Restriction digestion monitors facilitate plasmid construction and PCR cloning

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Plasmid construction by "forced" or "directional" ligation of fragments digested with two different restriction enzymes is highly efficient, except when inhibited digestion of one site favors vector recircularization. Such failures often result because incomplete double digestion is undetected in vector polylinkers or at terminal cloning sites on a PCR fragment. To test cleavage efficiency indirectly, a "monitor" plasmid is added to the digest. In a suitable monitor, the two test sites are separated by enough DNA (approximately 20% of full length) to distinguish the double digest from the failed single digest. To make this applicable to combinations of 32 popular cloning enzymes, we constructed a set of 4 monitors (pDM1, pDM2, pDM3, and pDM4). Each contains three polylinkers separated by stuffer segments of approximately 1 kb. The 32 sites are distributed in the polylinkers such that at least one plasmid in the set is diagnostic for each enzyme pair. The set is designed to be extended to up to 81 sites. A linearized version of the monitor allows for the determination of which of the two enzymes has failed in an incomplete double digest and is also useful when the target DNA is close to the size of the pDM backbone. The plasmids also serve as versatile self-monitoring cloning vectors for any site combination.

INTRODUCTION

Most plasmid construction techniques favor the insertion of a DNA fragment over the recircularization of an empty vector (1–5). Examples of these techniques include the use of dephosphorylated vectors (1,2); the use of TA™ and TOPO™ vectors (3,4) for cloning PCR products; ligation of partially end-filled vector restriction sites to other partially end-filled insert sites (e.g., *Xho*I and *Bam*HI end-filled with TC and GA, respectively); and the forced ligation of vector and insert fragments digested with a pair of enzymes, resulting in directional cloning (5).

Forced ligation is probably the most widely used method of plasmid construction, because of its versatility and efficiency and because it allows for control over the orientation of the insertion. It also results in a high percentage of plasmid clones with inserts (often >90%). However, if one of the two vector insertion sites is cleaved inefficiently, recircularization dominates, and empty vectors are disproportionately represented among transformants. The problem is aggravated when insert concentration is low or when three-fragment ligations are planned.

A successful construction is generally ensured if cleavage of both vector sites can be demonstrated. However, this is not possible if the sites are too close together (e.g., in a vector polylinker) because the linear molecules resulting from a single or double cut are unresolved on a gel. Similarly, digestion of cloning sites incorporated at the end of a PCR product is undetectable. Thus, it is helpful to have an indirect way of determining whether the four sites to be joined in a forced ligation have been cleaved efficiently.

A convenient test for the double digestion of a vector or PCR product is based on the addition of a monitor plasmid to the digest in question. In the monitor, the two sites are located far enough apart to detect both cleavages in the gel pattern of the mixed digest. The applicability of this method has been limited in the past because monitor plasmids suitable for most enzyme pairs were unavailable. Here we describe a set of four monitor plasmids containing 32 commonly used restriction sites, distributed in such a way that double digestion by nearly all combinations can be tested. The same plasmids can also be used as versatile cloning vectors.

MATERIALS AND METHODS

Construction of pDM1, pDM2, pDM3, and pDM4

Construction of the pDM plasmids is described at http://www.BioTechniques.com/June2004/AnandSupplementary.html. The pDM plasmids are available on request from the authors.

Enzyme digestion, Ligation, and Transformation

Restriction digests of vectors and PCR products were set up in 80 µL volumes, to one half of which was added 0.2 µg of the appropriate pDM monitor plasmid. pDM monitor plasmids were purified by alkaline lysis as previously described, with some modification. (For details of DNA purification, ligation, and transformation, see Supplementary Material.)

RESULTS AND DISCUSSION

Rationale for Monitoring Double Digestions

Successful application of forced ligation depends on efficient double digestion. This is quite difficult to demonstrate directly in a vector polylinker or at the ends of a PCR product. However, it is important to have some way of ensuring double cleavage because there are several potential causes of enzyme failure: DNA preparations may contain inhibitors, buffer conditions may not be optimal for both enzymes (despite vendor assurances), and dated enzymes may be inactive. We routinely test efficiency by including in the digest a monitor plasmid, in which the two cleavage sites are far enough apart to permit the resolution of the doubly and singly digested molecules on an agarose gel. The same technique is used to monitor the digestion of PCR products.

Construction of a Double Digest Monitor Plasmid Set

The four-plasmid pDM monitor set (Figure 1) includes 32 commonly used six-base restriction sites, including those in the pUC and pBS polylinkers, plus 15 others. The design criteria for a useful monitor set are (*i*) that the test sites be unique in each plasmid and (*ii*) that, for any given pair of restriction enzymes, there is at least one plasmid in the set in which the two sites are well separated. The 4.1-kb pDM construct contains three polylinker segments separated by approximately 1 kb restriction site-free segments, such that cleavage in two of the polylinkers yields approximately 1- and 3-kb fragments or two 2-kb fragments, all well-resolved from the 4.1-kb linear molecule.

In designing the pDM set, we noted that a set of "b" plasmids with "a" polylinkers is sufficient to monitor double digestion by all possible combinations of N sites, when $N \leq a^b$ (see supplementary Table S2 for template design algorithm). For example, for up to 27 (i.e., 3^3) sites, three plasmids with three polylinkers are needed, and for 81 sites (3^4), four plasmids with three polylinkers are required. The pDM set represents a partially filled version of the 81-site template (see supplementary Table S2), with 32 sites assigned to 26 positions. The restriction site-free DNA segments chosen for insertion between the three polylinker sites in pDM1-4 were two approximately 1 kb PCR-amplified segments from chromosome 5 of *Saccharomyces cerevisiae* (Figure 1) inserted between polylinker A and B, and B and C, respectively.

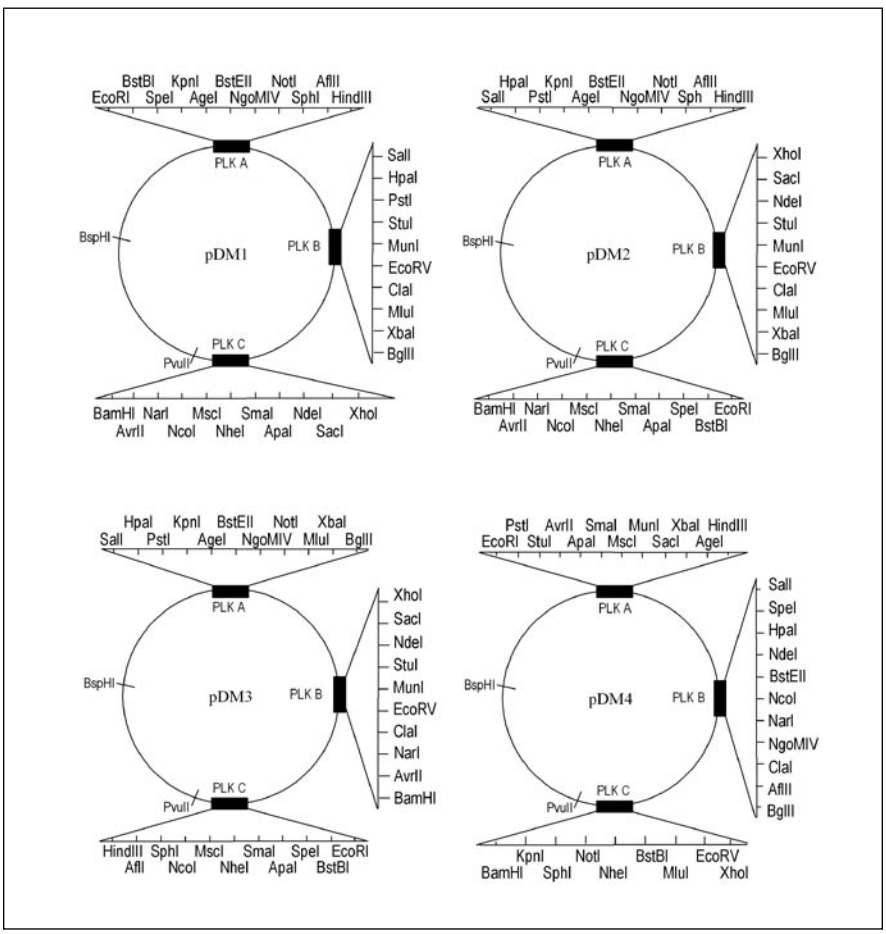


Figure 1. pDM1, pDM2, pDM3, and pDM4. The four plasmids are identical except for different distributions of 32 restriction sites in the three polylinkers. The common backbone consists of a 2.02-kb segment of pUC18 (6) (nucleotides 480–2501, between the *Ssp*I site and the *Hin*dIII sites) and two stuffer segments between the A and B, and B and C polylinkers of 1022 and 892 bp, respectively, derived from chromosome V of *Saccharomyces cerevisiae*. The polylinkers bring the size of the plasmids to from 4.09 to 4.15 kb.

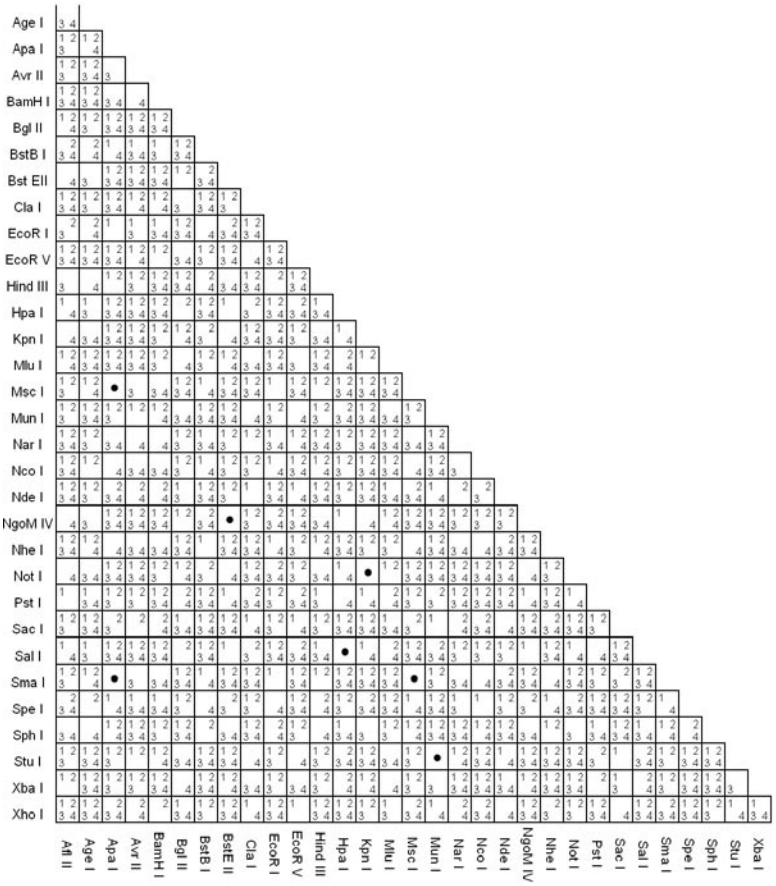
Monitoring Double Digestion of Plasmid Vectors or PCR Products

For the monitor test, a digestion reaction that is set up with target DNA and enzymes is divided, one half is "spiked" with a sample of the appropriate monitor plasmid (Table 1), and the two digests are run in parallel on a gel. A reliable criterion of efficient double digestion is the absence of a detectable singly cut full-length monitor (at 4.1 kb; Figure 2A). The mixed digestion is important; it is not sufficient to test the two enzymes together in the absence of the target DNA because the latter may contain inhibitors. Parallel digests with and without a monitor are preferable to a single mixed digest to avoid the contamination of the monitor in the excised vector band, but a single digest is sufficient if the target DNA runs well ahead of the monitor.

Figure 2B shows a digest in which one of the enzymes is partially inhibited. When detectable amounts of singly cut monitor are observed, it is necessary to optimize conditions for the double digestion. This can be done by (*i*) testing other buffers, (*ii*) reducing DNA or increasing enzyme, (*iii*) serial digestion in optimal buffers (achieved after incubation with the first enzyme by an of the salt concentration and/or pH before incubation with the second enzyme), (*iv*) replacing dated enzyme, or (*v*) repeating the plasmid preparation. It may also be helpful to test for the digestion of the monitor alone, to test enzyme activity and buffer conditions, and also to be sure that the monitor preparation itself is free of inhibitors. In rare cases, the order in which the enzymes are added may be important, where one enzyme preparation actually inhibits another (e.g., *Hin*dIII and *Bam*HI). One must also be aware of the possibility that cleavage of one of the vector cloning sites is inhibited by methylation, which would not be reflected in the monitor pattern.

Insertion efficiency can sometimes be assessed by a comparison of the number of transformants from ligations of the vector and insert with those of the vector alone. A large difference is observed for many enzyme pairs (e.g., *Hin*dIII and *Eco*RI), where the sticky ends are truly

Table 1. Choice of Plasmids to be Used for Monitoring Enzyme Pairs



The table indicates which of the four pDM plasmids are suitable monitors for each of the 496 possible pair-wise combinations of the 32 restriction enzymes represented in the three polylinkers. Monitoring is not possible for seven of these pairs whose recognition sites are in the same polylinker in each of the plasmids, indicated by a dot. Reference to plasmid maps in Figure 1 indicates whether the 2 + 2 or 3 + 1 cleavage pattern is observed.

incompatible, but for others, "illegitimate" recircularization readily occurs (e.g., with some combinations of blunt and sticky ends or with certain combinations such as *Bam*HI and *Xba*I), so that efficiently digested vectors give rise to comparable numbers of transformants with or without inserts. However, if the vector digest has passed the monitor test and if enough of an insertion fragment is added, we have generally observed a high insert frequency (apparently because compatible ends have a kinetic advantage).

PCR products purified by various means, including electrophoresis, often contain enzyme inhibitors, so it is important to determine whether the majority of PCR fragments to be used in a forced ligation are digested at both ends, as illustrated in Figure 2C. Less stringent criteria than for a vector apply because the presence of some partially digested fragments does not affect the outcome. The insert can be counted on for efficient ligation if the monitor test indicates greater than approximately 50% double cleavage, provided that the PCR primers are always designed with at least four extra bases 5′ to the restriction site to accommodate enzyme binding.

Use of Prelinearized Monitor to Identify Nonfunctioning Enzyme

A double digestion test using a monitor plasmid indicates whether one of the enzymes fails, but not which one. However, if the monitor is used in its prelinearized form (i.e., predigested with *Bsp*HI), the pattern of an incomplete digestion is diagnostic (Figure 3).

Use of Prelinearized Monitor with Target DNA of Similar Size

When the target DNA and the monitor DNA are of similar size (i.e., approximately 4.1 kb), co-migration is a problem because the linearized vector obscures the singly cut monitor, and failed digestion cannot be detected. In this case, the prelinearized monitor should be used because incomplete digestion of the prelinearized monitor produces less than 3 kb fragments (Figure 3), detectable ahead of the target band. Note that the circular monitor can be used if the target DNA is the same size as the doubly cut monitor DNA, since efficient digestion is still shown by the absence of the full-length linear monitor DNA. Furthermore there is a choice between the 1 + 3 or 2 + 2 pattern for many enzyme combinations (Figure 1).

Use of pDM Plasmids as Versatile Cloning Vectors

A monitor for a given pair of enzymes can also serve as a self-monitoring vector for the insertion of a fragment digested with the same enzymes because the doubly digested backbone is well separated from the full-length linear monitor DNA. Thus, the pDM plasmids are convenient vectors for cloning fragments digested with any pair of enzymes in the pDM site list, adding many options that are not available with pUC and pBS vectors, with a net efficiency that is comparable to blue-white selection (6). In case only the 2 + 2 configuration is available (e.g., *Xba*I-*Mlu*I), a third enzyme that cuts in polylinker B should be added to eliminate the co-migrating stuffer. Alternatively, the ligation should include at least approximate equimolar levels of the insert to compete with the stuffer fragment contaminating the excised vector band.

Design for a Comprehensive Monitor Set

By including additional sites in the polylinkers, the 3 × 4 template (Table S2) can accommodate 81 sites, which is enough for the 64 six-base palindromes, plus 17 other sites, in a universal monitor set. Even more could be added with a minor loss of utility, in that when two sites are in the same

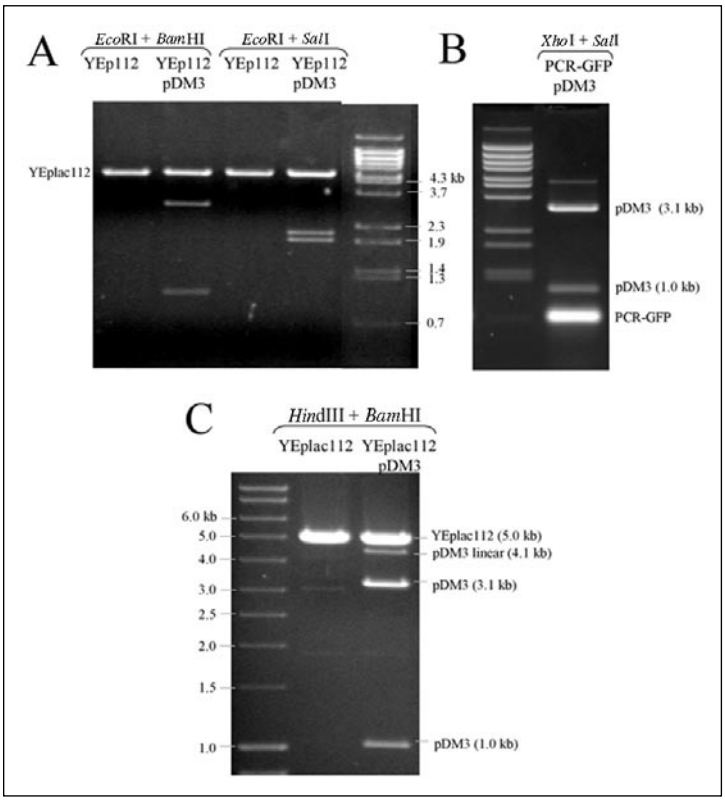


Figure 2. Monitoring vector digestion. (A) Plasmid YEplac112 was incubated with *Eco*RI and *Bam*HI or *Eco*RI and *Sal*I in the presence or absence of pDM3, which yields either a 3 + 1 or a 2 + 2 pattern, depending on the position of the sites in the three polylinkers. (B) The green fluorescent protein (GFP) open reading frame was amplified by PCR using primers containing *Xho*I and *Sal*I sites, respectively, and digested with those enzymes in the presence of pDM3. The marker ladder is λ bacteriophage DNA digested with *Bst*EII. (C) YEplac112 was incubated with *Hin*dIII and *Bam*HI in the presence or absence of pDM3. The presence of the 4.1-kb pDM3 linear reveals incomplete digestion. The marker ladder is the 1-kb ladder (Bioline USA, Randolph, MA, USA).

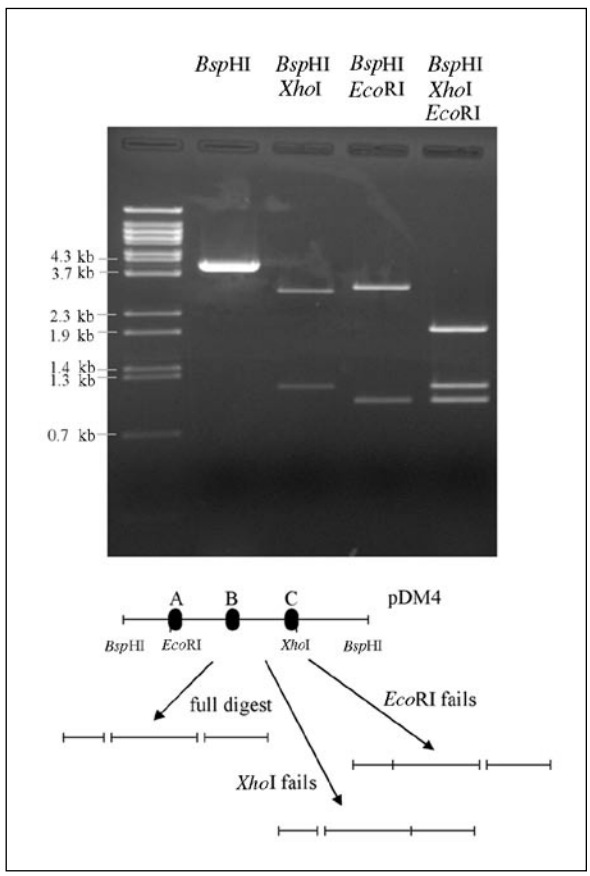


Figure 3. Prelinearized digestion monitor. pDM3 was digested with *Bsp*HI, followed by the indicated restriction enzymes, in three separate reactions. The diagram indicates the cutting patterns for each combination, and thus how failure by one enzyme can be distinguished from failure by the other.

polylinker in all four plasmids, they are the only pair that cannot be monitored. As replacements for the *S. cerevisiae* stuffers, which contain many six-base palindromes, a search of the human sequence database for restriction site-free sequences revealed numerous candidates of approximately 500 bp (1 kb site-free fragments are statistically unlikely), which can be co-ligated into pDM1-4, two at a time, in place of the yeast-derived stuffers.

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