A Modified Unique Site Elimination Mutagenesis in Constructing a Chloramphenicol Resistance-Encoding pGEM Vector

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Recombinant DNA research requires new and versatile cloning vectors. Many of the popular plasmid vectors, such as the pUC (4), pTZ (Amersham, Arlington Heights, IL, USA) and pGEM® (Promega, Madison, WI, USA) series, share common characteristics, including high copy number, sizes of around 3 kbp and the presence of a multiple cloning sequence (MCS) within the coding sequence for a part of the lacZ gene isolated from E. coli. Cloning a fragment using a restriction site within the MCS of one of these plasmids allows blue/white selection for recombinant molecules. These plasmids also carry a gene for ampicillin resistance. In our hands, selection for ampicillin-resistant bacteria can be problematic, as many nonresistant, satellite colonies are often found surrounding the actual resistant colonies. Although this problem can be overcome using shorter incubation periods, the use of chloramphenicol selection does not present such problems. Since the majority of these plasmids are around 3 kbp in size, this can lead to problems when trying to clone fragments, which are also close to 3 kbp. Subsequently, it can also be difficult to separate the desired cloned fragment from the vector fragment.

Site-directed mutagenesis has become a major force in the analysis of gene function. The ability to precisely change a single base pair within a segment of DNA has proven invaluable—illuminating the effects of single amino acid changes in a protein. One common method for site-directed mutagenesis is the unique site elimination (U.S.E.) protocol (1). We have introduced the gene encoding chloramphenicol resistance, chloramphenicol acetyl transferase (CAT), was isolated as a blunt-end SmaI fragment from pCAT19 (2) using a low-melting point agarose gel and β-agarase treatment. The vector pGEM-7Zf(+) was cut with ScaI within the bla gene encoding β-lactamase to give blunt ends. The SmaI CAT fragment was ligated to the ScaI vector ends, and the ligation mixture transformed into E. coli strain JM101 with selection for chloramphenicol resistance. Recombinant plasmids with the CAT gene inserted in both of the two possible orientations were isolated. One of these, pKM1 was chosen for the

This has increased the size of the plasmid to 4 kbp. However, the inserted fragment introduces a number of restriction sites rendering some sites in the MCS unsuitable for cloning. We describe the construction of a chloramphenicol resistance encoding pGEM-7Zf(+) derivative and a modification of the U.S.E. protocol that does not require elimination of a unique restriction site.

The gene encoding chloramphenicol resistance, chloramphenicol acetyl transferase (CAT), was isolated as a blunt-end SmaI fragment from pCAT19 (2) using a low-melting point agarose gel and β-agarase treatment. The vector pGEM-7Zf(+) was cut with ScaI within the bla gene encoding β-lactamase to give blunt ends. The SmaI CAT fragment was ligated to the ScaI vector ends, and the ligation mixture transformed into E. coli strain JM101 with selection for chloramphenicol resistance. Recombinant plasmids with the CAT gene inserted in both of the two possible orientations were isolated. One of these, pKM1 was chosen for the

Figure 1. Generalized representation of the mutagenesis protocol. The parental plasmid is represented at the top of the figure. The darkened part of the circle corresponds to the CAT gene. E and H refer to recognition sequences for the restriction enzymes EcoRI and HindIII, respectively. The "not E" refers to a sequence in which the EcoRI recognition sequence has been altered ([+]EcoRI in the text). The M refers to the site for mutagenesis. The left side of the figure depicts the situation where the "not E" and M primers are both incorporated into the newly synthesized strand. The right side of the figure depicts the situation when neither primer is incorporated into the newly synthesized strand.
rest of the procedure.

Introduction of the CAT gene into pKM1 gave a high copy number plasmid with most of the qualities of the original pGEM-7Zf(+) except that it encoded resistance to chloramphenicol rather than ampicillin. However, several of the restriction sites present in the MCS were now duplicated in pKM1 and were therefore not suitable for cloning (i.e., AsuII, BamHI, EcoRI, SphI, XhoI). Two mutagenic oligonucleotide primers were designed to eliminate the newly introduced EcoRI and SphI sites. The normal U.S.E. mutagenesis procedure requires the elimination of a unique restriction site (such as PstI) with the concomitant mutagenesis of the desired region. We reasoned that we could test a modification of this procedure that did not require the elimination of a unique site.

One of the EcoRI sites in pKM1 lies in the MCS; the other in the middle of the CAT gene reading frame. The (-) EcoRI (selection) primer was designed to eliminate the site without changing any of the encoded amino acids. The (-) PstI (mutagenic) primer was designed to eliminate the single PstI site and unwanted SphI site as well as introduce a second NaeI site into the plasmid. The mutagenesis was carried out as described in the instructions for the U.S.E. kit from Pharmacia Biotech (Piscataway, NJ, USA) (3) with several modifications.

The following steps are shown in Figure 1. The annealing of primers and synthesis of mutant DNA strands and the primary restriction enzyme selection were as described (3). Digestion with EcoRI at this point will cleave the mutant molecules, which have incorporated the (-)EcoRI primer once, producing a linear molecule. Digestion of any nonmutant molecules that have not incorporated the (-)EcoRI primer will be cleaved twice producing two linear molecules. If we dilute and ligate the mixture at this point, we would expect a predominance of intramolecular over intermolecular ligations. Intramolecular ligation of the mutant molecules will yield the original mutant plasmid (Figure 1, plasmid lower left). Intramolecular ligation of nonmutant molecules will not produce plasmids with the CAT gene intact (Figure 1,
lower right, plasmids A and B). Only intermolecular ligation of the nonmutant molecules can accomplish this and even then, there is only a 50% chance that this rare ligation event will occur in the correct orientation (Figure 1, lower right, plasmid C in correct orientation, plasmid D in incorrect orientation). Hence, the vast majority of nonmutant molecules will not yield plasmids with the CAT gene intact; all of the mutant molecules will. After digestion with EcoRI, the sample was diluted to 500 µL with T4 DNA ligation buffer, and 4 U of T4 DNA ligase were added. The ligation was run at 16°C overnight. The ligation mixture was used to transform E. coli strain BMH71-18 mutS, and the cells were plated on chloramphenicol-containing L plates. The DNA from the resultant colonies was screened for the presence of a single EcoRI site. Approximately 50% of the possible mutant plasmids contained a single EcoRI site and were screened further. These plasmids were not cleaved with PstI, cleaved once with SphI, and cleaved twice with NaeI confirming that they had also incorporated the (-)PstI mutagenic primer in the initial reactions. This new plasmid is pCKC1 (Figure 2). It is approximately 4 kbp in size, encodes chloramphenicol resistance and has the unique restriction enzyme sites suitable for cloning shown in the figure. Unlike the suggested protocol (3), we only used one round of restriction enzyme selection. A second round would increase the probability of isolating a mutant plasmid.

This modification of the U.S.E. protocol does not require the elimination of a unique restriction site. In this case, the unwanted site is in the CAT gene coding region, requiring the intermolecular ligation of two nonmutant fragments in the correct orientation to reconstruct the gene. Diluting the ligation reaction should enhance the frequency of intramolecular relative to intermolecular ligations. Although the elimination of a site within the coding sequence of a selectable marker should further enhance the frequency of obtaining mutants, this is not a necessity. If the two sites in question are far enough separated on the plasmid, and if cleavage of these sites leads to separation of important plasmid functions (such as antibiotic resistance and the origin of replication) the more probable intramolecular circularization of either of the two nonmutant fragments will not be able to produce a viable plasmid.

REFERENCES


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Bi-directional Dideoxy Fingerprinting (Bi-ddF): Rapid and Efficient Screening for Mutations in the Big Blue™ Transgenic Mouse Mutation Detection System

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Single-stranded conformation polymorphism (SSCP) (9) is currently the most widely used method for screening for mutations. SSCP does not detect all sequence changes, and its sensitivity depends on the sequence and size of the segment screened (3,6,8,11,12). For a sequence of about 200 bp, the observed sensitivities are often in the range of 60%–90%. For larger segments, the sensitivity is decreased. Dideoxy fingerprinting (ddF) is performed by electrophoresing one lane of a Sanger dideoxy termination reaction through a non-denaturing gel (11). ddF can screen a 250-bp segment with essentially 100% sensitivity (1,6,11). In bi-directional dideoxy fingerprinting (Bi-