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Lentiviral Vector Production at High Cell Density by Transient Transfection of Suspended Culture HEK Cells

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LENTIVIRAL VECTOR PRODUCTION AT HIGH CELL DENSITY BY TRANSIENT TRANSFECTION OF SUSPENDED CULTURE HEK CELLS

by

Jacob G. Accordino

A thesis submitted in partial fulfillment
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in

Biological Engineering

Approved:

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D. Keith Roper, Ph.D. Major Professor	Charles Miller, Ph.D. Committee Member
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Logan, Utah

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ABSTRACT

Lentiviral Vector Production at High Cell Density by Transient Transfection of Suspended Culture HEK Cells

by

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Department: Biological Engineering

Lentiviral vectors deliver functional genes to combat genetic diseases in seven FDA-approved treatments, a host of clinical studies, and research in e.g., optogenetics. Economic preparation of safe lentiviral vectors in quantities adequate to meet human health needs remains an ongoing challenge. Production of plasmids used to generate and package lentivirus via transient transfection of host cells is cost-prohibitive, transfection efficiency is reduced at high cell densities, and variability in lentivirus yield is pervasive in suspension production formats. This work investigates the transfection efficiencies and lentiviral vector titers attainable by transient transfection of Human Embryonic Kidney cells – cultured to high cell density in shake flasks – using commercially sourced cells, growth medium, transfection reagent, supplement and enhancer, and packaging plasmids.

Human Embryonic Kidney cells were cultured to varying cell densities between 5.0 and 7.0 million cells per milliliter, then diluted to a density of 4.7 million cells per milliliter with fresh culture medium to initiate the transfection process. Transfection was accomplished using a proprietary cationic lipid-based transfection reagent, supplement,

and enhancer. It was found that lentivirus produced per plasmid used can be increased beyond previously reported values where vector production occurred at cell densities above 4.0 million cells per milliliter. Transfection at 4.7 million cells per milliliter supported increases in lentiviral productivity from 7.3 to 154 viral gene copies per cell and specific plasmid productivity from approximately 7.3 million to 154 million viral gene copies per microgram of plasmid, respectively, as viable cell density pre-transfection increased from 5.1 million to 6.0 million cells per milliliter. Passaging of cells at a frequency that maintains viability and mid-exponential growth prior to transfection is important to achieve such increases.

The results of the present work provide a useful guide to intensify production of lentiviral gene vectors via transient transfection at high cell densities.

(71 pages)

PUBLIC ABSTRACT

Lentiviral Vector Production at High Cell Density by Transient Transfection of Suspended Culture HEK Cells

Jacob G. Accordino

Viral vectors are gene carriers that efficiently deliver therapeutic gene constructs to target cells. Viral vectors are frequently produced in suspension-growing cells of mammalian origin, referred to as packaging cells. Lentiviral vectors have become widely used as gene vectors since they were initially developed two and a half decades ago, particularly in research settings. The advantages of lentiviral vectors for treating diseases of genetic origin have driven research into their large-scale manufacture for clinical settings. Currently, three main challenges exist that limit mass production of lentiviral vectors. First, plasmid DNA used in the production process is very expensive. Second, high vector titers have historically only been achievable at low packaging cell densities, limiting the scalability of suspended-culture technologies. And third, vector production frequently suffers from batch-to-batch variability in vector yield. In conjunction, these challenges diminish the economic viability and practical implementation of industrially-scaled processes for lentiviral vector production.

Recent advances in commercial reagents can help mitigate these stated challenges. There is currently a knowledge gap in the utility of cationic lipid-based transfection reagents for producing lentivirus in suspended cultures at high cell density.

In the present work, we investigate the production of lentiviral vectors by transient transfection of high density Human Embryonic Kidney packaging cells with a commercial cationic lipid transfection reagent. Several parameters in the production process were monitored to identify sources of variability in vector titers. We found that as packaging cells were cultured to increasing densities pre-transfection, there was a corresponding increase in lentiviral vector yield. Further, we found that aside from pre-culture density, consistency in culture routine leading up to transfection had the most significant influence on lentiviral vector yields.

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And finally, I extend special thanks to my family and loved ones. I am especially grateful for my parents who supported me emotionally, and in many instances assisted me financially throughout my higher education. Most of all, I am grateful for and indebted to my beautiful wife, whose love, patience, encouragement, and belief sustained me through the long days of experiments, the late nights of writing, and all the highs and

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Jacob G. Accordino

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LIST OF ABBREVIATIONS AND NOTATIONS

LVV	Lentiviral vector.
RT-qPCR	Reverse-transcription quantitative polymerase chain reaction. A quantitative assay for determining concentrations of ribonucleic acids.
C_q	Quantification cycle. The number of PCR amplification cycles required to reach a preset threshold of fluorescence intensity.
NTC	Non-template Control. Samples in the PCR reaction that do not contain nucleic acid.
hpt	Hours post transfection.

CHAPTER 1

INTRODUCTION

Background

Gene therapy is a branch of medical biotechnology that seeks to treat disease at the level of the gene by introducing a functional gene (transgene) into a targeted cell. The transgene acts in place of a defective gene which may be the cause of diseases like muscular dystrophy or cystic fibrosis. Methods for delivering functional transgenes into a cell are of two primary types: nonviral DNA vectors (i.e., introduction of DNA into cells by synthetic means like a liposome), and viral vectors (i.e., incorporating the functional gene into a non-replicating viral host).[1] Viruses are useful gene therapy vehicles because of their innate ability to infect cells and efficiently deliver genetic material to the cell nucleus.

Viruses as gene transfer agents are generally more efficient in delivering a functional transgene to a host cell than non-viral methods.[2] Proteins on the outer surface of the virus interact specifically with cell surface receptor. This triggers endocytosis of the virus. Subsequent delivery of the transgene to the nucleus occurs for lentivirus as well as other gene vectors. In the case of retroviruses, the viral genome is reverse transcribed into complementary deoxyribonucleic acid (cDNA), which is then capable of being integrated into the host cell genome for long-term functionality. For some retroviruses, a state of cell division, which involves degradation of the nuclear envelope, is necessary for integration.[3]

Lentiviruses are a class of retrovirus that can transduce both replicating and non-replicating cells, enabling greater gene transfer efficiency compared to other retrovirus types. Lentiviral vectors (LVVs) as gene therapy vehicles allow for stable therapeutic expression in transduced cells and provide potentially inheritable expression in daughter cells.[4] Additionally, LVVs have larger payloads, as large as 10 kb,[5] compared to other common viral vectors such as adenovirus, which are limited to 5 kb.[6]

State of the art LVVs are at a third generation of design, characterized by (1) being self-inactivating vectors through deletion of promoter sequences in the 3' long terminal repeat (LTR) region, and (2) retaining only three of the nine genes present in wildtype lentivirus.[7] In transient LVV production systems, these three genes, *gag*, *pol*, and *rev*, are typically separated onto two packaging plasmids, with a third plasmid encoding genes to envelope the viral capsid (Figure 1).[5] The transgene is incorporated into a fourth plasmid, the backbone plasmid, between the LTR sequences.[8]

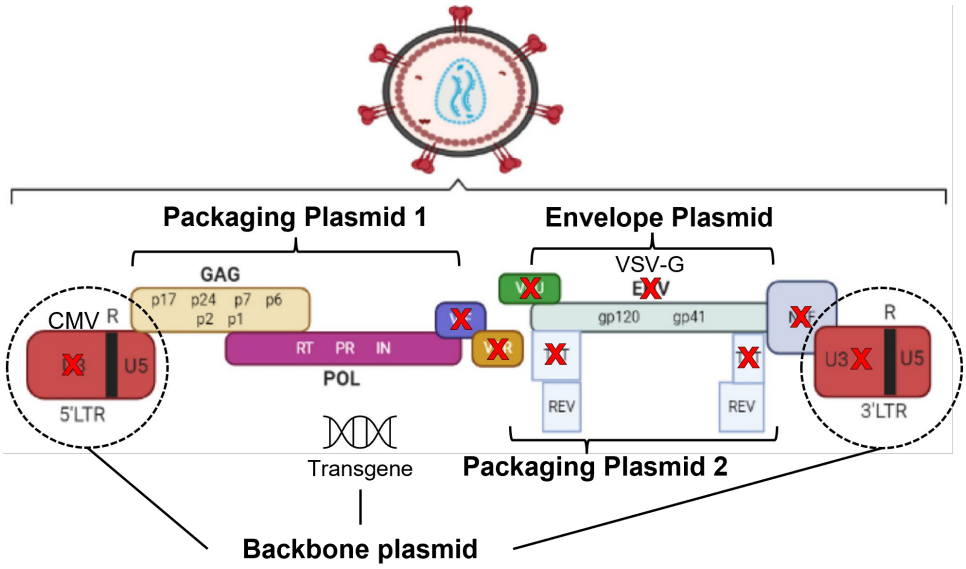

Figure 1. Third-generation lentiviral vector genome. Packaging and envelope genes from wildtype lentivirus are separated onto three plasmids with the gene of interest and promoter sequences contained in a fourth plasmid. Adapted from Ghaleh, et al..[9]

LVVs can be generated in stable producer or stable packaging cell lines. Host cells in these producer lines are often derived from Human Embryonic Kidney (HEK) cells. In stable producer cells, all genes necessary for vector production are inserted into the cells and either constitutively expressed or controlled by inducible systems.[10] In contrast, in stable packaging cells, only the gag-pol and rev genes are stably expressed, and the transfer plasmid is transiently transfected to initiate vector production.[11] While stable producer and stable packaging systems are useful for continuous generation of LVVs at high titers, they are prone to cytotoxicity from sustained expression of viral packaging and envelope genes. Moreover, their development is a slow process, resulting in lack of flexibility to changes in vector design.[12,13]

Transient transfection is an alternative to LVV production via stable producer or stable packaging cells. In this approach, LVVs are produced via transient co-transfection

of packaging and backbone plasmids into packaging cells. As with stable producer and stable packaging methods, HEK cell derivatives are the most common packaging cells. Advantages of transient transfection over stable cell methods include reduction of cytotoxicity, and greater flexibility and speed in pseudotyping or otherwise editing of vector constructs.[14] Figure 2 illustrates the differences between packaging cell formats.

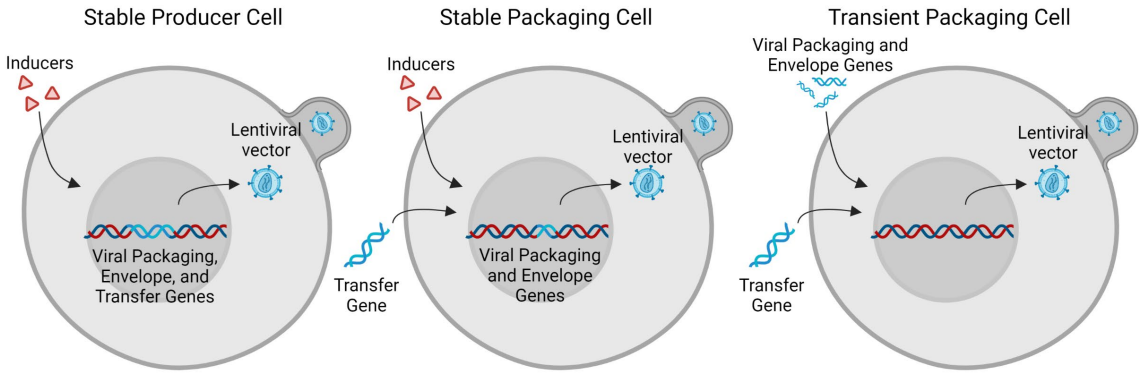

Figure 2. Comparison of lentiviral packaging cell types. Created with BioRender.com.

It is possible to produce LVV via transient transfection of packaging cells that are either adherent to a culture plate or suspended in a culture flask with the former being the most common method at developmental stages.[15] Transient transfection of suspended cells is more readily scalable to large stirred tank reactors to produce LVV at manufacturing scale. Table 1 summarizes previous reports using shake flasks for optimizing LVV production by transient transfection in batch or batch-replacement modes. Refereed reports to date all recommended initiating transfection when packaging cell densities were between 1 and 2 million cells per milliliter of medium. The most common transfection reagent used was polyethylenimine (PEI), a cationic polymer. Transfection with PEI is accomplished by mixing the negatively charged DNA vectors

with the positively charged polymer to form a positively charged complex. The complex is then added to the culture medium where the negatively charged cell membrane attracts and engulfs the DNA-polymer complex.[16] The procedure is less complicated than transfection by calcium phosphate precipitation, another common transfection method, which is highly sensitive to pH.[8] Though inexpensive and simple to use, some reports indicate transfection efficiency using PEI is lower than when DNA is complexed with a cationic lipid-based transfection reagent. This is termed lipofection. In shake flask LVV production, PEI-mediated transfection has achieved transfection efficiencies of up to 92% and reached functional LVV titers between 10^6 and 10^8 transduction-capable LVVs per milliliter (see Table 1). However, in adherent culture LVV production, lipofection can achieve transfection efficiencies between 60% and nearly 100%.[17,18] Little published data exists on the transfection efficiency of suspended culture HEK cells by lipofection for LVV production.

Currently, three main challenges exist to the large-scale manufacture of LVVs via transient transfection: (1) large batch-to-batch variability; (2) a need for large mass of plasmid to transfect the host cells; and (3) low cell densities used for transfection. Large variations in LVV titer are common when scaling production from one volume and format to another.[11,19] This occurs even in cases where transfection experiments are highly similar or identical.[20] It has been speculated that poor reproducibility in vector titers may come from the use of serum in culture medium,[21,22] transfection by calcium phosphate precipitation,[16,23] or from differences in cell physiological state between cultures.[14,19,24]

Current protocols for transient transfection typically call for 1 – 2 ug of plasmid per million cells (Table 1). For large plasmids (10,000+ bp), this means approximately 0.93 – 1.85 x 10^5 plasmid copies are required to transfect a single cell, though in reality up to 10^6 copies may be required.[25] Of those plasmids used in transfection, as few as 20 – 1,000 copies may successfully reach the cell nucleus following lipofection.[26] Finally, it has been observed that in transfection at densities higher than 1-2 million cells per milliliter, a reduction in specific productivity occurs.[19,27–29] Reduced specific LVV productivity at cell densities greater than 1 to 2 million cells per milliliter limits volumetric productivity of transient transfection. It presents a barrier to intensification of LVV production during transient transfection. This reduces the inherent scalability offered by suspended culture production formats.

The Thermo Fisher LV-MAX Lentiviral Production System could help address challenges to large-scale manufacture of LVVs. The LV-MAX platform generates LVVs through transient transfection of a suspension-adapted HEK293 packaging cell line with plasmids encoding the necessary proteins to assemble a functional viral vector. Through the use of chemically defined serum-free medium, transfection can be performed at higher cell densities (>4.0 x 10^6 cells/mL) than those previously reported in benchtop batch experiments (see Table 1). If transfection efficiency in LV-MAX media can be maintained at increased cell density, this could increase the volumetric titer of LVVs. A proprietary cationic lipid-based transfection reagent allows for high transfection efficiency of HEK cells with minimal cytotoxicity. This may enable reducing the mass of plasmid required for LVV production. The present study used the Thermo Fisher LV-MAX Lentiviral Production System to examine its potential to (1) increase the cell

density that can be efficiently transfected; (2) reduce the plasmid requirement; and (3) maintain consistent batch-to-batch LVV yield.

Table 1. Specific productivities of lentiviral vectors (LVV) or virus-like particles (VLP) produced in shake flasks by transient transfection of HEK cell derivatives.

Transfection Method	Plasmid (ug) per 10^6 Cells	Transfection Cell Density (cells/mL)	TU/mL	TU/ug plasmid	TU/cell	Ref.
PEI	1.0	5.0×10^5 1.0×10^6	1.2×10^6 2.3×10^6	*2.4×10^6* *2.3×10^6*	*2.4* *2.3*	24
PEI	1.0	1.0×10^6	8.0×10^6	*8.0×10^6*	2.0	19
PEI	*0.5*	2.0×10^6	2.8×10^6	*2.8×10^6*	*1.4*	28a
PEI	1.5	1.0×10^6 1.0×10^6	1.0×10^6 6.0×10^5	*6.7×10^5* *4.0×10^5*	*1.0* *0.6*	20
PEI	*1.2*	2.0×10^6	*2.1×10^8*	*4.4×10^6*	5.2	30a
PEI	0.6	2.0×10^6 2.0×10^6	3.7×10^8 5.3×10^7	*3.4×10^8* *4.8×10^7*	*185.0* *26.5*	27
PEI	*0.5*	2.0×10^6	*5.6×10^6*	*5.6×10^6*	*2.8*	29a
PEI	1.0	1.0×10^6	1.7×10^7	*1.7×10^7*	*17.0*	31
PEI	*2.0*	1.0×10^6	1.2×10^7	*6.0×10^6*	*12.0*	32
PEI	*1.9*	2.0×10^7	1.5×10^7	*3.95×10^5*	*0.8*	33

[a]Specific productivities in units of pg of Gag-GFP polyprotein per mL, ug plasmid, or cell.
Data are ordered by date of publication.
Italicized values were derived from reported transfection information and titers.
PEI = Polyethylenimine.

Objectives

We hypothesized that higher volumetric titers of lentivirus due to transfection at higher cell densities are supported by the ThermoFisher LV-MAX Production System using its proprietary culture medium, cationic lipid transfection reagent, transfection supplement, and transfection enhancer. The following research objectives were established to test this hypothesis:

1. Culture HEK293 cells in shake flasks to high cell densities using chemically-defined, serum-free medium.
2. Efficiently transfect HEK293 cells at high cell densities using lipofection.
3. Monitor variables in the transfection process that impact reproducibility.
4. Determine lentiviral vector titer and compare with previous reports in literature.

CHAPTER 2

METHODS

Overview

Lentiviral vectors were produced by transient transfection of HEK packaging cells in shake flasks. A schematic overview of the processes used for producing and quantifying the vectors is portrayed in Figure 3. Further details are provided in subsequent sections.

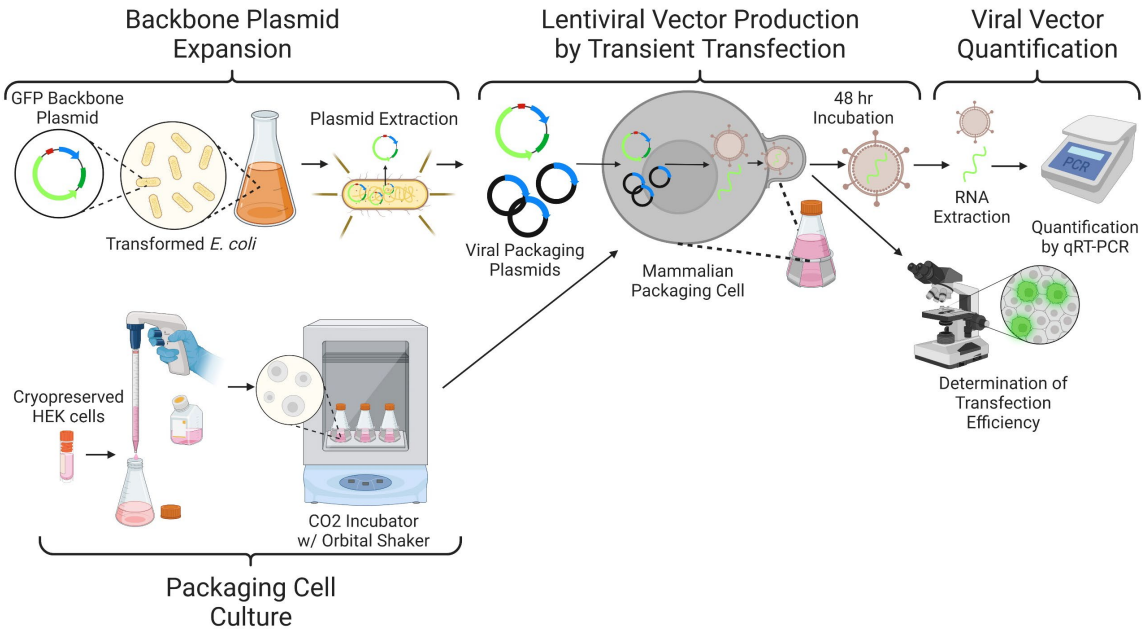

Figure 3. Schematic overview of lentiviral vector production and quantification. Created with BioRender.com

Preparation of Backbone Plasmid

The backbone plasmid, pLJM1-EGFP, was a gift from David Sabatini (Addgene plasmid # 19319; http://n2t.net/addgene:19319 ; RRID:Addgene_19319). This plasmid is

a third-generation high-copy plasmid containing genes for enhanced Green Fluorescent Protein (EGFP), puromycin resistance, and ampicillin resistance (Figure 11 – Appendix A).

The pLJM1-EGFP backbone plasmid was expanded in chemically competent DH5α cells generously provided by the Charles Miller Synthetic Biology Laboratory (Utah State University). All procedures associated with backbone plasmid expansion were performed in a class II biosafety cabinet in a biosafety level 1 laboratory.

Ampicillin and antibiotic-free LB-agar plates and TB broth were prepared according to Elbing and Brent.[34] Prior to the transformation procedure, LB plates were warmed in an incubator to 37 °C, and the chemically competent DH5α cells were thawed on ice (20-30 minutes). Stock pLJM1-EGFP (100 ng/uL) was diluted 100-fold in sterile nuclease-free water (Invitrogen, Grand Island, NY). 5 uL of diluted plasmid (1 ng/uL) was pipetted into 50 uL of competent *E. coli* in a microcentrifuge tube, and the tube was gently flicked to mix. A negative control was similarly prepared by replacing plasmid with 5 uL of nuclease free water; subsequent steps were performed to both mixtures. Plasmid was transformed into cells through heat shock by incubation in a 42 °C water bath for 45 sec., followed by immediate placement on ice for 2 min. 500 uL S.O.C. medium (Invitrogen 15544-034) was added to the cells, which were incubated for 15 min. at 37 °C in a shaking incubator at 250 RPM. 50 uL of transformed cells were pipetted onto ampicillin and ampicillin-free LB-agar plates and spread with a sterile spreader. Plates were inverted and incubated overnight at 37 °C.

Transformed colonies were present on the ampicillin plate while no colonies were visible on the negative control ampicillin plate, indicating successful transformation of

the backbone plasmid. A single transformed colony was selected from the ampicillin plate with a flame-sterilized wire loop and used to inoculate 40 mL TB broth in an autoclaved 125-mL glass shake flask. 40 uL ampicillin (100 mg/mL) was pipetted into the medium, and flasks were covered with autoclaved aluminum foil and placed in a shaking incubator (37 °C, 250 RPM) for 16 – 18 hours. Plasmid was extracted from bacterial fermentations by using a plasmid extraction kit.

Plasmid Extraction

High purity plasmid preparations were extracted from 100 mL bacterial fermentations (OD600 3.0-4.0) using the PureLink Expi Endotoxin-Free Maxi Plasmid Purification Kit (Thermo Fisher A31217) per the manufacturer's instructions (centrifuge method) with some optimized adjustments: Terrific broth (TB) was used to culture bacteria to maximize plasmid production. The harvested cell pellet was resuspended by inversion rather than by vortexing. A sample-free duplicate was prepared as a DNA-binding column pre-wash by combining the resuspension, lysis, precipitation, and endotoxin removal buffers in the same ratios used for the harvested cell pellet. The pre-wash was passed through a clean lysate clarification column, then added to the DNA-binding column and pulse-centrifuged until the entire column was wetted. Clarified bacterial lysate was loaded onto the column when only a sliver of pre-wash remained above the column header. The binding column was washed twice after loading with lysate. DNA was eluted by first centrifuging at 500 *x g* for 1 min, followed by 2,000 *x g* for 2 min. After elution, 0.7 times the sample volume of isopropanol was added and the sample was partitioned into microcentrifuge tubes, then centrifuged at 16,000 *x g* for 30 min. Samples were chilled overnight at -20 °C to enhance nucleic acid precipitation. The next day, samples were re-

centrifuged for 1 min. The supernatant was carefully discarded by micropipette. Pellets were washed with 70% ice-cold ethanol then re-centrifuged. The supernatant was carefully discarded, and nucleic acid pellets were air dried for 10 minutes. Pellets were resuspended in 10 mM tris buffer (pH 8.0)

Total DNA yields and purities were approximated by measuring sample absorbance at 260 and 280 nm. Backbone plasmid concentration was determined by quantitative PCR as described below.

After initial transformation, glycerol stocks of the transformed *E. coli* were prepared by adding 500 uL of overnight E. coli culture to 500 uL of 50 % glycerol solution (50% glycerol and 50% nuclease-free water). Glycerol stocks were stored at – 80 °C. Ampicillin plates with transformed *E. coli* were stored at 4 °C for up to 1 month and used for repeated preparations of backbone plasmid. Plates were discarded after 1 month, and new plates were prepared by partially thawing glycerol stocks and streaking cells onto the plates with a flame-sterilized wire inoculating loop.

HEK Cell Culture

All cell culture manipulations were conducted in a class II biosafety cabinet in a biosafety level 2 laboratory. Vials of cryopreserved HEK293F Viral Production Cells (Life Technologies, Grand Island, NY) were thawed in a water bath at 37 °C, then transferred by pipet into chemically defined, serum- and protein-free medium (LV-MAX™ Production Medium, Life Technologies). Cells were grown in 125-mL single-use PETG shake flasks (Thermo Fisher Scientific, USA, Cat. No. 41150125) in a high-humidity incubator set to 37 °C and 8% CO_2. Flasks were agitated on a 1.9 cm orbital shaker (Thermo Fisher Scientific, Cat. No. 88881103) at 110 rpm in the incubator. Viable cell density and

culture viability were determined by counting cells stained with 0.4% Trypan Blue (3- to 5-fold dilution) on a hemocytometer (200x magnification under inverted microscope). Cell density, viability, and growth rate were routinely determined in preparation for transfection experiments.

Cell culture was maintained according to the LV-MAX User Manual. Briefly, cells were counted every 3 – 5 days to determine cell density. Cells were typically passaged when a density of 3.5 – 5.5 x 10^6 cells/mL was reached by either directly diluting the culture with fresh medium, or by transferring cells to a new flask with fresh medium. Cells were cultured in 25 to 45 mL of medium and typically seeded at densities between 0.35 – 0.55 x 10^6 cells/mL. Cultures were discarded after approximately 40 passages.

Cells were regularly cryopreserved to maintain adequate stocks for viral vector production. Cultures that reached high cell densities (>3.5 x 10^6 cells/mL), exponential growth rates (doubling time approximately 26 – 32 hours), and high viabilities (>95%) were used for preservation. Cultures were centrifuged at 100 *x g* for 5 min. in a 50 mL centrifuge tube. Culture supernatants were removed by serological pipet, then cell pellets were resuspended by gentle inversion in sterile cryopreservation medium (10% v/v DMSO in LV-MAX production medium, 0.22 uM filtered) to a final density of 1 x 10^7 cells/mL. 1 mL aliquots were transferred to cryovials and frozen at – 80 °C for one day, then transferred to liquid nitrogen vapor space for long-term storage.

Viral Production by Transient Transfection

HEK293F Viral Production cells were transfected with the pLJM1-EGFP backbone plasmid expanded in *E. coli*, and packaging plasmids provided in the LV-MAX transfection kit (Thermo Fisher, A43237). Transfection was performed based on the LV-

MAX User Manual. Briefly, cells were cultured 3-6 days to a high density in LV-MAX Production Medium and diluted to 3.5-4.0 x 10^6 cells/mL with fresh medium at least 24 hours prior to transfection. The day of transfection, cell densities of several cultures were determined, and 2-4 cultures were combined at various ratios to achieve mixed densities of 5.0 – 7.0 x 10^6 cells/mL. Mixed cultures were then diluted with fresh medium in a new flask to 25.5 mL at a density of 4.7 x 10^6 cells/mL. 1.5 mL LV-MAX Supplement was added to diluted cultures, which were then returned to the orbital shaker in the incubator. Packaging and backbone plasmids were combined at a ratio of 3:5 in 1.5 mL OptiMEM I Reduced Serum Medium. Total specific plasmid mass used was 1.0 ug/10^6 cells to be transfected. 180 uL LV-MAX Transfection reagent (6 uL/mL) was mixed into 1.5 mL OptiMEM I Reduced Serum Medium and incubated at room temperature for 1 minute. The combined DNA was then pipet mixed with the transfection reagent and incubated for at least 10 minutes at room temperature to facilitate complexation of the plasmids and the lipid reagent. The DNA-lipid complex was then pipet mixed with the previously prepared flask of cells. Transfected cell cultures were then transferred to a separate BSL II lab to avoid cross-contamination of LVVs with non-transfected cell culture. The transfected cultures were placed on a 19-mm orbital shaker in an incubator under the same operating conditions as described previously. Two negative control groups were also prepared during each transfection experiment by transfecting with either packaging or backbone plasmids only. At between 5 and 14 hours post transfection (hpt), 1.2 mL of LV-MAX Enhancer was pipet mixed into each culture. At 46 – 49 hpt, the transfected cultures were harvested, and transfection efficiencies and LVV titers were determined.

Determination of Transfection Efficiency by GFP Expression

Expression of Enhanced Green Fluorescent Protein (EGFP) in transfected cells was determined at approximately 48 hpt to assess transfection efficiency. A 50 uL sample of transfected cell culture was diluted 1:10 in Hoechst 33342 nuclear stain (5 ug/mL) and incubated at room temperature for 5 min. Hoechst 33342 is a cell-permeant stain that has peak excitation and emission wavelengths at 350 and 461 nm, respectively. The blue emission can be detected through a DAPI filter. Stained cells were loaded into EVE disposable counting slides (NanEnTek, EVS-050) and imaged at 40x magnification using the Cytation 1 multi-mode plate reader (Biotek, VT, USA). Two randomly selected areas of each cell sample were imaged using the Brightfield detection channel as well as through DAPI and GFP filters. Camera exposure settings are listed in Table 2 below.

Table 2. Cytation 1 camera exposure settings for imaging of transfected cells.

Parameter	Brightfield	DAPI	GFP
LED Intensity	4	8	10
Integration Time (ms)	34	1000	800
Camera Gain	1	1	1

Cellular analysis was conducted using the Gen5 software to approximate total cell counts (DAPI detection channel) and GFP-expressing cell counts (GFP detection channel). Detection threshold was set to 13,000 intensity, primary edge objects were excluded from the count, and object size was set to 10-75 uM. Transfection efficiency was determined as the ratio of GFP-expressing cells to the total number of cells present in a sample image.

Determination of Lentiviral Vector Titer by RT-qPCR

RNA was extracted from transfected cell culture supernatants to approximate non-functional viral titer through reverse-transcription quantitative PCR (RT-qPCR).

RNA Extraction

The RNA extraction protocol was an adaption of Roper and VanRenslaar (in preparation), which provides a method of extracting viral RNA from natural environments by spin column extraction. Briefly, at approximately 48 hpt transfected cell cultures were centrifuged at 4696 *x g* for 10 minutes in a swinging bucket centrifuge at 4 ^{o}C. Transfected cell culture supernatants were transferred by pipet into 50 mL conical tubes containing 7.0 g NaCl and 300 uL TE Buffer (1 M Tris, 0.1 M EDTA, pH 7.2). The total volume of each supernatant was recorded prior to addition to the salt tubes (approximately 30 mL). Salinated supernatants were inverted to mix, then incubated in a 60 ^{o}C water bath for 1 hour to inactivate lentiviral particles. Samples were inverted again at 30, 45, and 60 minutes into inactivation to ensure total dissolution of the salt. Following lentiviral inactivation, supernatant samples were re-centrifuged to pellet residual debris. 7-10 mL of the inactivated supernatant was transferred to a new tube, and 100% ethanol was added dropwise while swirling to a concentration of 35% v/v. Samples were gently inverted 5-10x to mix.

A daisy chain assembly of two RNA-binding spin columns was connected to a vacuum manifold. 4 mL pre-wash was loaded into the column reservoir to prewet the spin columns. 5-10 mL of the ethanol containing sample was loaded into the reservoir before the column dried free of prewash. Reservoirs were covered with parafilm after adding sample to prevent ethanol evaporation. 2 mL of Wash Buffer 1 was added to the

column before it was dried, then 3 mL of Wash Buffer 2 (WB2) was similarly added, but the column was vacuumed dry. The primary spin column loaded with RNA was removed from the daisy chain assembly and inserted into a collection tube, then centrifuged at 10,000 *x g* for 2 minutes to elute residual WB2. The collection tube was discarded, and the spin column was heated uncapped in a culinary forced-convection oven at 160 ^{o}F for 60 seconds to evaporate residual ethanol. Spin columns were placed in sterile elution tubes, then 50 uL of 10 mM Tris (pH 8.0) prewarmed to 70 ^{o}C in a water bath was added to the column. The column was centrifuged for 1 minute at 0.5 *x g* to allow absorption but not elution of tris through the column. The column was reheated in the convection oven then centrifuged at 10,000 *x g* for 2 minutes to elute and collect the RNA. A second elution was pooled with the first by adding 100 uL of Tris to the column and repeating the centrifugation and heating steps. Total RNA concentrations in each sample were estimated by spectrophotometry.

Reverse-transcription qPCR

LVV gene copies in transfected cell cultures were quantified via RT-qPCR on a QuantStudio 3 PCR machine (Applied Biosystems). RNA extracts from culture supernatants were treated with DNase (Fisher Scientific, EN0525) according to manufacturer recommendations to degrade residual plasmid DNA carried over from the transfection process. The qScript One-Step SYBR Green RT-qPCR kit (Quantabio, 95089-200) was used as the reaction master mix. Lyophilized primers were ordered from Integrated DNA Technologies and were rehydrated in nuclease free water to a 100-uM stock concentration. Working concentration of primers in the master mix was 0.2 uM. Primer sequences were those identified by Butler et al. located in the 5’ LTR of the viral

genome,[35] and had the following positions and sequences on the pLJM1-EGFP backbone plasmid: Forward primer (103–122 bp) TGTGTGCCCGTCTGTTGTGT; Reverse primer (245–226 bp) GAGTCCTGCGTCGAGAGAGC. Total qPCR reaction volume contained 4.0 uL of DNase-treated RNA (diluted to approximately 1 ng/uL total RNA in 10 mM Tris, pH 8.0) and 6.0 uL of master mix prepared according to manufacturer recommendations. Non-template controls (NTCs) were prepared by substituting RNA samples with 10 mM Tris. Stock of pLJM1-EGFP plasmid (10 ng/uL) was serially diluted in 10 mM Tris from 1.2 ng/uL to 7.5 x 10^{-6} ng/uL to contribute five known concentrations of a calibration curve. The PCR reaction was as follows: Reverse Transcription stage – 10 min at 50 ^{o}C; PCR Stage – 40 cycles of 10 sec at 95 ^{o}C followed by 30 sec at 60 ^{o}C; Melt Curve Stage – 60 ^{o}C increased to 95 ^{o}C at 0.15 ^{o}C/s.

Plasmid copy number was calculated from plasmid mass based on length, assuming a molecular weight of 660 Da for dsDNA (see Equation 1 in Appendix A). Viral gene copies were considered analogous to plasmid copies such that LVV gene copies in a sample were determined by direct comparison to the quantification cycles of the backbone plasmid calibration curve. This PCR reaction method was also used to quantify backbone plasmid extracts.

Statistical Analysis

One-way ANOVA with Tukey's post hoc analysis was used to identify statistically significant relationships (p-value <0.05). Analysis was performed using SAS OnDemand for Academics.

CHAPTER 3

RESULTS

Determination of LVV Titer and Transfection Efficiency

Measurement of vector genomes in transfected culture supernatants through RT-qPCR provides a simple and rapid approach to quantification of LVV titer.[36] In our experiments, viral RNA was extracted from vector supernatants 48 hours post transfection (hpt) and quantified by RT-qPCR. A known concentration of backbone plasmid was used as the standard for the PCR assay and provided a reliable calibration curve across all experiments. Figure 4 shows the average quantification cycles (C_q) for the initial template concentrations in the calibration curve. Due to the exponential nature of PCR amplification, the base 10 logarithms of the template concentrations are graphed to linearize the calibration curve.

Amplification efficiency in a PCR reaction provides a measure of exponential replication, where 100% efficiency represents a 2-fold increase in nucleic acid concentration per cycle.[37] The average amplification efficiency across 10 PCR assays was 91.55%. Amplification efficiency was calculated using Equation 2 (Appendix A).

No degradation of the plasmid standard was observed over the duration of the transfection experiments. Non-template controls (NTCs) were used to detect PCR contamination and primer dimers. $C_q \geq 30.00$ for NTCs was considered the limit for accepting assay data.

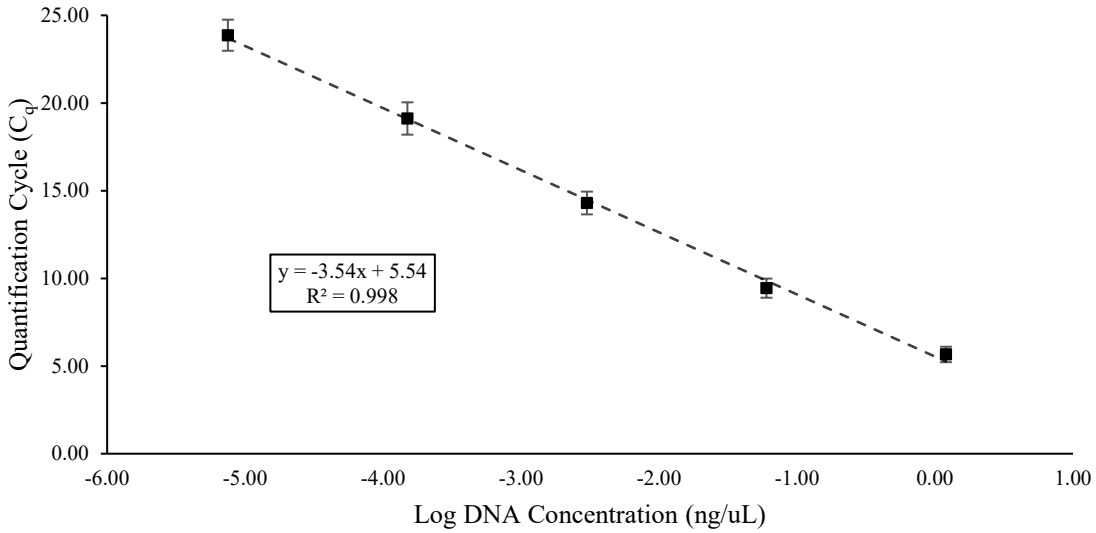

Figure 4. Average quantification cycles (Cq) for pLJM1-EGFP backbone plasmid calibration curve. Values represent the average of 10 RT-qPCR experiments with Cq standard deviations indicated as y-axis error bars. Average amplification efficiency was 91.55%.

The backbone used for this study was a simple reporter plasmid with a Green Fluorescent Protein (GFP) transgene. For some experiments, transfection efficiency was approximated by comparing GFP-expressing to total cell densities at 48 hpt. Measuring the percentage of GFP-expressing cells relative to total cells post-transfection, such as by flow cytometry or fluorescent microscopy, is commonly used to approximate transfection efficiency in LVV production.[38,39] Transfected cell culture samples were stained with Hoescht 33342 nuclear stain, loaded into a counting chamber, and viewed through DAPI and GFP filters in a Cytation 1 multi-mode imager. Gen5 software was used to automatically enumerate total cells (DAPI filter) and GFP-expressing cells (GFP filter) in a sample. Cell density was calculated by dividing the number of total or GFP-expressing cells in a sample by the depth of the chamber and the area of the image. The fluorescent

imager and software approximated cell density within 10% of the standard Trypan Blue exclusion assay and a hemocytometer (Figure 5).

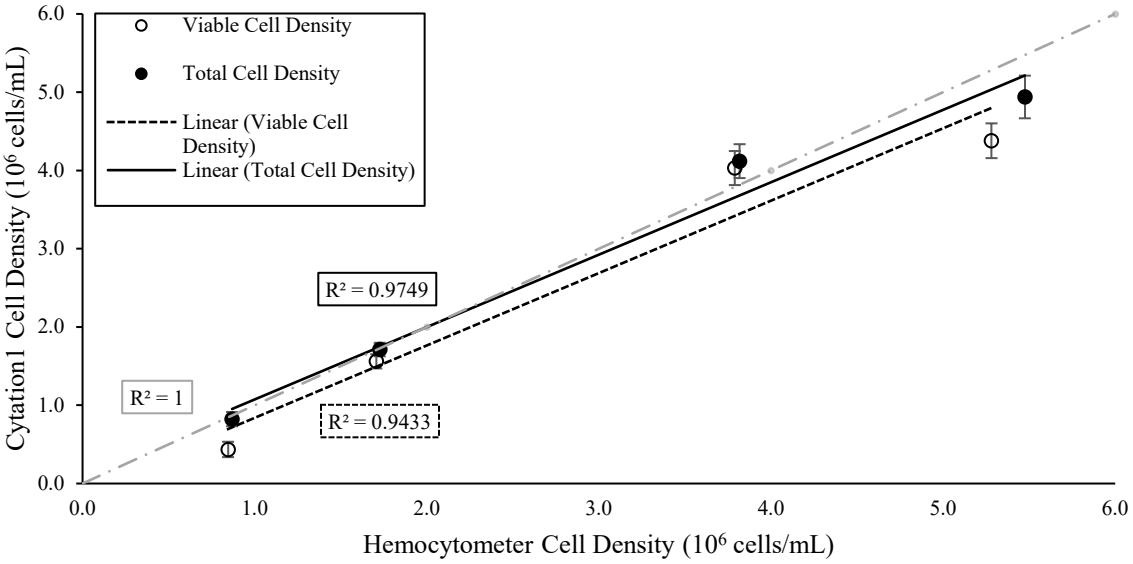

Figure 5. Comparison of cell densities determined by fluorescent microscopy and software to standard hemocytometer method. Duplicate samples of cell culture were stained with Hoechst 33342 (cell membrane-permeant) and SYTOX Green (cell membrane-impermeant) fluorescent nuclear stains, then imaged using a Cytation 1 multi-mode imager through DAPI and GFP filters, respectively. Gen5 software was used to generate total cells counts from DAPI images and dead cell counts from GFP images. Viable cell count was taken as the difference between total and dead cell counts. Cell counts were converted to density by dividing by the volume of the counting chamber. Error bars represent the standard deviation between cell counts for 6 images (3 images per cell culture sample). Total and viable cell densities were compared to densities determined by Trypan Blue exclusion assay with a hemocytometer. A line y=x representing perfect agreement between methods is also graphed for reference.

In addition to co-transfection of backbone and packaging plasmids into suspended culture HEK cells, control groups were included that were transfected with either backbone or packaging plasmids alone. Transgene expression on the backbone plasmid is controlled by the CMV promoter, which allows for constitutive expression in mammalian cells and high levels of expression specifically in HEK cell lines.[40] Consequently, GFP

expression was observed in cultures transfected with both backbone and packaging plasmids and cultures transfected with backbone plasmid only; no GFP expression was observed in groups transfected with packaging plasmid only (Figure 6). Slightly higher levels of GFP expression were observed for cultures transfected with both packaging and backbone plasmids over cultures containing backbone plasmid only. This suggests functional viral particles were produced in the cultures transfected with both plasmids, resulting in the infection and subsequent transgene expression of some non-transfected cells.

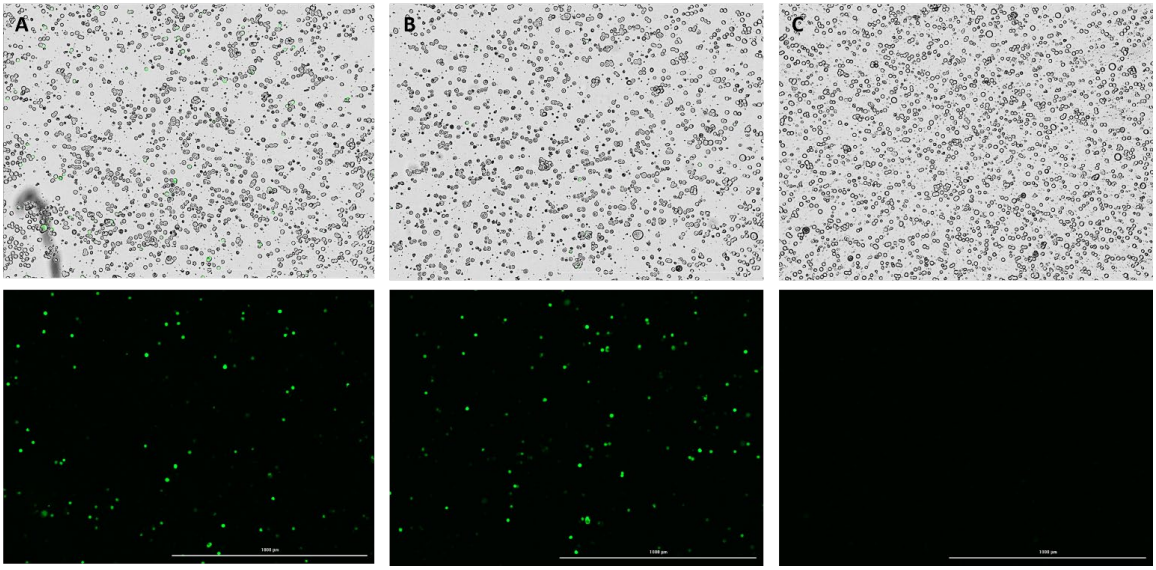

Figure 6. Expression of enhanced green fluorescent protein (GFP) in transfected HEK cells 48 hours post transfection. Images depict cultures transfected with (**A**) packaging and backbone plasmids, (**B**) backbone plasmid only, or (**C**) packaging plasmids only. Reduced levels of GFP expressing cells are identified in (**B**) (7.82%) compared to (**A**) (9.12%), while no expression is identified in sample (**C**). Cells were imaged using a Cytation 1 multi-mode imager at 40x magnification in Brightfield mode (upper images), DAPI mode (not shown), and GFP mode (lower images). All cultures used in this experiment were split from a mixed culture at 6.8 x 10^6 cells/mL prior to transfection.

LVV Titer Increases with Cell Density Pre-transfection

To examine the effect of cell density prior to transfection on LVV titer and transfection efficiency, cells were cultured to a high cell density, pooled with other flasks to provide homogeneous cultures, then diluted with fresh medium to 4.7 x 10^6 cells/mL to begin transfection.

Figure 7A shows viable cell densities (VCDs) prior to dilution for transfection and associated LVV titers 48 hpt of seven separate transfections. Up to 6.0 x 10^6 cells/mL, there was a positive correlation between increasing cell density and increasing LVV titer, with a 6.3-fold increase in LVV titer as VCD increased from approximately 5.0 to 6.0 x 10^6 cells/mL. Transfection experiments with statistically significant differences in LVV titer are indicated by brackets with the associated p-value. Above 6.0 x 10^6 cells/mL, LVV titer began to decline. Prior to transfection all cultures used maintained viabilities greater than 90% at the high cell densities. LVV gene copies collected 48 hpt per viable cell at the start of transfection increased from approximately 7.3 to 154.1 as cell density pre-transfection increased from 5.1 to 6.0 x 10^6 cells/mL.

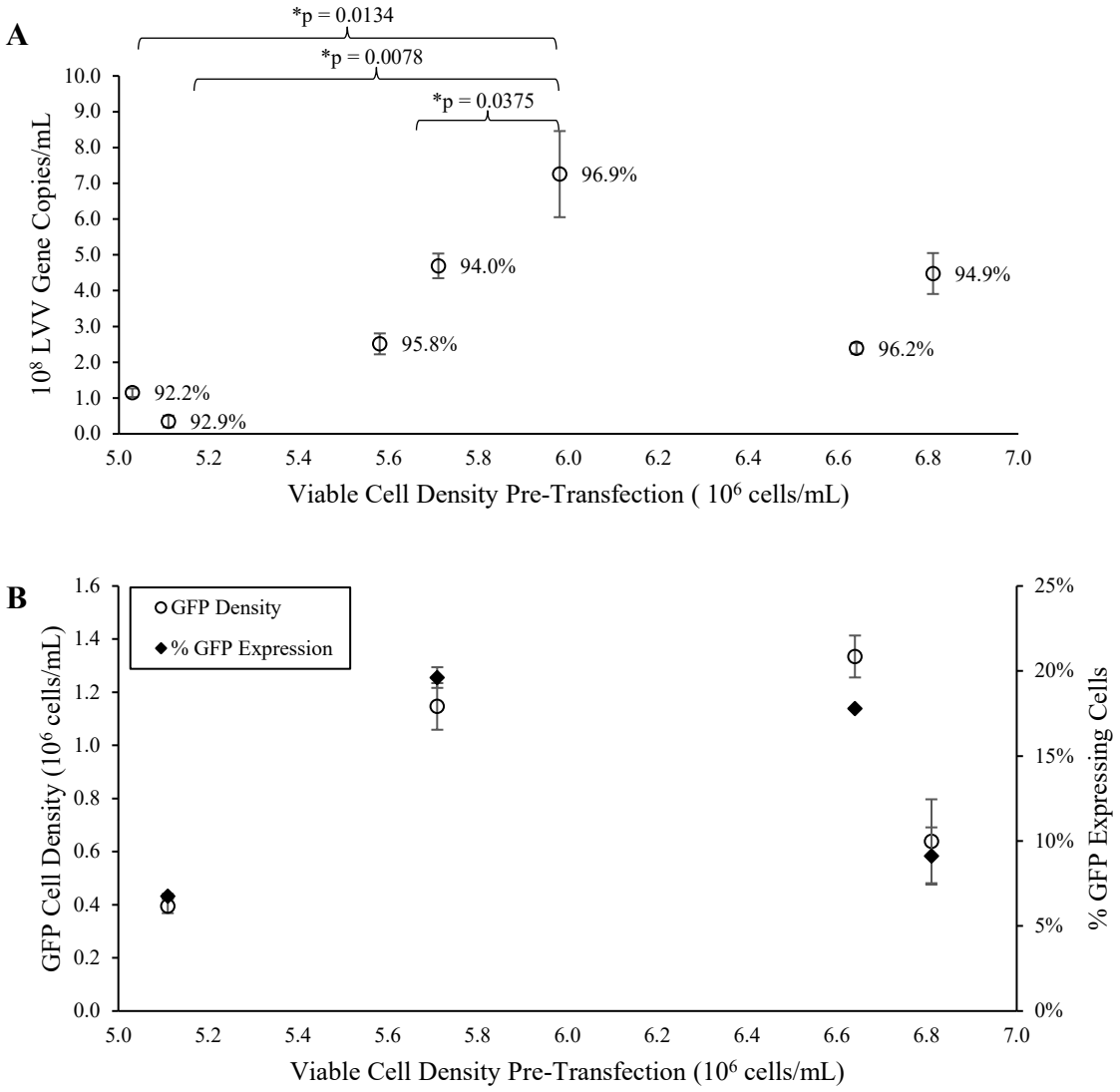

Figure 7. Relationship between viable cell density (VCD) prior to transfection to (A) LVV titer and (B) transfection efficiency. X-axis cell densities represent the combined VCD of 2 to 4 cell cultures mixed on the day of transfection before being diluted with fresh growth medium to the final density at which cells were transfected (4.7 x 10^6 cells/mL). (**A**) Percent viabilities for the combined cultures are reported adjacent to data markers. Vertical error bars represent the standard deviation of technical replicates from qPCR. Asterisks and brackets indicate significantly different titers and their associated p-values for VCDs less than 6.0 x 10^6 cells/mL. (**B**) % GFP expression was determined as the ratio of GFP expressing cells to total cells in a sample 48 hpt. Vertical error bars represent the standard deviation of duplicate images from the sample of a given cell culture.

Transfection of HEK cells cultured to high cell densities prior to transfection can increase volumetric expression of the transgene but may not improve transfection efficiency. Of the experiments where GFP-expressing and total cell densities were measured 48 hpt, the highest measured transfection efficiency was 19.6% from a culture with a pre-transfection density of 5.7 x 10^6 cells/mL (Figure 7B). The highest GFP-expressing cell density observed was 1.3 x 10^6 cells/mL from a culture with a pre-transfection density of 6.6 x 10^6 cells/mL. The density of GFP-expressing cells increases with increasing VCD pre-transfection up to about 6.6 x 10^6 cells/mL and then declines, suggesting that volumetric productivity as measured by GFP expression has a positive relationship with VCD pre-transfection up to 6.6 x 10^6 cells/mL. Percent GFP expression, however, decreases slightly at pre-transfection VCDs greater than approximately 6.0 x 10^6, indicative of lower transfection efficiency.

A comparison of cell densities pre-transfection to total cell densities 48 hpt is provided in Table 3. Note that viable density post-transfection was not determined, as the method for determining VCD using the fluorescent microscope required use of a green-fluorescent nuclear stain, which was indistinguishable from cells expressing GFP protein. Cells were diluted to 4.7 x 10^6 cells/mL to initiate transfection. After 48 hours, total cell densities had recovered to ±8.0% of their value before being diluted for transfection.

Table 3. Comparison of total cell densities prior to transfection to total cell densities 48 hours post-transfection.

VCD Pre-tfxn (10^6 cells/mL)	TCD Pre-tfxn (10^6 cells/mL)	TCD 48 hpt (10^6 cells/mL	% Difference in TCDs
5.1	5.5	5.9	5.9
5.7	6.1	5.9	-3.9
6.6	6.9	7.5	8.0
6.8	7.2	7.0	-2.3

Tfx = Transfection. VCD = Viable Cell Density. TCD = Total Cell Density.
Data are arranged by increasing VCD pre-transfection.

Specific LVV Productivity Increases with Cell Density Pre-transfection

Comparison of lentiviral vector titration methods has shown that measurement of viral gene copies in transfected culture supernatants by RT-qPCR overestimates functional viral titers.[36] The ratio of viral gene copies to transducing units ranges from approximately 10^2 to 10^4.[33,36,41,42] Our method of extracting RNA from vector supernatants includes a heat incubation step to inactivate viral particles, binding of RNA to spin columns, treatment of RNA preparations with DNase, and subsequent enzyme inactivation by both heating and EDTA addition prior to PCR. These processing steps diminish the risk of residual plasmid DNA from transfection contaminating the vector RNA preparations and inflating RNA titers. Additionally, our use of a plasmid standard for PCR likely underestimates viral RNA titers due to inherent inefficiencies of the reverse transcription step.[42] If the reverse transcription step isn't 100% efficient, less complimentary DNA will be present at the start of the PCR amplification cycles than what corresponds to the LVV RNA. Since the standard was plasmid, it would not require reverse transcription, so all plasmid present in the sample will presumably be amplified, thus underestimating RNA in the sample. Therefore, for the purpose of comparing our

results to those of others, we will assume a conservative ratio of 10^2 viral gene copies to an equivalent transducing unit (TU).

Specific volumetric, biomass, and plasmid productivities were calculated from equivalent TUs (see Equations 3-5 in Appendix A). Specific volumetric productivity is the ratio of TUs 48 hpt to the total culture volume at the time of transfection. Specific biomass productivity is the ratio of TUs 48 hpt to the number of viable cells present at the start of transfection. Specific plasmid productivity is the ratio of TUs 48 hpt to the mass of plasmid used to perform the transfection.

While the total volume and number of cells transfected were maintained between experiments, specific plasmid productivity increased from 7.3 x 10^6 to 1.5 x 10^8 viral gene copies per microgram of plasmid. This is equivalent to 0.73 x 10^5 TU/ug plasmid to a maximum of 1.5 x 10^6 TU/ug plasmid as the cell density pre-transfection increased from ~5.0 x 10^6 cells/mL to 6.0 x 10^6 cells/mL (Figure 8A). The important implication is that specific plasmid productivity can be increased 20-fold by simply culturing cells to higher densities (in our case up to 6.0 x 10^6 cells/mL) prior to diluting to the final density for transfection with fresh medium. Because volume and cell density at transfection were held constant, increases in specific biomass and volumetric productivities were proportional to specific plasmid productivity.

The highest volumetric productivity we observed (7.3 x 10^6 TU/mL) was similar to or higher than several previous reports, while specific plasmid and specific biomass productivities 1.5 x 10^6 TU/ug plasmid and 1.5 TU/cell, respectively, were generally lower (Figure 8B).

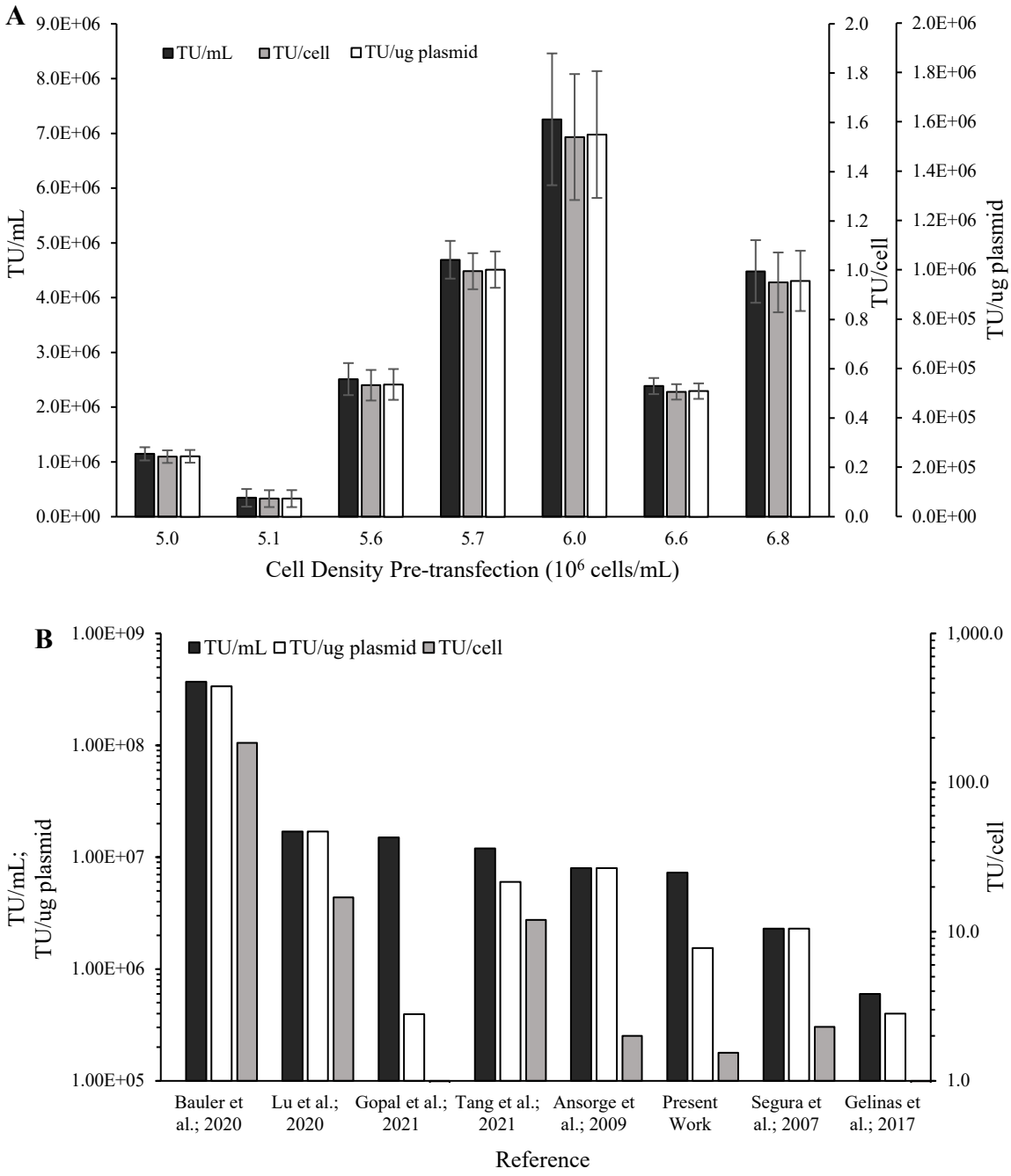

Figure 8. Comparison of Specific LVV Productivities. (**A**) Results of the present work compared to cell density pre-transfection. Transducing units were approximated from vector RNA titer, and specific volumetric, biomass, and plasmid productivities were calculated. (**B**) Specific productivities from studies where LVV production was performed in shake flasks. Productivities are calculated on the same basis as described in Equations 3-5 of Appendix A, except that Tang et al., Segura et al., and Gelinas et al. measured LVV titer 72 hpt instead of 48 hpt.[20,24,32] Optimized conditions are reported (see Table 1) and organized left to right by decreasing TU/mL. Studies on the production of lentivirus-like particles are excluded from this comparison.

Passage Routine Pre-transfection Affects LVV Titer

It is understood that cell physiological state can significantly influence transfection efficiency. Specifically, highest levels of transgene expression are strongly correlated with active cell division by cationic lipid-mediated transfection[43] as well as by polymer-mediated transfection.[44] To elucidate the relationship between high cell density and LVV titer, cell growth rate leading up to transfection was evaluated for all cultures used in transfection.

During routine cell culture, when cells are introduced to fresh growth medium a lag in growth rate is temporarily experienced as the cells acclimate to the new medium. We observed that the growth rate for HEK293 cells typically decreased to between 0.25 and 0.50 population doublings per day within 24 hours of dilution with fresh medium (data not shown) but recovered to maximum growth rates of 0.80 to 0.90 population doublings per day by day 3 following subculture. Figure 9A shows the velocity and VCDs of a culture used for a transfection where time between passages did not exceed 4 days. One day prior to transfection, the culture was diluted with fresh medium to a density of 4.0×10^6 cells/mL. On the day of transfection, VCD and growth rate were determined, then the culture was pooled with other cultures maintained on similar passaging schedules. The VCD of the combined cell cultures was 5.6×10^6 cells/mL prior to diluting with fresh medium for transfection. The pattern of velocity seen in this example was typical of other cultures whose combined density was less than 6.0×10^6 cells/mL pre-transfection. Notably, cell growth rate declined on the day of transfection in cultures partially diluted with fresh medium one day pre-transfection, similar to the lag cells experienced during routine passaging.

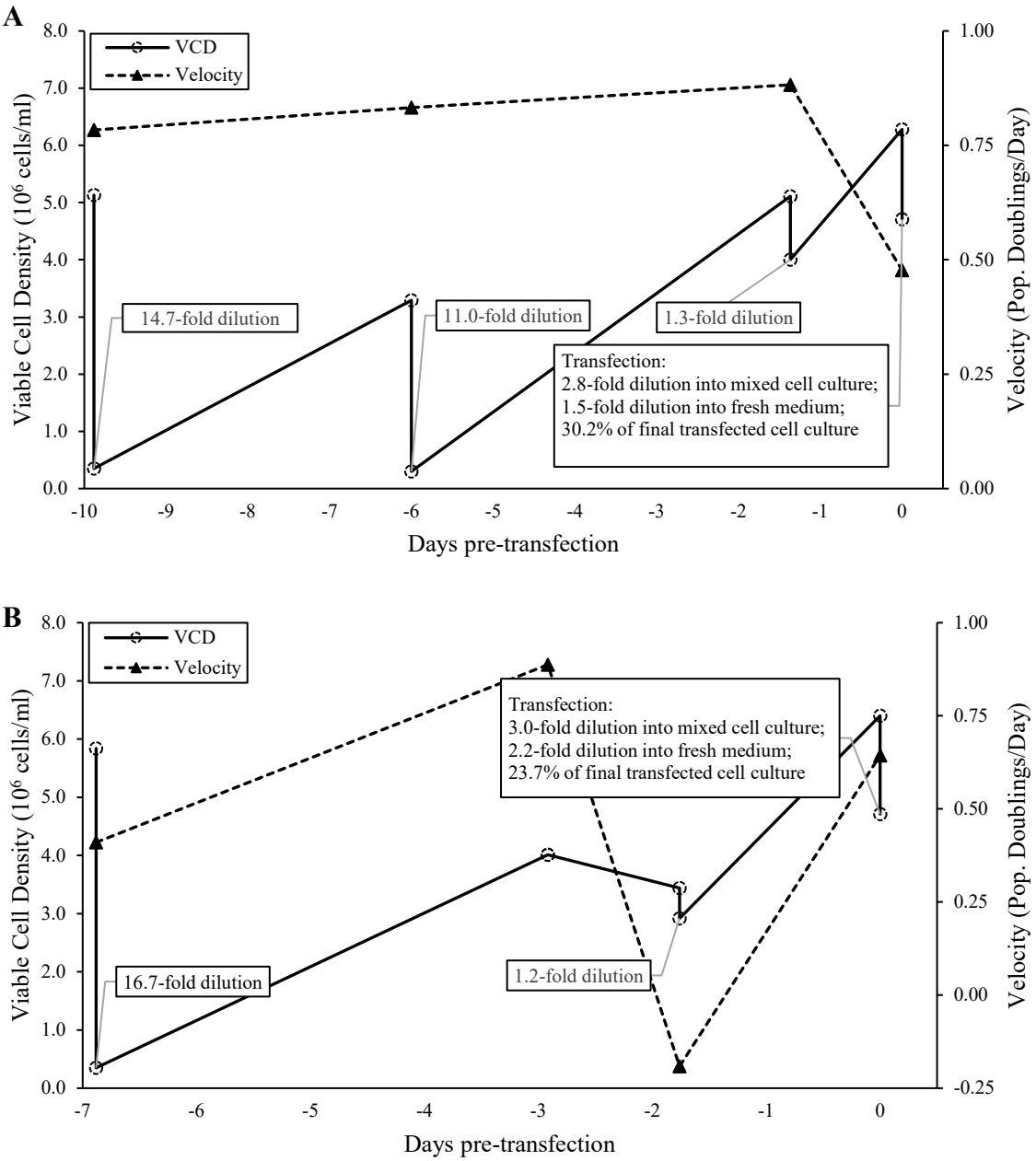

Figure 9. Viable cell density (VCD) and velocity of cell culture prior to transfection. Separate flasks of cells were passaged every three to five days, then diluted with fresh medium to a density between 3.0-4.0 x 10^6 cells/mL either (**A**) one day or (**B**) two days prior to transfection. On the day of transfection, cell cultures were mixed then diluted with fresh medium to the final density for transfection (4.7 x 10^6 cells/mL). Mixed cell densities on the day of transfection were (**A**) 5.6 x 10^6 and (**B**) 6.6 x 10^6 cells/mL. The far-right data label denotes the dilution factor from combining cell cultures, the dilution factor from addition of fresh medium to the combined cultures, and the percent the graphed cell line contributed to the total transfection volume.

In some instances, a culture was grown for more than 4 days without the addition of fresh medium. When this occurred, there was an immediate decline in growth velocity. Figure 9B shows an example of the velocity and VCDs of a culture used for transfection where the time between passages exceeded 4 days. The culture experienced a slight decline in VCD on the fifth day since passaging and was diluted with fresh medium. By the day of transfection, the growth rate of the culture had significantly recovered to 0.64 population doublings per day. This incidence of extended passage period before transfection occurred in cell cultures used for both transfection experiments where the combined VCD pre-transfection was greater than 6.0×10^6 cells/mL.

Although velocities recovered to higher levels by the day of transfection, it is possible that residual effects of the extended culturing contributed to the lower LVV titers. Alternatively, in both transfection experiments where the combined VCD pre-transfection surpassed 6.0×10^6 cells/mL, the cells were introduced to fresh medium two days prior to transfection, whereas in pooled cultures below 6.0×10^6 cells/mL on the day of transfection, fresh medium was introduced only a day before transfection. It is possible that although cells diluted 1 day prior to transfection were still experiencing lag in growth rates, the more recent introduction of medium provided sufficient nutrients to keep the cells in a state of active division through transfection. Cultures diluted more than 1 day prior to transfection may have reached the maximum density the growth medium could sustain without addition of nutrients by day 0. In either case, inconsistencies in the passaging routine leading up to transfection negatively influenced LVV titer.

Results Summary

The results of seven transfection experiments have been presented with emphasis on the effect of cell density pre-transfection and passage routine leading up to transfection on LVV titer and specific LVV productivity. We observed increases in LVV titer and specific LVV productivity associated with increases in cell density pre-transfection up to 6.0 x 10^6 cells/mL. We speculate that reductions in LVV titer and specific LVV productivity for pre-transfection densities greater than 6.0 x 10^6 cells/mL can be attributed to variations in passage routine several days prior to transfection.

CHAPTER 4

DISCUSSION

Challenges to the scaleup of lentiviral vector production by transient transfection include limitations to specific plasmid productivity, limitations to transfection efficiency at high cell density, and significant titer variability between production batches. The results of the present study can help overcome these challenges and will be discussed in the context of relevant findings from prior literature at three hierarchal scales: the cellular, the process, and the manufacturing scales.

Cellular Scale

Expression of transgenes following transfection is dependent on successful entry of the gene vector into the cell nucleus. Gene vectors, such as plasmids (pDNA), can enter the nucleus following mitotic dissociation of the nuclear membrane or, to a lesser degree, by import through nuclear pore complexes.[26] Higher transfection efficiencies are thus generally associated with active cell division when the disassembly of the nuclear envelope exposes gene vectors to the nuclear space. Following cytokinesis and reformation of the nuclear envelope, cytosolic pDNA can become localized in the nucleus alongside cell chromosomes.[26] The successful entry of pDNA into the nucleus is the most significant driver of transgene expression when considering other potential factors such as DNA topology, [31,45] DNA purification method,[46] concentration of pDNA in the cytoplasm,[43] or the type of transfection reagent used.[44] While some transiently transfected pDNA may enter the nucleus during interphase, as much as 83% of transgene expression from lipofection is associated with nuclear entry following mitosis.[47]

Interestingly, Kirchenbuechler et al. found that in HeLa cells transiently transfected by lipofection, equivalent levels of transgene expression can occur in cells entering mitosis up to 30 hours post-transfection as in those entering mitosis immediately following the addition of the transfection mixture.[47] By extension, if a given cell divides within 30 hours post-transfection, there is a possibility for the DNA lipoplexes to survive the residence time in the cytoplasm and be enclosed in the nucleus during the next mitotic event. Consequently, methods for maintaining cells in an actively dividing state following transient transfection can significantly increase levels of transgene expression.

In the present study, levels of GFP expression and LVV genomic RNA increased as cell densities prior to transfection increased from approximately 5.0 to 6.0 x 10^6 cells/mL. Given that all packaging cell cultures were diluted to the same density at the start of transfection, it is possible that increases in the density of pre-transfection cultures contributed to increased rates of cellular division post-transfection, thus improving LVV titer.

At a pre-transfection cell density of 6.0 x 10^6 cells/mL, we attained a LVV gene copy volumetric productivity approximately equivalent to 7.3 x 10^6 TU/mL. Of the previous studies where lentiviral vectors were produced in shake flasks, our results were 3 to 12-fold higher than two studies,[20,24] within 2.5-fold of four studies,[19,31–33] and approximately 50-fold lower than one study.[27] Gélinas et al. reported the lowest lentivirus titer, likely because their vector was pseudotyped with Sendai virus F/HN envelope proteins, which has been shown to reduce LVV titers by 20 to 40-fold compared to vectors pseudotyped with VSV-G.[20,48] Segura et al. reported the second lowest titer. Theirs was the first demonstration of LVV production in suspension cell culture format

and set the stage on which future studies built. Some studies reported volumetric productivities greater than our findings. In particular, Bauler et al. achieved specific productivities that were at a minimum 22-fold higher, and at a maximum 617-fold higher than previous studies.[27] They developed from HEK293T cells a suspension-adapted cell line that was conditioned to achieve fast growth rates and high cell densities in the absence of serum. Their process of converting adherent cells to grow in suspension may have selected for high LVV-producing cells. Additionally, they incorporated a complete medium replacement 24 hpt, which replenished nutrients and likely reduced cytotoxicity by removal of residual PEI-DNA polyplexes. While complete medium replacement post-transfection significantly increased LVV titer, this technique is not scalable and reduces the true volumetric titer when considering total medium used for post-transfection cell cultivation. When transfected cultures were diluted with an equal volume of fresh medium 24 hpt rather than complete exchange, Bauler et al. reported a 7-fold decrease in volumetric titer.

To date, several other studies on the optimization of lentiviral vector production have mainly addressed changing nutrient utilization of transfected cells by total replacement of the bulk medium prior to transfection,[30] after transfection,[20,28] or both.[19,33] Additionally, Gélinas et al. investigated the effect of supplementation by various lipid and peptide mixtures on LVV titers, but did not observe significant improvements.[20] The change in nutritional requirement of transfected cells for lentiviral vector production post-transfection has not been fully defined in literature, and should be the subject of future studies.

Those studies which did not use medium replacement are summarized in Table 4 and compared to our highest results. The average volumetric productivity of these three most comparable studies was 1.0 x 10^7 TU/mL. Our results are within 70% of this average.

Table 4. Volumetric productivities of lentiviral vectors (LVV) produced in shake flasks by transient transfection of HEK cell derivatives without medium replacement.

Transfection Method	Plasmid (ug) per 10^6 Cells	Transfection Cell Density (cells/mL)	TU/mL	TU/ug plasmid	TU/cell	Ref.
PEI	1.0	1.0 x 10^6	2.3 x 10^6	*2.3 x 10^6*	*2.3*	24
LP	1.0	4.7 x 10^6	*7.3 x 10^6*	*1.5 x 10^6*	*1.5*	PS
PEI	*2.0*	1.0 x 10^6	1.2 x 10^7	*6.0 x 10^6*	*12.0*	32
PEI	1.0	1.0 x 10^6	1.7 x 10^7	*1.7 x 10^7*	*17.0*	31

Italicized values were derived from reported transfection information and titers.
Data are reported by increasing volumetric productivity (TU/mL).
PEI = Polyethylenimine. LP = Lipofection. PS = Present study.

Despite attaining typical volumetric productivities, our specific plasmid and cell productivities were lower than most previous studies. Indeed, because our volumetric productivities were achieved at cell densities double to quadruple the density of all other comparable studies, the ratio of virus particles produced per plasmid and per cell are in most cases between 2 and 10-fold lower. This represents an area of substantial potential for growth, with even marginal increases translating to significant gains in volumetric productivity.

Process Scale

In the present work, transfection efficiency was estimated by the standard method used in literature, where efficiency is represented by the percentage of GFP-expressing

cells relative to the total cells present in the sample 48 hpt. We achieved transfection efficiencies of almost 20% at cell densities higher than previously reported for transfection of suspended cell cultures without complete medium replacement. This represents a significant advancement towards improving volumetric productivity of LVVs. Other studies have described transfection of HEK cells at high cell densities (HCD) (≥3.0 up to 20.0 x 10^6 cells/mL), however these processes employ special means of achieving elevated cell densities while maintaining cells in exponential growth, such as by perfusion [19,39,49,50] or by centrifugal concentration of low density (~2 x 10^6 cells/mL), log-phase cells with medium replacement.[51–55] While these methods increase volumetric productivity, continuous biomanufacturing processes present unique challenges in viral vector production (e.g., how to overcome packaging cell death from extended virus production following transient transfection), and economic advantages at manufacturing scale have yet to be demonstrated.[56] Additionally, concentration of cells by centrifugation and medium exchange become unrealistic at industrial scales. Batch processes for biotherapeutic production are well defined, but are generally unable to maintain high cell densities due to nutrient depletion and accumulation of toxic metabolites.[57] Further, a so-called "cell density effect" occurs in batch processes where increases in cell concentration at transfection are connected to significant reductions in transfection efficiency.[58] Several attempts to characterize this effect in various transfection/infection systems have been reported. Potential causes identified included changes to intracellular pH, smaller cell size and decreased protrusion limiting DNA uptake, and metabolic limitations at HCDs.[59–62] Additionally, osmolality and accumulation of lactate and ammonia were found to influence adenovirus titers at high

cell densities.[63] Interestingly, amino acid availability post-infection did not appear to constrain adenovirus titers, though post-infection supplementation with other essential nutrients (vitamins, sodium pyruvate and sodium acetate, lipids and precursors, and bases and nucleosides) increased efficiency in one commercial medium, suggesting nutrient limitations inhibit viral vector production in at least some formulations.

For production of HIV1 virus-like particles, Cervera et al. demonstrated that limitations to transfection efficiency at HCD were not exclusively driven by nutrient availability.[28] Lavado-García et al. performed a metabolic analysis that showed significant downregulation of nuclear transport machinery – importins and exportins that transport DNA into and proteins out of the nucleus, respectively – as cell density at transfection surpassed 3.0 x 10^6 cells/mL.[29] This downregulation may have contributed to the complete inhibition of transfection observed at 5.0 x 10^6 cells/mL. In contrast to previous reports, we observed transfection efficiencies as high as 19.6% when transfection was conducted at a density of 4.7 x 10^6 cells/mL. Further, a nearly 21-fold increase in specific productivities was achieved by increasing pre-culture densities from 5.0 to 6.0 x 10^6 cells/mL. To our knowledge, this is the first demonstration that an increase in transfection efficiency and cell specific viral productivity is associated with increasing cell density at transfection cell densities above 4.0 x 10^6 cells/mL for batch LVV production.

The actual cause of improved transfection efficiency at a density greater than 4.0 x 10^6 cells/mL is likely due to a combination of factors. Advances in chemically-defined serum-free medium formulations allow for high density mammalian cultures without the use of fed-batch or continuous feed supplementation.[64] Our work presents preliminary

evidence that a commercial medium can support increases in viral productivity at cell densities greater than 2.0 x 10^6 cells/mL, which has not previously been reported for batch processes.[58] Uses of the LV-MAX lentiviral production system have been recently published,[65–68] however these were gene expression studies and did not focus on optimizing LVV production for clinical applications.

The use of a commercial cationic-lipid transfection reagent may have also contributed to higher transfection efficiency despite the cytotoxic effects in some cases associated with lipofection.[69] Additionally, a proprietary supplement and enhancer may have worked in conjunction to overcome metabolic inhibitors to nuclear uptake of transfected DNA. Finally, it has been previously demonstrated that the commercially available LV-MAX cell line outperforms other HEK clones in LVV production, though this was achieved at different conditions than those used in the present study; LV-MAX cells were cultured in BalanCD with daily medium replacement.[33]

Regardless of cell density at the time of transfection, lot to lot variability is of concern in transient LVV production systems, where successful co-transfection of 3 to 4 plasmids is necessary for generation of functional viral particles.[70] Poor reproducibility in vector titers can be partially mitigated by selection of an optimized gene carrier. For example, transfection by cationic lipids tends to provide more reproducible efficiencies compared to calcium phosphate precipitation.[23] Among other potential factors, it is understood that cell culture maintenance is a critical parameter in the production of lentiviral vectors.[71] However, the significance of this effect has not been adequately investigated. In our experiments, lower vector titers were associated with cultures that had sustained irregular passaging schedules several days prior to transfection. Even

though the culture growth rates and viabilities had recovered and even surpassed those of the highest LVV producing cultures by the day of transfection, it is possible that variation in passage routine caused residual effects in metabolic processes important to high levels of transgene expression. Carpentier et al. suggested that some cellular physiological state exists that limits transport of pDNA to the nucleus and subsequent transcription.[72] Follow-up research is necessary to assess which physiological characteristics or metabolic pathways relevant to transfection efficiency might suffer from historical inconsistencies in passaging.

Manufacturing Scale

Lentiviral vectors are desirable as clinical gene therapies due to their large payloads, their ability to transduce non-dividing cells, and their integration of the therapeutic transgene into target cell genomes. Suspended culture manufacturing processes for production of lentiviral-based gene therapies could benefit from the findings of this study. Specifically, culturing packaging cells to HCD prior to transfection has practical advantages in addition to providing gains in volumetric titer. Traditional scaleup of suspended culture requires the transfer of seed culture to several intermediate vessels of increasing volumes. A final production-sized vessel is inoculated once enough seed culture is available to provide an appropriate seeding density (Figure 10). Increasing the maximum cell density a culture can support without losses to transfection efficiency could enable seed trains to skip intermediate vessels, leading to faster scale-up.

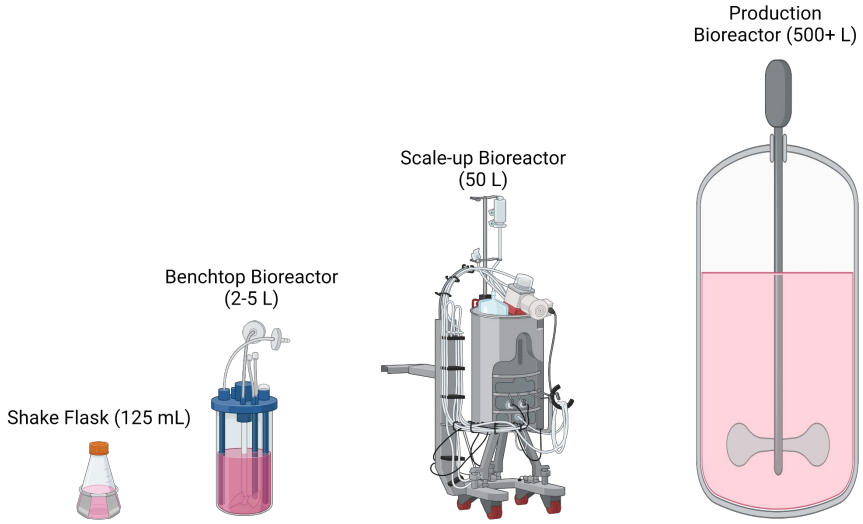

Figure 10. Example scaleup process for suspended culture. Created with BioRender.com

For scalable technologies (fixed bed bioreactors, rockers with microcarriers, and single-use stirred-tank bioreactors), pDNA production can account for as much as 28% of the yearly cost per dose of a LVV gene therapy.[73] This represents a basis for development of stable producer cell lines which reduce or eliminate the use of plasmid. However, advances in transient transfection processes to improve transfection efficiency would yield similar plasmid-reduction benefits while maintaining flexible platforms for rapid LVV design. In the present study, we demonstrated that for the same plasmid mass and cell concentration at transfection, specific plasmid productivity could be increased by culturing cells to higher densities pre-transfection. Implementation of this strategy – culturing of packaging cells to high densities and subsequent dilution with fresh medium immediately prior to transfection – could lead to higher specific plasmid productivities at production-scale transfections (e.g., >500 L).

New technologies for the large-scale production of DNA may further reduce the economic challenges to transient transfection. Recent studies have demonstrated the ability of DNA produced enzymatically by rolling circle amplification for use in LVV production[74] with infectious titers similar to those achieved by plasmid.[75] When coupled with production platforms optimized for transfection at HCD, the viability of transient transfection for large-scale manufacture of lentiviral-based gene therapies becomes more feasible.

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

In the present study, lentiviral vectors were produced by transient transfection in suspended culture HEK293F cells in 125-mL shake flasks. Transfection was performed at a high cell density of 4.7 x 10^6 cells/mL. The methods and reagents used to conduct transfection followed a specific vendor protocol. Volumetric productivity, viral gene copies per milliliter of culture, was 7.3 x 10^8, which is approximately equivalent to 7.3 x 10^6 transducing units per milliliter. This was within a range reported by three comparable studies, which ranged from 0.23 to 1.7 x 10^7 TU/mL. Specific plasmid productivity, 1.5 x 10^6 TU/ug plasmid, was lower than all three comparable reports which ranged from 0.23 to 1.7 x 10^7 TU/ug plasmid, suggesting that increases in plasmid productivity are possible, which would provide further increases to the volumetric and specific biomass productivities. Cells in which velocity and viability were maintained consistently prior to transfection without significant decreases due to exhaustion of growth medium provided continuous increases in vector titer with cell density pre-transfection up to 6.0 x 10^6 cells/mL, after which a decline in viral gene copies per culture volume was observed. This decline was observed up to pre-transfection cell densities of 6.8 x 10^6 cells/mL, which was the highest density tested in this study. To our knowledge, the LV-MAX lentiviral production system is the first commercial platform discussed in peer-reviewed literature to demonstrate volumetric titers of lentivirus produced by transient transfection of high density (>3.0 x 10^6 cells/mL) suspended cultures in batch mode comparable to titers from cells transfected at low density (<3.0 x 10^6 cells/mL).

Given the results of the present work, the following areas of investigation are warranted as the focus of future studies:

1. Determining transfection efficiencies of cells cultured to densities greater than 6.8 x 10^6 cells/mL prior to being diluted to 4.7 x 10^6 cells/mL for transfection, and efficiencies when transfecting at cell densities above 4.7 x 10^6 cells/mL. Are the declines in LVV titers we observed at higher densities due to nutrient exhaustion, build-up of toxic metabolites, cell death post-transfection, or some combination of these factors?
2. Examining the effect of dilution on transfection efficiency. Additionally, how does the nutrient requirement of the cells change following successful transfection versus non-transfected cells at high cell density? How does the level of dilution influence the viability and density of cells following transfection?
3. Elucidating the effect of passage routine on LVV titer. If a cell culture falls out of exponential growth several days prior to transfection but recovers by the day of transfection, are there metabolic pathways or physiological characteristics important to transfection and gene expression that might still be in an altered state independent of the cell growth rate or viability?
4. What other changes to the transfection process (adjuvants, nutrients in growth medium, etc.) can improve the specific plasmid productivity at high cell densities?

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APPENDICES

Appendix A: Supplementary Information

Supplemental Figures

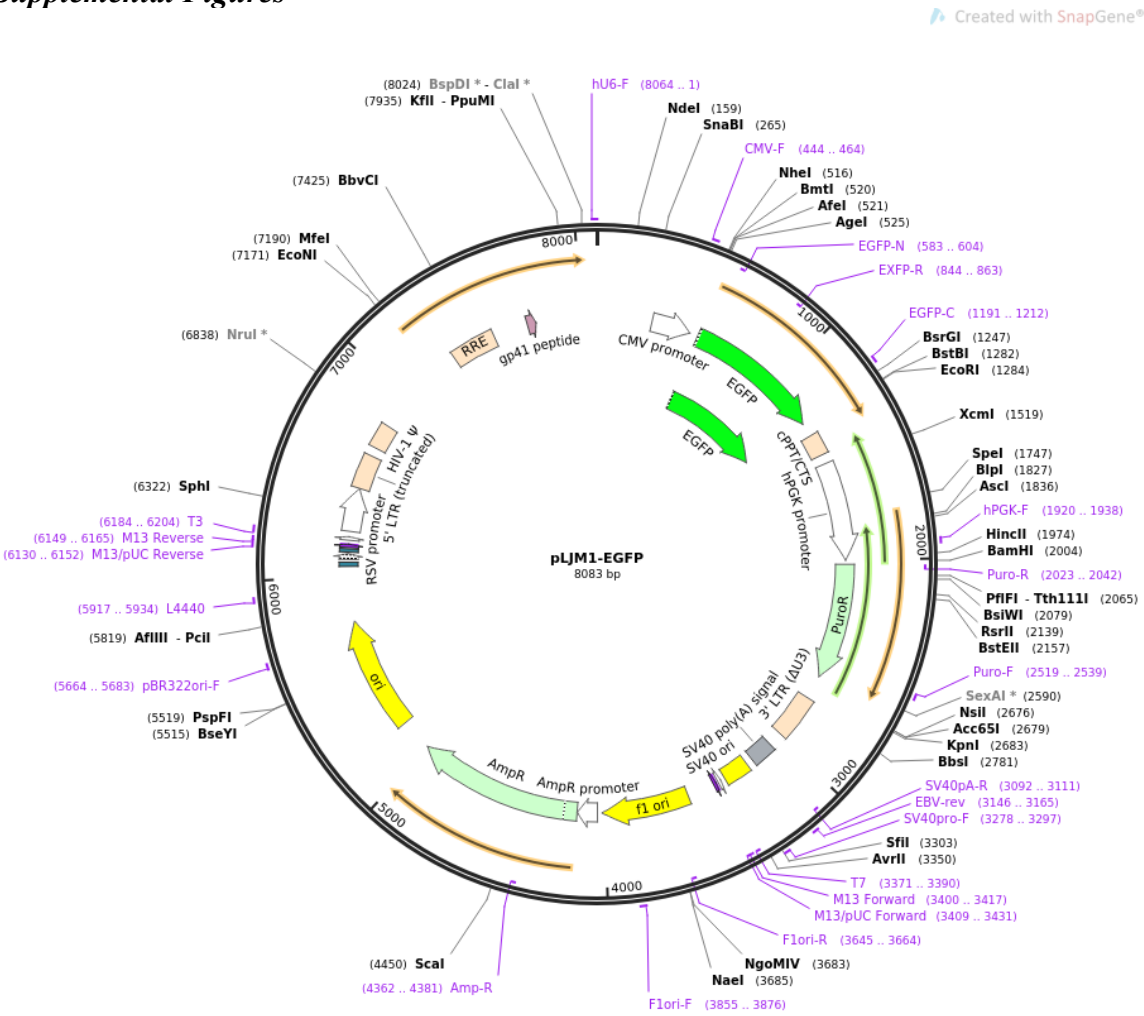

Figure 11. Plasmid map of pLJM1-EGFP lentiviral backbone plasmid. Retrieved from https://www.addgene.org/19319/#19319-DNA.cg.

Equation 1

Plasmid copy number was approximated from plasmid mass using the following equation:

$$Copy\ No. = \frac{(m * \frac{no.}{mol})}{bp * MW}$$

Where
m = mass of plasmid
$no./mol$ = 6.022 x 10^{23} molecules per mole (Avogadro's number)
bp = the length of the plasmid in base pairs

MW = 650 g/mol, or the average molecular weight of a base pair.

As an example, for 10 nanograms of the pLJM1-EGFP plasmid, which has a length of 8,083 bp, Equation 1 becomes:

$$Copy\ No. = \frac{(10\ ng * 6.022 * 10^{23})}{8{,}083 * 650} = 1.12 * 10^{9}\ copies$$

Equation 2

Plasmid copy number was approximated from plasmid mass using the following equation:

$$Efficiency\ (\%) = \left(10^{\frac{-1}{slope}} - 1\right) x\ 100$$

Equation 3

Specific volumetric productivity in transducing units per milliliter was calculated as follows:

$$Specific\ volumetric\ productivity = \frac{Lentiviral\ gene\ copies * \frac{10^2\ Lentiviral\ gene\ copies}{Transducing\ unit}}{volume\ of\ transfected\ culture}$$

Equation 4

Specific plasmid productivity in transducing units per microgram of plasmid used for transfection was calculated as follows:

$$Specific\ plasmid\ productivity = \frac{Specific\ volumetric\ productivity}{plasmid\ concentration * viable\ cell\ density\ at\ transfection}$$

$$= \frac{\frac{Transducing\ Units}{milliliter}}{micrograms\ plasmid * \frac{Viable\ cells}{millilter}}$$

Equation 5

Specific biomass productivity in transducing units per viable cell used for transfection was calculated as follows:

$$Specific\ biomass\ productivity\ = \frac{Specific\ volumetric\ productivity}{Viable\ cell\ density\ at\ transfection}$$

$$= \frac{\frac{Transducing\ Units}{milliliter}}{\frac{Viable\ cells}{millilter}}$$

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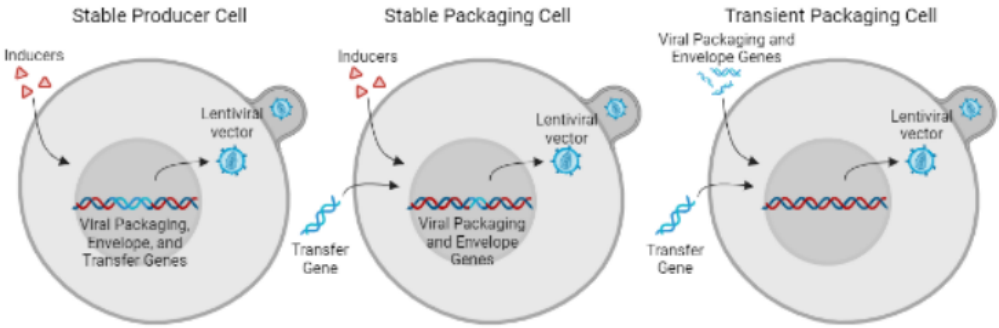

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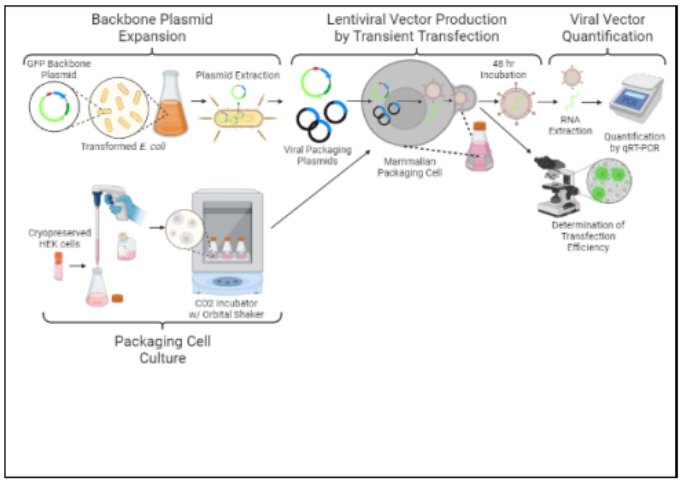

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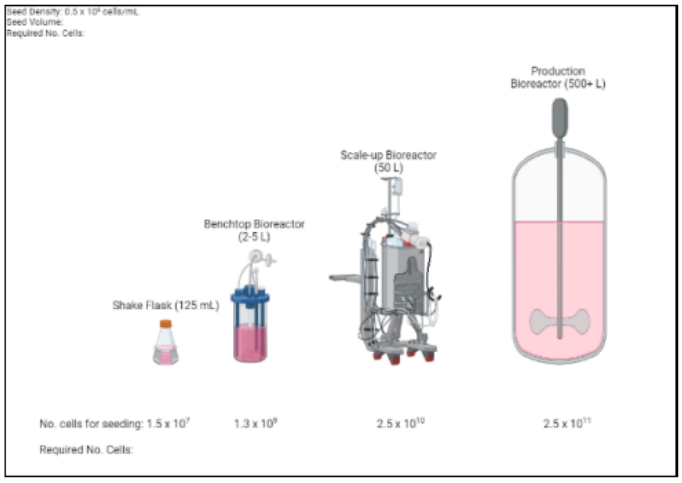

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