

Altered Sites® II in vitro Mutagenesis System

INSTRUCTIONS FOR USE OF PRODUCTS Q6210 AND Q6090.

Altered Sites® II in vitro Mutagenesis System

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of this system. E-mail: techserv@promega.com.

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1. Description

The Altered Sites® II in vitro Mutagenesis Systems^(a,b) provide a high-efficiency procedure for generation and selection of oligonucleotide-directed mutants. These second generation systems feature several improvements over the original Altered Sites® Systems, including the ability to mutagenize double-stranded template DNA, perform sequential rounds of mutagenesis without subcloning, and express the mutated gene products in vivo or in vitro. Each system uses one of two phagemids, described in Table 1 and Figures 2-4.

The systems use antibiotic selection as a means to obtain a high frequency of mutants (Figure 1). For example, the pALTER®-1 Vector^(a) contains genes for ampicillin and tetracycline resistance, but the ampicillin resistance gene has been inactivated. The Ampicillin Repair Oligonucleotide restores ampicillin resistance to the mutant strand during the mutagenesis reaction. This oligonucleotide is annealed to the single- or double-stranded DNA template at the same time as the mutagenic oligonucleotide. Subsequent synthesis and ligation of the mutant strand links the two. The appropriate oligonucleotides can be used simultaneously in the mutagenesis reaction to inactivate one resistance gene while repairing the other. In this way, subsequent rounds of mutagenesis and selection can be performed on the same plasmid without subcloning.

The pALTER®-Ex1 Vector^(a), a derivative of the pALTER®-1 Vector, contains appropriate transcription and translation signals for protein expression in vivo or in vitro. With each Altered Sites® II System, the appropriate mutagenic oligonucleotides are provided to both inactivate and repair the antibiotic resistance genes of the vector supplied.

Table 1. Properties of the pALTER® Family of Vectors.

Vector	pALTER®-1	pALTER®-Ex1
Selectable Markers ¹	amp, tet	amp, tet
Promoters Available	SP6, T7	SP6, T7, <i>tac</i>
Convenient Protein Expression in vitro and in vivo	No ²	Yes ³
Compatible with colE1 Plasmids	No	No
Blue/White Cloning	Yes	No

¹amp = ampicillin, tet = tetracycline

²Expression is possible, but the cloned sequence must contain the necessary translation start sites.

³Convenient cloning sites and translation start sites are provided in both directions for expression in vitro using the *E. coli* S30 Extract System for Circular DNA (Cat.# L1020) or for expression in vivo in *E. coli* using the *tac* promoter or another prokaryotic promoter.

Mutagenesis protocols are provided for both double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA). The mutagenesis reaction is initially transformed into a repair minus strain of *E. coli* (ES1301 *mutS*) to avoid selection against the desired mutation. A subsequent strain transfer into JM109 ensures proper segregation of mutant and wildtype plasmids and results in a high proportion of mutants. The Altered Sites® II Mutagenesis Systems allow consistently high mutagenesis frequencies (often >90%) using dsDNA or ssDNA.

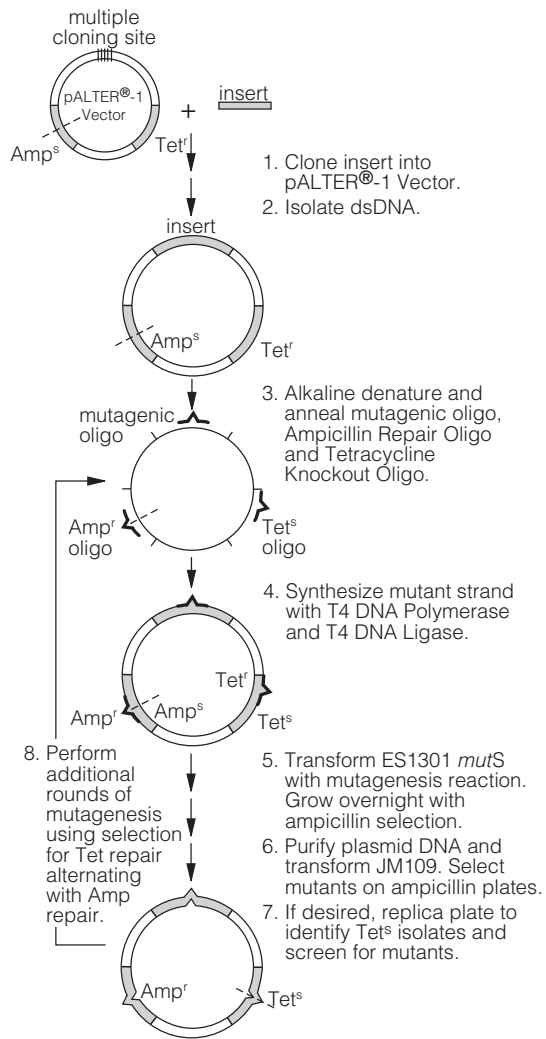
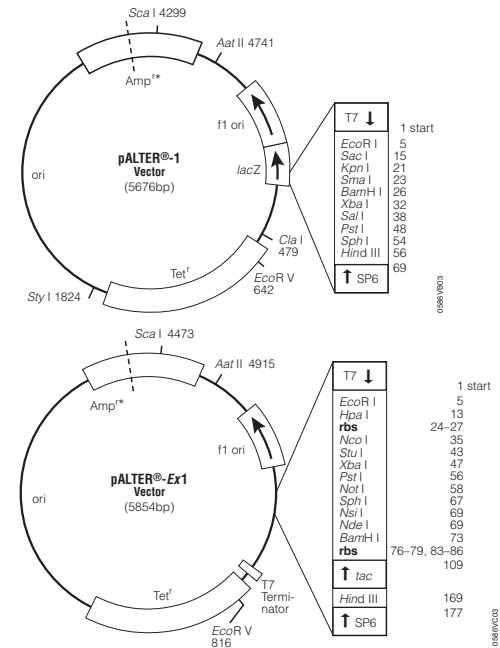


Figure 1. Schematic diagram of the Altered Sites® II in vitro mutagenesis procedure using the pALTER®-1 Vector as an example.



*As the vector is supplied, this antibiotic resistance gene is inactivated. It can be reactivated by using one of the mutagenesis oligonucleotides provided. See Section 5 for more details.

Figure 2. pALTER®-1 and pALTER®-Ex1 Vector circle maps and sequence reference points. Additional sequence and restriction site information for the pALTER® Vectors is provided in Section 9.E.

Note: For screening purposes, the Tetracycline Knockout Oligonucleotide removes the *EcoR V* site in the tetracycline gene, and the Ampicillin Repair Oligonucleotide restores a *Pst I* site in the ampicillin gene.

pALTER® Vectors Sequence Reference Points.

	pALTER®-1	pALTER®-Ex1
T7 RNA polymerase transcription initiation site	1	1
SP6 RNA polymerase transcription initiation site	69	177
<i>lac</i> transcription initiation site	N/A	113
T7 RNA polymerase promoter (-17 to +3)	5660-3	5838-3
SP6 RNA polymerase promoter (-17 to +3)	67-86	175-194
<i>lac</i> promoter	N/A	78-150
T7 translation start codon	N/A	37
<i>lac</i> and SP6 translation start codon	N/A	66, 70
multiple cloning regions	5-61	5-78
<i>lacZ</i> start codon	108	N/A
<i>lac</i> operon sequences	5497-5657, 94-323	*
<i>lac</i> operator	128-144	N/A
β-lactamase (Amp ^r) coding region	3748-4604	3926-4782
Tet ^r gene coding region	541-1728	719-1906
phage f1 region	5041-5496	5219-5674
binding site of pUC/M13 Forward Sequencing Primer	5617-5633	5795-5811
binding site of pUC/M13 Reverse Sequencing Primer	104-120	212-228
binding site of Ampicillin Repair Oligonucleotide	4052-4074	4230-4252
binding site of Tetracycline Knockout Oligonucleotide	624-654	802-832

Use the T7 or pUC/M13 Forward Primer to sequence ssDNA produced by the pALTER®-1 and pALTER®-Ex1 Vectors.

*While *lacZ* sequences are present in the uncut vector, colonies are light blue and thus may not be readily distinguished from white colonies. Therefore, blue/white cloning is not recommended.

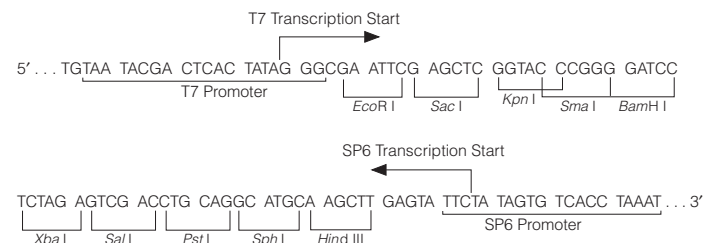


Figure 3. pALTER®-1 Vector promoter and multiple cloning site sequence. The sequence shown is complementary to the ssDNA produced upon infection with helper phage. This sequence corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by SP6 RNA polymerase.

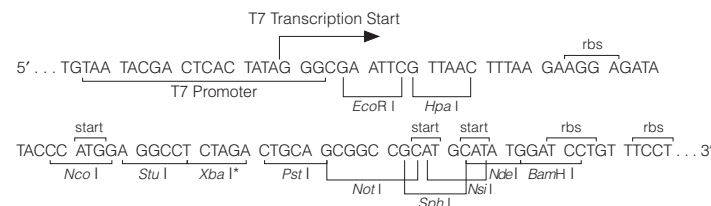


Figure 4. pALTER®-Ex1 Vector promoter and multiple cloning site sequence. The sequence shown is complementary to the ssDNA produced upon infection with helper phage. This sequence corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by SP6 RNA polymerase.

rbs = ribosome binding site

2. Product Components and Storage Conditions

Product	Size	Cat.#
Altered Sites® II in vitro Mutagenesis System	1 system	Q6210

Each system contains sufficient reagents to perform 25 mutagenesis reactions. Includes:


- 20µg pALTER®-1 Vector
- 30µl Ampicillin Repair Oligonucleotide
- 30µl Ampicillin Knockout Oligonucleotide
- 30µl Tetracycline Repair Oligonucleotide
- 30µl Tetracycline Knockout Oligonucleotide
- 75µl Annealing 10X Buffer
- 100µl Synthesis 10X Buffer
- 500u T4 DNA Polymerase
- 100u T4 DNA Ligase
- 0.2ml ES1301 *mutS* Bacterial Strain, Glycerol Stock
- 0.5ml JM109 Bacterial Strain, Glycerol Stock
- 1ml R408 Helper Phage
- 10µg R408 Helper Phage DNA

Product	Size	Cat.#
Altered Sites® II-Ex1 in vitro Mutagenesis/Expression System	1 system	Q6090

Each system contains sufficient reagents to perform 25 mutagenesis reactions. Includes the same reagents as Cat.# Q6210, except that the Vector and Oligonucleotides are as follows:

- 20µg pALTER®-Ex1 Vector
- 30µl Ampicillin Repair Oligonucleotide
- 30µl Ampicillin Knockout Oligonucleotide
- 30µl Tetracycline Repair Oligonucleotide
- 30µl Tetracycline Knockout Oligonucleotide

Storage Conditions: Store glycerol stocks of ES1301 *mutS* and JM109 at -70°C. Store all other system components at -20°C.

 The ES1301 *mutS* and JM109 bacterial strains supplied with the Altered Sites® II Systems are glycerol stocks and **not** competent cells.

3. General Considerations

Site-directed mutagenesis is a valuable tool for the study of DNA function and protein structure and function. A number of different mutagenesis methods have been reported (1,2). As introduced by Hutchison *et al.* (3), site-directed in vitro mutagenesis is accomplished by hybridizing a single-stranded DNA (ssDNA) to a synthetic oligonucleotide that is complementary to the ssDNA template except for a region of mismatch near the center. It is this region that contains the desired mutation. Following hybridization, the oligonucleotide is extended with DNA polymerase to create a double-stranded structure. The nick is then sealed with DNA ligase, and the duplex structure is transformed into an *E. coli* host.

If no selection method is used, the theoretical yield of mutants using this procedure is 50% (due to the semi-conservative mode of DNA replication). In practice, however, the mutant yield may be much lower, often only a few percent or less. This is assumed to be due to such factors as incomplete in vitro polymerization, primer displacement by the DNA polymerase used in the fill-in reaction, and in vivo host-directed mismatch repair mechanisms that favor repair of the unmethylated newly synthesized DNA strand (4). For this reason, the Altered Sites® II Systems use antibiotic selection to increase the yield of mutants.

3.A. pALTER®-1 Vector

The pALTER®-1 Vector contains a multiple cloning region flanked by the opposing SP6 and T7 RNA polymerase promoters and inserted into the DNA encoding the *lacZ* α -peptide. Cloning of a DNA insert into the multiple cloning region results in inactivation of the α -peptide. When plated on X-Gal indicator plates, colonies containing recombinant plasmids are white against a background of blue colonies. The SP6 and T7 promoters may be used to generate high-specific-activity RNA probes from either strand of the insert DNA. These promoters also serve as convenient priming sites for sequencing the insert. The pALTER®-1 Vector carries sequences for both ampicillin and tetracycline resistance. However, the plasmid is ampicillin-sensitive because a frameshift mutation has been introduced into the *Amp^r* gene by removing the *Pst* I site. Therefore, propagation of the plasmid and recombinants is performed under tetracycline selection. The pALTER®-1 Vector also contains the *f1* origin of replication, which allows for the production of ssDNA upon infection of the host cells with the helper phage R408 (5-7). The pALTER®-1 Vector has been used successfully with inserts up to 6kb in size.

 Propagation of the pALTER®-1 Vector and recombinants is performed under tetracycline selection.

3.B. pALTER[®]-Ex1 Vector

The pALTER[®]-Ex1 Vector differs from the pALTER[®]-1 Vector in that it contains a novel polylinker that allows protein expression in vivo and in vitro. Transcription of the insert is controlled by opposing T7 and SP6 promoters. A *tac* promoter is provided in the same orientation as the SP6 promoter. A T7 transcription terminator is located outside of the multiple cloning region to reduce transcription through the tetracycline gene. Ribosome binding sites are provided in both orientations, and convenient cloning sites are provided for cloning at the ATG initiation codons. The *tac* and T7 promoters can be used for expression in vivo. The *tac* promoter is induced by the addition of IPTG. The plasmid should be maintained in a *lacI^q* host, such as JM109, to reduce any toxic effects of low-level expression of the cloned protein. While *lacZ* sequences are present in the uncut pALTER[®]-Ex1 Vector, colonies are light blue and thus may not be readily distinguished from white colonies. Therefore, blue/white cloning is not recommended.


In vivo expression from the T7 promoter can be provided by strains that produce T7 RNA polymerase such as JM109(DE3) (Cat.# P9801) or BL21(DE3) (8). In our experience, the plasmid appears to be more stable in JM109(DE3) than in BL21(DE3), although it can be maintained in a BL21(DE3) strain containing the pLysS plasmid [such as Bacterial Strain BL21(DE3)pLysS (Cat.# P9811)], which contains T7 lysozyme to reduce the activity of the T7 RNA polymerase. Successful expression requires that the cloned fragment be inserted in the proper orientation for the desired promoter.

 Use of DH5 α [™] cells is not recommended with the Altered Sites[®] II Systems. DH5 α [™] cells transformed with the pALTER[®]-Ex1 Vector grow very slowly.

3.C. Important Features of the Altered Sites[®] II in vitro Mutagenesis Systems

dsDNA or ssDNA Templates

Mutagenesis can be performed using either dsDNA or ssDNA templates. The dsDNA procedure is faster and does not require the prior preparation of ssDNA. The ss procedure may be more useful, however, when trying to maximize the total number of transformants, such as for generating mutant libraries or when the mutagenic oligonucleotide is expected to have difficulty annealing to the template. Such annealing problems can be due to sequence mismatches at multiple locations, an AT-rich region or secondary structure. Poor-quality DNA can inhibit the second-strand reaction during mutagenesis. We recommend using sequencing-quality DNA.

 ES1301 *mutS* cells are restriction (+). Template DNA should be isolated from a modification (+) K12 strain, such as JM109, or it will be restricted by ES1301 *mutS*. For example, DNA isolated from HB101 or NM522 (modification minus strains) or BL21 (*E. coli* B strain) cells should not be used.

Note: Further information on the ES1301 *mutS* and JM109 strains is provided in Section 9.B.

High Yield of Mutants

The use of antibiotic selection for the mutant strand yields a high percentage of mutants. This high frequency enables identification of mutants by restriction analysis or direct sequencing of clones, eliminating the need to screen a large number of colonies by hybridization. Only a small amount of ds or ssDNA (0.1 μ g or less) is required to obtain many antibiotic-resistant colonies.

The ES1301 *mutS* strain (9) suppresses in vivo mismatch repair (10). It is used for the initial round of transformation to decrease the chance that the antibiotic repair mismatch or the mutagenic mismatch will be repaired. We do not recommend the use of the BMH 71-18 *mutS* strain because it is Tet^r and thus cannot be used for the secondary selection step.

Multiple Simultaneous Mutations

T4 DNA Polymerase is used in the synthesis reaction in place of Klenow fragment because it does not strand displace (11,12) and therefore does not displace the mutagenic oligonucleotide. As a result, multiple site-directed mutations may be introduced simultaneously simply by annealing additional mutagenic oligonucleotides to the DNA insert (13). We have performed up to four simultaneous mutations with >50% efficiency using this system.

Multiple Rounds of Mutagenesis

To perform multiple rounds of mutagenesis on a single construct without subsequent subcloning from the parental mutagenesis vector, one simultaneously inactivates the alternate antibiotic resistance on the mutagenesis vector while selecting for repair of the primary antibiotic resistance. The pALTER[®]-1 and pALTER[®]-Ex1 Vectors use ampicillin resistance as the primary selection for the mutant strand. If the Tetracycline Knockout Oligonucleotide is included in the mutagenesis reaction, a tetracycline-sensitive version of the mutant will be obtained. This plasmid then can be used in a subsequent mutagenesis reaction performed in the presence of the Tetracycline Repair Oligonucleotide. Mutants in this second round of mutagenesis are selected in the presence of tetracycline. Additional oligonucleotides are provided to alternately repair and inactivate each antibiotic resistance, so that an indefinite number of mutagenesis reactions can be performed on the same construct.

Expression in vivo or in vitro

The pALTER[®]-Ex1 Vector is constructed to allow both mutagenesis and protein expression. Several sites are provided for cloning to place the insert under control of the prokaryotic transcription and translation signals supplied in this vector. Inducible expression in vivo is available from the *tac* promoter when the vector is maintained in a *lacI^q* host strain such as JM109. The T7 promoter also can be utilized in vivo with an appropriate host such as JM109(DE3) to provide the T7 RNA polymerase.

3.C. Important Features of the Altered Sites® II in vitro Mutagenesis Systems (continued)

Mutagenized genes also can be conveniently expressed in vitro using coupled transcription/translation systems. The *E. coli* S30 Extract System for Circular DNA (Cat.# L1020) is useful for expression of inserts under control of the *tac* promoter and other prokaryotic promoters. Inserts under control of the T7 promoter can be expressed by addition of T7 RNA polymerase to the reaction.

Alternative Protocol: Cotransformation into ES1301 *mutS* and Transfer to JM109

Mutant plasmids may be rapidly transferred from the *mutS* host into a more suitable host for long-term maintenance and mutant segregation (see Section 8.F). This alternative to the standard procedure may be used when it is important to save time or minimize the chances of sequence rearrangements. ES1301 *mutS* is *recA*⁺, and as a result, inserts containing highly repetitive sequences may be unstable.

! Use this procedure only if you have highly competent ES1301 *mutS* cells (>10⁷cfu/μg DNA).

The mutagenesis reaction products are cotransformed into the ES1301 *mutS* strain along with R408 helper DNA. The cotransformed helper DNA causes the mutant phagemid to be replicated and packaged as an infectious particle that is secreted into the growth medium. These infectious particles are used to transfect a suitable (F⁺) host such as JM109, and the transfectants are selected by their antibiotic resistance encoded by the phagemid. The procedure requires only one transformation step into the ES1301 *mutS* strain, since the second host does not need to be competent. The procedure reduces the total time of the mutagenesis protocol and eliminates the plasmid miniprep and transformation steps for transfer into JM109.

! Do not use the BMH 71-18 *mutS* strain in place of the ES1301 *mutS* strain; BMH 71-18 *mutS* is F⁺ and will not work in this cotransformation procedure. Also, BMH 71-18 is Tet^r and thus cannot be used for multiple rounds of mutagenesis.

3.D. Design of Mutagenic Oligonucleotides

The mutagenic oligonucleotide must be complementary to the ssDNA strand produced by the mutagenesis vectors in the presence of helper phage. This is true for dsDNA mutagenesis as well, since the mutagenic oligonucleotide must hybridize to the same strand as the antibiotic repair oligonucleotide for the selection to be effective.

! Refer to Section 9.A for sequences of the oligonucleotides provided.

The stability of the complex between the oligonucleotide and the template is determined by the base composition of the oligonucleotide and the conditions under which it is annealed. In general, a 17- to 20-base oligonucleotide with the

mismatch located in the center will be sufficient for single-base mutations. This gives 8–10 perfectly matched nucleotides on either side of the mismatch. For mutations involving two or more mismatches, oligonucleotides 25 bases or longer are needed to allow for 12–15 perfectly matched nucleotides on either side of the mismatch. Oligonucleotides of 26 and 27 bases have been used successfully to perform four-base insertions and deletions. Larger deletions require an oligonucleotide having 20–30 matched bases on either side of the mismatched region.

The annealing conditions required may vary with the base composition of the oligonucleotide. AT-rich complexes tend to be less stable than GC-rich complexes and may require a lower annealing temperature to be stabilized. Routinely, oligonucleotides can be annealed by heating to 75°C for 5 minutes followed by slow cooling to room temperature. For more detailed discussions of oligonucleotide design and annealing conditions, refer to reference 14 and to reference 15, chapters 11 and 15.

3.E. Phosphorylation of Oligonucleotides

A significant increase in the number of mutants is observed when oligonucleotides are phosphorylated. Therefore, oligonucleotides included in this system are 5′-phosphorylated. We recommend phosphorylation of any mutagenic oligonucleotides used with this system (Section 8.D).

4. Cloning into the Mutagenesis Vectors

Clone the DNA to be mutated into the pALTER®-1 or pALTER®-Ex1 Vector using the multiple cloning regions shown in Figures 3 and 4. Transform the vector DNA into JM109 competent cells and select recombinant colonies by plating on LB plates containing 12.5μg/ml tetracycline. Competent JM109 cells are available from Promega. For the pALTER®-1 Vector, 0.5mM IPTG and 40μg/ml X-Gal can be added to screen for inactivation of the *lacZ* α-peptide by the insert. After incubation for 24 hours at 37°C, colonies containing recombinant plasmids will appear white. An alternative to preparing plates containing X-Gal and IPTG is to spread LB plates with 50μl of 50mg/ml X-Gal and 100μl of 100mM IPTG and allow these components to absorb for 30 minutes at 37°C prior to plating cells. Blue/white screening is not recommended for the pALTER®-Ex1 expression vector.

! Use of DH5α™ cells is not recommended with the Altered Sites® II Systems. DH5α™ cells transformed with the pALTER®-Ex1 Vector grow very slowly.

5. Mutagenesis Procedure

The mutagenesis reaction involves annealing of the antibiotic repair oligonucleotide (provided) and the mutagenic oligonucleotide(s) to the DNA template, followed by synthesis of the mutant strand with T4 DNA Polymerase and T4 DNA Ligase. The heteroduplex DNA is then transformed into the repair minus *E. coli* strain ES1301 *mutS*, and the mixture is grown in selective media to isolate clones containing the mutant plasmid. Antibiotic-resistant plasmids are isolated and transformed into the final host strain. Mutations can then be screened by restriction analysis or direct sequencing of the plasmid DNA from these isolates.

5.A. Before You Begin

Before starting the procedure, prepare all of the reagents needed, including ES1301 *mutS* and JM109 competent cells (see Section 8.B and 8.C). JM109 Competent Cells may be purchased from Promega (see Section 10). Calculate the amount of mutagenic oligonucleotide needed (Table 2), and select the appropriate repair and knockout oligonucleotides (Table 3) for your mutagenesis reaction.

! A cotransformation procedure is provided in Section 8.F as an alternative to the standard procedure described here. Use the alternative procedure only if you have highly competent ES1301 *mutS* cells ($>10^7$ cfu/ μ g DNA).

Table 2. Amount of Mutagenic Oligonucleotide Needed to Equal 1.25pmol.

Oligonucleotide Length	Oligonucleotide (in ng) Equal to 1.25pmol
17mer	7.0ng
20mer	8.3ng
23mer	9.5ng
26mer	10.8ng
29mer	12.0ng

In general: ng of oligonucleotide = pmol of oligonucleotide \times 0.33 \times N, where N = length of oligonucleotide in bases.

Table 3. Selection of Appropriate Repair and Knockout Oligonucleotides.

Vector Used	Mutagenesis Round	Repair Oligo ¹	Knockout Oligo	Phenotype Change	Antibiotic Selection
pALTER [®] -1 + Insert	#1	Amp ¹	Tet ¹	Amp ^s Tet ^r \rightarrow Amp ^r Tet ^s	Amp
pALTER [®] -1 + Insert	#2	Tet	Amp	Amp ^r Tet ^s \rightarrow Amp ^s Tet ^r	Tet
pALTER [®] -1 (no insert)	Positive Control	Amp	Tet	Amp ^s Tet ^r \rightarrow Amp ^r Tet ^s	Amp
pALTER [®] -Ex1 + Insert	#1	Amp	Tet	Amp ^s Tet ^r \rightarrow Amp ^r Tet ^s	Amp
pALTER [®] -Ex1 + Insert	#2	Tet	Amp	Amp ^r Tet ^s \rightarrow Amp ^s Tet ^r	Tet
pALTER [®] -Ex1 (no insert)	Positive Control	Amp	Tet	Amp ^s Tet ^r \rightarrow Amp ^r Tet ^s	Amp

¹oligo = oligonucleotide, Amp = ampicillin, Tet = tetracycline

Note: It is not necessary to include the antibiotic knockout oligonucleotide in the mutagenesis reaction if a second round of mutagenesis is not desired.

Materials to Be Supplied by the User

(Solution compositions are provided in Section 9.C.)

- ES1301 *mutS* and JM109 competent cells (see Sections 8.B and 8.C)
- mutagenic oligonucleotide, phosphorylated (see Section 8.D)
- sterile 17 \times 100mm polypropylene tubes
- 0.8% agarose gel
- heating block, thermal cycler or 75°C water bath
- 2M ammonium acetate (pH 4.6), freshly prepared
- 2M NaOH, 2mM EDTA, freshly prepared
- ethanol (100% and 70%), 4°C
- TE buffer (pH 8.0)
- sterile, deionized water
- ampicillin, tetracycline stock solutions
- LB medium
- LB plates containing either 125 μ g/ml ampicillin or 12.5 μ g/ml tetracycline
- SOC medium

5.B. Denaturation of Double-Stranded DNA Template

We recommend using DNA of sequencing quality. **Double-stranded DNA must be alkali-denatured before use.** Heat-denaturation does not work for this application because the two strands reanneal too quickly. Denaturation is not required when using a single-stranded DNA template for mutagenesis.

1. Set up the following alkaline denaturation reaction. This generates enough DNA for 10 mutagenesis reactions.

dsDNA template	0.5pmol (approx. 2µg)
2M NaOH, 2mM EDTA	2µl
sterile, deionized water to a final volume of	20µl

In general: $\text{ng of dsDNA} = \text{pmol of dsDNA} \times 0.66 \times N$,
where N = length of dsDNA in bases

! To ensure good DNA recovery, do not denature less than 0.5pmol DNA.

2. Incubate for 5 minutes at room temperature.
3. Add 2µl of 2M ammonium acetate (pH 4.6) and 75µl of 100% ethanol.
4. Incubate at -70°C for 30 minutes.
5. Precipitate the DNA by centrifugation at top speed in a microcentrifuge for 15 minutes.
6. Drain and wash the pellet with 200µl of 70% ethanol. Centrifuge again as in Step 5. Dry the pellet under vacuum.
7. Dissolve the pellet in 100µl of TE buffer (pH 8.0). Analyze a 10µl sample of the denatured DNA on an agarose gel to verify that no significant loss has occurred before proceeding to the annealing reaction.

5.C. Annealing Reaction and Mutant Strand Synthesis

The amount of oligonucleotide required for the annealing reaction may vary depending on the size and amount of the DNA template. Use the antibiotic repair and knockout oligonucleotides at a 5:1 oligo:template molar ratio and the mutagenic oligonucleotides at a 25:1 oligo:template molar ratio. A typical reaction may contain approximately 200ng (0.05pmol) of dsDNA or 100ng of ssDNA. Double-stranded DNA must be alkali-denatured (see Section 5.B) before it is used in the annealing reactions below. The Repair and Knockout Oligonucleotides supplied with the system are phosphorylated.

In the reaction examples shown in Step 1, both the antibiotic repair oligonucleotide and knockout oligonucleotide are included in the annealing reaction. It is not necessary to include the antibiotic knockout oligonucleotide in the mutagenesis reaction if a second round of mutagenesis is not desired.

Note: For more information on annealing and oligonucleotide design, see Section 3.D and references 14 and 15.

! This system contains several oligonucleotides with similar names. Please refer to Table 3 and read the vial labels carefully to make sure you are using the correct oligonucleotides.

1. Prepare the appropriate annealing reactions as described below.

Mutagenesis Reaction

alkaline-denatured dsDNA or ssDNA mutagenesis template	10µl (0.05pmol)
Repair Oligonucleotide (2.2ng/µl), phosphorylated	1µl (0.25pmol)
Knockout Oligonucleotide (2.2ng/µl), phosphorylated	1µl (0.25pmol)
mutagenic oligonucleotide, phosphorylated (see Table 2, Section 5.A)	1.25pmol
Annealing 10X Buffer	2µl
sterile, deionized water to a final volume of	20µl

Control Reaction

alkaline-denatured nonrecombinant pALTER® dsDNA	10µl (0.05pmol)
Repair Oligonucleotide (2.2ng/µl), phosphorylated	1µl (0.25pmol)
Knockout Oligonucleotide (2.2ng/µl), phosphorylated	5µl (1.25pmol)
Annealing 10X Buffer	2µl
sterile, deionized water to a final volume of	20µl

2. Heat the annealing reactions to 75°C for 5 minutes and allow them to cool slowly to room temperature. Slow cooling minimizes nonspecific annealing of the oligonucleotides. We recommend cooling the reactions at about 1°C per minute to 45°C, then more rapidly to room temperature (22°C).

Note: If you do not have access to a thermal cycler, the following annealing procedure may be used to slowly cool the annealing reactions.

- a. Heat reactions at 75°C for 5 minutes in a heating block or a beaker containing 300ml of water.
- b. Place heating block or beaker at room temperature until it reaches 45°C (30 minutes).
- c. Place heating block or beaker on ice until it reaches room temperature (10–15 minutes).

5.C. Annealing Reaction and Mutant Strand Synthesis (continued)

- Place the annealing reactions on ice and add these components **in the order listed**. The final volume will be 30µl.

sterile, deionized water	5µl
Synthesis 10X Buffer	3µl
T4 DNA Polymerase	1µl (5–10u)
T4 DNA Ligase	1µl (1–3u)

- Incubate the reaction at 37°C for 90 minutes to perform mutant strand synthesis and ligation. Do not incubate for longer than 90 minutes, as the DNA may be degraded by the T4 DNA Polymerase.

5.D. Transformation of ES1301 *mutS* Competent Cells

High-efficiency ES1301 *mutS* competent cells may be prepared using the procedures provided in Sections 8.B and 8.C. ES1301 *mutS* competent cells should yield >10⁷cfu/µg DNA for use in the following protocol. Follow this protocol as closely as possible to maximize the transformation efficiency of these cells.

! Do not use ES1301 *mutS* colonies as mutants. A second round of transformation (into JM109) should always be performed to avoid having a mixed population of mutants and wildtype DNA in the cell. ES1301 *mutS* is not a stable host for long-term maintenance of plasmids.

- Prechill sterile 17 × 100mm polypropylene culture tubes on ice, one for each annealing reaction.

Note: Use of standard microcentrifuge tubes, rather than the 17 × 100mm polypropylene tubes recommended, reduces the transformation efficiency by approximately 50% due to inefficient heat-shock treatment.

- Remove the frozen competent cells from -70°C and thaw on ice (approximately 5 minutes).
- Gently mix the cells by flicking the tube, then transfer 100µl of the thawed ES1301 *mutS* cells to each of the prechilled culture tubes.
- Add 1.5µl of each mutagenesis reaction or control reaction (approx. 10ng of template DNA) to 100µl of ES1301 *mutS* competent cells. Move the pipette tip through the cells while dispensing to mix. **Do not pipet or vortex to mix.** Quickly flick the tube several times.
- Immediately place the tubes on ice for 10 minutes.
- Heat-shock the cells for 45–50 seconds in a water bath at **exactly 42°C. Do Not Shake.**
- Immediately place the tubes on ice for 2 minutes.

- Add 900µl of room temperature LB broth without antibiotic to each transformation reaction and incubate for 30 minutes at 37°C with shaking (approximately 225rpm). Incubate for a minimum of 30 minutes if the cells will be subsequently selected with tetracycline.

Note: If you wish to check the ES1301 *mutS* transformation efficiency, plate 100µl of each transformation reaction onto each of 4–5 plates containing the appropriate selective media (LB + 125µg/ml ampicillin or 12.5µg/ml tetracycline) and incubate the plates at 37°C for 30–36 hours. Note that ES1301 *mutS* cells grow slowly and their colony size varies.

- Prepare overnight cultures by adding 500µl of each transformation to 4.5ml of the appropriate selective medium (LB + 125µg/ml ampicillin or 10µg/ml tetracycline). Incubate overnight at 37°C with shaking (approximately 225rpm). Use this culture for the plasmid miniprep and transformation described in Sections 5.E and 5.F.

Note: Cell growth in liquid medium is inhibited by tetracycline concentrations greater than 10µg/ml.

5.E. Plasmid Miniprep

Purify pALTER® plasmid DNA using the protocol provided in Section 8.E. Alternate purification methods, such as the Wizard® Plus SV Minipreps DNA Purification System (Cat.# A1340) or the Wizard® Plus Minipreps DNA Purification System (Cat.# A7100), also may be used for this step.

Note: The pALTER® Vectors tend to give lower yields than most high-copy-number plasmids (e.g., pUC18).

5.F. Transformation into JM109

JM109 competent cells may be purchased from Promega or prepared using the procedure provided in Sections 8.B and 8.C.

- Estimate the amount of DNA recovered from the plasmid miniprep. Run a portion of the DNA on an agarose gel and estimate the concentration by comparison to dsDNA markers. We recommend that you load 10µl of the 50µl plasmid solution obtained using the miniprep plasmid procedure described in Section 8.E.
Note: ES1301 *mutS* cells grow slowly; plasmid yields may be low.
- Prechill sterile 17 × 100mm polypropylene culture tubes on ice.
- Remove frozen JM109 competent cells from -70°C and thaw on ice (approximately 5 minutes).
- Gently mix the cells by flicking the tube, then transfer 100µl of the thawed JM109 cells to each of the prechilled culture tubes.

5.F. Transformation into JM109 (continued)

5. Add approximately 5–10ng of plasmid DNA to 100µl of JM109 competent cells. Move the pipette tip through the cells while dispensing. Quickly flick the tube several times.
6. Immediately place the tube on ice for 30 minutes.
7. Heat-shock the cells for 45–50 seconds in a water bath at **exactly 42°C**. **Do Not Shake.**
8. Immediately place the tubes on ice for 2 minutes.
9. Add 900µl of room temperature SOC medium to each transformation reaction and incubate for 60 minutes at 37°C with shaking (approximately 225rpm).
10. For each tube, plate 100µl of cells on each of two LB plates containing the appropriate selective medium (LB + 125µg/ml ampicillin or 12.5µg/ml tetracycline) and incubate at 37°C for 12–16 hours.

5.G. Analysis of Transformants

The Altered Sites® II mutagenesis procedure generally produces 60–90% mutants, so colonies may be screened by direct sequencing. Assuming that greater than 60% mutants are obtained, screening 5 colonies will give a greater than 95% chance of finding the mutation. If the mutation is located within 200–300 bases of either end of the DNA insert, the SP6 or T7 sequencing primers may be used for convenient priming of the sequencing reactions. Often restriction sites can be incorporated into the mutagenesis primers without altering the amino acid sequence. These sites can provide a quick screen to identify those clones containing the desired mutation.

When preparing antibiotic-resistant cells for plasmid minipreps, often it is convenient to screen simultaneously for antibiotic-sensitive isolates to be used for subsequent rounds of mutagenesis (e.g., Amp^rTet^s). Simply inoculate each isolate into two tubes of media, one containing each antibiotic, and it will be easy to identify those isolates that are Tet^s.

Alternatively, Amp^rTet^s colonies can be identified by picking and plating them in a grid format (see Section 9.D) on paired plates containing either ampicillin or tetracycline. Pick each colony with a sterile toothpick and inoculate the two plates in sequence.

Control mutagenesis reactions should give better than 50% tetracycline-sensitive colonies. **For screening purposes, the Tetracycline Knockout Oligonucleotide removes the EcoR V site in the tetracycline gene, and the Ampicillin Repair Oligonucleotide restores a Pst I site in the ampicillin gene.**

6. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: www.promega.com. E-mail: techserv@promega.com

Symptoms	Causes and Comments
No growth in overnight ESI301 <i>mutS</i> culture	Excessive antibiotic concentration. Check antibiotic concentration in selective media. See Section 5.D, Step 9, or Section 5.F, Step 10. Cells in liquid culture are especially sensitive to excess tetracycline.
	Incomplete denaturation of template DNA. Must use alkaline denaturation and not heat denaturation.
	Carryover of NaOH from the denaturation reaction. NaOH must be neutralized after the denaturation reaction. Use fresh ammonium acetate (pH 4.6) and check pH carefully.
	Poor recovery of DNA after alkaline denaturation. DNA not recovered after ethanol precipitation. Check denatured template on an agarose gel.
	Inaccurate or aberrant DNA concentration. Confirm concentration by comparison with known standards on an agarose gel.
	DNA contaminants present. Further purify DNA by PEG precipitation (15) if A_{260}/A_{280} ratio is less than 1.8.
	Inadequate oligonucleotide hybridization. Incorrect oligonucleotide:template nucleotide hybridization ratios. Check concentration of mutagenic oligonucleotide and DNA. Anneal more slowly (Section 5.C, Step 2).
	Wrong Antibiotic Repair Oligonucleotide added to annealing reaction. Refer to Table 3.
	Inefficient synthesis or ligation of second-strand DNA. Low T4 DNA Polymerase or T4 DNA Ligase activity due to poor quality DNA. Compare results to those of the control mutagenesis reaction.
	Contaminating DNA fragments in plasmid miniprep. Impure DNA may cause nonspecific priming.

6. Troubleshooting (continued)

Symptoms	Causes and Comments
No growth in overnight ES1301 <i>mutS</i> culture (continued)	DNA derived from a <i>hsdM</i> modification minus strain. ES1301 <i>mutS</i> is restriction (+). DNA should be isolated from a modification (+) K12 strain or it will be restricted by ES1301 <i>mutS</i> . For example, DNA isolated from HB101 or NM522 (modification minus strains) or BL21 (<i>E. coli</i> B strain) cells should not be used. Low competency of ES1301 <i>mutS</i> cells (<10 ⁷ cfu/μg). Check competency with pALTER [®] DNA provided using tetracycline selection.
JM109 antibiotic-resistant colonies, but no mutations	Mutagenic oligonucleotide not complementary to correct strand. Mutagenic oligonucleotide not synthesized to the same strand as the antibiotic repair oligo. Recheck the orientation of the cloned insert. Inadequate annealing of mutagenic oligonucleotide to template DNA. Wrong oligonucleotide:template ratios used in hybridization reaction. Check concentration and purity of mutagenic oligonucleotide by PAGE. Insufficient annealing time can result in poor annealing of mutagenic oligonucleotide to template. Secondary structure in cloned insert or mutagenic oligonucleotide can prevent annealing. Prepare template as single-stranded DNA (Section 8.A). If necessary, redesign mutagenic oligonucleotide. Mutagenic oligonucleotide not incorporated into newly synthesized strand. Make sure that mutagenic oligonucleotide is phosphorylated. 3' end of oligonucleotide not properly base-paired to template. Redesign oligonucleotide to incorporate into newly synthesized DNA strand. Initial selection not performed with <i>mutS</i> strain. <i>mutS</i> phenotype is required for efficient propagation of mutations. Check for kanamycin resistance, which is linked to the <i>mutS</i> mutation in ES1301 strain.

6. Troubleshooting (continued)

Symptoms	Causes and Comments
JM109 antibiotic-resistant colonies, but no mutations (continued)	Problem with cloned insert. The ES1301 strain is <i>recA</i> ⁺ and some constructs may undergo rearrangement. If possible, clone a small region for mutagenesis.
No JM109 colonies using alternative cotransformation protocol	Inefficient cotransformation. Follow back-up procedure (Section 8.F). Isolate plasmid DNA from back-up ES1301 <i>mutS</i> culture and use it to transform JM109 or final host. Used R408 helper phage particles rather than R408 rDNA in cotransformation. Check vial labels carefully. Insufficient phage infection of JM109. Incubate JM109 cells in phage supernatant for a longer period (try 1 hour) to increase the number of transfectants. JM109 cells have lost F' or final host is not F+. Select for F' by maintaining JM109 on minimal media. Assay JM109 or final host for plaque formation by infecting with R408 helper phage. See reference 16.

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8. Appendix A: Additional Protocols

8.A. Preparation of Phagemid Single-Stranded DNA

To produce single-stranded template for the mutagenesis reaction, grow individual colonies containing recombinant phagemids and infect the cultures with helper phage. **The single-stranded DNA produced is complementary to the strand of the multiple cloning site shown in Figures 3 and 4.**

The R408 Helper Phage is provided with this system. Differences in the yields of ssDNA have been observed to be dependent on the particular combination of host, vector and helper phage. In our experience, higher yields are obtained when the R408 Helper Phage is used in conjunction with the JM109 strain.

Materials to Be Supplied by the User

(Solution compositions are provided in Section 9.C.)

- LB broth
- phage precipitation solution
- TE buffer (pH 8.0)
- 0.8% agarose gel
- TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1)
- chloroform:isoamyl alcohol (24:1)
- 7.5M ammonium acetate (pH 7.5)
- ethanol (100% and 70%)
- LB broth containing either 125µg/ml ampicillin or 10µg/ml tetracycline

1. Prepare an overnight culture of JM109 cells containing recombinant phagemid DNA by picking individual antibiotic-resistant colonies from a fresh plate. Inoculate 1-2ml of LB broth containing the appropriate antibiotic (either 125µg/ml ampicillin or 10µg/ml tetracycline) and shake at 37°C.
2. The next morning, inoculate 25ml of LB broth containing the appropriate antibiotic with 0.5ml of the overnight culture. Shake vigorously at 37°C for 30 minutes in a 250ml flask.
3. Infect with R408 Helper Phage at a m.o.i. (multiplicity of infection) of 10-20; i.e., add 10-20 helper phage particles per cell. For the helper phage supplied with this system, add 200µl. Continue shaking for 6 hours with vigorous agitation.

Note: The volume of phage to add to arrive at a m.o.i. of 10-20 can be calculated by assuming that the cell concentrations of the starting cultures range from about 5×10^7 to 1×10^8 cells/ml ($OD_{600} = 0.1-0.3$). To infect at a m.o.i. of 10-20 requires 5×10^8 to 2×10^9 phage/ml or 10-40µl of phage/ml of cells using a 5×10^{10} pfu/ml stock of helper phage. A m.o.i. as high as 100 will not alter the yield of ssDNA.

4. Harvest the culture supernatant by pelleting the cells at $12,000 \times g$ for 15 minutes. Pour the supernatant into a new tube and spin again for 15 minutes.

8.A. Preparation of Phagemid Single-Stranded DNA (continued)

Note: Pelleting the cells twice is important for reducing the level of contaminating cellular nucleic acid in the supernatant. An additional level of purity of ssDNA can be attained by treating the supernatant (after Step 4 but before Step 5) with DNase I (10u/ml) and RNase A (10µg/ml) for 15 minutes at 37°C.

- Precipitate the phage by adding 0.25 volume of phage precipitation solution to the supernatant. Leave on ice for 60 minutes to overnight, then centrifuge for 15 minutes at 12,000 × *g*. Thoroughly drain the supernatant.
- Resuspend the pellet in 400µl of TE buffer (pH 8.0), then transfer the sample to a microcentrifuge tube.
- Add 0.4ml of chloroform:isoamyl alcohol (24:1) to lyse the phage, vortex for 1 full minute, and centrifuge at 12,000 × *g* in a microcentrifuge for 5 minutes. This step removes excess PEG.
- Transfer the upper, aqueous phase (containing phagemid DNA) to a new tube, leaving the interface behind. Add 0.4ml of TE-saturated phenol:chloroform:isoamyl alcohol to the aqueous phase, vortex for 1 full minute, and centrifuge as in Step 7.
- Transfer the upper, aqueous phase to a new tube and repeat the phenol extraction as in Step 8. If necessary, repeat this extraction several times until there is no visible material at the interface.
- Transfer the upper, aqueous phase to a new tube and add 0.4ml of chloroform. Vortex for 1 full minute and centrifuge as in Step 7.
- Transfer the upper, aqueous phase to a new tube and add 0.5 volume (200µl) of 7.5M ammonium acetate plus 2 volumes (1.2ml) of 100% ethanol. Mix and leave at -20°C for 30 minutes to precipitate the phagemid DNA.
- Centrifuge at 12,000 × *g* for 5 minutes, remove the supernatant, and carefully rinse the pellet with ice-cold 70% ethanol. If the pellet is disturbed, centrifuge again for 2 minutes. Drain the tube and dry the pellet under vacuum. The pellet may be difficult to see. A typical yield of ssDNA is 0.5µg per milliliter of culture.
- Resuspend the DNA in 20µl of water. The amount of DNA present can be estimated by agarose gel electrophoresis and ethidium bromide-staining of a 2µl sample.

Two major bands are usually seen on 1% agarose gels run in Tris-acetate buffer: helper phage DNA and single-stranded plasmid DNA. In some preparations, a small amount of chromosomal DNA may be present as well as some RNA resulting from cell lysis. In some cases where the recombinant is the same size as the helper phage, it may be difficult to distinguish between the two. In such cases, it may be advantageous to use an alternative helper phage. A 20µl sample of helper phage stock may be analyzed on the gel to show

where the helper phage DNA runs. Note that it is necessary to include 0.2% SDS in the sample of helper phage to disrupt the coat proteins before electrophoresis. The presence of the helper phage DNA does not interfere with the mutagenesis reaction. Additional tips for preparing ssDNA can be found in our *Protocols and Applications Guide* (17).

8.B. Preparation of ES1301 *mutS* and JM109 Competent Cells: Modified RbCl Method

This rubidium chloride protocol gives better transformation efficiencies than the CaCl₂ procedure for most strains. The procedure is an adaptation of one described in reference 18.

Note: The competency of ES1301 *mutS* cells should be checked with a known quantity of one of the pALTER® Vectors. We have tried several protocols to prepare competent ES1301 *mutS* cells, all of which typically yielded 10⁵-10⁸ cfu/µg of pALTER® DNA. The most consistent transformation results with ES1301 *mutS* have been obtained using electroporation (15; Section 8.C). Also, ES1301 *mutS* cells grow more slowly than other *E. coli* strains, and their colony size varies.

Materials to Be Supplied by the User

(Solution compositions are provided in Section 9.C.)

- LB medium and plates
 - LB + 20mM MgSO₄
 - TFB1, ice-cold
 - TFB2, ice-cold
 - dry ice/isopropanol bath
- Inoculate a single colony from an LB plate into 2.5ml of LB medium in a plating tube. Incubate overnight at 37°C with shaking (approximately 225rpm).
 - Subculture the overnight culture 1:100 by inoculating 2.5ml into 250ml of LB supplemented with 20mM MgSO₄. Grow the cells in a 1L flask until the OD₆₀₀ reaches 0.4-0.6 (usually 5-6 hours, but the time may vary).
 - 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.
 - 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.
 - Incubate the resuspended cells on ice for 5 minutes at 4°C.
 - Pellet the cells by centrifugation at 4,500 × *g* for 5 minutes at 4°C.
 - Gently resuspend the cells in 1/25 original volume of ice-cold TFB2. For a 250ml subculture, use 10ml of TFB2.

8.B. Preparation of ES1301 *mutS* and JM109 Competent Cells: Modified RbCl Method (continued)

- Incubate the cells on ice for 15–60 minutes, then aliquot 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. ES1301 *mutS* competent cells generally are stable for 3–6 months.

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 isopropanol bath in another. Place the top-labeled tubes in the rack with ice, aliquot 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 isopropanol, 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.

8.C. Electroporation Guidelines for ES1301 *mutS* Cells

ES1301 *mutS* cells may be transformed by electroporation as an alternative to the transformation protocols described in Sections 5.D and 5.F. In our experience, the following electroporation conditions consistently give effective transformation of ES1301 *mutS* cells. However, these are provided only as a general guideline in establishing conditions for your electroporation instrument. Perform electroporation according to the guidelines supplied by the instrument manufacturer.

Materials to Be Supplied by the User

(Solution compositions are provided in Section 9.C.)

- LB medium
- 10% glycerol, ice-cold
- dry ice/isopropanol bath

Preparing Electrocompetent Cells

- Inoculate 1 liter of LB with 10ml of an overnight culture of ES1301 *mutS* cells. Grow the cells at 37°C until the OD₆₀₀ reaches 0.5–0.7.
- Chill the culture on ice for 15–30 minutes. Perform all subsequent steps at 4°C.
- Harvest the cells by centrifugation at 4,000 × g for 15 minutes.
- Aspirate the supernatant to remove as much of the media as possible. Resuspend the cells in 1 liter of ice-cold 10% glycerol. Centrifuge as in Step 3.
- Remove the supernatant and resuspend the cells in 20ml of ice-cold 10% glycerol. Centrifuge as in Step 3.

- Resuspend the cells in a final volume of 2–3ml of ice-cold 10% glycerol. Freeze the cells in 100µl aliquots in a dry ice/ethanol bath and store at -70°C.

Electroporation Conditions

- Remove electrocompetent ES1301 *mutS* cells from -70°C storage and place on ice for 5 minutes or until just thawed. Transfer 50µl of cells into the electroporator cuvette.
- Add no more than 1µl of the ligation reaction products (from Section 5.C) to the 50µl of electrocompetent cells. Pre-incubate the cells with the DNA for 1 minute before electroporation.
- Electroporate the cells using conditions appropriate for your instrument. **The following conditions have been effective in our laboratory and are supplied only as a guideline.**

Instrument:	Bio-Rad Gene Pulser®
Cuvette Gap:	0.2cm
Voltage:	2.5kV
Capacitance:	25µF
Resistance:	200 ohms
Time Constant:	4.5–5.0msec

- After electroporation, resuspend the cells in 1ml LB medium and allow them to recover at 37°C with shaking (approximately 225rpm) as described in Section 5.D, Step 8, or Section 5.F, Step 9, before adding a selective antibiotic.

8.D. 5' Phosphorylation of Oligonucleotides

Materials to Be Supplied by the User

(Solution compositions are provided in Section 9.C.)

- oligonucleotide to be phosphorylated
- T4 Polynucleotide Kinase (Cat.# M4101)
- Kinase 10X buffer (supplied with T4 Polynucleotide Kinase, Cat.# M4101)
- ATP, 10mM

1. Add the following components to a microcentrifuge tube. Use the formula* below to calculate the nanograms of nucleotide equivalent to 100pmol (also see Table 2, Section 5.A).

oligonucleotide	100pmol
Kinase 10X buffer	2.5µl
T4 Polynucleotide Kinase	5.0u
ATP	2.5µl
sterile, deionized water to a final volume of	25.0µl

*In general: $\text{ng of oligonucleotide} = \text{pmol of oligonucleotide} \times 0.33 \times N$, where N = length of oligonucleotide in bases.

2. Incubate the reaction at 37°C for 30 minutes.
3. Incubate the reaction at 70°C for 10 minutes to inactivate the kinase.
4. The reaction products can be stored at -20°C or added directly to the annealing reaction (Section 5.C).

8.E. pALTER® Plasmid Miniprep Procedure

Purify pALTER®-MAX plasmid DNA using the protocol provided below. Alternate purification methods, such as the Wizard® Plus SV Minipreps DNA Purification System (Cat.# A1340) or the Wizard® Plus Minipreps DNA Purification System (Cat.# A7100), also may be used for this step.

Materials to Be Supplied by the User

(Solution compositions are provided in Section 9.C.)

- miniprep resuspension buffer
- 0.2N NaOH, 1% SDS (prepare fresh for each use)
- 3M potassium acetate solution (pH 4.8)
- TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1)
- chloroform:isoamyl alcohol (24:1)
- ethanol (100% and 70%)
- DNase-free RNase A
- JM109 competent cells (see Section 8.B and 8.C)
- DMSO, frozen in aliquots
- ampicillin or tetracycline
- LB medium
- LB plates containing either 125µg/ml ampicillin or 10µg/ml tetracycline

1. Place 1.5ml of the overnight culture from Section 5.D, Step 9, into a microcentrifuge tube and centrifuge at 12,000 × g for 1 minute. The remainder of the overnight culture can be stored at 4°C.
2. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.
3. Resuspend the pellet by vortexing in 100µl of ice-cold miniprep resuspension buffer.
4. Incubate for 5 minutes at room temperature.
5. Add 200µl of a freshly prepared solution containing 0.2N NaOH, 1% SDS to lyse the cells. Mix by inversion. **Do not vortex.** Incubate for 5 minutes on ice.
6. Add 150µl of ice-cold 3M potassium acetate solution (pH 4.8) to neutralize the lysate. Mix by inversion or gentle vortexing for 10 seconds. Incubate for 5 minutes on ice.
7. Centrifuge at 12,000 × g for 5 minutes.
8. Transfer the supernatant to a new tube, avoiding the white precipitate.
9. Add 1 volume of TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1). Vortex for 1 minute and centrifuge at 12,000 × g for 5 minutes.
10. Transfer the upper, aqueous phase to a new tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and centrifuge as in Step 9.
11. Transfer the upper, aqueous phase to a new tube and add 2.5 volumes of 100% ethanol. Mix and allow to precipitate 5 minutes on dry ice.
12. Centrifuge at 12,000 × g for 5 minutes. Rinse the pellet with 70% ethanol (prechilled) and dry the pellet under vacuum.
13. Dissolve the pellet in 50µl of sterile deionized water. Add 0.5µl of 100µg/µl DNase-free RNase A and incubate for 5 minutes at room temperature.
14. The yield of plasmid DNA can be determined by electrophoresis on an agarose gel. A yield of 0.2–3µg of plasmid DNA may be expected, depending on the plasmid copy number.

8.F. Alternative Protocol: Cotransformation into ES1301 *mutS* and Transfer to JM109 Strain

This procedure may be used as an alternative to the standard, two-step transformation of ES1301 *mutS* and JM109 cells (Sections 5.D-5.F) when it is important to save time or to minimize the chances of sequence rearrangements. ES1301 *mutS* is *recA+*, and as a result, inserts containing highly repetitive sequences may be unstable. **Use this procedure only if you have highly competent ES1301 *mutS* cells (>10⁷cfu/μg DNA).**

Before starting this procedure, have ready competent ES1301 *mutS* cells, prepared as described in Section 8.B or 8.C, and a 1–3ml overnight culture of JM109 cells.

1. Prechill sterile 17 × 100mm polypropylene culture tubes on ice, one for each annealing reaction.

Note: Use of standard microcentrifuge tubes, rather than the 17 × 100mm polypropylene tubes recommended, reduces the transformation efficiency by approximately 50% due to inefficient heat-shock treatment.

2. Remove competent ES1301 *mutS* cells from –70°C storage and thaw on ice (approximately 5 minutes).
3. Gently mix the cells by flicking the tube and then transfer 100μl of the thawed ES1301 *mutS* cells to each of the prechilled culture tubes.
4. Add 15μl of the synthesis reaction from Section 5.C, Step 4, and 100ng (1μl) of R408 Helper Phage DNA to each of the prechilled tubes. Move the pipette tip through the cells while dispensing. Quickly flick the tube several times.
5. Immediately place the tubes on 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 4ml of LB medium **without antibiotic** and incubate at 37°C for 3 hours with shaking (approximately 225rpm) to allow the cells to recover and produce infectious phagemid.
9.
 - a. Transfer 3ml of the transformed ES1301 *mutS* cells to 2 tubes and pellet the cells by centrifugation at top speed in a microcentrifuge for 3 minutes. Remove the supernatants and combine them. Add 100μl of an overnight culture of JM109 cells to the combined supernatants.
 - b. To the 1ml of unpelleted transformed ES1301 *mutS* cells, add 4ml of LB medium containing the appropriate antibiotic (either 125μg/ml ampicillin or 10μg/ml tetracycline) and incubate overnight at 37°C with shaking. This culture serves as a backup to be used if the cotransformation procedure yields too few colonies.

Note: Cell growth in liquid culture is inhibited by tetracycline concentrations greater than 10μg/ml.

10. Incubate the 3.1ml mixture of JM109 cells and phagemid from Step 9a for 30 minutes at 37°C with shaking and plate 100μl on each of 4–5 plates containing the appropriate selective medium (LB + 125μg/ml ampicillin or 12.5μg/ml tetracycline). A typical cotransformation experiment should yield approximately 50 colonies per plate.

To obtain more colonies, plate the entire 3ml JM109 culture. Pellet the cells by centrifugation at top speed for 1 minute in a microcentrifuge. Resuspend the cells in 500μl of LB and plate 100μl on each of 5 plates.

Notes:

1. The total number of colonies obtained from the cotransformation procedure is dependent on the competency of the ES1301 *mutS* cells; at least 10⁷cfu/μg DNA is required for efficient cotransformation. If insufficient colonies are obtained from 4–5 plates after cotransformation, perform a plasmid miniprep from the backup culture prepared in Step 9b and use this plasmid to transform competent JM109 cells as described in Section 5.F. The competency of ES1301 *mutS* cells should be checked using a known quantity of one of the pALTER® Vectors.
2. Removal of the ES1301 *mutS* cells is essential to ensure strain transfer and clonal selection. To confirm that mutant phagemids have been transferred to JM109, colonies from the selective plates can be grown on media containing nalidixic acid (10μg/ml). The *gyrA* chromosomal allele present in JM109 but not in ES1301 *mutS* confers resistance to nalidixic acid. Sensitivity to kanamycin also is a confirmation that the final host is JM109 rather than ES1301 *mutS*.
3. To measure the percent of mutants obtained in the control reactions, pick several Amp^r colonies and plate them in a grid format on paired plates containing either ampicillin or tetracycline (see Section 9.D for a grid template). Pick each colony with a sterile toothpick and inoculate the two plates in sequence.

Example:

$$[(\text{Amp}^r \text{ colonies} - \text{Tet}^r \text{ colonies}) \div \text{Amp}^r \text{ colonies}] \times 100 = \% \text{ mutants}$$

9. Appendix B: Reference Information

9.A. Sequences of the Repair and Knockout Oligonucleotides

These repair and knockout oligonucleotides are complementary to ssDNA produced by the pALTER® Vectors.

Description and Size	Sequence
Ampicillin Repair Oligonucleotide, Phosphorylated (27mer)	5'-d(pGTTGCCATTGCTGCAGGCATCGTGGTG)-3'
Ampicillin Knockout Oligonucleotide, Phosphorylated (27mer)	5'-d(pGTTGCCATTGCGGCATCGTGGTGTCAC)-3'
Tetracycline Repair Oligonucleotide, Phosphorylated (27mer)	5'-d(pGCCGGGCCTTTCGCGGATATCGTCCA)-3'
Tetracycline Knockout Oligonucleotide, Phosphorylated (27mer)	5'-d(pGCCGGGCCTTTCGCGGCGTCCATTC)-3'

9.B. Descriptions of Bacterial Strains

JM109

endA1, recA1, gyrA96, thi, hsdR17 (r_k^- , m_k^+), *relA1, supE44, λ*, Δ (*lac-proAB*), [*F'*, *traD36, proAB, lacI Δ ZAM15*]

JM109 (18) is a useful host in which to clone pALTER® and pGEM® Vectors and for production of single-stranded DNA from M13 or phagemid vectors. The strain grows well and is efficiently transformed by a variety of methods. Because JM109 is *recA*⁻ and lacks the *E. coli* K restriction system, undesirable restriction of cloned DNA and recombination with host chromosomal DNA are prevented. The *endA1* mutation leads to an improved yield and quality of isolated plasmid DNA. JM109 high-efficiency competent cells are available from Promega (Section 10).

JM109 always should be maintained on minimal plates (M-9) supplemented with 1mM thiamine-HCl. This selects for the presence of the *F'*, which carries a nutritional requirement for growth (proline biosynthesis). Maintenance of the *F'* is important for α -complementation, ssDNA yields and efficient strain transfer using the cotransformation procedure.

Blue/White Color Screening

JM109 can be used for blue/white color screening of the pALTER®-1 Vector. This strain is deficient in β -galactosidase activity due to deletions in both genomic and episomal copies of the *lacZ* gene. The deletion in the episomal (*F* factor) copy of the *lacZ* gene (*lacI Δ ZAM15*) is located in the α -peptide region and, as a result, β -galactosidase activity can be complemented by addition of a functional α -peptide. The pALTER®-1 Vector encodes the *lacZ* α -peptide, and cells carrying this plasmid are able to produce functional β -galactosidase.

When plated on indicator media containing X-Gal and IPTG, the host/plasmid combination will generate blue colonies. However, when the α -peptide is disrupted by cloning into the multiple cloning region of the pALTER®-1 Vector, complementation does not occur and no β -galactosidase activity is produced. Therefore, bacterial colonies harboring recombinant vector constructs remain white.

ES1301 *mutS*

lacZ53, mutS201::Tn5, thyA36, rha-5, metB1, deoC, IN(rmD-rrmE)

ES1301 (9) *mutS* is a mismatch repair minus strain of *E. coli*. Use of a *mutS* strain prevents repair of the newly synthesized unmethylated strand (4,10), leading to high mutation efficiencies. ES1301 *mutS* is *recA*⁺, and as a result, inserts containing highly repetitive sequences may be unstable. ES1301 *mutS* is kanamycin-resistant, due to the presence of Tn5, and tetracycline-sensitive, which is necessary for the selection (Tet repair) at the second round of mutagenesis. ES1301 *mutS* is also restriction (+). Template DNA should be isolated from a modification (+) K12 strain or it will be restricted by ES1301 *mutS*. For example, DNA isolated from HB101 or NM522 (modification minus strains) or BL21 (*E. coli* B strain) cells should not be used.

9.C. Composition of Buffers and Solutions

2M ammonium acetate (pH 4.6)

15.4g ammonium acetate
Dissolve in 50ml deionized water, bring to pH 4.6 with glacial acetic acid and bring final volume to 100ml with deionized water.

7.5M ammonium acetate (pH 7.5)

57.81g ammonium acetate
Dissolve in 50ml deionized water, bring to pH 7.5 with NaOH and bring final volume to 100ml with deionized water.

Annealing 10X Buffer

200mM Tris-HCl (pH 7.5)
100mM MgCl₂
500mM NaCl

antibiotic stock solutions

Ampicillin 125mg/ml in water
Tetracycline 12.5mg/ml in 80% ethanol

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

kinase 10X buffer

700mM Tris-HCl (pH 7.6)
100mM MgCl₂
50mM DTT

LB (Luria-Bertani) medium (1 liter)

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

Adjust pH to 7.5 with NaOH and autoclave.

9.C. Composition of Buffers and Solutions (continued)

LB plates plus antibiotic (1 liter)

Add 15g agar to 1 liter of LB medium. Adjust to pH 7.0 with NaOH. Autoclave. Allow the medium to cool to 55°C before adding antibiotic (either ampicillin, 125µg/ml final concentration or tetracycline, 12.5µg/ml final concentration). Pour 30–35ml of medium into 85mm petri dishes. If necessary, flame the surface of the medium with a Bunsen burner to eliminate bubbles. Let the agar harden. Store at 4°C for up to 1 month. Tetracycline is light sensitive, so cover plates with foil.

M-9 plates (1 liter)

15g agarose
Add 15 g agarose to 750ml water and autoclave. Cool to 50°C. Add:
2ml 1M MgSO₄
0.1ml 1M CaCl₂
10ml 20% glucose
1ml 1M thiamine-HCl
200ml 5X M-9 salts

5X M-9 salts (1 liter)

34g Na₂HPO₄
15g KH₂PO₄
2.5g NaCl
5g NH₄Cl

Dissolve in deionized water. Divide into 200ml aliquots and autoclave.

2M Mg²⁺ stock

20.33g MgCl₂ • 6H₂O
24.65g MgSO₄ • 7H₂O

Add distilled water to 100ml. Filter-sterilize.

miniprep resuspension buffer

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

2M NaOH 2mM EDTA

(prepare fresh for each use)

2ml 10M NaOH
40µl 500mM EDTA (pH 8.0)
7.96ml deionized water

phage precipitation solution

3.75M ammonium acetate (pH 7.5)
20% polyethylene glycol (MW 8,000)

Add equal volumes of 40% PEG-8000 stock solution and 7.5M ammonium acetate (pH 7.5).

3M potassium acetate

29.6g potassium acetate
11.5ml glacial acetic acid

Dissolve the potassium acetate in 50ml of distilled water. Add the glacial acetic acid. Add distilled water a final volume of 100ml. The resulting solution is 3M with respect to potassium and 5M with respect to acetate.

SOC medium (100ml)

2.0g Bacto[®]-tryptone
0.5g Bacto[®]-yeast extract
1ml 1M NaCl
0.25ml 1M KCl
1ml 2M Mg²⁺ stock, filter-sterilized (prepared as described above)
1ml 2M glucose, filter-sterilized

Add Bacto[®]-tryptone, Bacto[®]-yeast extract, NaCl and KCl to 97ml deionized water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg²⁺ stock and 2M glucose stock, each to a final concentration 20mM. Filter the complete medium through a 0.2µm filter unit. The pH should be 7.0.

9.C. Composition of Buffers and Solutions (continued)

Synthesis 10X Buffer

100mM Tris-HCl (pH 7.5)
5mM dNTPs
10mM ATP
20mM DTT

TE buffer

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

TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1)

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

TFB1

30mM potassium acetate
10mM CaCl₂
50mM MnCl₂
100mM RbCl
15% glycerol

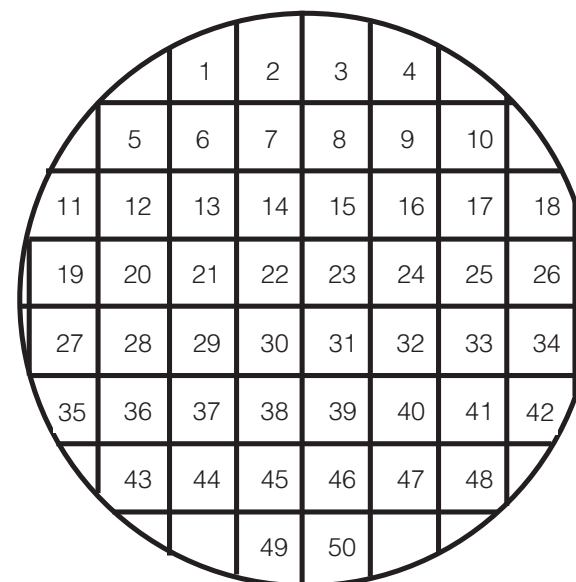
Adjust pH to 5.8 with 1M acetic acid. Filter-sterilize (0.45µM) and store at room temperature.

TFB2

100mM MOPS or PIPES (pH 6.5)
75mM CaCl₂
10mM RbCl
15% glycerol

Adjust the pH to 6.5 with 1M KOH. Filter-sterilize (0.45µM) and store at room temperature.

9.D. Template Grid for Plating Mutant Colonies



9.E. Vector Restriction Sites

For screening purposes, the Tetracycline Knockout Oligonucleotide removes the *EcoR V* site in the tetracycline gene and the Ampicillin Repair Oligonucleotide restores a *Pst I* site in the ampicillin gene.

pALTER®-1 Vector Restriction Sites

The following restriction enzyme tables were constructed using DNASTAR® software. Please note that we have not verified this information by restriction digestion with each enzyme listed. The location given specifies the 3' end of the cut DNA (the base to the left of the cut site). For more information on the cut sites of these enzymes or if you identify a discrepancy, please contact your local Promega Branch or Distributor. In the U.S., contact Promega Technical Services at 800-356-9526. Vector sequences are also available in the GenBank® database (GenBank®/EMBL Accession Number X65334) and on the Internet at: www.promega.com/vectors/

Table 4. Restriction Enzymes That Cut the pALTER®-1 Vector Between 1 and 5 Times.

Enzyme	# of Sites	Location	Enzyme	# of Sites	Location
<i>Aat II</i>	1	4739	<i>BsaM I</i>	1	1814
<i>Acc I</i>	2	39, 2700	<i>Bsm I</i>	1	1814
<i>Acc III</i>	1	2119	<i>BspH I</i>	4	944, 3648, 4652, 4757
<i>Acc65 I</i>	1	17	<i>BspM I</i>	2	51, 1509
<i>AccB7 I</i>	2	1776, 1825	<i>BsrBRI</i>	1	2127
<i>Afl III</i>	1	2929	<i>BssS I</i>	3	3101, 4481, 4788
<i>Alw26 I</i>	5	2571, 3882, 4654, 4807, 4849	<i>Bst1107 I</i>	1	2701
<i>Alw44 I</i>	4	2744, 3242, 4484, 4981	<i>BstZ I</i>	1	1394
<i>AlwN I</i>	1	3344	<i>Cla I</i>	1	479
<i>Ava I</i>	2	21, 1880	<i>Dra I</i>	3	3687, 3706, 4394
<i>Bal I</i>	1	1901	<i>Dra II</i>	4	979, 1894, 1936, 4793
<i>BamH I</i>	1	26	<i>Dra III</i>	1	5265
<i>Ban II</i>	4	15, 930, 944, 5340	<i>Drd I</i>	4	2623, 3036, 4901, 5220
<i>Bbe I</i>	4	872, 893, 1007, 1664	<i>Dsa I</i>	2	983, 1902
<i>Bbs I</i>	2	1185, 2048	<i>Eag I</i>	1	1394
<i>Bbu I</i>	1	54	<i>Ear I</i>	4	329, 2812, 4612, 5554
<i>Bgl I</i>	5	1390, 1624, 3941, 4061, 5509	<i>EcLHK I</i>	1	3821
<i>Bsa I</i>	1	3882	<i>Eco47 III</i>	4	689, 951, 1232, 2184
<i>BsaA I</i>	2	2682, 5265	<i>Eco52 I</i>	1	1394
<i>BsaB I</i>	1	2127			

Note: The enzymes listed in boldface type are available from Promega.

Table 4. Restriction Enzymes That Cut the pALTER®-1 Vector Between 1 and 5 Times (continued).

Enzyme	# of Sites	Location	Enzyme	# of Sites	Location
<i>EcoICR I</i>	1	13	<i>Psp5 II</i>	2	1894, 1936
<i>EcoN I</i>	1	1081	<i>PspA I</i>	1	21
<i>EcoR I</i>	1	5	<i>Pst I</i>	1	48
<i>EcoR V</i>	1	642	<i>Pvu I</i>	2	4187, 5537
<i>Ehe I</i>	4	870, 891, 1005, 1662	<i>Pvu II</i>	3	269, 2521, 5566
<i>Fsp I</i>	5	717, 1813, 1911, 4043, 5516	<i>Rsa I</i>	5	19, 620, 2736, 4297, 4973
<i>Hinc II</i>	2	40, 4358	<i>Sac I</i>	1	15
<i>Hind II</i>	2	40, 4358	<i>Sal I</i>	1	38
<i>Hind III</i>	1	56	<i>Sca I</i>	1	4297
<i>Kas I</i>	4	868, 889, 1003, 1660	<i>SgrA I</i>	1	865
<i>Kpn I</i>	1	21	<i>Sma I</i>	1	23
<i>Nae I</i>	5	858, 1226, 1386, 1740, 5368	<i>Sph I</i>	1	54
<i>Nar I</i>	4	869, 890, 1004, 1661	<i>Sse8387 I</i>	1	48
<i>Nde I</i>	2	2751, 4988	<i>Ssp I</i>	2	4621, 5057
<i>NgoM IV</i>	5	856, 1224, 1384, 1738, 5366	<i>Sty I</i>	1	1824
<i>Nhe I</i>	1	684	<i>Tth111 I</i>	1	2675
<i>Nru I</i>	1	1429	<i>Vsp I</i>	4	216, 275, 452, 3993
<i>PflM I</i>	2	1776, 1825	<i>Xba I</i>	1	32
<i>PshA I</i>	1	1171	<i>Xma I</i>	1	21
			<i>Xmn I</i>	3	453, 2488, 4416

Table 5. Restriction Enzymes That Do Not Cut the pALTER®-1 Vector.

<i>Afl II</i>	<i>Bsp120 I</i>	<i>Eco81 I</i>	<i>PinA I</i>	<i>Spl I</i>
<i>Age I</i>	<i>BsrG I</i>	<i>Fse I</i>	<i>Pme I</i>	<i>Srf I</i>
<i>Apa I</i>	<i>BssH II</i>	<i>Hpa I</i>	<i>Pml I</i>	<i>Stu I</i>
<i>Asc I</i>	<i>Bst98 I</i>	<i>I-Ppo I</i>	<i>Ppu10 I</i>	<i>Swa I</i>
<i>Avr II</i>	<i>BstE II</i>	<i>Mlu I</i>	<i>Rsr II</i>	<i>Xcm I</i>
<i>BbrP I</i>	<i>BstX I</i>	<i>Nco I</i>	<i>Sac II</i>	<i>Xho I</i>
<i>Bcl I</i>	<i>Bsu36 I</i>	<i>Not I</i>	<i>Sfi I</i>	
<i>Bgl II</i>	<i>Csp I</i>	<i>Nsi I</i>	<i>Sgf I</i>	
<i>Blp I</i>	<i>Csp45 I</i>	<i>Pac I</i>	<i>SnaB I</i>	
<i>Bpu1102 I</i>	<i>Eco72 I</i>	<i>PaeR7 I</i>	<i>Spe I</i>	

Note: The enzymes listed in boldface type are available from Promega.

Table 6. Restriction Enzymes That Cut the pALTER®-1 Vector 6 or More Times.

<i>Aci</i> I	<i>Bsr</i> S I	<i>Hae</i> II	<i>Mbo</i> I	<i>Sau</i> 3A I
<i>Acy</i> I	<i>Bst</i> 71 I	<i>Hae</i> III	<i>Mbo</i> II	<i>Sau</i> 96 I
<i>Alu</i> I	<i>Bst</i>O I	<i>Hga</i> I	<i>Mnl</i> I	<i>Scr</i> F I
<i>Asp</i> H I	<i>Bst</i> U I	<i>Hha</i> I	<i>Mse</i> I	<i>Sfa</i> N I
<i>Ava</i> II	<i>Cfo</i> I	<i>Hinf</i> I	<i>Msp</i> I	<i>Sin</i> I
<i>Ban</i> I	<i>Cfr</i> 10 I	<i>Hpa</i> II	<i>Msp</i>A1 I	<i>Taq</i> I
<i>Bbv</i> I	<i>Dde</i> I	<i>Hph</i> I	<i>Nci</i> I	<i>Tfi</i> I
<i>Bsa</i> H I	<i>Dpn</i> I	<i>Hsp</i>92 I	<i>Nde</i> II	<i>Tru</i>9 I
<i>Bsa</i> J I	<i>Dpn</i> II	<i>Hsp</i>92 II	<i>Nla</i> III	<i>Xho</i> II
<i>Bsa</i> O I	<i>Eae</i> I	<i>Mae</i> I	<i>Nla</i> IV	
<i>Bsp</i>1286 I	<i>Fnu</i> 4H I	<i>Mae</i> II	<i>Nsp</i> I	
<i>Bsr</i> I	<i>Fok</i> I	<i>Mae</i> III	<i>Ple</i> I	

Note: The enzymes listed in boldface type are available from Promega.

pALTER®-Ex1 Vector Restriction Sites

The following restriction enzyme tables were constructed using DNASTAR® sequence analysis software. Please note that we have not verified this information by restriction digestion with each enzyme listed. The location given specifies the 3' end of the cut DNA (the base to the left of the cut site). For more information on the cut sites of these enzymes or if you identify a discrepancy, please contact your local Promega Branch or Distributor. In the U.S., contact Promega Technical Services at 800-356-9526. Vector sequences are also available in the GenBank® database (GenBank®/EMBL Accession Number **U47102**) and on the Internet at: www.promega.com/vectors/

Table 7. Restriction Enzymes That Cut the pALTER®-Ex1 Vector Between 1 and 5 Times.

Enzyme	# of Sites	Location	Enzyme	# of Sites	Location
<i>Aat</i> II	1	4917	<i>Bss</i> S I	3	3279, 4659, 4966
<i>Acc</i> I	1	2878	<i>Bst</i> 1107 I	1	2879
<i>Acc</i> III	1	2297	<i>Bst</i>Z I	2	58, 1572
<i>Acc</i> B7 I	2	1954, 2003	<i>Dra</i> I	3	3865, 3884, 4572
<i>Afl</i> III	1	3106	<i>Dra</i> II	5	602, 1157, 2072, 2114, 4971
<i>Alw</i>26 I	5	2749, 4060, 4832, 4985, 5027	<i>Dra</i> III	1	5443
<i>Alw</i>44 I	4	2922, 3420, 4662, 5159	<i>Drd</i> I	4	2801, 3214, 5079, 5398
<i>Alw</i> N I	1	3522	<i>Dsa</i> I	3	35, 1161, 2080
<i>Ava</i> I	1	2058	<i>Eag</i> I	2	58, 1572
<i>Bal</i> I	1	20759	<i>Ear</i> I	4	437, 2990, 4790, 5732
<i>Bam</i>H I	1	73	<i>Ecl</i>HK I	1	3999
<i>Ban</i> II	3	1108, 1122, 5518	<i>Eco</i>47 III	4	867, 1129, 1410, 2362
<i>Bbe</i> I	4	1050, 1071, 1185, 1842	<i>Eco</i> 52 I	2	58, 1572
<i>Bbs</i> I	2	1363, 2226	<i>Eco</i> N I	1	1259
<i>Bbu</i> I	1	67	<i>Eco</i>R I	1	5
<i>Bgl</i> I	5	1568, 1802, 4119, 4239, 5687	<i>Eco</i>R V	1	820
<i>Bsa</i> I	1	4060	<i>Ehe</i> I	4	1048, 1069, 1183, 1840
<i>Bsa</i> A I	2	2860, 5443	<i>Fsp</i> I	5	895, 1991, 2089, 4221, 5694
<i>Bsa</i> B I	1	2305	<i>Hinc</i> II	3	13, 145, 4536
<i>Bsa</i>M I	1	1992	<i>Hind</i> II	3	13, 145, 4536
<i>Bsm</i> I	1	1992	<i>Hind</i> III	1	164
<i>Bsp</i> H I	4	1122, 3826, 4830, 4935	<i>Hpa</i> I	1	13
<i>Bsp</i> M I	1	1687			
<i>Bsr</i> BR I	1	2305			

Note: The enzymes listed in boldface type are available from Promega.

Table 7. Restriction Enzymes That Cut the pALTER®-Ex1 Vector Between 1 and 5 Times (continued).

Enzyme	# of Sites	Location	Enzyme	# of Sites	Location
<i>Kas I</i>	4	1046, 1067, 1181, 1838	<i>Psp5 II</i>	2	2072, 2114
<i>Nae I</i>	5	1036, 1404, 1564, 1918, 5546	<i>Pst I</i>	1	56
<i>Nar I</i>	4	1047, 1068, 1182, 1839	<i>Pvu I</i>	2	4365, 5715
<i>Nco I</i>	1	35	<i>Pvu II</i>	3	377, 2699, 5744
<i>Nde I</i>	1	69	<i>Rsa I</i>	4	798, 2914, 4475, 5151
<i>NgoM IV</i>	5	1034, 1402, 1562, 1916, 5544	<i>Sca I</i>	1	4475
<i>Nhe I</i>	1	862	<i>SgrA I</i>	1	1043
<i>Not I</i>	1	58	<i>Sph I</i>	1	67
<i>Nru I</i>	1	1607	<i>Ssp I</i>	2	4799, 5235
<i>Nsi I</i>	1	69	<i>Stu I</i>	1	43
<i>PflM I</i>	2	1954, 2003	<i>Sty I</i>	3	35, 597, 2002
<i>Ppu10 I</i>	1	65	<i>Tth111 I</i>	1	2853
<i>PpuM I</i>	2	2072, 2114	<i>Vsp I</i>	5	137, 324, 383, 560, 4171
<i>PshA I</i>	1	1349	<i>Xba I</i>	1	46
			<i>Xmn I</i>	3	56

Table 8. Restriction Enzymes That Do Not Cut the pALTER®-Ex1 Vector.

<i>Acc65 I</i>	<i>Bpu1102 I</i>	<i>Csp45 I</i>	<i>PinA I</i>	<i>Sma I</i>
<i>Afl II</i>	<i>Bsp120 I</i>	<i>Eco72 I</i>	<i>Pme I</i>	<i>SnaB I</i>
<i>Age I</i>	<i>BsrG I</i>	<i>Eco81 I</i>	<i>Pml I</i>	<i>Spe I</i>
<i>Apa I</i>	BssH II	EcoICR I	<i>PspA I</i>	<i>Spl I</i>
<i>Asc I</i>	Bst98 I	<i>Fse I</i>	<i>Rsr II</i>	<i>Srf I</i>
<i>Avr II</i>	BstE II	I-Ppo I	<i>Sac I</i>	<i>Sse8387 I</i>
<i>BbrP I</i>	BstX I	Kpn I	<i>Sac II</i>	<i>Swa I</i>
<i>Bcl I</i>	Bsu36 I	Mlu I	<i>Sal I</i>	<i>Xcm I</i>
Bgl II	<i>Cla I</i>	<i>Pac I</i>	Sfi I	Xho I
<i>Blp I</i>	Csp I	<i>PaeR7 I</i>	Sgf I	Xma I

Note: The enzymes listed in boldface type are available from Promega.

Table 9. Restriction Enzymes That Cut the pALTER®-Ex1 Vector 6 or More Times.

<i>Aci I</i>	<i>BsrS I</i>	Hae II	Mbo I	Sau3A I
<i>Acy I</i>	<i>Bst71 I</i>	Hae III	Mbo II	<i>Sau96 I</i>
Alu I	BstO I	<i>Hga I</i>	<i>Mnl I</i>	<i>ScrF I</i>
<i>AspH I</i>	<i>BstU I</i>	Hha I	<i>Mse I</i>	<i>SfaN I</i>
Ava II	Cfo I	Hinf I	Msp I	Sin I
Ban I	<i>Cfr10 I</i>	Hpa II	MspA1 I	Taq I
<i>Bbv I</i>	Dde I	<i>Hph I</i>	Nci I	<i>Tfi I</i>
<i>BsaH I</i>	Dpn I	Hsp92 I	Nde II	Tru9 I
<i>BsaJ I</i>	<i>Dpn II</i>	Hsp92 II	<i>Nla III</i>	Xho II
<i>BsaO I</i>	<i>Eae I</i>	<i>Mae I</i>	<i>Nla IV</i>	
Bsp1286 I	<i>Fnu4H I</i>	<i>Mae II</i>	<i>Nsp I</i>	
<i>Bsr I</i>	Fok I	<i>Mae III</i>	<i>Ple I</i>	

Note: The enzymes listed in boldface type are available from Promega.

10. Appendix C: Related Products

Components of the Altered Sites® Systems Available Separately

Product	Size	Cat.#
Ampicillin Repair Oligonucleotide	30µl	Q6311
T4 DNA Polymerase*	100u	M4211
T4 DNA Ligase*	100u (Weiss)	M1801
T4 DNA Ligase, High Concentration*	500u (Weiss)	M1794
ES1301 <i>mutS</i> Bacterial Strain, Glycerol Stock	200µl	Q6131
JM109 Bacterial Strain, Glycerol Stock	500µl	P9751
R408 Helper Phage	5ml	P2291
R408 Helper Phage DNA	10µg	P2341

*For Laboratory Use.

Other Related Products

Product	Size	Cat.#
Altered Sites® II Mammalian Mutagenesis System	1 system	Q5590
T4 Polynucleotide Kinase*	100u	M4101
JM109 Competent Cells, >10 ⁸ cfu/µg*	1ml (5 × 200µl)	L2001
JM109 Competent Cells, >10 ⁷ cfu/µg	1ml (5 × 200µl)	L1001
BL21(DE3)pLys Bacterial Strain, Glycerol Stock	500µl	P9811
<i>E. coli</i> S30 Extract System for Circular DNA	30 × 50µl reactions	L1020
<i>E. coli</i> S30 Extract System for Linear DNA	30 × 50µl reactions	L1030
<i>E. coli</i> T7 S30 Extract System for Circular DNA	30 × 50µl reactions	L1130
Wizard® Plus SV Minipreps DNA Purification System + Vacuum Adapters*	50 preps	A1340
	250 preps	A1470
Wizard® Plus Minipreps DNA Purification System*	50 preps	A7100
	100 preps	A7500
	250 preps	A7510

*For Laboratory Use.

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