

Review Article

# Utilization of Type IIS Endonucleases in Molecular Cloning

Jiayou Zhang\*

Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky, USA

\*Corresponding author: Jiayou Zhang, Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky, Medical Bldg, Room MN416, 800 Rose Street, Lexington, KY 40515, USA

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Type II endonucleases are powerful tools for molecular cloning. These endonucleases digest at a defined position within or close to their recognition sites. The Type II endonucleases used the most are those that digest DNA within their recognition sites, such as *EcoRI* (GAATTC) or *HindIII* (AAGCTT). Some literature refers to them as orthodox Type II endonucleases [1]. They usually recognize 6 or 8 bases as symmetrical sequences. A symmetrical sequence reads the same forward and backward. These endonucleases contain two identical subunits (homodimers). One binds a three (or four) base sequence while the other one binds to the other strand of DNA and the same three (or four) base sequence at the opposite direction, so that the two units are situated face to face and upside down in relation to each other [2]. The recognition and cleavage domains of these endonucleases are not organized as two distinguish part in their 3-D structure [2]. Orthodox Type II endonucleases cleave within their recognized sequences, creating a sticky end or a blunt. (The enzymes that recognize four bases will be not discussed here, because of the high frequency of their recognition sites, making it difficult to find a unique one within a DNA sequence.) *EcoRI*, a well-known orthodox Type II endonuclease, recognizes 5'-GAATTC-3', and digests within the sequence as



resulting in a 5' overhang 5'-AATT.

Digestion by orthodox Type II endonucleases that recognize a 6-base sequence makes it possible to create 5 different overhangs (Figure 1). So far, GAATTC is only recognized by *EcoRI*. However, the sequence GGCGCC could be recognized by 5 different orthodox Type II endonucleases: (1) Digestion with *SfoI* results in blunt end, and (2) *NarI* results in 5'-CG. An endonuclease that recognizes GGCGCC resulting in a (3) CG-3' overhang has not been discovered yet. Digestion with (4) *KasI* results in 5'-GCGC, and (5) *BbeI* results in GCGC-3'. These endonucleases that recognize the same sequence, but digest it differently are called "neoschizomers" [3].

In contrast, Type IIS endonucleases recognize asymmetrical sequences and digest outside of their recognition site [4]. Type IIS stands for a Type II endonuclease with an asymmetric target and

cleavage sites [5].

The Type IIS endonuclease *BsaI* recognizes the 6-base sequence 5'-GGTCTC-3' and digests this sequence downstream of the site, creating 4-base sticky ends.



Please note: This sequence can also be read as:



The distance between the recognition and cleavage sequences is long for many commercially available Type IIS endonucleases which limits their utilization for manipulation of DNA. Type IIS endonucleases are monomeric (single proteins) [6]. The recognition domain and cleavage domain are separated from each other. For *FokI*, a Type IIS endonuclease, two domains even can be separated into two fragments by trypsin digestion [6]. The cleavage domain of *FokI* has been linked to different specific DNA binding proteins, resulting in "artificial" endonucleases that cut DNA close to their predetermined site [7].

Type IIS endonucleases have not been used as popularly as orthodox Type II endonucleases. A significant reason for this is that the ends of *EcoRI* (a standard Type II endonuclease) are always compatible with ends of *BsaI*. However, the ends created by *BsaI*, a Type IIS endonuclease, would be not compatible with ends of *BsaI* from a different sequence or even from an identical sequence, in most cases (Figure 2B). However, Type IIS endonucleases are very useful in molecular cloning, as more and more people prefer to generate insertion DNA fragments by PCR amplification.

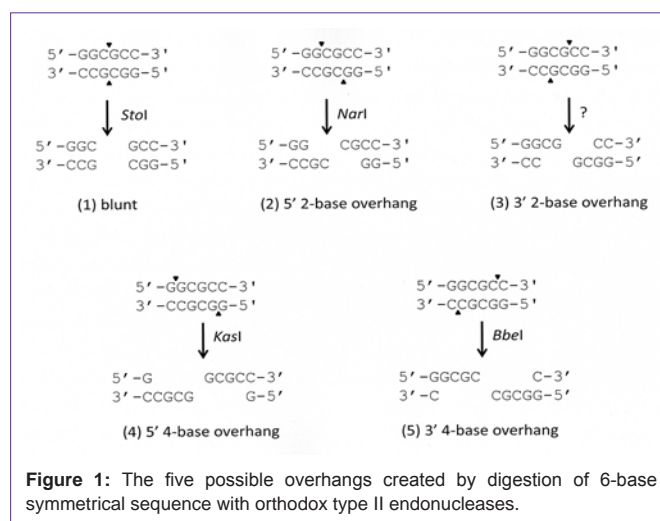


Figure 1: The five possible overhangs created by digestion of 6-base symmetrical sequence with orthodox type II endonucleases.

Since orthodox Type II recognition sequences of the four different kinds of overhangs are symmetrical, their overhangs exhibit symmetry too. Therefore, the ends digested by a specific standard Type II enzyme are compatible with its identical fragments (Figure 2A). In this case, an *EcoRI-HindIII* fragment A isolated using an agarose gel is designed to ligate a *HindIII-EcoRI* fragment B; only a small portion of ligations are between a single fragment A and single fragment B. Most ligations are between two identical fragments as or two fragment Bs, subsequent ligations result in a few very large fragments. As a result, most isolated fragments are wasted instead forming a circle of DNA.

In contrast, the sticky ends created by Type IIS endonucleases are usually not symmetrical. The overhangs resulting from digestion with *BsaI* usually would not be able to ligate the identical 5'-ATCG overhang, because they are neither symmetrical nor compatible as shown in Figure 2B.

This *BsaI* end only can ligate with a fragment with the 5'-CGAT overhang created by *BsaI* or another Type IIS endonuclease such as *BbsI*, *BsmBI*, and so on.

For example,

GGTCTCN/ATCGNNN  
CCAGA GNTAGC/NNN (Fragment C)

digested with *BsaI*, results in:

5'-ATCGNNN  
NNN (Fragment C digestion)

The overhang would not ligate with its identical overhang (Figure 2).

In the case of fragment D

5'-/CGATNGAGACC  
3'-GCTA/NCTCTGG (Fragment D)

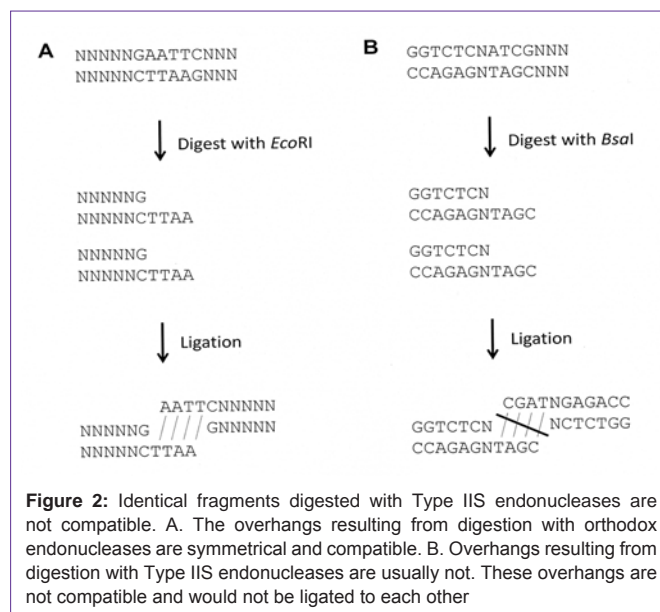
digested with *BsmBI*, results in:

5'-CGATNGAGACC  
NCTCTGG (Fragment D digestion)

Ligation between fragments C and D would not result in any head-to-head or tail-to-tail connection (if the other ends are not compatible). Therefore, ligations between two asymmetrical ends created by (the same or different) Type IIS endonucleases would not result in waste. Highly efficient ligation between two asymmetrical sticky-ends is very useful in molecular cloning. The general applications of Type IIS endonucleases are summarized below.

**i. Ligation among multiple fragments**

The efficiency of ligation between three fragments digested by orthodox endonucleases is low. Ligation between four is almost impossible to select for a correct connection, because most fragments ligate to the wrong one (Figure 2A). Since an asymmetrical overhang has to ligate to another, different asymmetrical overhang, the ligation efficiency between them can be almost 100% if the two fragments are equal in mole. Therefore, it would allow successful ligation among



**Figure 2:** Identical fragments digested with Type IIS endonucleases are not compatible. A. The overhangs resulting from digestion with orthodox endonucleases are symmetrical and compatible. B. Overhangs resulting from digestion with Type IIS endonucleases are usually not. These overhangs are not compatible and would not be ligated to each other

multiple fragments [8,9].

**ii. Creation of a large library due to high efficiency of ligation**

A fragment can be isolated by digestion, with one or two different Type IIS endonucleases creating two different asymmetrical overhangs. The two different overhangs can result from a single Type IIS endonuclease, because the Type IIS endonucleases cleavage sequences are outside their recognition sequence. These fragments are not ligated unless there are other fragments that contain two ends compatible with the first fragment. Since the second fragment would also not ligate itself, the product of ligation only can be between the two ones. Therefore, the ligation efficiency in this scenario would be greater than the one between fragments isolated by digestion with orthodox enzymes. It is possible to make a large plasmid library (>10<sup>6</sup>) by using Type IIS endonucleases and electroporation technology (Zhang, unpublished data).

**iii. Seamless connection of two sequences**

Traditional cloning requires a connection between two fragment digestions by two enzymes with compatible ends. In most cases, if there is not a restriction site, it would be very easy to design a PCR primer containing a restriction site within the primer. However, if a project does not allow us to mutate the sequence, it would be not difficult to design a primer that contains a Type IIS restriction site. As mentioned above, the ATCG/CGTA created by *BsaI* can be replaced to any four nucleotide overhangs, making it possible to seamlessly connect any two sequences without the insertion or deletion of a single base [10].

**iv. Creating an overhang compatible with ends digested by orthodox endonucleases**

In case a cloning strategy is planned, a fragment is needed to insert between the *EcoRI* and *HindIII* sites within a plasmid vector; the fragment can be amplified by PCR with two primers. The first one contains an *EcoRI* site, while the second one contains a *HindIII* site. The PCR product then can be digested with the two endonucleases and the *EcoRI-HindIII* fragments can be used for insertion. However,

attention must be paid to whether this fragment contains other *EcoRI* or *HindIII* sites, or the result would be in the digestion of this fragment into smaller pieces, making the product of ligation contain deletion (s). If there are more than one *EcoRI* or *HindIII* sites within this fragment, the first step can be checking whether there are *EcoRI* or *HindIII* compatible sites. The *MfeI* site (CAATTC) is compatible with the *EcoRI* site (GAATTC); both enzymes create the 5'- AATT overhang. No enzymes however, are compatible with *HindIII* (AAGCTT). If this fragment contains additional *HindIII* site (s), a 4-base 5' overhang of Type IIS endonucleases can be used to create the 5' overhang. There are at least three Type IIS endonucleases that recognize 6 bases (Table 1); in most cases, at least one from the three enzyme recognition sites would not be within this fragment. It is possible to design a primer that contains one of the three Type IIS sites. The digestion sites can be designed as 5'-AGCT (compatible with *HindIII* digestion) and the recognition sites can be designed outside the digested fragment. The fragment can be digested with *EcoRI* and the Type IIS endonuclease (instead of *HindIII*) and is used to insert between the *EcoRI* and *HindIII* sites of the plasmid. In addition, a choice can be made if the ligation site can be re-digested with *HindIII* by adding an A before the AGCT overhangs. If it is preferred that the ligation site not be digested with *HindIII*, the A can be replaced with T, C or G. Many Type IIS endonucleases are commercially available now. There are 5 possibilities resulting from

**Table 1:** Most useful Type IIS endonucleases.

3' end 2 base overhang:	<i>BsrDI</i>	G C A A T G N N / C G T T A C / N N
	<i>BtsI</i>	G C A G T G N N / C G T C A C / N N
5' end 2 base overhang:	<i>FauI</i>	C C C G C N N N N / N N G G G C G N N N N N /
5' end 3 base overhang:	<i>BspQI</i>	G C T C T T C N / N N N C G A G A A G N N N N /
	<i>EarI</i>	C T C T T C N / N N N G A G A A G N N N N /
5' end 4 base overhang:	<i>BsaI</i>	G G T C T C N / N N N N C C A G A G N N N N N /
	<i>BsmBI</i>	C G T C T C N / N N N N G C A G A G N N N N N /
	<i>BbsI</i>	G A A G A C N N / N N N N C T T C T G N N N N N N /
blunt	<i>MlyI</i>	G A G T C N N N N N / C T C A G N N N N N /
3' end 4 base overhang:	<i>BstXI*</i>	C C A N / N N N N N T G G G G T N N N N N / N A C C

\* *BstXI* is a Type IIP endonuclease. Type IIS endonucleases which would create 3' end 4 base overhang has not been discovered yet.

digestion with an orthodox restriction enzymes described above (Figure 1). Commercially available Type IIS endonucleases can create the 4 possible overhangs resulting from any orthodox endonucleases except the 3' 4 base overhang. *BstXI* is not a Type IIS but Type IIP endonuclease. Digestion with *BstXI* results in a 3' 4 base overhang; meanwhile three bases have to be added to the PCR fragment, which probably can serve most purposes.

Several factors need to be considered to choose a Type IIS endonuclease during DNA cloning. First, it is important that the recognition sequences will not be very far away from the digestion sequence, so that the primer containing a Type IIS restriction site will not be very long. Second, the recognition sequence will not be very short, with at least 5 bases or more, so that their restriction sites are less frequent within a DNA fragment. And finally, different cases may call for choices between different types of overhangs.

The most useful Type IIS enzymes are shown in Table 1.

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