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Dual Promoter Vector Construction for Simultaneous Gene Expression Using Spliced Overlap Extension by Polymerase Chain Reaction (SOE-PCR) Technique

Research Article

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ABSTRACT

There are two different co-expression systems including bicistronic; dual-vector or two-promoter to express two different genes simultaneously and also to study protein-protein interactions. Bicistronic system has disadvantages e.g. compared with two-promoter system. In this paper, a simple method based on spliced overlap extension by polymerase chain reaction (SOE-PCR) technique was demonstrated for construction of two-promoter vector to express two genes equally. To construct two-promoter vector, two pairs of mega-primer containing enzymatic restriction sites, T7 promoter, Lac operator and ribosome binding site (RBS) sequences were used in SOE-PCR. The constructed vector can be used in order to co-expression of other genes properly in a variety of bacterial expression hosts.

KEY WORDS co-expression, protein-protein interactions, SOE-PCR, two-promoter vector.

INTRODUCTION

Bicistronic and dual-vector are two commonly used systems for co-expression of two heterologous partner proteins (Kim *et al.* 2004). This system provides wealth information about protein-protein interaction analysis (Scheich *et al.* 2007). The bicistronic vector consists of a cluster of two genes under a single promoter. In this system, the expression of the second gene usually is much less than compared to the first gene (Rucker *et al.* 1997). The dual-vector system as another multiple protein expression system, may overcome this problem. In this system two target genes are co-expressed from two separate constructs (Dzivenu *et al.* 2004). However, the dominance of one vector over the another in the copy number and using vectors with the same compatible origin of replication are two main limitation associated with this method (Johnston *et al.* 2000). In this regards, these problems could be resolved by simply adding a promoter sequence in the front of the second target gene on a bicistronic vector, so the second target gene can be generated independently. Moreover, due to the two-promoter vector system is based on a single vector; it is also free of the major problem associated with the dual-vector system (Rucker *et al.* 1997; Kim *et al.* 2004). Brucellosis, also known as undulant fever or Mediterranean fever, is caused by Brucella bacteria and is still one of the major, highly transmissible and zoonotic diseases affecting people and animals. Economic and medical consequences of brucellosis have led to efforts to prevent the infection through the use of vaccines (Pappas *et al.* 2005). It is demonstrated that Omp25 and Omp31 are main antigens that causing the immune response. Moreover, these antigens

were found to be the most exposed outer membrane proteins (OMPs). Also, there are several reports which have shown that DNA vaccine and recombinant protein vaccine of Omp25 and Omp31 are protective against the virulent challenge of Brucella species in mice (Cassataro *et al.* 2005; Commander *et al.* 2007).

In this paper we described a simple method for construction of two-promoter vector which can be used for co-expression of two main *Brucella melitensis* antigens in order to study their protein-protein interaction. The spliced overlap extension by polymerase chain reaction (SOE-PCR) (Higuchi *et al.* 1988) was used for construction of the two-promoter vector containing all DNA elements for co-expression and protein purification. SOE-PCR is a suitable method in genetic engineering particularly in the multiple-site directed mutagenesis.

These approaches require fragment amplifications, overlap extensions, and purification of intermediate PCR products (Kumar *et al.* 2008).

MATERIALS AND METHODS

Primer design

Two pairs of megaprimers were designed for SOE-PCR reaction by Primer Premier 5 software. All DNA elements which were considered in megaprimers sequence were showed in Table1. These primers were designed based on amplification of two different sites of pET-32a (+) vector's sequence (Novagen, CA, USA).

Construction of two-promoter vector

Polymerase chain reaction (PCR)

Construction of two-promoter vector was carried out in five steps (Figure 1a). In the first step, two different fragments of PCR products were amplified by four megaprimers from pET-32a (+) vector as a template in PCR reactions.

Polymerase chain reaction (PCR) program was performed using the Personal Cycler™ thermo cycler (Biometra, Germany) with initial denaturation at 94 ˚C for 5 min followed by 22 cycles of denaturation, annealing and extension for 30 sec at 94 ˚C, 30 sec at 56 ˚C and 30 sec at 72 ˚C, respectively. Finally, an additional post-cycle extension was carried out for 10 min at 72 ˚C. The total volume of PCR reaction was 25 µL with the reaction mixture containing 2.5 µL of 10X PCR buffer, 2 µL $MgCl_2$ (50 m*M*), 2 µL dNTPs (2.5 pmol/µL), 0.3 µL of pET-32a (+) vector (50 to 100 ng/µL), 1.5 µL of mix primer (5 pmol/µL), 0.125 U/µL of *EX Taq* DNA polymerase (Takara, Japan) and deionised water up to 25 µL reaction volume.

The second step was "SOEing-assembly" that splicing PCR products were annealed together through their 58 bp shared overlapping fragments (Figure 1a, step 2).

P1 and P2 were used as templates in the PCR reaction mixture without primers. PCR components were considered like the first step. The PCR temperature program was initial denaturation for 5 min at 94 ˚C, 15 cycles of denaturation for 30 sec at 94 ˚C, annealing for 30 sec at 60 ˚C and extension for 30 sec at 72 ˚C. Final extension was performed for 5 min at 72 ˚C.

The last step of SOE-PCR was "PCR amplification" by TP-F1 and TP-R2 megaprimers and the full-length of fragment (TP) (Figure 1a, step 3). The PCR programs was carried out in 25 cycles of denaturation for 30 sec at 94 ˚C, annealing for 30 sec at 56 ˚C and extension for 30 sec at 72 ˚C. The initial denaturation and final extension were 5 min at 94 ˚C and 72 ˚C, respectively. The mixture of PCR containing 2.5 µL of 10X PCR buffer, 2 µL $MgCl_2$, 2 µL dNTPs, 0.5 µL of the second PCR reaction mix, 1 µL of mix primers (5 pmol/µL), 0.125 U/µL of *EX Taq* DNA polymerase (Takara, Japan) and deionised water up to 25 µL reaction volume.

T/A cloning and vector construction

The final full-length PCR product containing all considered elements (named TP) was purified by Ron`s Agarose Gel Mini prep Kit (BioRon, Germany) and then was cloned into pTZ57R/T cloning vector by Ins T/A clone™ PCR Product Cloning Kit (Thermo, USA) according to manufacturer's recommendations. The recombinant PTZ-TP plasmid (Figure 1a, step 4) was amplified in DH5α strain of *E. coli* (Invitrogen, USA) and sequenced. The PTZ-TP plasmid was digested by *NcoI* and *XhoI* (Thermo, USA) and then ligated with *NcoI* and *XhoI* digested pET-32a (+) expression vector using DNA ligation kit according to manufacturer's protocol. This recombinant vector was named pET-TP and used as a basic two-promoter vector (Figure 1a, step 5). After transformation, the positive colonies were confirmed by colony PCR using T7 universal primers, restriction endonuclease digestion and sequencing.

Construction of two-promoter vector containing Omp25 and Omp31 genes

In order to construction of two-promoter vector with ability to express simultaneously two main *Brucella melitensis* antigens, we used PTZ-Omp25 and PTZ-Omp31 vectors. These vectors were previously constructed in our lab and contain *Brucella melitensis*'s outer membrane protein 25 and 31, respectively. Briefly, two pairs of flanking primers with appropriate restriction site were designed to amplify Omp25 and 31 genes from *Brucella melitensis* Rev1 genomic DNA. The PCR products were separately cloned into pTZ57R/T vector. These constructs were digested using *NcoI/EcoRI* and *SacI/BamHI* to remove Omp25 and Omp31 open reading frame (ORF), respectively.

Table 1 The sequence of megaprimers

Primer name	Primer sequences (5´-3´)*	Length (bp)	PCR product (bp)	DNA element site (5´-3´)**
TP-F1	GTCGACCCATGGGATCGGCCTGTCGCTTGC	30		*SalI*, *NcoI*
TP-R1	GAGCTCCGCGGCCGCATGTA**TAT**CTCCTTCT TAAAGTTAAACAAAATTATTTCTAGAGGAAT TGTTATCCGCTCACAATTCCTATAGTGAGTC GTATTAAAGCTTGAATTCTATCGGTGATTCA TTCTGC	130	331	*EcoRI*, *HindIII*, stop codon, T7, Lac operator, RBS, *NotI*, *SacI*
TP-F2	CTCTAGAAATAATTTTGTTTAACTTTAAGAA GGAGATATACATGCGGCCGCGGAGCTCCACT GCGATGCTGGTTGC	76	440	RBS, *NotI*, *SacI*
TP-R2	CTCGAGCTTGTCGTCGTCGTCGATATCGGAT CCTCGGGAAACCTGTCGTGC	51		*BamHI*, *EcoRV*, enterokinase site, *XhoI*

* The linker sequence was highlighted in the megaprimers. The underlined sequence was annealed to pET-32a (+) vector in PCR reaction.
** Enzymatic restriction sites (*SalI*, *EncoI*, *EcoRI*, *HindIII*, *NotI*, *SacI*, *BamHI*, *EcoRV* and *XhoI*)
Stop codon: TAT
Lac operator sequence: AATTGTTATCCGCTCACAATTC.
Ribosomal binding site (RBS): GAAGGAGA.
Enterokinase site: GACGACGACGACAAG.

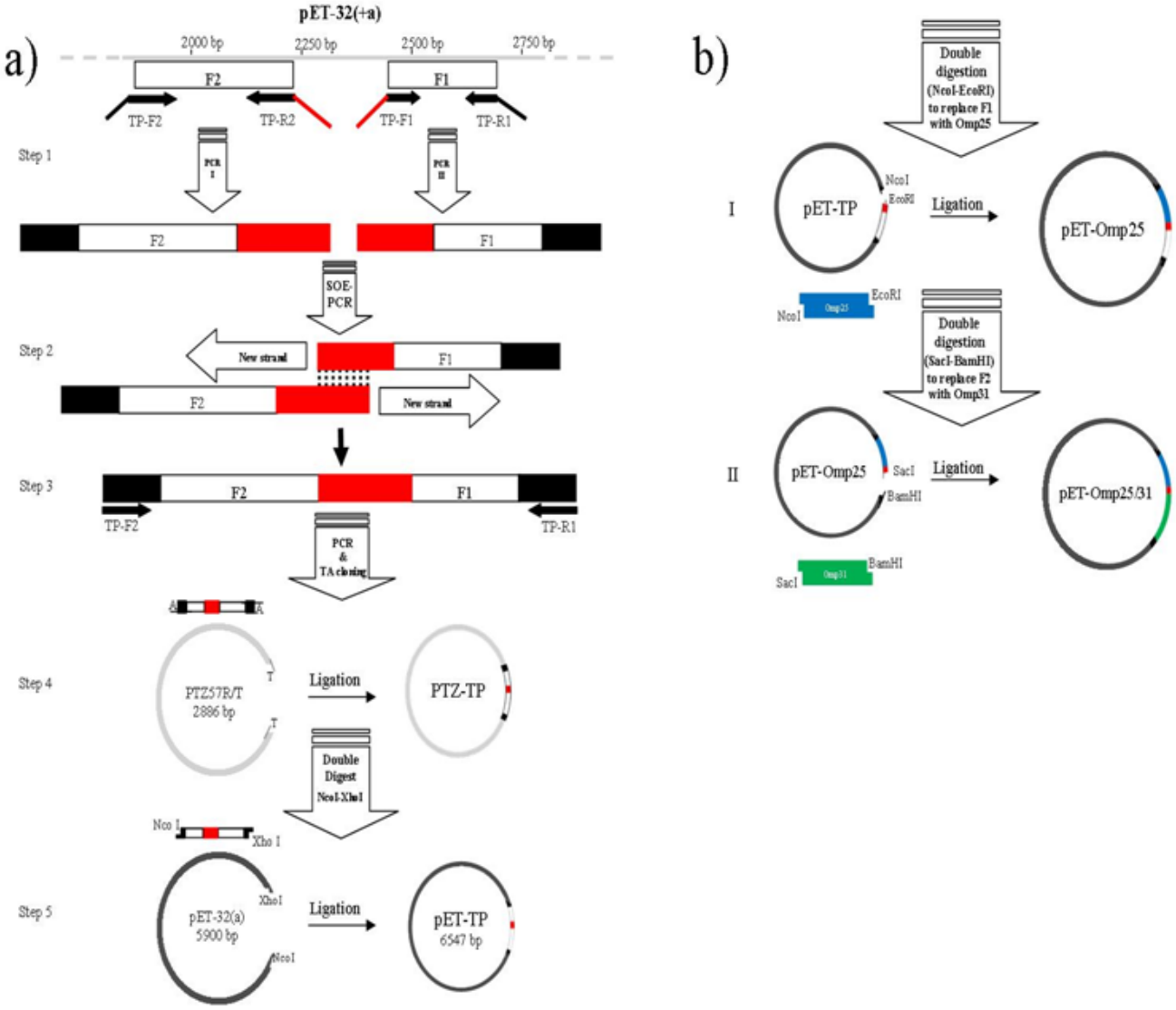

Figure 1 **a)** the schematic picture of different SOE-PCR steps and two-promoter vector construction; **b)** insertion of Omp25 and Omp31 antigens in pET-TP vector

The excised fragments were purified from agarose gel by Ron`s Agarose Gel Mini prep Kit (BioRon, Germany). The pET-TP vector was digested with *NcoI/EcoRI* to remove Fragment 1 (F1) and relegated with excised *NcoI/EcoRI* excised Omp25 ORF (this recombinant vector named pET-Omp25) (Figure 1b, part I). One positive DH5α clone was selected using colony PCR. The second Fragment (F2) was removed from pET-Omp25 using *SacI/BamHI* and relegated by *SacI/BamHI* excised Omp31 ORF. The positive selected recombinant vector named pET-Omp25/31 (Figure 1b, part II). The presence of DNA elements and Omp25/31 ORF was confirmed by restriction endonuclease analysis.

RESULTS AND DISCUSSION

Construction of two-promoter vector

The TP fragment sequence contains F1 fragment flanked with appropriate restriction enzyme sites, lac operator, T7 promoter, RBS, F2 fragment flanked with appropriated restriction enzyme sites and enterokinase sequence (Figure 2a) was successfully amplified in SOE–PCR reaction (Figure 3a). This SOE-PCR product (713 bp) was confirmed by sequencing (Figure 2b) and restriction mapping (Figure 3b). The sequencing results did not show any mutation in the TP fragment (Figure 2c).

TP Fragment was successfully cloned in pET-32a (+) expression vector. The promoter and lac operator of pET-32a (+) were located upstream of F1 fragment (Figure 4). The restriction mapping analysis showed that pET-TP recombinant vector could be used for replacing any gene of interest by F1 and F2 fragment (Figure 5).

Construction of expression vector

The F1 and F2 sequence were successfully replaced by Omp25 and Omp31 ORF, respectively. Colony PCR amplified expected fragment size (2161 bp) form positive DH5α selected colony (Figure 6). The restriction endonuclease analysis of pET-Omp25/31 recombinant expression vector confirmed *EX Taq* DNA polymerase proof of reading and the process of cloning (Figure 5).

Two promoter system is a suitable alternative for bicistronic system and could be the first choice in order to perform structural and biochemical investigation through the producing protein complexes and protein-protein interaction (Kim *et al.* 2004; Kholod *et al.* 2001). By simply adding a promoter sequence in front of the second target gene on a bicistronic vector, the mRNA transcripts of the second target gene can be generated independently of the "read-through" transcript from the first promoter (Kim *et al.* 2004).

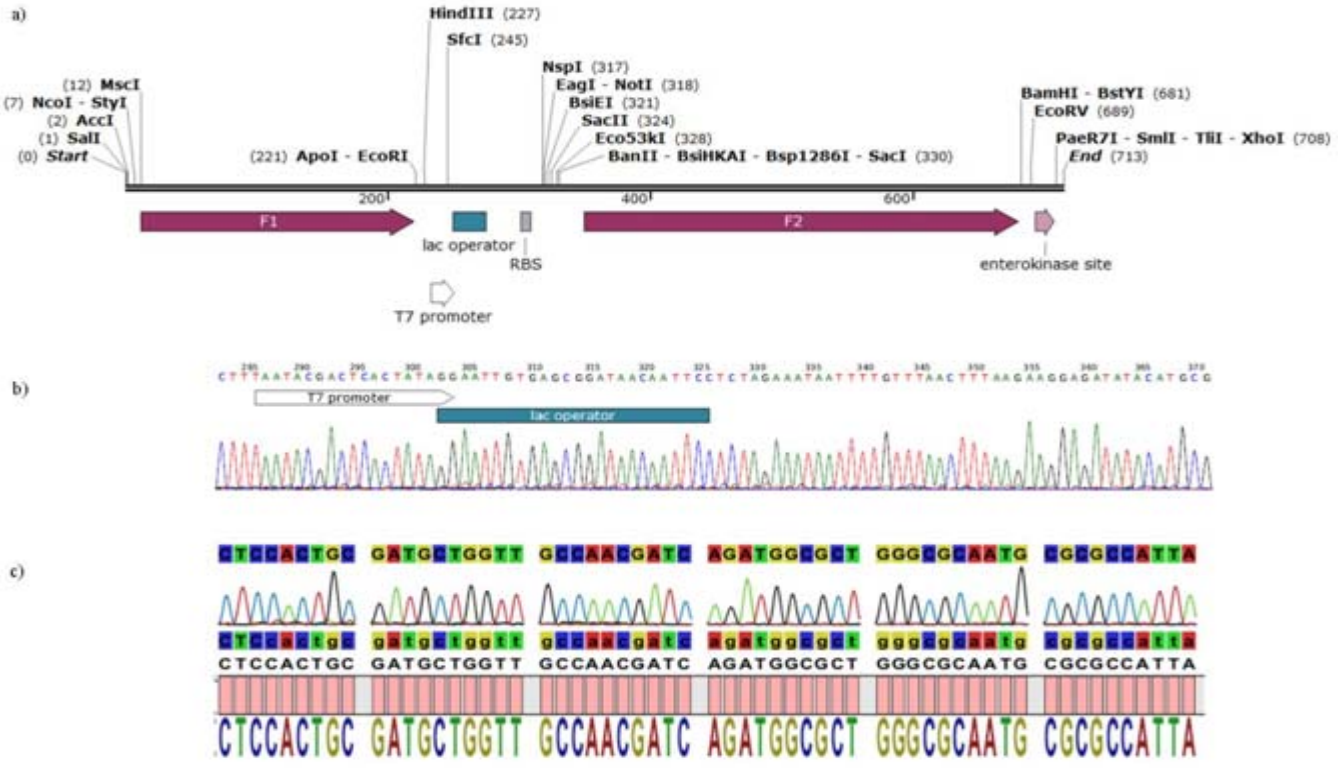

Figure 2 a) TP PCR product: different enzymatic restriction sites, lac operator sequence, ribosomal binding site (RBS), enterokinase site and T7 promoter were specified; **b)** the sequencing result of TP PCR product and **c)** the alignment of TP PCR product sequencing and expected sequence

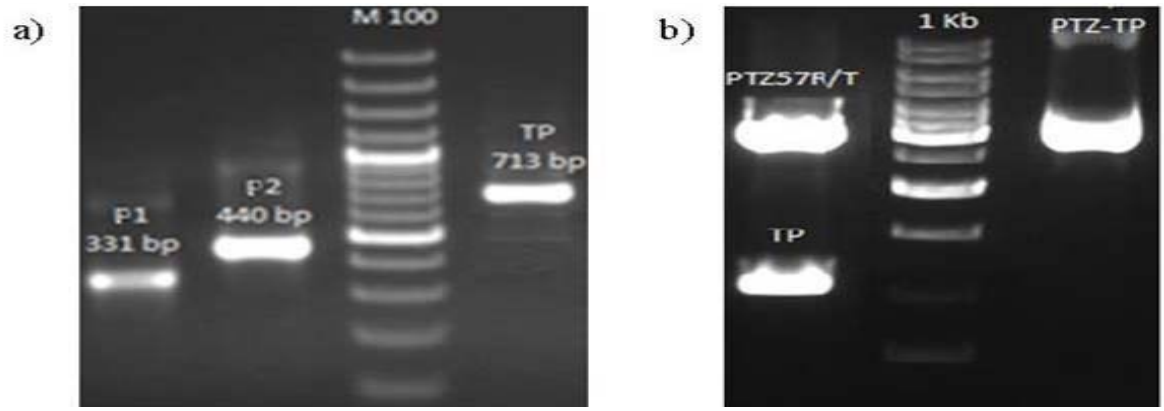

Figure 3 **a)** P1, P2 and TP products of SOE-PCR and **b)** undigested and double digested (with *NcoI*/*XhoI*) PTZ-TP

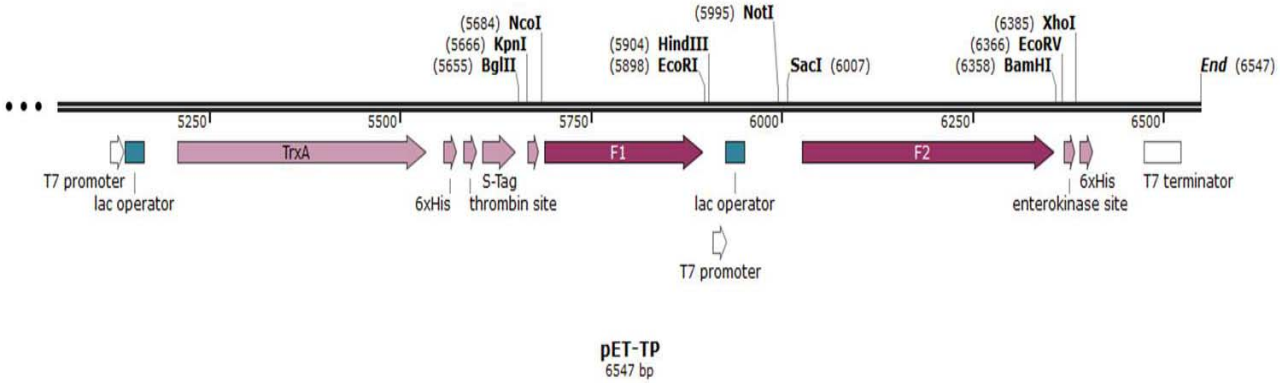

Figure 4 The schematic picture of pET-TP construct

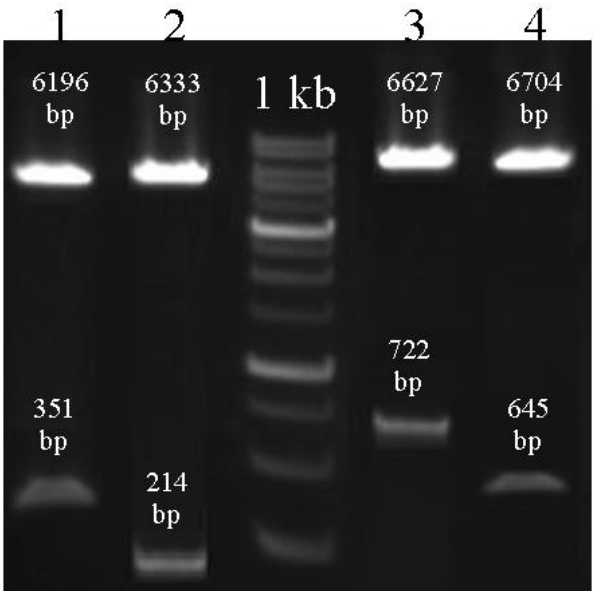

Figure 5 Line 1: double digestion of pET-TP vector with *SacI* and *BamHI* to extract F2 fragment; 2: double digestion of pET-TP vector with *NcoI* and *EcoRI* to extract F1 fragment; 3: double digestion of pET-Omp25/31 with *SacI* and *BamHI* to extract Omp31(723 bp) and 4: double digestion of pET-Omp25/31 with *NcoI* and *EcoRI* to extract Omp25 (642 bp)

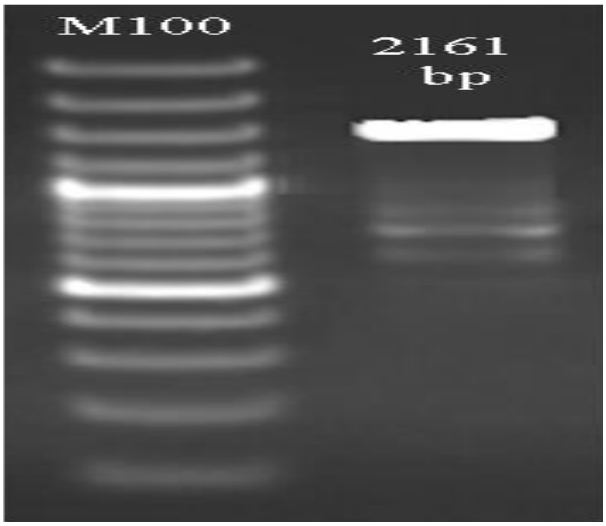

Figure 6 Colony PCR of pET-TP vector with T7 universal primers

Therefore, the resulting two-promoter vector should lead to an increased production of the protein encoded by the second target gene. Furthermore, because the two-promoter vector system is based on a single vector, it is free of the major problem associated with the dual-vector system (Rucker *et al.* 1997).

Several studies have shown that the use of multiple-promoter vectors increases the efficiency of co-expression and makes the better opportunity for protein interactions investigation (Scheich *et al*. 2007).

CONCLUSION

In this experiment, a two-promoter expression vector based on pET-32a (+) plasmid was successfully constructed by SOE-PCR approach. This vector has two versions of all DNA elements which are necessary for protein expression and purification in expression bacterial host systems (Figure 4). We designed appropriated restriction enzymatic sites flanks two different fragment of DNA which could be easily replaced by each ORF of interest. In the second step, we tried to examine the two-promoter vector (pET-TP) for replacing two main *Brucella melitensis* antigens by F1 and F2 fragments. The potency of pET-Omp25/31 vector for co-expression of these antigens has already initiated in our lab. Furthermore, pET-TP vector could be used for co-expressions of other genes in the variety bacterial expression hosts systems.

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