Type IIs gene assembly using PCR-generated inserts and vectors

PCR offers convenience, simplicity, and flexibility for type IIs cloning

Summary

Type IIs cloning enables assembly of multiple DNA fragments in a single digestion–ligation reaction using type IIs restriction endonucleases, since these enzymes' cleavage sites lie outside of their recognition sites. A type IIs cloning method using PCR-generated inserts and vectors is described here for its simplicity and flexibility. Generation of type IIs cloning fragments by PCR circumvents the need for a dedicated vector with built-in type IIs cloning sites. Using PCR amplicons generated using Thermo Scientific™ Phusion™ Plus DNA Polymerase, three genes were successfully assembled in two different vectors. Upon induction, clones with the created construct overexpressed the protein encoded by the assembled genes.

Introduction

Type IIs gene assembly, also known as Golden Gate cloning, relies on type IIs restriction endonucleases for seamless cloning and assembly of DNA fragments and combines restriction and ligation in a single reaction [1,2]. Type IIs restriction endonucleases (e.g., BpiI, AarI) recognize specific nonpalindromic sequences and cleave DNA at a defined distance from their recognition sites, in contrast to traditional type IIp restriction endonucleases (e.g., EcoRI) that recognize a palindromic sequence and cleave within the recognition sequence (Figure 1) [3]. Therefore, DNA ends can be designed with a type IIs recognition site such that restriction digestion and ligation reactions can take place in the same tube at the same time, because the type IIs recognition site is eliminated after cleavage while generating complementary overhangs (Figure 2). Such ends can be ligated seamlessly since the ligated junction lacks the original site or scars; hence, this method is also known as scarless cloning.

PCR is a popular and efficient approach to introduce type IIs cloning sites to inserts and vectors while generating sufficient amounts of DNA for cloning. PCR primers are designed with a desired type IIs recognition sequence, along with a desired add-on cleavage sequence at the 5′ end (Figure 3). Since there are no specific sequence requirements for the cleavage, the add-on sequence can be any combination of nucleotides that would be complementary for assembling PCR fragments in a specific order (Table 1, Figure 4).

Phusion Plus DNA Polymerase is an ideal choice for generating type IIs cloning fragments, due to its high fidelity, universal annealing feature, and robust performance. With its PCR sequence accuracy being >100x higher than that of *Taq* DNA polymerase, Phusion Plus DNA Polymerase helps reduce colony screening efforts and downstream troubleshooting. Additionally, Phusion Plus DNA Polymerase comes with a reaction buffer formulated for a universal annealing temperature of 60°C for all primers, offering convenience and simplicity when generating multiple PCR fragments for gene assembly. Moreover, the fast extension rate of Phusion Plus DNA Polymerase (15–30 sec/kb) helps save time amplifying long sequences such as vectors.

Figure 1. Comparison of the recognition site and cleavage site of type IIp versus type IIs restriction endonucleases. Type IIp restriction endonucleases (e.g., EcoRI) recognize specific 4- to 8-nucleotide sequences (red) that are typically palindromic, and cleave within the recognition site. In contrast, type IIs restriction endonucleases (e.g., BpiI, AarI) cleave DNA at a defined distance downstream of the recognition sequence. This is due to the enzyme structure—the catalytic and recognition domains are separated by a polypeptide linker.

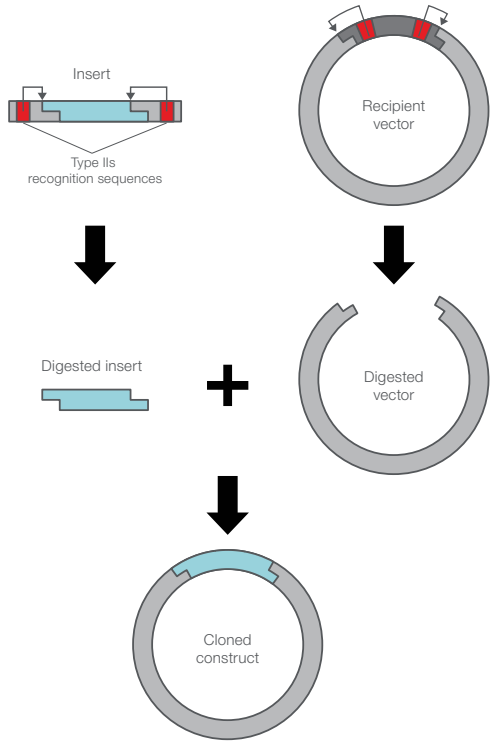

Figure 2. Schematic of type IIs cloning of one DNA fragment. The insert and vector DNA are digested with a type IIs restriction endonuclease. The digested insert and vector are then ligated to form a final cloned construct. All steps take place in a single-tube setup where repeated digestion and ligation cycles drive the reaction irreversibly toward generation of the correct construct.

Here we demonstrate generation of PCR fragments of three genes and two recipient vectors, followed by their assembly via type IIs cloning (Figure 3). We used Phusion Plus DNA Polymerase to amplify genes encoding three popular protein tags—glutathione (GST), green fluorescent protein (GFP), and thioredoxin (Trx)—from plasmids. We introduced desired type IIs cloning sites during PCR and used the resulting amplicons as cloning inserts. We also performed similar PCR with two vectors—pUC19 for cloning and pET15b for protein expression. We then assembled GST, GFP, and Trx sequences into pUC19 and pET15b separately by type IIs cloning. Finally, we confirmed their correct assembly in the plasmids using PCR and restriction digestion, and also showed overexpression of the fusion protein.

PCR with primers containing type IIs cloning sites | Type IIs restriction digestion and DNA ligation | Seamless gene assembly in the desired vector

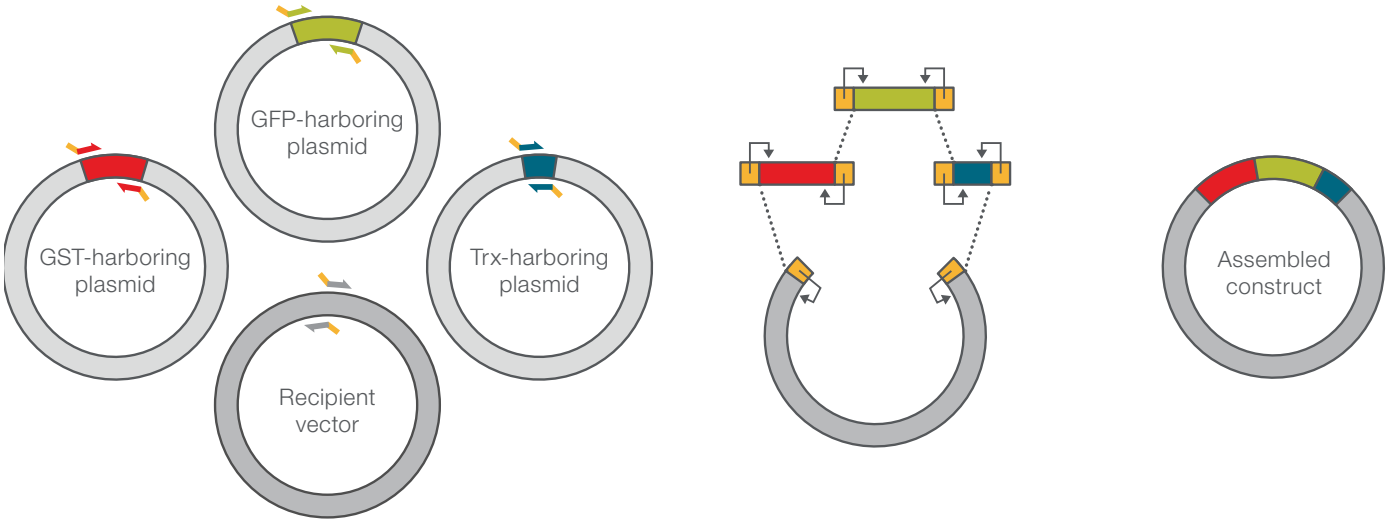

Figure 3. Schematic of assembly of PCR-generated inserts and vector. PCR fragments carrying target sequences and type IIs cloning sites were generated from their respective plasmids. A simultaneous reaction of type IIs restriction digestion and DNA ligation was carried out with the PCR fragments to assemble the desired construct.

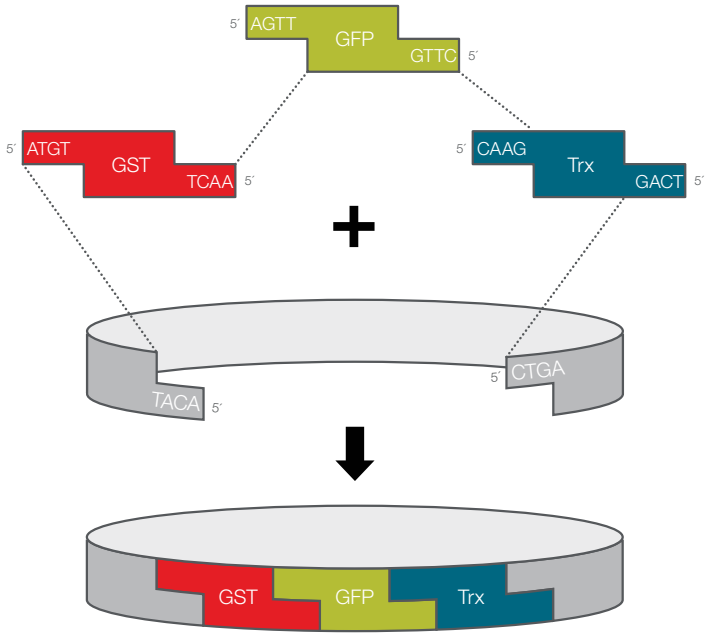

Figure 4. Overhangs of the inserts and vector after type IIs restriction digestion. The complementary overhangs shown are as a result of primer designs in Table 1. Fragments with the resulting complementary overhangs are assembled in the desired order during ligation.

Materials

PCR reagents

- Phusion Plus DNA Polymerase (**Cat. No. F630S**)
- Thermo Scientific™ 10 mM dNTP Mix (**Cat. No. R0191**)
- PCR primers with type IIs cloning sites
- Thermo Scientific™ DreamTaq™ Hot Start Green PCR Master Mix (**Cat. No. K9021**)

Template DNA

- Plasmids carrying genes of interest
- Thermo Scientific™ pUC19 (**Cat. No. SD0061**)
- Novagen™ pET15b (Fisher Scientific **Cat. No. 696613**)

DNA purification kits

- Thermo Scientific™ GeneJET™ Gel Extraction and DNA Cleanup Micro Kit (**Cat. No. K0832**)
- Thermo Scientific™ GeneJET™ Plasmid Miniprep Kit (**Cat. No. K0503**)

Electrophoresis reagents

- Thermo Scientific™ TopVision™ Agarose Tablets (**Cat. No. R2801**)
- Thermo Scientific™ GeneRuler™ 1 kb Plus DNA Ladder (**Cat. No. SM1333**)

Restriction endonucleases and DNA ligase

- Thermo Scientific™ FastDigest™ BpiI (**Cat. No. FD1014**)
- Thermo Scientific™ AarI (2 U/µL) (**Cat. No. ER1582**)
- Thermo Scientific™ T4 DNA Ligase (5 U/µL) (**Cat. No. EL0011**)
- Thermo Scientific™ FastDigest™ ScaI (**Cat. No. FD0434**)
- Thermo Scientific™ FastDigest™ Bsp1407I (**Cat. No. FD0934**)
- Thermo Scientific™ FastDigest™ CpoI (**Cat. No. FD0744**)

Competent cells

- Thermo Scientific™ DH10B Competent Cells (**Cat. No. EC0113**)
- Thermo Scientific™ BL21(DE3) Competent Cells (**Cat. No. EC0114**)

Protein analysis

- Thermo Scientific™ IPTG, dioxane-free (**Cat. No. R0392**)
- Thermo Scientific™ PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (**Cat. No. 26620**)
- Invitrogen™ Novex™ WedgeWell™ Tris-Glycine 10% Gel (**Cat. No. XP00100PK2**)

Methods

Generation of PCR fragments

PCR primers were designed to amplify GST, GFP, and Trx sequences as inserts and pUC19 and pET15b as recipient vectors for type IIs cloning. Type IIs recognition sites, along with add-on sequences to generate compatible overhangs after cleavage, were included in the 5′ end of the primers (Table 1). The PCR primers for subcloning into the pUC19 vector incorporated the BpiI recognition site since it is not present on the original vector. The PCR primers for subcloning into the pET15b vector incorporated the AarI recognition site instead, since the BpiI sites are already present on the vector and cannot be used for subcloning.

Each 50 µL PCR reaction included 1X reaction buffer, 0.2 mM of each dNTP, 0.5 µM of forward and reverse primers, 1 ng of the plasmid of interest, and 0.5 µL of Phusion Plus DNA polymerase. PCR cycling was performed according to the protocols in Table 2, using an Applied Biosystems™ ProFlex™ Thermal Cycler. The PCR products were analyzed using 1% agarose gels in TAE buffer and purified using the GeneJET Gel Extraction and DNA Cleanup Micro Kit. Concentrations of the purified DNA were determined on a Thermo Scientific™ NanoDrop™ spectrophotometer.

Table 1. PCR primer sequences. Type IIs recognition sequences are underlined, and the add-on sequences that would generate overhangs after cleavage are in bold (also see Figure 4). Nucleotides that are complementary to the template sequences are in lowercase.

PCR primers for cloning into the pUC19 vector using BpiI			
GST-for-BpiI	5′TATTATTTGAAGACTT**atgt**cccctatactaggttattgg	**GST-rev-BpiI**	5′TATTATTTGAAGACTT**aact**tccagatccgattttggag
GFP-for-BpiI	5′TATTATTTGAAGACTT**AGTT**atggtgagcaagggcgagga	**GFP-rev-BpiI**	5′TATTATTTGAAGACTT**cttg**tacagctcgtccatgcc
Trx-for-BpiI	5′TATTATTTGAAGACTT**CAAG**atgagcgataaaattattcacctgactg	**Trx-rev-BpiI**	5′TATTATTTGAAGACTT**tcag**gccaggttagcgtcga
pUC19-for-BpiI	5′TATTATTATTTGAAGACTT**ACAT**ttgcatgcctgcaggtcgac	**pUC19-rev-BpiI**	5′TATTATTATTTGAAGACTT**CTGA**gcttggcgtaatcatggtcatag
PCR primers for cloning into pET15b vector using AarI			
GST-for-AarI	5′ TATTATTTCACCTGCTTTT**atgt**cccctatactaggttattgg	**GST-rev-AarI**	5′ TATTATTTCACCTGCTTTT**aact**tccagatccgattttggag
GFP-for-AarI	5′ TATTATTTCACCTGCTTTT**AGTT**atggtgagcaagggcgagga	**GFP-rev-AarI**	5′ TATTATTTCACCTGCTTTT**cttg**tacagctcgtccatgcc
Trx-for-AarI	5′ TATTATTTCACCTGCTTTT**CAAG**atgagcgataaaattattcacctgactg	**Trx-rev-AarI**	5′ TATTATTTCACCTGCTTTT**tcag**gccaggttagcgtcga
pET15b-for-AarI	5′ TATTATTTCACCTGCTTTT**ACAT**ggtatatctccttcttaaagttaaac	**pET15b-rev-AarI**	5′TATTATTTCACCTGCTTTT**CTGA**atgctcgaggatccggctg

Table 2. PCR cycling protocols with Phusion Plus DNA Polymerase.

	GST (0.67 kb), GFP (0.72 kb), and Trx (0.33 kb) fragments			pUC19 fragment (2.7 kb)			pET15b fragment (5.7 kb)		
Cycling step	Temperature	Time	Cycles	Temperature	Time	Cycles	Temperature	Time	Cycles
Initial denaturation	98°C	30 sec	1	98°C	30 sec	1	98°C	30 sec	1
Denaturation **Annealing*** **Extension**	98°C 60°C 72°C	10 sec 10 sec 20 sec	30	98°C 60°C 72°C	10 sec 10 sec 80 sec	30	98°C 60°C 72°C	10 sec 10 sec 3 min	30
Final extension	72°C 4°C	5 min Hold	1	72°C 4°C	5 min Hold	1	72°C 4°C	5 min Hold	1

* The universal annealing feature of Phusion Plus DNA Polymerase enables annealing at 60°C for all targets.

Type IIs restriction digestion and ligation reaction

Following Table 3, 75 ng of each fragment (GST, GFP, Trx, and pUC19 or pET15b vector) was mixed with its respective type IIs restriction endonuclease, T4 DNA ligase, and other reaction components. The digestion–ligation reactions were performed in a ProFlex Thermal Cycler by cycling through the optimal temperatures for the restriction endonuclease (37°C) and the DNA ligase (23°C): 37°C for 5 min; 37°C for 1 min, 23°C for 1 min, and 37°C for 5 min, for 30 cycles; 65°C for 10 min (enzyme deactivation); hold at 4°C. After completion of the reactions, DNA was purified using the GeneJET Gel Extraction and DNA Cleanup Micro Kit.

Transformation and colony analysis

DNA purified from the digestion–ligation reactions of the pUC19 and pET15b constructs was used to transform DH10B competent cells. Colonies were screened for the presence of the three inserts (GST, GFP, and Trx) by PCR and restriction digestion.

For colony PCR, single colonies were picked and resuspended in 20 µL each of nuclease-free water. Then 1 µL of the colony suspension was added to a 19 µL PCR reaction containing 1X DreamTaq Hot Start Green PCR Master Mix, 0.5 µM forward primer, and 0.5 µM reverse primer (see Table 1 for primer sequences). PCR was run according to the protocols in Table 4. The PCR products were then analyzed using 1% agarose gels in TAE buffer.

For restriction digestion to verify correct assembly of GST, GFP, and Trx in the constructs, colonies formed after transformation were inoculated into 5 mL each of Luria-Bertani (LB) medium with ampicillin and grown overnight at 37°C. Plasmids were purified from overnight cultures using the GeneJET Plasmid Miniprep Kit. About 0.5 µg of the purified plasmid was digested using FastDigest ScaI, FastDigest Bsp1407I, and FastDigest CpoI in 20 µL reactions for 15 min at 37°C, followed by enzyme inactivation at 80°C for 10 min. The digested DNA was analyzed on 1% agarose gels in TAE buffer.

Protein expression analysis

The pET15b construct was purified from DH10B cells, then used for transformation of BL21(DE3) competent cells for protein expression analysis. pET15b plasmid without inserts was used in parallel for transformation as a negative control. Single colonies of BL21(DE3) transformants were picked and grown in LB medium with ampicillin to an optical density of 0.4. Protein expression was induced with 0.5 mM IPTG, and cells were grown for 3 hours. As a negative control for protein induction, some cells were grown without IPTG for the same duration. After the 3 hour incubation, the cells were harvested and disrupted by sonication. The cell lysates were analyzed for protein expression using Novex WedgeWell Tris-Glycine 10% SDS-PAGE Gels.

Table 3. Setup of type IIs restriction digestion and ligation reaction.

	For cloning into pUC19 with BpiI		For cloning into pET15b with AarI	
Component	**Final amount**	**In 20 µL reaction**	**Final amount**	**In 20 µL reaction**
Buffer for restriction enzyme	1X FastDigest Buffer	2 µL	1X Buffer AarI	2 µL
Restriction endonuclease	1 µL FastDigest BpiI	1 µL	1 µL AarI	1 µL
10X T4 DNA Ligase Buffer	1X T4 DNA Ligase Buffer	2 µL	1X T4 DNA Ligase Buffer	2 µL
50% PEG Solution*	5% PEG solution	2 µL	5% PEG solution	2 µL
T4 DNA Ligase	5 U T4 DNA Ligase	1 µL	5 U T4 DNA Ligase	1 µL
DNA fragments	75 ng each	Various	75 ng each	Various
Water, nuclease-free	—	Up to 20 µL	—	Up to 20 µL

* Part of the T4 DNA Ligase package (Cat. No. EL0011)

Table 4. PCR cycling protocols for colony PCR with DreamTaq Hot Start Green PCR Master Mix.

	GST fragment (0.67 kb)			GFP fragment (0.72 kb)			Trx fragment (0.33 kb)		
Cycling step	Temperature	Time	Cycles	Temperature	Time	Cycles	Temperature	Time	Cycles
Initial denaturation	95°C	3 min	1	95°C	3 min	1	98°C	3 min	1
Denaturation **Annealing** **Extension**	95°C 67°C 72°C	30 sec 30 sec 1 min	30	95°C 70°C 72°C	30 sec 30 sec 1 min	30	95°C 70°C 72°C	30 sec 30 sec 1 min	30
Final extension	72°C 4°C	5 min Hold	1	72°C 4°C	5 min Hold	1	72°C 4°C	5 min Hold	1

Results and discussion

GST, GFP, Trx, pUC19, and pET15b sequences were successfully amplified by PCR using Phusion Plus DNA Polymerase (Figure 5). All PCR amplicons were obtained at high yields and specificity using the 60°C annealing temperature for all primers, which is enabled by the hot-start and universal annealing features of Phusion Plus DNA Polymerase. The PCR fragments were then subjected to single-tube reactions of type IIs restriction digestion and DNA ligation.

Many colonies of pUC19 and pETb15 constructs were obtained after transformation of DH10B competent cells with DNA purified from the digestion–ligation reactions. Colony PCR with the primers for GST, GFP, and Trx sequences confirmed their presence in both constructs in all selected clones, as demonstrated by amplicons of expected sizes (Figure 6).

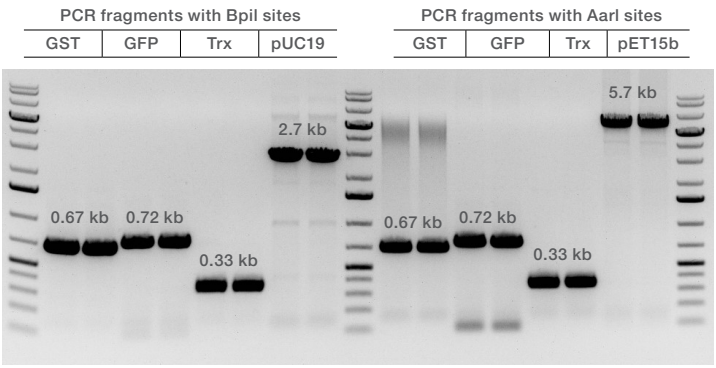

Figure 5. Generation of PCR fragments for type IIs cloning. Insert and vector fragments were amplified from 1 ng of their respective templates by PCR using Phusion Plus DNA polymerase. GeneRuler 1 kb Plus DNA Ladder was used as a size standard.

To verify the assembly of the fragments in the correct order, plasmids purified from two colonies each of pUC19 and pET15b transformants were analyzed by restriction digests. Digestion with ScaI, Bsp1407I, and CpoI resulted in four cleavages and fragments of expected sizes (Figure 7), confirming the correct order of gene assembly in both vectors.

Protein expression was also evaluated for the pET15b construct with the assembled genes, since the vector is capable of overexpressing proteins under control of the *lac* promoter. The pET15b construct, purified from DH10B cells, was used to transform BL21(DE3) competent cells, which are engineered for protein overexpression. After induction of the BL21(DE3) transformants with IPTG, the fusion protein of 65 kDa (combined molecular weight of the three individual proteins) was observed. No such band appeared for any of the negative controls (Figure 8).

These results demonstrate that type IIs cloning enables efficient assembly of multiple fragments into a recipient vector in a specific order. Using PCR, genes of interest and any vector can be easily amplified with desired type IIs cloning sites for their subsequent assembly. PCR with a high-fidelity enzyme like Phusion Plus DNA Polymerase offers high confidence in sequence accuracy (due to its fidelity being >100x higher than that of *Taq* polymerase). In addition, the universal annealing feature of Phusion Plus polymerase provides convenience in amplifying multiple fragments, while its fast DNA synthesis helps save time in PCR cycling.

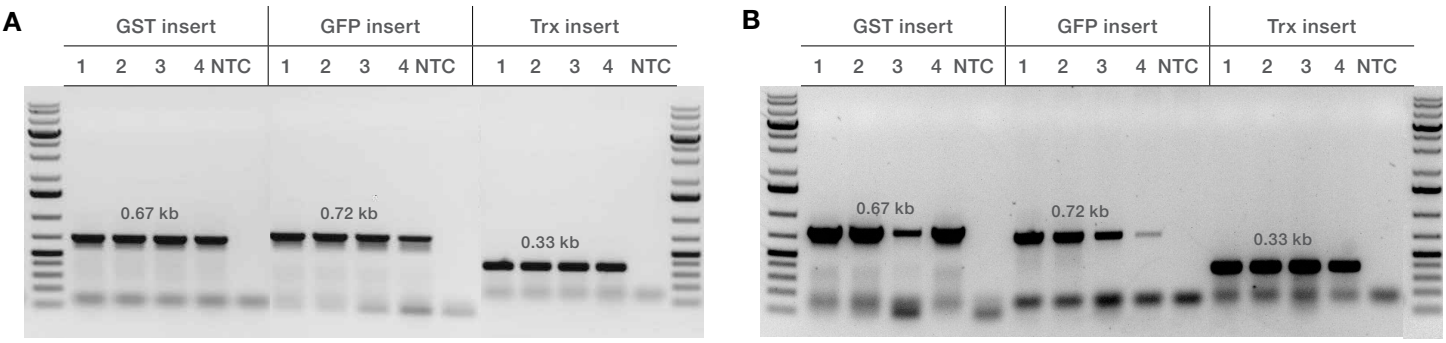

Figure 6. Analysis of transformants by colony PCR. Four colonies of **(A)** pUC19 construct and **(B)** pET15b construct were analyzed for the presence of the three inserts by PCR using DreamTaq Hot Start Green PCR Master Mix. Each colony was suspended in 20 µL of PCR-grade water, and 1 µL of cell suspension was added to the PCR reaction mix. The no-template control (NTC) contains no colony sample. GeneRuler 1 kb Plus DNA Ladder was used as a size standard.

thermo scientific

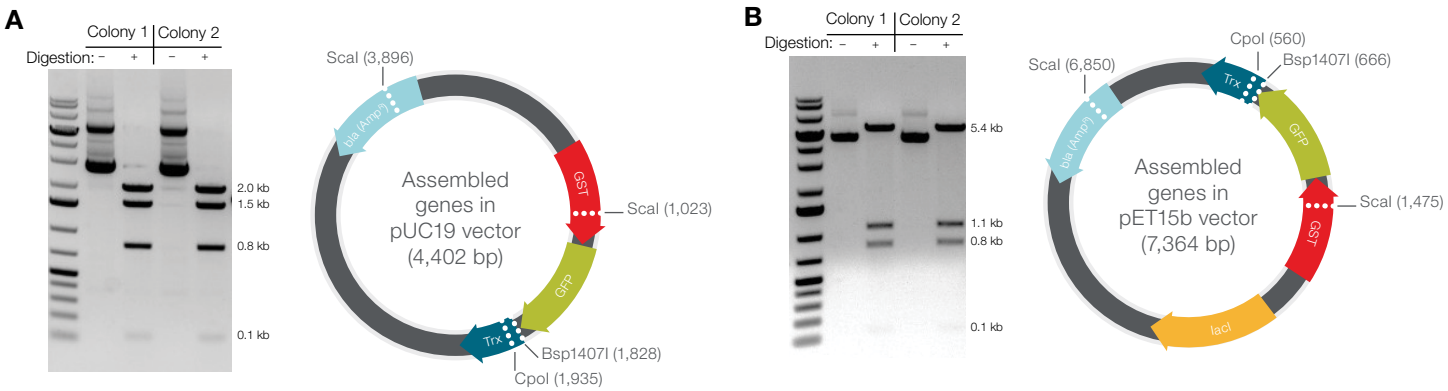

Figure 7. Analysis of transformants by restriction digestion. Purified plasmids from two colonies each of **(A)** pUC19 construct and **(B)** pET15b construct were digested with FastDigest ScaI, FastDigest Bsp1407I, and FastDigest CpoI. Expected fragments after digestion are 1,961 bp, 1,529 bp, 805 bp, and 107 bp for the pUC19 construct; and 5,375 bp, 1,074 bp, 809 bp, and 106 bp for the pET15b construct. GeneRuler 1 kb Plus DNA Ladder was used as a size standard.

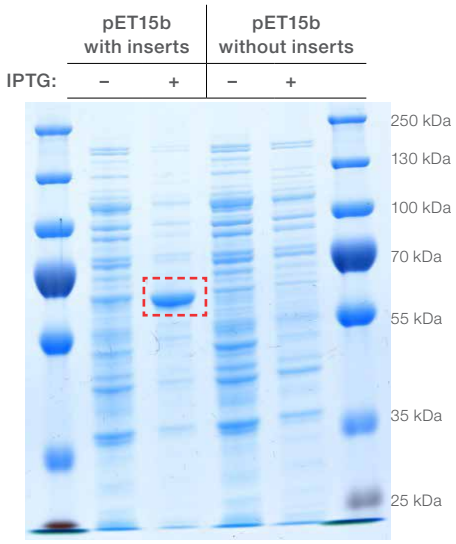

Figure 8. Analysis of protein overexpression in the pET15b construct. BL21(DE3) competent cells were transformed with pET15b with or without the inserts. Overexpression of the protein by the cells was analyzed after induction with IPTG (+ lanes), on a Novex WedgeWell Tris-Glycine 10% SDS-PAGE gel.

Conclusions

Type IIs cloning enables single-reaction assembly of multiple genes into a desired vector without introducing scars. PCR allows generation of insert and vector fragments with the desired type IIs cloning sites for their orderly assembly without the need for a specific vector or restriction sites. PCR experiments may be further simplified and improved upon by using an enzyme like Phusion Plus DNA Polymerase for its high fidelity, universal annealing conditions, and robustness.

Following this approach, genes of three proteins—GST, GFP, and Trx—were successfully assembled in pUC19 and pET15b vectors, separately. The overexpression of the fusion protein from the pET15b construct was also observed. A similar strategy may be considered for constructing recombinant clones to study oncogenic fusion proteins, to add signal peptides to genes of interest, or to incorporate desired tags into overexpressed proteins.

References

1. Engler C, Kandzia R, Marillonnet S (2008) A one pot, one step, precision cloning method with high throughput capability. *PLoS ONE* 3(11):e3647.
2. Engler C, Gruetzner R, Kandzia R et al. (2009) Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes. *PLoS ONE* 4(5):e5553.
3. Pingoud A, Jeltsch A (2001) Structure and function of type II restriction endonucleases. *Nucleic Acids Res* 29(18):3705–3727.

Find out more at **thermofisher.com/phusionplus**

ThermoFisher SCIENTIFIC

COL34189 0621