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Bioinformatics informations for Constructed Mammalian expression vector using nested PCR technique

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Abstract : Present study aims to used virtual design to transfer cytomegalovirus premotor (CMV) and red fluorescence protein gene (RFP) to mammalian expression vector by nested PCR technique, free bioinformatics soft wear were used to design primers, linkers and vector maps, the results show that DII design had 6179bp which created by insertion 1463 bp after cutting with *AflII* which insert in GFP- vector at site 1618 bp. The nested PCR product were 1473 bp ,1461 bp without any addition , 1481 bp after linker and supported nucleotides addition using nested PCR .1463 bp after cutting with *AflII* which insert in GFP- vector at site 1618 bp.
Key words : virtual design, nested PCR technique, free bioinformatics soft wear.

Introduction

Nested PCR was one of important technique used in genetic researches and genetic engineering laboratories, nested PCR based on amplified specific DNA sequence using 4 primer in to tow steps, sometimes the PCR product in the first step using as DNA tamplet in the second step. Nested PCR used for more fidelity amplification and for amplified target DNA sequence with additive nucleotides that added to primers with every steps[(1,2)]

The bioinformatics conceders as a main part of genetic engineering and genetic study programmers were Asttaau programmers that carry out specialized programs to deal with the DNA data, some of these programs was closed with company produced genetic materials and others are free online, these software have been updating to overcome problems occupant with genetic studies[(3,4)].

Mammalian expression vectorswere constructed into different patterns and the target gene was inserted in different sites in back bone vector and this insertion must be based on genetic engineering role in gene cloning.Constructed mammalian vector begins theoretically by bioinformatics software'sto design primers, linkers and vectors, Different strategies have been used DNA sequence manipulation for primers, linkers, probes and vectors for more specify and new designs which have new features suitable with target gene transcription, translation, regulation and post translation processing in transform cells, thus a new sequences are added to vectors for these aims like Internal ribosome entrysite IRES, inverted repeats IR, regulation sequences and restriction site sequence[(5-7)].

Subjected and software's

Data and raw DNA sequence: Turbo-GFP vector and Turbo-RFP sequence from Evrogen company http://www.evrogen.com/products/vectors/pTurboGFP-N/pTurboGFP-N.shtml and **Error! Hyperlink reference not valid.**, (2013)[(8-11)], these sequences were processed in the following software's

Table (1) online bioinformatics software's used in vector design

PCR primer design	**http://primer3.ut.ee/** **http://www.biomol.unb.br/sms2/pcr_primer_stats.html**
Vector graphic and design	**http://www.biomol.unb.br/sms2/rest_digest.html** **http://www.addgene.org/analyze-sequence/**
PCR amplification	**http://www.biomol.unb.br/sms2/pcr_products.html**

Vector Design (DII)

Red fluorescence protein gene and CMV promoter inserted in blank vector (GFP) in intragenic sequence as following.

1- Primer Design

1-The row data of RFP and GFP vector were download from evrogenewebsite , (Table 1)

2-RFP sequence transfer to primer3 software (table 1) to detected site of primers and design.

3-Primers ware choose, then it transfers to sms-primer state bioinformatics (table 1) for certification.

2- **Linker Design**.

1- Linker of *Afl*II restriction site was added in to two patterns:

5′-CTT AAG -3′

3′-GCC TTG-5′

A- Forward sequence of linker restriction site was added to forward primer.

B- Forward sequence of linker restriction site was added to revers primer.

C- Revers sequence linker restriction site was added to revers primer.

2- Supported sequence was added to primers according to *biolabs* guide for restriction enzyme activity support (https://www.neb.com/tools-and-resources/usage-guidelines/cleavage-close-to-the-end-of-dna-fragments)[12].

3- Complete primers (primer sequence, linker and supporting sequence) was transfer to sms-primer state bioinformatics (table 1) for certification.

3-Sequence Digest and Map Construction

1- Products transfer to Addgene site (table 1)for physical map construction .

2- Then Product was digested using restriction digest in sequence manipulation site (Table 1)by *Afl*II.

3- The GFP vector sequence was digested by *Afl*II, (Appendix ,3)

4- The RFP gene sequence was inserted in GFP- vector to constructed DII vector.

5- The sequence of DII vector transferred to addgene site to creation physical map and features of the new vector.

6- The DII sequence transfer to sequence manipulation suite for creation the complete map of DII vector (translation probability of proteins in vector with restriction site).

7-

4- Confirmatory Test

Confirmatory test performed by the same software in construction vector as a following:

1- Re-amplified target sequence in DII vector using same primer, positive results must be had longer than in blank vector (GFP).

2- Digested DII vector by *Afl*II , positive result must be give same sequence which used in creation vector D1.

Results

In this designRed florescence protein gene with CMV promoter were inserted in the GFP- vector, this design was performed using a nested PCR technique which based on added restricted site of *AflII* linker with addition supported bp to employ cutting by restriction enzyme into two steps as in Table (2). The results of primer design are in the Table (2)with linkers and supported sequence.

Table (2) Primers sequences Features of RFP gene with CMV premotor amplification in free bioinformatics software to created DII vector.

Single base run	Hairpin formation	Self-annealing	GC clump	GC%	TM	Primer sequences 5`→3`	Design
pass	pass	pass	pass	33.33	64.1	***F***- *CTT AAG TAA TAG TAA TCA ATT ACG GGG TCA	D3
pass	pass	pass	pass	54.55	69.5	***R***- *CTT AAG GGA GGT GTG GGA GGT T	
pass	pass	pass	pass	41.67	74.1	***F***- *CGG CGACTT AAG TAA TAG TAA TCA ATT ACG GGG TCA	D4
pass	pass	pass	pass	41.67	74.1	***F***- *CGG CGACTT AAG TAA TAG TAA TCA ATT ACG GGG TCA	
pass	pass	pass	pass	42.86	66.6 ◦	***F***- ACT CCT *CTT AAG TTT GTA TGC TCG TCA G	D5
Pass	pass	pass	pass	40.00	68.3	***R***- GAT ACA **GAA TTC ACT GGA ACA ACA CTC AAC C	

*linker sequence of *AflII* ; *supported sequence; *forward linker sequence of *AflII*;
** Revers Linker sequence of *AflII*

The Result of bioinformatics show that DII design had 6179 bp which consist of GFP gene, Kanamycine resistant gene and RFP gene as show in Figure (4-7) ,(4-9); some features in Table (4-7) and open reading frame in Table (4-9). After using D3 primer PCR product was 1473 bp (1461 bp without any addition , 1481 bp after second round amplified using D4 primer, 1463 bp after cutting with *AflII* which insert in GFP-vector at site 1618 bp as show in table (3) Figure (4-6) and (4-7) .Insertion of RFP-gene with CMV promoter may be with the same reading frame or in posit reading frame of GFP- vector genes as show inFigures (3 and 5).

Table (3) Nested PCR steps to created RFP –gene with linker of *AflII*

1	CGG CGA CTT AAG TAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGA GTTCCGCGTTACATAACTACGGTAAATGGCCCGCCTGGCTGACCGCC CAACGACCCCCGCCCATTGACGTCAATAATGACGTAGTTCCCATAGT AACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTAC GGTAAACGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTA CGCCCCCTATTGACGTCAATGACGGAAATGGCCCGCCTGGCATTATG CCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTAC	1461 bp product from linear template RFP-CMV base 8 to base 1481(D2 F - D2 R). (1473) bp for

	GTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACAT CAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCC ACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGG GACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGG CGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTGGTTTA GTGAACCGTCAGATCCGCTAGCGCTACCGGTCGCCACCATGGTGAGC GAGCTGATTAAGGAGAACATGCCCATGAAGCTGTACATGGAGGGCA CCGTGAACAACCACCACTTCAAGTGCACATCCGAGGGCGAAGGCAA GCCCTACGAGGGCACCCAGACCATGAGAATCAAGGTCGTCGAGGGC GGCCCTCTCCCCTTCGCCTTCGACATCCTGGCTACCAGCTTCATGTAC GGCAGCAGAACCTTCATCAAGCACCCTCCGGGCATCCCCGACTTCTT TAAGCAGTCCTTCCCTGAGGGCTTCACATGGGAGAGAGTCACCACAT ACGAAGACGGGGGCGTGCTGACCGCTACCCAGGACACCAGCCTCCA GGACGGCTGCCTCATCTACAACGTCAAGGTTAGAGGGGTGAACTTCC CAGCCAACGGCCCTGTGATGCAGAAGAAAACACTCGGCTGGGAGGC CTCCACCGAGACGATGTACCCCGCTGACGGCGGCCTGGAAGGCGCA TGTGACATGGCCCTGAAGCTCGTGGGCGGGGGCCACCTGATCTGCAA CCTTGAGACCACATACAGATCCAAGAAACCCGCTACGAACCTCAAG ATGCCCGGCGTCTACAACGTGGACCACAGACTGGAAAGAATCAAGG AGGCCGACGATGAGACCTACGTCGAGCAGCACGAGGTGGCTGTGGC CAGATACTCTACTGGTGGCGCTGGTGATGGAGGTAAAGGTGGAGGA GGTTCCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGA CGGTACCGCGGGCCCGGGATCCACCGGATCTAGATAACTGATCATA ATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCT CCCACACCTCCCTTAAGACATAG	D3 amplification and 1485 bp for D4 amplification
2	TTAAGTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATAT ATGGAGTTCCGCGTTACATAACTACGGTAAATGGCCCGCCTGGCTGA CCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTAGTTCCC ATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTA TTTACGGTAAACGCCCACTTGGCAGTACATCAAGTGTATCATATGCC AAGTACGCCCCCTATTGACGTCAATGACGGAAATGGCCCGCCTGGCA TTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACAT CTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGT ACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGT CTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCA ACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAA TGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTGG TTTAGTGAACCGTCAGATCCGCTAGCGCTACCGGTCGCCACCATGGT GAGCGAGCTGATTAAGGAGAACATGCCCATGAAGCTGTACATGGAG GGCACCGTGAACAACCACCACTTCAAGTGCACATCCGAGGGCGAAG GCAAGCCCTACGAGGGCACCCAGACCATGAGAATCAAGGTCGTCGA GGGCGGCCCTCTCCCCTTCGCCTTCGACATCCTGGCTACCAGCTTCAT GTACGGCAGCAGAACCTTCATCAAGCACCCTCCGGGCATCCCCGACT TCTTTAAGCAGTCCTTCCCTGAGGGCTTCACATGGGAGAGAGTCACC ACATACGAAGACGGGGGCGTGCTGACCGCTACCCAGGACACCAGCC TCCAGGACGGCTGCTCATCTACAACGTCAAGGTTAGAGGGGTGAACT TCCCAGCCAACGGCCCTGTGATGCAGAAGAAAACACTCGGCTGGGA GGCCTCCACCGAGACGATGTACCCCGCTGACGGCGGCCTGGAAGGC GCATGTGACATGGCCCTGAAGCTCGTGGGCGGGGGCCACCTGATCTG CAACCTTGAGACCACATACAGATCCAAGAAACCCGCTACGAACCTC AAGATGCCCGGCGTCTACAACGTGGACCACAGACTGGAAAGAATCA AGGAGGCCGACGATGAGACCTACGTCGAGCAGCACGAGGTGGCTGT GGCCAGATACTCTACTGGTGGCGCTGGTGATGGAGGTAAAGGTGGA GGAGGTTCCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCTGCAGT CGACGGTACCGCGGGCCCGGGATCCACCGGATCTAGATAACTGATC	1463 bp linear fragment from linear parent Untitled, base 8 to base 1470 (AflIIc|ttaag - AflIIc|ttaag).

	ATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAA CCTCCCACACCTCCC	
3	TTAAGACATAG	11 bp linear fragment from linear parent Untitled, base 1471 to base 1481 (AflIIc|ttaag - sequence end). TTAAGACATAG
4	CGGCGAC	7 bp linear fragment from linear parent Untitled, base 1 to base 7 (sequence start - AflIIc|ttaag)

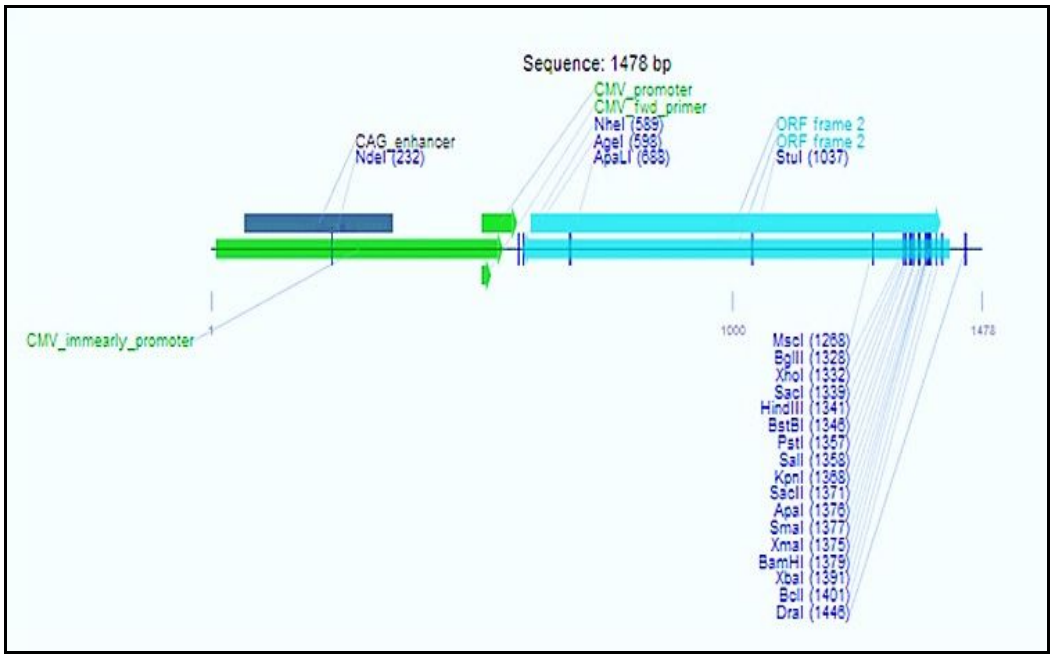

Figure (1) Physical map of RFP gene with CMV promoterin analysis sequence –addgene

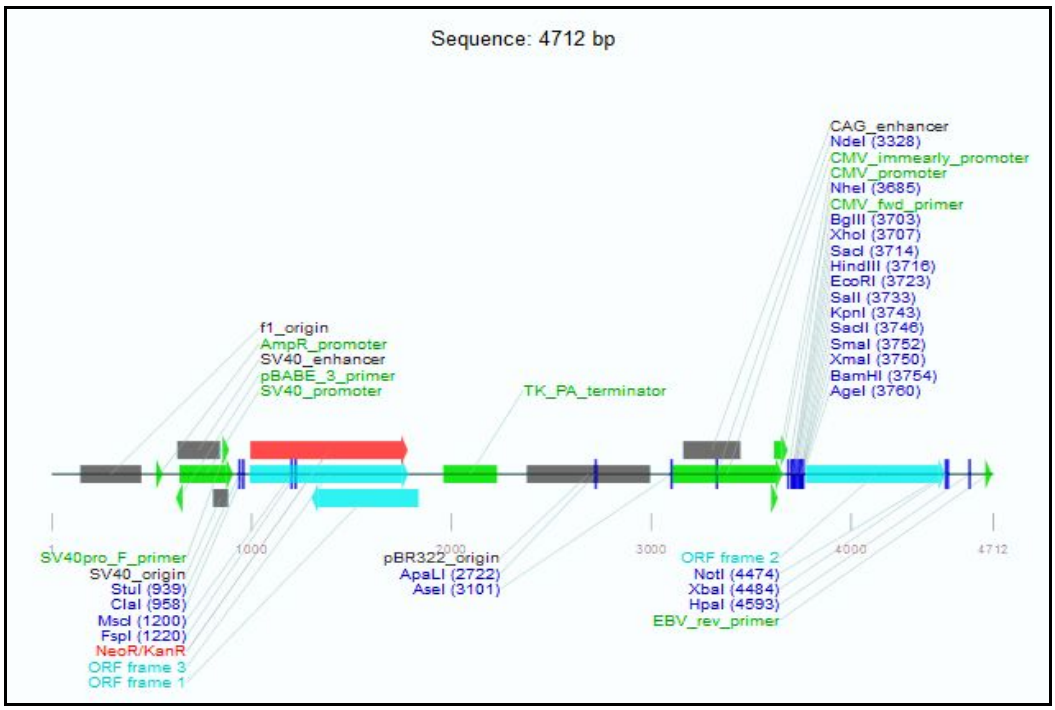

Figure (2) physical map of GFP- vector linearized using *AflII* restriction enzymein analysis sequence – addgene

Table(3) The sequence of DII vector

TTAAGGCGTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCA
GCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGAC
CGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGAC
TCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCAC
CCTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAG
CCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAA
AGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCAC
CACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCAGGTGGCACTTTTCGGGGAAAT
GTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGA
CAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTCCTGAGGCGGAAAGAA
CCAGCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGA
AGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCC
CAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCT
AACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGAC
TAATTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAG
TGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAGATCGATCAAGAGACAGGATGAGGA
TCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGA
GGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGG
CTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGA
ACTGCAAGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCT
GTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGC
AGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATG
CGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCA
TCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGA
GCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGAGCATGCCCGACGGC
GAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCC
GCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCG
TTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCT
TTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTT
CTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCAACCTGCCATCACGAG
ATTTCGATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCC
GGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCACCCTAGGGGGA
GGCTAACTGAAACACGGAAGGAGACAATACCGGAAGGAACCCGCGCTATGACGGCAATAA
AAAGACAGAATAAAACGCACGGTGTTGGGTCGTTTGTTCATAAACGCGGGGTTCGGTCCCA
GGGCTGGCACTCTGTCGATACCCCACCGAGACCCCATTGGGGCCAATACGCCCGCGTTTCTT
CCTTTTCCCCACCCCACCCCCCAAGTTCGGGTGAAGGCCCAGGGCTCGCAGCCAACGTCGG
GGCGGCAGGCCCTGCCATAGCCTCAGGTTACTCATATATACTTTAGATTGATTTAAAACTTC
ATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCT
TAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTG
AGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGG
TGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGA
GCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTC
TGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCG
ATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTC
GGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTG
AGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGAC
AGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGA
AACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTG
TGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGT
TCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGA
TAACCGTATTACCGCCATGCATTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCAT
AGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCC
CAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGG

ACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCA
AGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGC
ATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTA
GTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTT
TGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACC
AAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGG
TAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTGGTTTAGTGAACCGTCAGATCCGC
TAGCGCTACCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGACGGTACCGCG
GGCCCGGGATCCACCGGTCGCCACCATGGAGAGCGACGAGAGCGGCCTGCCCGCCATGGA
GATCGAGTGCCGCATCACCGGCACCCTGAACGGCGTGGAGTTCGAGCTGGTGGGCGGCGGA
GAGGGCACCCCCGAGCAGGGCCGCATGACCAACAAGATGAAGAGCACCAAAGGCGCCCTG
ACCTTCAGCCCCTACCTGCTGAGCCACGTGATGGGCTACGGCTTCTACCACTTCGGCACCTA
CCCCAGCGGCTACGAGAACCCCTTCCTGCACGCCATCAACAACGGCGGCTACACCAACACC
CGCATCGAGAAGTACGAGGACGGCGGCGTGCTGCACGTGAGCTTCAGCTACCGCTACGAGG
CCGGCCGCGTGATCGGCGACTTCAAGGTGATGGGCACCGGCTTCCCCGAGGACAGCGTGAT
CTTCACCGACAAGATCATCCGCAGCAACGCCACCGTGGAGCACCTGCACCCCATGGGCGAT
AACGATCTGGATGGCAGCTTCACCCGCACCTTCAGCCTGCGCGACGGCGGCTACTACAGCT
CCGTGGTGGACAGCCACATGCACTTCAAGAGCGCCATCCACCCCAGCATCCTGCAGAACGG
GGGCCCCATGTTCGCCTTCCGCCGCGTGGAGGAGGATCACAGCAACACCGAGCTGGGCATC
GTGGAGTACCAGCACGCCTTCAAGACCCCGGATGCAGATGCCGGTGAAGAATAAAGCGGCC
GCGACTCTAGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACC
TCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTT
ATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCAT
TTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTAAGTAATAGTAA
TCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGT
AAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTAT
GTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTA
AACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCA
ATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACT
TGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACAT
CAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCA
ATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCC
CCATTGAGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTGGTTT
AGTGAACCGTCAGATCCGCTAGCGCTACCGGTCGCCACCATGGTGAGCGAGCTGATTAAGG
AGAACATGCCCATGAAGCTGTACATGGAGGGCACCGTGAACAACCACCACTTCAAGTGCAC
ATCCGAGGGCGAAGGCAAGCCCTACGAGGGCACCCAGACCATGAGAATCAAGGTCGTCGA
GGGCGGCCCTCTCCCCTTCGCCTTCGACATCCTGGCTACCAGCTTCATGTACGGCAGCAGAA
CCTTCATCAAGCACCCTCCGGGCATCCCCGACTTCTTTAAGCAGTCCTTCCCTGAGGGCTTC
ACATGGGAGAGAGTCACCACATACGAAGACGGGGGCGTGCTGACCGCTACCCAGGACACC
AGCCTCCAGGACGGCTGCCTCATCTACAACGTCAAGGTTAGAGGGGTGAACTTCCCAGCCA
ACGGCCCTGTGATGCAGAAGAAAACACTCGGCTGGGAGGCCTCCACCGAGACGATGTACCC
CGCTGACGGCGGCCTGGAAGGCGCATGTGACATGGCCCTGAAGCTCGTGGGCGGGGGCCAC
CTGATCTGCAACCTTGAGACCACATACAGATCCAAGAAACCCGCTACGAACCTCAAGATGC
CCGGCGTCTACAACGTGGACCACAGACTGGAAAGAATCAAGGAGGCCGACGATGAGACCT
ACGTCGAGCAGCACGAGGTGGCTGTGGCCAGATACTCTACTGGTGGCGCTGGTGATGGAGG
TAAAGGTGGAGGAGGTTCCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGAC
GGTACCGCGGGCCCGGGATCCACCGGATCTAGATAACTGATCATAATCAGCCATACCACAT
TTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACCTCCGAATTCGATACA

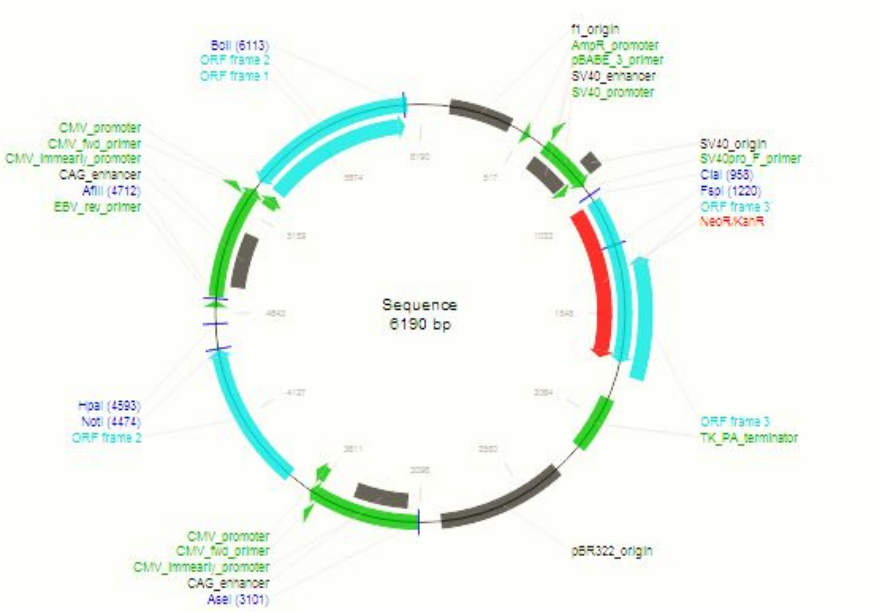

Figure (3) Physical map of DII vector show RFP- gene with the same direction of vector genesin analysis sequence –addgene.

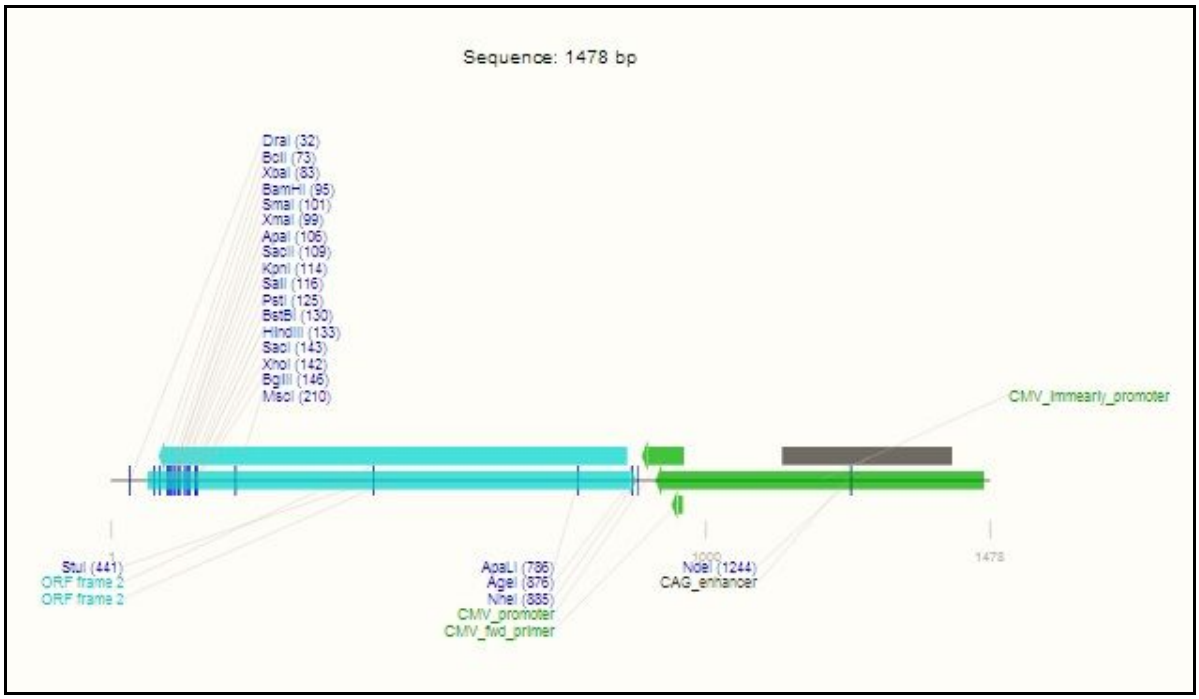

Figure (4) Physical map of DII vector in opposite direction of RFP-gene with CMV promoter in analysis sequence –addgene software.

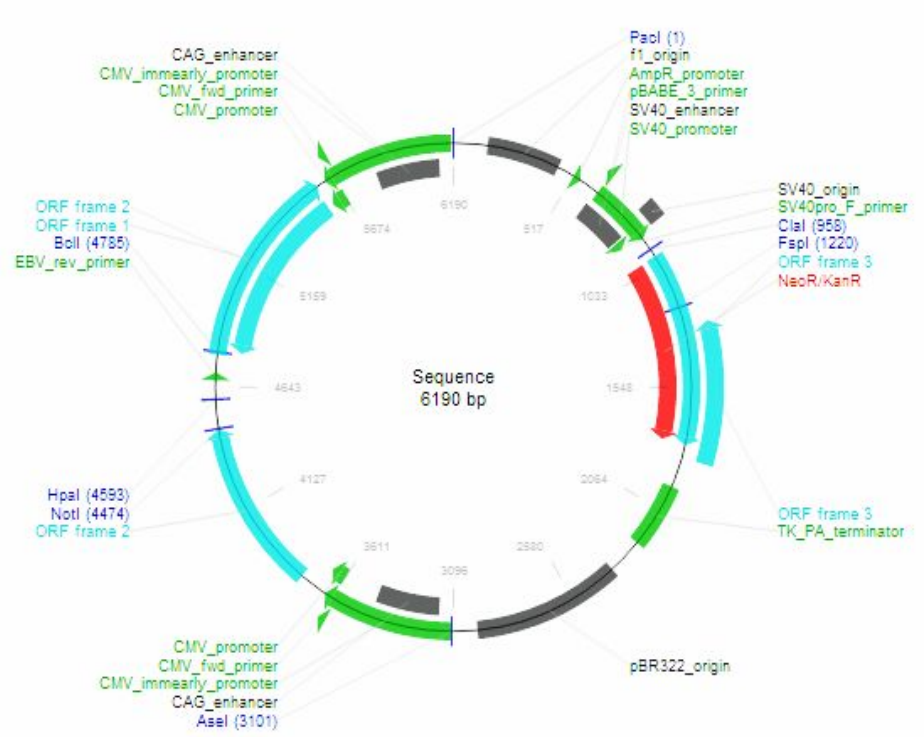

Figure (5*)* Physical map of DII vector when RFP- gene with CMV promoter insertion in opposite direction with the vector genes in analysis sequence – addgene software.

Table (4)Some features of DII vector with its positions

	SITE	
	From	to
f1_origin	448	142
AmpR_promoter	527	555
pBABE_3_primer	641	621
SV40_enhancer	842	627
SV40_promoter	639	907
SV40_origin	806	883
SV40pro_F_primer	868	887
NeoR/KanR	993	1781
TK_PA_terminator	1959	2228
pBR322_origin	2376	2995
CMV_immearly_promoter	3104	3656
CAG_enhancer	3159	3446
CMV_fwd_primer	3613	3633
CMV_promoter	3614	3683
EBV_rev_primer	4685	4704
CMV_immearly_promoter	4720	5271
CAG_enhancer	4775	5062
CMV_promoter	5229	5298

Table (5) Open reading frame position of DII vector

ORF frame 3	990	1784
ORF frame 2	1835	1299
ORF frame 2	3773	4471
ORF frame 2	6128	5307
ORF frame 3	5322	6110

Table (6) Confirmatory test sequence of DII using primer 1.

	TAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGA GTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGC CCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATA GTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTT ACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAA GTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCAT TATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACAT CTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGT ACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGT CTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCA ACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAA TGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTGG TTTAGTGAACCGTCAGATCCGCTAGCGCTACCGGACTCAGATCTCGA GCTCAAGCTTCGAATTCTGCAGTCGACGGTACCGCGGGCCCGGGATC CACCGGTCGCCACCATGGAGAGCGACGAGAGCGGCCTGCCCGCCAT GGAGATCGAGTGCCGCATCACCGGCACCCTGAACGGCGTGGAGTTC GAGCTGGTGGGCGGCGGAGAGGGCACCCCCGAGCAGGGCCGCATG ACCAACAAGATGAAGAGCACCAAAGGCGCCCTGACCTTCAGCCCCT ACCTGCTGAGCCACGTGATGGGCTACGGCTTCTACCACTTCGGCACC TACCCCAGCGGCTACGAGAACCCCTTCCTGCACGCCATCAACAACG GCGGCTACACCAACACCCGCATCGAGAAGTACGAGGACGGCGGCGT GCTGCACGTGAGCTTCAGCTACCGCTACGAGGCCGGCCGCGTGATC GGCGACTTCAAGGTGATGGGCACCGGCTTCCCCGAGGACAGCGTGA TCTTCACCGACAAGATCATCCGCAGCAACGCCACCGTGGAGCACCT GCACCCCATGGGCGATAACGATCTGGATGGCAGCTTCACCCGCACCT TCAGCCTGCGCGACGGCGGCTACTACAGCTCCGTGGTGGACAGCCA CATGCACTTCAAGAGCGCCATCCACCCCAGCATCCTGCAGAACGGG GGCCCCATGTTCGCCTTCCGCCGCGTGGAGGAGGATCACAGCAACA CCGAGCTGGGCATCGTGGAGTACCAGCACGCCTTCAAGACCCCGGA TGCAGATGCCGGTGAAGAATAAAGCGGCCGCGACTCTAGATCATAA TCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTC CCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGT TGTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATA GCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTT GTGGTTTGTCCAAACTCATCAATGTATCTTAAGTAATAGTAATCAAT TACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACAT AACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCG CCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAG GGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCC CACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTAT TGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACA TGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTC	3077 bp product from linear template Untitled, base 3102 to base 6178 (2 - 21).

	ATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCG TGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTG ACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCA AAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGC GTGTACGGTGGGAGGTCTATATAAGCAGAGCTGGTTTAGTGAACCGT CAGATCCGCTAGCGCTACCGGTCGCCACCATGGTGAGCGAGCTGATT AAGGAGAACATGCCCATGAAGCTGTACATGGAGGGCACCGTGAACA ACCACCACTTCAAGTGCACATCCGAGGGCGAAGGCAAGCCCTACGA GGGCACCCAGACCATGAGAATCAAGGTCGTCGAGGGCGGCCCTCTC CCCTTCGCCTTCGACATCCTGGCTACCAGCTTCATGTACGGCAGCAG AACCTTCATCAAGCACCCTCCGGGCATCCCCGACTTCTTTAAGCAGT CCTTCCCTGAGGGCTTCACATGGGAGAGAGTCACCACATACGAAGA CGGGGGCGTGCTGACCGCTACCCAGGACACCAGCCTCCAGGACGGC TGCCTCATCTACAACGTCAAGGTTAGAGGGGTGAACTTCCCAGCCAA CGGCCCTGTGATGCAGAAGAAAACACTCGGCTGGGAGGCCTCCACC GAGACGATGTACCCCGCTGACGGCGGCCTGGAAGGCGCATGTGACA TGGCCCTGAAGCTCGTGGGCGGGGGCCACCTGATCTGCAACCTTGA GACCACATACAGATCCAAGAAACCCGCTACGAACCTCAAGATGCCC GGCGTCTACAACGTGGACCACAGACTGGAAAGAATCAAGGAGGCCG ACGATGAGACCTACGTCGAGCAGCACGAGGTGGCTGTGGCCAGATA CTCTACTGGTGGCGCTGGTGATGGAGGTAAAGGTGGAGGAGGTTCC GGACTCAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGACGGTAC CGCGGGCCCGGGATCCACCGGATCTAGATAACTGATCATAATCAGC CATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACA CCTCC	

Discussion

The important part of vectors designs were primers design, (Table 4-1),some basics were dependent in it, most important was TM of annealing temperature, every primers set have approximate TM to employed accurate amplification it must be low than 68C°. Also GC% must be low than 60%[8-10] in present study two methods used in primer design, first software primer3 to design primer, forward primer when flanking sequences were dependent, while revers and linker that must be added to some revers primer manually as red sequence in Table (1), then every primer was pass in to software to make sure of its ability to accurate complement in amplification. Other company used special calculation of primer TM design it depended on salt concentration and pH of master mix buffer such as *biolab s*tools website [13](http://tmcalculator.neb.com/#!/)

Results show that this technology useful to detect primer TM as show in tables (1) and to avoid nonspecific amplification, The differences of TM between different softwrer because of the database of software's were designed according to materials of master mix of commercial company which design it, thus different annealing temperature can obtain for the same sequence in different website, also it is affected by salt concentration, type of amplification (hot start, touchdown, common amplification), type of DNA polymerase have main roles in annealing temperature also (*Taq*man, long patch amplification, hot start and proofreading activity, also primer concentration was effected in annealing temperature (*https://www.neb.com/*)[14].

In some primers; linker must be added to primer sequence in order to cutting target gene after amplification for suitable complement sequences to accurate insertion. Linker was added according to Dallas *et al*. (1998)[3] and https://www.addgene.org/plasmid-protocols/subcloning/ as in table(1) with modification, linker consist of 6 nucleotides followed by other 6 nucleotides for restriction enzyme activity according to *biolabs*(https://www.neb.com/tools-and-resources/usageguidelines/cleavageclose-to-the-end-of-dna-fragments)[15-19] some restriction enzyme need at least 3 nucleotides for high activity, the addition of these nucleotide dependent on GC percentage and TM calculation.

Other site can be used also but in present study reporter gene choses as a model for evaluate its expression in cells thus any site can be inactivated it or cause knock out gene must be discarded. The target

gene can be inserted in other site than multiple cloning site; from the lank vector (GFP) map free sequences were used for insertion it between genes in intragenic sequences that have restriction site, In present study DII design , *AflII*site choose to insert CMV promoter with RFP gene as in figure (1 and 2)The DII were created to introduced new features in expression vector and for processing some problems with a new molecular biology productions, Molecular cloning technology development a new vectors with different properties for cloning applications, commercial company of molecular production produce vectors for variety researches applications like prokaryotic vectors, eukaryotic vectors, vector have specific gene, and basic vectors (blank) , Al-Terehi et al., and another researchers [20-24] used MCS to inserted RFP gene using tow restriction enzyme.

All vectors have mutual elements such as origin of replication, promoters, reporter gene and marker gene, These vectors specific for expression in mammalian or prokaryotic cells, many researchers used it as blank vectors uses fluorescence protein as reporter gene or insert other genes for different applications[9, 25-28].

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