Construction of Double Gene Expression Vector

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Abstract- Recombinant vectors are valuable tools in the biopharmaceutical industry with a number of novel vectors being emerged every day. The aim of this project is to design a novel double gene bacterial expression vector where the two genes can be controlled individually. The double gene expression vector contains two independent transcriptional units. (a) The first transcriptional unit comprises sequences for the osmotic regulated promoter, restriction site for insertion of polypeptide and a transcription termination and (b) The second transcriptional unit comprises sequences for the T7 promoter, MCS for the insertion of polypeptide and a transcriptional termination sequence. The double gene expression vector (pUB-S-X-T7) was constructed by ligating the ClaI and PvuII fragment (4000bp) form the pUB-S-X plasmid and ClaI and ScaI fragment (1000bp) from pUB-T7 plasmid. The presence of two independent transcriptional units was confirmed by colony PCR.

Index Terms- PCR amplification, T end cloning vectors like pGEM-T ,expression vector ,pUB-S plasmid, Taq DNA Polymerase,pUB-S-X plasmid.

I. INTRODUCTION

Over the past few decades the rapid progress in biotechnology has made it possible to improve the expression of recombinant proteins. However, the current plasmid design still limits the controlled expression of multiple genes. Controlled expression of multiple genes is a useful approach for various purposes: 1. Selection of transgenic offspring with a marker gene, 2. Examination of relationship between two gene products. For eg. Many proteins exert their functions through Protein: Protein interactions. Three-dimensional structures of protein complexes usually provide a wealth of information regarding the action mechanism of constituent proteins. Protein complexes may be obtained by simply mixing individually purified proteins. However, expression of some proteins requires a binding protein for folding and stability, 3. Overcome the toxic effect of the recombinant protein on the host cell by co expression of the other second protein (Restriction modification system), 4. Genetic switches which permit the control of individual gene activities quantitatively and specifically will greatly facilitate the study of gene function in vivo.

Several strategies of multiple gene expression were tried in Bacteria, Mammalian and Plant cells (Table 1). However all these approaches suffer form advantages as well as disadvantages. Off these methods co-transformation with linked transgenes in single vector is a conventional and reliable approach in most of the cases. However, this approach is technically demanding. The lack of unique restriction cloning sites, loss of direct selection, as well as the relatively low efficiency of ligation of insert into larger vector. In order to overcome these problems a novel expression vector was developed with unique restriction sites and two different inducible promoters to control the expression of both the genes.

The current project is aimed at development of double gene bacterial expression vector with two independent regulated transcriptional units. The transcriptional unit I contains the (a) lac promoter, , (b) Multiple cloning site and rrnB/T1 and rrnB/T2 transcriptional terminators. The transcriptional unit II consists of (a) ProU promoter, an osmotic regulated promoter which expresses transgenes in the presence of high salt (Nacl) concentration, (b) Multiple cloning site (MCS) and (c) rrnB/T1 and rrnB/T2 transcriptional terminator. Cloning of BglII-Methylase under the contol of Lac promoter and BglII-Endonuclease under the control of ProU promoter the expression of both the genes can be controlled independently. Expression of Methylase ahead of endonuclease will protect t the host cell and facilitates the production of BglII restriction endonuclease

II. MATERIALS AND METHODS

METHODS

2.1: ISOLATION OF PLASMID USING MINI PREP KIT:

For the isolation of plasmid DNA single colony of bacteria was seeded in 10ml of LB media containing 100ug/ml Amp. Cultures were incubated in an orbital shaker (REMI) at 37°C and 200 rpm. Eighteen hours following incubation plasmid was isolated using UB-Plasmid Mini Prep Kit. Briefly, 1.5ml of culture was centrifuged at 13,000 rpm for 30 sec and media was discarded without disturbing the pellet. Bacterial pellet was resuspended with vortex in Solution 1 (100ul). Solution 2 (200ul) and Solution 3 (150ul) were then added to the cells suspension in sequence with gentle mixing of sample after each addition. A fluffy precipitate is formed. DNA binding buffer (450ul) was added to the lysate and centrifuged at 13,000 rpm for 30 sec. Clear lysate was transferred into a fresh eppendorf tube. DNA binding matrix (DBM) (10ul) was added to the clear lysate and incubated at room temperature with mixing. Samples were centrifuged at 13,000 rpm for 30sec and clear solution was discarded without disturbing the pellet. DBM was washed once with wash buffer (500ul) and centrifuged at 13000 rpm for 30 sec. Wash buffer was discarded without disturbing the pellet. DBM was resuspended in Elution buffer (50ul) and incubate at room temperature for 2min. Sample was centrifuged at 13000 rpm for 30 sec and elute was transferred in to a fresh eppendorf tube and stored at -20°C.

2.2: PURIFICATION OF PLASMID USING DESALTING KIT:

Restriction digestion, PCR samples were routinely purified using UB-Desalting Kit. Briefly, Equal volume of desalting buffer was added to the sample to be purified. DBM (10ul) was added to the sample and incubated at room temperature for 2min. Samples were centrifuged at 13000 rpm for 30 sec and clear solution was discarded without disturbing the pellet. DBM was washed with wash buffer (200ul) and centrifuged at 13000 rpm for 30 sec. Wash buffer was discarded without disturbing the pellet. DBM was resuspended in elution buffer (50ul) and incubated at room temperature for 2 min and then centrifuged at 13000 rpm for 30 sec. Elutes were collected in a fresh eppendorf tube and stored at -20°C.

2.3:GEL EXTRACTION:

DNA fragments separated on 1-2% agarose gels were purified using UB-Gel extraction kit. Briefly, the desired band was excised form the gel using scalpel and placed in an 1.5ml micro centrifuge tube. 3 volumes (300ul/100mg gel) of DNA binding buffer (G1) was added to the gel and incubated at 37°C for 5 min. Once the gel is completely melted DBM (10ul) was added to the sample and incubated at room temperature for 2min. Samples were centrifuged at 13000 rpm for 30 sec and G1 was discarded without disturbing the pellet. DBM was washed once with wash buffer (500ul) and centrifuged at 13000 rpm for 30 sec. Wash buffer was discarded and DBM was resuspended in 20-50ul of elution buffer and incubated at room temperature for 2 min. Samples were centrifuged at 13000 rpm for 30 sec and elute was transferred into a fresh microfuge tube and samples stored at -20°C.

2.4: RESTRICTION DIGESTION:

Restriction digestion was setup according to the manufacturers specifications. The usual reaction setup was given below.

Table 2.4: Restriction Digestion Reaction Set-up

Reaction components	Reaction volumes (20ul reaction)
10 x reaction buffer	2ul
Template DNA	5ul
Enzyme	1ul
H_2O	12ul

2.5:LIGATION:

50-100ng of DNA was routinely used to ligate with 1-5 U of T4-DNA ligase in 10 µl reaction volume under the reaction conditions specified by the manufacturer. The ratio of vector to insert was maintained between 1:3 and 1:5 for efficient ligation of insert with the vector. The reaction was routinely carried at 4˚C overnight in 10 µl reaction volume in 1.5 ml Eppendorf tube. Ligated samples were transformed in to JM109 (Promega Corporation, Madison, WI) as described

2.6:TRANSFORMATION:

Routinely, 2.5ul of DNA samples were transformed into 50ul of calcium chloride competent cells by heat shock method. Briefly, competent cells were quickly thawed in the palm of the hand. 2.5 ul of DNA was added to 50ul of competent cells and incubated on ice for 30 min. Following ice incubation cells were heat shocked at 42 °C for 90 sec and iced for 2 min. LB Media (800ul) was added to the cells and incubated at 37°C for 40min and 200ul was plated on the LB/Amp plate.

2.7:Colony-PCR:

Colony-PCR on recombinant clones was carried out according to the protocols given by sambrook and russle (Sambrok russel). Reaction was often carried out in 20ul reaction volumes using a single bacterial colony as a template, 50 pmol of O-X-Scr primers in the presence of 200mM dNTPs, 2mM $MgCl_2$ and 1 x concentration of PCR buffer and 1u of Taq. The reaction was carried out in a 0.2 ul PCR tube in a gradient PCR. The PCR program was as shown in (Table 4-3). Following PCR amplification PCR samples were separated on 1-2% agarose gel in TAE buffer (pH-8).

Table2.7: PCR Program For Colony PCR

Step 1	94°C	2 min
Step 2	94°C	1 min
Step 3	55°C	1 min
Step 4	72°C	1 min
Step 5	Repeat Step-2 to 4 (30 cycles)	
Step 6	72°C	10 min
Step 7	Hold 4°C	

2.8:AGAROSE GEL ELECTROPHORESIS:

DNA separation was routinely done in 0.8 to 1% agarose gel in 1 X TAE electrophoresis buffer pH 8.3 (2 mM Tris-Acetate/0.05M EDTA). Agarose gels were cast in 1 X TAE buffer containing 0.5 µg /ml of ethidium bromide. DNA samples were mixed with 1/6 volume of 6 X loading dye (Usha Biotech Pvt Ltd, Hyderabad, AP, India) and subjected to electrophoresis under controlled voltage of 5V/cm. Appropriate DNA size markers (1 kb or 100 bp DNA ladder) were run alongside the samples to estimate the size of DNA fragments. The DNA was visualized in an UV transilluminator and gel documentation system (syngene).

III. RESULTS

3.1:Isolation of pUB-S-X and pUB-T plasmids:

pUB-S-X (Figure 5-6) and pUB-T7 (Figure 5-2) were isolated using Ultra Pure Plasmid Purification kit (Usha Biotech) using the method described in section (4.2.1). Plasmid was eluted in 50ul of elution buffer. Two micro litres of each plasmid was run on 1% agarose gel in TAE buffer (Method 4.2.8). The plasmids were found to be of good quality which was represented by the appearance of plasmid with out any shearing (Figure 5-1).

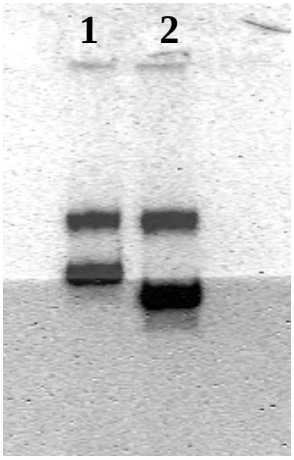


Figure3.2: Analysis of Mini-Prep Plasmid: Lane-1 (pUB-S-X) and Lane-2 (pUB-T7)

3.3:Isolation of T7-MCS-Termination sequences from pUB-T7:

pUB-T7 (Figure 5-2) is a bacterial expression plasmids. The recombinant gene that were cloned in this plasmid were under the control of a T7 RNA polymerase promoter. The induction of these genes occur in the presence of T7 RNA polymerase. For the construction of double gene vector T7 Promoter-MCS-rrnB/T1 and rrnB/T2 were isolated by sequential digestion of pUB-T7 Plasmids using CalI and ScaI enzymes. pUB-T7 was 1st digested with CalI as described in the method (4.2.4), the reaction was setup as shown in (Table 5-1). Two micro litres of the digested sample was analysed on 1% agarose gel as shown in method (4.2.8).

The presence of single linear band at 4.7 kb in Figure 5-3 indicated that ClaI digestion is complete. The digested plasmid was purified using the method described in (4.2.2) and was eluted in 10ul of double distilled water.

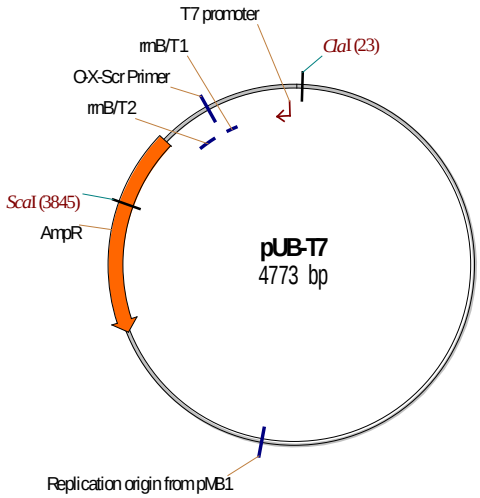


Figure3.4: pUB-T7 Plasmid Map

Table3.4: CalI digestion of pUB-T7

Components	20ul reaction
pUB-T7	10ul
10 x Buffer (NEB-3)	2ul
CalI (2u/ul)	1ul
H_2O	7ul

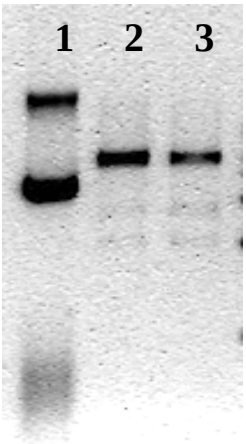
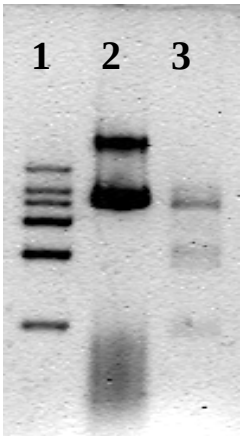


Figure3.5: Restriction digestion of pUB-T7 using ClaI: Lane-1 (Supercoiled pUB-T7), Lane-2 and 3 (2ul of digest) and Lane-4 (1kb ladder).

pUB-T7-CalI was digested with ScaI as described in the methods (4.2.4), the reaction was setup as shown in (Table 5-2). Double digested pUB-T7 CalI-ScaI was separated on 1% agarose gel as described in the method (4.2.8). The presence of multiple bands in (Figure 5-4) indicated the ScaI digestion was complete. 1000 bp fragment in (Figure 5-4) was gel extracted according to the method described in (4.2.3). The gel extracted samples was analysed on 1% gel as described in (4.2.8). A light band in Figure 5-5) indicated the presence of 1000bp fragment in gel extracted fragment.

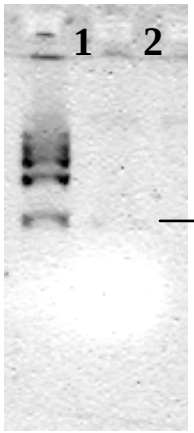
Table3.6: ScaI digestion of pUB-T7-CalI

Components	20ul reaction
pUB-T7	10ul
10 x Buffer (NEB-3)	2ul
ScaI (2u/ul)	1ul
H_2O	7ul



1000 bp fragment

Figure3.7: Restriction digestion of pUB-T7-CalI using ScaI: Lane-1 (1kb ladder), Lane-2 (supercoiled pUB-T7) and Lane-3 (Double digested pUB-T7).



1000 bp fragment

Figure3.8: Analysis of Gel Extracted 1000 bp ClaI-PvuII Fragment

3.9: Isolation of pUB-S-X back bone:

pUB-S-X (Figure 5-6) is a bacterial expression plasmids. The recombinant gene that were cloned in this plasmid were under the control of a ProU an osmotic regulated promoter. The promoter gets activated in the presence of increasing concentrations of NaCl. For the construction of double gene vector pUB-S-X was used as a back bone which carries the complete transcriptional unit (ProU-X-G-X- rrnB/T1 and rrnB/T2) for salt inducible expression and slectable marker gene (Ampicillin) and bacterial origin of replication (ColE1 Ori). The back bone was isolated by digesting pUB-S-X Plasmids using CalI and PvuII enzymes. pUB-S-X was 1st digested with CalI as described in the method (4.2.4), the reaction was setup as shown in (Table 5-3). Two micro litres of the digested sample was analysed on 1% agarose gel as shown in method (4.2.8).

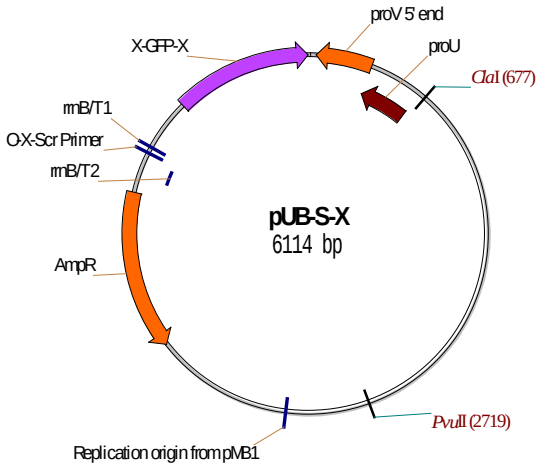


Figure3.10: pUB-S-X Plasmid Map

Table 3.11: CalI digestion of pUB-S-X plasmid

Components	20ul reaction
pUB-T7	10ul
10 x Buffer (NEB-3)	2ul
CalI (2u/ul)	1ul
H_2O	7ul

The presence of single linear band at 6.1 kb in (Figure 5-7) indicated that ClaI digestion is complete. The digested plasmid was purified using the method described in (4.2.2) and was eluted in 10ul of double distilled water.

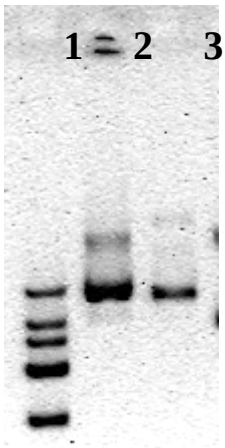


Figure 3.12: Restirction digestion of pUB-S-X using ClaI: Lane-1 (1kb ladder), Lane-2 (supercoiled pUB-S-X) and Lane-3 (CalI digested pUB-S-X)

Following digestion of pUB-S-X with Cl ClaI. The linear plasmid was digested with PvuII as described in the methods (4.2.4), the reaction was setup as shown in (Table 5-4). Double digested pUB-S-X CalI-PvuII was separated on 1% agarose gel as described in the method (4.2.8). The presence of multiple bands in (Figure 5-4) indicated the PvuII digestion was complete. 4000 bp fragment in (Figure 5-8) was gel extracted according to

the method described in (4.2.3), DNA was eluted in 10ul of H_2O. The gel extracted samples was analysed on 1% gel as described in (4.2.8). The presence of band at 4 kb in Figure 5-9 indicated the presence of pUB-S-X CalI-PvuII plasmid in gel extracted sample.

Table 3.13: PvuII digestion of pUB-S-X-CalI

Components	20ul reaction
pUB-T7	10ul
10 x Buffer (NEB-3)	2ul
PvuII (2u/ul)	1ul
H_2O	7ul

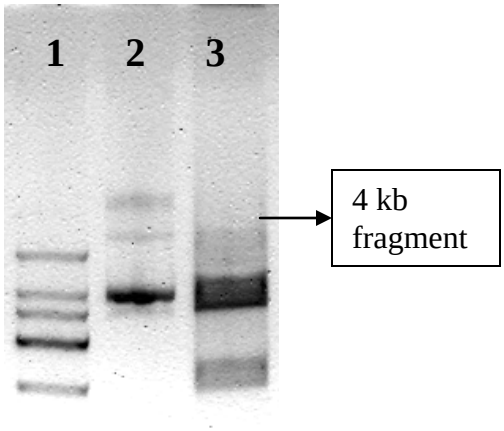


Figure 3.14: Restriction Digestion of pUB-S-X-CalI with PvuII: Lane-1 (1kb ladder), Lane-2 (supercoiled pUB-S-X) and Lane-3 (CalI and PvuII digested pUB-S-X plasmid)

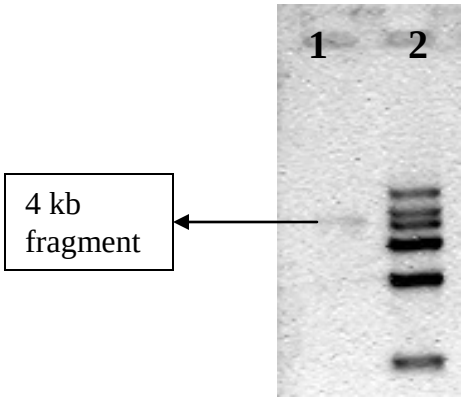


Figure 3.15: Analysis of Gel Extracted 4000 bp CalI-PvuII Fragment: Lane-1 (gel extracted pUB-S-X CalI-PvuII fragement) and Lane-2 (1kb ladder)

Ligation of pUB-S-X (PvuII/CalI) and T7-MCS-Termination sequences (CalI/ScaI) fragments:

pUB-C-X (ClaI-PvuII) fragment and T7 transcriptional unit (T7-MCS- rrnB/T1 and rrnB/T2) were ligated according to the method described in (4.2.5). The ligation reactions were setup as shown in

Table 3.16: Ligation reaction

Sample No.	**Back bone vector (pUB-S-X-CalI-PvuII)**	**Insert (T7-MCS-rrnB/T1 and rrnB/T2)**	**T4 Buffer**	**T4 enzyme**	**H_2O**
1	**2 ul**	**6 ul**	**1ul**	**1ul**	**-**
2	**2 ul**	**-**	**1ul**	**1ul**	**6ul**

Transformation of ligated samples in to JM109 competent cells:

2.5ul of ligated samples in Table 5-5 were transformed into 50 ul of JM109 competent cells. Transformation was carried out as described in (4.2.6). Following transformation 200 ul of transformed culture was plated on LB-Amp plates and incubated overnight at 37°C. Eighteen hours following incubation at 37°C, 3 colonies were observed in LB-Amp plate with Sample-1 in (Table 5-5). The transformation observations were shown in

Table 3.17: No. of surviving colonies folloing transformation in JM109 cells uing the ligated samples.

Ligated sample	No. of colonies
1	3
2	0

Analysis of pUB-S-X-T7 clones using colony PCR:

pUB-S-X-T7 has got two rrnB/T1 and rrnB/T2 sequences one on the sense stand and the other on the antisense stand. Prime O-X-Src (Table 4-1) was designed to anneal to the rrnBT1 and rrnB/T2 sequence. Use of this primer to amplify pUB-S-X-T7 DNA will give a band at 2300 bp. However, in pUB-S-X and pUB-T7 this prime does not amplify. The three clones in the (Table 5-6) were analysed by O-X-Src primer using the method described in (4.2.7). PCR master mix was prepared as shown in (Table 5-7) and 20 ul was aliquoted in to tube 1-6 shown in (Table 5-8).

Table 3.18: PCR master mix

Components	For 7 reactions
10 x PCR buffer	14 ul
10mM dNTP mix	5.6 ul
25mM $MgCl_2$	11.2 ul
O-X-Scr Primer (1:20 diluted)	14 ul
Taq (3u/ul)	2.5 ul
H_2O	92.7 ul
Total	140 ul

Table 3.19: PCR Reactions

Sample no.	Master mix	Template
1	20 ul	A pinch of bacterial colony (Clone 1)
2	20 ul	A pinch of bacterial

		colony (Clone 2)
3	20 ul	A pinch of bacterial colony (Clone 3)
4	20 ul	1ul of pUB-S-X
5	20 ul	1ul of pUB-T7
6	20 ul	-ve

Following PCR amplification 5ul of each PCR sample was analyzed on 1% agarose gel in TAE buffer as described in (4.2.8). Sample 1-3 (Lanes 2-4 in Figure 5-10) gave a band at 2300 indicating that the recombinant clones are a result of ligation of pUB-S-X (CalI-PvuII) and T7-MCS-rrnB/T1-rrnB/T2 (CalI-ScaI) fragments. The possibility of non-specific amplification was ruined by the lack of bands with samples 4-6 (Lanes 5-7 in Figure 5-10). Glycerol stock was prepared for clone-1,2 and 3 for use in further analysis.

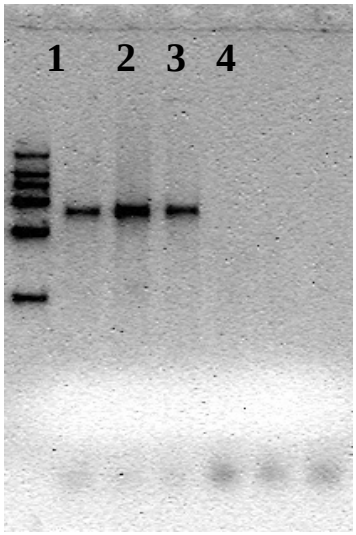


Figure 3.20: Analysis of pUB-S-X-T7 clones using colony PCR: Lane-1 (1kb ladder), Lane-2 (Clone-1), Lane-3 (clone-2), Lane-4 (clone-3), Lane-5 (pUB-S-X), Lane-6 (pUB-T7) and Lane-7 (-ve)

IV. DISCUSSION

Recombinant expression plasmids are valuable tools in life science research and bio-pharmaceutical industry. With the rapid progress in biotechnology a number of expression plasmids have been developed for the last few decades. However, the current plasmid design still limits the controlled expression of multiple genes. Controlled expression of multiple genes is a useful approach for the production of restriction endonucleases which demand the expression of restriction endonuclease and methylase simultaneously.A double gene expression vector with two independently regulated transcriptional units have been developed by cloning T7 promoter-MCS-rrnB/T1 and rrnB/T2 in to the pUB-S-X plasmid which already had a ProU Promoter-X-GX-rrnB/T1 and rrnB/T2 transcriptional unit. The cloning of one transcriptional unit into the other plasmid is achieved by restriction digestion and ligation (Section 5.2, 5.3, 5.4 and 5.5). The recombinant double gene expression vector was analyzed for the presence of both the transcriptional units using colony PCR. The appearance of 2.5kb fragment following colony PCR indicated that the T7 promoter-MCS-rrnB/T1 and rrnB/T2 was successfully ligated into pUB-S-X plasmid. The lack PCR amplification in pUB-S-X and pUB-T7 using the same primers indicated that the result of amplification is due to the presence of both the transcriptional unit

V. CONCLUSION

Double gene bacterial expression vector pUB-S-X-T7 was constructed and was analyzed for the presence of both the transcriptional units (PorU-X-G-X-rrnB/T1-rrnB/T2 and T7-MCS-rrnB/T1-rrnB/T2). With the use of the double gene expression vector two recombinant proteins can be expressed independently with the use of two different inducing agent Nacl for ProU promoter and T7 RNA polymerase for T7 promoter

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