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Designing and Reconstruction of pcDNA 3.0 Mammalian Expression Vector with its Multiple Cloning Sites by Directional Cloning Method

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Abstract

Traditional cloning is the cloning in which we use restriction endonucleases to produce DNA fragments with specific complementary end sequences that can be joined together with a DNA ligase enzyme, prior to the transformation. The purpose of this study was to reconstruct the mammalian expression vector by replacing the junk DNA with its natural multiple cloning sites. Plasmid was propagated and digested with Xho1 and Kpn1 enzymes from both sides of junk DNA a 600 bp DNA was cut from the vector and then ligation of 30 bp primer which was designed similar as its multiple sequence sites. Correct insertion was confirmed by plasmid isolation, plasmid colony PCR, PCR of cloned plasmid and comparison on gel electrophoresis with the original one and finally by sequencing. An extra DNA fragment of 600 bp was cut from the vector after restriction digestion. Ligated colonies appeared on the agar plate from which 60 bp colony PCR products was produced. Plasmid isolation after cloning and colony PCR and PCR of cloned plasmid confirmed the cloning. Cloning of multiple cloning sites in pcDNA3.0 mammalian expression vector was performed successfully. Ligation of multiple cloning sites in pcDNA vector provided various restriction enzymes recognition sites for simple and fast cloning.

Keywords: Cloning; Multiple cloning sites; Mammalian expression vector; pcDNA3.0 vector

Introduction

Gene cloning has made a unique impact on the speed of biological research and it is increasing its presence in several areas of everyday life. One of the reasons why biotechnology has received so much attention during the last decade is because of gene cloning [1]. Traditional Cloning is the cloning in which we use restriction endonucleases to produce DNA fragments with specific complementary end sequences that can be joined together with a DNA ligase enzyme, prior to the transformation [2]. This technique involves preparing both a DNA fragment which is going to be cloned (insert) and a DNA plasmid (vector) in which we are going to ligate our gene of interest, by cutting with two unique restriction enzymes that flank the DNA sequence on both sides with sticky ends, and whose cut sites are present at the preferred site of insertion of the vector, called the multiple cloning site (MCS) [3]. By using two different REs, two non-compatible ends are generated in the vector which were not ligated to itself, thus forcing the insert to be cloned directionally, and lowering the transformation background of re-ligated vector alone so this method is accurate at some extent. pcDNA3.0 is a mammalian expression vector of 5.4 kb which are specially designed for high-level stable and transient expression in mammalian hosts. pcDNA is available with the multiple cloning sites in the forward (+) and reverse (–) orientations to facilitate good cloning. High-level stable and non-replicative transient expression can be carried out in most mammalian cells. Many *E. coli* strains are suitable for the growth of this vector easily. For the most efficient selection is highly recommended choosing an *E. coli* strain. Its multiple cloning sites consist of HindIII, Kpn1, BamH1, BSTX1, ECORI. ECORV, Not1, and Xho1. The CMV promoter provides high-level expression in mammalian cells [4,5]. Multiple cloning sites are present in the forward (+) and reverse (–) orientations to facilitate cloning of gene of interest in which we restrict it by various enzyme.

The ampicillin and neomycin resistance genes in this plasmid pcDNA3.0 allow selection of this plasmid in *E. coli* and mammalian cells in the presence of the antibiotic ampicillin and neomycin [6].

Our initial aim was to restructured the pcDNA available in our laboratory, which is a mammalian expression vector, in which there was no multiple cloning site (MCS), instead of this a 600 bp extra DNA was present. Hence this 600 bp DNA was cut from the vector and then ligation of 30 bp primer which was designed similar as its multiple sequence sites.

The present study is aimed to generate a suitable mammalian expression vector in order to exploit it in future for further cloning.

Materials and Methods

Plasmid propagation

Competent cells of DH5-α strain was prepared. The bacterial cells were transformed by the original plasmid in which junk DNA was there and the transformed colonies were checked in ampicillin agar medium [7] (Figures 1-5).

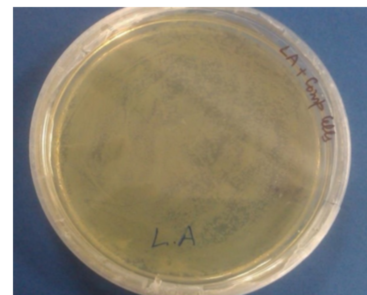

Figure 1: Freshly prepared competent cells growing in luria agar medium without ampicillin.

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Figure 2: Plate containing only DH5-α cells in ampicillin agar medium (-ve) control plate.

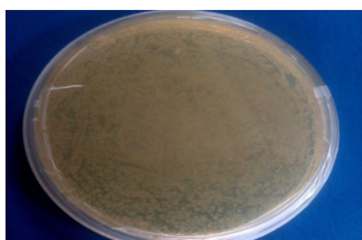

Figure 3: Transformed bacterial colonies growing in ampicillin agar plate.

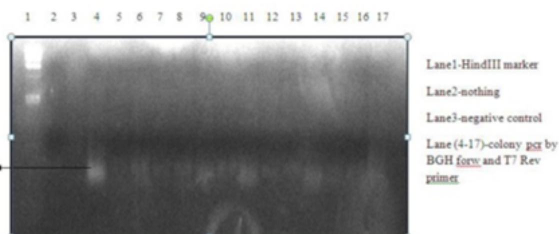

Figure 4: Agarose gel electrophoresis after colony PCR, lane 1- Hind 111 marker, lane 2 empty, lane 3-negative control, lanes (4-17) - colony PCR taking colony from ligation plate as template.

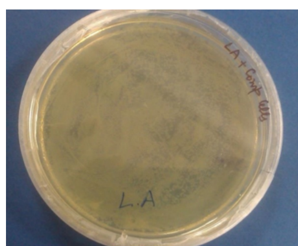

Figure 5: Freshly prepared competent cells growing in luria agar medium without ampicillin.

Plasmid isolation and DNA clean up

A single colony of transformed *E. coli* DH5α picked from the transformed plate and inoculated in 5 ml LB media containing ampicillin and grown overnight at 37°C at 200 rpm. DNA was isolated from the transformed bacterial colonies. The isolated DNA was run on 1% agarose gel. The propagated pcDNA multiple bands of 6 kb was clearly seen. The plasmid DNA was cleaned up. After DNA cleans up we make a 1% agarose gel and then loaded 1 µl of it to check whether our DNA cleaned up or not [8] (Figures 6-10).

Restriction digestion

After DNA clean up plasmid was digested with Xho1 and kpn1. Vortex and short spin was done after it. Eppendorf was placed in water bath set at 37°C for 1.5 hours. Plasmid was digested by enzymes such as Xho1 and Kpn1, these two restriction enzymes recognition sequences were present uniquely on both sides of extra DNA which were intended to replace with MCS. So they easily cut that extra fragment. Double digestion was performed to cut the extra DNA from both sides of circular plasmid by producing nicks from both sides of extra DNA [9] (Figures 11 and 12).

Gel extraction of DNA

The bands of DNA were excised from agarose gel using a scalpel and the DNA was extracted by sure Extract Gel Extraction kit. We cut

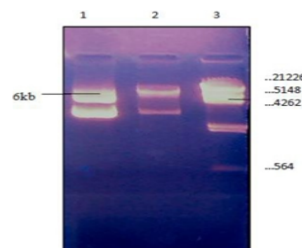

Figure 6: Agarose gel electrophoresis showing bands of pcDNA plasmid. Lane 1-isolated DNA of 6 kb, Lane 2- original DNA of 6 kb, Lane 3-Hind 111 ladder.

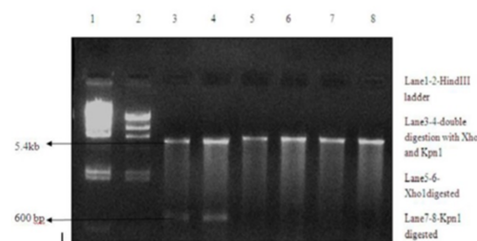

Figure 7: Agarose gel showing bands of restriction digestion of pcDNA , lanes (1-2)-hind111 ladder, lanes (3-4)-double digestion of pcDNA plasmid with Xho1 and Kpn1 enzymes producing 5.4 kb plasmid band and 600 kb junk DNA band, lane (5-6)-single digestion by Xho1, lane (7-8) single digestion with Kpn1 enzyme.

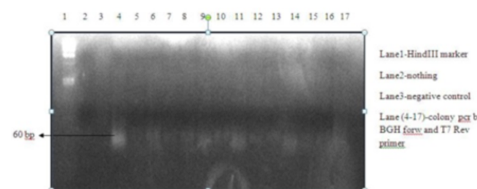

Figure 8: Agarose gel electrophoresis after colony PCR, lane 1-hind111 marker, lane 2-empty, lane 3-negative control, lanes (4-17)-colony PCR taking colony from ligation plate as template.

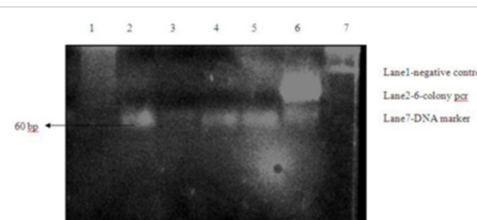

Figure 9: Colony PCR of selected colonies which show greater amplification in colony PCR above, lane 1-negative control, lanes (2-6)-colony PCR of selected colonies from secondary ligation plate of 60 bp band.

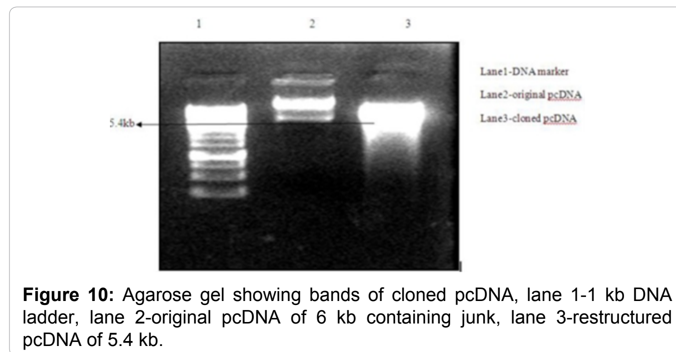

Figure 10: Agarose gel showing bands of cloned pcDNA, lane 1-1 kb DNA ladder, lane 2-original pcDNA of 6 kb containing junk, lane 3-restructured pcDNA of 5.4 kb.

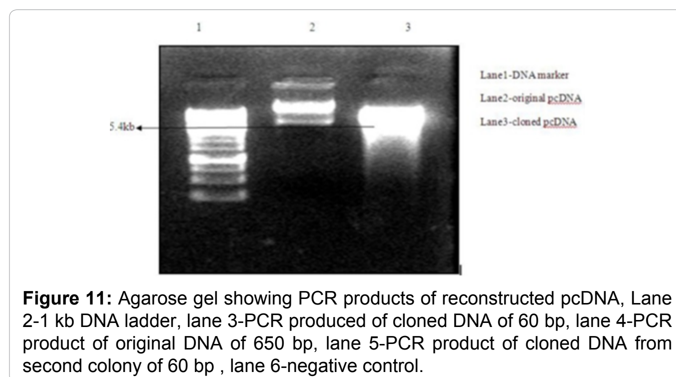

Figure 11: Agarose gel showing PCR products of reconstructed pcDNA, Lane 2-1 kb DNA ladder, lane 3-PCR produced of cloned DNA of 60 bp, lane 4-PCR product of original DNA of 650 bp, lane 5-PCR product of cloned DNA from second colony of 60 bp , lane 6-negative control.

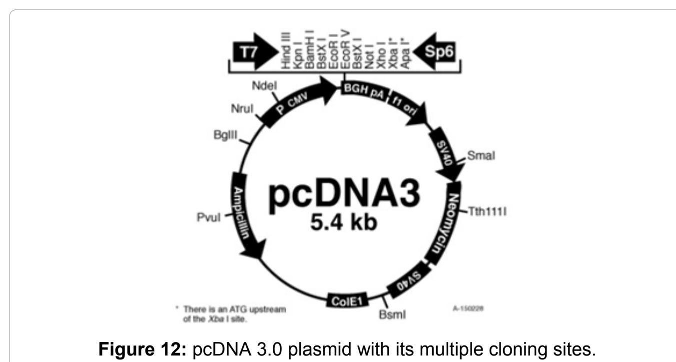

Figure 12: pcDNA 3.0 plasmid with its multiple cloning sites.

the desired bands of vector and extracted DNA from it which has sticky ends of enzyme recognition site of Xho1 and Kpn1 [10].

Primer designing and hydration

The primer was especially designed as same as multiple cloning sites which we were going to ligate in our linearized pcDNA vector. The forward and reverse primers were hydrated according to the common procedure told by the company who designed primers. In both reverse and forward primer tube add 229.3 and 275 nuclease free water was added respectively to make up the volume 100 µl placed it in incubator at 37°C for 30 minutes again incubated for 1 hour. Vortex and spin after every 10 minutes. In 20 µl of each tube a volume of 100 µl was made in each tube from nuclease free water. Mixed 10 µl of reverse primer with 10 µl of forward primer in an eppendorf and placed it in water bath for 1 hour to anneal.

5'' TACCGGATCCGAATTCATCGCGGCCGCC 3''

3'' GGCCTAGGCTTAAGTAGCGCCGGCGGAGCT 5''

Ligation

Next step was to ligate the digested and purified linearized pcDNA vector (without extra 600 bp DNA). All of the following components should be added in a small tube and mixed properly. The reaction mixture was wrapped in foil paper and incubated at 4°C overnight. Ligation mixture was ready to transform the bacteria.

Colony PCR

Colony PCR was used for colony screening by PCR. Different colonies were analyzed for the presence and orientation of the DNA insert using colony PCR. This was done to make sure about the insertions of 30 bp MCS by replacing 600 bp extra DNA in pcDNA. Colonies were taken as a template from the secondary plate and colony PCR was done. Clean PCR tubes were taken and putted it on stand. Reaction mixture mixed well and aliquoted in 18 PCR tubes placed on ice. Individual white colony was picked up and resuspended in each PCR tube to make a strike over the culture plate in order to save the plate. PCR performed at 94°C for 30 seconds, 45°C for 30 seconds and 72°C for 1 minute/kb and 30 cycles. The outcome of the PCR product of the expected length was analyzed on agarose gel [11,12].

DNA isolation after cloning

DNA isolation was done and then compared it with the original DNA in which extra DNA was there. Most potential colony of colony PCR should be picked and inoculated in 5 ml LB and incubated overnight at 37°C with vigorous shaking. Plasmid DNA was isolated from an overnight culture using a convenient plasmid miniprep method [13].

PCR of cloned plasmid with original plasmid

PCR amplification of both the original pcDNA vector and the cloned pcDNA vector after PCR the 30 bp MCS annealed with around 30 bp primer used in PCR such as T7 and BGH so a total of 60 bp product was produced in cloned plasmid, and 650 bp PCR product was produced with original plasmid. PCR is a rapid *in vitro* technique for amplifying defined target DNA sequences present within the source of DNA. This method was specially designed for selective amplification of a specific target DNA sequences within a diverse collection of DNA sequences. Amplification was usually carried out by the DNA polymerase I enzyme from Thermus aquaticus [14,15].

Results

In this study the 6 kb vector was propagated and colonies came in ampicillin agar plate from which plasmid was isolated, the plasmid contains an extra part of junk DNA of 600 bp present on the side of multiple cloning sites. So the plasmid was double digested with Xho1 and Kpn1 enzymes whose digestion site lies on both sides of junk DNA. After digestion the plasmid was cut and a 600 bp part of extra DNA came out in gel electrophoresis and 5.4 kb digested plasmid band also came in gel electrophoresis. The 5.4 kb gene extracted from gel extraction experiment and the designed primer of multiple cloning site of 30 bp was ligated with the sticky overhangs of digested plasmid. Ligated colonies checked in ampicillin agar medium. The cloned plasmid was rechecked in ampicillin agar secondary plate of transformation. Ligated colonies again appear to confirm cloning. After that colony PCR was done to make sure about the insertion of 30 bp MCS by replacing 600 bp extra DNA in pcDNA. Colony PCR was done with M7 and BGH primer of 30 bp so a total of 60 bp PCR product was amplified after pcr and gel electrophoresis in every lane 60 bp PCR products was occurred. The colonies which shows greater amplification in colony PCR should be selected and then further PCR was done to recheck whether the colonies which we select in colony PCR should be re amplified in the same amount or not. DNA was isolated from that colony which shows

greater amplification. DNA isolated cloned plasmid was compared with the original DNA in which extra DNA was there. The cloned DNA band in lane3 is slightly lower than the band of original DNA because instead of 600 bp extra DNA insert, ligated the 30 bp multiple cloning site so a total of around 5.4 kb plasmid was formed. The difference seen in the bands of cloned DNA with original DNA confirms our result. We finally checked the difference between clone and original DNA in agarose gel, this difference was also clearly seen after PCR amplification of both the original pcDNA vector and the cloned pcDNA vector that after amplification a measurable difference was seen in between them. After pcr the 30 bp MCS annealed with around 30 bp primer used in pcr such as T7 and BGH so a total of 60 bp product was produced in cloned plasmid, and 650 bp PCR products was produced with original plasmid. So the PCR result showed the amplified bands of cloned pcDNA with the original pcDNA. Gel showed the clear difference of 600 bp between the cloned DNA and original DNA which confirmed our result (Figures 11 and 12).

Discussion

The present study was aimed to generate a suitable mammalian expression vector in order to exploit it in future for further cloning of the Hepatitis B virus X gene [16]. Our initial aim was to restructured the pcDNA availabe in our lab, which is a mammalian expression vector [17,18]. in which there was no multiple cloning site (MCS), instead of this a 600 bp extra DNA was present. Hence this 600 bp DNA was cut from the vector and then ligation of 30 bp primer which was designed similar as its multiple sequence site Primers were designed in a way that they were complementary to each other providing the cohesive ends of restriction enzymes kpn1 and xhol on 5' and 3' ends for the purpose of directional cloning [19]. Bacterial colonies do not have any gene which was resistant to antibiotic so easily verified after transformation because pcDNA3.0 plasmid contains antibiotic resistant genes so they easily grown in antibiotic medium. Transformation result was confirmed by comparing bands of propagated pcDNA with the bands of original pcDNA both was of 6 kb. Plasmid was digested by enzymes such as Xho1 and Kpn1 because these two restriction enzymes recognition sequences were present uniquely on both sides of extra DNA which were intended to replace with MCS [17]. Both of these enzymes cut the DNA on the ends of the extra DNA which was inserted in pcDNA and produce sticky ends. We cut the desired bands of 5.4 kb of vector and extracted DNA from it which has sticky ends of enzyme recognition site of Xho1 and Kpn1. The transformed colonies came after ligation rechecked or verified by making their secondary [17]. From the colonies result we did the colony PCR. This was done to make sure about the insertions of 30 bp MCS by replacing 600 bp extra DNA in pcDNA.

All the white colonies appeared in this experiment theoretically must consist of the correct clone having inserted of 30 bp MCS, which was what theoretically expected and was obtained in colony PCR results [17]. This can be explained due to the presence of two different primers used such as M7 and BGH which was of 30 bp approximately so a total of 60 bp PCR products were produced. PCR result, plasmid should be isolated from the most potent colony. The cloned DNA band was slightly lower than the band of original DNA because instead of 600 bp extra DNA insert we ligated the 30 bp multiple cloning site. The difference seen in the bands of cloned DNA with original DNA confirmed the result. Difference between the cloned DNA with 5.4 kb and original DNA with 6 kb was seen clearly in gel electrophoresis. After PCR the 30 bp MCS annealed with around 30 bp primer used in PCR such as T7 and BGH so a total of 60 bp product was produced in cloned plasmid, and 650 bp PCR products was produced with original plasmid. Gel shows the clear difference of 600 bp between the cloned DNA and original DNA [10]. And finally sequencing of plasmid confirmed cloning. pcDNA3.0 plasmid consists of only one site of restriction enzymes such as xhol and kpn1 which lies on both the ends of extra DNA inserted, so these enzymes specifically cut the extra DNA from both the ends and produced sticky ends overhangs [9]. The multiple sequence site which is in primer form also consist of sticky overhangs of both the enzymes so it was expected that these sticky overhangs of primer get ligated with the sticky overhangs of the plasmid produced after restriction and a proper pcDNA3.0 plasmid clone was produced [8,9].

Conclusion

Reconstruction of a mammalian expression vector pcDNA3.0 was successfully done after ligation of its proper multiple cloning sites. It was confirmed by the generation of 60 bp band in colony PCR and generation of nearly 5.4 kb band after (DNA isolation). Cloning was also confirmed by difference produced in bands of cloned DNA with the original DNA in agarose gel electrophoresis. PCR results also showing clear difference between the cloned pcDNA with the original one. From these results we were successful to clone pcDNA3.0 with its natural structure.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgements

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