# A simple vector modification to facilitate oligonucleotide-directed mutagenesis

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## ABSTRACT

**We describe a simple modification of commonly used single-stranded cloning vectors that permits the efficient recovery of mutant DNA molecules in oligonucleotide-directed mutagenesis experiments, even when the absolute efficiency of mutagenesis is very low. The modification consists of the insertion of a short synthetic DNA fragment into the vector's polylinker and permits the identification of mutant clones based on a standard chromogenic plate assay for bacterial colonies or phage plaques producing functional beta-galactosidase. Other useful properties of the original vector are retained in the modified version. In vitro mutagenesis reactions are carried out with two oligonucleotides, one to introduce the mutation of interest, and the second to correct a frameshift mutation introduced into the beta-galactosidase gene of the modified vector. We have found that these two sequence changes are closely linked following transformation of an appropriate *E. coli* strain with the products of the in vitro mutagenesis reaction, and have thereby recovered desired mutations at a frequency of about 50% even when the overall mutagenesis efficiency is less than 1%. By alternately correcting and re-introducing the beta-galactosidase frameshift mutation, we have shown that multiple rounds of mutagenesis can be carried out on the same template with a high efficiency of mutant recovery in each step. Modifications similar or identical to those we describe here should be feasible for most commonly used single-stranded cloning vectors and should increase the usefulness of these vectors by providing an additional option for oligonucleotide-directed mutagenesis to be used in conjunction with or in lieu of other commonly used approaches.**

## INTRODUCTION

Oligonucleotide-directed mutagenesis has become a standard method in the in vitro manipulation of DNA sequences. The power and convenience of the approach has been enhanced by the development of methods that permit the efficient recovery of mutated sequences without screening large numbers of clones using hybridization techniques (1–3). We describe here a simple modification of single-stranded cloning vectors that permits the efficient recovery of mutant clones following oligonucleotide-directed mutagenesis even when the overall efficiency of mutagenesis is very low. The modification can easily be introduced into most single-stranded cloning vectors ultimately derived from the Messing M13 vectors (4–6), including a variety of 'phagemids'. These modified vectors can be used in conjunction with or in lieu of other methods for enhancing mutagenesis efficiency. We suggest that such a modification of standard phagemid and M13 cloning vectors should increase their general utility for most investigators.

## MATERIALS AND METHODS

### Vectors and Strains

Our specific vector constructs (pGP11, pGP12, and pGP76) have been prepared from the commercially available phagemids pBSM13$^-$ (Stratagene) and pIBI76 (International Biotechnologies, Inc.). The unique NdeI site of pBSM13$^-$ has been removed by digestion, fill-in, and religation; this change was made for specific purposes of our own and is not relevant to the general principle of vector modification described in this paper. Mutagenesis reactions have been carried out on three different phagemids. pST5RD contains a *X. borealis* somatic-type 5S RNA gene (7) (from pXbs201 (8)) cloned into the HincII site of pGP76. pGA1 contains the EcoRI fragment of pUC3a1.b (9), a *X. laevis* TFIIIA cDNA clone, inserted into the EcoRI site of pGP11. pGA11 contains the same fragment of pUC3a1.b inserted into the EcoRI site of pGP12. In general, plasmid and single-stranded DNA's have been prepared from strain NM522 (4,10, available from IBI). In some experiments, single-stranded DNA has been prepared from the dut$^-$ung$^-$ strain CJ236 (1). The products of in vitro mutagenesis reactions were used to transform competent NM522 cells; this strain expresses the beta fragment of beta-galactosidase from an episome and therefore permits alpha complementation by the mutagenesis vector.

### Oligonucleotide Synthesis and Purification

Oligonucleotides were synthesized on an Applied Biosystems 381A synthesizer, deprotected in $NH_4OH$, and purified by the OPC method as described by the manufacturer (Applied Biosystems). The specific oligonucleotides used in vector modification and beta-galactosidase frameshift mutagenesis were:

lac-start 1: 5′ AATTCAGGAAACAGCTATGACCATGATACA 3′
lac-start 2: 5′ AATTTGTATCATGGTCATAGCTGTTTCCTG 3′

promoter 1: 5' AATTCGTTGACAATTAATCATCGGCTCGGTAGATTGTGTGGA 3'
promoter 2: 5' AATTTCCACACAATCTACCGAGCCGATGATTAATTGTCAACG 3'
lac 12TI: 5' GACCATGATTACAAATTC 3'
lac 12TD: 5' GACCATGATACAAATTC 3'

### Vector Modification

To create pGP11 and pGP76, complementary oligonucleotides lac-start 1 and lac-start 2 were inserted at the unique EcoRI site of plasmids pBSM13$^-$ (modified as described above) and pIBI76, respectively. The desired product results in the regeneration of a unique EcoRI site at the end of the polylinker.

pGP12 was generated from pGP11 by insertion of the 42 base pair fragment consisting of the complementary oligonucleotides promoter 1 and promoter 2 at the unique EcoRI site of pGP11. Plasmids from blue colonies were screened to ensure insertion of the synthetic promoter fragment in the correct orientation with regeneration of the unique EcoRI site.

### In Vitro Mutagenesis Reactions

Single-stranded DNA was obtained from NM522 or CJ236 strains containing the phagemid of interest after superinfection with M13KO7 helper phage (IBI). 0.1−1.0 microgram single-stranded DNA (pST5RD, pGA1, or pGA11) in T4 DNA polymerase reaction buffer (33 mM Tris-acetate, pH 7.9; 66 mM $KCH_3COO$; 10 mM $Mg(CHI_3COO)_2$; 100 micrograms/ml bovine serum albumin; 0.5 mM dithiothreitol) was mixed with 30−300 fold molar excess of two different phosphorylated oligonucleotides: lac 12TI (or lac 12TD in some cases) and an oligonucleotide designed to introduce the mutation of interest. This mix was incubated at 65° for 2 minutes and then at 37° for 15 minutes. Additional dithiothreitol was added to a concentration of 1 mM, ATP to 1 mM, and dATP, dTTP, dGTP, and dCTP to 0.5 mM each. Approximately 1 unit each of T4 DNA polymerase and T4 DNA ligase were then added and incubated at 37° for approximately 2 hours. Aliquots of the reaction mix were used to transform competent NM522 cells which were then plated onto ampicillin/Xgal/IPTG plates. Appropriately colored colonies (blue for mutagenesis with lac 12TI; white for mutagenesis with lac 12 TD) were then picked and screened for the mutation of interest using either restriction enzyme digestion or direct sequencing.

## RESULTS

### Basic Vector Modification

Commonly used single-stranded cloning vectors are derived from the M13mp series developed by Messing and coworkers (4−6). The structure of these vectors in the polylinker region is shown schematically in figure 1. An important feature of these vectors is that they encode the alpha fragment of beta-galactosidase, permitting production of functional beta-galactosidase when expressed in a strain encoding the complementing beta fragment. The alpha fragment coding sequence, under control of the lac promoter, is interrupted by a polylinker sequence that maintains the proper open reading frame for production of functional alpha fragment. Insertion of DNA fragments into the polylinker usually disrupts this open reading frame, resulting in an inability to produce functional beta-galactosidase; thus, clones containing insertions can be easily identified on appropriate indicator plates. We have designed and synthesized a 30 base pair DNA fragment (sequence in figure 1; also see Materials and Methods) with overlapping ends that are suitable for insertion into the terminal restriction site in the polylinker of these vectors. The synthetic 30-mer contains a ribosome binding site with appropriate spacing to an ATG initiation codon followed by the first several codons for beta-galactosidase alpha fragment synthesis; these are the same codons that are used for alpha fragment synthesis when translation initiates upstream of the polylinker in these vectors. Thus, the modified vector contains two potential sites of translation initiation for beta-galactosidase alpha fragment synthesis: one upstream of the polylinker, and the second downstream. Translation initiation at the upstream site results in the production of functional beta-galactosidase alpha fragment because the inserted 30-mer was designed to maintain the appropriate open reading frame. The open reading frame resulting from initiation at the downstream site, however, is very short because of a deletion of one base pair in the fourth codon of beta-galactosidase, encoded in the inserted 30-mer. The insertion of this synthetic 30-mer in the correct orientation in the parent vector therefore has the following consequences: (1) The restriction site (an EcoRI site here) at the end of the polylinker will be regenerated as a unique site, since sequence asymmetry at the ends of the 30-mer results in the regeneration of the site at only one of the ligation joints; (2) The modified vector encodes functional beta-galactosidase alpha fragment, and will produce blue colonies on Xgal plates when introduced into a strain producing the beta fragment of beta-galactosidase; (3) Insertion of additional DNA sequences in the polylinker of the modified vector will result in the production of white colonies under the same circumstances, since translation initiated at the upstream initiation site will terminate in the inserted sequence (except in rare circumstances), and translation initiated at the downstream initiation site will terminate as a result of the one base pair deletion described above; (4) Insertion of the missing downstream fourth codon base pair by oligonucleotide-directed mutagenesis will result in production of functional beta-

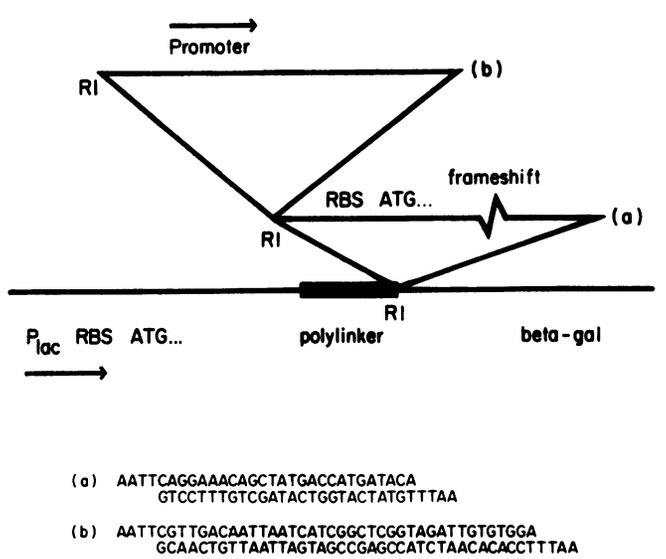


**Figure 1.** Polylinker Region of Modified Single-Stranded Cloning Vectors. A schematic representation of the polylinker region of the single-stranded cloning vectors used in these studies is shown. The synthetic DNA fragment used in the construction of pGP11 and pGP76 is shown as fragment (a) and the synthetic fragment used additionally in the construction of pGP12 is shown as fragment (b). Sequences of these fragments are given below. These fragments are inserted at a unique EcoRI site (RI in the figure). Other abbreviations are: $P_{lac}$, lac promoter; RBS, ribosome binding site; ATG, codon for initiator methionine (translation start site); beta-gal, sequence encoding alpha fragment of beta-galactosidase.

galactosidase alpha fragment and blue colonies on Xgal plates even when additional DNA fragments have been inserted into the polylinker, since all the signals for translation initiation at the downstream site are located downstream of the polylinker in the inserted 30-mer.

We have used phagemid vectors (that is, vectors containing both colE1 and phage f1 origins of replication) modified in this fashion (pGP11 and pGP76) for oligonucleotide-directed mutagenesis of sequences cloned into the polylinker region. Mutagenesis has been carried out by standard techniques (for details, see Materials and Methods), except that two different oligonucleotides have been included in the in vitro reactions. One of these introduces the desired mutation into the sequence of interest, and the second, a standard oligonucleotide used in all experiments (lac 12TI, see figure 1 and Materials and Methods), corrects the frame shift in the downstream open reading frame for beta-galactosidase alpha fragment synthesis by inserting the missing nucleotide in the fourth codon. Subsequent transformation of the in vitro reaction mix into an appropriate strain and plating on Xgal indicator plates permits the identification of clones containing this frameshift correction as blue colonies; the data of Table 1 demonstrate that the desired mutation in the sequence of interest is recovered at a high frequency (about 50%) in these clones. This is true even when the overall frequency of mutagenesis, measured by the frequency of blue colonies, is very low. This high frequency does not result from a disproportionately high efficiency of mutagenesis targeted at the sequence of interest; analysis of white colonies reveals no such mutations recovered in the absence of the frameshift correction directed by oligonucleotide lac 12TI (Table 1). The data of Table 1 show that this mutagenesis approach has proven successful in a large number of independent experiments.

We have also used this method in conjunction with that described by Kunkel (1,2), in which single-stranded templates are prepared from a dut$^-$ung$^-$ strain of *E. coli* (CJ236). In most cases, we find that while the overall efficiency of recovery of blue colonies is improved, as would be expected, the frequency with which the desired mutation is recovered from blue colonies is not greatly altered relative to that seen with single-stranded DNA prepared from NM522 cells (Table 1, experiment 11; other data not shown).

## Mutagenesis of Sequences Containing Transcription Terminators or Attenuators

A limitation of the vectors constructed as described above is that they depend upon transcription from the lac promoter located upstream of the polylinker to express beta-galactosidase sequences located downstream of the polylinker and any inserted DNA fragments. Usually, this poses no difficulty; in some cases, however, the cloned target fragment may contain sequences that act as transcriptional terminators or attenuators in *E. coli*. In this event, expression of functional beta-galactosidase may be minimal (because of low levels of transcript) even after frameshift correction by the lac 12TI oligonucleotide. In fact, we have observed this in one instance, making identification of positive colonies difficult because of their very faint blue color.

To correct this problem, we have synthesized a 42 base pair fragment (figure 1 and Materials and Methods) for insertion at the terminal restriction site of the polylinker of the modified vectors. As before, this fragment has asymmetric termini for the regeneration of a unique restriction site at the end of the polylinker when inserted in the correct orientation. This 42 base pair sequence includes a reasonably strong *E. coli* promoter, based on sequence similarity to consensus promoter elements. Insertion of this fragment therefore renders expression of RNA encoding beta-galactosidase alpha fragment independent of sequences cloned into the vector's polylinker. We have further modified phagemid pGP11 by the insertion of this promoter fragment to create the vector pGP12. The results of Table 2 demonstrate that this vector behaves similarly to pGP11 and pGP76 in oligonucleotide-directed mutagenesis experiments; it also permits the unambiguous identification of blue colonies in cases where the use of pGP11 was problematic for the reasons just described.

## Multiple Rounds of Mutagenesis

A consequence of the first round of mutagenesis as described above is that the resultant phagemid encodes a functional alpha fragment of beta-galactosidase. Therefore, one cannot simply repeat the same steps in subsequent rounds of mutagenesis to obtain multiple mutations in the sequence of interest. A simple modification of the protocol solves this problem, however. Instead of using oligonucleotide lac 12TI to correct the original frameshift in the beta-galactosidase sequence, we have used oligonucleotide lac 12TD (in combination with the mutating oligo of interest) to regenerate this frameshift by deletion of the T residue inserted by lac 12 TI in the first round of mutagenesis. Thus, in the second round of mutagenesis, beta-galactosidase mutation events are detected by a conversion of blue colonies to white ones on Xgal indicator plates. The results of Table 3 show that the desired mutations in the sequence of interest are recovered at frequencies similar to those observed in the first round of mutagenesis, even though the overall efficiency of mutagenesis is again low (number of white versus blue colonies) and no desired mutations are detected in blue colonies analyzed. By alternating the use of

**Table 1.** Oligonucleotide-directed Mutagenesis Using pGP76 and pGP11

| Vector | Mutation | # Colonies | % Blue | # Mutant/ # Blue | # Mutant/ # White |
|---|---|---|---|---|---|
| 1. pGP76 | 14 bp sub. | N.D. | N.D. | 4/7 | N.D. |
| 2. pGP76 | 12 bp sub. | N.D. | N.D. | 4/7 | N.D. |
| 3. pGP76 | 3 bp sub. | N.D. | N.D. | 4/6 | N.D. |
| 4. pGP11 | 4 bp ins. | 872 | 0.34 | 1/3 | N.D. |
| 5. pGP11 | 4 bp sub. | 791 | 0.38 | 2/3 | 0/12 |
| 6. pGP11 | 3 bp sub. | 328 | 1.2 | 2/4 | N.D. |
| 7. pGP11 | 4 bp sub. | 3950 | 0.15 | 2/7 | N.D. |
| 8. pGP11 | 1 bp sub. | N.D. | N.D. | 2/6 | N.D. |
| 9. pGP11 | 1 bp sub. | N.D. | N.D. | 1/6 | N.D. |
| 10. pGP11 | 6 bp sub. | N.D. | N.D. | 1/1 | N.D. |
| 11. pGP11/CJ236 | 35 bp ins. | 333 | 57.4 | 6/12 | N.D. |

**Table 2.** Oligonucleotide-directed Mutagenesis Using pGP12

| Vector | Mutation | # Colonies | % Blue | # Mutant/ # Blue | # Mutant/ # White |
|---|---|---|---|---|---|
| 1. pGP12 | 6 bp sub. | 71 | 2.8 | 3/6 | N.D. |
| 2. pGP12 | 7 bp sub. | 513 | 2.5 | 10/12 | N.D. |

**Table 3** Second Round of Oligonucleotide-directed Mutagenesis

| Vector | Mutation | # Colonies | % White | # Mutant/ # Blue | # Mutant/ # White |
|---|---|---|---|---|---|
| 1. pGP11 | 4 bp sub. | 876 | 1.3 | 0/12 | 5/11 |

oligonucleotides lac 12TI and lac 12TD in sequential rounds of mutagenesis, there should be no limit to the number of rounds of mutagenesis that can be conducted using this method. We have successfully used the approach through three rounds of mutagenesis.

## DISCUSSION

The method we have described above permits the efficient recovery of mutants in oligonucleotide-directed mutagenesis experiments even when the overall efficiency of mutagenesis is very low. We have efficiently obtained several different types of mutations using this method, including single base substitutions, clustered base substitutions, and small insertions and deletions. Because the method depends upon the modification of the single-stranded cloning vector used, it can be used in conjunction with other methods, such as that of Kunkel (1,2), that do not rely on vector modification. Alternatively, our approach can be used independently to efficiently recover mutants, as we have shown here, and there are some advantages to this approach. First, there is no requirement for preparation of single-stranded DNA from any particular strain, and this may make preparation of single-stranded templates easier in some cases. Second, in cases where it is necessary to carry out multiple rounds of mutagenesis, there is no need to cycle the phage or phagemid of interest through a special strain prior to the next round of mutagenesis; this eliminates an extra round of transformation for each mutagenesis step. Finally, since the method works even when the overall mutagenesis efficiency is very low, there is no particular need to optimize the in vitro steps of the mutagenesis reaction, and efficient recovery of mutants can be achieved even when reactions are carried out under suboptimal conditions. As a purely practical matter, we think this is an important consideration for the typical laboratory.

The success of the approach described here depends upon the recovery of the desired mutation linked to an easily screened mutation in the beta-galactosidase gene of the vector. Another option would have been to use the beta-lactamase gene (conferring ampicillin resistance) as a target for mutagenesis; an advantage to this approach would have been that desired mutations in the first round of mutagenesis could have been easily selected rather than identified by screening. In fact, such a vector has recently become commercially available (Promega). We chose the screening approach instead for two reasons: (1) the vector modification we suggest here can be made to the great majority of single-stranded cloning vectors that are currently in use, and modifications to inactivate the beta-lactamase gene are feasible only in the small percentage of vectors that contain a second selectable marker; (2) multiple round mutagenesis experiments are more easily carried out using a chromogenic screen for beta-galactosidase activity, since selection *against* beta-lactamase expression is not straightforward.

It is perhaps surprising that there is such a strong linkage between the beta-galactosidase frameshift mutation and the second, desired mutation in these experiments, even when the individual mutagenesis efficiencies are very low. Certainly, one contributing factor is that identification of clones containing the beta-galactosidase frameshift revertant helps to eliminate clones resulting from transformation with parent phagemid molecules that were not used as substrates in the in vitro mutagenesis reactions. It is also possible that some of the heteroduplex molecules are replicated without prior mismatch repair following transformation; this would account for strong linkage between the two mutations, since both occur on the same strand of the heteroduplex product of the in vitro reactions. It seems unlikely that this is the primary explanation for the strong linkage, however, since we rarely recover mixed populations of wild-type and mutant phagemids in single colonies, even though the vector used replicates as a high copy number plasmid. It is most likely that the strong linkage results from the fact that a major mechanism for mismatch repair in *E. coli* is methyl-directed mismatch repair, and such repair patches average roughly 3000 base pairs in length (11,12). The mutations we have generated all lie within about 1500 base pairs of the beta-galactosidase frameshift mutation used in the screen for mutants; perhaps the linkage frequency would be lower if mutations were introduced at more distant sites.

In conclusion, we would emphasize that while we have used only two particular parent vectors in the experiments described here, we can think of no reason why similar modifications could not be made to the great majority of single-stranded cloning vectors currently in use. Vectors prepared with the modifications we describe here should provide their users with an additional option for efficient oligonucleotide-directed mutagenesis.

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