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IMPROVED BACTOFECTION METHOD FOR ENHANCED DNA DELIVERY USING INVASIVE *E. COLI* DH10B IN HUMAN CELLS

Doctor of Philosophy

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ABSTRACT

Non-pathogenic *E. coli* vector is a safer alternative to viral vectors for gene delivery, but low proficiency remains a major drawback for their use as gene delivery vectors. This study presents a simple yet effective strategy for boosting up the invasive *E. coli* vector's efficiency in human cells. Firstly, *E. coli* bactofection without any reagent's addition was analyzed by detecting EGFP reporter gene's expression through flow cytometry, in nine varied human epithelial cells including lung, brain, kidney, and liver cells. Invasive *E. coli* displayed varying bactofection efficiency with the highest of 16% transgene delivery in cervical cells. Further, the bactofection efficiency of the *E. coli* vector was enhanced by adding commercially available transfection reagents known to help various vectors' entry (Trans-bactofection method), lysosomotropic reagents for vector's escape from lysosomal degradation or antibiotics for internalized bacterial cell lysis. Most eminently, the simple addition of Tetracycline (antibiotic) alone to invaded cells revealed transgene delivery up to ≈98% in HeLa and from 2% to 70% in MCF-7 cells. Moreover, combined administration of all the three reagents together with the invasive *E. coli* was also analyzed in certain cells, which resulted in ≈40% transgene expression in lung cells, in contrast to ≤1% GFP obtained by invasive *E. coli* alone.

Besides delivering reporter gene, bactofection of the 100 kb Bacterial Artificial Chromosome (BAC) DNA which carries human β-globin gene on it, resulted in ≈12% GFP expression without any treatment. Whereas, combined treatment method has helped to boost up globin BAC delivery up to 5 fold, resulting in ≈64% GFP in fibroblast cells.

The improved bactofection method which shows comparable efficiency to well-established viral or liposome-mediated gene transfers could be employed for escalating gene transferability of other bacterial vectors also, harboring therapeutic genes.

GENERAL DECLARATION

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis. This thesis contains data which has been used for manuscript preparation to be submitted soon to peer review journals for publication. Contents for prepared manuscript are presented in Sections 4.1.2, 4.2, 4.3 and 4.4. Section 4.5 of the thesis will also be used for another manuscript preparation and subsequent publication. My contribution to the work involved is as indicated:

Thesis Section	Title	Publication Status	Nature and extent of candidate's contribution
4.1.2, 4.2, 4.3 and 4.4	Tissue-dependent bactofection analysis and enhancement of *E. coli* DH10B mediated cytosolic transgene delivery in human epithelial cells	Manuscript in preparation	All experimentation; data collection, analysis & interpretation; writing and editing of the manuscript

Signed: Alviya Sultana

Date: 14/08/2017

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1. INTRODUCTION

Bacteria have been used as a gene carrier in various mammalian cells for over 3 decades still, it could not enter clinical trials of gene therapy due to its low transgene delivery efficiency. Hence, this project has been designed to focus on understanding transgene delivery behavior of non-pathogenic invasive *E. coli* DH10B in various human cell lines and further modification of existing bacteria mediated delivery (bactofection) method to improve its potential as a gene delivery vehicle in human cells.

Over the time various pathogenic bacterial species have been used in attenuated form for exogenous DNA and other molecules' delivery purposes and have shown efficient delivery in diversified mammalian cells (Bauer *et al.,* 2005; Vecino *et al.,* 2004; Krusch *et al.,* 2002; Darji *et al.,* 2000; Dietrich *et al.,* 1998; Sizemore *et al.,* 1997). But, since they are pathogenic strains and were used in attenuated forms there is always associated risk of virulence reverence causing infection or autoimmune reaction induction in the host (Yoo *et al.,* 2011; Vassaux *et al.,* 2006). This led to the development of non-pathogenic bacterial species i.e. *E. coli* which is otherwise non-invasive but was engineered to make it invasive and enter mammalian cells (Narayanan and Warburton, 2003; Grillot-Courvalin *et al.,* 1999; Grillot-Courvalin *et al.,* 1998). Although genetic modifications made *E. coli* invasive and help to enter into a diverse type of mammalian cells yet its efficiency remained low (Narayanan and Warburton, 2003; Grillot-Courvalin *et al.,* 1998). Nevertheless, efficiency obtained by invasive *E. coli* vectors suggest the requirement of further modifications, in order to achieve notable gene delivery efficiency by engineered invasive *E. coli*.

1.1 Gene Therapy

Genes encode proteins which have specialized functions in cells. Any sort of change in a gene (i.e. mutations in base sequence) could lead to disruption of particular protein functioning and eventually to disease. Examples of such diseases could be hemophilia, phenylketonuria and cystic fibrosis (Welch *et al.,* 2007; Mendell *et al.,* 2001). Gene therapy involves replacing the mutated gene with the normal functional gene in a human cell to compensate for the defective gene. Examples of such corrections are *ex vivo* studies for correction of X-linked human severe combined immunodeficiency (SCID)-X1 disease, treating an autosomal recessive disease β-thalassemia in an adult patient by delivering gene for β-globin and restoration of immunity in immunocompromised mice after delivery of IFN-γ encoding gene (Cavazzana-Calvo *et al.,* 2010; Cavazzana-Calvo *et al.,* 2000; Paglia *et al.,* 2000). Moreover, it can also be used in a way to transfer the DNA coding for therapeutic protein required for the treatment as done in the case of Cystic Fibrosis, which involved the transgene expression in target cells coding for functional CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) protein, whose malfunctioning is responsible for the disease (Rocchi *et al.,* 2010; Krusch *et al.,* 2002).

Overall, transferring the functional therapeutic genes efficiently into the target cells is a major goal for gene therapy approach. DNA entry into mammalian cells by spontaneous means has been shown to be inefficient due in large part to the negative charges present on both the mammalian cell membrane and on DNA molecule displayed by the phosphate backbone of DNA (Felgner and Ringold, 1989; Felgner *et al.,* 1987). Moreover, naked DNA being used for transfection is susceptible to mechanical stress and degradation/shearing as suggested by reports revealing the intramuscularly or intravenously injected plasmid DNA degradation of 70 - 90%

after 90 min. of injection (Seternes *et al.,* 2007; Barry *et al.,* 1999). Therefore, an efficient and effective vector for gene transfer is required for gene therapy applications.

Vectors for DNA delivery are categorized classically as, viral and nonviral comprising the two major classes of DNA delivery methods, infection (viral) (Gao *et al.,* 2007; Brunetti-Pierri *et al.,* 2005) and transfection (nonviral) (Seternes *et al.,* 2007; Estruch *et al.,* 2001). Despite the various qualities offered by viral vectors such as stable gene expression, capability of infecting dividing as well as non-dividing cells, still the low packaging DNA capacity, generation of immunogenicity, insertional mutagenesis and toxicity is a major concern with researchers using these vectors (Harvey *et al.,* 2011; Cohen, 2007; Wakimoto *et al.,* 2003).

To overcome the stated drawbacks, non-viral vectors are gaining popularity as a significant alternative (Yin *et al.,* 2014; Gao *et al.,* 2007; Magin-Lachmann *et al.,* 2004). The relatively simple and safer technique of non-viral gene transfer has been successfully used to combat inherent safety issues with viral vectors. Non-viral gene transfer can be carried out in various ways, which include but are not limited to physical, chemical and biological methods. Physical approach employs physical force which facilitates transgene delivery into cells by permeating the cell membrane, using methods such as micro-injection, electroporation, ultrasound, gene gun and hydrodynamic delivery (Heller *et al.,* 2005; Lawrie *et al.,* 2000; Liu and Fréchet, 1999; Zhang *et al.,* 1999; Sun *et al.,* 1995; Wolff *et al.,* 1990; Neumann *et al.,* 1982). Chemical methods involve the use of synthetic or natural compounds, including cationic liposomes, polymers, and polyamines, as carriers for intracellular gene transfer (Liu *et al.,* 2009; Neu *et al.,* 2005; Wang *et al.,* 2002; Roy *et al.,* 1999). Whereas, biological approaches utilize virulence-attenuated bacteria such as *Shigella flexneri*, *Salmonella typhimurium* and *Listeria monocytogenes*, to carry and deliver plasmid DNA into host cells (van Pijikeren *et al.,* 2010;

Bauer *et al.,* 2005; Vecino *et al.,* 2004; Grillot-Courvalin *et al.,* 2002; Dietrich *et al.,* 1998; Sizemore *et al.,* 1997). Injecting naked DNA is the simplest technique for non-viral mediated gene transfer. Uncomplexed DNA has been successfully injected into muscle cells but is associated with low transfection efficiency and rapid clearance (Seternes *et al.,* 2007; Zhang *et al.,* 1999; Wolff *et al.,* 1990). Particle bombardment using gene gun for mammalian cells has been shown to deliver the gene coated particles into those cells and have also been exploited as a DNA vaccination strategy for treating various human tumors (O'Brien and Lummis, 2005; Surman *et al.,* 1998). However, the process is limited to deliver genes in mucosa, skin or tissues which are surgically exposed (Yang *et al.,* 1990). Another process for delivering DNA into the target cells involves the increase in transmembrane potential by applying short electric impulse resulting in pore formation and DNA uptake by cells have been tested in many mammalian tissues (Heller *et al.,* 2005). Electroporation technique showed 2-3-fold higher reporter gene expression than naked DNA injection and delivered 100 kb DNA effectively into muscle cells and also showed stable expression up to 1 year (Magin-Lachmann *et al.,* 2004; Molnar *et al.,* 2004). *In vivo* application of the electroporation technique poses a few limitations such as a surgical process to keep electrodes in the tissue and high voltage application could damage tissue irreversibly (Gao *et al.,* 2007).

So far the most studied strategy of non-viral gene transfer is by either complexing the DNA with cationic lipids or cationic polymers or transfer by using the bacterial cell as a vector (bactofection). This approach has shown the functional transfer of large size genes in cultured human cells although with low efficiency (Rocchi *et al.,* 2010; Pérez-Luz *et al.,* 2007; Laner *et al.,* 2005; da Cruz *et al.,* 2004; Estruch *et al.,* 2001). Thus, the present study aims to imply and

improve the non-pathogenic bacterial strain (*E. coli*) as a gene delivery vehicle in different human cells derived from various tissues.

Potential of bacteria as gene delivery vehicle was demonstrated about two decades ago by Schaffner (1980). Initially, spheroplasts (bacteria without a cell wall) were used with chemical agents (Polyethylene glycol or calcium phosphate) for phagocytosis mediated DNA transfer in mammalian cells (Heitmann and Lopes-Pila, 1993; Schaffner, 1980). Later, various bacterial species have been used for delivering various drugs, DNA or for vaccination purposes as elaborated in section 1.1.2.

Amongst various non-viral DNA delivery methods, chemical and bacterial assisted gene delivery gained popularity due to certain limitations faced by the use of naked DNA such as mechanical stress, degradation/shearing, and involvement of complicated machines in physical methods (Gao *et al.*, 2007; Seternes *et al.*, 2007; Barry *et al.*, 1999). Transfection reagents, positively charged chemicals used for condensing negatively charged DNA molecule prior to delivery, are well-known enhancers of transfection efficiency and have been proven to potentially transfer nucleic acid in cultured mammalian cells (Nikcevic *et al.*, 2003; Felgner and Ringold, 1989). Bactofection referring to bacteria mediated gene delivery, on the other hand, employs attenuated and genetically modified strains of bacteria carrying the gene of interest to be delivered to the target tissue or an organism (Van Pijkeren *et al.*, 2010; Grillot-Courvalin *et al.*, 1998; Schaffner, 1980).

Summing up, issues of limited DNA packaging efficiency, production complexity and unfavorable toxic and immunological effects associated with viral methods can be avoided by adopting non-viral methods for gene delivery (Wu *et al.*, 2010; Seow and Wood, 2009). Non-

viral gene delivery besides being safer alternative, allows long-term and high levels of gene expression of delivered gene, such as in fibroblast cells for lysosomal storage disorder (Estruch *et al.,* 2001), in neural cells (da Cruz *et al.,* 2004), in glioblastoma cells for tumor suppression (Hsiao *et al.,* 1997). Hence, non-viral delivery vectors were exploited as gene delivery carriers in the current study and are discussed comprehensively in upcoming sections (Section 1.1.1 and Section 1.1.2).

1.1.1 Chemical/Synthetic Carriers

Liposomal or multicomponent transfection reagents i.e. transfection reagents derived from various components such as protein, carbohydrate, lipids have been proven as a suitable method of nucleic acid transfection into mammalian cell lines (Perez-Luz *et al.,* 2007; Magin-Lachmann *et al.,* 2004).

The principle involves the interaction of nucleic acids (negatively charged) with carrier molecules (positively charged), like polymers or lipids, enabling the nucleic acid to contact with the cellular membrane (containing negatively charged membrane components) followed by incorporation into the cell through endocytosis (Mehier-Humbert and Guy, 2005).

DNA/RNA complexed with a cationic liposome (e.g. DOTMA) which covered its negative charge has been used initially to transfect mammalian cells (Felgner and Ringold, 1989). Till date, numerous reagents have been used to successfully transfer the nucleic acid in various mammalian cells, such as the use of Lipofectamine and Effectene for delivering plasmid DNA into mammalian cell line with the average of 38% transfected cells without any cytotoxicity have been shown (Nikcevic *et al.,* 2003). High transfection efficiency, low toxicity, and cost-effectiveness made cationic liposomes an interesting reagent for gene delivery

(Sakaguchi *et al.,* 2008). Polyplexes, polymer-based vectors, have shown systemic gene delivery to pancreatic adenocarcinoma with appreciable transfection efficiency (Miyata *et al.,* 2008). Polyethylenimines, second cationic polymer based transfection reagent discovered after poly L-Lysine (Boussif *et al.,* 1995) promotes an elevated level of transgene expression in a target cell by helping in the endosomal escape of the DNA and its nuclear localization (Kichler *et al.,* 2001; Godbey *et al.,* 1999). The qualities offered by polyethylenimines renders them an elicit means of DNA transfection.

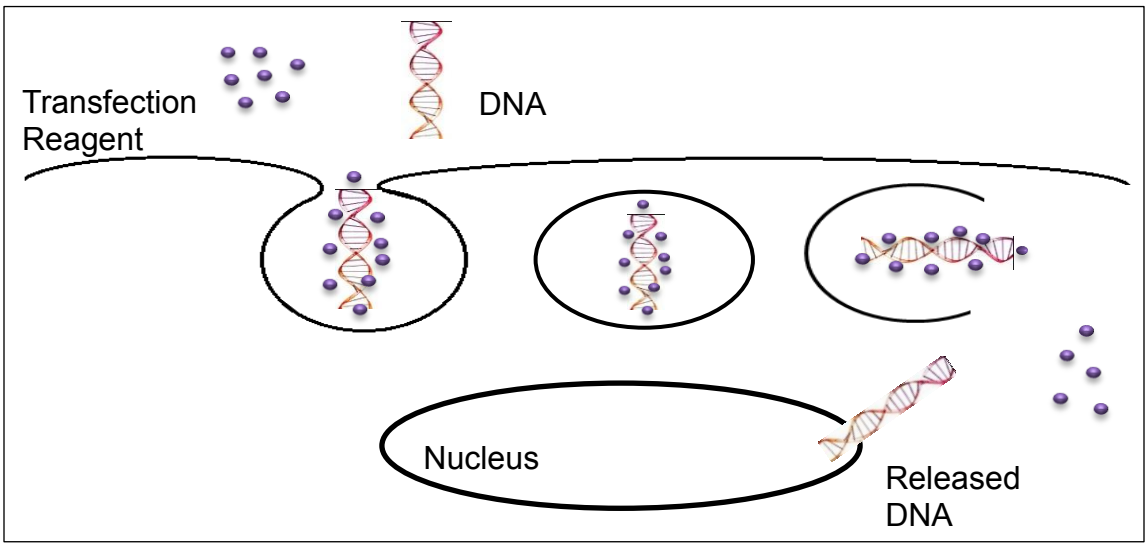


Figure 1.1. Exogenous DNA delivery into host mammalian cell carried by transfection reagent. Negatively charged DNA molecules upon incubation with an oppositely charged transfection reagent, results in the DNA-reagent complex formation. This helps in DNA condensation and further transportation into host cells via endocytosis, eventually leading to their cargo release in cell cytosol. Exogenous DNA is then transferred to the nucleus by various mechanisms for expression.

Cationic lipids have proven minimally toxic to mammalian cell without any compromise in DNA transfection efficiency (Mellet *et al.,* 2011; Shirazi *et al.,* 2011). Moreover, there is evidence of successful transfection of protein molecules ranging between 66 kDa - 540 kDa molecular weight producing up to four-fold protein transfection rate in mammalian cells (Loudet *et al.,* 2008; Mahlum *et al.,* 2007; Tinsley *et al.,* 1998).

In view of the capability of transfection reagents as a carrier of large molecules such as proteins, certain transfection reagents which are known for DNA and protein deliveries have been used in this project to study their effect in transferring bacterial cell in the cultured mammalian cell. Bacterial cell and cationic transfection reagent complex could result in increased transfection efficiency (Lonez *et al.,* 2008; Campanha *et al.,* 1999), presumably due to partial positive charge given by transfection reagent which may allow closer association of the complex with the mammalian cell membrane (negatively charged) and efficient transgene release in cell cytoplasm due to endosomal escape. This could aid in bacterial gene delivery capacity by enhancing the number of mammalian cells expressing transfected DNA.

1.1.2. Bacteria-mediated Gene Delivery/Bacterial Vectors

Since the use of *E. coli* for delivering DNA into mammalian cells by Schaffner in 1980, bacteria emerged as a promising alternative class of vectors for delivering the gene into mammalian cells. Bacterial species that have been employed to deliver gene in a variety of mammalian cells are *Shigella flexneri, Salmonella* spp., *E. coli, Yersinia enterocolitica* and *Listeria monocytogenes.* Attenuated strains of invasive *Shigella* and *Salmonella* have shown potential for gene delivery in mammalian cells (Michael *et al.,* 2004; Sizemore *et al.,* 1995). In due course, non-pathogenic and otherwise extracellular *E. coli* has been engineered and used for functional delivery of large DNA in non-phagocytic mammalian cells which could be proved

helpful in gene therapy where transfer of large size DNA containing the regulatory elements, is required for their appropriate expression (Narayanan and Warburton, 2003; Grillot-Courvalin *et al.,* 1998). Engineered *E. coli* has been successfully used in delivering functional DNA segment of up to 200 kb (Narayanan and Warburton, 2003).

Successful gene transfer from bacterial cell requires adhesion to host cells, cellular invasion followed by intracellular DNA release (Grillot-Courvalin *et al.,* 1998). Different strategies are executed by different species, such as *Salmonella* or *Shigella*, after contacting host cells cause bacteria to secrete a set of invasion proteins triggering intracellular signaling events, and eventual bacterial uptake by micropinocytosis (Galan, 1996). Whereas binding of *Yersinia pseudotuberculosis* invasin ligand to β1 integrins present on host cell results in its entry into the cell through a zipper-like mechanism (Isberg and Van Nhieu, 1994). Furthermore, the interaction of internalin with E-cadherin mediates entry of *Listeria monocytogenes* into certain cell types (Lecuit *et al.,* 1997).

Upon internalization, the bacterial cell is trapped in the endocytic vesicle. *Listeria* is capable of escaping from the endosome, triggered by the release of listeriolysin O (LLO) encoded by hemolysin gene (*hly*) shortly after uptake, causing the disruption of vacuolar membrane (Gaillard *et al.,* 1987). Moreover, *Shigella* is also capable of escaping from endocytic vesicle by toxin production, and this was first exploited by Sizemore *et al.,* (1995) to transfer DNA into mammalian cell cytoplasm, by using a diaminopimelate (DAP) auxotroph mutant of *Shigella.* The DAP auxotroph mutant was produced by deletion of the *asd* gene which encodes aspartate β-semialdehyde dehydrogenase, an essential enzyme required for synthesis of diaminopimelic acid (DAP) which forms a major constituent of bacterial cell membrane. The absence of DAP in mammalian cell cytoplasm renders *Shigella* incapable of cell division upon

entry into mammalian cell due to impaired cell-wall synthesis leading to bacterial death and subsequent release of DNA (Sizemore *et al.,* 1995).

The otherwise non-invasive *E. coli* was engineered and made invasive by transforming invasin gene (*inv*) of *Yersinia tuberculosis* into the strain (Narayanan and Warburton, 2003; Grillot-Courvalin *et al.,* 1998). Consequently, *E. coli* strain expresses invasin ligand on its surface which is detectable by integrin receptor (heterodimeric protein composed of various combinations of α and β chains) present on mammalian cell surface, leading to bacterial attachment and receptor-mediated endocytosis into mammalian cells upon simple co-incubation (Critchley *et al.,* 2004; Isberg and Van Nhieu, 1994) (Fig. 1.2). As depicted in Fig. 1.2, entry of *E. coli* in cultured mammalian cells by mere co-incubation is accredited to invasin ligand expression on the bacterial cell surface (Fig. 1.2 B). Amongst the various combinations of α-β heterodimers forming integrin receptors, multiple β1 chain integrins such as α3β1, α4β1, α5β1, α6β1 have shown binding with invasin immobilized on chemical surface (Isberg and Van Nhieu, 1994; Isberg and Leong, 1990) suggesting the selective binding of invasin to mammalian cell expressing specific combination of integrin receptors.

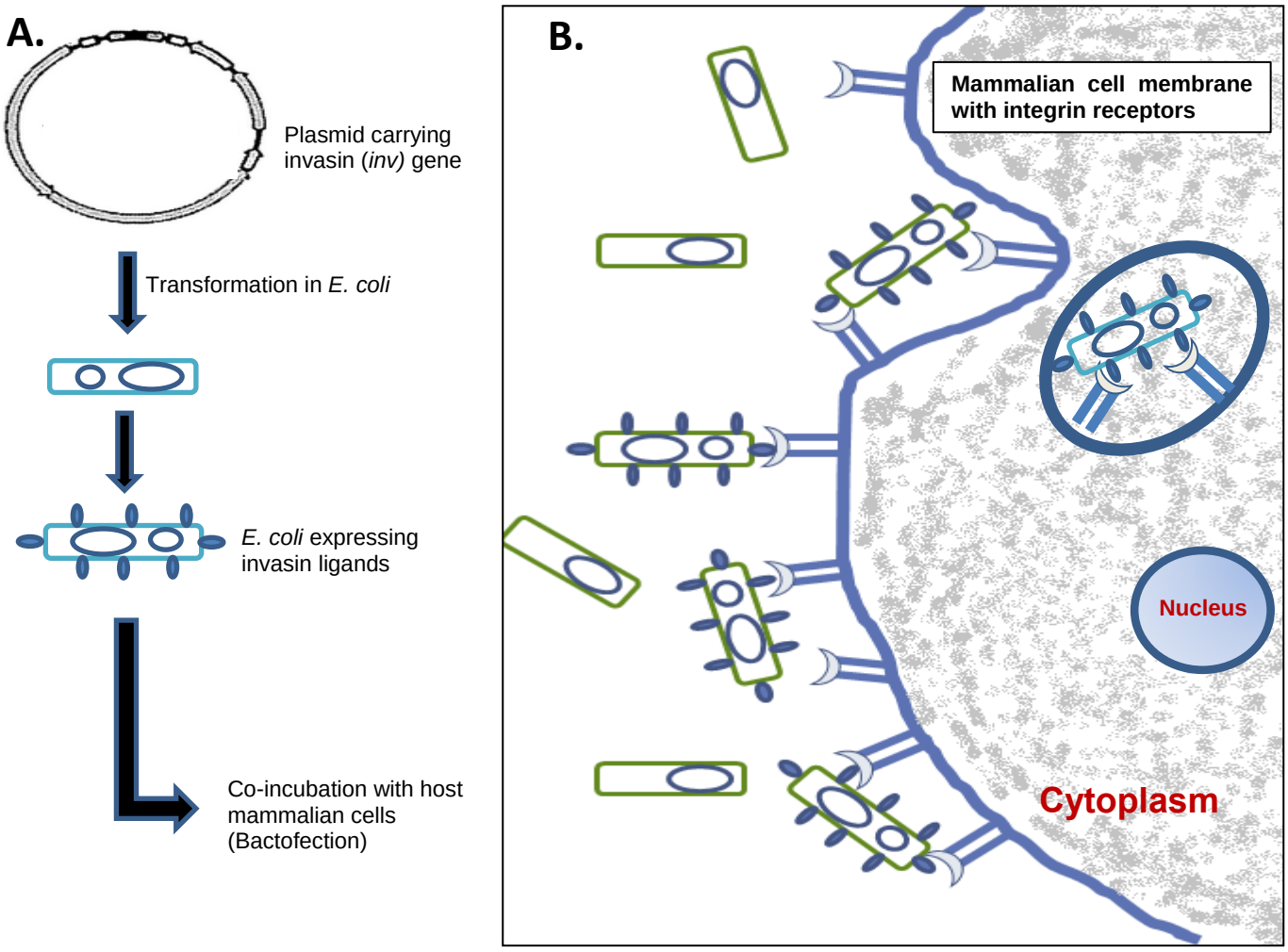


Figure 1.2. Bacterial cell attachment and invasion of the non-phagocytic cell. (A) Non-invasive *E. coli* strain was transformed with a plasmid carrying invasin gene from *Yersinia pseudotuberculosis*, which is responsible for invasin protein expression and helps the bacterial strain to become invasive (Narayanan and Warburton, 2003; Grillot-Courvalin *et al.*, 1998). Invasin binds to integrin receptors present on host cell surface, resulting in receptor-mediated endocytosis (RME). (B) Invasive bacterial strain successfully attaches to integrin receptors and eventually invades host cell via receptor-mediated endocytosis. Whereas, non-invasive bacterial cells without any invasin protein on its surface, could not attach to cell surface failing to cause any internalization in non-phagocytic host cells.

In the study conducted by Sizemore and colleagues, *Shigella* strain containing a plasmid encoding foreign antigen was inoculated intranasally in mice, which resulted in the generation of the immune response against the DNA (Sizemore *et al.,* 1997). Gene delivery through *Salmonella* has been studied using the auxotrophic *S. typhimurium* aroA strain SL7202, in epithelial and phagocytic cells together with *in vivo* administration of the strain orally in mice, resulting in genetic immunization (Grillot-Courvalin *et al.,* 2002; Paglia *et al.,* 1998; Darji *et al.,* 1997). Intragastric immunization of BALB/c mice using live attenuated *Yersinia enterocolitica* resulted in the induction of serum immunoglobulin and immune response (Al-Mariri *et al.,* 2002). Gram-positive bacterium *Listeria monocytogenes* showed proficient gene delivery through its internal suicidal mechanism by the expression of phage lysin leading to bacterial lysis in mammalian cells and the attenuated *L. monocytogenes* EGD strain achieved efficient expression of cloned genes together with antigen presentation by macrophage cells (Grillot-Courvalin *et al.,* 2002; Hense *et al.,* 2001). Also, *in vivo* studies carried out in mice using attenuated, self-destructing *L. monocytogenes* Δ2 strain, confirmed potential role of *Listeria* in oral vaccination (Dietrich *et al.,* 1998). The two invasive strains of *E. coli* i.e. BM2710 and DH10B *asd*$^{-}$ which are DAP auxotrophs, have been studied for their bactofection efficiency in various epithelial cell lines such as HeLa, CHO, COS, A549, HT1080 and a macrophage cell line J447 (Narayanan and Warburton, 2003; Grillot-Courvalin *et al.,* 1998; Courvalin *et al.,* 1995). The results showed varying gene expression (ranging from 2% - 20%) depending on cell line, HeLa and CHO cells resulted in efficiencies between 5% to 12% while 20% gene transfer efficiency was obtained with COS cells (Narayanan and Warburton, 2003; Grillot-Courvalin *et al.,* 1998). However, bacterial gene delivery efficiency remained below 5% in A549, HT1080 and a macrophage cell line J447 cells (Courvalin *et al.,* 1995).

There are several examples for therapeutic gene delivery in various cell line models using bactofection, such as *L. monocytogenes* used for functional delivery of CFTR gene involved in cystic fibrosis disease in CHO (Chinese Hamster Ovary) cells (Rocchi *et al.,* 2010; Krusch *et al.,* 2002). *S. typhimurium* has shown to be effective in therapeutic gene delivery for the diseases such as B-cell lymphoma, various carcinomas, and immunodeficiency in mice (Niethammer *et al.,* 2002; Paglia *et al.,* 2000; Urashima *et al.,* 2000). Furthermore, invasive *E. coli* BM4573 was successfully employed to deliver >300 kb BAC containing F8 gene and a 70 kb alphoid DNA for human artificial chromosome (HAC) formation, in human fibrosarcoma and kidney cells (Perez-Luz *et al.,* 2007). Similar to delivering BACs, invasive *E. coli* also showed the transfer of intact and functional CFTR locus together with alphoid DNA for HAC formation, contained in 160 kb P1-based artificial chromosomes (PACs) in human fibrosarcoma cells (Laner *et al.,* 2005). Another functional delivery of large-sized BACs of up to 200 kb containing genomic DNA using invasive *E. coli* in mammalian cells has proven bacterial delivery system to be a convenient and efficient method for therapeutic gene delivery in mammalian cells (Cheung *et al.,* 2012; Narayanan and Warburton, 2003).

To sum up, bacterial vectors have proven to be a strong candidate in the field of non-viral gene delivery vectors, as discussed above. They render appreciable qualities such as a safer alternative to viral vectors, simple in use, allow propagation and modification of carrier DNA and can carry intact genes for therapeutic purposes. However, despite all above-stated qualities, *E. coli* vectors are unable to enter into clinical trials which are primarily due to its low bactofection efficiency (Wiley *et al*., 2012; Larsen *et al.,* 2008). Figure 1.3 from the review article clearly indicates that *E. coli* contribute severely small fraction as a gene delivery vector among those which are exploited in clinical trials currently (Wiley *et al*., 2012). Therefore, to

address low gene delivery issue of various DNA carriers including bacterial vectors, several methods have been adopted in due course which focuses on enhancing vector's gene delivery capacity as discussed further in section 1.2.

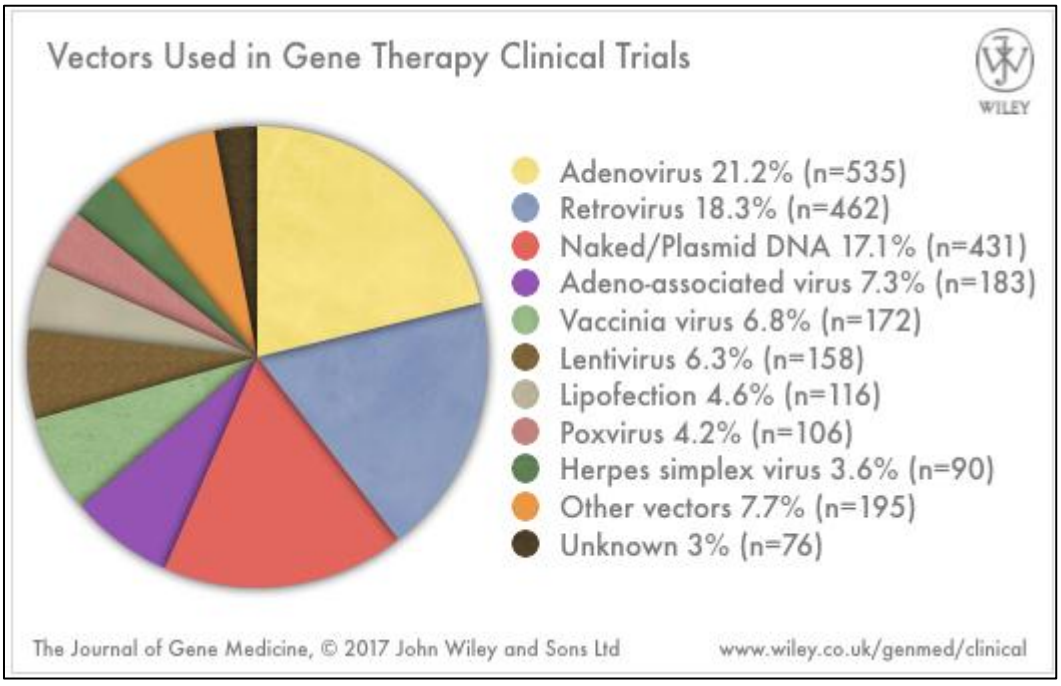


Figure 1.3. Gene delivery vectors currently employed in gene therapy clinical trials worldwide. Viral vectors (Adenovirus, Retrovirus, Vaccinia virus, Adeno-associated virus, Poxvirus, Lentivirus, and Herpes simplex virus) comprises a major portion of vectors utilized in clinical trials i.e. 66.8%, followed by naked/plasmid DNA vectors (17.7%), Lipofection (5.3%) and other vectors. *E. coli* vectors are included in "other vectors" category, which shares 7% of the total vectors' contribution in worldwide gene therapy clinical trials. http://www.wiley.com//legacy/wileychi/genmed/clinical/ (The Journal of Gene Medicine, 2017).

1.2 Potential Agents for Promoting Intracellular Gene Delivery

1.2.1 Increased Cytosolic Access by Endosomal Escape of Vectors

Receptor-mediated endocytosis leads to endosome formation and the content can be taken to lysosomes for degradation or recycled back to the plasma membrane for efflux to extracellular environment (Moore *et al.,* 2008; Roy *et al.,* 2005; Le Roy *et al.,* 2002). This poses a threat to successful gene expression inside the cell as the transferred DNA could lead to degradation upon entering the cell. Endosomal disruptive agents have been shown to facilitate endosomal escape through alteration of the acidic environment rendering the degradative enzymes inactive and overall help to increase gene expression (Moore *et al.,* 2008; Futai *et al.,* 2000; Drose *et al.,* 1997; Mellman *et al.,* 1986).

There are various lysosomotropic (LST) reagents known which act as inhibitors of endosomal/lysosomal acidification (Moore *et al.,* 2008; Futai *et al.,* 2000; Mallman *et al.,* 1986). Promoting endosomal escape, they may improve the chance of the DNA ending up in the nucleus (Fig. 1.4). Based on modes of action, lysosomotropic reagents are categorized mainly into three classes, they are as follows:

i. Lysophilic weak bases: These bases diffuse across the membrane and become protonated, resulting in neutralization of acidic environment of endocytic vesicles. The bases involved in neutralizing the acidosis of lysosomal vesicles are namely Chloroquine, Amantadine, and Ammonium Chloride (De Duve *et al.,* 1974). Inhibition of the acidic environment of endosome through the administration of Chloroquine, Amantadine, and Ammonium Chloride resulted in 15-fold increase in the viral infectivity in mammalian cells, suggesting their role in the endosomal escape of viral particle (Fredericksen *et al.,* 2002).

ii. Carboxylic ionophores: Ionophores are lipid soluble molecules used to transport ions across the cell membrane, and Porphyrin, Valinomycin, Ionomycin etc. are few examples. Among them Monensin has been reported to exchange protons for potassium and sodium, thereby causing the alkalization of endocytic vesicles (Mallman *et al.,* 1986). Monensin has shown the increased transfection efficiency approximately 10-fold, of the cationic lipoplex (DNA-lipid complex) in cultured vascular smooth muscle cells (Wang and McDonald, 2004).

iii. Inhibitor of vacuolar ATPases: Bafilomycin A1 and Concanamycin A act as inhibitors of V-ATPases and P-ATPases and have shown the endosomal escape of viral particle up to 50-fold in the host mammalian cell through their increased infectivity (Fredericksen *et al.,* 2002; Drose *et al.,* 1993 and Drose and Altendorf, 1997). Vacuolar ATPases acidify a wide array of intracellular organelles and pump protons across the plasma membrane by coupling the energy of ATP hydrolysis to proton transport (Futai *et al.*, 2000).

As discussed above and explained in Fig. 1.4, lysosomotropic reagents are known to alter the acidic environment of the endosome and rendering it incapable of maturation and fusion with a lysosome, thus allowing access of transfected DNA to the host cytoplasm effectively (Moore *et al.*, 2008; Drose and Altendorf, 1997). Implementation of the above-mentioned reagents together with the chemical assisted bactofection would probably be able to further increase the efficiency of the vector to deliver the DNA inside the mammalian cell without its degradation and eventually will lead to increased DNA transfer.

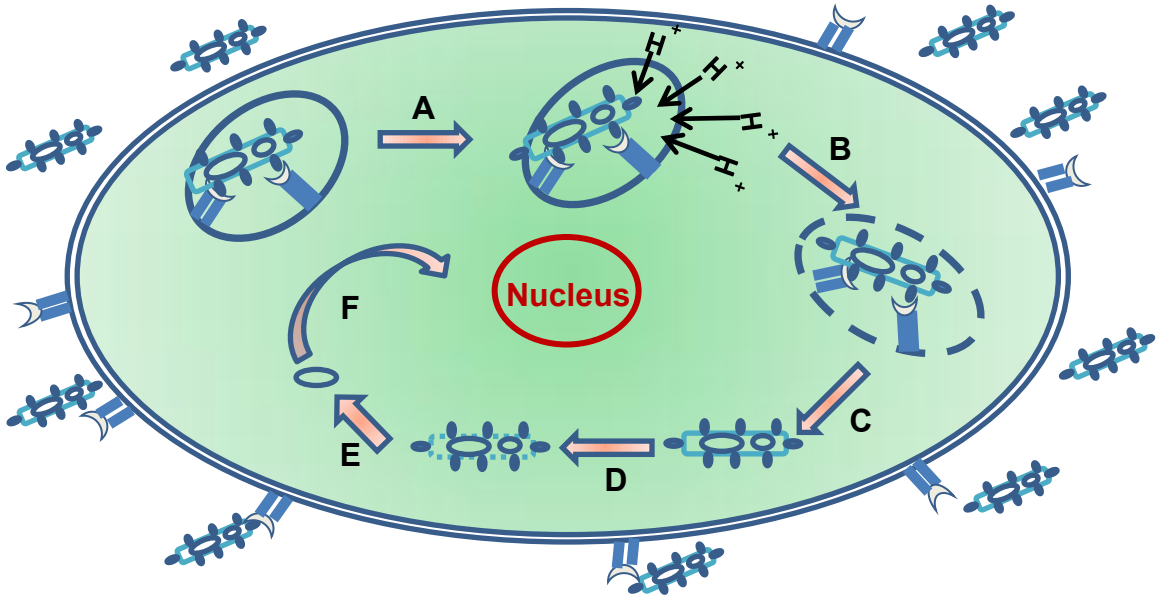


Figure 1.4. Key steps in the endosomal escape of internalized bacterial cell carrying the transgene. Receptor-mediated endocytosis (RME) into non-phagocytic mammalian cell through invasin-integrin interaction leads to formation of endosome, (A) acidification of endosome through influx of ions to the endosome, (B) endosomal acidification leads to vesicle swelling and eventually rupture of the membrane, (C) endosomal escape of bacterial cell containing the transgene increase the possibility of bacterial cell to release the DNA inside cytoplasm, (D,E) DAP auxotrophy leads to bacterial cell membrane rupture and intra-cytosolic release of transgene, (F) released transgene is then carried to the cell nucleus by unknown mechanism for its expression.

1.2.2 Promoting Disruption of Intracytosolic Bacterial Carriers

Besides escaping phagolysosomal entrapment of invaded bacterial cells, they also require to efficiently lyse for the cytosolic release of exogenous DNA and its subsequent expression. For the purpose, invasive *E. coli* strain was made DAP auxotroph mutant by deleting aspartate β-semialdehyde (*asd*) gene, which is an important component in bacterial membrane synthesis (Grillot-Courvalin *et al.*, 1998). Due to lack of DAP in mammalian cell environment internalized plasmid carrying bacterial cells cannot divide. Eventually, bacterial cell lyses releasing transgene inside the mammalian cell for expression. Nevertheless, up to 90% bacterial cell viability, 48 hr. post-infection of the invasive *E. coli* DH10B in mammalian cells has been observed (Laner *et al.*, 2005). Thus, a strategy involving antibiotic-mediated lysis of intracellular surviving bacterial vectors to promote the release of the transgene in human cells was utilized.

Antibiotics like Rifampicin, Polymyxin B, Rifamycin, Ampicillin act as the bactericidal agent causing bacteria cell death (Jones *et al.*, 2013; Kohanski *et al.*, 2007; Hopkins 2003; Sat *et al.*, 2001). Another set of antibiotics which includes Tetracycline, Chloramphenicol, Spectinomycin are bacteriostatic agents, which acts on bacterial systems differently to inhibit their replication (Pankey and Sabath, 2004; Hense *et al.*, 2001). Apart from their usage in the treatment of various diseases, lately antibiotics have also been implemented to aid in bacteria mediated gene delivery. Extensive studies were done to promote gene transferability of a facultative intracellular, Gram-positive bacterium *Listeria monocytogenes* by adapting antibiotic-mediated lysis *in vitro* (van Pijikeren *et al.*, 2010; Pilgrim *et al.*, 2003; Krusch *et al.*, 2002; Hense *et al.*, 2001). Tetracycline treatment of plasmid-harboring *Listeria* vectors following infection have positively shown DNA transfer to host eukaryotic cell lines (Hense *et al.*, 2001). Several antibiotics were employed to analyze their effect on gene transfer frequency of *L.*

monocytogenes in mammalian kidney (PtK2) cells. Among tested antibiotics, highest reduction of up to 97% in intracellular viable bacterial cells was observed upon addition of penicillin and penicillin + streptomycin combination (Krusch *et al.,* 2002). *In vitro* reporter gene delivery has been facilitated as a result of a 4-log reduction in viable bacterial vectors upon ampicillin treatment in MCF-7 cells (van Pijikeren *et al.,* 2010). Moreover, antibiotic-assisted plasmid delivery in *L. monocytogenes*-based bactofection system has also been reported in tumors in mice (Tangney *et al.,* 2010; van Pijikeren *et al.,* 2010). In addition, to lyse Gram-positive bacterial vector, the technique has also been successfully implemented in lysing Gram-negative bacterial vector *Escherichia coli* and help the release of carrier plasmid in host cytosol (Jones *et al.,* 2013). Post-internalization bacterial death has also been observed in HEp-2 cells in the presence of Chloramphenicol leading to the possibility of employing antibiotics for killing intracytosolic bacterial cells (Marouni and Sela, 2004).

For the purpose, two broad-spectrum antibiotics i.e. Tetracycline and Ciprofloxacin and an antibiotic for treating Gram-negative infections were used in the present study. Tetracycline primarily blocks protein synthesis of prokaryotes (Hense *et al.,* 2001). It binds to a 30S subunit of microbial ribosomes preventing attachment of charged aminoacyl-tRNA to the ribosome. Thus, it aborts synthesis and elongation of nascent peptide chain (Kohanski *et al.,* 2010; Chopra and Roberts, 2001). Ciprofloxacin, a bacteriostatic second-generation fluoroquinolone drug, functions against bacterial growth by inhibiting cell division. It inhibits DNA gyrase, an enzyme necessary for DNA separation during cell division (Kohanski *et al.,* 2010). On the other hand, Polymyxin B is a bactericidal antibiotic that works specifically against Gram-negative bacteria. It binds to the lipopolysaccharide layer and disrupts bacterial outer membrane permeability resulting membrane destabilization causing leakage of cellular molecules (Jones *et al.,* 2013).

1.3 Bacterial Artificial Chromosomes

The major implication in gene therapy includes functional supplementation of a flawed endogenous gene to restore its normal functioning in cells. Further, to match the physiological level of endogenous gene functions by heterologous gene delivery requires the complete gene to be delivered including, its long-range controlling elements. For the purpose, cloning vectors were constructed that can carry constructs ≥100 kb such as Bacterial artificial chromosomes (BACs), P-1 derived artificial chromosomes (PACs), Yeast artificial chromosomes (YACs) and Human artificial chromosomes (HACs) (Kazuki and Oshimura, 2011; Hoshiya *et al.,* 2009; Wild *et al.,* 2002; Giraldo and Montoliu, 2001; Ioannou *et al.,* 1994; Shizuya *et al.,* 1992). Low-copy bacterial artificial chromosome (or simply BAC) are fertility-plasmid (F-plasmid) vectors, has been developed and studied since the early 1990s (Fig. 1.5) (Shizuya *et al.,* 1992). Since then, BAC vectors have been extensively used for preparing DNA libraries such as BAC libraries of fungi, plants, mammals and human DNA and also for understanding normal gene functions by cloning novel genes provided by Genome Projects (Kamboj *et al.,* 2015; Tenzen *et al.,* 2010; Allen *et al.,* 2003; Jessen *et al.,* 1998; Mozo *et al.,* 1998; Schibler *et al.,* 1998; Asakawa *et al.,* 1997; Diaz-Perez *et al.,* 1996; Kim *et al.,* 1996; Cai *et al.,* 1995; Woo *et al.,* 1994).

To deliver BAC DNA for various purposes, several gene delivery vectors have been used. In the field of viral vectors, viral particles (particularly adenovirus) as DNA carriers have been generated (Curiel *et al.,* 1991). Adenovirus particles used for BAC delivery were made transcriptionally and replicational silent by inactivating them with the help of psoralen (Cotten *et al.,* 1996; Cotten *et al.,* 1994; Cotten *et al.,* 1992). These psoralen-inactivated adenovirus particles were linked to DNA with the help of polyethylenimine and used as DNA transfection carriers, which successfully resulted in BAC DNA delivery of up to 170 kb in human lung and

primary fibroblast cells, with transfection efficiencies comparable to that of 5-10 kb DNA delivery by viruses (Baker and Cotten, 1997; Cotten *et al.,* 1992). Nevertheless, the associated risks of immunogenicity generation and toxicity upon using viral vectors cannot be ignored (Harvey *et al.,* 2011; Cohen, 2007; Wakimoto *et al.,* 2003).

Amongst non-viral delivery systems, Lipofection of ≈150 kb BAC DNA carrying GFP, *oriP,* and *EBNA*-1 in mouse melanoma cells (B16F10) have produced intact BAC DNA after rescue from transfected cells after 5 weeks (Magin-Lachmann *et al.,* 2004; Magin-Lachmann *et al.,* 2003). Moreover, transfection of BAC DNA either carrying F8 gene responsible for Hemophilia A or CFTR gene associated with cystic fibrosis using Lipofectamine 2000 in mammalian cell lines have been reported (Kotzamanis *et al.,* 2009; Perez-Luz *et al.,* 2007). However, these studies suggest a high frequency of BAC DNA rearrangement that could possibly be due to the use of transfection method which requires preparation of good quality large size DNA (Cheung *et al.,* 2012; Kotzamanis *et al.,* 2009; Perez-Luz *et al.,* 2007).

Alongside the immunogenicity and toxicity risks, the major problem faced by gene therapy applications is to deliver intact therapeutic genes into mammalian cells efficiently. Thus, direct delivery of DNA using non-pathogenic invasive bacteria was used, which eliminates the major constraint of the requirement to prepare purified DNA. Invasive *E. coli* BM4573 and DAP auxotroph *E. coli BM*2710 strains were successfully employed in delivering BACs and PACs together with alphoid DNA to generate human artificial chromosomes *de novo* (Perez-Luz *et al.,* 2007; Laner *et al.,* 2005). Hemophilia A causing F8 gene was delivered on BAC of >300 kb size, which carried 70 kb alphoid DNA in human fibrosarcoma and kidney cells, resulting in expression of F8 mRNA greater than endogenous levels (Perez-Luz *et al.,* 2007). Similarly, bacterial delivery of large-sized BACs (up to 200 kb) containing genomic DNA yielded correctly

spliced product post-transcription in host mammalian cells, indicating the transfer of intact and functional gene (Cheung *et al.*, 2012; Narayanan and Warburton, 2003). These studies represent bacteria as an efficient carrier for intact and functional transfer of large genomic DNA fragments in host cells, for functional analysis.

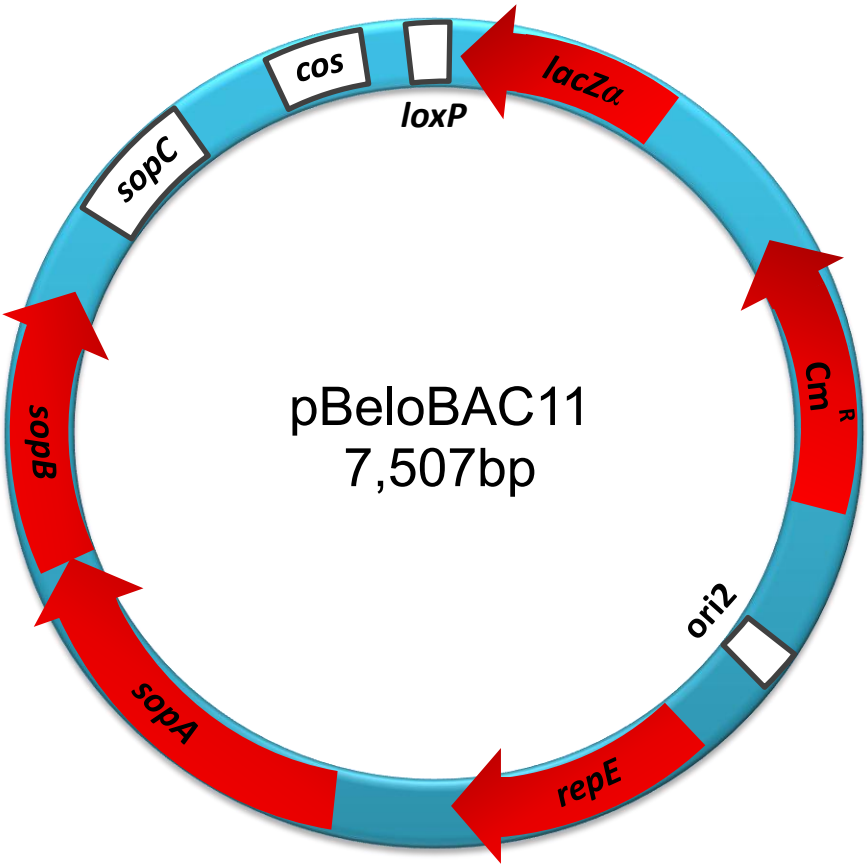


Figure 1.5. An *E. coli* plasmid cloning vector developed for constructing Bacterial Artificial Chromosomes (BACs). pBeloBAC11 is an *E. coli* cloning vector, which is capable of cloning large DNA fragments of up to 300 kb. It is generally maintained as single copy inside the cell. The vector contains unique cloning sites which are BamHI, SphI and HindIII and the gene for Chloramphenicol selection as a selectable marker (Cm^R). The cloning sites are present in a *lacZα* gene which allows screening of DNA insert by α-complementation.

2. PROJECT AIMS

In view of the capability of non-pathogenic bacteria as gene delivery vector described previously in section 1.1.2, the current study employs invasive *E. coli* DH10B for delivering exogenous DNA in various mammalian cells. This project focuses mainly on overcoming its major drawback i.e. low invading and gene delivery efficiency by modifying the bactofection method.

Genetically modified invasive *E. coli* strains have been studied for its bactofection efficiency assessment in various mammalian cells including HeLa, CHO, COS, A549, HT1080 (Narayanan and Warburton, 2003; Grillot-Courvalin *et al.,* 1998; Courvalin *et al.,* 1995). This study aims to study and evaluate bactofection efficiency of *E. coli* DH10B (pGB2Ωinv-hly, pEGFPN2) particularly in human cells derived from different tissues of human body. The purpose of bactofection in different cells is to analyze cells or tissues responding best to invasive *E. coli* so that the vector can be used in future for the correction of genetic disease associated with that particular tissue using gene therapy effectively.

Apart from studying bacterial vector's gene delivery efficiency in various cell lines, several efforts implying external addition of chemical reagents have also been conducted in order to improve non-viral gene delivery vehicles' efficiency. Recently, in an attempt to elevate invasive *E. coli* DH10B bactofection efficiency, bacterial vectors were coupled to a lipid-based transfection reagent i.e. Lipofectamine and have resulted in a 2.8-fold increase of transgene delivery in HeLa cells (Narayanan *et al.,* 2013). Furthermore, adoption of chemical agents such as lysosomotropic reagents and antibiotics are also been reported as capable of increasing several non-viral vectors' efficiency (Described in Section 1.2).

Thus, it has been hypothesized that along with testing *E. coli* DH10B (pGB2Ωinv-hly, pEGFPN2) vector's efficiency in various human cells, their gene delivery efficiency could possibly be enhanced by implementing various chemical agents commonly known for boosting up multiple vectors' efficiencies. Post-invasion, cytosolic access of DNA carriers and intracytosolic cargo release is a prerequisite for foreign DNA expression in host cells. Therefore, it was strategized to further introduce two different categories of reagents which would help to improve the cytosolic reach of invaded bacterial cells and efficient cargo release for expression in host cells. Further, it has been postulated that besides reporter gene delivery, external addition of chemical reagents could probably be analyzed for their effect on bacterial efficiency in delivering large size therapeutic gene also. Figure 2.1 reflects the stepwise summary of this project.

Hence, the objectives include assessment of bacterial vector's efficiency in delivering reporter gene in human cells (Aim 1). Further, in an attempt to study the effect of the external addition of chemical reagents, *E. coli* DH10B (pGB2Ωinv-hly, pEGFPN2) vector was conjugated with popular transfection agents prior to bactofection (Aim 2). Moreover, the impact of chemical reagents that affect intracellular pathway/fate of gene delivery vector in host cells was designed to study under Aim 3. Furthermore, chemical reagents which were added separately pre and post-invasion of the bacterial vector were postulated to produce a heightened effect if added together as depicted in figure 5.1 (Aim 4). Aim 5 was designed to study invasive *E. coli* DH10B efficiency in delivering large size gene i.e. human β-globin *in vitro,* with and without the external reagents' addition. Above listed aims are described as follows:

1) Testing efficiency in different cell lines derived from human tissues (Bactofection)

Testing gene delivery efficiency of the invasive *E. coli* vector in cell lines derived from nine different human tissues. This is to provide an insight into vector's gene delivery behavior in different tissues and also can help in understanding which tissue/s response best to this vector.

2) Enhancing the entry of bacteria by means of coupling them with transfection reagents (Trans-bactofection)

Transfection, when combined with the bactofection method, could help in increasing transgene delivery capacity of the vector. The method is termed as Trans-bactofection in this study. Transfection reagents of four kinds, differing in their chemical composition, when coupled with bacterial vectors could probably promote bacterial entry into nine different host human cells. Thus, trans-bactofection could result in higher number of invaded bacterial vectors and eventually higher transgene delivery.

3) Promoting endosomal escape and subsequent intracytosolic lysis of internalized bacterial vectors

This aim focuses on the cytosolic release of transgene carried by the vector to host cell after the process of invasion. As depicted in Fig. 1.2, invasive *E. coli* enters host cells through receptor-mediated endocytosis which leads to endosome formation (Critchley *et al.,* 2004; Isberg and Van Nhieu, 1994). These endosomes carrying foreign material are subjected to lysosomal degradation upon fusion with lysosomes and degraded products are effluxed back to extracellular environment (Moore *et al.,* 2008; Roy *et al.,* 2005; Le Roy *et al.,* 2002). Therefore, to prevent bacterial degradation and to promote the probability of transgene access to the host cell cytoplasm for its expression, chemical agents which are referred to as lysosomotropic (LST)

reagents such as Amantadine, Chloroquine, and Monensin will be used together with bactofection. These LST reagents are known for promoting bacterial escape from endosome in mammalian cells (Detailed under Section 1.2.1).

Besides, administration of antibiotics such as Ciprofloxacin, Tetracycline, and Polymyxin B will be done, which are well-known reagents for bacterial cell death in the host cell cytosol (Jones *et al.,* 2013; Krusch *et al.,* 2003; Hense *et al.,* 2001). However, DAP auxotrophic behavior of the vector renders bacterial cell division afflicted, due to the unavailability of DAP inside the mammalian cell which leads to the subsequent death of the bacterial cell and cytoplasmic release of the transgene (Narayanan and Warburton, 2003; Grillot-Courvalin *et al.,* 1998). Still, up to 90% bacterial cell viability, 48 hr. post-infection of the invasive *E. coli* DH10B in mammalian cells has been observed (Laner *et al.,* 2005). Intracellular bacterial vector viability eventually results in reduced number of transgene release to the cell cytoplasm for its expression. Hence, additional bacterial lysis method will be adopted which could help promote internalized bacterial lysis. Enhanced bacterial escape from lysosomal degradation and cytosolic degradation of invaded bacterial vectors would eventually lead to overall betterment in the gene delivery efficiency of the invasive *E.coli* vector.

4) Combined effect of externally added reagents for enhanced transgene delivery

Under this aim, best-performing reagents from three different categories i.e. transfection reagents, LST reagents, and antibiotics tested separately under aims 2 and 3 will be employed consecutively in a single experiment to analyze their combined effect on vector's efficiency. Diagrammatic illustration of the overall mode of action of combined treatment method is provided in Fig. 5.1. We hypothesized that the reagents which work at three different steps

during bactofection if employed together could boost up overall bacterial delivery efficiency in human cells.

5) Delivery of 100 kb β-globin BAC using the invasive *E. coli* vector

Ultimately, invasive *E. coli* containing approximately 100 kb globin BAC carrying human β-globin gene cluster, was used to analyze gene delivery efficiency of the vector.

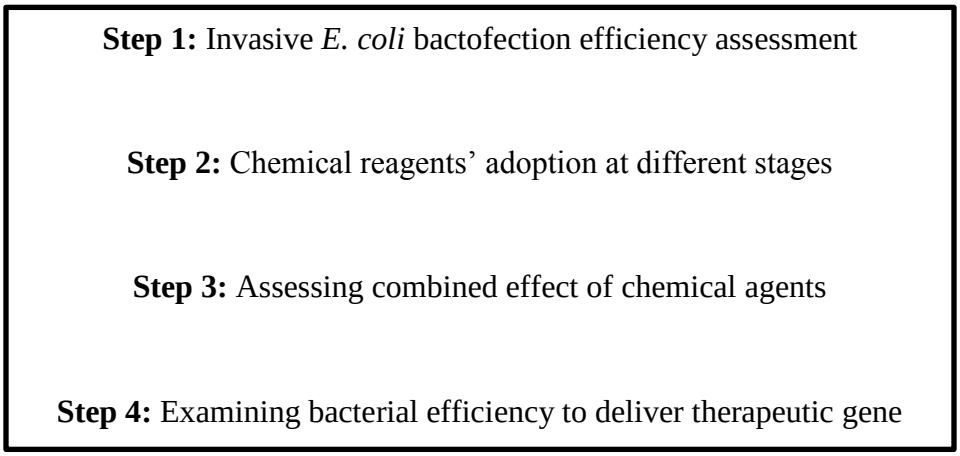


Figure 2.1. Project Outline. The entire project is being categorized mainly into four major steps. In the first step, the capacity of invasive *E. coli* as a vector will be assessed by quantifying GFP reporter gene delivery in varied human cell lines. Further, due to the low-efficiency expectancy of *E. coli* vectors, under Step 2 various reagents will be introduced separately at three different stages of bactofection. Step 3 is being designed to assess the synergistic effect of reagents which were employed at different steps of bactofection, on the overall improvement in bacterial DNA delivery capacity. Ultimately in Step 4, combined treatment method described under Step 3, will be used to analyze its effect on altering gene delivery capacity of invasive *E. coli* vector harboring large size therapeutic gene.

3. MATERIALS AND METHODS

3.1 *E. coli* Strains and Plasmids

3.1.1 Bacterial Strains

The current study employs *Escherichia coli* (*E. coli)* DH10B strains as gene delivery vehicle in human cells. *E. coli* DH10B is a non-pathogenic, gram-negative strain commonly used in laboratories. Genotype of *E. coli* DH10B is F^- *mcrA* Δ (mrr-hsdRMS-mcrBC) ϕ80dlacZΔM15 ΔlacX74 *deo*R *rec*A1 *end*A1 *ara*Δ139 Δ (*ara*, *leu*) 7697 *gal*U *gal*K λ^- *rps*L *nup*G (Grant *et al.*, 1990).

3.1.1.1 Engineered *E. coli* DH10B for Autolysis

Autolysis is required for intracellular release of foreign DNA in host cell carried by the bacterial vector. For the purpose, DH10B strain of *E. coli* mentioned above has been engineered to delete *asd* gene which is responsible for diaminopimelic acid (DAP) synthesis in a bacterial cell (Narayanan and Warburton, 2003). The gene was replaced by zeocin-containing cassette via GET recombination system rendering the strain auxotrophic for DAP resulting in bacterial cell wall lysis phenotype in the absence of DAP (Courvalin *et al.*, 1995; Narayanan and Warburton, 2003).

3.1.1.2 Invasive Autolyzing *E. coli* DH10B Carrying Reporter Gene

Auxotrophic *E. coli* DH10B strain (Section 3.1.1.1) which otherwise is non-invasive, was modified further so that it can invade a host cell and deliver foreign DNA into host cell cytosol. It was done by transforming invasin carrying plasmid i.e. pGB2Ωinv-hly into *E. coli* DH10B, resulting in expression of invasin ligands on the bacterial cell surface (Section 3.1.2.1; Fig. 1.2). Invasin ligands encoded by *inv* gene are recognized and bind by integrin receptors present on the mammalian cell surface, this interaction leads to receptor-mediated endocytosis causing bacterial

vector to enter host cell (Isberg and Van Nhieu, 1994) (Section 1.1.2; Fig. 1.2). *E. coli* DH10B was further transformed with a plasmid containing Green Fluorescent Gene (GFP) as pEGFP-N2 (Section 3.1.2.2).

Vectors carrying reporter gene are utilized to track the transgene delivery in the host cell (Narayanan and Warburton, 2003). The transformed auxotrophic *E. coli* DH10B strain is referred to as *E. coli* DH10B *asd*$^-$ pGB2Ωinv-hly, pEGFP-N2 or simply invasive *E. coli* (Narayanan *et al.*, 2013).

3.1.1.3 Autolyzing Invasive *E. coli* DH10B Carrying Human β-globin BAC

Auxotrophic *E. coli* DH10B strain containing invasive and EGFP reporter plasmid (Section 3.1.1.2) was further transformed with human globin gene carrying 100 kb globin BAC (BAC4396) (Kaufman *et al.*, 1999) (Section 3.1.2.3). This strain was used to assess *in vitro* gene delivery ability of bacterial vector. BAC4396-EGFPneo in addition to globin gene, carries reporter pEGFP-N2 gene under CMV promoter, to track its delivery by flow cytometer (Section 3.9.1) (Narayanan and Warburton, 2003). The strain was prepared by electroporating either BAC4396 or BAC4396-EGFPneo DNA into *E. coli* DH10B *asd*$^-$ (pGB2Ωinv-hly) strain as described in section 3.10.2 and strain details are provided in Table 3.1.

3.1.1.4 Non-invasive Autolyzing *E. coli* DH10B

Non-invasive *E. coli* is DAP auxotrophic DH10B strain devoid of invasive plasmid thus, incapable of invading host cells. However, the strain is transformed with reporter plasmid i.e. pEGFP-N2 to capture background fluorescence while analyzing bacterial invading capacity through flow cytometer (Section 3.9.1) (Narayanan and Warburton, 2003). Since the strain does not carry invasive plasmid it is unable to interact and invade host cell membrane. The strain was used as a negative control to study the extent of invasive bacterial internalization in the host cell.

The strain was obtained from A/P K. Narayanan's laboratory stock and used for the bactofection studies.

3.1.2 Plasmids

3.1.2.1 Invasive Plasmid

The Invasive plasmid pGB2Ωinv-hly is a kind gift from C. Grillot-Courvalin and S. Goussard (Grillot-Courvalin *et al.,* 1998). This 10.05 kb invasive plasmid is based on low copy number pGB2 plasmid (Churchward *et al.,* 1984), and carries invasin gene (*inv*, 3.2 kb) from *Yersinia pseudotuberculosis* together with hemolysin gene (*hly*, 1.5 kb) from *Listeria monocytogenes* which encodes listeriolysin O (Grillot-Courvalin *et al.,* 1998). Upon expression, invasin proteins facilitate receptor-mediated endocytosis by interacting with β1-integrin proteins on host cell surface (Critchley *et al.,* 2004; Isberg and Falkow, 1985). Further, listeriolysin O expression rescues and release bacteria from endocytic vesicles formed upon internalization which could otherwise lead to bacterial degradation by lysosomes (Bielecki *et al.,* 1990; Gaillard *et al.,* 1987).

3.1.2.2 Plasmid for Reporter Gene Expression (GFP)

EGFP reporter gene is carried by 4.7 kb pEGFP-N2 plasmid and was used for preparing invasive and non-invasive *E. coli* DH10B strains (Narayanan and Warburton, 2003). Reporter gene expression is driven by CMV promoter and the plasmid contains genes for kanamycin/neomycin antibiotic selectivity. These genes confer resistance to kanamycin and neomycin analogs, therefore, provides a selection for both prokaryotes and eukaryotes respectively (Narayanan and Warburton, 2003).

3.1.2.3 BAC Containing β-globin Gene Cluster

BAC4396 containing complete 100 kb human globin cluster (5' ε — γ-G — γ-A — δ — β 3') including 5'LCR and HS-1 site at 3' was carried by *E. coli* DH10B (Ooi *et al.,* 2008; Kaufman *et al.,* 1999). Further, EGFPneo cassette containing neomycin gene conferring resistance to neomycin analog in eukaryotes together with GFP reporter gene was retrofitted on 100 kb globin BAC4396 forming BAC4396-EGFPneo. *E. coli* DH10B strains containing BAC4396 and BAC4396-EGFPneo were provided by A/P Kumaran Narayanan for the preparation of invasive and non-invasive *E. coli* DH10B *asd*$^{-}$ carrying BAC DNA. BAC DNA was isolated and transferred to invasive *E. coli* DH10B vector using electroporation technique (Section 3.10.1.2 and Section 3.10.2.1). Later, the bacterial vector carrying globin BAC was used for bactofection of human fibroblast (HT1080) cells, to check vector's efficiency in delivering large DNA *in vitro*.

3.1.3 Growth Conditions and Selective Antibiotics for Strains and Plasmids

3.1.3.1 Bacterial Strains

Bacterial strains used in the current study are listed in Table 3.1. All the strains were grown and maintained at 37° C while 37° C and 220 rpm was provided for growing bacteria in liquid culture. Bacterial cultures were maintained either in Luria-Bertani (LB) or in Brain Heart Infusion (BHI) as indicated in Table below. All the cultures were provided with their respective antibiotic(s) selection (Table 3.1). As indicated in Table 3.1, certain strains require and were provided with additional media supplement i.e. DAP for their growth.

Table 3.1. Bacterial strains used in the present study with their growth conditions

No.	Strain	Selective Antibiotic(s)	Final Concentration (µg/ml)	Growth Media	Growth Requirement
1.	DH10B	Streptomycin	20	LB Broth & Agar (LabM)	No Additional Supplements
2.	Invasive autolyzing DH10B **{**DH10B *asd*$^-$ (pGB2Ωinv-hly)}	Zeocin	25	BHI Broth & Agar (Difco)	0.5 mM DAP (SIGMA)
		Spectinomycin	25		
3.	Invasive autolyzing DH10B with reporter gene **{DH10B *asd*$^-$ (pGB2Ωinv-hly, pEGFP-N2)}**	Spectinomycin	25	BHI Broth & Agar (Difco)	0.5 mM DAP (SIGMA)
		Kanamycin	20		
4.	Non-invasive autolyzing DH10B with reporter gene **{DH10B *asd*$^-$ (pEGFP-N2)}**	Zeocin	25	BHI Broth & Agar (Difco)	0.5 mM DAP (SIGMA)
		Kanamycin	20		
5.	DH10B containing BAC4396	Chloramphenicol	12.5	LB Broth & Agar (LabM)	No Additional Supplements
6.	DH10B containing BAC4396-EGFP	Chloramphenicol	12.5	LB Broth & Agar (LabM)	No Additional Supplements
		Kanamycin	20		
7.	Invasive autolyzing DH10B with BAC4396 {DH10B *asd*$^-$ (pGB2Ωinv-hly, BAC4396)}	Spectinomycin	25	BHI Broth & Agar (Difco)	0.5 mM DAP (SIGMA)
		Chloramphenicol	12.5		
8.	Invasive autolyzing DH10B with BAC4396-EGFPneo **{DH10B *asd*$^-$ (pGB2Ωinv-hly, BAC4396-EGFPneo)}**	Spectinomycin	25	BHI Broth & Agar (Difco)	0.5 mM DAP (SIGMA)
		Chloramphenicol	12.5		
		Kanamycin	20		

❖ Highlighted strains in the rows nos. 3 and 8 are the strains which have been used as gene delivery vehicles, to analyze bacterial vectors' efficiency in delivering either reporter gene or large sized DNA in human cells respectively. Strain mentioned in the row no. 4, has been used to determine background internalization. Whereas, non-highlighted strains are the ones which have not been used directly in the invasion studies. Rather, plasmids from these strains were isolated and used in preparing various invasive and non-invasive strains for this study.

3.1.3.2 Plasmids and BAC

Plasmids or BAC carried by invasive *E. coli* DH10B in the current study, are described below in Table 3.2.

Table 3.2. Details of plasmid/BAC used in the present study

No.	Plasmid/BAC	Antibiotic Selection	Final Concentration (µg/ml)
1.	pGB2Ωinv-hly	Spectinomycin	25
2.	pEGFP-N2	Kanamycin	20
3.	BAC4396	Chloramphenicol	12.5
4.	BAC4396-EGFPneo	Chloramphenicol	12.5
		Kanamycin	20

3.2 Mammalian Cell Culture

3.2.1 Human Non-Phagocytic Cell Lines

Adherent cells from nine different tissues of the human body (Section 3.2.2.1; Table 3.3) were obtained from different sources. The human brain (SK-N-SH) and fibroblast (HT1080) cancer cells were purchased from American Type Culture Collection (ATCC), while others were a kind gift from various neighboring laboratories in Monash University, Malaysia. Ovarian (SKOV-3), lung (A549) cells were contributed by Dr. Jaya Vejayan laboratory (School of Medicine). Human colon cells (Caco-2) were kindly provided by Dr. Uma Devi Palanisamy (School of Medicine and Health Sciences) and kidney cells (HEK-293) by Prof. Ishwar Parhar laboratory (School of Medicine and Health Sciences). Human liver (HepG2) and breast (MCF-7)

cell lines were acquired from laboratory stock of Prof. Sek Chuen Chow (School of Science) while HeLa was used from A/P Kumaran Narayanan's laboratory stock.

3.2.2 Cell Line's Growth and Maintenance

3.2.2.1 Cell Culture Media and Supplements

Cell lines were maintained in their respective media (Gibco, except EMEM which was purchased from ATCC) at 37° C, 5% CO_2 in T-75 tissue culture flask (Nunc). All of the purchased media were supplied with L-glutamine except EMEM and RPMI 1640. So, L-glutamine (Gibco) was added together with Fetal Bovine Serum (FBS) (Hyclone) at the indicated concentrations (Table 3.3), to prepare complete growth media which is referred to as (L-glut$^+$, FBS$^+$). All the cells were cultured and maintained in antibiotic-free media all the time. Detailed information on cell culture growth and maintenance is given in Table 3.3.

3.2.2.2 Stock Maintenance

Cultured cells were trypsinized using 0.05% trypsin-EDTA (Gibco) and collected. Cells were centrifuged and resuspended in freezing media containing 90% FBS and 10% dimethyl sulfoxide (DMSO) (Merck). Prepared frozen stocks were maintained in liquid nitrogen.

3.2.3 Cell Seeding

For invasion studies, cultured cells were removed from culture plates i.e. trypsinized using 0.05% trypsin-EDTA from T-75 tissue culture flask, mixed with trypan blue dye (SIGMA) and counted using hemocytometer. $1x10^5$cells/well density were seeded in 6-well plates (Greiner Bio-One) and incubated at 37° C, 5% CO_2 for the duration according to their respective doubling time (Table 3.3).

Table 3.3. Cell lines used in the present study and their growth conditions

Cell Line	Media	Supplements	Doubling Time†
HeLa (Cervical)	RPMI 1640	10% FBS; 4 mM L-glut	24 hr. (Chen *et al.,* 2014)
MCF-7 (Breast)	RPMI 1640	10% FBS; 4 mM L-glut	29 hr.*
SKOV-3 (Ovary)	McCoy's 5A	10% FBS; 4 mM L-glut	48 hr. (Hurst and Hooks, 2009)
HepG2 (Liver)	DMEM-LG	10% FBS; 2 mM L-glut	48 hr. (Aston *et al.,* 2000; Shafiee-Kermani *et al.,* 2013)
HEK-293 (Kidney)	DMEM-HG	10% FBS; 4 mM L-glut	24 hr. (Cervera *et al.,* 2011)
Caco-2 (Colon)	EMEM	20% FBS; 2 mM L-glut	72 hr.**
SK-N-SH (Brain)	EMEM	10% FBS; 2 mM L-glut	48 hr. (Ricordy *et al.,* 2002)
A549 (Lung)	F-12K Media	10% FBS; 2 mM L-glut	24 hr.*
HT1080 (Fibroblast)	EMEM	10% FBS; 2 mM L-glut	24 hr.**

† Doubling time indicated in the Table is given as approximate time ± 2 hr.

* Doubling time was used as mentioned by ATCC website

** Doubling time was calculated by culturing and calculating a number of cells after trypsinization using hemocytometer, at every 24 hr. interval. It was done in the case of cells for which no proper reference indicating their doubling time was available.

3.3 Bacterial Invasion (Bactofection)

3.3.1 Bacterial Culture for Invasion

3.3.1.1 Bacterial Inoculation and Growth

Bacterial strains were inoculated in 10 ml of respective media with selective antibiotics (Table 3.1) in 50 ml Falcon tubes. Inoculated cultures were then incubated in shaking condition i.e. 220 rpm for 18 hr. (Section 3.1.3.1).

3.3.1.2 Culture Dilution

Overnight grown bacterial cultures at a concentration of ~$1x10^9$ CFU/ml (Colony Forming Unit) were pelleted down at 4000 rpm speed for 7 min. in Universal 320 R centrifuge (Hettich Zentrifugen) at room temperature. Collected cells were resuspended in 1 ml of mammalian culture media devoid of FBS but containing 0.5 mM DAP for bacterial cell integrity maintenance and L-glutamine (FBS^-; $L\text{-}Glut^+$; DAP^+). Bacterial cells were further diluted in 2 ml of culture media (FBS^-; $L\text{-}Glut^+$; DAP^+) in 15 ml of Falcon tubes according to a required multiplicity of infection (MOI) ranging from 500-4000. MOI can be explained as the ratio of a number of bacterial cells to the number of target cells (Shabram and Aguilar-Cordova, 2000). Thus, it can be calculated as:

$$\frac{\text{CFU (colony forming unit) of bacteria}}{\text{Number of target cells}} = \text{MOI}$$

3.3.2 Invasion Procedure

Invasion in target cell using invasive *E. coli* occurs by simple co-incubation with the cultured cells (Grillot-Courvalin, 1998). Cultured human cells were washed twice with 1x phosphate buffered saline (PBS) (1^{st} BASE) and overlaid diluted bacterial culture at the desired MOI. Culture plates were centrifuged to maximize the vector-host contact, at 1000 x g using Heraeus® Megafuge® 40 R (Thermo Scientific™) for 10 min, 24 °C and incubated further in a

CO_2 incubator (RS Biotech Galaxy R+) for 1 hr. at 37° C, 5% CO_2 for internalization of the bacterial vector (Narayanan and Warburton, 2003).

Following the invasion, gentamicin protection assay was performed to kill extracellular bacterial cells (See Section 3.8.1). Basically, the post-invasion bacterial culture was removed by washing cultured human cells with 1x PBS and further to remove any non-internalized bacterial cells adhered to host cells, growth media (with serum) containing gentamicin (80 μg/ml) was added and incubated at 37° C, 5% CO_2. This was followed by either bacterial rescue assay in which the invaded host cells are lysed by Triton X-100, releasing internalized bacterial cells into the solution (Explained in Section 3.8.2). The collected lysate containing invaded bacterial cells was further diluted serially and the dilutions ranged from no dilution, in case of liver (HepG2) cells which showed very low bactofection efficiency by invasive *E. coli* (Fig. 4.6), up to four times dilution for other cells used in bactofection as well as trans-bactofection studies (Fig. 4.6; Fig. 4.8A, 4.9A, 4.10A and 4.11A). The lysate either undiluted or diluted was further plated onto selective media for viable internalized bacterial count determination (Narayanan and Warburton, 2003) (Refer to Table 3.1 and Table 3.2). The viable bacterial count is determined by calculating colony forming unit (CFU) on agar plates as detailed in section 3.8.2. Apart from the viable internalized bacterial count, flow cytometer analysis was performed to obtain quantitative data of bactofection efficiency achieved by delivered transgene (GFP) expression (See Section 3.9.1). For flow cytometer analysis, invaded host cells were trypsinized and washed with 1x PBS, further these live cells were analyzed by FACScan flow cytometer using an appropriate filter for quantification of delivered gene expression (Described in Section 3.9.1).

3.4 Coupling of Transfection Reagent with Invasive *E. coli* (Trans-bactofection)

3.4.1 List of Transfection Reagents

Bacterial invasion performed after complexing *E. coli* vector with a transfection reagent is termed and referred to as Trans-bactofection in the current study. Four types of transfection reagents differing in their chemical composition were used for the conducted study. The details of the reagents are as given in Table 3.4.

Table 3.4. Transfection reagents of varying chemical composition used in the study

Transfection Reagent	Nature	Company
Lipofectamine 2000	Cationic Liposome	Invitrogen
jetPEI	Cationic Polymer	Polyfect
PULSin	Cationic amphiphile	Polyfect
ExGen 500/TurboFect†	Linear Polyethylenimine	Fermentas

† ExGen 500 was used for initial studies while TurboFect for the later part since ExGen 500 supply was terminated and replaced with TurboFect by the manufacturing company. However, the company has assured about same chemical nature of both the reagents.

3.4.2 Trans-bactofection

Invasive *E. coli* was grown, the culture was pelleted and resuspended as described earlier (Section 3.3.1.1 and Section 3.3.1.2). Resuspended overnight grown culture and transfection reagent were further diluted as detailed in Table 3.5. Bacterial cultures were diluted to give MOI of 1000 bacteria/cell. Further, the diluted reagent was added to the bacterial tube, mixed gently and incubated for 30 min. at room temperature. Post-incubation the complex was diluted by

adding 2 ml of growth media (FBS^-; L-$Glut^+$; DAP^+). Dilution was followed by the invasion of the cultured mammalian cell as described above in section 3.3.2.

In order to decide single MOI for further work, certain points were mainly taken into consideration. Firstly, it has already been hypothesized that addition of chemical reagents and modification in bactofection method would possibly help to increase the invasion and overall gene delivery capacity of *E. coli* vector (Section 2; Fig. 2.1). Thus, the idea was to choose and start with the MOI where *E. coli* vector on its own is least capable, so that the effect of reagents' administration in improving bacterial DNA delivery capacity could be nicely studied. It could also be a good thing to monitor elevation in invasion capacity of invasive *E. coli* DH10B vector upon adding various reagents, where *E. coli* vector on its own is least capable. Nevertheless, using lower MOI could possibly help to eliminate the burden of high bacteria to host cell ratio which was observed to infer cellular toxicity to certain cells, used in this study. Based on these two parameters, 500 or 1000 MOIs seemed to be perfect options. Furthermore, based on bactofection studies 500 and 1000 MOI showed a minimum of approximately 0.5 and 1 bacteria per cell entry (Refer to Section 4.1.2.1; Fig. 4.6). As only one MOI was to be chosen among these two MOIs, 1000 was randomly picked. Thus, 1000 MOI was used for trans-bactofection and other reagents' (LST and Antibiotics) testing to maintain consistency for comparison purposes.

Table 3.5. Schematic illustration of bacterial culture and transfection reagent dilution for Trans-bactofection

	A	B	C	D	E	F
Media (+DAP; -FBS)	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl
Bacteria Volume (Tube 1)	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl
Reagent Volume (Tube 2)	0.0 µl	0.2 µl	0.4 µl	0.6 µl	0.8 µl	1.0 µl

3.5 Lysosomotropic Reagent (LST) Administration

3.5.1 List of LST Reagents and Concentrations

Listed compounds were purchased from SIGMA and used in the present study at the indicated range of concentrations given in Table 3.6 below.

Table 3.6. Lysosomotropic reagents used for endosomal escape of invaded bacteria

Reagent	Category	Concentration	Solvent
Chloroquine	Lysophilic weak bases	10 to 50 µM	Water
Monensin	Carboxylic ionophores	0.1 to 2 µM	DMSO
Amantadine	Lysophilic weak bases	0.2 to 1 mM	Water

3.5.2 LST Reagents' Addition

3.5.2.1 Time Duration Optimization for LST Administration

The optimum time duration for LST reagent's administration was first standardized using Chloroquine at various concentrations and 1000 MOI in HeLa cells. Cultured cells were pre-incubated with Chloroquine (10 – 50 µg/ml) for 2 hr. followed by washing cells with 1x PBS and bactofection using invasive *E. coli* DH10B. Later, cultured cells were again exposed to LST

agent for three different time durations to analyze most effective time point for drug treatment. For time duration optimization of drug treatment 1 hr., 18 hr. and 48 hr. time points were selected to analyze LST reagent's effect on improving bactofection efficiency in human cells. For the purpose, LST drug was added to cultured cells just after the addition of diluted bacterial culture and incubated for 1 hr. at 37° C, 5% CO_2. After incubation, the drug was washed away by 1x PBS along with bacterial culture, fresh complete media containing gentamicin (20 µg/ml) was added and incubated further for 48 hr. without any LST drug. Whereas, for 18 hr. treatment, Chloroquine was again added together with gentamicin (20 µg/ml) containing complete media and incubated at 37° C, 5% CO_2. This LST + gentamicin containing complete media was replaced with fresh gentamicin containing media after washing with 1x PBS, 18 hr. post-invasion and incubated further for 30 hr. at 37° C, 5% CO_2. However, for 48 hr. drug treatment, LST + gentamicin containing complete media was not replaced by fresh media post-invasion and the incubation at 37° C, 5% CO_2 continued for 48 hr. All the treatments were followed by FACS analysis of trypsinized HeLa cells after 48 hr. post-invasion (Section 3.9.1).

3.5.2.2 LST Reagents' Administration

Cultured cells were pre-incubated with lysosomotropic (LST) drugs for 2h prior to bactofection (Fredericksen *et al.*, 2002). Later, cells were washed with 1x PBS and diluted bacterial cells at MOI 1000 CFU/cell were added together with the LST drugs and incubated at 37° C, 5% CO_2. Post-incubation cells were washed with 1x PBS, added gentamicin (20 µg/ml) containing media and LST reagents and further incubated at 37° C, 5% CO_2, 48 hr. for FACS analysis.

3.6 Post-invasion Antibiotic Treatment

3.6.1 List of Antibiotics

Antibiotic was added to the invaded mammalian culture for killing internalized surviving bacterial cells. Listed compounds in Table 3.7 were purchased from SIGMA and used within the range of concentrations indicated in the Table, in four different cell lines. The range of concentration indicated for each reagent, are the final concentrations used in the present study.

Table 3.7. Antibiotics from different categories used for invaded bacterial lysis

Reagent	Category	Concentration	Solvent
Tetracycline	Broad spectrum	10-50 µg/ml	Water
Ciprofloxacin	Fluoroquinolone	10-100 µg/ml	Dil. HCl
Polymyxin B	Pore-forming	60-100 µg/ml	Water

3.6.2 Antibiotic Treatment

3.6.2.1 Time Duration Optimization for Antibiotic Administration

For optimizing time duration of antibiotic treatment, two set of treatments were examined. In one set, invaded cells were incubated with the antibiotic (Merck) containing growth media for 48 hr. However, 20 µg/ml of gentamicin was added to the same growth media after 24 hr. incubation. In another set, antibiotic-containing growth media was replaced with the fresh growth media containing 20 µg/ml of gentamicin only, after 24 hr. incubation. Both the sets were incubated at 37° C, 5% CO_2 for 48 hr. before proceeding with the FACS analysis.

3.6.2.2 Antibiotic Administration for Effective Internal Bacterial Lysis

Bactofection of cultured mammalian cells at MOI 1000 CFU/cell was followed by 1 hr. of gentamicin treatment (20 µg/ml) to kill non-internalized bacteria. Following gentamicin treatment, antibiotics i.e. Tetracycline, Ciprofloxacin, or Polymyxin B were added to the growth medium containing gentamicin and the culture was further incubated at 37° C, 5% CO_2 for 48 hr. for flow cytometric analysis (Birmingham *et al.*, 2006; Krusch *et al.*, 2002).

3.7 Combined Treatment of Reagents

3.7.1 Combined Treatment of Transfection, LST Reagent, and Antibiotic

Chemical reagents adapted to enhance vector's efficiency were all treated together to cultured and invaded cells to overall enhance gene delivery. Concentrations showed the highest responses for each of the reagents separately were used to study the combined effect. Methods of each treatment remained same as used individually (Section 3.4.2, 3.5.2.2 and 3.6.2.2) however, they were added stepwise in a single invasion to elevate gene delivery by helping vector's entry, escape from lysosomal degradation and intracellular lysis for transgene release.

3.8 Bacterial Assays

3.8.1 Gentamicin Protection Assay

Post-invasion incubation was followed by aspirating off the media containing non-invaded cells, washing thrice the plates by 1x PBS and adding the growth media (with serum) containing gentamicin (80 µg/ml) to remove non-internalized bacteria (Gentamicin protection assay) (Isberg *et al.*, 1985; Grillot-Courvalin *et al.*, 1998). Plates were again incubated at 37° C, 5% CO_2 for 1 hr. followed by a bacterial rescue (Section 3.8.2).

3.8.2 Bacterial Rescue Assay

The mammalian cells were washed again after 1 hr. (continued from Section 3.8.1) and lysed by treating with freshly prepared 1% Triton X-100 (+DAP) for 5 min. at 37° C, 5% CO_2, and collected the lysate in the Eppendorf tubes and stored on ice. Further, the lysate was serially diluted in BHI (+DAP) and plated onto the selective medium to determine the viable intracellular bacterial count (Narayanan and Warburton, 2003). Colony forming unit (CFU) on an agar plate was determined by:

$$\frac{\text{Number of colonies (CFUs)}}{\text{Dilution factor x Amount plated (ml)}} = \text{Number of bacteria/ml}$$

The obtained bacterial count was further calculated as a number of internalized bacteria per mammalian cell. This was obtained by dividing the CFU count with the number of the cultured mammalian cell.

$$\frac{\text{Bacterial CFU Count}}{\text{Number of cultured cell/well}} = \text{Number of internalized bacteria / Cell}$$

3.8.3 Spontaneous Bacterial Lysis Assay Post-invasion in HeLa

The rate of spontaneous lysis of intracellular *E. coli* DH10B *asd*$^-$ (pGB2Ωinv-hly, pEGFP-N2) after internalization in HeLa was determined over a period of 3 days. This was to assess if post-invasion, DAP auxotrophic bacterial cells are autolyzing completely over time or require external reagents to promote lysis for maximal gene delivery as suggested by Chen and colleagues (2014). Hence, spontaneous lysis rate was tested to obtain basal values of intracellular bacterial survival in HeLa cells before antibiotic treatment (Section 3.6.2.2 and Section 4.3.2). Among the four tested cells, HeLa was used as a model cell to study spontaneous intracellular bacterial death rate at 1000 MOI. As described earlier in section 3.4.2, 1000 MOI was used for

all kinds of chemical reagent assisted bactofection performed in this study i.e. transfection reagent, lysosomotropic reagent, and antibiotic treatment. Therefore, the spontaneous death rate was also assessed at the same MOI to analyze bacterial survival in host cells and to see if antibiotic treatment was required for effective transgene release.

Therefore, HeLa cells were invaded by invasive *E. coli* at 1000 MOI, which was further treated with gentamicin (80 µg/ml) and incubated at 37° C, 5% CO_2. Later, cultures were lysed at 24 hr. intervals scraped and plated onto selective media (Refer to Table 3.1). CFU values obtained initially after 1 hr. of gentamicin treatment was used as a reference value.

3.9 Transgene Delivery Efficiency Analyses

3.9.1 Quantitative Analysis of Reporter Gene (GFP) Expression using FACS

Invaded mammalian cells post-invasion was treated with gentamicin (20 µg/ml) and incubated further at 37° C, 5% CO_2 for 48 hr. (Gentamicin Protection Assay). Later, the cells were washed twice with 1x PBS and trypsinized. Trypsinized cells were pelleted down at 1200 rpm, 10 min. and washed twice with 1x filtered PBS. Finally, the cells were pelleted down and resuspended in filtered (through 0.22 µM) 1x PBS. The samples were then quantified by counting 10,000 cells using FACScan flow cytometer and CellQuest software (Laner *et al.*, 2005; Narayanan and Warburton, 2003). The population of cells with fluorescence was counted and given as percentage count by the software.

3.9.2 Qualitative Analysis of GFP Expression using Fluorescence Microscope

Cultured human cells invaded using *E. coli* vector carrying and delivering reporter gene which expresses green fluorescent protein were viewed under a microscope for qualitative

analysis of vector's gene delivery capacity. Culture plates after 48 hr. of gentamicin treatment were directly viewed under Fluorescence microscope (Nikon Eclipse TS100) using FITC filter. The FITC filter has excitation spectrum peak approximately at the wavelength of 495 nm while the emission spectrum peak at 519 nm (Khalfan *et. al.,* 1986; Riggs, *et. al.,* 1958). The attached cells from culture plates were then trypsinized (using 0.05% Trypsin-EDTA) and prepared for FACS analysis (Refer to Section 3.9.1) to correlate between quantitative and qualitative observation.

3.10 Molecular Biology Techniques

3.10.1 DNA Isolation

3.10.1.1 Plasmid DNA Extraction from Invasive and Non-invasive *E. coli*

Plasmid DNA was obtained using modified alkaline lysis miniprep method (Narayanan *et al.,* 1999; Ioannou and DeJong, 1996), for the confirmation of *E. coli* DH10B *asd*$^-$ (pEGFP-N2) and *E. coli* DH10B *asd*$^-$ (pGB2Ωinv-hly, pEGFP-N2) bacterial strains using restriction digestion method (Section 3.10.6). Plasmid DNA from *E. coli* DH10B *asd*$^-$ (pEGFP-N2) was also isolated for GFP DNA transfection purpose in human cells (Section 3.10.5.1). Bacterial culture was grown in 5 ml of growth media with required supplements and antibiotic selection (Section 3.1.3.1). 1.5 ml of overnight grown culture was centrifuged at 15,000 x g, 4 °C, 5 min. Obtained pellet was separated by discarding the supernatant and it was resuspended in 300 µl of the P1 buffer which contains 15 mM Tris-HCl (pH 7.6), 10 mM EDTA (pH 7.6). To the suspension, 300 µl of P2 buffer (0.2 N NaOH and 1% SDS) was added and mixed by inverting. Further, 300 µl of chilled P3 buffer (3 M CH_3COOK) was added and mixed by inversion. The tube containing mixture was incubated on ice for 10 min. and centrifuged later 15,000 x g, for 20 min. at room

temperature. From the obtained supernatant, 750 µl was added to the tube containing an equal amount of isopropanol (i.e. 750 µl) and mixed gently by inverting the tube. The tube was incubated on ice for 7 min. before being centrifuged at 15,000 x g, 20 min. at room temperature. The supernatant was carefully drained and the pellet was washed using 500 µl of 70% ethanol and centrifuging at same condition for 5 min. The supernatant was removed and the pellet was air dried before dissolving it in 20 µl of TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) containing RNase (20 µg/ml).

3.10.1.2 DNA Extraction from BAC Clone

BAC4396 clones described in Table 3.1 were used for BAC DNA isolation, and BAC DNA from these strains was prepared using alkaline lysis method as described above (Section 3.10.1.1.). However, to prevent shearing of large size DNA being isolated which is ≈80 kb, pipette tips were cut from the front to make it broad. This helped to maintain BAC DNA integrity during the isolation procedure.

3.10.2 Bacterial DNA Transformation

3.10.2.1 BAC DNA Electroporation into Invasive *E. coli* Vector

Electrocompetent cells of invasive *E. coli* strain DH10B *asd*$^{-}$ (pGB2Ωinv-hly) (Table 3.1) was prepared freshly and used for electroporation of BAC DNA. 40 ng of transforming DNA i.e. BAC4396-EGFPneo or BAC4396 was added to electrocompetent cells and was transferred to a chilled 2 mm cuvette (Bio-Rad). Electroporation was performed by pulsing cells at 2.5 kV, 200 Ω, and 25 µF settings using GenePulser® Xcell (Bio-Rad). Immediately after giving a pulse to cells, 1 ml of BHI media + 0.5 mM DAP was added which was then transferred gently using a 1 ml pipette, to a 1.5 ml centrifuge tube and incubated at 37° C, 220 rpm for 30-40 min. At the

end, cells were pelleted down by centrifuging at 5000 rpm, 5 min. and pelleted cells were resuspended in BHI media containing 0.5 mM DAP. 1/5 and 1/10 dilutions of resuspended pellet were plated separately onto growth media containing selective antibiotics while rest of it was plated on another plate. This was done to ensure low-density colonies on plates carrying diluted transformed cells while high-density colonies on the other. Low density enables picking up of single colonies easily for further screening of obtained colonies, to confirm the presence of transformed DNA. The screening was done by firstly extracting plasmid DNA from an overnight grown bacterial culture of transformed colonies (Section 3.10.1.2). Isolated plasmids were further digested with suitable restriction enzymes (Section 3.10.6) and electrophoresed using PFGE for analyzing digested DNA (Section 3.10.4.2).

3.10.3 β-Globin Expression Screening in Human Cell Lines

β-globin expression in cultured human cells was examined by measuring mRNA levels, using two-step Reverse Transcriptase PCR (RT-PCR) assay. Under two-step protocol, reverse transcription of isolated RNA is done first and then the PCR amplification of synthesized complementary DNA (cDNA) follows. Reverse transcription involves steps explained under sections 3.10.3.1 – 3.10.3.3 while PCR was done as described in section 3.10.3.4. Transcriptor Reverse Transcriptase cDNA synthesis kit (Roche) was used for the purpose, in this study.

RT-PCR was done to detect endogenous β-globin gene expression in all the nine human cells used for the current study (Table 3.3) and also the transcription of human β-globin transgene carried and delivered by invasive *E. coli* DH10B vector in cultured human fibroblasts (HT1080) (Results are described in Sections 4.5.1.2 and 4.5.2.3 respectively).

3.10.3.1 Total RNA Extraction

RNA was isolated from nine cultured human cell lines used in this study (Table 3.3), to synthesize cDNA for RT-PCR. Cells were either grown in culture dishes or 6-well plates and were trypsinized using 0.05% Trypsin-EDTA, $1x10^7$ cells were collected and counted for RNA extraction. For the purpose, RNeasy Mini Kit (Qiagen) was used and followed the manufacturer's protocol for extracting total RNA. RNA was isolated in 30 µl of RNase-free water and the isolated RNA was either used for RT-PCR directly or stored at -80 °C for long-term storage.

3.10.3.2 DNase I Treatment

Isolated RNA was firstly treated with DNase to remove any DNA contamination before proceeding with cDNA synthesis. 0.5 unit of DNase I from genomic DNA removal kit (Thermo Scientific) was used for treating 2 µg of RNA. The enzyme was added to RNA and incubated tube at 37° C for 30 min. Further, the enzyme was removed by adding 1 µl of DNase Removal Reagent (DRR) from the kit and incubated the mixture for 2 min. at room temperature. Following which the tube was centrifuged at 1000 rpm for 1 min. to collect the supernatant containing DNA-free RNA in a separate fresh Eppendorf tube to be used for cDNA synthesis.

3.10.3.3 cDNA Synthesis

Complementary DNA (cDNA) was synthesized using RNA transcripts obtained from nine different cell lines used in this study (Table 3.3), separately for globin gene expression analysis. Total RNA was used for preparing cDNA using Transcriptor Reverse Transcriptase cDNA synthesis kit (Roche) as per manufacturer's instructions. Total RNA yield was found to be ≈1 µg/µl upon RNA extraction from human cells, for endogenous globin expression screening

(Section 4.5.1.2). Therefore, 2 µl of the extracted total RNA was used for the Reverse Transcriptase (RT) reaction. However, very low RNA concentration was noted when total RNA was extracted after bactofection of BAC DNA in HT1080 cells i.e. <10 ng/µl. Hence, no water was added in the reaction and entire volume of total RNA was used. First, RT reaction was prepared on ice, by mixing the components as indicated below:

No.	Components	Volume (µl)
1	Total RNA	10 ng-5 µg
2	Anchored-oligo$(dT)_{18}$ Primer (50 pmol/µl)	1
3	Water, PCR Grade	13 (Add up to)
	Final Volume	**13**

The mix was incubated at 65 °C for 10 min. and placed immediately on ice, to denature secondary structures of RNA, if present. To this mix, following components were further added while keeping the tube on ice:

No.	Components	Volume (µl)
1	Transcriptor RT Reaction Buffer (5x)	4
2	Protector RNase Inhibitor (40 U/µl)	0.5
3	Deoxynucleotide Mix (10 mM each)	2
4	Transcriptor Reverse Transcriptase	0.5
	Final Volume	**20**

The components were mixed and the tube was spun briefly in a microfuge. The tube was further incubated at 55 °C for 30 min. Post-incubation, the tube was put in an incubator at 85 °C for 5 min. to inactivate Transcriptor Reverse Transcriptase and shifted the tube to the ice after 5 min. Amplified cDNA was either used for immediate PCR amplification (Described under Section 3.10.3.4) or stored at -20 °C until further use.

3.10.3.4 Polymerase Chain Reaction (PCR)

Prepared cDNA was further used as a template to amplify the obtained product using PCR technique. Two set of amplification reactions were prepared, of these one contained primers for actin gene while the other for the β-globin gene (Detailed in Section 3.10.3.5). Actin was used as a housekeeping gene to confirm that mRNA was present in PCR reactions (Hutvagner *et al.,* 2001). For globin amplification, a set of primer spanning between exons 2 and 3 of the human β-globin gene was used (Ooi *et al.,* 2008; Black and Vos, 2002). For expression analysis of delivered globin gene by invasive *E. coli* in human fibroblast cells (HT1080), total RNA was isolated from invaded cells (Section 3.10.3.1). Isolated RNA was then transcribed into complementary DNA by using Transcriptor Reverse Transcriptase cDNA synthesis kit (Roche) (Section 3.10.3.3). Synthesized cDNA was further used as a template for PCR amplification using actin and globin specific primer sets (Described in 3.10.3.5). Correctly spliced RNA transcript of human β-globin would generate the unique 231-bp β-globin gene product whereas any genomic DNA contamination would yield 1 kb amplification product (Ooi *et al.,* 2008; Black and Vos, 2002; Sierakowska *et al.,* 1996). Amplified products were further analyzed and observed by gel electrophoresis on agarose gel for β-globin gene expression (Section 3.10.4.1) PCR was performed using MyTaq DNA polymerase (Bioline).

Components for PCR mix (per reaction) were as follows:

No.	Components	Volume (µl)
1	MyTaq Buffer (5x)	10
2	cDNA Template	1
3	Forward Primer (10 µM)	2
4	Reverse Primer (10 µM)	2
5	MyTaq DNA Polymerase	0.5
6	Nuclease-free water	34.5
	Total	**50**

Amplification conditions used are as follows:

Steps	Temperature (°C)	Time Duration (Seconds)	
1	95	300	
2	94	30	**34 Cycles**
3	55	30	
4	72	30	
5	72	600	
6	4	Infinite	

3.10.3.5 Details of Primers Used

In the present work, primarily two sets of primers were used for β-globin expression screening in cultured human cells which were synthesized by AITbiotech PTE LTD, Singapore. Actin was used as housekeeping gene and primer sequence was obtained from the study conducted by Hutvagner and colleagues (2001). The human β-globin gene contains a total of 3 exons and 2 introns, intron 1 lies between exons 1 and 2 while intron 2 between exons 2 and 3 (Antoniou *et. al.,* 1988; Collins and Weissman, 1984). Gene expression screening in human cells was performed by using a set of primers spanning from exon 2 to exon 3, which was used earlier also in different studies (Ooi *et al.,* 2008; Black and Vos, 2002). The same sets of primers were used as they already have shown successful amplification from transfected clones thus, eliminating any chance of amplification error due to primer design.

Table 3.8. Primers used for actin and β-globin expression in human cells

No.	Primer Name	Primer Sequence	Tm (°C)	Annealing Temperature*
		Actin Primers		
1.	ACTF	5'-CGTGATGGTGGGCATGGGTCAG	55.3	T_A = 55 °C
2.	ACTR	5'-CTTAATGTCACGCACGATTTCC	47.9	
		β-Globin Primers		
3.	HBB-e2F	5'-GGACCCAGAGGTTCTTTGAGTCC	53.7	T_A = 55 °C
4.	HBB-e3R	5'-GCACACAGACCAGCACGTTGCCC	57.3	

* To evaluate the annealing temperature of primers, gradient PCR was performed in the range of melting temperature (Tm) for each set of primers i.e. 47° to 57 °C. Tm was provided by the oligonucleotide manufacturing company (AITbiotech PTE LTD, Singapore) along with the other details in Oligo Technical Data Sheet.

3.10.4 Gel Electrophoresis

3.10.4.1 Agarose Gel Electrophoresis

Electrophoresis was performed to visualize DNA molecules either after plasmid isolation (Section 3.10.1.1) to check its integrity or after restriction digestion to confirm the plasmid contained in the vector (Section 3.10.6). Also, the technique has been used to visualize band patterns of PCR amplified products (Section 3.10.3.4).

1% agarose gel was prepared in 0.5x TBE buffer (22.5 mM Tris-Borate, 0.5 mM EDTA) using UltraPure™ agarose (Invitrogen). Ethidium bromide (Sigma) was added to molten agarose to make DNA bands visible under ultraviolet (UV) light after the run and samples were mixed with 6x loading dye (Fermentas) prior to loading onto gel. 1 kb Plus DNA ladder (Fermentas) was used to load in wells together with samples for comparing DNA bands' sizes. Gel run was performed in 0.5x TBE buffer at 80 V for ≈1 hr. and separated DNA bands were viewed under UV light using gel imager (BioRad).

3.10.4.2 Pulsed Field Gel Electrophoresis (PFGE)

Apart from typical electrophoresis used for detecting and analyzing small sized DNA molecules, PFGE was performed by using CHEF-DR® III Pulsed Field Electrophoresis System for detecting large sized BAC clones (≈ 100 kb). 1% agarose gel (Roche) was cast using 0.5x TBE buffer (Section 3.10.4.1) in the tray for PFG without ethidium bromide addition and placed at 4 °C after solidification for cooling. Later, a thin slice of DNA ladder (Low Range PFG Marker, New England Biolabs) was cut from the plug using a sharp razor and placed vertically in one of the wells and the well was sealed using molten agarose. 0.5x TBE buffer was again used for electrophoresis, but firstly the buffer was filled in the tank and cooled down to 10 °C by running it through the cooling module. Further, the gel containing sealed DNA ladder was placed

in the tank and samples mixed with 6x loading dye (Fermentas) were loaded into remaining wells.

The gel was electrophoresed at 4.5 V/cm for 16 hr. with the buffer maintained at 4 °C through cooling module throughout the run to avoid overheating. Initial switch time was 0.1 seconds and final switch time used was 10 seconds. After electrophoresis, the gel was stained using ethidium bromide (Sigma), viewed under UV light for DNA bands and captured the pictures using gel imager (BioRad).

3.10.5 Cell Line Transfection

3.10.5.1 GFP DNA Transfection using Lipofectamine 2000

Amongst all the tested cell lines in this study, HepG2, MCF-7, and SKOV-3 cells were obtained from users who have not maintained any record of their cell line's passage number. Therefore, to be sure that these cell lines still maintain their transfectibility efficiency and are healthy to be used for further experimentation, their DNA uptake ability was assessed. It was done by transfecting reporter gene i.e. EGFP DNA in these cells, using Lipofectamine 2000. DNA uptake efficiency was then quantified by the level of GFP expression using FACS analysis (Section 3.9.1). Obtained values were then compared with the reported literature for each cell giving us the idea of DNA uptake capacity of tested cells. Comparable DNA uptake efficiencies would suggest that the cells are in healthy state and can be further used for bacterial invasion studies (Section 4.1.1.3; Fig. 4.3 – Fig. 4.5). Results as described in section 4.1.1.3, suggests that obtained DNA uptake efficiencies by tested cells are quite comparable to their expected values and hence, reflects their healthy state. Thus, these cells were further used in the current study for various bactofection studies.

Transfection is the method to deliver DNA using chemical reagent which binds to and delivers DNA into target cells. For the purpose, Lipofectamine 2000 reagent was selected as it is the most commonly used reagent and the cited literature also used the same with the respective cell lines which make the result comparable.

Cells were cultured in 6-well plates at $1x10^5$ cells/well density in the appropriate media (Table 3.3) and incubated at 37° C, 5% CO_2, 24 hr. DNA transfection was performed after 24 hr. incubation. For transfection process, plasmid DNA (pEGFP-N2) was isolated from *E. coli* DH10B *asd*$^-$ (pEGFP-N2) strain using conventional alkaline lysis method (See Section 3.10.1.1). Isolated plasmid DNA and transfection reagent (Lipofectamine 2000) were diluted in serum-free media in separate tubes (Table 3.9). Diluted DNA from Tube 1 was mixed into Tube 2 and the mix was incubated at room temperature for 20 min. Post-incubation, the complexed DNA was added into the cultured mammalian cells (in 6-well plates) and incubated in a CO_2 incubator at 37° C, 5% CO_2. After 4 hr. of incubation, media in the plates was replaced with fresh media and the plates were incubated for 48 hr. at 37° C & 5% CO_2. The samples were then analyzed for reporter gene expression by FACS (Section 3.9.1).

Table 3.9. Schematic illustration of dilution preparation of DNA and transfection reagent for reporter DNA transfection

	A	**B**	**C**	**D**	**E**	**F**	**G**
Media for dilution (FBS$^-$)	250 µl	250 µl	250 µl	250 µl	250 µl	250 µl	250 µl
DNA Concentration (Tube 1)	1 µg	1 µg	1 µg	1 µg	1 µg	1 µg	1 µg
Lipofectamine Concentration (Tube 2)	0 µg/µl	1 µg/µl	2 µg/µl	4 µg/µl	6 µg/µl	10 µg/µl	14 µg/µl

3.10.5.2 BAC DNA Transfection using Lipofectamine 2000

Cells were cultured in 6-well plates at $1x10^5$ cells/well density in the appropriate media (Table 3.3) and incubated in a CO_2 incubator at 37° C, 5% CO_2 for 24 hr. DNA was isolated as given in section 3.10.1.2, transfection was performed after 24 hr. incubation. For transfection process, BAC DNA and transfection reagent were diluted in reduced serum media Opti-MEM® (Life Technologies) in separate tubes (Table 3.10). Diluted DNA from Tube 1 was mixed into Tube 2 and the mix was incubated at room temperature for 20 min. Post-incubation, the complexed DNA was added into the cultured mammalian cells (in 6-well plates) and incubated at 37° C, 5% CO_2. After 4 hr. of incubation, media in the plates was replaced with fresh media and the plates were incubated for 48 hr. at 37° C & 5% CO_2. The samples were then analyzed for reporter gene expression by FACS (Section 3.9.1).

Table 3.10. Schematic description of dilution preparation of DNA and transfection reagent for BAC DNA transfection

	A	**B**	**C**	**D**	**E**	**F**
Opti-MEM for Dilution (FBS^-)	250 µl	250 µl	250 µl	250 µl	250 µl	250 µl
BAC DNA Concentration (Tube 1)	2 µg	2 µg	2 µg	2 µg	2 µg	2 µg
Lipofectamine Concentration (Tube 2)	0 µg/µl	2 µg/µl	4 µg/µl	6 µg/µl	8 µg/µl	10 µg/µl

3.10.6 Restriction Digestion

Isolated plasmid DNAs were digested using specific restriction enzymes (Roche) for confirming clone used in this study (Sections 3.1.3.1 and 3.10.1). Target DNA was subjected to digestion by the appropriate enzyme and analyzed the restricted fragments using gel

electrophoresis (Section 3.10.4). The reaction mixture containing buffer and enzyme together with the template was incubated at 37° C for 1 hr.

Restriction digestion reaction contains the following components in each reaction:

No.	Components	Volume (µl)
1	10x Buffer	2
2	Restriction Enzyme	1
3	Template DNA	2
4	Distilled Water	15
	Total	**20**

3.10.7 DNA Sequencing

BAC clones containing beta-globin gene were also confirmed by sequencing analysis. DNA was isolated using conventional method (Section 3.10.1.2) and was sequenced using next-generation sequencing method using MiSeq Sequencer. Sequencing results were obtained in FASTA format which was further analyzed by doing BLAST with the available sequence from NCBI.

3.10.8 Statistical Analysis

GraphPad Prism 5 software was used to perform statistical analysis in this current study. Mainly One-Way ANOVA (ANalysis Of VAriance) was performed followed by two kinds of post-hoc tests i.e. either Tukey's or Dunnett's Multiple Comparison Test. Tukey's test was used for statistical analysis of "combined treatment" experiments which involves two parameters i.e. different MOIs and separate control for each MOI (Section 4.4 and Section 4.5.2.3). While Dunnett's Multiple Comparison Test was performed for all the other conducted experiments, as they involved single parameter only and obtained values were compared with the control value. Particular post-hoc test for each experiment set is mentioned in the relative section/figure.

4. RESULTS

4.1 Tissue-dependent Bactofection of Invasive *E.coli* in Varied Human Cell Lines

4.1.1 Bacterial Strains' Confirmation and Preliminary Studies for Invasion

4.1.1.1 Genotypic Verification of *E. coli* Strains

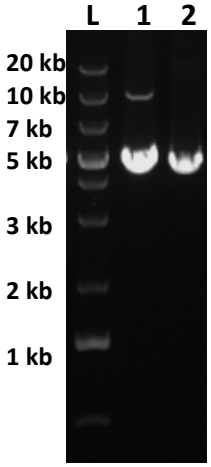
Genotypes of bacterial strains obtained from A/P K. Narayanan's lab stock was confirmed by their size and antibiotic resistance carried by plasmids (Section 3.1.3). Digested plasmids from invasive i.e. *E. coli* DH10B *asd*$^-$ (pGB2Ωinv-hly, pEGFP-N2) and non-invasive i.e. *E. coli* DH10B *asd*$^-$ (pEGFP-N2) *E. coli* strains were analyzed on an agarose gel (Sections 3.10.6 and 3.10.4.1). Plasmid from invasive *E. coli* upon restriction digestion and gel electrophoresis produced DNA bands of 10 kb and ≈5 kb, which confirmed the presence of pGB2Ωinv-hly (10 kb) and pEGFP-N2 plasmids (4.7 kb) (Fig. 4.1A; Lane 1). Similarly, restriction digestion and subsequent agarose gel analysis of non-invasive plasmid resulted in a single band of ≈5 kb showing the presence of pEGFP-N2 plasmid alone and absence of invasin plasmid (Fig. 4.1A; Lane 2).

Additionally, strains were confirmed by antibiotic selectivity based on specific resistance genes carried by each plasmid. For the verification, 20 different colonies of each of the bacterial strain were picked from an agar plate using sterilized toothpicks and, grown on separate growth media containing specific selective antibiotic/growth condition, simultaneously (Fig. 4.1B). Both invasive and non-invasive *E. coli* DH10B strains showed nil growth on media which was deprived of DAP supplement, but positive growth by all the colonies on DAP supplemented media, indicating DAP auxotrophy due to the aspartate β-semialdehyde (*asd*) gene deletion in both the strains. Deletion of aspartate β-semialdehyde gene cause DAP auxotrophy in *E. coli*

strains i.e. bacterial cell lyse upon cell division in the absence of DAP supplementation (Narayanan and Warburton, 2003). Simultaneous positive and negative growth by all the picked colonies on DAP supplement and DAP non-supplemented growth media respectively, further confirms that the picked colonies from invasive and non-invasive *E. coli* DH10B *asd*$^-$ strains, were all viable and that the negative growth of these colonies was solely due to the DAP auxotrophy. Positive growth by all the picked colonies on zeocin-containing medium, further confirmed the *asd* gene deletion in the tested invasive and non-invasive *E. coli* DH10B *asd*$^-$ strains, as the *asd* gene was replaced by zeocin resistance gene (Narayanan and Warburton, 2003).

Furthermore, positive growth of all the picked colonies from both the invasive and non-invasive *E. coli* strains on kanamycin-containing growth media, confirmed the presence of pEGFP-N2 plasmid which carried the reporter green fluorescent protein (GFP), in both the strains. Moreover, spectinomycin resistance-conferring gene is present on the low copy number plasmid pGB2Ωinv-hly plasmid. Therefore, the positive growth of picked colonies was shown only by the invasive *E. coli* DH10B strain only, due to the presence of pGB2Ωinv-hly plasmid and not by the non-invasive *E. coli* DH10B strain, which lacks the invasive plasmid (Fig. 4.1B). Hence, the strains were confirmed to be the appropriate one containing the required plasmids.

A.



B.

	BHI only	BHI + DAP	BHI +DAP+Zeo	BHI +DAP+Kan	BHI +DAP+Spec	Genotype
DH10B (Non-invasive)	X	√	√	√	X	*asd*$^-$ *zeo*^{r} *kan*^{r}
DH10B (Invasive)	X	√	√	√	√	*asd*$^-$ *spec*^{r} *kan*^{r} *zeo*^{r}

Figure 4.1. Genotype verification of invasive and non-invasive *E. coli* DH10B *asd*$^-$ strains. A. Digested plasmids from *E. coli* DH10B *asd*$^-$ *zeo*^{r} (pGB2Ωinv-hly, pEGFP-N2) (Lane 1) and *E. coli* DH10B *asd*$^-$ (pEGFP-N2) (Lane 2) was run on an agarose gel, L- 20 kb DNA ladder. B. 20 colonies from each strain were picked and grown on growth media under the conditions as indicated in the chart above. Both the invasive and non-invasive bacterial strains mentioned above showed positive growth on culture media supplemented with DAP and Zeocin, but no growth on media without DAP supplement. This confirms deletion and replacement of aspartate β-semialdehyde (*asd*) gene with zeocin resistance gene. Further, spectinomycin resistance confirmed the presence of pGB2Ωinv-hly plasmid and kanamycin resistance showed the presence of the pEGFP-N2 plasmid in both the invasive and non-invasive strains.

4.1.1.2 Appropriate Seeding Density of Cultured Cells for Invasion

After strain confirmation, next step was to analyze appropriate seeding density for performing invasion studies. Invasion studies required quantitative analysis of transgene expression using the flow cytometry (FACS) method. Moreover, for FACS analysis, cultured cells were required to be incubated further for 48 hr. post-invasion, to allow appropriate protein expression from the delivered gene. Cultured cells were required not to be densely packed after 48 hr. of the incubation period for FACS analysis, which affects GFP expression (based on observation). Thus, varying concentrations of cultured cells were tested to obtain suitable cell density for further experimental studies.

A varying number of cells were seeded in a 6-well plate using cervical cell line (HeLa) and observed after 2 days under a microscope (Fig. 4.2). The cell density of 10^4 did not show confluence at the end of 48 hr. but cell density of 10^5 revealed comparatively higher density. Among them, $2x10^5$ cell density shows densely packed cells whereas, $1x10^5$ cells density shows the appropriate population of cells required for *in vitro* expression studies. Hence, $1x10^5$ cells per well (6-well plate) was the seeding density which was used in further studies throughout the project.

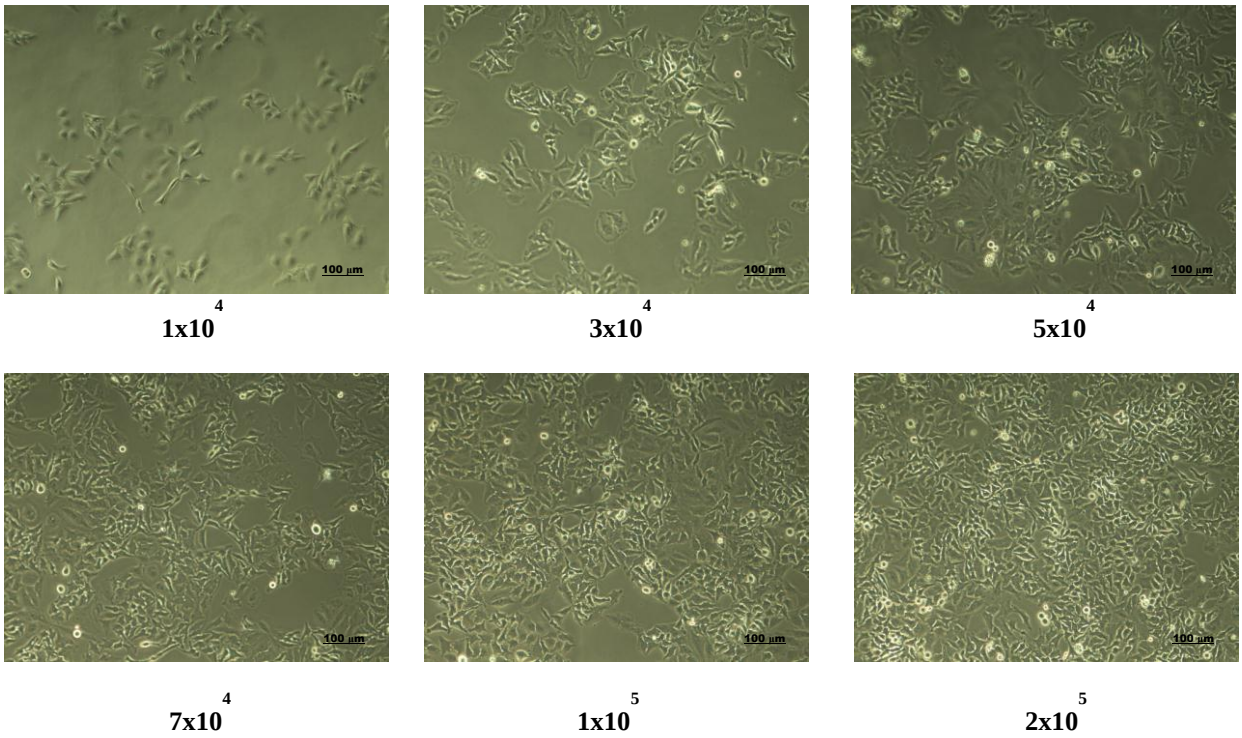


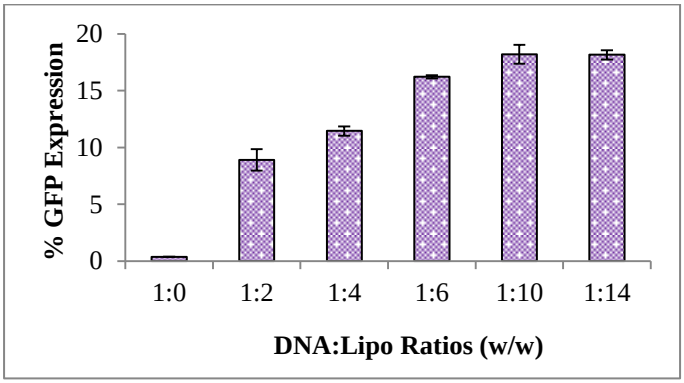
Figure 4.2. Seeding density test for invasion studies. HeLa (Cervical) cells were cultured in a 6-well plate at different densities as indicated above. After doubling time of 24 hr. for HeLa cells, media was replaced by fresh media to imitate the invasion method and further plates were incubated at 37° C, 5% CO_2 for 48 hr. Incubated cells were later viewed under a light microscope using 10x magnification and captured images; Scale: 100 µm.

4.1.1.3 Investigation of DNA Uptake Efficiency of Cell Lines

Mammalian cells with high passage number i.e. ≥35 were tested for their transfection capability by comparing the obtained gene expression with the literature. This was done to assure the uptake ability of cells and confirm their healthiness for further invasion studies.

DNA to transfection reagent ratios were prepared as described in section 3.10.5.1, Table 3.9. Transfection efficiency was obtained by GFP expression quantification using flow cytometer

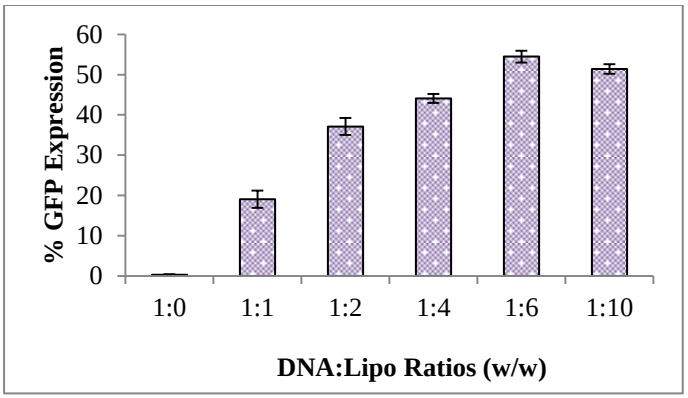
and obtained values were then compared with the cited literature. The comparison was performed based on the fact that cited literature and conducted study both have used same transfection reagent i.e. Lipofectamine 2000 and have followed manufacturer's protocol, for all the tested cells. Moreover, no clear ratios of DNA and reagent are mentioned in the cited literature thus, a range of ratios was tested in the present study. Overall, the basic aim was to get roughly comparable values so as to confirm its DNA uptake potential.



Transfection Efficiency

Expected	Observed
18%	19%

Figure 4.3. DNA transfection in HepG2 (Liver) cells using Lipofectamine 2000. Transfection efficiency was analyzed by GFP expression by FACS. Plasmid DNA (pEGFP-N2) 1 µg was used for transfection, whereas Lipofectamine 2000 was used in varying concentrations (µg/µl). Diluted DNA without any transfection reagent was used as negative control. Transfection efficiency as expected from literature study and observed gene expression from the present study is shown in tabulated form (Min *et al.*, 2005; Kim *et al.*, 2004). Error bars represent mean ±SEM (n=3). One-Way ANOVA was performed using Dunnett's Multiple Comparison Test as a post-hoc test, $p<0.0001$.

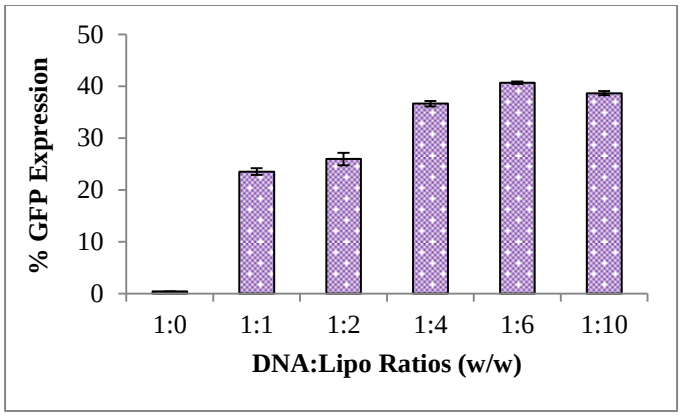


Transfection Efficiency

Expected	Observed
50%	54%

Figure 4.4. DNA transfection in MCF-7 (Breast) cells using Lipofectamine 2000. Transfection efficiency was analyzed by GFP expression by FACS. Plasmid DNA (pEGFP-N2) 1 µg was used for transfection, whereas Lipofectamine 2000 was used in varying concentrations (µg/µl). Diluted DNA without any transfection reagent was used as negative control. Transfection efficiency as expected from literature study and observed gene expression from the present study is shown in tabulated form (Wurzer *et al.,* 2003). Error bars represent mean ±SEM (n=3). One-Way ANOVA was performed using Dunnett's Multiple Comparison Test as a post-hoc test, $p<0.0001$.

Obtained gene expression was found to be comparable with the expected value at the ratio of 1:10 (w/w) for liver cells (Fig. 4.3) whereas for breast (Fig. 4.4) and ovarian (Fig. 4.5) cancer cells maximum gene expression was achieved at 1:6 ratios. The observed and expected transfection values for each cell were shown in tabulated form next to the graph. Comparing the transfecting capability of the three cell lines, breast cancer cells showed maximum transfectibility with Lipofectamine 2000 which was 54% ($p<0.0001$) (Wurzer *et al.,* 2003) followed by ovarian cells showing 40% transfection efficiency ($p<0.0001$) (Canine *et al.,* 2009) and liver cells with 19% transfection ($p<0.0001$) (Min *et al.,* 2005; Kim *et al.,* 2004).



Transfection Efficiency

Expected	Observed
37%	40%

Figure 4.5. DNA transfection in SKOV-3 (Ovarian) cells using Lipofectamine 2000. Transfection efficiency was analyzed by GFP expression by FACS. Plasmid DNA (pEGFP-N2) 1 µg was used for transfection, whereas Lipofectamine 2000 was used in varying concentrations (µg/µl). Diluted DNA without any transfection reagent was used as negative control. Transfection efficiency as expected from literature study and observed gene expression from the present study is shown in tabulated form (Canine *et al.*, 2009). Error bars represent mean ±SEM (n=3). One-Way ANOVA was performed using Dunnett's Multiple Comparison Test as a post-hoc test, $p<0.0001$.

4.1.2 Invasive *E. coli* Behavior in Nine Different Cell Lines from Human Tissues

Gene delivery capacity of invasive *E. coli* in cell lines derived from different human tissues was analyzed. A wide array of cell lines derived from various human tissues including lung, liver, and brain was chosen to study bacterial vector's capability of invading these tissues *in vitro.* This could provide an insight into bacterial vector's behavior in various tissues of the human body and also, specific tissues responding best towards the vector. Invasion capacity was analyzed by bacteria per cell count, after rescuing the internalized bacteria from mammalian cells using gentamicin (Gm) protection assay (Isberg *et al.*, 1985; Section 3.8). Bacteria per cell count

indicates a number of the internalized bacterial cell by each host human cell, thus calculating the number of rescued bacterial cells after host cell lysis indicates the invasion or bactofection capacity of invasive *E. coli* vector towards a particular host cell. However, the expression level of the transgene in host cells 48 hr. post-invasion was quantified by a flow cytometer, to evaluate gene delivery efficiency of invasive *E. coli* DH10B (Section 3.9.1). FACS evaluates gene delivery efficiency based on green fluorescence protein produced by pEGFP-N2 gene expression, which is carried and delivered to host cell by the invasive *E. coli* vector (Detailed in Section 3.1.1.2 and Section 3.9.1).

4.1.2.1 Internalized Invasive *E. coli* Quantification by Bacterial Rescue at Different MOIs

Bacterial rescue represents the number of live, internalized bacterial cells post-invasion in a host cell. Comparing among tested cells cervical and ovarian cells showed a maximum number of internalized bacteria i.e. 11 and 10 bacteria per mammalian cell respectively ($p<0.0001$ and $p=0.0003$ respectively) (Fig. 4.6). Kidney cells have shown on average 5 bacteria per mammalian cell ($p<0.0001$) and fibroblast cells resulted in highest of ≈2 invaded bacteria per cell ($p<0.0001$) (Fig. 4.6). However, other cell lines such as breast, brain, lung, and colon cells have revealed less than 1 bacterial entry per cell ($p=0.0021$, $p<0.0001$, $p<0.0001$ and $p<0.0001$ respectively). Liver cells did not show any significant bacterial entry ($p>0.05$).

Increasing MOI have resulted in increasing number of invaded bacteria in HeLa (Cervical; $p<0.0001$) and MCF-7 (Breast; $p=0.0021$) cell lines, unlike other cells which revealed highest bacterial entry at 1000 or 1500 MOI and then decrease in number of viable count at higher MOI i.e. 2000 and 4000 MOI (Fig. 4.6). Ovarian cells showed a maximum number of internalized bacteria at 1000 MOI ($p<0.05$) while kidney cells have shown highest bacterial entry

at 1500 MOI and a further decline in the number of invaded bacteria ($p<0.05$) (Fig. 4.6). Similarly, fibroblast cells revealed peaked entry of bacterial cells at 2000 MOI ($p<0.0001$) and a drop at higher MOI ($p<0.001$). Whereas, brain cells revealed non-significant bacterial entry at 500 and 1000 MOIs ($p>0.05$) but maximum internalized bacterial cells at 1500 MOI ($p<0.0001$) and further decline at 2000 and 4000 MOIs in comparison to control were observed ($p<0.01$ and $p<0.001$ respectively). Similar to ovarian and kidney cells, colon cells also did not show any significant difference in internalized bacteria count at MOIs higher than 1500 ($p>0.05$). Moreover, a similar pattern of reduced viable bacteria counts at 2000 ($p<0.01$) and 4000 MOI ($p>0.05$) was again observed in lung cells.

The exact mechanism for the discrepancy in viable bacteria count with increasing MOI is unknown but it can be correlated to host cell response towards the foreign body. Bacterial entry into host cell occurs through receptor-mediated endocytosis, which leads to endosome formation (Grillot-Courvalin *et al.*, 1998). Endosome formation leads to its fusion with lysosome resulting in degradation of the engulfed material. This lysosomal fusion and degradation are prevented by hemolysin protein in the vector which helps its escape from endosome. Nevertheless increased bacterial entry at higher MOI results in increased endocytic activity and rapid lysosomal membrane fusion (Cataldo *et al.*, 1997). This will result in endosomal degradation of bacteria thus, reducing the viable bacteria count i.e. CFU.

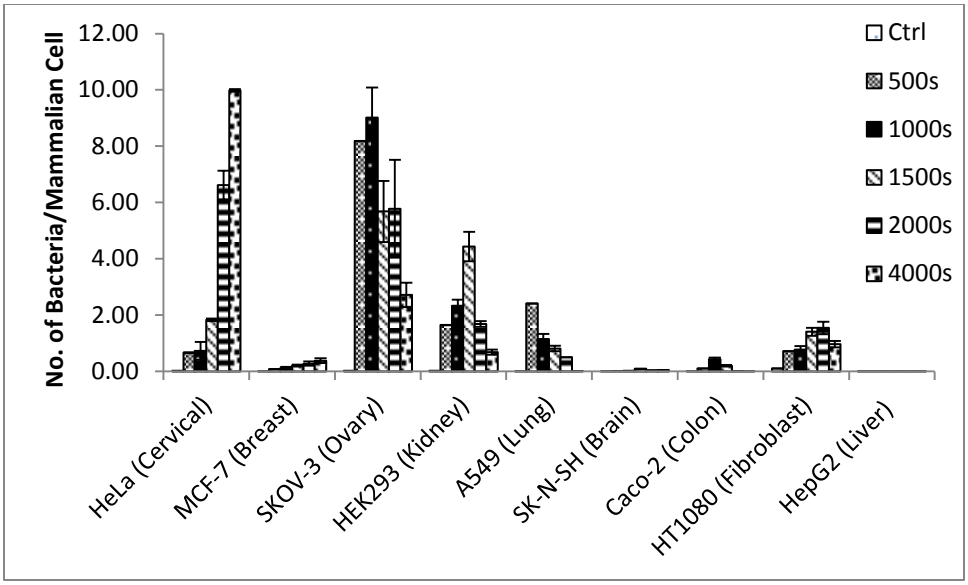


Figure 4.6. Bacterial invading capacity in different cell lines using invasive *E. coli* at different MOIs and non-invasive as a negative control at maximum MOI. Internalized bacterial rescue count in terms of bacteria per cell at various MOIs (Multiplicity of Infection) ranging from 500-4000, and control strain (Ctrl) at 4000 MOI. The non-invasive strain which was used as a control was tested at highest MOI i.e. 4000 to determine background invasion while comparing CFU counts of invasive strain with control strain, after the bacterial rescue. Error bars represent mean ±SEM (n=3). One-Way ANOVA was performed using Dunnett's Multiple Comparison Test as a post-hoc test for each cell separately.

4.1.2.2 Transgene Delivery Quantification by GFP Expression Analysis using FACS at Different MOIs

Gene delivery efficiency of the invasive *E. coli* vector was analyzed using flow cytometry technique by quantifying reporter gene expression i.e. GFP carried by the vector in mammalian cells. Transgene delivery by invasive *E. coli* was quantified based on the difference in gene expression compared to non-invasive *E. coli* also carrying GFP as a reporter gene.

Amongst the tested range of cell lines derived from various human tissues, bacterial vector showed highest gene delivery capacity in three cell lines i.e. cervical, ovarian and kidney cells with 16 fold ($p<0.0001$), 13 fold ($p<0.0001$) and 13 fold ($p<0.0001$) respectively (Fig. 4.7). These cells also showed the highest number of internalized bacteria among tested cell lines, which correlates with their high transgene delivery (Fig. 4.6). Gene delivery capacity by invasive vector showed a 4-fold increase in brain cells ($p<0.0001$) and a 3-fold increase in fibroblast cells ($p<0.0001$), over non-invasive counterpart (Fig. 4.7). Whereas, in other tested cell lines gene expression remained very low with less than 2-fold increase (Fig. 4.7). Moreover, liver cells were found to be somewhat resistant towards bacterial invasion because neither the internalized bacteria count nor the transgene delivery showed any statistically significant values ($p>0.05$ and $p=0.02$ respectively). In addition to flow cytometer analysis, HeLa cells were viewed under fluorescence microscope before trypsinizing invaded host cells 48 hr. post-invasion, for FACS sample preparation (Section 3.9.2). Fluorescence microscopy was done to assess if fluorescent green host cells, by virtue of successful gene expression from the delivered transgene, could be visualized under a microscope. For the purpose, HeLa was used as reference cell line and qualitative analysis of gene delivery by bacterial vector alone (without any treatment) was performed with HeLa only (Fig. 4.7 B).

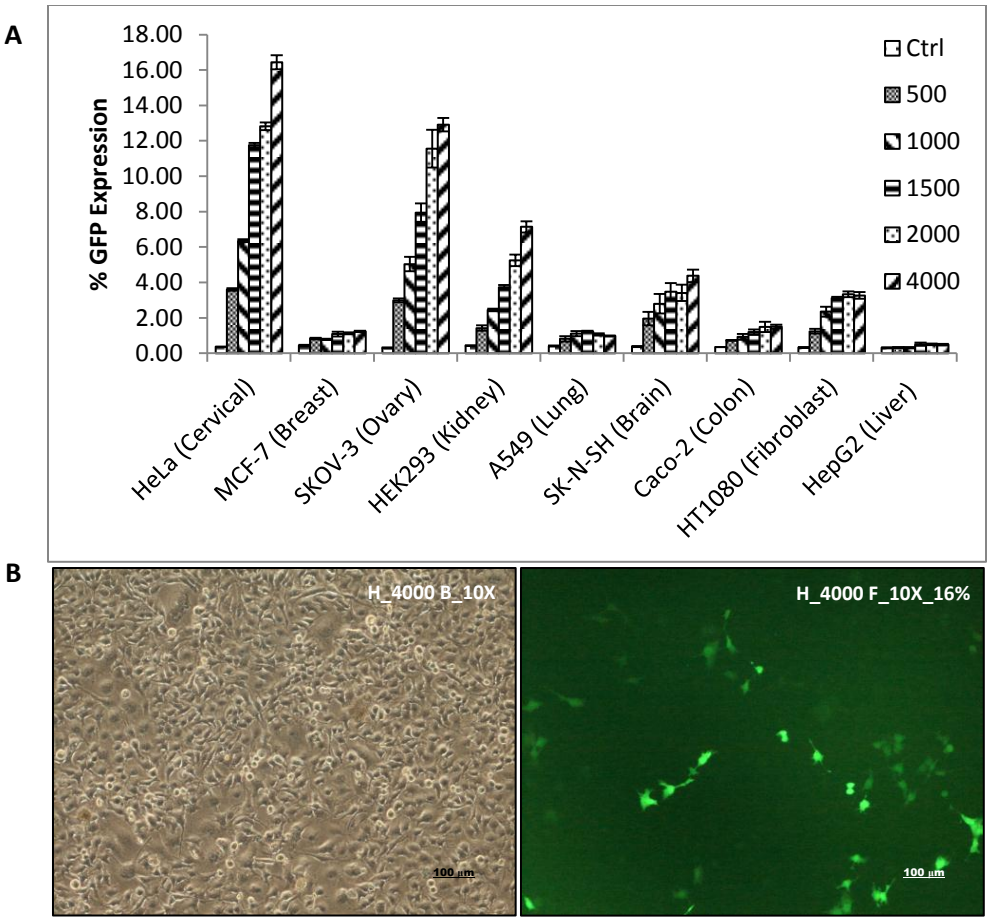


Figure 4.7. Invasive *E. coli* bactofection efficiency quantification in various human-derived epithelial cell lines. (A) Bactofection efficiency was quantified based on intracellular transgene expression carried by vector i.e. pEGFP-N2 48 hr. post-invasion. GFP expression was determined in percentage using FACScan Calibur at various MOIs ranging from 500-4000 for invasive *E. coli*, Ctrl - MOI of 4000 for control strain i.e. non-invasive *E. coli* in all the tested cell lines. Error bars represent mean ±SEM (n=3). One-Way ANOVA was performed using Dunnett's Multiple Comparison Test as a post-hoc test for each cell separately. (B) Fluorescence microscopy pictures of HeLa cells showing the highest number of GFP-expressing cells among tested cells i.e. ≈16% GFP, together with bright field image. H - HeLa (Cervical cells). 4000 – 4000 MOI. B and F- are the bright field and fluorescence view respectively. % values- show the obtained percentage values of same using flow cytometer; Scale: 100 µm.

In contrast to the viable bacteria count, GFP expression in every tested cell line showed a pattern of increasing gene expression with increasing MOI. The differential behavior of internalized bacteria counts and gene delivery in bactofection studies has been reported by other researchers also (Laner *et al.*, 2005; Grillot-Courvalin *et al.*, 1998). Hypothesized explanations states two possible conditions: (1) dead or dying bacteria still can be GFP positive; and (2) during phagosomal degradation of bacteria, DNA finds its way to cytosol contributing to gene expression (Laner *et al.*, 2005; Dietrich *et al.*, 1998; De Chastelliar *et al.*, 1994).

4.2 Enhanced Bactofection by Combining Bacterial Invading Capacity with Transfecting Ability of Chemical Reagents (Trans-bactofection)

4.2.1 Invasion and Transgene Delivery Quantification of jetPEI Coupled Invasive *E. coli* by CFU Count and Flow Cytometry

Polyethylenimine based transfection reagent, jetPEI have helped in aiding bacteria mediated gene delivery in some of the studied cell lines. In the presented study, jetPEI and other selected transfection reagents were coupled with invasive *E. coli* DH10B *asd*$^{-}$ (pGB2Ωinv-hly, pEGFP-N2) at different concentrations and used for invading cultured mammalian cells as described above (Section 3.4.2 and Section 3.3; Table 3.5). Invaded bacteria count and gene expression analyses were performed as mentioned in material and methods' sections 3.8.2 and 3.9.1. Results are compared with the values obtained with invasion carried out without any addition of reagent i.e. invasive *E. coli* DH10B alone.

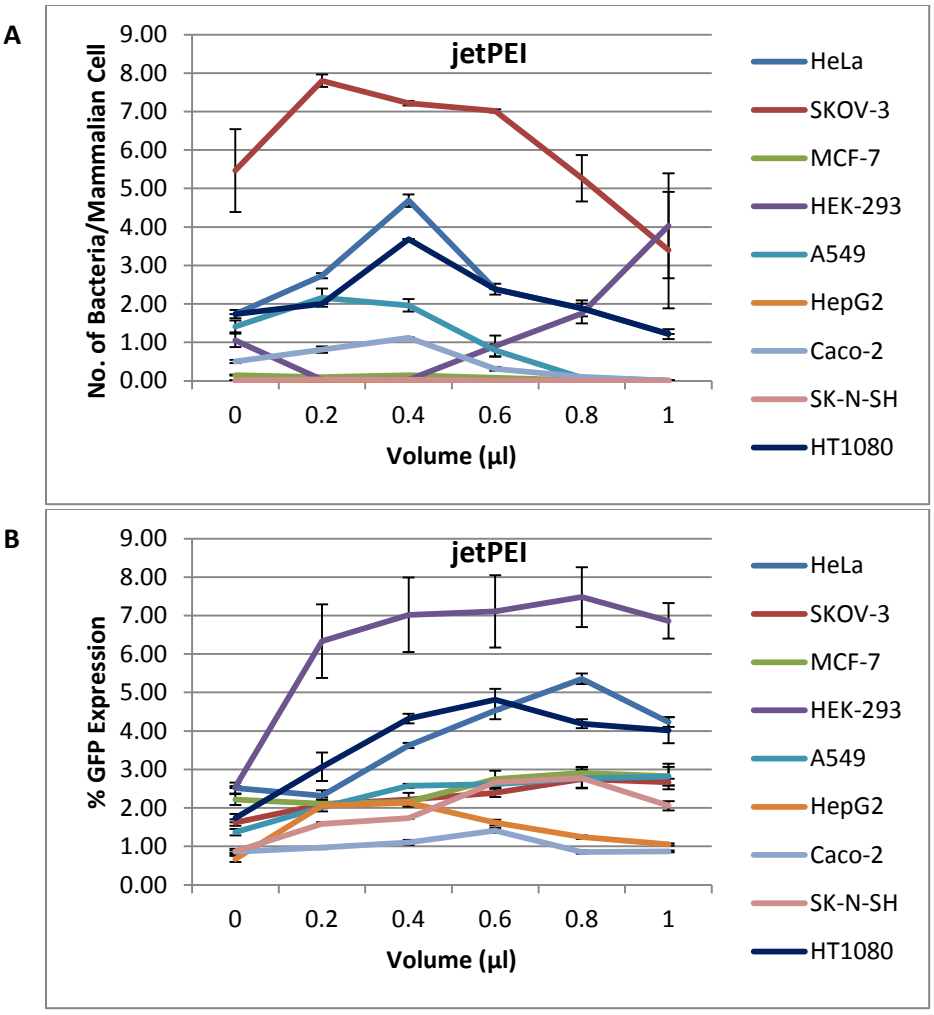


Figure 4.8. Trans-bactofection analysis using jetPEI in human-derived cell lines. (A) Internalized bacterial count in terms of bacteria per cell at various concentrations of the transfection reagent coupled with the diluted bacterial culture at 1000 MOI. Invasive *E.coli* alone without any transfection reagent was used as a control, (B) GFP expression determination in percentage using FACScan Calibur at various concentrations of transfection reagent in the diluted bacterial culture of 1000 MOI. Cultured mammalian cells were used as negative control for FACS analysis. Error bars represent mean ±SEM (n=3). One-Way ANOVA was performed using Dunnett's Multiple Comparison Test as a post-hoc test.

Bacteria CFU count after invasion has been increased from ≈1 to 5 bacteria per HeLa cell, ≈1 to 4 bacteria per HT1080 cell and from ≈5 to 8 bacteria per SKOV-3 cell at reagent volume of as low as 0.2 µl for HeLa and HT1080 cells while 0.4 µl for SKOV-3 cells ($p<0.0001$; $p<0.0001$ and $p=0.0200$ respectively) (Fig. 4.8A). Further increasing volume resulted in a decrease of CFU count in both the cells. However, HEK-293 cells showed no high increase at lower volumes but the internalized bacteria CFU count peaked at highest volume (1µl) i.e. increased from 1 to 4 bacteria per cell ($p=0.0037$). Caco-2 and A549 cells showed a significant increase of ≈1 bacteria per cell entry, upon reagent addition prior to bactofection ($p<0.0001$; $p<0.0001$). MCF-7, SK-N-SH, and HepG2cells revealed a small but significant increase in the bacterial entry ($p<0.0001$; $p<0.0001$; $p<0.0001$) (Fig. 4.8A). Reduced viable bacteria count was noted with increasing jetPEI concentration i.e. 0.6 µl – 1 µl in Caco-2, A549, MCF-7 and HepG2 cells.

Gene expression usually showed maximum value at a lower volume of reagent and at the highest volume, it showed a decline or no further increase in expression (Fig. 4.8B). In agreement with the highest increase in bacterial entry among tested cell lines, HEK-293 cells also showed highest of a 3-fold increase in GFP expression ($p=0.0063$). Brain (SK-N-SH) cells also showed 3-fold enhanced gene expression ($p<0.0001$), while HeLa and A549 cells gained an increase of 2 fold in GFP expression ($p<0.0001$; $p=0.0001$ respectively). Amazingly, HepG2 (Liver) cells which are hard to transfect and showed almost no significant gene expression with 0.6% GFP using invasive *E. coli* alone, have resulted in 2% GFP expression (3 fold; $p<0.0001$) after using jetPEI (Fig. 4.8B). Except for HepG2 which attained a maximum enhancement of gene expression at the lowest volume of 0.2 µl, HeLa, and A549 cells showed their peak in expression at the reagent volume of 0.8 µl followed with a slight decrease in values. HT1080, on

the other hand, revealed an increase of GFP expression from ≈1% to ≈5% at 0.6 µl jetPEI concentration and a further decrease in reporter gene expression at 0.8 and 1 µl reagent concentrations ($p<0.0001$). Remaining cells which include MCF-7, SKOV-3 and Caco-2 attained very low but significant increase in gene expression with the addition of reagent ($p=0.0109$; $p=0.0026$; $p<0.0001$ respectively).

4.2.2 Invasion and Transgene Delivery Quantification of Lipofectamine 2000 Coupled Invasive *E. coli* by CFU Count and Flow Cytometry

Cationic liposomes are well-known reagents to deliver nucleic acid in a variety of cells as described in section 1.1.1. Their contribution in mediating transgene delivery in mammalian cells is well recognized (Nikcevic *et al.,* 2003; Mortimer *et al.,* 1999). In this study, Lipofectamine 2000 was used which is a liposome-based DNA transfection reagent. Lipofectamine 2000 mediated trans-bactofection did not result in much increase of internalized bacteria count in few cell lines. Instead, for the majority of cell lines, the CFU count either remained same or dropped as compared to invasion by invasive *E. coli* alone. Coupling of Lipofectamine 2000 to bacterial vector produced a non-significant change in internalized bacteria count in HeLa and MCF-7 cell lines ($p=0.1881$ and $p=0.6477$ respectively) (Fig. 4.9A). Statistical analysis for trans-bactofection of HEK-293 using Lipofectamine 2000, revealed significantly different means ($p=0.0253$) however no significant difference could be observed in bacteria per cell count in comparison to control value, as indicated by Dunnett's Multiple Comparison Test ($p>0.05$) (Fig. 4.9A). HepG2 and SK-N-SH cells could achieve a significant but very slight increase in viable bacteria count upon reagent addition only at 0.2 and 0.4 µl concentrations respectively ($p=0.0016$; $p<0.0001$ respectively). Likewise, a slight increase of 0.8 to 1.5 bacteria per cell was noted in Caco-2 cells at 0.4 µl reagent volume with no further increase in bacteria count at higher volumes ($p<0.0001$).

On the contrary, in ovarian cells (SKOV 3) there is a sharp decrease in CFU count with the addition of reagent, a number of internalized bacteria count dropped from 5 bacteria per mammalian cell to less than 1 bacterium per cell ($p<0.0001$) (Fig. 4.9A). Similarly, in A549 and HT1080 cell lines, the addition of higher volumes of reagent i.e. 0.8 and 1 µl led to reduced bacteria per mammalian cell count as compared to control ($p=0.0013$ and $p=0.001$ respectively). Whereas at lower volumes of reagent coupling to bacterial vector, pre-invasion to both A549 and HT1080 cells, a non-significant difference was observed in CFU count ($p>0.05$) (Fig. 4.9A).

However, gene delivery was enhanced by coupling bacteria with the reagent. Among the tested cell lines with Lipofectamine 2000, cervical cells (HeLa) showed the best response of 2.6-fold increase ($p<0.0001$) in gene delivery at the lowest volume of 0.2 µl of reagent and no further increase with the increasing volume (Fig. 4.9B). This suggests that only a small amount of reagent is sufficient to aid in transgene expression. This result is comparable to another study conducted by our group to invade HeLa cells using the same reagent in combination with invasive *E. coli* DH10B containing invasin gene but no hemolysin gene (pGB2Ωinv, pEGFP-N2). The study showed an increase of 2.8 fold in GFP expression (Narayanan *et al.*, 2013). Apart from cervical cells, three other cell lines i.e. lung ($p=0.0020$), brain ($p=0.0008$) and breast ($p=0.0193$) showed an increase in gene delivery by coupling the reagent with bacteria although the increase remained under two-fold (1.4 fold, 1.2 fold, and 1.3 fold respectively). Fibroblast cells also showed a statistically significant increment of about 1.4 fold at low concentration of 0.4 µl ($p=0.0001$). Other cells i.e. ovary, HEK-293, Caco-2, and liver did not show any statistically significant difference in transgene expression upon reagent addition ($p=0.6219$; $p=0.0932$; $p=0.1390$ and $p=0.1009$ respectively).

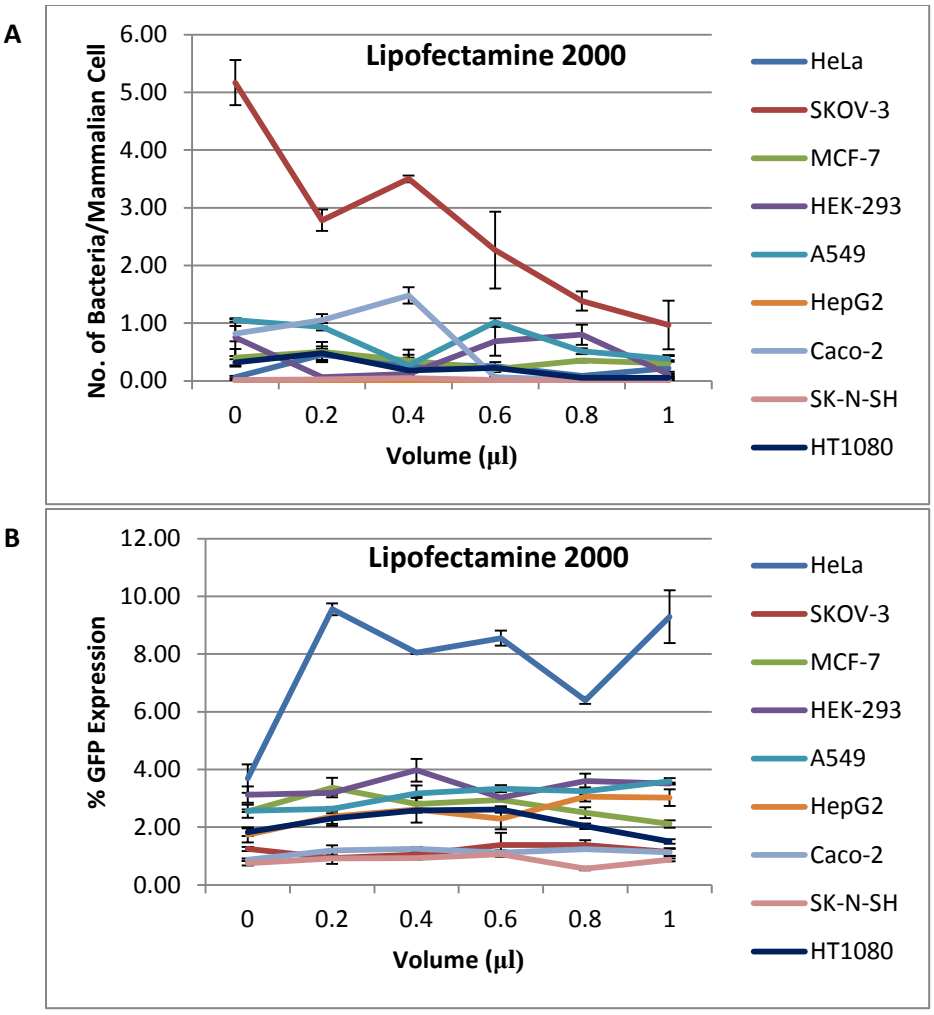


Figure 4.9. Trans-bactofection analysis using Lipofectamine 2000 in human-derived cell lines. (A) Internalized bacterial count in terms of bacteria per cell at various concentrations of the transfection reagent coupled with the diluted bacterial culture at 1000 MOI. Invasive *E.coli* alone without any transfection reagent was used as a control, (B) GFP expression determination in percentage using FACScan Calibur at various concentrations of transfection reagent in the diluted bacterial culture of 1000 MOI. Cultured mammalian cells were used as negative control for FACS analysis. Error bars represent mean ±SEM (n=3). One-Way ANOVA was performed using Dunnett's Multiple Comparison Test as a post-hoc test.

4.2.3. Invasion and Transgene Delivery Quantification of PULSin Coupled Invasive *E. coli* by CFU Count and Flow Cytometry

PULSin, a transfection reagent known for protein or antibody delivery was used in this study to assess its ability if any, in enhancing bacteria mediated gene delivery. Surprisingly, PULSin was able to elevate the transgene expression in all of the tested cell lines. The reagent helped in increasing the bacterial entry in the cervical (HeLa), ovarian (SKOV-3), kidney (HEK-293) and colon (Caco-2) cells (Fig. 4.10A). Hela cells revealed the highest increase in bacterial entry while HepG2 cells could not produce any viable bacteria per cell count upon bacterial rescue ($p<0.0001$; $p=0.0973$ respectively). In HeLa cells increase of 4 invaded bacteria/cell upon addition of reagent i.e. from ≈1 CFU/HeLa cell to ≈5 CFU/HeLa cell at highest volume of PULSin (1µl) was observed ($p<0.0001$). Bacterial entry into kidney, ovarian and colon cell lines was observed to be approximately elevated by 3, 1 and 1 CFU/mammalian cell ($p=0.0002$; $p<0.0001$; $p<0.0001$ respectively). In colon cells, lowest quantity of 0.2 µl was sufficient to help in enhancing bacteria entry with no further increase, in fact, increasing concentration resulted in a consistent reduction in CFU ($p=0.0001$). On the contrary, the highest volume of the reagent resulted in the highest increase in the bacterial entry, in HeLa and HEK-293 cell lines. They both showed an increase in lowest volume i.e. 0.2 µl followed by a reduction in entry and then a sudden increase at the highest reagent volume i.e. 1 µl. Also, in breast cells (MCF-7) addition of 0.4 µl reagent resulted in approximately 0.5 bacteria per cell entry in contrast to <0.1 bacteria per cell count ($p<0.0001$). Furthermore, in SK-N-SH cells 0.4 µl of PULSin produced very small but significant difference in bacterial entry upon coupling of the reagent ($p<0.0001$). However, 0.8 µl of the reagent was required to produce ≈1 bacteria/cell entry in A549 and HT1080 cells ($p<0.0001$; $p<0.0001$ respectively). These results suggest that optimum quantity of the reagent for aiding bacterial entry in mammalian cells upon invasion differs with the different cell line.

While the bacterial entry did not reveal any specific trend with increasing reagent concentration, gene expression on the other hand in the majority of cells was found to be concentration dependent (Fig. 4.10B). The highest increase of ≈10 fold GFP expression (from ≈2% to 20% GFP) in breast cell line was noted ($p<0.0001$). PULSin assisted invasion also resulted in a noteworthy increase in transgene expression of 6 fold and 5.2 fold in the brain and ovarian cell lines respectively ($p<0.0001$ and $p<0.0001$). HeLa cells showed comparatively lower but significant increase in GFP expression (3.4 fold; $p<0.0001$). Moreover, PULSin addition prior to the invasion in HT1080 cells revealed highest of 3-fold increase with 6% GFP expression at 0.8 µl ($p<0.0001$). Other cells such as Caco-2 (2.2 fold), A549 (2.1 fold) and HEK-293 (1.7 fold) also showed statistically significant increase in gene expression after coupling bacteria with the transfection reagent ($p=0.0073$; $p<0.0001$; $p=0.0009$ respectively). Although, HepG2 cells could not produce any viable bacteria per cell count upon bacterial rescue yet resulted in a 3.2-fold increase ($p=0.0002$) in GFP expression as compared to bactofection without any transfection reagent (Fig. 4.10A and B). This suggests that it might be due to high lysosomal activity in liver cells causing bacterial degradation but transgene's cytosolic access for its expression.

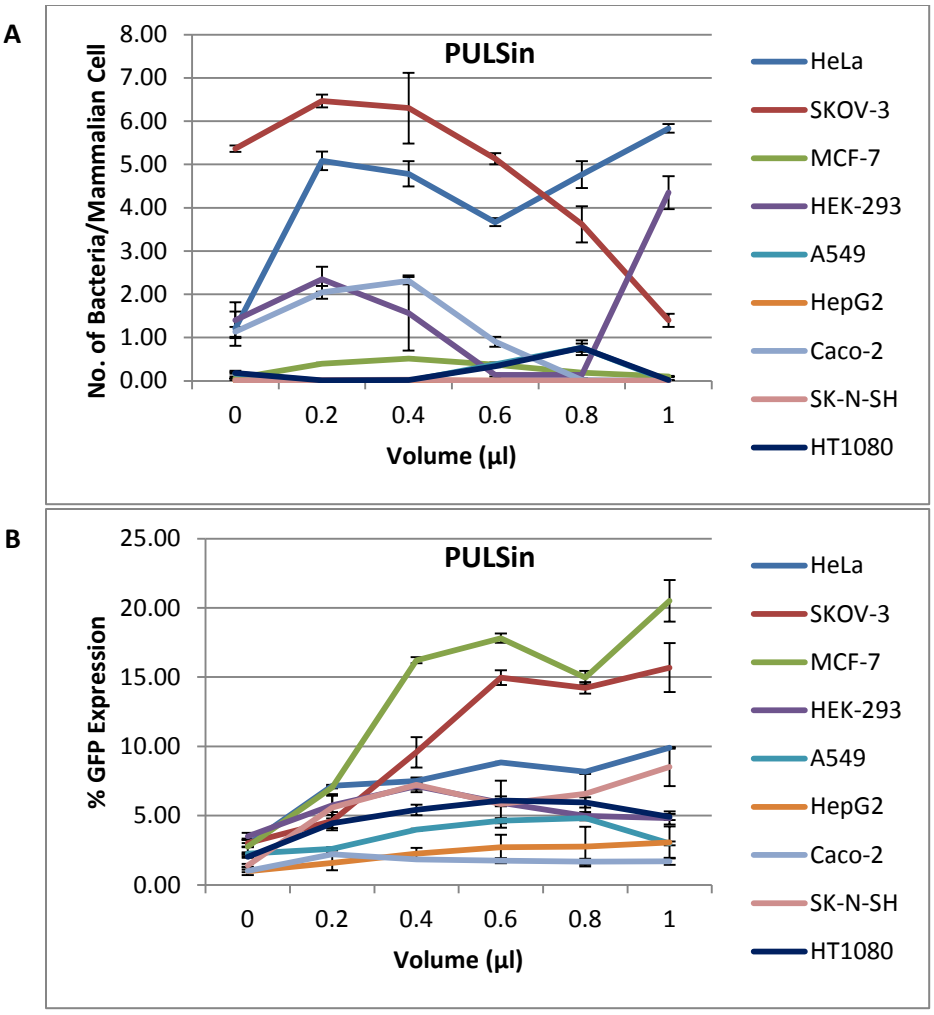


Figure 4.10. Trans-bactofection analysis using PULSin in human-derived cell lines. (A) Internalized bacterial count in terms of bacteria per cell at various concentrations of the transfection reagent coupled with the diluted bacterial culture at 1000 MOI. Invasive *E.coli* alone without any transfection reagent was used as a control, (B) GFP expression determination in percentage using FACScan Calibur at various concentrations of transfection reagent in the diluted bacterial culture of 1000 MOI. Cultured mammalian cells were used as negative control for FACS analysis. Error bars represent mean ±SEM (n=3). One-Way ANOVA was performed using Dunnett's Multiple Comparison Test as a post-hoc test.

4.2.4. Invasion and Transgene Delivery Quantification of ExGen 500/TurboFect Coupled Invasive *E. coli* by CFU Count and Flow Cytometry

Cationic Polymer-based transfection reagent, ExGen 500/TurboFect in conjunction with invasive *E. coli* yielded similar bacterial entry pattern as with Lipofectamine 2000 i.e. lower volumes of reagent are more effective in elevating bacteria per cell count (Section 4.2.1, Fig. 4.9A). Moreover, reduced viable bacteria count at higher volumes was noted. No significant bacterial entry enhancement was shown by HeLa and HEK-293 cells upon coupling the transfection reagent pre-invasion ($p=0.9368$ and $p=0.0253$ respectively). Although Dunnett's Multiple Comparison Test resulted in no significant values in HEK-293 cells yet, significantly different means were obtained upon statistical analysis by one-way analysis of variance (Fig. 4.11A). In HepG2, SK-N-SH and HT1080 cells small but significant difference in the internalized bacteria count were noted at lower volumes of the reagent i.e. 0.2 µl and 0.4 µl ($p=0.0002$; $p<0.0001$; $p<0.0001$ respectively). Internalized bacteria count has been increased up to approximately 3 fold, 2 fold and 2 fold in MCF-7, A549 and Caco-2 cells respectively ($p=0.0003$; $p=0.0001$; $p<0.0001$ respectively). In SKOV-3 cells, a steep decrease in CFU count after addition of the reagent was observed and the decrease is almost increasing in parallel to the increasing concentration of the reagent ($p<0.0001$).

Despite the reduced CFU count, gene expression continued to remain high as observed in other tested reagents also (Fig. 4.8, 4.9, 4.10). Gene expression in SK-N-SH cells was raised from 1.8% to 5.34% GFP (≈3 fold; $p<0.0001$) at as low as the 0.4 µl volume of the reagent (Fig. 4.11B). A549 cells also showed the highest gene expression at a 0.4 µl volume of reagent (2.2 fold; $p<0.0001$). Whereas, ovarian (1.6 fold) and breast (1.3 fold) cells showed their peaked gene expression at 0.8 and 0.6 µl volume of transfection reagent ($p=0.0090$; $p=0.0077$ respectively). In

accordance with no significant bacteria/cell count, HEK-293 cells also resulted in a non-significant change in GFP expression upon FACS analysis ($p=0.0932$). On the contrary, although Caco-2 cells produced significant internalized bacteria count yet no significant value could be obtained from Caco-2 GFP expression data analysis by using Dunnett's Multiple Comparison Test ($p>0.05$). Statistical analysis of HeLa cells, suggests a small but significant change in GFP expression after bacterial vector coupling to the transfection reagent ($p<0.0001$). TurboFect reagent's coupling to invasive *E. coli* resulted in 3.6 and 2.3-fold increase in GFP expression in Hep-G2 and HT1080 cell lines respectively, at volumes less than 0.8 µl ($p=0.0021$; $p=0.0004$ respectively). None of the tested cells showed any increased GFP expression at higher volumes of reagent i.e. 0.8 and 1 µl volumes, suggesting that the highest volume of ExGen 500/TurboFect reagent used in this study have not assisted in any further enhancement of transgene delivery (Fig. 4.11B).

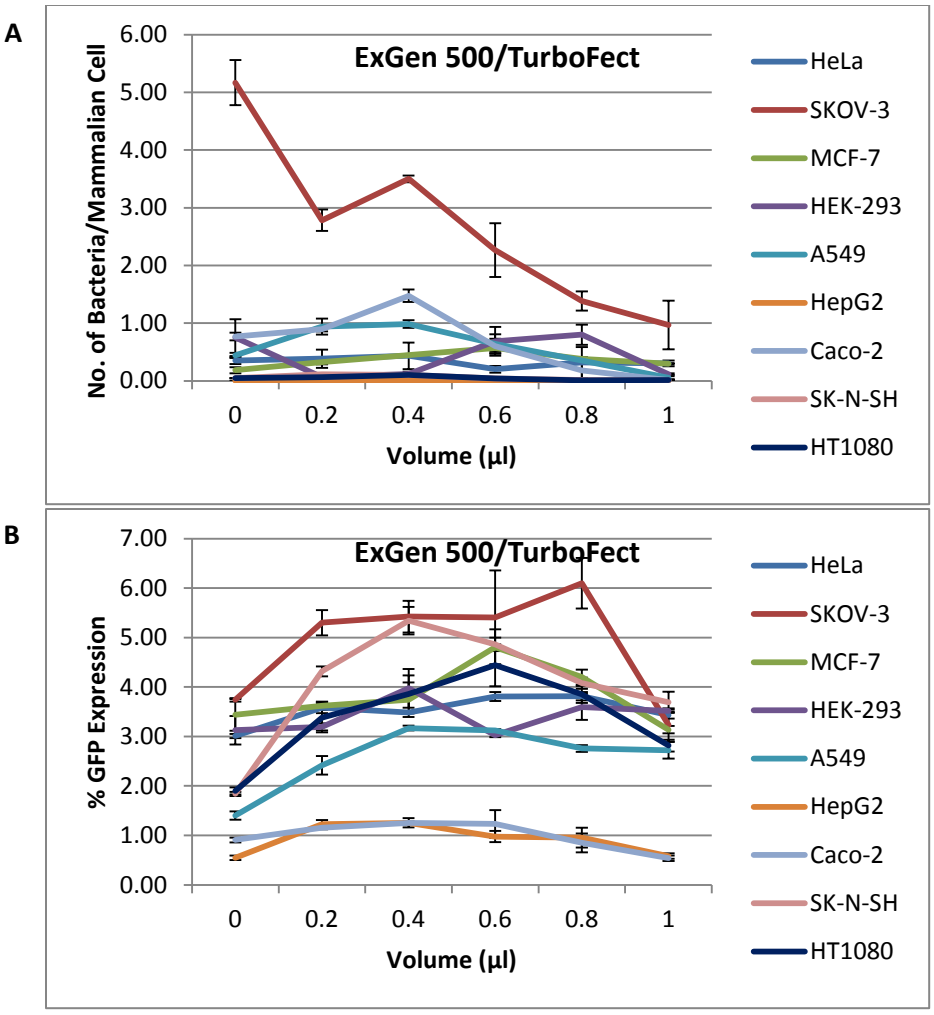


Figure 4.11. Trans-bactofection analysis using ExGen 500/TurboFect in human-derived cell lines. (A) Internalized bacterial count in terms of bacteria per cell at various concentrations of the transfection reagent coupled with the diluted bacterial culture at 1000 MOI. Invasive *E.coli* alone without any transfection reagent was used as a control, (B) GFP expression determination in percentage using FACScan Calibur at various concentrations of transfection reagent in the diluted bacterial culture of 1000 MOI. Cultured mammalian cells were used as negative control for FACS analysis. Error bars represent mean ±SEM (n=3). One-Way ANOVA was performed using Dunnett's Multiple Comparison Test as a post-hoc test.

4.3 Post-invasion Methods Assisting Cytosolic Entry and Lysis of Vector Ameliorates Transgene Delivery in Human Cells

Endo-lysosomal escape of bacterial vectors is a crucial step for successfully delivering the transgene to host cells. Endosomal disruptive agents have been shown to facilitate endosomal escape through alteration of the acidic environment rendering the degradative enzymes inactive and overall help to increase gene expression (Moore *et al.,* 2008; Futai *et al.,* 2000; Drose *et al.,* 1997). Three reagents were assessed for their probable effect in elevating bacterial delivery. Varying range of concentration for each reagent was used based on their earlier implementation in enhancing different viral and bacterial vector's efficiency in mammalian cells in different studies (Cheung *et al.,* 2012; Wang and MacDonald, 2004; Fredericksen *et al.,* 2002).

DAP auxotrophy renders bacterial cell division afflicted due to the unavailability of DAP inside mammalian cell leading to the subsequent death of bacterial cell resulting in the cytoplasmic release of DNA (Narayanan and Warburton, 2003; Grillot-Courvalin *et al.,* 1998). However, up to 90% bacterial cell viability, 48 hr. post-infection of the invasive *E. coli* DH10B in mammalian cells has been observed (Laner *et al.,* 2005). In the present study, spontaneous bacterial lysis rate post-invasion was also analyzed over a period of three days and found viable bacteria count up to 4 log units (Data not shown), agreeing to observation reported earlier (Chen *et al.,* 2014). Thus, suggesting an adaptation of steps for promoting lysis of these viable intracellular bacterial cells which hinders transgene release in host cellular environment for its expression. To promote bacterial lysis, a different class of antibiotics was used which are known to be effective against gram-negative bacterial species (See Section 3.6.1). Different concentrations of each reagent were selected and used based on published articles related to internalized bacterial lysis in mammalian cells (Jones *et al.,* 2013; Krusch *et al.,* 2002).

4.3.1 Lysosomotropic Reagents' Administration for Endosomal Escape of Internalized Bacterial Cells Prevents their Lysosomal Degradation

Endosomal escape mediated by lysosomotropic reagents helped enhanced transgene expression in tested cell lines. Amongst lysosomotropic reagents used, Chloroquine which is a lysophilic weak base showed the highest increase in transgene delivery in all four tested cell lines (Fig. 4.12). Maximum of 7.9-fold increase over control in breast cells was observed using as low as 20 μM of Chloroquine ($p<0.0001$) (Fig. 4.12C). Moreover, increase in GFP expression from 7% to 48% upon 20 μM Chloroquine addition was seen in cervical cells ($p<0.0001$) (Fig. 4.12A). A 4.3-fold increase in transgene expression was obtained after adding even 10 μM Chloroquine in lung cells ($p<0.0001$), resulting in 6% expression in contrast to 1.4% expression without reagent addition (Fig. 4.12B). In contrast, Amantadine which is another lysosomotropic reagent under the category of lysophilic weak bases did not show remarkable results as Chloroquine except in cervical cells but are statistically significant.

Amantadine addition in cervical cells resulted in high transgene expression similar to Chloroquine addition i.e. ≈7-fold increase ($p<0.0001$). However, the concentration of the drug used was quite high (800 μM) in comparison to Chloroquine (20 μM) which makes it a preferable drug to be used (Fig. 4.12A). Whereas, the concentration of Amantadine even as high as 100 μM resulted only in 2-fold and 3-fold transgene delivery increase in lung and breast cells, $p<0.001$ and $p<0.0001$ respectively (Fig. 4.12B; Fig. 4.12C). Monensin, on the other hand, a carboxylic ionophore also helped increasing vector's transgene delivery efficiency and highest of a 3.7-fold increase in reporter gene expression was achieved at 1 μM concentration in cervical cells ($p<0.01$) (Fig. 4.12A). Whereas, the increment of 2.5 fold was attained in lung cells at 1 and 0.5 μM concentrations ($p<0.0001$) and the statistically slight increase was obtained in breast cells at 0.1 and 1.5 μM with the p-value of 0.0039 (Fig. 4.12B; Fig. 4.12C). Overall amongst the three

tested lysosomotropic reagents, Monensin appeared to be least effective while Chloroquine showed the highest effect in assisting *E. coli* mediated transgene delivery.

In fibroblast cells also, Chloroquine and Amantadine showed significant GFP expression increment (Fig. 4.12D). Although, Amantadine resulted in a 13-fold increase ($p<0.0001$) as compared to 5-fold increase obtained by Chloroquine ($p<0.0001$). Yet, Chloroquine is preferable since Amantadine gave high GFP expression at very high concentration (1 mM) which resulted in considerable cytotoxicity as observed under light microscopy after 48 hr. drug treatment and before FACS analysis. Monensin also gave considerably high transgene delivery in HT1080 cells (Fig. 4.12D). It also gave about 4-fold increase at highest of 2 μM concentration which is also close to Chloroquine efficiency ($p<0.0001$). However, Chloroquine is being selected since it has shown high efficiency consistently in all the tested cells.

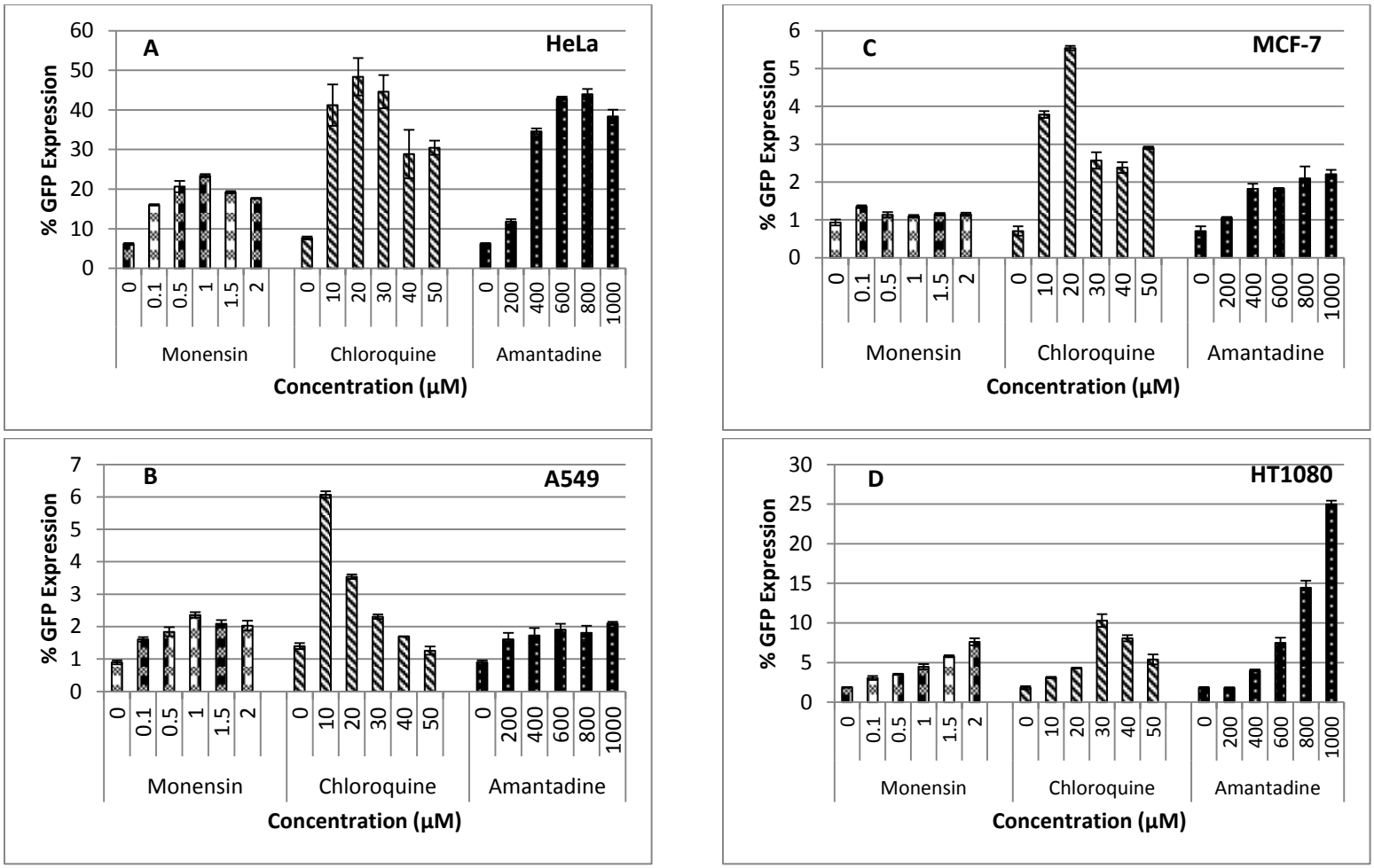


Figure 4.12. Effect of lysosomotropic reagents treatment for the endosomal escape of bacterial vector and enhanced transgene delivery in cultured cells at different concentrations. Efficiency quantification was based on transgene delivery and expression (pEGFP-N2) resulted by vector's successful escape and lysis. GFP expression was determined in percentage using FACScan Calibur at various concentrations, invasion carried out using invasive *E. coli* without LST reagent treatment was used as negative control. A. HeLa (Cervical cell); B. A549 (Lung cell); C. MCF-7 (Breast cell); D. HT1080 (Fibroblast cell). Data represents the average of duplets in three independent experiments. Error bars represent mean ±SEM (n=3). One-Way ANOVA was performed using Dunnett's Multiple Comparison Test as a post-hoc test.

4.3.2 Antibiotic Administration for Sustaining Cytosolic Bacterial Vectors' Lysis Promotes Transgene Release in the Host Cell

Tetracycline, a broad spectrum antibiotic has proven to be a most promising reagent for promoting intracellular surviving bacterial lysis in comparison to Ciprofloxacin (fluoroquinolones) and Polymyxin B (pore-forming antibiotic) (Fig. 4.13).

Tetracycline (Referred to as Tetra) mediated bacterial lysis upon entry have shown as high as 14-fold increase causing tremendously high GFP expression of 87% in cervical cells at the concentration as low as 10 μg/ml with no significant increase with increasing concentration (Fig. 4.13A). Similarly, a 30-fold increase in GFP expression upon 10 μg/ml Tetracycline addition to invaded breast cells was observed resulting in 75% GFP in contrast to less than 1% expression without antibiotic addition (Fig. 4.13C). On the contrary, in lung cells, the merely 2-fold increase was noted at the lowest concentration of the antibiotic whereas an increase of as high as ≈80 fold was observed at the highest concentration i.e. 50 μg/ml (Fig. 4.13B). Moreover, fibroblast cells also showed high transgene expression at the highest concentration i.e. 50 μg/ml only with a very low difference at lower concentrations (Fig. 4.13D). Though highest concentration seemed more effective for some cells yet, lower concentrations of the drug opted for further studies since the idea was not to burden cells with high drug dosage. Polymyxin B (Referred to as Poly B) and Ciprofloxacin (Referred to as Cipro) drugs, on the other hand, did not contribute much to internalized bacterial lysis in tested cells. The effect observed by these two antibiotics remained under 2 fold even at the highest concentration used for these drugs i.e. 100 μg/ml in cervical, lung and breast cells (Fig. 4.13A; Fig. 4.13B; Fig. 4.13C). Nevertheless, Ciprofloxacin surprisingly showed a tremendous increase in transgene release in fibroblast cells (Fig. 4.13D).

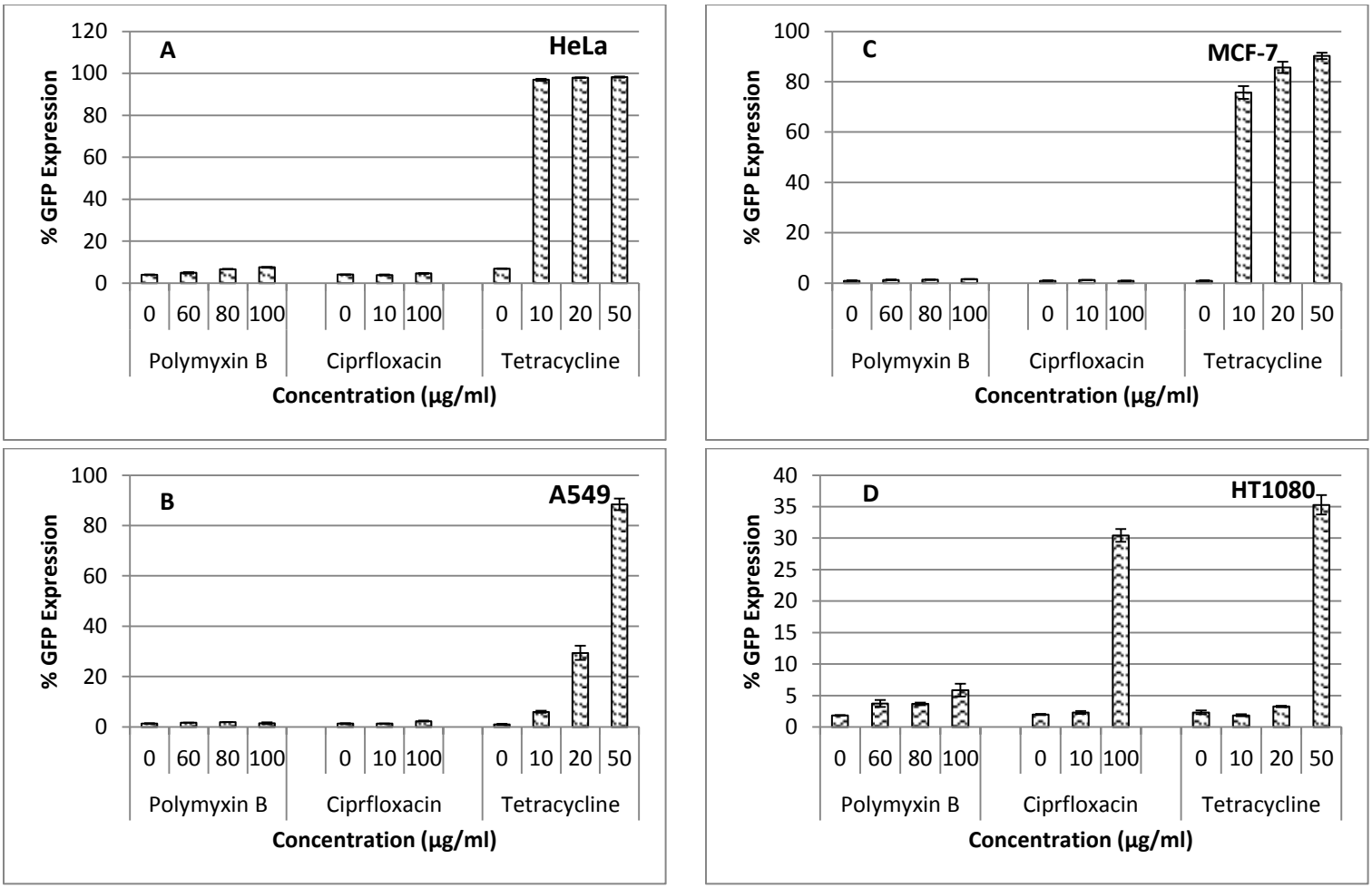


Figure 4.13. Antibiotic efficacy analysis in reducing the intracellular viable bacterial number in human epithelial cells by using flow cytometer. Efficiency quantification was based on transgene delivery and expression (pEGFP-N2) resulted by vector's lysis. Antibiotics at the indicated concentrations in the graph were used for 48 hr. to kill invaded bacterial cells. GFP expression quantification in HeLa (Cervical), A549 (Lung), MCF-7 (Breast) and HT1080 (Fibroblast) cells using FACScan Calibur at various concentrations. Data represent the average of three independent experiments. Error bars represent mean ±SEM (n=3). One-Way ANOVA was performed using Dunnett's Multiple Comparison Test as a post-hoc test.

The drug resulted in 16-fold increase in HT1080 cells but at the concentration 100 μg/ml, which is quite high as compared to other tested antibiotics (Fig. 4.13). Statistically, Tetra addition resulted in significantly achieving increased transgene delivery in all tested cells at generally all concentrations ($p<0.0001$; Fig. 4.13). Cipro addition also yielded significant difference with a p-value <0.05 in HeLa, A549, and MCF-7 cells while in HT1080 it yielded $p<0.0001$ at 100 μg/ml concentration. However, PolyB addition to A549 cells resulted in a non-significant difference in internalized bacterial lysis ($p>0.05$) (Fig. 4.13B). Nonetheless, it resulted in significant bacterial lysis in MCF-7, HT1080 cells ($p<0.05$) and also in HeLa cells ($p<0.0001$) at higher concentrations i.e. 80 and 100 μg/ml concentrations.

4.4 *In vitro* Analysis of Combined Effect of Pre and Post-invasion Methods on Overall Enhancement of Invasive *E. coli* Gene Delivery Efficiency

Since *E. coli* is not a natural pathogen they lack the capability of invading mammalian cells (Grant *et al.,* 1990). Due to their harmless nature in comparison to widely used viral vectors, bacterial strains were engineered to make invasive and to be used as a vector (Narayanan and Warburton, 2003; Grillot-Courvalin *et al.,* 1998). However, *E. coli* vectors did not show remarkable delivery capacity as compared to viral or chemical mediated deliveries (Narayanan and Warburton, 2003; Grillot-Courvalin *et al.,* 1998; Courvalin *et al.,* 1995). Three basic steps which affect vector's efficiency in delivering gene are, an efficient entry into the host cell, escape from lysosomal degradation and finally, the ability to release cargo in host cell environment for its expression. Thus, to develop *E. coli* as an efficient vector these three steps were focused and methods were implemented at each step to help bacteria deliver more transgene in the host cell. Firstly, transfection reagents were added to aid vector's entry into the

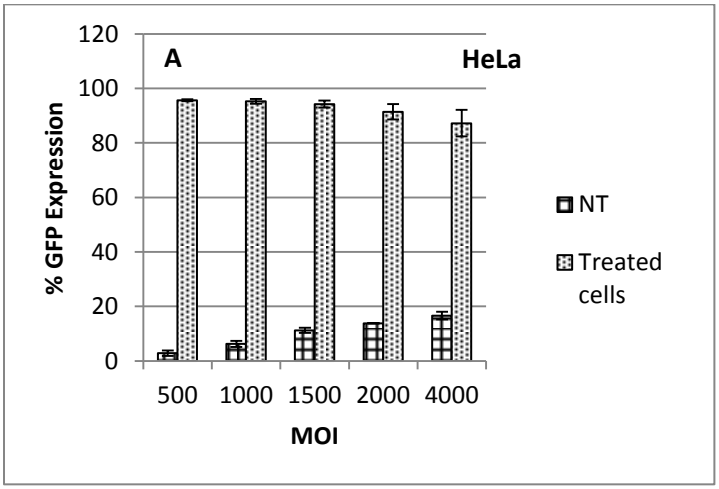
host cell (See Section 4.2). Moreover, lysosomotropic (LST) reagents were added to facilitate bacterial escape from endosomal degradation (See Section 4.3). Finally, antibiotics were used to reduce intracellular surviving bacterial cells in the host cell which could help in higher transgene release in the cytosolic environment for its expression (See Section 4.3). External additions of reagents/drugs have successfully shown increment in vector's transgene delivery capacity by increasing reporter gene expression (Fig. 4.8 - 4.13).

Lastly, above-mentioned methods which were used separately for developing vector's efficiency were combined together to study their effect on transgene delivery in various cells. Chemical agent at the concentration which showed the highest response in each category was selected and used together in a single experiment to analyze its effect in four different cells namely cervical, lung, breast and fibroblast cells.

The combination of PULSin (1 µl), Chloroquine (20 µM) and Tetracycline (10 µg/ml) in cervical cells have shown tremendous increment in GFP expression which is close to 100% (Fig. 4.14A). High transgene delivery and the resultant increase in GFP expression in HeLa cells could be further confirmed by the respective histogram, which shows peak shifting towards the extreme right as compared to histogram obtained from control cells' FACS analysis (Fig. 4.14E1-E2). Likewise, a combination of reagents in breast cells has also resulted in an amazing increase of ≈70% GFP expression in comparison to 2% of GFP expression using invasive *E. coli* alone and no reagent addition (Fig. 4.14C). Similar to HeLa cells, increase in bactofection efficiency upon combined treatment in breast cells is clearly evident from its histogram which also shows a shift in peak towards the right, suggesting an increased number of host cells expressing green fluorescent protein (Fig. 4.14G1-G2). For lung and fibroblast cells, a combination of reagents' concentration varied from others. A combination of PULSin (0.8 µl),

Chloroquine (10 μM) and Tetracycline (10 μg/ml) was used for lung cells and it has successfully resulted in transgene expression up to 70% in comparison to >1% using invasive *E. coli* alone for transgene delivery (Fig. 4.14B). Whereas in fibroblast cells, a combination of PULSin (0.6 μl), Chloroquine (30 μM) and Tetracycline (20 μg/ml) was used and also have resulted in 70% GFP expression (Fig. 4.14D). In lung and fibroblast cells also, their respective histogram supports increased GFP expression as a result of enhanced bactofection efficiency of invasive *E. coli* DH10B in host cells, upon combined treatment (Fig. 4.14F1-F2 and H1-H2 respectively).

Statistical analysis shows a significant difference of all the treated MOIs compared to their non-treated counterparts, for all four tested cells i.e. HeLa, A549, MCF-7 and HT1080 ($p<0.0001$) (Fig. 4.14A-D). On the other hand, no significant difference was noted among treated MOIs in HeLa, MCF-7 and HT1080 cells (Fig. 4.14A; Fig. 4.14C; Fig. 4.14D). However, in lung cells statistical difference was observed between following treated MOIs' pairs i.e. 500 - 4000 ($p<0.01$), 1000 - 4000 ($p<0.0001$), 1500 - 4000 ($p<0.0001$) and 2000 – 4000 ($p<0.01$) (Fig. 4.14B).


A
HeLa
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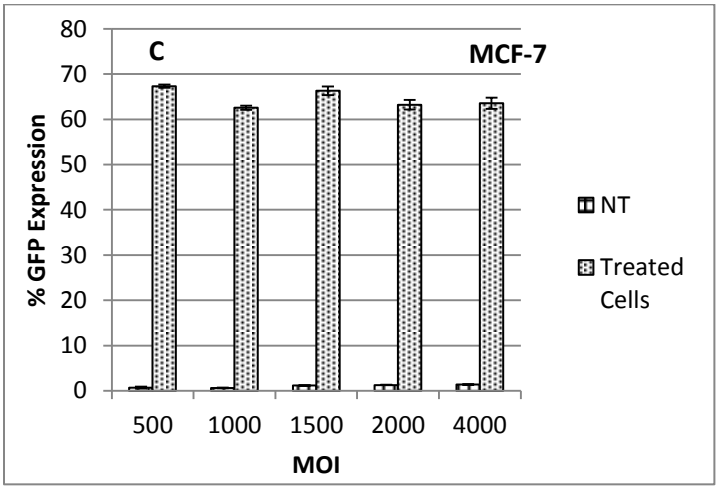
120
100
80
60
40
20
0
500
1000
1500
2000
4000
MOI
NT
Treated cells


C
MCF-7
% GFP Expression
80
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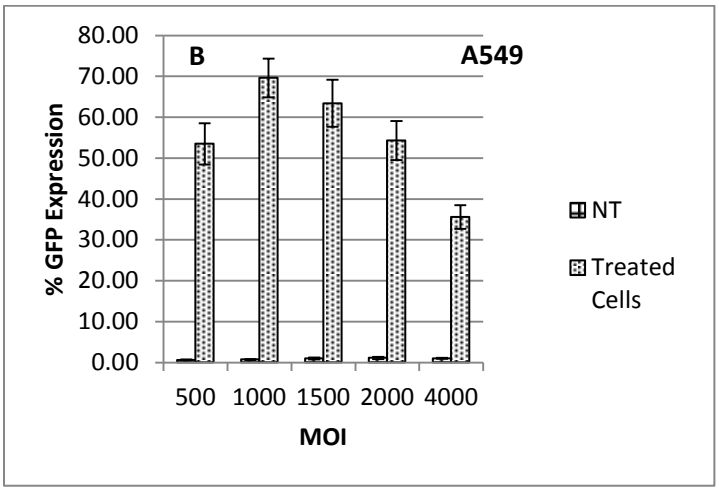
B
A549
% GFP Expression
80.00
70.00
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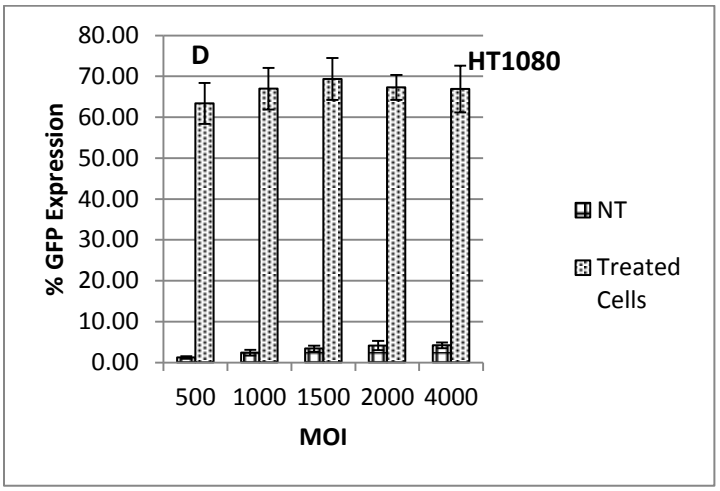
D
HT1080
% GFP Expression
80.00
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MOI
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Treated Cells

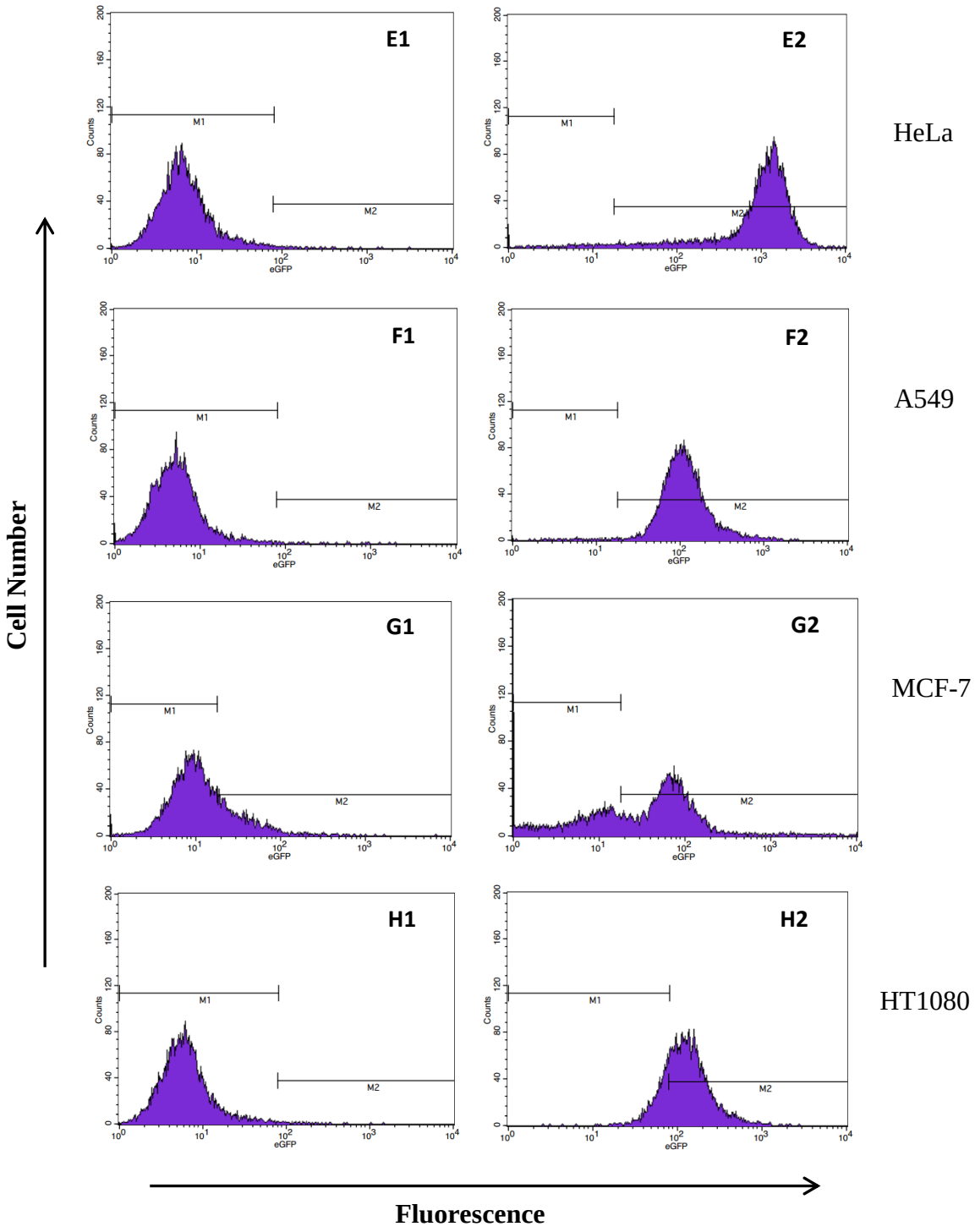

Figure 4.14. Flow cytometric analysis of the combined effect of pre and post-invasion methods for enhancing bacterial vector's transgene delivery capacity *in vitro*. Three

different methods were adapted to enhance bacterial vector's efficiency i.e. coupling bacteria with transfection reagents pre-invasion, the addition of lysosomotropic (LST) reagent and antibiotic administration post-invasion separately. Highest effective reagents and drugs from each category were later used in a single experiment to study their combined effect in overall boosting up vector's efficacy. A-D: graphical representation of transgene delivery efficiencies obtained by using flow cytometer, at various MOIs in different human cells. NT- corresponds to expression values obtained using invasive *E. coli* mediated gene delivery, without any treatment of reagents/drugs. Treated cells- represent values obtained as the combined effect of PULSin (Transfection reagent), Chloroquine (LST reagent) and Tetracycline (Antibiotic). Efficiency quantification was based on transgene delivery and expression (pEGFP-N2) analyzed using FACScan Calibur. HeLa (Cervical), A549 (Lung), MCF-7 (Breast) and HT1080 (Fibroblast) cells at different MOIs (500-4000), invaded mammalian cells using invasive *E. coli* with no treatment were used as negative control. Data represent the average of three independent experiments. Error bars represent mean ±SEM (n=3). One-Way ANOVA was performed using Tukey as a post-hoc test, p-value < 0.0001 for all four cells. E1-H1: histogram showing the population of respective host cells expressing green fluorescent protein, 48 hr. post-invasion by bacteria alone; E2-H2: represents a shift in the population of green fluorescent protein-expressing host cells upon using combined treatment method, 48 hr. post-invasion. Histograms shown here are representative of three independent experiments.

Host cells which were invaded employing combined treatment method underwent simultaneous quantitative and qualitative analysis, using flow cytometry and fluorescence microscopy, respectively. Among the four tested cells, HeLa resulted in close to 100% GFP whereas all the other three cells (i.e. A549, MCF-7, and HT1080) showed a maximum of ≈70% transgene expression (Fig. 4.14A-D). Therefore, HeLa was selected to confirm such a high expression of GFP and among the other three cells, A549 was picked as a representative cell for qualitative discrimination in GFP expression upon combined treatment (Fig. 4.15). GFP-expressing host cells attached to culture plates were viewed directly under a fluorescence microscope using FITC filter, before trypsinizing GFP cells for FACS sample preparation (Section 3.9.2). It was done to support high GFP expression data obtained from flow cytometer and pictures were captured randomly to verify a large number of the population expressing green fluorescent protein (Fig. 4.14). It was hypothesized that, if cells expressing fluorescent protein are so high in number, then an abundant number of green cells should be visible under a microscope. This hypothesis was proven correct by HeLa cells, which showed a high number of green cells, scattered all over the culture plate at 1000 MOI and can be seen in Fig. 4.15 A. On the contrary, HeLa cells invaded by bacteria alone without any treatment, showed far less GFP-expressing cells at the highest MOI of 4000 by viewing under fluorescence microscope under similar conditions (Fig. 4.7 B). Thus, further confirming exceptionally high transgene delivery achieved by invasive *E. coli* DH10B vector upon combined treatment.

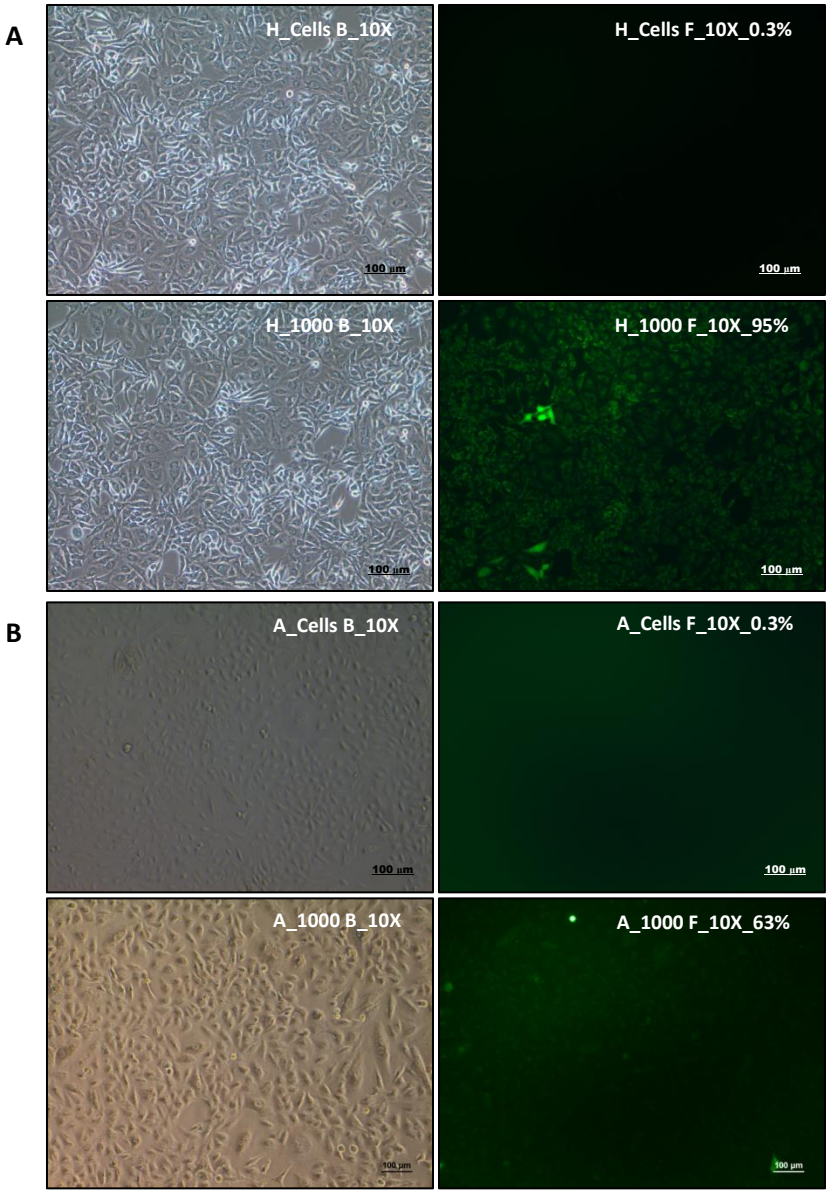

Figure 4.15. Microscopic analysis of high GFP expression after combined treatment. Cultured cells were invaded using invasive *E. coli* together with combined treatment method. Cultured cells with no bacterial invasion were used as background to confirm that fluorescence is only from GFP-positive cells. H, A- HeLa, A549 cells. Cells - Bright field and fluorescence view of control. B and F- are the bright field and fluorescence view respectively. 1000- 1000 MOI of bacteria used to invade the cultured mammalian cell. % values- show the obtained percentage values of same using flow cytometer; Scale: 100 µm.

Whereas, A549 cells which also successfully achieved ≈60% GFP expression upon combined treatment, could not be viewed as clearly as HeLa cells under a fluorescence microscope (Fig. 4.15 B). This could probably be due to the reason that HeLa cells showed a higher number of internalized bacteria per cells i.e. up to 10 in comparison to A549 cells which is up to 2 (Fig. 4.6). Moreover, peak intensity in histogram obtained from FACS analysis reflects the fluorescence intensity from sample cells. Comparative analysis of histogram from HeLa and A549 cells after combined treatment reveals slightly intense peak in HeLa cells which is in accordance with microscopic analysis (Fig. 4.14E2 and F2; Fig. 4.15). Thus, it can be deduced that possibly multiple numbers of transgene copies per HeLa cells delivered by carrier bacteria, showed higher intensity green cells than A549. Additionally, it is well established that GFP expression is heterogeneous i.e. protein expression intensity varies with cell type (Zhao *et al.*, 1998; Prasher, 1995; Cubitt *et al.*, 1995). It is also being reviewed by Tsien (1998) that there are several factors which affect the GFP expression and its detectability level, such as a number of copies of gene, temperature, autofluorescence of cells etc.

4.5 Functional Delivery of 100 kb BAC Clone using Invasive *E. coli* Vector in Human Cells

4.5.1 Preparation of Invasive *E. coli* Containing 100 kb Human Globin BAC

4.5.1.1 BAC DNA Confirmation

E. coli DH10B strains containing circular BAC4396 and BAC4396-EGFPneo were used to obtain globin BAC DNA for electroporation in invasive *E. coli* for analyzing its gene delivery efficiency in human cells. Before proceeding further for bactofection, DNA was firstly confirmed by digesting the isolated DNA from two random clones and analyzing its band pattern

through PFGE (Sections 3.10.1.2; 3.10.6 and 3.10.4.2). Amongst other enzymes that have their restriction sites in the globin BAC sequence, XhoI was selected since it has a maximum number of restriction sites in the sequence (Ooi *et al.*, 2008). Therefore, digesting the DNA with XhoI could confirm sequence integrity over a long stretch of the DNA.

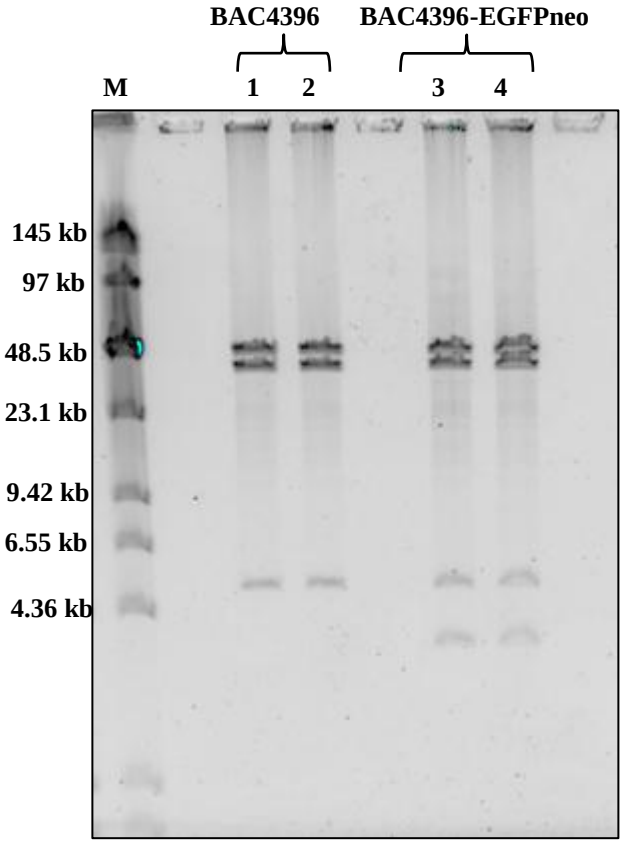

Figure 4.16. Pulsed Field Gel Electrophoresis (PFGE) to confirm BAC clones. Gel shows required band pattern upon XhoI digestion. Two separate bacterial plasmid isolation from each clone was used to determine the intactness of BAC DNA. Lanes 1 and 2, show digested bands from BAC4396; lanes 3 and 4, show digested bands from BAC4396-EGFPneo DNA. M- PFG low range ladder from NEB. The PFG run was performed at 4.5 V/cm with a switch time of 0.1 - 10 seconds, at 4° C for 16 hr. using 0.5x TBE buffer.

Digestion with the selected enzyme would result in 3 bands from circular BAC4396 (while 4 bands from linear BAC4396) in the case of intact and non-rearranged DNA sequence as reported previously by using linear BAC4396 (Ooi *et al.,* 2008). Expected bands of sizes 50.9 kb, 43.4 kb and 4.9 kb from BAC4396 plasmid digestion confirmed the presence of intact, non-rearranged BAC DNA (Fig. 4.16; Lanes 1 and 2). An additional band of size 3.8 kb was obtained as expected from BAC4396-EGFPneo clone due to the presence of EGFP plasmid (Fig. 4.16; Lanes 3 and 4).

4.5.1.2 Screening of Cell Lines for Endogenous β-globin Expression

Human β-globin gene was chosen for heterologous gene delivery *in vitro* using invasive *E. coli*. Thus, the cell line was required which lacks globin gene expression endogenously so that bacteria mediated functional gene delivery can be analyzed. For the purpose, all available nine cell lines were screened for globin expression. Total RNA was isolated and RT-PCR was performed to assess gene expression using a primer set for exon 2 and exon 3 of β-globin (Table 3.8) (Section 3.10.3.1 and Section 3.10.3.4). The amplified product was run and analyzed by gel electrophoresis together with actin, which was used as housekeeping gene (Hutvagner *et al.,* 2001). Expected band size for globin gene is 231 bp and 500 bp for actin gene amplification (Ooi *et al.,* 2008; Black and Vos, 2002; Hutvagner *et al.,* 2001). Except for fibroblast cells, screening of cell lines showed positive globin expression for rest of the eight tested cell lines, four of which are shown as a representative in Fig. 4.17 (The gel included the mock sample, not presented here). In the globin amplified product, some non-specific amplifications were obtained as could be seen in the globin lanes of the tested cells i.e. HeLa, HEK-293, A549 and SK-N-SH (Fig. 4.17). These non-specific amplified bands in the gel could probably be the isoform of the globin gene, as the additional band can be seen in all the tested cell lines and at the same position.

Moreover, the band is clearly absent in the actin amplified product. Nevertheless, the additional band amplification was not taken into consideration because the motive was to check the presence or absence of endogenous β-globin expression, only.

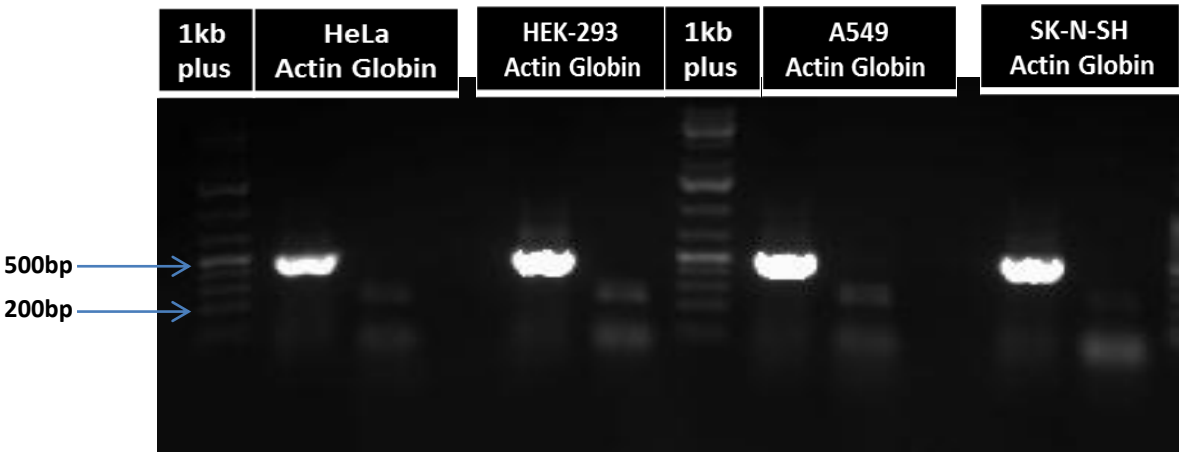

Figure 4.17. Agarose gel electrophoresis showing expression of the β-globin gene in tested cell lines. RT-PCR was performed to analyze globin gene expression in available cell lines. Amplification from four of the cell lines which was run on 1% agarose gel electrophoresis, is shown here as representative cells. HeLa: Cervical; HEK-293: Kidney; A549: Lung; SK-N-SH: Brain cells. Actin: Housekeeping gene; Globin: β-globin amplified product; 1 kb plus: 1 kb plus DNA ladder (Invitrogen).

Fibroblast cells (HT1080) were further extracted and analyzed separately to assure the absence of endogenous globin expression in these cells. For the confirmation, an amplified product from the BAC4396 clone (which carries the β-globin gene) using globin specific primers, was also included and the agarose gel showed positive amplification of globin gene

from BAC clone while no globin amplification from fibroblast cells, was obtained (Fig. 4.18). Thus, HT1080 was further used for gene delivery capacity assessment, of invasive *E. coli* containing 100 kb globin BAC.

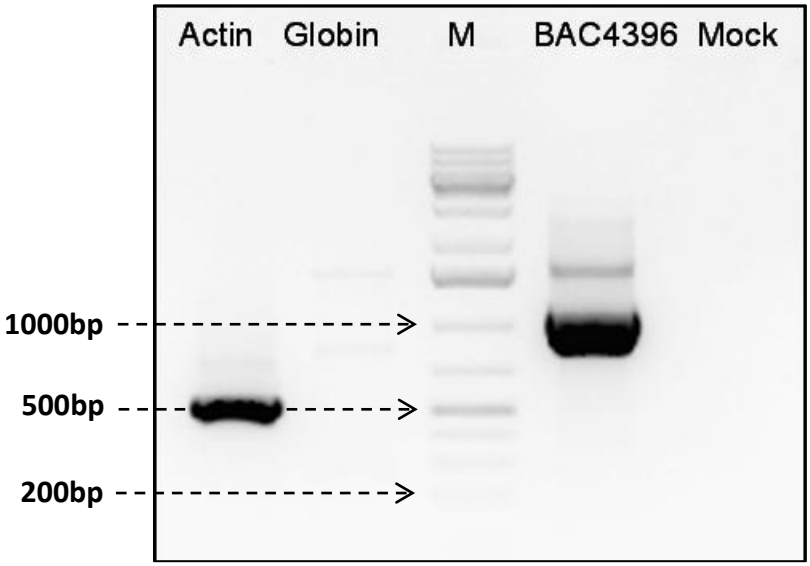

Figure 4.18. Agarose gel electrophoresis showing the absence of β-globin expression in HT1080 cells. RT-PCR was performed to analyze globin gene expression. Amplification from fibroblast cell lines was run on 1% agarose gel electrophoresis. Actin: Housekeeping gene; Globin: β-globin amplified product; M: 1 kb plus DNA ladder (Invitrogen); BAC4396: amplification from plasmid DNA isolated from *E. coli* DH10B carrying BAC4396 clone; Mock: PCR amplified product of reaction mixture without any template DNA, to confirm absence of any background amplification and used as negative control.

4.5.2 Delivery of β-globin Gene using Invasive *E. coli* Vector

4.5.2.1 Construction of Invasive *E. coli* Carrying 100 kb Globin BAC

Invasive *E. coli* with no reporter gene was required so that reporter gene on BAC plasmid when delivered by *E. coli* vector to human cells could be tracked by GFP expression. Invasive *E. coli* DH10B *asd*$^-$ (pGB2Ωinv-hly, pEGFP-N2) was negatively selected for aborting reporter plasmid i.e. pEGFP-N2. It was done by streaking invasive strain containing reporter plasmid, several rounds on the growth media without selective antibiotic i.e. kanamycin for the EGFP-N2 plasmid. After up to 10 rounds of streaking, pEGFP-N2 was aborted. The absence of reporter gene from invasive *E. coli* strain was confirmed by screening 15 colonies on kanamycin-containing media. No growth of colonies confirmed the absence of pEGFP-N2 carrying kanamycin resistance gene (Data not shown). This strain was further used for electrocompetent cells preparation and electroporation of BAC4396-EGFPneo (Section 3.10.2.1).

The transformation of BAC4396-EGFPneo into invasive *E. coli* DH10B *asd*$^-$ (pGB2Ωinv-hly) strain was confirmed by double digestion (Section 3.10.6). Plasmid pGB2Ωinv-hly was digested and linearized by BamHI (Roche) enzyme since it cuts once within the sequence, based on restriction site and expected fragments analysis using NeBcutter V 2.0 software (New England BioLabs). While, XhoI (Roche) was used for BAC4396-EGFPneo and BAC4396 plasmids' digestion as described earlier in section 4.5.1.1, Fig. 4.16 (Ooi *et al.*, 2008). Therefore, to confirm the presence of both plasmids in pGB2Ωinv-hly, BAC4396-EGFPneo clones, plasmids were subjected to both the restriction enzymes together. To confirm clones after double digestion, separate pGB2Ωinv-hly (Fig. 4.19; Lane 9) and BAC4396-EGFPneo plasmids (Fig. 4.19; Lane 8) were also double digested to be used as controls and run along with the transformed ones (Fig. 4.19; Lanes 1-6). Double digestion of isolated plasmid DNA from *E. coli*

DH10B (pGB2Ωinv-hly, BAC4396-EGFPneo) strain and control strain i.e. *E. coli* DH10B (BAC4396-EGFPneo) showed same band patterns on agarose gel after performing PFGE (Fig. 4.19). Thus, it confirms the successful and non-rearranged BAC4396-EGFPneo DNA electroporation into invasive *E. coli* DH10B (pGB2Ωinv-hly) strain forming *E. coli* DH10B (pGB2Ωinv-hly, BAC4396-EGFPneo) for further gene delivery purpose in human cells (Fig. 4.19).

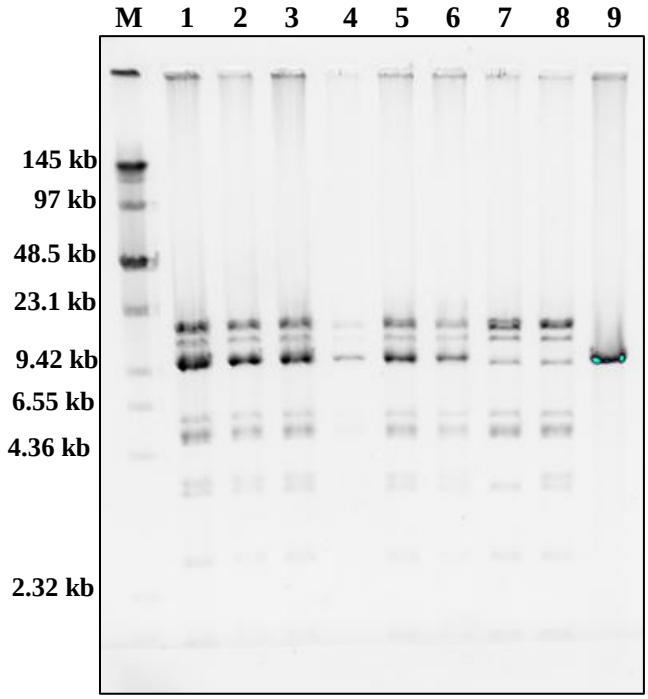

Figure 4.19. Pulsed Field Gel Electrophoresis (PFGE) to confirm *E. coli* DH10B *asd*⁻ (pGB2Ωinv-hly, BAC4396-EGFPneo) clones. Gel shows required band pattern after double digestion of 6 different colonies obtained after electroporation. Plasmids were digested using BamHI and XhoI, Buffer B was used to perform the reaction. Digestion was performed in a usual manner i.e. incubating at 37° C for 1 hr. The digested product was then loaded on to 1% pulsed field gel and ran for 16 hr. with a pulse time of 0.1-10 sec. M: PFG low range ladder from NEB; 1-6: Double digested BAC DNA from six different transformed colonies, clone no. 4 showed similar band pattern but faint bands due to less DNA quantity; 7: Double digested DNA from

E.coli DH10B *asd*$^-$ (pGB2Ωinv-hly, BAC4396) clone; 8: Double digested DNA from *E.coli* DH10B *asd*$^-$ (pGB2Ωinv-hly, BAC4396-EGFPneo) clone; 9: Double digested plasmid of pGB2Ωinv-hly from *E.coli* DH10B *asd*$^-$ (pGB2Ωinv-hly) clone.

4.5.2.2 Transfection of BAC4396-EGFPneo using Lipofectamine 2000 in HT1080 Cells

Purified DNA of BAC4396-EGFPneo was transfected using Lipofectamine 2000 to assess transfection efficiency of 100 kb DNA in human fibroblast cell (Section 3.10.5.2; Table 3.10). It was done, to later compare bacterial gene delivery efficiency with commercial transfection reagent and also to assess if pre and post-invasion chemical addition could enhance the bacterial vector's efficiency up to the transfection level (Discussed in Section 4.5.2.3).

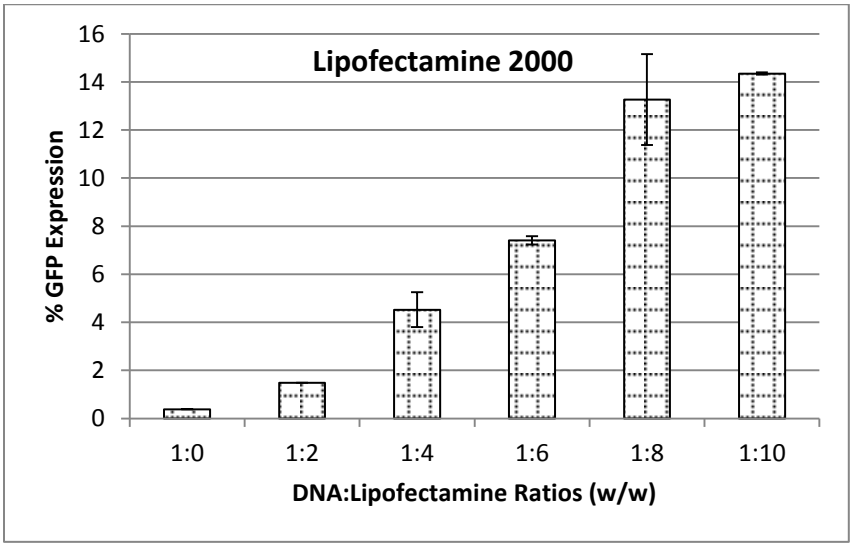

Figure 4.20. Quantification of BAC4396-EGFPneo DNA transfection in HT1080 (Fibroblast) cells by Lipofectamine 2000 using flow cytometry. Transfection efficiency was

analyzed by GFP expression by FACS. Plasmid DNA (BAC4396-EGFneo) 2 μg was used for transfection, whereas Lipofectamine 2000 was used in varying concentrations (μg/μl). Diluted DNA without any transfection reagent was used as negative control. Error bars represent mean ±SEM (n=3). One-Way ANOVA was performed using Dunnett's Multiple Comparison Test as a post-hoc test, p-value < 0.0001.

BAC DNA was isolated as given in section 3.10.1.2, 2 μg of which was combined with different Lipofectamine 2000 ratios and analyzed transfection efficiency by FACS (Section 3.9.1). All the ratios are represented as DNA: Lipofectamine 2000 as shown in Fig. 4.20. Highest of 14% GFP expression was obtained at the highest ratio of 1:10 (w/w) (Fig. 4.20). Statistical analysis to compare tested ratios (w/w) with the control revealed significant difference at all the ratios except 1:2 ($p<0.001$ for the ratio 1:4; $p<0.0001$ for 1:6 – 1:10 ratios).

Furthermore, the functionality of BAC DNA delivered using Lipofectamine 2000, was also analyzed in HT1080 cells by performing RT-PCR of RNA from transfected cells. For the purpose, the BAC4396-EGFneo plasmid was transfected using 1:2 ratios (w/w) of DNA: Lipofectamine 2000 in three of 10 cm dishes. Transfected cells were then pooled from the three dishes and used for RNA extraction and RT-PCR subsequently (Section 3.10.5.2 and Section 3.10.3). 10 μl of amplified product was then electrophoresed and visualized on an agarose gel (Section 3.10.4.1). Gel electrophoresis showed a 231 bp band of globin amplified product in transfected cells confirming functional delivery of BAC DNA using Lipofectamine 2000 (Fig. 4.21; Lane G1).

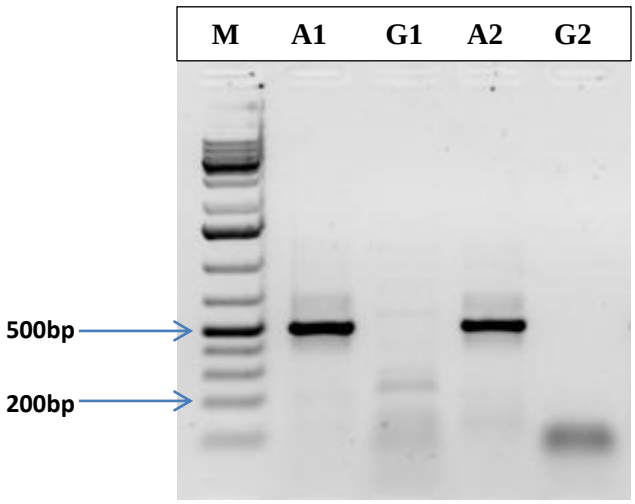

Figure 4.21. Agarose gel electrophoresis showing functional β-globin BAC delivery by Lipofectamine 2000 in HT1080 (Fibroblast) cells. RT-PCR was performed to analyze globin gene expression. Amplification from fibroblast cell lines was run on 1% agarose gel electrophoresis. M: 1 kb plus DNA ladder (Invitrogen); A1: Actin amplified product from Lipofectamine 2000 transfected cells; G1: β-globin amplified product from Lipofectamine 2000 transfected cells; A2: Actin amplified product from non-transfected cells; G2: β-globin amplified product from non-transfected cells.

4.5.2.3 Bactofection of BAC4396-EGFPneo using Invasive *E. coli* in HT1080 Cells

100 kb globin BAC delivery was further assessed by using invasive *E. coli* (pGB2Ωinv-hly, BAC4396-EGFPneo). Bacterial gene delivery efficiency in human cells was analyzed with and without any chemical reagents' addition. Bacterial delivery without any treatment involved only bacterial vector addition at different MOIs to cultured fibroblast cells similar to bactofection using invasive *E. coli* strain carrying reporter plasmid i.e. DH10B *asd*$^{-}$ (pGB2Ωinv-hly, pEGFP-

N2) (Section 3.3). Further, the effect of chemical reagents' addition on the bactofection efficiency of BAC carrying vector was also assessed (Section 3.7).

GFP expression carried by BAC plasmid showed highest of 12% expression upon bacterial delivery at 1500 MOI in HT1080 cells (Fig. 4.22). Upon comparing bactofection efficiency with that of transfection efficiency in delivering BAC DNA, both the vectors revealed comparable proficiencies i.e. a maximum of 12% and 15% GFP expression respectively (Fig. 4.20 and Fig. 4.22). Thus, suggesting bactofection to be a considerable alternative to transfection in terms of exogenous DNA delivery efficacy in human cells. However, combined treatment of chemical reagents for enhancement of bactofection efficiency had successfully led to significant enhancement of BAC DNA delivery and yielded ≈65% GFP carried by BAC at 1500 MOI (Fig. 4.22). All MOIs upon combined treatment resulted in statistically significant increase compared to their control values ($p<0.0001$). Bars which are referred as "No Treatment" and placed alongside the bars representing "Treated Cells" at each MOI in Fig. 4.22 are used as controls. Amongst the controls, non-significant difference in GFP expression between different MOIs was obtained i.e. without any treatment bactofection of BAC DNA was almost similar and resulted in non-significant p-values at all the tested MOIs in human fibroblast cells ($p>0.05$) (Fig. 4.22). On the contrary, upon combined treatment bacterial delivery of BAC DNA showed a significant increase at 1500 MOI as compared to 500 MOI ($p<0.001$). No further increase in gene delivery was noticed rather, a significant decrement was obtained at 4000 MOI in comparison to 1500 MOI, upon statistical analysis ($p<0.001$). The increment in GFP expression upon combined treatment, to bactofection of GFP carrying BAC DNA, is similar to that obtained for invasive *E. coli* mediated delivery of pEGFP-N2 plasmid. Both reporter gene and BAC DNA delivery by invasive *E. coli* DH10B in human fibroblast cells resulted in maximum gene delivery of 69% and

64% respectively at the same MOI i.e. 1500 (Fig. 4.14D; Fig. 4.22). Therefore, it suggests that the effect of combined treatment on the bacterial delivery system is not much altered or affected by carrier DNA in the vector.

BAC DNA is a single copy and about 17 times bigger while pEGFP-N2 is a high copy and small plasmid nevertheless, gene delivery efficiencies of both vectors are found to be same. Generally, with bigger constructs, the gene delivery efficiency is reduced. However, PEI-mediated DNA transfection studies also reported unusually high gene transfer and subsequent expression efficiency with large transfection complexes in contrast to smaller complexes (Ogris *et al.*, 2001; Ogris *et al.*, 1998). Furthermore, in accordance to the present study PEI22 and Lipofectamine 2000 mediated transfection studies using GFP-positive cells (%) assay, revealed 2-9 times more delivery of 150 kb BAC than plasmid DNA of ≈5 kb size while taking copy number into consideration (Magin-Lachmann *et al.*, 2004). Although, the exact mechanism for this discrepancy could not be stated it can be hypothesized that besides DAP auxotrophy, BAC DNA cargo exerts additional burden on bacterial vector multiplication inside the host cell. This would eventually cause increased cytosolic bacterial lysis and intracellular cargo release for its expression.

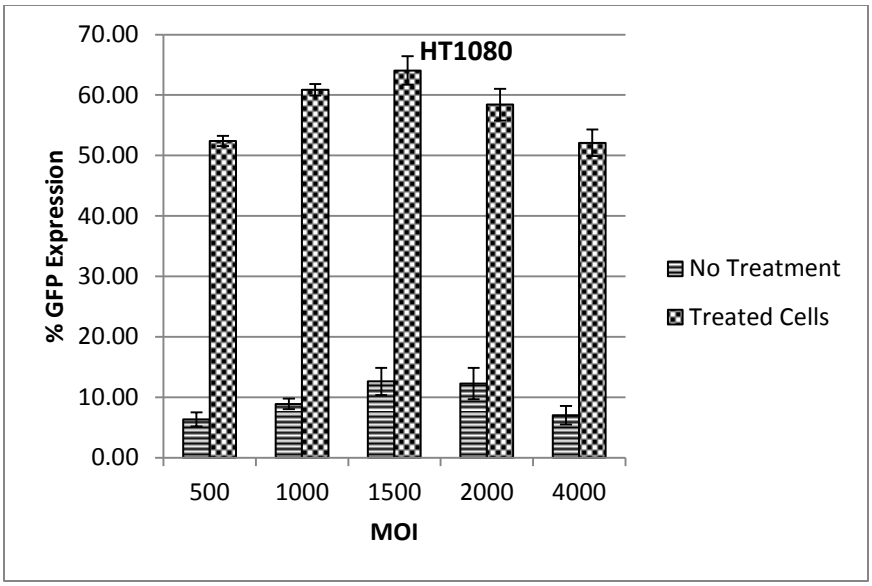

Figure 4.22. The combined effect of pre and post-invasion methods on bactofection of BAC DNA in HT1080 (Fibroblast) cell line. Three different methods were adapted to enhance bacterial vector's efficiency i.e. coupling bacteria with transfection reagents pre-invasion, the addition of lysosomotropic (LST) reagent and antibiotic administration post-invasion separately. Highest effective reagents and drugs from each category were later used in a single experiment to study their combined effect in overall boosting up vector's efficacy. No Treatment - corresponds to expression values obtained using invasive *E. coli* mediated gene delivery, without any treatment of reagents/drugs. Treated cells- represent values obtained as the combined effect of PULSin (Transfection reagent), Chloroquine (LST reagent) and Tetracycline (Antibiotic). Efficiency quantification was based on gene delivery and expression (BAC4396-EGFPneo) analyzed using FACScan Calibur at different MOIs (500-4000) in HT1080 cells. Invaded mammalian cells using invasive *E. coli* with no treatment were used as negative control. Data represent the average of three independent experiments. Error bars represent mean ±SEM (n=3). One-Way ANOVA was performed using Tukey as a post-hoc test, p-value < 0.0001.

Similar to functionality assessment of transfected BAC DNA using RT-PCR, the intention was to analyze functional bactofection of BAC DNA *in vitro*. Cultured fibroblast (HT1080) cells were invaded using the combined method by invasive *E. coli* DH10B (pGB2Ωinv-hly, BAC4396-EGFPneo) at 1500 MOI which showed significant GFP expression (Fig. 4.22). Invaded cells after 48 hr. of invasion, from 10 of 6-well plates were collected and used for RNA extraction (Section 3.10.3.1). The entire quantity of extracted RNA i.e. 10 µl was used for cDNA preparation and about 1 µl of cDNA was used further for RT-PCR. Complete amplified product (50 µl) was loaded onto 1% agarose gel and electrophoresed. No clearly visible bands upon electrophoresis could be obtained (Data not presented). Therefore, the amount of cDNA template was increased to 5 µl and 10 µl to see any effect on the amplified product. This time, after loading even 10 µl of the amplified product could give visible bands of 231bp product (indicated by an arrow in Fig. 4.23) from globin DNA (Lanes G1 and G2), which infer the possibility to consider it as the outcome of functional BAC DNA delivery in the host cell. However, this data exhibits few shortcomings which make it inappropriate to conclude as proof of bacteria-mediated functional globin delivery.

Firstly, increase in template DNA led to non-specific DNA amplification during PCR which could be seen as several background bands along with the target gene on an agarose gel (Fig. 4.23; Arrows in Lanes G1 and G2 points towards the target gene amplification among the non-specific bands). Besides multiple non-specific bands, acquired bands from globin amplification display low intensity as compared to actin bands (Fig. 4.23; Lanes A1-A2 and G1-G2). This could possibly be due to loss/degradation of the product while RNA isolation and handling at each step. Furthermore, actin is a housekeeping gene and are expressed in all cells constituting to its high expression while BAC is a single copy DNA and expressed only in host

cells which have been successfully invaded by the bacterial vector (Fig. 4.23; Lanes A1 and A2). HeLa cells are known for endogenous globin expression hence, including a positive control of globin amplification from HeLa cells could have been a promising confirmation for *in vitro* functional delivery of β-globin in human fibroblast cells.

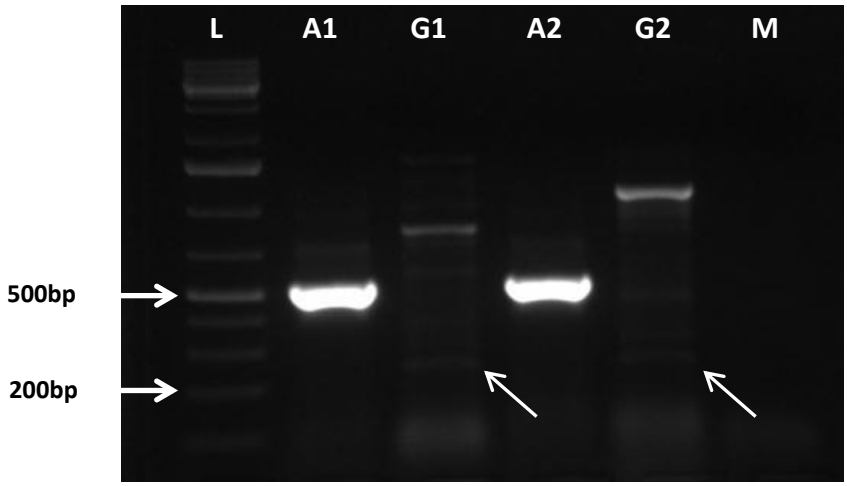

Figure 4.23. Functional β-globin BAC delivery using invasive *E. coli* DH10B (pGB2Ωinv-hly, BAC4396-EGFPneo) in HT1080 cells. RT-PCR was performed to analyze globin gene expression. Electrophoresis performed after RT-PCR amplification of RNA from invaded fibroblast cell lines and ran on 1% agarose gel. L: 1 kb plus DNA ladder (Invitrogen); A1: Actin amplified product when 5 µl template cDNA was used; G1: β-globin amplified product when 5 µl template cDNA was used; A2: Actin amplified product when 10 µl template cDNA was used; G2: β-globin amplified product when 10 µl template cDNA was used; M: Mock sample in which all PCR components were added except template DNA, to check any background amplification or presence of any contamination; Arrows in Lanes G1 and G2 points towards the β-globin gene amplification among the non-specific bands.

Gel picture presented here is definitely not the best one, but due to the time constraint, the experiment could not be repeated further to achieve the PCR amplification without any non-specific bands upon agarose gel electrophoresis. Nevertheless, this figure is being presented here as a preliminary data which could be used as a reference for further refining of the experiment and confirm the potential use of *E. coli* as a gene delivery vector in functional therapeutic gene transfer in human cells (Fig. 4.23).

5. DISCUSSION

The project aims at the development of bacteria as gene delivery vector for human cells. To develop the vector, firstly its invading capacity was assessed using GFP as a reporter gene in different cells derived from various human tissues. This could give an insight into tissues behavior towards the vector and which tissues can be targeted in future studies for best results. Human epithelial cell lines derived from various tissues covering major organs of the human body such as brain, breast, lung, liver, kidney, colon, ovary, cervix and fibroblast were used.

Bacterial delivery was found to be tissue dependent and have shown successful invasion in a range of non-phagocytic cells. Bactofection efficiency can be categorized in highest, medium and least GFP-expressing cells with cervical and ovarian being in the first category with highest of 16% and 13% respectively (Fig. 4.7). Kidney, brain, and fibroblast cells showed medium GFP expression levels i.e. 7%, 4%, and 3% respectively. Following are breast, lung and colon cells whose transgene expression levels remained below 2%. Liver cells, however, did not show any significant increase in foreign DNA expression levels over background upon *E.coli* invasion. A similar pattern of cell-dependent bactofection efficiency using *E.coli* was previously also reported using cells such as HeLa, CHO, Cos, A549 (Grillot-Courvalin *et al.,* 1998; Grillot-Courvalin *et al.,* 1995). Varying efficiency can be probably due to different cells expressing varying levels of β1 integrin which is required for bacterial internalization (Fajac *et al.*, 2004).

Due to different types of integrin receptors on each of the cell membrane, it is apparent that the cells expressing more sub-types of β1-integrin receptors on their membrane will result in higher invasion by the vector. Reporter gene expression was as high as 16% in cervical and 13% in ovarian cell lines, expressing maximum (seven) β1-integrin subtypes among tested cell lines followed by kidney cells expressing six β1-integrin subtypes (7% GFP expression). Expression

of lesser β1-integrin subtypes (three) on brain cells as compared to cells mentioned above might have resulted in lower GFP expression of 4%. However, this trend was overruled in breast cells which express three sub-types of β1-integrin (α2β1, α3β1, αvβ1) showed GFP expression under 2%. Similarly, lung and colon cells also showed lower gene expression regardless of high integrin sub-types on their cell membranes. Among all tested cell lines, liver cells were the only one which showed no invasion by bacteria (Fig. 4.7). Lipofection in liver cells also gave the very low efficiency of 19% GFP expression only (Fig. 4.3) as compared to other cells showing around 50% (Fig. 4.4 and 4.5). Likewise, another study using Lipofectamine to transfect liver cells reported only 18% of exogenous DNA expression supporting that these are hard to transfect cells (Wang *et al.,* 2006).

Another observed trend was the difference in observed numbers of invaded cells and GFP-expressing cells which have also been reported earlier (Dietrich *et al.,* 1998). This could be explained on the basis of two possibilities:

1) Not all of the invading bacteria are capable of escaping from the endosome and reach cytosol, rather some of them are dead in the antimicrobial environment of the phagosome and are digested by lysosome together with the transgene (De Chastellier and Berche, 1994).
2) Only some of the internalized invasive *E.coli* reach the mammalian cell cytoplasm as an intact viable bacteria, but still, the DNA is released into the host cytoplasm (Dietrich *et al.,* 1998).

Besides *in vitro* transfer of reporter gene, invasive *E. coli* strains have been successfully used for functional delivery of large size genes containing regulatory elements. Functional delivery of therapeutic genes such as 200 kb β-globin gene and artificial chromosomes

containing CFTR gene locus have been reported in human cells using invasive *E. coli* vector (Ooi *et al.,* 2008; Laner *et al.,* 2005). Furthermore, *in vivo* studies have also been conducted using recombinant *E. coli* for delivering CFTR gene in lung epithelial cells but have reported showing low gene delivery ability as compared to cationic lipid-mediated gene transfer (Larsen *et al.,* 2008). These studies suggest, that *E. coli* vectors despite being an excellent choice for carrying large size gene with no immune reaction in host cells, lacks the efficiency of achieving high gene delivery. Thus, in an effort to enhance bacterial gene delivery capacity recently, invasive *E. coli* (pGB2Ωinv, pEGFP-N2) vector has been coupled with transfection reagent Lipofectamine 2000 yielding increase of 2.8 fold GFP expression (Narayanan *et al.,* 2013).

Further, various transfection reagents in addition to Lipofectamine 2000 were studied to evaluate their effect in transgene delivery by *E. coli* vector containing hemolysin gene in addition to invasin i.e. invasive *E. coli* (pGB2Ωinv-hly, pEGFP-N2) (See Section 3.4 and 4.2). Like bactofection, comparative study of transfection reagents used also showed varying efficiency in different cell lines. Highest efficiency was obtained by coupling bacterial cells with protein delivery agent (PULSin) which successfully have resulted in obtaining significantly higher GFP expression over control in all the tested cell lines (See Section 4.2.3). Highest of the 7.5-fold increase was noted in breast cells upon using PULSin (Fig. 4.10B). Except for kidney cells, an increase in transgene expression in all the other tested cell lines ranged from 2 – 6 fold. PULSin showed a dose-dependent GFP expression profile with the highest increase at 1 µl reagent volume addition in tested cells exceptions were lung and fibroblast cells where 0.8 µl volume additions have shown peak expression. Moreover, colon cells have shown highest transgene expression at as low as the 0.2 µl addition of the reagent. jetPEI on the other hand, have resulted in 3 fold higher GFP in the brain, kidney, and liver cells and approximately 2-fold

increase in cervical and lung cells (Fig. 4.8B). ExGen 500 also helped in elevating bacteria mediated heterologous DNA expression in brain, lung, and liver with efficiencies ranging from a 2-3-fold increase (Fig. 4.11B). However, Lipofectamine 2000 have resulted in nearly 3-fold increase only in cervical cells while other tested cells revealed efficiencies less than 1 fold (Fig. 4.9).

The suggestive mechanism behind trans-bactofection method depicts involvement of integrin-mediated endocytosis but the theory does not hold any prove so far. Recently it has been shown that Lipofectamine coupling has resulted in obtaining equivalent internalized non-invasive *E. coli* count as its invasive counterpart in human cervical cells (HeLa). However, instead of a high number of internalized bacterial cells, gene expression levels of non-invasive *E. coli* remained remarkably low compared to invasive *E. coli* expression upon Lipofectamine coupled invasion (Narayanan *et al.,* 2013). Similarly, jetPEI mediated trans-bactofection in ovarian cells resulted in an increase of 2 internalized bacteria per cell yet transgene expression did not show any considerable increase. Likewise, PULSin assisted bactofection in breast cells did not show any significant increase in internalized bacteria count but have resulted in highest gene expression with the 7-fold increase among tested cell lines (Fig. 4.10A and B). Furthermore, no bacteria were rescued post-invasion from liver cells upon trans-bactofection by either of reagents, still, 3.2 folds' increase producing 3% GFP each by jetPEI and PULSin addition in comparison to 0.6% GFP expression was achieved (Fig. 4.8B and 4.10B). So far, intracellular bacteria count data did not yield any conclusive results thus, it is not conceivable to summarize that transfection reagents helped in bacterial entry into the mammalian host. As discussed earlier, discrepancies in internalized bacteria count and GFP expression profile could be attributed to various factors listed above. Moreover, there could be two possible ways

transfection reagents helped elevating bacterial gene delivery capacity 1) either by enhancing interaction between and mammalian cell membrane and helping more condensation of bacterial vectors on host cell surface resulting in more endocytosis 2) or by altering pH of endosome helping more bacterial release for cytosolic reach.

High charge on PEI has been reported to condense DNA particles subsequently leading to endocytosis mediated internalization in the target cell (Benjaminsen *et al.,* 2013; Kunath *et al.,* 2003; Godbey *et al.,* 1999). Furthermore, polyethylenimine reagents have shown to form bigger aggregates during transfection which could hinder endocytosis (Varga *et al.,* 2005). Meanwhile, pH measurement of endosomal compartment after cationic liposome-mediated transfection showed pH of 7.1 explaining buffering of the acidic environment resulting in endosomal membrane destabilization and cytosolic release of cargo (Akinc and Langer 2002; El Ouahabi *et al.,* 1997; Xu and Szoka 1996). PEI reagents contain both tertiary and secondary amines which make them a suitable medium for altering endosomal pH levels through "proton sponge" effect which causes endosomal vesicle swelling and rupture with subsequent release of transfected particles (Benjaminsen *et al.,* 2013; Varga *et al.,* 2005; Akinc and Langer 2002). Exceptional behavior of PULSin assisted bactofection of causing significantly high GFP expression in all the cells in contrast to other transfection reagents could be attributed to its nature i.e. cationic amphiphile. Cationic amphiphilic peptides have been implemented earlier for DNA deliveries and resulted in effects similar to popular commercial transfection reagents such as Lipofectamine Plus, SuperFect, GenePorter and FuGene in COS-7 cells (Monkey Kidney Cells) (Fominaya *et al.,* 2000). Underlying mechanism states that amphiphilic peptides are capable of both condensing the DNA molecules and modify membrane permeability avoiding endocytic route to reach cell interior (Fominaya *et al.,* 2000). In addition, PULSin has been reported to mediate

partial escape of semiconductor nanocrystals or quantum dots in mammalian cells (Bradburne *et al.,* 2013; Delehanty *et al.,* 2010).

Although it has been established that commercial transfection reagents mentioned above have shown the capacity of altering lysosomal pH, their buffering capacity is known not to be dominant effect in the release of transfection complexes (Benjaminsen *et al.,* 2013; Bradburne *et al.,* 2013; Delehanty *et al.,* 2010). Nevertheless, possibly the synergistic effect of transfection reagents' buffering capacity together with the hemolysin activity contained by the pGB2Ωinv-hly plasmid in invasive *E. coli*, have resulted in an increased bacterial efficiency in epithelial cells.

Still, to address the endo-lysosomal degradation issue post-invasion, professional drugs were used which are known to enhance various viral and non-viral vector's gene delivery efficiency in mammalian cells (Margus *et al.,* 2012; Andaloussi *et al.,* 2011; Cheng *et al.,* 2006; Fredericksen *et al.,* 2002). Among tested reagents, Chloroquine which is a lysophilic weak base has emerged as a most promising drug to enhance bacteria mediated DNA delivery in human cells (Fig. 4.12A, B, C). Chloroquine has previously also proved to be a better lysosomotropic agent over methylamine with the 6-fold increase in contrast to the 3-fold increase in bacterial delivery to human fibroblast cells, respectively (Cheung *et al.,* 2012). In the present study, Chloroquine addition revealed up to 7-fold increase in cervical cells showing ≈50% GFP expression (Fig. 4.12A). Results are comparable to previous findings where Chloroquine addition enhanced gene delivery up to 6 fold from bacterial and viral vectors in human cervical and fibrosarcoma cell lines (Cheung *et al.,* 2012; Fredericksen *et al.,* 2002). Also, it has successfully helped in invasive *E. coli* mediated functional delivery of CFTR gene in airway epithelial cells (Kotzamanis *et al.,* 2009).

Implementations of bactericidal antibiotics to lyse intracellular surviving bacterial carriers, post-invasion for cargo release have been reported. This approach has successfully aborted intracellular growth of gram-positive bacterial strain *Listeria monocytogenes* used either in cancer gene therapy or for CFTR DNA delivery *in vitro* and *in vivo* (van Pijikeren *et al.,* 2010; Tangney *et al.,* 2010; Krusch *et al.,* 2002). Ampicillin addition upon Listeria-mediated invasion in breast cells (MCF-7) has shown a 6-fold increase in transgene expression (van Pijikeren *et al.,* 2010). Antibiotic-mediated intracellular lysis of *E. coli* vector has also been published causing effective transgene delivery *in vitro* (Jones *et al.,* 2013). Among tested antibiotics in the present study, Tetracycline which is a broad spectrum bactericidal reagent has shown highest and most significant transgene release and expression post-invasion (Fig. 4.13A, B, C). It has shown more than 60% GFP expression at as low as 10 μg/ml concentration in breast cells, which showed even less than 1% GFP expression without any antibiotic addition (Fig. 4.13C). Comparatively in another study also Tetracycline treatment resulted in significant reduction of viable intracellular *L. monocytogene* causing high transgene expression in CHO cells (Krusch *et al.,* 2003; Hense *et al.,* 2001).

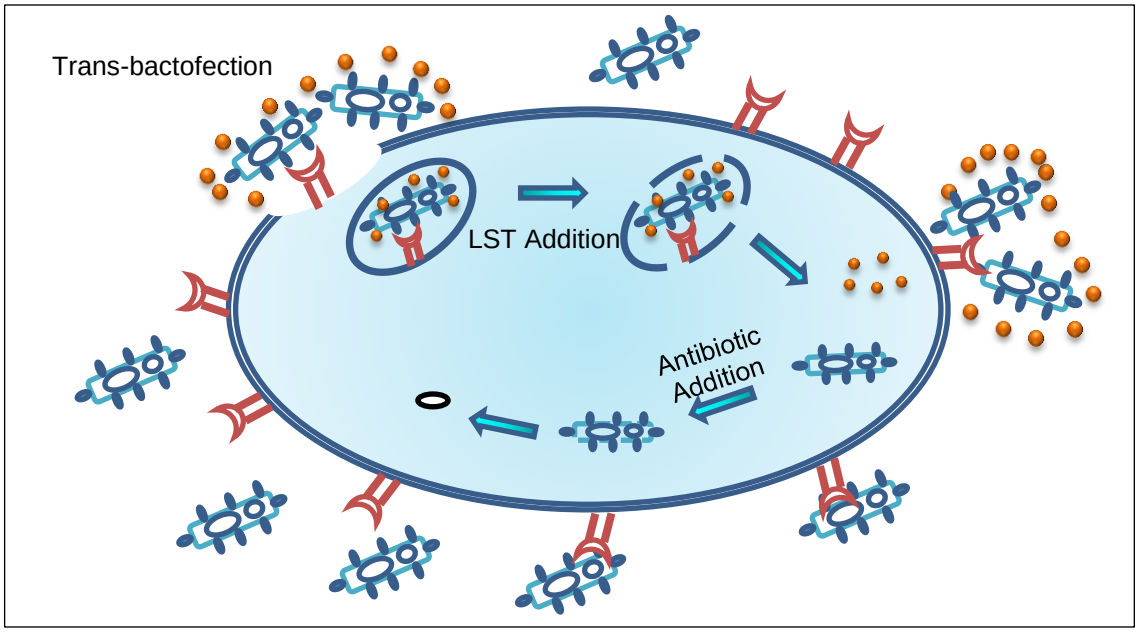

Figure 5.1. Diagrammatic illustration of overall steps adapted during bactofection to enhance vector's efficiency. External reagents were added at three different steps: firstly, bacterial cells were complexed with commercial transfection reagents to perform trans-bactofection, secondly, reagents were added to help bacterial vector escape lysosomal degradation and finally, antibiotics were added to lyse bacteria post-invasion for effective cytosolic transgene release. Eventually, all the steps were combined i.e. best effective reagent from all the three categories were added together to study their synergistic effect on boosting *E. coli* vector's gene delivery efficiency.

Finally, it was investigated that how would be the efficiency of DNA delivery if all the barriers reducing *E. coli* capability are removed altogether (Fig. 5.1). Thus, a combinatorial effect of best reagent from coupled transfection, endosomal escape, and the antibiotic category was chosen and administered in the single invasion. Figure 5.1 depicts the various points of the

target used in this study for the combined bactofection method with the aim to overall increase the bacterial vector's efficiency. In cervical and breast cells, even adding reagents together resulted in similar GFP expression as Tetracycline addition alone gave (Fig. 4.14A, B). Whereas, in lung cells, the combined treatment resulted in around 49-fold increase (Fig. 4.14C). These results suggest major limiting step in cervical and breast cells was intracellular surviving bacterial cells which resulted in the limited release of the transgene in host cellular environment. While, in lung cells enhanced GFP expression was gained as a result of higher entry, escape and release of cargo due to combined treatment. High GFP expression in all the cells was further confirmed by qualitative analysis using fluorescence microscopy, which also has shown a high number of GFP-expressing cells (Fig. 4.15).

Apart from reporter gene delivery, the bacterial vector was tested for large size gene delivery in human cells which is the ultimate aim of developing a gene delivery vector. Functional delivery of therapeutic genes such as β-globin gene and artificial chromosomes containing CFTR gene locus have been reported in human cells using invasive *E. coli* vector (Ooi *et al.,* 2008; Laner *et al.,* 2005). Furthermore, *in vivo* studies have also been conducted using recombinant *E. coli* for delivering CFTR gene in lung epithelial cells but have reported showing low gene delivery ability as compared to cationic lipid-mediated gene transfer (Larsen *et al.,* 2008). These studies suggest that *E. coli* vectors despite being an excellent choice for carrying large size gene with no immune reaction in host cells lacks the efficiency of achieving high gene delivery. Invasive *E. coli* in the present study has shown comparable gene delivery efficiency with that of transfection reagent Lipofectamine 2000 which is quite appreciable (Fig. 4.17 and 4.19). Further, gene delivery up to 70% was obtained by applying combined reagent addition method developed in the present study (Fig. 4.19). Lipofectamine 2000 and invasive *E.*

coli DH10B successfully showed delivery of globin BAC in HT1080 cells, however, presented data for bacteria mediated functional globin delivery is not quite conclusive (Fig. 4.21; Fig. 4.23). Still, it can be used as a preliminary data to further repeat the experiment and show bacterial vector's capability in delivering large therapeutic gene. On comparing functional globin delivery data obtained upon RT-PCR with that of gene expression values upon FACS analysis in both Lipofectamine 2000 and bacterial delivery case, it can be noted that in both the cases globin DNA bands showed weak intensity on agarose gel while flow cytometry revealed high values of GFP expression carried by BAC [Fig. 4.20 and Fig. 4.21 (Lanes G1 & G2); Fig. 4.22 and Fig. 4.23 (Lanes G1 & G2)]. This discrepancy of low DNA band intensity upon RT-PCR while a high percentage of gene expression upon FACS analysis, could have probably been avoided by analyzing globin gene expression kinetics i.e. time duration optimization for maximal RNA expression as was done for FACS analysis but the experiment could not be done for present study due to time limitation.

6. CONCLUSION

Overall concluding presented results, it demonstrates an improved bactofection method for delivering exogenous DNA in human cells at high efficiency. This was successfully attained by stepwise bacterial efficiency enhancement method. Moreover, invasive *E.coli* DH10B has been reported for the first time to deliver a therapeutic gene (β-globin) responsible for hereditary disorders such as β-thalassemia, with efficiency comparable to that of transfection method.

In the current study, invasive *E.coli* has shown successful delivery of exogenous DNA in a wide range of human cells, albeit with low efficiency and in a tissue-dependent manner. Additionally, it would be quite helpful to conduct cytotoxicity assay in order to assess the safety levels of the vector, towards the cells. Since the invasin ligand carried by the vector binds to the integrin receptors present on the host cells. It would be noteworthy to inspect the type and abundance of the integrin receptor on mammalian cells, in order to understand the tissue-dependent behavior of the invasive *E.coli* vector.

In an attempt to boost up vector's efficacy also, the simple stepwise addition of commercial reagents during bactofection have resulted in significant efficiency enhancement of bacterial vector. Similar to bactofection, different tissues behaved differently towards the chemical reagents' treatments. Although it is not very conclusive to say still, endosomal escape and post-invasion bacterial lysis were proven to be the limiting steps rather than the bacterial entry. Yet, it was quite evident by exceptionally high transgene expression in HeLa cells i.e. up to 50% and 90%, upon LST reagent and antibiotic addition, respectively. On the contrary, highest of 10% expression was obtained by the trans-bactofection method in HeLa cells. The above-stated hypothesis of endosomal escape and intracellular bacterial lysis being the major limiting steps could be established by treating host cells with the combination of LST reagent

and antibiotic but no transfection reagents, during the bactofection. More specifically, it can be rightly said that intracytosolic transgene release by the vector is proven to be the key limiting factor as perceptible by the highest transgene delivery, obtained upon antibiotic treatment in tested cells. However, in HT1080 cells highest GFP expression or in other words maximum transgene delivery, was obtained when all the treatments were given altogether during bactofection. Thus, it can be established that different cells have different mechanisms and behave differently towards external treatments. So, combined treatment method could give best bacterial vector efficiency in a wide array of cells. External reagents' administration has shown selective toxicity towards human cells. Certain cells are prone towards bacterial delivery while others showed a minimal or no toxicity such as HeLa and A549 cells showed more than 80% cell survival upon reagents' treatments whereas MCF-7 and HT1080 cells' survival was found to be less than 50%. Nevertheless, a proper cytotoxicity assay over the range of cells would yield a definitive list of the cells, which could be utilized for the combined treatment method with least toxicity.

Improved bactofection method has been successfully implemented in delivering GFP from a 100 kb BAC DNA in HT1080 cells using invasive *E.coli* with high efficiency. It has also been established (based on FACS analysis) that the combined treatment method is highly efficient in enhancing invasive *E.coli* bactofection capacity irrespective of its cargo size, be it plasmid or large size DNA. Hence, the improved bactofection method has proven its capability in developing invasive *E.coli* as an efficient gene delivery vector for human cells. Functional DNA delivery by the vector, which is still to be examined, would further strengthen the invasive *E.coli* vector's role in the field of gene delivery.

7. SIGNIFICANCE AND FUTURE DIRECTIONS

Under this study, the following objectives were attained:

a) Invasive *E.coli* mediated bactofection in a wide array of human cells and also a manifestation of cell lines responding best and least towards the vector.

b) Trans-bactofection by using various transfection reagents among which, protein delivery reagent displayed maximum effect in enhancing bacterial delivery capacity in all the tested cells.

c) The bacterial invasion followed by the administration of chemical reagents to the invaded host cells, for increased cytosolic access of DNA cargo carried by *E.coli* vector led to enhanced bacterial delivery in four tested cells.

d) Implementation of trans-bactofection together with post-invasion chemical reagents produced a synergistic effect and elevated GFP reporter delivery drastically in all of the four tested cells.

e) Lastly, the developed method of combined administration of chemical agents pre and post-invasion successfully showed GFP delivery from 100 kb human β-globin BAC in human fibroblast cells.

For the first time, comparative bactofection efficiency of invasive *E.coli* DH10B in nine different non-phagocytic human cell lines was demonstrated in this study. This provides an insight into the behavior of human tissues towards the bacterial vector and hence, the tissues responded effectively could be targeted in future for gene therapy by using the invasive *E.coli* DH10B vector. We also have successfully shown the development of a simple yet, effective bactofection method which helped to overcome the major drawback of a bacterial vector i.e. low transgene delivery efficiency in comparison to viral vectors and transfection method, *in vitro*. These studies reveal the great potential of the developed bactofection method in elevating

invasive *E.coli* DH10B vector's gene delivery ability, *in vivo*. Moreover, the quantitative evaluation to check the efficacy of the improved bactofection method in delivering large DNA molecule (≈100 Kb β-globin BAC), was proven to be equally effective as for small sized GFP gene. Therefore, suggesting the implementation of the developed bactofection method in transferring other DNAs carrying therapeutic genes together with its regulatory elements.

For further studies, in addition to the tested cell lines, other cell lines which are of significant importance in view of gene therapy such as muscle cells, heart cells etc., could be studied for gene delivery using invasive *E.coli* DH10B (Long *et al.,* 2016; Carroll *et al.,* 2016). Also apart from adherent cells, assessing gene delivery capacity by invasive *E.coli* in suspension cells such as blood cells, could be a promising strategy to target diseases related to these cells e.g. Sickle-cell disease (Urbinati *et al.,* 2015). Moreover, trans-bactofection could further be tested with other popular and commercially available transfection reagents. In fact, other DNA carriers such as nanoparticles, adenovirus particles etc. could also be combined with the *E.coli* DH10B vector and study their effect on gene delivery in host cells (Akin *et al.,* 2007). However, a certain level of cellular toxicity upon implying chemical reagents during bactofection was observed, which needs to be further monitored and addressed.

Functional globin delivery by using invasive *E.coli* need to be accurately detected with proper controls to verify the amplification. As evident from *in vitro* studies, invasive *E.coli* shows the great possibility for highly efficient bacteria mediated gene delivery *in vivo.* Additionally, the modified bactofection method could also be employed for other well known bacterial vectors, that are carrying therapeutic gene together with alphoid DNA for HAC formation (Rocchi *et al.,* 2010; Laner *et al.,* 2005; Krusch *et al.,* 2002). Apart from gene delivery, the developed bactofection method could also be employed for delivering proteins,

RNA molecules etc. in host cells efficiently, and serve as a potential replacement for viral or complicated non-viral vectors (Carroll *et al.,* 2016; Severino *et al.,* 2015). In view of high gene transfer capability of *E.coli* DH10B vector, upon using the improved bactofection method *in vitro*, it can be rightly said that more preclinical researches on the vector would affirm its use in clinical research.

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