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Introduction of Multiple Restriction Enzyme Sites by In Vitro Mutagenesis Using the Polymerase Chain Reaction

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The development of the polymerase chain reaction (PCR) has facilitated the process of site-directed mutagenesis. A frequent application of this technology is using mutant oligonucleotides, to produce changes in the target DNA, that introduce restriction enzyme recognition sites. Among the several methods that have been reported for in vitro mutagenesis using PCR, the production of single-stranded amplification products[1,2] remains an important method. However, the efficiency of mutagenesis with these procedures decreases with the increase in length of the DNA that needs to be manipulated. We describe a simple and straightforward strategy for the introduction of multiple restriction enzyme sites as an alternative to the existing methods. This strategy involves the production of double-stranded amplification products, and therefore is not constrained by the limitations associated with the production of single-stranded molecules. The chief requirement of this procedure is the need for a unique overhang for each of the restriction enzyme sites desired to be introduced. We have demonstrated the use of this strategy by introducing two restriction enzyme sites. The principle can be extended for the introduction of additional sites.

In this strategy, the entire targeted region, flanked by the preexisting restriction enzyme sites, is segmentally amplified using multiple sets of primers. All of the primers, with the exception of the outermost pair, are synthesized to contain the desired changes. This procedure will segmentally generate $n + 1$ amplification products, where n is the number of restriction enzyme sites to be introduced. In the present study, three fragments were generated for the introduction of two restriction enzyme sites (Fig. 1). The middle fragment contains the two unique restriction enzyme sites at its respective termini, while the 5′- and 3′-flanking fragments contain the desired restriction enzyme sites at their 3′ and 5′ termini, respectively. Digestion of these fragments with the appropriate restriction enzymes will generate cohesive termini facilitating directed ligation. The presence of blunt or nonhomologous termini at the outside ends of the flanking fragments prevent the formation of undesirable concatamers. The resultant large fragment is subsequently digested at the preexisting native restriction enzyme recognition sites, purified, and ligated to the similarly digested parental vector fragment.

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TABLE 1 Oligonucleotide Primers Utilized in the Study

Primer Number	Name	Sequence
201	Sal5367(+)	5′-CAGAATTGGGTGTCGACATAGCAGAATAGG
170	Mlu6709(−)	5′-ATTGTTGTTGGGACGCGTACAATTAATTTCTA
209	Mlu6709(+)	5′-GAAATTAATTGTACGCGTCCCAACAAC
210	Eag7225(−)	5′-ATCTCCTCCTCCCGGCCGGAAGATCTCGGA
171	Bam8071(−)	5′-GATAAGTGCTAAGGATCCGTTCACTAATCG
172	Eag7225(+)	5′-TCCGAGATCTTCCGGCCGGGAGGAGGAGATATG

The highlighted bases indicate the substitutions made to incorporate the restriction enzyme recognition sites. The *Mlu*I and *Eag*I sites were designed to be unique in the proviral pBRU3 plasmid and these sites were identified by translating their respective recognition sites in three reading frames and identifying a match for the amino acid motifs[4] with the amino acid sequence of the protein. The numbers in the name of the primers indicate the nucleotide position in the HIV-1_{Bru} proviral genome.[5]

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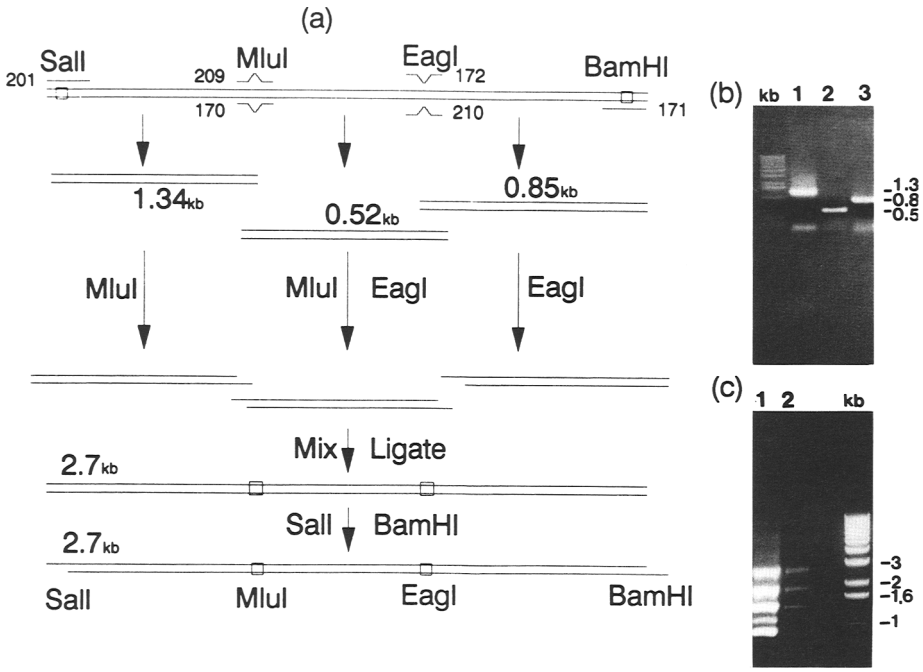

FIGURE 1 The strategy for the introduction of *Mlu*I and *Eag*I sites in the 2.7-kb *Sal*I–*Bam*HI fragment of the pBRU3 plasmid. These sites were designed to flank the V_3–V_5 regions of the HIV-1 *env* gene. The strategy is illustrated in *a* and the successful amplification to obtain the appropriately sized fragments is presented in *b*. Lanes *1*, *2*, and *3* indicate the amplification products from primer pairs 201–170, 209–210, and 171–172, respectively. The middle fragment was digested with both *Mlu*I and *Eag*I, while the outer two fragments were digested with either *Mlu*I or *Eag*I, respectively, as detailed in (*a*). Following ligation, the products were digested with *Sal*I and *Bam*HI and electrophoresed as illustrated in (*c*). The 2.7-kb fragment was eluted from the gel and used for ligation into the parental vector. Lanes *1* and *2* contain differing amounts of the ligated product; the smaller-sized bands are the partial and unligated products; and lanes *kb* indicate the kilobase DNA size markers (GIBCO-BRL). PCR has been described previously[3] and other DNA manipulations were by standard protocols. The successful introduction of the desired mutations was ascertained by restriction mapping and sequencing the target areas. The biological properties of the parental and the mutant viral clones were ascertained by estimation of p24 antigen in the culture supernatant following transfection of the viral constructs into H9 cells.

SUGGESTED PROTOCOL

1. Phenol-chloroform extract 200–300 µl of the amplification reactions and ethanol-precipitate the DNA.
2. Dissolve the DNA in 50 µl of appropriate 1x restriction enzyme buffer solution and digest with the appropriate restriction enzymes as described in Figure 1.
3. Electroelute the fragment following agarose gel electrophoresis and resuspend the DNA precipitate in 20 µl of 10 mM Tris (pH 8), 0.1 mM EDTA.
4. Mix 10 µl of each of the fragments, add 3 µl of 10x T4 DNA ligase buffer, 1–2 units of the enzyme, and ligate at 4°C overnight.
5. Stop the reaction by adding 55 µl of distilled water and heating to 65°C for 10 min. Add the appropriate restriction enzyme buffer to 1x and digest the ligated DNA with restriction enzymes specific to the ends (Fig. 1).
6. Electrophorese the above digestion mixture and electroelute the appropriately sized band.
7. Ligate into the parental vector fragment and transform to obtain the mutant vector. Analyze by restriction mapping or other suitable method.

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