Efficient site directed *in vitro* mutagenesis using ampicillin selection

Martin K.Lewis* and David V.Thompson
Promega Corporation, Madison, WI 53711, USA

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ABSTRACT

A novel plasmid vector pSELECT-1 is described which can be used for highly efficient site-directed *in vitro* mutagenesis. The mutagenesis method is based on the use of single-stranded DNA and two primers, one mutagenic primer and a second correction primer which corrects a defect in the ampicillin resistance gene on the vector and reverts the vector to ampicillin resistance. Using T4 DNA polymerase and T4 DNA ligase the two primers are physically linked on the template. The non-mutant DNA strand is selected against by growth in the presence of ampicillin. In tests of the vector, highly efficient (60 – 90%) mutagenesis was obtained.

INTRODUCTION

Site-directed *in vitro* mutagenesis is a valuable technique for among other things the study of critical amino acid residues involved in enzymatic activity, the study of DNA promoter and enhancer function and structure, the study of residues important in protein folding, the study of the structure of DNA binding sites for proteins, the study of functions of particular residues or domains in protein stability, the creation of mutant proteins with increased stability or resistance to environmental agents, the study of effects of removing sites for protein modification, such as phosphorylation or glycosylation and for engineering of expression clones.

Hutchison *et al.* (1) introduced a general method to obtain site-specific changes in DNA sequences using single-stranded DNA (ssDNA) and a synthetic oligonucleotide. The oligonucleotide is complementary to the single-stranded template DNA except for a region of mismatch in the center. Following hybridization, the oligonucleotide is extended with DNA polymerase to create a double-strand structure. The nick is sealed and the resulting heteroduplex is transformed into an *E. coli* host. Upon DNA replication and strand segregation, the cell contains a mixture of wild-type and mutant templates. Because mutant and wild-type plasmids are present in the same cell, a second round of transformation is generally employed to ensure genetic purity.

Though theoretically the yield of mutants in the above procedure should be 50% in practice, it is generally much lower, often only a few percent. Various selection techniques have been employed to increase the efficiency of site-directed *in vitro* mutagenesis (2,3). We describe a novel phagemid vector and selection technique which results in a high proportion (60–90%) of mutants.

MATERIALS AND METHODS

Materials

All restriction enzymes and DNA modifying enzymes were obtained from Promega Corporation. Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer using phosphoramidite chemistry. All chemicals were of reagent grade.

Construction of pSELECT-1

pSELECT-1 is a cloning vector specifically constructed for use in *in vitro* mutagenesis. The vector is a hybrid of the plasmids pBR322 (4,5) and pGEM-3Zf(+) (Promega Corporation, Madison, Wisconsin). The vector carries modified ampicillin and tetracycline resistance genes derived from pBR322 and in addition carries the polylinker and fl replication origin from pGEM-3Zf(+).

To construct pSELECT-1 (see Figure 1), the ampicillin resistance gene of pBR322 was inactivated by digesting the DNA with Pst I, blunting the ends using the Klenow fragment of DNA polymerase I and recircularizing the vector using T4 DNA ligase. This introduced a four-base frameshift which was checked by DNA sequencing and was found to make the vector ampicillin sensitive.

Ligation mixes were transformed into *E. coli* JM109 and plated on LB plates containing 15 μg/ml tetracycline. To clone the segments of pGEM-3Zf(+) into this modified pBR322, the former was digested with Aat II and Afl III and the latter with Aat II and Eco R1. The digests were mixed together and ligated for two hours, allowing the Aat II end of the pGEM-3Zf(+) fragment to ligate to the Aat II end of the modified pBR322. The DNA ends were then blunted by filling in with Klenow and the ligation then allowed to proceed overnight.

This step allows the recircularization of the recombinant plasmid by blunt end ligation of the filled Afl III and Eco R1 ends. The ligation mix was plated on LB plates containing tetracycline, IPTG and X-Gal and scored for tetracycline resistant

* To whom correspondence should be addressed

blue colonies. To obtain a colony which is both tetracycline resistant and blue would indicate the successful cloning of the pGEM −3Zf(+) Aat II-Afl III fragment (which carries the lac alpha peptide and hence confers blue color to JM109) into the tetracycline resistant modified pBR322 between the Aat II and Eco R1 sites. A blue tetracycline resistant colony was found and the structure of the resident plasmid was checked and found to be the correct fragment inserted into the modified pBR322. This plasmid was named pBR322ZF. It was predicted that the Eco R1 site should have been reformed at the Afl III-Eco R1 junction, and in fact restriction mapping indicated that this was the case.

Though the construct now contained the pGEM-3Zf(+) polylinker, many of these sites were no longer unique. In particular, the Hind III, Bam H1, Sph I and Sal I sites in the linker were also present in the tetracycline resistance (tet) gene. In order to remove these sites from the tet gene, another derivative of pBR322 was constructed. In this case only the F1 origin region from pGEM-3Zf(+) was cloned into the ampicillin sensitive pBR322 derivative on an Aat II-Eco R1 fragment between the Aat II and Eco R1 sites on this vector. This allowed one to make single-stranded DNA (ssDNA) containing the tet gene and hence modify this gene by site-specific *in vitro* mutagenesis. This vector was named pBR322F1. Single-stranded DNA was made from this vector by propagating the plasmid in *E. coli* NM522 and infecting with M13K07 helper phage. *In vitro* mutagenesis to remove the Hind III site was performed by hybridizing an oligonucleotide having the sequence pGCTTATCATCGATTA-GCTTTAATGCGG to the ssDNA. This oligonucleotide removes the Hind III site present in the tetracycline resistance gene promoter by changing the first A in the sequence AAGCTT to a T. About 0.1 μg of single-stranded template was used and an oligonucleotide:vector ratio of about 15. The hybridization conditions were 25 mM Tris-HCl pH 7.3, 12 mM MgCl2 and 60 mM NaCl in a volume of 25 μl. The annealing reaction was heated to 70°C for 5 minutes and then cooled to room temperature over the course of 15 minutes. Then all four deoxyribonucleotides (dATP, dCTP, dGTP, dTTP) were added to the reaction to a final concentration of 1 mM, 10 units of T4 DNA polymerase and 2 units of T4 DNA ligase. These additions increased the reaction volume to 35 μl. The fill in reaction was allowed to proceed for 90 minutes at 37°C at which point the entire reaction was transformed into competent BMH 71−18 mutS *E. coli* and the transformation mixture added to a 50 ml LB culture containing 15 μg/ml tetracycline and the culture grown up overnight.

Plasmid DNA was then prepared from this culture using a mini-prep procedure, the DNA was restricted with Hind III (to select for those mutants missing the Hind III site), transformed into *E. coli* JM109 and the cells plated on LB plates containing 15 μg/ml tetracycline. Two tetracycline resistant colonies were isolated and plasmid DNA prepared from these isolates. Restriction enzyme digestion indicated that both isolates had in fact deleted the Hind III site.

To delete the Bam H1, Sph I and Sal I sites from the tetracycline resistance gene, oligonucleotides were designed which removed each restriction site while keeping the amino acid sequence of the tet protein unchanged. The respective oligonucleotides used were pCCCGTCCTGTGGATTCTCTA-CGCCGG, pGGCGCCATCTCCTTACATGCACCATTCCT-TGCG and pTCGCATAAGGGAGAGCGCCGACCCATGC-CCTTG. In each case the mutagenesis procedure was followed essentially as above and basically involved a hybridization, an *in vitro* fill in, a transformation, a plasmid preparation, a restriction enzyme recut and a retransformation. This completed the engineering of the tetracycline resistance gene so that it would be useful when incorporated into the mutagenesis vector.

To transfer the modified tet gene into pBR322ZF, the gene was excised on a Cla I-Sty I fragment, gel purified and cloned into pBR322ZF between the Cla I and Sty I sites. Next, one of the two Eco R1 sites in the resulting vector was removed. The site removed was the one outside the polylinker and it was destroyed by partial Eco R1 digestion, filling with Klenow and religating, followed by restriction enzyme digestion to map which site was removed from isolates which cut only once with Eco R1. The resulting vector was named pSELECT-1.

RESULTS

Reversion of pSELECT-1 to Ampicillin Resistance

pSELECT-1 is a plasmid specially engineered for use in *in vitro* site-directed mutagenesis. The plasmid (see Figure 1) carries two antibiotic resistance markers. The plasmid carries an active tetracycline resistance gene and is initially propagated in a host in the presence of tetracycline. The ampicillin resistance gene on the vector has been inactivated by cutting at the Pst I site, blunting the ends with Klenow and religating to introduce a four base frameshift. We asked whether an oligonucleotide could be used in an *in vitro* mutagenesis protocol to revert the vector to ampicillin resistance. Using the oligonucleotide pGTTGCCATTGCTGCAGGCATCGTGGTG, which restores the Pst I site and the natural sequence to the ampicillin resistance gene, we found we could generate many ampicillin resistant colonies starting from single-stranded DNA and following the *in vitro* fill in reaction outlined in Materials and Methods. When the complement of the above oligonucleotide was used, no ampicillin resistant colonies were obtained.

Coupling the Ampicillin Repair Oligonucleotide to a Second Mutagenic Oligonucleotide

We sought next to test the idea that the ampicillin resistance oligonucleotide could be used as a tag for a second mutagenic oligonucleotide. This would provide an absolute selection against the parental DNA strand and assuming linkage between the two oligonucleotides only mutants would be ampicillin resistant. We chose first to test whether pSELECT-1 could be reverted to ampicillin resistance at the same time as a second oligonucleotide was incorporated which changed the phenotype of the plasmid from blue to white. Using a 27mer oligonucleotide which is complementary to a portion of the polylinker in pSELECT-1 and disrupts by frameshifting the lac alpha peptide, we performed mutagenesis to examine the linkage between this oligonucleotide and the ampicillin repair oligonucleotide. Since the parental strand (encoding a good lac alpha peptide) upon one round of transformation can coexist in the same cell as the mutated (white) newly synthesized strand, we expected blue color to be dominant and white mutants not to be evident until a second round of transformation. To our surprise, we found about twenty percent white colonies upon one round of transformation and to our satisfaction, about eighty-five percent whites upon two rounds of transformation. These values greatly exceeded the F′ loss rate of the strain which was estimated to be less than two percent. Apparently we had successfully coupled a second mutagenic oligonucleotide to the ampicillin repair oligonucleotide with high efficiency.

The basic scheme of the mutagenesis procedure is shown in

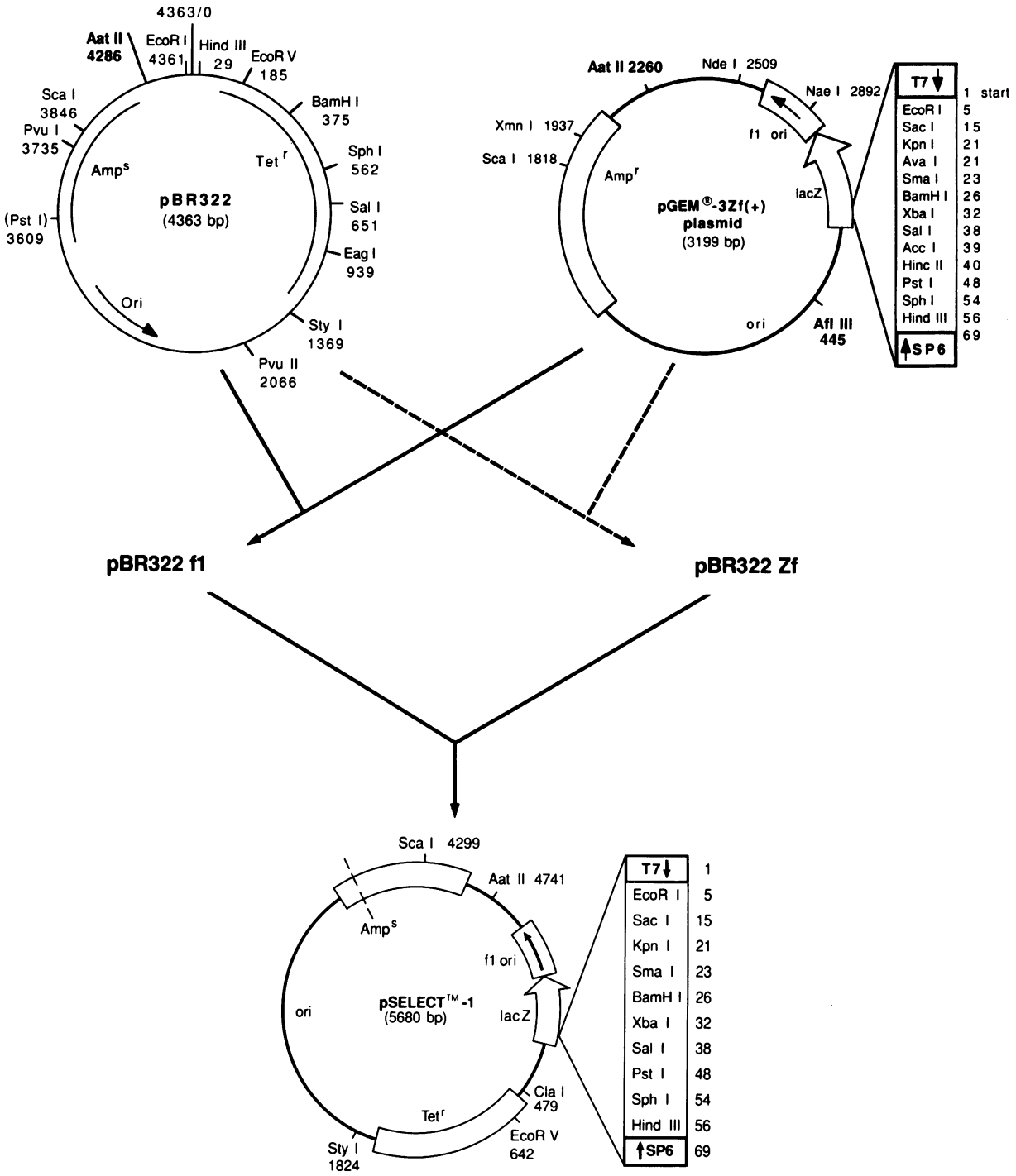

Figure 1. Diagram of the steps involved in the construction of the pSELECT-1 plasmid vector. pSELECT-1 is derived from pBR322 and pGEM-3Zf(+) via the intermediate plasmids pBR322F1 and pBR322ZF.

Figure 2. Both the ampicillin repair oligonucleotide and the second mutagenic oligonucleotide are annealed to a single-stranded DNA template. These two oligonucleotides are linked as the second strand is filled in using T4 DNA polymerase. Unlike the Klenow enzyme, T4 DNA polymerase does not perform strand displacement (6,7). First round transformation is then performed into a mismatch repair minus *E. coli* host such as BMH 71−18 mutS (8,9). Use of a mismatch repair minus strain is very important for achieving high mutation efficiencies since if the mismatch at the position of the second mutagenic oligonucleotide is repaired and the mismatch in the ampicillin resistance gene is not, then ampicillin resistant non-mutant colonies will appear.

Testing the Mutagenesis System with pSELECT-Control

Because the blue to white phenotypic change described above results from a loss of function mutation and could possibly have resulted from nucleotide changes other than that desired, we chose to construct a new vector called pSELECT-Control. pSELECT-

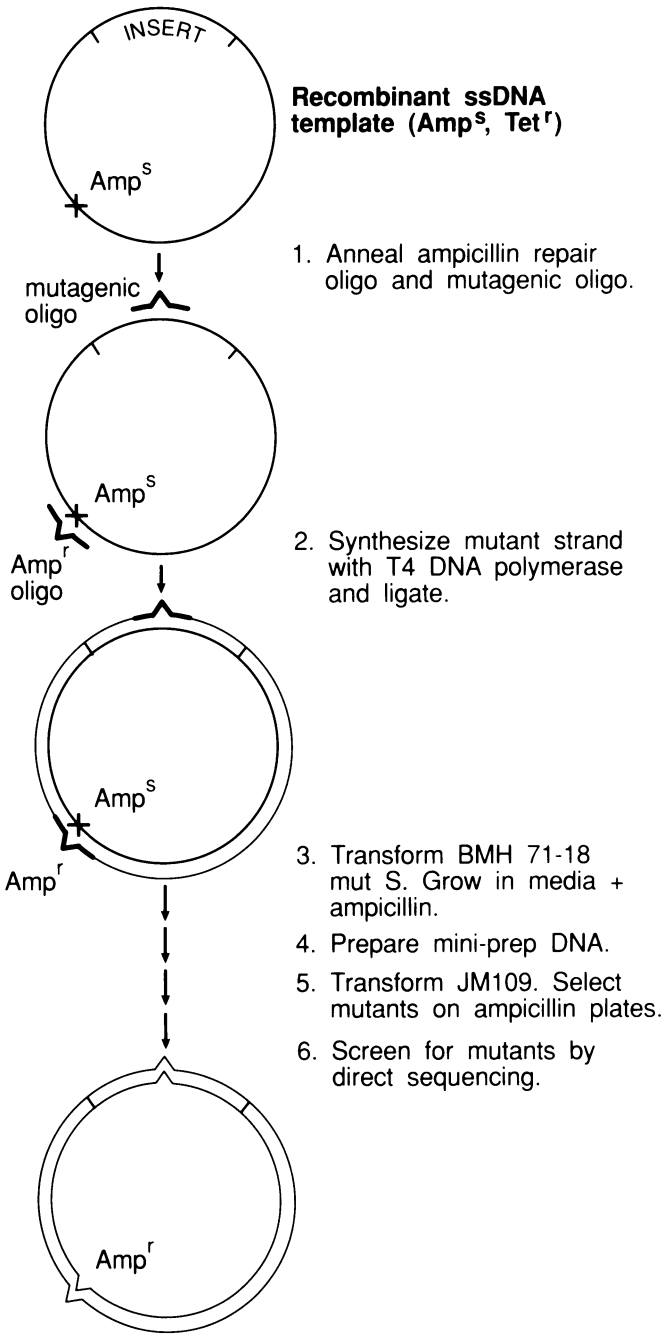

Figure 2. Diagram of the basic procedure for performing mutagenesis using the pSELECT-1 vector.

Control derives from pSELECT-1 and was constructed by cutting at the Pst I site in the polylinker of pSELECT-1, removing the overhang with Klenow and religating. This introduced a four base deletion in the lac alpha peptide gene which frameshifted the gene product resulting in a white (lac minus) phenotype. Using a lac repair oligonucleotide of sequence pTAGAGTCGACCTGCA-GGCATGCAAGC which restores the natural sequence to the polylinker, we performed *in vitro* mutagenesis using the ampicillin repair oligonucleotide along with this lac repair oligonucleotide. We routinely obtained 65−75% blue colonies upon one round of transformation and 80−90% blue colonies upon two rounds of transformation. Thus the method gave high efficiency mutagenesis in a restoration of function mutation. When the ampicillin repair oligonucleotide was used alone, only white colonies resulted.

Testing pSELECT-1 with an Insert

In order to test the functioning of pSELECT-1 with an insert, we first deleted the Eco R1 site in the linker by cutting with this enzyme, filling in the ends with Klenow and religating. A 792 b.p. Hind III fragment containing a promoterless gene for chloramphenicol acetyl transferase (CAT) was then cloned into the Hind III site of the vector. Each colony found to grow on medium containing both tetracycline and chloramphenicol was found to contain the insert oriented for expression by the lac promoter on the vector. This insert carries a single unique Eco R1 site in the coding region and we inactivated the CAT gene by cutting at this site, filling with Klenow and religating. We used a synthetic oligonucleotide 30 bases long to repair the insertion in the CAT gene coupling this oligonucleotide to the ampicillin repair oligonucleotide. Following two rounds of transformation we picked colonies from an ampicillin plate and tested them for growth on chloramphenicol. Fifty-five percent of the ampicillin resistant colonies were found now to be chloramphenicol resistant. This frequency in mutagenesis is still high enough to identify mutants by direct DNA sequencing.

In another experiment, a 3 Kb fragment of the virulence region of *Agrobacterium tumefaciens* was cloned into pSELECT-1. A 31 bp oligonucleotide was designed to introduce two new restriction sites near the middle of the cloned fragment. Two separate base changes were made using one mutagenic oligo. A single base deletion creating an Eco R1 site and a single base insertion creating a Cla 1 site. The insertion and deletion were separated on the mutagenic oligo by 7 bases. After single strand DNA was prepared, the mutagenic oligo and the ampicillin repair oligo were annealed and the mutagenic strand was synthesized. After two rounds of transformation, ten random colonies were selected and mini-prep DNA was prepared. Sequencing was performed on five of the mini-preps. Sequence data indicated that four of the five clones contained both of the mutations. Restriction analysis of the ten clones indicated that eight of the ten clones contained both mutations.

The Effect of Oligonucleotide Phosphorylation

All the oligonucleotides used in the above experiments were synthesized containing a 5′ phosphate. We examined the effect of using an unphosphorylated oligonucleotide. Phosphorylated ampicillin repair oligonucleotide was used with pSELECT-Control single-stranded DNA and an unphosphorylated lac repair oligonucleotide. In this case, a mutagenesis frequency of only 38% was obtained (38% blue colonies). We found that phosphorylation of this oligonucleotide using polynucleotide kinase restored the mutation frequency to 83%.

Multiple Simultaneous Mutations

We tested whether our system could be used to introduce more than one mutation at once. We used pSELECT-Control single-stranded DNA with both the ampicillin repair and the lac repair oligonucleotides and a third oligonucleotide designed to reintroduce the Bam H1 site into the tetracycline resistance gene. Upon two rounds of transformation—first into BMH 71−18 mutS and then into JM109—eighty-six percent blue colonies was obtained. Fifteen of these were picked and plasmid DNA prepared from them. Restriction mapping indicated that all fifteen colonies had introduced the new Bam H1 site, demonstrating the utility of the system in performing multiple simultaneous mutations.

DISCUSSION

We describe a highly efficient procedure for performing *in vitro* site-directed mutagenesis. The method is based on the coupling of a mutagenic oligonucleotide to another oligonucleotide which restores ampicillin resistance to the mutagenesis vector. The vector pSELECT-1 is described which carries a faulty ampicillin resistance gene which is restored to function via an oligonucleotide. The linking of this oligonucleotide to a mutagenic oligonucleotide of interest provides a powerful selection for mutation. In tests of the mutagenesis system using a control vector, highly efficient (80−90%) mutagenesis was observed.

To perform mutagenesis, two rounds of transformation are required. This is because the ampicillin sensitive parental strand can co-exist in the same cell as the ampicillin resistant mutant strand. Two rounds of transformation are required to isolate pure mutant DNA. We generally employ the mismatch repair minus strain BMH 71−18 mutS for the first round of transformation and JM109 for the second. The use of the mismatch repair minus host in the first round of transformation is crucial for achieving high frequencies of mutation. Using JM109 in the first round reduces the mutagenesis efficiency to only about twenty percent. Oddly, when performing mutagenesis using the ampicillin repair oligonucleotide and an oligonucleotide which disrupts the reading frame of the lac alpha peptide changing the phenotype of the plasmid from blue to white, some white colonies are observed in the first round of transformation, though the percent of whites is always higher with two rounds of transformation. Apparently in some fraction of the cells the parental (blue ampicillin sensitive) strand segregates and is lost from the cell even before it is eliminated by a second round of transformation. Also, we have found that when mutating the pSELECT-Control vector from white to blue, a somewhat higher percentage of blue colonies is obtained on the second round as compared to the first round of transformation. In most cells the blue color is dominant and is expressed upon a single round of transformation. However a small fraction of cells (10−15%) appears to suppress blue coloration when containing both parental (white) and mutant (blue) plasmid in the same cell. The mechanism of this effect is unknown but may relate to interference caused by the presence in the cell of a non-functional lac alpha peptide fragment.

Construction of pSELECT-1 involved the removal of four restriction sites located in the tetracycline resistance gene on the vector. The Hind III site was located between the −35 and −10 regions of the tetracycline resistance promoter. Deletion of this site was achieved by changing an AT base pair to a TA base pair and did not affect the ability of the vector to confer tetracycline resistance. Each of the Bam H1, Sph I and Sal I sites lay within the coding region of the gene and was removed by site-directed mutagenesis by changing a base in the wobble position of the appropriate codon and leaving the amino acid sequence of the tetracycline resistance protein unchanged. These changes also had no measurable effect on the ability of the vector to confer resistance to tetracycline.

Our method of *in vitro* site-directed mutagenesis is simple to perform and requires only a small amount of single-stranded DNA template (0.1 μg) to obtain many ampicillin resistant colonies. The method is thus ideally suited to a phagemid such as pSELECT-1 in which certain recombinants may produce only small amounts of single-stranded DNA. The ampicillin selection against the parental strand is absolute and does not require a complicated series of enzymatic steps such as in other methods to select against the parental strand. Furthermore, it is possible that the requirement for ampicillin resistance selects for a fully copied mutant strand.

We have demonstrated the feasibility of performing more than one mutation at once in our system by using a single oligonucleotide to introduce more than one mutation or by simply adding more than one mutagenic oligonucleotide to the hybridization and fill in reaction. This ability obviates the need to reclone into the ampicillin sensitive vector if it is desired to create more than one mutation within a given target gene.

The requirements for a vector of the pSELECT type are an inactive first genetic marker which is capable of being restored to functional expression, an active second genetic marker, a polylinker region and an f1 replication origin. We speculate that vectors could be built which were based on markers other than ampicillin resistance. For instance neomycin, streptomycin or chloramphenicol acetyl transferase genes might be used in the same manner as the ampicillin resistance gene of pSELECT-1. We have, however, been unsuccessful in our attempts to use the tetracycline resistance gene as an inactivated restorable marker. We inactivated the tetracycline resistance gene of a pBR322 derivative carrying an f1 replication origin by cutting at the Bam H1 site, blunting the ends with Klenow and religating. Using single-stranded DNA from this vector, we attempted to use an oligonucleotide to revert the vector to tetracycline resistance. We were not successful in doing so and concluded that tetracycline sensitivity of this particular mutant was dominant. It may be that having a non-functional tet protein in the cell interferes with the action of functional tet protein in the same cell.

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