Chapter 4
Selection, Isolation and Characterization of pDNA

It’s the thought that counts

-From "Polar Express”

4.1 Selection of pDNAs and their properties

Selection of an appropriate target gene for gene delivery research is an important aspect for the success of gene delivery systems. Osteoporosis is a metabolic disorder of bone and hence involves a large number of growth factors, cytokines and hormones playing role in the pathophysiology of disease. Hence, appropriate selection of a gene is required for development of gene delivery system. Among various genes implicated, BMP-9 was selected for development of formulation for treatment of osteoporosis.

In order to evaluate the therapeutic potential of gene delivery systems, it is essential to determine the in vitro expression of the genes after transfection with the delivery system. This requires either direct estimation of the therapeutic gene expression in the cells or evaluation of the expression of a reporter gene that can be easily estimated through a suitable analytical tool. Estimating the expression of therapeutic gene usually requires more sophisticated tools such as western blotting, ELISA and requires use of protein specific antibodies. However, it is possible to use alternative reporter genes expression of which can easily be estimated qualitatively as well as quantitatively using easily available techniques such as fluorescence microscopy, confocal laser scanning microscopy and fluorescence activated cell sorting. Such reporter genes include several examples such as green fluorescent protein, yellow fluorescent protein, red fluorescent protein etc.

4.1.1 Rationale for selection of BMP-9 gene:

In the past decade various gene delivery approaches have been studied for the treatment of osteoporosis. Such gene delivery approaches particularly act either by inducing or suppressing one or other growth factors, cytokines, transcription factors, other mediators or their receptors that are implicated in osteoporosis. **Table 4.1** below details various gene (pDNA) and gene delivery approaches (vectors) used for treatment of osteoporosis.

Various cytokines, particularly interleukin-1 (IL-1) and tumor necrosis factor (TNF), have been strongly implicated in postmenopausal osteoporosis occurring due to estrogen deficiency. Both of these cytokines are powerful inducers of bone resorption. From this information, it follows that inhibiting the biological activities of IL-1 and TNF should reduce bone loss under conditions of estrogen deficiency. Genes encoding for IL-1

receptor antagonist (IL-1Ra) or soluble form of TNF receptors would ameliorate the osteoporotic bone loss by inhibiting osteoclastic activity. [1, 2]

Bone morphogenetic factors (BMPs), mainly BMP-2, BMP-4, BMP-6, BMP-7 and BMP-9, are other osteogenic proteins that have been studied for bone regeneration in fractured bone healing, osteoporosis and osteomalasia. [3] Recombinant human bone morphogenetic protein-2 and -7 are recently granted United States Food and Drug Administration approval for select clinical applications in bone repair. [2, 3] These BMPs act primarily as differentiation factors, turning responsive mesenchymal cells into cartilage- and bone-forming cells. [4] While significant progress has been made in the delivery of recombinant osteogenic proteins to promote bone healing, the short half-life and instability of the protein requires the delivery of milligram quantities of factor or multiple dosages. [2] So delivery of genes encoding for various BMPs have been investigated in various studies. Various transcription factors and growth factors have also been found to enhance the effects of various BMPs. Such factors include VEGF, RunX2, TGF etc.

Intravenous delivery of human osteoprotegerin (hOPG) gene using viral vectors results in systemic circulation of the OPG which in turn inhibits osteoclastic activity. The mechanism involves the binding of OPG to RANKL (receptor activator of nuclear factor κβ ligand) which prevents the binding of latter to RANK. This in turn suppresses its ability to increase bone resorption by osteoclasts. [5, 6]

LIM mineralization protein (LMP) which induces the bone mineralization and expression of various osteogenic genes, BMP-2, RunX2 (Runt related transcription factor X2), OSX (Osterix) etc., and thereby promotes the osteoblast differentiation. One study has also shown that it induces bone formation more efficiently than even BMP-2 [7].

Accounting for the FDA approval status of two BMPs (BMP-2 and BMP-7) for clinical applications, BMPs have been shown to be effective treatment alternatives for treatment of osteoporosis. Additionally, virus based gene delivery of BMP-9 gene has shown the most effective and robust bone formation over delivery of other BMP genes. From this, BMP-9 pDNA was selected for development of non-viral gene delivery vector for osteoporosis treatment.

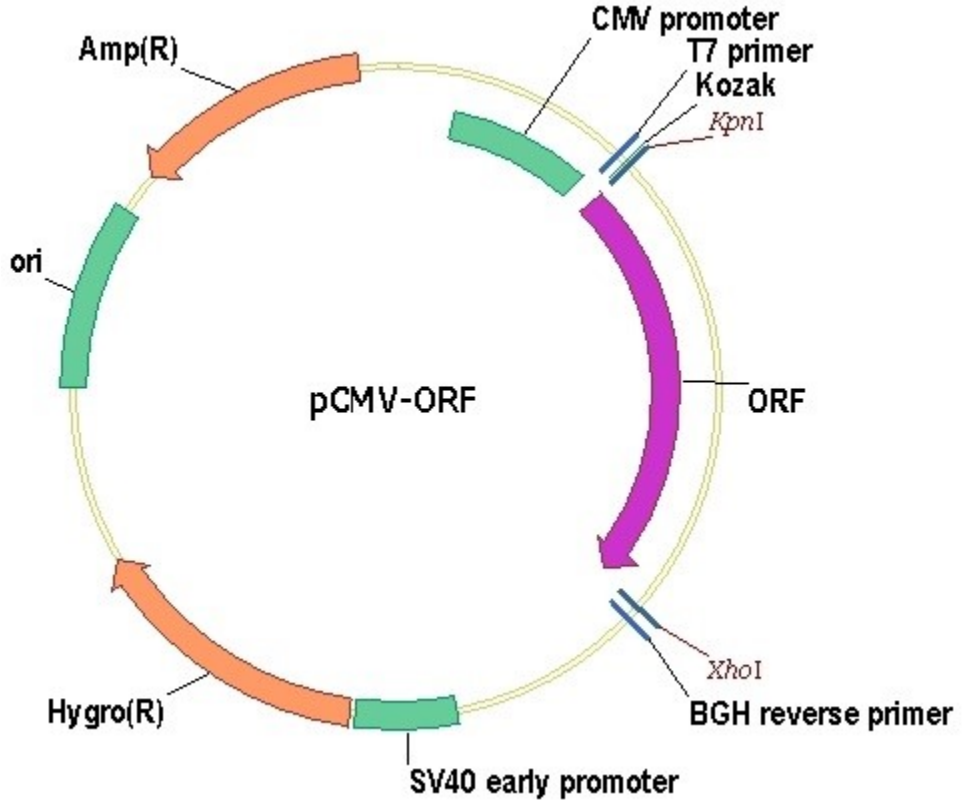
Table 4.1 Various gene delivery approaches used in osteoporosis

Gene therapy with	Encoded protein		Vector for transfection	Use of	Route of administration	Remarks	Ref.
pDNA	IL-1Ra(interleukin-1 receptor antagonist		Adenovirus	Recombinant adenovirus	Intramedullary injection	-	[1]
pDNA	BMP-2		Adenovirus	Recombinant adenovirus	--	-	[8]
pDNA	Bone morpho-genetic proteins (BMPs)	BMP-2	Adenovirus	Recombinant adenovirus and AdBMP-transduced osteoblast progenitors	Intramuscular injection (in quadriceps)	Activity inhibited by BMP-3	[9]
		BMP-3				Negative regulator of bone formation	
		BMP-6				Most robust and mature ossification, Activity inhibited by BMP-3	
		BMP-7				Activity inhibited by BMP-3	
		BMP-9				Most robust and mature ossification, Activity inhibited by BMP-3	
pDNA	BMP-2		Baculovirus	Recombinant baculovirus-transduced hMSCs (mesenchymal stem cells)	Injection into back subcutis	-	[10]
pDNA	BMP-2		Adenovirus with RGD tripeptide containing coat	Recombinant adenovirus-transduced hMSCs		Coat with RGD peptide enhances interaction with MSCs' surface integrins and thus enhance transfaction.	[11, 12]
pDNA	BMP-2 and RunX2		Adenovirus	Recombinant adenovirus-transduced pluripotent C3H10T1/2 cell	Subcutaneous implant	Complementary effect of RunX2 and BMP-2 on bone formation	[13]
pDNA	BMP-4 and vascular endothelial growth factor (VGEF)		Retrovirus	Recombinant retrovirus	Implantation into defect	VEGF and BMP-4 appeared to act synergistically to enhance bone healing	[14]
pDNA	BMP-9		Adenovirus	Recombinant adenovirus-transduced hMSCs	Intramuscular injection	-	[15]

Gene therapy with	Encoded protein	Vector for transfection	Use of	Route of administration	Remarks	Ref.
pDNA and fusion construct of pDNA with immune-globulin constant domain (pDNA-Fc)	Human osteoprotegerin (hOPG)	Adenovirus	Adenovirus	Intravenous injection	-	[16]
pDNA	Human osteoprotegerin (hOPG)	Adenoassociated virus	Recombinant adenoassociated virus	Intravenous injection	-	[5]
pDNA	Human osteoprotegerin (hOPG)	Adenoassociated virus	Recombinant adenoassociated virus	Intramuscular injection	-	[6]
pDNA	BMP-2 and VGEF	Nonviral gene transfer	Gene transfer	Intramuscular injection	VGEF synergized the effect of BMP-2 on ossification	[17]
pDNA	BMP-7	Nanostructured calcium phosphate (NanoCaP)	Fibrin gel matrix of pDNA- NanoCaP	Intramuscular implantation	-	[18]
pDNA	BMP-2	Nanostructured calcium phosphate (NanoCaP)	Collagen Gene activated matrix of pDNA or pDNA-NanoCaP	Subcutaneous transplant or injection in bone-marrow	Modification of GAM with CaP effective in tissue regeneration at lower pDNA level	[19, 20]
pDNA	BMP-2	Nonviral gene transfer	BMP-2 gene-modified autologous MSCs or β-tricalcium phosphate	--	-	[21]
pDNA	LMP-3	Adenovirus	Recombinant adenovirus	Intramuscular injection	More efficient ectopic bone formation in-vivo than BMP-2	[7]

4.1.2 Properties of BMP-9 pDNA and protein

4.1.2.1 Vector map and pDNA description



Gene_bank_ref_id:	NM_016204.1
cDNA Description:	ORF Clone of Homo sapiens growth differentiation factor 2 DNA.
Gene Synonym:	BMP9, BMP-9
Species:	Human
Vector:	pCMV/hygro
Restriction Site:	KpnI + XhoI
Sequence Description:	Identical with the Gene Bank Ref. ID sequence.
Shipping_carrier:	Each tube contains approximately 10 µg of lyophilized plasmid.
Storage:	The lyophilized plasmid can be stored at ambient temperature for three months.

4.1.2.2 pCMV / hygro Plasmid Complete Sequence-Sino Biological Inc.

1 GACGGATCGG GAGATCTCCC GATCCCCTAT GGTGCACTCT CAGTACAATC TGCTCTGATG
61 CCGCATAGTT AAGCCAGTAT CTGCTCCCTG CTTGTGTGTT GGAGGTCGCT GAGTAGTGCG
121 CGAGCAAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA CAATTGCATG AAGAATCTGC
181 TTAGGGTTAG GCGTTTTGCG CTGCTTCGCG ATGTACGGGC CAGATATACG CGTTGACATT
241 GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA
301 TGGAGTTCCG CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC
361 CCCGCCCATT GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC
421 ATTGACGTCA ATGGGTGGAG TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT
481 ATCATATGCC AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT
541 ATGCCCAGTA CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA
601 TCGCTATTAC CATGGTGATG CGGTTTTGGC AGTACATCAA TGGGCGTGGA TAGCGGTTTG
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721 AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG
781 GTAGGCGTGT ACGGTGGGAG GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCCA
841 CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG GGAGACCCAA GCTGGCTAGG
901 CCGCCACCAA GCTTGGTACC GCTAGCGGAT CCGTTAACCT TAAGACCGGT ATGGGCTGGT
961 CCTGCATCAT CCTGTTCCTC GTGGCGACCG CGACCGGGGT CCACAGCGAT ATCATCGATA
1021 GCGCTTAAAC TCGAGTCTAG AGCGGCCGCC GAATTCGGGC CCGTTTAAAC CCGCTGATCA
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1261 GAGGATTGGG AAGACAATAG CAGGCATGCT GGGGATGCGG TGGGCTCTAT GGCTTCTGAG
1321 GCGGAAAGAA CCAGCTGGGG CTCTAGGGGG TATCCCCACG CGCCCTGTAG CGGCGCATTA
1381 AGCGCGGCGG GTGTGGTGGT TACGCGCAGC GTGACCGCTA CACTTGCCAG CGCCCTAGCG
1441 CCCGCTCCTT TCGCTTTCTT CCCTTCCTTT CTCGCCACGT TCGCCGGCTT TCCCCGTCAA
1501 GCTCTAAATC GGGGGCTCCC TTTAGGGTTC CGATTTAGTG CTTTACGGCA CCTCGACCCC
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1621 CGCCCTTTGA CGTTGGAGTC CACGTTCTTT AATAGTGGAC TCTTGTTCCA AACTGGAACA
1681 ACACTCAACC CTATCTCGGT CTATTCTTTT GATTTATAAG GGATTTTGCC GATTTCGGCC
1741 TATTGGTTAA AAAATGAGCT GATTTAACAA AAATTTAACG CGAATTAATT CTGTGGAATG
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1861 TGCATCTCAA TTAGTCAGCA ACCAGGTGTG GAAAGTCCCC AGGCTCCCCA GCAGGCAGAA
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2101 GCTTTTTTGG AGGCCTAGGC TTTTGCAAAA AGCTCCCGGG AGCTTGTATA TCCATTTTCG
2161 GATCTGATCA GCACGTGATG AAAAAGCCTG AACTCACCGC GACGTCTGTC GAGAAGTTTC
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2701 CGCAGGCTCT CGATGAGCTG ATGCTTTGGG CCGAGGACTG CCCCGAAGTC CGGCACCTCG
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3301 GGGATCTCAT GCTGGAGTTC TTCGCCCACC CCAACTTGTT TATTGCAGCT TATAATGGTT
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3421 GTTGTGGTTT GTCCAAACTC ATCAATGTAT CTTATCATGT CTGTATACCG TCGACCTCTA
3481 GCTAGAGCTT GGCGTAATCA TGGTCATAGC TGTTTCCTGT GTGAAATTGT TATCCGCTCA
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3601 TGAGCTAACT CACATTAATT GCGTTGCGCT CACTGCCCGC TTTCCAGTCG GGAAACCTGT
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4381 TTACCTTCGG AAAAAGAGTT GGTAGCTCTT GATCCGGCAA ACAAACCACC GCTGGTAGCG
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4561 TCATGAGATT ATCAAAAAGG ATCTTCACCT AGATCCTTTT AAATTAAAAA TGAAGTTTTA
4621 AATCAATCTA AAGTATATAT GAGTAAACTT GGTCTGACAG TTACCAATGC TTAATCAGTG
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5341 CGGGGCGAAA ACTCTCAAGG ATCTTACCGC TGTTGAGATC CAGTTCGATG TAACCCACTC
5401 GTGCACCCAA CTGATCTTCA GCATCTTTTA CTTTCACCAG CGTTTCTGGG TGAGCAAAAA
5461 CAGGAAGGCA AAATGCCGCA AAAAAGGGAA TAAGGGCGAC ACGGAAATGT TGAATACTCA
5521 TACTCTTCCT TTTTCAATAT TATTGAAGCA TTTATCAGGG TTATTGTCTC ATGAGCGGAT
5581 ACATATTTGA ATGTATTTAG AAAAATAAAC AAATAGGGGT TCCGCGCACA TTTCCCCGAA
5641 AAGTGCCACC TGACGTC

cDNA sequence of the BMP-9 pDNA obtained from SinoBiologicals is shown below which matches with the Homo Sapiens GDF2 (BMP-9) sequence (NM_016204.1)

ATGTGTCCTGGGGCACTGTGGGTGGCCCTGCCCCTGCTGTCCCTGCTGGCT
GGCTCCCTACAGGGGAAGCCACTGCAGAGCTGGGGACGAGGGTCTGCTG
GGGGAAACGCCCACAGCCCACTGGGGGTGCCTGGAGGTGGGCTGCCTGA
GCACACCTTCAACCTGAAGATGTTTCTGGAGAACGTGAAGGTGGATTTCC
TGCGCAGCCTTAACCTGAGTGGGGTCCCTTCGCAGGACAAAACCAGGGTG
GAGCCGCCGCAGTACATGATTGACCTGTACAACAGGTACACGTCCGATAA
GTCGACTACGCCAGCGTCCAACATTGTGCGGAGCTTCAGCATGGAAGATG
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GTTCCCCACAAAGGTGGGCAAGGCCTGCTGTGTGCCCACCAAACTGAGCC
CCATCTCCGTCCTCTACAAGGATGACATGGGGGTGCCCACCCTCAAGTAC
CATTACGAGGGCATGAGCGTGGCAGAGTGTGGGTGCAGGTAA

4.1.2.3 BMP-9 amino acid sequence

BMP-9 amino acid sequence translated from the above gene is shown below.

MCPGALWVALPLLSLLAGSLQGKPLQSWGRGSAGGNAHSPLGVPGGGLPEH
TFNLKMFLENVKVDFLRSLNLSGVPSQDKTRVEPPQYMIDLYNRYTSDKSTT
PASNIVRSFSMEDAISITATEDFPFQKHILLFNISIPRHEQITRAELRLYVSCQNH
VDPSHDLKGSVVIYDVLDGTDAWDSATETKTFLVSQDIQDEGWETLEVSSAV
KRWVRSDSTKSKNKLEVTVESHRKGCDTLDISVPPGSRNLPFFVVFSNDHSSG
TKETRLELREMISHEQESVLKKLSKDGSTEAGESSHEEDTDGHVAAGSTLARR
KRSAGAGSHCQKTSLRVNFEDIGWDSWIIAPKEYEAYECKGGCFFPLADDVT
PTKHAIVQTLVHLKFPTKVGKACCVPTKLSPISVLYKDDMGVPTLKYHYEGM
SVAECGCR

4.1.2.4 Cloning sites/restriction digestion sites

Cloning and restriction digestion sites of the selected vector are shown in **Figure 4.1.**

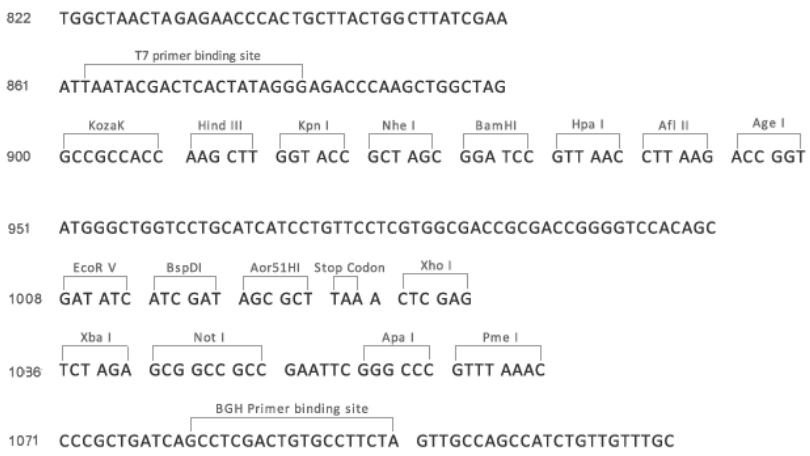


Figure 4.1 Cloning and restriction digestion sites of BMP-9 pDNA

The BMP-9 gene (/mRNA/protein) is conserved indifferent species including rat, mice, chimpanzee, monkeys etc. The sequence is orthologous between different species meaning the gene preserves its activity in all the species.

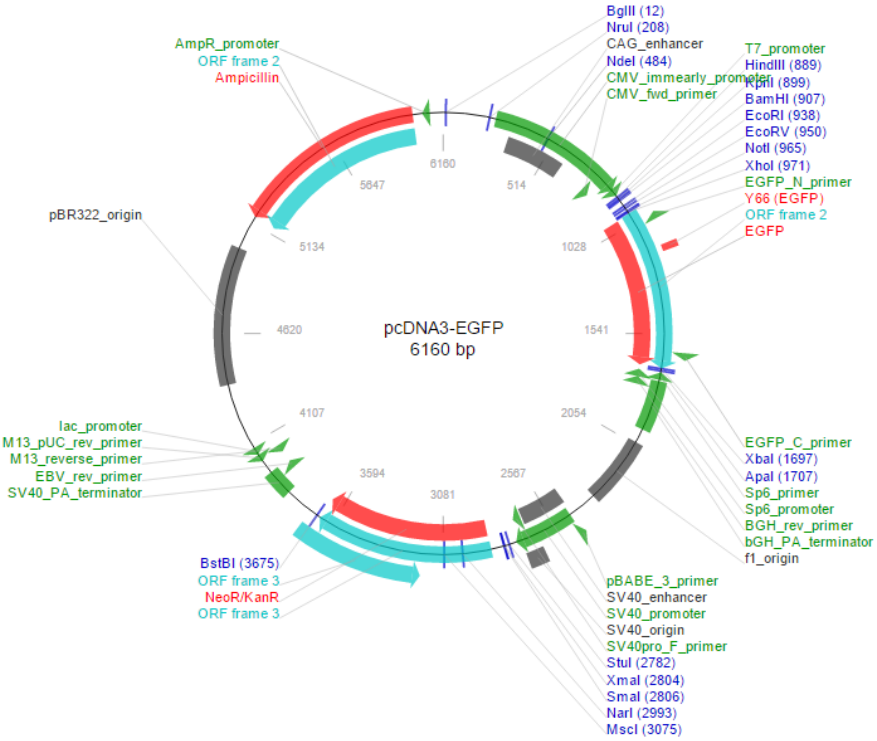
4.1.3 Rationale for selection of eGFP gene

Among these reporter genes, green fluorescent protein has been employed most commonly due to their wide spread established use and ease of availability. Hence, in context of development of gene delivery systems, green fluorescent proteins have become essential tools in order to evaluate the intracellular delivery and expression of genes through easy fluorescence detection systems. Similar to the therapeutic pDNA, the pDNA for GFP can be used to develop lipoplex systems which can be used for in vitro cell line studies in order to evaluate the transfection potential of lipoplex based gene delivery system. Genes for green fluorescent protein are easily available at cost effective rates. Among the variants of Green fluorescent proteins, eGFP gene gives more robust expression of green fluorescence and as the name suggests the enhanced fluorescence activity allows sensitive detection of the gene

4.1.4 Properties of eGFP gene

pcDNA3-EGFP was a gift from Doug Golenbock (Addgene plasmid # 13031). Properties of eGFP plasmid are discussed in coming sections.

4.1.4.1 Vector map and pDNA description



cDNA Description:	**ORF clone of enhanced green fluorescent protein**
Gene Synonym:	Enhanced GFP, eGFP
Vector:	pcDNA3
Restriction Site:	XhoI + XbaI
Shipping_carrier:	pcDNA3 transformed *E.Coli* in agar slab.
Storage:	Plasmid stored at below -20°C for long term storage.

4.1.4.2 pCDNA3/EGFP plasmid complete sequence

GACGGATCGGGAGATCTCCCGATCCCCTATGGTCGACTCTCAGTACAATCTGCTCTGATGCCGCATAGTT
AAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGCGAGCAAAATTTAAGCTACA
ACAAGGCAAGGCTTGACCGACAATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCG
ATGTACGGGCCAGATATACGCGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTC
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GGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATT
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GGATGCGGTGGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGCTGGGGCTCTAGGGGGTATCCCCACGCG
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CCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGC
TCTAAATCGGGGGCATCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGAT
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TTTATAAGGGATTTTGGGGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCG
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GAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCAC
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GGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCT
TCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCAACCTGCCAT
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TCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATT
TGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTC

cDNA sequence of the eGFP pDNA obtained from AddGene is shown below

ACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTGGGGA
TACCCCCTAGAGCCCCAGCTGGTTCTTTCCGCCTCAGAAGCCATAGAGCCCACCGCATCCC
CAGCATGCCTGCTATTGTCTTCCCAATCCTCCCCCTTGCTGTCCTGCCCCACCCCACCCCCC

AGAATAGAATGACACCTACTCAGACAATGCGATGCAATTTCCTCATTTTATTAGGAAAGG
ACAGTGGGAGTGGCACCTTCCAGGGTCAAGGAAGGCACGGGGGAGGGGCAAACAACAGA
TGGCTGGCAACTAGAAGGCACAGTCGAGGCTGATCAGCGAGCTCTAGCATTTAGGTGACA
CTATAGAATAGGGCCCTCTAGATTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGC
GGCGGTCACGAACTCCAGCAGGACCATGTGATCGCGCTTCTCGTTGGGGTCTTTGCTCAGG
GCGGACTGGGTGCTCAGGTAGTGGTTGTCGGGCAGCAGCACGGGGCCGTCGCCGATGGGG
GTGTTCTGCTGGTAGTGGTCGGCGAGCTGCACGCTGCCGTCCTCGATGTTGTGGCGGATCT
TGAAGTTCACCTTGATGCCGTTCTTCTGCTTGTCGGCCATGATATAGACGTTGTGGCTGTTG
TAGTTGTACTCCAGCTTGTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCTTCA
GCTCGATGCGGTTCACCAGGGTGTCGCCCTCGAACTTCACCTCGGCGCGGGTCTTGTAGTT
GCCGTCGTCCTTGAAGAAGATGGTGCGCTCCTGGACGTAGCCTTCGGGCATGGCGGACTT
GAAGAAGTCGTGCTGCTTCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTA

4.1.4.3 eGFP amino acid sequence

Fluorescent Green Protein amino acid sequence translated from the above gene is shown below:

MKQHDFFKSA MPEGYVQERT IFFKDDGNYK TRAEVKFEGD TLVNRIELKG
IDFKEDGNIL GHKLEYNYNS HNVYIMADKQ KNGIKVNFKI RHNIEDGSVQ
LADHYQQNTP IGDGPVLLPD NHYLSTQSAL SKDPNEKRDH MVLLEFVTAA
GITLGMDELY K*

4.2 Transformation of pDNA

Transformation is the process in which exogenous DNA is introduced into the cell. The purpose of transformation of bacteria with foreign plasmid is to use that bacterial cell for storage and amplification of plasmid DNA in order to produce large quantities of it.

4.2.1 Transformation of competent *E. coli* using magnesium chloride

Transformation of plasmid DNA of interest into *E. coli* using the calcium chloride/magnesium chloride - heat shock method is a basic, simple, rapid and widely used technique. As *Escherichia coli* or *E.coli* cells are actively growing bacterial cells, they are commonly used for transformation procedures. Bacterial cell membrane is composed of phospholipid bilayer in which the hydrophilic and electronegative head groups are at the outside of the membrane. Since plasmid DNA is negatively charged and hydrophilic, it cannot pass through a bacterial cell membrane normally. Therefore, the bacteria must be made competent in order to take up the plasmid. Magnesium chloride salt solution solves this problem by neutralizing negative charges

through interaction of positively charged calcium ions with negatively charged phosphates and by creating small holes in bacterial cell membrane. Then the plasmid DNA can be forced into the cell by incubating it with competent bacterial cells stored in calcium chloride solution and given a heat shock by suddenly rising the temperature. The sudden increase in temperature could push a plasmid into a bacterial cell and the cells are then plated out on antibiotic containing media for which they have resistant gene.

4.2.1.1 Material

Bacterial Strain: *Escherichia Coli* strain (E Coli DH 5 α)

Vectors for transformation: BMP-9 pCMV/Hygro vector and eGFP pcDNA3 vector (Profiles shown in **Table 4.2**)

Table 4.2 Profile of Plamid DNA used in the study

Name plasmid	*BMP9*	*eGFP*
Obtain from cell	*E. coli*	*E. coli*
Strain of E coli	DH5α	DH5α
Vector	pCMV vector	pcDNA3-cytomegalovirus
Size of vector	5657 bp	5446 bp
Size of cDNA sequence	1290 bp	700 bp
Selection marker	Ampicillin resistance gene	Neomycin resistance gene

Chemicals: Luria broth and agar were purchased from Himedia, Mumbai.

Media: 2% w/v of sterile Luria veg Broth (LB) in water for initial growth of culture.

Sterile Luria broth agar plates (2% w/v LB and 2% w/v Agar in water).

Sterile Luria broth agar plates (2% w/v LB and 2% w/v Agar in water) with appropriate antibiotic (Ampicillin- 100 µg /ml).

Instruments and special equipments: Centrifuge, Water bath, Laminar Air Flow, Shaker, Incubator.

Buffer solutions:

Transformation Buffer (freshly prepared *E. Coli* transformation buffer). Recipe for 5 mL buffer is shown in **Table 4.3.**

Table 4.3 Recipe for the transformation buffer

Ingredient	Quantity for 5ml
PEG 8000 10%W/V	0.5 gm
1M $MgCl_2$ 50mM mg+2	0.15ml
DMSO 5% W/V	0.25ml
LB	q.s

Required quantity of 1M $MgCl_2$ was prepared. Specified quantity of PEG8000 was added to that and the mixture was then autoclave. After autoclaving required amount of previously filter sterilized DMSO and autoclaved LB were added to prepare buffer.

4.2.1.2 Method

4.2.1.2 a) Preparation of competent cell

a. 25ml culture of DH5α was allowed to grow over night in 2% w/v of sterile Luria veg Broth.
b. 200-400 µl of that culture was taken and inoculated in another 10 ml of sterile Luria veg Broth media. Culture was allowed to grow in shaker incubator at 37°C until OD reaches 0.4-0.5.
c. Meanwhile autoclaved eppendorf was label and kept in ice for pre-cooling.
d. After reaching the specified OD culture was centrifuged at 3000 rpm, 4°C for 10 min to settle down the cells in the pre-cooled round bottom tubes.
e. The cell pellet was re-suspended in $1/10^{th}$ volume of transformation buffer to make them competent (If initial volume was 10 ml, 100 µl buffer was used).
f. These competent cells were stored in 20% of glycerol in previously cooled eppendorf tubes at -70°C.

4.2.1.2 b) Transformation of competent cell (Heat shock method)

a. In two eppendorf tubes, 100 µl of competent cells were taken and to one eppendorf tube 100 ng of specified plasmid DNA was added. One tube is negative control without plasmid and other serves as positive control with plasmid.
b. Both the tubes were kept on ice for 30min.

c. After 30 min, tubes were kept at 42°C for 90 sec in water bath.
d. After this sudden heat shock cells were kept on ice for 2min.
e. To these tubes 300µl of sterile LB was added individually.
f. Cells in the tubes were kept in shaker incubator with mild stirring conditions at 37°C for 45 min and then centrifuged at 5000 rpm, at 4°C for 2 min.
g. Supernatant was removed after centrifugation 100 µl and of sterile LB was added to it for re-suspension of cells.
h. Cells were poured and spread over on agar plates in presence and in absence of antibiotic.
i. Cells were allowed to grow on plates over night at 37 °C in incubator.

4.2.1.2 c) Storage of bacterial cells transformed with plasmid

a. A single colony of plasmid transfected cells was selected from the antibiotic containing agar plate. The cells were allowed to grow in sterile Luria veg Broth containing ampicillin (100 µg /ml of culture) as an antibiotic over night at 37 °C in shaker incubator (200 rpm).
b. Plasmid transfected ells were stored in 20 % of glycerol at -70°C.

4.2.1.3 Result & Discussion

The transformation of both the plasmids into prepared competent cells was confirmed by the growth of the competent cells on agar plates with and without antibiotic to which the plasmid DNA is resistant, which acts as positive and negative control. During transformation of each plasmid, 4 agar plates were streaked (**Figure 4.2**) and observations are noted in **Table 4.4**.

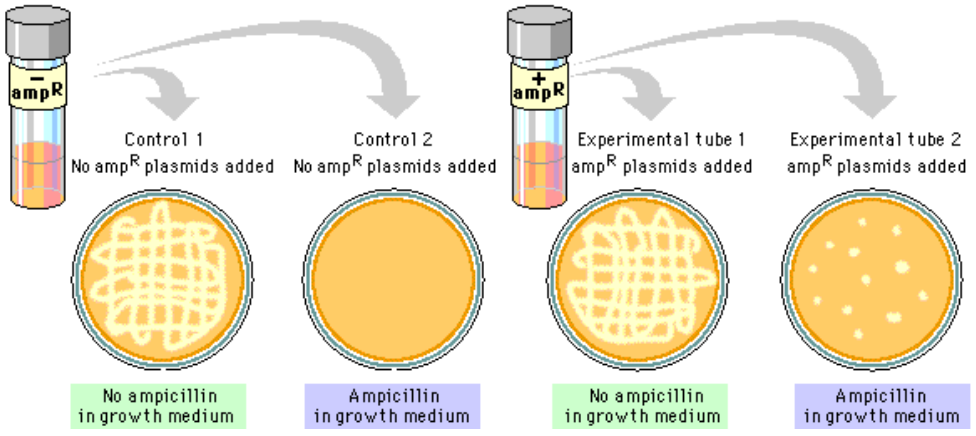

Figure 4.2 Schematic diagram representing the streaked agar plates with different *E. Coli*

Table 4.4 Results observed during transformation of plasmid DNA (BMP9 & EGFP) on agar plate

Agar Plate	Observation	Inference
+ Control: Prepared competent cells were allowed to grow on agar plate in absence of antibiotic.	High cell growth was observed throughout the plate.	Competent cells were intact and able to grow on agar plate.
– Control: Prepared competent cells were allowed to grow on agar plate in presence of antibiotic.	No Cell growth was observed throughout the plate.	Competent cells were sensitive to the antibiotic used and not able to survive.
+ Control Test Plate: Plasmid Transformation Plate: Competent cells were transformed with BMP9 & EGFP plasmids and allowed to grow on agar plate in absence of antibiotic individually.	High cell growth was observed throughout the plate but no discrete colonies were observed.	After transformation cells were intact and able to grow on agar plate. Transformed as well as non-transformed cells were able to grow and hence no separate colonies were found.
– Control Test Plate: Plasmid Transformation Plate: Competent cells were transformed with BMP9 & EGFP plasmids and allowed to grow on agar plate in presence of antibiotic individually.	Separate colonies of cells were observed throughout the plate.	Transformed cells acquiring plasmid DNA with antibiotic resistance were able to grow. This confirms successful transformation of required plasmid DNA into bacterial cells.

The prepared competent cells were found to grow on agar plate without Ampicillin; however, on Amp containing plates, no cell growth was observed indicating complete suppression of cells by Ampicillin. When transformed cells (i.e competent cells treated with plasmid containing Antibiotic resistance marker base pairs) were allowed to grow on Ampicillin containing plates, the actually transformed cells were found to grow because of the Amp resistance acquired by the cells. These transformed cells were developed as small colonies and were further sub-cultured and used or cell multiplication and DNA isolation.

4.2.2. Isolation and purification of plasmid DNA

Plasmids are self-replicating extra-chromosomal DNA molecules found in virtually all bacterial species. Most prokaryotic plasmids are double stranded circular DNA molecules; however, linear plasmids have been identified in both gram-positive and gram-negative bacteria. The size of plasmids varies widely, from several kilo-bases to hundreds of kilo-bases. Plasmid replication may be synchronized with the bacterial cell cycle, resulting in a low number of plasmid molecules per bacterial cell, or independent of the host cell cycle, allowing for the proliferation of hundreds of plasmid copies per cell. Plasmids carry genes that specify a wide variety of functions. DH5α is the most commonly used *E.coli* strain for routine cloning applications because its high transformation efficiency that leads to amplification of plasmid DNA which can be then isolated and purified for therapeutic use. All plasmid vectors contain three common features: a replicator, a selectable marker, and a cloning site. The replicator is a stretch of DNA that contains the site at which DNA replication begins (the origin of replication or ori), and that also includes genes encoding whatever plasmid-encoded RNAs and proteins are necessary for replication. The selectable marker, necessary for following and maintaining the presence of the plasmid in cells is usually dominant and is usually a gene encoding resistance to some antibiotic. The cloning site is a restriction endonuclease cleavage site into which foreign DNA can be inserted without interfering with the plasmid's ability to replicate or to confer the selectable phenotype on its host.

The alkaline lysis method for MaxiPrep® kit (Qiagen, Germany) was used for *plasmid DNA* isolation. Alkaline lysis is probably the most generally useful plasmid preparation procedure. It is fairly rapid, very reliable, and yields crude DNA that can be further purified. Plasmid-bearing E. coli cells are lysed with lysozyme. The lysate is treated with NaOH/SDS solution and potassium acetate then centrifuged to separate plasmid DNA from proteins and chromosomal DNA. The supernatant is treated with isopropanol to precipitate plasmid DNA.

QIAGEN Plasmid Purification Kits are based on the remarkable selectivity of patented QIAGEN resin, allowing purification of ultrapure supercoiled plasmid DNA with high yields. QIAGEN plasmid purification protocols are based on a modified alkaline lysis procedure (**Figure 4.3**), followed by binding of plasmid DNA to

QIAGEN resin under appropriate low-salt and pH conditions (**Figure 4.4**). RNA, proteins, dyes, and low-molecular–weight impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation.

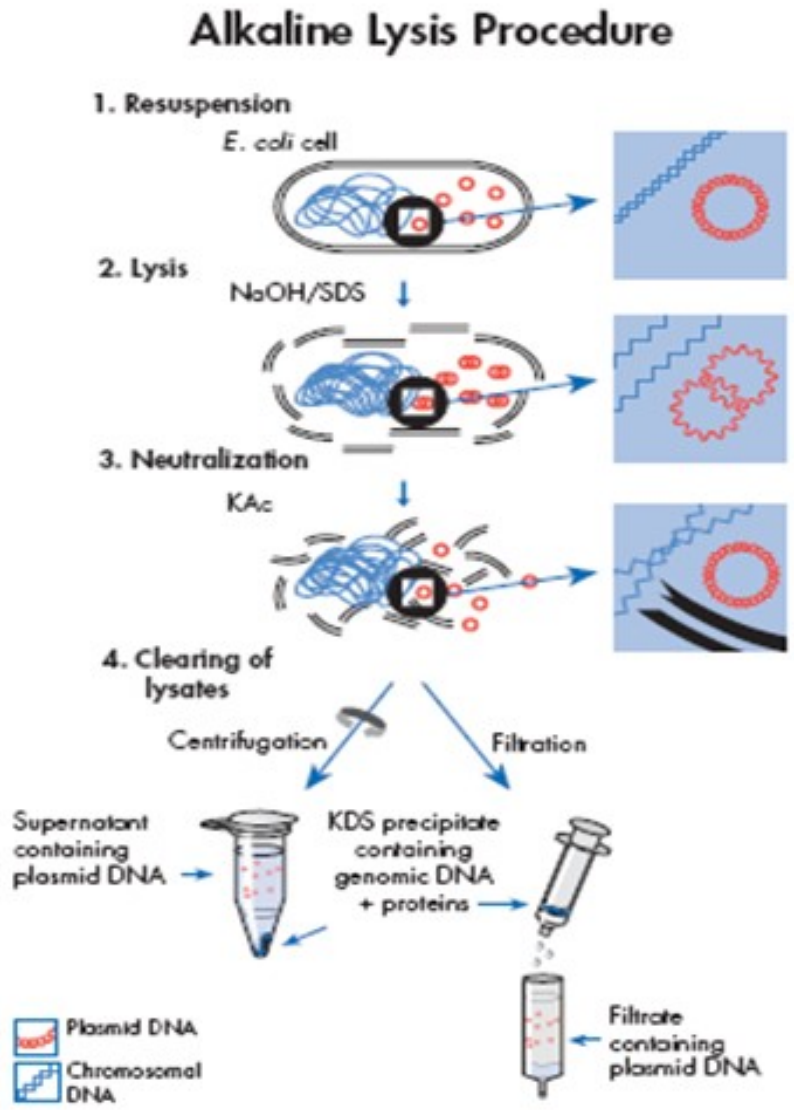

Figure 4.3 Flow chart representing the alkaline lysis and isolation method of Qiagen kit.

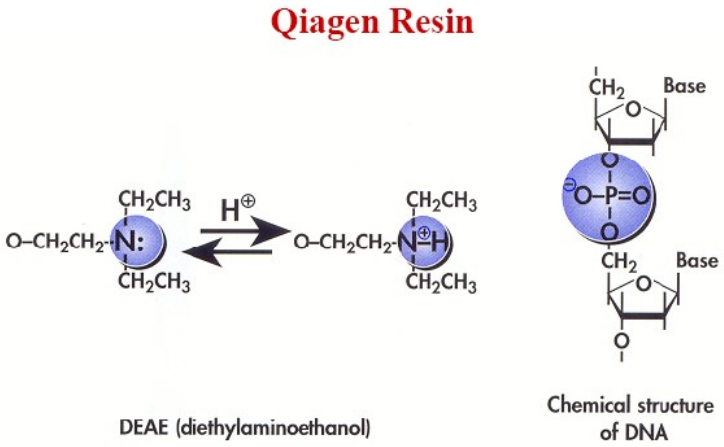

Figure 4.4 Chemical structures of the Qiagen anion exchange resin and DNA showing their respective binding sites

4.2.2.1 Material

Chemicals

Maxi plasmid isolation and purification kit, EDTA, Ethidium bromide, RNAs A solution, Ampicillin, Bromophenol blue, Agarose gel M, LB

Instruments

BOD Incubator with shaker (ThermoScientific, India), Gel electrophoresis (GeneI, India), Gel Doc (Bio Red , USA), Laminar air flow unit (Swastika Pvt. Ltd., Ahmedabad), Centrifuge (Remi centrifuge, India)

Buffer solutions

Buffer compositions are given per liter of solution. Do not autoclave MOPS- or isopropanol containing buffers; sterilize by filtration instead.

Buffer calculations are based on Tris base adjusted to pH with HCl (Tris·Cl). If using Tris·HCl reagent, the quantities used should be recalculated.

- **P1:** Dissolve 6.06 g Tris base, 3.72 g $Na_2EDTA{\cdot}2H_2O$ in 800 ml distilled water. Adjust the pH to 8.0 with HCl. Adjust the volume to 1 liter with distilled water. Add 100 mg RNase A per liter of P1.
- **P2:** Dissolve 8.0 g NaOH pellets in 950 ml distilled water, 50 ml 20% SDS (w/v) solution. The final volume should be 1 liter.

- **P3:** Dissolve 294.5 g potassium acetate in 500 ml distilled water. Adjust the pH to 5.5 with glacial acetic acid (~110 ml). Adjust the volume to 1 liter with distilled water.
- **QBT:** Dissolve 43.83 g NaCl, 10.46 g MOPS (free acid) in 800 ml distilled water. Adjust the pH to 7.0 with NaOH. Add 150 ml pure isopropanol and 15 ml 10% Triton X-100 solution (v/v). Adjust the volume to 1 liter with distilled water.
- **QC:** Dissolve 58.44 g NaCl and 10.46 g MOPS (free acid) in 800 ml distilled water. Adjust the pH to 7.0 with NaOH. Add 150 ml pure isopropanol. Adjust the volume to 1 liter with distilled water.
- **QF:** Dissolve 73.05 g NaCl and 6.06 g Tris base in 800 ml distilled water and adjust the pH to 8.5 with HCl. Add 150 ml pure isopropanol. Adjust the volume to 1 liter with distilled water.
- **Tris-HCl (1 M):** Dissolve121.1 g of Tris base in 800 ml of water. Adjusted pH to 8.0 by adding 42 ml of concentrated Hydrochloric acid. Adjust final pH and make up the volume with water to 1 liter and sterilize by autoclaving.
- **EDTA 0.5 M (pH 8.0):** Dissolve 186.1 g of disodium EDTA. $2H_2O$ in 800 ml of water with vigorous stirring on a magnetic stirrer, adjust pH to 8.0 with sodium hydroxide and make up the volume with water to 1 liter and sterilize by autoclaving.
- **10X Tris-EDTA (TE):** 100 mM of Tris-Cl (pH 8.0) and 10 mM EDTA (pH 8.0) in water was prepared and the solution was sterilized by autoclaving and stored at room temperature.
- **50 X Tris-Acetate-EDTA buffer (TAE):** Dissolve 242 g of Tris base in 500 ml of autoclaved water and mix with 100 ml of 0.5 M EDTA (pH 8.0) and 37.1 ml of glacial acetic acid. Make up the volume to1 liter with autoclaved water and store at 4^0 C
- **Ethidium Bromide:** Dissolve 10 mg of Ethidium Bromide in 1 ml of sterile water. Cover the solution in eppendorf tube by aluminum foil and store in cool and dark place.
- **Gel Loading Dye (Bromophenol Blue):** The gel loading dye is prepared by preparing 0.25 % w/v solution of Bromophenol Blue in 30 % v/v glycerol in water.

- **10 mM Tris (pH 8.0):** 1 ml of 1 M Tris buffer was diluted to 100 ml with sterile water with pH maintained to 8.0.
- **70 % Ethanol:** Absolute ethanol diluted to 70% v/v by water.

Media

2% w/v of sterile Luria veg Broth (LB) in water with appropriate antibiotic (Ampicillin- 100 µg/ml).

Culture

E. Coli culture

4.2.2.2 Method

1. E.coli DH5α cells were previously transformed with plasmid of interest and a single colony of those cells from a freshly streaked agar plate was selected.
2. That colony was used to inoculate the starter culture of 5 ml LB medium containing the 100 mcg/ml ampiciline as an antibiotic. The culture was incubated for approx 8 hr at 37°C with vigorous shaking (approx. 200 rpm) in shaker incubator.
3. 100µl of starter culture was used to inoculate 100ml of LB containing the same antibiotic as starter culture and allowed to grow at 37°C for 12–16 hr with vigorous shaking (approx 200 rpm) in shaker incubator. (For optimum growth and cell density of approximately 3-4 x 10^9 cells per milliliter a flask with a volume at least 4-5 times the volume of culture was used).
4. The bacterial cells from this culture were harvested by centrifugation at 6000 x g for 15 min at 4°C.
5. The bacterial cell pellet was then re-suspended using the 10 ml of Buffer P1 in which RNAse A was previously added. The bacterial cells were re-suspended properly in sealed tube by vortexing or pipetting up and down until no cell clumps in the culture remain.
6. Then in the same suspension 10 ml of Buffer P2 was added and mixed thoroughly by vigorously inverting the sealed tube 4–6 times, and incubated at room temperature (15–25°C) for 5 min.
7. To this 10 ml of chilled Buffer P3 was added, mixed immediately and thoroughly by vigorously inverting the tubes 4–6 times, and incubated on ice for 20 min.
8. The suspension was centrifuged at 20,000 x *g* for 30min at 4°C and supernatant containing plasmid DNA promptly was collected.

9. The supernatant was again centrifuged at 20,000 x *g* for 15 min at 4°C and collected.
10. QIAGEN-tip 500 was equilibrated by applying 10 ml of QBT buffer, and the column was allowed to empty by gravity flow.
11. The supernatant from step 9 was applied to the QIAGEN-tip and was allowed to enter the resin by gravity flow promptly.
12. The QIAGEN-tip column was then washed with 30 ml of QC buffer twice to remove contaminants.
13. The plasmid DNA of our interest was collected in 15 ml of Buffer QF. Collect the elute in a 15 ml or 50 ml tube.
14. DNA was then precipitated by adding 10.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA. It was mixed and centrifuged immediately at ≥15,000 x *g* for 30 min at 4°C. The supernatant was carefully decanted.
15. Obtained DNA pellet was washed with 5 ml of room-temperature 70% ethanol, and centrifuged at ≥15,000 x *g* for 10 min. The supernatant was carefully decanted without disturbing the pellet.
16. The pellet was allowed to air-dry for 5–10 min, and the DNA was redissolved in a suiTable 4.volume of buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris·Cl, pH 8.5). Purified pDNA was stored in aliquots at -20°C until further use.

4.2.2.3 Result & Discussion

The plasmid DNA was isolated from the working culture of the E. Coli transformed strains using the alkaline lysis method. At each step of isolation procedure, samples were collected and used for gel electrophoresis to track the process outcome i.e. removal of protein impurities, siRNA etc according to the manufacturer's protocol. The purity of the plasmid was ascertained by agarose gel assay and UV spectrophotometry for absorbance detection at 260 and 280 nm. The ratio of absorbance between 1.8-2.0 at 260 nm and 280 nm indicated pure plasmid devoid of protein and RNA. The concentration of plasmid is determined by absorbance at 260 nm by comparing with the standard calibration curve or by the equation:

$$1\ OD_{260} = 50\ \mu g \text{ of plasmid DNA/ml.}$$

Different culture conditions i.e. culture volume, culture vessel volume, inoculum size, antibiotic concentration and agitation speed were evaluated for preliminary optimization of the transformation and isolation process. The results in terms of pDNA recovery are shown in **Table 4.5**.

Table 4.5 Effect of various culture parameters on the plasmid recovery

Volume of culture (mL)	Flask volume (mL)	Inoculum (ml)	Ampicillin concentration (100 µg/mL)	Rotaion speed (rpm)	OD (@600 nm)
100	250	1	100	150	2.6
100	250	1	100	200	3.1
100	250	2	100	200	3.6
100	500	1	100	200	5.3
200	1000	2	200	200	4.08

From preliminary observations, some general conclusions can be drawn such as increasing in flast rotation speed led to increase in OD indicating higher plasmid. Increase in the flask volume from 250 mL to 500 mL for a fixed volume of culture (100 mL) led to increased BMP-9 pDNA content. However, further increase in the flask to a 1000 mL doesn't provide additional advantage and rather, reduced the plasmid content. Based on the afore said screening 100 mL culture volume in a 5 times flask volume (500 mL) with 1 mL inoculum size at 200 rpm was chosen for subsequent isolation of plasmid. Process parameters optimized for BMP-9 pDNA transformation and isolation were used for eGFP pcDNA isolation as well.

4.3 Plasmid Digestion

Plasmid digestion studies are helpful in determining the identity of the isolated pDNA. Isolated plasmid DNA can be exposed to different restriction digestion enzymes and then can be loaded on the agarose gel against the DNA molecular weight marker. The molecular weight of the isolated pDNA can be confirmed by comparing the distanced traveled by pDNA with the corresponding molecular weight marker.

4.3.1 Materials

1. BMP-9 and eGFP purified pDNA
2. Molecular Markers (1kb DNA molecular weight marker, Himedia, India)

3. Restriction enzyme, HindIII and XhoI for BMP9 and HindIII for eGFP Fermentas, USA)
4. Restriction Endonuclease Buffer. (HindIII buffer from Fermentas, USA and V2 buffer for XhoI, Vivantis, USA)

4.3.2 Method

The digestion of the isolated plasmid was carried out by restriction endonuclease enzyme. Briefly, To the 1 µg of plasmid DNA, 14.5 µl of autoclaved distilled water, 2 µl of 10 X buffer solution, 1 µl of restriction enonuclease buffer and 1 unit (0.5 µl) of restriction endonuclease enzyme was added. The mixture was allowed to incubate overnight at 37°C. (For detailed protocol, refer manufacturer's manuals)

The enzymes linearize the plasmid and it was allowed to run along with the linear molecular marker on 1.2% w/v agarose gel. Briefly, the above cocktail was loaded along with molecular marker and supercoiled pDNA in three different wells into 1.2 % agarose gel containing ethidium bromide and the fluoroscence was detected under UV transilluminator (GelDoc Image XR+, BioRad, USA). Images were captured by ImageLab software ver. 5 (BioRad, USA). The band position of our isolated plasmid is compared with the band of known molecular weight of marker to verify molecular weight of isolated plasmid.

4.3.3 Result & Discussion

The digestion of the isolated plasmid was carried out by restriction endonuclease enzyme for confirming the pDNA with the tansformed DNA. The isolated pDNA after linearizing with restriction digestion showed a single strong band on the agarose gel while undigested intact pDNA showed typical two band characteristic (**Figure 4.5** and **Figure 4.6**). BMP-9 and eGFP pDNA when transformed, isolated, and digested by enzymes showed migration on the agarose gel corresponding to their theoretical weights on basis of the map provided by supplier. The molecular weights were confirmed by the molecular weight markers, which were run alongside the plasmids. The BMP-9 plasmid showed a linear band corresponding to molecular weight of 5657 bp (**Figure 4.5**) and eGFP plasmid showed the linear band corresponding to its molecular weight of 5446 bp (**Figure 4.6**) theirby confirming the plasmid DNA.

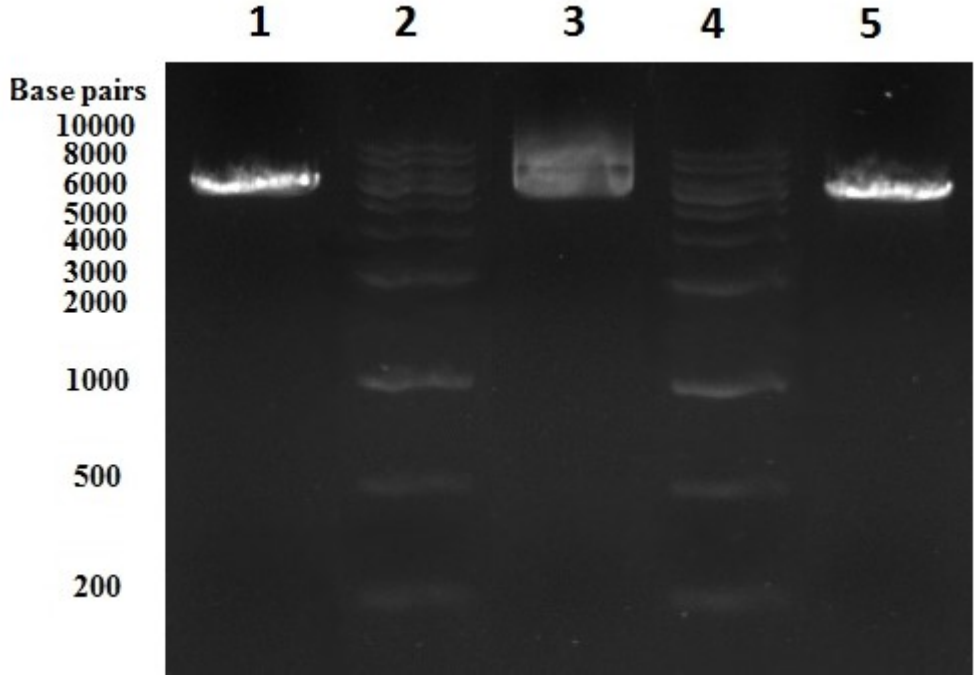

Figure 4.5 Restriction Digestion of BMP-9 pDNA by HindIII and XhoI enzyme

(Lane 1: HindIII digested pDNA, Lanes 2,4=1 kb DNA ladder, Lane 3=naked pDNA, Lane 5= XhoI digested pDNA)

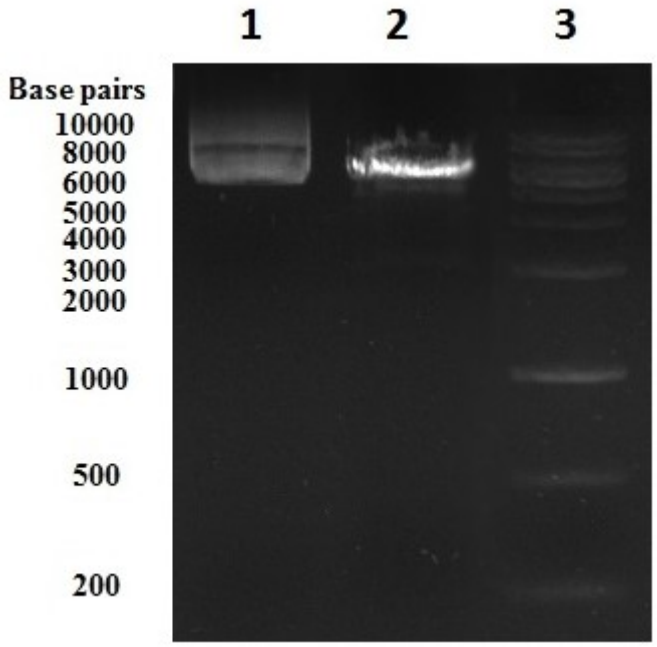

Figure 4.6 Restriction digestion of eGFP pDNA by HindIII enzyme
(Lane 1: naked pDNA, Lane 2= HindIII digested pDNA, Lanes 3 =1 kb DNA ladder)

Agarose gel electrophoresis after restriction digestion confirmed the identity of the isolated BMP-9 and eGFP pDNA.

4.3 References

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