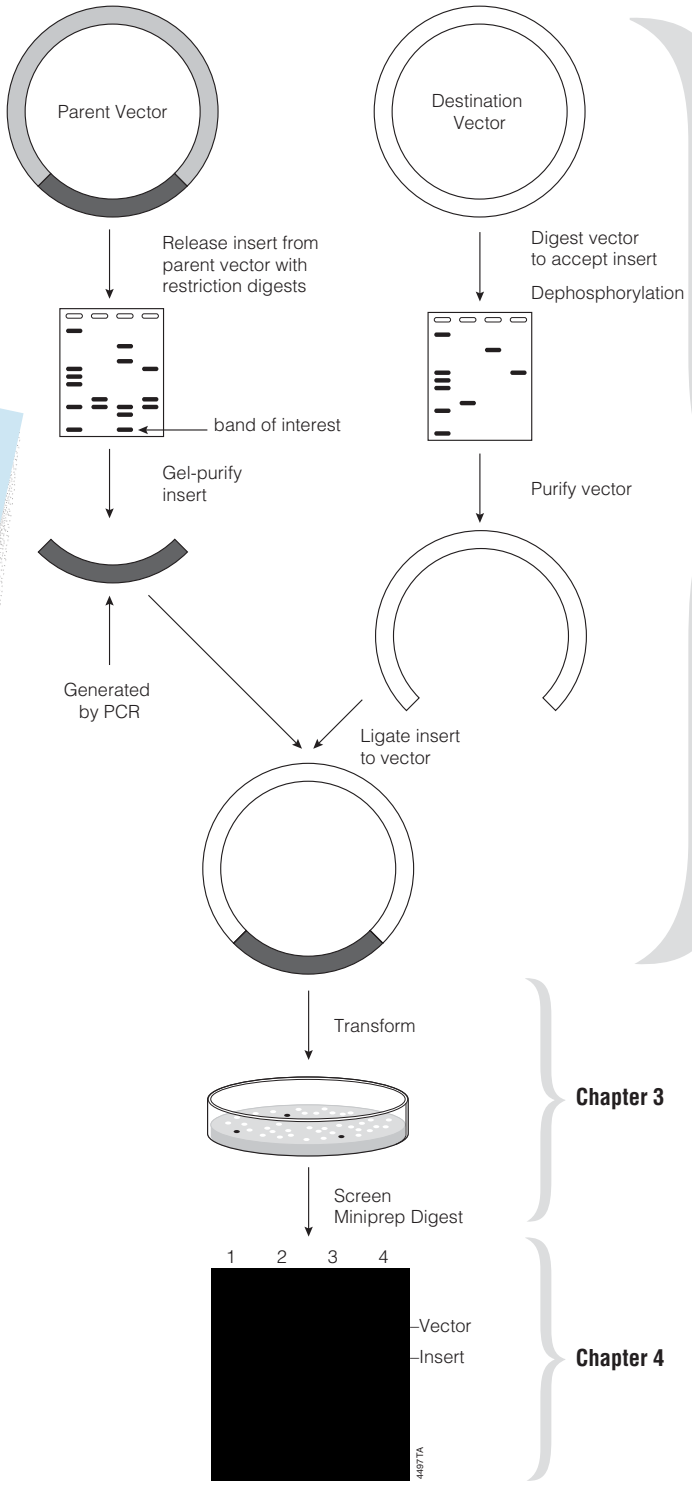
Classic Subcloning

Basic Steps for Subcloning

Subcloning is a basic procedure in molecular biology required to move inserts from one vector to another to gain the desired functionality to study your insert. Essentially all subcloning reactions proceed the same way as illustrated in the figure below. You release and purify your insert from the parent vector, ligate this insert into a prepared destination vector, transform this ligation reaction into competent bacterial cells. Then you screen the transformed cells for the insert. This *Subcloning Notebook* will guide you through every step in the process.



Dephosphorylation reduces the chance of vector self-ligation to virtually zero.

Gel isolation is a practical necessity in subcloning. You get the fragment you need.

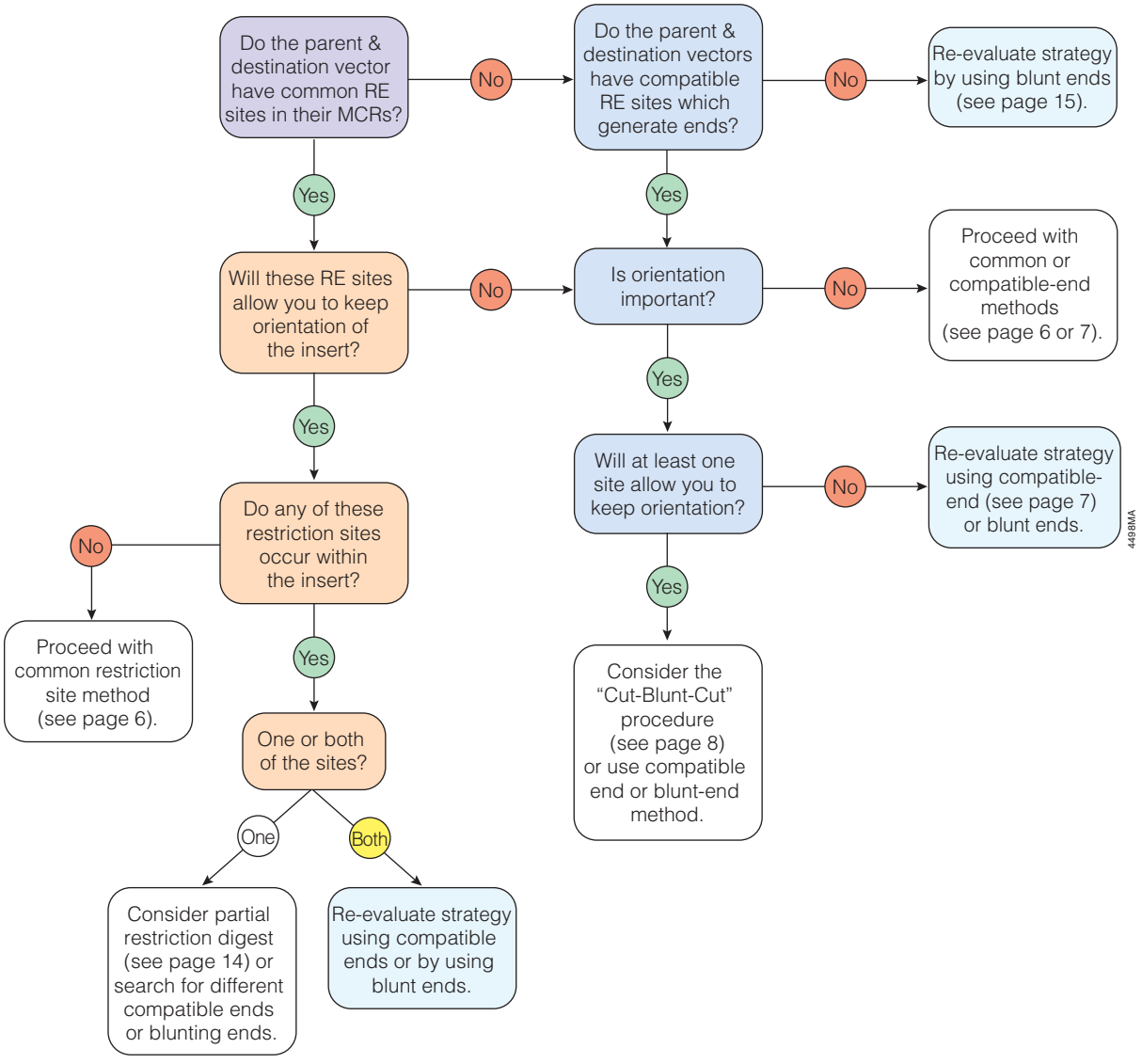
Gel isolation of vector reduces background by eliminating uncut vector from the transformation.

Classic Subcloning

Subcloning Strategy

Before you begin your subcloning, you need to know: The restriction enzyme (RE) sites available for subcloning in your parent vector multiple cloning region (or in the insert if you need to digest the insert); the RE sites available in the destination vector multiple cloning region (MCR); and if these same sites also occur in your insert. Once you know this information, you can begin to ask questions about which subcloning strategy to use.

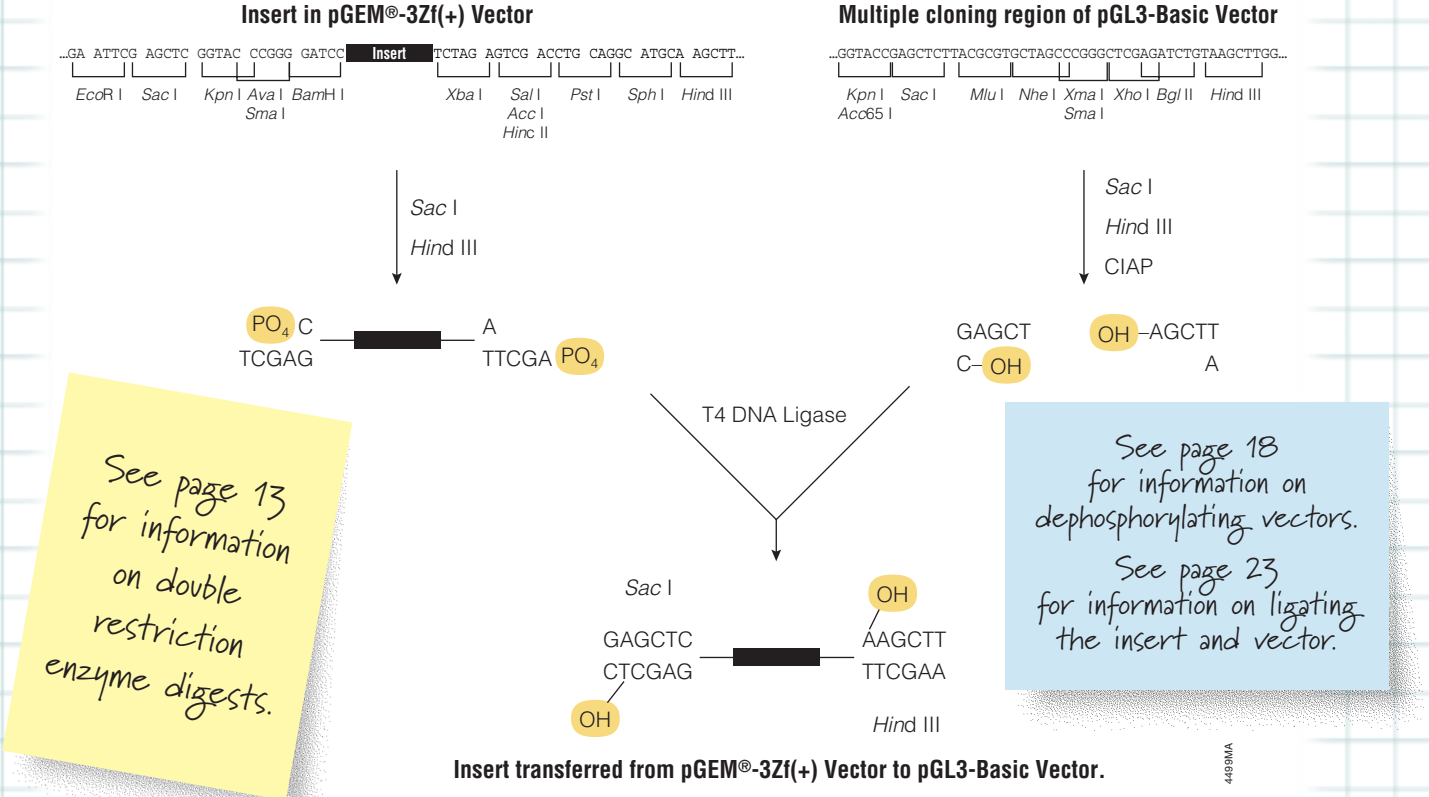
See the Compatible Ends Table on page 61 for a listing of overhangs compatible with Promega enzymes.



Subcloning Strategy: Common Restriction Sites

If your parent and destination vector multiple cloning regions contain common restriction sites and neither of these restriction sites occur within your insert, you have a very straightforward subcloning process. You digest your parent and destination vectors with the same two enzymes followed by dephosphorylation of the destination vector. The insert and the dephosphorylated vector are then separated on an agarose gel and purified using a system such as the Wizard® SV Gel and PCR Clean-Up System (see page 28) and ligated.

The T4 DNA Ligase will join the DNA through reforming the bond between the 5′-PO_4 coming from the insert and the 3′-OH of the vector. The vector has been dephosphorylated so the second bond will not be formed in vitro (indicated by the OH). These nicks will be repaired in the bacteria upon transformation.



Insert transferred from pGEM®-3Zf(+) Vector to pGL3-Basic Vector.

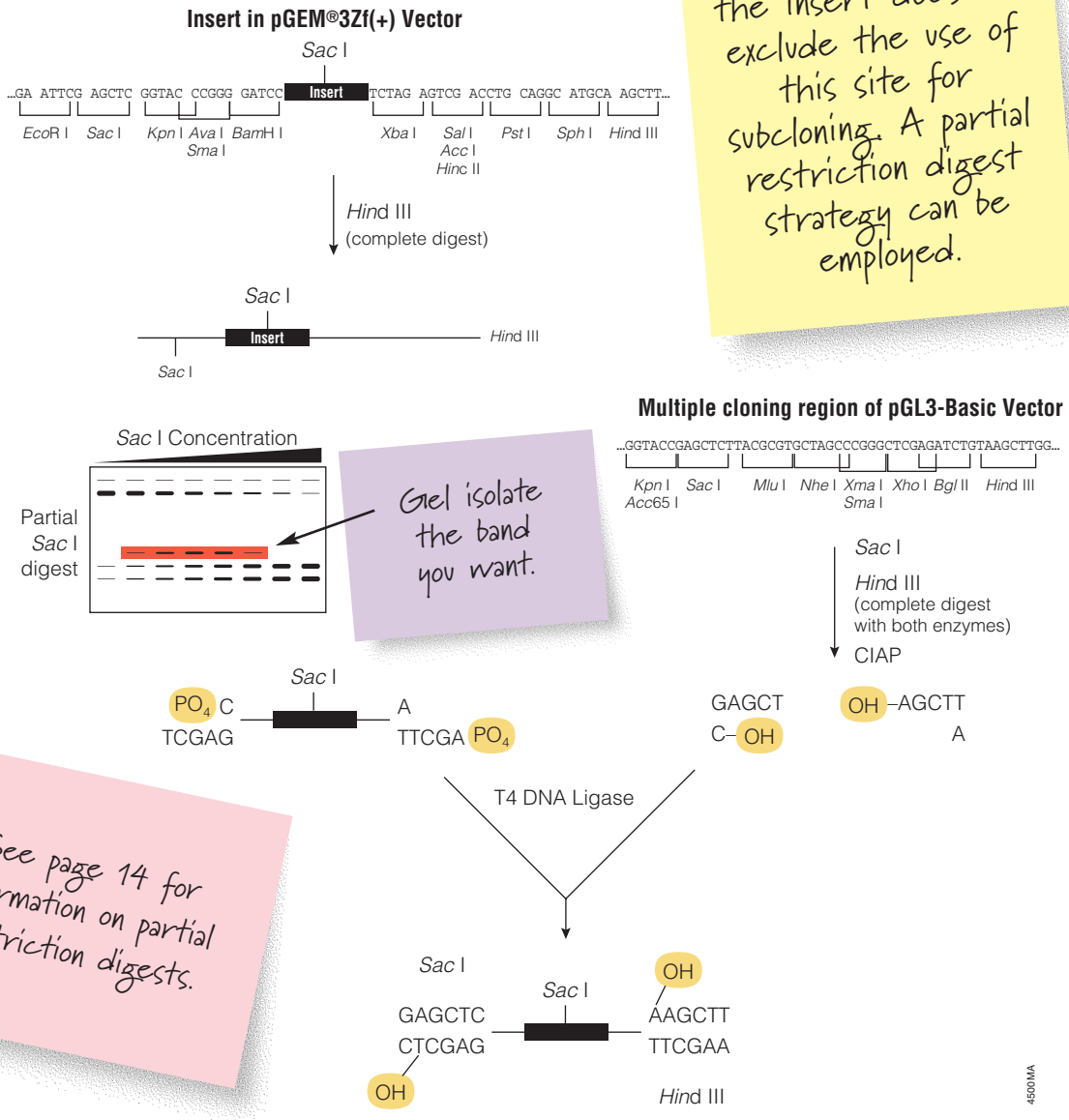
4499MA

See page 13 for information on double restriction enzyme digests.

See page 18 for information on dephosphorylating vectors.
See page 23 for information on ligating the insert and vector.

Subcloning Strategy: Common Restriction Sites with Partial Digests

Having a restriction site in both the multiple cloning region and the insert does not exclude the use of this site for subcloning. A partial restriction digest strategy can be employed.



Insert transferred from pGEM®-3Zf(+) Vector to pGL3-Basic Vector.

See page 14 for information on partial restriction digests.

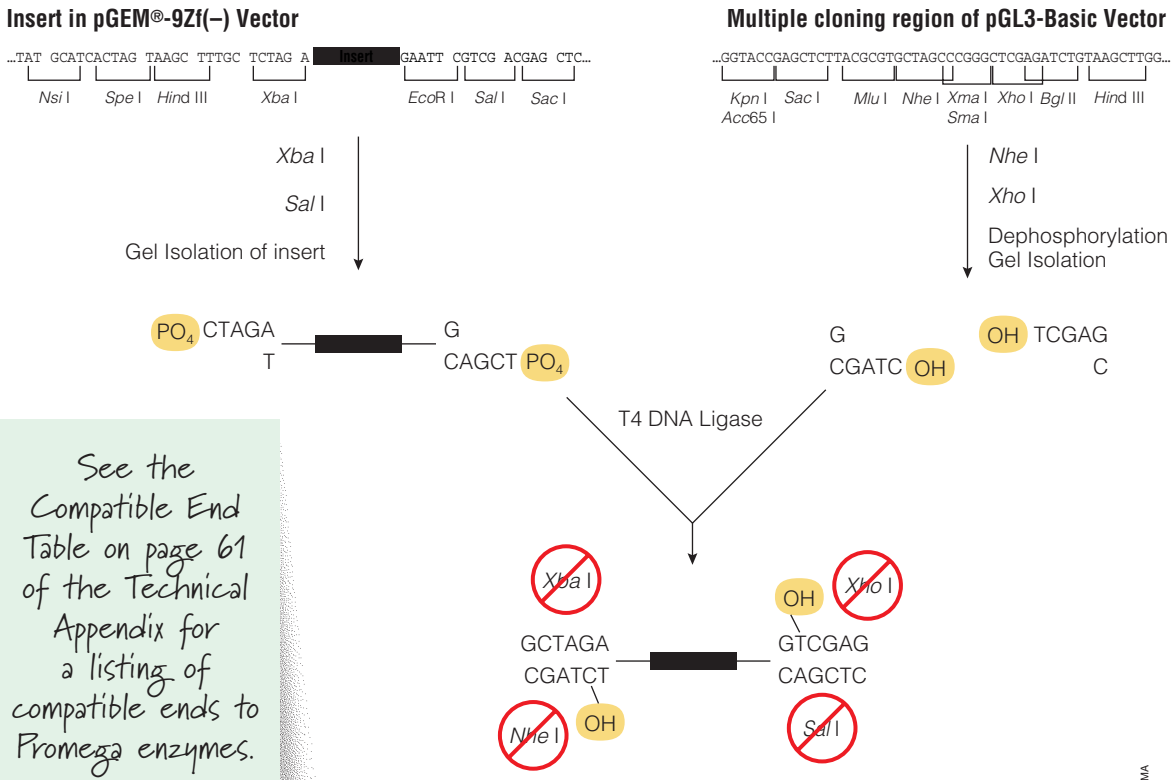
Subcloning Strategy: Moving Inserts with Compatible Restriction Sites

If you don't have common restriction sites in the parent and destination vector multiple cloning regions, you may have compatible restriction sites. Compatible restriction sites have the same overhang sequence and can be ligated together. In this example, *Xba* I and *Nhe* I both produce the same 5′ overhang sequence. Cut sites from these two are exactly matching and ligate well. However, neither the *Xba* I or *Nhe* I sites are regenerated in the ligation. A table of compatible ends is present on page 61 of this Notebook. Compatible end ligation is straightforward after the enzymes are identified.

Xba I or Spe I is compatible with the Nhe I site of the pGL3-Basic Vector.

Xba I and Sal I have better buffer compatibility for the double digest than Spe I and Sal I.

Sal I is compatible with the Xho I site in the pGL3-Basic Vector.



Insert transferred from pGEM-9Zf(–) Vector to pGL3-Basic Vector.

4501MA

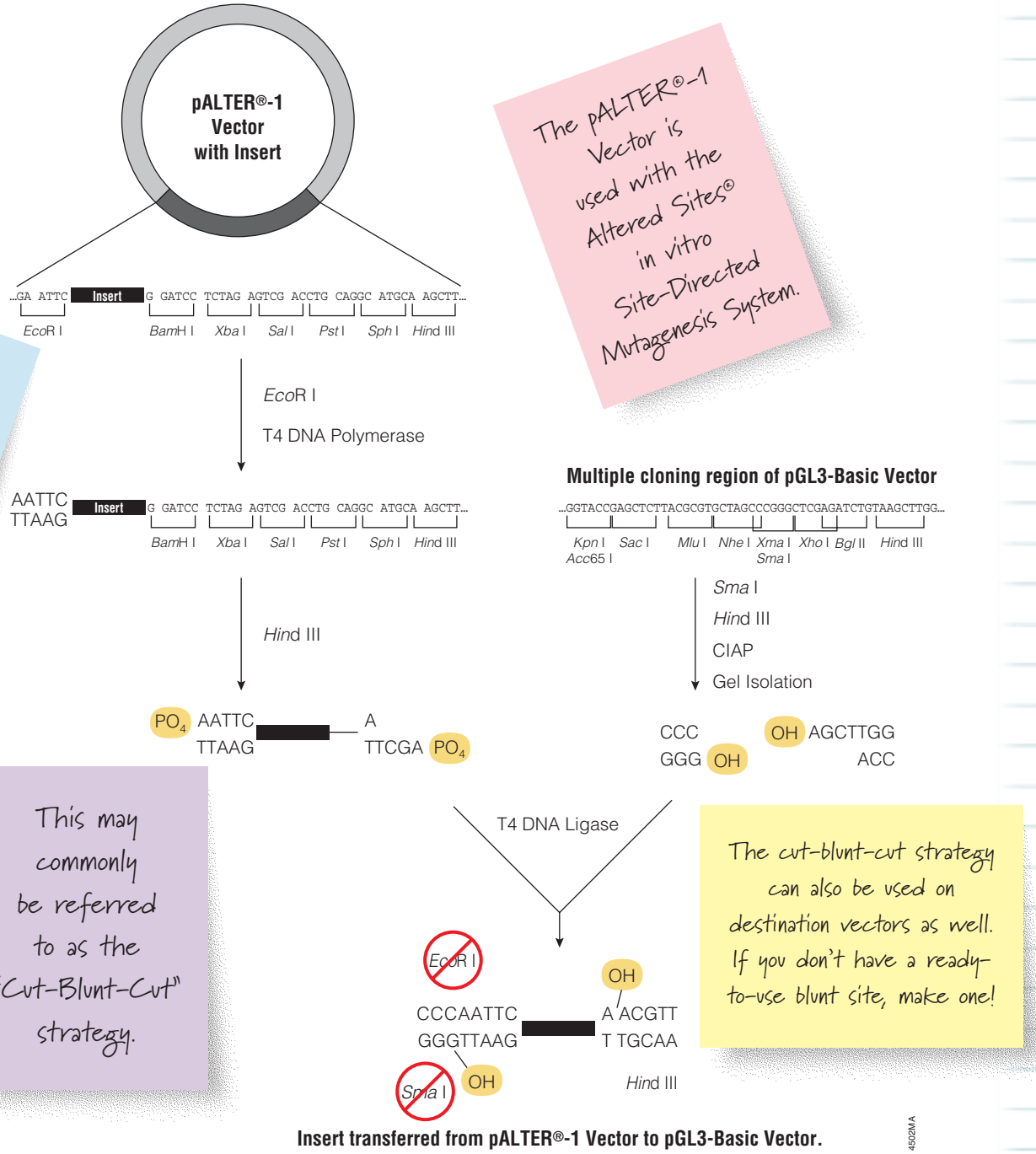
See the Compatible End Table on page 61 of the Technical Appendix for a listing of compatible ends to Promega enzymes.

In this example, none of the restriction sites used for the compatible-end subcloning are regenerated in the final ligation product.

Classic Subcloning

Subcloning Strategy: Moving Inserts with Only One Common Site

You've looked for common sites or compatible sites and you can find only one match on one side of your insert. What do you do about the other side of the insert? You can use a method commonly referred to as "cut-blunt-cut". Any restriction site can be made blunt through the action of T4 DNA Polymerase. Simply digest the parent vector and blunt that site with T4 DNA Polymerase (protocols on page 16), run the products on a gel, purify and proceed with the common or compatible end restriction enzyme digestion. In this example, the destination vector has *Sma* I site, which leaves a blunt end. Most vectors have at least one blunt-ended restriction site that can accept the newly created blunt end from the insert. If you don't have such a site or the site would not be in the correct orientation, the same "cut-blunt-cut" strategy may be applied to the destination vector as well.



Insert transferred from pALTER®-1 Vector to pGL3-Basic Vector.

4502MA

See page 16 for T4 DNA Polymerase procedure.

The pALTER®-1 Vector is used with the Altered Sites® in vitro Site-Directed Mutagenesis System.

This may commonly be referred to as the "Cut-Blunt-Cut" strategy.

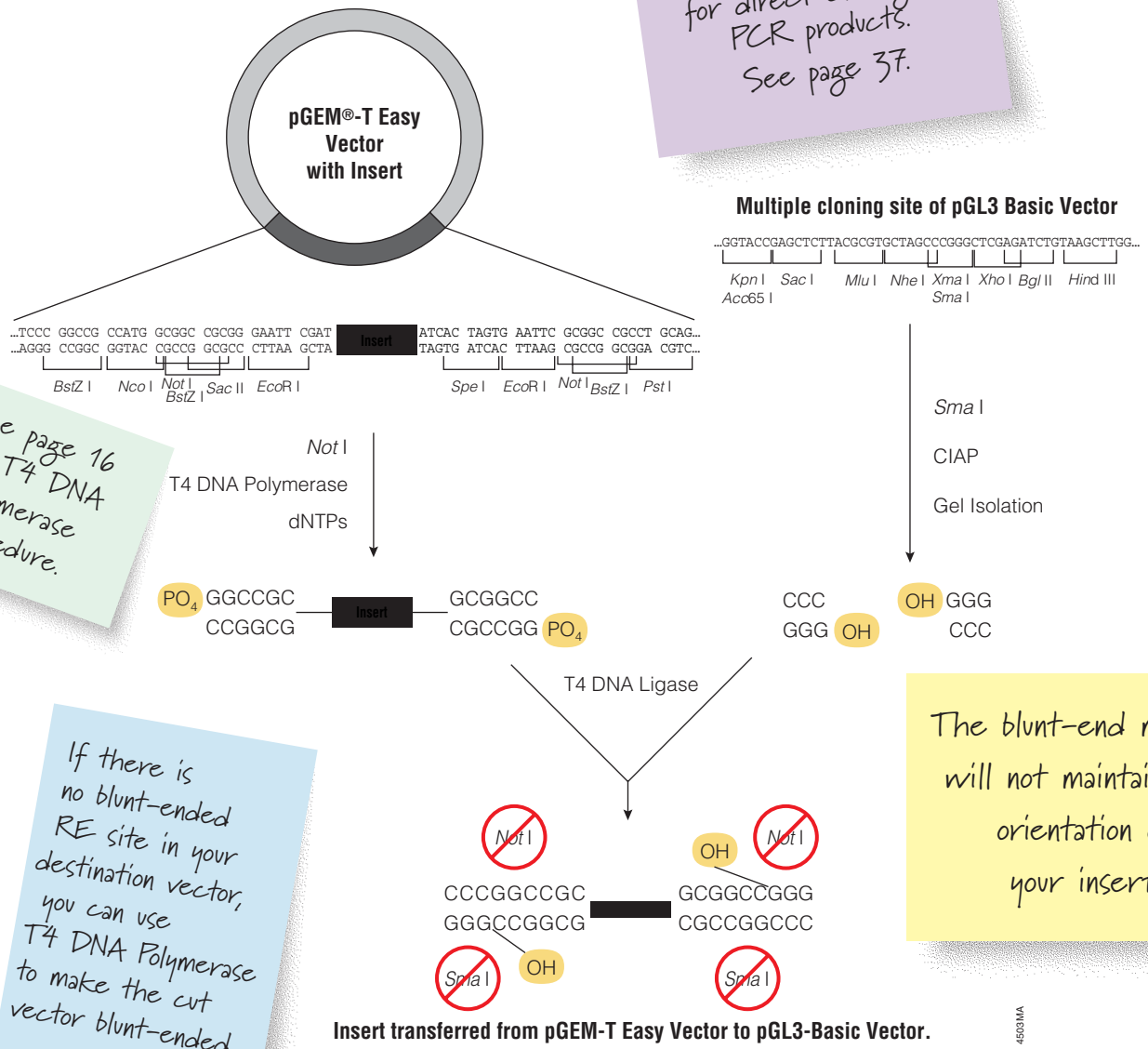
The cut-blunt-cut strategy can also be used on destination vectors as well. If you don't have a ready-to-use blunt site, make one!

Classic Subcloning

Subcloning Strategy: Blunt-End Method

You can't find a single common site or compatible site in the parent or destination vector. What do you do? Many people resort to amplifying the insert with restriction sites in the primers to provide the compatibility, but this strategy may cause some problems (i.e., introduction of mutations, difficulty digesting PCR products [see page 40]). Another method involves straight blunt-end cloning. You cut out your insert with whichever enzymes you desire. Treat with T4 DNA Polymerase to blunt either 5′ or 3′ overhangs and ligate into the destination vector opened with a blunt-end cutter or made blunt by T4 DNA Polymerase. Remember though, this method will not retain orientation of your insert so you will have to screen for orientation by methods like those outlined on page 50.

The pGEM-T Easy Vector is designed for direct cloning of PCR products. See page 37.



Insert transferred from pGEM-T Easy Vector to pGL3-Basic Vector.

4503MA

See page 16 for T4 DNA Polymerase procedure.

If there is no blunt-ended RE site in your destination vector, you can use T4 DNA Polymerase to make the cut vector blunt-ended.

The blunt-end method will not maintain the orientation of your insert.

Classic Subcloning

Restriction Digestion

Restriction endonucleases (RE), also referred to as restriction enzymes, are proteins that recognize short, specific (often palindromic) DNA sequences. Type II REs cleave double-stranded DNA (dsDNA) at specific sites within or adjacent to their recognition sequences. Many restriction enzymes will not cut DNA that is methylated on one or both strands of the recognition site, although some require substrate methylation (see page 62).

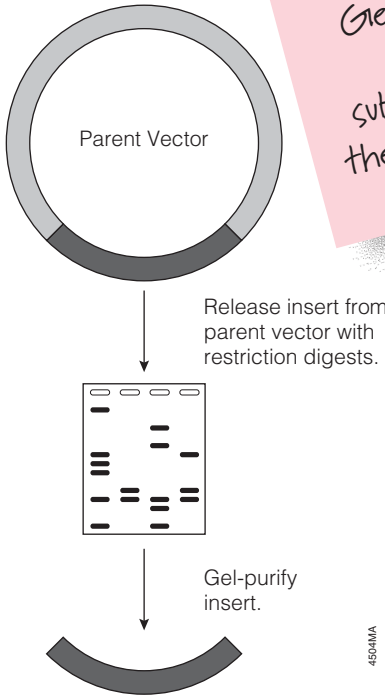
Restriction digestion is one of the most common reactions performed in molecular biology. For a digestion with a single RE the reaction is very simple:

Nuclease-Free Water	14µl
10X Restriction Buffer	2µl
Acetylated BSA (1mg/ml)	2µl
DNA (~1µg)	1µl
Restriction Enzyme (10u)	1µl
Final Volume	**20µl**

Mix by pipetting and collect the contents at the bottom of the tube. Incubate at the appropriate temperature for the enzyme for 1–4 hours. Add 4µl of 6X Blue/Orange Loading Dye and analyze digested DNA by gel electrophoresis.

Preparing an insert for transfer from one vector to another usually requires a **double digest** (digest with two different REs). If both restriction enzymes work in the same restriction enzyme buffer, the reaction is straight-forward. **Simply add 1µl of the second restriction enzyme and adjust the amount of water used**.

Remember, restriction enzymes are commonly stabilized in 50% glycerol solution. Do not exceed 5% glycerol in final digest with the two enzymes. Glycerol concentrations >5% may lead to star activity (see page 63).



Gel isolation is a necessity in subcloning. You get the insert you need.

Learn more about the history and enzymology of restriction enzymes with the Promega Restriction Enzyme Resource located at: www.promega.com/guides

Look at these search tools to help you plan your experiments. www.promega.com/guides/re_guide/default.htm

Restriction Digestion

What is supplied with Promega Restriction Enzymes?

Each RE has specific requirements for optimal activity. Ideal storage and assay conditions favor the highest activity and highest fidelity in a particular enzyme's function. Conditions such as temperature, pH, enzyme cofactors, salt composition and ionic strength affect enzyme activity and stability.

Each Promega Restriction Enzyme is supplied with:

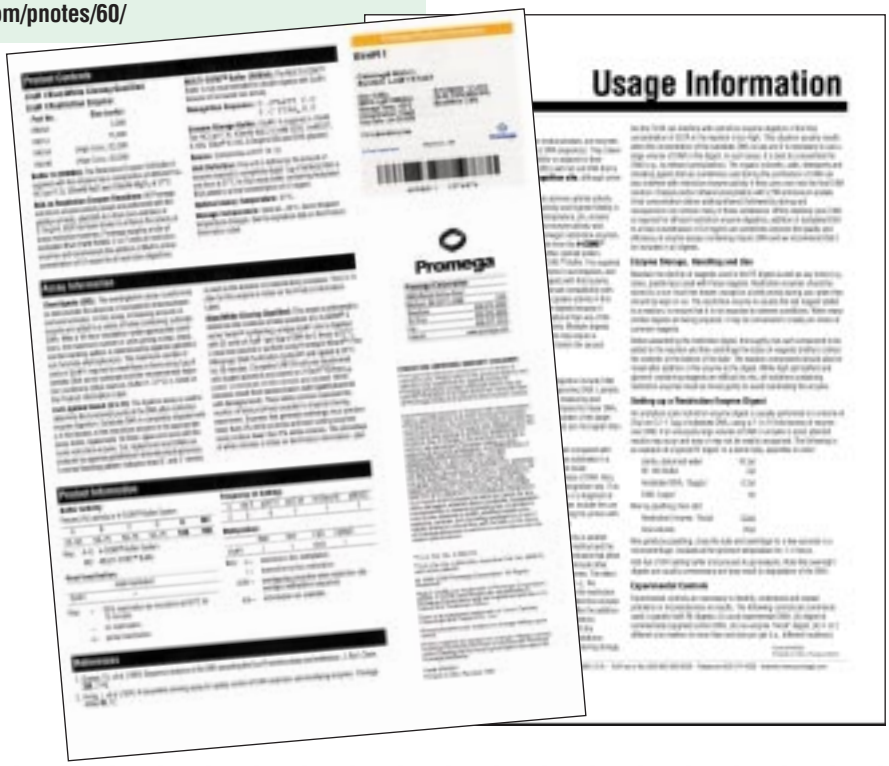
- The optimal reaction buffer
  This may be from the 4-CORE® System (Reaction Buffers A, B, C, D) or one of the other optimal buffers (Reaction Buffers E–L). This buffer always yields 100% activity for the enzyme that it accompanies, and serves as the specific reaction buffer for single digests.
- MULTI-CORE™ Buffer
  This is designed for broad compatibility and is provided with enzymes that have 25% or greater activity in this buffer. The MULTI-CORE™ Buffer is useful for multiple digests because it generally yields more activity for more enzyme combinations than any of the other buffers, but sometimes using the MULTI-CORE™ Buffer can compromise enzyme activity. Multiple digests using REs with significantly different buffer requirements may require a sequential reaction with the addition of RE buffer or salt before the second enzyme is used.
- 100X Acetylated BSA
  We recommend adding 0.1mg/ml acetylated BSA to every reaction. The acetylated BSA improves the stability of the enzyme in the reaction.

For more information on the use of acetylated BSA in restriction digests, see "BSA and Restriction Enzyme Digestions" in *Promega Notes* **60** at:
www.promega.com/pnotes/60/

For a recent review on restriction enzymes see:
Williams, R.J. (2003) Restriction Endonucleases: Classification, properties and applications. Mol. Biotechnol. **23,** 225–43.

Easily locate usage and lot information

Each enzyme comes with a Promega Product Information Sheet (PPI) that contains details of quality control assays performed, lot-specific information and usage information. The sheet also has protocol information and references. The lot-specific information is printed on a removable sticker that can be pasted into a notebook or logbook, making your record keeping easier.



Restriction Digestion: Other Considerations

Do both enzymes work at the same temperature?

The majority of restriction enzymes work best at 37°C, but those isolated from thermophilic bacteria require higher temperatures for maximal activity (e.g., *Bst*X I and *Bst*Z I work best at 50°C). Some work below 37°C like *Sma* I (25°C) and *Csp* I (30°C). If you must work with two enzymes with different optimum temperatures, you can use the sequential digest method (assemble all components, perform for the lower-temperature digest first, then digest at the higher temperature second). Usually an hour at each temperature will work fine.

When working with an enzyme that requires a temperature above 37°C, evaporation of the reaction can lead to increased glycerol concentration, which can in turn lead to star activity. Evaporation can be avoided in such reactions by applying a few drops of molecular biology grade mineral oil above the reaction. Clean up with the Wizard® SV Gel and PCR Clean-Up System to remove the mineral oil and recover the pure DNA.

See the tables on pages 57–58 for optimal reaction temperatures of Promega Restriction Enzymes.

Do my enzymes exhibit methylation sensitivity?

An often overlooked reason for a restriction enzyme failure is sensitivity to *dam* and *dcm* methylation. Many common bacterial strains like JM109, XL1-Blue, and DH5α™ are positive for these two genes. The *dam* gene encodes a DNA adenosine methylase that methylates the N6 position of the adenine residue in the sequence: 5′…GATC…3′, a common sequence within many restriction sites. The *dcm* gene encodes a DNA cytosine methylase that methylates the C5 position of the internal cytosine residue in the sequence: 5′…CCAGG…3′. Some restriction enzymes are sensitive to these methylations and will not cut their recognition sequence if the methylation occurs within the recognition site (e.g., *Bcl* I and *dam* methylation) or overlaps the recognition site (e.g., the ATCGAT recognition site falling within the context of …**G**ATCGAT… or …ATCGAT**C**…for *dam* methylation).

See the table on page 62 for methylation sensitivities of Promega Restriction Enzymes.

Need to digest a piece of DNA with a dam or dcm sensitive enzyme?

- Check to see if the enzyme has an isoschizomer or neoschizomer. The isoschizomer or neoschizomer may not be sensitive to the methylation.
- Transform the plasmid into a dam/dcm minus bacterial strain like JM110.

See the tables on pages 59–60 for listings of isoschizomers and neoschizomers.

Double Enzyme Digests

Double Digests with a Common Buffer

In many cases, the enzymes are not supplied with the same reaction buffer, and another buffer may be appropriate. In these cases, activities in other buffers must be assessed by consulting buffer activity charts like those on pages 57–58. In this chart, all Promega REs are tested in Buffers A, B, C, D and MULTI-CORE™ Buffers. Promega Blue/White Cloning-Qualified REs are also assayed in Buffers E and H. Ideally you want to choose a buffer in which each enzyme retains at least 75%. For instance, if you were to perform a double digest with *Eco*R I (optimal in Buffer H) and *Bam*H I (optimal in Buffer E) you would choose in Buffer E because the *Bam*H I has 100% activity and *Eco*R I has 75–100% activity. Both enzymes will maintain acceptable levels of activity in this buffer. Promega has developed an online restriction enzyme compatible buffer search engine available at: **www.promega.com/guides/re_guide/** to assist you in finding the right buffer for double digests with all Promega Restriction Enzymes.

A table that describes activity of Promega REs in Promega Restriction Buffers is located on pages 57–58 of this notebook and in the Promega Catalog Appendix.

Compare conditions for two Promega REs quickly online. See the RE resource tools at: www.promega.com/techserv/tools

Double Digests without a Common Buffer

Some enzymes just do not partner well [e.g., double digest with *Pst* I (optimal in Buffer H) and *Spe* I (optimal in Buffer B)]. A review of the tables on pages 57–58 shows that the best-case scenario is provided by Buffer B. *Spe* I of course is optimal in B (100%) but *Pst* I has only 50–75% activity. Three choices are available.

Sequential Method: Perform sequential digests: First digest with *Spe* I in Buffer B, purify DNA, and then perform the *Pst* I digest in Buffer H.

Incubate Longer: Assemble the reaction as usual in Buffer B and incubate 2–4 hours.

Add More Enzyme: Add 1.5–2.0µl of *Pst* I and incubate 1–2 hours.

All three methods work. The first scenario seems intensive, but systems like the Wizard® SV Gel and PCR Clean-Up System make the process very easy (see page 28). The entire reaction can be cleaned and eluted in 15µl of water, and the buffer, enzyme and BSA can be added to bring the reaction to 20µl for the second optimal digest. This is really your only option if both enzymes have no compatibility (i.e., activity in buffer less than 25%).

The second and third methods may provide alternatives to performing sequential digests, depending on the enzymes involved. The second method simply takes more time. The activities in the tables on pages 57–58 are based on a 1-hour incubation. Longer incubation can improve the percent cleavage of the template. This is useful if the two enzymes have a buffer capable of at least 50% activity for both enzymes. The third method is tricky, especially if one of the enzymes is prone to star activity in higher glycerol concentrations. Remember, restriction enzymes are usually stabilized by 50% glycerol so they do not freeze in –20°C storage. Star activity (see page 63) may occur when the digestion glycerol concentration in the reaction rises above 5%. This method is usually only acceptable for two enzymes that have more than 50% activity in the same buffer.

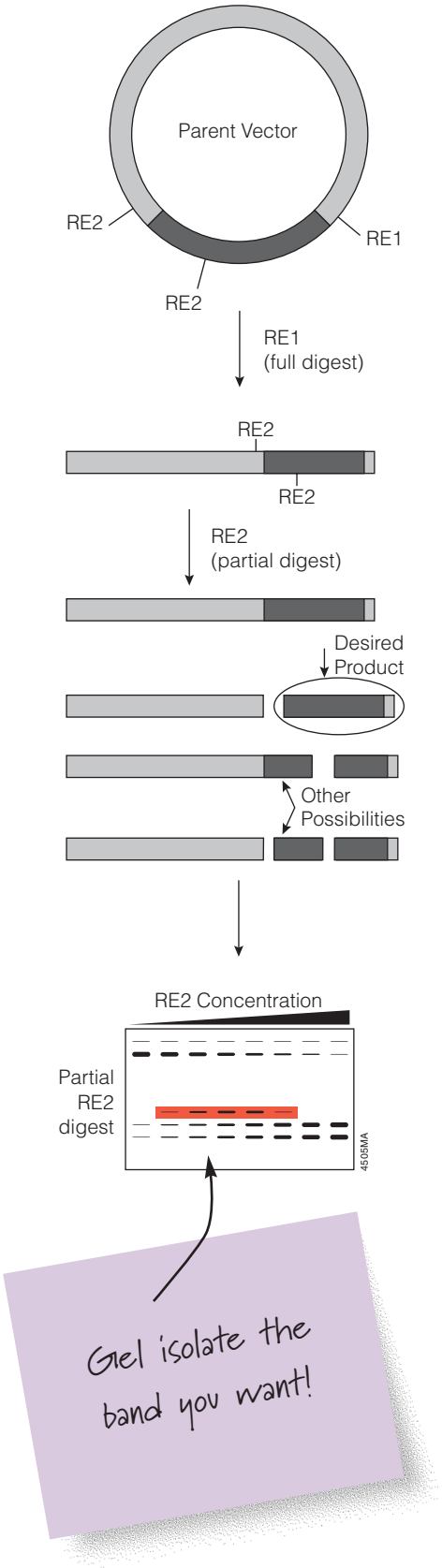
Partial Restriction Digestion

Controlling Cut Frequency in Restriction Digestion

The presence of a restriction recognition site in the insert and the multiple cloning region does not necessarily preclude use of that restriction site in a subcloning strategy. Under normal restriction digest conditions, the enzyme is in excess so that all recognition sites in the plasmid can be cleaved. You can manipulate the restriction digest conditions such that you will digest only a subset of sites. Many strategies have been employed to do partial digests: Decreasing reaction temperature, using a non-optimal buffer, and decreasing units of enzyme. The method presented here uses dilutions of enzyme in the optimal buffer.

A key to doing partial digests is to have a way in which you can differentiate partial digests from complete digests. In other words, you must have a discernable base pair-size difference on the agarose gel so you can cut out the band and perform gel isolation to purify the fragment for ligation into the destination vector. In the following example, the parent vector is first linearized and a partial digest performed on the linearized vector.

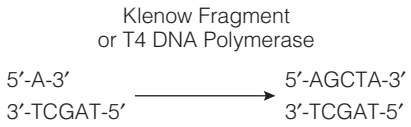
1. Digest 10µg of parent vector to completion to linearize (i.e., RE1; 50µl reaction).
2. Purify vector with the Wizard® SV Gel and PCR Clean-Up System directly from the reaction. Elute in 20µl nuclease-free water.
3. On ice, create serial dilutions of RE2 in 1X RE Buffer containing 0.1mg/ml Acetylated BSA (e.g. to yield 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039u of RE per 18µl of solution).
4. Add 2µl of the purified vector to each tube.
5. Incubate all reactions at 37°C for 30–45 minutes.
6. Add loading dye to each reaction and analyze digests by agarose gel electrophoresis.
7. Identify and cut bands from the gel containing the DNA fragment of interest.
8. Purify insert using the Wizard® SV Gel and PCR Clean-Up System. Elute in 15–20µl nuclease-free water.
9. Proceed to ligation reaction.



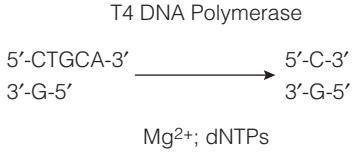
Creating Blunt Ends

Turning an Overhang into a Blunt End

Occasionally you encounter a subcloning application where the choice of restriction sites you can use is limited or where no restriction sites exist in common between vectors and insert. Blunt-ended ligation is an option in these situations. Most vectors contain a blunt cutter like *Eco*R V or *Sma* I in the multiple cloning region, but the parent vector containing your insert may not contain a blunt-cutter site. A blunting reaction can come in handy. Two enzymes are commonly used to generate blunt ends: T4 DNA Polymerase (see page 16) and the Klenow Fragment of DNA Polymerase I (see page 17). The T4 DNA Polymerase is useful for blunting both 5′ and 3′ overhangs. Klenow works best with 5′ overhangs.



5′ Overhang Fill-In Reaction



3′ Overhang Blunting Reaction

4506MA

Creating Blunt Ends

Blunting a 5′ Overhang

T4 DNA Polymerase Method

T4 DNA Polymerase has excellent activity in Promega Restriction Enzyme Buffers B, C, E, and MULTI-CORE™, displaying more than 70% activity. The protocol below is for an integrated blunting reaction following the restriction digestion, and has been tested with the buffers listed above. The following protocol works from a 50µl digestion. The 50µl digestion is recommended to reduce the concentration of glycerol coming from both the restriction enzymes and the T4 DNA Polymerase. Reducing the glycerol concentration prevents potential star activity that may be associated with some restriction enzymes.

1. Digest DNA (0.5–2.0µg) in a 50µl volume.*
2. Add 5u of T4 DNA Polymerase/µg DNA.
3. Add dNTPs to a final concentration of 100µM (e.g., 0.5µl of dNTP Mix [Cat.# U1511]).
4. Incubate at 37°C for 10 minutes.
5. Purify DNA with the Wizard® SV Gel and PCR Clean-Up System direct purification protocol. If both ends of the DNA are being blunted in this reaction, use gel electrophoresis followed by the gel purification protocol to purify the DNA from the enzymes.

*Restriction digest should contain 0.1µg/µl acetylated BSA.

Blunting a 3′ Overhang

T4 DNA Polymerase Method

T4 DNA Polymerase has excellent activity in Promega Restriction Enzyme Buffers B, C, E, and MULTI-CORE™, displaying more than 70% activity. The protocol below is for an integrated blunting reaction following the restriction digestion and has been tested with the buffers listed above. The following protocol works from a 50µl digestion. The 50µl digestion is recommended to reduce the concentration of glycerol coming from both the restriction enzymes and the T4 DNA Polymerase. Reducing the glycerol concentration prevents potential star activity that may be associated with some restriction enzymes.

1. Digest DNA (0.5–2.0µg) in a 50µl volume.*
2. Add 5u of T4 DNA Polymerase/µg DNA.
3. Add dNTPs to a final concentration of 100µM (e.g., 0.5µl of dNTP Mix [Cat.# U1511]).
4. Incubate at 37°C for 5 minutes.
5. Purify DNA with the Wizard® SV Gel and PCR Clean-Up System direct purification protocol. If both ends of the DNA are being blunted in this reaction, use gel electrophoresis followed by the gel purification protocol to purify the DNA from the enzymes.

*Restriction digest should contain 0.1µg/µl acetylated BSA.

T4 DNA Polymerase

Cat.# M4211 100u 5–10u/µl

Cat.# M4215 500u 5–10u/µl

See the Product Information Sheet at: www.promega.com/tbs

Note: With high concentrations of dNTPs (i.e., 100µM), degradation of the DNA will stop at the duplex DNA. However, if the dNTPs are exhausted, the highly active exonuclease activity (200 times more active than that of DNA polymerase I) of T4 DNA Polymerase will degrade the dsDNA.

Creating Blunt Ends

Blunting a 5′ Overhang

Klenow Polymerase Method

Following the restriction enzyme digestion that generated the 5′-protruding ends, purify the DNA from the reaction with a system like the Wizard® SV Gel and PCR Clean-Up System (see page 28 for more information).

1. Assemble the following reaction:

DNA template	1–4µg
10X Klenow Buffer	2µl
Acetylated BSA (10µg/µl)	0.2µl
dNTPs (1mM each)*	0.8µl
Klenow Polymerase	1µl
Nuclease-Free Water	to 20µl

* A 1:10 dilution of the dNTP Mix (Cat.# U1511) in water.

2. Incubate at ambient room temperature for 10 minutes.
3. Purify the DNA from the reaction using the Wizard® SV Gel and PCR Clean-Up System with the direct purification protocol. If both ends of the DNA are being blunted in this reaction, use gel electrophoresis followed by the gel purification protocol.

Note: Promega Restriction Enzyme Buffers A, B, C, D, E, and H may be substituted for the 10X Klenow Buffer, but polymerase activity is 27–43% of the 10X Klenow Buffer.

Note: This method will not work for 3′ overhangs.

DNA Polymerase I Large (Klenow) Fragment

Cat.# M2201 150u 5–10u/µl

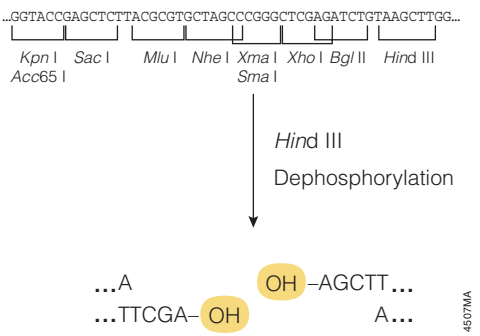
Cat.# M2206 500u 5–10u/µl

See the Product Information Sheet at: www.promega.com/tbs

Dephosphorylating Vectors to Limit Self-Ligation

Preventing vector self-ligation is critical for reducing subcloning background. The efficiency of ligating the plasmid to itself is far better than ligating a separate piece of DNA into the vector and is the favored reaction. Removing the 5′ phosphates of the linearized vector will prevent T4 DNA Ligase from recircularizing the vector. Calf Intestinal Alkaline Phosphatase is the classic enzyme for vector dephosphorylation. The enzyme can be used on 5′ recessed ends (i.e., results from an enzyme leaving a 3′ overhang), 5′ overhangs and blunt-ends. After dephosphorylation, the enzyme must be removed either by direct purification or gel electrophoresis and gel isolation with DNA purification systems like the Wizard® SV Gel and PCR Clean-Up System. Shrimp Alkaline Phosphatase can be used in place of Calf Intestinal Alkaline Phosphatase and offers the advantage of simple heat denaturation to inactivate the enzyme without the need for further purification.

Multiple cloning region of pGL3-Basic Vector



Dephosphorylation can reduce the chance of vector self-ligation to virtually zero.

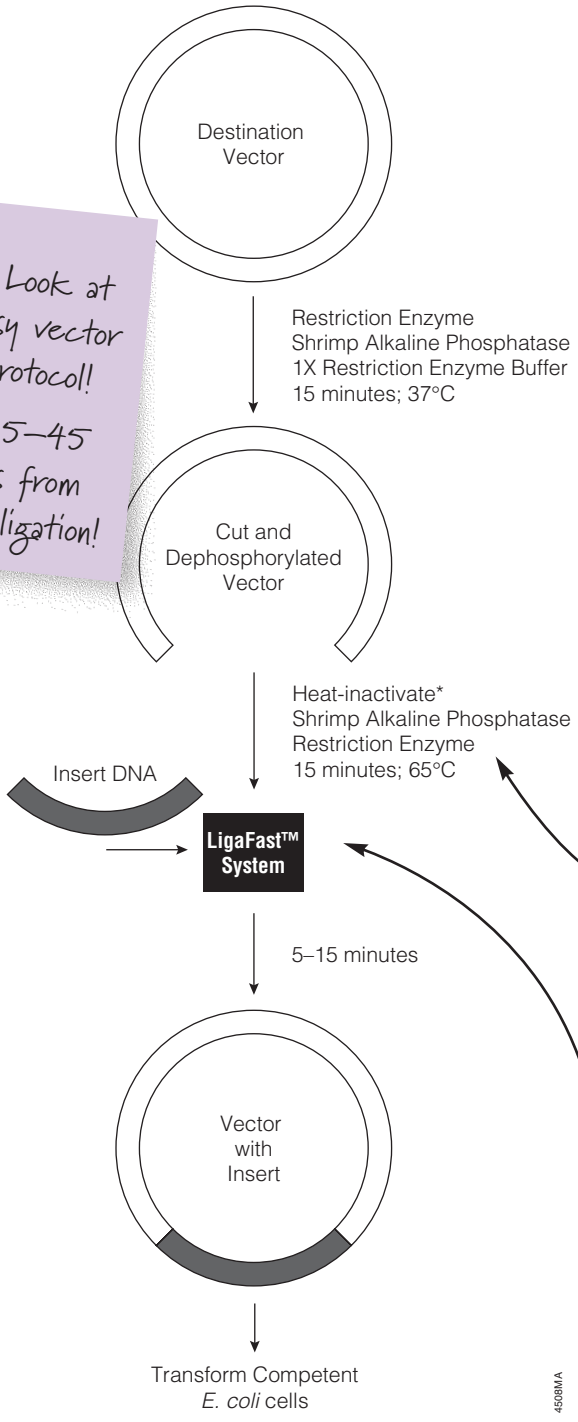
Is it necessary to dephosphorylate linearized vectors before performing the insert ligation?

If the plasmid vector being used was linearized with a single restriction enzyme (generating either a blunt or overhanging end), then dephosphorylation of the vector is a prerequisite to reduce religated vector background. However, if the vector was cut with two different restriction enzymes that leave incompatible ends (this does not include two different enzymes that each leave blunt ends), then dephosphorylation may be omitted. One exception to this is when the selected restriction sites lie close to one another in the vector. In this case, it is still advisable to dephosphorylate the vector, because you cannot be certain from looking at the digested plasmids on the gel if both enzymes cut the plasmid to completion. The presence of a small amount of singly cut plasmid vector in the subsequent ligation reaction can dramatically increase background, which could make it difficult to identify your desired recombinant.

Gel purification of the processed destination vector before ligation ensures that uncut and partially cut vectors are removed from the subcloning reaction.

Classic Subcloning

Dephosphorylating Vectors: Shrimp Alkaline Phosphatase



* Not all restriction enzymes can be heat-inactivated.

Wow! Look at this easy vector prep protocol! Only 35–45 minutes from start to ligation!

Streamlined Restriction Digestion, Dephosphorylation and Ligation Procedure

1. Combine restriction digestion and dephosphorylation of DNA vector in 1X restriction enzyme buffer. Use 15 units of restriction enzyme/µg vector and 10 units Shrimp Alkaline Phosphatase (SAP)/µg vector in a final volume of 30–50µl. Incubate at 37°C for 15 minutes. This is a sufficient amount of SAP to completely dephosphorylate the vector regardless of overhang type (5′, 3′, or blunt) in any Promega RE buffer.
2. Heat-inactivate both restriction enzyme and SAP for 15 minutes at 65°C.
   Note: Not all restriction enzymes can be heat inactivated (see pages 57–58).
3. Centrifuge and remove 1–2µl of vector for ligation with appropriate DNA insert using T4 DNA Ligase and 2X Rapid Ligation Buffer from LigaFast™ Rapid DNA Ligation System at 15°C for 5 minutes (3′ or 5′ ends) or 15 minutes for blunt ends in a final reaction volume of 10–50µl. We recommend starting with a 1:2 molar ratio of vector:insert DNA.
4. Transform the ligated material directly into competent *E. coli* cells.

If your restriction enzyme cannot be heat-inactivated, use the Wizard® SV Gel and PCR Clean-Up System for direct purification. Full purification in just 15 minutes, and you can elute the DNA in as little as 15µl of water.

Contains the Promega Blue/White Cloning–Qualified T4 DNA Ligase and 2X Rapid Ligation Buffer.

Five-minute ligations for sticky ends; 15-minute ligations for blunt ends.

Dephosphorylating Vectors: Shrimp Alkaline Phosphatase

Dephosphorylation of Purified DNA

1. Purify vector from restriction digest using the Wizard® SV Gel and PCR Clean-Up System.
2. Combine the following:

DNA (1–2µg)	Xµl
10X SAP Buffer	3–5µl
SAP (1u/µl)	1µl/µg DNA
Nuclease-Free Water	to 30–50µl

3. Incubate at 37°C for 15 minutes (works for both 5′ and 3′ overhangs or blunt ends).
4. Inactivate SAP by heating to 65°C for 15 minutes or purify with the Wizard® SV Gel and PCR Clean-Up System. Proceed to ligation.

Shrimp Alkaline Phosphatase
Cat.# M8201
500u
1u/µl

See the Product Information Sheet at:
www.promega.com/tbs

SAP Activity in Promega RE Buffers

Buffer	% Activity of SAP
A	20%
B	20%
C	25%
D	35%
E	20%
F	60%
G	30%
H	30%
J	30%
K	20%
L	30%
MULTI-CORE™ Buffer	10%

This protocol is designed to handle most situations with 5′, 3′ and blunt ends on the DNA.

Below are the minimal unit requirements for the various ends in 1X SAP Buffer:

5′ Overhang: 0.015u SAP/pmol ends

Blunt Overhang: 0.03u SAP/pmol ends

3′ Overhang: 0.4u SAP/pmol ends

Using the protocol above with MULTI-CORE™ Buffer in place of SAP Buffer and blunt-ended ligation, greater than 90% of the transformants contained inserts.

Dephosphoylating Vectors: Calf Intestinal Alkaline Phosphatase

Dephosphorylation Immediately After Restriction Digestion

1. Add the following components directly to the digested DNA. The CIAP may be diluted on ice in 1X CIAP Buffer immediately before use. Discard any unused, diluted enzyme.

CIAP 10X Reaction Buffer	10µl
CIAP (0.01u/pmol of ends*)	1–2µl
Nuclease-Free Water	to 100µl

   *For pmol of ends, simply multiply the pmol of DNA by 2. For example, 1µg of a 1kb DNA fragment will convert to 1.52pmol of DNA and converts to 3pmol of ends.

   Note: Dilution of the standard CIAP (1u/µl) is not absolutely necessary, but these are the conditions under which we test the enzyme.

2. Incubate using one of the following conditions, depending on the type of ends present:

   5′ Overhangs: Incubate for 30 minutes at 37°C. Add another 0.01u CIAP/pmol ends and incubate an additional 30 minutes at 37°C.
   3′ Overhangs or Blunt Ends: Incubate for 15 minutes at 37°C, then for 15 minutes at 56°C. Add another 0.01u CIAP/pmol ends and repeat incubations at both temperatures.

3. Purify DNA using the Wizard® SV Gel and PCR Clean-Up System and proceed to ligation.

The CIAP Buffer must be added to the reaction for efficient dephosphorylation. The diluted CIAP needs the Zn^{2+} from the buffer to work effectively.

Calculating pmol of DNA from micrograms of DNA.

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

N is the number of nucleotides and 660pg/pmol is the average molecular weight of a nucleotide pair.

Online calculators for this equation and many other useful equations are available on the Promega BioMath page: www.promega.com/biomath

Dephosphorylating Vectors: Calf Intestinal Alkaline Phosphatase

Dephosphorylation of Purified DNA

1. Dilute sufficient 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.
2. Assemble the following reaction:

DNA (up to 10pmol of ends)	40µl
CIAP 10X Reaction Buffer	5µl
diluted CIAP (0.01u/µl)	up to 5µl
Nuclease-Free Water to	50µl

   See previous page for calculation of pmol of ends.

   Note: Diluting the standard CIAP (1u/µl) is not absolutely necessary, but these are the conditions under which we test the enzyme.
3. Incubate using one of the following conditions, depending on the type of ends present:

   5′ Overhangs: Incubate for 30 minutes at 37°C, add another 0.01u/pmol of ends of CIAP and repeat incubation.

   3′ Overhangs or Blunt Ends: Incubate for 15 minutes at 37°C then for 15 minutes at 56°C. Add another 0.01u CIAP/pmol ends and repeat incubations at both temperatures.
4. Purify DNA using the Wizard® SV Gel and PCR Clean-Up System and proceed to ligation.

Alkaline Phosphatase, Calf Intestinal
Cat.# M1821 — 1,000u, 1u/µl
Cat.# M2825 — 1,000u, 20u/µl
See the Product Information Sheet at: www.promega.com/tbs

Calf Intestinal Alkaline Phosphatase must be removed prior to the ligation reaction. The Wizard® SV Gel and PCR Clean-Up System can do the purification in 15 minutes, and the dephosphorylated vector can be eluted from the membrane in as little as 15µl of water.

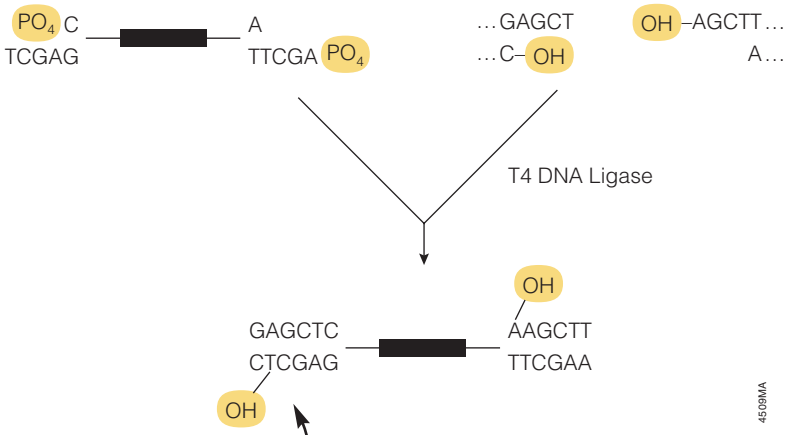
Ligation: Ligating Vector and Insert

Molecular biologists have exploited DNA ligases to insert pieces of DNA into vectors for decades. The enzyme most commonly used is derived from bacteriophage T4. T4 DNA Ligase is about 400-fold more active than *E. coli* DNA ligase for ligating blunt ends, and thus is the enzyme of choice for all molecular biology requirements. Promega offers **T4 DNA Ligase** in standard or high-concentrate form (see page 25), with the standard Ligase Buffer or with the 2X Rapid Ligation Buffer offered in the **LigaFast™ Rapid DNA Ligation System** (see page 24). The LigaFast™ System allows rapid, 5-minute ligations for 5′ or 3′ overhang cohesive ends or 15-minute ligations for blunt ends.

How Does DNA Ligase Work?

DNA ligases are responsible for joining gaps that form in DNA during replication, DNA repair and recombination (1). DNA ligases catalyze the formation of a phosphodiester bond between adjacent nucleotides with the concomitant hydrolysis of ATP to AMP and inorganic phosphate. DNA ligases will only form this covalent linkage in a duplex molecule (e.g., at a nick in dsDNA or when joining cohesive- or blunt-ended dsDNAs; 2). The ligation mechanism occurs in three stages. First is the formation of an enzyme-nucleotide intermediate through transfer of an adnenylyl group (AMP) from ATP to the ε-amine group of a lysine residue in the enzyme. This results in the release of pyrophosphate from ATP. Second, the adenylyl group is transferred from the enzyme to the 5′-phosphate of the DNA, thereby activating it. Third, a phosphodiester bond is formed by nucleophilic attack of the 3′-hydroxyl group of the DNA with concomitant release of AMP.

1. Okazaki, R. *et al.* (1968) *Proc. Natl. Acad. Sci. USA* **59**, 598.
2. Higgins, N.P. and Cozzarelli, R. (1989) In: *Recombinan DNA Methodology* Wu, R., Grossman, L. and Moldave, K., eds. Academic Press, Inc., San Diego, California.



These nicks will be repaired within the host bacteria upon transformation.

Ligation

LigaFast™ Rapid DNA Ligation System

We recommend starting with a 1:2 molar ratio of vector:insert DNA when cloning a fragment into a plasmid vector. The following example illustrates the conversion of molar ratios to mass ratios for a 3.0kb plasmid and a 0.5kb insert DNA fragment

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

Example:

How much 0.5kb insert DNA should be added to a ligation in which 100ng of 3kb vector will be used? The desired vector:insert ratio will be 1:2.

$$\frac{100\text{ng vector} \times 0.5\text{kb insert}}{3\text{kb vector}} \times \frac{2}{1} = 33.3\text{ng insert}$$

The following ligation reaction of a 3kb vector and a 0.5kb insert DNA uses the 1:2 vector:insert ratio. Typical ligation reactions use 100–200ng of vector DNA.

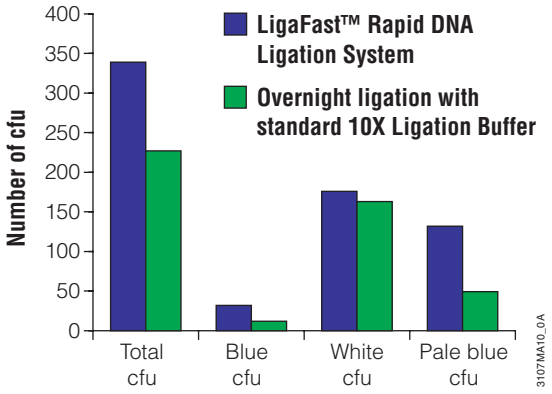
1. Assemble the following reaction in a sterile microcentrifuge tube:

vector DNA	100ng
insert DNA	33ng
2X Rapid Ligation Buffer	5µl
T4 DNA Ligase (3u/µl)	1µl
nuclease-free water to	10µl

2. Incubate the reaction at room temperature for 5 minutes for cohesive-ended ligations, or 15 minutes for blunt-ended ligations.

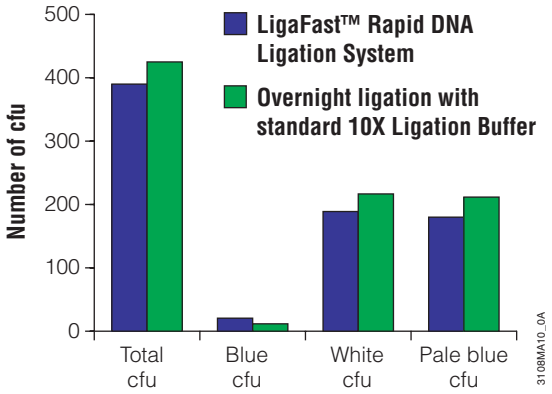
LigaFast™ Rapid DNA Ligation System
Cat.# M8221 30 reactions
Cat.# M8225 150 reactions
See the Product Information Sheet at:
www.promega.com/tbs

Blunt-ended Ligation = 15 minutes



Comparison of overnight ligations and the LigaFast™ Rapid DNA Ligation System using blunt-ended DNA inserts. Experiment performed with blunt-end insert ligated into an *Eco*R V-cut, dephosphorylated pGEM® Vector. Ligations were performed under standard conditions (see pages 24 and 25) using 4°C overnight for the T4 DNA Ligase (3u with standard 10X Ligation Buffer) or 15 minutes at room temperature for the LigaFast™ System. Ligated DNA was transformed into High Competency JM109 cellls and plated on indicator media. White and pale blue colonies were confirmed to contain recombinant vector by restriction enzyme analysis.

Sticky-ended Ligation = 5 minutes



Comparison of overnight ligations and the LigaFast™ Rapid DNA Ligation System using a DNA insert with 5′ overhangs. Experiment performed with blunt-end insert ligated into an *Sal* I-cut, dephosphorylated pGEM® Vector. Ligations were performed under standard conditions using 4°C overnight for the T4 DNA Ligase (3u in with standard 10X Buffer) or 5 minutes at room temperature for the LigaFast™ System. Ligated DNA was transformed into High Competency JM109 cells and plated on indicator media. White and pale blue colonies were confirmed to contain recombinant vector by restriction enzyme analysis.

Ligation

T4 DNA Ligase

We recommend using a 1:1, 1:3 or 3:1 molar ratio of vector:insert DNA when cloning a fragment into a plasmid vector.

The following ligation reaction of a 3.0kb vector and a 0.5kb insert DNA uses the 1:3 vector:insert ratio. Typical ligation reactions use 100–200ng of vector DNA.

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

vector DNA	100ng
insert DNA	50ng
Ligase 10X Buffer	1µl
T4 DNA Ligase (3u/µl)	1µl
Nuclease-Free Water to	10µl

2. Incubate the reaction:

22–25°C	3 hours	Cohesive ends
4°C	Overnight	Cohesive ends
15°C	4–18 hours	Blunt ends

T4 DNA Ligase
Blue/White Cloning Qualified

Cat.# M1801	100u 1–3u/µl
Cat.# M1804	500u 1–3u/µl
Cat.# M1794	500u 10–20u/µl

See the Product Information Sheet at: **www.promega.com/tbs**

Ligation temperature and duration vary widely in the scientific literature. These are the conditions we use when testing the enzyme.

Standard T4 DNA Ligase methods are more forgiving toward dilute DNA concentrations. Vector and insert can make up 80% of the final volume.

Ligase Buffers contain ATP to drive the reaction. Try to avoid multiple freeze–thaw cycles of the buffer. Dispense the buffer into smaller volumes to minimize the freeze–thaw cycles on each aliquot.

Ligation: Control Reaction

Controls help ensure that everything is functioning normally in your subcloning reaction. If something does go wrong, you can use your controls to figure out where a problem might have occurred.

When ligating insert and vector, you can do a control ligation of vector with no insert. Carry this reaction through transformation and plating. The number of colonies you see can be a good indicator of how a ligation reaction performed and how many background colonies you will have on your plate.

Quick Checks of T4 DNA Ligase

You can always do a quick test of your ligase by simply taking 1µg of a DNA digest marker (e.g., Lambda DNA *Hin*d III Markers [Cat.# G1711]) and performing a 15- to 30-minute ligation reaction under normal conditions. Run the ligation reaction on a gel in comparison to the standard marker. You should see DNA of much higher molecular weight on the gel in comparison to the marker.

Another quick test is to cut a plasmid with a single restriction enzyme. Add this vector to a ligation reaction and transform.

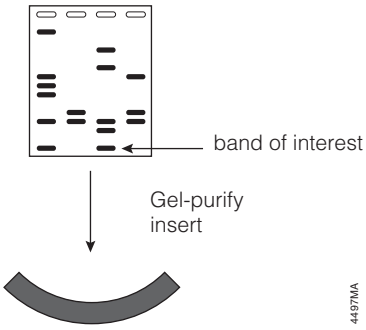
Questions on Subcloning? Call Promega Technical Services

The Promega Worldwide Technical Service Group, Field Applications Specialists, and Distributors are committed to providing you with the highest quality products available to ensure your success. Each of these individuals has an extensive background in molecular biology research, hands-on bench experience with Promega products, and training in problem solving and troubleshooting. Additionally, the full resources of our R&D, Quality Assurance and Production Scientists are available to help increase your laboratory's productivity.

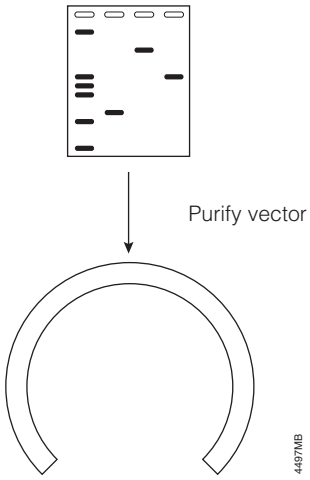
Contact Promega Technical Services directly or through your Branch Office or by email at: **techserv@promega.com**

Purifying Vector and Insert

Purification of the insert and destination vector are absolutely critical for success in subcloning applications. Years ago, each step called for phenol:chloroform extractions followed by ethanol precipitation to remove enzymes such as calf intestinal alkaline phosphatase from enzymatic vector manipulations. Guanidine-based nucleic acid clean-up systems greatly simplified the removal of enzymes. Gel isolation methods further improved the efficiency of subcloning by segregating the wanted reactants from the unwanted reactants.



Gel isolation is a practical necessity in subcloning. You get the insert you need.



Gel isolation of vector reduces background by eliminating uncut vector from the ligation.

Purifying Vector and Insert

Wizard® SV Gel and PCR Clean-Up System

The Wizard SV Gel and PCR Clean-Up System is designed to extract and purify DNA fragments directly from PCR[a] or from agarose gels. Fragments of 100bp to 10kb can be recovered from standard or low-melt agarose gels in either Tris acetate (TAE) buffer or Tris borate buffer (TBE). Up to 95% recovery is achieved, depending upon the DNA fragment size. This membrane-based system, which can bind up to 40µg of DNA, allows recovery of isolated DNA fragments or PCR products in as little as 15 minutes, depending on the number of samples processed and the protocol used. Samples can be eluted in as little as 15µl of nuclease-free water. The purified DNA can be used for automated fluorescent sequencing, cloning, labeling, restriction enzyme digestion or in vitro transcription/ translation without further manipulation.

Wizard® SV Gel and PCR Clean-Up System

Cat.# A9281 50 preps

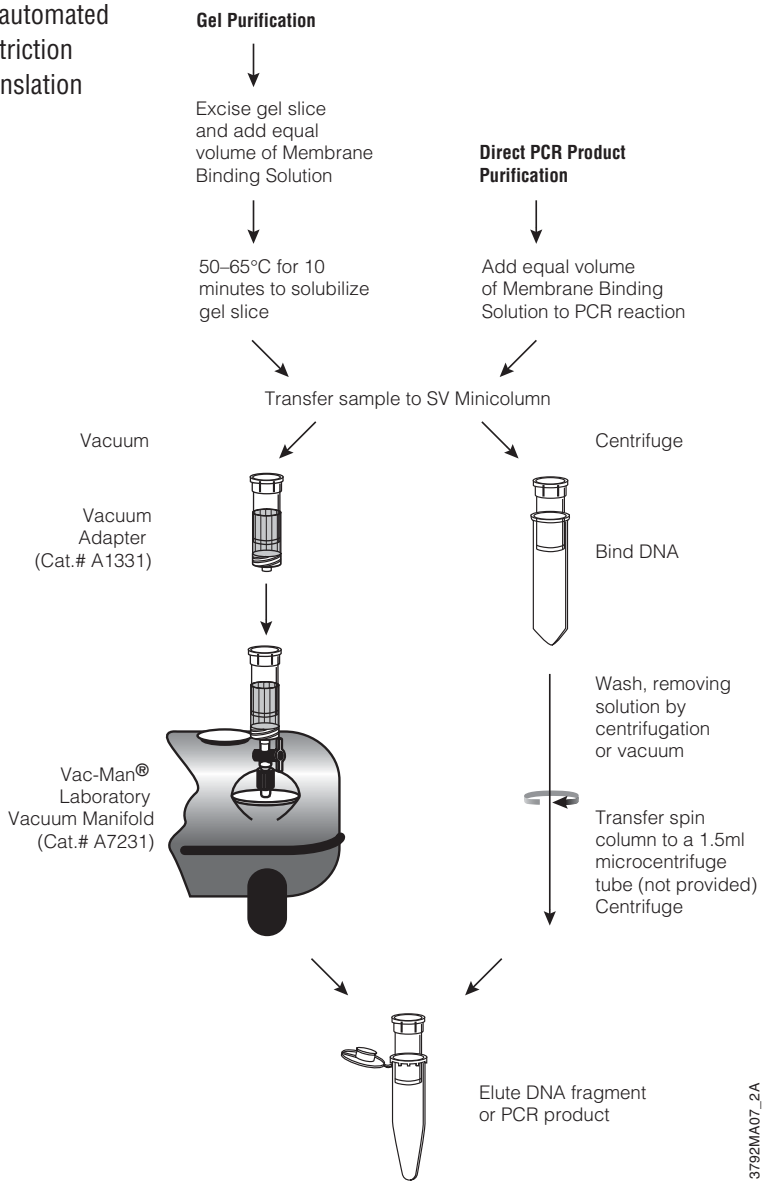
Cat.# A9282 250 preps

Protocol available at:
www.promega.com/tbs/tb308/tb308.html

Process up to 10 gel slices (3.5g total) on a single column with sequential loading.

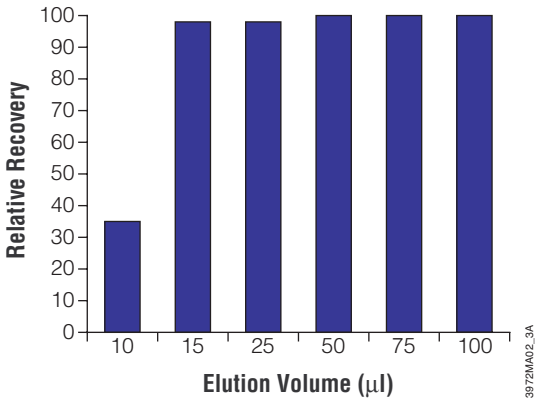
Capture up to 40µg of DNA on a single column!

From start to purified DNA in 15 minutes!



Flow chart of DNA fragment gel purification or direct PCR product purification using the Wizard SV Gel and PCR Clean-Up System.

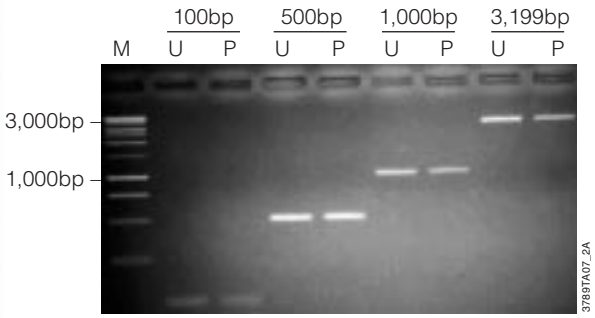
Purifying Vector and Insert



Elution volume versus recovery for a 700bp PCR product. One hundred percent is based on recovery with 50µl elution. Adapted from Table 4 in Betz, N. and Strader, T. (2002) Clean Up with Wizard® SV for Gel and PCR. *Promega Notes* **82**, 2–5.

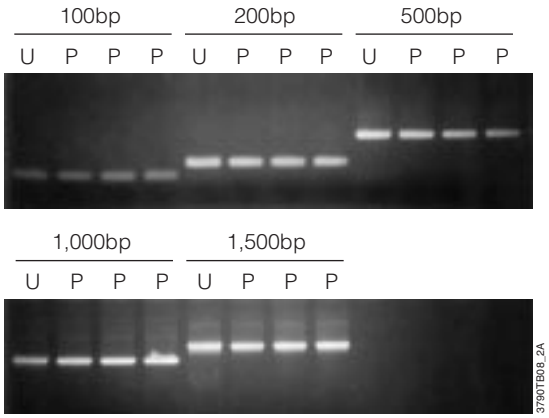
Concentrate DNA by eluting in as little as 15µl.

Linear DNA as big as 10kb can be purified with the system with up to 95% recovery.



U: Unpurified P: Purified

Recovery of various sized unpurified (U) and purified (P) PCR products. Purified lanes were extracted from a 1% agarose gel run with TAE buffer.



Recovery comparison of various sized unpurified (U) and purified (P) PCR products directly purified from PCR amplifications.

Wizard SV Gel and PCR Clean-Up System can remove ethidium bromide and tough enzymes like calf intestinal alkaline phosphatase. See Buros, M. and Betz, N. (2002) Removal of ethidium bromide and calf intestinal alkaline phosphatase using the Wizard SV Gel and PCR Clean-Up System. This can be viewed online at: www.promega.com/enotes/applications/ap0045_tabs.htm

Wizard SV Gel and PCR Clean-Up System is tested for purification from up to 3% agarose gels.

Gel Percentages and Resolution of Linear DNA on Agarose Gels.

% Agarose	Resolution
0.8	800bp–10kb+
1.0	400bp–8kb+
1.2	300bp–7kb
1.5	200bp–4kb
2.0	100bp–3kb
3.0	100bp–1kb

Adapted from Brown, T.A. (1998) In: *Molecular Biology LABFAX II: Gene Analysis.* 2nd ed. Academic Press, **90**.

Gel Electrophoresis

Agarose Gel Electrophoresis of DNA

Running double-stranded, linear DNA (like plasmid DNA from restriction enzyme digests) on an agarose gel is a routine activity in molecular biology laboratories. The basic method is very straightforward:

1. Set up the minigel apparatus as recommended by the manufacturer.
2. Weigh the required amount of agarose and add it to the appropriate amount of TAE or TBE 1X Buffer in a flask or bottle. For example, to prepare a 1% agarose gel, add 1.0g of agarose to 100ml of buffer. **Note:** The volume of buffer and agarose should not exceed half the volume of the container.
3. Heat the mixture in a microwave oven or on a hot plate for the minimum time required to allow all the agarose to dissolve. Interrupt the heating at regular intervals and swirl the container to mix the contents. Do not allow the solution to boil over.
   CAUTION: The container and contents will be hot! Swirling may also cause solution to boil vigorously. Use adequate precautions.
4. Cool the solution to 50–60°C and pour the gel. Allow the gel to form completely (typically, 30 minutes at room temperature is sufficient). Remove the comb from the gel, place it in the electrophoresis chamber and add a sufficient volume of TAE or TBE 1X buffer to just cover the surface of the gel.
5. Load samples with 1X Blue/Orange Loading Dye into the wells.
6. Connect the gel apparatus to an electrical power supply and apply an appropriate voltage to the gel. For minigels, typical gradients used are between 1–5 volts/cm. Higher voltages and shorter runs will decrease the resolution of the gel and may also cause overheating that may melt the agarose.
7. After electrophoresis is complete, remove the gel and stain it by soaking it in a solution of 0.5µg/ml ethidium bromide for 30 minutes at room temperature. **Note:** Ethidium bromide may also be incorporated in the gel and electrophoresis buffer, at a concentration of 0.5µg/ml, during gel preparation. This eliminates the need for post-electrophoretic staining but may interfere with accurate size determination of DNA fragments. **CAUTION:** Always wear gloves when working with ethidium bromide.
8. Place the gel on a UV lightbox and photograph the gel according to the specification recommended for your camera and film type. **CAUTION:** Use protective eyewear when working with a UV light source. **Note:** You may wish to destain or rinse the gel in fresh 1X running buffer prior to viewing it on the UV lightbox.

Recipes

Nearly all of these reagents can be purchased premade including the agarose gels. Here are the directions if you wish to prepare your own reagents.

Blue/Orange Loading Dye, 6X
(available from Promega [Cat.# G1881])

10mM	Tris-HCl, pH 7.5
50mM	EDTA
15%	Ficoll® 400
0.03%	bromophenol blue
0.03%	xylene cyanol FF
0.4%	orange G

One or more dyes can be left out of the recipe to create a custom loading dye.

TAE 50X Buffer (1L)
(Available in a 10X or 40X solution from Promega [Cat.# V4271 and V4281, respectively])

Dissolve 242g Tris base and 37.2g disodium EDTA, dihydrate in 900ml of deionized water. Add 57.1ml glacial acetic acid and adjust the final volume with water to 1 liter. Store at room temperature or 4°C.

TBE 10X Buffer (1L)
(Available in a 10X solution from Promega [Cat.# V4251])

Dissolve 108g of Tris base and 55g boric acid in 900ml deionized water. Add 40ml 0.5M EDTA (pH 8.0) and increase the final volume to 1L. Store at room temperature or 4°C.

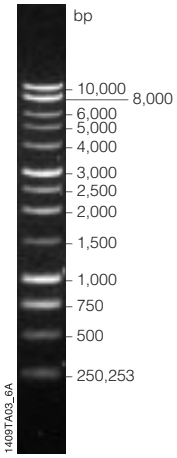
Ethidium bromide can detect as little as 1ng of dsDNA in a band.
Brown, T.A. (1998) In: Molecular Biology LABFAX II: Gene Analysis. 2nd ed. Academic Press, 101.

DNA Markers

DNA markers should always be run on agarose gels to aid in identifying bands of interest. This is especially true if you are performing applications such as partial restriction digestion. Promega offers a wide variety of DNA markers to fit your needs. Below is a sampling of marker options available from Promega. BenchTop Markers come premixed with Blue/Orange Loading Dye ready to load onto the gel. As the name implies, you can store them on your benchtop, no need to freeze and thaw every time you need it. Conventional markers are pure DNA solutions and come with a tube of 6X Blue/Orange Loading Dye for use with the marker and your samples.

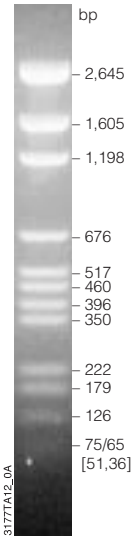
Each of these markers is available in a ready-to-use BenchTop version or in a conventional version.

1kb DNA Ladder
BenchTop Cat.# G7541
Conventional Cat.# G5711



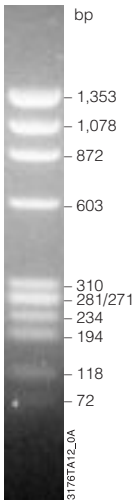
0.7% agarose

pGEM® DNA Markers
BenchTop Cat.# G7521
Conventional Cat.# G1741



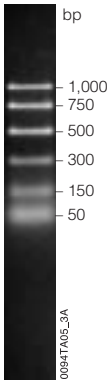
2% agarose

ϕX174 DNA/ *Hae* III Markers
BenchTop Cat.# G7511
Conventional Cat.# G1761



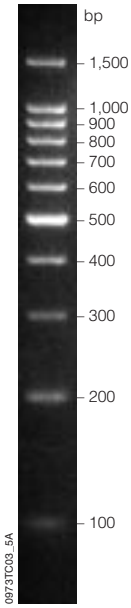
2% agarose

PCR Markers
BenchTop Cat.# G7531
Conventional Cat.# G3161



2% agarose

100bp DNA Ladder
BenchTop Cat.# G8291
Conventional Cat.# G2101



2% agarose

Classic Subcloning: Ordering Information

Enzyme	Heat Inactivated	Buffer	Recognition Site	Size (u)	Conc. (u/µl)	Cat.#
Aat II	+	J	GACGT▼C	50	3–5	R6541
				250	3–5	R6545
Acc I	–	G	GT▼(A/C)(T/G)AC	100	3–10	R6411
				500	3–10	R6415
Acc III	–	F	T▼CCGGA	200	10	R6581
*Acc*65 I (*Kpn* I)	+	D	G▼GTACC	1,500	10	R6921
*Acc*B7 I	+	E	CCANNNN▼NTGG	200	10	R7081
Age I	+	K	A▼CCGGT	100	3–10	R7251
Alu I	+	B	AG▼CT	500	10	R6281
*Alw*26 I	+	C	GTCTC(N)$_1$▼	100	8–12	R6761
			GTCTC(N)$_5$	500	8–12	R6765
*Alw*44 I	+	C	G▼TGCAC	1,000	10	R6771
Apa I	+	A	GGGCC▼C	5,000	10	R6361
				25,000	40–80	R4364
Ava I	+/–	B	C▼(T/C)CG(A/G)G	200	8–12	R6091
				1,000	8–12	R6095
Ava II (*Sin* I)	+	C	G▼G(A/T)CC	100	1–10	R6131
				1,000	1–10	R6135
Bal I	+	G	TGG▼CCA	50	2–10	R6691
				250	2–10	R6695
*Bam*H I	+	E	G▼GATCC	2,500	10	R6021
				12,500	10	R6025
				12,500	40–80	R4024
				50,000	40–80	R4027
Ban I	–	G	G▼G(T/C)(A/G)CC	200	8–12	R6891
Ban II	+	E	G(A/G)GC(T/C)▼C	1,000	8–12	R6561
Bbu I (*Sph* I)	+	A	GCATG▼C	200	10	R6621
				1,000	40–80	R4624
Bcl I	–	C	T▼GATCA	1,000	10	R6651
				5,000	40–80	R4654
Bgl I	+	D	GCCNNNN▼NGGC	1,000	10	R6071
				5,000	10	R6077
				5,000	40–80	R4074
Bgl II	–	D	A▼GATCT	500	10	R6081
				2,500	10	R6085
				10,000	10	R6087
				2,500	40–80	R4084
*Bsa*M I	–	D	GAATGCN▼	500	10	R6991
*Bsp*1286 I	+		AG(G/A/T)GC(C/A/T)▼C	500	10	R6741
*Bsr*S I	–	D	ACTGGN	500	10	R7241
*Bss*H II	–	H	G▼CGCGC	100	10	R6831
				500	10	R6835
*Bst*98 I	–	D	C▼TTAAG	500	8–12	R7141
*Bst*E II	–	D	G▼GTNACC	2,000	10	R6641
*Bst*O I	–	C	CC▼(A/T)GG	2,000	10	R6931
*Bst*X I	+/–	D	CCANNNNN▼NTGG	250	8–12	R6471
				1,000	8–12	R6475
*Bst*Z I	–	D	C▼GGCCG	500	10	R6881
*Bsu*36 I	–	E	CC▼TNAGG	500	10	R6821
Cfo I (*Hha* I)	+/–	B	GCG▼C	3,000	10	R6241
Cla I	+	C	AT▼CGAT	500	10	R6551
				2,500	10	R6555
Csp I	+	K	CG▼G(A/T)CCG	100	10	R6671
				500	10	R6675
*Csp*45 I	+	B	TT▼CGAA	2,500	10	R6571
Dde I	+/–	D	C▼TNAG	200	10	R6291
				1,000	10	R6295
Dpn I (*Sau*3A I)	+	B	G^{me}A▼TC	200	10	R6231
Dra I	+	B	TTT▼AAA	2,000	10	R6271
Ecl HK I	+	E	GACNNN▼NNGTC	100	10	R7111
*Eco*47 III	+	D	AGC▼GCT	50	2–5	R6731
*Eco*52 I (*Bst*Z I)	+	L	C▼GGCCG	50	1–5	R6751
*Eco*ICR I (*Sac* I)	+	B	GAG▼CTC	1,000	10	R6951
				5,000	40–80	R4954
*Eco*R I	+	H	G▼AATTC	5,000	12	R6011
				15,000	12	R6017
				25,000	40–80	R4014
				50,000	40–80	R4017
*Eco*R V	+	D	GAT▼ATC	2,000	10	R6351
				10,000	10	R6355
				10,000	40–80	R4354
Fok I	+	B	GGATG(N)9 GGATG(N)(13)▼	100	2–10	R6781
Hae II	–	B	(A/G)GCGC▼(T/C)	1,000	10	R6661
Hae III	–	C	GG▼CC	2,500	10	R6171
				10,000	10	R6175
				12,500	40–80	R4174
Hha I (*Cfo* I)	+	C	GCG▼C	1,000	10	R6441
Hinc II	+	B	GT(T/C)▼(A/G)AC	200	10	R6031
				1,000	10	R6035
				5,000	10	R6037
				1,000	40–80	R4034
Hind III	+	E	A▼AGCTT	5,000	10	R6041
				15,000	10	R6045
				25,000	40–80	R4044
				50,000	40–80	R4047
Hinf I	–	B	G▼ANTC	1,000	10	R6201
				5,000	10	R6205
				5,000	40–80	R4204
Hpa I	–	J	GTT▼AAC	100	3–10	R6301
				500	3–10	R6305
Hpa II (*Msp* I)	–	A	C▼CGG	1,000	10	R6311
				5,000	10	R6315
*Hsp*92 I	+	F	G(A/G)▼CG(T/C)C	500	10	R7151
*Hsp*92 II	+	K	CATG▼	1,000	10	R7161
I-*Ppo* I (Intron-Encoded Endonuclease)	+	10X I-*Ppo* I	CTCTCTTAA▼GGTAGC	10,000	100–200	R7031
Kpn I[(b)] (*Acc*65 I)	+/–	J	GGTAC▼C	2,500	8–12	R6341
				10,000	8–12	R6345
				12,500	40–80	R4344
Mbo II	+	B	GAAGA(N)$_8$ GAAGA(N)7▲	100	2–10	R6723
Mlu I	+/–	D	A▼CGCGT	1,000	10	R6381
Msp I (*Hpa* II)	+	B	C▼CGG	2,000	10	R6401
				10,000	10	R6405
				10,000	40–80	R4404
*Msp*A1 I	+	C	C(A/C)G▼C(G/T)G	1,000	10	R7021
Turbo™ *Nae* I[(c)]	+	Turbo™	GCC▼GGC	250	4	R7231

Indicates Genome Qualified.

Indicates Blue/White Cloning Qualified.

Classic Subcloning: Ordering Information

Enzyme	Heat Inactivated	Buffer	Recognition Site	Size (u)	Conc. (u/µl)	Cat.#
Nae I (*Ngo* M IV)	+	A	GCC▼GGC	250	4	R7131
				1,000	4	R7135
Turbo™ *Nar* I[c]	+	Turbo™	GG▼CGCC	200	10	R7261
Nar I	+	G	GG▼CGCC	200	10	R6861
Nci I	+	B	CC▼(C/G)GG	1,000	10	R7061
Nco I	+	D	C▼CATGG	200	10	R6513
				1,000	10	R6515
Nde I	+	D	CA▼TATG	500	10	R6801
Nde II (*Dpn* I, *Sau*3A I)	+	D	▼GATC	200	10	R7291
				1,000	10	R7295
Ngo M IV (*Nae* I)	+	MULTI-CORE™	G▼CCGGC	500	10	R7171
Nhe I	+	B	G▼CTAGC	250	10	R6501
				1,250	10	R6505
Not I	+	D	GC▼GGCCGC	200	10	R6431
				1,000	10	R6435
				1,000	40–80	R4434
Nru I	+	K	TCG▼CGA	200	10	R7091
Nsi I	+/–	D	ATGCA▼T	250	10	R6531
Pst I	+/–	H	CTGCA▼G	3,000	10	R6111
				15,000	10	R6115
				15,000	40–80	R4114
				50,000	40–80	R4117
Pvu I	–	D	CGAT▼CG	100	2–10	R6321
				500	2–10	R6325
Pvu II	+	B	CAG▼CTG	1,000	8–12	R6331
				5,000	8–12	R6335
Rsa I	+	C	GT▼AC	1,000	10	R6371
				5,000	40–80	R4374
Sac I (*Eco*ICR I)	+	J	GAGCT▼C	1,000	10	R6061
				5,000	10	R6065
				5,000	40–80	R4064
Sac II	+	C	CCGC▼GG	500	10	R6221
Sal I	+	D	G▼TCGAC	2,000	10	R6051
				10,000	10	R6055
				10,000	40–80	R4054
*Sau*3A I (*Dpn* I, *Nde* II)	+	B	▼GATC	100	3–10	R6191
				500	3–10	R6195
Sca I	+	K	AGT▼ACT	1,000	8–12	R6211
				5,000	40–80	R4214
Sfi I[d]	+	B	GGCCNNNN▼NGGCC	250	10	R6391
				1,250	40–80	R4394
Sgf I	+/–	C	GCGAT▼CGC	250	8–12	R7103
				1,250	40–80	R5104
Sin I (*Ava* II)	+	A	G▼G(A/T)CC	200	8–12	R6141
				1,000	40–80	R4144
Sma I (*Xma* I)	+	J	CCC▼GGG	1,000	8–12	R6121
				5,000	8–12	R6125
				5,000	40–80	R4124
*Sna*B I	–	B	TAC▼GTA	100	2–10	R6791
				500	2–10	R6795
Spe I	+	B	A▼CTAGT	200	10	R6591
				1,000	10	R6595

Indicates Genome Qualified.

Indicates Blue/White Cloning Qualified.

Enzyme	Heat Inactivated	Buffer	Recognition Site	Size (u)	Conc. (u/µl)	Cat.#
Sph I (*Bbu* I)	+	K	GCATG▼C	200	10	R6261
				1,000	10	R6265
Ssp I	+	E	AAT▼ATT	500	10	R6601
				2,500	40–80	R4604
Stu I	+	B	AGG▼CCT	400	10	R6421
Sty I	+	F	C▼C(A/T)(T/A)GG	2,000	10	R6481
Taq I	–s	E	T▼CGA	1,000	10	R6151
				10,000	10	R6155
				5,000	40–80	R4154
*Tru*9 I	–	F	T▼TAA	200	8–12	R7011
*Tth*111 I	–	B	GACN▼NNGTC	500	8–12	R6841
Vsp I	+	D	AT▼TAAT	500	8–12	R6851
Xba I	–	D	T▼CTAGA	2,000	8–12	R6181
				10,000	8–12	R6185
				10,000	40–80	R4184
Xho I	+	D	C▼TCGAG	3,000	10	R6161
				10,000	10	R6165
				15,000	40–80	R4164
Xho II	+	C	(A/G)▼GATC(T/C)	100	5–10	R6811
				500	5–10	R6815
Xma I (*Sma* I)	+	B	C▼CCGGG	50	1–5	R6491
				250	1–5	R6495
Xmn I	+	B	GAANN▼NNTTC	500	10	R7271
				2,500	10	R7273

Product	Size	Conc. (mg/ml)	Cat.#
BSA, (Bovine Serum Albumin) Acetylated	400µl	1	R9461
	1ml	10	R3961
MULTI-CORE™ Buffer Pack	3 × 1ml	—	R9991
4-CORE® Buffer Pack (1 each A-D)	4 × 1ml	—	R9921

For Laboratory Use.

Turbo™ Enzymes are provided with a reaction buffer containing a noncleavable affector sequence that facilitates efficient digestion of slow and resistant sites.

Restriction enzymes are shown to be heat inactivated (+) if they show >95% loss of activity after a 15 minute incubation at 65°C.

Enzymes followed by another enzyme name in parentheses indicate that the enzyme is an isoschizomer or neoschizomer of the enzyme in parentheses.

Restriction Enzyme Buffer Composition (1X).

Buffer	pH (at 37°C)	Tris-HCl (mM)	$MgCl_2$ (mM)	NaCl (mM)	KCl (mM)	DTT (mM)
A	7.5	6	6	6	–	1
B	7.5	6	6	50	–	1
C	7.9	10	10	50	–	1
D	7.9	6	6	150	–	1
E	7.5	6	6	100	–	1
F	8.5	10	10	100	–	1
G	8.2	50	5	–	–	–
H	7.5	90	10	50	–	–
J	7.5	10	7	–	50	1
K	7.4	10	10	–	150	–
L	9.0	10	3	100	–	–

MULTI-CORE™ Buffer (1X): 25mM Tris-Acetate (pH 7.8 @ 25°C), 100mM potassium acetate, 10mM magnesium acetate, 1mM DTT.

1. For each 10°C rise in temperature between 0°C and 25°C, the pH of Tris buffers decreases 0.31 pH units.
2. For each 10°C rise in temperature between 25°C and 37°C, the pH of Tris buffers decreases 0.25 pH units.

Classic Subcloning: Ordering Information

Enzymes	Size	Conc.	Cat.#
T4 DNA Polymerase[d]	100u	5–10u/µl	M4211
	500u	5–10u/µl	M4215
DNA Polymerase I Large (Klenow) Fragments	150u	5–10u/µl	M2201
	500u	5–10u/µl	M2206
Shrimp Alkaline Phosphatase	500u	1u/µl	M8201
Alkaline Phosphatase, Calf Intestinal	1,000u	1u/µl	M1821
	1,000u	20u/µl	M2825
LigaFast™ Rapid DNA Ligation System	30 reactions	—	M8221
	150 reactions	—	M8225
T4 DNA Ligase	100u	1–3u/µl	M1801
	500u	1–3u/µl	M1804
	500u	10–20u/µl	M1794

For Laboratory Use.

Purification Systems	Size	Cat.#
Wizard® SV Gel and PCR Clean-Up System*	50 preps	A9281
(ready for spin protocol)	250 preps	A9282
Vac-Man® Laboratory Vacuum Manifold, 20-sample capacity (required for vacuum protocol)	1	A7231
Vacuum Adapters (required for vacuum protocol)	20	A1331

*For Laboratory Use.

Ready-to-Load BenchTop DNA Markers	Size	Cat.#
BenchTop 100bp DNA Ladder	50 lanes	G8291
BenchTop 1kb DNA Ladder	100 lanes	G7541
BenchTop PCR Markers	50 lanes	G7531
BenchTop pGEM® DNA Markers	50 lanes	G7521
BenchTop ϕX174 DNA/*Hae* III Markers	50 lanes	G7511

For Laboratory Use.

Conventional DNA Markers (supplied with 6X Blue/Orange Loading Dye)	Size	Cat.#
100bp DNA Ladder	50 lanes	G2101
1kb DNA Ladder	100 lanes	G5711
PCR Markers	50 lanes	G3161
pGEM® DNA Markers	50 lanes	G1741
ϕX174 DNA/*Hae* III Markers	50 lanes	G1761

For Laboratory Use.

Accessory Items	Size	Conc.	Cat.#
4-CORE® Buffer Pack*	4 × 1ml	—	R9921
MULTI-CORE™ Buffer Pack*	3 × 1ml	—	R9991
Bovine Serum Albumin, Acetylated*	400µl	1µg/µl	R9461
	1,000µl	10mg/ml	R3961
T4 DNA Ligase Buffer Pack*	3 × 500µl	—	C1263
CIAP Buffer Pack*	3 × 500µl	—	M1833
dNTP Mix*	200µl	10mM	U1511
	1,000µl	10mM	U1515
Agarose, LE, Analytical Grade	100g	—	V3121
	500g	—	V3125
Blue/Orange Loading Dye, 6X*	3 × 1ml	—	G1881
TAE Buffer, 10X	1,000ml	10X	V4271
TAE Buffer, 40X	1,000ml	40X	V4281
TBE Buffer, 10X	1,000ml	10X	V4251
Ethidium Bromide Solution, Molecular Grade	10ml	10mg/ml	H5041
Mineral Oil*	12ml	—	DY1151

*For Laboratory Use.