ARTYKUŁ PROMOCYJNY

Artificially engineered specific nucleases – a breakthrough in RE research – Rewiev

Eglė Česnavičienė
Fermentas UAB, Vilnius, Lithuania

During the last few decades, several hundred type II restriction enzymes have been isolated and characterized from various bacterial strains, and yet, many specificities are still unavailable. There is an increasing demand for a wider selection of restriction enzymes with varying recognition sequences, which has stimulated efforts to produce artificial restriction enzymes. The rapidly expanding field of protein engineering couples studies of protein structure and function with molecular evolution based on random mutagenesis and high throughput screening to create enzymes having desired properties.

There have been numerous achievements that have contributed to the improvement of biochemical properties of biocatalysts, such as specific activity, substrate specificity, enantioselectivity and stability (1,2). In spite of these advancements, many efforts that sought to alter the mode of cleavage or recognition specificity of restriction endonucleases have, so far, been unsuccessful (3). A probable reason for this failure is that RE DNA recognition and catalysis are tightly linked; therefore, alterations to the DNA recognition domain may require exchange or alteration of other amino acids throughout the enzyme in order to maintain activity. Until now, mutagenesis/selection techniques were of limited use for identification of REs with altered specificity since a powerful selection method was unavailable.

Over the last 20 years at Fermentas, the knowledge and understanding of restriction endonucleases has continued to expand. Our expertise, combined with ongoing screening for micro-

Adres do korespondencji
Eglė Česnavičienė,
Fermentas UAB,
Graičiūno 8,
2028 Vilnius,
Lithuania;
e-mail: egle@fermentas.lt

biotechnologia
2 (61) 7–11 2003

organisms that possess unique R-M systems, has resulted in a breakthrough in RE engineering. Fermentas scientists have successfully created two, man-made, specific endonucleases: *Eco57IM*, a restriction endonuclease and *N.Bpu10I*, a site and strand specific nicking enzyme.

Eco57MI restriction endonuclease (recognition sequence 5' CTGRAG(N)16/14i) is a mutant version of *Eco57I* (recognition sequence 5' CTGAAG(N)16/14i). *Eco57I* is a bifunctional restriction enzyme that possesses both restriction endonuclease and DNA methyltransferase activities (4-6). *Eco57MI* was isolated from the mutagenized *eco57I* gene library using a novel, patent-pending method for altered specificity of restriction endonucleases. This method is applicable to all bifunctional type II restriction endonucleases that exhibit DNA methyltransferase activity. The biochemical properties of *Eco57MI* are similar to those of its progenitor *Eco57I*: maximal enzyme activity is achieved in low ionic strength buffers, in addition to the absolute requirement for Mg^{+2}; the enzyme cleavage activity is increased 100-fold by S-adenosylmethionine (SAM).

N.Bpu10I nicking enzyme (recognition sequence 5' GCiTNAGG) is a site and strand specific endonuclease engineered from the restriction endonuclease *Bpu10I*. *N.Bpu10I* nicking enzyme cleaves only one DNA strand within its quasi-symmetric recognition sequence on its double-stranded DNA substrate. *Bpu10I* restriction endonuclease is a heteromeric enzyme made up of two, non-identical subunits that recognizes the asymmetric DNA sequence 5'CCTNAGC(-5/-2)i and cleaves DNA on both strands within its recognition sequence (7).

It was proposed, and later experimentally confirmed, that *Bpu10I* subunits are enzymatically active only when complexed and that each subunit nicks a different strand of DNA within the recognition sequence (7). The discovery of such a uniquely organized RE as *Bpu10I* created the possibility of engineering a site specific nicking enzyme by introduction of strategic mutations which abolish the cleavage activity of only one of the heteromeric units, while preserving the DNA recognition function of the heteromeric complex. This patent-pending method has been developed by Fermentas to create *N.Bpu10I*, the first artificial nicking enzyme.

Currently available nicking enzymes have several shortcomings that limit their use. One class of naturally occurring site-specific nicking enzymes appear to originate from Restriction-Modification systems. These naturally occurring enzymes recognize short trinucleotide to pentanucleotide DNA sequences, resulting in a high frequency of cleavage (8-10).

Another commercially available nicking enzyme is the bacteriophage fd gene II protein (gpII protein). This enzyme nicks one DNA strand in the phage origin of replication, but unlike other similar enzymes involved in phage replication, gpII protein does not form a covalent protein complex with the 5'-end of the nicked strand during DNA hydrolysis (11,12). Commercial applications of this enzyme include the production of single-stranded DNA, labeling of DNA probes and for the introduction of unidirectional nested deletions (13). One major drawback with gpII protein is that

DNA nicking is inefficient making it impossible to achieve complete nicking of a substrate. The resulting high background requires an additional purification step to separate nicked DNA from the non-nicked form.

N.Bpu10I recognizes a heptanucleotide DNA sequence that occurs infrequently in substrate DNA, and nicks DNA much more efficiently than gpII protein, making it useful in a wider variety of applications as detailed below. See **www.fermentas.com/catalog/re/nbpu10i.htm** for a listing of protocols.

Ligation-independent cloning eliminates the ligation step in recombinant molecule construction as, for example; in the preparation of a plasmid vector with a DNA insert (14). The PCR fragment to be cloned is incorporated into the vector *via* complementary, single-stranded nucleotide sequences, which are introduced using uridinilated primers/adapters and uracil DNA glycosylase (UDG) from *E. coli* (15). The ability of *N.Bpu10I* to specifically nick pre-selected DNA strands may be used for the efficient preparation of vectors for ligation-independent cloning, thereby success-

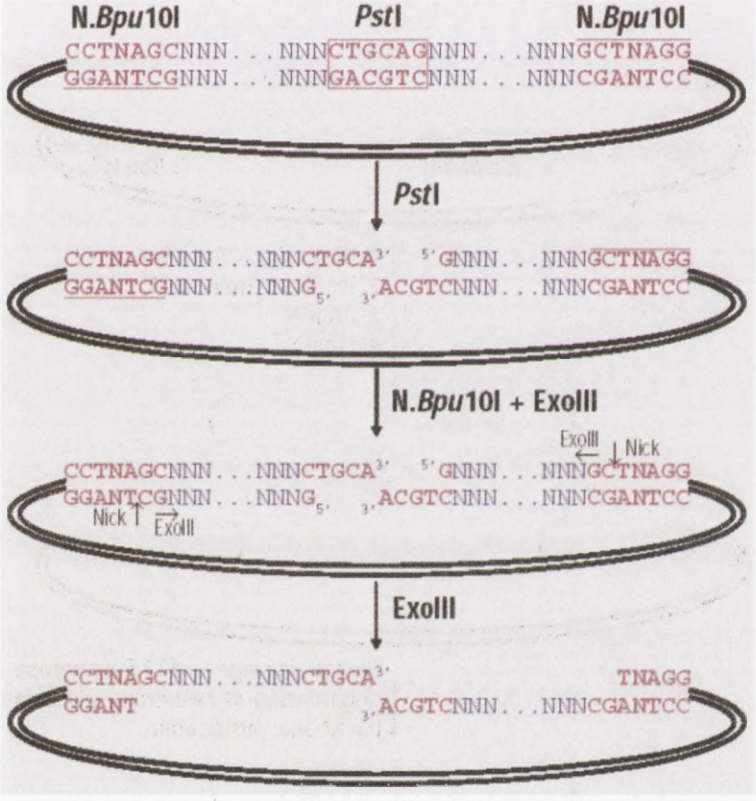

Fig. 1. Scheme of vector preparation for ligase independent cloning method using site-specific nickase *N.Bpu10I*.

fully eliminating the vector amplification/UDG treatment step in this procedure. For this method, a unique site for a restriction endonuclease which generates a 3'-protruding end must be present in the cloning vector, flanked by inverted N.*Bpu10I*, recognition sequences (see Fig.1). Such a sequence can be created as a cassette, which may be ligated into any desired vector; the resulting vector can then be amplified *in vivo*.

Following isolation and purification, the vector is linearized by the restriction endonuclease to produce 3'-overhangs. Treatment with *N.Bpu10I* allows for the introduction of nicks into selected opposite DNA strands. Short, nicked DNA strands are then degraded from the 3'-end by *E. coli* exonuclease III, producing 3'-overhangs of desired length at the ends of the linearized vector.

Specific DNA nickases, such as *N.Bpu10I* (see **www.fermentas.com/catalog/re/bpu10i.htm** for protocol) may also be used in the preparation of covalently closed linear DNA molecules. Linear covalently closed molecules appear to be very promising as minimalistic transfection vectors for gene therapy or vaccine development. As an example, the Minimalistic Immunologically Defined Gene Expression

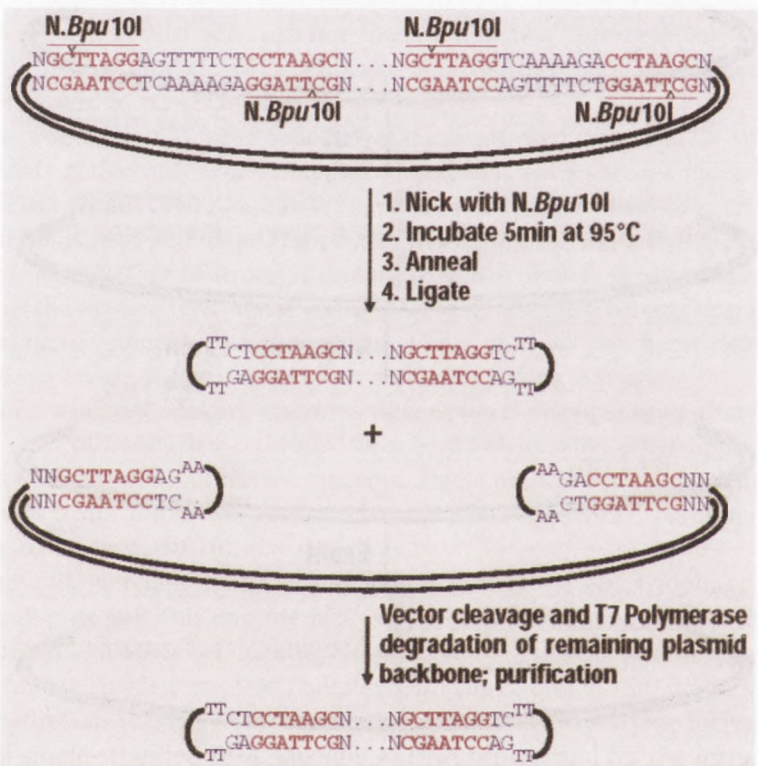

Fig. 2. Preparation of covalently closed DNA using site-specific nickase *N.Bpu10I*.

(**MIDGE**®) vectors can be produced using Fermentas MIDGE® Vector Production Kit. *N.Bpu10I* can replace the combination of restriction endonuclease/oligonucleotide ligation for construction of covalently-closed linear DNA molecules (see Fig. 2). Use of a site-and-strand specific enzyme is even more advantageous when compared to conventional techniques, because it tolerates the presence of nickase recognition sites in the desired DNA insert. In the subsequent ligation step, not only are DNA ends covalently closed, but all additional nicks in the DNA fragment are efficiently ligated at the same time. This feature allows the use of standardized vectors for the creation of any desired, covalently-closed linear DNA insert. The use of restriction endonucleases for excision of a desired DNA insert requires the construction of a vector backbone for each application by introducing recognition sequences to flank the cloning site for that have no targets in the insert DNA.

Literature

1. Arnold F. H., Volkov A. A., (1999), Curr. Opin. Chem. Biol., 3, 54-59.
2. Petrounia I. P., Arnold F. H., (2000), Curr. Opin. Biotechnol., 11, 325-330.
3. Lanio T., Jeltsch A., Pingoud A., (2000), Protein Eng., 13, 275-281.
4. Janulaitis A., Petrusyte M., Maneliene Z., Klimasauskas S., Butkus V., (1992), Nucleic Acids Res., 20, 6043-6049.
5. Janulaitis A., Vaisvila R., Timinskas A., Klimasauskas S., Butkus V., (1992), Nucleic Acids Res., 20, 6051-6056.
6. Rimseliene R., Janulaitis A., (2001), J. Biol. Chem., 276, 10492-10497.
7. Stankevicius K., et al., (1998), Nucleic Acids Res., 26, 1084-1091.
8. Zhang Y., et al., (1998), Virology, 240, 366-375.
9. Xia, Y., et al., (1988), Nucleic Acid Res., 16, 9477-9487.
10. Abdurashitov M. A., et al., (1996), Mol. Biol. (Mosk), 30, 1261-1267.
11. Meyer T. F., et al., (1979), Nature, 278, 365-367.
12. Geider K., et al., (1982), J. Biol. Chem., 257, 6488-6493.
13. Quick – Strand TM Site-specific mutagenesis kit, NBL Gene Sciences, Inc. advertisement leaflet.
14. Aslanidis C., et al., (1990), Nucleic Acids Res., 18, 6069-6074.
15. Rashtchian A., et al., (1992), Anal. Biochem., 206, 91-97.