Technical Manual

Altered Sites® II Mammalian Mutagenesis System

INSTRUCTIONS FOR USE OF PRODUCT Q5590.

PRINTED IN USA.
Revised 4/05

AF9TM041 0405TM041

Part# TM041

Altered Sites® II Mammalian Mutagenesis System

All technical literature is available on the Internet at www.promega.com/tbs Please visit the web site to verify that you are using the most current version of this Technical Manual. Please contact Promega Technical Services if you have questions on use of this system. E-mail techserv@promega.com.

I. Description

The Altered Sites® II Mammalian Mutagenesis System[(a,b,c)] provides a high-efficiency procedure for the generation and selection of oligonucleotide-directed mutants. The system provides reagents to mutagenize double-stranded (ds) or single-stranded DNA (ssDNA) templates and to perform sequential rounds of mutagenesis without subcloning into another vector. Expression of sequences in mammalian cells is also possible without further subcloning.

The system uses antibiotic selection to obtain a high frequency of mutants (Figure 1). The pALTER®-MAX Vector[(b,c,d)] (Section III.A) contains the genes for ampicillin and chloramphenicol resistance, but the ampicillin resistance gene has been inactivated. In the mutagenesis reaction, the Ampicillin Repair Oligonucleotide and the specific mutagenesis oligonucleotide anneal to the same strand of the DNA template. Subsequent synthesis and ligation of the mutant strand links the two regions, resulting in restoration of ampicillin resistance and introduction of the desired mutation. The Chloramphenicol Knockout Oligonucleotide can also be included in the initial reaction to inactivate the chloramphenicol acetyltransferase gene, thus allowing a second round of mutagenesis and antibiotic selection without the need to subclone.

Mutagenesis protocols are provided for both dsDNA and ssDNA. Initially, the repair minus strain of *E. coli* (ES1301 *mut*S) is transformed to avoid selection against the desired mutation. A subsequent transfer into strain JM109 ensures proper segregation of mutant and wildtype plasmids and results in a high proportion of mutant to wildtype clones.

II. Product Components

Product	Size	Cat.#
Altered Sites® II Mammalian Mutagenesis System	1 system	Q5590

Each system contains sufficient reagents to perform 25 mutagenesis reactions and is provided with glycerol stocks of bacterial strains ES1301 *mut*S and JM109. **The ES1301 *mut*S and JM109 cells are not competent.** Includes:

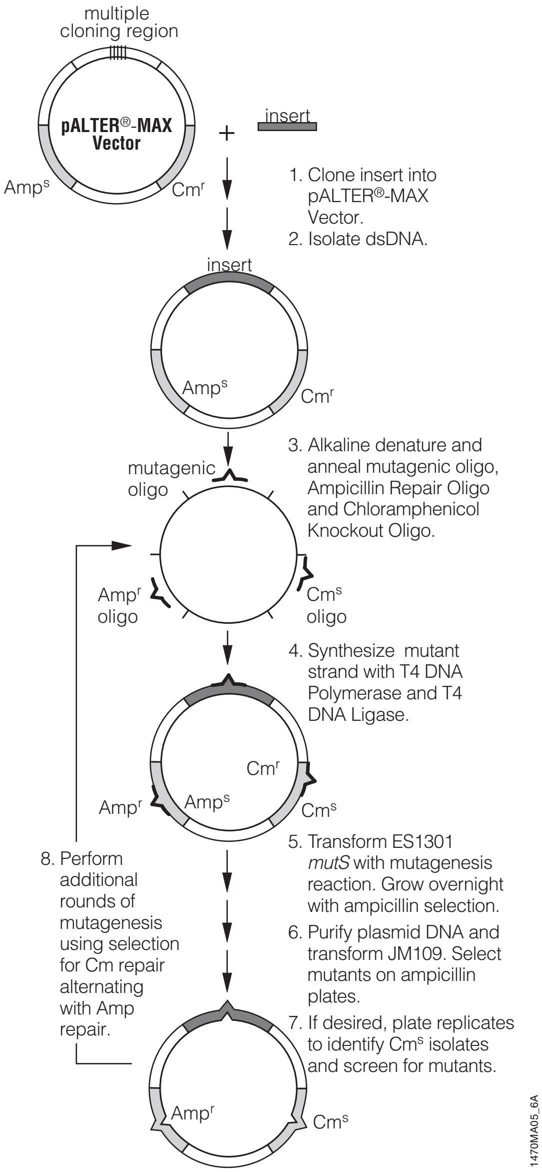
- 20µg pALTER®-MAX Vector
- 30µl Ampicillin Repair Oligonucleotide
- 30µl Ampicillin Knockout Oligonucleotide
- 30µl Chloramphenicol Repair Oligonucleotide
- 30µl Chloramphenicol Knockout Oligonucleotide
- 75µl Annealing 10X Buffer
- 100µl Synthesis 10X Buffer
- 500u T4 DNA Polymerase
- 100u T4 DNA Ligase
- 200µl ES1301 *mut*S Bacterial Strain, Glycerol Stock
- 500µl JM109 Bacterial Strain, Glycerol Stock
- 1ml R408 Helper Phage
- 10µg R408 Helper Phage DNA
- 1 Protocol

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

III. General Considerations

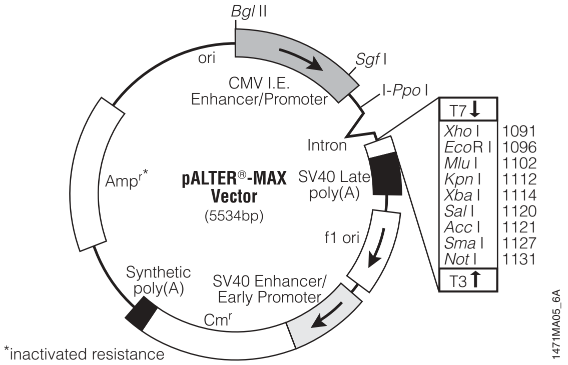
Site-directed mutagenesis is a powerful tool for the study of DNA structure and protein structure and function. A number of different mutagenesis methods are in common use (1,2). Site-directed in vitro mutagenesis, introduced by Hutchison *et al.* (3), entails hybridizing a mismatch-containing synthetic oligonucleotide to the ssDNA template. The user designs a mismatch in the oligonucleotide to produce the desired mutation in the gene of interest. Following hybridization, DNA polymerase extends the oligonucleotide to create a duplex structure. DNA ligase seals the 'nicks', and the double-stranded plasmid is then transformed into a suitable *E. coli* host.

In the absence of any screening method, 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 is often much lower, often only a few percent or less. Many factors may account for this, including incomplete in vitro polymerization, primer displacement by the DNA polymerase, 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 Mammalian Mutagenesis System uses antibiotic screening to increase the yield of mutants.



1. Clone insert into pALTER®-MAX Vector.
2. Isolate dsDNA.
3. Alkaline denature and anneal mutagenic oligo, Ampicillin Repair Oligo and Chloramphenicol Knockout Oligo.
4. Synthesize mutant strand with T4 DNA Polymerase and T4 DNA Ligase.
5. Transform ES1301 *mutS* with mutagenesis reaction. Grow overnight with ampicillin selection.
6. Purify plasmid DNA and transform JM109. Select mutants on ampicillin plates.
7. If desired, plate replicates to identify Cm^s isolates and screen for mutants.
8. Perform additional rounds of mutagenesis using selection for Cm repair alternating with Amp repair.

Figure 1. Schematic diagram of the Altered Sites® II Mammalian Mutagenesis System procedure.



*As the vector is supplied, this antibiotic resistance gene is inactivated. It can be reactivated by using the ampicillin repair oligonucleotide provided. See Section V for more details.

Figure 2. pALTER®-MAX Vector circle map and sequence reference points. Additional description: _∧_, position of intron. Arrows indicate the direction of transcription except for f1 ori where the arrow indicates the direction of ssDNA synthesis. Additional sequence and restriction site information for the pALTER®-MAX Vector is provided in Section IX.E.

Note: For screening purposes, the Ampicillin Repair Oligonucleotide restores a *Pst* I recognition site in the ampicillin gene; the Chloramphenicol Repair Oligonucleotide adds an *Acc* III site.

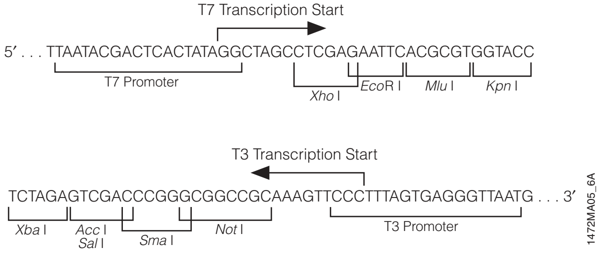


Figure 3. pALTER®-MAX Vector promoters and multiple cloning region sequence. The sequence shown corresponds to the strand produced upon infection with helper phage. Also, this sequence corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by T3 RNA polymerase.

pALTER®-MAX Vector Sequence Reference Points.

CMV I.E. (immediate/early) enhancer	1–659
CMV I.E. (immediate/early) promoter	669–750
chimeric intron	890–1022
T7 RNA polymerase promoter (–17 to +2)	1067–1085
T7 RNA polymerase transcription initiation site	1084
multiple cloning region	1091–1137
T3 RNA polymerase promoter (–17 to +3)	1143–1162
T3 RNA polymerase transcription initiation site	1146
SV40 late polyadenylation signal	1171–1392
f1 origin	1487–1942
SV40 enhancer/early promoter	2007–2377
minimal SV40 origin of replication	2273–2338
chloramphenicol acetyltransferase gene (Cm^r)	2623–3281
synthetic polyadenylation signal	3379–3427
β-lactamase gene (Amp^r)	3846–4706
Ampicillin Repair** Oligonucleotide binding site	4376–4398
Chloramphenicol Repair Oligonucleotide binding site	2827–2853
T7 EEV*** (forward) sequencing primer binding site	1053–1074
T3 (reverse) sequencing primer binding site	1143–1162
transcription start sites	2312, 2318, 2323

**The Ampicillin Knockout Oligonucleotide binds to 4372–4398.

***The T7 EEV (Eukaryotic Expression Vector) Promoter Primer (Cat.# Q6700) is designed specifically to prime sequencing reactions from the mammalian expression vectors pCI-neo (Cat.# E1841), pCI (Cat.# E1731), pSI (Cat.# E1721) and pALTER®-MAX (Cat.# Q5761).

❗ The T7 Promoter Primer (Cat.# Q5021) cannot be used to sequence from the pALTER®-MAX Vector due to a single base difference at the 3′-end of the primer.

III.A. Important Features of the Altered Sites® II Mammalian Mutagenesis System

dsDNA or ssDNA Templates

Mutagenesis can be performed on either dsDNA or ssDNA templates. The procedure for dsDNA is faster and does not require prior template preparation of ssDNA. The procedure for ssDNA 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, presence of an AT-rich region, or secondary structure. Poor quality DNA can inhibit the second-strand reaction during mutagenesis. Therefore, we recommend using sequencing-quality DNA.

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

High Yield of Mutants

Antibiotic screening of the mutant strand yields a greater percentage of mutants in cases where the frequency without screening is, at best, only a few percent. This high frequency enables identification of mutants by restriction analysis or direct sequencing of clones, eliminating the need to screen large numbers of clones by colony hybridization. Only a small amount of dsDNA or ssDNA (0.1µg or less) is required to obtain multiple antibiotic-resistant colonies.

The ES1301 *mut*S strain (5) suppresses in vivo mismatch repair (6) and is used for the initial round of transformation specifically to decrease the chance of repair to the oligonucleotide-directed mutation of the antibiotic or insert gene.

Note: Further information on the ES1301 *mut*S and JM109 strains is provided in Section IX.C.

Multiple Simultaneous Mutations

T4 DNA Polymerase is used in the synthesis reaction in place of Klenow because the T4 DNA polymerase does not strand displace (7,8) and, therefore, will not remove the mutagenic oligonucleotide from the template strand. DNA ligase then seals the nicks present between the end of the newly synthesized strand and the mismatched oligonucleotide. As a result, multiple site-directed mutations may be introduced simultaneously simply by annealing additional mutagenic oligonucleotides to the DNA insert (9). We have performed up to four simultaneous mutations with >50% efficiency using this system.

Multiple Rounds of Mutagenesis

To perform multiple rounds of mutagenesis without additional subcloning, the antibiotic-sensitive gene is activated (sensitive → resistant) and the antibiotic-resistant gene is inactivated (resistant → sensitive) with each round of mutagenesis. In addition to introducing a specific desired mutation in the gene of interest, the first mutagenesis reaction alters the selective antibiotic gene from chloramphenicol- to ampicillin-resistant. The ampicillin resistance present in the pALTER®-MAX Vector is then used to screen for the first mutant strand. A second mutagenesis reaction is used to restore chloramphenicol resistance and ampicillin sensitivity. Include the Chloramphenicol Knockout Oligonucleotide in the first mutagenesis reaction, in addition to the Ampicillin Repair Oligonucleotide, to obtain chloramphenicol-sensitive clones. This plasmid then can be used in a second mutagenesis reaction performed in the presence of the Chloramphenicol Repair Oligonucleotide and the Ampicillin Knockout Oligonucleotide. The Altered Sites® II Mammalian Mutagenesis System provides oligonucleotides, four in all, to alternately repair and knockout the two genes for antibiotic resistance. In this manner, an indefinite number of mutagenesis reactions are possible using the same construct.

Alternative Protocol: Cotransformation of ES1301 *mut*S and Transfer to JM109

Mutant plasmids may be transferred rapidly from the *mut*S host into a more suitable host for long-term maintenance and mutant segregation (Section VIII.F). Use this alternative procedure when it is important to save time or to minimize the chances of sequence rearrangements. ES1301 *mut*S is *rec*A+, and inserts containing highly repetitive sequences are sensitive to recombination. Use only high-efficiency competent ES1301 *mut*S cells (>10^7cfu/µg DNA) with this alternative procedure. We have found that electroporation works best for transforming this strain.

Note: Do not use the F^+ strain BMH 71-18 *mut*S (catalog# Q6321) in place of the ES1301 *mut*S strain; the co-transformation procedure described here will not function with BMH 71-18 *mut*S *E. coli*.

Cotransform the mutagenesis reaction products in ES1301 *mut*S with R408 helper phage DNA. The helper phage promotes replication of the mutant phagemid and its secretion into the medium as an infectious particle. These infectious particles are then transfected into a suitable (F^+) host such as JM109, and the transfectants are screened for phagemid-encoded antibiotic resistance. The procedure requires only one transformation step into the ES1301 *mut*S 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.

III.B. Design of Mutagenic Oligonucleotides

Each mutagenic oligonucleotide must be complementary to the ssDNA strand produced by the mutagenesis vector after helper phage-induced replication. 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 screening to be effective.

! Refer to Section IX.B for sequences of the provided antibiotic oligonucleotides.

The stability of the oligonucleotide:template complex is determined by the base composition of the oligonucleotide and the annealing conditions. A 17–20mer, with the desired mismatch located in the center, is sufficient to anneal and generate single-base mutations. This design allows 8–10 matched nucleotides to flank the mismatch. For mutations involving two or more mismatches, oligonucleotides 25 bases or longer are needed to allow for 12–15 matched, flanking nucleotides. Oligonucleotides of 26 and 27 bases have been used to perform four-base insertions or deletions. Larger deletions require an oligonucleotide of 20–30 matched bases flanking the mismatched region. The annealing conditions required vary with the base composition of the oligonucleotide. AT-rich complexes are less stable than GC-rich complexes and require lower annealing temperatures. For most situations, anneal oligonucleotides by heating to 75°C for 5 minutes followed by slow cooling to room temperature. For a more detailed discussion of oligonucleotide design and annealing conditions, refer to reference 10, and to chapters 11 and 15 of reference 11.

If multiple rounds of mutagenesis will be performed, include the Chloramphenicol Knockout Oligonucleotide and the Ampicillin Repair Oligonucleotide with the specific mutagenic oligonucleotide. The vector will become ampicillin-resistant and chloramphenicol-sensitive.

Note: Once the Chloramphenicol Knockout Oligonucleotide renders the vector sensitive to chloramphenicol, CAT expression can no longer serve as a control for transfection in mammalian cells. CAT activity may not be detectable if the vector contains the mutation and is sensitive to chloramphenicol. Convert the vector back to chloramphenicol-resistant prior to CAT detection studies in mammalian cells.

III.C. Phosphorylation of Oligonucleotides

Phosphorylated oligonucleotides significantly increase the number of mutants generated. A 5′ phosphate group is required for DNA ligase to seal the nick following oligonucleotide hybridization and DNA polymerization. Therefore, this system includes oligonucleotides that are phosphorylated at the 5′-end. We recommend using only phosphorylated oligonucleotides with this system (Section VIII.D).

IV. Cloning into the Mutagenesis Vector

Ligate the DNA to be mutated into the multiple cloning region of the pALTER®-MAX Vector (Figure 3). Refer to the *Protocols and Applications Guide* (12) for general information on cloning. Transform JM109 competent cells and screen for recombinant colonies on LB plates containing 20μg/ml chloramphenicol. Competent JM109 cells are available from Promega.

! DH5α™ cells transformed with the pALTER®-MAX Vector grow quite slowly. We do not recommend using this strain with the Altered Sites® II Mammalian Mutagenesis System.

V. Mutagenesis Procedure

The mutagenesis reaction involves annealing of the antibiotic repair oligonucleotide (and the alternate antibiotic knockout oligonucleotide if additional rounds of mutagenesis are to be performed) and the mutagenic oligonucleotide(s) to the DNA template followed by synthesis of the mutant strand with T4 DNA Polymerase and T4 DNA Ligase. Transform the circular, heteroduplex DNA into the repair minus *E. coli* strain ES1301 *mut*S and grow in selective medium to isolate clones containing the mutant plasmid. Identify antibiotic-resistant plasmids and transform them into the final host strain. Screen mutations by restriction analysis or direct sequencing of the plasmid DNA.

Materials to Be Supplied by the User
(Solution compositions are provided in Section IX.D.)

- ES1301 mutS and JM109 competent cells (see Sections VIII.B and VIII.C)
- mutagenic oligonucleotide, phosphorylated (see Section VIII.D)
- sterile 17 × 100mm polypropylene tubes (Falcon #2059 or equivalent)
- 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
- antibiotic stock solutions
- LB medium
- LB plates containing antibiotic
- SOC medium

V.A. Before You Begin

Before starting the procedure, prepare all of the materials needed, including ES1301 *mut*S and JM109 competent cells (see Section VIII.B and VIII.C). Competent JM109 cells may be purchased from Promega (see Section X). Calculate the amount of mutagenic oligonucleotide needed (Table 1) and select the appropriate repair and knockout oligonucleotides (Table 2) for your mutagenesis reaction.

Note: A cotransformation procedure is provided in Section VIII.F as an alternative to the standard procedure described below. This procedure may be used when it is important to save time or to minimize the chances of sequence rearrangements. ES1301 *mut*S is *rec*A+, therefore, inserts containing highly repetitive sequences may be unstable in this strain. **Use the alternative procedure only with high-efficiency competent ES1301 *mut*S cells (>10^7cfu/μg DNA)**.

Table 1. 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 × 0.33 × N, where N = length of oligonucleotide in bases.

Table 2. Selection of Appropriate Repair and Knockout Oligonucleotides.

DNA Used	Mutagenesis Round	Repair Oligo[1]	Knockout Oligo	Phenotype Change	Antibiotic Selection
pALTER®-MAX Vector + insert	#1	Amp[1]	Cm[1]	$\text{Amp}^s\text{Cm}^r \rightarrow \text{Amp}^r\text{Cm}^s$	Amp
pALTER®-MAX Vector + insert	#2	Cm	Amp	$\text{Amp}^r\text{Cm}^s \rightarrow \text{Amp}^s\text{Cm}^r$	Cm
pALTER®-MAX Vector only	Positive Control	Amp	Cm	$\text{Amp}^s\text{Cm}^r \rightarrow \text{Amp}^r\text{Cm}^s$	Amp

[1] oligo = oligonucleotide, Amp = ampicillin, Cm = chloramphenicol

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

V.B. Denaturation of Double-Stranded DNA Template

We recommend using sequencing quality DNA. Double-stranded DNA must be alkali-denatured before use. Heat-denaturation does not work for this application because the two strands reanneal too quickly.

1. Set up the following alkaline denaturation reaction. This generates enough template 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: ng of dsDNA = pmol of dsDNA × 0.66 × N, where N = length of dsDNA in bases.

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

2. Incubate for 5 minutes at room temperature.
3. Add 2µl of 2M ammonium acetate (pH 4.6) and 75µl of 100% ethanol. Mix well.
4. Precipitate at –70°C for 30 minutes.
5. Pellet the DNA by centrifugation at maximum speed (≥12,000 × *g*) 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.

V.C. Annealing Reaction and Mutant Strand Synthesis

The amount of oligonucleotide required for the annealing reaction will vary depending on the size and amount of the DNA template. Use the antibiotic repair and knockout oligonucleotides at a 5:1 oligonucleotide:template molar ratio and the mutagenic oligonucleotides at a 25:1 oligonucleotide:template molar ratio. A typical reaction will contain approximately 200ng (0.05pmol) of dsDNA or 100ng of ssDNA.

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.

This system contains several oligonucleotides with similar names. Please refer to Table 2 (Section V.A) and check the vial labels for the correct oligonucleotides.

1. Prepare the appropriate annealing reactions as described below. Double-stranded DNA must be alkali-denatured before use. Heat-denaturation does not work for this application because the two strands reanneal too quickly. Please refer to Section V.C. Denaturation is not required for ssDNA.

   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 1, Section V.A)	1.25pmol
Annealing 10X Buffer	2µl
sterile, deionized water to a final volume of	**20µl**

   Control Reaction

alkaline-denatured nonrecombinant pALTER®-MAX vector 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). A thermal cycler programmed to cool the tubes works well. For more information on oligonucleotide design and annealing conditions, see Section III.B and references 10 and 11.

   Note: If a thermal cycler is not available, the following annealing procedure may be used to slowly cool the annealing reactions.

   a. Heat the 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).

3. Place the annealing reactions on ice, and add these components **in the order listed:**

annealing reaction (above)	20µl
sterile, deionized water	5µl
Synthesis 10X Buffer	3µl
T4 DNA Polymerase	1µl (5–10u)
T4 DNA Ligase	1µl (1–3u)
final volume	**30µl**

4. Incubate the reaction at 37°C for 90 minutes to perform mutant strand synthesis and ligation.

V.D. Transformation of ES1301 *mut*S Competent Cells

High-efficiency ES1301 *mut*S competent cells can be prepared using the procedures provided in Sections VIII.B and VIII.C. ES1301 *mut*S competent cells should yield >10^7cfu/µg DNA for use in the following protocol. Follow this protocol as closely as possible to maximize the transformation efficiency of these cells.

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

   Note: Use of a standard microcentrifuge tube reduces the transformation efficiency approximately 50% due to inefficient heat-shock treatment.

2. Remove the frozen competent cells from –70°C and place on ice until just thawed (approximately 5 minutes).
3. Gently mix the cells by flicking the tube and transfer 100µl of the ES1301 *mut*S cells to each of the prechilled culture tubes.
4. Add 1.5µl of each mutagenesis reaction or control reaction (~10ng of template DNA) to 100µl of ES1301 *mut*S 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.
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 900µl of room temperature LB broth without antibiotic to each transformation reaction and incubate for 30 minutes at 37°C with shaking (approximately 225rpm).

9. Prepare overnight cultures by adding 500µl of each transformation culture to 4.5ml of the appropriate selective medium (LB + 100µg/ml ampicillin or 20µg/ml chloramphenicol). Incubate at 37°C with shaking (approximately 225rpm) for 12–24 hours. Use this culture for the plasmid miniprep and transformation procedure in Section V.E.

   Note: ES1301 *mut*S cells grow slowly, and yields of plasmid may be low. We recommend analyzing 20% (10µl) of the plasmid solution obtained from either miniprep plasmid procedure in Section V.E.

   ❗ Do not use ES1301 *mut*S colonies as source of 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 *mut*S is not a stable host for long-term maintenance of plasmids.

V.E. Plasmid Miniprep

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

V.F. Transformation of JM109 with pALTER®-MAX Plasmid

JM109 competent cells are available from Promega (see Section X) or can be prepared using the procedures provided in Sections VIII.B and VIII.C.

1. Prechill sterile 17 × 100mm polypropylene culture tubes on ice.
2. Remove frozen JM109 competent cells from –70°C and place them on ice until just thawed (approximately 5 minutes).
3. Gently mix the cells by flicking the tube and transfer 100µl of the thawed JM109 cells to each of the prechilled culture tubes.
4. Add 10µl of plasmid DNA (10ng minimum) to 100µl of JM109 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.
5. Immediately place the tube on ice for 30 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 room temperature SOC medium to each transformation reaction and incubate for 60 minutes at 37°C with shaking (approximately 225rpm).
9. For each tube, plate 100µl of cells on each of two LB plates containing the appropriate selective medium (LB + 100µg/ml ampicillin or 20µg/ml chloramphenicol) and incubate at 37°C for 12–14 hours.

V.G. Analysis of Transformants

The Altered Sites® II Mammalian Mutagenesis System procedure generally produces 50–90% positive mutants, so colonies may be screened by direct sequencing. Assuming that greater than 50% mutants are obtained, screening just 5 colonies insures a greater than 95% chance of identifying a clone with the desired mutation. A mutation located within 200–300 bases of either end of the DNA insert is optimal for sequencing reactions using either the T3 Promoter Primer (Cat.# Q5741) or the T7 Eukaryotic Expression Vector Primer (Cat.# Q6700). Sequencing reactions performed by an automated sequencer typically generate base reads further from the primer than by manual methods but require DNA of greater purity. Alternatively, restriction sites may be incorporated into the mutagenesis primers without altering the desired amino acid sequence. Restriction enzyme digestions that target these sites then can be used to identify positive clones.

When preparing antibiotic-resistant cells for plasmid minipreps, it is often convenient to screen simultaneously for antibiotic-sensitive isolates to be used for subsequent rounds of mutagenesis. Simply inoculate each isolate into two tubes of medium, one containing each antibiotic, and it will be easy to identify those isolates that are sensitive to chloramphenicol.

Alternatively, ampicillin-resistant (chloramphenicol-sensitive) colonies can be identified by picking and plating in a grid format on paired plates containing either antibiotic. Pick each colony and inoculate the two plates in identical fashion.

Control mutagenesis reactions should give at least 50% chloramphenicol-sensitive colonies. **For screening purposes, the Ampicillin Repair Oligonucleotide restores a *Pst* I recognition site in the ampicillin gene; the Chloramphenicol Repair Oligonucleotide adds an *Acc* III site.** The pALTER®-MAX Vector, as supplied, contains a single *Pst* I recognition site at position 838.

V.H. Transfection of Eukaryotic Cells with pALTER®-MAX Vector

The pALTER®-MAX Vector is a mammalian expression vector and is suitable for expression of genes in mammalian cells. Transfection of DNA into mammalian cells may be mediated by cationic lipids (13,14), calcium phosphate (15,16), DEAE-dextran (17–19), polybrene-DMSO (20,21) or electroporation (22,23). Transfection systems based on cationic lipids (TransFast™ Transfection Reagent, Transfectam® Reagent and Tfx™ Reagents), calcium phosphate and DEAE-dextran (ProFection® Mammalian Transfection Systems) are available from Promega. Stable expression will require cotransfection of a vector with a selectable marker for mammalian cells.

More information on the use of these reagents is available in the *TransFast™ Transfection Reagent Technical Bulletin* (#TB260), the *Transfectam® Reagent Technical Bulletin* (#TB116), the *Tfx™-10, Tfx™-20 and Tfx™-50 Reagents Technical Bulletin* (#TB216) and the *ProFection® Mammalian Transfection Systems Technical Manual* (#TM012), respectively.

VI. 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 ES1301 *mut*S culture	Excessive antibiotic concentrations. Check antibiotic concentration in selective medium (see Section V.D, Step 9, or Section V.F, Step 9).
	Incomplete denaturation of template DNA. Alkaline denaturation is required; heat denaturation will not work.
	Residual NaOH from the denaturation reaction. NaOH must be neutralized after template denaturation. Use fresh 2M ammonium acetate (pH 4.6), and check pH carefully.
	Poor recovery of DNA after alkaline denaturation. DNA not recovered after ethanol precipitation. Check recovery of denatured template on an agarose gel.
	Inaccurate DNA concentration. Confirm concentration by comparison with known standards on an agarose gel.
	Contaminants present in DNA. Further purify DNA by PEG precipitation (38) if A_{260}/A_{280} ratio is less than 1.8.

VI. Troubleshooting (continued)

Symptoms	Causes and Comments
No growth in overnight ES1301 *mut*S culture (continued)	Inadequate oligonucleotide hybridization. Incorrect oligonucleotide to template ratios. Check concentration of mutagenic oligonucleotide and DNA. Anneal more slowly (Section V.C, Step 2).
	Wrong antibiotic repair oligonucleotide added to annealing reaction. Refer to Table 2, Section V.A.
	Inefficient synthesis or ligation of second-strand. 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.
	DNA derived from a *hsd*M modification minus strain. ES1301 *mut*S is restriction (+). DNA should be isolated from a modification (+) K12 strain, or it will be restricted by ES1301 *mut*S. For example, DNA isolated from HB101 or NM522 modification (–) strains or BL21 (*E. coli* B strain) cells should not be used.
	Low competency of ES1301 *mut*S cells ($<10^7$cfu/µg). Check competency with pALTER®-MAX Vector DNA provided using chloramphenicol selection.
JM109 antibiotic-resistant colonies but no mutations	Mutagenic oligonucleotide not complementary to correct strand. Mutagenic oligonucleotide was not designed to the same strand as the antibiotic repair oligonucleotide. Recheck the orientation of the cloned insert.
	Inadequate annealing of mutagenic oligonucleotide to template DNA. Wrong oligonucleotide:template molar ratios were used in hybridization reaction. Check concentration and purity of mutagenic oligonucleotide by PAGE.
	Insufficient annealing time for oligonucleotide and DNA template. See Section V.C.

VI. Troubleshooting (continued)

Symptoms	Causes and Comments
JM109 antibiotic-resistant colonies but no mutations (continued)	Secondary structure in cloned insert or mutagenic oligonucleotide. Prepare template as ssDNA (Section VIII.A). If necessary, redesign the mutagenic oligonucleotide.
	Mutagenic oligonucleotide not incorporated into newly synthesized strand. Make sure mutagenic oligonucleotide is phosphorylated.
	3′-end of oligonucleotide does not properly base-pair to template, preventing incorporation into synthesized strand. Redesign oligonucleotide.
	Initial selection not performed with *mut*S strain. The *mut*S phenotype is required for efficient propagation of mutations. Check ES1301 strain for kanamycin resistance that is linked to the *mut*S mutation.
	Problem with cloned insert. The ES1301 strain is *rec*A+, and some constructs may undergo rearrangement. If possible, clone a smaller region for mutagenesis.
No JM109 colonies using alternative cotransformation	Inefficient cotransformation. Perform the back-up procedure described in Section VIII.F, Step 9b. Isolate plasmid DNA from ES1301 *mut*S culture and use it to transform JM109 or other final host.
	R408 helper phage particles used instead of R408 helper phage DNA 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 medium. Assay JM109 or final host for plaque formation by infecting with R408 helper phage. See reference 40.

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VIII. Appendix A: Additional Protocols

VIII.A. Preparation of Phagemid Single-Stranded DNA

To produce ssDNA template for the mutagenesis reaction, grow individual colonies containing recombinant phagemids and infect the cultures with helper phage. **The exported ssDNA molecule is the same as the strand shown in Figure 3 (Section III).**

Helper Phage R408 is provided with this system to optimize yields of ssDNA. Differences in the yields of ssDNA are dependent on the particular combination of host, vector and helper phage. In our experience, best results are obtained when R408 helper phage is used in conjunction with JM109 bacteria.

Materials to Be Supplied by the User
(Solution compositions are provided in Section IX.D.)

- 7.5M ammonium acetate (pH 7.5)
- antibiotic stock solutions
- ethanol (100% and 70%)
- NZYM broth
- phage precipitation solution
- TE buffer (pH 8.0)
- TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1)

Day 1:

1. Inoculate a 1ml culture of NZYM broth containing 20µg/ml chloramphenicol with a single antibiotic-resistant colony of pALTER®-MAX-transformed JM109 from a recent transformation.
2. Grow the culture at 37°C with shaking (about 250rpm) to an $O.D._{600}$ of 2.0 (approximately 6 hours).
3. Inoculate 25ml of NZYM broth containing 20µg/ml chloramphenicol with 0.5ml of the above culture. Use a 250ml Erlenmeyer flask for adequate aeration.
4. Incubate for 1 hour with shaking at 37°C.
5. Infect with helper phage R408 at an m.o.i. (multiplicity of infection) of 10–20 (i.e., add 10–20 helper phage particles/cell). **Add 200µl of the helper phage supplied with this system.**

   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 ($OD_{660} = 0.1$–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.
6. Continue incubating overnight with shaking at 37°C.

Day 2:

1. Harvest the cells by centrifuging at 12,000 × *g* (10,000rpm in Beckman JA-20 or equivalent) at 4°C for 15 minutes.
2. Transfer the supernatant to a new tube. Do not disturb the pellet.
3. Repeat the Step 1 spin on the supernatant and transfer again to a new tube.
4. Precipitate the phage by adding 0.25 volume of phage precipitation solution to the supernatant. Leave on ice for at least 1 hour, or overnight at 4°C.
5. Centrifuge at 12,000 × *g* for 20 minutes at 4°C.
6. As soon as the centrifugation is complete, remove the supernatant and resuspend the pellet in 400µl of TE buffer (pH 8.0) and transfer to a 1.5ml microcentrifuge tube.
7. Add one volume of TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1) to the sample, vortex at least 1 minute and centrifuge at maximum speed (≥12,000 × *g*) in a microcentrifuge for 5 minutes.
8. Transfer the upper, aqueous phase (containing phagemid DNA) to a new tube without disturbing the interface. Repeat the organic solvent extraction until there is no visible material at the interface.

9. Add 0.5 volume (200µl) of 7.5M ammonium acetate plus two volumes (1.2ml) of 100% ethanol. Mix and leave at –20°C for 30 minutes to precipitate the phagemid DNA.
10. Centrifuge at maximum speed 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.
11. 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, corresponding to helper phage DNA and ssDNA, are usually seen on 1% agarose gels in TAE buffer. In some preparations, a small amount of chromosomal DNA and RNA may be present from cell lysis. If the recombinant is the same size as the helper phage, it may be difficult to distinguish between the two species on a gel. R408 helper phage ssDNA (6.4kb) appears to be 3kb when compared to dsDNA markers. Analyze a 20µl sample of helper phage DNA on a gel to show where it migrates. Include 0.2% SDS in the sample of helper phage to disrupt the coat proteins before electrophoresis. Occasionally we have observed the presence of helper phage bands at approximately 1kb and 700bp when compared to dsDNA markers. The presence of the helper phage DNA does not interfere with the mutagenesis reaction.

VIII.B. Preparation of ES1301 *mut*S and JM109 Competent Cells: Modified RbCl Method

Rubidium chloride gives better transformation efficiencies than calcium chloride for most strains. This procedure is an adaptation of one described in reference 24.

Note: Check the competency of ES1301 *mut*S cells with a known quantity of pALTER®-MAX Vector. We have tried several protocols to prepare competent ES1301 *mut*S cells, all of which typically yield 10^5–10^8 cfu/µg of pALTER®-MAX DNA. Electroporation with ES1301 *mut*S yields the most consistent transformation results (12) (see Section VIII.C). Also, ES1301 *mut*S 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 IX.D.)

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

1. Inoculate a single colony from a plate in 2.5ml of LB medium in a plating tube. Incubate overnight at 37°C with shaking (approximately 225rpm).

2. Subculture the overnight culture at 1:100 by inoculating 2.5ml into 250ml of LB + 20mM $MgSO_4$. Grow the cells in a 1L flask until the A_{600} reaches 0.4–0.6 (5–6 hours).
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.
4. Gently resuspend the cell pellet in 0.4X the 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 0.04X the original volume of ice-cold TFB2. For a 250ml subculture, use 10ml of TFB2.
8. Incubate the cells on ice for 15–60 minutes and 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 *mut*S 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 a dry ice/isopropanol bath in another. Place the tubes (label the tops) in the rack with ice, aliquot 100µl cells per tube and then close the tubes. Add the dry ice to the ethanol bath, wait for it to stop bubbling, and 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 cells, but not with these racks. Use only plasticware designed for liquid nitrogen.

VIII.C. Electroporation Guidelines for ES1301 *mut*S Cells

Alternatively, electroporation of the ES1301 *mut*S cells may be performed in place of the transformation protocols. In our experience, the following conditions consistently give effective transformation of ES1301 *mut*S cells. We provide these conditions only as general guidelines in establishing conditions for your electroporation instrument. Otherwise, perform electroporation according to the guidelines supplied by the instrument manufacturer.

Materials to Be Supplied by the User
(Solution compositions are provided in Section IX.D.)

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

Preparing Electrocompetent Cells

1. Inoculate 1L of LB with 10ml of an overnight culture of ES1301 *mut*S cells. Grow the cells with shaking at 37°C until the A_{600} reaches 0.5–0.7.
2. Chill the culture on ice for 15–30 minutes. Perform all subsequent steps at 4°C.
3. Harvest the cells by centrifugation at 4,000 × *g* for 15 minutes.
4. Aspirate the supernatant to remove as much of the medium as possible. Resuspend the cells in an equal volume of ice-cold 10% glycerol. Centrifuge as in Step 3.
5. Remove the supernatant and resuspend the cells in 0.02X the volume of ice-cold 10% glycerol. Centrifuge as in Step 3.
6. Resuspend the cells in 0.002–0.003X the final volume (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

1. Remove electrocompetent ES1301 *mut*S cells from –70°C storage and place on ice until just thawed. Transfer 50µl of cells into the electroporation cuvette.
2. Add no more than 1µl of the ligation reaction product (from Section V.C) to the 50µl of electrocompetent cells. Pre-incubate the cells with the DNA for 1 minute before electroporation.
3. Electroporate the cells using conditions appropriate for your instrument. The following conditions are 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

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

VIII.D. 5′ Phosphorylation of Oligonucleotides

Materials to Be Supplied by the User
(Solution compositions are provided in Section IX.D.)

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

1. Add the following components to a microcentrifuge tube. Use the formula* below to calculate the nanogram equivalents of 100pmol (also see Table 1, Section V.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: ng of oligonucleotide = pmol of oligonucleotide × 0.33 × N, where N = length of oligonucleotide in bases.

2. Incubate the reaction at 37°C for 30 minutes.
3. Inactivate the kinase by incubating the reaction at 70°C for 10 minutes.
4. The reaction products can be stored at –20°C or added directly to the annealing reaction (Section V.C).

VIII.E. pALTER®-MAX Plasmid Miniprep Procedure

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

Materials to Be Supplied by the User
(Solution compositions are provided in Section IX.D.)

- miniprep lysis buffer
- NaOH/SDS solution (prepare fresh for each use)
- 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 VIII.B)
- DMSO, frozen in aliquots
- antibiotic stock solutions
- LB medium
- LB plates + antibiotic

1. Place 1.5ml of the overnight culture from Section V.D, Step 9, into a microcentrifuge tube and centrifuge at maximum speed (≥12,000 × *g*) for 1 minute. Store the remainder of the overnight culture at 4°C.
2. Remove the medium by aspiration; leave the bacterial pellet as dry as possible.
3. Resuspend the pellet by vortexing in 100µl of ice-cold miniprep lysis buffer.
4. Incubate for 5 minutes at room temperature.
5. Add 200µl of a freshly prepared lysis solution (0.2N NaOH, 1% SDS). Mix by inversion. **Do not vortex.** Incubate for 5 minutes on ice.
6. Add 150µl of ice-cold 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. Place a drop of chloroform in each tube prior to the spin. This helps to separate the phases during centrifugation. Centrifuge at maximum speed for 5 minutes.
8. Transfer the supernatant to a new tube. Avoid the white precipitate.
9. Add an equal volume of TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1). Vortex at least 1 minute and centrifuge at maximum speed 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 maximum speed for 5 minutes. Rinse the pellet with 70% ethanol (prechilled to 4°C) 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. Determine the yield of plasmid DNA by electrophoresis on an agarose gel. Expect a yield of 0.2–3µg of plasmid DNA depending on the plasmid copy number.

VIII.F. Alternative Protocol: Cotransformation of ES1301 *mut*S and Transfer to JM109

This procedure may be used as an alternative to the standard, two-step transformation of ES1301 *mut*S and JM109 cells (Sections V.D–V.F) when it is important to save time or to minimize the chances of sequence rearrangements. ES1301 *mut*S is *rec*A+, and as a result, inserts containing highly repetitive sequences may be unstable. Use this procedure with only high-efficiency competent ES1301 *mut*S cells (>10^7cfu/µg DNA).

Before starting this procedure, prepare competent ES1301 *mut*S cells as described in Section VIII.B or VIII.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 a standard microcentrifuge tube reduces the transformation efficiency approximately 50% due to inefficient heat-shock treatment.

2. Remove competent ES1301 *mut*S cells from –70°C storage and place on ice until just thawed (approximately 5 minutes).
3. Gently mix the cells by flicking the tube, and transfer 100µl of ES1301 *mut*S cells to each of the prechilled culture tubes.
4. Add 15µl of the synthesis reaction from Section V.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 to mix. **Do not pipet or vortex to mix.** 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 *mut*S cells to two microcentrifuge tubes and pellet the cells by centrifugation at top speed in a microcentrifuge for 3 minutes. Remove and combine the supernatants. Add 100µl of an overnight culture of JM109 cells to the combined supernatants.

   b. To the 1ml of unpelleted transformed ES1301 *mut*S cells, add 4ml of LB medium containing the appropriate antibiotic (either 100µg/ml ampicillin or 20µg/ml chloramphenicol) 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.

10. Incubate the 3ml 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 + 100µg/ml ampicillin or 20µg/ml chloramphenicol). 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 maximum 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 highly dependent on the competency of the ES1301 *mut*S cells; at least 10^7cfu/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 V.F. The competency of ES1301 *mut*S cells should be checked using a known quantity of the pALTER®-MAX Vector.

2. Removal of the ES1301 *mut*S 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 medium containing nalidixic acid (10µg/ml, Sigma Cat.# N4382). The *gyr*A chromosomal allele, present in JM109 and absent in ES1301 *mut*S, confers resistance to nalidixic acid. Sensitivity to kanamycin also is a confirmation that the final host is JM109 rather than ES1301 *mut*S.

3. To measure the percent of mutants obtained in the control reactions, pick several ampicillin-resistant colonies and plate them in a grid format on paired plates containing either ampicillin or chloramphenicol. Pick each colony with a sterile toothpick, and inoculate the two plates in sequence.

   Example:

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

IX. Appendix B: Reference Information

IX.A. pALTER®-MAX Vector and Its Components

The pALTER®-MAX Vector circle map and sequence reference points can be found in Figure 2 and on the following page (Section III). The pALTER®-MAX Vector promoters and multiple cloning region sequence can be found in Section III, Figure 3.

Enhancer/Promoter Regions

The vector contains the human cytomegalovirus (CMV) immediate-early enhancer/promoter region to promote strong, constitutive expression of cloned DNA inserts in a variety of mammalian cell types. In transgenic mice, CMV enhancer/promoter regulated CAT gene expression in 24 of 28 tissues examined (25), demonstrating the promiscuous activity of this control element. The pALTER®-MAX Vector also contains the SV40 enhancer and early promoter regions upstream of the CAT gene. The SV40 origin of replication, contained in the SV40 early promoter, induces transient episomal replication of the vector in cells that express the SV40 large T antigen such as COS-1 or COS-7 cells (26).

Chimeric Intron

A chimeric intron, composed of the 5′-donor splice site from human β-globin gene intron one and the branch plus the 3′-acceptor splice site from the intron of an immunoglobulin gene heavy chain variable region, resides downstream of the CMV enhancer/promoter region (27). The sequences of the donor and acceptor splice sites, along with the branchpoint site, were modified to match the consensus sequence for splicing (28). The intron is located upstream of the cDNA insert to prevent the use of cryptic 5′-donor splice sites residing within the cDNA sequence.

An intron flanking the cDNA insert frequently increases the level of gene expression in transfection studies (29–33), although the increase in expression depends on the particular cDNA insert. For example, in transient transfections of 293 cells, the presence of the chimeric intron results in an approximate 20-fold increase in expression of the CAT gene (34). In contrast, the chimeric intron increases the gene expression level from the luciferase cDNA by only threefold (34). In transgenic experiments, an intron is necessary to promote a high level of expression for virtually all cDNA inserts tested (35–37).

T7 and T3 RNA Polymerase Promoters

In the pALTER®-MAX Vector, T7 and T3 RNA Polymerase promoters flank the multiple cloning region. These promoters will direct RNA synthesis from the sense or antisense strand of the cloned DNA insert. Please see Section V.G for information on sequencing inserts in the pALTER®-MAX Vector.

The T7 Promoter Primer (Cat.# Q5021) cannot be used to sequence from the pALTER®-MAX Vector due to a single base difference at the 3′-end of the primer.

Multiple Cloning Region and Convenient Restriction Enzyme Sites

The multiple cloning region in the pALTER®-MAX Vector is nearly identical to that found in the pCI-neo (Cat.# E1841), pCI (Cat.# E1731) and pSI (Cat.# E1721) Mammalian Expression Vectors. The multiple cloning region is compatible with cDNAs generated by the Universal RiboClone® cDNA Synthesis System (Cat.# C4360).

Large hairpin structures in the 5′-end of untranslated mRNAs reduce the level of in vitro and in vivo translation in higher eukaryotes (38–42). RNA transcribed from the multiple cloning region of the pALTER®-MAX Vector is predicted to contain no hairpin structures that would interfere with translation (41). There are no ATG sequences between the transcription start site and the T3 RNA Polymerase promoter; **the inserted DNA must contain an ATG for the initiation of translation.**

Restriction sites flanking the CMV enhancer (*Bgl* II and *Sgf* I) and the CMV promoter (*Sgf* I and I-*Ppo* I) allow easy replacement of these regions by alternative regulatory regions.

SV40 Late Polyadenylation Signal

Polyadenylation signals trigger the addition of approximately 200–250 adenosine residues to the 3′-end of the RNA transcript and lead to the termination of transcription by RNA polymerase II (43). Polyadenylation enhances RNA stability and translation (44,45). The SV40 late polyadenylation signal, positioned downstream of the multiple cloning region, facilitates the efficient processing of cloned DNA inserts lacking such signals. The SV40 late polyadenylation signal is extremely efficient and increases the steady-state level of RNA approximately fivefold more than the SV40 early polyadenylation signal (46).

CAT Gene

The CAT gene can be expressed in both bacterial and mammalian cells. It is used as a selectable marker in bacteria, as expression of CAT confers resistance to the antibiotic chloramphenicol. The simian virus 40 (SV40) enhancer/early promoter drives expression of CAT in the pALTER®-MAX Vector.

The level of CAT expression from the pALTER®-MAX Vector, though still easily detectable using standard CAT assay procedures, may be lower than other CAT vectors. Lack of an intron upstream of the CAT gene leads to decreased expression of CAT protein. Since CAT expression from this vector is used as a transfection control, high levels of expression are unnecessary. Also, the decreased expression of CAT protein in the cell is less likely to have an adverse effect on cell physiology. For information on CAT assay systems, refer to the *CAT Enzyme Assay System with Reporter Lysis Buffer Technical Bulletin* #TB084.

f1 Origin of Replication and Plasmid Replicon

The pALTER®-MAX Vector contains the origin of replication of the filamentous phage f1 and is a high-copy plasmid. To generate ssDNA from the f1 origin, bacteria transformed with the pALTER®-MAX Vector carrying the DNA insert of interest are infected with an appropriate helper phage. The plasmid then undergoes f1 replication, and the resulting ssDNA is exported from the cell as an encapsidated virus particle. The ssDNA molecule exported has the sequence of the strand shown for the multiple cloning region in Figure 3.

The pALTER®-MAX Vector does not contain the "poison" sequence present in pBR322 that inhibits replication of SV40 origin-containing vectors in COS cells (47). This results in more efficient expression of the cloned cDNAs in COS cells and other SV40 large T antigen-transformed cells.

IX.B. Sequences of the Repair and Knockout Oligonucleotides

These repair and knockout oligonucleotides are complementary to the ssDNA produced by the pALTER®-MAX Vector.

Description and Size	Sequence
Ampicillin Repair Oligonucleotide, Phosphorylated (27mer)	5´-d(pGTTGCCATTGCTGCAGGCATCGTGGTG)-3´
Ampicillin Knockout Oligonucleotide, Phosphorylated (27mer)	5´-d(pGTTGCCATTGCGGCATCGTGGTGTCAC)-3´
Chloramphenicol Repair Oligonucleotide, Phosphorylated (27mer)	5´-d(pCATTGCCATACGGAGTTCCGGATGAGC)-3´
Chloramphenicol Knockout Oligonucleotide, Phosphorylated (25mer)	5´-d(pCATTGCCATACGGAACCGGATGAGC)-3´

IX.C. Descriptions of Bacterial Strains

JM109

*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k-, m_k+), *rel*A1, *sup*E44, λ^-, Δ(*lac-pro*AB),
[F´, *tra*D36, *pro*AB, *lac*I^{q}ZΔM15]

JM109 is a useful host in which to transform pALTER® and pGEM® Vectors and to produce ssDNA from M13 or phagemid vectors. The strain grows well and is transformed efficiently by a variety of methods. Because JM109 is *rec*A– and lacks the *E. coli* K restriction system (e.g., *end*A–), undesirable restriction of cloned DNA and recombination with host chromosomal DNA are prevented. The *end*A1 mutation leads to an improved yield and quality of isolated plasmid DNA. JM109 high-efficiency competent cells are available from Promega (Section X).

Propagate JM109 on minimal plates (M–9) supplemented with 1mM thiamine-HCl to maintain 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.

ES1301 *mut*S

*lac*Z53, *mut*S201::Tn5, *thy*A36, *rha*-5, *met*B1, *deo*C, IN(*rrn*D-*rrn*E)

ES1301 (6) *mut*S is a mismatch repair strain of *E. coli*. Use of a *mut*S strain prevents repair of the newly synthesized unmethylated strand (4,7) allowing for high mutation efficiencies. ES1301 *mut*S is *rec*A+; therefore, inserts containing highly repetitive sequences may be unstable. ES1301 *mut*S is kanamycin-resistant due to the Tn5 transposon. ES1301 *mut*S is also restriction (+). Template DNA should be isolated from a modification (+) K12 strain or it will be restricted by ES1301 *mut*S. For example, DNA isolated from HB101 or NM522, modification (–) strains, or BL21 (*E. coli* B strain) cells should not be used.

IX.D. Composition of Buffers, Solutions and Media

ammonium acetate, 2M (pH 4.6)

15.4g	ammonium acetate

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

ammonium acetate, 7.5M (pH 7.5)

57.75g	ammonium acetate

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

Annealing 10X Buffer

200mM	Tris-HCl (pH 7.5)
100mM	$MgCl_2$
500mM	NaCl

antibiotic stock solutions (1,000X)

Ampicillin	100mg/ml in water
Chloramphenicol	20mg/ml in 80% ethanol

Store at –20°C.

IX.D. Composition of Buffers and Solutions (continued)

chloroform:isoamyl alcohol (24:1)

Mix 24 parts of chloroform with 1 part of isoamyl alcohol (24:1). Store protected from direct light at room temperature.

Kinase 10X Buffer

700mM	Tris-HCl (pH 7.6)
100mM	$MgCl_2$
50mM	DTT

LB (Luria-Bertani) medium (1 liter)

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

Adjust pH to 7.5 with NaOH. Autoclave.

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 at 100µg/ml or chloramphenicol at 20µg/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 ≤1 month.

M-9 plates (1 liter)

15g	agarose

Add 15 g agarose to 750ml water and autoclave. Cool to 50°C. Add:

2ml	1M $MgSO_4$
0.1ml	1M $CaCl_2$
10ml	20% glucose
1ml	1M thiamine-HCl
200ml	5X M-9 salts

5X M-9 salts (1 liter)

34g	Na_2HPO_4
15g	KH_2PO_4
2.5g	NaCl
5g	NH_4Cl

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

miniprep lysis buffer

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

NaOH/SDS solution

0.2N	NaOH
1%	SDS

Prepare fresh using 10M NaOH and 10% SDS.

NZYM broth

5g	NaCl
2g	$MgSO_4 \cdot 7H_2O$
5g	yeast extract
10g	NZ Amine (casein hydrolysate)

Adjust to pH 7.5 with NaOH. Autoclave.

phage precipitation solution

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

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

IX.D. Composition of Buffers and Solutions (continued)

potassium acetate

60ml	5M potassium acetate
11.5ml	glacial acetic acid
28.5ml	H_2O

Add the glacial acetic acid and water to the potassium acetate. 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 Mg^{2+} stock, filter-sterilized (prepared as described below)
1ml	2M glucose, filter-sterilized

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

2M Mg^{2+} stock

20.33g	$MgCl_2 \bullet 6H_2O$
24.65g	$MgSO_4 \bullet 7H_2O$

Add distilled water to 100ml. Filter-sterilize.

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

Adjust pH to 5.8 with 1M acetic acid. Filter-sterilize (0.45µm). Store at room temperature.

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). Store protected from direct light at 4°C.

TFB1

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

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

TFB2

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

Adjust pH to 6.5 with 1M KOH. Filter-sterilize (0.45µm). Store at room temperature.

IX.E. pALTER®-MAX Vector Restriction Sites and Sequence Accession Number

For screening purposes, the Ampicillin Repair Oligonucleotide restores a *Pst* I recognition site in the ampicillin gene; the Chloramphenicol Repair Oligonucleotide adds an *Acc* III site.

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. The vector sequence is available in the GenBank® database (**GenBank®/EMBL Accession Number AF361302**) and on the Internet at: **www.promega.com/vectors/**

Table 3. Restriction Enzymes That Cut the pALTER®-MAX Vector Between 1 and 5 Times.

Enzyme	# of Sites	Location
Aat II	5	278, 331, 414, 600, 3714
_Acc_B7 I	2	2501, 3068
Acc I	1	1121
Acc III	1	2832
_Acc_65 I	1	1108
Afl II	4	828, 847, 1050, 2404
Afl III	1	1102
_Alw_44 I	3	3464, 3961, 5203
*Alw*N I	1	5108
*Asp*H I	5	729, 3468, 3965, 4050, 5207
Ava I	2	1091, 1125
Ava II	2	4269, 4487
Avr II	1	2356
Bal I	3	10, 64, 3103
Ban II	2	729, 1646
Bbs I	1	961
Bbu I	2	2104, 2176
Bgl II	1	5529
Bsa I	2	915, 4563
*Bsa*A I	3	493, 1717, 2529
*Bsa*B I	2	1403, 3442
_Bsa_M I	4	1222, 1315, 2829, 3236
Bsm I	4	1222, 1315, 2829, 3236
*Bsp*H I	3	3688, 3793, 4797
*Bsp*M I	1	877
*Bsr*G I	1	96
*Bss*S I	3	3657, 3964, 5344
_Bst_98 I	4	828, 847, 1050, 2404
_Bst_Z I	1	1131
*Cfr*10 I	2	1612, 4544
Cla I	2	1407, 3430
Dra II	1	3653
Dra III	1	1720
Drd I	5	817, 1764, 2393, 3550, 5415
Dsa I	3	513, 2263, 3137
Eae I	5	8, 62, 1131, 3101, 4240
Eag I	1	1131
Ear I	2	1425, 3834
*Ecl*HK I	1	4629
*Eco*52 I	1	1131
_Eco_ICR I	1	727
_Eco_R I	1	1096
Fsp I	2	1466, 4406
Hae II	3	1562, 1570, 5277
_Hin_c II	4	677, 1122, 1301, 4091
*Hin*d II	4	677, 1122, 1301, 4091

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

Table 3. Restriction Enzymes That Cut the pALTER®-MAX Vector Between 1 and 5 Times (continued).

Enzyme	# of Sites	Location	Enzyme	# of Sites	Location
_Hin_d III	2	756, 2372	**_Sal_ I**	1	1120
Hpa I	1	1301	**_Sca_ I**	3	1063, 3253, 4152
I-_Ppo_ I	1	851	**_Sfi_ I**	1	2309
Kpn I	1	1112	**_Sgf_ I**	1	664
Mlu I	1	1102	**_Sin_ I**	2	4269, 4487
Nae I	1	1614	**_Sma_ I**	1	1127
Nco I	3	513, 2263, 3137	**_Sna_B I**	1	493
Nde I	1	387	**_Spe_ I**	1	152
_Ngo_M IV	1	1612	**_Sph_ I**	2	2104, 2176
Nhe I	2	1085, 2410	**_Ssp_ I**	5	5, 52, 1925, 3148, 3828
Not I	1	1131			
Nsi I	2	2106, 2178	**_Stu_ I**	1	2355
Nsp I	3	2104, 2176, 3608	**_Sty_ I**	4	513, 2263, 2356, 3137
PaeR7I	1	1091			
_Pfl_M I	2	2501, 3068	_Tfi_ I	3	2378, 3186, 3427
_Ppu_10 I	2	2102, 2174	**_Vsp_ I**	2	160, 4454
_Psp_A I	1	1125	**_Xba_ I**	1	1114
Pst I	1	838	**_Xho_ I**	1	1091
Pvu I	3	664, 1447, 4264	**_Xma_ I**	1	1125
Pvu II	2	2032, 2736	**_Xmn_ I**	1	4033
Sac I	1	729			

Table 4. Restriction Enzymes That Do Not Cut the pALTER®-MAX Vector.

Age I
Apa I
Asc I
_Bam_H I
Bbe I
_Bbr_P I
Bcl I
Blp I
_Bpu_1102 I
_Bsp_120 I
_Bss_H II
_Bst_1107 I
_Bst_E II
_Bst_X I
_Bsu_36 I
Csp I
_Csp_45 I
_Eco_47 III
_Eco_72 I
_Eco_81 I
_Eco_N I
_Eco_R V
Ehe I
Fse I
Kas I
Nar I
Nru I
Pac I
_Pin_A I
Pme I
Pml I
_Ppu_M I
_Psh_A I
_Psp_5 II
Rsr II
Sac II
_Sgr_A I
Spl I
Srf I
_Sse_8387 I
Swa I
_Tth_111 I
Xcm I

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

Table 5. Restriction Enzymes That Cut the pALTER®-MAX Vector 6 or More Times

Aci I
Acy I
***Alu* I**
***Alw*26 I**
***Ban* I**
Bbv I
***Bgl* I**
*Bsa*O I
*Bsa*H I
*Bsa*J I
***Bsp*1286 I**
Bsr I
***Bsr*S I**
*Bst*71 I
***Bst*O I**
*Bst*U I
***Cfo* I**
***Dde* I**
***Dpn* I**
Dpn II
***Dra* I**
*Fnu*4H I
***Fok* I**
***Hae* III**
Hga I
***Hha* I**
***Hinf* I**
***Hpa* II**
Hph I
***Hsp*92 I**
***Hsp*92 II**
Mae I
Mae II
Mae III
***Mbo* I**
***Mbo* II**
Mnl I
Mse I
***Msp* I**
***Msp*A1 I**
***Nci* I**
***Nde* II**
Nla III
Nla IV
Ple I
***Rsa* I**
***Sau*3A I**
*Sau*96 I
*Scr*F I
*Sfa*N I
***Taq* I**
***Tru*9 I**
***Xho* II**

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

X. Appendix C: Related Products

Components of the Altered Sites® System Available Separately

Product	Size	Cat.#
pALTER®-MAX Vector	20µg	Q5761
Ampicillin Repair Oligonucleotide	30µl	Q6311
T4 DNA Polymerase*	100u	M4211
	500u	M4215
T4 DNA Ligase*	100u (Weiss)	M1801
	500u (Weiss)	M1804
T4 Polynucleotide Kinase*	100u	M4101
	1,000u	M4103
R408 Helper Phage	5ml	P2291
R408 Helper Phage DNA	10µg	P2341

Other Related Products

Product	Size	Cat.#
T3 Promoter Primer	2µg	Q5741
T7 EEV Promoter Primer	2µg	Q6700
Wizard® *Plus* SV Minipreps DNA Purification System + Vacuum Adapters*	50 preps	A1340
	250 preps	A1470

*For Laboratory Use.

Mutagenesis Systems

Product	Size	Cat.#
Altered Sites® II in vitro Mutagenesis System	1 system	Q6210
Altered Sites® II-*Ex*1 in vitro Mutagenesis System	1 system	Q6090
Altered Sites® II-*Ex*2 in vitro Mutagenesis System	1 system	Q6080
GeneEditor™ in vitro Site-Directed Mutagenesis System	30 reactions	Q9280

Bacterial Strains

Product	Size	Cat.#
ES1301 *mut*S Bacterial Strain, Glycerol Stock (noncompetent)	200µl	Q6131
JM109 Bacterial Strain, Glycerol Stock (noncompetent)	500µl	P9751
JM109 Competent Cells, High Efficiency (>10^8 cfu/µg)*	1ml (5 × 200µl)	L2001
JM109 Competent Cells, Low Efficiency (>10^7 cfu/µg)	1ml (5 × 200µl)	L1001

*For Laboratory Use.

[a] U.S. Pat. No. 5,955,363 has been issued to Promega Corporation for a vector for in vitro mutagenesis and use thereof.

[b] The CMV promoter and its use are covered under U.S. Pat. Nos. 5,168,062 and 5,385,839 owned by the University of Iowa Research Foundation, Iowa City, Iowa, and licensed FOR RESEARCH USE ONLY. Commercial users must obtain a license to these patents directly from the University of Iowa Research Foundation.

[c] Licensed under U.S. Pat. No. 4,935,361.

[d] U.S. Pat. No. 4,766,072 has been issued to Promega Corporation for transcription vectors having two different bacteriophage RNA polymerase promoter sequences separated by a series of unique restriction sites into which foreign DNA can be inserted.

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