

We Claim:

1. A method for directional cloning of a PCR product into a pre-digested vector comprising:
 - a) generating a PCR product of two genes, wherein the PCR product has sticky ends complementary to overhangs of a pre-digested vector; where said sticky ends are produced by the steps comprising:
 - i. designing two sets of primers-normal primers and tailed primers;
 - ii. performing two parallel PCRs using the primers of step (i) and Pfu DNA polymerase for the desired fragment of gene to obtain the two PCR products;
 - iii. purifying the two PCR products of step (ii) to remove Pfu DNA polymerase;
 - iv. mixing the two purified PCR products of step (iii) in equal proportions and subjecting to denaturation followed by annealing;
 - b) ligating the sticky ends PCR product of step (iv) and the pre-digested vector and transforming into a host cell.
2. The method as claimed in claim 1, wherein the normal primers are designed in frame to the open reading frame of a vector and the tailed primers are designed to carry an overhang on the 5' end complementary to the overhang on the pre-digested vector.
3. The method as claimed in claim 2, wherein in PCR, tailed forward primer and reverse normal primer are used in one PCR and a normal forward primer and a tailed reverse primer are used in the other PCR.
4. A method for preparation of chimeric fusion product comprising:
 - amplifying each gene fragment using two parallel PCRs to obtain PCR product;
 - purifying the products of the two PCRs of both fragments to remove the Pfu DNA polymerase;
 - mixing the PCR products of the two parallel PCRs of each fragment in equal proportions and subjecting to denaturation, followed by annealing;

ligating the gene fragments at room temperature;

performing nested PCR with the ligation mixture as a template and normal forward primer of first gene and normal reverse primer of second gene as primers to generate the chimeric fusion product.

5. The method as claimed in claim 4, wherein for amplification, tailing bases of reverse primer of one gene and forward primer of the other gene are designed so as to produce PCR fragments with overhangs complementary to each other for splicing.
6. The method as claimed in claim 4, wherein in the chimeric fusion product, the two gene fragments are separated by a linker region.
7. The method as claimed in claim 4 wherein the method comprises the step of creating sticky ends on the fusion product using the method as claimed in claim 1.

Dated: 26th day of March 2012

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To:
The Controller of Patents
The Patent Office,
New Delhi

We Claim:

1. A method for directional cloning of a PCR products into a pre-digested vector comprising:

(a) generating a PCR product of two genes, wherein the PCR product has
~~with~~ sticky ends complementary to ~~the~~ over hangs of ~~the~~ pre-digested
 vector; where said sticky ends are produced by the steps comprising:

(i) designing two sets of primers-normal primers and tailed primers;

(ii) performing two parallel PCRs using the primers of step (i) and Pfu DNA polymerase for the desired fragment of gene to obtain the two PCR products;

(iii) purifying the two PCR products of step (ii) to remove Pfu DNA polymerase;

(iv) mixing the two purified PCR products of step (iii) in equal proportions and subjecting to denaturation followed by annealing;

(b) ligating the sticky ends PCR product of step (iv) and the pre-digested vector and transforming into a host cell.

- ~~2. The method as claimed in claim 1, wherein PCR products with sticky ends are produced by the steps comprising:~~

~~designing two sets of primers-normal primers and tailed primers;~~

~~performing two parallel PCRs for the desired fragment;~~

~~purifying the products of the two PCRs to remove the Pfu DNA polymerase;~~

~~mixing the purified PCR products in equal proportions and subjecting to denaturation followed by annealing.~~

- ~~3.2.~~ The method as claimed in claims ~~1 and 2~~, wherein the normal primers are designed in frame to the open reading frame of ~~at~~ the vector and the tailed primers are designed to carry an overhang on the 5' end complementary to the overhang on the pre-digested vector.

4.3. The method as claimed in claim 2, wherein in ~~step (b), PCR~~, tailed forward primer and reverse normal primer are used in one PCR and a normal forward primer and a tailed reverse primer are used in the other PCR.

5.4. A method for preparation of chimeric fusion product comprising:

amplifying each gene fragment using two parallel PCRs to obtain PCR product;

purifying the products of the two PCRs of both fragments to remove the Pfu DNA polymerase;

mixing the PCR products of the two parallel PCRs of each fragment in equal proportions and subjecting to denaturation, followed by annealing;

ligating the gene fragments at room temperature;

performing nested PCR with the ligation mixture as a template and normal forward primer of first ~~gene~~ and normal reverse primer of second gene as primers to generate the chimeric fusion product.

6.5. The method as claimed in claim ~~45~~, wherein for amplification, tailing bases of reverse primer of one gene and forward primer of the other gene are designed so as to produce PCR fragments with overhangs complementary to each other for splicing.

7.6. The method as claimed in claim ~~45~~, wherein in the chimeric fusion product, the two gene fragments are separated by a linker region.

8.7. The method as claimed in claim ~~45~~ ~~further comprising~~ wherein the method comprises the step of creating sticky ends on the fusion product using the method as claimed in claim 1.

Dated: 26th day of March 2012

FIELD OF THE INVENTION

The present invention relates to the field of molecular biology. More particularly, the present invention provides for a method of directional cloning of Polymerase Chain Reaction (PCR) products with preformed cohesive ends for construction of fusion or chimeric genes.

DESCRIPTION OF RELATED ART

There are various types of cloning methods used world over, which can be categorized into two groups: Ligation-dependent and Ligation-independent cloning. The former method is used more widely than latter. According to DNA ends, the existing ligation-dependent cloning methods can be further divided into three types: Blunt end cloning, sticky end cloning, and T-A cloning, while the Sticky-end cloning is routinely used method to clone PCR products.

All the above mentioned cloning methods have certain drawbacks. Blunt end cloning is not very efficient due to the inefficiency of blunt end ligation and high tendency of self-ligation of vectors, which yields lower recombinant clones. Sticky end cloning also has drawbacks. Many restriction endonucleases fail to efficiently cleave the restriction recognition sequences located at the 5' termini, which may be the result of the unstable binding of restriction enzymes to the DNA termini. Another problem encountered with sticky end cloning is the undesirable cleavage of internal restriction site(s) identical to those in primers. Although T-A cloning is simple, reliable and more efficient than blunt-end cloning, it has the disadvantage of being a non-directional cloning strategy and requires a T-vector. T-A cloning also has the drawback of variable cloning efficiencies. This may be a consequence of variability in 3' dA addition to PCR products mediated by Taq polymerase. It has also been observed that not only dA but also dG, dC, or dT can be added to the PCR products and dT overhangs in the T-vector and dA overhang in the PCR product are unstable and may get lost during storage, especially in room temperature.

Gal, J et al., 1999, have reported the cloning method called autosticky PCR, which uses two special primers that contain tetrahydrofuran derivative abasic sites, which makes DNA polymerase stall and the PCR products obtained carry different single stranded DNA 5'-overhangs at both ends, corresponding to sticky ends of two different restriction sites. The intact PCR products can be directionally cloned into correspondingly cut plasmid vectors. In the resulting clones, the abasic position is either substituted by a nucleotide, frequently a dTMP residue, or is deleted. At times, the deletion of the abasic site is accompanied by a minor deletion in the vicinity of the position as well. These deletions are problematic if the purpose of cloning is to get a recombinant protein from the cloned PCR fragment.

Bao-Li Wang, et al., 2009, reported cloning PCR products with preformed sticky ends, where the target sequence is amplified in two parallel asymmetric PCRs using specially but simply designed primers. Two single-stranded products are produced and they are annealed to form a double-stranded DNA fragment bearing overhangs at both ends that correspond to the restriction overhangs of certain restriction enzymes. This fragment can then be cloned into any vector previously treated with the corresponding enzymes without the restriction of the inserted fragment. One of the drawbacks of this method is that the single stranded PCR product produced in two parallel asymmetric PCRs is very much susceptible to digestion by 3'-5' exonuclease activity of the thermostable *Pfu* DNA polymerase enzyme. Another drawback is the standardization of high concentration and low concentration primers in the asymmetric PCR reaction in order to obtain the majority of PCR product which is single stranded. Moreover, the amplification in this method is linear unlike logarithmic in normal PCR and hence the number of required single-stranded PCR products is minimal by this method.

There is hence a need for developing newer techniques for overcoming the drawbacks in the above mentioned methods.

SUMMARY OF THE INVENTION

In one embodiment, the present invention provides a method for directional cloning of PCR products into a pre-digested vector which includes generating a PCR product with sticky ends complementary to the over hangs of the pre-digested vector; and ligating the PCR product and pre-digested vector and transforming into a host cell.

In another embodiment, the present invention provides a method for preparation of a chimeric fusion product which includes amplifying each gene fragment using two parallel PCRs; purifying the products of the two PCRs of both fragments to remove the Pfu DNA polymerase; mixing the PCR products of the two parallel PCRs of each fragment in equal proportions and subjecting to denaturation, followed by annealing; ligating the gene fragments at room temperature; and performing nested PCR with the ligation mixture as template and normal forward primer of first gene and normal reverse primer of second gene to generate the chimeric fusion product.

DESCRIPTION OF ACCOMPANYING DRAWINGS

Figure 1 illustrates an Agarose gel showing PCR amplified products from the colony PCR of the recombinant clones to check for the presence of cloned PCR insert.

Figure 2 illustrates a Polyacrylamide gel (12%) stained with coomassie brilliant blue showing the expression of the recombinant protein from the IPTG induced clone.

Figure 3 is a Polyacrylamide gel (12%) photograph showing the IMAC purified recombinant protein from the induced culture under denaturation conditions. A single protein was present at the required region from elution 3 onwards.

Figure 4 illustrates the western blot results

Figure 5 is a diagrammatic representation of design of tailing primers for the cloning strategy

Figure 6 is a diagrammatic representation of the detailed procedure of the generation of sticky PCR fragment utilizing two sets of primers amplified from a distinct gene.

Figure 7 is a diagrammatic representation showing the ligation of compatible pre-digested vector and sticky PCR insert for transformation into appropriate vector for expression of recombinant protein from PCR insert.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for directional cloning of PCR products into a pre-digested vector comprising:

- (a) generating PCR product with sticky ends complementary to the overhangs of the pre-digested vector;
- (b) ligating the PCR product and pre-digested vector and transforming into a host cell.

PCR products with sticky ends are produced by the steps comprising:

- (a) designing two sets of primers-normal primers and tailed primers;
- (b) performing two parallel PCRs for the desired fragment;
- (c) purifying the products of the two PCRs to remove the Pfu DNA polymerase;
- (d) mixing the purified PCR products in equal proportions and subjecting to denaturation followed by annealing.

The normal primers are designed in frame to the open reading frame of the vector and the tailed primers are designed to carry an overhang on the 5' end complementary to the overhang on the pre-digested vector such that at the end of the denaturation and annealing step, approximately 25% of products would be having overhangs that are complementary to the overhangs present after digestion of the vector. A tailed forward primer and reverse normal primer are used in one PCR and a normal forward primer and a tailed reverse primer are used in the other PCR in the parallel PCR step. All PCRs are carried out with thermostable pfu DNA polymerase. Removal of the Pfu DNA polymerase is carried out using a PCR clean up kit as the Polymerase may degrade or polish the overhangs generated by its 3'-5' exonuclease activity.

The present invention also provides a method for preparation of chimeric fusion product comprising:

- (a) amplifying each gene fragment using two parallel PCRs;
- (b) purifying the products of the two PCRs of both fragments to remove the Pfu DNA polymerase;
- (c) mixing the PCR products of the two parallel PCRs of each fragment in equal proportions and subjecting to denaturation, followed by annealing;
- (d) ligating the gene fragments at room temperature;
- (e) performing nested PCR with the ligation mixture as template and normal forward primer of first gene and normal reverse primer of second gene to generate the complete PCR fragment with all the gene fragments.

For the amplification reaction, two sets of primers are designed for each gene. The tailing bases of reverse primer of one gene and forward primer of the other gene are designed so as to produce PCR fragments with overhangs complementary to each other for splicing. Additionally, the forward tail primer of first gene is designed to carry an overhang corresponding to the cut site of one restriction recognition site

while the reverse tail primer of second gene is designed to carry an overhang corresponding to the cut site of another restriction recognition site. Further the primers are designed in such a way that the two truncated gene portions are separated by a region of DNA sequence that codes for a flexible linker region, generally a flexible poly-glycine serine linker (G4S)₃ to physically separate the two protein domains of the expressed fusion proteins apart, so that they can function as two different independent functional genes linked together as a single chimeric gene. This is found to increase the immunogenicity of the recombinant protein produced when the fusion gene is cloned into a vector and transformed into host cells. Once the fusion gene is formed, a sticky fusion PCR product may be produced using the process of the invention.

The present invention further provides for purification of recombinant protein produced from the fusion gene product using affinity chromatography and quantifying the protein and carrying out immunization of mice to assess the antigenic potential of the recombinant protein.

The present invention is explained further with the help of the following examples which are only by way of illustration and do not limit the scope of the invention.

EXAMPLES

EXAMPLE 1- Fusion product Preparation

For explaining the above invention, two fragments from Staphylococcal enterotoxin A (SEA) and Toxic Shock Syndrome Toxin (TSST) genes were amplified, ligated, cloned into pRSET A and expressed in E.coli BL21 (DE3)pLysS host cells:

1.1 Materials and methods:

1. *Staphylococcus aureus* strains positive for SEA and TSST toxins were obtained from Dept. of Microbiology, SDM medical college, Dharwad.

2. Reagents required in all steps of cloning namely PCR (pfu polymerase, dNTPs), restriction digestion of vector (BamHI, EcoRI), and ligation (T4 DNA ligase with 10x ligase buffer) were obtained from MBI, Fermentas.
3. Other reagents of DNA extraction kit from agarose gel, PCR clean-up kit, Plasmid extraction kit, trima, urea, and IPTG were procured from Sigma Aldrich, USA.
4. Ni-NTA agarose for affinity purification of recombinant protein was obtained from Quiagen Company.
5. All dehydrated media and antibiotics were obtained from Himedia laboratories, India.

1.2 Designing two sets of primers

Two sets of primers were prepared for each region (SEA and TSST) of the fusion gene to be cloned. One set of primers (normal primers) were designed in-frame to the open reading frame of the vector, whereas other set of primers (tailed primers) were designed to carry an overhang on the 5' end of the first set of primers that are complimentary to the overhangs carried by the digested vector. The forward tail primer of the first gene carried an overhang corresponding to the cut site of BamHI recognition sequence and the reverse tail primer of the second gene carried an overhang corresponding to the cut site of EcoRI (Figure 5). The tailing bases of the reverse primer of the first gene and the forward primer of the second gene were designed in a special way such that, both the PCR fragments can have overhangs complimentary to each other and thus can be spliced as shown in Table 1 below:

Primer	Primer Sequence (5'-3')
SEA-forward	*AGGCTTTTTTACAGATCATTCG
SEA-forward	*GATCAGGCTTTTTTACAGATCATTCG

tail	
SEA- reverse	<i>!GCCAGAGCCACCGCCGCCCCCATCAAAAACATCAGAGT</i>
SEA- reverse tail	<u>&ACCACC</u> <i>GCCAGAGCCACCGCCGCCCCCATCAAAAACATCAGAGT</i>
TSST- forward	<i>!GGCTCGGGCGGCGGTGGTTCAACAGATGGCAGCATCAGC</i>
TSST- forward tail	<u>&GGTGGT</u> <i>GGCTCGGGCGGCGGTGGTTCAACAGATGGCAGCATCAGC</i>
TSST- reverse	<i>*ATGTGGATCCGTCATTCATT</i>
TSST- reverse tail	<i>*AATTATGTGGATCCGTCATTCATT</i>

In addition these two primers were designed in such a way that the two gene fragments were separated by a region of flexible poly-glycine serine linker (G₄S)₃.

1.3 Preparing the sticky insert by PCR

The antigenic determinant regions of SEA and TSST genes were amplified by PCR, using whole genomic DNA of *S.aureus* clinical strains E-2215 and E-2147 respectively as template. All the PCRs were performed in 20µl reaction volume in triplicates containing 0.5 pM of each primer, 200µM of each dNTP, 0.9U Pfu polymerase (MBI Fermentas), and 1.5mM of MgSO₄ in 1X pfu PCR buffer (MBI Fermentas) with 50-100ng of template DNA without any contaminants for which pfu

polymerase is sensitive to. PCR was carried out in Eppendorf master gradient (Hamberg, Germany) thermal cycler with initial denaturation of 95°C for 3 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 59°C for 30 seconds and an extension time of 72°C for 2 minutes followed by 5 minutes of final extension at 72°C.

Two parallel PCRs were performed for both the fragments (SEA and TSST) of the fusion gene. In one, PCR tailed forward primer and a reverse normal primer was used and in other PCR normal forward primer and a tailed reverse primer were used for amplification of the desired gene fragment. All PCRs were carried out with thermostable pfu DNA polymerase. Later the products of the two PCRs for both the fragments were purified with PCR clean up kit to remove the pfu DNA polymerase which may degrade or polish the overhangs that will be generated by its 3'-5' exonuclease activity. The two parallel purified PCR products for each gene fragment were mixed in equal proportions and subjected to three cycles of denaturation for 2 minutes followed by annealing for 2 minutes (Figure 6). At this stage, approximately 50% of PCR products of one gene fragment were found to be having overhangs that are complimentary to the overhangs present in the second gene fragment. Subsequently the two gene fragments were ligated using T4 DNA ligase at room temperature over night.

In the next stage, a nested PCR was performed with ligation mixture as template with normal forward primer of first gene and the normal reverse primer of second gene, which generated a fusion product with both the gene fragments. Later a sticky fusion PCR product was made by using the above fusion PCR product as described by the above strategy of generating sticky ends.

1.4 Preparation of vector for cloning of sticky fusion inserts:

The pRSET A vector was subjected to overnight double digestion with BamHI and EcoRI at 37°C in 2x concentration of tango buffer and two-fold excess of BamHI.

1.5 Cloning and transformation of the fusion product of SEA and TSST:

The sticky fusion PCR fragment was ligated into a pre-digested vector pRSET A (Bam HI and Eco RI double digest cuts) by T4 DNA ligase (Figure 7) and later transformed into plys S host cells.

The pRSET vectors are pUC-derived expression vectors designed for high-level protein expression and purification from cloned genes in E.coli. High levels of expression of DNA sequences cloned into the pRSET vectors were made possible by the presence of the T7 promoter. In addition, DNA inserts were positioned downstream and in frame with a sequence that encodes an N-terminal fusion peptide. This sequence includes an ATG translation initiation codon, a polyhistidine tag that functions as a metal binding domain in the translated protein, a transcript stabilizing sequence from gene 10 of phase T7, the Xpress, epitope, and the enterokinase cleavage recognition sequence. The metal binding domain of the fusion peptide allows simple purification of recombinant proteins by Immobilized Metal Affinity Chromatography. The enterokinase cleavage recognition site in the fusion peptide located between the metal binding domain and the recombinant protein allows for subsequent removal of this N-terminal fusion peptide from the purified recombinant protein. The transformed recombinant clones were checked for the presence of the insert in right kind of orientation by performing a colony PCR (Figure 1) with T7 promoter primer and the reverse primer of second gene of fusion product namely TSST.

EXAMPLE 2: Protein Expression Studies

The protein expression of fusion gene was carried out in BL21 (DE3) Plys S E.coli host cells. This host contains copy of T7 RNA polymerase gene under control of lacUV5 promoter and hence expression of genes cloned under T7 promoter could be induced with inducer such as IPTG.

Briefly, following protocol was followed for checking the expression of the recombinant protein: Luria Bertani broth supplemented with 50µg/ml of ampicillin and 35µg/ml chloramphenicol and induced with a final concentration of 1mM inducer IPTG.

The E.coli host carrying the recombinant plasmid was grown at 37°C along with proper controls in Luria Bertani broth (5 ml) with 50µg/ml of ampicillin and 35µg/ml of chloramphenicol at 200 rpm. When A600 reached 0.6, IPTG was added to a final concentration of 1mM. After 5 hours of induction, 1.5ml of broth was harvested by centrifugation at 12,000 rpm for 5 minutes.

2.1 PAGE analysis for expression of recombinant protein:

The harvested cell pellet samples were then run on four 12% SDS PAGE gels. One gel was subjected to coomassie-brilliant blue staining of proteins. Later the gel was subjected to destaining by immersing the gel in destaining solution (methanol:acetic acid: water in 4:1:5 ratio). A thick stained protein was observed in all the IPTG induced clones at the expected region (32.8 kD) which was not present in other controls like plain host cells and host cells carrying pRSET A without the insert (Figure 2).

2.2 Bulk expression of recombinant protein and purification:

One of the recombinant clones positive for expression of the recombinant protein was selected for bulk purification of recombinant protein. From this 5ml of LB containing 50-100 µg/ml ampicillin and 34µg/ml chloramphenicol was inoculated with a single recombinant E.coli colony and grown overnight at 37°C with vigorous shaking (225-250rpm). The next day, 100ml of LB broth (stored at 37°C) containing 50-100µg/ml ampicillin was inoculated with 2 ml of the overnight culture in 500ml conical flask and grown the culture at 37°C with vigorous shaking (225-250 rpm) to an OD600= 0.5 (approx) for 2-3 hours. When the cells are in mid-log phase, 1mM IPTG solution was added to induce expression of the recombinant protein. This induced culture was allowed to grow at 37°C with vigorous shaking for another 5 hours. Later the cells were harvested by centrifugation (7000 rpm for 10 minutes at

+4°C). The pellet was stored at -80°C for future use. The recombinant protein was purified by IMAC standard protocol under denaturation conditions using Ni-NTA agarose as chelating agent and hexa-histidine residues as affinity tag. The purified recombinant protein was run on a polyacrylamide gel along with flow-through, washes and elutions. The gel was stained with coomassie-brilliant blue stain to visualize the purified recombinant protein (Figure 3). The recombinant protein was quantified by Lowry's calorimetric method with bovine serum albumin as standard. It was determined to be 4.2 mg/ml.

Example 3: Western Blot analysis

Proteins run on other gels were transferred onto nitrocellulose membrane by Western blot analysis. The nitrocellulose membrane was later blocked with 5% milk at 4°C overnight and later probed with anti-polyhistidine antibodies raised in mouse, polyclonal anti-staphylococcal enterotoxin A antibodies raised in rabbit obtained from Sigma Aldrich, USA and also with anti-TSST antibodies raised in rabbit to confirm the antigenic nature of the recombinant protein. Later these blots were probed with appropriate (anti-rabbit or anti-mouse) HRP labeled antibodies. The blots were washed in PBST solution (tween 0.05%) and later incubated in substrate solution (TMB from Bangalore Genie) for visualization of recombinant protein at 32.8 KD region (Figure 4).

ABSTRACT

A METHOD FOR DIRECTIONAL CLONING OF PCR PRODUCTS

The present invention provides a cloning method for directional cloning of PCR fragments with preformed sticky ends. Said PCR fragments can then be directly cloned into a compatible pre-digested vector with corresponding restriction overhangs that are complimentary to the overhangs in the PCR product. The invention also provides for inframe fusion of PCR fragments from two or more distinct genes by generating PCR fragments with sticky ends ligating them and cloning the PCR product into a pre-digested vector.

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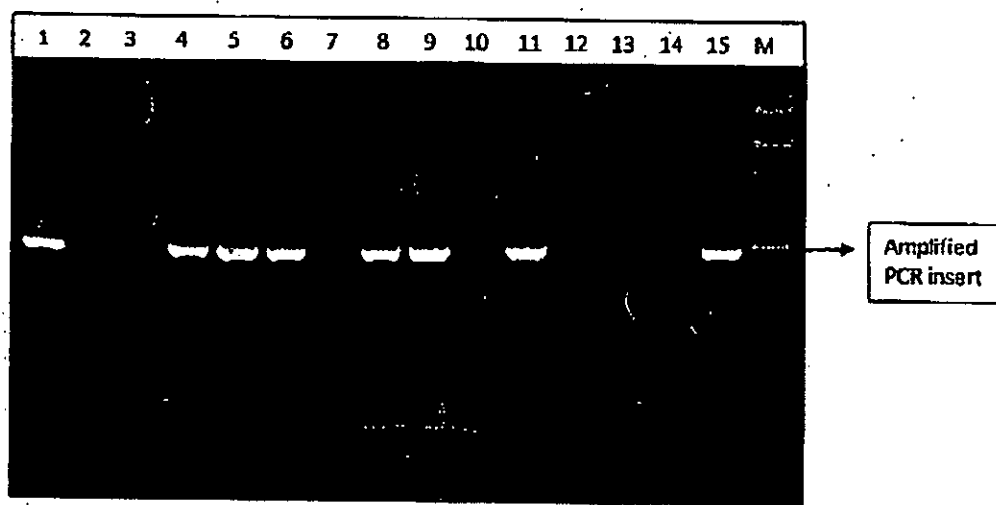


FIGURE 1

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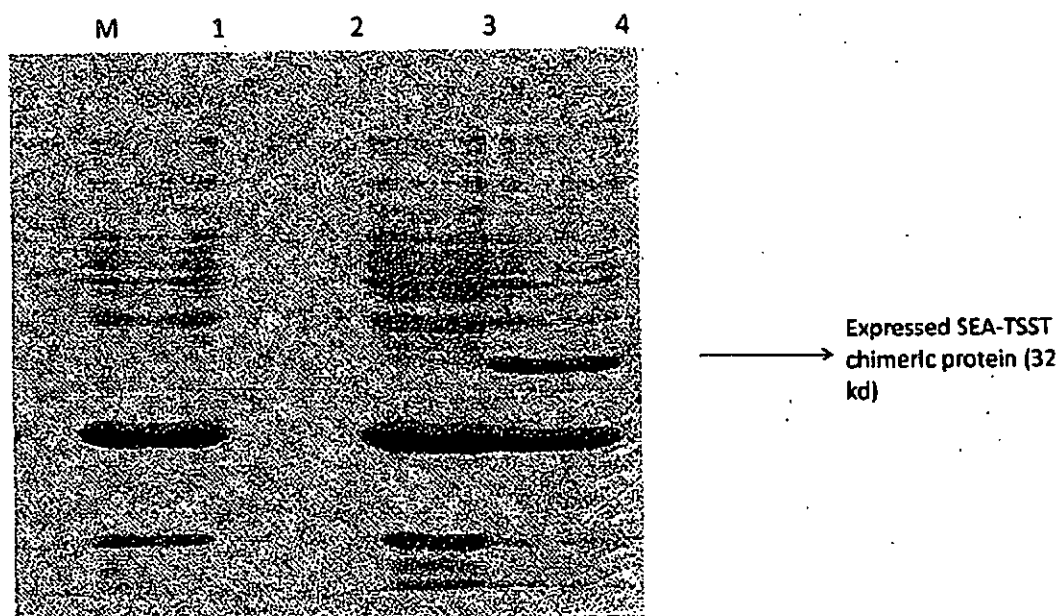


Figure 2: M- Prestained ladder, 1. plys-S with prsetA with out insert (uninduced). 2. plys-S with prset A with out insert (induced), 3. plys-S with prset A carrying SEA-TSST Insert (uninduced), 4. plys-S with prset A carrying SEA-TSST insert (induced).

FIGURE 2

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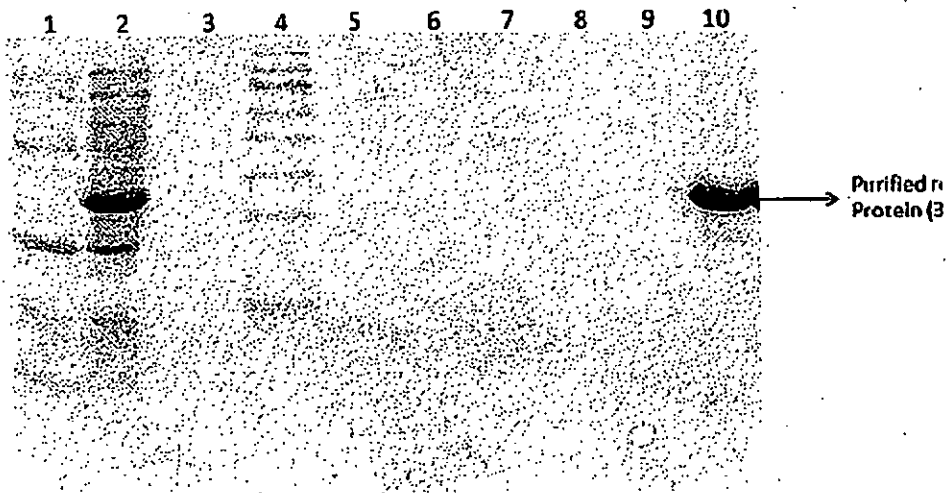


Figure 3: 1. Uninduced. 2. Induced. 3. Flow through. 4. Prestain marker. 5. Wash (1). 6. Wash-2.
7. Wash-3. 8. Elution-1. 9. Elution-2. 10. Elution-3.

FIGURE 3

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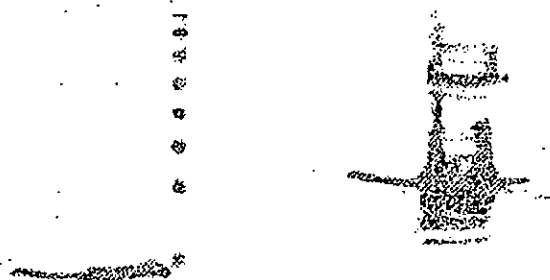
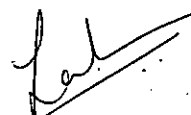


Figure: 4. 1. plysS cells uninduced, 2. plys S cells induced, 3. +ve control uninduced, 4. +ve control induced, 5. prestained marker, 6. plys S with prset A uninduced, 7. plys S with prset A induced, 8. plys S (prset A+ Insert) uninduced, 9. plys S (prset A+ Insert) induced 10. empty well.

FIGURE 4

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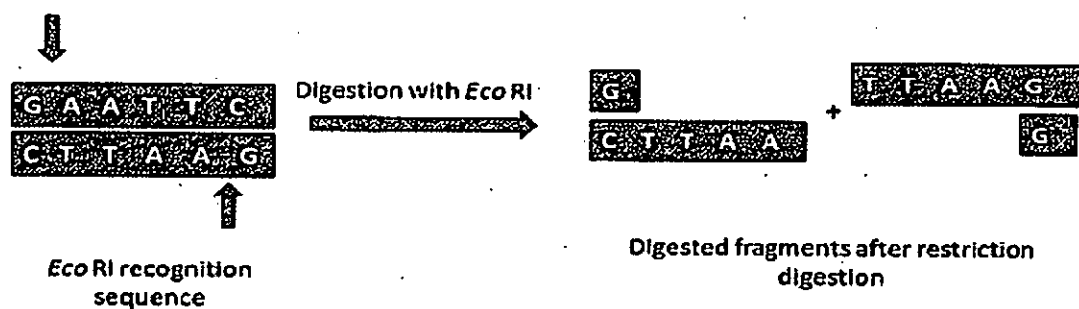
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5' TTAAG Primer sequence 3'

Tailing primer to be synthesized with 5' overhang
corresponding to cut site of any restriction
sequence

FIGURE 5

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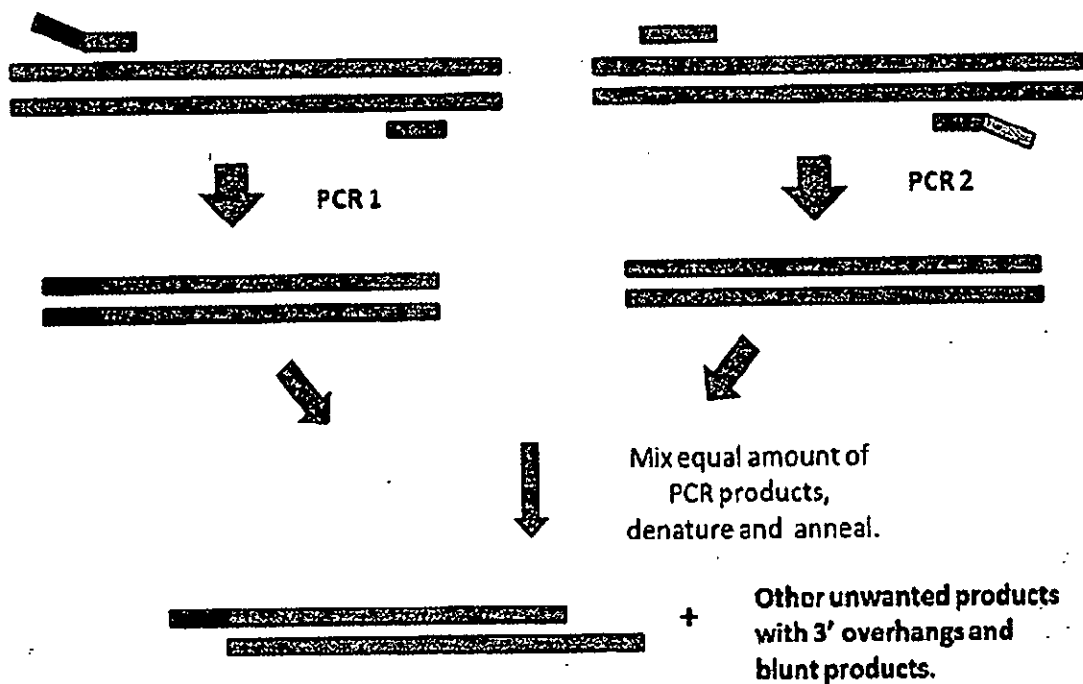


FIGURE 6

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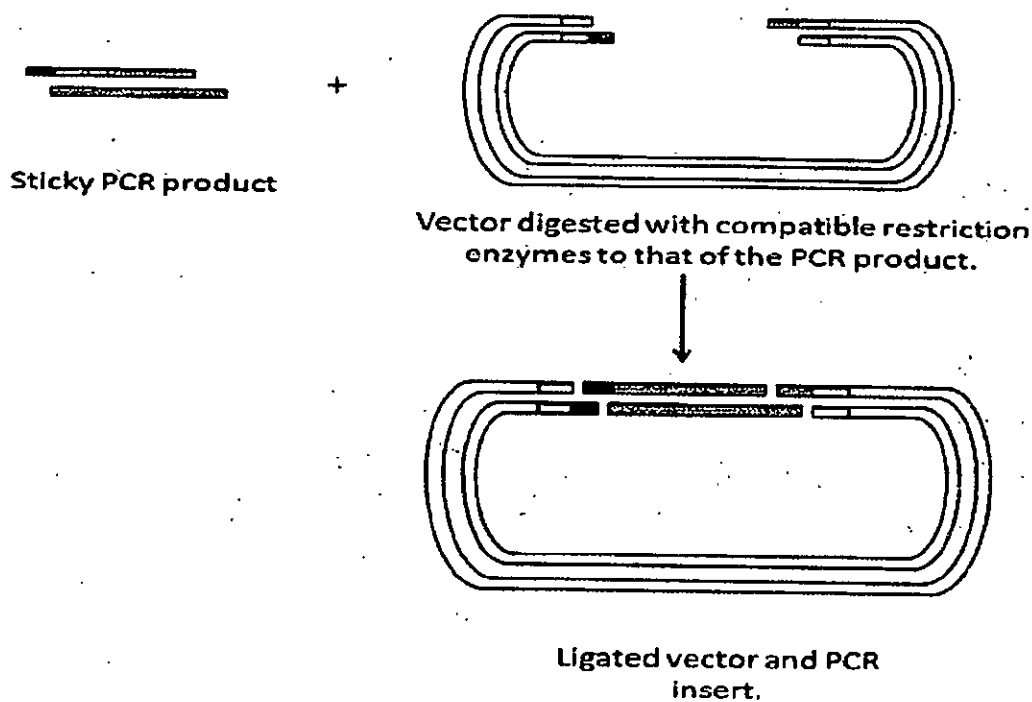


FIGURE 7

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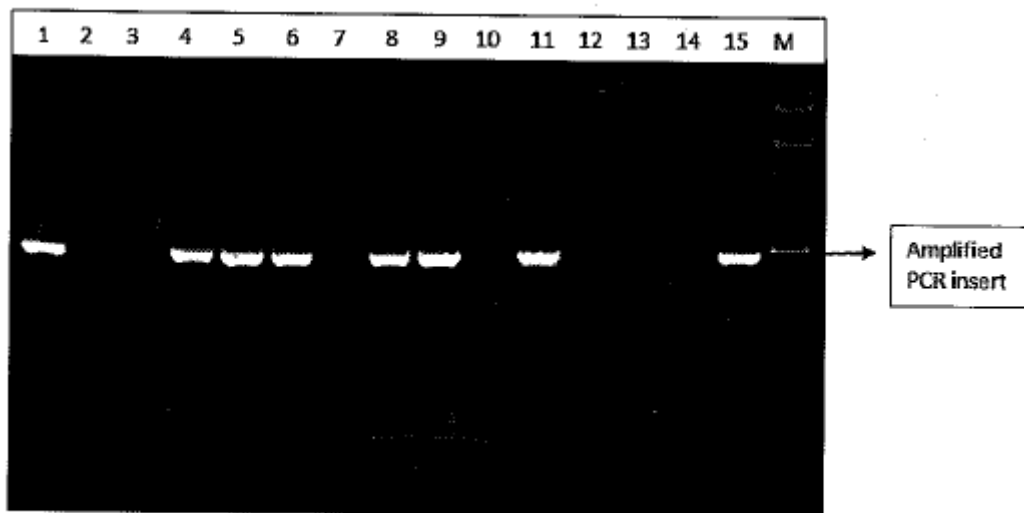


FIGURE 1

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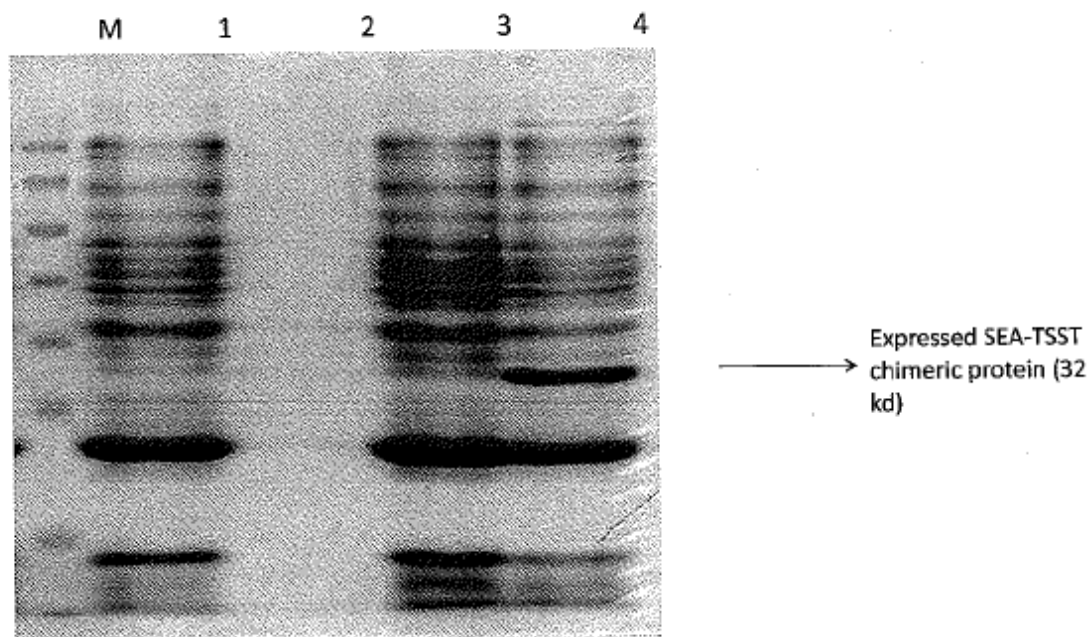


Figure 2: M- Prestained ladder, 1. plys-S with prsetA with out insert (uninduced). 2. plys-S with prset A with out insert (induced), 3. plys-S with prset A carrying SEA-TSST Insert (uninduced), 4. plys-S with prset A carrying SEA-TSST insert (induced).

FIGURE 2

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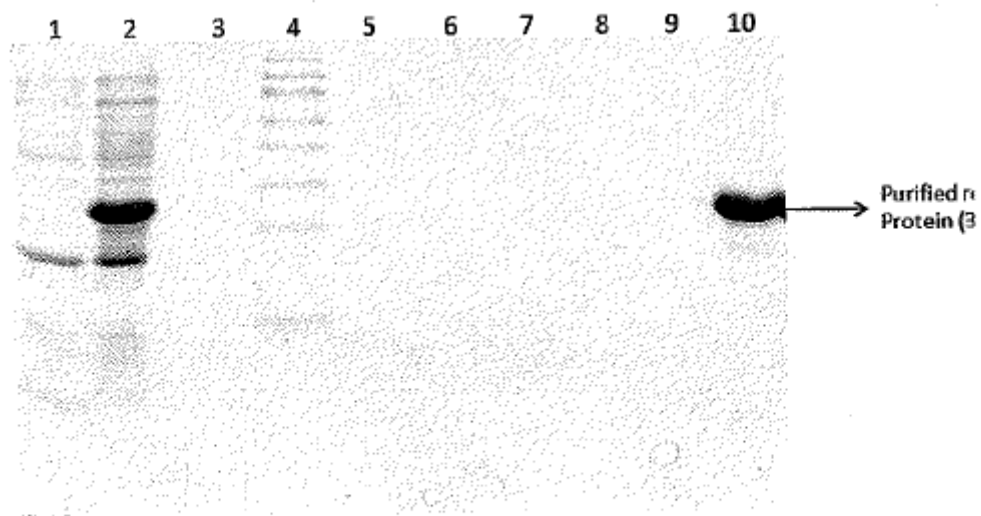


Figure 3: 1. Uninduced. 2. Induced. 3. Flow through. 4. Prestain marker. 5. Wash (1). 6. Wash-2.
7. Wash-3. 8. Elution-1. 9. Elution-2. 10. Elution-3.

FIGURE 3

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1 2 3 4 5 6 7 8 9 10

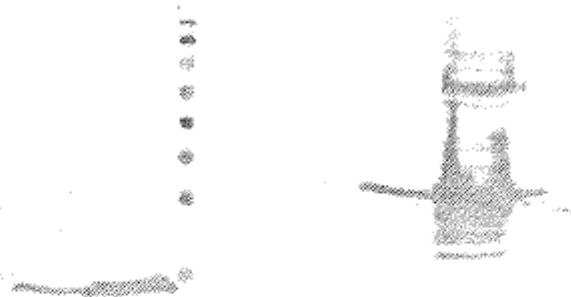


Figure: 4. 1. plysS cells uninduced, 2. plys S cells induced, 3. +ve control uninduced, 4. +ve control induced, 5. prestained marker, 6. plys S with prset A uninduced, 7. plys S with prset A induced, 8. plys S (prset A+ Insert) uninduced, 9. plys S (prset A+ Insert) induced 10. empty well.

FIGURE 4

Signature

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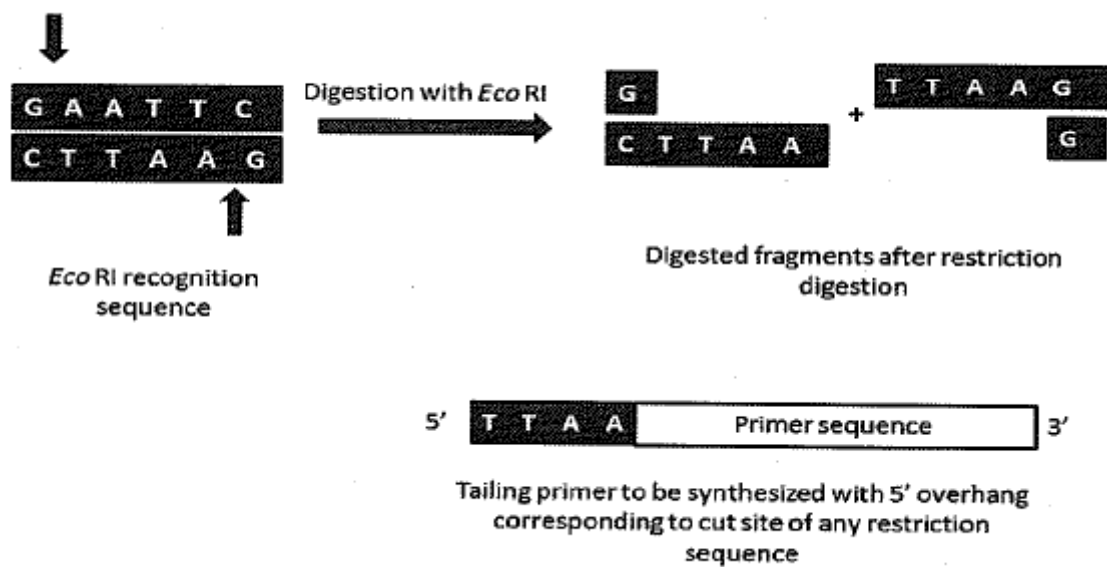


FIGURE 5

Signature

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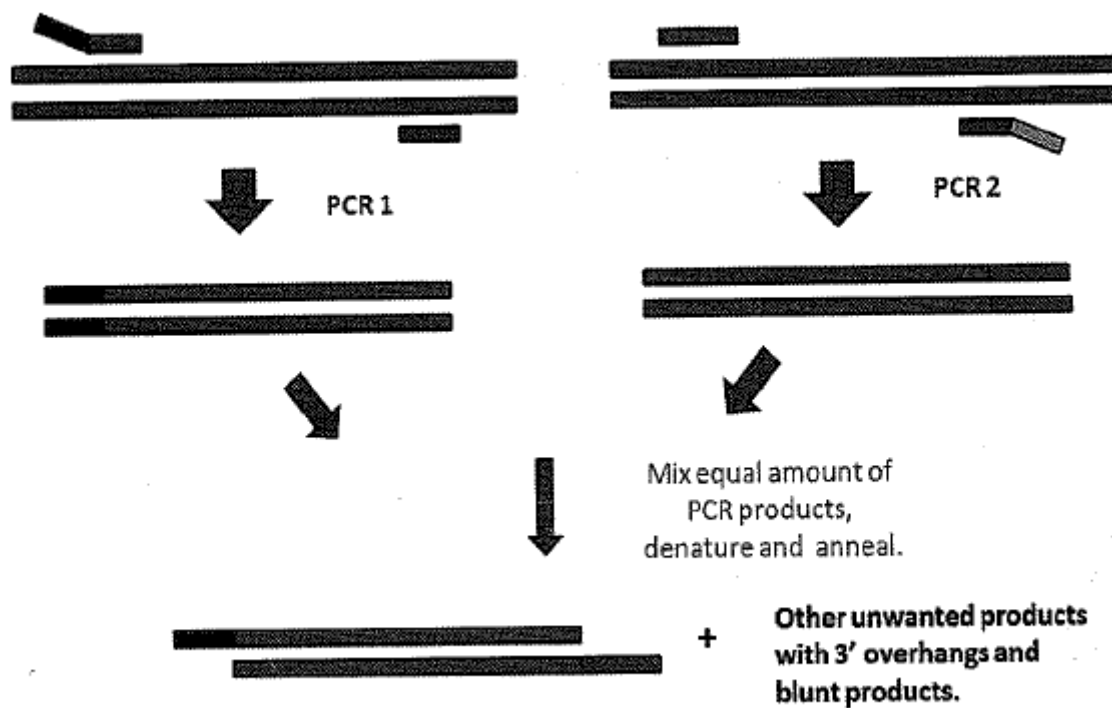


FIGURE 6

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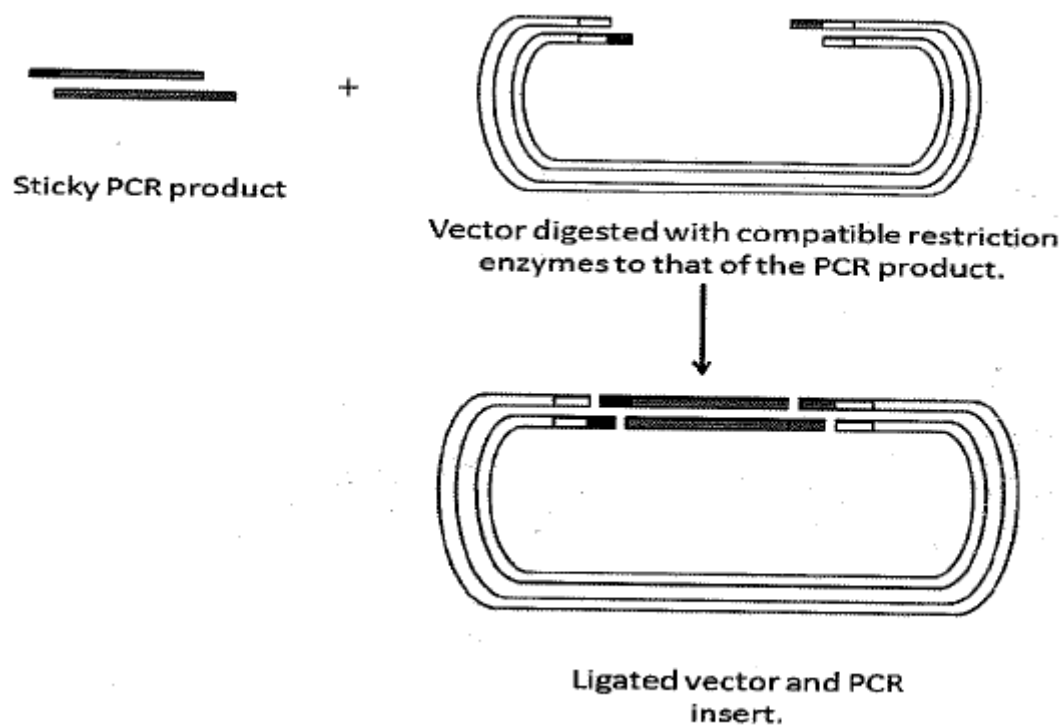


FIGURE 7

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FORM 2

THE PATENTS ACT, 1970

[39 of 1970]

&

THE PATENTS RULES, 2003

COMPLETE SPECIFICATION

(Section 10; Rule 13)

A METHOD FOR DIRECTIONAL CLONING OF PCR PRODUCTS

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**The following Specification particularly describes the invention and the
manner in which it is to be performed**

FIELD OF THE INVENTION

The present invention relates to the field of molecular biology. More particularly, the present invention provides for a method of directional cloning of Polymerase Chain Reaction (PCR) products with preformed cohesive ends for construction of fusion or chimeric genes.

DESCRIPTION OF RELATED ART

There are various types of cloning methods used world over, which can be categorized into two groups: Ligation-dependent and Ligation-independent cloning. The former method is used more widely than latter. According to DNA ends, the existing ligation-dependent cloning methods can be further divided into three types: Blunt end cloning, sticky end cloning, and T-A cloning, while the Sticky-end cloning is routinely used method to clone PCR products.

All the above mentioned cloning methods have certain drawbacks. Blunt end cloning is not very efficient due to the inefficiency of blunt end ligation and high tendency of self-ligation of vectors, which yields lower recombinant clones. Sticky end cloning also has drawbacks. Many restriction endonucleases fail to efficiently cleave the restriction recognition sequences located at the 5' termini, which may be the result of the unstable binding of restriction enzymes to the DNA termini. Another problem encountered with sticky end cloning is the undesirable cleavage of internal restriction site(s) identical to those in primers. Although T-A cloning is simple, reliable and more efficient than blunt-end cloning, it has the disadvantage of being a non-directional cloning strategy and requires a T-vector. T-A cloning also has the drawback of variable cloning efficiencies. This may be a consequence of variability in 3' dA addition to PCR products mediated by Taq polymerase. It has also been observed that not only dA but also dG, dC, or dT can be added to the PCR products and dT overhangs in the T-vector and dA overhang in the PCR product are unstable and may get lost during storage, especially in room temperature.

Gal, J et al., 1999, have reported the cloning method called autosticky PCR, which uses two special primers that contain tetrahydrofuran derivative abasic sites, which makes DNA polymerase stall and the PCR products obtained carry different single stranded DNA 5'-overhangs at both ends, corresponding to sticky ends of two different restriction sites. The intact PCR products can be directionally cloned into correspondingly cut plasmid vectors. In the resulting clones, the abasic position is either substituted by a nucleotide, frequently a dTMP residue, or is deleted. At times, the deletion of the abasic site is accompanied by a minor deletion in the vicinity of the position as well. These deletions are problematic if the purpose of cloning is to get a recombinant protein from the cloned PCR fragment.

Bao-Li Wang, et al., 2009, reported cloning PCR products with preformed sticky ends, where the target sequence is amplified in two parallel asymmetric PCRs using specially but simply designed primers. Two single-stranded products are produced and they are annealed to form a double-stranded DNA fragment bearing overhangs at both ends that correspond to the restriction overhangs of certain restriction enzymes. This fragment can then be cloned into any vector previously treated with the corresponding enzymes without the restriction of the inserted fragment. One of the drawbacks of this method is that the single stranded PCR product produced in two parallel asymmetric PCRs is very much susceptible to digestion by 3'-5' exonuclease activity of the thermostable *Pfu* DNA polymerase enzyme. Another drawback is the standardization of high concentration and low concentration primers in the asymmetric PCR reaction in order to obtain the majority of PCR product which is single stranded. Moreover, the amplification in this method is linear unlike logarithmic in normal PCR and hence the number of required single-stranded PCR products is minimal by this method.

There is hence a need for developing newer techniques for overcoming the drawbacks in the above mentioned methods.

SUMMARY OF THE INVENTION

In one embodiment, the present invention provides a method for directional cloning of PCR products into a pre-digested vector which includes generating a PCR product with sticky ends complementary to the overhangs of the pre-digested vector; and ligating the PCR product and pre-digested vector and transforming into a host cell.

In another embodiment, the present invention provides a method for preparation of a chimeric fusion product which includes amplifying each gene fragment using two parallel PCRs; purifying the products of the two PCRs of both fragments to remove the Pfu DNA polymerase; mixing the PCR products of the two parallel PCRs of each fragment in equal proportions and subjecting to denaturation, followed by annealing; ligating the gene fragments at room temperature; and performing nested PCR with the ligation mixture as template and normal forward primer of first gene and normal reverse primer of second gene to generate the chimeric fusion product.

DESCRIPTION OF ACCOMPANYING DRAWINGS

Figure 1 illustrates an Agarose gel showing PCR amplified products from the colony PCR of the recombinant clones to check for the presence of cloned PCR insert.

Figure 2 illustrates a Polyacrylamide gel (12%) stained with coomassie brilliant blue showing the expression of the recombinant protein from the IPTG induced clone.

Figure 3 is a Polyacrylamide gel (12%) photograph showing the IMAC purified recombinant protein from the induced culture under denaturation conditions. A single protein was present at the required region from elution 3 onwards.

Figure 4 illustrates the western blot results

Figure 5 is a diagrammatic representation of design of tailing primers for the cloning strategy

Figure 6 is a diagrammatic representation of the detailed procedure of the generation of sticky PCR fragment utilizing two sets of primers amplified from a distinct gene.

Figure 7 is a diagrammatic representation showing the ligation of compatible pre-digested vector and sticky PCR insert for transformation into appropriate vector for expression of recombinant protein from PCR insert.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for directional cloning of PCR products into a pre-digested vector comprising:

- (a) generating PCR product with sticky ends complementary to the overhangs of the pre-digested vector;
- (b) ligating the PCR product and pre-digested vector and transforming into a host cell.

PCR products with sticky ends are produced by the steps comprising:

- (a) designing two sets of primers-normal primers and tailed primers;
- (b) performing two parallel PCRs for the desired fragment;
- (c) purifying the products of the two PCRs to remove the Pfu DNA polymerase;
- (d) mixing the purified PCR products in equal proportions and subjecting to denaturation followed by annealing.

The normal primers are designed in frame to the open reading frame of the vector and the tailed primers are designed to carry an overhang on the 5' end complementary to the overhang on the pre-digested vector such that at the end of the denaturation and annealing step, approximately 25% of products would be having overhangs that are complementary to the overhangs present after digestion of the vector. A tailed forward primer and reverse normal primer are used in one PCR and a normal forward primer and a tailed reverse primer are used in the other PCR in the parallel PCR step. All PCRs are carried out with thermostable pfu DNA polymerase. Removal of the Pfu DNA polymerase is carried out using a PCR clean up kit as the Polymerase may degrade or polish the overhangs generated by its 3'-5' exonuclease activity.

The present invention also provides a method for preparation of chimeric fusion product comprising:

- (a) amplifying each gene fragment using two parallel PCRs;
- (b) purifying the products of the two PCRs of both fragments to remove the Pfu DNA polymerase;
- (c) mixing the PCR products of the two parallel PCRs of each fragment in equal proportions and subjecting to denaturation, followed by annealing;
- (d) ligating the gene fragments at room temperature;
- (e) performing nested PCR with the ligation mixture as template and normal forward primer of first gene and normal reverse primer of second gene to generate the complete PCR fragment with all the gene fragments.

For the amplification reaction, two sets of primers are designed for each gene. The tailing bases of reverse primer of one gene and forward primer of the other gene are designed so as to produce PCR fragments with overhangs complementary to each other for splicing. Additionally, the forward tail primer of first gene is designed to carry an overhang corresponding to the cut site of one restriction recognition site

while the reverse tail primer of second gene is designed to carry an overhang corresponding to the cut site of another restriction recognition site. Further the primers are designed in such a way that the two truncated gene portions are separated by a region of DNA sequence that codes for a flexible linker region, generally a flexible poly-glycine serine linker (G4S)₃ to physically separate the two protein domains of the expressed fusion proteins apart, so that they can function as two different independent functional genes linked together as a single chimeric gene. This is found to increase the immunogenicity of the recombinant protein produced when the fusion gene is cloned into a vector and transformed into host cells. Once the fusion gene is formed, a sticky fusion PCR product may be produced using the process of the invention.

The present invention further provides for purification of recombinant protein produced from the fusion gene product using affinity chromatography and quantifying the protein and carrying out immunization of mice to assess the antigenic potential of the recombinant protein.

The present invention is explained further with the help of the following examples which are only by way of illustration and do not limit the scope of the invention.

EXAMPLES

EXAMPLE 1- Fusion product Preparation

For explaining the above invention, two fragments from Staphylococcal enterotoxin A (SEA) and Toxic Shock Syndrome Toxin (TSST) genes were amplified, ligated, cloned into pRSET A and expressed in E.coli BL21 (DE3)pLysS host cells:

1.1 Materials and methods:

1. *Staphylococcus aureus* strains positive for SEA and TSST toxins were obtained from Dept. of Microbiology, SDM medical college, Dharwad.

2. Reagents required in all steps of cloning namely PCR (pfu polymerase, dNTPs), restriction digestion of vector (BamHI, EcoRI), and ligation (T4 DNA ligase with 10x ligase buffer) were obtained from MBI, Fermentas.
3. Other reagents of DNA extraction kit from agarose gel, PCR clean-up kit, Plasmid extraction kit, trima, urea, and IPTG were procured from Sigma Aldrich, USA.
4. Ni-NTA agarose for affinity purification of recombinant protein was obtained from Quiagen Company.
5. All dehydrated media and antibiotics were obtained from Himedia laboratories, India.

1.2 Designing two sets of primers

Two sets of primers were prepared for each region (SEA and TSST) of the fusion gene to be cloned. One set of primers (normal primers) were designed in-frame to the open reading frame of the vector, whereas other set of primers (tailed primers) were designed to carry an overhang on the 5' end of the first set of primers that are complimentary to the overhangs carried by the digested vector. The forward tail primer of the first gene carried an overhang corresponding to the cut site of BamHI recognition sequence and the reverse tail primer of the second gene carried an overhang corresponding to the cut site of EcoRI (Figure 5). The tailing bases of the reverse primer of the first gene and the forward primer of the second gene were designed in a special way such that, both the PCR fragments can have overhangs complimentary to each other and thus can be spliced as shown in Table 1 below:

Primer	Primer Sequence (5'-3')
SEA-forward	*AGGCTTTTTTACAGATCATTCG
SEA-forward	*GATCAGGCTTTTTTACAGATCATTCG

tail	
SEA- reverse	!GCCAGAGCCACCGCCGCCCCCATCAAAAACATCAGAGT
SEA- reverse tail	<u>&ACCACC</u>GCCAGAGCCACCGCCGCCCCCATCAAAAACATCAGAGT
TSST- forward	!GGCTCGGGCGGCGGTGGTTCAACAGATGGCAGCATCAGC
TSST- forward tail	<u>&GGTGGT</u>GGCTCGGGCGGCGGTGGTTCAACAGATGGCAGCATCAGC
TSST- reverse	*ATGTGGATCCGTCATTCATT
TSST- reverse tail	*AATTATGTGGATCCGTCATTCATT

In addition these two primers were designed in such a way that the two gene fragments were separated by a region of flexible poly-glycine serine linker (G₄S)₃.

1.3 Preparing the sticky insert by PCR

The antigenic determinant regions of SEA and TSST genes were amplified by PCR, using whole genomic DNA of *S.aureus* clinical strains E-2215 and E-2147 respectively as template. All the PCRs were performed in 20µl reaction volume in triplicates containing 0.5 pM of each primer, 200µM of each dNTP, 0.9U Pfu polymerase (MBI Fermentas), and 1.5mM of MgSO₄ in 1X pfu PCR buffer (MBI Fermentas) with 50-100ng of template DNA without any contaminants for which pfu

polymerase is sensitive to. PCR was carried out in Eppendorf master gradient (Hamberg, Germany) thermal cycler with initial denaturation of 95°C for 3 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 59°C for 30 seconds and an extension time of 72°C for 2 minutes followed by 5 minutes of final extension at 72°C.

Two parallel PCRs were performed for both the fragments (SEA and TSST) of the fusion gene. In one, PCR tailed forward primer and a reverse normal primer was used and in other PCR normal forward primer and a tailed reverse primer were used for amplification of the desired gene fragment. All PCRs were carried out with thermostable pfu DNA polymerase. Later the products of the two PCRs for both the fragments were purified with PCR clean up kit to remove the pfu DNA polymerase which may degrade or polish the overhangs that will be generated by its 3'-5' exonuclease activity. The two parallel purified PCR products for each gene fragment were mixed in equal proportions and subjected to three cycles of denaturation for 2 minutes followed by annealing for 2 minutes (Figure 6). At this stage, approximately 50% of PCR products of one gene fragment were found to be having overhangs that are complimentary to the overhangs present in the second gene fragment. Subsequently the two gene fragments were ligated using T4 DNA ligase at room temperature over night.

In the next stage, a nested PCR was performed with ligation mixture as template with normal forward primer of first gene and the normal reverse primer of second gene, which generated a fusion product with both the gene fragments. Later a sticky fusion PCR product was made by using the above fusion PCR product as described by the above strategy of generating sticky ends.

1.4 Preparation of vector for cloning of sticky fusion inserts:

The pRSET A vector was subjected to overnight double digestion with BamHI and EcoRI at 37°C in 2x concentration of tango buffer and two-fold excess of BamHI.

1.5 Cloning and transformation of the fusion product of SEA and TSST:

The sticky fusion PCR fragment was ligated into a pre-digested vector pRSET A (Bam HI and Eco RI double digest cuts) by T4 DNA ligase (Figure 7) and later transformed into plys S host cells.

The pRSET vectors are pUC-derived expression vectors designed for high-level protein expression and purification from cloned genes in E.coli. High levels of expression of DNA sequences cloned into the pRSET vectors were made possible by the presence of the T7 promoter. In addition, DNA inserts were positioned downstream and in frame with a sequence that encodes an N-terminal fusion peptide. This sequence includes an ATG translation initiation codon, a polyhistidine tag that functions as a metal binding domain in the translated protein, a transcript stabilizing sequence from gene 10 of phase T7, the Xpress, epitope, and the enterokinase cleavage recognition sequence. The metal binding domain of the fusion peptide allows simple purification of recombinant proteins by Immobilized Metal Affinity Chromatography. The enterokinase cleavage recognition site in the fusion peptide located between the metal binding domain and the recombinant protein allows for subsequent removal of this N-terminal fusion peptide from the purified recombinant protein. The transformed recombinant clones were checked for the presence of the insert in right kind of orientation by performing a colony PCR (Figure 1) with T7 promoter primer and the reverse primer of second gene of fusion product namely TSST.

EXAMPLE 2: Protein Expression Studies

The protein expression of fusion gene was carried out in BL21 (DE3) Plys S E.coli host cells. This host contains copy of T7 RNA polymerase gene under control of lacUV5 promoter and hence expression of genes cloned under T7 promoter could be induced with inducer such as IPTG.

Briefly, following protocol was followed for checking the expression of the recombinant protein: Luria Bertani broth supplemented with 50µg/ml of ampicillin and 35µg/ml chloramphenicol and induced with a final concentration of 1mM inducer IPTG.

The E.coli host carrying the recombinant plasmid was grown at 37°C along with proper controls in Luria Bertani broth (5 ml) with 50µg/ml of ampicillin and 35µg/ml of chloramphenicol at 200 rpm. When A600 reached 0.6, IPTG was added to a final concentration of 1mM. After 5 hours of induction, 1.5ml of broth was harvested by centrifugation at 12,000 rpm for 5 minutes.

2.1 PAGE analysis for expression of recombinant protein:

The harvested cell pellet samples were then run on four 12% SDS PAGE gels. One gel was subjected to coomassie-brilliant blue staining of proteins. Later the gel was subjected to destaining by immersing the gel in destaining solution (methanol:acetic acid: water in 4:1:5 ratio). A thick stained protein was observed in all the IPTG induced clones at the expected region (32.8 kD) which was not present in other controls like plain host cells and host cells carrying pRSET A without the insert (Figure 2).

2.2 Bulk expression of recombinant protein and purification:

One of the recombinant clones positive for expression of the recombinant protein was selected for bulk purification of recombinant protein. From this 5ml of LB containing 50-100 µg/ml ampicillin and 34µg/ml chloramphenicol was inoculated with a single recombinant E.coli colony and grown overnight at 37°C with vigorous shaking (225-250rpm). The next day, 100ml of LB broth (stored at 37°C) containing 50-100µg/ml ampicillin was inoculated with 2 ml of the overnight culture in 500ml conical flask and grown the culture at 37°C with vigorous shaking (225-250 rpm) to an OD600= 0.5 (approx) for 2-3 hours. When the cells are in mid-log phase, 1mM IPTG solution was added to induce expression of the recombinant protein. This induced culture was allowed to grow at 37°C with vigorous shaking for another 5 hours. Later the cells were harvested by centrifugation (7000 rpm for 10 minutes at

+4°C). The pellet was stored at -80°C for future use. The recombinant protein was purified by IMAC standard protocol under denaturation conditions using Ni-NTA agarose as chelating agent and hexa-histidine residues as affinity tag. The purified recombinant protein was run on a polyacrylamide gel along with flow-through, washes and elutions. The gel was stained with coomassie-brilliant blue stain to visualize the purified recombinant protein (Figure 3). The recombinant protein was quantified by Lowry's calorimetric method with bovine serum albumin as standard. It was determined to be 4.2 mg/ml.

Example 3: Western Blot analysis

Proteins run on other gels were transferred onto nitrocellulose membrane by Western blot analysis. The nitrocellulose membrane was later blocked with 5% milk at 4°C overnight and later probed with anti-polyhistidine antibodies raised in mouse, polyclonal anti-staphylococcal enterotoxin A antibodies raised in rabbit obtained from Sigma Aldrich, USA and also with anti-TSST antibodies raised in rabbit to confirm the antigenic nature of the recombinant protein. Later these blots were probed with appropriate (anti-rabbit or anti-mouse) HRP labeled antibodies. The blots were washed in PBST solution (tween 0.05%) and later incubated in substrate solution (TMB from Bangalore Genie) for visualization of recombinant protein at 32.8 KD region (Figure 4).

WE CLAIM:

1. A method for directional cloning of PCR products into a pre-digested vector comprising:

generating a PCR product with sticky ends complementary to the overhangs of the pre-digested vector;

ligating the PCR product and pre-digested vector and transforming into a host cell.

2. The method as claimed in claim 1, wherein PCR products with sticky ends are produced by the steps comprising:

designing two sets of primers-normal primers and tailed primers;

performing two parallel PCRs for the desired fragment;

purifying the products of the two PCRs to remove the Pfu DNA polymerase;

mixing the purified PCR products in equal proportions and subjecting to denaturation followed by annealing.

3. The method as claimed in claims 1 and 2, wherein the normal primers are designed in frame to the open reading frame of the vector and the tailed primers are designed to carry an overhang on the 5' end complementary to the overhang on the pre-digested vector.

4. The method as claimed in claim 2, wherein in step (b), tailed forward primer and reverse normal primer are used in one PCR and a normal forward primer and a tailed reverse primer are used in the other PCR.

5. A method for preparation of chimeric fusion product comprising:

amplifying each gene fragment using two parallel PCRs;

purifying the products of the two PCRs of both fragments to remove the Pfu DNA polymerase;

mixing the PCR products of the two parallel PCRs of each fragment in equal proportions and subjecting to denaturation, followed by annealing;

ligating the gene fragments at room temperature;

performing nested PCR with the ligation mixture as template and normal forward primer of first gene and normal reverse primer of second gene to generate the chimeric fusion product.

6. The method as claimed in claim 5, wherein for amplification, tailing bases of reverse primer of one gene and forward primer of the other gene are designed so as to produce PCR fragments with overhangs complementary to each other for splicing.

7. The method as claimed in claim 5, wherein in the chimeric fusion product, the two gene fragments are separated by a linker region.

8. The method as claimed in claim 5 further comprising the step of creating sticky ends on the fusion product using the method as claimed in claim 1.

Dated this 26th day of March 2012

Signature

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ABSTRACT

The present invention provides a cloning method for directional cloning of PCR fragments with preformed sticky ends. Said PCR fragments can then be directly cloned into a compatible pre-digested vector with corresponding restriction overhangs that are complimentary to the overhangs in the PCR product. The invention also provides for inframe fusion of PCR fragments from two or more distinct genes by generating PCR fragments with sticky ends, ligating them and cloning the PCR product into a pre-digested vector.