Muta-Gene® M13 *in vitro* Mutagenesis Kit, Version 2

Instruction Manual

Catalog Number 170-3580

For Technical Service
Call Your Local Bio-Rad Office or
in the U.S. Call **1-800-4BIORAD**
(1-800-424-6723)

BIO-RAD

Table of Contents

Foreword

This manual contains useful background information and detailed protocols for performing *in vitro* mutagenesis with the Muta-Gene® M13 *in vitro* mutagenesis kit. The mutagenesis technique described is intrinsically very easy to use. It requires only one enzymatic step; the two bacterial strains are healthy and easy to grow; and the efficiency is high enough that mutants can be identified by DNA sequence analysis rather than a phenotypic or hybridization screen.

Bio-Rad's kit provides reagents of the highest quality. All components have been rigorously tested in *in vitro* mutagenesis experiments. If you have any questions regarding the use of these products, please contact your local Bio-Rad representative or Technical Services at 1-800-4BIORAD.

These products are for research use only and not for use in humans or for diagnostic procedures.

Safety precautions should be used when handling hazardous materials (radioactive nucleotides, acrylamide, ethidium bromide, phenol, chloroform, ether).

Users of this kit should comply with NIH or other relevant guidelines for recombinant DNA work.

Section 1
Introduction

Oligonucleotide-directed *in vitro* mutagenesis is a widely used procedure for the study of the structure and function of DNA and the protein for which it codes. A wide variety of techniques are available for performing *in vitro* mutagenesis.[1] A typical strategy is to clone the segment of DNA to be mutated into a vector whose DNA exists in both single- and double-stranded forms. An oligonucleotide complementary to the region to be altered, except for a limited internal mismatch, is hybridized to a single-strand copy of the DNA. A complementary strand is then synthesized by DNA polymerase using the oligonucleotide as primer. Ligase is used to seal the new strand to the 5' end of the oligonucleotide. The double-stranded DNA, completely homologous except for the intended mutation, is then transformed into *E. coli*, resulting in two classes of progeny—the parental and those carrying the oligonucleotide-directed mutation. Since there are both parental and mutant progeny, no more than half of the progeny will be mutant. In practice, a much lower fraction is usually obtained.

The Muta-Gene M13 *in vitro* mutagenesis kit is based on a method described by Kunkel[2,3] which provides a very strong selection against the non-mutagenized strand of a double-stranded DNA. When DNA is synthesized in a *dut, ung* double mutant bacterium, the nascent DNA carries a number of uracils in thymine positions as a result of the *dut* mutation which inactivates the enzyme dUTPase and results in high intracellular levels of dUTP. The *ung* mutation inactivates uracil N-glycosylase which allows the incorporated uracil to remain in the DNA. This uracil-containing strand is then used as the template for the synthesis *in vitro* of a complementary strand primed by an oligonucleotide containing the desired mutation. When the resulting double-stranded DNA is transformed into a cell with a proficient uracil N-glycosylase, the uracil-containing strand is inactivated with high efficiency, leaving the non-uracil-containing survivor to replicate. Typical mutagenesis frequencies obtained with the Muta-Gene M13 kit are greater than 50%, a rate high enough to allow identification of mutants by sequence analysis.

To take full advantage of this method, a vector which can exist in both double- and single-stranded forms is needed. The Muta-Gene M13 kit provides the M13-based cloning vectors, M13mp18 and M13mp19, developed by Messing and collaborators.[4] The M13 virion is a filamentous phage consisting of a protein-encased circular single-stranded DNA molecule. The replicative form (RF) of the

bacteriophage, on the other hand, is a circular double-stranded DNA molecule and allows cloning by standard restriction endonuclease methods. The filamentous nature of the virion allows varying lengths of DNA to be encapsulated. Finally, the vector carries a region containing many restriction sites that lie within a segment of the ß-galactosidase gene which allows detection of cloned inserts. M13 and its variants have been described in detail by Messing.[5]

The approach used in the Muta-Gene M13 kit, outlined in Figure 1, is to clone the DNA to be mutated into the RF form of M13mp18 (or M13mp19) and introduce this recombinant vector into the *E. coli dut, ung* strain. Phage particles whose single strand of DNA contains some uracils are produced from these cells. The uracil-containing DNA is purified, used as template in an *in vitro* mutagenesis reaction, and transformed into a strain with a functional uracil N-glycosylase, thus selecting against the parental strand. Phage particles containing single-stranded DNA can then be produced for sequence analysis.

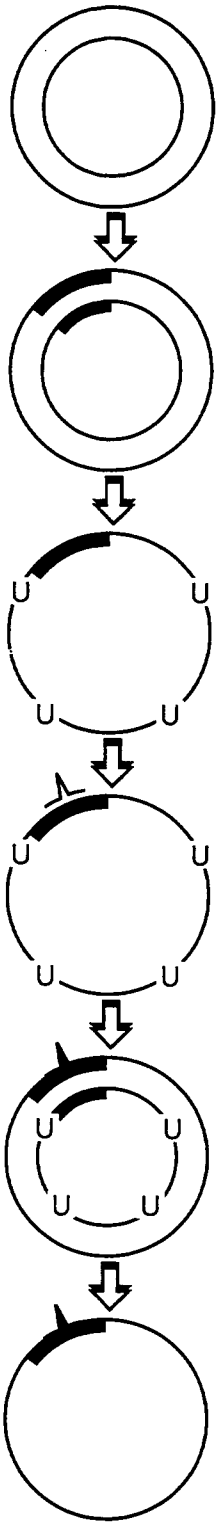


Fig. 1. Steps involved in *in vitro* mutagenesis using the Muta-Gene M13 kit.

Table 1 Kit Components

1. M13mp18 and M13mp19 RF DNAs: 5 µg of each at a concentration of 0.1 µg/µl in TE (10 mM Tris, pH 8.0, 1 mM EDTA).
2. *E. coli* CJ236 *dut, ung, thi, rel* A; pCJ105 (*Cm*^{r}): glycerol stock.
3. *E. coli* MV1190 Δ(*lac-pro* AB), *thi, sup* E, Δ(*srl-rec* A)306::Tn10 (*tet*^{r}) [F': *tra* D36, *pro* AB, *lac* I^{q}ZΔM15]: glycerol stock.
4. 10x Annealing Buffer: 200 mM Tris pH 7.4, 20 mM $MgCl_2$, 500 mM NaCl, 50 ml.
5. 10x Synthesis Buffer: 5 mM each deoxynucleotide triphosphate, 10 mM ATP, 100 mM Tris pH 7.4, 50 mM $MgCl_2$, 20 mM dithiothreitol, 50 µl.
6. T7 DNA Polymerase: 20 units in 20 mM potassium phosphate, pH 7.4, 1 mM dithiothreitol, 0.1 mM EDTA, 50% glycerol.
7. T4 DNA Ligase: 75 units in 10 mM Tris, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 50% glycerol.
8. Control reagents consisting of: a. **Amber phage**, a clear plaque mutant M13 amber-1 phage; b. **U-amber DNA**, purified uracil-containing DNA of the clear plaque forming amber phage; and c. **Control primer**, a 16-base oligonucleotide which reverts the amber mutation.
9. Specifications sheet.
10. Instruction manual.
11. Laminated short protocol.
12. Instructions for use of the control reagents.

Store the kit at -20 °C. The amber phage, U-amber DNA and RF DNAs should not be thawed and refrozen repeatedly; once thawed, they should be kept at 4 °C. Immediately before use, spin the tubes for a few seconds in a microcentrifuge to bring the contents to the bottom of the tube. The T7 DNA polymerase and T4 DNA ligase should be stored at -20 °C where they are stable for at least 6 months and should not be thawed and refrozen repeatedly.

Section 2
Kit Components

All of the components of the kit are listed in Table 1. The kit contains sufficient reagents to perform 25 oligonucleotide-directed *in vitro* mutagenesis reactions and enough M13mp18 and M13mp19 RF DNAs for about 20 cloning reactions. A detailed description of these components is presented in this section.

2.1 M13mp18 and M13mp19 RF DNAs

M13mp18 and M13mp19 are genetically engineered cloning vectors. DNA containing the lac operator and promoter and the first 145 codons of the ß-galactosidase gene was inserted into the intergenic region between genes IV and II. This protein fragment can complement a truncated host fragment, producing a functional ß-galactosidase molecule. In addition, a segment called a polylinker which carries numerous restriction enzyme recognition sites was incorporated near the beginning of the lac DNA. This polylinker contains the single cloning sites of *Eco*RI, *Sst* I, *Kpn* I, *Xma* I, *Sma* I, *Bam*HI, *Xba* I, *Sal* I, *Acc* I, *Hinc* II, *Pst* I, *Sph* I, and *Hind* III (Figure 2). Insertion of DNA into these sites disrupts the DNA coding for the ß-galactosidase fragment and results in colorless plaques when plated in the presence of IPTG and X-gal. The only difference between M13mp18 and M13mp19 is the orientation of the polylinker.

2.2 Bacterial Strains *E. coli* CJ236 and MV1190

E. coli CJ236 carries the following markers: *dut*-1, *ung*-1, *thi*-1, *rel* A-1; pCJ105(Cm^r). The *dut* and *ung* phenotypes are non-reverting and result in occasional uracils substituted for thymine in all DNA synthesized in the bacterium.[3] F' plasmid pCJ105[6] carries the information for pili construction and provides chloramphenicol resistance. Pili are necessary for phage attatchment to, and entry into, the bacterial cell. Since F' plasmids tend to be lost if not under selective pressure, this strain should be grown in the presence of chloramphenicol.

E. coli MV1190 is Δ(*lac-pro* AB), thi, sup E, Δ(*srl-rec* A) 306::Tn10(tet^r)[F':tra D36, *pro* AB, *lac* I^{q}ZΔM15]. In this strain, DNA coding for lactose utilization and proline biosynthesis has been deleted from the chromosome. The F' plasmid carries the proline synthesis genes and a truncated ß-galactosidase gene that produces a protein fragment which can be complemented by the fragment coded for by the M13 phage. Therefore, cells carrying phage with an intact fragment sequence will produce blue plaques in the presence

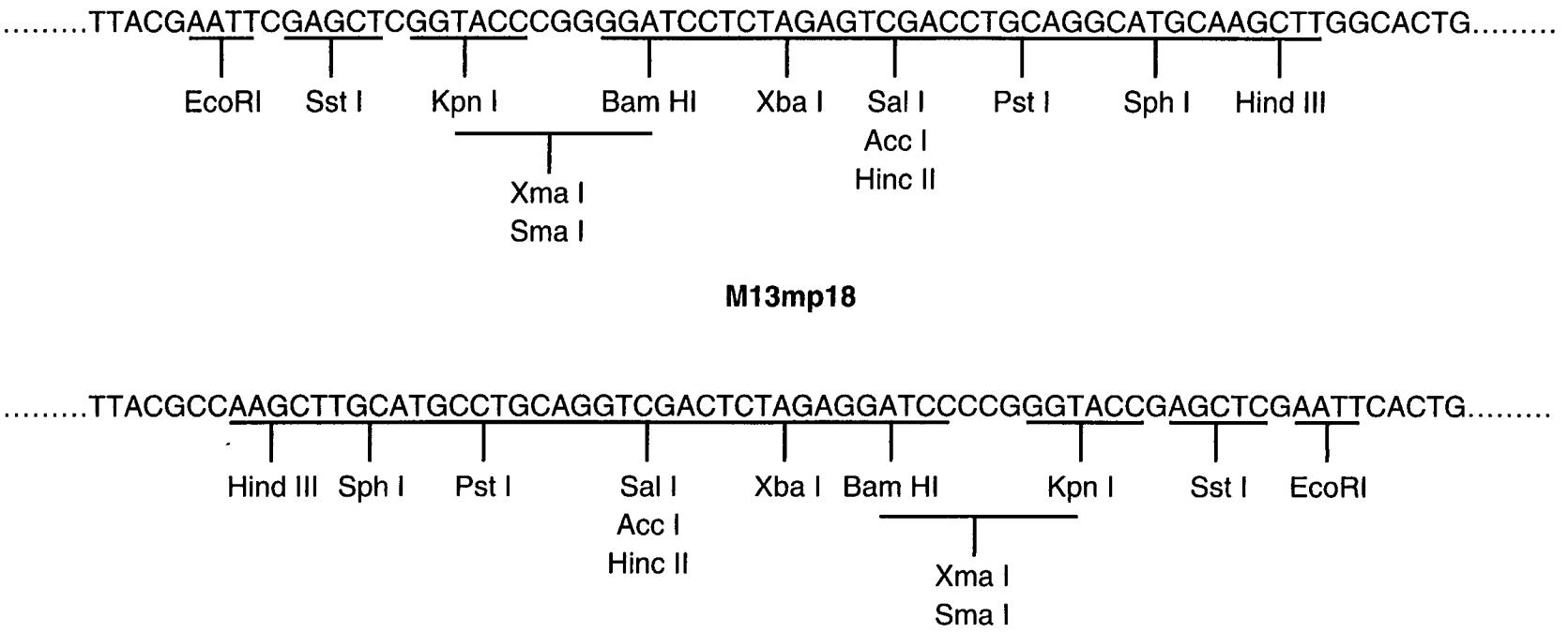


Fig. 2. Cloning sites in M13mp18 and M13mp19.

of the inducer IPTG and the indicator dye X-gal. Cells carrying phage with DNA inserted into the fragment sequence will not produce a functional fragment (or produce a poorly functioning fragment) and, hence, will generate colorless plaques. The presence of the proline synthesis genes on the F' allows growth in the absence of proline. Therefore, a positive selection exists for its presence.[5]

2.3 T7 DNA Polymerase

Native T7 DNA polymerase consists of two subunits—a 84,000 dalton polypeptide derived from gene 5 of T7 bacteriophage and a 12,000 dalton polypeptide, thioredoxin, coded by *E. coli*.[7,8] Native T7 DNA polymerase has 5' to 3' polymerase and 3' to 5' exonuclease activity and has recently been shown to be highly effective in *in vitro* mutagenesis reactions.[9,10] It is a highly processive enzyme and completely copies the uracil-containing template. Its high rate of polymerization allows mutagenic reactions to be performed in less time than with T4 DNA polymerase. Figure 3 shows a comparison of the rate of polymerization of T4 and T7 polymerase. T7 DNA polymerase completely copies the template within 30 minutes, as evidenced by the presence of covalently closed circular (ccc) DNA, while it takes T4 DNA polymerase one hour for the same result. Mutagenic efficiencies are comparable with both enzymes.

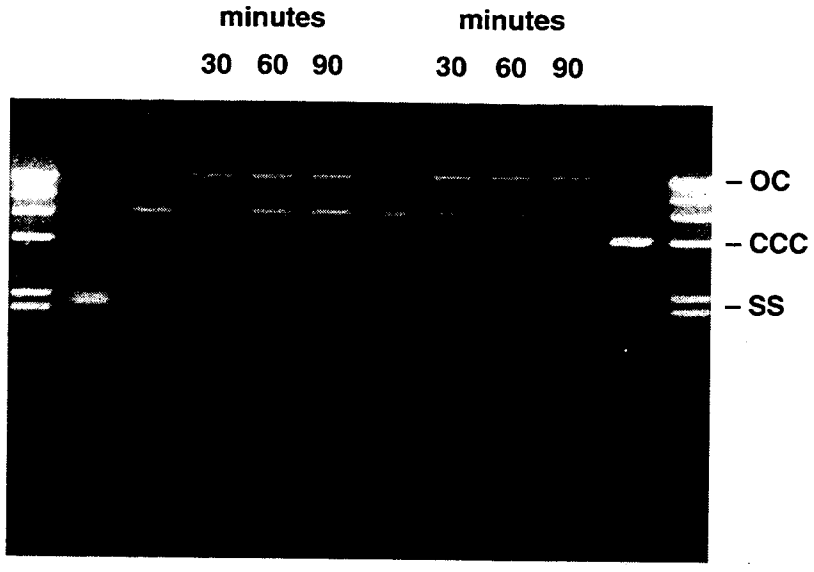
T7 DNA polymerase can also alleviate the need for T4 gene 32 protein. When copying templates with secondary structure, T4 DNA polymerase requires gene 32 protein to completely copy the template. Figure 4 shows the synthesis of T4 and T7 DNA polymerase on a M13mp7 uracil-containing template. M13mp7 contains a hairpin structure through which T4 DNA polymerase cannot synthesize. Addition of gene 32 protein allows complete copying of the template as evidenced by the presence of ccc DNA. T7 DNA polymerase can completely copy the template without gene 32 protein.

Like T4 DNA polymerase, T7 DNA polymerase does not perform strand displacement[11] and, therefore will not readily remove the hybridized primer. The Klenow fragment of *E. coli* DNA polymerase I, an enzyme commonly used in *in vitro* mutagenesis, on the other hand, does exhibit some strand displacement activity. We have found a substantial decrease in the mutation efficiency when using the Klenow polymerase.[12] The difference in the mutation efficiency may be due to the ability of the Klenow polymerase to peel off the mutagenic primer in a strand displacement reaction[13] and, hence, copy the template strand rather than leave in the desired mutation.

We have found that modified T7 DNA polymerase (Sequenase®), which has been chemically treated to inactivate the 3' to 5' exonuclease activity, is not effective in *in vitro* mutagenesis reactions (data not shown). It is also noteworthy that some sources of unmodified T7 DNA polymerase also do not work in these reactions (data not shown), which indicates that the enzyme may be inadvertantly modified during the purification procedure.

2.4 T4 DNA Ligase

T4 DNA ligase is used to ligate the newly synthesized DNA strand to the 5' end of the oligonucleotide primer. The importance of this step has been demonstrated.[12] Using the control reagents supplied with the kit, in the absence of ligase we observed no covalently closed circular DNA on ethidium bromide-containing agarose gels (see Section 3.5), low biological activity, and a low frequency of mutation. Although in some experiments, it is possible to get good mutagenesis without *in vitro* ligation,[3,14] we recommend its use since, in at least the experiments described in Reference 12, it is necessary.

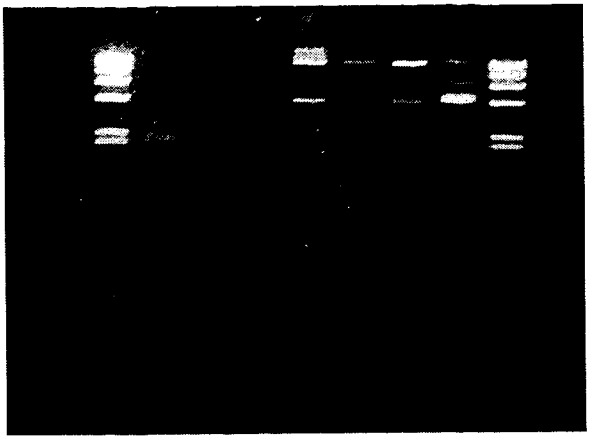


lane	1	2	3*	4	5	6	7*	8	9	10	11	12
pfu/ng	–	–	5	30	74	77	22	106	86	94	–	–
%mutants	–	–	0	47	54	63	0	52	54	57	–	–

*no primer

Fig. 3. Comparison of the rates of synthesis of T4 DNA polymerase vs. T7 DNA polymerase. Buffers, enzymes, template, and primer were from Muta-Gene *in vitro* mutagenesis kits. Annealing and synthesis conditions were as described in the manual. The reactions represented in lanes 3–6 contained 1 unit of T4 DNA polymerase; and the reactions represented in lanes 7–10 contained 0.5 unit of T7 DNA polymerase. Lanes 3 and 7 were no primer controls. All reactions contained 3 units of T4 DNA ligase and were incubated for the times shown. The template was M13mp11 uracil-containing DNA with an amber mutation at codon 16 of the *lacZ'* fragment, yielding colorless plaques in the presence of X-gal and IPTG. The mutagenic primer was a 16-mer (5' pGGTTTT CCCAGTCACG 3') that reverts the amber mutation back to wild-type, yielding blue plaques in the presence of X-gal and IPTG. Mutants are scored by counting the number of blue vs. white plaques. Lanes 1 and 12 are λ-*Hin*dIII fragments as relative size standards. Lane 2 is M13mp11 single-stranded uracil-containing template. Lane 11 is double-stranded M13mp11 DNA. The gel was 1% agarose containing 0.5 μg/ml ethidium bromide.

oc: open circles; ccc: covalently closed circular DNA; ss: single-stranded template.



lane 1 2 3* 4 5 6 7* 8 9

*no primer

Fig. 4. Comparison of T4 DNA polymerase and T7 DNA polymerase on synthesis around M13mp7 DNA. Annealing and synthesis conditions were as described for Figure 3. The reactions represented in lanes 3–5 contained 1 unit of T4 DNA polymerase. The reaction represented in lane 5 contained 2 µg of gene 32 protein. The reactions in lanes 6 and 7 contained 0.5 unit of T7 DNA polymerase. Lanes 3 and 6 were no primer controls. All reactions contained 3 units of T4 DNA ligase and were incubated for 90 minutes. The template was M13mp7 uracil-containing DNA. The primer was a 16-mer (5'pGGTTTTCCTAGTCACG3') which changes the UGC codon (trp) in *lacZ'* to UAG (amber). Lanes 1 and 9 are λ-*Hin*dIII fragments as relative size standards. Lane 2 is M13mp7 single-stranded uracil-containing template. Lane 8 is double-stranded M13mp7 DNA. The gel was 1% agarose containing 0.5 µg/ml ethidium bromide.

oc: open circles; ccc: covalently closed circular DNA; ss: single-stranded template.

2.5 Annealing Buffer and Synthesis Buffer

These buffers contain all components necessary for annealing the mutagenic oligonucleotide and for synthesizing the complementary strand from the U-containing template. The buffers are functionally tested in oligonucleotide-directed mutagenesis assays.

10x Annealing Buffer is 200 mM Tris, pH 7.4 (at 37 °C), 20 mM $MgCl_2$, 500 mM NaCl.

10x Synthesis Buffer is 5 mM each dATP, dCTP, dGTP and TTP; 10 mM ATP, 100 mM Tris, pH 7.4 (at 37 °C), 50 mM $MgCl_2$, 20 mM DTT.

2.6 Control Reagents

Several reagents in the kit allow you to functionally test the various procedures used with the Muta-Gene M13 kit. Detailed protocols are given in Section 3.7 and the accompanying specifications sheets.

Amber Phage. This is a phage which was constructed, by oligonucleotide mutagenesis, to contain an amber mutation at codon 16 of the *lacZ'* fragment of M13mp11. The UGG codon (tryptophan) was converted to the chain terminating codon UAG. Since the phage contains an amber mutation in the *lacZ'* fragment and is, therefore, unable to complement *lacZ'* host strains, it yields clear plaques on plates containing IPTG and X-gal (the amber mutation is not significantly suppressed by the *sup* E in MV1190 to give a blue plaque). This phage stock was prepared on *E. coli* MV1190 and therefore does not contain uracils in the packaged DNA. This phage allows you to test the functioning of the *dut, ung* double mutant CJ236 strain. The M13 amber-1 phage are used to infect CJ236. Phage recovered from the CJ236 are then transformed into MV1190 and CJ236 in side-by-side reactions. If the CJ236 is funtioning properly, there should be 10^2 to 10^4 fold fewer plaques on the MV1190 plate. The DNA of this stock can then be extracted and used as a template in a mutagenesis experiment using the control primer (see below) as a test of the DNA purification procedure.

U-amber DNA. This DNA was purified from M13 amber-1 after growth on CJ236 and, therefore, contains uracils. This DNA is included to serve as a control for the *in vitro* mutagenesis DNA synthesis reactions, in conjunction with the control primer (see below), as well as for transformation of the reaction product in *E. coli* MV1190.

Control Primer. A phosphorylated 16-base oligonucleotide which will revert the mutation in the M13 amber-1 phage is included in the kit. This oligonucleotide has the sequence 5'pGGTTTTCCCAGTCACG3' and will revert the amber mutation UAG back to the wild-type UGG codon for tryptophan. The underlined base is that which is reverted in M13 amber-1 phage by this primer. This oligonucleotide is included in the kit for use as the primer for the control *in vitro* mutagenesis reaction on M13 amber-1 DNA.

Section 3
Protocols

This section describes various procedures used with the Muta-Gene kit. When using new kit reagents, or if you have any question about any of the reagents that you have prepared, a control reaction using the test reagents included with the Muta-Gene kit should be performed. Instructions for use of the test reagents are given in Section 3.7. Troubleshooting hints are included with each section.

3.1 Bacteriology

For detailed instructions on preparation of media, sterile techniques, titering of phage, etc., two manuals from Cold Spring Harbor Laboratory are very useful.[15,16]

Media

LB (L broth)
10 g Bactotryptone
5 g yeast extract
10 g NaCl
Deionized H_2O to 1 liter

2x YT
16 g Bactotryptone
10 g yeast extract
5 g NaCl
Deionized H_2O to 1 liter

H Medium
10 g Bactotryptone
5 g NaCl
Deionized H_2O to 1 liter

Glucose-Minimal Media
6 g Na_2HPO_4
3 g KH_2PO_4
0.5 g NaCl
1 g NH_4Cl
Deionized H_2O to 1 liter
After autoclaving, add the following filter-sterilized solutions:
1 ml of 1 M $MgSO_4$
0.5 ml of 2% thiamine HCl in deionized H_2O
10 ml of 20% glucose

To prepare agar plates, add 15 g Bacto-Agar to the mixture (1 liter) before autoclaving, cool to ≈60 °C before pouring. To prepare top agar, add 0.7 g Bacto-Agar/100 ml and autoclave.

Chloramphenicol: The stock solution is 30 mg/ml in 100% ethanol. Store in aliquots at -20 °C. The working concentration is 30 µg/ml for solid media and 15 µl/ml for liquid. Add just before pouring plates or after liquid medium has cooled below 60 °C.

IPTG (isopropyl ß-D thiogalactopyranoside): The stock solution is 100 mM in deionized water. Filter sterilize.

X-gal (5-bromo 4-chloro 3-indolyl ß-D-galactoside): The stock solution is 2% in dimethylformamide.

Growth of Bacterial Strains

M13 phage require pili for entry into the cell. These pili are part of the fertility function of E. coli. **Since pili are not formed below 35 °C, all growth should take place at 37 °C.** Both the cloning/selecting strain MV1190 and the uracil-inserting strain CJ236 contain plasmids, called F' plasmids, which code for the functions required to manufacture pili. Since the cell must exert energy to maintain these plasmids, they will be lost if selective pressure is not maintained.

E. coli MV1190 has lost some of the chromosomal genes for synthesis of proline and the entire *lac* operon. The F' carries the *lac* operon except for a small segment of the ß-galactosidase gene and the missing genes for proline synthesis. By requiring synthesis of proline, selection is maintained for the F'. Because of this, MV1190 should be streaked on a glucose-minimal medium plate, and well-isolated colonies on this plate should be used to inoculate liquid cultures. The defective ß-galactosidase gene can be complemented by the *lacZ'* segment coded for on the M13 phage, thus allowing blue-white screening for inserts.

The F' in *E. coli* CJ236 has the genes for chloramphenicol resistance inserted into it. Therefore, in order to maintain the F', CJ236 should always be grown in the presence of chloramphenicol. It should be streaked on an LB (or H) plate containing chloramphenicol, and colonies from this culture should be used to inoculate liquid medium containing chloramphenicol. A working culture is obtained by streaking from the glycerol stock supplied onto an LB (or H) plate containing chloramphenicol. Well-isolated single colonies from this plate are used to inoculate every overnight culture. These plates can be used for 2–3 months, then a new plate is

made by streaking from the original stock supplies, NOT from the plate. The concentration of chloramphenicol should not exceed the recommended 30 µg/ml in solid media or 15 µl/ml in liquid media. CJ236 has a doubling time about twice that of MV1190.

Both cultures are supplied in 15% glycerol and should be stored frozen (-20 °C or lower).

Titering Phage

The following protocol may be used to titer non-uracil containing phage on MV1190 or on CJ236 and uracil-containing phage on CJ236. Because uracil-containing phage are inactivated on MV1190, to titer these phage on MV1190, it is necessary to plate the three most concentrated dilutions.

1. From a glucose-minimal medium plate with MV1190, inoculate 20 ml of LB. For CJ236, inoculate 20 ml of LB containing chloramphenicol with a colony from a chloramphenicol-containing plate. Grow overnight at 37 °C with shaking.

2. Prepare four tubes of top agar by melting it in boiling water or in a microwave oven, pipetting 2.5–3 ml of agar into sterile tubes, and keeping these tubes at 50–55 °C until use.

3. Prepare four serial 100-fold dilutions of the phage stock (10^2-fold, 10^4-fold, 10^6-fold, 10^8-fold) in sterile tubes in LB.

4. Place 0.2 ml of the overnight culture of *E. coli* into each of four sterile tubes. Add 100 µl of the 10^4 dilution to the first tube, 100 µl of the 10^6 dilution to the second and 100 µl of the 10^8 dilution to the third. No addition is made to the fourth tube (it is a control). If titering uracil-containing phage on MV1190, use the 10^2 dilution instead of the 10^8 dilution.

5. After 5 minutes at room temperature, pour a tube of top agar into one of the tubes containing culture and phage, mix thoroughly by inverting or gently vortexing, and pour onto a plate, swirling to cover the entire plate with top agar. Repeat with the other tubes.

6. Allow the top agar to harden for 15 minutes, then invert the plates and incubate at 37 °C overnight.

7. The following morning, count the plaques on the plate which has a countable number on it. Since the phages do not kill the cells extruding them, only slow their growth, the plaques will be turbid circles on a denser background.

   titer = (# of plaques x 10 x dilution factor) pfu/ml

3.2 Cloning into M13 Vectors

The first step in the oligonucleotide-directed mutagenesis process is to clone the DNA to be mutated into the M13 vector. DNA molecules may be cloned into M13mp18 and M13mp19 (M13 vectors carrying amber mutations such as M13mp8 cannot grow on CJ236) by using standard protocols such as those found in Maniatis *et al.*[17] or Bio-Rad's M13 Cloning/Sequencing Instruction Manual.

The cloning reaction should then be transformed into MV1190. Two protocols are given for the preparation of cells competent to take up DNA. Both rely on the cell wall disruption caused by high concentrations of calcium ions. In the first method, cells are prepared on the day they are to be used. When kept on ice, these cells will retain their competence for about 24 hours. Alternatively, cells can be resuspended in 15% glycerol at the final step and frozen in liquid nitrogen or dry ice/ethanol. If kept at -70 °C, these cells will retain their competence for several months.

Alternatively the phagemid DNA may be transformed into the host cells by electroporation. Electrocompetent MV1190 (catalog number 170-3115) and CJ 236 (catalog number 170-3114) may be purchased from Bio-Rad and used in conjunction with the Bio-Rad Gene Pulser II (catalog number 165-2105) and Pulse Controller II or Pulse Controller PLUS (catalog number 165-2109 or 165-2110, respectively). Another option is the use of the E coli Pulser (catalog number 165-2102). Efficiencies on the order of 10^8-10^{10} colonies/μg are routinely attained via electroporation. Call Bio-Rad's technical service at 1-800-4BIORAD for more information.

It is very important to keep the bacteria ice-cold during and after the first resuspension step in both protocols. All pipets and solutions should be pre-chilled to ice-water (not just refrigerator) temperature.

Competent Cell Preparation

Procedure A

1. Streak MV1190 on a glucose-minimal medium plate. Grow at 37 °C for 1–2 days until well-defined colonies appear. This plate can be stored in the refrigerator and used to inoculate liquid cultures for 1–2 months. The night before you plan to do the transformations and platings, inoculate 10 ml of LB medium. Grow overnight at 37 °C with shaking. This overnight culture (ONC) will be used both as a plating culture and as inoculum for competent cell production. As a plating culture, it will remain viable for at least 1 week if kept refrigerated except when in use.

2. Read the $O.D._{600}$ of the overnight culture. (A 1/10 dilution gives an accurate reading.)

3. To prepare competent cells, inoculate 40 ml of LB medium with enough of the overnight culture to give an initial absorbance reading of approximately 0.1. Save the rest on ice or in a refrigerator for use as a plating culture. Incubate with shaking at 37 °C.

4. When the culture reaches an absorbance of 0.8–0.9 (approximately 2 hours—dilute 1/3 for accurate reading), harvest the culture by centrifugation at 0 °C. For easier resuspension, centrifuge only long enough to pellet the cells. (For example, 5 minutes at 5,000 rpm in a Sorvall Superspeed centrifuge.) Carefully pour off the supernatant and drain well.

5. With a pre-chilled pipet, resuspend the pellet in 1 ml of cold 50 mM $CaCl_2$. Resuspend gently by swirling. If it does not easily resuspend, gently resuspend by pipetting up and down. The cells are very fragile at this stage. Add an additional 19 ml of cold $CaCl_2$ (final volume of 20 ml).

6. Hold on ice 20–30 minutes.

7. Harvest the cells again by gentle centrifugation. Resuspend in 1 ml 50 mM $CaCl_2$. Again, use a pre-chilled pipet and resuspend gently. Add an additional 3 ml of cold $CaCl_2$ (final volume of 4 ml). The cells are now competent to take up DNA and will remain so for about 24 hours. This procedure yeilds sufficient competent cells for about 20 transformations.

Procedure B (Cells prepared for storage at -70 °C)

1. Inoculate 200-250 ml of LB in a 500 ml Erlenmeyer flask to an $O.D._{600}$ of 0.1 from an overnight culture of MV1190, prepared as described in the previous procedure. Incubate with shaking at 37 °C.

2. When the $O.D._{600}$ reaches 0.9, harvest the culture by centrifugation at 0 °C. For easier resuspension, centrifuge only long enough to pellet the cells (5 minutes at 5,000 rpm in a Sorvall Superspeed centrifuge). Carefully pour off the supernatant and drain well. Resuspend the cells very gently in 50 ml of ice-cold 100 mM $MgCl_2$. If it is difficult to obtain complete suspension, the cells may be gently pipetted up and down with a chilled pipet.

3. Again harvest the cells and drain the pellets well. Resuspend the cells gently in 10 ml of 100 mM $CaCl_2$ until a smooth suspension is obtained. Add 100 ml of 100 mM $CaCl_2$, mix, and keep the cells on ice 30-90 minutes.

4. Again harvest the cells and drain the pellets well. Resuspend in 12.5 ml of 85 mM $CaCl_2$ and 15% glycerol.

5. Immediately aliquot the cell suspension in 0.5-0.6 ml portions and freeze in either dry ice/ethanol or liquid N_2. These cells will retain competence for at least 6-9 months if kept at -70 °C. To use, simply thaw on ice and proceed as below.

Transformation

The following protocol may be used to transform the competent MV1190 prepared in the preceding protocol. Transformations are performed at two stages of the *in vitro* mutagenesis procedure: i) when initially cloning the DNA fragment to be mutagenized into M13 phages; ii) after synthesis of the mutagenized strand on the uracil-containing template. The transformation protocol described here may be used in either case. Slight differences for the two situations are noted in the instructions.

1. For each transformation, place 0.3 ml of competent cells in a cold, 1.5 ml sterile polypropylene tube. Keep the tube on ice. If frozen competent cells are used, thaw them **on ice**. Do not allow the temperature of the cell suspension to rise above that of the ice, as competence will be rapidly lost.

2. Add 1–10 ng of a ligation reaction when cloning into M13, or 3–10 µl of synthesis reaction after dilution with stop buffer as described in Section 3.5; mix **gently**, and hold on ice for 30–90 minutes.

4. Heat shock the cells by floating the tubes in water at 42 °C for 3 minutes, and return to ice.

5. Immediately proceed to plating the transformants:

a. Mix well. Add 10, 50, or 100 µl of the transformed cells to 0.3 ml of an MV1190 ONC. (Prepared as described in "Competent Cell Preparation," Section 3.2, Procedure A, Step 3.) The mixture is conveniently set up in a sterile 13 x 100 mm test tube.

b. Add 50 µl 2% X-gal (dissolved in dimethyl formamide) and 20 µl 100 mM IPTG to 2.5 ml of molten top agar that has been cooled to about 50 °C. Add this to the cells, mix by vortexing, and immediately pour on H agar plates.

c. Allow the top agar to solidify for about 10 minutes, invert and incubate at 37 °C overnight.

The following morning, plaques should be visible. Those without inserts will be blue; those with inserts will be clear. Pick clear plaques by inserting a sterile Pasteur pipet through the plaque and blowing the entire plug into 1 ml of TE. This will transfer 10^7 or more phage. The phage will be stable at 4 °C for several months. (It is important to pick fresh plaques. Don't allow the plates to remain in the incubator longer than overnight.)

A stock of recombinant phage should be grown and titered. To verify that the insert is of the proper size, a small DNA preparation (miniprep) from phage-infected cells can be made by one of several methods.[18] The DNA is then cut with the same enzyme as that used to clone the segment, and the reaction electrophoresed on an agarose gel. The remainder of the phage stock is used to prepare uracil-containing phage DNA.

3.3 Growth of Uracil-Containing Phage

One of the major factors in performing successful mutagenesis experiments using this system is the preparation of uracil-containing phage carrying the fragment to be mutagenized. We have determined through rigorous testing that the strain CJ236 included in the Muta-Gene kit is *dut, ung*. Controls for checking the phenotype of the strain are described in Section 3.7. In growing phage on this strain for the purpose of obtaining uracil-containing DNA, the most important factor is to keep the multiplicity of infection low so that progeny phage are not contaminated by unadsorbed phage from the inoculum. These will not have been passaged through the *dut, ung* strain and therefore will not be selected against in the subsequent transformation. This will result in a decreased frequency of mutagenesis. The following protocol insures the appropriate multiplicity of infection and results in an amount of DNA sufficient for at least 10 mutagenesis experiments.

1. Streak out CJ236 onto an LB plate containing chloramphenicol. Grow at 37 °C until distinct colonies appear.

2. Pick an isolated colony and place in 20 ml of LB containing 15 µg/ml chloramphenicol. Incubate with shaking at 37 °C overnight.

3. Inoculate 50 ml of 2xYT medium containing15 µg/ml chloramphenicol with 1 ml of the overnight culture of CJ236 in a 250 ml flask. Incubate with shaking at 37 °C. Only 30 ml of this culture will be used to isolate uracil-containing DNA. The remainder is surplus for reading O.D.s.

4. Grow to an $O.D._{600}$ of 0.3, which will take from 1 to 4 hours. This corresponds to **approximately** $1x10^8$ cfu/ml. Add the phage to obtain an M.O.I. (multiplicity of infection) of 0.2 or less (*i.e.*, 0.2 phage/cell).

5. Incubate with shaking at 37 °C for 4–6 hours.

6. Transfer 30 ml of the culture to a 50 ml centrifuge. Centrifuge at 17,000 x g (12K rpm in the Sorvall SS-34 rotor) for 15 minutes. Transfer supernatant containing the phage particles to a fresh centrifuge tube. Recentrifuge this at 17,000 x g for 15 minutes at 0–4 °C.

7. Transfer the second supernatant to a fresh polyallomer centrifuge tube and add 150 µg of RNase A. (See Reference 16 for details on preparing DNase-free RNase.) Incubate at room temperature for 30 minutes.

8. To the supernatant, add 1/4 vol. of a solution containing 3.5 M ammonium acetate and 20% PEG 8000, mix thoroughly, and hold on ice for at least 30 minutes. **Note:** It is important to use a polyallomer tube since the PEG precipitate does not stick to polycarbonate.

9. Centrifuge at 17,000 x g for 15 minutes at 4 °C, pour off the supernatant carefully, and drain thoroughly. Wipe off surplus fluid.

10. Resuspend in 200 µl of high salt buffer (300 mM NaCl, 100 mM Tris, pH 8.0, 1 mM EDTA). Hold on ice for 30 minutes. Centrifuge for 2 minutes in a microcentrifuge to remove insoluble material. Transfer supernatant to a fresh tube. Store at 4 °C. DNA from this preparation should be extracted within 1 week.

11. Titer the stock on CJ236 and MV1190 (see Section 3.1). If infection was productive, there will be at least 5 x 10^{11} pfu/ml. If the phage DNA contains sufficient uracil to be inactivated in MV1190, the efficiency of titer on MV1190 will be 10^4-fold lower or less than on CJ236. Low phage yields may be obtained if the inserted DNA is large (3 kb or more). In this case, it may be necessary to grow 100 ml or more instead of 20 (resuspend the PEG-precipitated phage in the same volume (200 µl) of TE). Alternativley, the insert can be cloned into a phagemid vector (refer to Muta-Gene Phagemid *In Vitro* Mutagenesis Kit Manual and Reference 22). If the efficiency of growth on the *dut+ ung+* strain MV1190 is too high, either the multiplicity of infection was too high or the *dut- ung-* strain CJ236 was contaminated. The presence of chloramphenicol in the growth medium prevents the growth of most other bacteria, but it may be necessary to go back to the original culture from the kit and grow from a single colony on a chloramphenicol-containing plate.

3.4 Extraction of DNA

After a suitable phage stock that has uracil-containing viral DNA has been obtained, the DNA must be purified by extraction prior to its use as template in the *in vitro* mutagenesis reactions. The procedure for extracting the viral DNA is described in this section. The extractions are conveniently performed in standard 1.5 ml microcentrifuge tubes.

Alternatively, mutagenesis-grade templates may be purified without organic extraction with Bio-Rad's Prep-A-Gene DNA purification kit.

1. Extract the entire 200 µl phage stock 2x with an equal volume of neutralized phenol (see Reference 16 for neutralization procedures), 1x with phenol/chloroform (1:1:1/48 phenol: chloroform:isoamyl alcohol), and several times with chloroform/isoamyl alcohol. Continue the chloroform extractions until there is no visible interface, then once more. It is important to vortex at least the first three extractions vigorously for 30 seconds or more. The yield can be increased 30–50% by back-extracting each step: add 100 µl of TE (10 mM Tris, pH 8.0, 1 mM EDTA) to the first phenol extraction tube, vortex, add resultant aqueous phase to the next phenol extraction tube, vortex, and so on.
2. Pool the aqueous phases, add 1/10 vol. 7.8 M ammonium acetate and 2.5 vol. ethanol. Keep at -70 °C at least 30 minutes.

3. Centrifuge 15 minutes in the cold, carefully remove supernatant, wash gently with 70% ethanol, and resuspend the pellet in 20 µl TE. Avoid dissolving any residue that may cling to the side of the tube.

4. Transfer to a fresh tube. Run a small aliquot on an agarose gel with a known amount of single-stranded DNA to detemine concentration. Typically, a few µg DNA are obtained. Since only 0.2 µg is used in an *in vitro* synthesis reaction and this reaction should yield tens of thousands of transformants, it is not necessary to isolate large amounts of DNA.

3.5 Synthesis of the Mutagenic Strand

After preparation of single-stranded uracil-containing DNA from the M13 phage, the mutagenic strand may be synthesized. This is accomplished by priming the synthesis from the single-stranded DNA with the oligonucleotide containing the sequence of the mutation(s) that you desire to insert. For discussions of various strategies for designing oligonucleotides for the purpose of inserting mutations, consult References 19 and 20.

After synthesis and purification of the oligonucleotide, it must be phosphorylated prior to its use as a primer in the synthesis reaction. Using the 16-base single-mismatch primer in the test reagents, the efficiency of mutagnesis was decreased three-fold when using a non-phosphorylated primer. Instructions for phosphorylating the oligonucleotide and for synthesis of the complementary strand are given in this section.

Phosphorylation of the Oligonucleotide

The oligonucleotide should be lyophylized after synthesis and resuspended in water at 10–20 pmol/µl. The following procedure was taken from Zoller and Smith[21] and been succesfully used at Bio-Rad.

1. Prepare the following reaction in a sterile 500 µl microcentrifuge tube:

Component	Volume	Total concentration/mass
oligonucleotide	varies	200 pmol
1 M Tris, pH 8.0	3 µl	100 mM
0.2 M $MgCl_2$	1.5 µl	10 mM
0.1 M DTT	1.5 µl	5 mM
1 mM ATP (neutralized)	13 µl	0.4 mM
sterile water	varies	to a total volume of 30 µl

2. Mix.
3. Add 4.5 units of T4 polynucleotide kinase.
4. Mix and incubate at 37 °C for 45 minutes.
5. Stop by heating at 65 °C for 10 minutes.
6. Dilute the oligonucleotide to 6 pmol/µl with TE. Store frozen.

Annealing of the Primer to the Template

1. Prepare the following reaction mix in a 500 µl microcentrifuge tube:

Component	Volume	Final concentration/mass
uracil-containing DNA	varies	200 ng (0.1 pmol)
mutagenic oligonucleotide	varies	2–3 pmol
10x annealing buffer	1 µl	20 mM Tris, pH 7.4 2 mM $MgCl_2$ 50 mM NaCl
water	varies	to a total volume of 10 µl

Note: The molar ratio of primer to template in this reaction is between 20:1 and 30:1 for a 16-mer oligonucleotide and a 7,200 base template. These are the sizes of the primer and template included in the kit. Higher ratios can interfere with subsequent ligation.[12] Also, very high ratios of primer to template can result in a significant level of spurious priming from secondary hybridization of primer to the template.

2. Prepare a second reaction mixture containing all the above ingredients except the primer. This is a control reaction which will test for non-specific endogenous priming caused by contaminating nucleic acids in the template preparation. This test is important because endogenous priming may result in reduced mutation efficiency. Properly prepared templates should result in little, if any, covalently closed circular DNA synthesized in the absence of added primer (see Figures 3 and 4).
3. Place the reaction in a 70 °C water bath. Allow it to cool in the water bath at a rate of approximately 1 °C/minute to 30 °C over a 40-minute period. After this, place the reactions in an ice-water bath. **These annealing conditions have been optimized for use with the control reagents supplied with the kit. It will be necessary to optimize conditions for the particular oligonucleotide and template used. These conditions are expected to vary widely.**[3]

Synthesis of the Complementary DNA Strand

1. The T7 DNA polymerase is supplied at 1 unit/µl and must be diluted to 0.5 unit/µl for use. Make a 1:2 dilution of the T7 DNA polymerase using cold 20 mM potassium phosphate, pH 7.4, 1 mM dithiothreitol, 0.1 mM EDTA, 50% glycerol as the dilution buffer. Since the enzyme is not as stable at the lower dilution, dilute an amount necessary for immediate use only.

2. With the reaction still in the ice-water bath, add the following components in the order listed:

Component	Volume	Final concentration/mass
10x synthesis buffer	1 µl	0.4 mM each dNTP
		0.75 mM ATP
		7.5 mM Tris, pH 7.4
		3.75 mM $MgCl_2$
		1.5 mM DTT
T4 DNA ligase	1µl	2–5 units
T7 DNA polymerase	1µl	0.5 unit

Note: The final reaction conditions used for DNA synthesis are: 23 mM Tris (7.4), 5 mM $MgCl_2$, 35 mM NaCl, 1.5 mM DTT, 0.4 mM dATP, dCTP, dGTP, and TTP, 0.75 mM ATP, plus the nucleic acids and enzymes, as given in the chart above.

3. Incubate the reaction on ice for 5 minutes (to stabilize the primer by initiation of DNA synthesis under conditions that favor binding of the primer to the template3) then, incubate at 25 °C for 5 minutes, and finally at 37 °C for 30 minutes.

4. After 30 minutes, add 90 µl of stop buffer (10 mM Tris, pH 8.0, 10 mM EDTA) to the reaction and stop the reaction by freezing. The reaction is stable at -20 °C for at least 1 month for use in the subsequent transformation.

Gel Analysis of the Reaction Products

The reaction products should be analyzed on a 1% agarose gel in 1x Tris-acetate buffer that contains 0.5 µg/ml ethidium bromide. The ethidium bromide binds to covalently closed circular (ccc), relaxed DNA and causes positive supercoils. This condensation causes the DNA to migrate more rapidly through the gel. The second strand synthesis reaction results in the formation of relaxed ccc DNA, and, hence, a band migrating slower than single-stranded (ss) plasmid DNA indicates successful conversion to the biologically

active ccc DNA (see Sections 2.3 and 2.4). An example of an ethidium bromide-agarose gel analysis of an *in vitro* mutagenesis experiment is presented in Figure 3. In the absence of primer, little single-stranded template is converted to ccc DNA, indicating that the template is not contaminated with impurities which can prime complementary strand DNA synthesis. At primer-to-template ratios of 20:1 to 30:1 we typically convert 50–80% of the ss DNA template to primarily double-stranded material which includes both ccc and oc (open circle) DNA forms, 10–50% of this material is converted to ccc DNA when ligase joins the newly synthesized strand to the 5' end of the mutagenic oligonucleotide. Production of ccc DNA in the absence of added primer is indicative of contaminated DNA template, and new template should be purified from a fresh phagemid stock. Failure to produce ccc DNA when primer has been added can result from a variety of causes. Common problems include inactive T7 DNA polymerase, failure of the primer to hybridize to the template, and improperly set-up reactions (see reference 3 for a complete discussion of possible causes).

Gel analysis of the reaction products may be performed as follows.

1. Add to 9 µl of each *in vitro* mutagenesis reaction, 1 µl of gel loading buffer (1% SDS, 0.25% bromophenol blue, 50% glycerol). Reactions that should be analyzed on the gel include the no-primer control and the complete reaction.

2. Set up one microcentrifuge tube containing 100 ng of the single-stranded form and another tube containing 100 ng of the double-stranded (ccc) form of the phagemid that is being mutagenized. Bring the volume of each tube to 9 µl with TE and add 1 µl of gel loading buffer. This DNA will serve as the marker to aid in identification of ccc DNA. If the control reactions have also been performed (as described in section 3.8), they should be electrophoresed on the same gel.

3. Run the samples on a 1% agarose gel containing 0.5 µg/ml ethidium bromide. A mini-gel is convenient for this purpose and electrophoresis is carried out until the bromophenol blue dye has migrated about halfway through the gel. It is not necessary to add ethidium bromide to the electrophoresis buffer if the gel is not run any longer than this. If electrophoresis is carried out beyond this point, the front of ethidium in the gel (which travels toward the negative pole) passes the fastest-migrating DNA bands and the ethidium bromide bound to the DNA is stripped off.

4. Destain the gel for 15 minutes in distilled water (there is no need to stain the gel, due to the presence of ethidium bromide in the gel) and then photograph the gel. Interpretation of the pattern has been discussed previously in this section and will be aided by reference to Figure 3. If little or no ccc DNA is observed in the reaction with added primer, refer to Section 3.7 (Use of Muta-Gene Phagemid Control Reagents). If there is a substantial amount of ccc DNA present in the reaction with primer added, compared to the no-primer control, the reaction products may be transformed as described later in this section.

3.6 Analysis of Transformants by DNA Sequencing

Since this procedure generally produces at least 50% mutants, screening is typically not necessary to find the desired mutation. For example, if the frequency is exactly 50%, and three plaques are picked, there is a 90% chance that one of them will be mutant. A good strategy would be to pick a fairly large number of plaques (ten or twelve), but, start by analyzing four. Once picked, the plaques can be stored indefinitely (see following protocol). It is possible to isolate enough DNA from an overnight miniprep to run 10 or more sequencing reactions. If the mutation is not too many bases away from the boundary between the insert and the polylinker region, the universal primer can be used for sequencing. If the mutation is farther away, however, it is best to synthesize a sequencing primer complementary to a more advantageous location. In any case, a properly performed sequencing reaction will reveal whether the desired mutation has been introduced.

1. In the morning, pick a fresh (less than 24-hours-old) plaque into 1 ml of TE. (A plug removed by a Pasteur pipet will transfer 10^7 or more phage.) Vortex briefly to resuspend phage. This resuspended plaque will remain viable for several months if kept refrigerated, so it is not necessary to analyze all plaques at once. It is important to plaque-purify the isolates, since if a large amount of DNA was used in the transformation reaction, one competent cell could have been transformed by more than one M13 DNA molecule. In this case, dilute the plaque suspension 10^3 and plate 100 µl. The next morning, pick one plaque again into 1 ml of TE buffer, and proceed as described below.

2. Streak some solution across an LB plate, allow to dry for 2 or 3 minutes, then pour top agar containing 0.3 ml of an MV1190 ONC across the plate starting at the more concentrated side. Incubate at 37 °C until plaques become visible (about 6 hours). It is important that the plates be relatively fresh.

3. Add a liberal scoop (1/8–1/4 of the top agar on the plate) from an area with many closely spaced plaques to 20 ml LB, and grow 4–6 hours at 37 °C with shaking. Remove the bacteria by centrifugation and precipitate with PEG as described in Section 3.3. This is a stock of presumptive mutant phage. Set aside an aliquot for further manipulation. The remainder, when processed as in Section 3.4, yields DNA of sufficient purity to be sequenced.

3.7 Use of the Muta-Gene M13 Control Reagents

These reagents are included so that the user can test all components of the *in vitro* mutagenesis procedure and try the protocols before committing valuable experimental material. Table 2 lists the reagents and their properties and uses.

Table 2 Muta-Gene M13 Control Reagents

Control Reagent	Description	Purpose
Amber Phage	M13 phage with an amber mutation in the *lacZ'* region. Plaques are clear on IPTG/X-gal plates. Grown on MV1190, so do not contain uracil in their DNA.	Test of the *dut, ung* phenotype of CJ236 and the complete *in vitro* mutagenesis procedure. The amber mutation will be reverted.
U-amber DNA	Uracil-containing single-stranded DNA of the amber Phage. Grown on CJ236 and purified.	Test of the *in vitro* mutagenesis synthesis reagents and the presence of active uracil N-glycosylase in MV 1190.
Control Primer	Phosphorylated 16-base oligonucleotide which will revert the amber mutation in *lacZ'*.	Used in conjunction with U-amber DNA to test the functioning of synthetic reagents.

Use of the Amber Phage to Test the *dut, ung* Phenotype of CJ236

1. From a chloramphenicol-containing plate with CJ236, inoculate 20 ml of LB. From a glucose-minimal medium plate with MV1190, inoculate 20 ml of LB. Grow both cultures overnight with shaking at 37 °C.
2. Inoculate 50 ml of 2xYT in a 250 ml flask with 1 ml of the overnight culture of CJ236 and incubate at 37 °C until early log phase (OD_{600} of 0.3–0.35). Save the remainder of both overnight cultures for plating.
3. Add 10 µl of the amber phage and incubate at 37 °C for a further 2 hours.
4. Prepare eight tubes of top agar by melting it in boiling water or in a microwave oven, pipetting 2.5–3 ml of agar into sterile tubes, and keeping these tubes at 50°–55 °C until use.
5. Spin 1 ml of the culture in a microcentrifuge for 2–3 minutes to remove bacteria and save the supernatant which contains the infectious phage particles.
6. Prepare four serial 100-fold dilutions of the phage stock (10^2-fold, 10^4-fold, 10^6-fold, 10^8-fold) in sterile tubes in LB.
7. Place 0.2 ml of the overnight culture of CJ236 into each of four sterile tubes. Add 100 µl of the 10^4 dilution to the first tube, 100 µl of the 10^6 dilution to the second and 100 µl of the 10^8 dilutions to the third. The fourth is a control; no phage are added.
8. Place 0.2 ml of the overnight culture of MV1190 into each of four sterile tubes. Add 100 µl of the 10^2 dilution to the first tube, 100 µl of the 10^4 dilution to the second and 100 µl of the 10^6 dilutions to the third. The fourth is a control; no phage are added.
9. After 5 minutes at room temperature, pour a tube of top agar into one of the tubes containing culture and phage, mix thoroughly by vortexing, and pour onto a plate, swirling to cover the entire plate with top agar. Repeat with the other tubes.
10. Allow the top agar to harden for 15 minutes, then invert the plates and incubate at 37 °C overnight.
11. The following morning, count the plaques on the plate which has a countable number on it. Since the phages do not kill the cells extruding them, only slow their growth, the plaques will be turbid circles on a denser background.

titer = (# of plaques x 10 x dilution factor) pfu/ml

If the titer is high enough on CJ236 and low enough on MV1190 (indicating sufficient uracil in the phage DNA), the phenotype of CJ236 is *dut*, *ung* and the F' is present.

12. If desired, the phage can be PEG-precipitated, the DNA extracted, and this DNA used as template in an *in vitro* mutagenesis reaction (see below).

Use of the U-amber DNA and the Control Primer

1. Follow the protocol in Section 3.5 for annealing of the primer and synthesis of the mutagenic strand. The Quality Control test of these reagents consists of four reactions: one without primer, the other three with increasing amounts of primer. The specifications sheet which comes with the Muta-Gene M13 kit gives the number of plaques obtained from each reaction and the percentage which were mutant using the batch of reagents contained in the kit. From these data, the optimal amount of primer can be chosen. These instructions are found on a separate sheet entitled "Use of Muta-Gene M13 Control Reagents."

2. Analyze the reactions on an agarose gel as described in Section 3.5.

3. If the reactions are successful, transform the reaction products into competent MV1190. Prepare competent cells, and transform the reaction products as described in Section 3.2. The plaques containing reverted (mutated) amber phage will be blue, those with non-mutated phages will be white.

   We typically obtain between 60 and 80% mutants with this particular reaction, and 100–200 pfu/ng of template.

Section 4
References

1. Leatherbarrow, R. J. and Fersht, A. R., *Prot. Eng.*, **1**, 67–74 (1986).
2. Kunkel, T. A., *Proc. Natl. Acad. Sci. USA*, **82**, 488–492 (1985).
3. Kunkel, T. A., Roberts, J. D. and Zabour, R. A., *Methods Enzymol.*, **154**, 367–382 (1987).
4. Norrander, J., Kempe, T. and Messing, J., *Gene.*, **26**, 101–106 (1983).
5. Messing, J., *Methods Enzymol.*, **101**, 20–78 (1983).
6. Joyce, C. M. and Grindley, N. D. F., *J. Bacteriol.*, **158**, 636–643 (1984).
7. Modrich, P. and Richardson, C. C. J., *Mol. Biol.*, **250**, 5515–5522 (1975).
8. Mark, D. F. and Richardson, C. C., *Proc. Natl. Acad. Sci. USA*, **73**, 780–781 (1976).
9. Kunkel, T. A. in Nucleic Acids and Molecular Biology, eds. Eckstein, F. and Lilly, D. M. J., Springer–Verlag, Berlin Heidelberg, vol. 2, pp. 124–135 (1988).
10. Bebeneck, K. and Kunkel, T. A., *Nuc. Acids Res.*, **17**, 5408 (1989).
11. Lechner, R. L., Engler, M. J. and Richardson, C. C., *J. Biol. Chem.*, **258**, 11174–11184 (1983).
12. Geisselsoder, J., Witney, R. and Yuckenberg, P., *BioTechniques.*, **5**, 786–791 (1987).
13. Kramer, W., Drutsa, V. Jansen, G. W., Kramer, B., Pflugfelder, M. and Fritz, J., *J. Nucleic Acids Res.*, **12**, 9441–9456 (1984).
14. Kunkel, T. A., personal communication.
15. Miller, J. H., *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724 (1972).
16. Davis, R. W., Botstein, D. and Roth, J., *Advanced Bacterial Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724 (1981).
17. Maniatis, T., Fritsch, E. F. and Sambrook, J., *Molecular Cloning*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724 (1982).
18. Birnboim, H. C. and Doly, J., *Nucleic Acids Res.*, **7**, 1513–1523 (1979).
19. Botstein, D. and Shortle, D., *Science*, **229**, 1193–1201 (1985).
20. Craik, C. S., *BioTechniques.*, **3**, 10–19 (1985).
21. Zoller, M. J. and Smith, M., *Methods Enzymol.*, **100**, 468–500 (1983).
22. Mead, D. A., Szozesna-Skorupa, E. and Kemper, B., *Prot. Eng.*, **1**, 67–74 (1986).

BIO-RAD

Bio-Rad Laboratories

Molecular Bioscience Group

U.S. (800) 4BIORAD ***California*** *(510) 741-1000* ***Australia*** *02-9914-2800* ***Austria*** *(1) 877 89 01* ***Belgium*** *09-385 55 11* ***Canada*** *(905) 712-2771* ***China*** *(01)2046622* ***Denmark*** *39 17 9947* ***Finland*** *90 804 2200* ***France*** *(1) 49 60 68 34* ***Germany*** *089 31884-0* ***India*** *91-11-461-0103* ***Italy*** *02-21609 1* ***Japan*** *03-5811-6270* ***Hong Kong*** *7893300* ***The Netherlands*** *0318-540666* ***New Zealand*** *09-443 3099* ***Singapore*** *(65) 272-9877* ***Spain*** *(91) 661 70 85* ***Sweden*** *46 (0) 735 83 00* ***Switzerland*** *01-809 55 55* ***United Kingdom*** *0800 181134*

LIT-244 Rev B

SIG 070196 Printed in USA