Consecutive Cycles of Precise, Unidirectional 14-bp Deletions Using a BseRI/BsgI Trimming Plasmid

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ABSTRACT

A straightforward method for generating precise, consecutive, unidirectional 14-bp deletions into cloned DNA, adopted from the trimming principle developed by Szybalski and his colleagues, is presented. The method utilizes pTRIM14, a plasmid constructed with the class-II restriction enzyme recognition sites for BseRI and BsgI arranged in the form of a cassette, just upstream from the cloned DNA. Class-II restriction enzymes cleave DNA downstream of their recognition sites. pTRIM14, containing the cloned DNA, is processed through a trimming cycle that involves sequential restriction digestions with BsgI and then BseRI, followed by treatment with Mung Bean nuclease and then with ligase. One trimming cycle results in a net 14-bp deletion. We demonstrate precise, consecutive deletions at very high efficiency.

INTRODUCTION

Trimming plasmids, as developed by Szybalski and his colleagues (1-3), can be used to produce small, precise, consecutive, unidirectional deletions into cloned DNA. They thus lend themselves well to various projects requiring the generation of such consecutive deletions, such as removal of unwanted restriction sites problematic for a particular biotechnological purpose or fine-mapping of promoter regions. Trimming plasmids are based on class-II restriction endonucleases that cleave DNA at a precise distance in the 3' direction from their recognition sites (reviewed in Reference 3). A plasmid containing one or a combination of class-II restriction enzyme recognition sites, in the form of a cassette, digested with the proper class-II enzyme(s), followed by removal of the single-stranded DNA staggered ends and religation results in a net deletion of DNA on the 3' side of the cassette. Trimming plasmids have been constructed using BspMI (1) or a cassette of MboII and FokI (2) recognition sites to generate deletions up to 4 bp or 12 bp, respectively. However, the MboII/FokI 12-bp trimming plasmid is based on 5-bp recognition sites, which are likely to be present at fairly high frequency in cloned DNA, and uses a dihydrofolate reductase gene as a selectable marker against the antibiotic trimethoprim. This is an uncommon procedure in many laboratories.

We have constructed a new trimming plasmid, termed pTRIM14, that contains a cassette designed with BseRI and BsgI 6-bp recognition sites to facilitate 14-bp deletions into cloned DNA and uses ampicillin resistance as a selectable marker. BseRI (recognition site 5'-GAGGAG-3') and BsgI (recognition site 5'-GTGCAG-3') are class-II restriction enzymes that cleave DNA at 10/8 and 16/14 nucleotides (nt), respectively, downstream of their recognition sites to generate staggered ends. These recognition site sequences were arranged into a BseRI/BsgI trimming cassette so that the BseRI site is at the 5'-end directly adjacent to the BsgI site at the 3'-end (5'-GAGGAG GTGCAG-3') and subcloned between the HindIII and EcoRI sites of pUC18's multiple cloning site (MCS), yielding pTRIM14 (Figure 1). Cloned DNA may then be inserted into the EcoRI site of pTRIM14 for subsequent processing through deletion or trimming cycles. The trimming cycle begins by stepwise digestion first with BsgI and then BseRI to cleave 16/14 nt distal and 4/2 nt proximal, respectively, relative to the 3'-end of the cassette. This thus conserves the trimming cassette but removes the intervening 6-bp EcoRI site and 8-bp at the 5'-end of the cloned DNA fragment. The trimming cycle proceeds by treatment with Mung Bean nuclease to generate blunt ends and finally recircularization with T4 DNA ligase back into a plasmid. In the end, the trimming cycle results in a precise 14-bp deletion, which can be repeated as often as necessary (Figure 1).

MATERIALS AND METHODS

pTRIM14 was built as follows: the oligodeoxynucleotides TRIM14α (5'-AGCTTGAGGAAGGTCAGTAG3') and TRIM14β (5'-AATTCTACTGCACCTCCCTA-3') were synthesized (University of Wisconsin Biotechnology Center, Madison, WI, USA) and annealed at 37°C to form the BseRI/BsgI trimming cassette with 5' HindIII and 3' EcoRI staggered ends. The intervening sequence between the HindIII and EcoRI sites in the MCS of pUC18 was removed by restriction digestion, and the resulting linearized plasmid was ligated to the BseRI/BsgI cassette to give pTRIM14.

To test pTRIM14, it was necessary to insert a cloned DNA into the pTRIM14 EcoRI site (Figure 1). For such a purpose, a 313-bp fragment of the rat sperm membrane protein YWK-II cDNA (4) corresponding to nucleotide positions 1074-1386 was used. The YWK-II cDNA fragment was isolated during screening of a subtracted cDNA library (unpublished data) and had oligodeoxynucleotide linkers containing EcoRI sites ligated onto its termini, which facilitated subcloning of the YWK-II cDNA fragment into the EcoRI site of pTRIM14 (Figure 1). However, the YWK-II cDNA fragment contains one BseRI recognition site in reverse orientation centered at nucleotide position 1306. Therefore, the YWK-II fragment was subcloned in reverse orientation into pTRIM14, yielding pT14Δ0. Consequently, when pT14Δ0 is subjected to its first trimming cycle, there is a deletion of 110 bp because of the internal BseRI site within the subcloned YWK-II cDNA fragment (Figure 3); this is addressed under Results and Discussion.

Each trimming cycle requires consecutive digestions with BsgI and BseRI, which to the best of our knowledge, are only commercially available from New England Biolabs (Beverly, MA, USA). Also, reaction conditions for BsgI and BseRI digestions are critical for optimal efficiency of cleavage. The enzymes cleave DNA efficiently only with their supplied reaction buffers, as any contaminating salt significantly reduces cleavage efficiency. As a result, before plasmid DNA is used as substrate for BsgI and BseRI digestions, it is important to first remove contaminating salts by passing the plasmid DNA through a Chroma Spin™ 400 centri-
fuge column (CLONTECH Laboratories, Palo Alto, CA, USA). Bsg1 and BseRI digestions are carried out using 5 U enzyme/µg DNA, since star activity may occur at 10 U enzyme/µg DNA. Additionally, Bsg1 and BseRI have a short half-life and are inactive after 2 h at 37°C. To ensure complete digestion, an additional aliquot of fresh enzyme is added to the reaction after two hours, and the digestion then proceeds for another two hours. Similarly, an additional aliquot of Mung Bean nuclease is added after its initial 1-h digestion, which then proceeds for another hour to ensure blunted DNA ends.

Plasmid DNA for each trimming cycle was prepared with QIAGEN® plasmid preps (Qiagen, Chatsworth, CA, USA). An initial 10 µg of plasmid DNA were sufficient to complete one cycle. Before a plasmid was subjected to a trimming cycle and after each of the Bsg1, BseRI and Mung Bean nuclease digestions, any contaminating salts and proteins were removed by a Chroma Spin 400 centrifuge column. Digestions with BseRI, Bsg1 and Mung Bean nuclease (New England Biolabs) were performed according to the manufacturer’s protocol, with the above modifications. Ligations were completed with the Ligation Express™ kit (CLONTECH) using 0.5 µg of plasmid DNA in a total ligation volume of 28.4 µL and incubated at 16°C for 30 min. After each trimming cycle, 3 µL of ligation mixture were transformed into subcloning efficiency DH5α™ bacterial cells (Life Technologies, Gaithersburg, MD, USA) using the manufacturer’s protocol. Resulting bacterial colonies were cultured in LB medium supplemented with 50 µg/mL ampicillin (Sigma Chemical, St. Louis, MO, USA).

Deletions resulting from each trimming cycle were characterized initially by polymerase chain reaction (PCR) and finally by DNA sequencing of the YWK-II fragment. PCR was performed using 2 µL of bacterial culture directly as the DNA template source and M13/pUC forward and reverse primers (Life Technologies) for 30 cycles (94°C for 1 min, 55°C for 1 min and 72°C for 45 s) of amplification. The M13/pUC primers flank the trimming cassette and cloned DNA (Figure 1), resulting in amplification of the cloned DNA. PCR products generated after each trimming cycle were evaluated by electrophoresis through a 3% Trevigen™ 500 agarose gel (Trevigen, Gaithersburg, MD, USA). Plasmid DNA was isolated (QIAGEN plasmid preps) and sequenced (Sequenase™ Quick-Denature plasmid sequencing kit; United States Biochemical [USB], Cleveland, OH, USA) from 5 samples after each trimming cycle. Sequencing products ([35S]-labeled) were resolved on a standard 5% sequencing gel and exposed to X-OMAT AR film (Eastman Kodak, Rochester, NY, USA).

RESULTS AND DISCUSSION

Three sets of experiments were conducted to characterize trimming. The first set of experiments evaluated trimming precision. The second set of experiments determined trimming efficiency for 1 cycle on a single plasmid clone. The third set of experiments determined trimming efficiency for 2 cycles carried out consecutively, that is, without isolating an intermediate single plasmid clone between trimming cycles and instead using the entire population of plasmids generated from the first trimming cycle to begin a second trimming cycle. Data from each set of experiments are verified on independent gels; however, in an effort to
conserv e publication size, data from all three sets of experiments are presented in Figure 2.

Trimming was initially characterized by subjecting pT14A0 to three consecutive trimming cycles (Δ1, Δ2 and Δ3) generating pT14Δ1, pT14Δ2 and pT14Δ3, respectively. Trimming cycle Δ1 resulted in the deletion of 110 bp from pT14Δ0, due to the internal BseRI cleavage site in the cloned DNA, generating pT14Δ1 (compare Figure 2, lanes labeled T14Δ0 with T14Δ1). This demonstrates that if a BseRI or BsgI site is present within the cloned DNA, the first trimming cycle will result in a deletion extending 10 or 16 bp on the 3' side of the internal BseRI or BsgI site, respectively, and thus a net deletion of more than 14 bp (Figure 3B). Trimming cycle Δ2 was carried out on pT14Δ1, producing pT14Δ2, which in turn was used for trimming cycle Δ3 to generate pT14Δ3. Trimming cycles Δ2 and Δ3 resulted in two consecutive 14-bp deletions as shown in Figure 2 (compare lanes labeled T14Δ2, T14Δ2 and T14Δ3) and were further demonstrated as precise 14-bp unidirectional deletions by sequence analysis in Figure 3A. Trimming cycles Δ1, Δ2 and Δ3 and their resultant deletions are schematized in Figure 3B.

Trimming efficiency for 1 cycle was determined using PCR to score precise deletions (Figure 2, lanes labeled 1–20 and 1 Cycle). Plasmid was prepared from a single isolated pT14Δ1 clone and processed through one trimming cycle. The resulting population of plasmids were transformed into DH5α cells, from which 20 bacterial colonies were isolated. PCR was performed directly from the bacterial clones as the source of DNA template using M13/pUC forward and reverse primers to amplify the cloned DNA (Figure 1). Because the resulting PCR products after each trimming cycle differ by only 14 bp, small anomalies in DNA migration during electrophoresis become significant and may affect data analysis. Therefore, independent PCR products resulting from the trimming efficiency experiments were mixed with a PCR product from the parental pT14Δ1 (T14Δ1 Mr, Figure 2). T14Δ1 Mr serves only as a molecular weight marker or as an internal reference for each lane to determine that a deletion occurred as opposed to an anomaly in DNA migration. Twenty independent PCR products resulting from 1 trimming cycle [T14Δ2 (Figure 2, middle bands labeled 1 Cycle)] and from 2 consecutive trimming cycles [T14Δ3, (Figure 2, bottom bands labeled 2 Cycles)] as discussed below were mixed with T14Δ1 Mr and resolved on a 3% TreviGel 500 agarose gel, thus displaying three bands in one lane if the plasmids were properly trimmed. All 20 T14Δ2 PCR products from individual bacterial colonies show a 14-bp deletion as compared to T14Δ1 Mr [(Figure 2, lanes 1–20; compare migration of T14Δ2 (middle band labeled 1 Cycle) with T14Δ1 Mr (top band)]. As stated above, this result was verified on an independent gel using only samples generated from the efficiency of 1 trimming cycle experiment. These results indicate a trimming efficiency up to 100% from a single plasmid clone after 1 trimming cycle.

A similar set of experiments were performed to determine the efficiency of 2 consecutive trimming cycles without isolating a unique plasmid between cycles. Plasmid DNA was prepared from a single pT14Δ1 clone, processed through 1 trimming cycle, and the resulting plasmids transformed into DH5α cells. Plasmid DNA was then prepared from the entire population of transformed cells, processed through a second trimming cycle and again transformed into DH5α cells. PCR was performed directly on 20 isolated bacterial colonies using M13/pUC primers to amplify the cloned DNA. As above, the 20 independent PCR products derived from 2 consecutive trimming cycles [T14Δ3 (2 Cycles, Figure 2)] were mixed with the reference molecular weight marker T14Δ1 Mr and T14Δ2 PCR products and resolved on a 3% TreviGel 500 agarose gel. T14Δ3 PCR products are scored for a 28-bp deletion or two cycles of 14-bp deletions as compared with T14Δ1 Mr [Figure 2, lanes 1–20; compare migration of T14Δ3 (bottom band labeled 2 Cycles) with T14Δ1 Mr (top band)]. Eighteen of the 20 T14Δ3 PCR products show a 28-bp deletion, while the T14Δ3 PCR products in lanes 9 and 14 are absent and instead show a 14-bp deletion since densitometric analysis of the gel indicates that the T14Δ2 PCR product in lanes 9 and 14 are twice the intensity as the other lanes. Again, as stated above, this result was verified on an independent gel using only samples generated from the efficiency of 2 consecutive trimming cycles experiment. These results indicate a trimming efficiency up to 90% for two consecutive cycles without an isolated intermediate.

The trimming efficiencies of 100% for 1 cycle and 90% for 2 consecutive cycles should be considered as esti-
mates because of the limited sample size of 20 colonies. Furthermore, when the BsgI and BseRI digestions are performed for only 2 h each without adding a fresh aliquot of enzyme for digestion of an additional 2 h as detailed under Materials and Methods, the trimming efficiency for 1 cycle drops to 65% and for 2 consecutive cycles to 30% (data not shown).

We did not observe 12- or 13-bp deletions resulting from inefficient digestion of the staggered DNA ends by Mung Bean nuclease after restriction endonuclease treatment. However, Mung Bean nuclease blunting efficiency was maximized by essentially carrying out the reaction twice, that is, adding a fresh aliquot of the enzyme to the reaction and allowing it to digest for another hour, as stated in the Materials and Methods. Therefore, if the Mung Bean step is performed twice, we would not expect to generate a subset of clones with 12-bp deletions, which could occur if the staggered ends were complementary and incompletely removed. But, if T4 DNA polymerase is substituted for Mung Bean nuclease to fill in the staggered ends instead of removing the staggered ends, a net deletion of 10 bp should occur, although we did not characterize this protocol modification.

It should be noted that when a cloned DNA is inserted into pTRIM14, the cloned DNA will be 8 bp on the 3' side of the trimming cassette due to the two nucleotides, TA (Figure 3) and the regeneration of EcoRI sites flanking the cloned DNA (Figure 1). As a result, the first trimming cycle will delete 14 bp—6 bp of which are from the EcoRI site plus 8 bp of cloned DNA. However, subsequent trimming cycles will delete 14 bp of cloned DNA.

In like manner to pTRIM14, we have also built the 8-bp precise trimming plasmids pTRIM8B, based on a cassette of the 6-bp recognition site enzymes BsgI/BbsI (5'-GTGCAG GAAGAC-3'), and pTRIM8S, based on a cassette of the 6- and 7-bp recognition site enzymes, respectively, BsgI/SapI (5'-GTGCAG GCTTCTTC-3').

In short, pTRIM14 is a precise trimming plasmid based on a cassette designed with BseRI and BsgI recognition sites and generates consecutive, precise, 14-bp unidirectional deletions in cloned DNA at high efficiency.

REFERENCES


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