Scaling-up The Production and Purification of HSV-based Gene Therapy Vectors and Development of GLP Assay System to Analyze Quantity and Quality of Clinical-grade HSV Vector Stocks

by

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ABSTRACT

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Scaling-up The Production and Purification of HSV-based Gene Therapy Vectors and Development of GLP Assay System to Analyze Quantity and Quality of Clinical-grade HSV Vector Stocks

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Herpes simplex virus type-1 (HSV-1) represents an ideal vector for use in many gene therapy applications. HSV-based vectors have recently been employed in human clinical trials to treat malignant brain tumors. The production of HSV-1 vectors must now be scaled-up to meet the demand for large volumes of high-titer clinical-grade vector

stocks. However, in order to develop efficient large-scale production and purification methods, not only the factors affecting the efficiency of HSV-1 vector production have to be systematically investigated but also new Good Laboratory Practices (GLP) vector assay systems needs to developed.

We evaluated the dependence of viral yield on genetic background, culture pH, glucose and lactate concentration as well as serum content. High-level glucose consumption, with the corresponding increase in lactate synthesis, observed within the first 24 hours post infection period. Media replacement at various times following infection resulted in a 2-fold overall increase. The influence of culture pH on viral yield and virus stability was also investigated. Lower pH (6.8) not only enhanced the viral yield but also increased the half-life of virus compare to higher pH values (7.4 and 8.0). The use of serum-free media did not result in high viral yield. However, our results show that serum content of 5% provided similar viral yields as that of 10%, suggesting a potential cost reduction and ease in the purification of vector. Then, we successfully scaled-up the vector production by employing CellCube bioreactor.

Finally, we developed and a very sensitive assay system relevant for meeting and surpassing current FDA requirements for clinical grade viral vector stocks. Our real time PCR assay is linear from 10 to 10^7 copies of HSV and 1 to 10^5 copies of host cell genomic DNA. Our PicoGreen micro-plate assay is fast and accurate, with a detection limit as low as 0.5 ng of HSV DNA corresponding to ~$3x10^6$ HSV particles. The resultant combination of real-time PCR and PicoGreen micro-plate DNA quantitation assays represents a standard in the field of HSV gene therapy vector quality assessment.

DESCRIPTORS

Cell Culture

GLP Assay Development

Optimization of Culture Conditions

Gene Therapy

Herpes Simplex Virus Type 1

Viral Vector Production

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NOMENCLATURE

6-PG	6-Phosphogluconate
AAV	Adeno-Associated Virus
ADV	Adenovirus
ATP	Adenosine Triphosphate
cDNA	Complimentary Deoxyribonucleic Acid
CNS	Central Nervous System
D	Defective particles
DI	Defective Interfering Particles
DMEM	Dulbecco's Minimum Essential Medium
DNA	Deoxyribonucleic Acid
E	Early Viral Genes Expressed
EBV	Epstein-Barr Virus
EDTA	Ethylenediaminetetraacetic Acid
f	Fraction of Cells Infected
G-6-P	Glucose-6-Phosphate
gB	Glycoprotein B
GLP	Good Laboratory Practices
GMP	Good Manufacturing Practices
HIV	Human Immuno-deficiency Virus
HPI	Hours Post Infection

HSV-1	Herpes Simplex Type 1 Virus
I	Number of Infected Cells
ICP	Infected Cell Protein
IE	Immediate Early Viral Gene Expressed
L	Late Viral Gene Expressed
LAT	Latency-Associated Transcript
MOI	Multiplicity of Infection
MEM	Minimum Essential Medium
NAD	Nicotinamide Adenine Dinucleotide
NADH	NAD, Reduced
NGF	Nerve Growth Factor
NMDA	N-Methyl-D-Aspartate
PFU	Plaque-Forming Units
SOD-1	Superoxide Dismutase
SDS	Sodium Dodecyl Sulfate
SV40	Simian Virus 40
$TCID_{50}$	50% Tissue Culture Infectious Dose
TH	Tyrosine Hydroxylase
TK	Thymidine Kinase
VEGF	Vascular Endothelial Growth Factor
VP16	Virion Protein 16

1.0 BACKGROUND

1.1 Gene Therapy

1.1.1 Introduction

Gene transfer may be defined as a strategy in which defective or missing genetic information of patients' cell is replaced with the insertion of new genetic information[(1,2)*]. Because it may become a promising treatment for prevention, diagnosis and treatment of many complex diseases including cancer, Parkinson Disease, and AIDS, there has been tremendous increase in clinical gene transfer applications[(3,4)]. Efficient gene therapy requires three elements: an appropriate disease model, a target cell, and a gene transfer method. The disease model determines the type of target cell and the target cell determines the appropriate gene transfer method[(5)].

Viruses are known as one of the best vectors for gene therapy[(6,7)]. Unlike other gene transfer systems such as liposome mediated gene transfer, receptor mediated gene transfer, and direct injection of naked DNA, viruses are the only vector system available for use in cases where a large number of target cells are required to be treated[(7)]. In addition, viruses are extremely efficient for delivering the nucleic acids to the target cell as well as evading immune response. Since most of the gene transfer applications require no secondary virus infection after the initial cell infection, viral vectors are generally

* Parenthetical references placed superior to the line of text refer to the bibliography

derived from replication-incompetent (replication-defective) viruses. Replication incompetent viruses are those in which most or all of the viral coding regions have been replaced with the gene(s) of interest [8]. Because the coding has been modified, the viruses are incapable of making proteins needed for viral replication. In this situation, viral proteins required for initial cell infection are provided by genetically engineered cell lines called packaging cell lines.

The host range of a virus can be defined as the range of cell types or species in which the viruses can infect and replicate. The host range depends on the viral glycoprotein, which determines the efficiency of virus binding, and entrance to the target cell [8].

The titer (or concentration) of virus expressed as plaque forming units per milliliter is an important parameter affecting the efficiency of gene transfer applications[8]. The titer of vector is very important especially for *in vivo* type transfer applications in which volume of virus solution that can be injected is limited. Thus, stable and efficient transduction of genes into cells can only be accomplished with high titer viral vectors[7,8].

Helper viruses are replication-competent viruses that enable a replication incompetent virus present in the same cell to replicate[7,8,9]. Their existence in stocks of replication-incompetent viruses is an important safety problem for gene transfer applications. Because such viruses may help replication-incompetent viruses to replicate and may cause an overwhelming viral infection or even death of the target cells.

1.1.2 Methods of Gene Transfection

Gene delivery systems can be categorized as viral and non-viral[1,9]. Non-viral delivery systems are limited by the low efficiency of gene transfer but very attractive because of their non- pathogenicity, limitless foreign gene capacity and ease of production[10-19]. Either chemical means such as the use of calcium phosphate and liposomes, or physical means such as electroporation and microinjection may achieve non-viral gene delivery.

DNA can pass through the plasma membrane when the target cell is treated with calcium phosphate or when DNA is encapsulated into lipid bilayers called liposomes[13,14,15]. Basically, in chemical approaches many copies of DNA carrying the gene of interest are mixed with charged substances such as calcium phosphate or liposomes and applied onto target cells. The chemical puts stress on cell membrane and DNA moves into target cell. These chemical methods are very simple and safe, however, they may not provide stable long-term expression of healthy genes and may not reach the targeted cells[15-18].

Electroporation, a physical gene delivery method, is the transport of the gene of interest into target cell by means of an electric field[16]. This procedure is simple, but it provides short-term transient expression of therapeutic gene. This technique has not been used for any clinical applications. Though another physical method called microinjection provides very efficient gene delivery, it also can not provide stable, long-term expression

and this technique is very labor-intensive[17,18,19] (each individual cell must be injected one by one).

1.1.3 Gene Transfection by Viruses

Viruses are small intracellular parasites that contain either DNA or RNA as genetic material[20,21]. The structure of virion is composed of DNA or RNA, a capsid, and sometimes an envelope. They are classified into different categories according to their nucleic acid type, size, and the presence or absence of an envelope[22]. Viruses can grow and replicate only in living cells. In the viral replication cycle, viruses enter the cell, and transcribe the viral genes in the host cell. Naturally, genetically engineered viruses have a promising potential to carry any gene of interest into a target cell by using current advanced molecular genetics methods [6].

1.1.4 *Ex Vivo* and *In Vivo* Gene Transfer

Gene transfer can be carried out in two ways; *ex vivo* or *in vivo* methods[23]. In *ex vivo* transfer applications, the gene of interest is inserted into the patient's cells in the laboratory, which are then re-administered to the patient. Alternatively, gene transfer can be accomplished by *in vivo* transfers in which the gene of interest is directly introduced into target cells inside the patient's body.

1.1.5 Strategies for Gene Transfer

Gene transfer strategies are disease specific[2]. For single-gene disorders such as cystic fibrosis, gene augmentation is the method of choice[9,24]. In this method, a

functional copy of the gene of interest is introduced into the target cell. Cancer treatment can be achieved by various gene transfer strategies such as tumor vaccines, suicide genes, multiple drug resistance genes, and tumor suppressor genes[25,26,27]. HIV treatment with gene transfer can be done in two ways; intracellular and extracellular[9,24]. Goals of intracellular gene transfer are either to kill infected cells by inserting cytotoxic genes or to interfere with the HIV life cycle by inserting genes which inactivate the regulatory proteins required for virus replication [9,27].

1.1.6 Current State of Gene Therapy

Table 1 shows the delivery systems and number of protocols approved by the National Institute of Health (NIH)[28]. Most of the gene transfer applications employ viral vector systems. Retroviral vectors are the most commonly used delivery system (71%), and are being used in the majority of the approved clinical gene transfer trials. Adenovirus based vectors follow retroviral vectors as a second common vector of clinical gene transfer applications (14 %). Adeno-associated vector is approved only once for an clinical application to date. Herpes vectors, on the other hand, have not been approved for clinical gene transfer applications yet. Non-viral delivery systems such as cationic liposomes, plasmid DNA, and particle mediated delivery covers only the 13% of gene transfer protocols approved to date.

Table 1

Delivery Vehicles for Gene Transfer [28]

Delivery Vehicle	Number of approved protocols	Advantages of vector	Disadvantages of vector
Viral			
Retrovirus vectors	76	Large capacity Easy to produce Efficient transfer	Targets only dividing cells Risk of replication Random DNA insertion
Adenovirus	15	Large capacity Targets non dividing cells Efficient transfer	Possible host-immune reactions Risk of replication
Adeno-associated virus	1	Less likely to produce immune reactions Targets non dividing cells Persistent delivery capacity	Possible host-immune reactions Risk of replication
Nonviral			
Cationic liposome complex	12	Easy to produce Less likely to produce immune reactions	Low efficiency
Plasmid DNA Particle-mediated	2	No size limitation Easy to produce No replication risk Less likely to produce immune reactions	Low efficiency

1.1.7 Common Gene Therapy Vectors

1.1.7.1 Retroviral Vectors. Retroviruses are enveloped Class V viruses with a single envelope glycoprotein. Retroviruses are small particles with a diameter of 80 to 130 nm[20,21,22]. These viruses contain reverse transcriptase, which is essential for reverse transcription, i.e., the production of a molecule of DNA from an RNA template. After penetration into the target cell, the viral RNA is converted into a double stranded RNA by the reverse transcriptase enzyme. Viral DNA enters the nucleus and integrates into the host genome[6,7]. The integrated viral DNA is called a provirus, which is the template for the expression of the viral genes and synthesis of progeny virion RNA. Virions, which are assembled by the viral proteins and progeny RNA, leave the host cell by budding through the plasma membrane[6,7].

A typical retroviral genome structure contains three genes: 1) gag, which encodes the proteins that form the viral envelope protein; 2) pol, which encodes the reverse transcriptase and viral DNA polymerase; 3) env, which encodes the viral envelope glycoproteins that are inserted into host cell membrane [6,7]. Major advantages of retroviral vectors over other delivery systems are the stable and efficient integration into host cell, the ability to infect a broad range of cell types, the lack of vector spread after initial infection, the lack of toxicity of retroviral vectors in the target cells, and the obligatory integration step in their life cycle[29,30,31]. However, they have some potential problems due to their biology and unique life cycle. Their capacity for carrying a gene of interest is less than 10 kb, and they have not yet been produced at a high enough virus

titer that would allow them to be appropriate for in vivo transfer applications. Also, retroviral vectors target only dividing cells. They are, thus, not an appropriate choice when the target cell is non–dividing[29,30,31].

1.1.7.2 Adenoviral Vectors. Adenoviruses are large, naked, Class I DNA viruses that are stable from pH 5 to 9 and from 36°C to 47°C[20,21,22]. Adenoviruses are the first alternative to retroviruses when the target cells are non-dividing cells since their life cycle does not require target cell division. This vector does not integrate into target cell chromosome. Moreover, adenoviruses are well suited as vectors for in vivo gene transfer trials because they can be produced in high titers and can be easily purified. They can also maintain a long-term association with target cells[6,9,32,33]. The infection cycle of adenoviruses contains early and late phase. Early phase is composed of attachment of virus to host cell membrane and its fusion into cell before the viral DNA synthesis. Late phase contains the production of viral DNA and virions. Adenovirus genomes are linear and are divided into early (E1 to E4) and late (L1 to L5) genes[34,35]. Each end of the genome has a short sequence responsible for viral replication called inverse terminal repeat (ITR)[34,35].

Adenovirus derived vectors have been successfully used for gene transfer trials. They are one of the most promising vector candidates for in vivo gene delivery. This vector has high capacity for foreign genes and can target non-dividing cells. A high titer of viral stock can be obtained in cell culture, and nonpathogenic replication-defective mutants of adenovirus derived vectors are available[36,37]. However, one shortcoming of

vector is the high risk of immune response. Furthermore, adenoviral vector design is more complicated than that of other viruses because of its large and complex genome structure[34-37].

1.1.7.3 Adeno-associated Viral Vectors. Adeno-Associated Viruses (AAV) are very small, naked Class III DNA viruses with a diameter of 18 to 26 nm[20,21,22]. They do not cause any known diseases. In order to replicate, AAV needs to be co-infected with an unrelated virus (adenovirus or herpes virus)[6,36,37]. In the absence of helper virus, AAV can integrate into specific regions of host cell chromosomes. The genome structure is very simple and contains a 4.7 kb single stranded DNA with two genes (Rep, Cap), three promoters and the inverted terminal repeats (ITR). Rep and Cap are the coding sequences responsible for non-structural and structural proteins respectively. The integration process does not require any viral gene, but does require the internal terminal repeats. ITRs are the only sequences required for replication, packaging, and integration[6,36,37].

The adeno-associated virus (AAV) based vector is the last viral vector approved for clinical trials. The major advantage of AAV vectors is their ability to integrate into a specific site of the target genome and provide long-term expression in non-dividing cells[37,38]. They have a broad host range and simple genome. Furthermore, they do not have pathogenicity in humans as well. AAV vector applications are limited due to the small size of the viral genome, and the difficulty in the production of high titer stocks[37].

1.1.7.4 Herpes Simplex Virus Type 1 Vectors. Due to their unique biology, herpes virus vectors are considered one of the most promising vector systems for gene therapy[38-47]. Their potential as a viral vector comes from their ability to become latent in non-dividing cells and provide long-term stable heterogeneous gene expressions in target cells. Other advantages of herpesvirus based viral vectors are (i) large gene loading capacity, and (ii) broad host range including non-dividing neural cells. Major limitations for herpesvirus based gene transfer applications are excessive defective particle formation, cytotoxicity of herpes vectors and difficulty in mass production[43-47].

1.2 HSV-1 Biology

1.2.1 Classification of Herpesviruses

Based on the biological properties, the Herpesviridae family can be divided into three subfamilies[48]: the Alphaherpesvirinae, the Betaherpesvirinae and Gammaherpesvirinae. Alphavirinae, which has a wide host range and short replication cycle, contains Simplex Virus (HSV-1, HSV-2, circoptheane herpesvirus1, and bovine manilitis virus) and Varicellovirus genera (VZV, pseudorabies virus, and equine herpesvirus1). Betaherpesvirinae can be categorized as Cytomegalovirus (HCMV) and Muromegalovirus genera (murine cytomegalovirus). This subfamily has a relatively small host range and a long replication cycle, and may have potential for gene delivery but has not yet been used as a gene delivery method. Gammaherpesvirinae is composed of two genera, Lympryptovirus (EBV) and Rhadinovirus (herpes saimiri). Gamma-

herpesviruses have a potential to be used as vector since the members of this subfamily can establish a lytic infection in dividing cells although it has a limited host range. For example, Eppstein–Barr virus is the viral vector of choice in the treatment of cystic fibrosis.

Based on the replication cycle followed, human herpes viruses can be classified as[49,50] lymphotropic and neurotropic. Lymphotropic herpes viruses undergo lytic infection in which host cell is destroyed at the end of the replication cycle. After each cell division, lymphotoric herpes viruses retain their genomes in daughter cells. Neurotropic herpes viruses can form latent infections in non-dividing cells like neurons where they do not need to replicate to maintain their latent state. Herpes viruses are large enveloped Class I DNA viruses with a 150 - 200 nm diameter[20,21,22].

1.2.2 HSV-1 Structure

The herpes virion contains a linear double-stranded DNA genome at the center, or the core, enclosed by an icosadeltahedral protein shell called the capsid[49,50]. The tegument, a protein matrix, surrounds the capsid. A lipid–containing membrane called the envelope protects the entire structure. The capsid contains 162 capsomers and its diameter is approximately 100-110 nm[49,50]. The tegument is composed of a number of proteins, some of them involved in shut-off of protein synthesis, activation of immediate early viral gene expression and assembly functions. The envelope contains 12

glycoproteins and these glycoproteins function in the attachment and the penetration of the virion into the cell nucleus.

1.2.3 Molecular Biology of HSV-1

Genomes of herpes viruses vary in size from 125 Kb to 229 Kb, and encode approximately 75 genes[20]. Among different types of herpes viruses, the herpes simplex virus type 1 (HSV-1) is the most commonly used herpes virus as a vector candidate in gene therapy [51]. HSV-1 genome contains three origins of replication and a packaging signal ("a" sequence) and is divided into L (large, M.W. 67.2 $x10^6$) and S (small, 5.8 $x10^6$) components separated by inverted repeat sequences[49,50]. According to the time that they are expressed during the productive infection, viral genes can be divided into three subgroups: immediate early, early, and late.

Immediate early (IE or α) genes are expressed in the absence of new viral protein synthesis. IE genes encode major proteins that mainly regulate the expression of other viral genes necessary for the rest of the replication cycle. There are five infected cell polypeptides (ICPs) expressed by IE genes: ICP0, ICP4, ICP22, ICP27, and ICP47 [52,53,54]. Among IE genes, two of them are essential for replication (ICP4 and ICP27)[54]. ICP22 and ICP47 are non-essential for viral replication but the deletion of ICP22 helps to decrease the toxicity of HSV vectors[51]. Although ICPO is considered a non-essential gene for the replication, the deletion of ICP0 has decreased the production of viral vector significantly [55]. ICP0 has been shown to associate with ICP4 in some regulatory functions.

In the next step, early genes encode viral proteins required for DNA replication. Early (E or β) genes are expressed only in the presence of IE proteins but does not depend on viral DNA synthesis[49,50,51]. Products of E genes, namely Tymidine Kinase (TK), DNA polymerase, Dnase, Ribonucleotide reductase, and UTPase, are primarily involved in nucleic acid metabolism and viral DNA synthesis. Most of these gene products duplicate cellular proteins.

The late genes (L or γ) are expressed after DNA replication. The main role of the late genes is to produce virion proteins and enzymes required for assembling the virus particle[50]. Expression of these three groups of genes is regulated by a series of positive and negative feedback loops. An IE gene stimulates the transcription of E genes, and E genes can both inhibit and stimulate the L genes which therefore inhibits IE and E gene transcription based on the time of the replication process[49,50,51].

1.2.4 Life Cycle of HSV

Following the attachment of the virus into peripheral sites such as epithelial or mucosal cells, the virus starts to replicate[47]. Then, the virus infects the nerve terminals of infected peripheral cells and moves to sensor ganglia by retrograde axonal transport [56,57]. When virus reach sensor ganglia, there are two pathways that the virus can follow; lytic infection or latency[44,48,58,59].

1.2.4.1 Latency. A common feature of herpes viruses is latency, the presence of a virus in host cells, usually in neurons, in the absence of clinically apparent

infection[44,48]. When a virus particle enters the latency cycle, only a specific part of the HSV-1 genome can be transcribed. This is the part of genome that encodes a set of RNAs, called latency-associated transcripts (LATs). The roles of LATs are not clearly understood. Recent studies speculate that LATs may play an important role in replication, regulation and pathogenicity of virus[44,48].

1.2.4.2 Lytic cycle. Several types of stimulation such as physical or emotional stress, drug, hormonal intake, sunlight, menstruation, etc. can reactivate herpesviruses in the latent state[48]. Following the reactivation of the virus, new infectious viruses are produced in the ganglion and then, new infectious particles moves to neighboring epithelial cells where primary infection was established previously (anterograde transport)[56,60]. The infection cycle of the herpes viruses contains several steps. First, the virus attaches to the cell surface then, the viral particle (virion) fuses through the cell membrane and the tegument proteins and nucleocapsid enter the cell[48]. Attachment is a receptor-mediated binding process mediated by gG, gE, gI, and gL glycoproteins[61-64]. Surface receptor for binding is heparin sulfate[65]. After attaching to the cell, the viral genome is transported by the nucleocapsid to the nucleus where the viral genes are transcribed[66].

1.2.5 Syncytia

Syncytia, formation of multinucleated cells (polykaryocytes), is a hallmark of herpetic lesions in vivo[66]. However, only spontaneous fusing-inducing mutants (synctial or Syn mutants) lead to formation of polykaryocytes[67,68,69]. HSV–induced cell fusion is

believed to be the reason why polykaryocytes form. Some of syn mutants are incapable of producing infectious viruses while the others are very efficient in infectious virus production. Infections of syn mutants are dependent upon the type of cell. Some of these mutants can cause polykaryocytes formation in some cell types but not others. The effects of cell types on HSV-induced cell fusion have not been clarified yet[66]. The syn mutants can be constructed by the deletion of gene(s) classified as "modulators" of cell fusion[66]. Modulators of HSV-induced cell fusion are UL20, UL24, UL1, UL27, and UL53. Unlike UL20 and UL24, UL1, UL27, and UL53 genes encode membrane glycoproteins gL, gB and gK respectively[66].

1.3 Defective Interfering Particles

1.3.1 Introduction

Defective (D) virus particles are the mutants of standard virus particles[70-74]. When defective particles interfere with the viral replication, they are called defective interfering particles (DI). Discovery of DI particles was based on the observations that many researchers have faced during the propagation of viruses at high multiplicity of infection. In 1957, Von Magnus demonstrated the existence of DI particles, and this phenomenon was named after him[75]. Following studies on many virus systems, contamination due to DI particles became well-known phenomena for many virus systems[70,71,76]. There are several reasons that make the studies on the evaluation and replication of DI particles very interesting[74,77,78]. First, DI particles may play an

important role in natural virus infection as they do in in vitro infections. Perhaps, DI particles could provide protection from many viral diseases. Due to the technical difficulties, presence of DI particles in natural infections has not been proved yet. However, many investigators have shown that DI particles can be generated or amplified in laboratory animal[74]. Second, studies of the evaluation and replication of DI particles may help characterizing the mechanism of replication and propagation of non-defective (standard) viruses. Third, comparative analysis of viral gene expression in cells infected by DI particles with cells infected by non-defective viruses may explain the influence of specific gene alterations on viral gene expression.

There are several unique properties of DI particles such as their dependence on helper virus, or defectiveness, their ability to decrease the standard virus (SV) yield or interference, and their ability to increase the DI /SV ratio or enrichment[70,71,72].

The defectiveness of viral particle means that the virus genome has lost or rearranged genes. Deletion appears to be main reason for defectiveness rather than point mutations or DNA rearrangement. The mechanism of gene(s) deletion has not been understood yet but appears to be virus specific[74].

Interference is the most notable property of DI particles. Basically, DI particles inhibit the replication of standard virus which DI particles use as a helper virus to replicate[74,79]. The mechanism of interference is not well understood, but it is believed to vary depending on the virus system. The input quantity and the quality of the infecting virus are two important parameters that affect the interference capacity of DI particles in

a given undiluted propagation application. The input quantity of the infecting virus or MOI strongly affects the generation of DI particles[74,80]. High MOI causes more DI particle production. The quality of the infecting viruses or the proportion of defective particles also strongly influences the DI particle generation[74].

Another interesting characteristic of DI particles is enrichment [70,74]. Enrichment can be defined as the ability of DI particles to increase their number at the expense of standard viruses. Thus, the proportion of DI particles to standard viruses increases as the passage number increases. The mechanism of enrichment involves a preferential replication or a preferential encapsidation of DI genome. In this mechanism, the nucleic acid of DI particles must compete with that of standard virus for the rate limiting factor of viral DNA replication and somehow the pathway toward production of DI particle must be more favorable than that of the production of standard virus.

1.3.2 Defective Herpes Viruses

Due to their very large and complex genome structure, herpes viruses are good candidates to generate DI particles[70,71]. DI particles derived from herpes viruses contain rearranged, deleted, or reiterated viral sequence[70.71,78-82]. HSV-1 and HSV-2 type herpes viruses have been investigated very intensively in terms of their DI particle production [79-82]. Structural features of defective HSV genomes are[82,83,84] (i) defective HSV genomes are less complex than standard virus genome and contain multiple repeated DNA sequences; (ii) each defective HSV genome is composed of a variety of DNA molecules of different size and repeat units; and (iii) based on the origin of DNA

sequences contained in their DNA repeat units, defective HSV genomes can be divided into Class I and Class II defective genomes[74-76]. Class I defective genomes originate from S component of the parental DNA and have high G+C content. In contrast, Class II defective genomes originate from the end or within the S component of DNA, and have lower buoyant density than Class I defective genomes due to their low G+C content.

Two models have been proposed to explain the evaluation of defective HSV genomes[74,83,84]. The first model proposes that intramolecular recombination events favor the production defective genome production while the second model proposes that defective DNA molecules possess a preferential advantage in replication since there is a limited diversity in defective HSV genomes.

Furthermore, two routes have been proposed to explain the replication of defective HSV genomes[74,83,84]. First, defective genomes of HSV may replicate without any physical interaction with the standard virus. Defective HSV genomes may contain necessary parts for the replication and propagation. Second, defective HSV genomes may attach to helper virus genomes by a covalent bond and cause defective HSV genomes to be produced.

Studies have demonstrated that infections with HSV viruses containing DI particles cause dramatic changes in the viral gene expressions[74,83,84]. The ICP productions at different DI particle concentrations have shown a considerable difference. According to these studies, ICPs can be classified into three categories based on the effect of the concentration of the DI particles on the synthesis of ICPs:

- first group ICPs (ICP8 and ICP21) is not affected,
- second group ICPs (ICP4) is over-produced (a linear relationship between production of ICP4 and defective particle concentration was observed),
- third group ICPs (ICP5, ICP6, ICP11, ICP15, ICP18.5, and ICP25) are under-produced at different defective viral particle concentrations.

1.4 Animal Cell Nutrients

A number of chemically defined cell culture mediums has been developed to provide the complex nutritional requirements of cells. A typical growth medium contains amino acids, vitamins, hormones, growth factors, mineral salts and carbon sources. Glucose and glutamine are the common source of energy for most of the commercial growth medium formulations[85,86]. Their concentrations in medium formulations vary between 5.5-25mM and 1-5mM respectively[87,88]. The addition of mammalian blood serum to growth medium is necessary for many cells types[89]. Serum provides the growth factors necessary for culture viability. The concentration of serum in growth medium generally changes between 2 to 10% dependent upon the cell type. Since serum is expensive and complicates the downstream purification processes, serum-free media have been formulated and used for cell culturing[90,91,92].

1.5 Requirement of High Concentration of Vectors

The use of highly concentrated vectors in gene transfer applications is very crucial since the volume of vector solution that can be injected into target cells is limited[8,9]. In cases where a large number of cells must be infected uniformly with a virus, high titer virus stock must be used for a stable and efficient transduction of genes into target cells. Generally, increase in the titer of the viral vector increases the efficacy of the gene transfer[1,2,8,9].

Cytotoxicity of viral vector to target cells and immune response due the injection of vector also limit the dose of viral vector use in gene transfer[51,93-96]. Cytotoxicity of vector is related to viral gene expressions and can be reduced by the deletion of gene(s) responsible for the production of these toxic gene expressions. Thus, the larger number of deleted genes the lower will be the cytotoxicity. On the other hand, there is an inverse relation between the number of genes deleted from a virus and its yield in cell cultures.

1.6 Large-scale Production of Viral Vectors

1.6.1 General Issues

Early viral vector based gene therapy trials have generally required small quantities of viral vectors and these viral vector requirements have been supplied by laboratory scale production. Consequently objectives of developments in viral vectors have been mainly concentrated on new vector constructs, product quality and safety

rather than process optimization, scale-up and operability for expanded manufacturing. Today, along with the expanded studies in the development and clinical applications of viral vectors, the demand for large volumes of high titer viral vectors has skyrocketed. Since conventional production methods including tissue culture flasks and roller bottles are not efficient enough to provide these significant quantities of high quality herpes vectors, the use of bioreactor systems is becoming a necessity. However, there are several critical issues that must be addressed to design an efficient large-scale viral production system including: contamination, the optimization of a number of interactive process parameters including mixing, aeration, oxygen demand, the selection of type of reactor and mode of reactor operation, the selection of culture media, the control of toxic product accumulation and several operation parameters such as temperature and pH[97, 98]. Contamination may occur during the reactor run. This is the main problem of many large-scale cell-culturing applications. Various parts of the bioreactors carry high risk of contamination including inlet and outlet filters, bioreactor internals, inlet and outlet ports, valves and connection lines. Hence reactors should be designed to allow sterile operations and sterilization procedures should be validated prior to reactor runs.

It is essential but difficult to optimize interactive reactor parameters such as mixing, aeration, and oxygen demand. Homogenous distribution of nutrients and oxygen throughout the reactor is necessary. However, physical stress due to mixing may cause damage to sensitive host cells or viruses. For this reason, physical stress due to mixing should be minimized by optimization of mixing equipment and conditions.

The oxygen demand of mammalian cells are relatively low (0.05-0.5 μmole oxygen /10^6 cells/h) compare to that of microorganisms[97]. However, the low solubility of oxygen (0.2 mmole per liter culture) in culture environment makes the supply of adequate oxygen difficult[97]. It becomes more of a problem as the volume of the culture increases with decreasing surface to volume ratio.

For anchorage dependent cultures, fixed bed bioreactors employing microcarrier systems are most commonly used. Fixed bed reactors have been developed to minimize the effects of physical stress on productivity and maximize the scale-up potential of anchorage dependent cells. These reactors can be operated in various modes: batch, fed-batch, and continuous.

Batch culture is a closed process where following to inoculation of cells with fresh media, nothing is added or removed from the system. In this operation mode, essential nutrients deplete and toxic by-products accumulate. Fed-batch culture, on the other hand, is an open system where small volumes of essential nutrients are added to the reactor during the culture period.

Continuous culture is a process where fresh medium is continuously added to system while the same amount of spent culture continuously removed from the system. This culture type can be divided into chemostat culture and perfusion culture. The difference between these two continuous culture types is the removal of cells from the reactor. In chemostat culture, cells are removed continuously from the reactor while in perfusion culture cells are retained in the reactor. Continuous mode of culture operation

offers several advantages over conventional batch culture, including higher yields and productivity, reduced raw material costs but it suffers from operation complexity, and excessive media consumption[98,99].

The concentrations of toxic by-products such as ammonia and lactic acid should be controlled very efficiently to prevent any inhibition potentially caused by these materials. Accumulation of lactate and ammonia usually causes inhibition of growth and promotion of cell death. Critical inhibitory levels of these two toxic metabolites are cell dependent but are about 40mM and 3mM for lactate and ammonia respectively[99].

1.6.2 Prior Work on Vector Production

Most of the process optimization and scale-up studies have been focused on retroviral vectors. In optimization studies, the influence of temperature,[100-102] infection time,[102,103] serum and polycation concentrations[100-102.] and freezing/thawing[100,101] were examined. Results of these studies have shown that up to 100 fold increase in production can be accomplished by optimization of these parameters. Kotani *et al*[100] (1994) studied 22 producer cell lines and optimized retroviral vector production and transduction. They found that higher stability and 5 to 15 fold increase of retrovirus vector production at 32°C than 37°C. They also studied the large scale production of retroviral vectors and postulated that the production of retroviral vector is scalable by roller bottles or CellCube bioreactor system (Figure 1). In scaling–up studies the CellCube Bioreactor System provided very promising results. CellCube bioreactor system provided 4-5 times higher vector titers than roller bottle method. Morgan *et al*.[101] (1995) investigated the effects of

time and target cell number on retroviral infection. They reported that efficiency of infection is limited by short half-life of the retrovirus (8-9 hours at 37 °C) and can be maximized by prolonging the exposure of cells to the retrovirus. They also observed that virus infection was dependent on virus concentration and independent of target cell number.

Lee *et al.*[102] (1996) worked on the optimization of certain steps of retrovirus vector production including culture and handling conditions. In an agreement with Kotani *et al*. (1994), they observed 2-fold increase in vector production at 32 °C compared to 37 °C. They also found that a loss of 30% to 50% viral infectivity during a freezing/thawing step. They investigated the effect of polycation use in retroviral vector production and found about 3-fold increase in efficiency of infection when using DEAE-dextran instead of polyberene as a polycation agent during the infection. In another study, the stability and the production efficiency of retroviral vectors at various temperatures, packaging cell line, and culture volumes were investigated. In this study, Kaptein *et al.*[103] (1997) observed about a 4-fold increased half life and 5 to 10 fold increased titers at 32 °C compared to 37 °C.

Some efforts have also been directed at the replacement of serum media with serum free media[102-106]. In particular, Lee *et al.* (1996) investigated the effect of serum concentration on the production of retroviral vectors and found 50% reduction in vector production due to the replacement of serum media with serum free media. They observed no significant influence of serum concentration (1% to 20 %) on vector production.

Relatively little process optimization and scale-up studies have been accomplished for herpes vectors. Several methods have been investigated for the production of HSV vaccines including the use of microcarriers and glass beads in fixed bed bioreactor systems [107,108]. Griffiths *et al.*[107] (1982) studied the use of microcarrier and glass sphere culture techniques for the production of Herpes Simplex Virus. They observed similar cell growth and production efficiency in both systems and postulated that the use of glass bead columns rather than microcarrier systems is more efficient in the production of herpes virus. In another study, Thornton *et al.*[108] (1985) used a glass bead culture system for the production of Herpes Simplex Virus. Their system provided 12000-cm^2 growth surface for virus production. The efficiency of production was similar to that of roller bottle cultures. In addition, this system suffered from complex set-up procedure and an inability to recover intracellular virus.

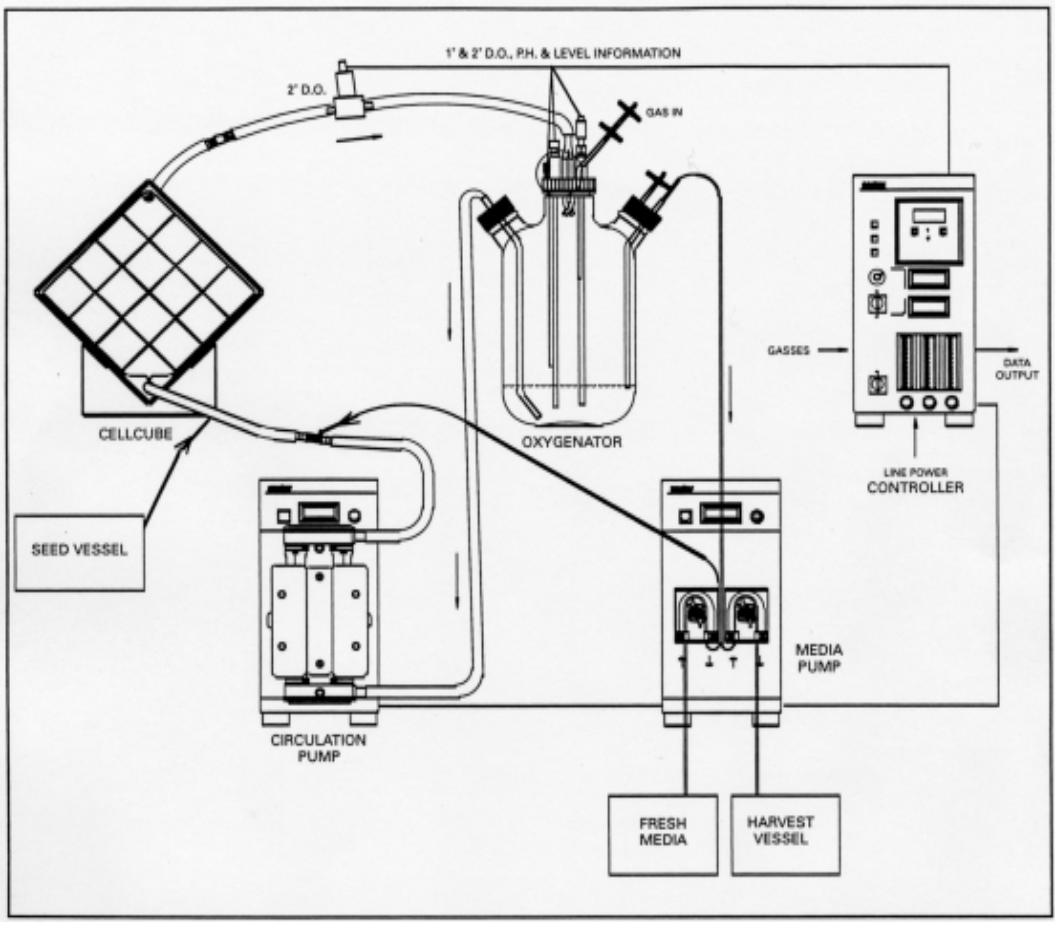


Figure 1 CellCube Bioreactor System

2.0 MATERIAL AND METHODS

2.1 HSV-1 Vectors

The replication-defective HSV-1 vectors used in this study were kindly provided by Dr. J. Glorioso, University of Pittsburgh, Department of Molecular Genetics and Biochemistry. The D.2 (Figure 2) construct is a double-mutant virus that contains the deletion of only two IE genes (ICP4 and ICP27)[51,109]. DOZ.1 (Figure 2) is an ICP4/ICP27 IE gene deletion mutant that contains an ICP0 IEp-lacZ (Immediate Early promoter driving lacZ) expression cassette inserted into the UL41 virion host shut-off (vhs) gene[109]. DOZ.2 (Figure 2) is identical to DOZ.1 except that the normal early gene promoter for thymidine kinase (UL23) has been altered by the substitution of the ICP4 IE gene promoter which also affects the downstream reading frame encoding UL24. Mutation of UL24 results in the virus developing a syncytial plaque (syn^-) phenotype. The syncytial plaques are readily distinguishable from the wild-type non-syn plaque phenotype by light microscopy. This mutation generally results in the virus being cell-associated with a reduction in the number of particles released into the cell culture supernatant. TOZ.1 (Figure 2), a triple IE gene deletion mutant virus with the deletion of three IE genes encoding proteins ICP4, ICP27, ICP22, contains the *lac*Z gene inserted under control of the ICP0 promoter[51,110] in the UL41 locus. QOZHG (Figure 2) lacks two essential viral genes(ICP4, ICP27) and contains a HCMV IEp-GFP reporter gene cassette present in the ICP27 locus. The ICP22 and ICP47 IE gene promoters have been mutated so they are no longer recognized and activated by VP16, and hence are

expressed as early, or late genes. The virion host shutoff (vhs) gene was disrupted by the insertion of an HSV-1 ICP0 IE promoter-lacZ expression cassette into the U_L41 locus.

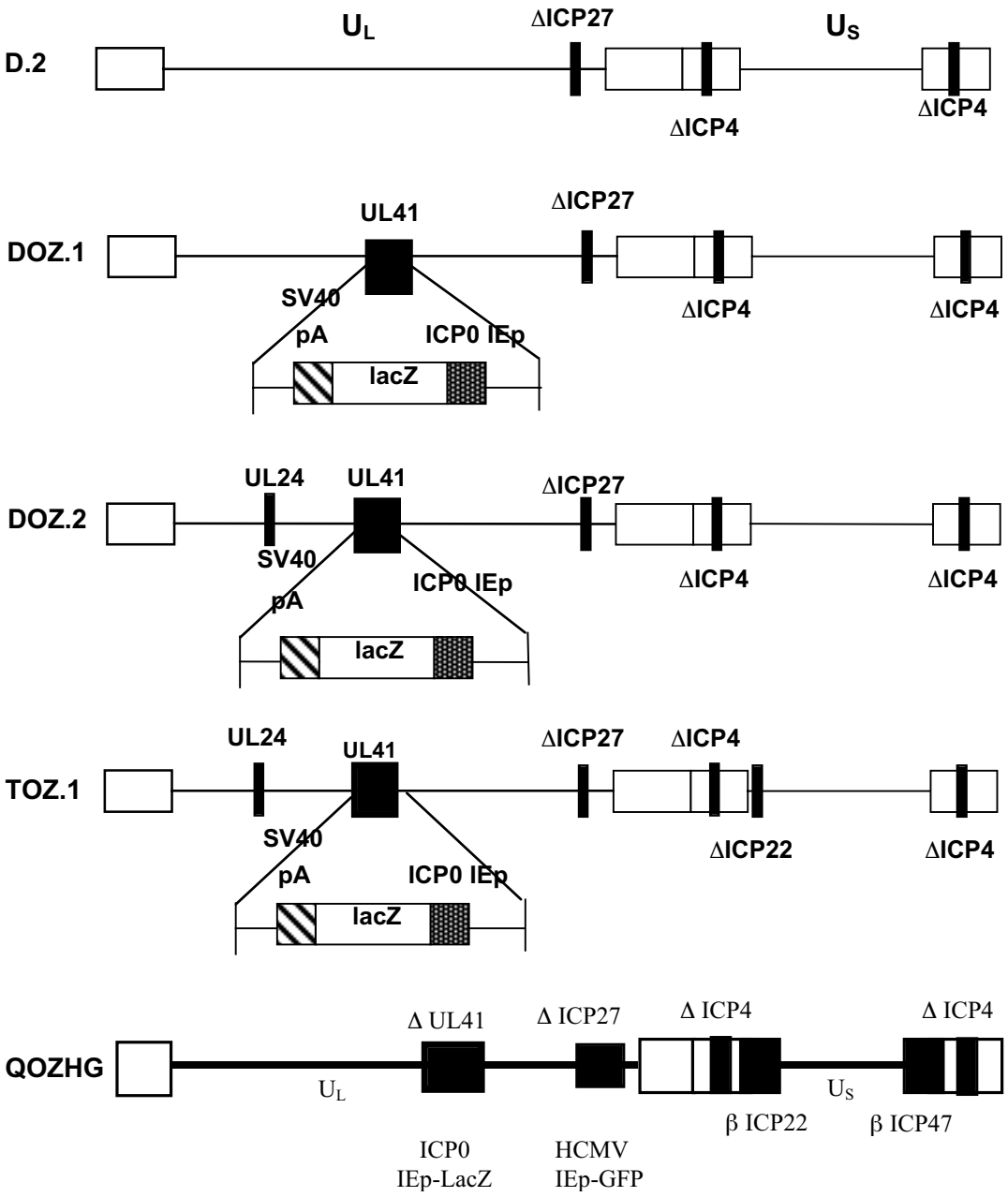


Figure 2 Schematic Diagram of HSV-1 Mutant Virus Genomes

2.2 Complementary Vero Cell Line

The Vero cell line was isolated from the kidney of an African Green Monkey in 1969 and has been used for production of human polio vaccine, viral studies and plaque assays since then[111,112,113]. The 7b complementing cell line[51] derived from Vero cells, provides the essential ICP4 and ICP27 IE gene functions *in trans* to support the in vitro propagation of replication defective vectors lacking ICP4 and ICP27.

2.3 Cell Culture

7b cells were grown and maintained in Minimum Essential Medium (MEM:Life Technologies Inc., Gaithersburg, MD) supplemented with 10% Fetal Bovine Serum, 2 mM L-glutamine, and 100 U/mL Penicillin/Streptomycin (All media supplements: Life Technologies Inc.). Cells were sub-cultured in 150-cm^2 vented cap polystyrene tissue culture flasks (Falcon/Becton Dickinson, San Diego, CA) and incubated at 37°C in a humidified 5% CO_2 incubator. Upon confluency ($1x10^5$ cells/cm^2), cells were harvested with trypsin (Life Technologies Inc.) and sub-cultured into new flasks at an initial cell density of 1-$5x10^4$cells/cm^2.

2.3.1 Media for pH Experiments**.** The pH levels of Minimum Essential Medium without bicarbonate buffer (Life Technologies Inc.) containing 10% Fetal Bovine Serum, 2mM L-glutamine, and 100U/mL Penicillin/Streptomycin were adjusted to 6.8, 7.4, and 8.0 by the addition of appropriate amounts of either 0.1M HCl or 0.1 M NaOH solutions and then also supplemented with 0.025 M Hepes buffer.

2.4 Virus Infection

All infections, unless otherwise specified, were conducted at 80% confluency. The titer of various viral stocks was measured by standard plaque assays using the 7b cell line prior to each experiment. Cells were diluted to an initial cell density of $1x10^6$ cells/mL and infected with varying virus multiplicities of infection (MOI). Following a 1 hour adsorption period at 37°C, infected cells were placed into 6-well plates (Falcon/Becton Dickinson) and the plates were incubated at 37°C .

2.5 Viral Plaque Assay

The quantification of infectious particles was accomplished by standard viral plaque assay. Samples frozen at –70 °C were thawed quickly in 36 °C water bath. The quantification of infectious particles was accomplished by standard viral plaque assay. Serial virus dilutions were added to $1x10^6$ cells in 1.7 mL safe-lock eppendorf tubes and rocked on a Nutator rocker (Becton Dickinson) for 1 hour at 37°C. Following a 1 hour adsorption period, infected cells were plated in 6-well plates. After 12 hours, the innoculum was replaced with media containing 0.05% methyl cellulose (Aldrich, Milwaukee,WI) and incubated for an additional 3 days. The plates were then stained with 1% crystal violet (Sigma, St. Louis, MO) in 50% MeOH/50% water and the number of plaques counted. Titers were calculated as plaque forming units (pfu) per ml virus suspension. All viral plaque assays were minimally performed in duplicate

2.6 Infection Parameters

Infection parameters investigated in this work are the multiplicity of infection (MOI), the confluency of cells at the time of infection, pH and the temperature of infection. The MOI, the infection concentration expressed as plaque or colony forming units per ml (PFU/ml) has a potential to influence the vector yield, and the defective particle formation. The confluency is the degree of coverage of a growth surface by cells. Confluency may affect cell physiology and virus induced cell fusion. The temperature and pH are critical infection parameters due to their strong influence on host cell and virus metabolism.

2.7 Infection Procedure

All infections, unless otherwise specified, were conducted at 80% confluency. The titer of various viral stocks was measured by standard plaque assays using the 7b cell line prior to each experiment. Cells were diluted to an initial cell density of $1x10^6$ cells/mL and infected with varying virus multiplicities of infection (MOI). Following a 1 hour adsorption period at 37°C, infected cells were placed into 6-well plates (Falcon/Becton Dickinson) and the plates were incubated at 37°C or 33°C.

2.8 Ammonia Assay

Ammonia concentrations were determined by an enzymatic assay at 340 nm. (Sigma Diagnostic, Procedure No. 171-UV). The basis of ammonia assay is the

reductive amination of 2-oxoglurate by the enzyme glutamate dehydrogenase. During this reaction nicotinamide adenine dinucleotide hydrophophate(NADPH) is reduced to nicotinamide adenine dinucleotide phosphate(NADP). The decrease in absorbency due to this oxidation reaction is proportional to ammonia concentration.

2.9 Glucose Assay

The glucose concentrations were measured by an enzymatic assay kit (Sigma Diagnostic, Procedure No. 16-UV). The principle of glucose assay kit depends on the two coupled reactions: (1) Glucose is converted to glucose-6-phosphate by the expense of adenosine triphosphate (ATP) (2) Glucose-6-phosphate is oxidized to 6-phosphogluconate in the presence of nicotiamide adenine dehydrogenase. The amount of NAD reduction to NADH is directly proportional to glucose concentration and can be measured by spectrophotometer at a wavelength of 340 nm. An increase in absorbency is directly proportional to an increase in glucose concentration.

2.10 Lactate Assay

Another enzymatic diagnostic kit (Sigma Diagnostics, Procedure No. 735) was used to measure the lactate concentration. This enzymatic assay kit employs lactic oxidase to catalyze conversion of lactic acid to pyruvate and hydrogen peroxide (H_2O_2). Another enzyme, proxidase catalyzes the oxidative condensation of chromogen precursor to give color to the sample solution in the presence of an H_2O_2 formed. The change in absorbency at 540 nm. is directly proportional to lactate concentration in the sample.

2.11 PicoGreen Assay

PicoGreen dsDNA quantitation kit (Molecular Probes Inc., Eugene, OR) was used to estimate the purity and amount of HSV particles in stocks. The labeled concentration of λ DNA stock provided in PicoGreen assay kit was reconfirmed by UV and PAGE gel measurements. All assay buffers, reagents and λ DNA standard curves were prepared using the conditions specified by the manufacturer except that 20 μL of sample was mixed with 180 μL of working PicoGreen solution and spectrofluoremeter (Perkin-Elmer) readings of 96-well microplates (Becton Dickinson, NJ) were performed at excitation and emission values of 485 and 540 nm, respectively. In order to measure the viral DNA concentrations; viral samples were lysed with 0.05 SDS. Basically, 25 μL of viral sample was mixed with, 25 μL of 0.1% SDS in TE buffer and incubated 15 minutes at room temperature. PicoGreen signals of each viral sample were fitted to linear equation obtained by DNA standards to calculate corresponding DNA concentrations for viral samples. To avoid the errors coming from the presence of SDS in viral sample preparations, equal amount of SDS concentrations were added to each DNA standard. Finally, the viral DNA values determined from calibration curve were converted to viral particle concentration.

2.12 Real-time Quantitative PCR

Viral preparations were quantitated for two separate viral genes present in the replication-defective vectors including the glycoprotein D (gD) and the immediate-early

gene (ICP27). Contamination of viral preparations by Vero packaging cell genomic DNA was quantitated using the human apolipoprotein B (H-ApoB) sequences. Except H-ApoB, all assays were conducted in 50 μL PCR volumes containing 10^7-10^8 PFU viral preparations (2 μL), 200 nM of each primer, 200 nM Probe, and 25 μL TaqMan 2X Universal Master Mix (PE Applied Biosystem, Foster City, CA) including 8% glycerol, 1X TaqMan buffer A, 5 mM $MgCl_2$, 400 μM dUTP, 200 μM dATP, dCTP, dGTP (each), AmpliTaq Gold (0.025 U/μL), and AmpErase UNG (0.01 U/μL). Assays for H-ApoB were conducted under similar conditions using primer concentrations of 40 nM each that allows simultaneous detection of H-ApoB in the same reaction as any of the other gene sets (multiplex). Primer sequences for gD (sense- CCC CGC TGG AAC TAC TAT GAC A, and antisense- GCA TCA GGA ACC CCA GGT T), ICP27 (sense- GGG CCT GAT CGA AAT CCT AGA, and anitsense- GCC GTC AAC TCG CAG ACA), and H-ApoB (sense- TGA AGG TGG AGG ACA TTC CTC TA, and antisense- CTG GAA TTG CGA TTT CTG GTA A) were designed using the Primer Express program (PE Applied Biosystem). The TaqMan probes for detection of gD (TTC AGC GCC GTC AGC GAG GA), and ICP27 (CGC ACC GCC AGG AGT GTT CGA G) were labeled with fluorescent reporter dye 6-FAM at the 5' end and the quencher dye TAMRA at the 3' end (PE Applied Biosystem). The H-ApoB probe (CGA GAA TCA CCC TGC CAG ACT TCC GT) differed by the use of the VIC 5' reporter dye (PE Applied Biosystem). All PCR reactions were set up in a MicroAmp Optical 96-well Reaction Plate (PE Applied Biosystem). Amplification conditions were 2 min at 50°C and 10 min at 95°C for the first cycle, followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min. The

TaqMan probes were cleaved during the amplification of target sequence generating fluorescent emission specific for FAM-labeled or VIC-labeled probes. All samples and standards were run at least in duplicate. Standards curves for viral genes gD, ICP27, were generated using 10-fold serial dilutions of plasmids known to contain the respective target sequences. Comparison of these plasmid standard curves to electron microscopicly quantitated viral particles dilution series confirmed accuracy. A standard curve for the H-ApoB assay was generated using a 10 fold dilution series (10^5–1 genome copies) of Vero cell genomic DNA determined by UV absorbance. Additionally, the target sequence of the African Green Monkey ApoB gene was confirmed by PCR cloning and sequencing. The emission data was collected in real-time from an ABI PRIME 7700 Sequence Detector and transferred to a Macintosh 7100 for analysis using Sequence Detector V1.6 (PE Applied Biosystem).

2.13 Sonication

Release of cell–associated virus was accomplished by using an ultrasonic cell disrupter (The Virsonic 475 sonicator; dissipating 475 watts at 20kHz). This sonicator contains a 250-ml cup-horn water bath and a regulated power supply. Infected Vero cell monolayers were scraped by the use of cell scrapers(policeman). Samples were then centrifuged at low speed (1400 rpm) for 7 min at 4 °C. Pellets were resuspended in 1 ml of complete media containing no serum and transfered into 7 ml sonicator tubes (Falcon). The sonicator tubes were then placed in the water bath and sonicated for 30 seconds at the power of 47.5 watts. This procedure was repeated three times.

2.14 CellCube Bioreactor System

The CellCube (Corning, Acton, MA) is an integrated modular bioreactor system for the mass culture, growth, and process control of anchorage dependent cells. The CellCube system (see Figure 1) is composed of five parts: a system controller, an oxygenator, a circulation pump, a media pump and a CellCube culture module. The CellCube module is an integrally encapsulated, sterile single-use device providing a large, stable surface area for the immobilization of anchorage dependent cells containing a series of parallel, tissue-culture treated styrene plates with a laminar flow spaces between adjacent plates. The area of one CellCube (21,250 cm^2) is equivalent to 25 standard roller bottles (850-cm^2) or 150 standard tissue culture flasks (150-cm^2).

The operational procedure for CellCube experiments can be summarized as follows: 7b cells grown in roller bottles are trypsinized and suspended in 1.5 L of culture media. The first side of the CellCube module was seeded with $2x10^8$ cells overnight at 37^oC. The other side of the CellCube module was seeded with the same amount of cells the following day. After the seeding period oxygenator and connection lines are filled with 1.0 L of culture media and the media in the system was re-circulated to start the growth period. Following the growth period, cells were infected with appropriate number of virus particles by direct injection of virus into the system. Media perfusion throughout the process was provided by a media pump which supplies continuous introduction of fresh medium and removal of spent medium at the same time (Figure 1). Throughout the process different volumes of media were perfused

3.0 OBJECTIVES

Since herpesvirus based gene therapy vectors have been widely used in research applications and have recently been employed in human clinical trials it now becomes critical to produce large volumes of clinical-grade herpes vectors. In order to scale-up the production of HSV vectors, first important production parameters such as culture conditions, the growth medium and infection parameters must be investigated. Then, new large-scale production configurations together with new quality control and assurance strategies must be developed to meet the FDA regulations for viral vector production.

Objectives of this research can be summarized as :

1. characterizing the effects of production parameters on viral yield
2. scaling-up the production and purification of HSV vectors
3. developing rapid, accurate and precise assay systems to monitor and control the quantity and quality of HSV vectors throughout the manufacturing process
4. implementation of Good Manufacturing Pratices (GMP) rules for HSV vector production

4.0 EFFECT OF GENETIC BACKGROUND AND CULTURE CONDITIONS ON PRODUCTION OF HERPESVIRUS - BASED GENE THERAPY VECTORS

Herpes simplex virus type-1 (HSV-1) represents a unique vehicle for the introduction of foreign DNA to cells of a variety of tissues. The nature of the vector, the cell line used for propagation of the vector, and the culture conditions will significantly impact vector yield. An ideal vector should be deficient in genes essential for replication as well as those that contribute to its cytotoxicity. Advances in the engineering of replication-defective HSV-1 vectors to reduce vector-associated cytotoxicity and attain sustained expression of target genes make HSV-1 an attractive gene delivery vehicle. However, the yield of the less cytotoxic vectors produced in standard tissue culture systems is at least three order of magnitudes lower than that achieved with wild-type virus. The low overall yield and the complexity involved in the preparation of HSV vectors at high concentrations represent major obstacles in use of replication-defective HSV-derived vectors in gene therapy applications. In this work, the dependence of the vector yield on the genetic background of the virus is examined. In addition, we investigated the production of the least toxic, lowest yield vector in a CellCube bioreactor system. After initial optimization of the operational parameters of the cell cube system, we were able to attain virus yields similar to or better than those values attained using the tissue culture flask system for vector production with significant savings with respect to time, labor, and cost.

4.1 Introduction

Recombinant herpes simplex virus type 1 (HSV-1) vectors have recently emerged as potentially important vectors for gene delivery and expression[51,114-116]. Advances in the engineering of HSV-1 based vectors along with its intrinsic advantages make HSV-1 a widely applicable vector for gene transfer applications. These advantages include: (i) its broad host range demonstrated by its ability to infect a wide range of cell types and tissues including postmitotic neurons; (ii) its large genome (over 150 kb) allows incorporation of foreign DNA for gene transfer strategies involving either large[117] or multiple[110] transgenes; and (iii) its ability to establish life-long latency in neurons, during which lytic HSV-1 gene expression is silenced except for the expression of a group of co-linear RNAs designated as the latency-associated transcripts or LATs[118-120]. The latency active promoter system of HSV-1 can be employed for sustained expression of therapeutic genes[121,122]. Following infection, the HSV genome is circularized and remains episomal[123] eliminating the risk of inactivating host genes through integration of viral DNA into the host genome.

Viral gene expression is tightly regulated during productive HSV-1 infection. HSV-1 genes can be categorized into three groups: immediate early (IE or α), early (E or β) and late (L or γ) according to the time at which they are expressed[124]. The IE genes activate the expression of early genes[55,125,126] which encode functions involved in viral DNA replication. After the initiation of viral DNA replication, late genes are

expressed[127,128]. Most late genes encode HSV structural proteins or proteins that facilitate virion assembly and maturation.

The expression of the five immediate early genes, ICP0, ICP4, ICP22, ICP27, and ICP47 does not require any *de novo* viral gene synthesis. All IE proteins except for ICP47 have regulatory functions coordinating the expression of the early and late genes. ICP4 and ICP27 are essential for viral replication[55,126] and most HSV-1 vectors have these essential genes deleted from virus genome thereby rendering these viruses replication-defective. The vectors lacking ICP4 and ICP27 must be produced in complementary cell lines providing these essential proteins in trans for viral replication.

After viral infection of cells in culture with replication-defective virus, a wide range of cytopathegenic effects have been observed including cytoplasmic blebbing, chromasomal aberration, and host cell DNA fragmentation[94]. Since UV-irradiated virus is basically apathogenic[129,130] the cytotoxicity observed with replication defective virus can be attributed to de novo HSV-1 gene expression, especially to the IE regulatory proteins. The IE genes play a prominent role in cytotoxicity because cells infected with single (ICP4) or double (ICP4 and ICP27) IE gene deletion mutants have decreased toxicity yet still demonstrate cytopathogenic effects[131-134]. Deletion of IE genes[131,135,136] dramatically reduced the vector-associated toxicity and resulted in enhanced transgene expression levels and duration. It is now well established that HSV-1 IE genes ICP0, ICP4, ICP22 and ICP27 are responsible for the cytotoxicity observed in virus infected cells.

In this chapter, the yield of several recombinant HSV-1 vectors with deletion/inactivation of the various viral genes is presented. We examined the growth and propagation of a replication-defective vector designated TOZ.1 deleted for three IE gene products[110]. The yield of vector TOZ.1 propagated in complementary cells is very low achieving about 1 virus particle/cell, approximately 0.1% of that obtained using wild-type virus. The low yield of these less cytotoxic vectors is a major obstacle in evaluating the potential use of the HSV-based vectors for gene therapy applications. To overcome this fundamental problem, production of HSV-based vectors should be scaled-up from inefficient conventional production systems (i.e. tissue culture flasks and roller bottles) to high-capacity bioreactor systems. A CellCube bioreactor system was employed to evaluate the potential for large-scale production of the prototypic TOZ.1 vector. After initial optimization of the operational parameters of the cell cube system, we successfully scaled-up the production of the TOZ.1 vector.

4.2 Results and Discussions

4.2.1 Factors Affecting Vector Yield

Initial experiments were designed to optimize the vector MOI required to achieve the greatest vector yield. First we examined the effect of MOI on propagation of the D.2 vector (Figure 3A). Experiments were performed over a range of MOI from 0.002 to 2. The maximum yield of virus released into the supernatant was observed at about 60 hours post-infection (hpi) for MOI=0.02. Several other vectors confirmed that the highest virus

yield was attained using MOIs in the range of 0.01 to 0.05 (Figure 3B). At MOI=2 essentially all the cells are infected at the time of introduction of the vector, hence the number of infected cells able to produce infectious virus will be about the same as the initial cell number. Moreover, the use of higher MOIs to infect cells for virus production results in an increase in the number of defective non-infectious particles, and thus higher MOIs must be avoided. Lower vector yield observed at MOI=0.002 may be the result of nutrient depletion and/or by-product accumulation prior to the time when most cells are infected. Thus, an intermediate MOI value may allow for sufficient cell growth and virus production yielding an expansion. All the experiments described next were performed using the range of MOI determined above.

Next, we compared the yields obtained using vector constructs that differ according to the number of viral genes deleted from the vector backbone (Table 2). The vectors examined includes D.2 (syn^+), DOZ.1 (syn^+) DOZ.2 (syn^-) and TOZ.1 (syn^-) (Figure 2). The yield values given in Table 2 represent the maximum extracellular virus obtained during the course of infection (MOI=0.02 to 0.05). The pattern of virus production during the course of infection is displayed in Figure 4. Several observations can be noted regarding the effect of specific gene deletions on viral yield.

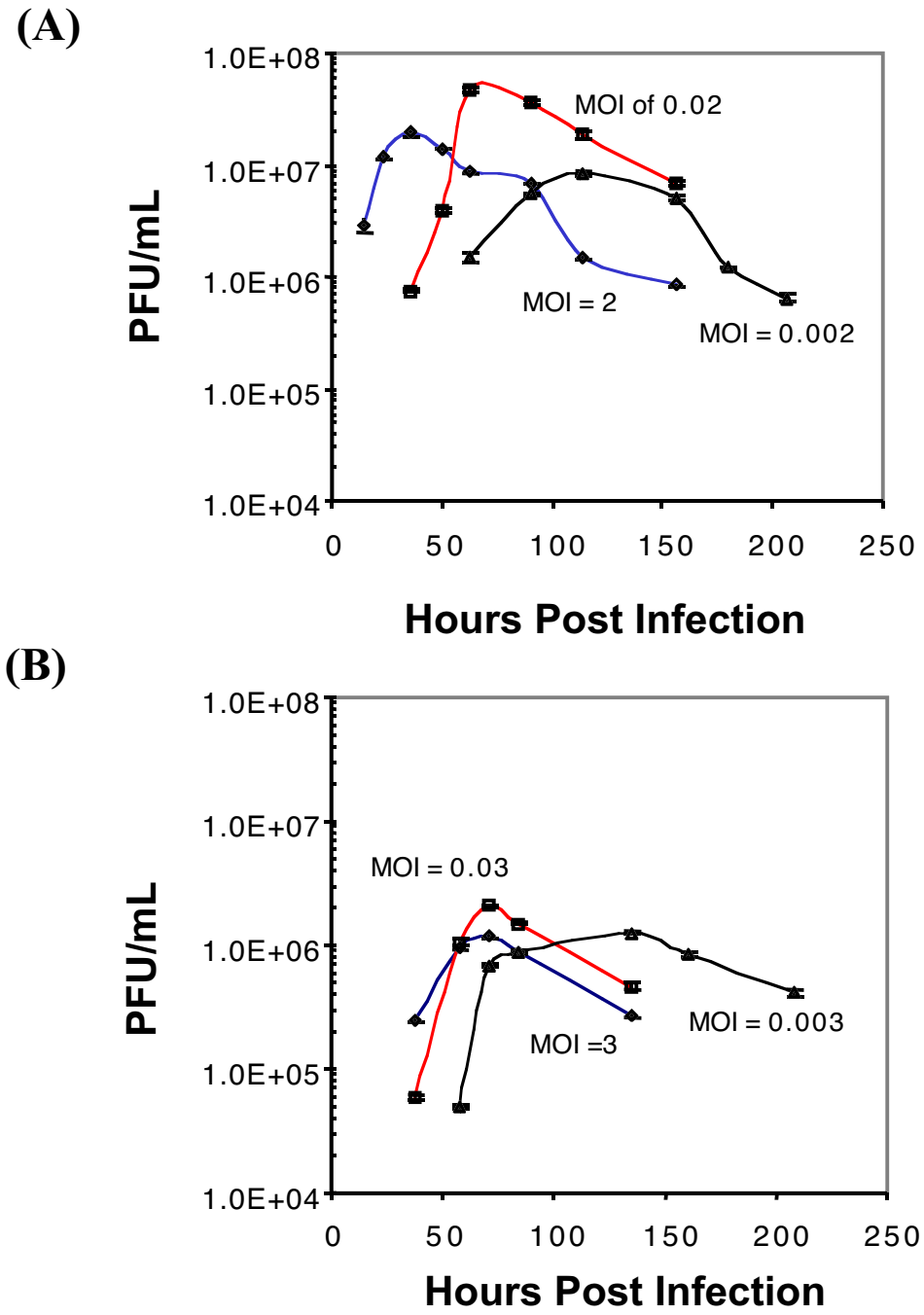


Figure 3 Effect of MOI Viral Vector Yield

First, comparison of the DOZ.1 to D.2 vectors (Figure 4A) showed that deletion of UL41 (virion host shut-off gene) resulted in a 5-fold reduction in vector yield. Since, UL41 has been shown to contribute to the reduction in the translation of specific host messages[137-140] these host gene products may be needed to obtain optimal vector yields in the background of an ICP4/ICP27 double gene mutant virus. Second, comparison of DOZ.1 to DOZ.2 (Figure 4B) revealed that the disruption of UL24 (syn^- phenotype) had no pronounced effect on vector yield. This result suggests that the syn^- phenotype does not reduce the yield of virus released from the infected cell in a double IE gene mutant virus, an unexpected finding in contrast to that seen comparing a triple IE gene mutant virus with other single and double IE mutants that are wild-type for UL24[131].

Third, comparison of DOZ.2 to TOZ.1 (Figure 4C), demonstrated that deletion of ICP22 substantially reduced the vector yield (5-10 fold) as well as increased the time required for maximal yield. This result suggests that finding ways to complement the function of ICP22 will greatly enhance the yield of the IE triple gene deletion vectors. It has been proven to be difficult to produce cell lines that complement ICP22 in addition to ICP4 and ICP27. To this end, quadruple IE gene vectors have been constructed[135,136]. In these vectors, in addition to deletions of both ICP4 and ICP27, the promoters controlling the expression of the ICP22 and ICP47 IE genes have been altered so that these genes are only expressed when the virus is propagated in the 7b complementing cell line. Finally, the comparison of D.2 to TOZ.1 (Figure 4D) showed that the combined effect of the ICP22, UL24, and UL41 mutations dramatically reduces the vector yield (~50 fold). Although the combination of these mutations led to dramatically reduced vector-

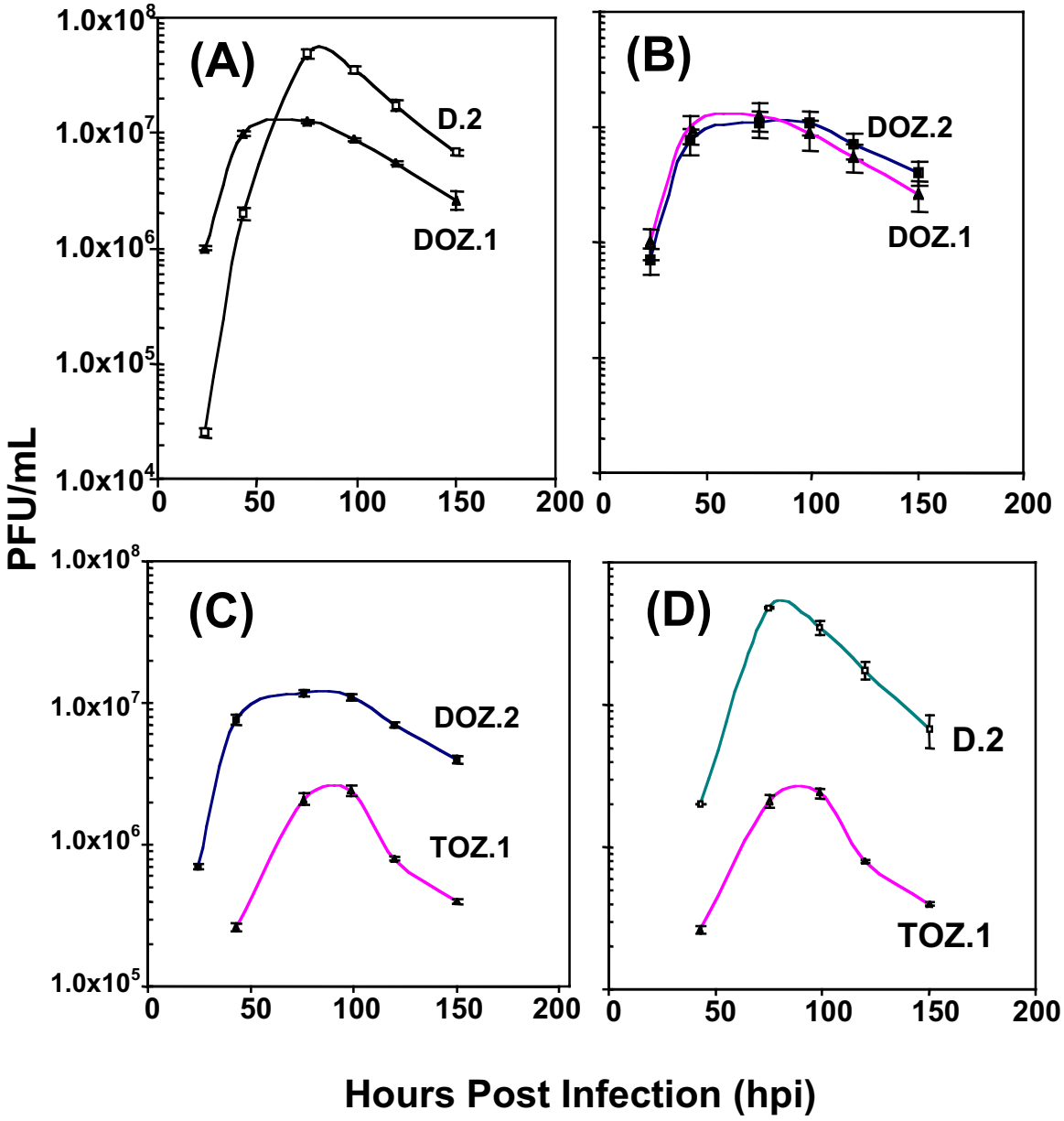


Figure 4 Effect of Vector Backbone on Vector Yield

associated toxicity[133], they have considerably compromised vector yield. Thus, their functions may require complementation to achieve optimal vector titers required for human clinical trials in gene therapy applications employing HSV vectors

4.2.2 The CellCube Bioreactor Experiments

Table 2 highlights the finding that the final yield achieved using TOZ.1 was very low in cultures of complementing cells. This vector was selected for our scale-up studies using the CellCube Bioreactor since this vector displayed the lowest toxicity of all the vectors employed[131]. The CellCube bioreactor system (Figure 1) provides homogenous distribution of oxygen and nutrients over the cell's surface with low diffusional gradients. The CellCube bioreactor system has been successfully used for production of retroviral vectors[100].

While the CellCube system may be operated in a continuous perfusion mode for the production of retroviral vector which is readily released into the culture fluid from the producer cell line that continuously produces the vector, continuous operation is not appropriate for large-scale HSV vector production due to the lytic nature of the HSV infection process in the 7b complementing cell line. Thus, a fed-batch operation was considered. The operational parameters of this system included seeding density, length of growth period, duration and rate of medium perfusion during the growth period, and multiplicity of infection. We have conducted several experimental runs to investigate these parameters (Table 3). The first experiment was conducted with an initial seeding of 20% confluency, followed by a growth period of 49 hours as recommended by the

manufacturer. During the first 30 hours of the growth period, the medium provided during seeding was circulated without infusion of fresh medium. After this period 2.3 L of medium was perfused for the remaining 19 hours of the growth period. After a total of 49 hours of growth, cells were infected with virus at an MOI of 0.05. At 26 hours post infection, medium perfusion was re-initiated. About 12.2 L of medium were perfused during the next 100 hours of operation. The total PFU obtained in this initial run was $8.75x10^8$ corresponding to $4.12x10^4$ PFU/cm^2. This yield was about 25% of that obtained in tissue culture flasks. This lower yield might be due to insufficient cell growth prior to infection, thereby altering the actual number of cells at infection and ultimately the MOI.

Because it is not feasible to have on-line information on cell density at the time of infection and even to observe the density of the culture in the CellCube, it was critical to assess the significance of cell density at the time of infection on virus production using tissue culture flasks. To investigate the effect of confluency at the time of infection on virus yield, cells were infected in 6-well plates at different confluency levels. The results for (A) 20%, 50%, 80% and (B) 1-day and 2-day over confluent culture infections are given in Figure 5.

The results of these experiments revealed that the lower confluencies of 20% and 50% resulted in lower vector yield and confluencies of about 80% or higher produced about the same vector yield. Since the longer pre-infection growth period did not lower the final vector yield, the second experiment with the CellCube was performed with a pre-infection growth period of about 5 days. Other parameters essentially remained

unaltered. During this run a vector yield of $1.14x10^5$ PFU/cm^2 was obtained, almost 3-fold higher than was attained with the first run.

Although the amount of growth medium used in the first two runs was approximately 22 L, this amount may be reduced if fresh media perfusion was reduced during infection. Results from experiments performed in T-150 tissue culture flasks suggested that all the cells will be infected at about 50 hours post infection at MOI of 0.05 (Figure 3B). After that point the change in extracellular virus concentration reflects the release of virus. For this last experiment, one day prior to the termination of the growth phase and initiation of infection, 3.7 L (equal to reactor working volume) of medium was perfused to refresh the medium. The culture was then infected with virus at an MOI of 0.05. At the time of the infection medium perfusion was stopped completely, but medium circulation continued for 10 hours to allow virus adsorption onto the cells. Metabolic analysis of infected cultures (Figure 6) indicated a substantial increase in glucose consumption and lactate production, suggesting that further medium perfusion might be useful. Thus, an additional 1 L of medium was perfused during the following 24 hours. The third and final CellCube run produced the highest virus yield per surface area, corresponding to that obtained using tissue culture flasks and roller bottles (Table 4). Total pfu per cm^2 obtained in this run was $1.83x10^5$ compared to values of $1.67x10^5$ and $1.18\ x10^5$ for 150-cm^2 tissue culture flasks and 850-cm^2 roller bottles respectively. In addition we have significantly decreased the media consumption from 22 L to 7.2 L (Table 3) in these initial runs and the amount of virus produced per media volume used in the final CellCube run was higher than those of tissue culture flasks and roller bottles.

This indicates successful scale-up of the production of HSV-1 vectors from tissue culture flask to reactor scale. It should be noted that we achieved vector yields with the CellCube system similar to those observed in tissue culture flask experiments. This is very encouraging due to the fact that in large-scale production systems limitation in mass-transfer, even distribution of nutrients, adequate supply of oxygen and the formation of considerable amount of toxic and inhibitory products ultimately limits the efficiency of production. Even though the CellCube system requires a comparable amount of labor as infections in roller bottles, the CellCube system can be easily scaled-up and used for the development of GMP methods for HSV vector production.

Table 2

Yield Attained Using HSV Vectors of Different Genetic Backgrounds

Virus Name	**Mutated or Deactivated Genes**	**Transgenes**	**Morphology**	**PFU/mL**	**Productivity (particle/cell)**
Wild Type	–	–	–	$1.0x10^9$	1000
D.2	ICP 4, ICP27	–	syn^+	$6x10^7$	50
DOZ	ICP4, ICP27, *UL41*	ICP0-lacZ	syn^+	$1.1x10^7$	10
DOZ	ICP4, ICP27, *UL24, UL41*	ICP0-lacZ	syn^-	$1.2x10^7$	10
TOZ.	ICP4, ICP27, *ICP22, UL24,UL41*	ICP0-lacZ	syn^-	$1.1x10^6$	1

Table 3

Data for CellCube Runs

	Run A	Run B	Run C
Seeding Cell Number	$4x10^8$	$4x10^8$	$4x10^8$
Length of Cell Growth Period (h)	49	120	120
Media Perfused During Cell Growth Period (L)	2.3	6.4	6.2
Media Perfused After Infection (L)	12.19	10.2	1
Total Media Used (L)*	18.3	20.3	7.2
Post-infection Period (h)	126	91	76
Total Virus Collected (PFU)	$8.75x10^8$	$2.4x10^9$	$3.89x10^9$

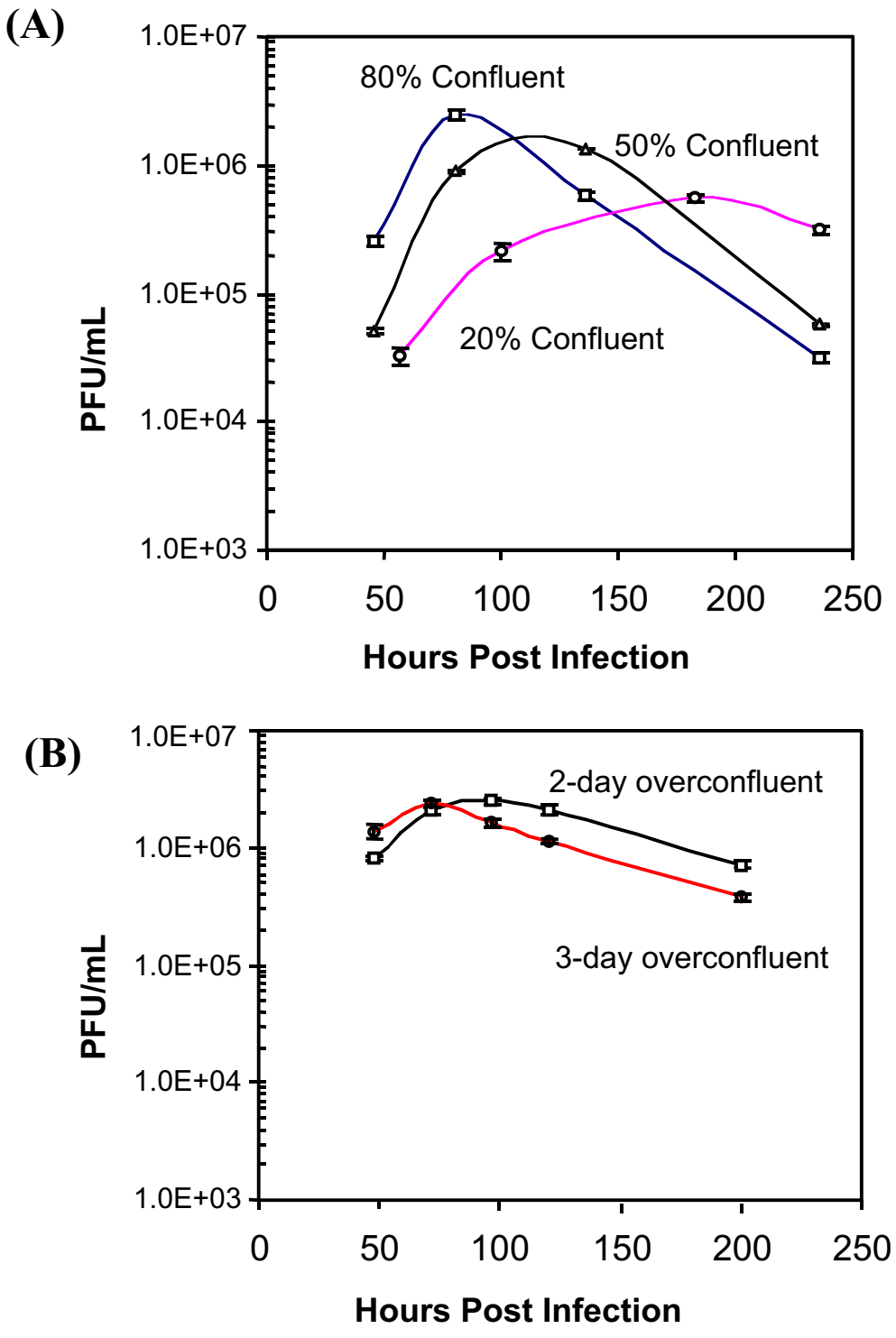


Figure 5 Effect of Confluency on Viral Vector Yield

Table 4

Comparison of Viral Production Methods

	Surface Area (cm^2)	**Yield (PFU/cm^2)**
Tissue Culture Flask	150	$1.67x10^5$
Roller Bottle	850	$1.18x10^5$
CellCube	21,250	$1.83x10^5$

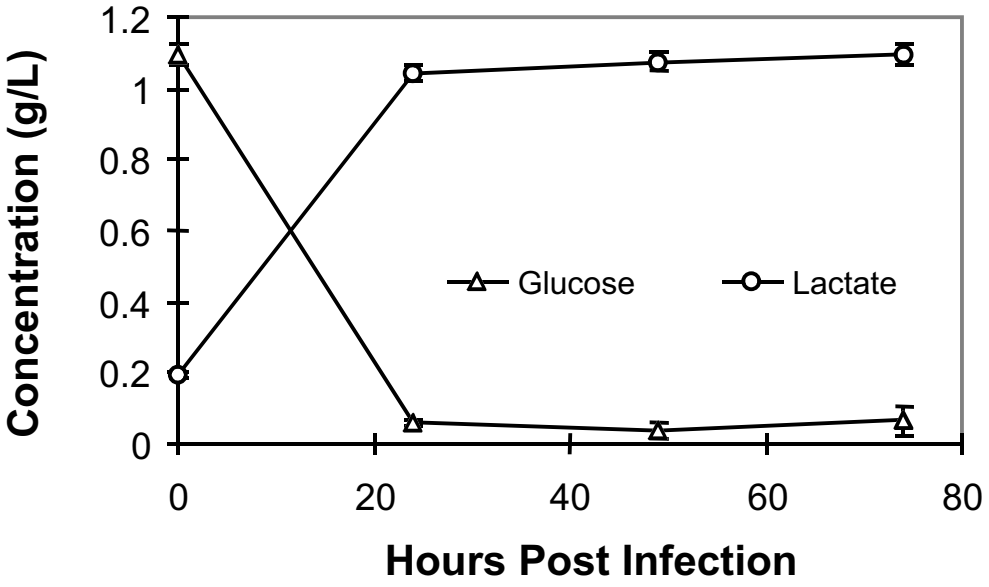


Figure 6 Effect of Virus Infection on Cellular Glucose and Lactate Metabolism

4.3 Conclusions

In this chapter we have demonstrated the correlation between vector backbone and vector yield during production. Our results revealed that as we further crippled the virus by deleting viral genes from the base virus to reduce toxicity, lower overall yields were observed due to the lack of complete complementation. Construction of double IE gene deletion mutant DOZ.1 and triple IE gene deletion mutant TOZ.1 resulted in 100-fold and 1000-fold respective decrease in vector yield compared to wild-type virus. Due to this significant loss in virus yield using the reduced cytotoxicity vectors, scaling-up the production of herpes based vectors has become a requirement for the progress of herpes virus-based gene therapy applications. We have successfully scaled-up the production of HSV-based gene therapy vectors from 20 mL/150-cm^2 tissue culture flasks or 100 mL/850-cm^2 roller bottles to the 4 L/21,250-cm^2 CellCube Bioreactor System with a higher production efficiency per media consumption than possible using tissue culture flasks and roller bottles, the standard systems employed in this field of research.

5.0 EVALUATION OF CULTURE PARAMETERS IN THE PRODUCTION OF HSV-1 VIRAL VECTORS

Herpes simplex virus type-1 (HSV-1) represents an ideal vector for use in many gene therapy applications. HSV-based vectors have recently been employed in human clinical trials to treat malignant brain tumors. The production of HSV-1 vectors must now be scaled-up to meet the demand for large volumes of high-titer clinical-grade vector stocks. However, in order to develop efficient large-scale production and purification methods, factors affecting the efficiency of HSV-1 vector production have to be systematically investigated. In this work, we evaluated the dependence of viral yield on culture pH, glucose and lactate concentration as well as serum content. High level glucose consumption, with the corresponding increase in lactate synthesis, observed within the first 24 hours post infection suggested that replacing culture medium at some point of post-infection might increase overall viral yield. Culture medium was replaced with fresh media at various times post-infection and 2-fold increase in overall viral yield was obtained. Potential influence of culture pH on viral yield and virus stability was also examined. Low pH (6.8) not only enhanced the viral yield but also increased the half-life of virus compare to higher pH values (7.4 and 8.0). Even though serum-free media offers many advantages over serum containing media in virus purification schemes, our results suggest that serum-free media did not support high viral yield. However, our results shown that serum content of 5% provided similar viral yields as that of 10%, suggesting a potential cost reduction and ease in the purification of vector. Overall, our results suggest

that several infection parameters dramatically affect the yield of replication-defective HSV vectors and will have a direct impact on the large-scale production of these vectors using bio-reactor systems to produce clinical-grade vectors.

5.1 Introduction

Recombinant viral vectors are created by the deletion or disruption of one or more genes from the genome of the corresponding virus. *In vitro* propagation of replication-defective vectors, that lack one or more genes that are essential to virus replication, can be achieved in host cells expressing the deleted essential genes of the virus in trans. Viral vectors may also lack one or more non-essential genes, that may contribute to the replication or spread of the virus *in vivo*. The removal of non-essential genes extends the vector's capacity for therapeutic genes and could lead to reduced toxicity of the engineered vector[131,132].

The first generation replication-defective HSV-1 based vectors carried a deletion of the ICP4 immediate early (IE) gene[126]. Both ICP4 and ICP27 are essential for replication of the virus in standard cell lines[139,140]. The other IE genes expressed in ICP4 mutants include ICP0, ICP27, ICP22, ICP47 and ICP6, an IE/E gene that encodes the large subunit of ribonucleotide reductase[141]. Some viral early genes are also expressed, but at greatly reduced levels[126]. Expression of the ICP0, ICP22, and ICP27 IE genes decreases cell survival and hence contribute to the overall cytotoxic properties exhibited by cells transfected with these genes[94]. We[51,110] and others[135,136] have shown that the

subsequent deletion of multiple IE genes dramatically reduces the virus-associated toxicity and extends transgene expression in the second and third generation replication-defective vectors. Therefore, to minimize vector-associated cytotoxicity it is necessary to delete multiple genes from the virus backbone. Unfortunately, there is an inverse relation between the number of genes deleted from a virus and the yield of infectious particles produced in cell culture. Thus, the parameters involved in infection must be carefully optimized to develop effective production schemes for vectors displaying diminished toxicity. The production of high titer viral vector stocks will depend on several factors including the nature of the host cells, the ability of complementing cell lines to express the deleted viral gene products, the vector construct backbone including the nature and the number of deleted genes, as well as the culture conditions, the growth medium and infection parameters.

This chapter evaluates the effects of several culture parameters on the yield of a third generation replication-defective HSV-1 vector (TOZ.1) using a complementing Vero cell line that expresses essential IE gene products in trans. TOZ.1 [51,110] is a triple IE gene deletion mutant virus (Figure 2) with the deletion of three IE genes encoding proteins ICP4, ICP27, and ICP22. This vector exhibits low toxicity due to its lack of expression of a large number of viral genes[131], and its production yield is relatively low compared to most first and second generation vectors deleted for one or two IE genes.

In this part of thesis, the effects of culture pH, glucose concentration, and serum content during the post-infection period on virus yield were investigated. The results

demonstrated that a significant increase in vector yield was attained when the infected cell medium was replaced with fresh medium at some point after infection. The effect was more pronounced at lower multiplicities of infection (MOI) indicating possible inhibition due to accumulation of metabolic by-products. Culture pH also demonstrated a significant effect on vector yield. Not only was the peak vector yield substantially higher at lower pH (pH=6.8), but the rate of decline in overall titer was also significantly lower. While experiments in culture with different serum concentrations showed similar levels of vector yield for a serum content of 5 or 10%, vector yield was lower for serum levels less than 5%. Together, these data suggest that simple infection parameters in toto impact in a significant manner on the overall yield in the production of replication-defective HSV-1 vectors.

5.2 Results

Since herpesvirus based gene therapy vectors have been widely used in research applications and have recently been employed in human clinical trials [142,143] it now becomes critical to produce large volumes of clinical-grade herpes vectors. Culture conditions, the growth medium and infection parameters play an important role in vector production. In this work the effect of several culture parameters on the production yield of a replication-defective HSV vector, TOZ.1 (Figure 2), was investigated.

The concentration of extra-cellular virus and culture glucose concentration during the period of the first several days post-infection were analyzed using TOZ.1 at a multiplicity of infection (MOI) of 0.05 (Figure 7). The results showed that glucose was

almost completely consumed by about 24 hours post-infection (hpi). We hypothesized that higher glucose concentrations in the culture at the time of infection might lead to higher vector production. Therefore, we examined virus yield in the cultures possessing higher starting glucose levels.

The glucose concentration of MEM (1 g/L) was adjusted to 3 g/L and 4.5 g/L by the addition of appropriate amounts of glucose. The maximum levels extra-cellular virus produced in cultures with the initial glucose concentration at time of infection of 1, 3, and 4.5 g/L were $2.1x10^6$ pfu/mL, $9.6x10^5$ pfu/mL, and $8.9x10^5$ pfu/mL, respectively. These results indicate that virus production was lower in cultures with higher starting glucose concentrations. Figures 8A and 8B depict the glucose and lactate concentrations for initial glucose concentrations of 3 and 4.5 g/L, respectively. These results demonstrated that the glucose was not completely utilized and higher amounts of lactate were present in cultures with higher initial glucose concentrations.

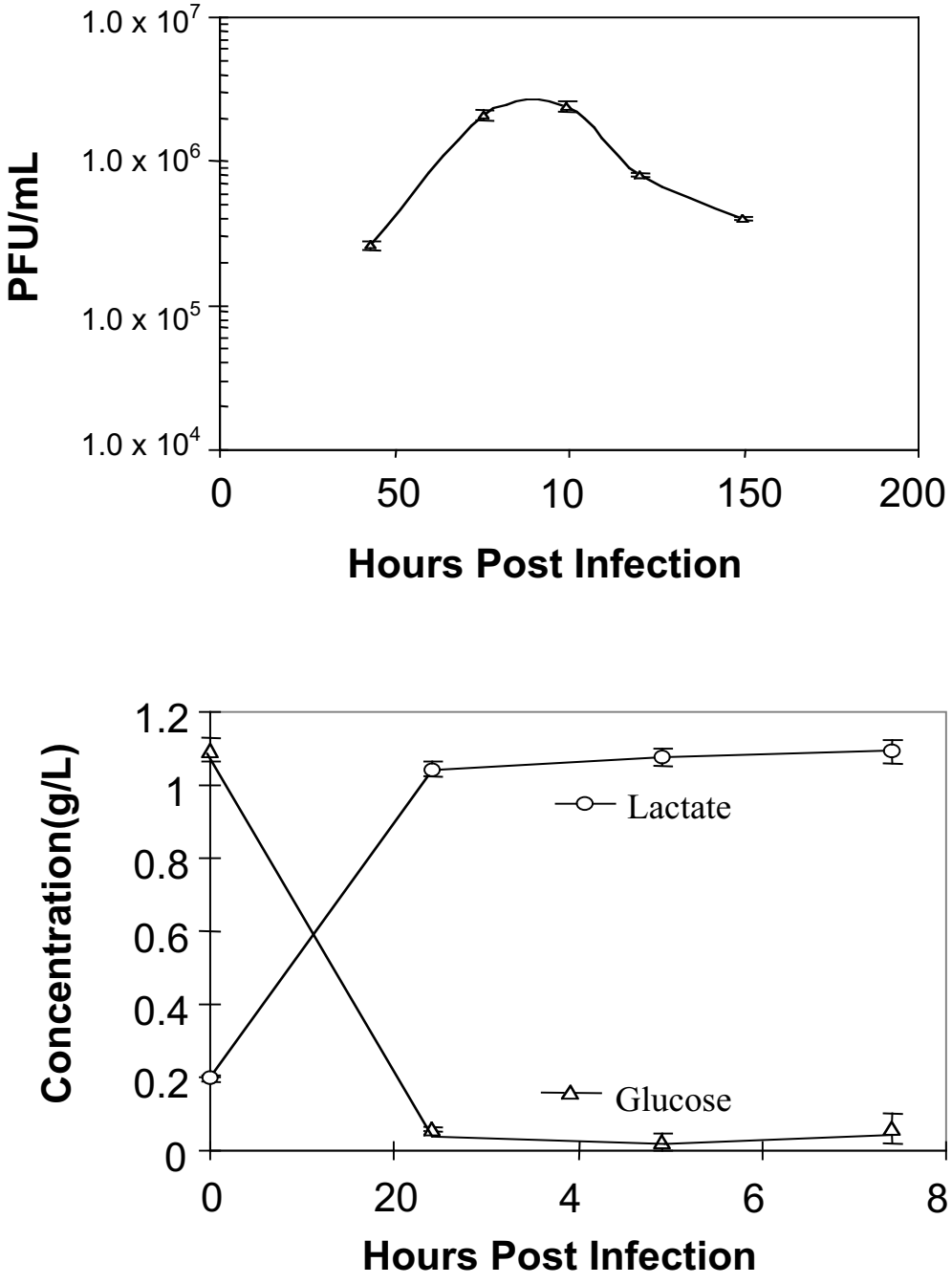


Figure 7 The concentration of Extra-cellular Virus and Culture Glucose Concentration During The Post-infection Period

Since significant lactic acid formation occurred during infection (Figure 8), replacing the growth medium at some point post-infection would increase virus yield. Thus, we initiated experiments to evaluate the effects of media change on overall vector yield. The effect of media replacement for infections employing MOIs of 0.004 and 0.04 was examined (Figure 9). The time of medium replacement 24 hpi (MOI=0.04) and 50 hpi (MOI=0.004) was based on our visual observations demonstrating that at these time points of post-infection the majority of the cells have been infected using a vector such as TOZHG that expresses GFP (Figure 10). A two-fold increase in virus yield was observed due to the media replacement at MOI of 0.004 (Figure 9A), whereas infection at an MOI of 0.04 led to only a 55% increase in virus yield. The greater increase in overall virus yield observed with medium replacement at the MOI of 0.004 compared to 0.04 may reflect a higher utilization of growth medium due to the longer cell growth period required before the complete spread of the virus to all cells at the lower MOI.

To assess whether the media change resulted in an overall increase in the production of viral particles or just increased the rate of virus release from infected cell membranes, the total virus concentration (cell-associated + supernatant) was measured in experiments with and without media change. At various times post-infection, cells were scraped from the culture plates and sonicated to release membrane-bound virus particles and the total amount of virus determined. We observed that the total number of viral particles produced by 48-60 hpi was about 2-fold higher when the medium was replaced at 24 hpi (Figure 9B).

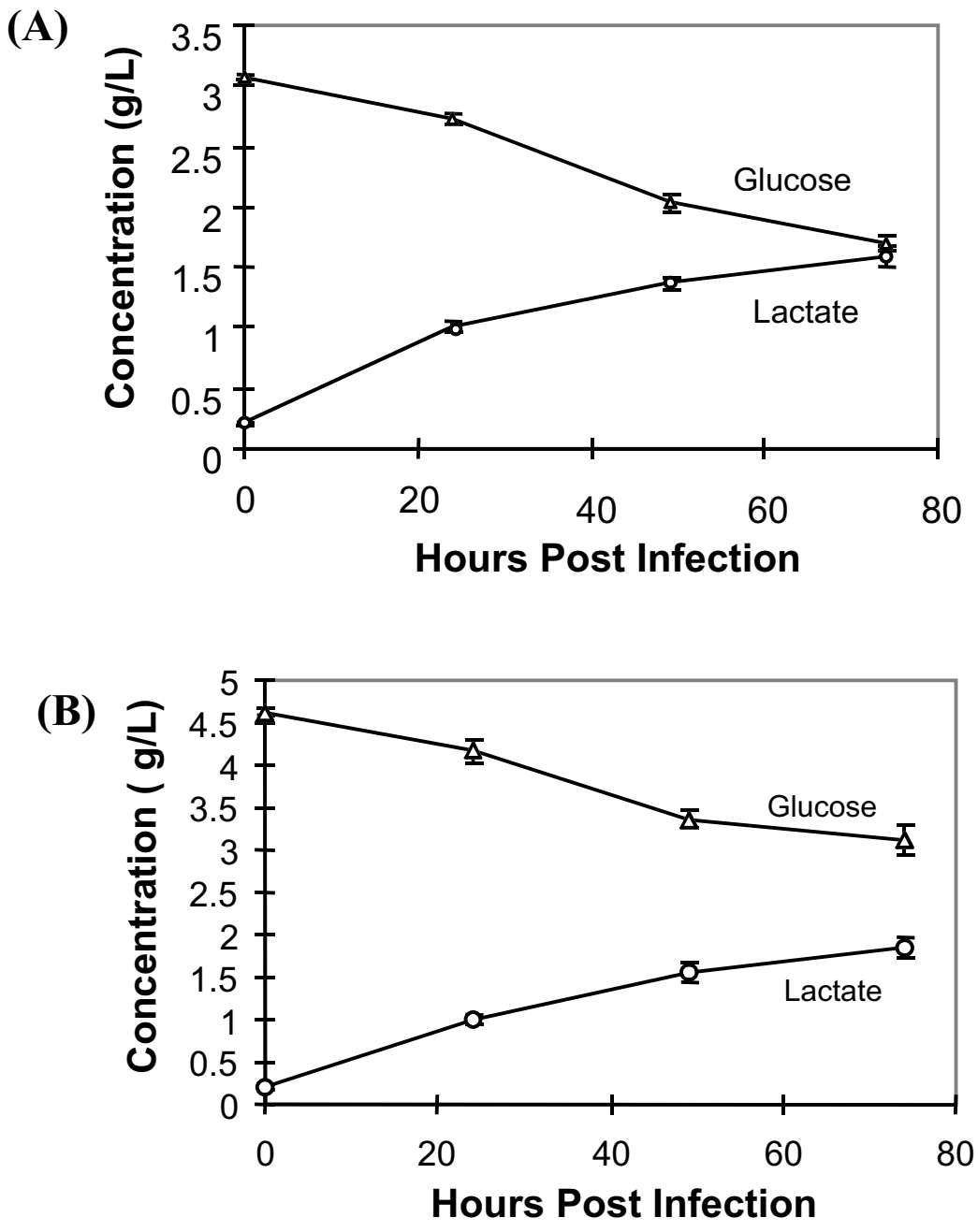


Figure 8 The Effect of Glucose on Vector Production

Thus, it is likely that media change leads to higher production levels rather than a higher rate of release. This result showing that higher levels of virus were observed following media replacement led us to examine the effect of culture pH on virus yield. The optimum pH range for herpesvirus production is strain dependent [144] and it may lie between 6.8 to 7.5. We decided to monitor pH during the post-infection period and assess the buffering capacity of our growth medium that contains sodium bicarbonate buffer. 7b complementing cells at an initial cell density of $1x10^6$/mL were either mock-infected or infected with TOZ.1 vector at (MOI=0.05). The pH values of both infected and uninfected cells were measured daily. We observed that the pH of the culture remained constant within the expected range of 7.0 to 7.6 for both infected and uninfected (control) cultures (Figure 11). Next, cells were diluted to an initial cell density of $1x10^6$/mL and infected with TOZ.1 (MOI=0.05) in media of three different pH values (6.8, 7.4 and 8.0). We found that cultures infected in media at lower pH values (pH=6.8) resulted in higher virus production (Figure 12).

To further assess the possible effect of pH on virus production, glucose consumption and lactate production, virus stability and spread of virus were examined. We observed no significant difference in the patterns of glucose consumption and lactate production in these cultures (Figure 13).

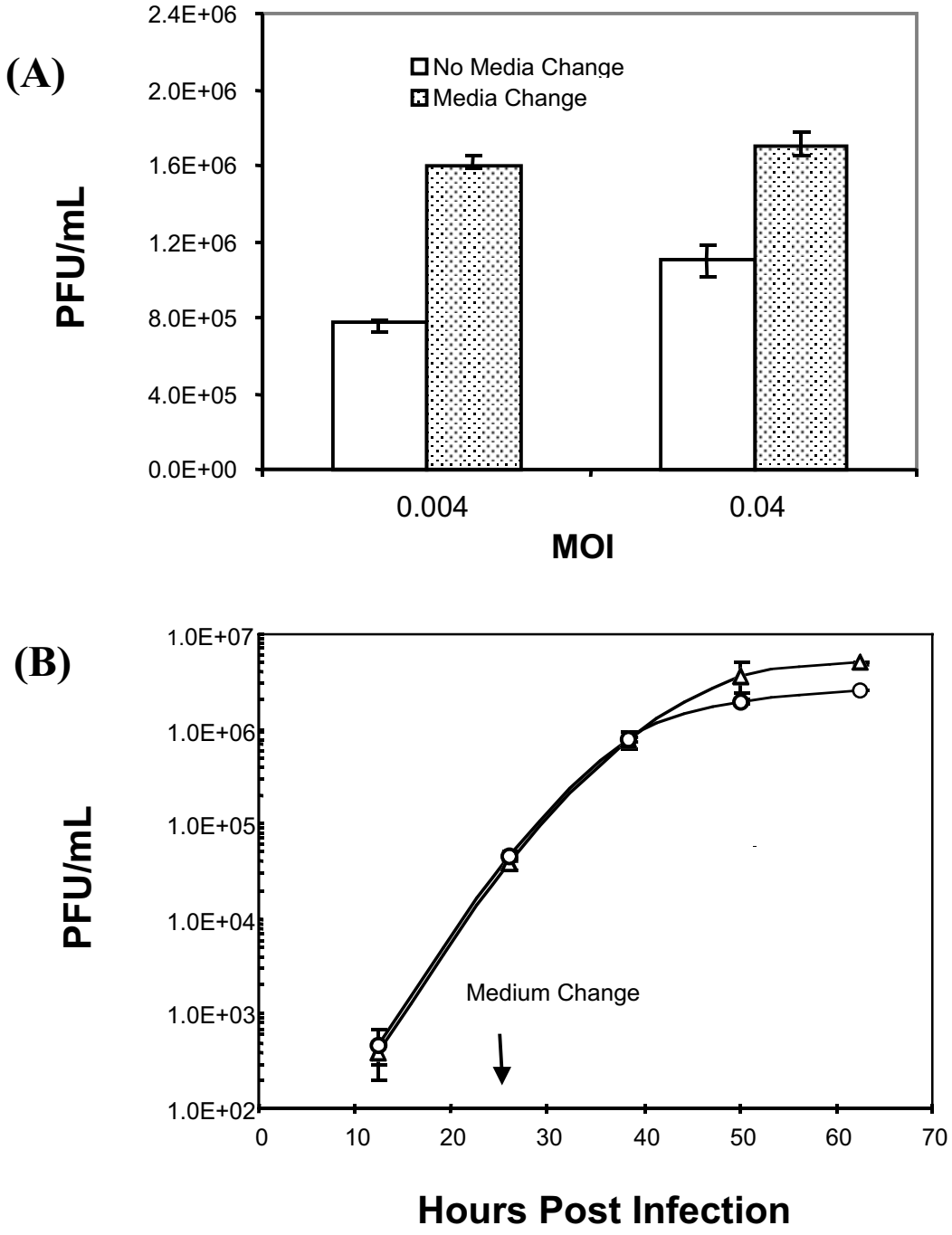


Figure 9 The effect of Media Change on Extra-cellular Viral Production

The effect of pH on cell-to-cell spread of the virus was investigated by employing the TOZHG vector (see Figure 10) carrying a GFP reporter gene. Fluorescent microscopic observation of the infected cells at 24 hpi demonstrated that the spread of infection also does not vary significantly with pH at MOI of 0.05 (Figure 14). The pH-dependent stability of the virus was determined by the addition of virus to growth media of different pHs and changes in the level of functional virus capable of infecting cells and forming plaques was determined as a function of time. The results (Figure 15) demonstrate that vector stability increases with a corresponding decrease in pH. The half-life of the virus is 6.7 hours at pH 6.8. This value is significantly longer than the half-life at pH 7.4 ($t_{1/2}$ =4.7 h) and pH 8 ($t_{1/2}$ =3.6 h). Thus, it seems likely that the pH effect on production results primarily from its effect on virus half-life.

Finally, the effect of serum content on virus yield was examined. Cells were infected in culture media with different serum content as well as a serum-free medium (Figure 16). The peak vector yield in serum-free medium was 7-fold lower than in medium with high serum content. Our results with serum-free medium are in accord with those reported for production of retrovirus and adenovirus vectors[102,145]. Moreover, virus concentration decreased more rapidly in the serum-free medium than in medium with high serum content indicating a higher decay rate. Furthermore, the results illustrate that the virus production in the presence of 5% serum in culture is similar to the medium with a serum content of 10%. Thus, 5% serum can be considered as the optimum serum content

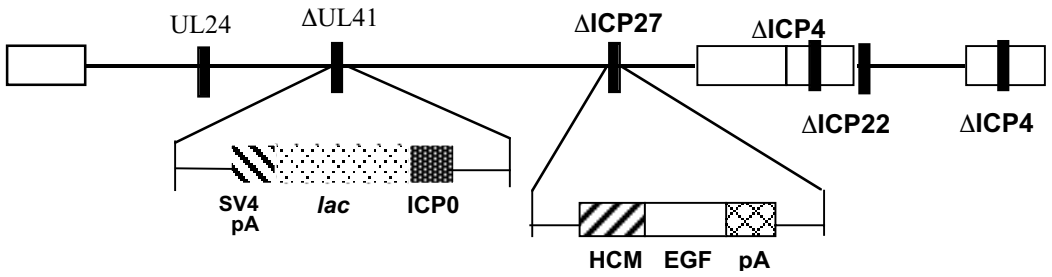


Figure 10 Schematic Diagram of TOZHG Vector

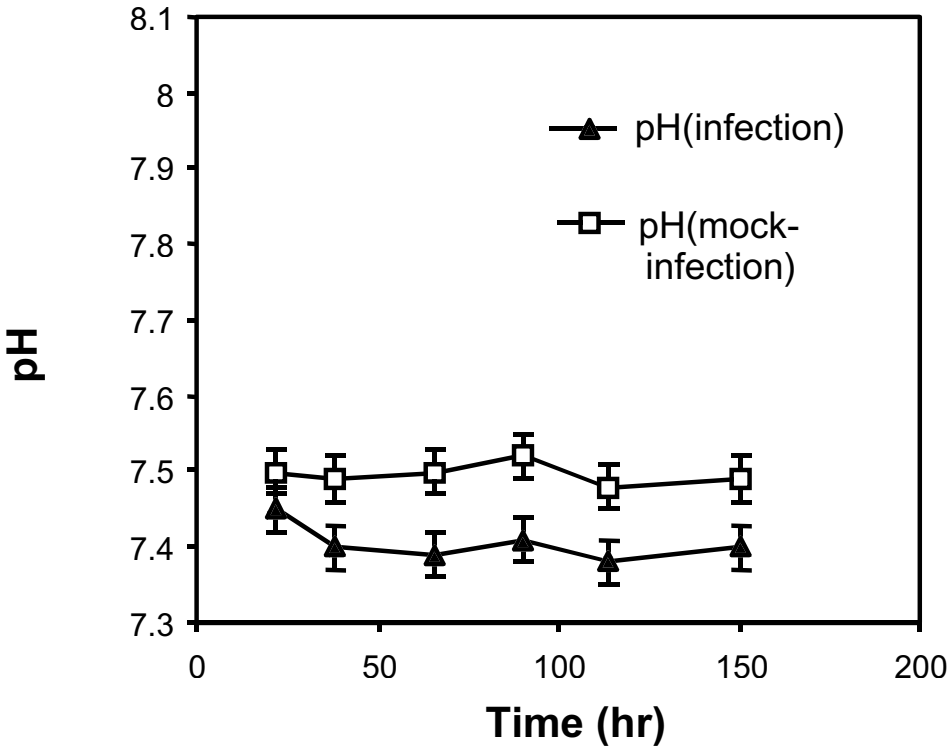


Figure 11 The Effect of Infection on pH.

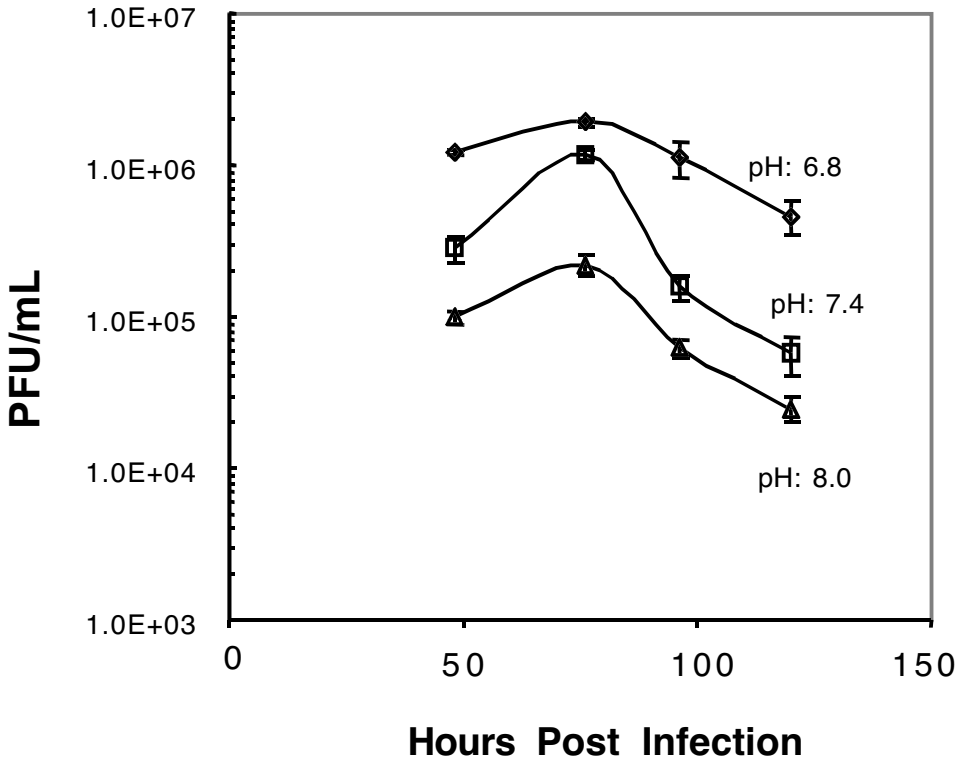


Figure 12 The Effect of pH on Extra-cellular Vector Production

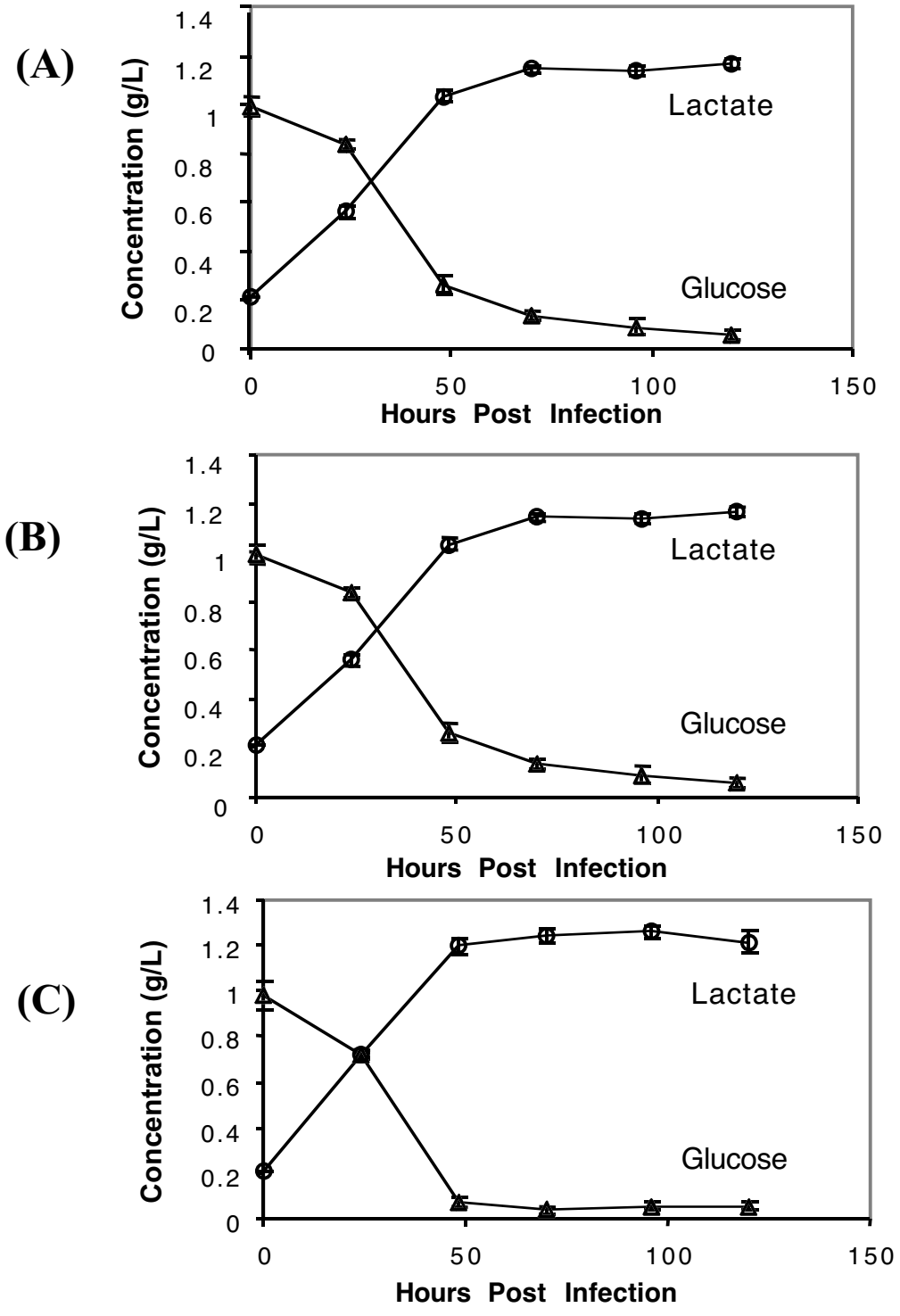


Figure 13 The Effect of pH on Post-infection Glucose and Lactate Metabolism

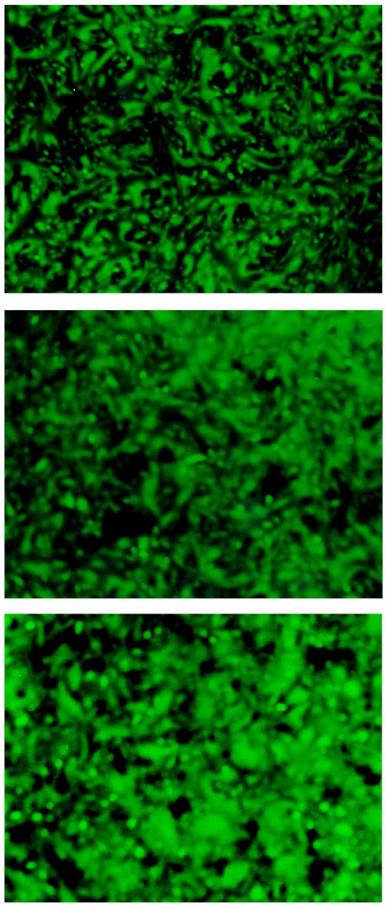


Figure 14 The Effect of pH on Virus Spread at MOI=0.1

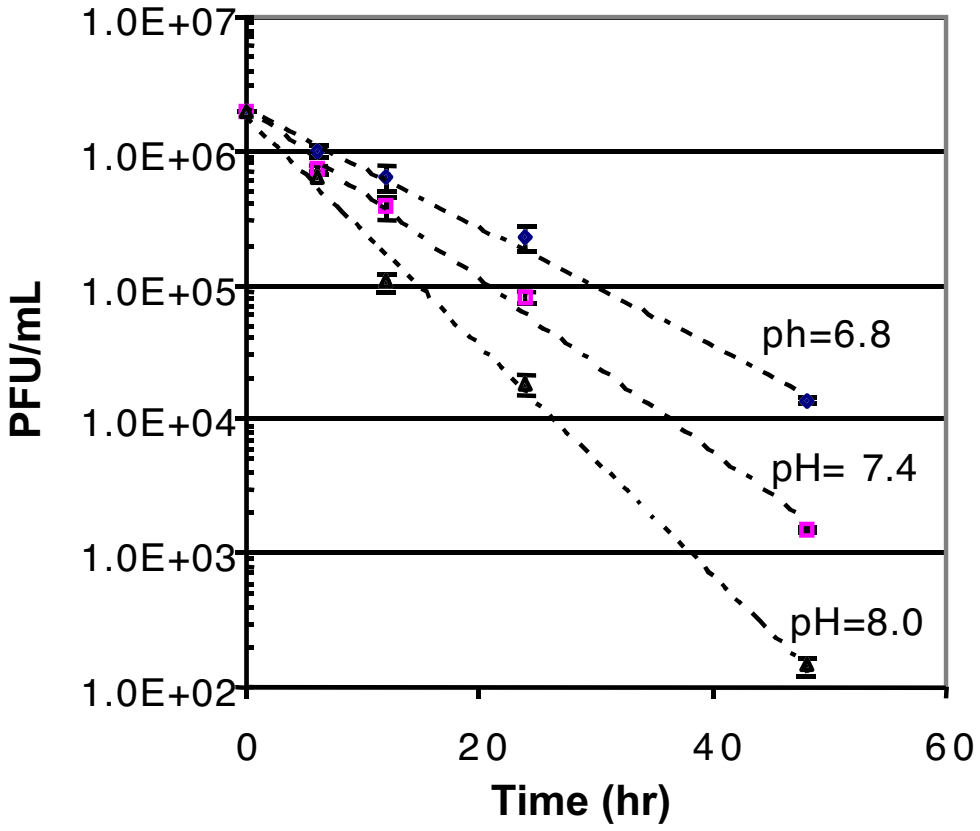


Figure 15 The Effect of pH on Virus Stability

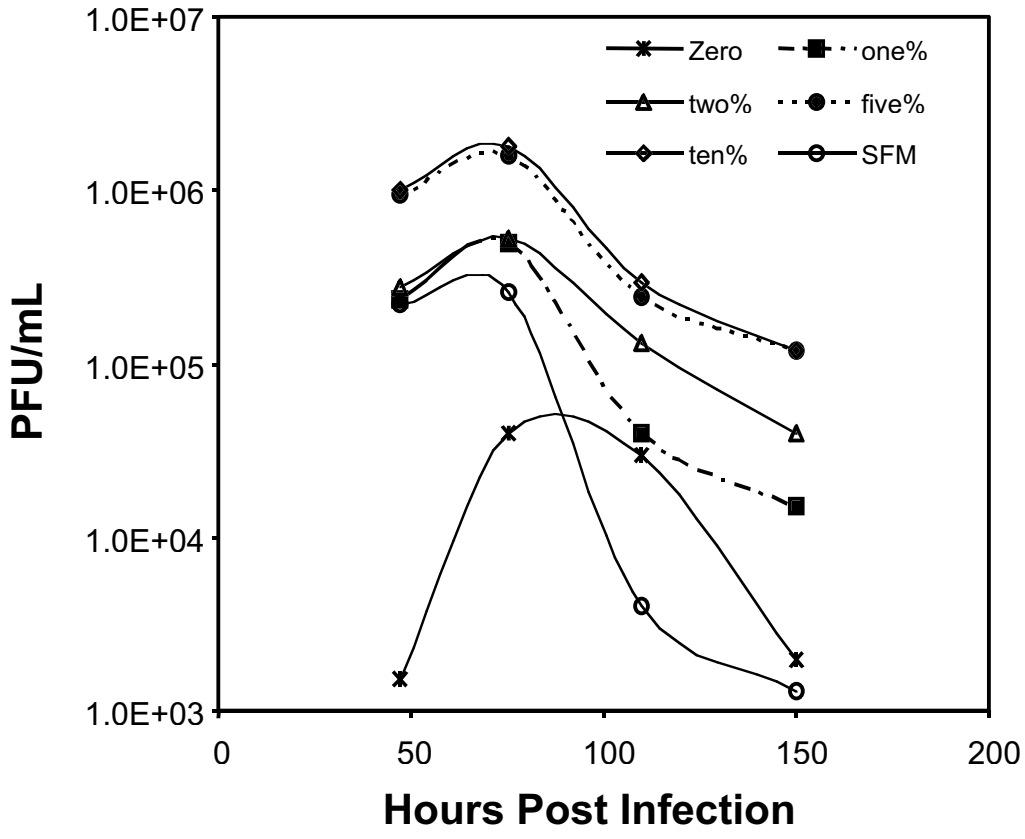


Figure 16 The Effect of Serum on Vector Yield

5.3 Discussion

Many factors must be taken into account in order to achieve maximum yields of viral vectors in the various production strategies required for the large-scale production of gene therapy vectors. In this study we have endeavored to systematically assess the multiple variables that may effect overall yield of the production of HSV replication-defective vectors in culture.

There are several important observations that can help increase overall vector yield. First, medium replacement during the post-infection period led to a higher yield of both extra- and intra-cellular vector. However, the implementation of total medium replacement could prove costly and create logistical difficulties in a large-scale bio-reactor system. Therefore, it is feasible to provide for some perfusion of fresh medium during the post-infection period instead of complete replacement. Second, lower pH values at the time of infection and during the post-infection period significantly increased the overall virus yield. This finding has significant impact on the production of the vector in large-scale bio-reactor systems. Since a rapid method for measuring the virus yield in the reactor at any specific time does not exist, the time of the harvesting of the culture will be primarily based on prior observations and experience. The results of the pH experiment (Figure 11) show that there is a significantly longer time period during which the culture can be harvested with relatively high virus yield when infected at low pH. However, cultures infected at higher pHs demonstrated a rapid decline in virus yield, hence if such cultures are not harvested at a time point very close to the time of the peak

virus yield, the production yield is significantly compromised. Moreover, based on our results (Figure 7, 11, 12) one can accurately estimate ultimate harvest time at which the highest overall viral yield was achieved. Unlike vector yield, electronic monitoring devices exist to accurately measure on-line both glucose and lactate concentrations in large-scale bio-reactor systems. Thus, we may be able to readily determine the optimal time of virus harvesting based on these readings. Third, Figure 16 depicts a substantially lower virus yield in the serum-free medium. Unless the composition of the serum-free medium is improved to support higher overall yield, the use of serum-free medium for HSV production will be limited despite the advantage it offers in downstream processing of the vector. Additionally, our results show that serum content of about 5% is sufficient to capture the enhancement in virus yield due to the presence of serum, suggesting a potential cost reduction that may also be beneficial to the overall virus purification schemes.

5.4 Conclusions

The use of HSV-based vectors in clinical trials has made development of large-scale production and purification methods a necessity. Prior to use of large-scale production systems, culture parameters affecting the yield of HSV vectors should be intensively investigated. Here, the effect of culture pH, serum content and the use of medium replacement on viral yield were evaluated. Viral yield and stability was greater at pH 6.8 than 7.4 and 8.0. Media replacement at various time points of post-infection improved viral yield considerably. Our serum experiments have shown that we still can

not replace serum containing media with a serum-free media but serum content of current culture media can be reduced from 10% to 5% without losing any viral yield which suggests a potential cost reduction and ease in the purification of vector.

In this chapter, we have shown that, by proper choice of culture pH, serum content and the use of medium replacement, viral yields can be enhanced. However, the final yields are still low. Higher titers will be needed for efficient use of these vectors for various gene therapy applications. New molecular designs have to be developed for constructions of vectors allowing for high production levels while still exhibiting diminished cytotoxicity. The ability to expand virus production in large-scale bio-reactors may compensate for the reduced yields obtained with the current vectors. Use of these bio-reactor systems in combination with efficient vector purification procedures will enable the realistic use of replication-defective HSV vectors in human clinical trials in the near future.

6.0 FURTHER EVALUATION OF CULTURE PARAMETERS IN THE PRODUCTION OF HSV-1 VIRAL VECTORS

Infectivity and/or stability of enveloped virions has been shown to be affected by various conditions such as medium components, pH, and temperature[100,102,103,146,147-150]. It has been also shown that thermal or pH inactivation of HSV results in particles with a slightly increased density compared to untreated virus[149]. Inactivated particles were found to retain the ability to adsorb to the surface of cells but were greatly reduced in productive entry[149,151]. In this chapter, we examined the effect of temperature, cell passage number and expanded our previous work on pH.

6.1 Effect of Temperature on Viral Yield

Temperature affects cell and viral growth as well as virus stability[100-103]. First, increase in temperature results in increased rates of enzyme-catalyzed reactions up to certain temperature values. Above such temperature values, because of enzyme denaturation, enzyme activity decreases with increasing temperature. Optimum temperature values for cell growth and virus production vary dependent upon the types of host cells and viruses employed. For herpesviruses, the optimum values of incubation temperature is between 32 °C and 37°C[152]. Release of virus from the host cell is also temperature dependent and is slower at 34°C or below. Furthermore, herpes viruses are very heat labile and less stable at high temperatures[152]. Temperature may also have an

effect on defective viral particle formation. For these reasons, temperature is a critical environmental parameter that should be optimized for efficient viral vector production.

We have conducted experiments to analyze the effect of temperature on vector production by employing both D.2 and TOZ.1 vectors. Cells were infected in duplicate 6-well plates at MOI of 0.05 and incubated at either 37°C or 33°C. Representative results are shown in Figure 17. In both vector infections, 33°C provided considerably higher vector yields compared to 37 °C. Moreover, the peak of virus yield at 33°C was greatly extended compared to 37°C, suggesting that lowering temperature after infection provides not only a substantial increase in vector yield but also a considerable increase in optimum harvesting time window for bioreactor operations.

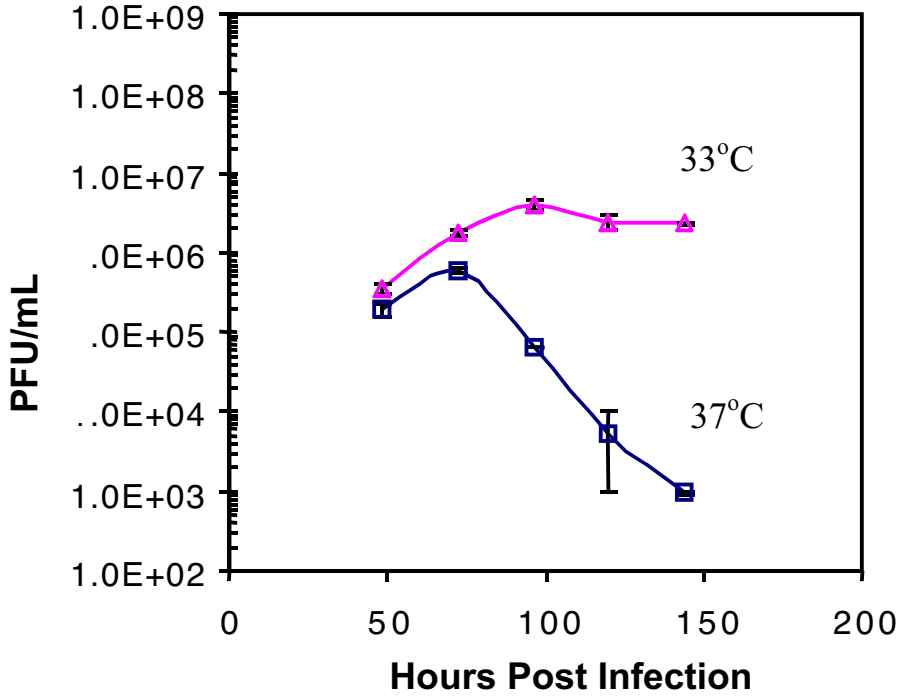
6.2 Effect of Passage Number on Viral Yield

Long-term passaging of cell lines may have detrimental effects on cell productivity[153]. Complementing cells that express gene products absent in replication-defective vectors are continuously passaged usually in the absence of drug selection due to expense and reduced growth rate. We had previously observed that cell passage number played some role in vector production. In order to determine the effect of cell passage on the production of vector, infections were performed on high passage number cells. The concentration of extracellular virus (PFU/mL) was determined (Fig. 18) for infections at an MOI of 0.05 using cells of (A) 30 and (B) 80, passages. Cells of indicated passage number were infected and incubated at 37°C. Samples of supernatant

were removed over time and analyzed for the presence of infectious virus by standard plaque assay. We observed a 3-fold increase in overall vector yield on lower (30) passage cells, suggesting that early passage number cells should be used in large-scale vector production studies. A possible explanation for the difference incurred by higher passage cells may be of a metabolic nature. To see if there are substantial variations in cell metabolism as a function of passage number, glucose consumption and lactate formation were measured for cells of 30 and 80-passage numbers.

Cells were infected at MOI=0.05, as in the previous production experiments, and supernatant samples removed over time. A substantially higher rate of glucose utilization and lactate formation was observed for the longer-passaged cells (Fig. 19). Long-term passaged cells consumed glucose completely in the first 24 hours of post-infection (Figure 19). The shift observed in these curves is important since in most experiments, the optimal time of peak viral harvest is 48 to 72 hours post infection.

A-) TOZ.1



B-) D.2

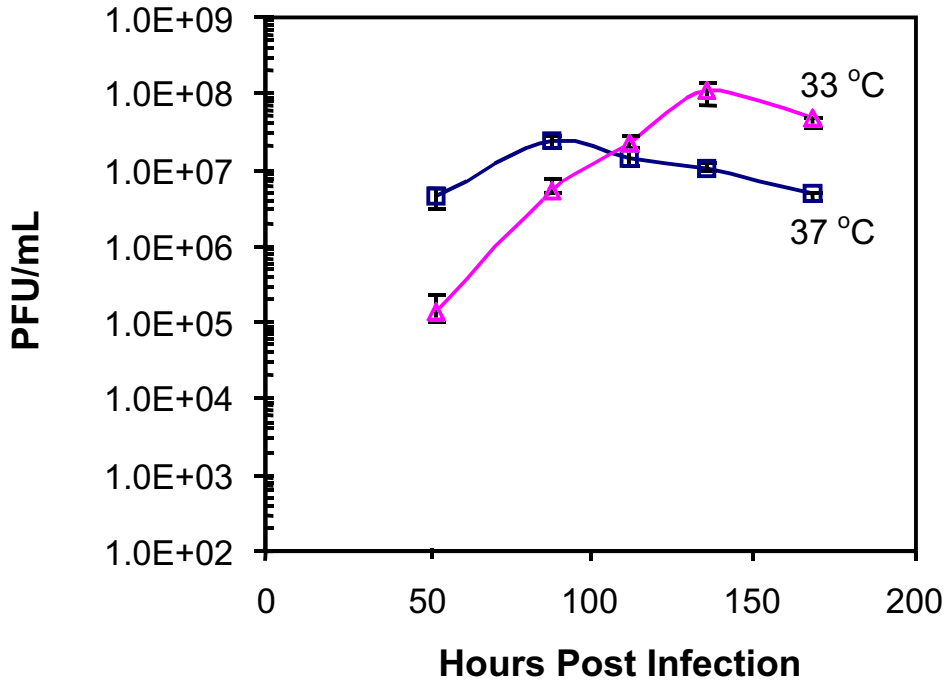


Figure 17 Effect of Temperature on Vector Yield

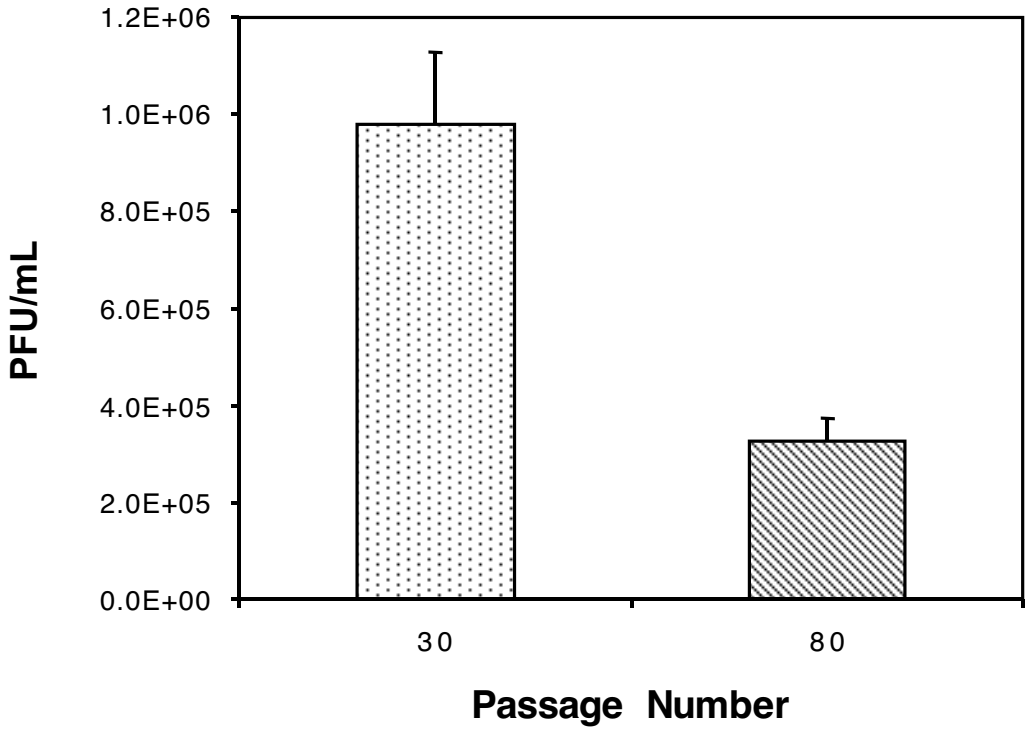
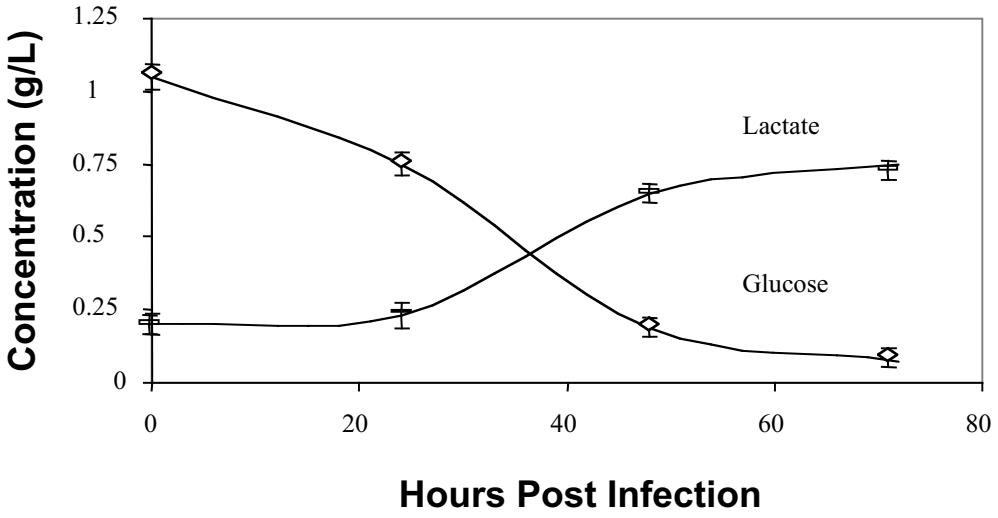


Figure 18 Effect of Passage Number on Vector Yield

(A) Passage 30



(B) Passage 80

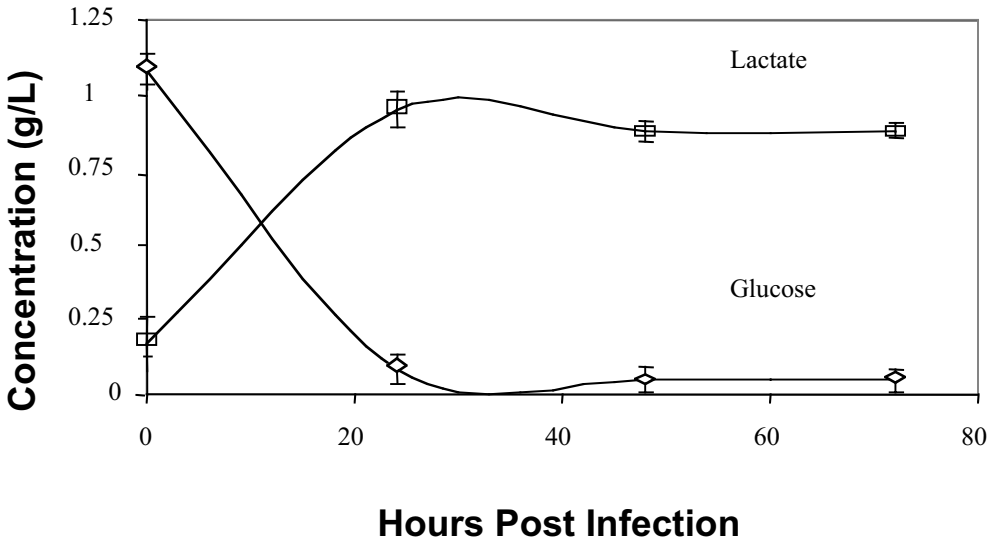


Figure 19 Effect of Passage Number on Extracellular Glucose and Lactate Levels.

6.3 Effect of pH on the Cell-to-Cell Spread

pH is an important factor affecting viral vector production. The influence of medium pH on mammalian cell culture has been investigated for a number of different cell types[144,147-150]. Viruses usually prefer isotonic conditions at physiological pH but their tolerance limits are strain dependent. The optimum pH range for herpesvirus production is between 6.8 and 7.5[144].

In chapter 4, we demonstrated the substantial effect of pH on virus production. Now, we would like to examine the effect of pH on cell-to-cell virus spread which would be an important issue during large-scale bioreactor runs. Therefore, we designed new pH experiments employing the TOZHG vector (see Figure 10) that carries a GFP reporter gene. 7b cells were diluted to an initial cell density of $1x10^6$/mL and infected with TOZHG (MOI=0.001) in media of three different pH values (6.8, 7.4 and 8.0). Fluorescent microscopic observation of the infected cells at 24 hpi shown that the spread of infection varies significantly with pH (Figure 20) and the effect is more pronounced at low MOI values (Figure 14 and 20).

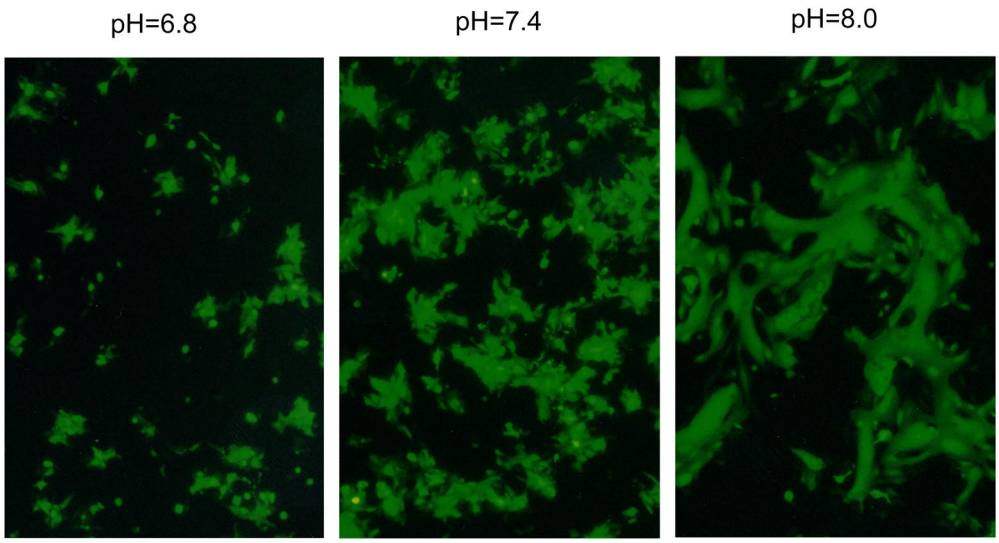


Figure 20 The effect of pH on the Cell-to-Cell Spread

6.4 Conclusions

We have shown a significantly longer virus half-life at 4°C or 33°C than at 37°C, as well as increased vector stability in conditioned culture medium compared to the fresh medium with or without serum. Complementing cells displayed a longer doubling time when cultured at 33°C and showed a 16-24 hour lag in virus production and release when compared to 37°C. However, 2-fold greater vector levels were produced from a synchronous infection at 33°C. To integrate these findings into a scaleable production scheme, cells infected at 37°C were shifted to 33°C at 24 hours post infection to enhance initial viral spread, viral production, as well as vector stability. We found that early passage cells showed a 3-fold increase in extracellular virus at the reduced temperature. This difference increased up to 10-fold as the cell passage number increased. One

notable difference between these cell populations is that glucose is utilized much quicker in high passage cells, possibly leading to decreased vector production.

The higher HSV vector production levels achieved at the lower temperature of 33°C are in accord with the reports on retrovirus vectors [100-103]. To our knowledge, this is the first report on the effect of passage number on vector yield at different temperatures, and only limited data exist on the effect of long-term culturing on cell growth, metabolic, and genetics changes. However, a recent work with insect cells revealed some insights into cell growth and recombinant protein production in the insect-baculovirus expression system[153]. Early passage cells grew to a 2- to 3-fold higher cell density and displayed higher levels of recombinant protein production. In addition, the older-passaged cells grew to a lower density, although the cells showed a shorter lag phase and about 20% faster growth rate initially. Hence, higher rates of glucose consumption and lactate formation may indeed lead to a lower final cell density, higher initial growth rate, and perhaps even a shorter lag time. Thus, it is possible that the higher glycolytic activities displayed by the Vero cell cultures in Figure 5, are the common characteristics of longer-passaged cells of different origins and may contribute to decreased vector production at 37°C.

7.0 DEVELOPMENT OF RAPID, ACCURATE AND PRECISE ASSAY SYSTEM TO ANALYZE THE QUALITY AND QUANTITY OF CLINICAL-GRADE HSV VECTOR STOCKS

As the number of clinical and research applications of herpesvirus-based vectors increase, it becomes critical to develop rapid, accurate and precise assay systems to analyze the quality and the quantity of clinical grade vector stocks. Current HSV quantitation strategies such as gel-based liquid hybridization and electron microscopy are accurate, however, they are both time and labor intensive. We have developed and are optimizing a sensitive assay system relevant for meeting and surpassing current FDA requirements for clinical grade viral vector stocks. Our assay system contains two independent and complementary DNA quantification methods: a real-time quantitative PCR system using the 5' exonuclease activity of Taq polymerase and a micro-plate assay using PicoGreen dsDNA quantification reagent. This assay system is being optimized to quantify the amount of infectious versus defective HSV particles, and the purity of HSV vector stocks. Our real-time quantitative PCR assay employs two independent primer sets. The first set, specific for glycoprotein D sequences present in all HSV genomes, allows for the quantification of total viral particles and for defective DNA containing particles when compared with the number of plaque forming units generated by standard virological plaque assay. A second primer set, specific for the human Apo-B gene, enables the estimation of purity of gene therapy vector stocks. Our real time PCR assay is linear from 10 to 10^7 copies of HSV and 1 to 10^5 copies of host cell genomic DNA. In

contrast to our PCR method that quantifies the viral and host cellular DNA concentration, we developed an independent micro-plate assay measuring the total DNA concentration of vector stocks. PicoGreen is an ultra-sensitive nucleic acid stain that selectively binds to double-stranded DNA in solution. Our PicoGreen micro-plate assay is fast and accurate, with a detection limit as low as 0.5 ng of HSV DNA corresponding to ~$3x10^6$ HSV particles. The resultant combination of real-time PCR and PicoGreen micro-plate DNA quantitation assays represents a standard in the field of HSV vector quality assessment.

7.1 Introduction

Recombinant herpes simplex virus type 1 (HSV-1) vectors have gained increased attention as promising vectors for gene therapy applications due to their unique advantages over other viral vectors. As the number of clinical-trials using HSV based vectors increases, the demand for clinical-grade HSV vector also increases. One of the most important challenges in the success of gene therapy applications is to develop large-scale production and purification strategies that can satisfy the demand for vectors. However, scaling up the production of viral vectors suffers from the lack of available bio-reactor types that can support the growth of anchorage-dependent cell growth. Additionally, the loss of high amounts of virus particles during the rough purification steps due to the FDA regulations requiring the removal of adventitious agents as well as host cell proteins and DNA has limited the large-scale production of these vectors. In order to adequately respond to FDA requirements for the quality assurance (QA) and

control (QC) of gene therapy stocks, rapid analysis methods that eliminate long and labor intensive analysis methods such as electron microscopy (EM) will be required.

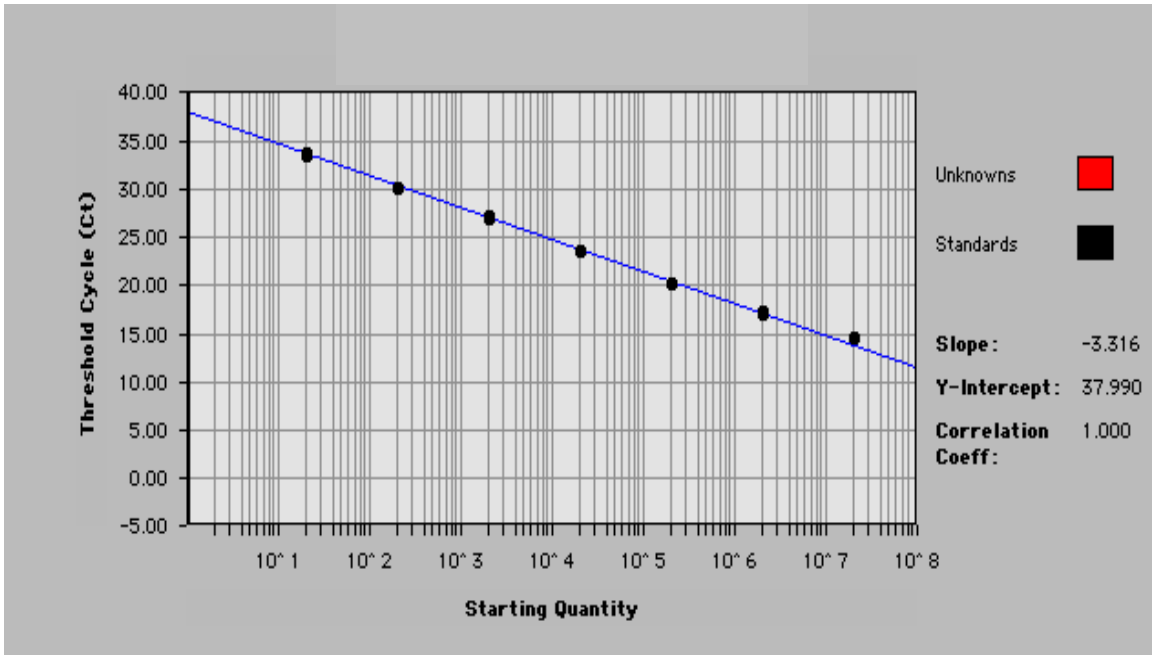
Current HSV-based vector quantitation strategies such as gel-based liquid hybridization and electron microscopy are accurate, however, they are time and labor intensive. A standard analysis method that can provide complete information on host cell and extra-virion virus DNA contamination, as well as infectious to total particle ratio is still unavailable. In order to develop validated upstream and downstream processes that can satisfy the tough FDA regulations it is critical to possess fast, accurate analysis methods. Our viral assay system (VAS) combining real-time PCR with the highly sensitive PicoGreen microassay will allow rapid comparisons of different production and purification methodologies to measure rapid progress in the development of better production/purification strategies. Our overall goal is to establish a more accurate, less time consuming, inexpensive and comprehensive clinical-grade vector analysis system to determine; (i) infectious/defective particle ratio; (ii) extra-virion DNA contamination; and (iii) the effect of different production and purification schemes on quality and quantity of clinical preparations.

7.2 Results and Discussions

There are strict regulations established by the FDA regarding the amounts of contaminating DNA amounts in clinical-grade vector stocks since contaminating DNA could elicit an unwanted immune response following injection of the vector into patients.

For that reason, detection and purification of clinical-grade vector stocks is one of the most critical steps in viral vector preparations. Electron microscopy, a laborious and time-consuming analysis method, has been required to measure total HSV particles and to assess the integrity of the vector preparation in terms of the number of intact enveloped virion particles and the amount of contaminating cellular debris. Thus, we have decided to develop rapid and sensitive assays instead of EM analysis to more accurately assess viral stock purity. Development of such high-throughput assays would also enhance the sensitivity for the measurement of HSV DNA and accelerate the progress in the development of large-scale production and purification methodologies. In addition, it would be very easy to detect batch-to-batch variation of viral vector preparations. Initially, a large stock of QOZHG vector (Figure 2) was propagated, purified and aliquoted as it was described in Materials and Methods section. Then, several vials were analyzed by standard plague assay, electron microscopy and used for the development of Q-PCR and PicoGreen total DNA quantitation assays.

TaqMan. In order to establish a reliable quantitative protocol to assess the number of virus particles within a virus preparation, we decided to use the TaqMan real-time quantitative PCR (Q-PCR) detection system for the development of this assay. TaqMan PCR techniques have shown to be very successful in the detection of virus particles in clinical samples [(154)]. The Q-PCR detection system is based upon digestion of an internal probe containing a fluorescent and a quencher dye by the 5' exonuclease activity of Taq polymerase.



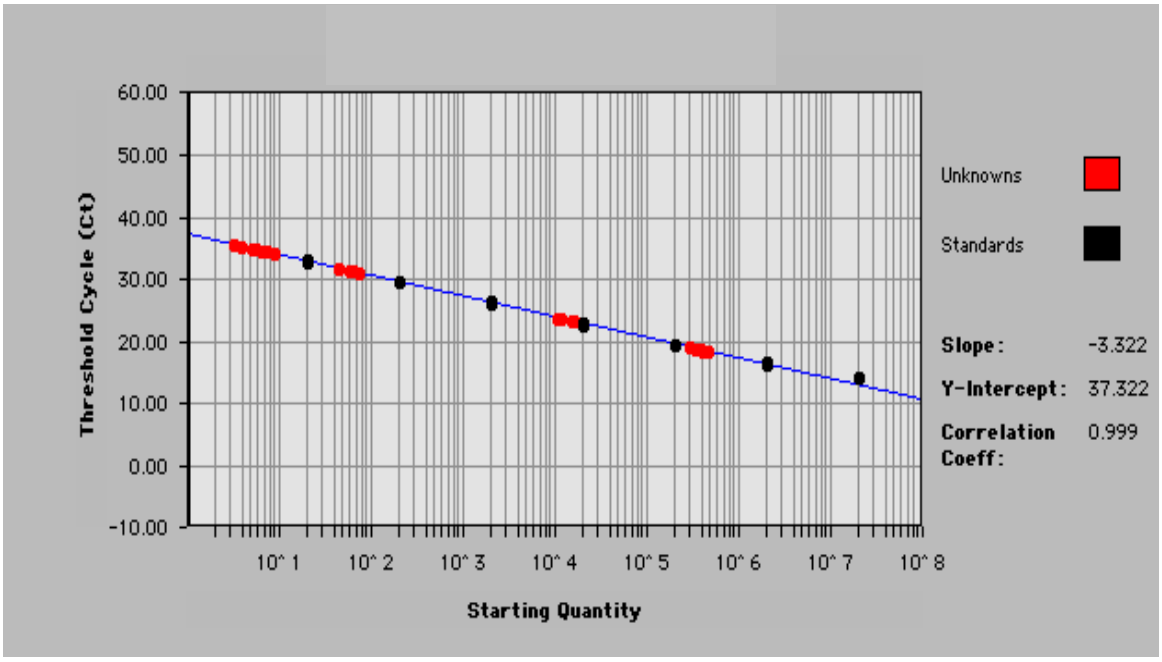


Figure 21 Q-PCR Calibration curve for A) HSV plasmid and B) Vector dilutions

The probe hybridizes to a specific region of the target DNA and during the amplification of the target DNA from the PCR primers the reporter dye is released in every cycle of the PCR. Then, the viral genome copy number can be calculated by the comparative analysis of fluorescence obtained by using viral DNA preparations made from purified virus stocks to a known amount of plasmid DNA representing specific regions of the HSV genome. Selection of specific primer pairs and the operation parameters of PCR machine are two key factors affecting the sensitivity of this assay.

TaqMan analyses have been performed using two primer sets that were constructed from the ICP27 and gD gene segments of the HSV genome. In the first three runs, serial dilutions of HSV virus stocks were used to plot a standard curve. In the fourth run, the standard curve was prepared by serial dilution of HSV plasmid pgD-Sac since plasmid dilutions provide more reliable quantitation readings compared to virus dilutions, which might contain considerable numbers of defective particles.

The reliability of chosen primer pairs also has been tested in these runs by testing the amplification efficiency (slope) and duplicate sample reproducibility (R^2) values of the standard curves. The correlation coefficients of 0.983 and 0.994 were observed using the ICP27 and gD primers, respectively. Parameters of both assays were very close to each other suggesting that both primers estimated equivalent values for the number of HSV particles. We have recently obtained correlation coefficients of 0.999 and 1.000 for gD plasmid (Figure 21A) and virus standards (Figure 21B) respectively. Using these

standards we have now shown that the assay is linear over a range from 10 to 10^7 copies of HSV genome. This is by far the most sensitive assay for detecting the HSV genome.

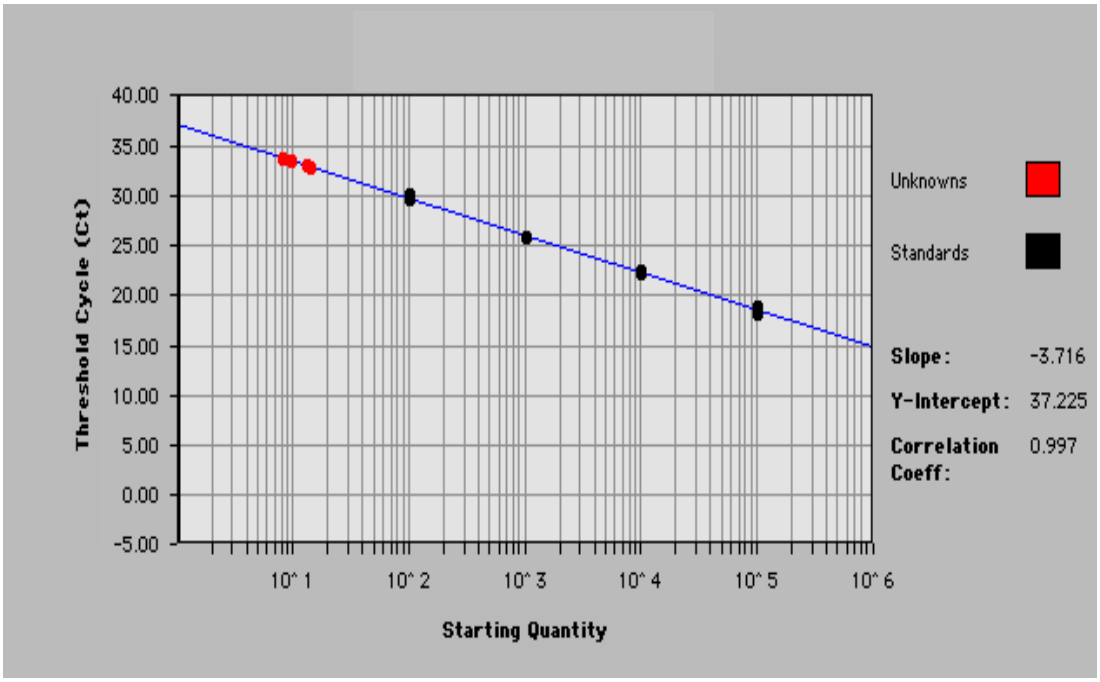


Figure 22 Q-PCR Calibration Curve for Host Cell DNA Contamination

In addition, we are now using an Apo-B probe to quantitate the amount of cellular DNA in the virus preparations to determine the level of cell contaminant in each stock. This method should be more sensitive than comparing the total amount of protein in each preparation to the number of pfu determined in a standard plaque assay. Using a second primer set which is specific for the human Apo-B gene, we can estimate the purity of gene therapy vector stocks. As shown in Figure 22, as low as one host cell DNA genome equivalent (Vero cells) can be detected by this assay and the assay is linear from 1 to 10^5

copies of host cell genomic DNA with a correlation coefficient of 0.997. Overall, our Q-PCR system allows us to quickly and easily quantify the number of HSV genomes in sample with an exceptional reliability and reproducibility. Sensitivity, precision and the accuracy of this assay appears to be superior to any other method of HSV genome quantitation.

PicoGreen. In addition to the real-time PCR method, we have investigated another quantitation method, which does not rely on any specific part of a DNA sequence as does Q-PCR. A microplate assay using PicoGreen dsDNA quantitation reagent has been evaluated for this purpose. PicoGreen reagent is a very sensitive fluorescent nucleic acid stain for quantitating small amounts of dsDNA in solution[155].

This reagent does not bind to any single-stranded DNA or RNA. The assay procedure is simple and requires a PicoGreen quantitation kit (Molecular Probes Inc., Eugene, OR), a microplate and a fluorescent microplate reader. Murakami *et al* (1999) showed that this assay can be used to quantitate as little as 10.3 ng/ml of Adenovirus DNA in a 200 µl assay volume. We conducted five experiments to find the optimum wavelength, total assay and sample reagent volumes. Optimum excitation and emission wavelengths were found as 485 nm and 540 nm while optimum sample volume and total assay volumes were 20 µl, and 200 µl, respectively.

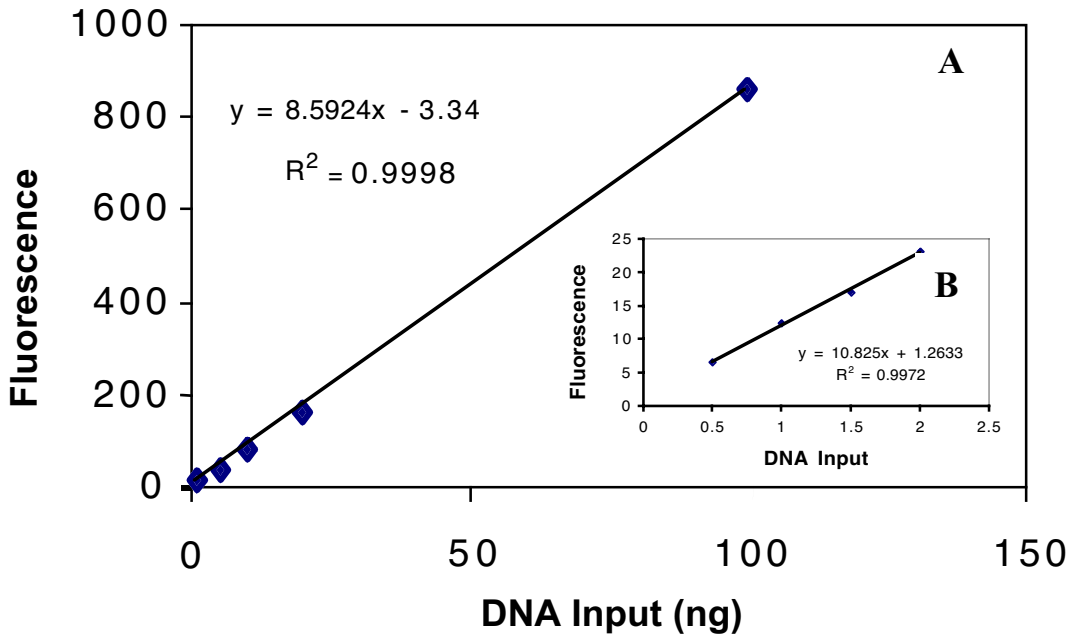


Figure 23 PicoGreen Assay DNA Calibration Curves

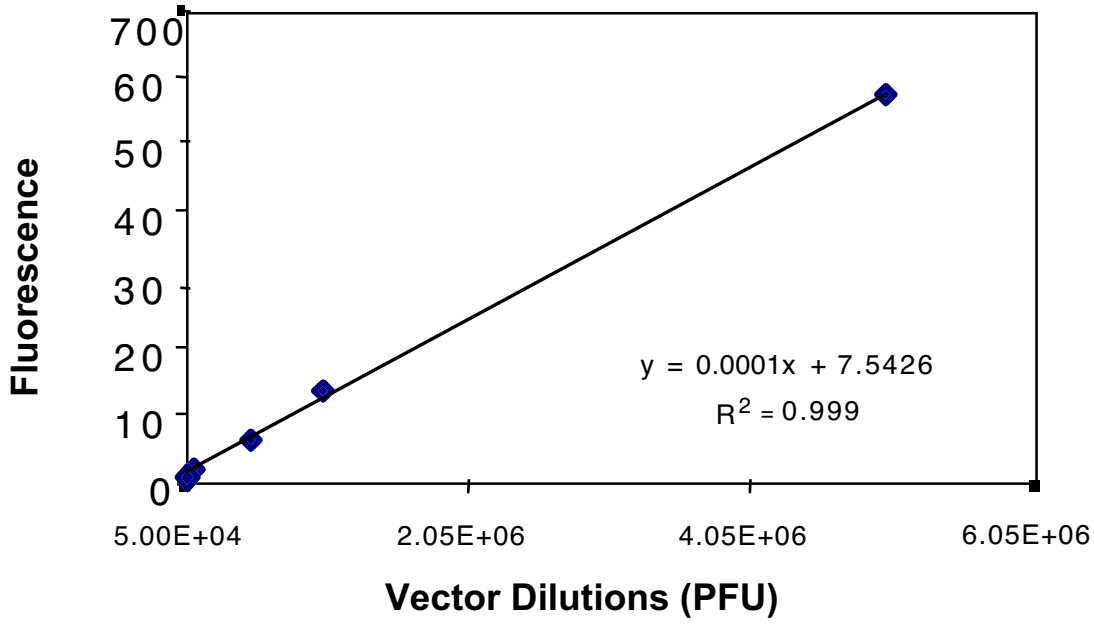
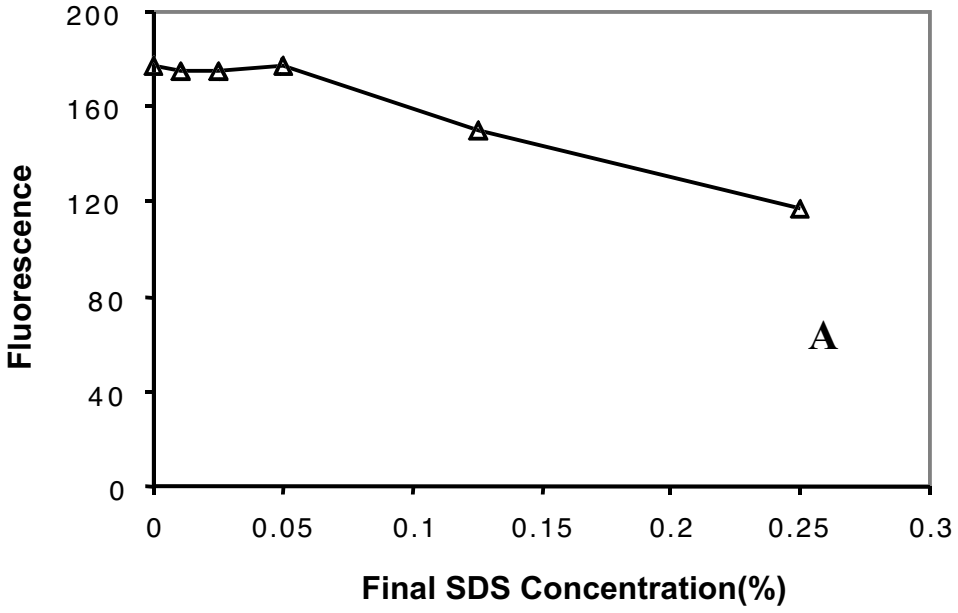


Figure 24 PicoGreen Assay Calibration curves for Vector Dilutions



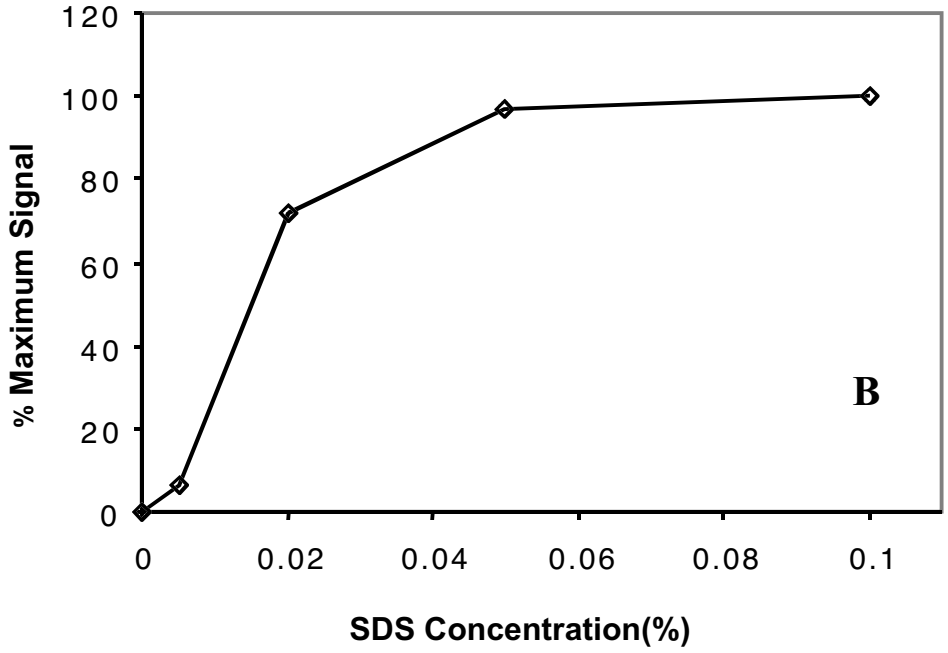


Figure 25 Required SDS Concentrations for PicoGreen Assay

To test the dynamic range and sensitivity of the assay, low and high range calibration curves have been prepared. Figure 23 shows the low and high- range calibration curves obtained by using dilutions of λ DNA stock. Low- (Fig. 23A) and high-range (Fig. 23B) calibration curves provided good correlation coefficients with the values of 0.9998 and 0.9951, respectively. A linear correlation between 0.5 to 100 ng was observed. The limit of DNA quantitation i.e. the DNA amount sufficient to generate 10-fold higher fluorescence signal than assay noise was determined to be 0.5 ng. The standard deviation of replicates of PicoGreen reagent only samples was used to measure the assay noise. The limit of detection, (the DNA amount sufficient to generate a 3-fold higher PicoGreen signal than assay noise) was found to be 0.2 ng.

The effects of SDS (Sodium Dodecyl Sulfate), a compound used in the preparation of viral DNA samples, on the sensitivity of PicoGreen microassay was investigated. Even though it is known that ionic detergents may substantially inhibit the sensitivity of PicoGreen assay, SDS is used to for the lysis of herpesvirus particles to obtain virion DNA. The effects of SDS inhibition on PicoGreen assay was tested by assaying bacteriophage (lambda) DNA at various SDS concentrations. Figure 24A demonstrates that presence of SDS concentration up to 0.05% in the sample did not affect (interfere with the assay) the PicoGreen assay. To test whether SDS concentrations of 0.05% can provide complete lysis of herpesvirus particles, viral samples were treated with SDS concentrations varying from 0 to 0.1% for 15 minutes at room temperature. A plateau in signal recovery (Figure 24B) obtained after the SDS concentration of 0.05%

has shown that herpesvirus particles can be prepared for the PicoGreen assay with a negligible interference at 0.05% SDS concentration.

Reproducibility and reliability of our PicoGreen assay has been tested by six independent triplicate measurements of two different concentrations of DNA at determined on different days (Table 5). Since the determination of the samples was done with a fresh standard curve for each analysis, the standard deviation and coefficient of variation values are a good representation of reproducibility and reliability of the method. Several stocks with defined virus concentrations were subjected to the assay.

The PicoGreen micro-plate DNA quantitation assay is fast, and accurate with a detection limit of 0.25 ng λ DNA (Fig. 25A) or $1x10^6$ HSV particles (Fig. 25B). This assay is very convenient for the rapid analysis of herpesvirus samples. The sensitivity of assay is adequate for our clinical-grade herpes virus-based vector stocks, ranging from 10^8 to 10^{11} particles/mL. Depending on the lysis of the viral particles (w/o/SDS) the total amount of DNA or only the extra-virion viral and host cell DNA contaminants can be measured with this assay.

We found that the Pico Green assay is not affected by SDS concentrations needed to isolate viral DNA from purified virions and that the assay is linear from 10^4 to 10^7 pfu/ml. As with the TaqMan assay, we are now using an Apo-B probe to quantitate the amount of cellular DNA in the virus preparations to determine the level of cell contaminant in each stock. Instead of using the standard BioRad protein assay to assess the level of protein in each vector preparation, we have now developed an assay that

employs the NanoOrange protein quantitation kit (Molecular Probes). This assay system is considerably more sensitive than the BCA, Bradford or Lowry methods and can accurately detect protein in the range of 10 ng/mL to 10 µg/mL. In addition, the assay is easy and shows less protein-to-protein variability than other standard protein assays.

Table 6 compares the assay results of a HSV vector stock (QOZHG) using different methods including EM (Figure 26). The titers represent infectious particles determined by using standard plaque assay. Q-PCR results reveal the total number of HSV genomes present within the stock, which includes intact particles and those that have lost their integrity and lost some/all of their genome. On the other hand Electron Microscopy (EM) gives the total number of intact particles. However, this assay is highly subjective to individuals that may result dramatic variation in the standard deviation of the assay. PicoGreen measures total DNA concentration whether the DNA represents HSV DNA or host/contaminant DNAs.

Table 5
Statistical Analysis of PicoGreen Assay

DNA Amount (ng)	45	11	2.5
# of replicates	6	6	6
average	45.06	10.94	2.58
Ave. Deviation	1.44	0.29	0.16
Max	47.21	11.35	2.77
Min	43.66	10.31	2.37
Median	43.97	11.04	2.59
STDEV	1.61	0.37	0.192
% CV	3.57	3.44	7.846

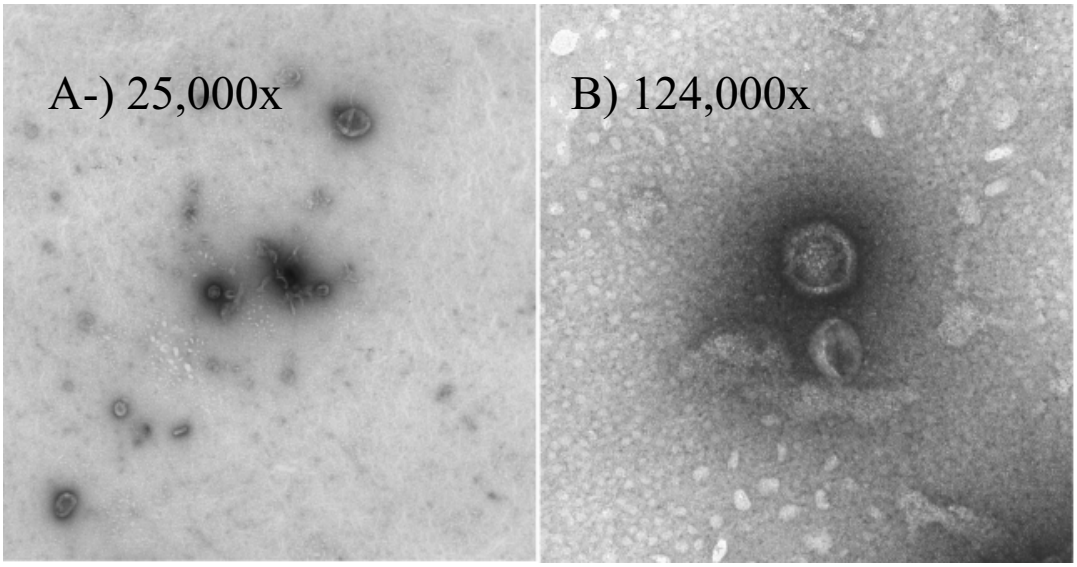


Figure 26 Measurement of Total Virion Number by EM

Table 6
Comparison of Assay Results

Assay	Results (Number of HSV particles/μl)
Titer	1.95×10^4
EM	3.4×10^5
TaqMan	1.16×10^6
PicoGreen	2.85×10^6

As expected if one uses the PicoGreen results and assumes that all of the DNA present is from HSV, the total number of particles will be overestimated. In other words,

the difference between the PicoGreen and Q-PCR results represents the amount of host/contaminating DNA.

7.3 Conclusions

Our assay system is fast, precise and accurate to analyze clinical-grade HSV-vector stocks. In the case of measurement of defective to infectious particle ratio; Q-PCR with gD and ICP27 primer/probe and PicoGreen with SDS solution for the comparative determination of total HSV particles and standard plaque assay for the determination of infectious particles were used. In order to determine the purity of stocks Q-PCR with H-Apo-B primer probe set, PicoGreen without SDS solution and NanoOrange protein quantitation assay were used. Our assay system represents a standard in the field of HSV vector quality assessment

8.0 CONCLUSIONS AND FURTHER RESEARCH

8.1 Conclusions

Recombinant herpes simplex virus type 1 (HSV-1) vectors have become one of the most promising vectors for gene therapy applications due to their unique advantages over other viral vector systems. As the number of clinical-trials using HSV based vectors increases, the demand for clinical-grade HSV vector also increases and large-scale production and purification of HSV vectors become a requirement for success . Here, we have shown that production of HSV-based vectors can be efficiently scaled-up. Outcomes of this study can be summarized as following:

- there is a correlation between vector backbone and optimal vector yield during production. Our results revealed that as we further crippled the virus by deleting viral genes from the base virus backbone to reduce toxicity, lower overall yields were observed due to the lack of complementation

- we can scale-up the production of HSV-based gene therapy vectors from 20 mL/150-cm^2 tissue culture flasks or 100 mL/850-cm^2 roller bottles to the 4 L/21,250-cm^2 CellCube Bioreactor System with a higher production efficiency per media unit consumption than possible using tissue culture flasks and roller bottles, the standard systems employed in this field of research

- by correct choice of many culture parameters such as pH, temperature, MOI, initial cell density, serum content, and the use of medium replacement, viral yields can be substantially enhanced (10-15-fold)

- we developed a vector assay system that is fast, precise and accurate method to analyze clinical-grade HSV-vector stocks. Our assay system represents a new standard in the field of HSV vector quality assessment and applicable to GMP production guidelines determined by the FDA.

8.2 Further Research

In these studies, we have shown that we can develop efficient production strategies for HSV-based gene therapy vectors. We are now planning to test several other bioreactor configurations compared to the efficiency of our CellCube Bioreactor System and validate our manufacturing process for Good Manufacturing Practice applications (GMP).

8.2.1 CelliGen^{+} Bioreactor System

CelliGen^{+} Bioreactor System (Figure 28, New Brunswick Scientific Co, Edison NJ) is a fixed bed reactor that employs Fibra-Cel polyester disks (New Brunswick Scientific Co, Edison NJ) as microcarriers to support the growth of anchorage-dependent cells. This system is scalable from 1.5-liter to 10-liter perfused bioreactor with a surface area

equivalent to 28 CellCube bioreactor that makes CelliGen^{+} a promising configuration for scale-up studies since this would be equivalent to 700 850-cm^{2} roller bottles.

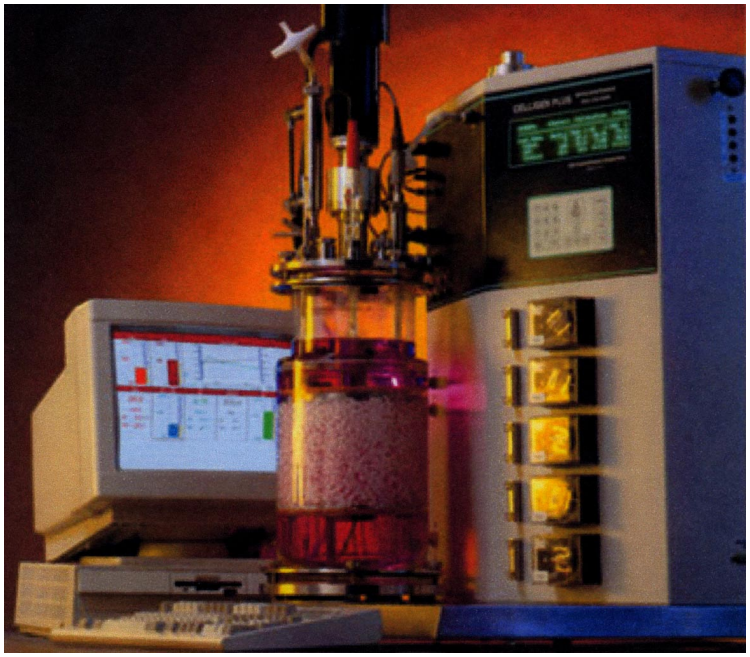


Figure 27 CelliGen^{+} Bioreactor System

8.2.2 Selective Elimination of Extra-virion DNA Contamination from Clinical-Grade HSV vector Stocks

DNase I can potentially be used to digest the extra-virion contaminating DNA present in HSV vectors stocks. However, potential interaction of DNase I with infectious vector particles that may cause loss of infectious virus particles should be prevented. To overcome this problem we used immobilized DNase I enzymes (MoBiTec, FL) for selective elimination of extra-virion DNA contaminants. In this system, DNase I molecules are covalently linked to inner surface of porous polyvinyl beads. Since the

size of pores is smaller than virus particles, any contact of virus with DNase molecules is avoided. Immobilized DNase I beads are suspended in reaction buffer and mixed with the appropriate virus and DNA stocks. Preparations are incubated at 37 ^{o}C. for several hours. As shown in Figure 28, we were able to eliminate >99% of contaminating DNA from both virus and lambda DNA stocks. During the DNase I treatment process, infectious particle number of vector stock decreased 15%. Results are promising but conditions of DNase I treatment process needs to be optimized to lower the loss of infectious virus particles.

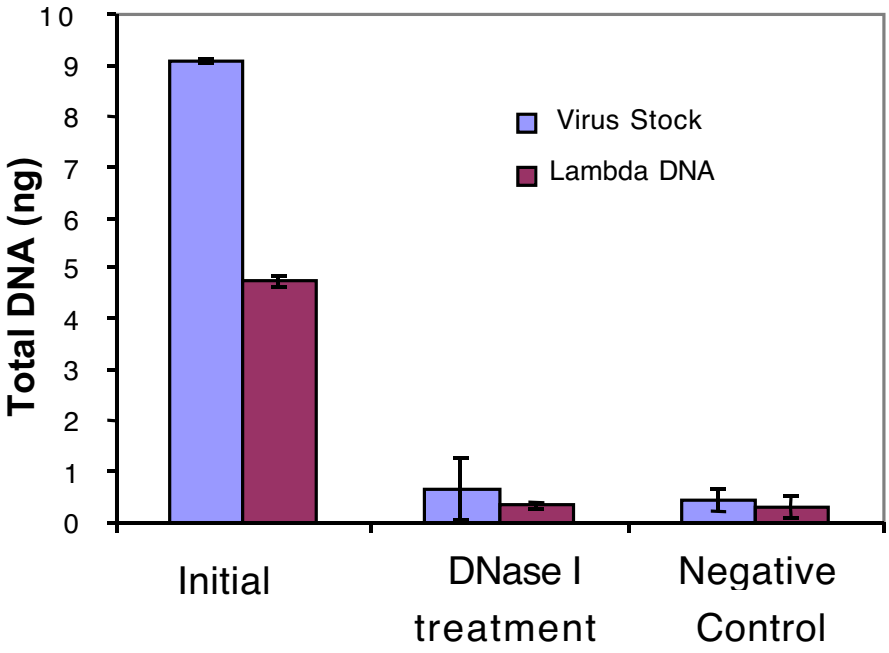


Figure 28 Selective Elimination of Extra-virion contamination in HSV-based vector stocks

8.2.3 Development of Current Good Manufacturing Practices (cGMP) for the Production of HSV Vector

Prior to human clinical trials, the manufacture of HSV vectors must be licenced by the FDA. Production of HSV-based vectors as gene therapeutics must be accomplished under cGMP conditions. cGMP is "a set of current, scientifically sound methods, practices or principles that are implemented and documented during product developments and production to ensure consistent manufacture of safe, pure and potent products"[156] and applies to both the manufacturing process and facilities. cGMP is important in all phases of development but can be implemented in a step-wise approach (Figure 29) meaning that the level of compliance should increase as the phases progress. Elements of cGMP can be summarized as[156]:

- facility design / adequate documentation and recording
- production and process controls
- quality control and assurance
- validation
- equipment calibration and qualification
- personel training and certification
- environmental monitoring

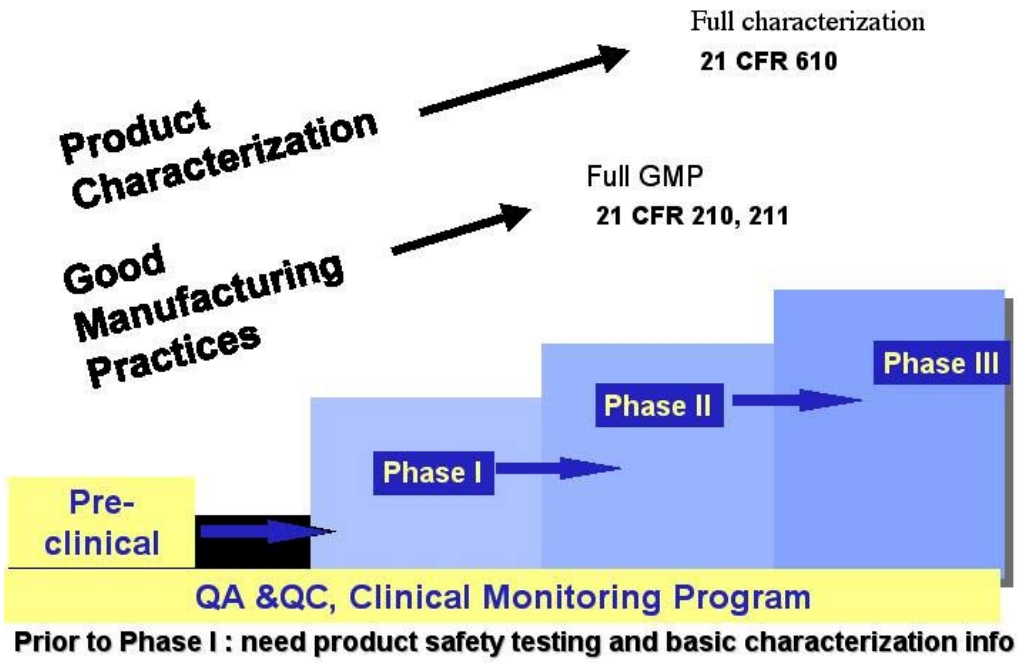


Figure 29 Step-wise Approach to Application of Regulatory Requirements [156]

Since early phase cGMP requires attention to the documentation, facility, quality control (QC) and quality assurance (QA), we have concentrated on development of validated large-scale production and purification strategies and viral assay system for QC / QA. Summary of production and purification methods that we are planning to run under cGMP conditions is given in Figures 30-33. Currently, we are writing detailed instructions and protocols for each step in the manufacturing process known as Standard Operating Procedures (SOP).

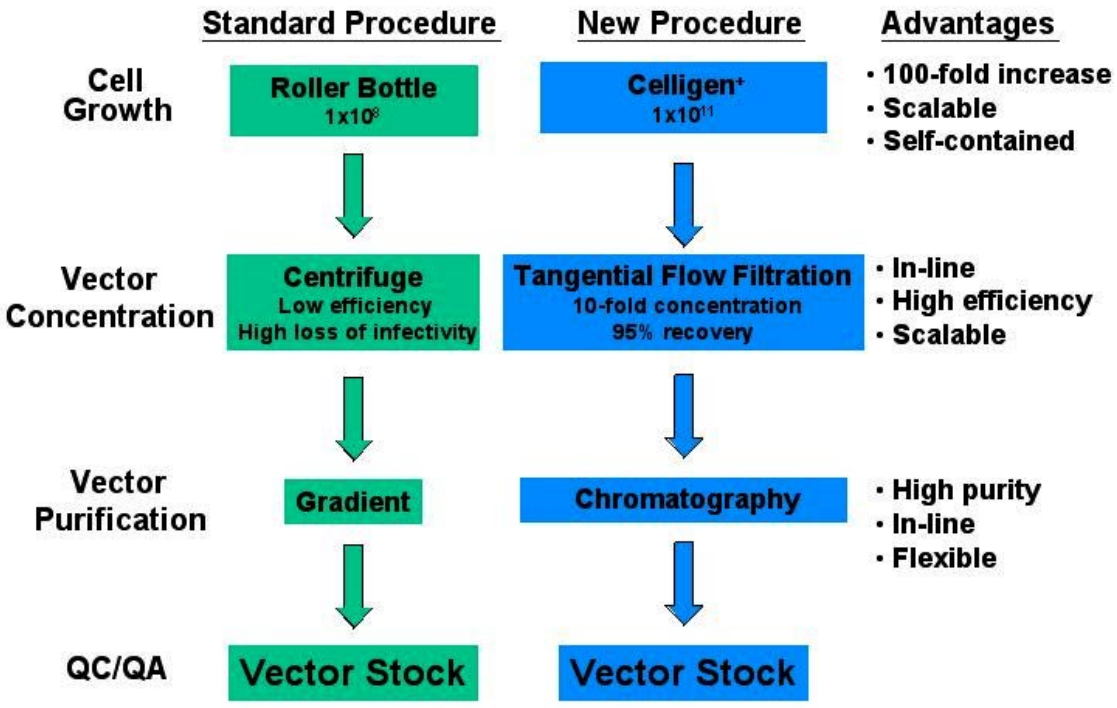


Figure 30 Comparison of Standard and New Procedures of HSV Production and Purification methods

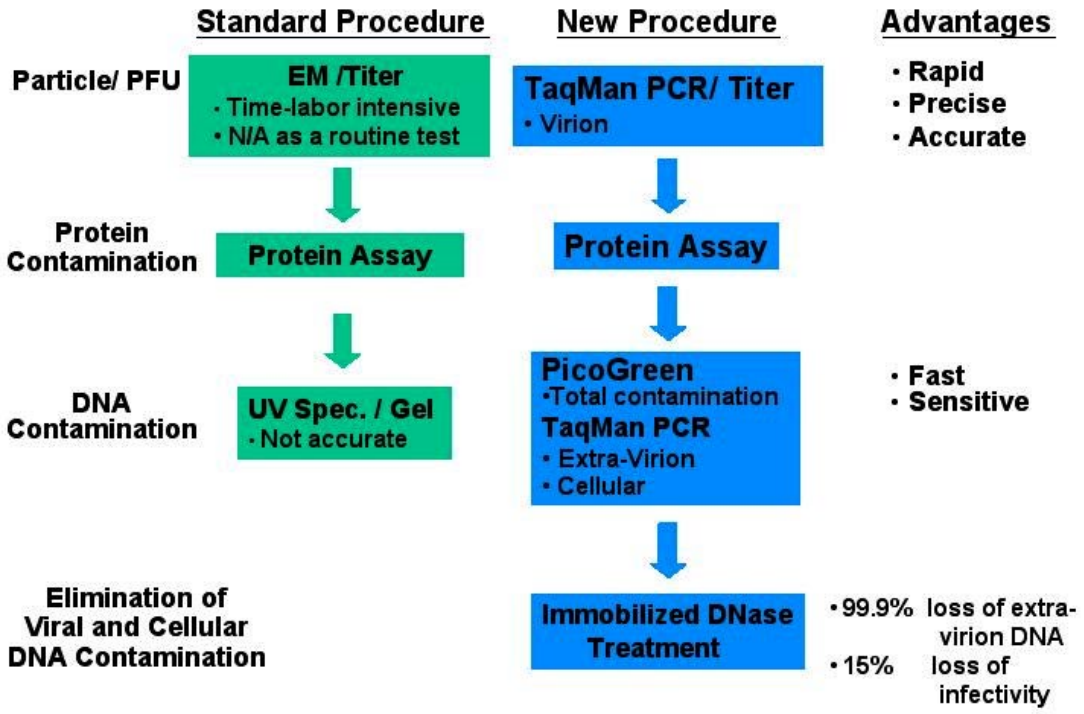


Figure 31 Comparison of Standard and New Procedures of Process and Product Control Methods

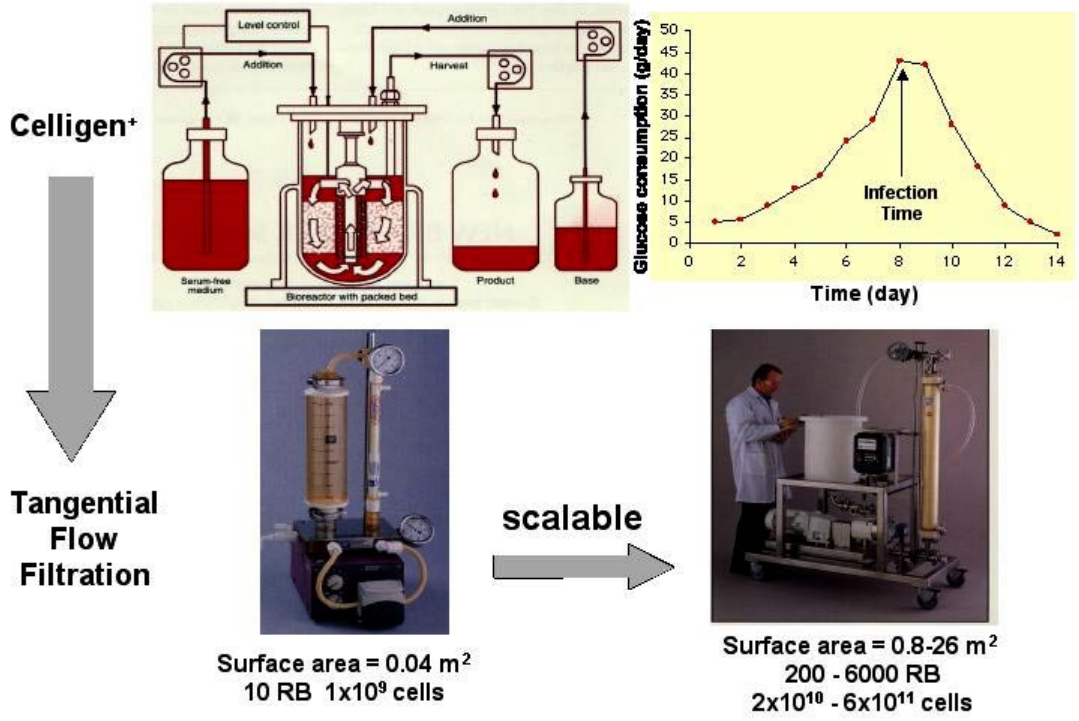


Figure 32 cGMP Production and Purification Methods

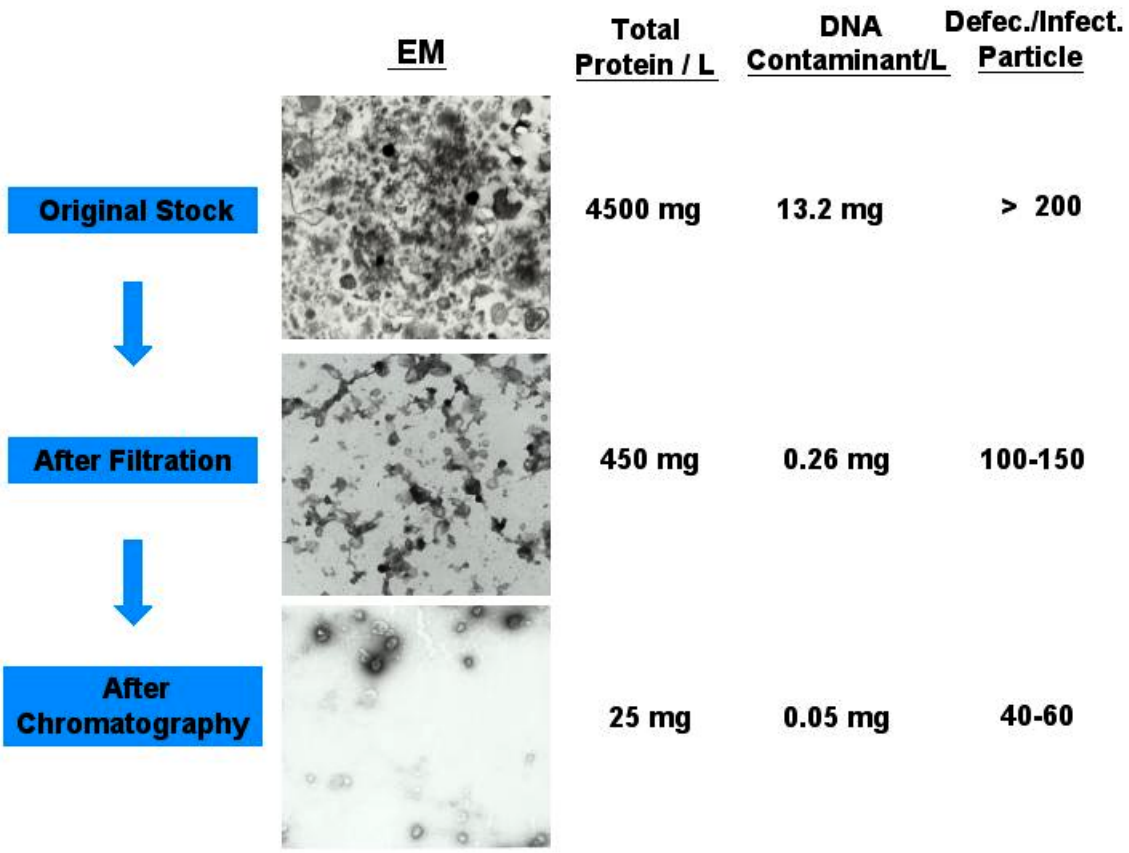


Figure 33 cGMP Purification Method and Purity Levels of Each Step

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