 5.2), mix, then add 2 volumes of room temperature 95% ethanol (2X the volume after NaOAc addition). For ammonium acetate precipitation, add 0.5 volume of 7.5M NH<sub>4</sub>OAc, mix, then add 2.5 volumes of room temperature 95% ethanol (2.5X the volume after NH<sub>4</sub>OAc addition).
- Allow the mixture to stand 1–2 hours (2 hours for small amounts of DNA in NH<sub>4</sub>OAc) at room temperature (do not incubate in the cold), then centrifuge for 15 minutes at room temperature in a microcentrifuge at maximum speed.
- Immediately decant the supernatant, orienting the tube so that the pellet is on the upper side of the tube. Centrifuge the tube for 1–2 seconds in a microcentrifuge to bring down residual supernatant. Remove the remaining supernatant.
- Wash the pellet by adding 200–500µl of cold (<10°C) 70% ethanol, agitating briefly, centrifuging for 5 minutes and decanting as above.
- Dry the pellet in a Speed-Vac® instrument if desired, then reconstitute the pellet in an appropriate volume of water or buffer. Allow at least 15 minutes for the nucleic acid to redissolve.

**Table 6. Troubleshooting AgarACE® Enzyme.**

**Symptom:**

Large quantities of white or translucent precipitate in tube when ethanol precipitated; pellet resuspends poorly

**Possible Causes:**

Incomplete digestion of agarose prior to precipitation; possible loss of enzyme activity.

Incomplete digestion of agarose prior to precipitation; agarose concentration higher than intended.

Incomplete digestion of agarose prior to precipitation; possible buffer incompatibility.

**Comments:**

Resuspend the sample, remelt residual agarose and retreat with AgarACE® Enzyme.

Resuspend sample, remelt residual agarose and retreat with AgarACE® Enzyme.

Repeat using a different buffer, and a longer incubation time.

**Table 7. DNA Recovery from AgarACE® Enzyme-Hydrolyzed Agarose after Ethanol Precipitation (6).**

DNA Size (bp)	DNA Recovery (%)		
	Sodium Acetate, 1 hour at 25°C	Ammonium Acetate, 2 hours at 25°C	Ammonium Acetate, 1 hour at 25°C
296	122 ± 4	105 ± 2	39 ± 3
344	115 ± 4	95 ± 3	27 ± 2
395	nd	100 ± 1	54 ± 2
500	104 ± 4	99 ± 1	74 ± 2
1,000	115 ± 4	96 ± 1	65 ± 2
1,600	107 ± 3	94 ± 1	80 ± 4
2,000	94 ± 3	83 ± 1	62 ± 3
3,000	106 ± 1	77 ± 3	78 ± 3

Recovery, using AgarACE® Enzyme, of 100ng total DNA from a DNA ladder, as quantified by image analysis of a SYBR® Green I-stained gel. Starting amounts of DNA for the smaller bands (296bp, 344bp) were approximately 2–4ng and apparent recoveries >100% were due to gel contributions. Results demonstrate DNA recoveries at or near 100% for sodium and ammonium acetate precipitations after 1 and 2 hours, respectively, but lower recoveries with ammonium acetate for 1 hour.





## Compatibility of AgarACE® Enzyme with Downstream Applications

**Table 8. Summary of the Compatibility of Nucleic Acid Isolated Using AgarACE® Enzyme With Subsequent Applications.**

Application (running buffer)	Can Be Used Directly in Application?	Required Pre-Treatment	Comments
<b>Ligation</b>			
(TAE)	Yes	None	TBE lowers transformation efficiency (6).
(TBE)	Yes	None	
<b>Cloning of DNA Fragments</b>			
(TAE)	Yes	None	TBE can greatly lower transformation efficiency (Table 10; 6).
(TBE)	Yes	None	
<b>Transformation of <i>E. coli</i></b>			
(TAE)	No	Add ligase buffer	For TBE, the DNA volume is important. Tables 9 and 10 and Figure 20 show that addition of ligase buffer is important.
(TBE)	No	Add ligase buffer	
<b>Electroporation of <i>E. coli</i></b>			
(TAE)	No	Ethanol precipitation	Slightly fewer colonies obtained.
(TBE)	No	Ethanol precipitation	
<b>pGEM®-T Vector Cloning</b>			
(TAE)	Yes	None	The number of colonies is somewhat variable.
(TBE)	Yes	Soak gel in water	
<b>Labeling with T4 PNK</b>			
(TAE)	Yes, but lower labeling	Add 2X kinase (optional)	Soaking the gel in water can improve labeling (Figure 18).
(TBE)	Yes, but lower labeling	Add 2X kinase (optional)	
<b>Dephosphorylation (AP)</b>			
(TAE)	Yes	None	Borate ions inhibit CIAP (Figure 17).
(TBE)	No	Ethanol precipitation	
<b>Digestion with Restriction Enzymes</b>			
(TAE)	Yes, but more enzyme	Yes, in certain cases	Important! Varies with restriction enzyme used (Table 11).
(TBE)	Yes, but more enzyme	Yes, in certain cases	
<b>Prime-a-Gene® Labeling</b>			
(TAE)	Yes	None	See reference 7.
(TBE)	Yes	None	
<b>Nick Translation</b>			
(TAE)	Yes	None	See reference 7.
(TBE)	Yes	None	
<b><i>fmoI</i>® DNA Sequencing</b>			
(TAE)	Yes	Ethanol precipitation	Ethanol precipitation before sequencing is useful for eliminating "hard stops" (Figure 19; 6).
(TBE)	No	Ethanol precipitation	
<b>PCR</b>			
(TAE)	Yes	None	See Figure 19 and reference 6.
(TBE)	Yes, but 40–60% yield (variable)	Ethanol precipitation	

### Labeling with either Prime-a-Gene® Labeling System or Nick Translation System

Use AgarACE® Enzyme-isolated DNA directly, or if it is necessary, concentrate the DNA by performing an ethanol precipitation. Cool DNA solution to room temperature before adding labeling reactants (7).

### *fmoI*® DNA Sequencing System

Use AgarACE® Enzyme-isolated DNA directly if TAE is used as the running buffer, or if TBE is the buffer or it is necessary to concentrate the DNA, perform an ethanol precipitation. In some cases "hard stops" have been observed at various locations when the DNA is not first ethanol precipitated (6).

### Transformation of Cells by Electroporation

Before using the DNA for electroporation, ethanol precipitate it using sodium acetate, and wash the DNA pellet at least once with cold (<10°C) 70% ethanol to remove traces of the sodium acetate. The total number of colonies observed will be approximately 2–3-fold lower than observed for DNA in water.



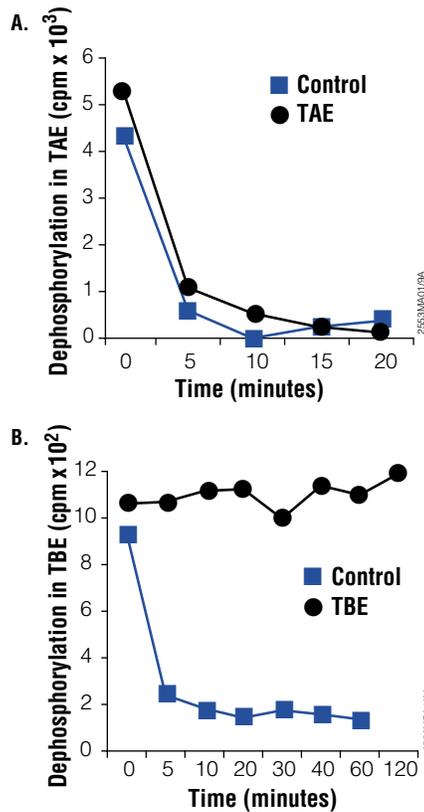
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**Compatibility of AgarACE® Enzyme with Downstream Applications (continued).**

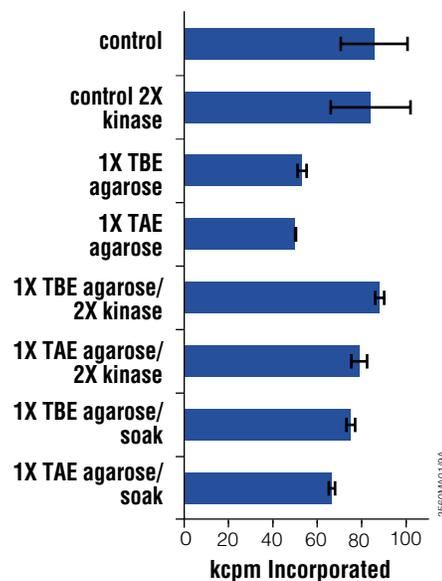
**Figure 17. Performance of AgarACE® Enzyme-harvested DNA in dephosphorylation reactions with calf intestinal alkaline phosphatase (CIAP).** For TAE gels (Panel A), the DNA is efficiently dephosphorylated relative to control, but the borate ion in TBE gels (Panel B) completely inhibits the CIAP enzyme.



**Recommendation: Dephosphorylation with Calf Intestinal Alkaline Phosphatase**

Use isolated DNA directly if TAE is used as the running buffer. If it is necessary to concentrate the DNA or if TBE is used, perform an ethanol precipitation. If ethanol precipitation is not done for samples from TBE, the borate in the TBE will completely inhibit the CIAP.

**Figure 18. Kinase reactions with AgarACE® Enzyme-harvested DNA.** The figure demonstrates that while performing a kinase reaction with control DNA in water, using twice the normal amount of kinase does not increase labeling. However, it does increase labeling of AgarACE® Enzyme-harvested DNA (from TBE or TAE gels) up to control levels. Alternatively, soaking the gel to remove some of the buffer has a similar benefit.



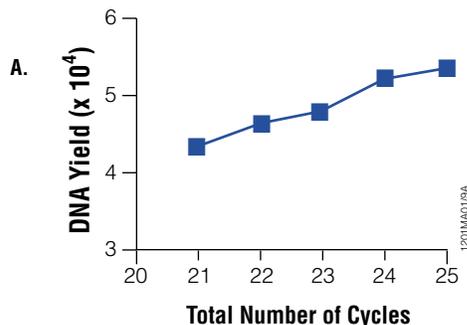
**Recommendation: 5' End-Labeling with T4 Polynucleotide Kinase**

Perform either an ethanol precipitation (using sodium acetate; ammonium ion inhibits kinase) prior to labeling or double the amount of kinase added to obtain the highest specific activity possible. Alternatively, soak the gel in water (Figure 16) prior to digesting with AgarACE® Enzyme to get DNA that can be labeled to at least one-half the specific activity of DNA in water. DNA harvested from unsoaked gels and not ethanol precipitated will label to at least one-fourth to one-third the specific activity of DNA in water.



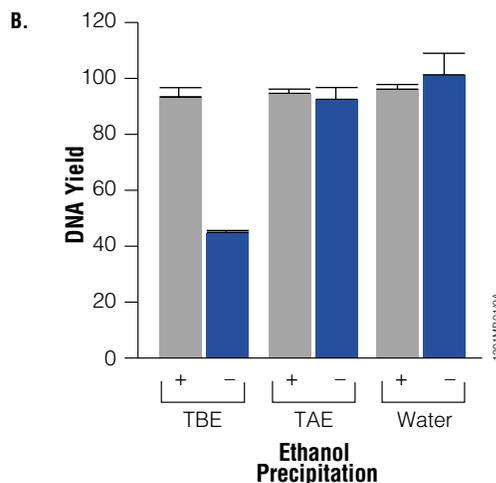
Compatibility of AgarACE® Enzyme with Downstream Applications (continued).

**Figure 19. Performance of AgarACE® Enzyme-harvested DNA in PCR. Panel A** demonstrates that for template DNA harvested from a TAE-buffered gel using AgarACE® Enzyme, the yield of PCR product (measured in a fluorescent dye assay) increased linearly with increasing numbers of cycles. **Panel B** demonstrates that template DNA harvested from TAE gels using AgarACE® Enzyme resulted in PCR product yields similar to those observed with control DNA resuspended in water. Template DNA harvested from TBE gels needed to be ethanol-precipitated to perform well in PCR.

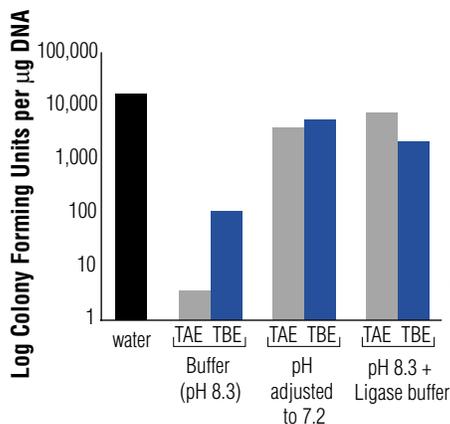


**Recommendation: PCR from Isolated Fragment**

AgarACE® Enzyme-isolated DNA may be used directly if TAE is used as the running buffer. If TBE is used or if it is necessary to concentrate the DNA, an ethanol precipitation should be performed. TBE results in 2–3-fold lower PCR yields unless DNA is first ethanol-precipitated.



**Figure 20. Transformation efficiency is greatly dependent on the pH of the gel-running buffer.** When DNA is used in buffer at pH 8.3 (TAE or TBE) low transformation efficiency results, but when the pH is adjusted to 7.2 the transformation efficiency increases dramatically. Addition of ligase buffer decreases the interference caused by high pH electrophoresis buffers, provided the volume used in a 100µl transformation is minimal.



**Recommendation: Transformation of E. coli.**

We recommend using DNA harvested from TAE, setting up a normal ligation and transforming cells with ≤5µl DNA per 100µl competent cells. If TBE is used, run the gel in 0.5X buffer or soak the gel in water prior to digestion, and then use ≤8µl of DNA per 100µl cells. In all cases, for high colony yields ethanol precipitation of DNA will remove the buffer and concentrate the DNA.



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**Table 9. Transformation Efficiency of DNA Isolated from Agarose Gels using AgarACE® Enzyme (6).**

Treatment	cfu/μg
water control	6.4 x 10 <sup>7</sup>
TAE (pH 8.3)	7.5 x 10 <sup>7</sup>
TAE (pH 8.3) + AgarACE® Enzyme	8.1 x 10 <sup>7</sup>
TAE (pH 8.3) + agarose + AgarACE® Enzyme	1.3 x 10 <sup>8</sup>
TBE (pH 8.3)	2.2 x 10 <sup>7</sup>
TBE (pH 8.3) + AgarACE® Enzyme	2.5 x 10 <sup>7</sup>
TBE (pH 8.3) + agarose + AgarACE® Enzyme	4.6 x 10 <sup>7</sup>
TAE (pH 7.2)	6.5 x 10 <sup>7</sup>
TAE (pH 7.2) + AgarACE® Enzyme	6.8 x 10 <sup>7</sup>
TAE (pH 7.2) + agarose + AgarACE® Enzyme	7.7 x 10 <sup>7</sup>

pGEM®-3Zf(+) Vector DNA was treated in one of three ways: 1) suspended in 1X gel buffer; 2) diluted in 1X gel buffer and AgarACE® Enzyme (1u/200μl) added; or 3) embedded in 1% SeaPlaque® GTG® agarose cast in 1X gel buffer, melted and digested with AgarACE® Enzyme as described in the DNA Recovery Protocol (Figure 10). Pseudoligations contained 5ng of plasmid in 5μl of one of the above mixtures, 1μl of 10X Ligase Buffer and 4μl water. In each case, 2μl of the pseudoligation (1ng DNA) was transformed into low efficiency competent JM109 Cells and plated on LB/Amp/IPTG/X-Gal plates. Colonies were counted and cfu/μg were calculated.

**Table 10. Luciferase Activity and Transformation Efficiency of pBEST-*luc*™ Vector DNA After Ligation (6).**

Treatment	μl DNA Used in Ligation	cfu/μg	% Colonies Expressing Luciferase
TAE (pH 8.3)	3μl	1 x 10 <sup>6</sup>	97%
TAE (pH 8.3)	8μl	3.1 x 10 <sup>4</sup>	95%
no ligase control	8μl	<limits	—
TBE (pH 8.3), water-soaked	3μl	1 x 10 <sup>5</sup>	100%
TBE (pH 8.3), water-soaked	8μl	7.4 x 10 <sup>3</sup>	98%
no ligase control	8μl	<limits	—
TBE (pH 8.3)	3μl	<limits	—
TBE (pH 8.3)	8μl	<limits	—
no ligase control	8μl	<limits	—

The pBEST *luc*™ Vector<sup>®</sup> was digested with *Bam*H I and *Cla* I and 2μg was loaded onto a 1% SeaPlaque® GTG® gel cast in either 1X TAE or 1X TBE (pH 8.3). The *luc* gene and vector fragments were excised from the gel, pre-soaked in water where noted and the DNA isolated as described in the DNA Recovery Protocol (Figure 10). Equal molar ratios of the two fragments were mixed adding either 3 or 8μl of the hydrolyzed agarose (20–22ng DNA and 51–54ng DNA, respectively) to the ligation. Fragments were ligated in a 10μl reaction using 3 units of T4 DNA Ligase, overnight at 15°C. Part of the ligation reaction (5μl) was transformed into 50μl Subcloning Efficiency JM109 Competent Cells, plated on nitrocellulose filters on LB/Amp plates and incubated at 37°C overnight. Colonies were counted for transformation efficiency. Luciferase activity was assayed by transferring the nitrocellulose filter to a dish containing 1mM luciferin in 0.1M sodium citrate buffer (pH 5.5) at 45–50°C and observing in the dark at room temperature. The limits of detection were 4.5 x 10<sup>2</sup> cfu/μg.

**Table 11. Effect of AgarACE® Digestion on Restriction Enzyme Activity.**

Enzyme	Units Required if Isolated from 1X TBE*	Units Required if Isolated from 1X TAE*	Notes
<i>Acc</i> I	3.5X	7X	Purify with Wizard® PCR Preps**
<i>Apa</i> I	3X	3X	
<i>Bam</i> H I	4X	4X	
<i>Eco</i> R I	6X	6X	Purify with Wizard® PCR Preps**
<i>Hind</i> III	8.5X	8.5X	Purify with Wizard® PCR Preps**
<i>Kpn</i> I	4X	4X	
<i>Not</i> I	3.5X	2X	
<i>Pst</i> I	7X	17X	Purify with Wizard® PCR Preps**
<i>Sac</i> I	1X	1.5X	
<i>Sal</i> I	1.5X	2X	
<i>Sph</i> I	1X	1X	

\*The amount of restriction enzyme required (X-fold) to digest the DNA relative to a control digestion containing DNA in water. The DNA was isolated from *Promega LMP* (Cat.# V2831, V3841), Preparative Grade Agarose; other agaroses may give different results.

\*\*The use of TBE buffer is not recommended with the Wizard® PCR Preps DNA Purification System.





## Technical Appendix

### Composition of Solutions

**Note:** Solution compositions for the Cloning Enzyme 10X reaction buffers are listed in Table 15.

#### Antibiotic Stock Solutions

**Ampicillin:** 100mg/ml in deionized water (filter-sterilized).

**Tetracycline:** 10mg/ml in 80% ethanol.

**Kanamycin:** 30mg/ml kanamycin sulfate in deionized water (filter-sterilized).

**Chloramphenicol:** 20mg/ml in 80% ethanol.

Store at -20°C. **Note:** Cell growth in liquid culture is inhibited by tetracycline concentrations greater than 10mg/ml.

#### Cell Lysis Solution

0.2N NaOH, 1% SDS in deionized water. Prepare fresh for each use.

#### Cell Resuspension Solution

25mM	Tris-HCl (pH 8.0)
10mM	EDTA
50mM	glucose

#### IPTG Stock Solution (0.1M)

1.2g	isopropyl β-D-thiogalactopyranoside (IPTG) (Cat.# V3951)
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Add deionized water to 50ml final volume. Filter-sterilize (0.2µm) and store at 4°C.

#### LB Medium

10g	Bacto®-tryptone
5g	Bacto®-yeast extract
5g	NaCl

Add deionized water to approximately 1L. Adjust pH to 7.5 with 10N NaOH and autoclave.

#### LB/Antibiotic Plates (1L)

Add 15g of agar to 1 liter of LB medium and autoclave. Allow the medium to cool to 55°C before adding antibiotic to the specified final concentration (ampicillin: 100µg/ml; tetracycline: 12.5µg/ml; kanamycin: 30µg/ml; chloramphenicol: 20µg/ml). Pour 30–35ml of medium into 85mm petri dishes. If necessary, flame the surface of the medium with a Bunsen burner to eliminate bubbles. Let the agar harden overnight. Store at 4°C for <1 month. Tetracycline is light-sensitive; LB/tetracycline plates should be covered with foil.

#### M-9 Plates

6g	Na <sub>2</sub> HPO <sub>4</sub>
3g	KH <sub>2</sub> PO <sub>4</sub>
0.5g	NaCl
1g	NH <sub>4</sub> Cl
15g	agar

Add deionized water to approximately 1L. Adjust pH to 7.4 with 10N NaOH. Autoclave. Cool to 50°C. Then add:

2.0ml	1M MgSO <sub>4</sub>
0.1ml	1M CaCl <sub>2</sub>
10.0ml	20% glucose
1.0ml	1M thiamine-HCl

Filter the medium through a 0.2µm filter unit.

#### Phenol:Chloroform:Isoamyl Alcohol (25:24:1)

Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

#### Potassium Acetate Solution (pH 4.8)

Prepare 60ml of 5M potassium acetate. Add 11.5ml of glacial acetic acid and 28.5ml of deionized water. This solution will be 3M with respect to potassium and 5M with respect to acetate. Store at 4°C.

#### SOC Medium

2.0g	Bacto®-tryptone
0.5g	Bacto®-yeast extract
1ml	1M NaCl
0.25ml	1M KCl
1ml	2M Mg <sup>2+</sup> stock (1M MgCl <sub>2</sub> • 6H <sub>2</sub> O, 1M MgSO <sub>4</sub> • 7H <sub>2</sub> O), filter-sterilized
1ml	2M glucose, filter-sterilized

Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97ml deionized water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg<sup>2+</sup> stock and 2M glucose stock, each to a final concentration of 20µM. Filter the complete medium through a 0.2µm filter unit. The pH should be 7.0.

#### TAE 10X Buffer

400mM	Tris base
200mM	Sodium acetate
10mM	EDTA

Adjust pH to 8.2 with glacial acetic acid.

#### TBE 10X Buffer

890mM	Tris base
890mM	Boric acid
19mM	EDTA

Adjust pH to 8.3.

#### TE Buffer

10mM	Tris-HCl (pH 8.0)
1mM	EDTA

#### X-Gal Stock Solution (50mg/ml)

100mg	5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal)
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Dissolve in 2ml of N,N'-dimethylformamide. This stock solution is available from Promega (Cat.# V3941).

**Table 12. Cloning Enzymes: Promega's Quality Acceptance Criteria.**

Enzyme	Concentration (u/μl)	SDS-PAGE Purity	Contaminant Activity		
			Endonuclease: Supercoiled DNA, 1 μg	dsDNase: 50ng Radiolabeled DNA	RNase: 50ng Radiolabeled RNA
AgarACE® Enzyme (1)	0.15–0.30	>90%	Gel analysis, absence of visible nicking or cutting, 5u/4hr at 42°C	<1% release, 5u/4hr at 42°C	<3% release, 5u/4hr at 42°C
Alkaline Phosphatase, Calf Intestinal (2)	1	N/A	Gel analysis, absence of visible nicking or cutting, 5u/1hr at 37°C	<3% release, 5u/1hr at 37°C	<3% release, 5u/1hr at 37°C
RecA Protein (3,4)	N/A	>95%	Gel analysis, absence of visible nicking or cutting, 15μg/16hr at 37°C	<1% release, 5μg/4hr at 37°C	N/A
T4 DNA Ligase (2,5)	1–3	>90%	Gel analysis, absence of visible nicking or cutting, 5u/16hr at 37°C	<1% release, 20u/16hr at 37°C	<3% release, 20u/5hr at 37°C
T4 Polynucleotide Kinase (6)	5–10	>90%	Gel analysis, absence of visible nicking or cutting, 25u/5hr at 37°C	<3% release, 25u/3hr at 37°C	<3% release, 25u/3hr at 37°C
T4 RNA Ligase	9–12	>90%	20u/3hr at 37°C	<1% release, 20u/3hr at 37°C	<1% release, 20u/3hr at 37°C

N/A – Not Applicable

1. T-Vector, Blue/White, DNA recovery of 2μg of DNA ladder.

2. Blue/White Assay is performed.

3. ssDNA-dependent ATPase activity.

4. Strand exchange.

5. T-Vector, Lambda ligation and packaging, ssDNase.

6. Oligo 5' end-labeled to high specific activity.

**Table 13. Uses and Genotypes of Various Strains of *E. coli*.**

Strain	Genotype	Comments
BL21(DE3)	F <sup>-</sup> , ompT, hsdS <sub>B</sub> , (r <sub>B</sub> <sup>-</sup> , m <sub>B</sub> <sup>-</sup> ), dcm, gal, λ(DE3)	<i>In vivo</i> expression of T7 Promoter-driven genes (e.g., pET-5, pGEM®).
<b>BL21(DE3)pLysS<sup>(e)</sup></b>	F <sup>-</sup> , ompT, hsdS <sub>B</sub> , (r <sub>B</sub> <sup>-</sup> , m <sub>B</sub> <sup>-</sup> ), dcm, gal, λ(DE3), pLysS CmR	<i>In vivo</i> expression of T7 Promoter-driven genes (e.g., pET-5, pGEM®), pLysS provides tighter control of T7 RNA Pol expression.
<b>BMH 71-18 mutS*</b>	thi, supE, Δ(lac-proAB), [mutS::Tn10] [F <sup>-</sup> , proAB, laq1 <sup>α</sup> ZΔM15]	GeneEditor™ Site-Directed Mutagenesis System; mismatch repair deficient strain.
<b>CJ236</b>	F <sup>-</sup> , cat(pCJ105; M13 <sup>α</sup> Cm <sup>r</sup> )/dut, ung1, thi-1, relA1, spoT1, mcrA	Kunkel mutagenesis; dut(-), ung(-).
<b>C600</b>	thi-1, thr-1, leuB6, lacY1, tonA21, supE44	λgt10, Permissive host; allows both parental and recombinant phage to grow.
<b>C600hfl</b>	thi-1, thr-1, leuB6, lacY1, tonA21, supE44, hflA150, [chr::Tn10]	λgt10, Restrictive host; allows recombinant phage to grow in preference to parental phage.
DH1	recA1, endA1, gyrA96, thi-1, hsdR17 (r <sub>K</sub> <sup>-</sup> , m <sub>K</sub> <sup>+</sup> ), supE44, relA1	Parent of DH5. DH5 more efficiently transformed by large (40 - 60kb) plasmids.
DH5α®	θ80dlacZΔM15, recA1, endA1, gyrA96, thi-1, hsdR17 (r <sub>K</sub> <sup>-</sup> , m <sub>K</sub> <sup>+</sup> ), supE44, relA1, deoR, Δ(lacZYA-argF)U169	Common host for cDNA cloning; supports α-complementation, recA(-) and endA(-).
DH5α®F'	F <sup>-</sup> , θ80dlacZΔM15, recA1, endA1, gyrA96, thi-1, hsdR17 (r <sub>K</sub> <sup>-</sup> , m <sub>K</sub> <sup>+</sup> ), supE44, relA1, deoR, Δ(lacZYA-argF)U169	Single-stranded DNA synthesis.
<b>ES1301 mutS*</b>	lacZ53, thyA36, rha-5, metB1, deoC, IN(rrnD-rrnE), [mutS201::Tn5]	Provided with the Altered Sites® Mutagenesis Systems; mismatch repair deficient (kan <sup>r</sup> ).
<b>HB101*</b>	thi-1, hsdS20 (r <sub>B</sub> <sup>-</sup> , m <sub>B</sub> <sup>-</sup> ), supE44, recA13, ara-14, leuB6, proA2, lacY1, rpsL20 (str <sup>r</sup> ), xyl-5, mtl-1, galK2	Common strain for propagating plasmids that do not allow α-complementation.
JM83	ara, Δ(lac-proAB), rpsL, θ80dlacZΔM15	Host for pUC plasmids; pBR322- recA(+), r <sub>K</sub> <sup>+</sup> , m <sub>K</sub> <sup>+</sup> .
JM101	supE, thi, Δ(lac-proAB), [F <sup>-</sup> , traD36, proAB, lacI <sup>α</sup> ZΔM15]	Host for M13mp vectors; recA(+), r <sub>K</sub> <sup>+</sup> .

Strains listed in boldface are available from Promega.

\* Indicates strains available as competent cells.

(continued)



**Table 13. Uses and Genotypes of Various Strains of *E. coli* (continued).**

Strain	Genotype	Comments
JM103	<i>endA1, hsdR, supE, sbcB15, thi-1, strA, Δ(lac-proAB)</i> , [F', <i>traD36, proAB, lacI<sup>q</sup>ZΔM15</i> ]	Host for M13mp vectors; <i>recA</i> (+), <i>r<sub>K</sub></i> <sup>+</sup> .
JM105	<i>endA1, thi, rpsL, sbcB15, hsdR4, Δ(lac-proAB)</i> , [F', <i>traD36, proAB, lacI<sup>q</sup>ZΔM15</i> ]	Host for M13mp vectors; <i>recA</i> (+), <i>r<sub>K</sub></i> <sup>+</sup> .
JM107	<i>endA1, thi, gyrA96, hsdR17 (r<sub>K</sub><sup>-</sup>, m<sub>K</sub><sup>+</sup>), relA1, supE44, Δ(lac-proAB)</i> , [F', <i>traD36, proAB, lacI<sup>q</sup>ZΔM15</i> ]	Host for M13mp vectors; <i>recA</i> (+), <i>r<sub>K</sub></i> <sup>+</sup> .
JM108	<i>endA1, recA1, gyrA96, thi, hsdR17 (r<sub>K</sub><sup>-</sup>, m<sub>K</sub><sup>+</sup>), relA1, supE44, Δ(lac-proAB)</i>	Recombination deficient mutation in <i>rec A</i> increases stability of plasmids.
<b>JM109*</b>	<i>endA1, recA1, gyrA96, thi, hsdR17 (r<sub>K</sub><sup>-</sup>, m<sub>K</sub><sup>+</sup>), relA1, supE44, Δ(lac-proAB)</i> , [F', <i>traD36, proAB, lacI<sup>q</sup>ZΔM15</i> ]	Common host for cloning. ssDNA synthesis; restriction(-), <i>rec</i> (-), allows $\alpha$ -complementation: Included with the majority of our plasmids; Maintain on M9 plates supplemented with thiamine (to maintain F' episome).
<b>JM109(DE3)</b>	<i>endA1, recA1, gyrA96, thi, hsdR17 (r<sub>K</sub><sup>-</sup>, m<sub>K</sub><sup>+</sup>), relA1, supE44, Δ(lac-proAB)</i> , [F', <i>traD36, proAB, lacI<sup>q</sup>ZΔM15</i> ], $\lambda$ (DE3)	<i>In vivo</i> expression of T7 Promoter-driven genes (e.g., pET-5, pGEM <sup>®</sup> ), allows $\alpha$ -complementation.
JM110	<i>rpsL, thr, leu, thi, hsdR17 (r<sub>K</sub><sup>-</sup>, m<sub>K</sub><sup>+</sup>), lacY, galK, galT, ara, tonA, tsx, dam, dcm, supE44, Δ(lac-proAB)</i> , [F', <i>traD36, proAB, lacI<sup>q</sup>ZΔM15</i> ]	<i>dam</i> (-), <i>dcm</i> (-) strain, allows $\alpha$ -complementation.
<b>LE392</b>	<i>hsdR514, (r<sub>K</sub><sup>-</sup>, m<sub>K</sub><sup>+</sup>), supE44, supF58, lacY1 or Δ(lacIZY)6, galK2, galT22, metB1, trpR55</i>	Genomic and cDNA cloning; restriction(-), <i>rec</i> (+), permissive host, no color selection, <i>lon</i> (+). Recommended (if no color selection needed) as primary strain for amplification of recombinant phage and screening of cDNA library with nucleic acid probe.
<b>KW251</b>	<i>supE44, galK2, galT22, metB1, hsdR2, mcrB1, mcrA, [argA81::Tn10], recD1014</i>	Genomic cloning; permissive host, used as alternative to LE392, <i>rec</i> (-) strain, restriction (-), <i>tet</i> <sup>r</sup> .
MB408	<i>recF, recB21, recC22, sbcB15, hflA, hflB, hsdR, (tet<sup>r</sup>)</i>	
MC1061	F-, <i>araD139, Δ(ara-leu)7696, galE15, galK16, Δ(lac)X74, rpsL(Str), hsdR2(r<sub>K</sub><sup>-</sup>, m<sub>K</sub><sup>+</sup>), mcrA, mcrB1</i>	
<b>NM522</b>	<i>supE, thi, Δ(lac-proAB), Δhsd5 (r-, m-)</i> , [F', <i>proAB, lacI<sup>q</sup>ZΔM15</i> ]	ssDNA synthesis; restriction (-), <i>rec</i> (+), F'. Grow on M9 plates to maintain F' episome.
<b>NM538</b>	<i>supF, hsdR (r<sub>K</sub><sup>-</sup>, m<sub>K</sub><sup>+</sup>), trpR, lacY</i>	Genomic cloning; permissive host.
<b>NM539</b>	<i>supF, hsdR (r<sub>K</sub><sup>-</sup>, m<sub>K</sub><sup>+</sup>), lacY, (P2)</i>	Restrictive host; used for <i>Spi</i> selection of recombinant phage.
P2392	LE392 (P2)	
RR1	<i>hsdS20, (r<sub>B</sub><sup>-</sup>, m<sub>B</sub><sup>+</sup>), supE44, ara-14, proA2, rpsL20 (str), lacY1, galK2, xyl-5, mtl-1, supE44</i>	<i>Rec A</i> (+) version of HBI01.
$\chi$ 1776	<i>tonA53, dapD8, minA1, glnV44, supE44, Δ(gal-uvrB)40, min82, rib-2, gyrA25, thyA142, oms-2, metC65, oms-1, (tte-1), Δ(bioH-asd)29, cyc82, cycA1, hsdR2</i>	Debilitated strain used in early work with recombinant DNA.
XL1-Blue	<i>recA1, endA1, gyrA96, thi, hsdR17(r<sub>K</sub><sup>-</sup>, m<sub>K</sub><sup>+</sup>), supE44, relA1, lac, [F', <i>proAB<sup>+</sup>, lacI<sup>q</sup>ZΔM15, ::Tn10(Tet<sup>r</sup>)</i>]</i>	Common host for cloning.
SURE Cells	<i>E14-(McrA-) Δ(mcrCB-hsdSMR-mrr) 171 endA1, supE44, thi-1, gyrA96, relA1, lac, recB, recJ, sbcC, umuC::Tn5(kan<sup>r</sup>), uvrC, [F', <i>proAB, lacI<sup>q</sup>ZΔM15 [Tn10(Tet<sup>r</sup>)</i>]</i>	Increased stability of DNA containing inverted repeats or Z-DNA (Stratagene); F' episome- able to make ssDNA from phagemid DNA.
Y1088	$\Delta$ ( <i>lacU169</i> ), <i>supE, supF, hsdR (r<sub>K</sub><sup>-</sup>, m<sub>K</sub><sup>+</sup>), metB, trpR, tonA21, [proC::Tn5] (pMC9)</i>	Host for amplification of cDNA libraries. pMC9 confers <i>amp</i> <sup>r</sup> , <i>tet</i> <sup>r</sup> .
<b>Y1089</b>	$\Delta$ ( <i>lacU169</i> ), <i>proA<sup>+</sup>, Δ(lon), araD139, strA, hflA150, [chr:Tn10(tet<sup>r</sup>)</i> , (pMC9)	cDNA cloning- <i>lon</i> (-), <i>hflA150</i> (enhances lysogeny), pMC9- maintain on <i>amp</i> / <i>tet</i> plates: Used primarily for generation of preparative amounts of recombinant fusion protein. pMC9 confers <i>amp</i> <sup>r</sup> , <i>tet</i> <sup>r</sup> .
<b>Y1090</b>	$\Delta$ ( <i>lacU169</i> ), <i>proA<sup>+</sup>, Δ(lon), araA139, strA, supF, [trpC22::Tn10 (tet<sup>r</sup>)</i> , (pMC9), <i>hsdR (r<sub>K</sub><sup>-</sup>, m<sub>K</sub><sup>+</sup>)</i>	cDNA cloning. Useful for screening expression cDNA libraries. pMC9 confers <i>amp</i> <sup>r</sup> , <i>tet</i> <sup>r</sup> .

Strains listed in boldface are available from Promega.

\* Indicates strains available as competent cells.



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**Table 14. Applications of Promega's Competent Cells.**

Applications	Cells	Genotype	>1 x 10 <sup>8</sup> cfu/μg	>1 x 10 <sup>7</sup> cfu/μg	>1 x 10 <sup>6</sup> cfu/μg
Cloning T-Vector Cloning Blue/White Screening	JM109	<i>endA1, recA1, gyrA96, thi hsdR17</i> ( <i>r<sub>K</sub><sup>-</sup>, m<sub>K</sub><sup>+</sup></i> ), <i>relA1, supE44 Δ(lac- proAB)</i> , [ <i>F' traD36, proAB</i> <i>lacI<sup>q</sup>ZΔM15</i> ].	✓	✓	
Cloning	HB101	<i>F', thi-1, hsdS20 (r<sub>B</sub><sup>-</sup>, m<sub>B</sub><sup>-</sup>)</i> , <i>supE44, recA13, ara-14, leuB6</i> , <i>proA2, lacY1, galK2 rpsL20, (str<sup>r</sup>)</i> , <i>xyl-5, mtl-1</i>	✓	✓	
Site-Directed Mutagenesis	BMH 71-18 <i>mutS</i>	<i>thi, supE, Δ(lac-proAB)</i> , [ <i>mutS::Tn10</i> ], [ <i>F' proAB</i> , <i>lacI<sup>q</sup>ZΔM15</i> ].Mutagenesis		✓	
	ES1301 <i>mutS</i>	<i>lacZ53, mutS201::Tn5, thyA36</i> , <i>rha-5, metB1, decC1, IN(rrnD- rrnE)</i> .		✓	
Protein Expression	BL21 (DE3)pLysS <sup>(e)</sup>	<i>F', ompT, hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup>, m<sub>B</sub><sup>+</sup>)</i> , <i>dcm</i> , <i>gal</i> , (DE3), pLysS, Cmr <sup>r</sup>		✓	✓

**Table 15. Cloning Enzyme 10X Reaction Buffer Formulations.**

Enzyme	10X Formulation
T4 DNA Ligase*	300mM Tris-HCl (pH 7.8), 100mM MgCl <sub>2</sub> , 100mM DTT, 10mM ATP
T4 RNA Ligase	500mM Tris-HCl (pH 7.8), 100mM MgCl <sub>2</sub> , 50mM DTT, 10mM ATP
T4 Polynucleotide Kinase (PNK)	700mM Tris-HCl (pH 7.6), 100mM MgCl <sub>2</sub> , 50mM DTT
Calf Intestinal Alkaline Phosphatase (CIAP)	500mM Tris-HCl (pH 9.3), 10mM MgCl <sub>2</sub> , 1mM ZnCl <sub>2</sub> , 10mM spermidine

\* pGEM<sup>®</sup>-T and pGEM<sup>®</sup>-T Easy Vector Systems (Cat.# A1360, A1380, A3600, A3610) provide a 2X Rapid Ligation Buffer for T4 DNA Ligase with a formulation of: 60mM Tris-HCl (pH 7.8), 20mM MgCl<sub>2</sub>, 20mM DTT, 2mM ATP, 10% polyethylene glycol (MW 8000).

The Rapid Ligation Buffer enables performance of ligation reactions in as little as 1 hour.



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**Additional Literature**

**Technical Manuals and Product Information Sheets**

- #9PIM180 T4 DNA Ligase
- #9PIM182 Calf Intestinal Alkaline Phosphatase
- #9PIM181 T4 DNA Polymerase
- #9PIM220 DNA Polymerase I Large (Klenow) Fragment
- #TM042 pGEM®-T and pGEM®-T Easy Vector Systems

**Promega Notes Articles**

- PN032 pGEM®-Zf Vector update: Considerations for optimal single-stranded DNA production
- PN045 pGEM®-T Vector Systems troubleshooting guide
- PN051 pGEM®-T Vector: Technically Speaking
- PN054 pGEM®-T Vector: Cloning of modified blunt-ended DNA fragments
- PN071 Rapid Ligation for the pGEM®-T and pGEM®-T Easy Vector Systems  
Cloning Blunt End *Pfu* DNA Polymerase-Generated PCR Fragments into pGEM®-T Vector



## Glossary

**5'-RACE:** Rapid Amplification of cDNA 5'-Ends.

**Activated Calf Thymus DNA:** Nicked and gapped dsDNA prepared by treatment with DNase I; a substrate for many DNA polymerases.

**Alkaline Phosphatase (AP):** An enzyme that catalyzes the removal of a phosphate group from a substrate. This property is used in colorimetric and chemiluminescent detection reagents.

**Alpha Complementation:** The process by which a functional  $\beta$ -galactosidase (*lacZ*) gene is generated when the *lacZ*  $\alpha$ -peptide (N-terminus) complements the  $\omega$  fragment of *lacZ* (C-terminus). In general, a cloning vector contributes the *lacZ*  $\alpha$ -peptide and the host bacterial strain provides the  $\omega$  fragment. See also blue/white cloning.

**Blue/White Cloning:** A technique used to identify recombinant (positive) clones in cloning experiments. Specially constructed cloning vectors will produce  $\beta$ -galactosidase by  $\alpha$ -complementation when transformed into the appropriate host strain after exposure to the chemical IPTG. This will cause the cells to exhibit a blue color in the presence of the substrate X-Gal. Foreign DNA inserted into the cloning vector will interrupt the *lacZ* gene, preventing  $\alpha$ -complementation and producing white colonies.

**Blunt Ends:** DNA ends that do not possess short, single-stranded overhangs. See also sticky ends.

**Cloning:** The production of a large number of identical DNA molecules from a single DNA molecule through replication of the DNA within a cell. Cloning also refers to the production of large numbers of identical cells from a single cell.

**Cohesive Ends:** Two DNA ends, in the same or different molecules, which have short, single-stranded overhangs that are complementary to one another. Cohesive ends allow comparatively efficient ligation of DNA molecules. See also sticky ends.

**Competent Cells:** Bacterial cells that are able to take in exogenous DNA.

**Distributive:** Enzyme dissociates from template after a single nucleotide addition.

**DNA (Deoxyribonucleic Acid):** A polymeric molecule composed of deoxyribonucleotide units joined in a specific sequence through the formation of 3'→5' phosphodiester bonds.

**DNase (Deoxyribonuclease):** An enzyme that breaks down DNA into small fragments or deoxyribonucleotides.

**DNA Ligase:** An enzyme that joins two DNA molecules (or two ends of the same DNA molecule) by forming a phosphodiester bond between the 3'- and 5'-ends.

**dNTP:** Deoxyribonucleotide 5'-triphosphate.

**ds:** Double-stranded.

**DTT:** Dithiothreitol.

**End-Labeling:** The addition of a labeled group (radioactive or nonradioactive) to the 5'- or 3'-end of DNA or RNA. This is typically accomplished by using a kinase to label the 5'-end, or a DNA polymerase or terminal deoxynucleotidyl transferase to label the 3'-end.

**Endonuclease:** An enzyme that hydrolyzes phosphodiester bonds at internal locations within a DNA or RNA molecule.

**Exonuclease:** An enzyme that hydrolyzes phosphodiester bonds at the ends of DNA or RNA molecules, resulting in the stepwise removal of nucleotides.

**Gene:** A segment of DNA that encodes a polypeptide, protein or RNA molecule.

**Genotype:** The specific genes (which may or may not be expressed) that are present in an organism.

**Heat-Inactivation:** Destroying an enzyme's activity by heating to a high temperature (typically 60–70°C) for an extended length of time. Not all enzymes can be heat-inactivated.

**In vitro:** A reaction or experiment performed in the absence of living cells, typically using conditions that attempt to mimic those found within cells.

**In vivo:** A reaction or experiment performed in a living organism or cell.

**kb:** Kilobase or kilobase pairs.

**$k_{cat}$ :** Maximum number of substrate molecules converted to products per active site per unit time.

**kDa:** KiloDalton.

**$K_m$ :** The Michaelis constant; the concentration of substrate that an enzyme can convert to product at half its maximal rate.

**Labeling:** A process in which nucleic acids or proteins are tagged with a radioactive or nonradioactive marker.

**Ligase:** An enzyme that catalyzes DNA or RNA linkage, generally splitting off a pyrophosphate group from ATP concurrently.

**Multiple Cloning Site (MCS):** The region of a DNA vector that contains unique restriction enzyme recognition sites into which foreign DNA can be inserted; also called a polylinker.

**Nuclease:** An enzyme that degrades nucleic acids.

**Nucleotide:** A molecule composed of an organic base, sugar and phosphate group, which constitutes the "building blocks" of nucleic acids (DNA and RNA).

**Oligonucleotide (Oligo):** A short (typically <50 nucleotides), single-stranded DNA or RNA molecule.

**PCR:** Polymerase Chain Reaction.

**PEG:** Polyethylene glycol.

**Phosphatase:** An enzyme that removes a phosphate group from a protein, nucleic acid or other molecule.

**$PP_i$ :** Inorganic pyrophosphate.

**Primer:** An oligonucleotide or short single-stranded nucleic acid that acts as a starting point for the synthesis of nucleic acids from a template.

**Promoter:** DNA sequence for the initiation of RNA transcription by RNA polymerase.

**RNA (Ribonucleic Acid):** A polymeric molecule composed of ribonucleotide units joined in a specific sequence through the formation of 3'→5' phosphodiester bonds.

**RNase (Ribonuclease):** An enzyme that breaks down RNA into smaller RNA fragments or ribonucleotides.

**rNTP:** Ribonucleotide 5'-triphosphate.

**ss:** Single-stranded.

**Sticky Ends:** Two DNA ends, in the same or different molecules, that have short, single-stranded overhangs that are complementary to one another. Sticky ends allow comparatively efficient ligation of DNA molecules. See also Cohesive Ends.

**TAE:** Tris Acetate EDTA.

**TCA:** Trichloroacetic acid.

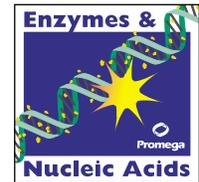
**Terminator:** DNA sequence for the termination of RNA transcription by RNA polymerase.

**Transformation:** The process during which a plasmid DNA is inserted into a bacterial cell.

**Turnover Rate ( $k_{cat}$ ):** Maximum number of substrate molecules converted to products per active site per unit time.

**Vector:** A DNA molecule that can replicate within a host cell and that allows the insertion of foreign DNA sequences. Vectors commonly used may be plasmids, phagemids or bacteriophage.





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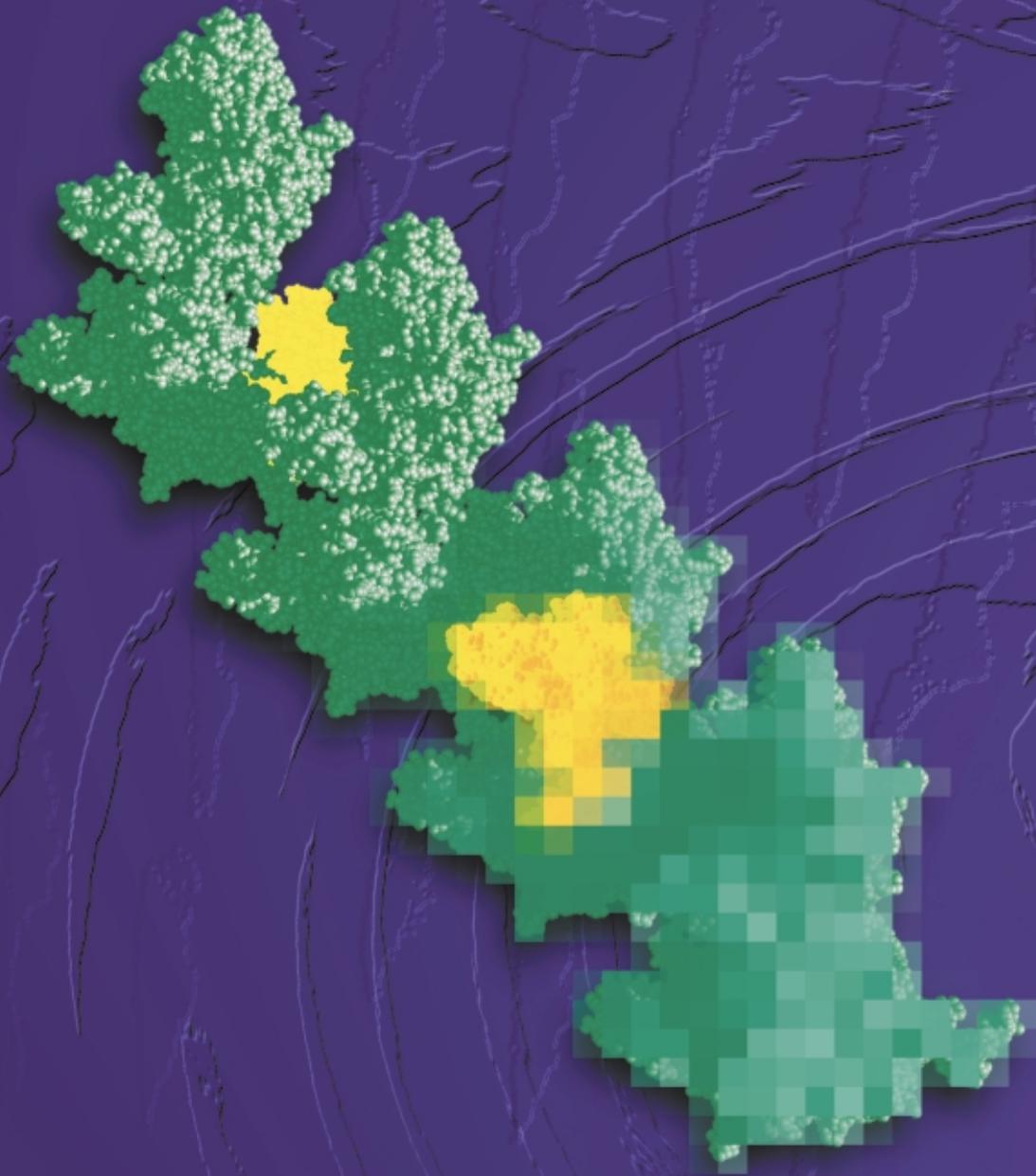


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