Cloning with Anza type IIs restriction endonucleases

Abstract

Traditional cloning workflows involve multiple hands-on steps: a recipient vector is digested with one or more restriction enzymes, and in some cases, dephosphorylated to minimize religation of the linearized vector; then the linearized plasmid is gel purified. In parallel, the fragment to be cloned is also subjected to digestion and gel purification, or alternatively, PCR amplified, digested, and spin-column–purified. The two components are then joined together in a ligation reaction and transformed into *E. coli*. Restriction enzyme cloning is usually limited to inserting a single DNA fragment into a recipient vector. In contrast, Golden Gate cloning [1]utilizes type IIs restriction enzymes in combination with DNA ligase in a single reaction tube to drive the insertion of one or several DNA fragments into a recipient vector, without the need for multiple hands-on steps and often without the need for agarose gel purification. Furthermore, cloning products can be obtained without the inclusion of residual restriction enzyme sites and other unwanted DNA sequences at fragment junctions (scarless cloning). Here, we describe a process using Golden Gate cloning, in which high-efficiency Invitrogen™ Anza™ type IIs restriction enzymes and Anza™ T4 DNA Ligase Master Mix are combined in a single reaction. We provide two step-by-step examples—both at 100% cloning efficiency—of the utility of this rapid and straightforward method, without the necessity for gel purification of vector fragments. The first example details the transfer of 6 precloned PCR fragments into a single recipient vector in order to assemble a 1.8 kb fragment, and the second example demonstrates the precise insertion of a 20 bp sequence into a recipient vector, a workflow useful for the rapid generation of CRISPR guide RNA (gRNA) expression clones [2].

Introduction

Over several decades, an increasingly broad range of restriction endonucleases have been repurposed from bacteria to serve as workhorse tools for the manipulation of DNA. The typical restriction enzymes familiar to most researchers are the type II enzymes that bind to specific double-stranded DNA sequences and cut at those sequences in a symmetrical fashion, leaving blunt or protruding ends. Perhaps the prototypical example is EcoRI, which recognizes the palindromic sequence GAATTC, and introduces a double-strand break within that sequence, leaving a 5′ protruding “sticky end” on each fragment that can subsequently be ligated to any other compatible DNA fragment (usually another DNA fragment digested by EcoRI). Type IIs endonucleases are a variation on this theme. They bind to specific nonpalindromic DNA sequences and introduce a double-strand break outside this recognition sequence in a directional manner [3] (Figure 1).

One effect of this offset cleavage is that the still-intact recognition sequence remains with one fragment while the other fragment loses the recognition sequence. A second effect is that while the precise distance of the cleavage site from the recognition site is enzyme-dependent, as is the type of cleavage (blunt, 3´ protruding, or 5´ protruding), the sequence at the cleavage site is typically irrelevant; once the enzyme binds to its recognition site, it will cleave any DNA sequence the appropriate distance away. The result of this mechanism is that two different DNA fragments cleaved by a given type IIs restriction enzyme that leaves protruding ends will not have compatible overhangs—unless the two fragments have been intentionally designed to be compatible. With such upfront design (done by choosing or creating type IIs–compatible cloning vectors and generating compatible cloning fragments via gene synthesis or PCR primer design), workflows can be simplified and cloning products can be scarless, which can be an important consideration when dealing with open reading frames. It is also possible to simultaneously assemble multiple inserts in a specific order into a single vector [4].

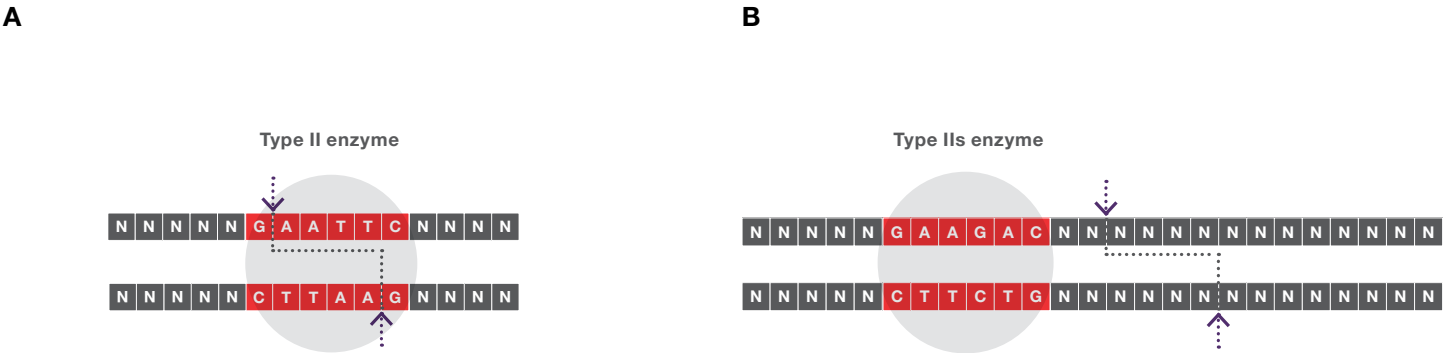

Figure 1. Type II vs. type IIs restriction endonucleases. Representative **(A)** type II (EcoRI) and **(B)** type IIs (BpiI) enzymes are shown. The DNA binding and DNA cleavage activities of typical type II enzymes are closely tied together in a single protein domain, as is reflected in their cleavage patterns. Type IIs enzymes bind at one DNA sequence, but a second protein domain cleaves DNA at a specified distance in a sequence-independent manner.

To further illustrate the logic behind Golden Gate cloning for the transfer of a single DNA fragment (which could be a PCR product, an Invitrogen™ TOPO™–cloned PCR product, or a synthetic DNA fragment) into a recipient vector, type IIs recognition sites are designed on the fragment ends such that cleavage leaves the fragment with two different sticky ends, each compatible with the recipient vector, but removes the enzyme-binding sites (Figure 2A). The recipient vector must be similarly designed—the two enzyme-binding sites are oriented such that the offset cleavage of the type IIs enzymes removes the sites from the now-linearized vector, and the two sticky ends of the vector are compatible with the directional cloning of the insert, but not with religation of the vector itself.

The key is that during repeated cycles between the temperatures optimal for digestion (37°C) and ligation (23°C), the desired final product loses its type IIs recognition sites when it is generated, while undesired cloning products or uncut vector and insert fragments retain the recognition sites and are subject to digestion in subsequent cycles. During ligation cycles, potential nonproductive outcomes include the possibility that an excised vector fragment is religated into the same vector it was liberated from or the recipient vector is cleaved at only one of its two type IIs sites and is religated. In these cases, the enzyme-binding sites would once again be present in the starting material and these molecules would be available for cleavage in the next digestion cycle. However, if the digested insert is ligated to the recipient vector as desired, the recombinant molecule does not contain the type IIs binding sites and the reaction is irreversible. The

overall result of multiple cycles is to drive the reaction toward the production of the correct DNA fragment [1,4]. The efficiency of the reaction is very high, eliminating the need for gel purification or sequential digestion and ligation steps.

With careful design of fragment ends, multiple fragments can be inserted into a recipient vector simultaneously with this method (Figure 2B). The same type IIs restriction enzyme recognition site must be present at the ends of each fragment, and each sticky end of a given fragment can be designed to be compatible with only one other sticky end in the reaction.

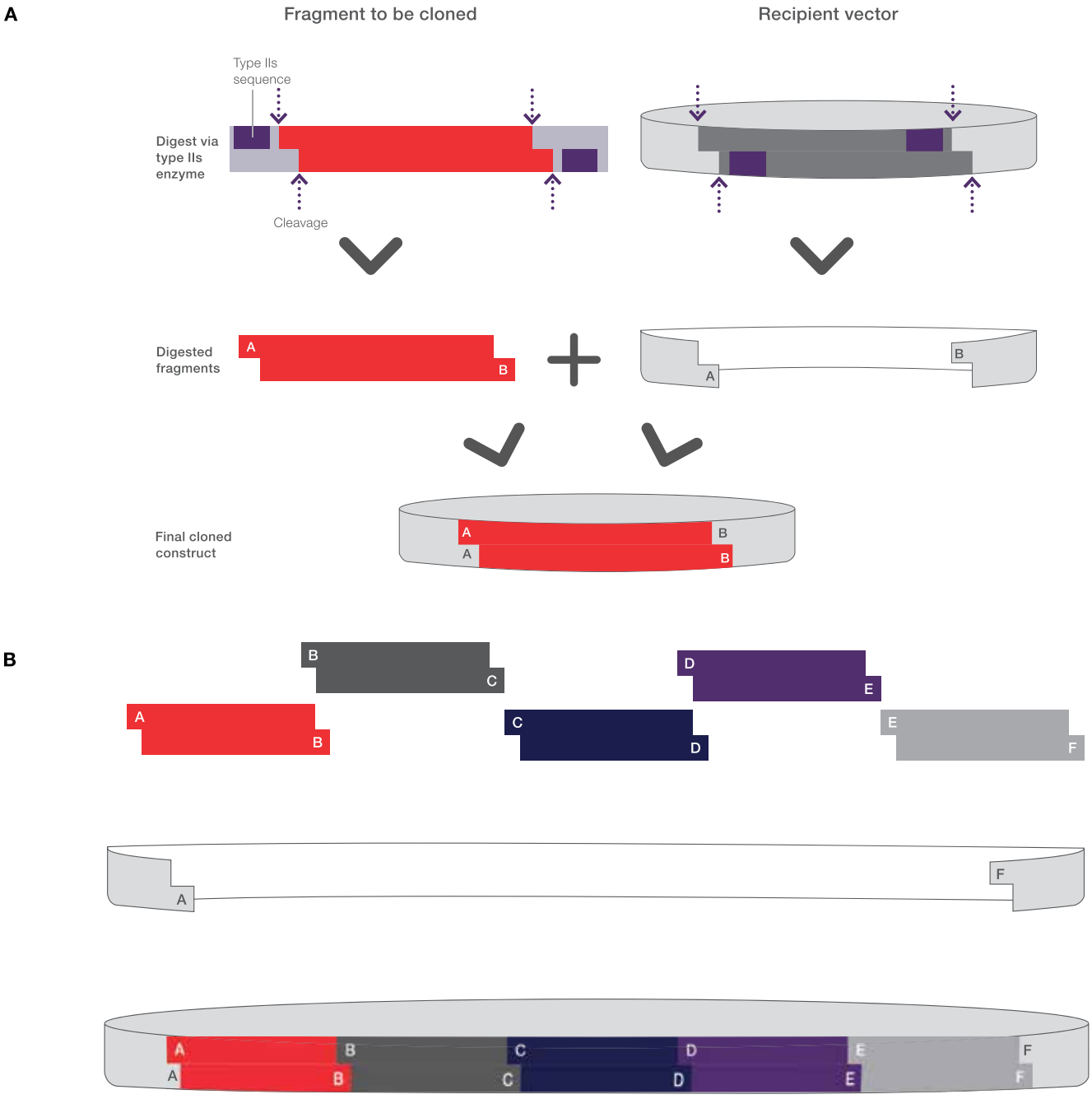

Figure 2. Schematic workflows of type IIs cloning. (A) Directional cloning of one DNA fragment using type IIs restriction enzymes. Repeated digestion and ligation cycles drive the reaction irreversibly toward generation of the correct clone. Note that incorrect ligation events recapitulate the original molecules, complete with type IIs binding sites (and will be redigested in the next cycle), while correct ligation events generate a molecule that does not contain the type IIs binding sites (and are therefore irreversible). **(B)** Multifragment assembly using Golden Gate cloning. As with single-fragment assembly, repeated digestion and ligation cycles drive the reaction irreversibly toward generation of the correct clone. While a single type IIs restriction enzyme (plus DNA ligase) is used in the cloning reaction, the expected overhangs after digestion are systematically varied such that only correctly ordered assembly of the fragments is possible.

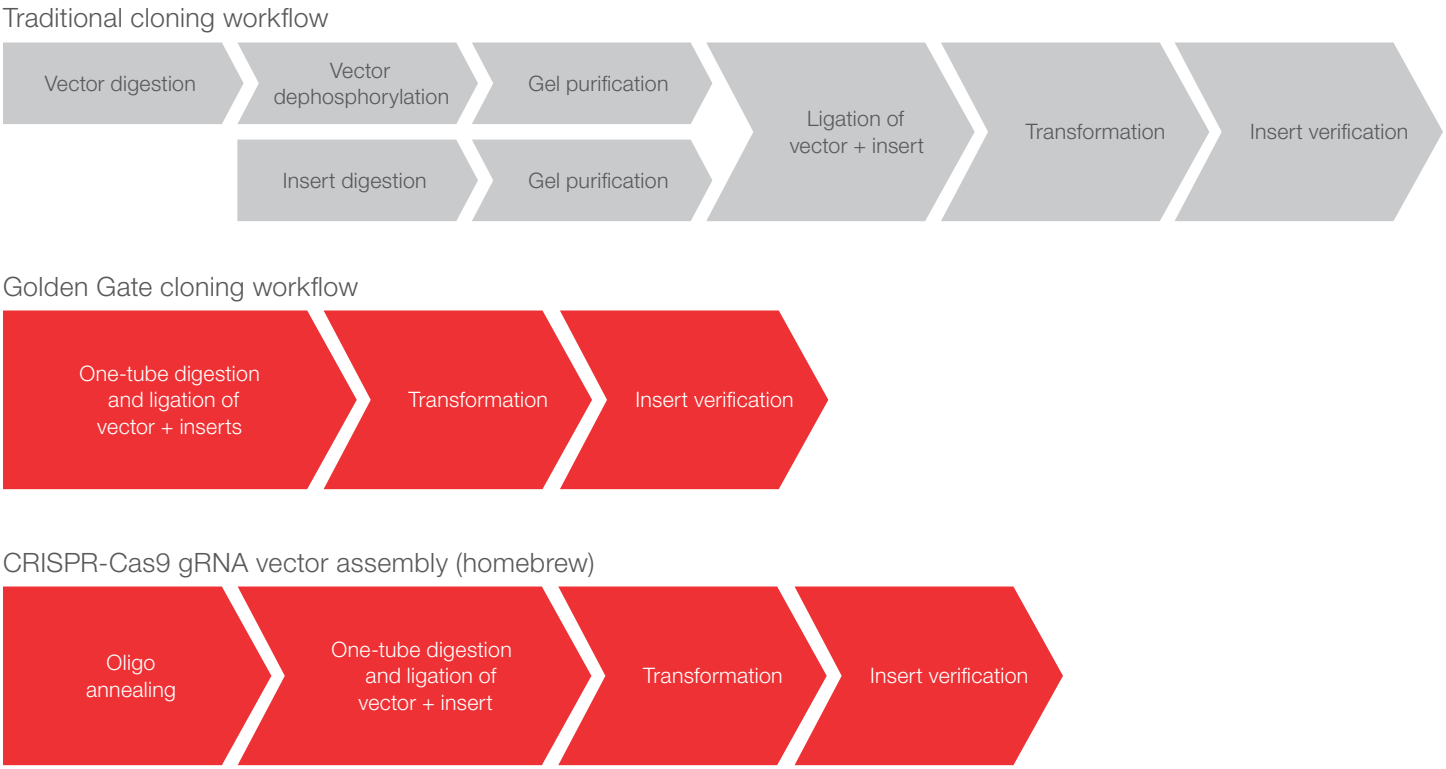

Figure 3. Comparison of Golden Gate workflow and CRISPR-Cas9 homebrew gRNA vector assembly to a traditional cloning workflow.

This cloning strategy enables much simpler workflows for multifragment cloning and for single-fragment cloning of gRNA sequences in the CRISPR-Cas9 system (Figure 3). Here, we provide examples of scarless Golden Gate cloning using high-efficiency Anza type IIs restriction endonucleases and Anza T4 DNA Ligase Master Mix.

Material and methods

Six-fragment assembly

A 1.8 kb sequence for assembly was designed. This sequence was broken into 6 fragments, and each fragment was synthesized using Invitrogen™ GeneArt™ Gene Synthesis and obtained as a clone, with Anza 36 Eco31I (BsaI isoschizomer) recognition sites immediately flanking the insert, such that digestion would liberate the fragments but not the recognition sites from the parent plasmid. Each fragment was designed so that the resulting 4 nt overhang would be complementary with only one other 4 nt overhang in the reaction, out of a total of 12 insert ends and 2 preset vector ends, such that only a single assembly of the 6 fragments would be possible and that no extraneous DNA sequences would remain at the fragment junctions. Reactions were assembled in 20 µL and contained: 5 µL water, 2 µL Anza 10X Buffer, 1 µL (75 ng) recipient vector, 1 µL (75 ng) each GeneArt clone, 1 µL Anza 36 Eco31I, and 5 µL Anza T4 DNA Ligase Master Mix. Thermal cycler conditions were as follows: 37°C for 5 min; 30 cycles at 37°C for 1 min, 23°C for 1 min; 37°C for 5 min; 65°C for 10 min; hold at 4°C.

CRISPR-Cas9 gRNA vector assembly

Two 24 nt DNA oligonucleotides with 20 bp of complementarity encoding a 20 nt CRISPR gRNA target sequence were designed and resuspended in water at 100 µM. After annealing, the resulting 4 nt overhangs on each side were compatible with precise directional insertion into 3 versions of a recipient vector encoding the remainder of the gRNA sequence (each vector utilizing a different type IIs restriction enzyme, but all three designed to yield overhangs compatible with the same annealed oligo pair). Oligonucleotides were annealed in 20 µL (2 µL each oligo in a final solution of 10 mM Tris, pH 7.8; 1 mM EDTA; 50 mM NaCl) in a thermal cycler (95°C for 5 min; ramp to 25°C at 0.1°C/sec; 25°C for 5 min). The completed reaction was stored at –20°C or used immediately. The reaction was diluted 1:100 to create a 100 nM working stock for subsequent cloning. Three different type IIs enzymes (Anza 36 Eco31I [BsaI isoschizomer], Anza 13 Esp3I [BsmBI isoschizomer], and Anza 4 BpiI [BbsI isoschizomer]) were used in cloning reactions under the

following conditions: 10 µL water; 2 µL Anza 10X Buffer, 1 µL (100 ng) recipient vector, 1 µL annealed oligos, 5 µL Anza T4 DNA Ligase Master Mix, and 1 µL Anza type IIs restriction endonuclease. Cycling conditions were as with 6-fragment assembly but with a reduced number of digestion and annealing cycles: 37°C for 5 min; 10 cycles at 37°C for 1 min, 23°C for 1 min; 37°C for 5 min; 65°C for 10 min; hold at 4°C.

Transformation and insert verification

A portion of each reaction was transformed into chemically competent Invitrogen™ One Shot™ DH10B™ cells according to the provided protocols. Colonies produced from the 6-fragment assembly were assessed by restriction digestion with Anza 6 NheI and analyzed on 1% Invitrogen™ E-Gel™ agarose gels for bands of the appropriate size (~4.2 kb and ~2.3 kb). gRNA clones were analyzed by colony PCR using the following reaction mixture: 25 µL Invitrogen™ Platinum™ Green Hot Start PCR Master Mix (2X), 1 µL each 10 µM primer, and 23 µL water. Thermal cycler conditions were as follows: 94°C for 4 min; 25 cycles at 94°C for 30 sec, 52°C for 30 sec, 72°C for 1 min; hold at 4°C. The expected band size was 550 bp. Six-fragment and gRNA assemblies were submitted for Sanger sequencing.

Results and discussion

For the 1.8 kb 6-fragment assembly utilizing Anza 36 Eco31I (BsaI isoschizomer), 10 clones were analyzed by restriction digestion, with all 10 displaying the expected banding pattern (Figure 4). Three of these clones were further confirmed as correct by Sanger sequencing. For insertion of the 20 bp gRNA target sequence into the 3 recipient vectors, 10 colonies from each of the 3 plates were analyzed by colony PCR, with 100% cloning efficiency in each case (Figure 5). All 30 gRNA clones were confirmed as correct by Sanger sequencing.

Multifragment assembly using type IIs restriction endonucleases and Golden Gate cloning can be a highly effective and streamlined method for constructing clones incorporating multiple elements in a scarless (if desired) fashion. While other methods are available (e.g., seamless methods using enzyme mixes and gene synthesis), these methods can prove troublesome when dealing with repetitive or otherwise complex DNA sequences. One limitation to using Golden Gate cloning is that it is first necessary to identify recipient cloning vectors dedicated to type IIs cloning (e.g., the paired type IIs restriction sites are contained only within the multiple cloning site). While these vectors exist and in many cases are available at a nominal cost, this does not include all vectors. In workflows where specialized vectors are repeatedly used, it may be well worth the effort upfront to modify the vector or design a similar vector containing the same specialized elements that is compatible with type IIs cloning enzymes, with the expectation that significant time savings and efficiencies will be reaped in the future.

In the case of gene editing using the CRISPR-Cas9 system, there are a number of options: highly efficient workflows using commercial recombinant Cas9 protein and custom gRNAs are available, as are dual expression plasmids that contain the Cas9 open reading frame as well as a second site for precise oligo-based type IIs cloning of target sequences into the preexisting framework of the gRNA constant region.

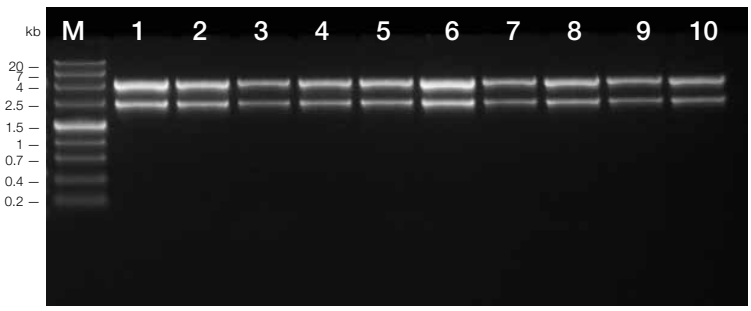

Figure 4. Restriction analysis of clones obtained with 6-fragment assembly. Ten clones analyzed by digestion with Anza 6 NheI were all correct (expected fragment sizes of ~4.2 kb and ~2.3 kb).

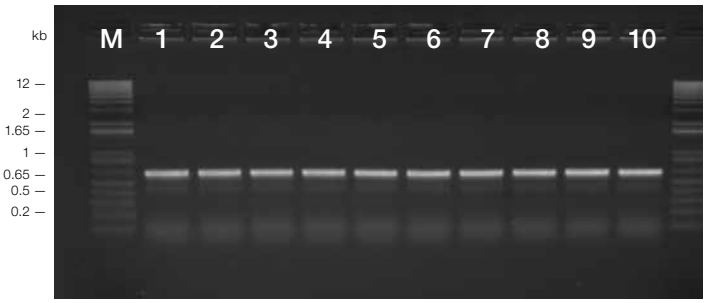

Figure 5. Analysis of gRNA transformants by colony PCR. Ten transformants cloned via Anza 4 BpiI (BbsI isoschizomer) were analyzed by colony PCR with an expected amplicon size of 550 bp. Identical results were obtained with Anza 36 Eco31I (BsaI isoschizomer) and Anza 13 Esp3I (BsmBI isoschizomer).

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Conclusion

Whether using annealed oligonucleotides, single-fragment insertions, or complex multiple-fragment assemblies, Golden Gate cloning can be highly advantageous, both from the standpoint of cloning efficiency as well as from the perspective of hands-on time savings. Anza type IIs restriction enzymes and Anza T4 DNA Ligase Master Mix enable this cloning method with a simple, rapid protocol that uses a single buffer.

Ordering information

Product	Isoschizomer	Cut site	Quantity	Cat. No.
Restriction enzymes				
Anza 36 Eco31I	BsaI	5′...G G T C T C (N)$_1$↓...3′ 3′...C C A G A G (N)$_5$↑...5′	800 units	IVGN0366
Anza 13 Esp3I	BsmBI	5′...C G T C T C (N)$_1$↓...3′ 3′...G C A G A G (N)$_5$↑...5′	800 units	IVGN0136
Anza 4 BpiI	BbsI	5′...G A A G A C (N)$_2$↓...3′ 3′...C T T C T G (N)$_6$↑...5′	250 units	IVGN0044
Anza 33 LguI	SapI	5′...G C T C T T C (N)$_1$↓...3′ 3′...C G A G A A G (N)$_4$↑...5′	400 units	IVGN0334
Anza 48 MnlI	MnlI	5′...C C T C (N)$_7$↓...3′ 3′...G G A G (N)$_6$↑...5′	400 units	IVGN0484
Anza 109 Alw26I	BsmAI	5′...G T C T C (N)$_1$↓...3′ 3′...C A G A G (N)$_5$↑...5′	800 units	IVGN1096
Anza 104 MboII	MboII	5′...G A A G A (N)$_8$↓...3′ 3′...C T T C T (N)$_7$↑...5′	250 units	IVGN1044
Anza 119 BmsI	SfaNI	5′...G C A T C (N)$_5$↓...3′ 3′...C G T A G (N)$_9$↑...5′	250 units	IVGN1194
Anza 89 Mva1269I	BsmI	5′...G A A T G C N↓...3′ 3′...C T T A C↑G N ...5′	400 units	IVGN0894
Anza 112 BseGI	BtsCI	5′...G G A T G N N↓...3′ 3′...C C T A C↑N N ...5′	800 units	IVGN1126
Modifying enzymes				
Anza T4 DNA Ligase Master Mix			50 rxns	IVGN2104
			200 rxns	IVGN2108

References

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