we estimate more than 80% of embryos are devitellinized. In some cases, more than 95% of embryos are devitellinized in our hands. The general morphology of the devitellinized embryos is unaltered and can be used for antibody staining or in situ hybridization directly. We have used this method to detect the expression of *lacZ* under the control of a tissue-specific enhancer (Figure 1B), double labeling of *Drosophila* embryos with X-gal and digoxigenin-labeled probe (Figure 1C) and X-gal and antibodies to facilitate identification of null mutant embryos (Figure 1D) with great success. Most antibodies we have used are not affected by X-gal staining. However, in our hands, the anti-*even-skipped* antibody does not work well with X-gal-stained embryos. We overcame this problem by amplifying the signal with the VECTASTAIN® Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA). With the versatility of X-gal staining, we believe that this protocol could provide a useful alternative for labeling *Drosophila* embryos.

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## pMECA: A Cloning Plasmid with 44 Unique Restriction Sites that Allows Selection of Recombinants Based on Colony Size

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Advances in recombinant DNA technology have increased the demand for novel vectors, particularly those that facilitate the cloning process. In particular, the manipulation of large segments of cloned DNA has been limited by the availability of vectors containing versatile polylinkers. Most of the current plasmid vectors contain a limited number of hexanucleotide restriction sites and one or two rare restriction sites in the polylinker region. Usually, these cloning vectors are highcopy-number plasmids that are built from a pUC (7) backbone that has the *lac*Z gene fused to the multiple cloning site for blue/white screening.

Overlapping extension polymerase chain reaction (OE-PCR) has been used to generate synthetic DNA without the aid of an available template (2,3,6). This allows for the construction of computer-designed DNA of known sequence by synthesis of complementary overlapping oligonucleotides and subsequent PCR amplification. Ideally, a polylinker would incorporate several unique sites, both common and rare. This limitation is generally a function of sites absent in the vector backbone. However, in a *lac*Z fusion polylinker, considerations must also be given to length and maintenance of the open reading frame (ORF). The limitations to length can be overcome by the use of sites with opposite polarity (SWOP) pairs to create a compact multiple cloning site (5). This depends on the creation of adjacent restriction sites that share half or portions of their palindromic recognition sequence. Though many sites can be incorporated into a limited space, some distribution must be allowed for adequate restriction site availability and maintenance of the *lac*Z ORF. Thus, our goal was to develop a versatile cloning vector with a large polylinker region that would facilitate the manipulation of large segments of cloned DNA.

Eight synthetic oligonucleotides and the OE-PCR technique were used to generate a pUC19 (7) based plasmid vector that contained a *lac*Z-fused polylinker with 44 unique restriction sites, nine of which represent rare-cutter restriction sites. In addition, the multiple cloning site is flanked by the T3 and T7 bacteriophage promoters. Positive transformants can be determined by either disruption of the *lac*Z gene for blue/white screening or by a characteristic change in colony size from small to large.

A 230-bp synthetic polylinker was designed by computer analysis using the software packages MacDNASIS®

2.0 (Hitachi Software Engineering America, San Bruno, CA, USA) and OLIGO™ 4.0 (National Biosciences, Plymouth, MN, USA) as a *lac*Z gene fusion into the *Eco*RI/*Hin*dIII sites of pUC19 (7). Six oligonucleotides of 57–59 bases containing 23-nucleotide overlaps were synthesized (Operon Technologies, Alameda, CA, USA) and used in a two-step OE-PCR as defined by Dillon and Rosen (3) with a RoboCycler™ 9600 Gradient Thermal Cycler (Stratagene, La Jolla, CA, USA). The reaction mixture consisted of 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.75), 2 mM $MgSO_4$, 0.1% Triton® X-100, 100 µg/mL bovine serum albumin (BSA), 200 µM dNTPs, 400 µM each oligonucleotide (P1–P6) and 2.5 U cloned *Pfu* DNA Polymerase (Stratagene) in a 50-µL volume. Thermal cycling conditions were as follows: initial denaturation at 94°C for 5 min and seven cycles at 94°C for 1.5 min and 75°C for 3 min. One microliter of the PCR was used for the second round of amplification with flanking oligonucleotides FP1 and FP2

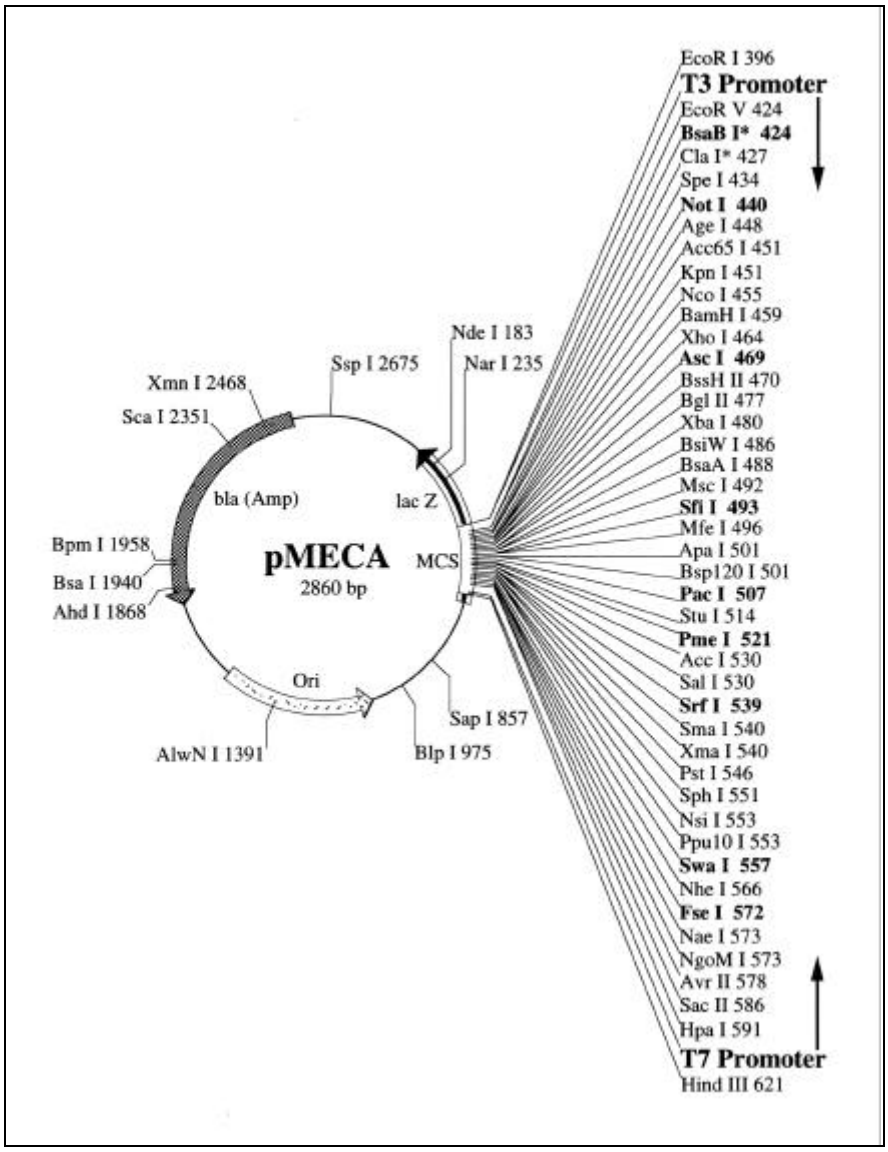


**Figure 1. Physical map of pMECA.** The multiple cloning site is revealed as an *Eco*RI/*Hin*dIII fusion with the *lac*Z gene. Unique restriction sites are shown, and the same numbers are used for neoschizomers (isoschizomers that cut at different sites within the same sequence). Rare-cutter restriction sites are shown in bold. Sites with an asterisk are blocked by overlapping *Dam* methylation. The T3 and T7 bacteriophage promoters are shown, with direction of transcription indicated by arrows.

(Operon Technologies). Reaction conditions were as previously stated, except that 200 µM each oligonucleotide (FP1 and FP2) were substituted for oligonucleotides P1–P6. Thermal cycling conditions were as follows: initial denaturation at 94°C for 5 min and 20 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 7 min. The PCR product was digested with *Eco*RI/*Hin*dIII and purified by agarose gel electrophoresis (4). The purified DNA fragment was ligated to the *Eco*RI and *Hin*dIII sites of pUC19 to create a multiple enzymatic cloning area (pMECA).

Colonies containing the insert were confirmed by PCR using the T3 and T7 bacteriophage promoter primers (Operon Technologies), which are absent in pUC19. Positive clones were confirmed through restriction digestion followed by gel electrophoresis and subsequent sequencing using a Model 394 Automated Sequencer (PE Applied Biosystems, Foster City, CA, USA) at the University of Georgia Molecular Genetics Instrumentation Facility.

To test cloning efficiency, a fragment containing the neomycinphosphotransferase II gene (*npt*II) was used as an indicator of positive transformants into the pMECA vector. Briefly, pBSL86 (1) was digested with either *Pst*I or double-digested with *Kpn*I/*Nhe*I, and the approximately 1.2-kb fragment containing the *npt*II fragment was gel-purified as before. The fragments were ligated into either *Pst*I or double-digested *Kpn*I/*Nhe*I pMECA and transformed into DH5α™ (Life Technologies, Gaithersburg, MD, USA). Bacteria were selected on LB agar plates supplemented with 100 µg/mL ampicillin alone or with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and 20 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The efficiency of selection for positive transformants by colony size was compared to that of blue/white screening by selecting 700 small and 700 large colonies from the ampicillin plates. In addition, 700 blue and 700 white colonies were selected from the ampicillin plates supplemented with IPTG/X-gal. All selected colonies were transferred to grid plates containing LB agar supplemented with 30 µg/mL kanamycin and scored for growth after

**Table 1. Comparison between Colony Size and Colony Color as Parameters for the Selection of Colonies with Inserts Successfully Ligated into the Polylinker**

| Vector | Insert | N[c] | Morphology[a] | Growth[b] (%) |
|---|---|---|---|---|
| pMECA/*Pst*I | *npt*II/*Pst*I | 700 | Small | 0 |
| | | 700 | Large | 682 (97.4) |
| pMECA/*Kpn*I/*Nhe*I | *npt*II/*Kpn*I/*Nhe*I | 700 | Small | 0 |
| | | 700 | Large | 694 (99.1) |
| pMECA/*Pst*I | *npt*II/*Pst*I | 700 | Blue | 0 |
| | | 700 | White | 675 (96.4) |
| pMECA/*Kpn*I/*Nhe*I | *npt*II/*Kpn*I/*Nhe*I | 700 | Blue | 0 |
| | | 700 | White | 697 (99.6) |

Vector to insert ratio was 1:1 for all clonings.

[a]Morphology of selected colonies is indicated as size for those on ampicillin plates only, or color for those initially selected on ampicillin supplemented with IPTG and X-gal.

[b]Growth represents number and percentage of 700 initially chosen clones that grew on agar plates supplemented with kanamycin.

[c]Number of colonies tested.

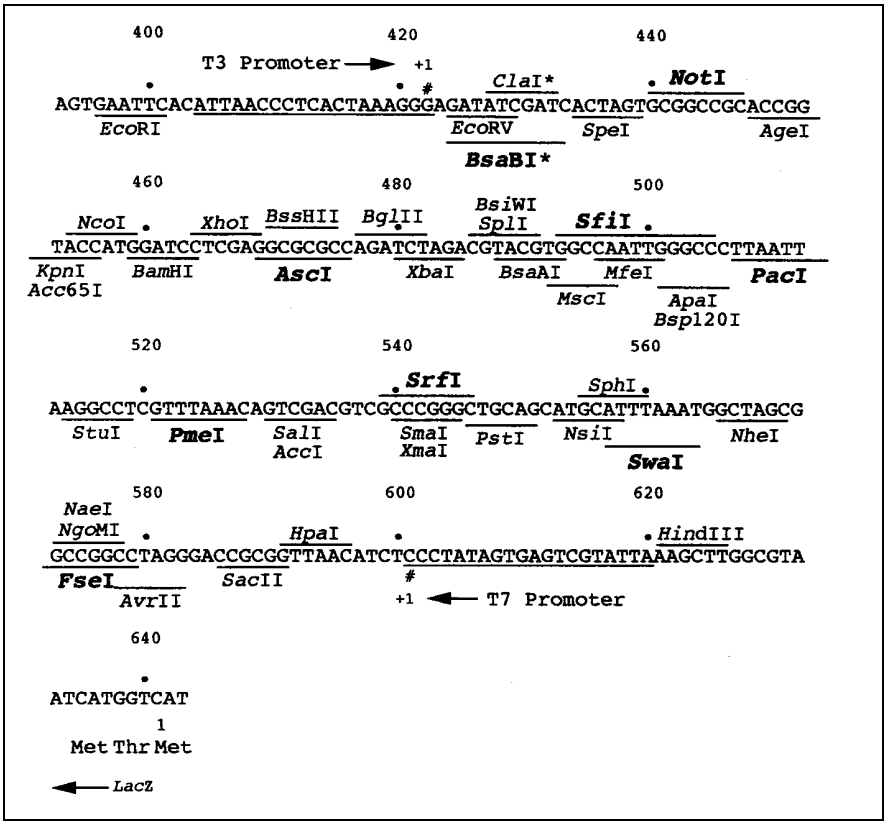


**Figure 2. Nucleotide sequence of the pMECA polylinker.** Nucleotide sequence of the polylinker is shown extending from the *Eco*RI to *Hin*dIII sites of pMECA. Unique restriction sites are underlined and labeled. Underlined sites with multiple labels represent neoschizomers. Rare-cutter restriction sites are shown in bold. Sites with an asterisk are blocked by overlapping *Dam* methylation. The T3 and T7 bacteriophage promoters are shown underlined and with arrows to indicate orientation. Start sites for transcription of the T3 and T7 bacteriophage promoters are indicated by the symbol (#) and +1 above and below the first nucleotide, respectively. The *lac*Z orientation and translation start site are indicated below the sequence with an arrow and the first three amino acids, respectively.

16 h of incubation at 37°C.

The result is a new vector designed for ease of DNA manipulation that has been designated pMECA (Figures 1 and 2). Nucleotide sequence analysis revealed the existence of all 44 restriction sites with no mutations. In this report, we have shown the use of OE-PCR to generate a synthetic *Lac*Z fusion polylinker in pUC19. Interestingly, bacterial clones that are transformed by this plasmid vector are light blue in color compared to those transformed by the parental pUC19 plasmid (data not shown). However, an analysis of the polylinker for RNA folding patterns using MacDNASIS 2.0 has revealed a large hairpin, which may be responsible for this reduction in β-galactosidase activity. Furthermore, bacterial colonies are significantly larger when the polylinker is disrupted by insertion of a DNA fragment (Figure 3). Cloning of the *npt*II gene into pMECA and selection on kanamycin has shown that this

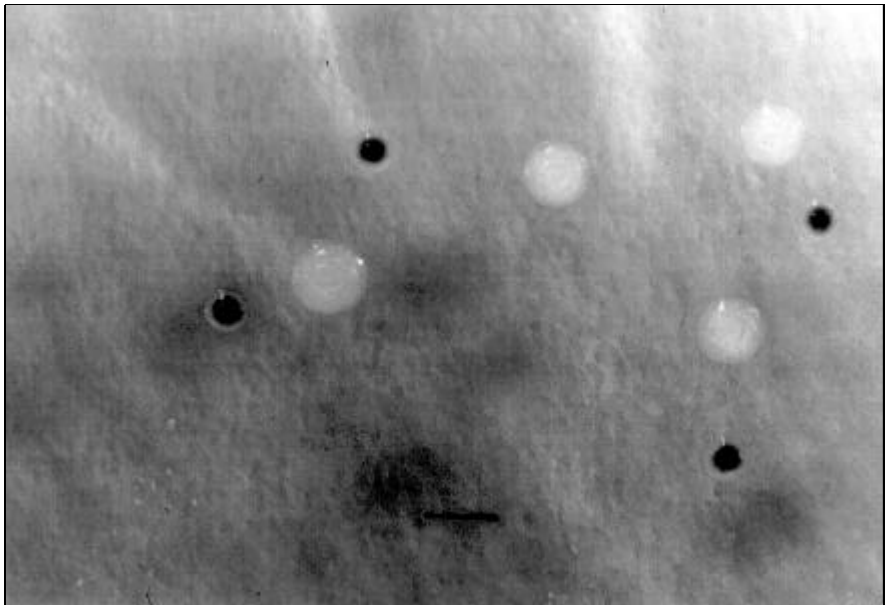

**Figure 3. Colony morphology of bacteria transformed with pMECA.** Morphological changes are shown by bacterial colonies transformed with pMECA either disrupted or not by insertion of the *npt*II-resistance cassette into the polylinker. Colonies are shown on LB agar plates supplemented with 100 μg/mL ampicillin, 0.1 mM IPTG and 20 μg/mL X-gal. Blue colonies that represent nondisrupted polylinkers appear small as compared to the large, white colonies containing disrupted polylinkers. Scale bar equals 1 mM.

distinct morphological change in bacterial colony size can be used as an indicator of positive transformants with a success rate of nearly 100% (Table 1). The observed reduction in bacterial colony size has not affected plasmid yields, leading us to speculate that the polylinker hairpin or the synthetic protein product of the polylinker in pMECA is affecting bacterial colony size. It should be emphasized that while this vector can be used for blue/white screening by conventional techniques, colonies containing an insert can be identified just as effectively by simply selecting the larger colonies, thus eliminating the need for IPTG and X-gal. The complete nucleotide sequence of pMECA is available from GenBank® as Accession No. AF017063.

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