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Reliable Scarless Fusion of Genes through Overlap Extension PCR to Develop Chimeric Genes

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IG, MSA, IT and RM performed the experiments and wrote the manuscript. MAA designed the study and critical review the manuscript.

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ABSTRACT

Overlap extension PCR is used to make versatile chimeric gene products. However, the results of this technique are not always reliable due to inefficient overlapping of the sequences especially those derived from authenticated native sequences. Certain forbidden sequences do appear at junction of spliced genes whenever efficiency of gene fusion is improved by inserting GC rich sequences. In this study, reliable scar-less gene fusion with the insertion of sequence of choice at junction sites has been described by optimization of various parameters of overlap extension PCR. The fusion of IFNα2b gene to the gene of human latency associated protein with insertion of HCV NS3 protease cleavage site at splicing junction is reported.

INTRODUCTION

Splicing of genes or DNA fragments is of great importance in the field of molecular biology and biotechnology. It is used for structural and functional analysis of proteins (Elleuche *et al*., 2011; Henness and Broom-Smith, 1993; Aslam *et al*., 2018), for studying the effect of different regulatory sequences on gene expression (Haoug *et al*., 2004; Nemetz *et al*., 2002), for protein purification (Waugh, 2005), for targeted drug delivery (Lu *et al*., 2014), removal of introns from genes (Mitani *et al*., 2004; Mergulhao *et al*., 1999), tagging of reporter/marker gene (Hobert, 2002; Siegel and Isacoff, 1997) and for many other purposes. The conventional methods of gene fusion rely on restriction enzymes and subsequent ligation that introduce undesired sequences at the junction of spliced genes (Lu, 2005). Alternatively, overlap extension PCR is a useful procedure where genes can be spliced directly or by inserting sequence of choice at splicing point (Horton *et al*., 2013). Specially designed overlapping primers incorporating the changes are the backbone of procedure. However, this method is not considered as method of immediate choice as the results are not always reliable, especially when the overlapping sequences are derived from natural gene sequences to be spliced (Cha-aim *et al*., 2012). The success of splicing by overlap extension (SOE) depends upon the efficient gene fusion after annealing of overlapping sequences. Different strategies can be employed to improve the efficiency of SOE PCR such as optimizing the length of overlapping primers, use of poly GC rich overlap sequences (Cha-aim *et al*., 2009) or controlling the PCR reaction by optimizing different parameters (Bryksin and Matsumura, 2010). To avoid the complexities of various modifications of overlap extension PCR, inventors of overlap extension PCR suggest that following all the intermediate steps of basic overlap extension PCR protocol, efficiency and accuracy of ultimately generated product can be maximized (Heckman and Pease, 2007).

In case, where a specific sequence has to be inserted at junction of spliced gene, option of poly GC overlapping sequence is considered unreliable. Nonetheless, fusion through G/C stretches improves gene fusion but likewise the restriction enzyme digestion-DNA ligation, fusion through G/C stretches leave scars of unwanted sequences at junction site. For reliable scar less fusion without any forbidden sequences, the efficiency of fusion has been improved in the present study by controlling the PCR conditions.

In the present study, various parameters of splicing by overlap extension PCR has been optimized to efficiently develop chimeric gene products (IFNα2b-NS3-LAP and LAP-NS3-IFNα2b) by splicing interferon alpha 2b (IFNα2b) gene with latency associated protein (LAP) gene of human transforming growth factor β (TGFβ) in both

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orientations with the insertion of hepatitis C virus (HCV) NS3 protease cleavage site at splicing junction. In addition overlap primer walk PCR (OPW-PCR) was also performed to introduce Tag sequences at 5' end of chimeric gene products. To our knowledge, these novel chimeric gene products have not been developed so far.

MATERIALS AND METHODS

The list of primers used for chimeric genes development by SOE-PCR and OPW-PCR is given in Table I. The primers were designed manually and analyzed by free online program ''Oligocalculator''. The IFNα2b-NS3-LAP chimeric gene was developed by using chimeric primers (F3, $F2_{30}$, $F2_{27}$, $F2_{24}$, $F2_{21}$, $F2_{18}$, $F2_{15}$) and non-chimeric primers (F1 and F4). Whereas, for the development of LAP-NS3-IFNα2b chimeric gene, $F3^{/}$ and $F2^{/}_{21}$ primer were used as chimeric while $F1^{/}$ and $F4^{/}$ were used as non-chimeric primer pair. The primers F5, $F5^{/}$, F6, F4 and $F4^{/}$ were used in OPW-PCR. F4 and $F4^{/}$ primers had restriction site of *XhoI* whereas F6 primer had *XbaI* restriction site.

IFNα2b and LAP genes were amplified from the plasmids pIFN and pLAP respectively containing IFNα2b and LAP genes.

SOE-PCR was performed in two steps (Fig. 1). In the first step, IFNα2b was amplified from pIFN plasmid using primer pairs F1/ $F2_{30}$, $F1/F2_{27}$, $F1/F2_{24}$, $F1/F2_{21}$, $F1/F2_{18}$, $F1/F2_{15}$ while LAP gene was amplified using F3 and F4 primer from pLAP plasmid. The reaction mixture of PCR was prepared using 0.2 mM dNTPs, 0.5µM of each primer, 2 mM $MgSO_4$, 50 ng of plasmid DNA as template, 2.5 U of Pfu DNA polymerase and 1X Pfu buffer (50 mM KCl, 10 mM Tris-Cl pH 8.0, 1.5 mM $MgSO_4$) and subjected to the 35 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C (1 min for IFNα2b; 2 min for LAP) followed by final extension of 7 min at 72 °C. Another PCR reaction was also performed under similar reaction conditions except using $F1^{/}/F2^{/}_{21}$ primer pair for amplification of LAP gene and $F3^{/}/F4^{/}$ primer pair for amplification of IFNα2b. The amplicons were analyzed by 1 % agarose gel electrophoresis and purified using Thermoscientific Gel Extraction Kit following the protocol of manufacturer. The purified amplicons were quantified using nanodrop.

In the second step, first10 cycles of SOE-PCR were performed in reaction mixture without adding primers to the reaction mixture and containing equimolar concentration (0.8 pmoles-0.00625 pmoles) of purified IFNα2b and LAP amplicon from 1st step of PCR as template, 0.2 mM dNTPs, 2 mM $MgSO_4$, 2.5 U of Pfu DNA polymerase and 1X Pfu buffer (50 mM KCl, 10 mM Tris-Cl pH 8.0, 1.5 mM $MgSO_4$) under conditions of denaturation at 95 °C for 1 min, annealing at 45 °C-60 °C for 1 min and extension at 72 °C for 3 min. After 10 cycles of each reaction, primer pairs F1/F4 and $F1^{/}/F4^{/}$ were added in respective reaction vials for amplification of IFN-NS3-LAP and LAP-NS3-IFN chimeric gene products respectively and further subjected to 30 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 3 min followed by final extension at 72 °C for 7 min.

Table I.- List of primers used for overlap extension PCR and OPW-PCR.

Name	Sequence	GC % of overlapping region
F1	5' TGTGACTTGCCACAAACTCACTCCTTGGGTTCC 3'	-
$F2_{30}$	5'GTAGGACATAGAACAACAAACAACATCTTCCTCCTTGGATCTCAAGGACT 3'	37
$F2_{27}$	5' GGACATAGAACAACAAACAACATCTTCCTCCTTGGATCTCAAGGACT 3'	37
$F2_{24}$	5' CATAGAACAACAAACAACATCTTCCTCCTTGGATCTCAAGGACT 3'	33
$F2_{21}$	5' AGAACAACAAACAACATCTTCCTCCTTGGATCTCAAGGACT 3'	33
$F2_{18}$	5' ACAACAAACAACATCTTCCTCCTTGGATCTCAAGGACT 3'	33
$F2_{15}$	5' ACAAACAACATCTTCCTCCTTGGATCTCAAGGACT 3'	33
F3	5' GAAGATGTTGTTTGTTGTTCTATGTCCTACTTGTCTACTTGTAAGACTAT 3'	37-33
F4	5' GTTCTAGATTACCTCCTATGACGGGAAGATTGCAAATGTTGGGCT 3'	-
F1'	5'TTGTCTACTTGTAAGACTATTGATATGGAATTGGTTAAGAGAAAGAGA ATTGAAG 3'	-
$F2'_{21}$	5' AGAACAACAAACAACATCTTCCCTCCTATGACGGGAAGATTG 3'	33
F3'	5'GAAGATGTTGTTTGTTGTTCTATGTCCTACTGTGACTTGCCACAAACTCA 3'	33
F4'	5' GCCTTCTAGATTACTCCTTGGATCTCAAGGACTCTTGCAAGTTGGTAG 3'	-
F5	5' CACCACGGATCCGATGATGATGATAAGTGTGACTTGCCACAAACTCA 3'	48
F5'	5' CACCACGGATCCGATGATGATGATAAG TTGTCTACTTGTAAGACTATT GATATG 3'	48
F6	5' GCCTCGAGAAAAGACACCACCACCACCACCACGGATCCGATGATGAT 3'	52

Underlined portions indicate the overlapping regions.

In order to introduce the additional sequences (His-Tag, Glycine-Serine spacer and Enterokinase cleavage site) at the 5' end of the each chimeric product, overlap primer walk PCR (OPW-PCR) was performed (Fig. 2). In 1st round of PCR, SOE-PCR products (templates) were amplified to introduce enterokinase cleavage site and Gly-Ser spacer by using primer pair F5/F4 and F5/F4/. In the 2nd round of PCR, OPW-PCR products of 1st round were amplified to introduce His-Tag, Kex2 cleavage site and *XhoI* site by using primer pair F6/F4 and F6/F4/ for IFN-NS3-LAP and LAP-NS3-IFN chimeric genes, respectively. The reaction was performed using 0.5 µM of each primer along with 0.2 mM dNTPs, 100 ng of template (for step 1 gene product of SOE-PCR; for step 2 gene product of 1st step of OPW-PCR), 2 mM $MgSO_4$, 2.5 U of Pfu DNA polymerase and 1X Pfu buffer (50 mM KCl, 10 mM Tris-Cl pH 8.0, 1.5 mM $MgSO_4$). Each reaction was performed for 35 cycles under the following conditions: denaturation at 95 °C for 1 min, annealing at 62 °C for 1 min and extension at 72 °C for 4 min followed by final extension at 72 °C for 7 min.

RESULTS AND DISCUSSION

In this study, a simple procedure of overlap extension PCR was used to fuse human IFNα2b gene to the LAP gene of human TGFβ in both orientations along with insertion of HCV NS3 protease cleavage site sequence at the splicing junction of genes.

For splicing of genes and insertion of HCV NS3 cleavage site sequence simultaneously, chimeric primers ($F2_{30}$, $F2_{27}$, $F2_{24}$, $F2_{21}$, $F2_{18}$, $F2_{15}$, F3, F2/,F3/) along with non-chimeric primers were used in first step of SOE-PCR. Chimeric primers have anchoring sequence (20 nts) at 3' end (derived from the template gene) and flanking sequence having HCV NS3 protease cleavage site sequence ranging from 30 nts ($F2_{30}$, F/2) to 15 nts ($F2_{15}$) at 5' end. Using Pfu

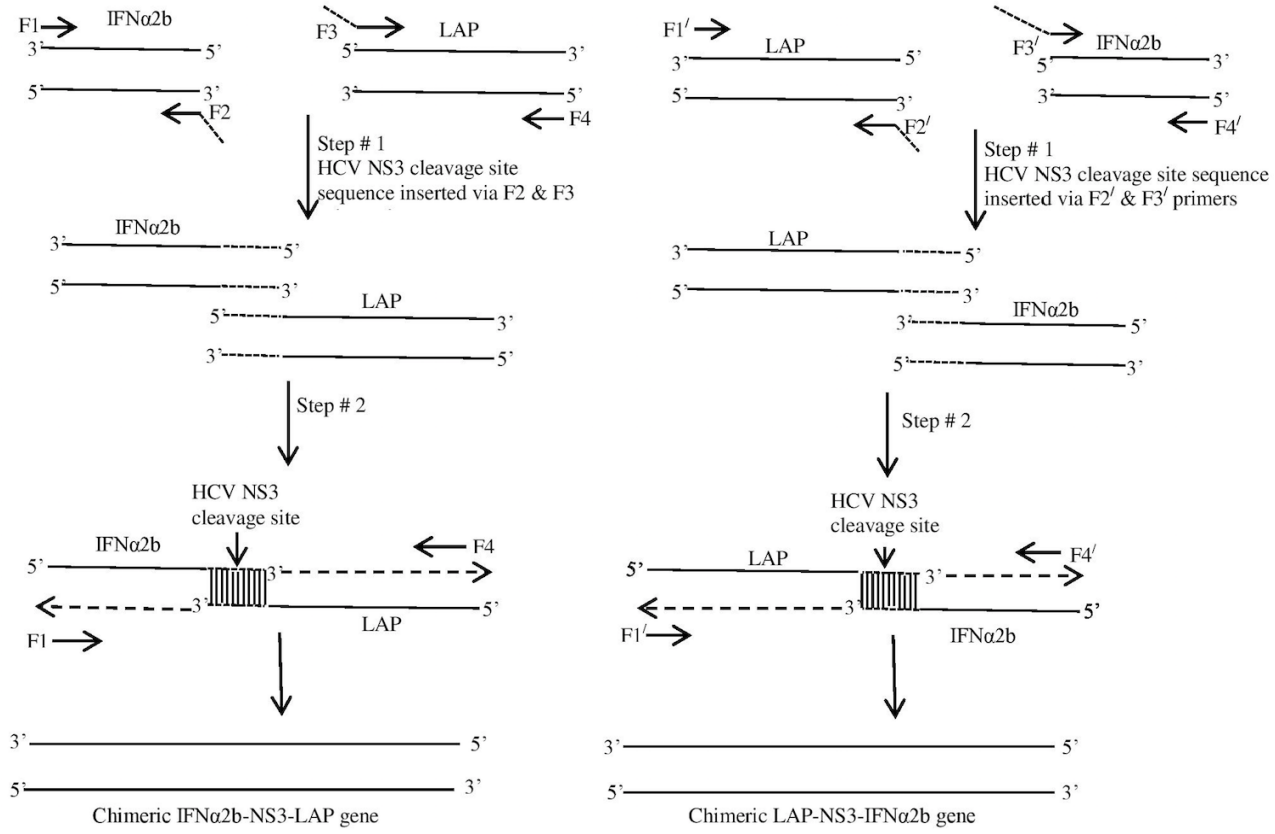

Fig. 1. Two step overlap extension PCR for construction of chimeric gene products (IFNα2b-NS3-LAP and LAP-NS3-IFNα2b). F2, F3 and F2/, F3/ were chimeric primers that introduced the overhangs of HCV NS3 protease cleavage site sequence when used with non-chimeric primers F1, F1/ and F4, F4/ in step # 1 of SOE-PCR. Overhangs introduced overlapping regions that act as primer in initial 10 cycles of second step of PCR. First 10 cycles of step # 2 were performed without addition of any primer and strands were extended from 3' ends of the overlapping region. In the later 30 cycles, non-chimeric primers F1/F4 and F1//F4/ were added in reaction vial to amplify the full length chimeric gene products.

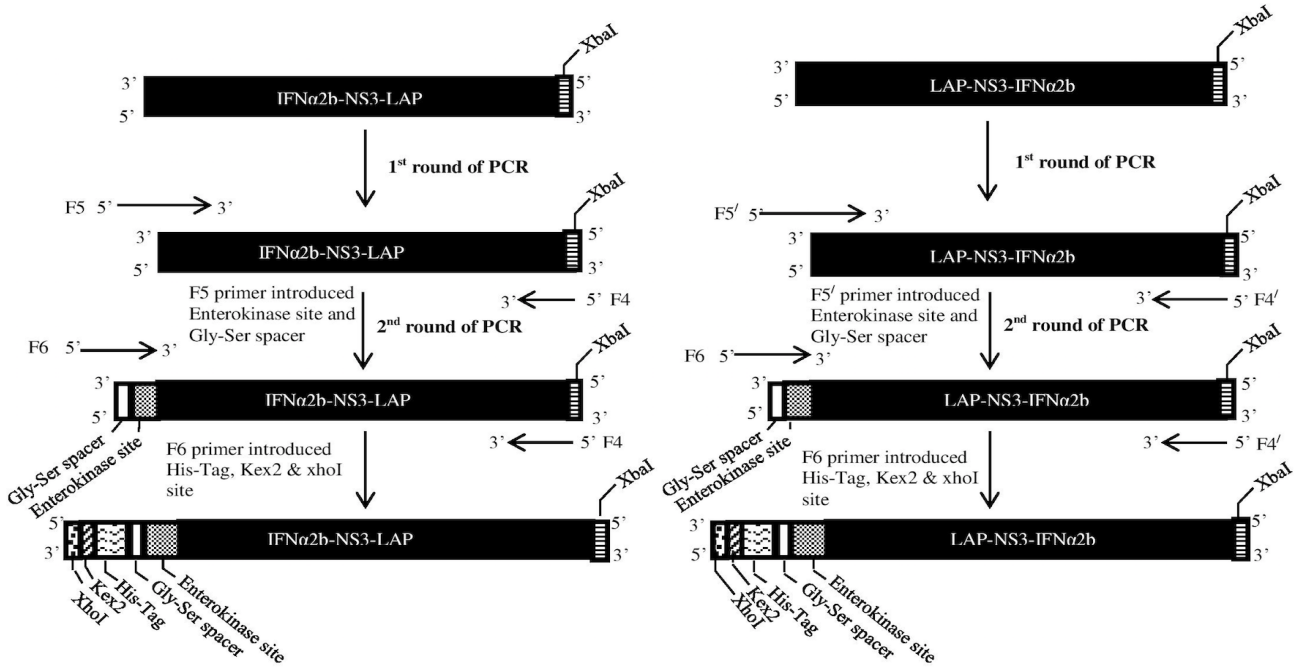

Fig. 2. Overlap Primer Walk PCR (OPW-PCR). In 1st round of PCR, Primer F5 and F5/ were used to introduce enterokinase cleavage site at 5' end of chimeric gene products IFNα2b-NS3-LAP and LAP-NS3-IFNα2b respectively. In 2nd round of PCR, primer F6 introduced Gly-Ser spacer, His-Tag, Kex2 and XhoI restriction site at 5' end of the PCR product of 1st round.

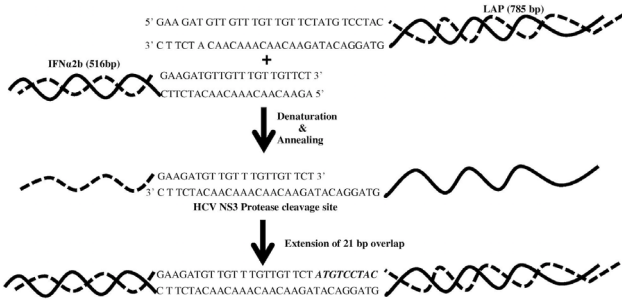

Fig. 3. Splicing during the first 10 cycles of step # 2 of SOE- PCR driven without addition of primers. Inserted overlapping region of HCV NS3 protease cleavage site sequence acts as primers for strands extension. HCV NS3 protease cleavage site sequence is highlighted by alphabets.

DNA polymerase in the 1st step of SOE-PCR, IFNα2b gene (495 bp) was amplified by primer pairs F1/$F2_{30}$, F1/$F2_{27}$, F1/$F2_{24}$, F1/$F2_{21}$, F1/$F2_{18}$ and F1/$F2_{15}$ whereas F3/F4 primer pair was used to amplify LAP gene (747bp). This PCR amplification introduced overhangs of 30-15 nts of HCV NS3 protease cleavage site sequence at 3' end of IFNα2b gene (525-510 bp) by $F2_{(30\text{-}15)}$ primers and 30 nts overhang at 5' end of LAP gene (785 bp) by F3 primer (Fig. 4A). $F2_{(30\text{-}15)}$ primers refer to the length of 5' flanking sequence sharing the overlapping region with F3 primer ranging from 30 nucleotides to the 15 nucleotides. At this step, it is very critical to use the DNA polymerase which has low error rate, high fidelity and proofreading activity as addition or substitution of a single nucleotide at 3' end of the overlapping regions of genes results in complete failure of splicing. In the first 10 cycles of second step of SOE-PCR, purified IFNα2b and LAP gene from 1st step with various equimolar concentrations (0.8 pmoles-0.00625 pmoles) were used without addition of any primer for amplification of full length chimeric gene (1.280 kb) with insertion of 30 nucleotide of HCV NS3 protease cleavage site sequence (IFNα2b-NS3-LAP). The 3' ends of antisense strand of IFNα2b and sense strand of LAP had overlapping region of maximum length 30 nucleotides to minimum length of 15 nucleotides. The efficiently overlapped region resulted in extension from 3' end of each strand and synthesize full length chimeric gene (Fig. 3). In our case, overlapping region of at least 21 nucleotides with 33% GC content resulted in successful fusion of 516 bp IFNα2b gene to 785 bp LAP gene with insertion of 30 nucleotide HCV NS3 protease cleavage site sequence to develop 1.280 kb chimeric gene product (IFNα2b-NS3-LAP) (Figs. 3, 4C). Purity of template, concentration of template, annealing temperature of overlapping region and number of nucleotides in overlapping region are important factors at 2nd step that

affect the efficient overlapping and further success of procedure. Long overlapping sequences can be selected but as the length increase, the chances for the presence of palindromic sequences may also increase that can reduce the efficient overlapping by developing the secondary structures (Cha-aim *et al*., 2009). High GC content in overlapping region improves base pairing during annealing step. In this study efficient base pairing with as minimum as 33% GC content in overlapping region resulted in successful fusion of genes. In case of overlapping regions derived from native sequences, GC content of the overlapping region can be increased by using the narrow window of codon redundancy *i.e.* by selecting the codons of required amino acid with more G/C contents. The same strategy can also be applied to avoid palindromic sequences if present in overlapping region without any change in the amino acid sequence of protein.

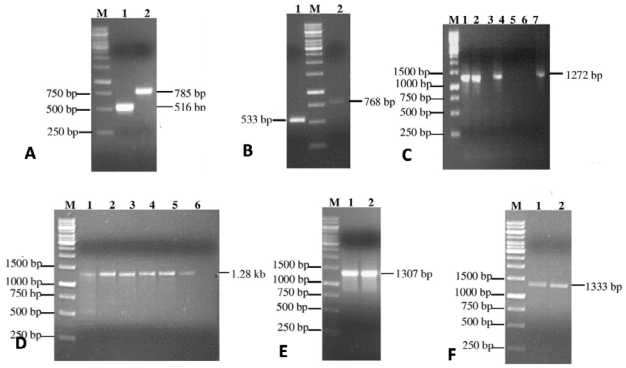

Fig. 4. Analysis of PCR products by 1% agarose gel electrophoresis. Gels were visualized by staining with ethidium bromide; **A**, Gel purified PCR products of steps # 1 of SOE-PCR. Lane M: DNA size marker, Lane 1: IFNα2b gene (516 bp) amplified using primer pair F1 and F221, Lane 2: LAP gene (785 bp) amplified using primer pair F3 and F4; **B**, Gel purified PCR products of steps # 1 of SOE-PCR . Lane M: DNA size marker, Lane 1: IFNα2b gene (533 bp) amplified using primer pair F3/ and F4/, Lane 2: LAP gene (768 bp) amplified using primer pair F1/ and F2/21; **C**, Optimization of annealing temperature (44-60°C) in first 10 cycles of step # 2 of SOE-PCR during which 30 bp overhang (HCV NS3 protease cleavage site sequence) at the 3' end of IFNα2b gene (516 bp) overlap by 21 bp with 5' end overhang of LAP gene (774 bp). In later 30 cycles, 58 °C annealing temperature was used with primer pair F1 and F4 to get 1.28 kb chimeric gene product. Lane M: DNA size marker, Annealing temperatures in Lane 1: 50 °C, Lane 2: 55 °C, Lane 3: 60 °C, Lane 4: 58 °C, Lane 5: 48 °C, Lane 6: 44 °C and in Lane 7: 58 °C; **D**, Optimization of minimum required equimolar concentration of genes for SOE-PCR. Lane M: DNA size marker, PCR product using equimolar concentration in Lane 1: 0.80 pmoles, Lane 2: 0.20 pmoles, Lane 3: 0.10 pmoles, Lane 4: 0.05 pmoles, Lane 5: 0.025 pmoles, Lane 6: 0.0125 pmoles and Lane 7: 0.00625 pmoles; **E**, 1st step of OPW-PCR with F5/F4 and F5//F4/ primer using chimeric genes IFNα2b-NS3-LAP and LAP-NS3-IFNα2b respectively introduced enterikinase cleavage site and Gly-Ser spacer at 5' end; **F**, 2nd round of OPW-PCR with F6/F4 and F6/F4/ primer using PCR products of 1st round of OPW-PCR as templates to introduce His-Tag sequence and XhoI cleavage site.

The annealing temperature of first 10 cycles was also optimized to ensure efficient and accurate overlapping. Although the calculated annealing temperature of the overlapping region was 44 °C, annealing at 58 °C showed a clear sharp band of chimeric gene product. Annealing temperatures < 58 °C resulted in non-specific amplifications as wells while no amplification at all at annealing temperatures > 58 °C (Fig. 4C). The results corroborates with the studies of Heckman and Pease (2007) and Cha-aim *et al*. (2009) who reported that higher annealing temperatures increase the efficiency and accuracy of the PCR while low annealing temperatures result in amplification of artifact products. It was also observed that increase in annealing time at high annealing temperature further improves the chances of precise annealing. It is important to mention that increase in annealing time, from 30 sec to 2 min, was effective in getting the specific PCR product (Fig. 4C). Wurch *et al*. (1998) extended annealing time to 25 minutes to improve overlapping between 24 bp region but in our study 2 min annealing time was sufficient for successful overlapping of 21 bp region. To further improve the efficiency of SOE-PCR, minimum equimolar concentration of genes required for fusion was also optimized. With overlap of 21 nucleotides, 1.280 kb chimeric gene was successfully amplified by decreasing the equimolar concentration of genes to 0.0125 pmoles. Below this concentration, no amplification was observed (Fig. 4D). Hence, it is established that minimum 0.0125 pmoles of each gene are required to perform SOE-PCR regardless of how much is available. After 10 cycles, non-chimeric primers (F1/F4) were added in reaction vials and chimeric product (1.280 kb) was successfully amplified.

In parallel experiment, for development of chimeric gene product in opposite direction (LAP-NS3-IFNα2b), same procedure and conditions were followed except amplification of LAP and IFNα2b genes with F1/F2/ and F3/F4/ primers respectively in 1st step while F1/F4/ primer pair in second step of SOE-PCR (Fig. 4B).[21]

Additional sequences at 5' end of chimeric genes were introduced by overlap primer walk PCR (OPW-PCR). In 1st round of PCR, F5/F4 and F5/F4/ primer pairs while in 2nd round of PCR F6/F4 and F6/F4/ primer pairs were used for IFNα2b-NS3-LAP and LAP-NS3-IFNα2b chimeric

genes, respectively to introduce enterokinase cleavage site (DDDDK), glycine-serine spacer, six amino acid His-Tag, two amino acid Kex2 protease cleavage site and *XhoI* restriction enzyme site in stepwise manner (Fig. 4E, F). Primer F5 and F5/ had 20 nts and 24 nts region respectively complementary to the templates along with 5' overhangs of 27 nts that share overlapping region of 21 nts with F6 plasmid. F4 and F4/ primers introduced *XbaI* restriction enzyme site at 3' end of chimeric genes. Chimeric genes, restricted with *XbaI* and *XhoI* restriction enzymes were ligated with the linearized pPICZαA plasmid restricted with the same enzymes and the recombinant plasmids were cloned in *E. coli* DH5α cells.

CONCLUSIONS

Using overlap extension PCR, a novel chimeric gene was constructed in both orientations (IFNα2b-NS3-LAP and LAP-NS3-IFNα2b) with insertion of HCV NS3 protease cleavage site by the overlap of only 21 bp (33% GC content) using as low as 0.0125 pmoles of each template. In addition, by overlap primer walk PCR, 45 nucleotide sequence was introduced at 5' end of chimeric genes. Using 2 steps of SOE-PCR and primer walk PCR two novel chimeric genes can be constructed and cloned just within one week. The accuracy and successful splicing the genes with insertion of desired sequences depends on: 1) the precise designing of overlapping primers, 2) purification of SOE-PCR product of step #1, 3) minimal required equimolar concentration of templates, 4) use of DNA polymerase high fidelity, 5) length of overlapping region between splicing genes, 6) GC content of overlapping region and 7) annealing temperature of overlapping region. Keeping these parameters in mind, the simple technique of SOE-PCR can be used with high rate of success to make versatile scarless chimeric genes along with insertional mutagenesis to introduce any sequence of choice and length at desirable position.

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Statement of conflict of interest

The authors declare that they have no competing interests.

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