Subcloning Notebook

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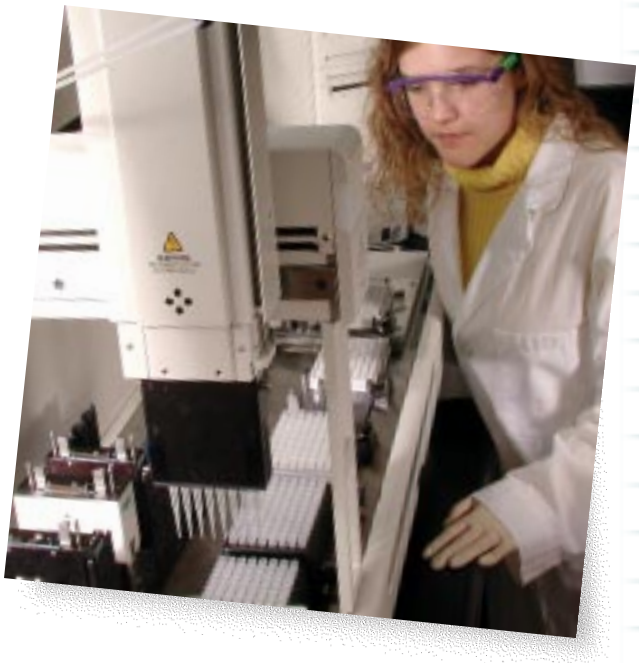

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Subcloning Notebook

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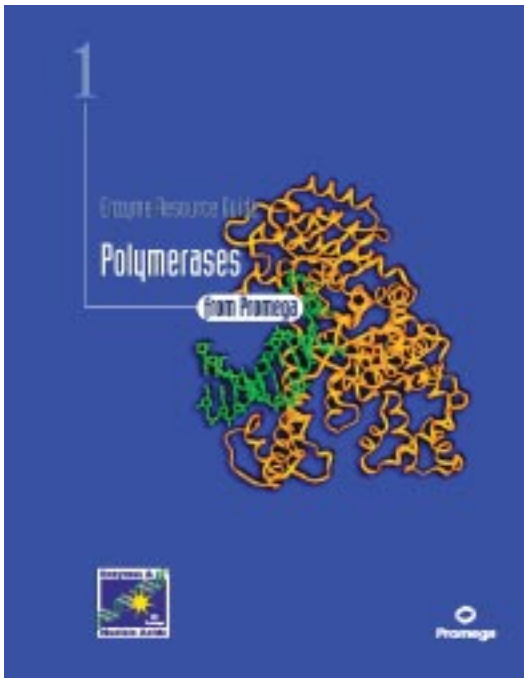
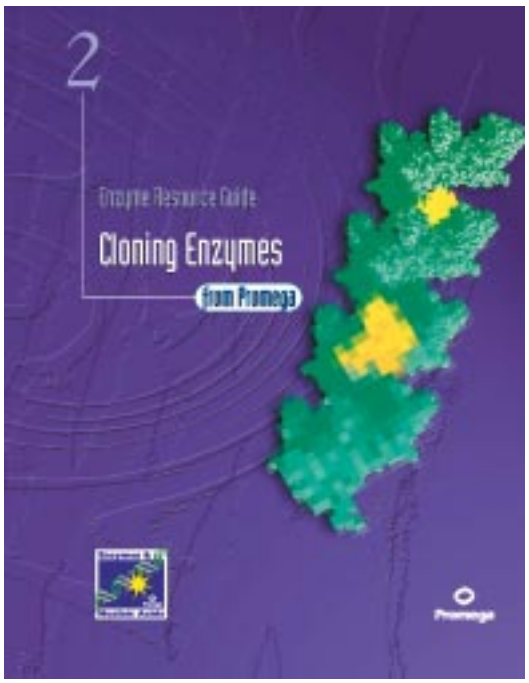
Starving for more in-depth knowledge of the enzymes mentioned in this notebook?

Savor these tasty offerings at the Promega Web site:



The **Polymerases Guide** features mesophilic and thermophilic DNA polymerases, RNA polymerases, reverse transcriptases and terminal transferase. The guide includes background information, a list of applications for each polymerase, enzyme properties and other product information such as the quality control assays performed to ensure high-quality enzymes. Figures and tables enhance the text reference information. The guide is available in pdf format online at: **www.promega.com/guides/polym_guide/**

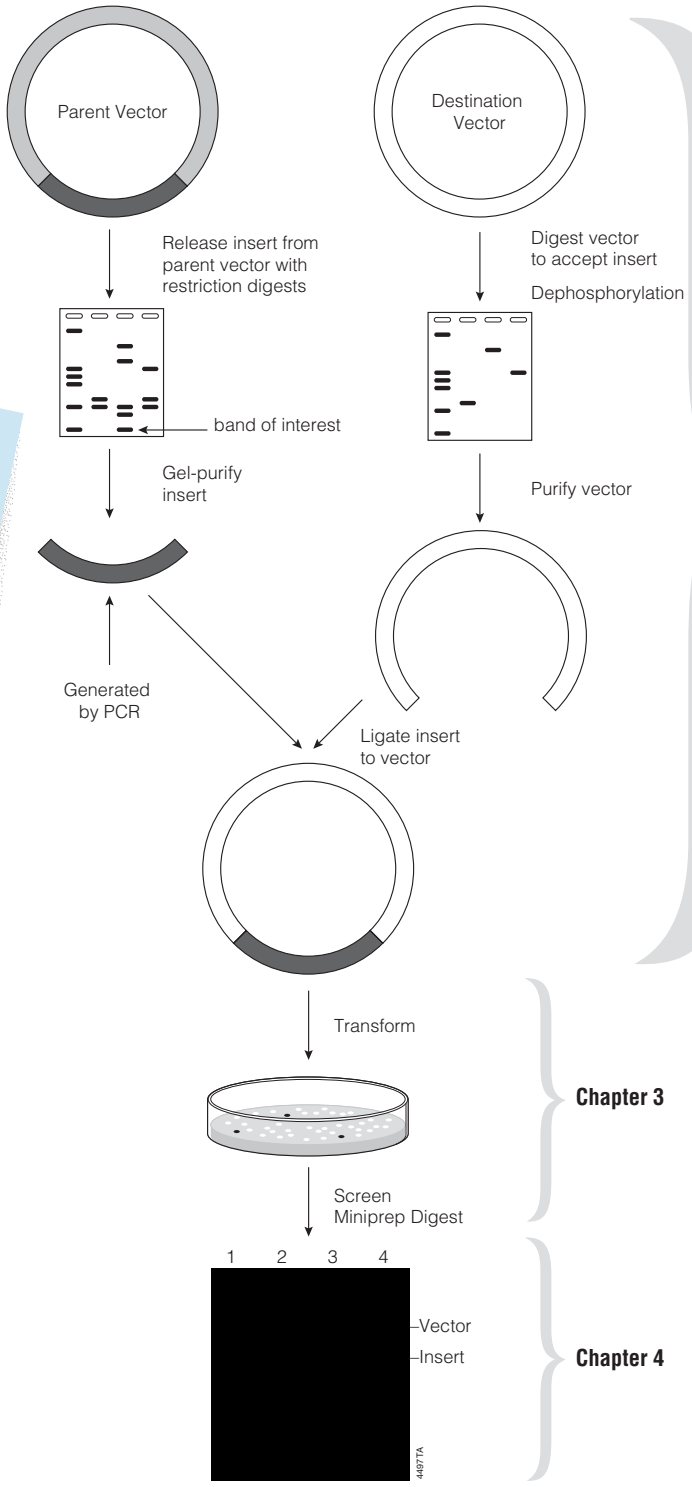


Cloning Enzymes, in the Enzyme Resource Guide series, highlights those enzymes important in nucleic acid cloning procedures. Enzymes that modify nucleic acids provide the foundation for many molecular biology techniques. Specific features of the in vivo functions of these enzymes have been exploited in vitro to provide many of the protocols currently used in nucleic acid manipulations. The guide is available in pdf format online at: **www.promega.com/guides/cloning_guide/**

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.

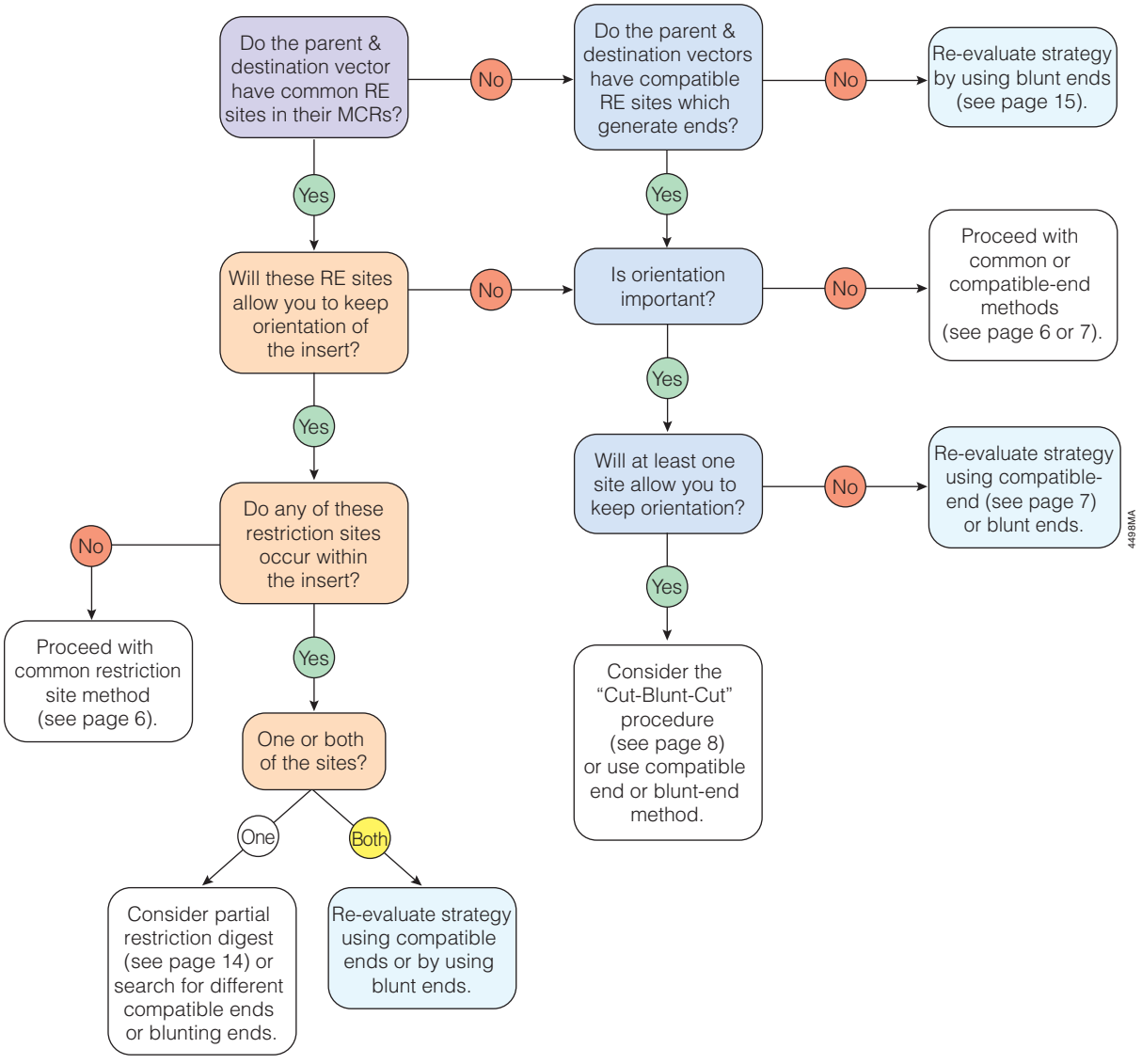
Gel isolation of vector reduces background by eliminating uncut vector from the transformation.

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.

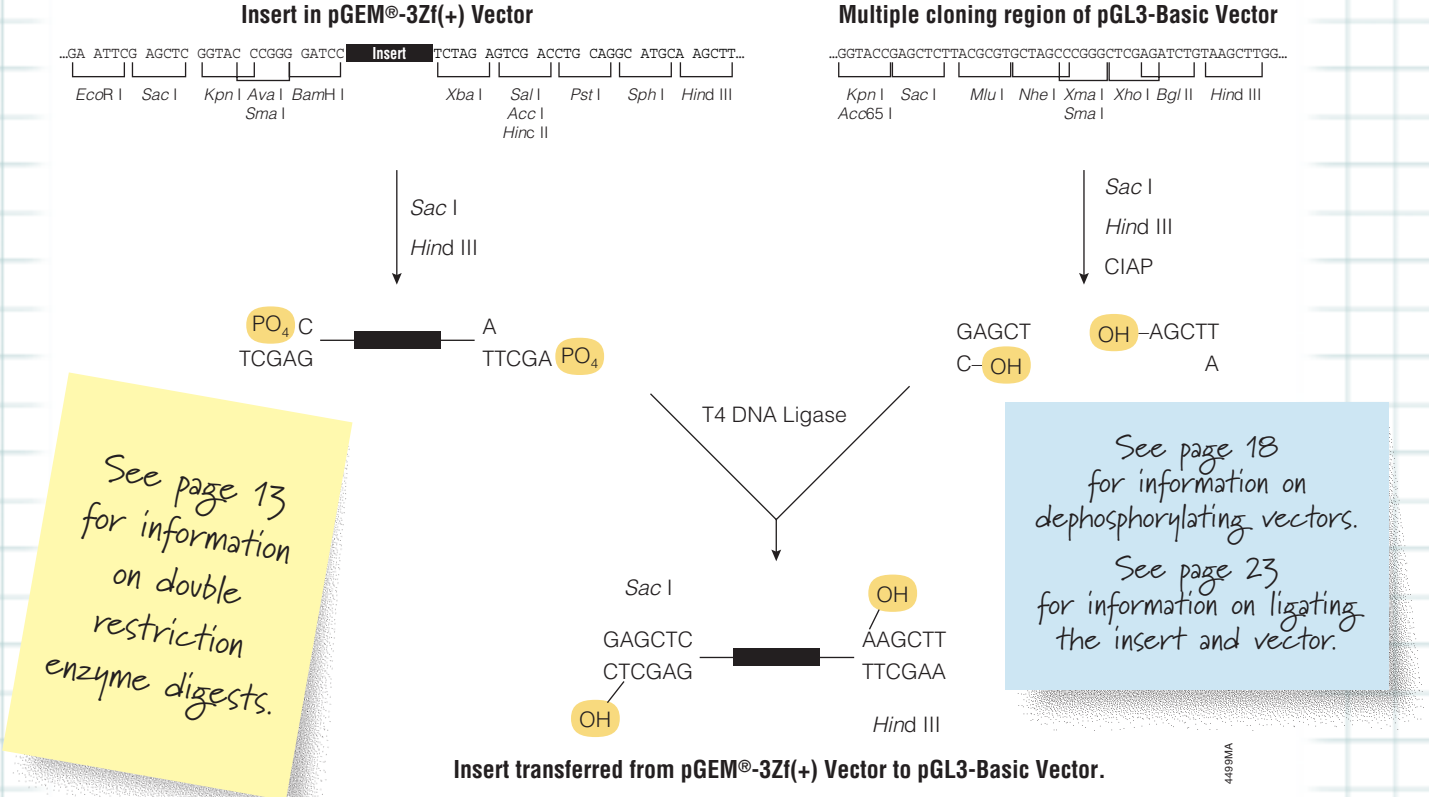
See the Compatible Ends Table on page 61 for a listing of overhangs compatible with Promega enzymes.



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.

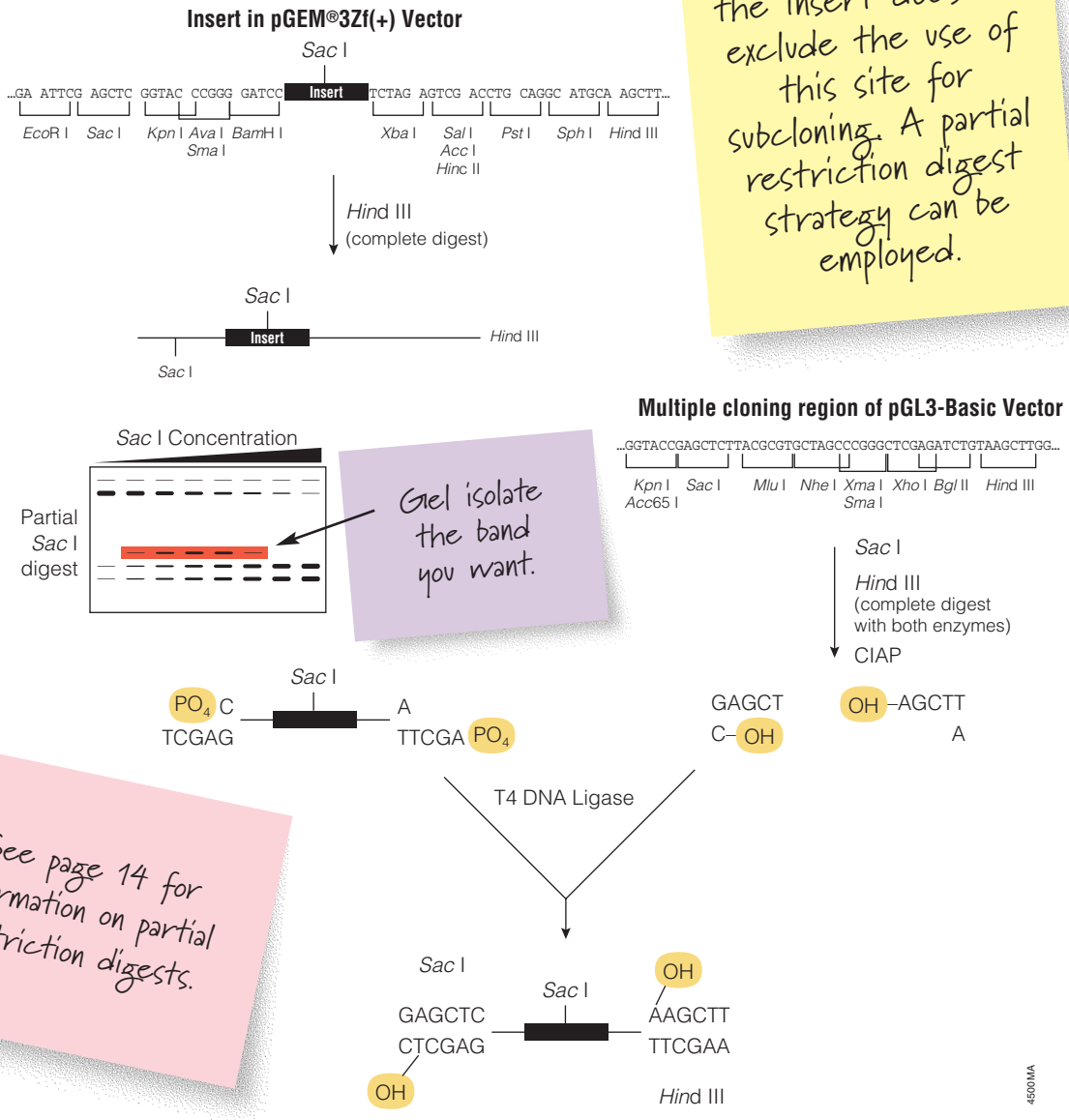
4499MA

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.

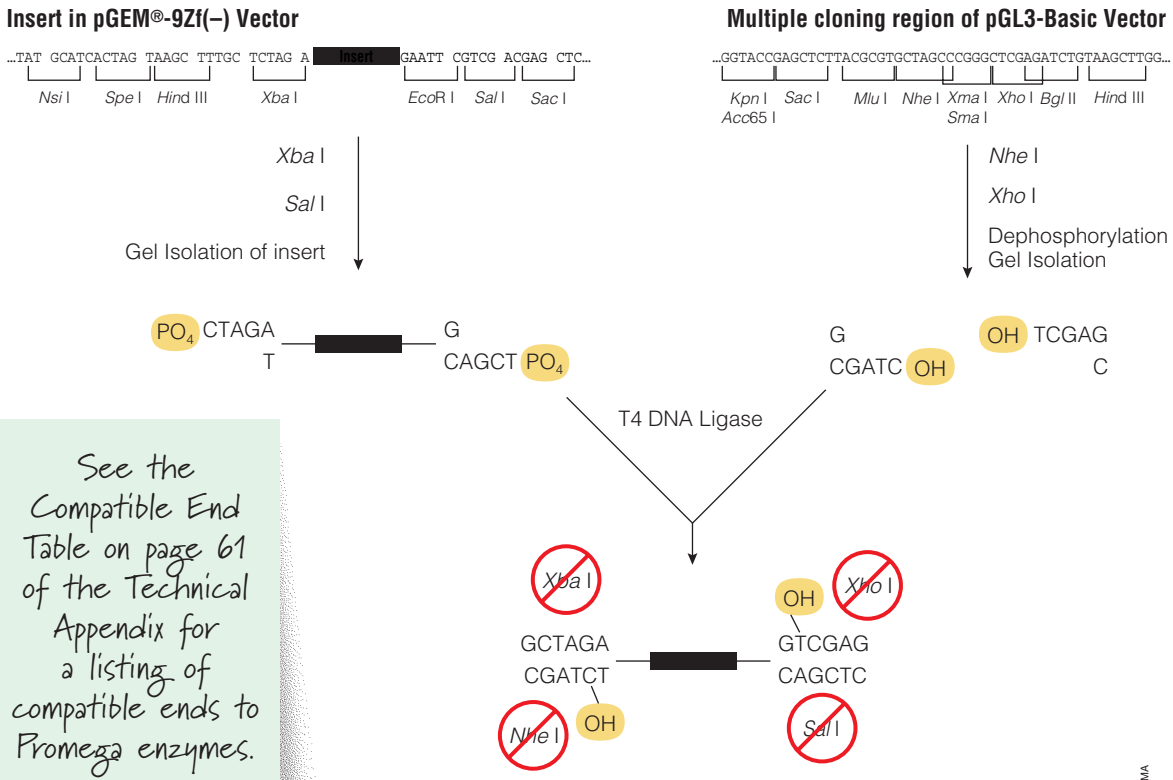
Subcloning Strategy: Moving Inserts with Compatible Restriction Sites

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

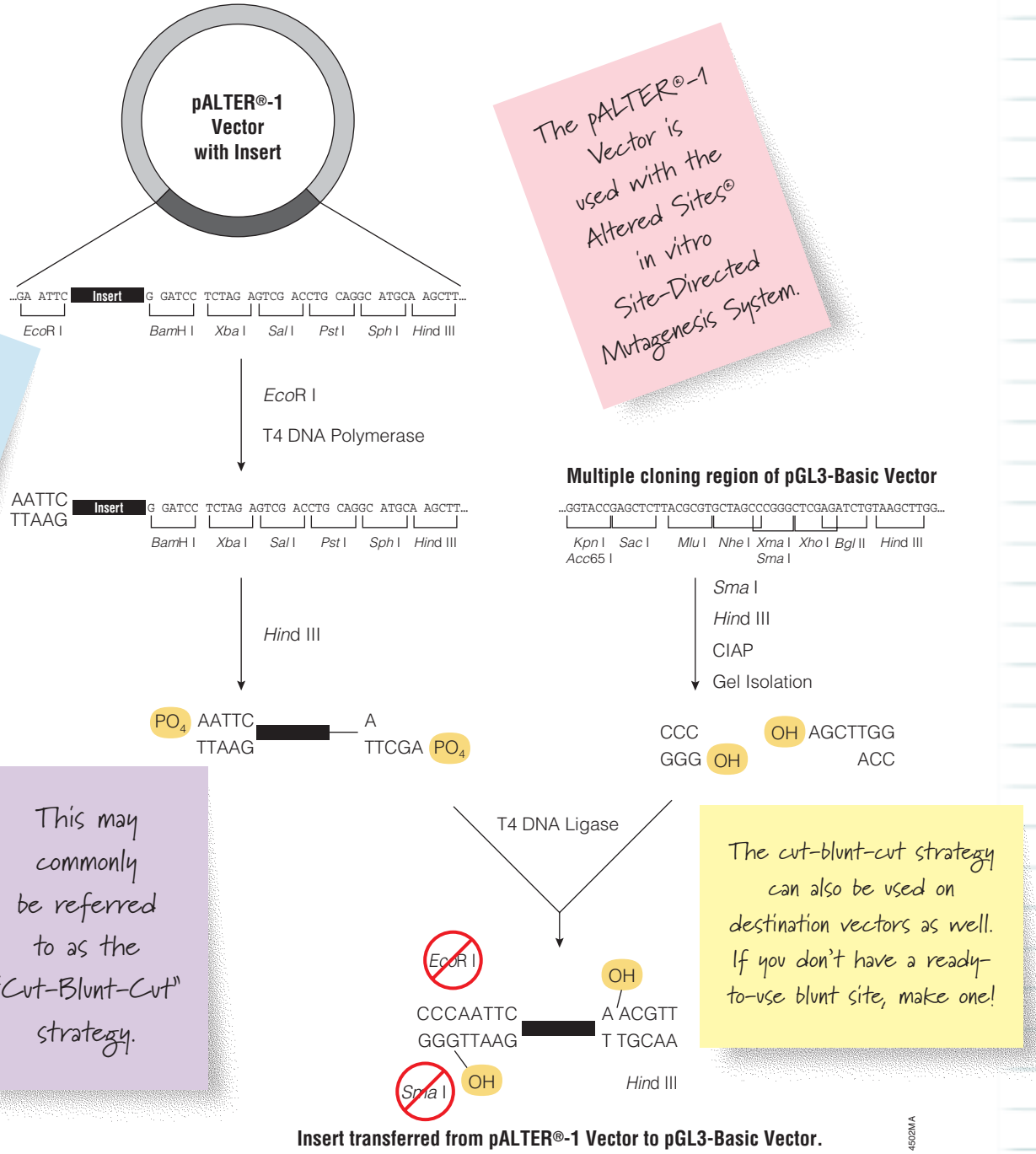
See the Compatible End Table on page 61 of the Technical Appendix for a listing of compatible ends to Promega enzymes.

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.

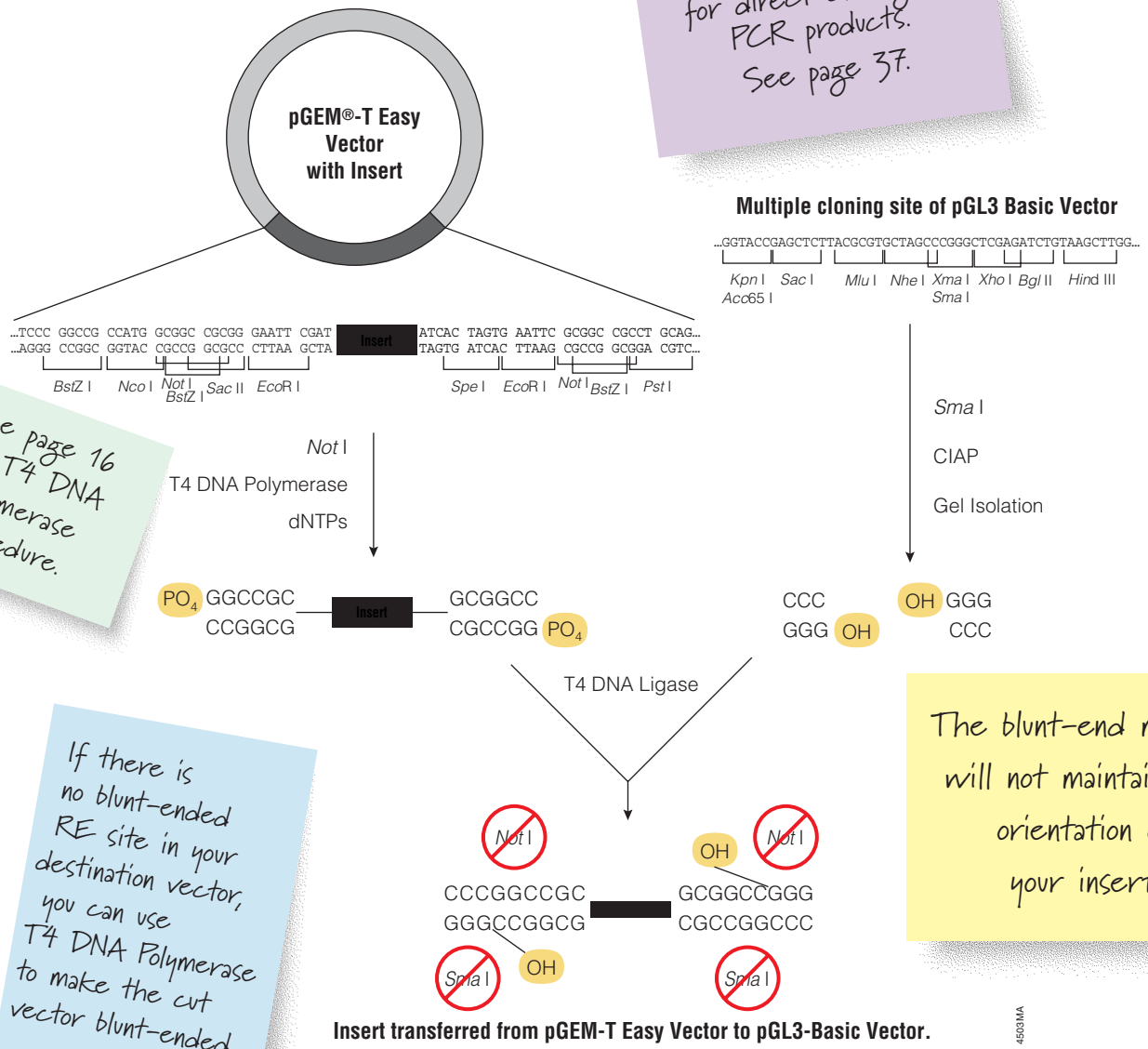
The cut-blunt-cut strategy can also be used on destination vectors as well. If you don't have a ready-to-use blunt site, make one!

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).

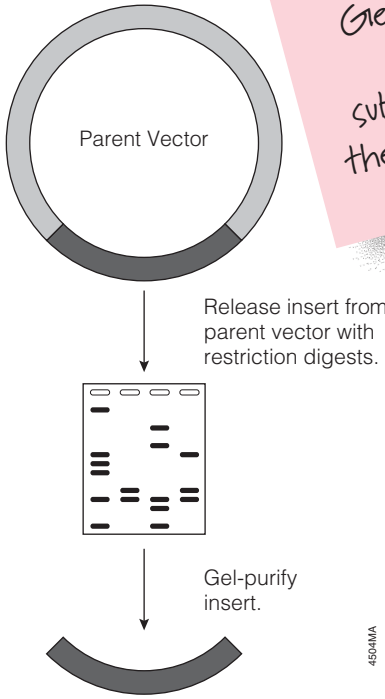
Restriction digestion is one of the most common reactions performed in molecular biology. For a digestion with a single RE the reaction is very simple:

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:

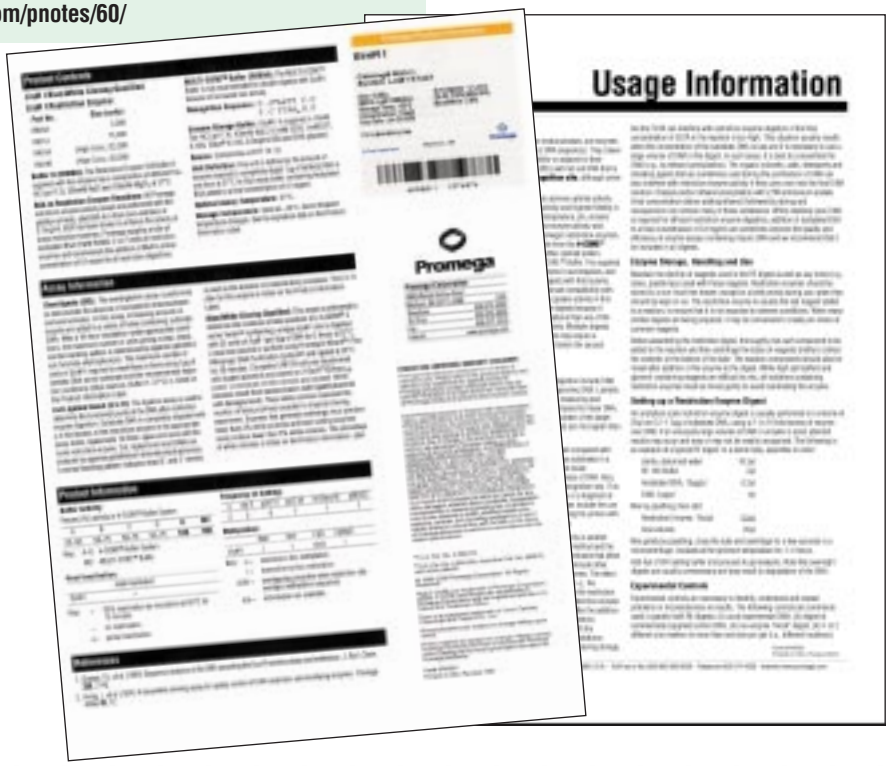
- The optimal reaction buffer
  This may be from the 4-CORE® System (Reaction Buffers A, B, C, D) or one of the other optimal buffers (Reaction Buffers E–L). This buffer always yields 100% activity for the enzyme that it accompanies, and serves as the specific reaction buffer for single digests.
- MULTI-CORE™ 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.

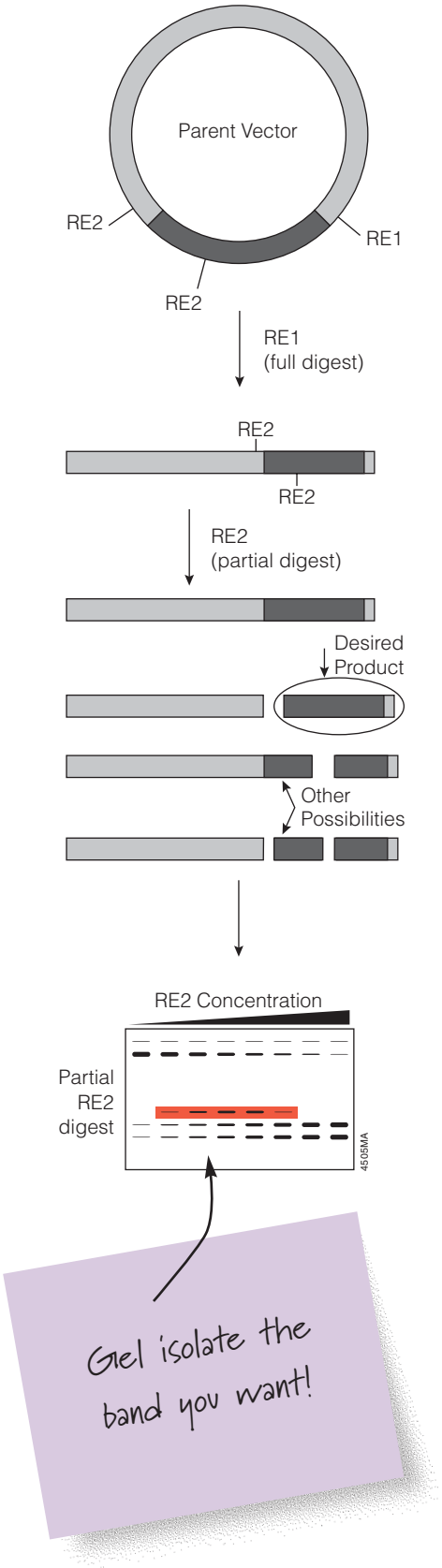
Partial Restriction Digestion

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.

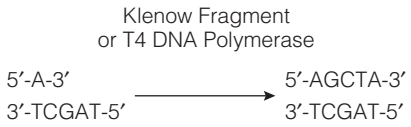
1. Digest 10µg of parent vector to completion to linearize (i.e., RE1; 50µl reaction).
2. Purify vector with the Wizard® SV Gel and PCR Clean-Up System directly from the reaction. Elute in 20µl nuclease-free water.
3. On ice, create serial dilutions of RE2 in 1X RE Buffer containing 0.1mg/ml Acetylated 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.



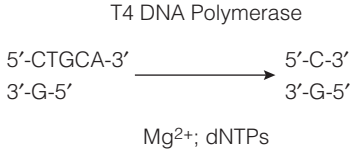
Creating Blunt Ends

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

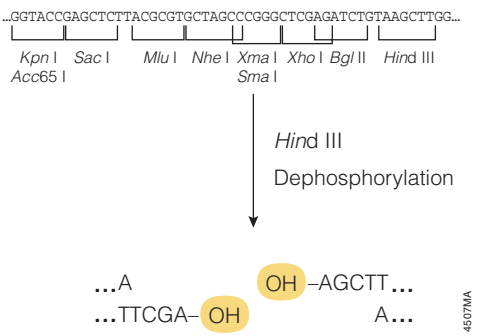
Cat.# M2206 500u 5–10u/µl

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.

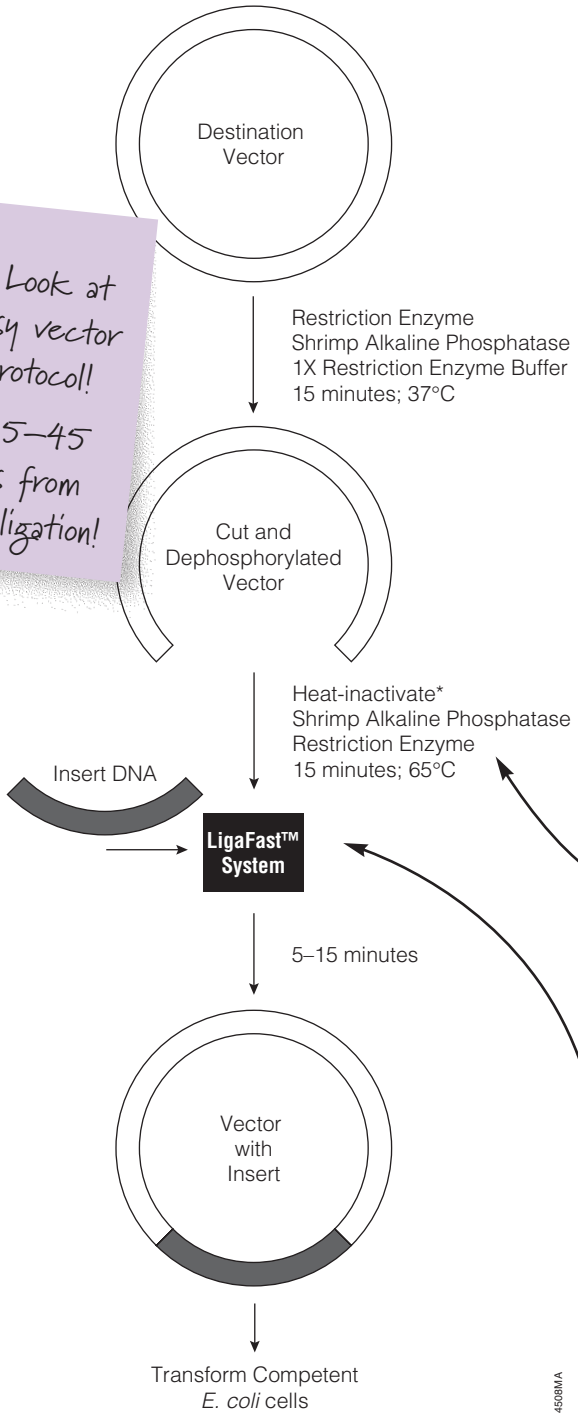
Is it necessary to dephosphorylate linearized vectors before performing the insert ligation?

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.

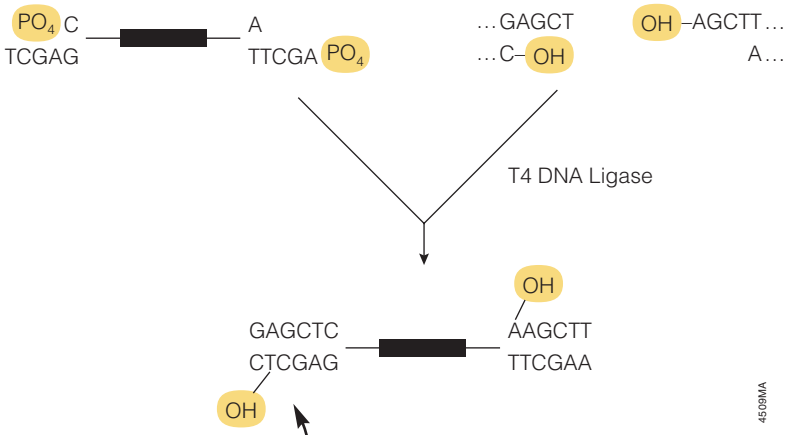
Ligation: Ligating Vector and Insert

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.

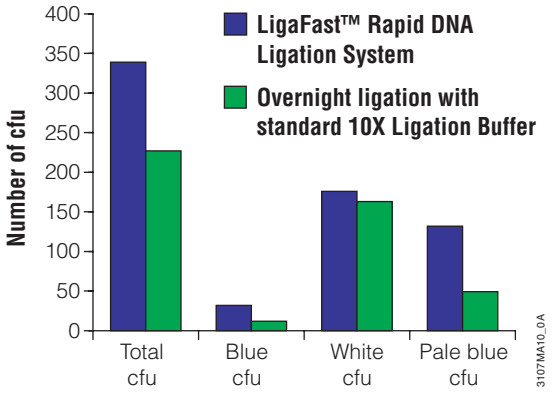
1. Assemble the following reaction in a sterile microcentrifuge tube:

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.

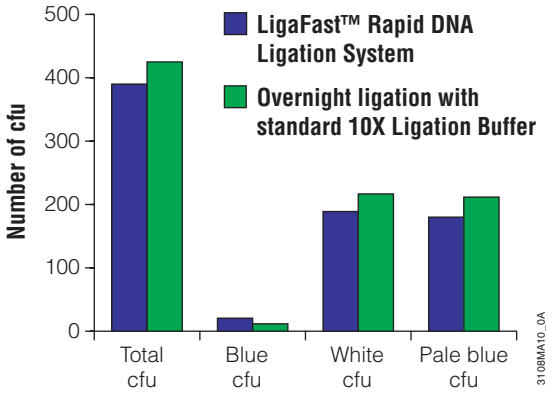
LigaFast™ Rapid DNA Ligation System
Cat.# M8221 30 reactions
Cat.# M8225 150 reactions
See the Product Information Sheet at:
www.promega.com/tbs

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.

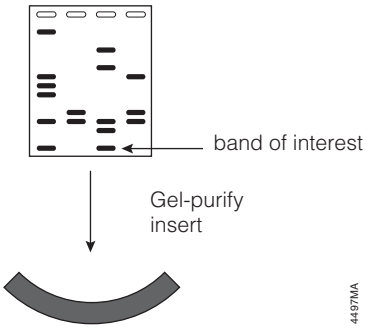
Questions on Subcloning? Call Promega Technical Services

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.

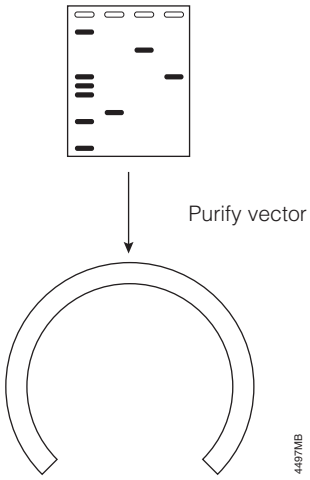
Contact Promega Technical Services directly or through your Branch Office or by email at: **techserv@promega.com**

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

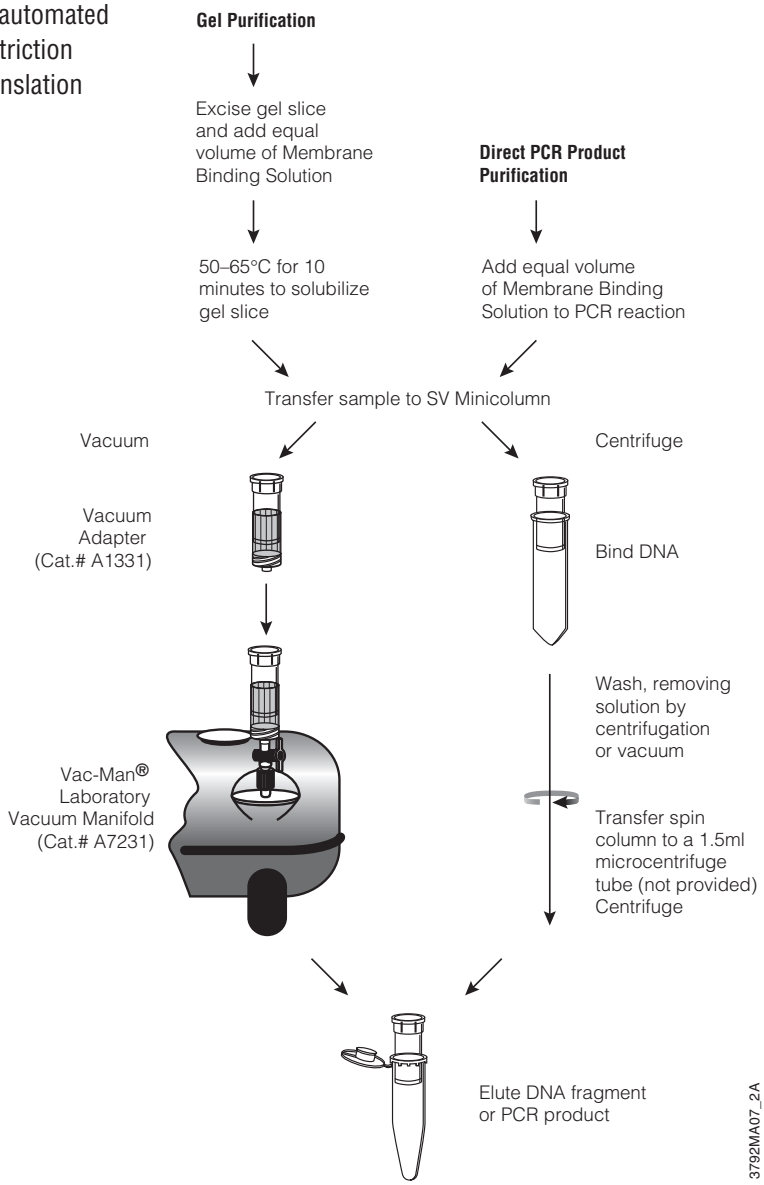
Cat.# A9282 250 preps

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.

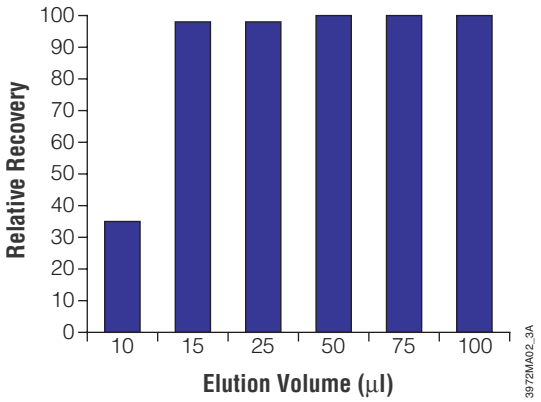
Capture up to 40µg of DNA on a single column!

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.

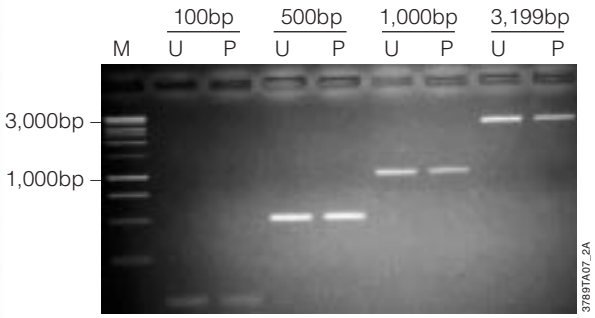
Purifying Vector and Insert



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.

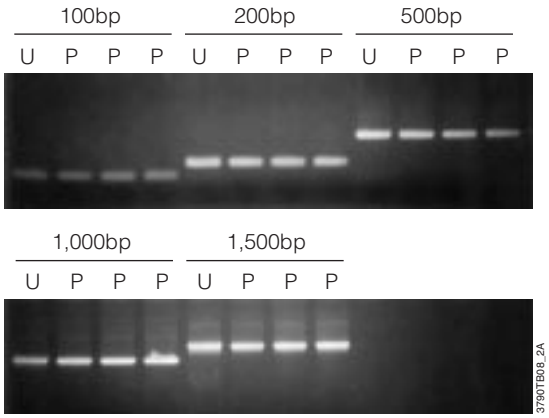
Concentrate DNA by eluting in as little as 15µl.

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.

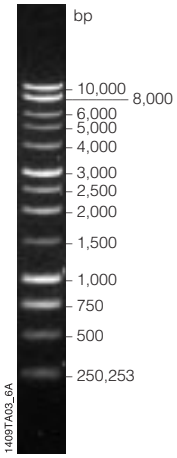
Ethidium bromide can detect as little as 1ng of dsDNA in a band.
Brown, T.A. (1998) In: Molecular Biology LABFAX II: Gene Analysis. 2nd ed. Academic Press, 101.

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.

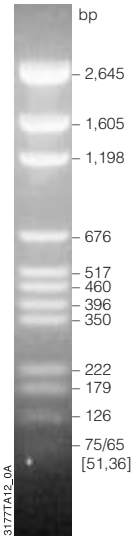
Each of these markers is available in a ready-to-use BenchTop version or in a conventional version.

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



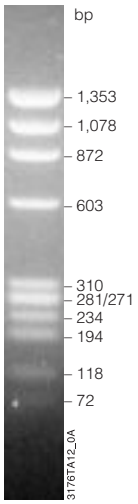
0.7% agarose

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



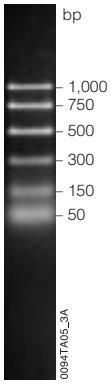
2% agarose

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



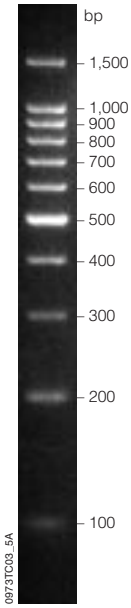
2% agarose

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.

PCR Subcloning

Introduction

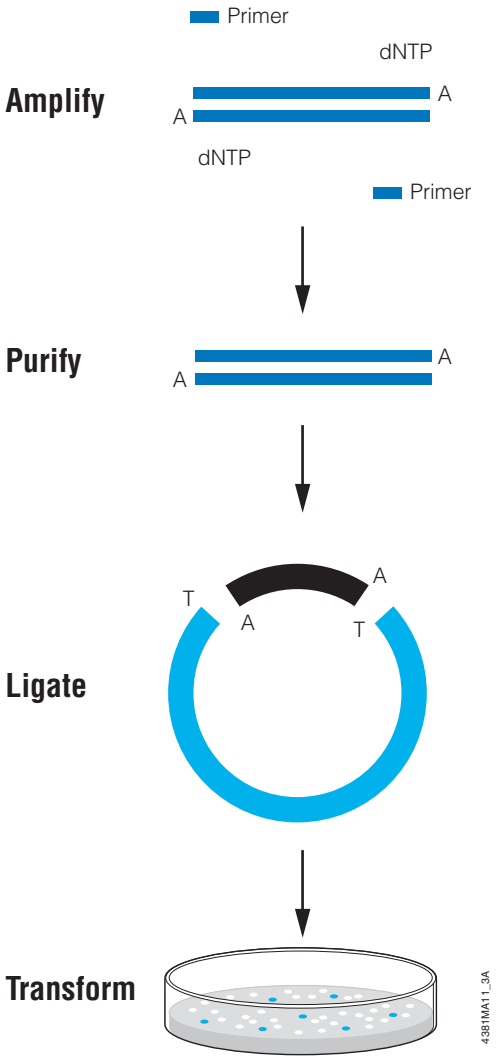
You may wish to subclone your PCR product into a plasmid cloning vector. When PCR was in its infancy, researchers found that subcloning PCR products by simple blunt-ended ligation into blunt-ended plasmid cloning vectors was not easy. Thermostable DNA polymerases, like *Taq* DNA polymerase, add a single nucleotide base extension to the 3′ end of blunt DNA in a template-independent fashion (1,2). These polymerases usually add an adenine, leaving an "A overhang."

Historically, researchers have used several approaches to overcome the cloning difficulties presented by the presence of A overhangs on PCR products. One method involves treating the product with the Klenow fragment of *E. coli* DNA Polymerase I to create a blunt-ended fragment for subcloning. However this technique is not particularly efficient.

Another method commonly used by researchers is to add restriction enzyme recognition sites to the ends of the PCR primers (3). The PCR product is then digested and subcloned into the desired plasmid cloning vector in a desired orientation. Care must be exercised in primer design when using this method, as not all REs cleave efficiently at the ends of DNA, and you may not be able to use every RE you desire (4). Some REs require extra bases outside the recognition site (see page 40), adding further expense to the PCR primers as well as risk of priming to unrelated sequences in the genome.

A method of choice for cloning PCR products is T-Vector cloning. In essence, the plasmid cloning vector is treated to contain a 3′ T overhang to match the 3′ A overhang of the amplicon (5). The A-tailed amplicon is directly ligated to the T-tailed plasmid vector with no need for further enzymatic treatment of the amplicon other than the action of T4 DNA ligase. Promega has systems based on this technology for routine subcloning, and direct mammalian expression.

4 Simple Steps to Success



References

1. Clark, J.M. (1988) Novel non-template nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. *Nucl. Acids Res.* **16**, 9677–86.
2. Mole, S.E., Iggo, R.D. and Lane, D.P. (1989) Using the polymerase chain reaction to modify expression plasmids for epitope mapping. *Nucl. Acids Res.* **17**, 3319.
3. Scharf, S.J., Horn, G.T. and Erlich, H.A. (1986) Direct cloning and sequence analysis of enzymatically amplified genomic sequences. *Science* **233**, 1076–8.
4. Kaufman, D.L. and Evans, G.A. (1990) Restriction endonuclease cleavage at the termini of PCR products. *BioTechniques* **9**, 304–6.
5. Mezei, L.M. and Storts, D.R. (1994) Cloning PCR Products. In: *PCR Technology Current Innovations*. Griffin, H.G. and Griffin, A.M. (eds). CRC Press, 21–7.

T-Vector Systems

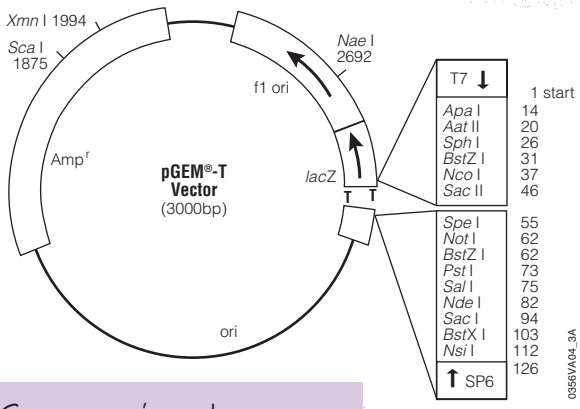
pGEM®-T and pGEM®-T Easy Vector Systems

The most basic need in PCR subcloning is a simple, general cloning vector. The pGEM-T and pGEM-T Easy Vector Systems[e,f,g] are designed for just that purpose. The vectors are based on the pGEM-5Zf(+) Vector[g] backbone. Each provide convenient T7 and SP6 promoters to serve as sequencing primer binding sites or for in vitro transcription of either strand of the insert with the appropriate RNA polymerase. The vectors have the *lac*Zα, allowing easy blue/white screening of the inserts with an appropriate bacterial strain (e.g., JM109, DH5α™, XL1 Blue, etc). To speed your research, these vectors are provided with 2X Rapid Ligation Buffer, allowing efficient ligation in just 1 hour with the supplied T4 DNA Ligase. You can either supply your own favorite *E. coli* cells or purchase the system with Promega JM109 Competent Cells. The choice is yours.

pGEM®-T Vector System I
(you supply competent cells)
Cat.# A3600 20 reactions

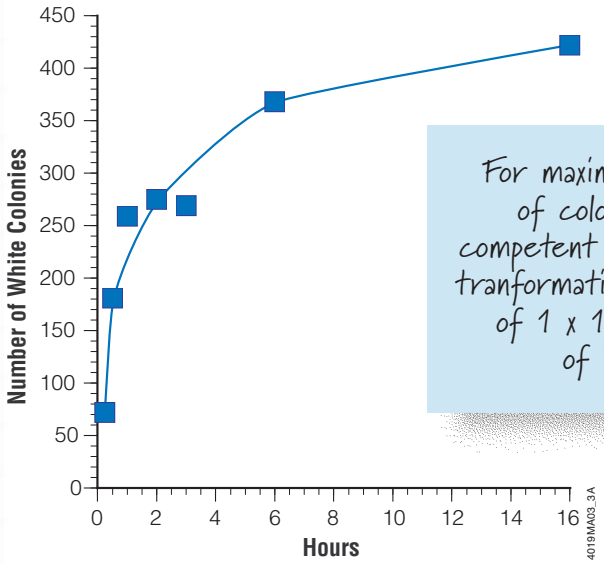
pGEM®-T Vector System II
(supplied with High Efficiency JM109 Competent Cells)
Cat.# A3610 20 reactions

Protocol available at:
www.promega.com/tbs/tm042/tm042.html



Select recombinants by blue/white selection.

Sequence inserts with the following:
- SP6 Promoter Primer
- T7 Promoter Primer
- M13 Forward Primer
- M13 Reverse Primer



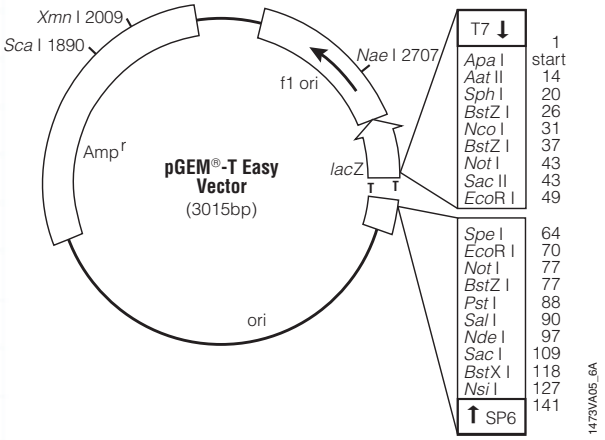
For maximum number of colonies, use competent cells with a tranformation efficiency of 1 x 10^8 cfu/µg of DNA.

Drop out insert with a single BstZ I digest.

Number of white colonies (transformants) versus time of ligation. Control pGEM-T Easy ligation reactions were set up at room temperature (24°C) and allowed to proceed from 0.25 to 16 hours using the methods described in Technical Manual #TM042. Graph was adapted from Table 2 in Frackman, S. and Kephart, D. (1999) Rapid ligation for the pGEM-T and pGEM-T Easy Vector Systems. *Promega Notes* **71,** 8–10.

PCR Subcloning

T-Vector Systems



pGEM®-T Easy Vector System I
(you supply competent cells)
Cat.# A1360 20 reactions

pGEM®-T Easy Vector System II
(supplied with High Efficiency JM109 Competent Cells)
Cat.# A1380 20 reactions

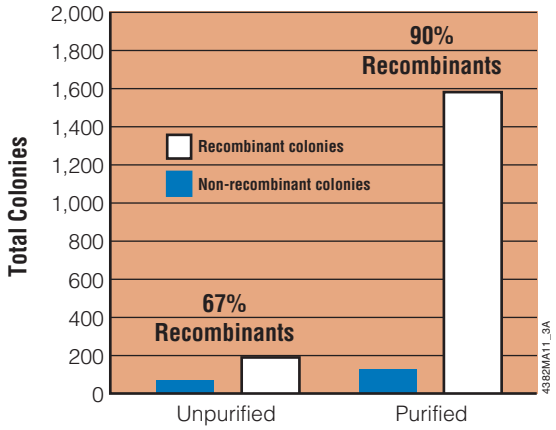
Protocol available at:
www.promega.com/tbs/tm042/tm042.html

Sequence inserts with the following:
- SP6 Promoter Primer
- T7 Promoter Primer
- M13 Forward Primer
- M13 Reverse Primer

Drop out insert with a single BstZ I, EcoR I or Not I digest.

For maximum subcloning efficiency, purify the PCR product before subcloning. The presence of PCR primers and primer dimers can reduce subcloning efficiency.

Purification achieved with Wizard® SV Gel and PCR Clean-Up System. For more information, see page 28.

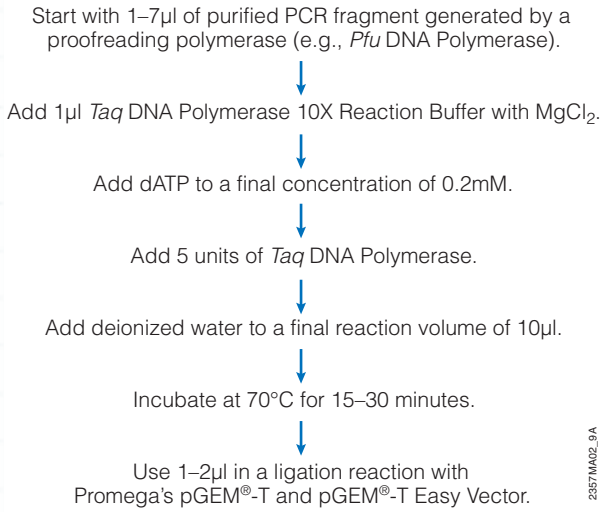


Cloning efficiency of a purified PCR product and an unpurified PCR product.

PCR Subcloning

Giving Blunt-Ended DNA an A-tail for T-Vector Subcloning

PCR amplicons generated with proofreading polymerases like *Pfu* or *Tli* DNA Polymerase are blunt-ended. Promega has developed an easy method to add an A-Tail to the DNA so that it can be used for T-Vector cloning.



Ends Left on PCR Products by Thermostable Enzymes.

Polymerase	Type of End*
Taq DNA Polymerase	3′ A overhang
GoTaq® DNA Polymerase	3′ A overhang
Tfl DNA Polymerase	3′ A overhang
Tth DNA Polymerase	3′ A overhang
Pfu DNA Polymerase	Blunt end
Tli DNA Polymerase	Blunt end
Long PCR mixes	Blunt end
Other Proofreading Polymerases	Blunt end

*All bases may be found at 3′ overhang; adenine tends to be encountered most often.

Full details of the protocol are available in the *pGEM®-T* and *pGEM®-T Easy Vector Systems Technical Manual*, TM042. The proofreading enzyme must be removed using a system like the Wizard® SV Gel and PCR Clean-Up System prior to the A-tailing procedure. Any remaining proofreading enzyme in the PCR will remove the A-overhangs created by the *Taq* DNA polymerase.

For more information and techniques for cloning PCR DNA, check out Promega Frequently Asked Questions for the T-Vector cloning systems at: **www.promega.com/faq**

Promega recommends a guanidine-based purification method, like the Wizard SV Gel and PCR Clean-Up System, to remove proofreading polymerases.

Subcloning with RE Sites

What PCR Cloning Controls Can Do for You

Each Promega PCR cloning system is provided with a control insert. The ligation and subsequent transformation of this positive control can give you a lot of information with regard to the ligation and transformation of your insert.

Typical Results

	Efficiency*	% White
Control insert	1110	92%
Control insert	1125	92%
No insert	92	—
No insert	109	—

*cfu/ng control insert DNA; JM109 cells at 1.5 × 10^8 cfu/µg; pGEM®-T Easy Vector System II using room temperature ligation for 1 hour.

The total number of blue colonies obtained with positive control insert and no-insert controls should be approximately equal. The negative control may have some white colonies as well.

Interpreting Results from T-Cloning

Experimental insert looks like control insert in efficiency and percent white colonies.

Successful experiment. Greater than 80% of the white colonies should contain inserts

Experimental insert and control insert look like negative control.

Ligation has failed. Avoid multiple freeze/thaws of the ligation buffer. Ligase buffer contains ATP and could be damaged by freeze/thaw cycles. You may need to dispense the ligase buffer into smaller aliquots for your experimental needs.

No colonies with experimental insert, control insert or negative control.

Transformation has failed. Reassess the competent cells with an intact, supercoiled plasmid and determine the transformation efficiency. Use cells >1 × 10^8 cfu/µg to insure >100 colonies from the control insert ligation.

Experimental insert has more blue colonies than control insert or negative control and fewer white colonies than control insert.

In-frame insertion, no interruption of α-fragment. Although the pGEM®-T Vector Control DNA will produce recombinants that generate white colonies, the insertion of other DNA fragments into the *lac*Z coding sequence may not result in white colonies unless the fragments disrupt the *lac*Z reading frame. Although this tends to occur most frequently with PCR products of 500bp or less, inserts of up to 2kb can result in blue colonies. Moreover, some insert DNAs can also result in pale blue colonies or "bull's-eye" colonies with a blue center and a white perimeter. In one case in particular, we found that a 1.8kb insert when oriented in one direction produced white colonies and in the other produced bull's-eye colonies [Knoche, K. and Kephart, D. (1999) Cloning blunt-end *Pfu* DNA polymerase-generated PCR fragments into pGEM®-T Vector Systems. *Promega Notes* **71**, 10–13.].

Subcloning Using PCR Primers Containing Restriction Sites

Frequently, the ends of insert DNA do not contain a suitable restriction enzyme site. The problem can be solved by using PCR to generate a site at the desired location. For this technique, the restriction enzyme site is designed into the 5′-end of the PCR primer. Because certain restriction enzymes inefficiently cleave recognition sequences located at the end of a DNA fragment, it is advisable to include at least four additional bases in front of the restriction recognition site. For the majority of restriction enzymes this will result in efficient cleavage.

Success in digesting PCR products can depend on the purity of the PCR product. Primers and primer dimers are present in overwhelming quantities when compared to the actual PCR product. Your PCR product will be competing with primers and primer dimers for the attention of the restriction enzyme, resulting in conditions favoring partial restriction digest. A simple clean-up of the reaction with the Wizard® SV Gel and PCR Clean-Up System can improve RE cleavage.

If you encounter a situation where the PCR product will not subclone, the digest may be adversely affected by proximity to the end of the PCR product. To improve the "placement" of the restriction site, the PCR product can be subcloned into the pGEM®-T Easy Vector. The restriction site should be readily cleavable in the context of the vector.

Ability of Restriction Enzymes to Cut PCR Products With RE Sites Near the End of the Fragment.

Enzyme	Distance (in bp) from the end of the PCR Fragment			
	0	1	2	3
Apa I	–	–	+/–	+
*Bam*H I	–	+/–	+	+
*Bst*X I	–	+/–	+	+
Cla I	–	+/–	+	+
*Eco*R I	–	+/–	+	+
*Eco*R V	–	+	+	+
*Hin*d III	–	–	+	+
Not I	–	–	+	+
Pst I	–	–	+/–	+
Sac I	–	+/–	+	+
Sal I	+	+	+	+
Sma I	–	+/-	+	+
Spe I	+	+	+	+
Xba I	–	+/–	+	+
Xho I	–	–	+/–	+

PCR products in which the end of the restriction enzyme recognition sequence was flush with the end of the product or 1, 2, or 3 base pairs away from the end of the product were digested with a variety of enzymes. Purified PCR fragments (10–50ng) were digested with 0.5units of RE in 10µl of the appropriate reaction buffer for 45 minutes. Digestion is indicated as follows: Cleavable (+), not cleavable (–) and not reproducible (+/–). Data are the result of at least duplicate experiments and are reproduced by permission of Eaton Publishing. Taken from Simmermann, K. *et al.* (1998) Digestion of terminal restriction endonuclease recognition sites on PCR products. *BioTechniques* **24**, 582–4.

PCR Subcloning: Ordering Information

Basic PCR Cloning Systems

Product	Size	Cat.#
pGEM®-T Vector System I[e,f,g]	20 reactions	A3600
Supplied with linearized, ligation-ready pGEM®-T Vector, 2X Rapid Ligation Buffer, T4 DNA Ligase and Positive Control Insert.		
pGEM®-T Vector System II[e,f,g]	20 reactions	A3610
Same contents as System I with 6 × 200µl JM109 High Efficiency Competent Cells.		
pGEM®-T Easy Vector System I[e,f,g]	20 reactions	A1360
Supplied with linearized, ligation-ready pGEM®-T Easy Vector, 2X Rapid Ligation Buffer, T4 DNA Ligase and Positive Control Insert.		
pGEM®-T Easy Vector System II[e,f,g]	20 reactions	A1380
Same contents as System I with 6 × 200µl JM109 High Efficiency Competent Cells.		

For Laboratory Use.

Sequencing Primers

Product	Conc.	Size	Cat.#
T7 Promoter Primer [5′-d(TAATACGACTCACTATAGGG)-3′]	10µg/ml	2µg	Q5021
SP6 Promoter Primer [5′-d(TATTTAGGTGACACTATAG)-3′]	10µg/ml	2µg	Q5011
pUC/M13 Primer, Forward (24 mer) [5′-d(CGCCAGGGTTTTCCCAGTCACGAC)-3′]	10µg/ml	2µg	Q5601
pUC/M13 Primer, Reverse (22 mer) [5′-d(TCACACAGGAAACAGCTATGAC)-3′]	10µg/ml	2µg	Q5421

Thermostable DNA Polymerases

Product	Conc.	Size	Cat.#
PCR Master Mix[h]	2X	100 reactions	M7502
	2X	1,000 reactions	M7505
PCR Master Mix contains dNTPs, buffer, Mg^{2+} and *Taq* DNA Polymerase. A standard reaction contiains 25µl of PCR Master Mix giving 1.5mM Mg^{2+}, 200µM each dNTP and 1.25u of *Taq* DNA Polymerase in the final 50µl reaction.			
GoTaq® DNA Polymerase[i]	100u	5u/µl	M3001
	500u	5u/µl	M3005
	2,500u	5u/µl	M3008
Supplied with 5X Green and 5X Colorless GoTaq® Reaction Buffer. Each contain 1.5mM $MgCl_2$ in the final 1X concentration. Use the Green Buffer for direct gel analysis of amplification reactions. Use the Colorless Buffer for any reaction requiring absorbance or fluorescence measurements without prior PCR clean-up.			
Taq DNA Polymerase in Storage Buffer B[i]	5u/µl	100u	M1661
(Supplied with 10X Thermophillic Reaction Buffer and 25mM $MgCl_2$ Solution.)	5u/µl	500u	M1665
	5u/µl	2,500u	M1668
Taq DNA Polymerase in Storage Buffer B[i]	5u/µl	100u	M2661
(Supplied with 10X Thermophillic Reaction Buffer containing 15mM $MgCl_2$.)	5u/µl	500u	M2665
	5u/µl	2,500u	M2668
Taq DNA Polymerase in Storage Buffer A[i]	5u/µl	100u	M1861
(Supplied with 10X Thermophillic Reaction Buffer and 25mM $MgCl_2$ Solution.)	5u/µl	500u	M1865
	5u/µl	2,500u	M1868
Taq DNA Polymerase in Storage Buffer A[i]	5u/µl	100u	M2861
(Supplied with 10X Thermophilic Reaction Buffer containing 15mM $MgCl_2$.)	5u/µl	500u	M2865
	5u/µl	2,500u	M2868

For Laboratory Use.

PCR Subcloning: Ordering Information

Thermostable DNA Polymerases

Product	Size	Conc.	Cat.#
*Taq*Bead™ Hot Start Polymerase[f]*			
(Supplied with 10X Thermophillic Reaction Buffer and 25mM $MgCl_2$ Solution.)	100 reactions	1.25u/bead	M5661
Tfl DNA Polymerase[f]*	100u	5u/µl	M1941
(Supplied with *Tfl* 10X Reaction Buffer and 25mM $MgSO_4$ Solution.)	1,000u	5u/µl	M1945
Tth DNA Polymerase[f]*	100u	5u/µl	M2101
(Supplied with 10X Reverse Transcription Buffer, 10X Chelate Buffer, 10X Thermophillic Reaction Buffer, 25mM MgCl2 and 25mM $MnCl_2$.)	500u	5u/µl	M2105
Pfu DNA Polymerase[f]*	100u	2–3u/µl	M7741
(Supplied with *Pfu* 10X Reaction Buffer containing $MgSO_4$. Not available in North America.)	500u	2–3u/µl	M7745
Tli DNA Polymerase[f]*			
(Supplied with 10X Thermophillic Reaction Buffer and 25mM $MgCl_2$.)	50u	3u/µl	M7101

*For Laboratory Use.

PCR-qualified Nucleotides

Product	Conc.	Size	Cat.#
Set of dATP, dCTP, dGTP, dTTP[f]	100mM	10µmol each	U1330
	100mM	25µmol each	U1420
	100mM	40µmol each	U1240
	100mM	200µmol each	U1410
PCR Nucleotide Mix[f]	10mM	200µl	C1141
	10mM	1,000µl	C1145

Equal mixture of dATP, dCTP, dGTP and dTTP. Use 1µl per 50µl reaction for a final dNTP concentration of 200µM each.

For Laboratory Use.

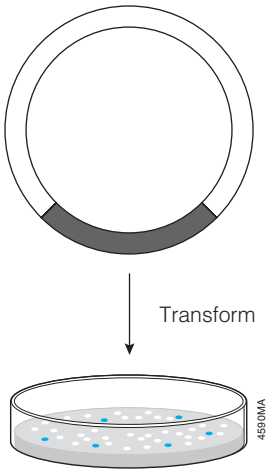
Transforming Bacteria

Properties of E. coli Strains for Subcloning

Common laboratory strains of *E. coli,* like JM109, DH5α™, and XL-1 Blue, are different from their wildtype counterparts. These strains carry some mutations designed to help you propagate plasmids. Typically laboratory strains have a mutation in the *rec*A gene (*rec*A1), a gene involved in recombination. The mutant gene limits recombination of the plasmid with the *E. coli* genome so that the plasmid inserts are more stable (the *rec*A1 mutation is more effective than the *rec*A13 mutation). Each of these strains also carries the *end*A1 mutation that inactivates a nuclease that might copurify with plasmids during purification. This mutation helps you to purify higher quality plasmids. Special treatments must be performed on plasmids from strains that do not have this mutation (e.g., RR1, HB101, etc.) to eliminate the nuclease from the plasmid prep (e.g., the Alkaline Protease digestion in the Wizard® *Plus* SV Miniprep protocol).

Common laboratory strains of *E. coli* are typically defined as K strains or B strains based on the presence of the restriction and modification system that functions around *Eco*K I or *Eco*B I, respectively. In a wildtype K strain, the *E. coli* will have both the *Eco*K I restriction enzyme to cleave foreign DNA and *Eco*K I methylase to protect and mask host DNA recognition sequences. In B strains, the *Eco*B I restriction enzyme and methylase serve the same purpose. Strains like JM109, DH5α™ and XL-1 Blue are K strains but carry the *hsd*R17 (r_{K^-}, m_{K^+}) mutation. This mutation knocks out the *Eco*K I restriction enzyme but leaves the methylase intact. Therefore, these strains will not degrade plasmid DNA isolated from a B or K strain but will methylate it. This is useful if the DNA must be transferred to a K strain with an intact K restriction and methylation system.

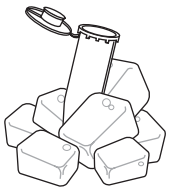
If you wish to incorporate blue/white selection into your subcloning scheme, you need to transform *E. coli* carrying a *lac*ZΔ. This mutation deletes a portion of the β-galactosidase gene leaving what is termed the ω-fragment. The plasmid vector supplies this deleted portion, or α-fragment. Once inside the bacterium, the plasmid produces the α-fragment and the *E. coli* produces the ω-fragment, which combine to make a



functional β-galactosidase. If grown on plate containing 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal), the colony will turn blue as a result of β-galactosidase activity and indicate full complementation of the bacterium by the plasmid. This is termed α-complementation. Blue/White cloning methods use plasmids with a multiple cloning region within the coding sequence of the α-fragment. Disruption of the reading frame due to the presence of the insert will produce a non-functional α-fragment incapable of α-complementation. These disrupted plasmids are differentiated from the plasmids without insert by the color of the colony (white versus blue), hence the term blue/white selection. Strains like JM109, DH5α™ and XL-1 Blue have the necessary deletion. One difference between these strains lies in how you get the bacterium to produce the ω-fragment. Both JM109 and XL-1 Blue have a second mutation call *lac I*^{q}. This mutation leads to increased production of the *lacI* repressor that stops transcription from the *lac* operon until substrate is present. To relieve this repression, these strains are grown on media containing the non-cleavable lactose analog, isopropyl-β-D-thiogalactopyranoside (IPTG). DH5α™ does not have the *lac I*^{q} mutation and constantly produces a low level of the ω-fragment through leaky transcription of the *lac* operon and therefore does not require IPTG for blue/white selection.

Transforming Bacteria

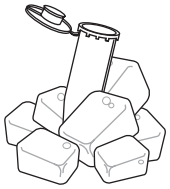
Ready-to-Use Competent Cells



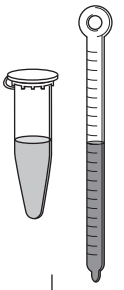
Thaw on crushed ice.

Add 1–50ng of DNA from ligation reacton.

Use in blue/white selection cloning procedures!

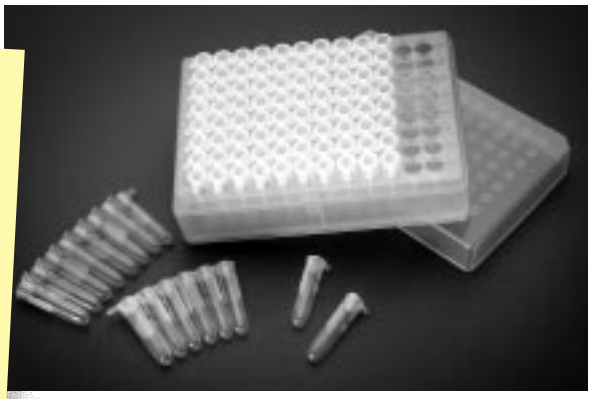


Incubate on crushed ice for 30 minutes.

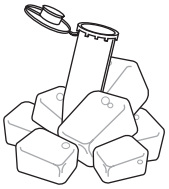


Heat shock at 42°C for 30 seconds.

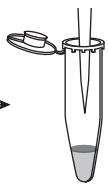
Single-use aliquots! Use one-at-a-time or all 96 at one time.



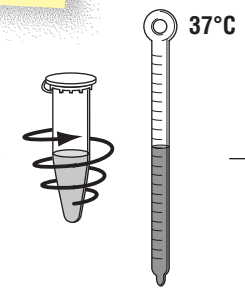
3722CA05_2A



Place on crushed ice for 2 minutes.



Add 250µl room temperature SOC Media.



Incubate at 37°C; with shaking (225–250rpm) for 60 minutes.

4591MA

Spread 100µl per plate. Incubate at 37°C overnight.

Select96™ Competent Cells

Select96™ Competent *E. coli* cells are single-use aliquots of high competency cells provided in 12 eight-strip tubes ready for use. Simply snip off the number of transformations you need, thaw on ice and proceed. Handle all steps in the tubes from beginning to end. No need to transfer to other tubes. Cells are guaranteed to provide at least 1 × 10^8 cfu/µg of supercoiled DNA. The cells are the *rec*A1, *end*A1, and *lac*ZΔM15. Blue/White selection does not require IPTG induction. For more information on genotype, see the table on page 64.

Select96™ Competent Cells
Cat.# L3300 1 x 96 reactions

Full protocol available at:
www.promega.com/tbs/tb301/tb301.html

Transforming Bacteria

Ready-to-Use Competent Cells

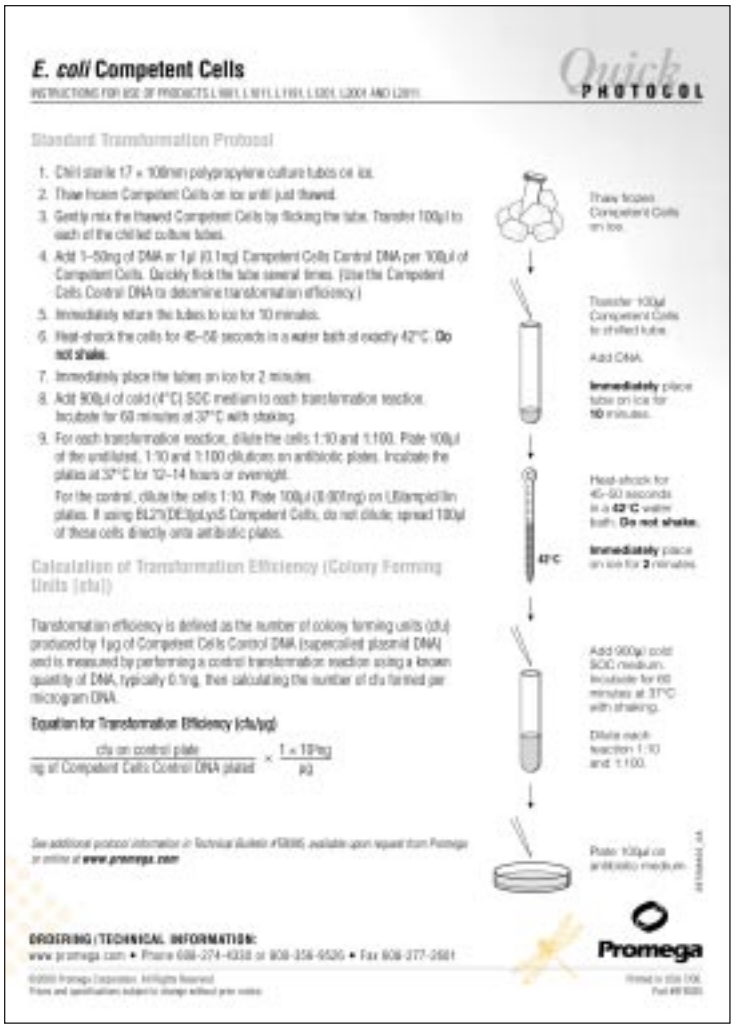
JM109 Competent Cells, >10^8 cfu/µg
Cat.# L2001 5 x 200µl

JM109 Competent Cells, >10^7 cfu/µg
Cat.# L1001 5 x 200µl

Protocol available at:
www.promega.com/tbs/tb095/tb095.html

JM109 Competent Cells

Available in two efficiencies: High Efficiency at greater than 10^8cfu/µg and Subcloning Efficiency at greater than 10^7cfu/µg. JM109 cells are an ideal host for many molecular biology applications including standard subcloning that requires blue/white screening, scale-up for large plasmid preparations and routine minipreps. The cells are *rec*A1, *end*A1, and *lac*Z∆*M*15.



***E. coli* Competent Cells**

Quick PROTOCOL

Standard Transformation Protocol

1. Chill sterile 17 x 100mm polypropylene culture tubes on ice.
2. Thaw frozen Competent Cells on ice until just thawed.
3. Gently mix the thawed Competent Cells by flicking the tube. Transfer 100µl to each of the chilled culture tubes.
4. Add 1–50ng of DNA or 1µl (0.1ng) Competent Cells Control DNA per 100µl of Competent Cells. Quickly flick the tube several times. (Use the Competent Cells Control DNA to determine transformation efficiency.)
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 900µl of cold (4°C) SOC medium to each transformation reaction. Incubate for 60 minutes at 37°C with shaking.
9. For each transformation reaction, dilute the cells 1:10 and 1:100. Plate 100µl of the undiluted, 1:10 and 1:100 dilutions on antibiotic plates. Incubate the plates at 37°C for 12–14 hours or overnight.

   For the control, dilute the cells 1:10. Plate 100µl (0.001ng) on LB/ampicillin plates. If using B121(DE3)pLysS Competent Cells, do not dilute; spread 100µl of these cells directly onto antibiotic plates.

Calculation of Transformation Efficiency (Colony Forming Units [cfu])

Transformation efficiency is defined as the number of colony forming units (cfu) produced by 1µg of Competent Cells Control DNA (supercoiled plasmid DNA) and is measured by performing a control transformation reaction using a known quantity of DNA, typically 0.1ng, then calculating the number of cfu formed per microgram DNA.

Equation for Transformation Efficiency (cfu/µg)

$$\frac{\text{cfu on control plate}}{\text{ng of Competent Cells Control DNA plated}} \times \frac{1 \times 10^3\text{ng}}{\text{µg}}$$

See additional protocol information in Technical Bulletin #TB095, available upon request from Promega or online at www.promega.com

ORDERING / TECHNICAL INFORMATION:
www.promega.com • Phone 608-274-4330 or 800-356-9526 • Fax 608-277-2601

Promega

Transforming Bacteria

Making Your Own Competent Cells

Preparation of Competent Cells: Modified RbCl Method

This rubidium chloride protocol gives better transformation efficiencies than the $CaCl_2$ procedure for most strains. The procedure is an adaptation of one described in Hanahan, D. (1985) In: *DNA Cloning*, Volume 1, D. Glover, ed., IRL Press, Ltd., London, 109.

Materials to Be Supplied by the User
(Solution compositions are provided on page 48.)

- LB medium and plates
- LB + 20mM $MgSO_4$
- TFB1, ice-cold
- TFB2, ice-cold
- dry ice/isopropanol bath

1. Inoculate a single colony from an LB plate (for JM109, use M9 + B1 plate so that F′ episome is maintained) into 2.5ml of LB medium in a plating tube. Incubate overnight at 37°C with shaking (approximately 225rpm).
2. Subculture the overnight culture at a 1:100 dilution by inoculating 2.5ml into 250ml of LB supplemented with 20mM $MgSO_4$. Grow the cells in a 1L flask until the OD_{600} reaches 0.4–0.6 (usually 5–6 hours, but the time may vary).
3. Pellet the cells by centrifugation at 4,500 × *g* for 5 minutes at 4°C. For a 250ml culture, use two 250ml centrifuge bottles in a large rotor.
4. Gently resuspend the cell pellet in 0.4 original volume of ice-cold TFB1. For a 250ml subculture, use 100ml of TFB1 (50ml/bottle). Combine the resuspended cells in one bottle. For the remaining steps, keep the cells on ice and chill all pipettes, tubes and flasks.
5. Incubate the resuspended cells on ice for 5 minutes at 4°C.
6. Pellet the cells by centrifugation at 4,500 × *g* for 5 minutes at 4°C.
7. Gently resuspend the cells in 1/25 original volume of ice-cold TFB2. For a 250ml subculture, use 10ml of TFB2.
8. Incubate the cells on ice for 15–60 minutes, then dispense 100µl/tube for storage at –70°C. Quick-freeze the tubes in a dry ice/isopropanol bath. JM109 competent cells prepared by this method are stable for 1 year.

Many *E. coli* strains carry episomes (e.g., F′ and P2) expanding the capabilities of the bacterium for use in subcloning applications. For example, the XL1-Blue and JM109 strains carry the *lacI*^{q}ΔM15 mutation on the F′ episome. The episomes are extrachromosomal, replicating plasmids with a selectable marker (page 64). When making competent cells from strains with episomes, the bacteria must first be plated on selective media. For XL1-Blue, colonies are selected on tetracycline plate since the episome contains the Tet^R gene. Due to this, however, the strain cannot be used with subcloning plasmids containing the Tet^R gene for selection. JM109 cells should be selected first on M9 minimal media containing thiamine (vitamin B1). The bacterial chromosome lacks the biosynthetic genes for proline synthesis (*proAB*) but the episome carries those genes. Colonies grown on the M9 + B1 plates (recipe on page 48) can then be processed into competent cells ready for blue/white selection.

Note: Competent cells may be conveniently quick-frozen using ice bath racks, which have an ice compartment bottom and a removable rack (American Scientific Products, Cat.# S9233-1). Set up an ice bath in one rack and an ethanol bath in another. Place the top-labeled tubes in the rack with ice, dispense 100µl cells per tube, then close the tubes. Add the dry ice to the ethanol bath, wait for it to stop bubbling, then transfer the rack and tubes to the dry ice bath for about 15 seconds. Drain the ethanol, wipe with a tissue, and transfer to an empty bottom compartment and place in a –70°C freezer. Do not get alcohol on the lips of the tubes. Liquid nitrogen also can be used for quick-freezing, but not with these racks. Use only plasticware designed for liquid nitrogen.

Note: Be careful not to get alcohol on the labels because it will remove them.

Transforming Bacteria

Determining Transformation Efficiency of Competent Cells

This is a general protocol for use with the procedure for producing competent cells that is provided on page 46. Please follow manufacturers' instructions when using purchased competent cells.

1. Thaw a 100µl aliquot of competent cells on ice.
2. Transfer 100µl of the cells to a 17 × 100mm polypropylene tube prechilled on ice.
3. Add 0.1ng of a supercoiled plasmid [e.g., pGEM®-3Zf(+) Vector] in a 10µl volume to the competent cells and gently mix by swirling the pipet tip (do not mix by pipetting).
4. Transfer the tubes from ice to a 42°C water bath and heat shock for 45–60 seconds. Place on ice immediately to cool for 2 minutes.
5. Add 890µl of SOC medium (giving a concentration of 0.1ng DNA/ml) and incubate for 45 minutes at 37°C with shaking (~150rpm).
6. Transfer 100µl of cells to 900µl of SOC medium (0.01ng DNA/ml) and plate 100µl of this (0.001ng/100µl) onto LB plates with the appropriate antibiotic. You may wish to plate 100µl of undiluted cells for determining efficiency as well. The 100µl aliquot will contain 0.01ng DNA.
7. Incubate the plates overnight in a 37°C incubator and count the number of colonies obtained. For example, if 200 colonies were obtained:

$$\frac{200\text{cfu}}{0.001\text{ng}} = 2 \times 10^5\text{cfu/ng} = 2 \times 10^8\text{cfu/µg DNA}$$

Competencies below 10^6 may not be useful for subcloning applications.

Transforming Ligation Reactions

This is a general protocol for use with the procedure for producing competent cells that is provided on page 46. Please follow manufacturers' instructions when using purchased competent cells.

1. Thaw a 100µl aliquot of competent cells on ice.
2. Transfer 100µl aliquot of the competent cells to a 17 × 100mm polypropylene tube prechilled on ice.
3. Add no more than 10ng of DNA in a maximum of 10µl from a ligation reaction to the cells and gently swirling the pipet tip (do not mix by pipetting). Incubate on ice for 30 minutes.
4. Transfer the tubes from ice to a 42°C water bath and heat-shock for 45–60 seconds. Place on ice immediately to cool for 2 minutes.
5. Add 1ml of LB or SOC medium and incubate for 45 minutes at 37°C with shaking (~150rpm).
6. Plate 100–200µl of the transformation mix, or an appropriate dilution onto selection plates. If you suspect low ligation efficiency, take the remaining cells and pellet by a quick 10–20 second spin in a microcentrifuge. Pour off media and resuspend pellet in about 200µl of SOC and plate.

Transformation Controls

Controls help you figure out where things may have gone wrong with the subcloning procedure. When transforming bacteria with your subcloning reaction DNA, also determine transformation and efficiency.

Transforming a ligation control (see page 26) of cut, dephosphorylated vector without insert can tell you how many background colonies you can expect in your actual vector + insert ligation.

Transforming more than 10ng of DNA from a ligation reaction may actually decrease transformation efficiency.

Transforming Bacteria

Media and Solutions

LB (Luria-Bertani) medium (1 liter)

10g tryptone
5g yeast extract
5g NaCl

Adjust pH to 7.5 with NaOH and autoclave.

LB plates (1 liter)

Add 15g agar to 1 liter of LB medium. Adjust to pH 7.5 with NaOH. Autoclave. Pour 30–35ml of medium into 85mm petri dishes. If necessary, flame the surface of the medium with a Bunsen burner to remove bubbles.

LB Medium

Allow the medium to cool to 55°C before adding antibiotic (either ampicillin, 125µg/ml final concentration; tetracycline, 12.5µg/ml final concentration; or chloramphenicol, 20µg/ml final concentration).

LB plates plus antibiotic (1 liter)

Add 15g agar to 1 liter of LB medium. Adjust to pH 7.5 with NaOH. Autoclave. Pour 30–35ml of medium into 85mm petri dishes. If necessary, flame the surface of the medium with a Bunsen burner to remove bubbles.

SOC medium

2.0g tryptone
0.5g yeast extract
1ml 1M NaCl
0.25ml 1M KCl
1ml Mg^{2+} stock
(1M $MgCl_2 \cdot 6H_2O$, 1M $MgSO_4 \cdot 7H_2O$) filter-sterilized
1ml 2M glucose, filter-sterilized

Add tryptone, yeast extract, NaCl and KCl to 97ml distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg^{2+} stock and 2M glucose stock, each to a final concentration 20mM. Adjust to pH 7.0. Filter the complete medium through a 0.2µm filter unit.

M9 + B1 plates

6g Na_2HPO_4
3g KH_2PO_4
0.5g NaCl
1g NH_4Cl
15g Agar

Add deionized water to approximately 1L. Adjust to pH 7.4 with 10N NaOH. Autoclave and cool to 50°C. Then add the following sterile solutions:

2.0ml 1M $MgSO_4$
0.1ml 1M $CaCl_2$
10.0ml 20% Glucose (Filter Sterilized)
1.0ml 1M Thiamine-KCl (Filter Sterilized)

Pour 30–35ml of medium into 85mm petri dishes.

Bacterial Plates for Blue/White Selection.

Ampicillin Stock Solution

Dissolve at 50mg/ml in water, filter sterilize, store in aliquots at –20°C.

IPTG stock solution (0.1M)

1.2g IPTG (Cat.# V3951)

Add water to 50ml final volume. Filter-sterilize and store at 4°C.

X-Gal (2ml)

100mg X-gal (Cat.# V3941)

Dissolved at 50mg/ml in N,N′-dimethyl-formamide.
Cover with aluminum foil and store at –20°C.

LB plates with ampicillin/IPTG/X-Gal

Add 15g agar to 1 liter of LB medium. Autoclave. Allow the medium to cool to 50°C before adding ampicillin to a final concentration of 100µg/ml then supplement with 0.5mM IPTG and 80µg/ml X-Gal and pour the plates. Pour 30–35ml of medium into 85mm petri dishes. Let the agar harden. Store at 4°C for up to 1 month or at room temperature for up to 1 week.

TFB1

30mM potassium acetate
10mM $CaCl_2$
50mM $MnCl_2$
100mM RbCl
15% glycerol

Adjust pH to 5.8 with 1M acetic acid. Be very careful as you approach 5.8; if the pH drops lower than 5.8, a black precipitate may form. Filter sterilize (0.2µM) and store at room temperature.

TFB2

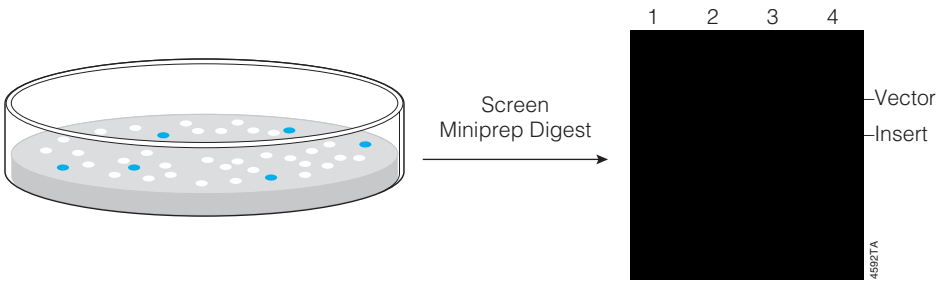
100mM MOPS or PIPES (pH 6.5)
75mM $CaCl_2$
10mM RbCl
15% glycerol

Adjust the pH to 6.5 with 1M KOH. Filter sterilize (0.2µM) and store at room temperature.

Screening for Recombinants

Introduction

Now that you've transformed your DNA and allowed the colonies to grow overnight, you need to determine if they contain the insert of interest. You can either screen them by colony PCR or the more traditional plasmid miniprep followed by restriction digestion. Colony PCR is the most rapid initial screen. A plasmid miniprep will take an extra day to grow up the culture but will provide a lot of material for further analysis. Some people do both and simply do not put the negative colonies that were identified through the colony PCR through the full miniprep procedure. The choice is yours.



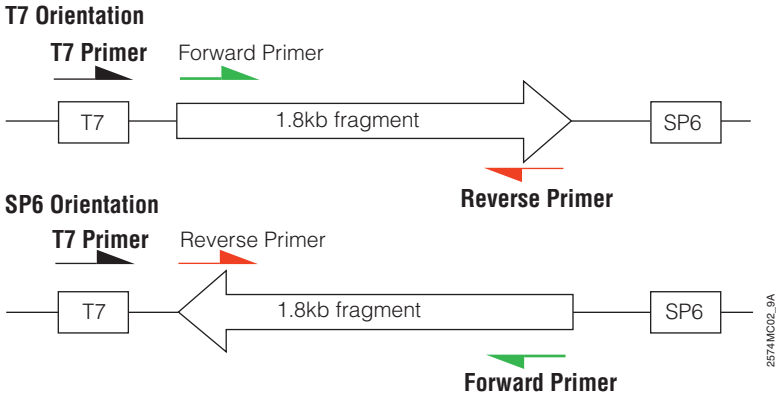
Screening for Recombinants

Colony PCR

Colony PCR involves lysing the bacteria and amplifying a portion of the plasmid. You can use either insert-specific primers or vector-specific primers to screen for recombinant plasmids. If your subcloning scheme will not maintain the orientation of the insert, you can use colony PCR to screen for orientation. Simply combine a vector-specific primer with an insert-specific primer.

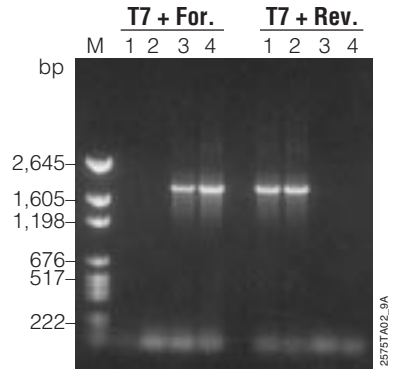
PCR cloning using the A-overhangs left by *Taq* DNA Polymerase(f) and an appropriately T-tailed vector (e.g., pGEM®-T Easy Vector) is not a technique that will retain orientation. The orientation can be rapidly assessed with colony PCR using vector-specific primers and insert-specific primers as detailed below.

This technique was used when screening for orientation of a 1.8kb insert into the pGEM-T Easy Vector. Colony PCR was performed with the T7 Promoter Primer and either the insert-specific forward or reverse PCR primer. Eight white colonies were chosen from the cloning experiment for analysis. Clones with the T7 orientation will produce the fragment only with the T7 primer and reverse PCR primer, and clones in the opposite (SP6) orientation will only produce a product with the forward PCR primer as illustrated below.



Colony Prep for Colony PCR

1. Pick a well isolated colony and transfer to 50µl of sterile water. Part of the colony may be transferred to LB media containing the appropriate antibiotic for over night culture and miniprep if desired.
2. Boil for 10 minutes.
3. Centrifuge at 16,000 x g for 5 minutes.
4. Use 5µl of the supernatant in a 50µl PCR.



Colony PCR. Colonies were suspended in 50µl sterile water, boiled for 10 minutes, centrifuged at 16,000 × *g* for 5 minutes, and 5µl of the supernatant was used in each amplification. The DNA was amplified by PCR in 50µl volumes with 50pmol of each primer and 1.25 units of *Taq* DNA Polymerase (Cat.# M1661). After an initial denaturation of 2 minutes at 94°C, the amplification profile was 35 cycles of denaturation (94°C for 30 seconds), annealing (55°C for 1 minute) and extension (72°C for 2.5 minutes); PCR was concluded with 1 cycle of 72°C for 10 minutes. Amplification products (8µl) were analyzed on a 1% agarose gel containing ethidium bromide.

Screening for Recombinants

Go Directly to Gel

GoTaq® DNA Polymerase is the ideal choice for colony PCR applications. The enhanced buffer can handle the "dirty" template better than a conventional reaction buffer, and the Green GoTaq Reaction Buffer allows you to load the PCR products directly onto a gel after amplification. No need to add loading dye.

Colony PCR with GoTaq® DNA Polymerase Typical Reaction.

Nuclease-Free Water	to 50µl
5X Green GoTaq® Reaction Buffer	10µl
PCR Nucleotide Mix (Cat.# C1141)	1µl
GoTaq® DNA Polymerase (5u/µl)	1.25u
Downstream Primer	50pmol
Upstream Primer	50pmol
Colony Lysate	5µl

Assemble the reaction on ice in the order listed. A master mix containing everything but the colony lysate can be prepared and dispensed into reaction tubes ready to accept the colony lysate.

GoTaq® DNA Polymerase

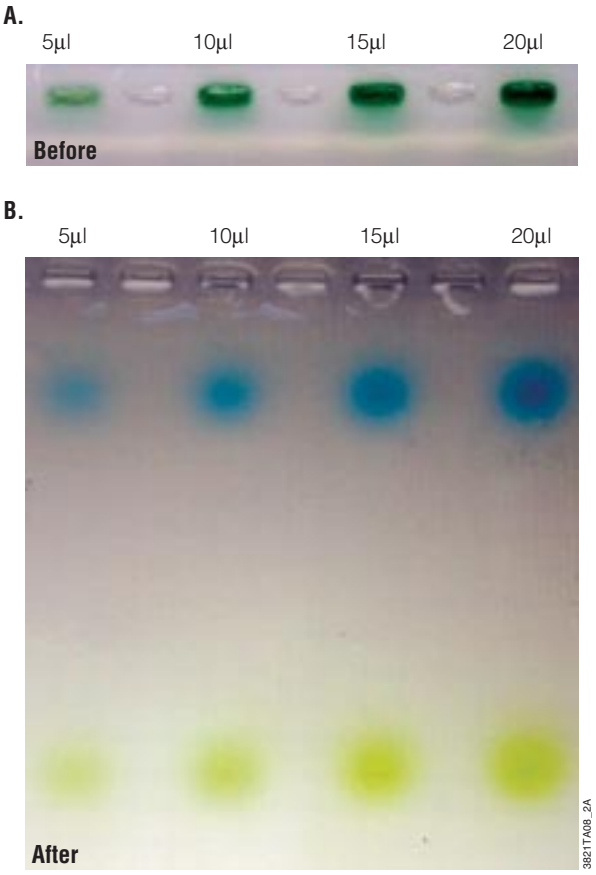
Cat.# M3001 100u; 80 reactions

Cat.# M3005 500u; 400 reactions

Cat.# M3008 2,500u; 2,000 reactions

Supplied with enzyme (5u/µl), 5X Green GoTaq® Reaction Buffer and 5X Colorless GoTaq® Reaction Buffer. Sufficient to give the indicated number of 50µl reactions using 1.25u of enzyme per reaction.

Protocol available at:
www.promega.com/tbs/9pim300/9pim300.html



Amplification reactions using GoTaq DNA Polymerase with Green GoTaq Reaction Buffer. Panel A shows loaded wills of an agarose gel. **Panel B** shows the blue and yellow dyes after electrophoresis. Volumes of 5, 10, 15 and 20µl of the amplification reactions were loaded into a 1% agarose gel with TBE buffer and subjected to electrophoresis.

Cycling Conditions For GoTaq® Reactions.

Step	Temp	Time	Cycles
Initial Denaturation	94°C	2	1
Denaturation	94°C	0.5–1.0	25–35
Annealing	42–65°C*	0.5–1.0	
Extension	72°C	1 minute/kb	
Final Extension	72°C	5	1
Soak	4°C	Indefinite	1

Reactions are placed in a thermal cycler that has been preheated to 94°C.

*Annealing temperature should be optimized for each primer set based on the primer melting temperature (T_m). An online calculator for melting temperatures of primers in GoTaq® Reaction Buffer is available at: **www.promega.com/biomath**

The extension time should be at least 1 minute/kilobase of target. Typically, anything smaller than 1kb uses a 1-minute extension.

Screening for Recombinants

Screening by Plasmid Miniprep and RE Digests

The classic method for screening colonies involves performing a plasmid miniprep followed by restriction digestion. Well-isolated colonies are picked from a plate and transferred to culture medium containing the appropriate antibiotic for selection. Proper sterile technique is important. Many different culture media formulations are commonly used for minipreps. Promega recommends LB media supplemented with antibiotics (see page 48) for miniprep cultures to insure that the bacteria do not outgrow the ability of the antibiotic to select for the plasmid. If a rich medium like Terrific Broth is used, the bacteria can grow to very high cell densities and deplete the antibiotic. Once the antibiotic is depleted, the selection pressure to keep the plasmid is removed, and the plasmid may be lost.

You can inoculate the colony into 1–10ml of culture medium. If using a high-copy plasmid, 1–5ml (more typically, 1–2ml) is plenty. If you are using a low-copy plasmid, inoculate 10ml. Aerating the culture is very important for maximum cell density. A 17 × 100mm culture tube is fine for 1–2ml. If growing a larger volume, a 50ml sterile, disposable culture tube is better. Incubate the culture overnight (12–16 hours) with shaking (~250rpm). Remember, the greater the surface area, the greater the aeration. You can even grow miniprep cultures in sterile 25–50ml Erlenmeyer flasks.

Once the DNA is purified, a portion of the plasmid is screened by restriction digestion. For high-copy plasmids, you can obtain 4–10µg plasmid DNA per purification (1–5ml). For low-copy plasmids, you will obtain 1–3µg plasmid DNA per purification (10ml). Use 0.5–1µg of plasmid in your digest. Design the digest so that you can easily determine if your plasmid contains insert.

Note: Be sure to run uncut plasmid on the same gel for comparison.

Antibiotics: Mode of Action and Mechanism of Resistance.

Antibiotic	Mode of Action	Mechanism of Resistance	Working Concentration	Stock Solution
Ampicillin (Amp)	A derivative of penicillin that kills growing cells by interfering with bacterial cell wall synthesis.	The resistance gene (*bla*) specifies a periplasmic enzyme, β-lactamase, which cleaves the β-lactam ring of the antibiotic.	50–125µg/ml in water	50mg/ml
Chloramphenicol (Cm)	A bacteriostatic agent that interferes with bacterial protein synthesis by binding to the 50S subunit of ribosomes and preventing peptide bond formation.	The resistance gene (*caf*) specifies an acetyltransferase that acetylates, and thereby inactivates, the antibiotic.	20–170µg/ml in ethanol	34mg/ml
Kanamycin (Kan)	A bactericidal agent that binds to 70S ribosomes and causes misreading of messenger RNA.	The resistance gene (*kan*) specifies an enzyme (aminoglycoside phosphotransferase) that modifies the antibiotic and prevents its interaction with ribosomes.	30µg/ml in water	50mg/ml
Streptomycin (Sm)	A bactericidal agent that binds to the 30S subunit of ribosomes and causes misreading of the messenger RNA.	The resistance gene (*str*) specifies an enzyme that modifies the antibiotic and inhibits its binding to the ribosome.	30µg/ml in water	50mg/ml
Tetracycline (Tet)	A light-sensitive bacteriostatic agent that prevents bacterial protein synthesis by binding to the 30S subunit of ribosomes.	The resistance gene (*tet*) specifies a protein that modifies the bacterial membrane and prevents transport of the antibiotic into the cell.	10µg/ml in liquid culture; 12.5µg/ml in plates	12.5mg/ml in ethanol

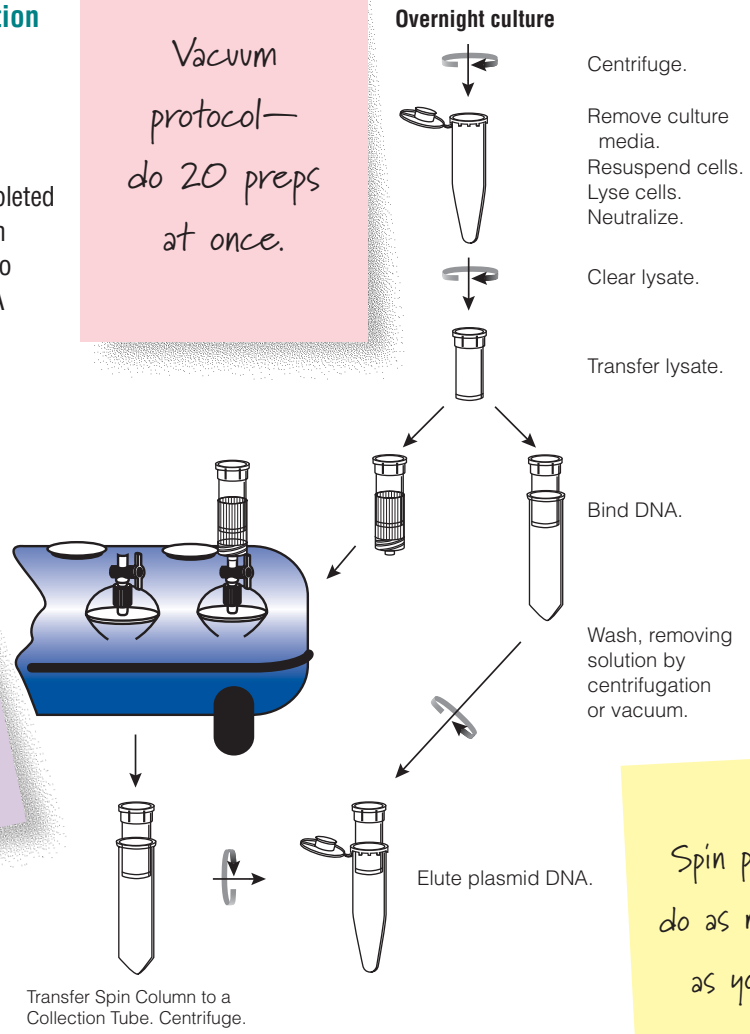
Screening for Recombinants

Plasmid Minipreps

Wizard® *Plus* SV Minipreps DNA Purification System

The Wizard *Plus* SV Minipreps DNA Purification System[h,i], a simple membrane-based system, provides a reliable method for rapidly isolating plasmid DNA. The entire procedure can be completed in 45 minutes or less. Work with up to 5ml of an overnight culture of a high-copy plasmid or up to 10ml of low-copy plasmid. Use the isolated DNA directly for applications such as automated fluorescent sequencing and restriction digests.

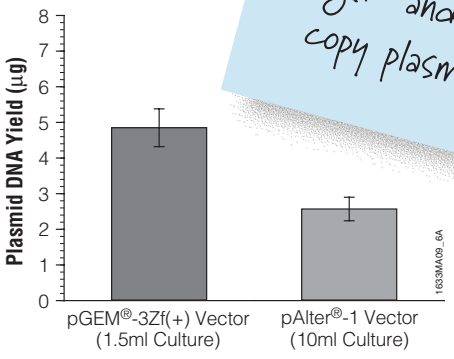
Vacuum protocol— do 20 preps at once.



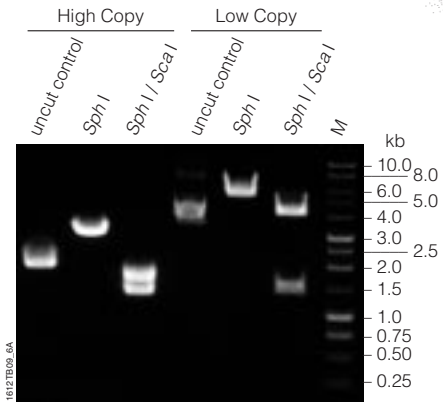
Wizard® Plus SV Minipreps
Ready for spin protocols:
A1330 50 preps
A1460 250 preps
Ready for spin or vacuum protocols:
A1340 50 preps
A1470 250 preps
Protocol available at:
www.promega.com/tbs/tb225/tb225.html

Spin protocol— do as many preps as your rotor can hold.

Works with high- and low-copy plasmids!



Plasmid DNA yield from high and low copy plasmids using the Wizard *Plus* SV Minipreps DNA Purification System. *E. coli* DH5α™ cells were transformed with either the pGEM®-3Zf(+) Vector (high-copy number plasmid) or the pALTER®-1 Vector (low-copy number plasmid) and grown in LB medium containing 50µg/ml of ampicillin (16 hours at 37°C, 200rpm). Plasmid DNA was isolated in sets of 42 on three consecutive days (126 total samples each) from 1.5ml (pGEM-3Zf(+) Vector) and 10ml (pALTER-1 Amp^r Vector) cultures using the Wizard® *Plus* SV Minipreps DNA Purification System.



Restriction enzyme digestion of high- and low-copy number plasmids. The plasmid vectors pGEM-3Zf(+)(high-copy) and pALTER-1 (low copy), isolated from *E. coli* DH5α using the Wizard *Plus* SV Minipreps DNA Purification System, were digested with 10 units of the indicated enzymes for 1 hour at 37°C. The digested samples were resolved on a 1% agarose gel and stained with ethidium bromide. The marker is the 1kb DNA Ladder (Cat.# G5711).

Troubleshooting Subcloning Experiments

Symptoms	Possible Causes	Comments
Few or no colonies obtained after transformation	Cells not competent	Competent cells may exhibit lower transformation efficiencies 5–6 weeks after preparation. To verify that bacteria are competent, perform a test transformation using a known amount of a standard supercoiled plasmid (see page 47).
	Unsuccessful ligation	Analyze samples of a linearized vector and the vector + insert ligation on an 0.8% agarose gel. If ligation was successful, the banding pattern of the ligation products should be different from that of the unligated sample.
	Inactive T4 DNA Ligase	Verify that the T4 DNA Ligase is active; perform a control ligation reaction with linear plasmid DNA.
	Inactive T4 DNA Ligase Buffer	Store T4 DNA Ligase 10X Reaction Buffer in small aliquots at –20°C to minimize freeze-thaw cycles of the buffer. Multiple freeze-thaw cycles may degrade the ATP in the buffer.
	Digested vector ends are not compatible with the fragment	Restriction enzyme sites that are adjacent within the multiple cloning region or near the ends can prove difficult to digest completely. See page 40 for a method to create PCR products with restriction sites in the primers.
	Excess ligation products added to competent cells	The added ligation products should not exceed 0.5% of the transformation reaction volume. Excess DNA (>10ng) may also inhibit the transformation.
High Background	Unsuccessful dephosphorylation of vector DNA	Attempt to religate the dephosphorylated vector. It should religate with low efficiency.
	Plates lack the correct antibiotic; the antibiotic is inactive	Perform a mock transformation with no DNA added. If colonies grow, discard the plates.
	Ratio of linearized, phosphorylated vector to insert DNA is too high	Reduce the amount of linearized vector in the reaction. Religation of the vector is favored when the vector:insert ratio is too high.

Troubleshooting Subcloning Experiments—Deletions of the Insert

During screening of your recombinant plasmids, you may encounter a situation where part of your insert is deleted. Perhaps careful examination of the subcloning strategy has not identified steps that may have led to this deletion. You attempt to sequence these clones and find that a portion of the insert has been deleted along with part of the vector. How did this happen? Two possibilities come to mind that could lead to such an event.

Symptoms	Possible Causes	Comments
Recombinants are isolated but contain deletions (usually unidirectional deletions of insert and part of vector)	Insert is unstable in the host strain	The insert may have been a substrate for recombination by recombinases in the most bacterium (remember most common laboratory strains are *rec*A minus, but there are other recombinases present). You can transform the plasmid into an *E. coli* strain deficient in more recombinases than just the recA. Some strains like SURE® cells from Stratagene are deficient in recombinases *rec*B and *rec*J and may allow you to propagate the unstable insert. Also try growing at a lower temperature.
	Insert is toxic to the host strain	Certain inserts may produce toxic gene products. The bacteria responds by deleting a portion of either the plasmid, the insert or both. High copy-number plasmids will tend to produce more toxin and thus be more prone to deletion or rearrangement. Most cloning plasmids carry the modified ColE1 origin of replication derived from pUC vectors, which maintain the copy number of the plasmid as high as 100–400 copies per bacterium. Low-level, leaky transcription in this high copy number plasmid can yield significant quantities of the toxic product. One solution is to transfer the insert to a different vector with a lower copy number. Promega has some vectors with lower copy numbers available like the pALTER®-1 (based on the pBR322 ColE1 origin; as few as 25 copies per cell) and the pALTER®-*Ex*2 (based on the pACYC origin of replication; ~10 copies per cell). This solution requires moving to a new vector. If you need to stay with the same vector (e.g., a mammalian expression vector) there are *E. coli* strains that have mutations that limit the copy number of pUC-based ColE1 origin of replication. The ABLE® strains from Stratagene will reduce copy number four- to ten-fold compared to more common laboratory strains.

Screening for Recombinants: Ordering Information

Competent Bacteria

Product	Size	Cat.#
Select96™ Competent Cells (>10^8cfu/µg)	1 × 96 reactions	L3300
JM109 Competent Cells, >10^8cfu/µg*	5 × 200µl	L2001
JM109 Competent Cells, >10^7cfu/µg	5 × 200µl	L1001

*For Laboratory Use.

Chemicals for Blue/White Screening

Product	Size	Conc.	Cat.#
IPTG, Dioxane-Free	1g	—	V3955
	5g	—	V3951
	50g	—	V3953
X-Gal	100mg	50mg/ml	V3941

For Laboratory Use.

Reagents for Colony PCR

Product	Conc.	Size	Cat.#
GoTaq® DNA Polymerase	5u/µl	100u	M3001
	5u/µl	500u	M3005
	5u/µl	2,500u	M3008
PCR Nucleotide Mix	10mM	200µl	C1141
	10mM	1,000µl	C1145
Set of dATP, dCTP, dGTP and dTTP	100mM	10µmol	U1330
	100mM	25µmol	U1420
	100mM	40µmol	U1240
	100mM	200µmol	U1410

For Laboratory Use.

Plasmid DNA Purification

Product	Size	Cat.#
Wizard® *Plus* SV Minipreps DNA Purification System[l,m]*	50 preps	A1330
	250 preps	A1460

Ready for use as a spin prep requiring only a microcentrifuge.

Product	Size	Cat.#
Wizard® *Plus* SV Minipreps DNA Purification System plus Vacuum Adapters[l,m]*	50 preps	A1340
	250 preps	A1470

Ready for use as a spin prep or a vacuum prep. Spin protocol requires only a microcentrifuge. Vacuum protocol requires Vac-Man® Laboratory Vacuum Manifold and a microcentrifuge.

Product	Size	Cat.#
Vac-Man® Laboratory Vacuum Manifold, 20-sample capacity	1 each	A7231

*For Laboratory Use.

Restriction Enzyme Activity in Promega 10X Buffers, Reaction Temperature and Heat Inactivation.

The 10X Reaction Buffer supplied with each restriction enzyme is optimized to give 100% activity. In many cases good activity is also obtained using one of Promega's 4-CORE® 10X Buffers. Many commonly used cloning enzymes (i.e. restriction sites are found in vector multiple cloning sites) have buffers E and H as their optimal buffer and so we have determined the activity of many of our other restriction enzymes in those buffers as well. This table may be used to select the best buffer for digestion with multiple restriction enzymes. Enzyme activity is expressed as a percent of the activity obtained with the optimal buffer for each enzyme in a one-hour digest. Enzymes with 100% activity (green) perform as well as the optimal buffer. Enzymes with 50–75% or 75–100% (yellow) will give acceptable activity in that buffer. Enzymes with <10%, 10–25%, or 25–50% (pink) activity generally do not have acceptable activity in that buffer. Also, buffers leading to star activity of the enzyme (*; pink) should be avoided. If compatible buffers cannot be identified with acceptable activity for both enzymes, each digest should be performed separately in the optimal buffer for each enzyme.

Promega Enzyme	Cat.#	Buffer Supplied with Enzyme	Activity in							Heat Inactivation	Enzyme Assay Temperature
			A	B	C	D	E	H	MULTI-CORE™		
Aat II	R6541	J	50–75%	10–25%	<10%	<10%	10–25%	<10%	<10%	+	37°C
Acc I	R6411	G	50–75%	25–50%	25–50%	10–25%	<10%	<10%	25–50%	–	37°C
Acc III	R6581	F	<10%	10–25%	25–50%	25–50%	n.d.	n.d.	<10%	–	65°C
*Acc*65 I	R6921	D	10–25%	50–75%	75–100%	100%	75–100%	100–125%**	100%	+	37°C
*Acc*B7 I	R7081	E	10–25%	50–75%	100%*	<10%	100%	n.d.	100%	+	37°C
Age I	R7251	K	25–50%	25–50%	25–50%	50–75%	n.d.	n.d.	100%	+	37°C
Alu I	R6281	B	75–100%	100%	75–100%	10–25%	n.d.	n.d.	10–25%	+	37°C
*Alw*26 I	R6761	C	10–25%	25–50%	100%	10–25%	n.d.	n.d.	75–100%	+	37°C
*Alw*44 I	R6771	C	<10%	25–50%	100%	25–50%	n.d.	n.d.	100%	+	37°C
Apa I	R6361	A	100%	50–75%	50–75%	<10%	10–25%	<10%	75–100%	+	37°C
Ava I	R6091	B	10–25%	100%	50–75%	25–50%	100%	10–25%	<10%	+/–	37°C
Ava II	R6131	C	50–75%	50–75%	100%	25–50%	n.d.	n.d.	25–50%	+	37°C
Bal I	R6691	G	10–25%	<10%	<10%	<10%	n.d.	n.d.	<10%	+	37°C
*Bam*H I	R6021	E	75–100%*	75–100%	75–100%	50–75%	100%	50–75%	75–100%	+	37°C
Ban I	R6891	G	25–50%	25–50%	10–25%	<10%	n.d.	n.d.	100%	–	50°C
Ban II	R6561	E	75–100%	75–100%	75–100%	25–50%	n.d.	n.d.	100%	+	37°C
Bbu I	R6621	A	100%	75–100%	75–100%	<10%	10–25%	10–25%	100%	+	37°C
Bcl I	R6651	C	10–25%	75–100%	100%	50–75%	50–75%	50–75%	10–25%	–	50°C
Bgl I	R6071	D	10–25%	25–50%	75–100%	100%	25–50%	75–100%	100%	+	37°C
Bgl II	R6081	D	25–50%	75–100%	75–100%	100%	n.d.	n.d.	<10%	–	37°C
*Bsa*M I	R6991	D	10–25%	25–50%	50–75%	100%	n.d.	n.d.	25–50%	–	65°C
*Bsp*1286 I	R6741	A	100%	50–75%	25–50%	10–25%	n.d.	n.d.	75–100%	+	37°C
*Bsr*S I	R7241	D	10–25%	25–50%	10–25%	100%	n.d.	n.d.	100%	–	65°C
*Bss*H II	R6831	H	75–100%	50–75%	75–100%	50–75%	n.d.	100%	75–100%	–	50°C
*Bst*98 I	R7141	D	<10%	10–25%	10–25%	100%	n.d.	n.d.	25–50%	–	37°C
*Bst*E II	R6641	D	25–50%	50–75%	50–75%	100%	n.d.	n.d.	100%	–	60°C
*Bst*O I	R6931	C	10–25%	25–50%	100%	25–50%	n.d.	n.d.	<10%	–	60°C
*Bst*X I	R6471	D	<10%	10–25%	25–50%	100%	100%	75–100%	10–25%	+/–	50°C
*Bst*Z I	R6881	D	<10%	<10%	10–25%	100%	10–25%	75–100%	10–25%	–	50°C
*Bsu*36 I	R6821	E	<10%	25–50%	50–75%	25–50%	100%	n.d.	50–75%	–	37°C
Cfo I	R6241	B	75–100%	100%	75–100%	25–50%	n.d.	n.d.	100%	+/–	37°C
Cla I	R6551	C	75–100%	75–100%	100%	75–100%	100%	50–75%	100%	+	37°C
Csp I	R6671	K	<10%	10–25%	25–50%	50–75%	100%	100–125%**	10–25%	+	30°C
*Csp*45 I	R6571	B	25–50%	100%	50–75%	25–50%	100%	25–50%	50–75%	+	37°C
Dde I	R6291	D	25–50%	25–50%	50–75%	100%	n.d.	n.d.	25–50%	+/–	37°C
Dpn I	R6231	B	50–75%	100%	75–100%	50–75%	n.d.	n.d.	100%	+	37°C
Dra I	R6271	B	75–100%	100%	75–100%	50–75%	n.d.	n.d.	25–50%	+	37°C
*Ecl*HK I	R7111	E	<10%	<10%	75–100%	10–25%	100%	n.d.	50–75%	+	37°C
*Eco*47 III	R6731	D	<10%	25–50%	50–75%	100%	n.d.	n.d.	25–50%	+	37°C
*Eco*52 I	R6751	L	<10%	<10%	10–25%	25–50%	25–50%	50–75%	<10%	+	37°C
*Eco*ICR I	R6951	B	10–25%	100%	75–100%	<10%	25–50%	n.d.	100%	+	37°C
*Eco*R I	R6011	H	25–50%	50–75%	50–75%	50–75%	75–100%	100%	100%*	+	37°C
*Eco*R V	R6351	D	10–25%	25–50%	50–75%	100%	25–50%	50–75%	100%	+	37°C
Fok I	R6781	B	75–100%	100%	75–100%	25–50%	n.d.	n.d.	50–75%	+	37°C
Hae II	R6661	B	50–75%	100%	50–75%	10–25%	n.d.	n.d.	100%	–	37°C
Hae III	R6171	C	75–100%	75–100%	100%	50–75%	n.d.	n.d.	100%	–	37°C
Hha I	R6441	C	50–75%	75–100%	100%	50–75%	n.d.	n.d.	75–100%	+	37°C
Hinc II	R6031	B	25–50%	100%	25–50%	50–75%	75–100%	50–75%	100%	+	37°C
Hind III	R6041	E	25–50%	100%	75–100%	10–25%	100%	25–50%	50–75%	+	37°C
*Hin*f I	R6201	B	50–75%	100%	75–100%	75–100%	n.d.	n.d.	50–75%	–	37°C

Restriction Enzyme Activity in Promega 10X Buffers, Reaction Temperature and Heat Inactivation (continued).

Promega Enzyme	Cat.#	Buffer Supplied with Enzyme	Activity in A	B	C	D	E	H	MULTI-CORE™	Heat Inactivation	Enzyme Assay Temperature
Hpa I	R6301	J	25–50%	50–75%	25–50%	10–25%	n.d.	n.d.	100%	–	37°C
Hpa II	R6311	A	100%	50–75%	50–75%	10–25%	n.d.	n.d.	100%	–	37°C
*Hsp*92 I	R7151	F	10–25%	75–100%	50–75%	25–50%	n.d.	n.d.	10–25%	+	37°C
*Hsp*92 II	R7161	K	10–25%	25–50%	25–50%	<10%	n.d.	n.d.	<10%	+	37°C
I-*Ppo* I	R7031	NA	10–25%	25–50%	25–50%	25–50%	n.d.	n.d.	—	+	37°C
Kpn I	R6341	J	100%*	25–50%	25–50%	<10%	25–50%	<10%	75–100%	+/–	37°C
Mbo I	R6711	C	10–25%	75–100%	100%	50–75%	n.d.	n.d.	<10%	+	37°C
Mbo II	R6723	B	10–25%	100%	50–75%	75–100%	n.d.	n.d.	100%	+	37°C
Mlu I	R6381	D	10–25%	25–50%	50–75%	100%	25–50%	100–125%**	10–25%	+/–	37°C
Msp I	R6401	B	75–100%	100%	75–100%	25–50%	n.d.	n.d.	25–50%	+	37°C
*Msp*A1 I	R7021	C	25–50%	100%*	100%	10–25%	n.d.	n.d.	100%	+	37°C
Nae I	R7131	A	100%	50–75%	25–50%	<10%	n.d.	n.d.	50–75%	+	37°C
Nar I	R6861	G	75–100%	50–75%	75–100%	25–50%	n.d.	n.d.	50–75%	+	37°C
Nci I	R7061	B	100%*	100%	25–50%	25–50%	n.d.	n.d.	50–75%	+	37°C
Nco I	R6513	D	50–75%	75–100%	75–100%	100%	100%	100–125%**	75–100%	+	37°C
Nde I	R6801	D	<10%	<10%	25–50%	100%	n.d.	n.d.	25–50%	+	37°C
Nde II	R7291	D	<10%	<10%	10–25%	100%	n.d.	n.d.	25–50%	+	37°C
*Ngo*M IV	R7171	MULTI-CORE™	100%*	100%*	100%*	<10%	n.d.	n.d.	100%	+	37°C
Nhe I	R6501	B	75–100%	100%	75–100%	10–25%	75–100%	10–25%	100%	+	37°C
Not I	R6431	D	<10%	10–25%	25–50%	100%	25–50%	100–125%**	25–50%	+	37°C
Nru I	R7091	K	<10%	<10%	<10%	50–75%	n.d.	n.d.	10–25%	+	37°C
Nsi I	R6531	D	10–25%	50–75%	50–75%	100%	25–50%	>125%**	10–25%	+/–	37°C
Pst I	R6111	H	10–25%	50–75%	50–75%	50–75%	25–50%	100%	25–50%	+	37°C
Pvu I	R6321	D	10–25%	25–50%	50–75%	100%	n.d.	n.d.	<10%	–	37°C
Pvu II	R6331	B	25–50%	100%	50–75%	25–50%	n.d.	n.d.	50–75%	+	37°C
Rsa I	R6371	C	75–100%	75–100%	100%	<10%	n.d.	n.d.	<10%	+	37°C
Sac I	R6061	J	75–100%	25–50%	25–50%	<10%	100%	25–50%	100%	+	37°C
Sac II	R6221	C	100%	50–75%	100%	50–75%	25–50%	>125%**	<10%	+	37°C
Sal I	R6051	D	<10%	10–25%	25–50%	100%	25–50%	25–50%	<10%	+	37°C
*Sau*3A I	R6191	B	25–50%	100%	75–100%	<10%	n.d.	n.d.	100%	+	37°C
Sca I	R6211	K	<10%	100%*	50–75%	75–100%	n.d.	n.d.	10–25%	+	37°C
Sfi I	R6391	B	75–100%	100%	75–100%	25–50%	75–100%	50–75%	75–100%	–	50°C
Sgf I	R7103	C	25–50%	25–50%	100%	<10%	n.d.	n.d.	<10%	+/–	37°C
Sin I	R6141	A	100%	75–100%	50–75%	10–25%	n.d.	n.d.	100%	+	37°C
Sma I	R6121	J	<10%	<10%	<10%	<10%	<10%	<10%	100%	+	25°C
*Sna*B I	R6791	B	50–75%	100%	50–75%	<10%	n.d.	n.d.	100%	–	37°C
Spe I	R6591	B	75–100%	100%	75–100%	75–100%	100%	25–50%	100%	+	37°C
Sph I	R6261	K	75–100%	75–100%	100%*	75–100%	100%	>125%**	10–25%	+	37°C
Ssp I	R6601	E	10–25%	50–75%	50–75%	75–100%	100%	100–125%**	50–75%	+	37°C
Stu I	R6421	B	75–100%	100%	75–100%	50–75%	n.d.	n.d.	50–75%	+	37°C
Sty I	R6481	F	25–50%	75–100%	75–100%	75–100%	10–25%	50–75%	<10%	+	37°C
Taq I	R6151	E	10–25%	25–50%	50–75%	50–75%	100%	n.d.	100%	–	65°C
*Tru*9 I	R7011	F	75–100%	50–75%	75–100%	25–50%	n.d.	n.d.	25–50%	–	65°C
*Tth*111 I	R6841	B	50–75%	100%	75–100%	25–50%	n.d.	n.d.	100%	–	65°C
Vsp I	R6851	D	<10%	25–50%	75–100%	100%	n.d.	n.d.	<10%	+	37°C
Xba I	R6181	D	50–75%	75–100%	75–100%	100%	100%	100–125%**	100%	–	37°C
Xho I	R6161	D	25–50%	75–100%	75–100%	100%	25–50%	100–125%**	10–25%	+	37°C
Xho II	R6811	C	25–50%	25–50%	100%	10–25%	n.d.	n.d.	<10%	+	37°C
Xma I	R6491	B	50–75%	100%	25–50%	<10%	25–50%	<10%	50–75%	+	37°C
Xmn I	R7271	B	75–100%	100%	75–100%	10–25%	n.d.	n.d.	75–100%	+	37°C

* Not recommended due to potential star activity.
** Unit activity is based on recommended buffer. In Buffer H, some enzymes have enhanced activity.
n.d. = Not determined.

Heat Inactivation Key:

+	=greater than 95% inactivation (DNA is undigested)
–	=less than 95% inactivation (DNA digest is complete, i.e., ≥5% of the initial 20 activity units [≥1 unit] remains)
+/–	=partial inactivation (DNA is partially digested)

Heat Inactivation Conditions:
Twenty units of enzyme in 50µl of its optimal buffer were heated at 65°C for 15 minutes.
One microgram of DNA was added and incubated for 1 hour in accordance with the unit definition, then analyzed by agarose gel electrophoresis.

Isoschizomers. **The enzymes in boldface type are available from Promega.**

Enzyme	Isoschizomer(s)	Recognition Sequence
mAat I	***Stu* I**, *Eco*147 I, *Pme*55 I, *Sse*B I	AGG▼CCT
***Aat* II**	—	GACGT▼C
***Acc* I**	*Fbl* I, *Xmi* I	GT▼(A/C)(G/T)AC
***Acc* III**	*Bsp*E II, *Mro* I	T▼CCGGA
***Acc*65 I**	*Asp*718 I	G▼GTACC
	Kpn* I	GGTAC▼C
*Acc*B1 I	***Ban* I**, *Bsh*N I, *Eco*64 I	G▼G(C/T)(G/A)CC
***Acc*B7 I**	*Pfl*M I, *Van*91 I	CCAN$_4$▼NTGG
*Acl*N I	***Spe* I**	A▼CTAGT
*Acl*W I	*Alw* I	GGATCNNNN▼
Acy I	*Bbi* II, *Hin*1 I, ***Hsp*92 I**, *Bsa*H I, *Msp*171 I	G(A/G)▼CG(T/C)C
Acs I	*Apo* I	(G/A)▼AATT(C/T)
Afa I	*Csp*6 I*, ***Rsa* I**	GT▼AC
Afe I	***Eco*47 III**	AGC▼GCT
Afl II	***Bst*98 I**	C▼TTAAGG
***Age* I**	*Pin*A I	A▼CCGGT
Aha III	***Dra* I**	TTT▼AAA
Ahd I	***Ecl*HK I**	GACNNN▼NNGTC
***Alu* I**	—	AG▼CT
Alw I	*Acl*W I	GGATCNNNN▼
***Alw*26 I**[1]	*Bsm*A I	GTCTC(1/5)
***Alw*44 I**	*Apa*L I	G▼TGCAC
Aoc I	***Bsu*36 I**, *Cvn* I	CC▼TNAGG
***Apa* I**	*Bsp*120 I	GGGCC▼C
*Apa*L I	***Alw*44 I**, *Vne* I	G▼TGCAC
Apo I	*Acs* I	(G/A)▼AATT(C/T)
Ase I	***Vsp* I**, *Asn* I	AT▼TAAT
Asn I	***Vsp* I**, *Ase* I	AT▼TAAT
Asp I	***Tth*111 I**	GACN▼NNGTC
*Asp*E I	*Ahd* I, *Eam*1105 I, ***Ecl*HK I**	GACNNN/NNGTC
*Asp*700 I	***Xmn* I**	GAANN▼NNTTC
*Asp*718 I	***Acc*65 I**	G▼GTACC
	Kpn* I	GGTAC▼C
Asu I	*Sau*96 I, *Cfr*13 I	G▼GNCC
Asu II	*Csp*45 I, *Bst*B I	TT▼CGAA
*Asu*HP I	*Hph* I	GGTGAN$_8$▼
***Ava* I**	*Ama*87 I, *Bco* I, *Bso*B I, *Eco*88 I	C▼(C/T)CG(G/A)G
***Ava* II**	***Sin* I**, *Eco*47 I, *Hgi*E I	G▼G(A/T)CC
Axy I	***Bsu*36 I**	CC▼TNAGG
***Bal* I**	*Msc* I, *Mlu*N I	TGG▼CCA
***Bam*H I**	—	G▼GATCC
***Ban* I**	*Acc*BI, *Bsh*N I, *Eco*64 I	G▼G(T/C)(A/G)CC
***Ban* II**	*Eco*24 I	G(A/G)GC(T/C)▼C
Bbe I	—	GGCGC▼C
	Nar* I	GG▼CGCC
*Bbr*P I	*Eco*72 I, *Pml* I	CAC▼GTG
Bbs I[1]	*Bsc*91 I, *Bpi* I	GAAGAC(2/6)
***Bbu* I**	*Pae* I, ***Sph* I**	GCATG▼C
***Bcl* I**	*Bsi*Q I, *Fba* I	T▼GATCA
Bcn I	***Nci* I**	CC▼(C/G)GG
Bfr I	***Bst*98 I**	C▼TTAAG
***Bgl* I**	—	GCCNNNN▼NGGC
***Bgl* II**	—	A▼GATCT
Bmy I	***Bsp*1286 I**	G(G/A/T)GC(C/A/T)▼C
Bpm I	*Gsu* I	CTGGAG(16/14)
*Bsa*H I	***Hsp*92 I**	G(A/G)▼CG(T/C)C
***Bsa*M I**	*Bsm* I	GAATGC(1/–1)
*Bsa*O I	*Bsh*1285 I, *Bsi*E I	CG(A/G)(T/C)▼C
*Bse*A I	***Acc* III**	T▼CCGGA
*Bse*N I	***Bsr*S I**, *Bsr* I	ACTGGN (1/–1)
*Bse*P I	***Bss*H II**, *Pau* I	G▼CGCGC
*Bsh*1285 I	*Bsa*O I	CG(A/G)(T/C)▼CG
*Bsh*N I	***Ban* I**, *Acc*B1 I, *Eco*64 I	G▼G(T/C)(A/G)CC
*Bsh*1365 I	*Bsr*BR I	GATNN▼NNATC
*Bsi*E I	*Bsa*O I	CG(A/G)(T/C)▼CG

Enzyme	Isoschizomer(s)	Recognition Sequence
Bsm I	***Bsa*MI**	GAATGCN▼
*Bsm*A I[1]	***Alw*26 I**	GTCTC(1/5)
*Bso*B I	***Ava* I**, *Ama*87 I, *Bco* I, *Eco*88 I	C(C/T)CG(G/A)G
*Bsp*19 I	***Nco* I**	C▼CATGG
*Bsp*68 I	***Nru* I**	TCG▼CGA
*Bsp*106 I	***Cla* I**, *Bsa*D I	AT▼CGAT
*Bsp*119 I	***Csp*45 I**, *Nsp* V, *Bst* B I	TT▼CGAA
*Bsp*120 I	***Apa* I**	G▼GGCCC
*Bsp*143 I	***Mbo* I**, ***Sau*3A I**, ***Nde* II**	▼GATC
*Bsp*143 II	***Hae* II**	(A/G)GCGC▼(T/C)
***Bsp*1286 I**	*Bmy* I, *Sdu* I	G(G/A/T)GC(C/A/T)▼C
*Bsp*C I	***Pvu* I**	CGAT▼CG
*Bsp*D I	***Cla* I**	AT▼CGAT
*Bsp*E I	***Acc* III**	T▼CCGGA
Bsr I[1]	***Bsr*S I**, *Bse*N I	ACTGGN(1/-1)
***Bsr*S I**[1]	*Bse*N I, *Bsr* I	ACTGGN(1/-1)
***Bss*H II**	*Bse*P I, *Pau* I	G▼CGCGC
***Bst*98 I**	*Afl* II, *Bfr* I	C▼TTAAG
*Bst*B I	***Csp*45 I**, *Nsp* V, *Bsp*119 I	TT▼CGAA
***Bst*E II**	*Bst*P I, *Eco*91 I, *Psp*E I	G▼GTNACC
*Bst*N I	***Bst*O I**, *Mva* I, *Eco*R II	CC▼(A/T)GG
***Bst*O I**	*Bst*N I, *Eco*R II, *Mva* I	CC▼(A/T)GG
***Bst*X I**	–	CCANNNNN▼NTGG
*Bst*Y I	***Xho* II**, *Mfl* I	(A/G)▼GATC(T/C)
***Bst*Z I**	***Eco*52 I**, *Eag* I, *Xma* III, *Ecl*X I	C▼GGCCG
*Bsu*15 I	***Cla* I**	AT▼CGAT
***Bsu*36 I**	*Cvn* I, *Aoc* I, *Eco*81 I	CC▼TNAGG
*Bsu*R I	***Hae* III**, *Pal* I	GG▼CC
***Cfo* I**	***Hha* I**	GCG▼C
	*Hin*6 I	GCG▼C
	*Hin*P1 I*	G▼CGC
*Cfr*9 I	***Xma* I**	C▼CCGGG
	Sma* I	CCC▼GGG
*Cfr*13 I	*Sau*96 I	G▼GNCC
*Cfr*42 I	***Sac* II**	CCGC▼GG
***Cla* I**	*Ban* III, *Bsp*106 I, *Bsp*D I, *Bsu*15 I	AT▼CGAT
Cpo I	***Csp* I**, *Rsr* II	CG▼G(A/T)CCG
***Csp* I**	Cpo I, *Rsr* II	CG▼G(A/T)CCG
*Csp*6 I	***Rsa* I***, *Afa* I*	GT▼AC
***Csp*45 I**	*Bst*B I, *Nsp* V, *Bsp*119 I	TT▼CGAA
Cvn I	***Bsu*36 I**	CC▼TNAGG
***Dde* I**	*Bst*DE I	C▼TNAG
***Dpn* I**[2]	*Dpn* II*	G^{me}A▼TC
Dpn II	***Mbo* I**, ***Sau*3A I**, ***Nde* II**, ***Dpn* I***	▼GATC
***Dra* I**	—	TTT▼AAA
Eag I	***Eco*52 I**, ***Bst*Z I**, *Ecl*X I, *Xma* III	C▼GGCCG
*Eam*1105 I	***Ecl*HK I**, *Ahd* I, *Asp*E I	GACNNN▼NNGTC
*Ecl*136 II	***Eco*ICR I**	GAG▼CTC
	Sac* I	GAGCT▼C
***Ecl*HK I**	*Ahd* I, *Eam*1105 I, *Asp*E I	GACNNN▼NNGTC
*Ecl*X I	***Bst*Z I**, *Eag* I, ***Eco*52 I**, *Xma* III	C▼GGCCG
*Eco*24 I	***Ban* II**, *Fri*O I	G(AG)GC(TC)▼C
*Eco*32 I	***Eco*R V**	GAT▼ATC
*Eco*47 I	***Ava* II**, ***Sin* I**	G▼G(A/T)CC
***Eco*47 III**	*Afe* I	AGC▼GCT
***Eco*52 I**	***Bst*Z I**, *Xma* III, *Eag* I, *Ecl*X I	C▼GGCCG
*Eco*64 I	***Ban* I**, *Bsh*N I, *Eco*64 I	G▼G(TC)(AG)CC
*Eco*81 I	***Bsu*36 I**	CC▼TNAGG
*Eco*88 I	***Ava* I**	C▼(TC)CG(AG)G
*Eco*91 I	***Bst*E II**	G▼GTNACC
*Eco*105 I	***Sna*B I**	TAC▼CTA
*Eco*130 I	***Sty* I**	C▼C(A/T)(T/A)GG
*Eco*147 I	***Stu* I**	AGG▼CCT
***Eco*ICR I**	*Ecl*136 II	GAG▼CTC
	Sac* I	GAGCT▼C
	Sst I*	GAGCT▼C

Isoschizomers (continued). **The enzymes in boldface type are available from Promega.**

Enzyme	Isoschizomer(s)	Recognition Sequence
***Eco*R I**	—	G▼AATTC
*Eco*R II	***Bst*O I**, *Bst*N I, *Mva* I	CC▼(A/T)GG
***Eco*R V**	*Eco*32 I	GAT▼ATC
*Eco*T14 I	***Sty* I**	C▼C(A/T)(A/T)GG
*Eco*T22 I	***Nsi* I**	ATGCA▼T
Ehe I	***Nar* I***	GG▼CGCC
***Fok* I**[2]	—	GGATG(9/13)
***Hae* II**	*Bsp*143 II	(A/G)GCGC▼(T/C)
***Hae* III**	*Bsu*R I, *Pal* I	GG▼CC
Hap II	***Hpa* II**, ***Msp* I**	C▼CGG
*Hgi*E I	*Eco*47 I, ***Sin* I**, ***Ava* II**	G▼G(A/T)CC
***Hha* I**	***Cfo* I**	GCG▼C
	*Hin*P1 I*, *Hin*6 I*	G▼CGC
*Hin*1 I	*Acy* I, ***Hsp*92 I**	G(A/G)▼CG(T/C)C
***Hin*c II**	*Hin*d II	GT(T/C)▼(A/G)AC
*Hin*d II	***Hin*c II**	GT(T/C)▼(A/G)AC
***Hin*d III**	—	A▼AGCTT
***Hin*f I**	—	G▼ANTC
*Hin*P1 I	—	G▼CGC
	Hha* I, ***Cfo* I***	GCG▼C
***Hpa* I**	*Ksp*A I	GTT▼AAC
***Hpa* II**[3]	***Msp* I**, *Hap* II	C▼CGG
***Hsp*92 I**	*Acy* I, *Bsa*H I, *Hin*1 I	G(A/G)▼CG(C/T)
***Hsp*92 II**	*Nla* III	CATG▼
***I-Ppo* I**	—	CTCTCTTAA▼GGTAGC
Kas I	***Nar* I***	GG▼CGCC
***Kpn* I**	—	GGTAC▼C
	Acc*65 I, *Asp*718 I*	G▼GTACC
Ksp I	***Sac* II**	CCGC▼GG
***Mbo* I**	***Sau*3A I**, ***Nde* II**, *Dpn* II	▼GATC
***Mbo* II**[1]	—	GAAGA(8/7)
Mfl I	***Xho* II**	(A/G)▼GATC(T/C)
***Mlu* I**	—	A▼CGCGT
*Mlu*N I	***Bal* I**, *Msc* I	TGG▼CCA
Mro I	***Acc* III**	T▼CCGGA
Msc I	***Bal* I**, *Mlu*N I	TGG▼CCA
Mse I	***Tru*9 I**	T▼TAA
***Msp* I**[3]	***Hpa* II**, *Hap* II	C▼CGG
***Msp*A1 I**	*Nsp*B II	C(A/C)G▼C(G/T)G
Mst II	***Bsu*36 I**	CC▼TNAGG
Mva I	***Bst*O I**, *Eco*R II, *Bst*N I	CC▼(A/T)GG
***Nae* I**	***Ngo*M IV**	G▼CCGGC
***Nar* I**	—	GG▼CGCC
	Ehe I*	GGC▼GCC
	Kas I*	G▼GCGCC
	Bbe I*	GGCGC▼C
***Nci* I**	*Bcn* I	CC▼(C/G)GG
***Nco* I**	*Bsp*19 I	C▼CATGG
***Nde* I**	—	CA▼TATG
***Nde* II**	***Mbo* I**, ***Sau*3A I**, *Dpn* II	▼GATC
***Ngo*M IV**	***Nae* I**	G▼CCGGC
***Nhe* I**	—	G▼CTAGC
Nla III	***Hsp*92 II**	CATG▼
***Not* I**	—	GC▼GGCCGC
***Nru* I**	*Bsp*68 I	TCG▼CGA
***Nsi* I**	*Eco*T22 I, *Mph*1103 I	ATGCA▼T
Nsp V	***Csp*45 I**, *Bst*B I, *Bsp*119 I	TT▼CGAA
*Nsp*B II	***Msp*A1 I**	C(A/C)G▼C(G/T)G
Pae I	***Bbu* I**, ***Sph* I**	GCATG▼C
*Pae*R7 I	***Xho* I**	C▼TCGAG
Pal I	***Hae* III**, *Bsu*R I	GG▼CC
*Pfl*M I	***Acc*B7 I**, *Vau*91 I	CCAN$_4$▼NTGG
*Pin*A I	***Age* I**	A▼CCGGT
***Pst* I**	—	CTGCA▼G
***Pvu* I**	*Bsp*C I	CGAT▼CG
***Pvu* II**	—	CAG▼CTG
***Rsa* I**	*Afa* I	GT▼AC
Rsr II	***Csp* I**, *Cpo* I	CG▼G(A/T)CCG
***Sac* I**	*Sst* I	GAGCT▼C
	*Ecl*136 II*, ***Eco*ICR I***	GAG▼CTC
***Sac* II**	*Sst* II, *Ksp* I, *Cfr*42 I	CCGC▼GG
***Sal* I**	—	G▼TCGAC
***Sau*3A I**	***Mbo* I**, ***Nde* II**, *Dpn* II	▼GATC
*Sau*96 I	*Cfr*13 I	G▼GNCC
***Sca* I**	—	AGT▼ACT
Sdu I	***Bsp*1286 I**	G(G/A/T)GC(C/A/T)▼C
***Sfi* I**	—	GGCCNNNN▼NGGCC
Sfu I	***Csp*45 I**	TT▼CGAA
***Sgf* I**	—	GCGAT▼CGC
***Sin* I**	***Ava* II**, *Eco*47 I	G▼G(A/T)CC
***Sma* I**	—	CCC▼GGG
	Xma* I, *Cfr*9 I*	C▼CCGGG
***Sna*B I**	*Eco*105 I	TAC▼GTA
***Spe* I**	*Acl*N I	A▼CTAGT
***Sph* I**	***Bbu* I**, *Pae* I	GCATG▼C
***Ssp* I**	—	AAT▼ATT
Sst I	***Sac* I**	GAGCT▼C
	Eco*ICR I	GAG▼CTC
Sst II	***Sac* II**	CCGC▼GG
***Stu* I**	*Aat* I, *Eco*147 I	AGG▼CCT
***Sty* I**	*Eco*T14 I	C▼C(A/T)(A/T)GG
***Taq* I**	*Tth*HB8 I	T▼CGA
***Tru*9 I**	*Mse* I	T▼TAA
***Tth*111 I**	*Asp* I	GACN▼NNGTC
*Tth*HB8 I	***Taq* I**	T▼CGA
*Van*91 I	***Acc*B7 I**, *Pfl*M I	CCAN$_4$▼NTGG
Vne I	*Apa*L I, ***Alw*44 I**	G▼TGCAC
***Vsp* I**	*Ase* I, *Asn* I	AT▼TAAT
***Xba* I**	—	T▼CTAGA
***Xho* I**	*Pae*R7 I	C▼TCGAG
***Xho* II**	*Bst*Y I, *Mfl* I	(A/G)▼GATC(T/C)
***Xma* I**	*Cfr*9 I, *Xma*C I,	C▼CCGGG
	Sma* I	CCC▼GGG
Xma III	***Eco*52 I**, ***Bst*Z I**, *Eag* I, *Ecl*X I	C▼GGCCG
*Xma*C I	***Xma* I**	C▼CCGGG
	Sma* I	CCC▼GGG
***Xmn* I**	*Asp*700 I	GAANN▼NNTTC

Key:

N = A, C, G or T
* = neoschizomer

Notes:

1. The locations of cleavage sites falling outside the recognition site are indicated in parentheses. For example, GTCTC(1/5) indicates cleavage at:

   5′...GTCTCN▼...3′
   3′...CAGAGNNNNN▲...5′

2. *Dpn* I is unique among commercially available restriction enzymes in requiring methylation of a nucleotide (adenine) in its recognition sequence in order to cut. Therefore, *Dpn* I cannot be substituted for other enzymes recognizing the GATC sequence (e.g., *Mbo* I and *Sau*3A I).
3. Although *Hpa* II and *Msp* I recognize the same nucleotide sequence, *Hpa* II is sensitive to methylation of either cytosine in its recognition sequence, while *Msp* I is sensitive only to methylation of the external cytosine. These enzymes may not be interchanged for all applications.

Reference

Roberts, R.J. (1991) *Nucl. Acids Res.* **19** (supp), 2077–109.

Compatible Ends.

Promega Restriction Enzymes That Generate 5′ Overhangs

Overhang	Definite Compatible Ends	Possible Compatible Ends
5′-N		*Tth*111 I
5′-S		*Nci* I
5′-W		*Bst*O I
5′-AT	*Acc* I	
5′-CG	*Nar* I, *Msp* I, *Hsp*92 I, *Taq* I, *Cla* I, *Csp*45 I, *Hpa* II	
5′-GN		*Bsr*S I
5′-MK		*Acc* I
5′-TA	*Vsp* I, *Nde* I, *Tru*9 I	
5′-ANT		*Hin*f I
5′-GNC		*Sau*96 I
5′-GWC		*Ava* II, *Csp* I, *Sin* I
5′-TNA		*Dde* I, *Bsu*36 I
5′-AATT	*Eco*R I	
5′-AGCT	*Hin*d III	
5′-CATG	*Nco* I	*Sty* I
5′-CCGG	*Age* I, *Xma* I, *Acc* III, *Ngo*M IV	*Ava* I
5′-CGCG	*Mlu* I, *Bss*H I	
5′-CTAG	*Spe* I, *Nhe* I, *Xba* I	*Sty* I
5′-CWWG		*Sty* I
5′-GATC	*Mbo* I, *Sau*3A I, *Bam*H I, *Bgl* II, *Xho* II, *Bcl* I, *Nde* II	
5′-GCGC	*Ban* I	
5′-GGCC	*Not* I, *Bst*Z I, *Eco*52 I	
5′-GTAC	*Acc*65 I	*Ban* I
5′-GTNAC		*Bst*E II
5′-GYRC		*Ban* I
5′-TCGA	*Sal* I, *Xho* I	*Ava* I
5′-TGCA	*Alw*44 I	
5′-TTAA	*Bst*98 I	
5′-YCGR		*Ava* I

Promega Restriction Enzymes That Generate 3′ Overhangs

Overhang	Definite Compatible Ends	Possible Compatible Ends
N-3′		*Ecl*HK I
AT-3′	*Sgf* I, *Pvu* I	
CG-3′	*Cfo* I, *Hha* I	
CN-3′		*Bsa*M I
GC-3′	*Sac* II	*Bsa*O I
NNN-3′		*Acc*B7 I, *Bgl* I, *Sfi* I
ACGT-3′	*Aat* II	
AGCT-3′	*Sac* I	*Ban* II, *Bsp*1286 I
CATG-3′	*Hsp*92 II, *Sph* I, *Bbu* I	
DGCH-3′		*Bsp*1286 I
GCGC-3′	*Hae* II	
GGCC-3′	*Apa* I	*Ban* II, *Bsp*1286 I
GTAC-3′	*Kpn* I	
NNNN-3′		*Bst*X I
RGCY-3′		*Ban* II, *Bsp*1286 I
TGCA-3′	*Nsi* I, *Pst* I	*Bsp*1286 I
TTAA-3′	I-*Ppo* I	

Key:

D = A or G or T
H = A or C or T
K = G or T
M = A or C
N = A or C or G or T
R = A or G
S = C or G
W = A or T
Y = C or T

Site-Specific Methylation Sensitivity of Promega Restriction Enzymes.

This table lists the sensitivities of several Promega restriction enzymes to site-specific methylation at *dam*, *dcm*, CpG and CpNpG sites (p = phosphoryl group). These four modifications are frequently found in DNA of bacteria, eukaryotes or their viruses. Many strains of *E. coli* contain the site-specific *dam* and *dcm* DNA methylases. Higher eukaryotes contain the site-specific CpG and CpNpG DNA methylases. In mammalian genomes, methylation occurs mainly at the CG dinucleotide. In plant genomes, methylation may occur at both the CG and CNG sequences.

Prokaryotic Methylation

dcm Cytosine methylase mutation—methylates the C5 position of the internal cytosine residue in the sequence 5′...CCTGG...3′.

dam Adenine methylase mutation—methylates the N6 position of the adenine residue in the sequence 5′....GATC...3′.

Eukaryotic Methylation

CpG Methylates the C5 position of the cytosine residue in the dinucleotide recognition sequence 5′...CG...3′.

CpNpGp Methylates the C5 position of the cytosine residue in the trinucleotide recognition sequence 5′...CNG...3′ (N = any base).

For further information regarding site-specific methylation, refer to McClelland, M., Nelson, M. and Raschke, E. (1994) *Nucl. Acids Res.* **22**, 3640–59.

Key:

s = sensitive to this methylation
i = insensitive to this methylation
s(ol) = overlapping (sensitive when restriction site overlaps methylation sequence)
n/a = information not available

Enzyme	Recognition Sequence	*dam*	*dcm*	CpG	CpNpG
Aat II	GACGTC	i	i	s	i
*Acc*B7 I	CCANNNNNTGG	i	s(ol)	i	i
Acc III	TCCGGA	s(ol)	i	i	i
*Acc*65 I	GGTACC	i	s(ol)	i	i
Apa I	GGGCCC	i	s(ol)	s(ol)	i
Ava I	CYCGRG	i	i	s	i
Ava II	GGWCC	i	s(ol)	s(ol)	s(ol)
Bal I	TGGCCA	i	s(ol)	i	s(ol)
*Bam*H I	GGATCC	i	i	i	s(ol)
Ban II	GRGCYC	i	i	i	i
Bbu I	GCATGC	i	i	i	i
Bcl I	TGATCA	s	i	i	i
Bgl I	GCCNNNNNGGC	i	i	s(ol)	s(ol)
Bgl II	AGATCT	i	i	i	s(ol)
*Bsp*1286 I	GDGCHC	i	i	i	i
*Bss*H II	GCGCGC	i	i	s	i
*Bst*E II	GGTNACC	i	i	i	i
*Bst*O I	CCWGG	i	i	i	n/a
*Bst*X I	CCANNNNNNTGG	i	i	i	i
*Bst*Z I	CGGCCG	i	i	s(ol)	s(ol)
Cfo I	GCGC	i	i	s	n/a
Cla I	ATCGAT	s(ol)	i	s	i
Csp I	CGGWCCG	i	i	i	s
*Csp*45 I	TTCGAA	i	i	s	i
Dde I	CTNAG	i	i	i	s(ol)
*Eco*47 III	AGCGCT	i	i	s	i
*Eco*52 I	CGGCCG	i	i	s	i
*Eco*R I	GAATTC	i	i	s(ol)	i
Fok I	GGATC	i	i	i	i
Hae III	GGCC	i	i	i	s(ol)
Hha I	GCGC	i	i	s	s(ol)
Hinc II	GTYRAC	i	i	i	i
Hind III	AAGCTT	i	i	i	i
Hpa II	CCGG	i	i	s	s

Enzyme	Recognition Sequence	*dam*	*dcm*	CpG	CpNpG
Kpn I	GGTACC	i	i	i	i
Mbo II	GAAGA(8/7)	s(ol)	i	i	i
Mlu I	ACGCGT	i	i	s	i
Msp I	CCGG	i	i	i	s
Nae I	GCCGGC	i	i	s	s
Nar I	GGCGCC	i	i	s	i
Nde II	GATC	s	i	i	i
*Ngo*M IV	GCCGGC	i	i	s	s
Nhe I	GCTAGC	i	i	s(ol)	s(ol)
Not I	GCGGCCGC	i	i	s	s
Nru I	TCGCGA	s(ol)	i	s	i
Pst I	CTGCAG	i	i	i	s
Pvu I	CGATCG	i	i	s	s(ol)
Pvu II	CAGCTG	i	i	i	s
Sac I	GAGCTC	i	i	i	i
Sac II	CCGCGG	i	i	s	s
Sal I	GTCGAC	i	i	s	n/a
*Sau*3A I	GATC	i	i	s(ol)	s(ol)
*Sau*96 I	GGNCC	i	s(ol)	s(ol)	s(ol)
Sca I	AGTACT	i	i	i	i
Sfi I	GGCCNNNNNGGCC	i	s(ol)	s(ol)	s(ol)
Sgf I	GCGATCGC	i	i	s	n/a
Sin I	GGWCC	i	i	i	s(ol)
Sma I	CCCGGG	i	i	s	s
*Sna*B I	TACGTA	i	i	s	i
Sph I	GCATGC	i	i	i	i
Stu I	AGGCCT	i	s(ol)	i	s(ol)
Taq I	TCGA	s(ol)	i	i	i
Xba I	TCTAGA	s(ol)	i	i	i
Xho I	CTCGAG	i	i	s	i
Xho II	RGATCY	i	i	i	s(ol)
Xma I	CCCGGG	i	i	i	n/a
Xmn I	GAANNNN	i	i	n/a	n/a

Restriction Enzyme Buffer Composition.

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 7.5 at 37°C), 100mM potassium acetate, 10mM magnesium acetate, 1mM DTT.

Notes:

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.
3. All of Promega's Restriction enzymes are supplied with 10mg/ml Acetylated BSA. Although BSA is not absolutely required for activity, it has been shown to enhance activity of many restriction enzymes. We recommend adding BSA to all restriction digests at a final concentration of 0.1mg.ml.

Copy Number of Commonly Used Plasmids.

Plasmid	Plasmid Size (approx.)	Origin of Replication*	Copy Number	**Yield per ml of Culture	Reference
pGEM®	2,700bp	mutated pMB1	300–700	1.8–4.1µg	1
pUC	2,700bp	mutated pMB1	500–700	2.9–4.1µg	1
pBR322	4,400bp	pMB1	>25	>0.23µg	2
ColE1	4,500bp	ColE1	>15	>0.15µg	3
pACYC	4,000bp	p15A	~10	~0.09µg	4
pSC101	9,000bp	pSC101	~6	~0.12µg	5
pGL Series	5,000bp	mutated pMB1	300–700	3.3–7.6µg	1
pRL Series	4,000bp	mutated pMB1	300–700	2.7–6.0µg	1
phRL Series	4,000bp	mutated pMB1	300–700	2.7–6.0µg	1
phRG Series	4,000bp	mutated pMB1	300–700	2.7–6.0µg	1
pGEM®-T/ T easy	3,000bp	mutated pMB1	300–700	2.0–4.6µg	1
psiLentGene™ Series	4,000bp	mutated pMB1	300–700	2.7–6.0µg	1
psiCHECK™ 1/2	3,500bp	mutated pMB1	300–700	2.7–5.3µg	1
psiSTRIKE™ Series	4,000bp	mutated pMB1	300-700	2.7–6.0µg	1
pALTER®-1/*Ex*1	5,800bp	pMB1	>25	>0.3µg	3
pALTER®-*Ex*2	5,800bp	p15A	~10	~0.13µg	4
pSP	2,500bp	mutated pMB1	300–700	1.6–3.8µg	1
pCI, pSI	3,600bp	mutated pMB1	300–700	2.4–5.5µg	1

* Plasmids carrying the pMB1, mutated pMBI and ColE1 belong to the same incompatibility group, so they are not compatible with one another, but they are fully compatible with those carrying p15A and pSC101 replicons.

** Theoretical plasmid yields were calculated from the reported copy number and size of each plasmid assuming 2.0×10^9 cells per milliliter of culture grown for 16 hours at 37°C.

References

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4. Birnboim, H.C. and Doly, J. (1979) *Nucl. Acids Res.* **7**, 1513–23.
5. Birnboim, H.C. (1983) *Meth. Enzymol.* **100**, 243–55.

Star Activity.

Restriction enzymes, under nonstandard conditions, can demonstrate the ability to cleave DNA at sequences different from their defined recognition sites. The term "star activity" has been given to this nonsequence-specific cleavage of DNA under nonoptimal reaction conditions. The most common types of altered activity are single-base substitutions, truncation of the outer bases in the recognition sequence and single-strand nicking (1). In general, star activity is not a concern if restriction endonucleases are used in the recommended buffers at the appropriate temperatures. Star activity is evident with a number of restriction enzymes when the following parameters are altered in the reaction environment (2):

- High enzyme concentration (generally >100 units/µg).
- High glycerol content (>5% v/v).
- Substitution of Mn^{2+} for Mg^{2+} (or substitution of other divalent cations).
- Low salt concentration (generally <25mM).
- Extremes of pH, especially pH>8.0.
- Presence of DMSO, ethanol or other organic solvents.

References

1. Barany, F. (1988) *Gene* **65**, 149–65.
2. Brown, T.A., Hames, B.D. and Rickwood, D. (1991) In: *Molecular Biology Lab Fax,* BIOS Scientific Publishers Limited, Oxford, United Kingdom.

Genotypes of Frequently Used Bacterial Strains.

All genes in the bacterium are presumed to be in the wildtype state, except for those listed, which are mutant alleles carried by that bacterium. Genes listed on the F′ episome, however, represent wildtype alleles unless specified otherwise. Strains are λ– unless specified otherwise. *Strains available from Promega as competent cells are indicated by an asterisk. Strains shown in **bold** are available from Promega as glycerol freezer stocks.

Strain	Genotype
BL21(DE3)	F–, *omp*T, *hsd*S_B (r_B^-, m_B^-), *dcm*, *gal*, λ(DE3)
***BL21(DE3)pLysS**	F–, *omp*T, *hsd*S_B (r_B^-, m_B^-), *dcm*, *gal*, λ(DE3), pLysS (Cm^r)
***BMH 71-18 *mut*S**	*thi*, *sup*E, Δ(*lac-proAB*), [*mutS*::Tn10(tet^r)] [F′, tra D36, *proAB*, *lacI*^{q}ZΔM15]
C600 (1)	*thi*-1, *thr*-1, *leuB*6, *lacY*1, *tonA*21, *supE*44
C600*hfl* (1)	*thi*-1, *thr*-1, *leuB*6, *lacY*1, *tonA*21, *supE*44, *hfl*A150::Tn10(tet^r)
DH1 (2)	*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17 (r_K^-, m_K+), *supE*44, *relA*1
DH10B	F–, *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *end*A1, *ara*D139, Δ(*ara*, *leu*)7697, *gal*U, *gal*K, λ-, *rps*L(str^r), *nup*G
DH5α™	ϕ80d*lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17 (r_K^-, m_K+), *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*) U169, *pho*A
DM1 (3)	F′, *dam*-13::Tn9(Cm^r) *dcm*, *mcrB*, *hsdr-M*+, *gal*1, *gal*2, ara-, *lac*-, *thr*-, *leu*-, *ton*^{R}, *tsx*^{R}, *Su*^{0}
ES1301 *mut*S	*lacZ*53, *thyA*36, *rha*-5, *metB*1, *deo*C, IN(*rrn*D-*rrn*E), [*mutS*201::Tn5]
*HB101 (4)	*thi*-1, *hsdS*20 (r_B^-, m_B^-), *supE*44, *recA*13, *ara*-14, *leuB*6, *proA*2, *lacY*1, *galK*2, *rpsL*20(str^r), *xyl*-5, *mtl*-1
JM101 (5)	*supE*, *thi*, Δ(*lac-proAB*), F′ (*traD*36, *proAB*, *lacI*^{q}ZΔM15)
***JM109** (5)	*endA*1, *recA*1, *gyrA*96, *thi*-1, *hsdR*17 (r_K^-, m_K+), *relA*1, *supE*44, Δ(*lac-proAB*), [F′, *traD*36, *proAB*, *lacI*^{q}ZΔM15]
JM109(DE3) (5)	*endA*1, *recA*1, *gyrA*96, *thi*-1, *hsdR*17 (r_K^-, m_K+), *relA*1, *supE*44, Δ(*lac-proAB*), [F′, *traD*36, *proAB*, *lacI*^{q}ZΔM15], λ(DE3)
JM110 (5)	*rpsL*(str^r), *thr*, *leu*, *thi*, *hsdR*17 (r_K^-, m_K+), *lacY*, *galK*, *galT*, *ara*, *tonA*, *tsx*, *dam*, *dcm*, *supE*44, Δ(*lac-proAB*), [F′, *traD*36, *proAB*, *lacI*^{q}ZΔM15]
KW251	*supE*44, *galK*2, *galT*22, *metB*1, *hsdR*2, *mcrB*1, *mcrA*, [*argA*81::Tn10(tet^r)], *rec*D1014
LE392 (6)	*hsdR*514, (r_K^-, m_K+), *supE*44, *supF*58, *lacY*1 or Δ(*lacIZY*)6, *galK*2, *galT*22, *metB*1, *trpR*55
NM522 (7)	*supE*, *thi*, Δ(*lac-proAB*), Δ*hsd*5 (r_K^-, m_K^-), [F′, *proAB*, *lacI*^{q}ZΔM15]
NM538 (8)	*supF*, *hsdR* (r_K^-, m_K+), *trpR*, *lacY*
NM539 (8)	*supF*, *hsdR* (r_K^-, m_K+), *lacY*, (P2)
*Select96™	*mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), ϕ80lacZΔM15, Δ*lac*X74, *rec*A1, *ara*D139 (*ara-leu*)7697, *gal*U, *gal*K, *rsp*L, *end*A1, *nup*G
Stbl2™	F–, *mcr*A, Δ(*mcr*BC-*hsd*RMS-*mrr*), *rec*A1, *end*A1, *gyr*A96, *thi*-1, *sup*E44, *rel*A1, λ–, Δ(*lac-pro*AB)
Stbl4™	*mcr*A, Δ(*mcr*BC-*hsd*RMS-*mrr*), *rec*A1, *end*A1, *gyr*A96, *thi*-1, *sup*E44, *rel*A1, λ–, Δ(*lac-pro*AB), *gal*, F′{*pro*AB+, *lacI*^{q}, ZΔM15, *Tn*10(*tet*^{R})}
SURE®	*e*14–, (*mcr*A–) Δ(*mcr*CB-*hsd*SMR-*mrr*)171, *end*A1, *sup*E44, *thi*-1, *gyr*A96, *rel*A1, *lac*, *rec*B, *rec*J, *sbc*C, *umu*C::Tn5 (*kan*^{r}), *uvr*C, [F′ *pro*AB, *lacI*^{q}ZΔM15::Tn10 (*tet*^{r})]
TOP10	F–, *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), ϕ80*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *ara*D139, Δ(*ara*, *leu*)7697, *gal*U, *gal*K, *rps*L (str^R), *end*A1, *nup*G
TOP10F′	F′{*lacI*^{q} Tn10 (tet^R)}, *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), ϕ80*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *ara*D139, Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L (str^r), *end*A1, *nup*G
XL1-Blue	*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17(r_K^-, m_K+), *supE*44, *relA*1, *lac*, [F′, *proAB*, *lacI*^{q}ZΔM15::Tn10(tet^r)]
Y1089 (9)	Δ(*lac*U169), *proA*+, Δ(*lon*), *araD*139, *strA*, *hflA*150, [chr::Tn10(tet^r)], (pMC9)
Y1090 (9)	Δ(*lac*U169), *proA*+, Δ(*lon*), *araD*139, *strA*, *supF*, rpsL(str^r), [*trpC*22::Tn10 (tet^r)], (pMC9), *hsdR* (r_K^-, m_K+)

Miscellaneous

F′ Host contains an F′ episome with the stated features.

λ(DE3) Bacteriophage λ carrying the gene for T7 RNA polymerase is integrated into the host genome.

pMC9 is pBR322 with *lacI*^{q} inserted and confers amp and tet resistance.

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Genetic Markers in *E. coli*.

Symbol	Description	Effect of Mutation
ara-14	Mutation in arabinose metabolism	Blocks arabinose catabolism.
*ara*D	L-ribulose phosphate 4-epimerase mutation; part of an inducible operon araBAD repressed by L-arabinose	Blocks arabinose catabolism.
*arg*A	N-Acetylglutamate synthase mutation; inhibited by the presence of arginine	Arginine required from growth in minimal media.
*cyc*A	Involved in D-alanine, glycine, D-serine and D-cycloserine transport, and an L-alanine carrier	Mutants cannot use D-alanine as a carbon source.
dam	DNA adenine methylase mutation	Blocks methylation of adenine residues in the sequence 5′…G^{m}ATC…3′.
*dap*D	Succinyl-diaminopimelate aminotransferase mutation	Mutant reflects impaired synthesis of succinyl CoA and needs to be supplemented with succinate or lysine + methionine.
dcm	DNA cytosine methylase mutation	Blocks methylation of cytosine in the sequence 5′…C^{m}CAGG…3′ or 5′…C^{m}CTGG…3′.
*deo*C	Deoxyribose-phosphate aldolase mutation	
*deo*R	Regulatory gene mutation allowing constitutive expression of genes for deoxyribose synthesis	Allows efficient propagation of large plasmids.
*dut*1	Mutation of deoxyuridine triphosphatase, which catalyzes dUTP the conversion to dUMP and PPi	Mutants are impaired in conversion of dUTP to dUMP, leading to higher dUTP pools that can lead to misincorporation of uracil instead of thymidine. Stable incorporation of dUTP needs mutation in ung gene.
*end*A1	DNA-specific endonuclease I mutation	Improves quality of plasmid DNA isolations.
*gal*E	Part of the galETK operon that encodes UDP galactose-4-epimerase	Mutant is more resistant to bacteriophage P1 infection.
*gal*K	Galactokinase mutation	Blocks catabolism of galactose.
*gal*T	Galactose-1-phosphate uridylyltransferase mutation	Blocks catabolism of galactose.
*gyr*A96	DNA gyrase mutation	Confers resistance to nalidixic acid.
*hflA*150	Protease mutation that leads to stabilization of cII gene products	Leads to high frequency of lysogeny by λ phages (1).
*hfl*B	Gene encodes a possible protease component	Mutations lead to high frequency of bacteriophage lambda lysogenization.
hsdR (r_K^-, m_K+)	Host DNA restriction and methylation system mutation: Restriction minus, modification positive for the *E. coli* K strain methylation system	Allows cloning without cleavage of transformed DNA by endogenous restriction endonucleases. DNA prepared from this strain can be used to transform r_K+ *E. coli* strains.
*hsdS*20 (r_B^-, m_B^-)	Mutation of specificity determinant for host DNA restriction and methylation system. Restriction minus, modification minus for the *E. coli* B strain methylation system	Allows cloning without cleavage of transformed DNA by endogenous restriction endonucleases. DNA prepared from this strain is unmethylated by the *hsdS*20 methylases.
lacI^{q}	Overproduction of the *lac* repressor protein	Leads to high levels of the *lac* repressor protein, inhibiting transcription from the *lac* promoter.
lacY	Galactoside permease mutation	Blocks lactose utilization.
*lacZ*ΔM15	Partial deletion of β-D-galactosidase gene	Allows complementation of β-galactosidase activity by α-complementation sequence in pGEM®-Z Vectors. Allows blue/white selection for recombinant colonies when plated on X-Gal.
leuB	β-isopropylmalate dehydrogenase mutation	Requires leucine for growth on minimal media.
Δ(*lon*)	Deletion of *lon* protease	Reduces proteolysis of expressed proteins.
*Lys*S	pLysS plasmid is integrated into the host genome	Strains carrying this plasmid will be tet resistant and produce T7 lysozyme, a natural inhibitor of T7 RNA polymerase, thus lowering background transcription of sequences under the control of the T7 RNA polymerase promoter (2).
mcrA	Mutation in methylcytosine restriction system	Blocks restriction of DNA methylated at the sequence 5′…G^{m}CGC…3′.
mcrB	Mutation in methylcytosine restriction system	Blocks restriction of DNA methylated at the sequence 5′…AG^{m}CT…3′.
metB	Cystathionine γ-synthase mutation	Requires methionine for growth on minimal media.
*met*C	Cystathionine beta-lyase mutation; involved in methionine biosynthesis	Methionine required from growth in minimal media.
mtl	Mutation in mannitol metabolism	Blocks catabolism of mannitol.
mutS	Methyl-directed mismatch repair mutation	Prevents repair of the newly synthesized, unmethylated strand.
ompT	Mutation of protease VII, an outer membrane protein	Reduces proteolysis of expressed proteins.
P2	P2 bacteriophage lysogen present in host	λ phages containing the *red* and *gam* genes of λ are growth inhibited by P2 lysogens (3).
*pro*A	γ-glutamyl phoshate reductase mutation	proA/argD mutant will not block proline synthesis, but will be repressed by arginine. Mutants excrete proline on minimal media and are resistant to proline analogs. proA/argD/argR triple mutant grows slowly on minimal media + arginine.
proAB	Mutations in proline metabolism	Requires proline for growth in minimal media.

Genetic Markers in *E. coli* (continued).

Symbol	Description	Effect of Mutation
*recA*1, *recA*13	Mutation in recombination	Minimizes recombination of introduced DNA with host DNA, increasing stability of inserts. Inserts are more stable in *recA*1 than *recA*13 hosts.
recB, *recC* *rec*D	Exonuclease V mutations The Rec BCD trimer (exonuclease V) progressively degrades ssDNA and dsDNA in an ATP-dependent manner to form oligonucleotides; implicated in homologous recombination	Reduces general recombination and affects repair of radiation damage. Allows easier propagation of sequences with inverted repeats.
*rec*F	Recombination and repair mutation	Mutant cannot repair daughter strand gaps (post-replicational repair).
*rel*A	ppGpp synthetase I mutation, a novel nucleotide guanosine 5′-diphosphate-3′-diphosphate produced in response to starvation by relA ribosomal protein sensing uncharged tRNA	Allows RNA synthesis in the absence of protein synthesis.
rha	Utilization of L-rhamnose, a methylpentose	Blocks rhamnose catabolism.
rpsL	Mutation in subunit S12 of 30S ribosome	Confers resistance to streptomycin.
sbcB	Exonuclease I mutation	Allows general recombination in *rec*BC mutant strains.
*str*A	Mutant alters ribosome protein S12	Confers resistance to streptomycin
supB, *supC*, *supG*, *supL*, *supM*, *supN*, *supO*	Suppressor mutations	Suppresses ochre (UAA) and amber (UAG) mutations.
supD, *supE*, *supF*	Suppressor mutations	Suppresses amber (UAG) mutations.
thi-1	Mutation in thiamine metabolism	Thiamine required for growth in minimal media.
thr	Threonine biosynthesis mutation	Mutants are obligate threonine auxotrophs.
*thy*A	Thymidylate synthase; dTTP biosynthesis	Mutants are obligate thymidine auxotrophs.
Tn5	Transposon	Encodes resistance to kanamycin.
Tn10	Transposon	Encodes resistance to tetracycline.
tonA	Mutation in outer membrane protein	Confers resistance to bacteriophage T1.
*traD*36	Transfer factor mutation	Prevents transfer of F′ episome.
*trp*C	Phosphoribosyl anthranilate isomerase mutation; part of tryptophan biosynthesis pathway	
*trp*R	trpR aporepressor; regulates the biosynthesis of tryptophan and its transport	
tsx	T6 and colicin K phage receptor; outer membrane protein involved in specific diffusion of nucleosides; transports the antibotic albicidin	Resistant to bacteriophage T6 and colicin K.
*ung*1	Uracil-DNA N-glycosylase	Allows uracil to exist in plasmid DNA.
xyl-5	Mutation in xylose metabolism	Blocks catabolism of xylose.

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Technical Appendix

Nucleic Acids and Proteins: Calculations.

An online calculator for these values is available in the "tools" section of Promega's Web site at: **www.promega.com/techserv/tools/**

Metric Prefixes

Prefix	Symbol	Factor
kilo	k	10^{3}
centi	c	10^{-2}
milli	m	10^{-3}
micro	µ	10^{-6}
nano	n	10^{-9}
pico	p	10^{-12}
femto	f	10^{-15}
atto	a	10^{-18}
zepto	z	10^{-21}

Spectrophotometric Conversions

1 A_{260} unit of double-stranded DNA = 50µg/ml
1 A_{260} unit of single-stranded DNA = 33µg/ml
1 A_{260} unit of single-stranded RNA = 40µg/ml

DNA Molar Conversions

1µg of 1,000bp DNA = 1.52pmol (3.03pmol of ends)
1µg of pBR322 DNA = 0.36pmol DNA
1pmol of 1,000bp DNA = 0.66µg
1pmol of pBR322 DNA = 2.8µg

Formulas for DNA Molar Conversions

For dsDNA:

To convert pmol to µg:

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

To convert µg to pmol:

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

where N is the number of nucleotide pairs and 660pg/pmol is the average MW of a nucleotide pair.

For ssDNA:

To convert pmol to µg:

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

To convert µg to pmol:

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

where N is the number of nucleotides and 330pg/pmol is the average MW of a nucleotide.

Dalton (Da) is an alternate name for the atomic mass unit, and kiloDalton (kDa) is 1,000 Daltons. Thus a protein with a mass of 64kDa has a molecular weight of 64,000 grams per mole.

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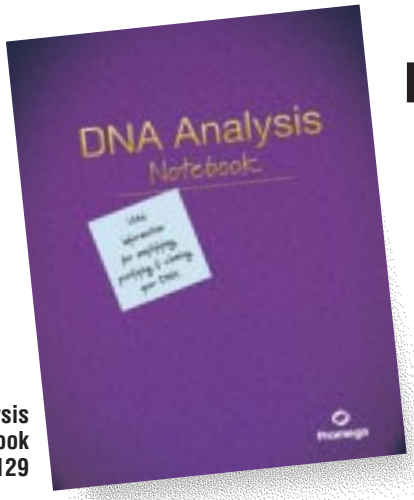

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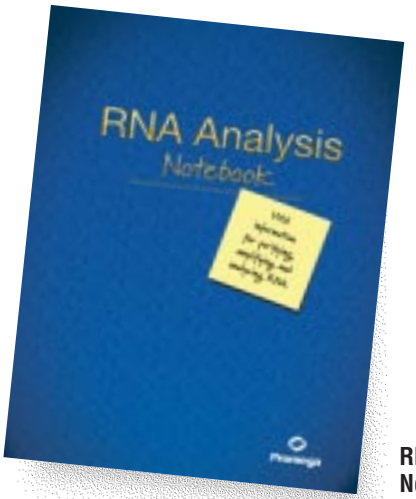

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