Title

Large-scale production of lentiviral vector in a closed system hollow fiber bioreactor

Authors

Sheu, Jonathan
Beltzer, Jim
Fury, Brian
et al.

Publication Date

2015

DOI

10.1038/mtm.2015.20

Peer reviewed

Citation: Molecular Therapy — Methods & Clinical Development (2015) **2,** 15020; doi:10.1038/mtm.2015.20

www.nature.com/mtm

ARTICLE

Large-scale production of lentiviral vector in a closed system hollow fiber bioreactor

Jonathan Sheu[1], Jim Beltzer[2], Brian Fury[1], Katarzyna Wilczek[1], Steve Tobin[1], Danny Falconer[1], Jan Nolta[1] and Gerhard Bauer[1]

Lentiviral vectors are widely used in the field of gene therapy as an effective method for permanent gene delivery. While current methods of producing small scale vector batches for research purposes depend largely on culture flasks, the emergence and popularity of lentiviral vectors in translational, preclinical and clinical research has demanded their production on a much larger scale, a task that can be difficult to manage with the numbers of producer cell culture flasks required for large volumes of vector. To generate a large scale, partially closed system method for the manufacturing of clinical grade lentiviral vector suitable for the generation of induced pluripotent stem cells (iPSCs), we developed a method employing a hollow fiber bioreactor traditionally used for cell expansion. We have demonstrated the growth, transfection, and vector-producing capability of 293T producer cells in this system. Vector particle RNA titers after subsequent vector concentration yielded values comparable to lentiviral iPSC induction vector batches produced using traditional culture methods in 225 cm^2 flasks (T225s) and in 10-layer cell factories (CF10s), while yielding a volume nearly 145 times larger than the yield from a T225 flask and nearly three times larger than the yield from a CF10. Employing a closed system hollow fiber bioreactor for vector production offers the possibility of manufacturing large quantities of gene therapy vector while minimizing reagent usage, equipment footprint, and open system manipulation.

Molecular Therapy — Methods & Clinical Development (2015) **2**, 15020; doi:10.1038/mtm.2015.20; published online 17 June 2015

INTRODUCTION

Clinical gene therapy has historically been plagued with many obstacles, primarily due to the inefficient nature of gene modification by physical means of DNA transfer. Beginning in the 1980s, hematopoietic stem cell and T-cell gene therapy[1–3] for the treatment of leukemia and severe combined immunodeficiency (SCID) were developed.[4,5] Research and clinical trials utilizing non-viral DNA delivery methods such as lipofection,[6] electroporation,[7] and simple naked DNA injections[8] yielded some promising results, but the levels of gene expression were consistently low.[9] However, with the emergence of gamma-retroviral vectors based on the Moloney leukemia virus as gene delivery vehicles in the late 1980s,[10] gene therapy obtained a powerful tool. These vectors allowed for an increase in the amount of DNA to be transferred, permanent gene modification due to integration into the target cells' DNA and the possibility of a lasting treatment or even cure for genetic diseases due to durable gene expression.[11] Subsequently, γ-retroviral vectors were successfully used to cure children of adenosine deaminase (ADA) SCID applying stem cell gene therapy.[12] γ-retroviral vectors, however, have the drawback of only being able to transduce dividing cells, and they also preferentially integrate into transcriptional start regions of active genes. This integration pattern can potentially lead to insertional oncogenesis due to the activation of nearby oncogenes. A new development in the late 1990s was the emergence of lentiviral vectors based on HIV. These vectors are not limited to transducing dividing cells, and do not preferentially integrate into transcriptional start regions of active genes, making them safer. However, multiple-copy and semi-random vector insertions into the target cells still pose the potential risk of insertional mutagenesis. To curb this risk, both lowering the transferred copy number and deleting the Moloney leukemia virus LTR U3 enhancer to create a self-inactivating γ-retrovirus[13] could lead to lower chances of insertional mutagenesis. Nevertheless, due to their advantages, lentiviral vectors are being used in clinical trials worldwide.

A relatively recent development is the generation of induced pluripotent stem cells (iPSCs).[14] Lentiviral vectors can be used to insert the genes for early acting transcription factors such as *Sox2*, *Klf4*, *c-myc*, and *Oct4*, into somatic cells to revert them back into a pluripotent state. While considerable efforts have gone into reverting somatic cells back to not only a phenotypically but also a genotypically similar state as embryonic stem cells,[15,16] epigenetic memory is still a challenge for iPSC differentiation.[17,18] In spite of this drawback, iPSCs are seen as an excellent target for gene correction of cells coming from patients with genetic diseases.[19,20] Lentiviral vectors, due to their large gene carrying capacity (up to 9kb, as opposed to 5kb for adeno-associated viral vectors)[21–23] and high transduction efficiency are still a popular method of iPSC generation; however, for clinical purposes, these vectors need to be removable after reliable iPSC generation, otherwise the early acting transcription factors will continue to be produced. Such a removable lentiviral vector can

[1]Good Manufacturing Practice Facility, Institute for Regenerative Cures, University of California Davis School of Medicine, Sacramento, California, USA; [2]Cell Processing, Terumo BCT, Inc., Lakewood, Colorado, USA. Correspondence: J Sheu (jonathan.sheu@ucdmc.ucdavis.edu)
Received 27 January 2015; accepted 16 April 2015

be created by use of the Cre-lox system.[24] By exposing transduced cells to cre-recombinase, the integrated vector can be excised at the loxP sites it carries. As an example, this system was used to generate iPSCs from patients with a genetic disease such as recessive dystrophic epidermolysis bullosa that could be gene corrected using homologous recombination and then differentiated into keratinocytes.[25] In the future, such gene corrected differentiated cells can be expanded greatly and used in autologous, clinical transplantation procedures to replace diseased tissue with healthy tissue.

Lentiviral vector is mostly generated by use of a transient transfection system applying plasmids and producer cells. Both three and four plasmid systems are in use, where the structural, polymerase, envelope and genes of interest are split up into multiple plasmids and transfected into a producer cell line such as 293T cells, a highly transfectable derivative of human embryonic kidney cells that contains the SV40 T-antigen. Traditional culture flask methods for growing producer cells and their subsequent transfection using lipofection or calcium phosphate precipitation is convenient for small-scale preparations used in basic research; however, translational research demanding larger scale toxicity studies also warrants larger volumes of vector while maintaining a high titer. Large-scale lentiviral vector production can become quite cumbersome when flasks are used, as it requires the growth and transfection of billions of cells, which can equate to 40 or more T225 flasks. 10- and 40-layer cell factories provide larger surface areas for cell growth; however, they come with their own challenges of cell visualization, uniform cell distribution and adequate gas exchange throughout all layers, and an extraordinary volume of media. Achieving a consistently high vector titer is also a major challenge for these culture systems.

New methods of large-scale vector production have therefore been contemplated, and one of the most prominent concepts is a closed system hollow fiber bioreactor. The Terumo Quantum Cell Expansion System is a hollow fiber bioreactor designed to expand cells to the 10^9 range in a manageable and compact culture vessel. The Quantum system is comprised of a computer interface and a tubing adapter panel containing peristaltic pumps, fluid sensors and tubing clamps placed at various locations to control fluid flow. The disposable cell expansion set, which contains tubing, inlet bags, waste bags and the hollow fiber bioreactor cartridge itself, is manually loaded onto the tubing adapter panel prior to use. This cartridge is comprised of 11,100 individual fibers, totaling 2.1 square meters of surface area available for cell culture, while occupying a total volume of only 180 ml. Additionally, the entire set contains two mutually exclusive feed loops—an intracapillary (IC) loop, which flows through the fibers' interiors, and an extracapillary (EC) loop, which flows through the space between the fibers. The fibers are porous, with the pores being so small that cells cannot be transported through these pores. Metabolites and fresh nutrients, however, can be transported through the pores. Each of these loops can be accessed independently through selective task programming, depending on the tasks required for any particular run. Inlet lines coming off the expansion set are available for loading IC media, EC media, cells, and reagents; two outlet lines are available for waste and cell harvest.

Additional features of the Quantum system include the following: To assess the rate of cell growth, a syringe septum allows access to the EC loop for taking media samples. The front door to the Quantum system creates an airtight seal that maintains a 37 °C environment around the expansion set; internal sensors alert the operator if the intra-tubular pressure exceeds 1,000 mm Hg. While the Quantum system has been used in studies for both mesenchymal stem cell[26] and embryonic stem cell (ESC)[27] expansions, we have shown for the first time that we can utilize the capabilities of this system to produce large-scale lentiviral vector with titers comparable to those obtained using conventional flask cultures and multi-layer cell factories.

Table 1 Hollow-fiber bioreactor lentiviral vector production statistics

	First run	*Second run*	*Third run*
Cells seeded	1.00×10^9	1.00×10^9	1.87×10^9
% Viability of seeded cells	97%	97%	98%
Viral titer (vg/ml)	1.915×10^8	2.790×10^8	1.025×10^8
Cells harvested	1.77×10^9	1.77×10^9	2.02×10^9
% Viability of harvested cells	94%	95%	98%

HEK 293T cells were expanded to the 10^9 range in T225 flasks prior to seeding the bioreactor and underwent a partial doubling during the expansion period. Cell counts and viability assays were performed before and after each run via Trypan Blue exclusion dye in a hemacytometer as described. Total particle titers were measured using a qPCR lentivector titration kit.

RESULTS

Three vector production runs were performed in the hollow-fiber bioreactor, each time using identical ratios of the plasmids and accompanying reagents as described above. For comparison

Table 2 Volume and particle yield comparison between lentiviral vector production methods

Mean yield, Bioreactor[a] (vg)	Mean yield, per T225[b] (vg)	Mean yield, CF10[a] (vg)	Bioreactor to T225 Ratio[d] (viral yield)	Bioreactor to CF10 Ratio[d] (viral yield)
3.82×10^9	2.06×10^7	1.75×10^9	185.21	2.18
Mean vector volume, Bioreactor (ml)[a]	Mean vector volume, per T225[c] (ml)	Mean vector volume, CF10 (ml)[a]	Bioreactor to T225 ratio[d] (volume)	Bioreactor to CF10 Ratio[d] (volume)
20.00	0.14	6.75	145.45	2.96

[a]Mean yields and volumes for large systems (bioreactor, CF10) were calculated by multiplying the titer by the postconcentration volume and averaging the resulting values from each run. [b]Mean per T225 flask yields were calculated by multiplying the titer by the postconcentration volume and averaging the resulting values, then dividing the total calculated viral output by the number of vessels used in that run. [c]Mean per flask concentrated vector volume was calculated by dividing the total volume of the concentrate by the number of vessels used in that run, since the viral supernatant from multiple flasks was required to fill a single Centricon Plus-70. [d]Ratio values were calculated by dividing the mean bioreactor yield by the respective mean per flask and mean per CF10 yields.

studies, two runs were performed in CF10s. Five runs were performed in T225 flasks as controls. The results of the bioreactor runs are summarized in Table 1.

Transfecting and harvesting 293T cells using the hollow fiber system does not affect viability

First, we assessed the amount of expansion achieved by using HEK 293T cells in the pretransfection expansion period as well as cell viability after vector production and cell harvest. Cells were expanded in T225 flasks for approximately 6 days in order to obtain roughly 1 billion cells, which were counted and assayed for initial viability via Trypan Blue exclusion prior to seeding the bioreactor. Immediately after concluding the 36-hour harvest period and removing the vector collection bag, the 293T cells were trypsinized and harvest off the bioreactor to assess expansion and viability. Harvested cells were counted and assayed for viability using the same Trypan Blue exclusion method mentioned above. Cell counts pre- and post-run show appreciable cell expansion during the 24-hour attachment phase. During transfection, we anticipated a decrease in the rate of cell division, so a complete doubling was not expected. Viral titer was performed on the harvested supernatant via qPCR, and the viral genomes per ml (vg/ml) were used to approximate the total viral yield (Table 2).

Different manufacturing platforms obtain comparable viral titers

Across all three manufacturing platforms, viral titers achieved fell between 1.7×10^8 vg/ml and 2.6×10^8 vg/ml, demonstrating that the bioreactor produces titers comparable to those obtained in conventional static cultures. This suggests that 293Ts as producer cells can easily adapt from conventional static culture platforms to a dynamic, constant-perfusion system with a vastly increased surface area and small overall volume, such as the hollow-fiber bioreactor, and that viral output is not affected by this culture environment.

For statistical analysis, due to unequal variance values, Welch's *t*-test was chosen to compare production methods. In comparing the bioreactor to CF10s, a df = 2 was used to calculate a *t*-score of 1.3052, yielding a two-tailed *P* value of $0.15 < P < 0.20$, indicating that there is no statistically significant difference between the bioreactor and CF10 methods with respect to titer. The 95% confidence interval for this comparison was calculated to be -4.21×10^8 vg/ml $< \mu < 1.76 \times 10^8$ vg/ml.

In comparing the bioreactor to T225 flasks, a df = 2 was used to calculate a *t*-score of 0.4758, yielding a two-tailed *P* value of $P > 0.20$, indicating that there is no statistically significant difference between the bioreactor and CF10 methods with respect to titer. The 95% confidence interval for this comparison was calculated to be -2.20×10^8 vg/ml $< \mu < 1.49 \times 10^8$ vg/ml.

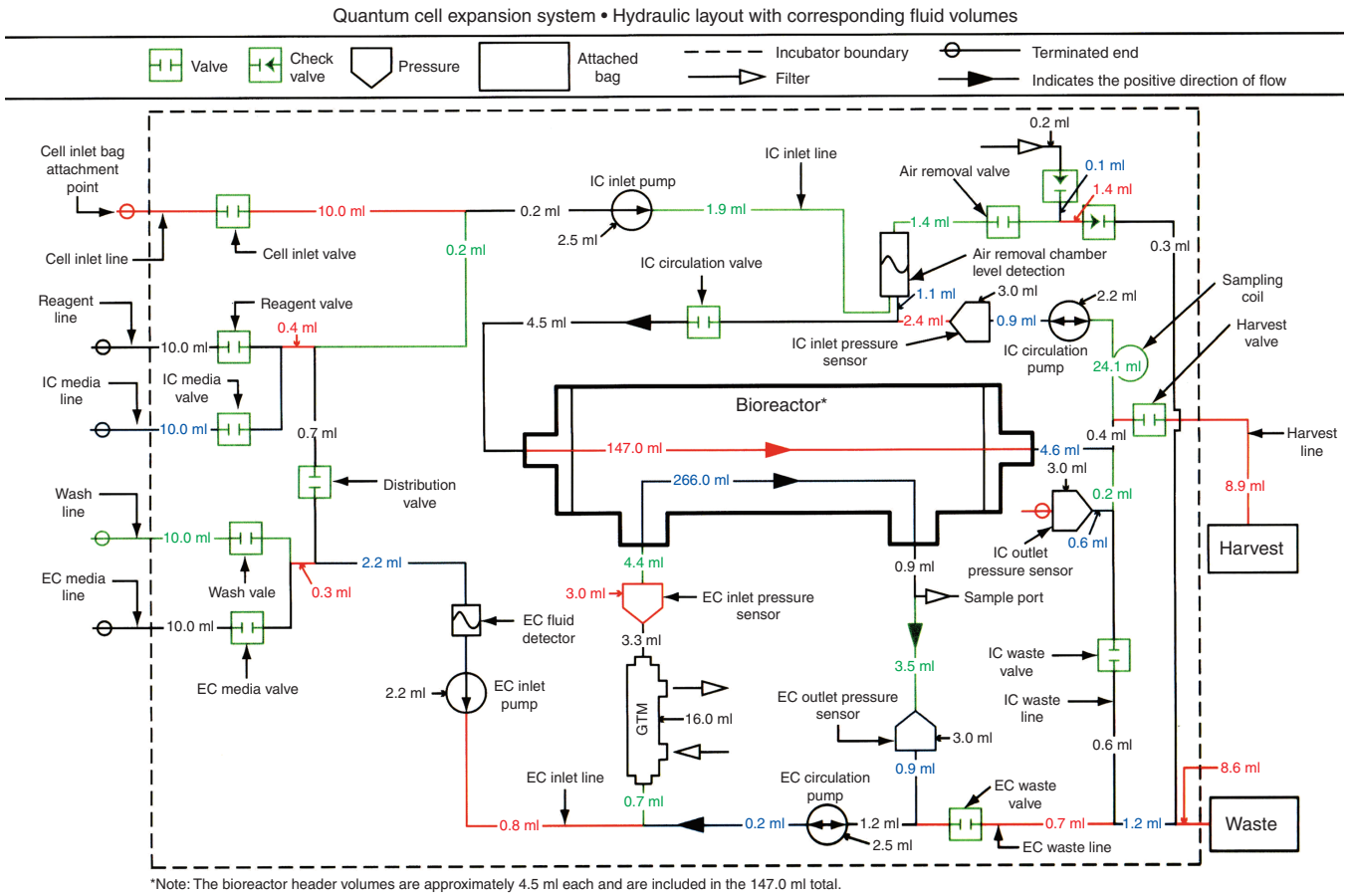

Figure 1 Quantum hollow fiber bioreactor hydraulic layout. This layout details the fluid flow, vessel schematics, and approximate volumes of the bioreactor cartridge itself and the accessory tubing system surrounding it. This illustrates the dynamic-perfusion concept of the bioreactor and allows one to follow the flow of fluid through the system, permitting the engineering of custom tasks to direct fluid through a specific, user-defined path if desired.

Viral output from the hollow fiber bioreactor outweighs yields from T225 flasks and CF10s

Owing to its largely increased surface area, constant-perfusion system, and greater harvest volume capacity, the hollow fiber bioreactor's yield far outweighed that of flasks and CF10s. While the number of viral genomes per ml remained comparable between platforms, the sheer amount of viral supernatant obtained from the bioreactor allowed for greater viral output than either static culture platform.

DISCUSSION

Since lentiviral vectors have become important tools for gene therapy, particularly for clinical applications of gene therapy, is has also become necessary to manufacture affordable and high quality lentiviral vector while minimizing reagent use and personnel time required. CF10s have previously been explored as culture vessels for clinical-grade vector production,[28–30] yielding titer values similar to the ones we achieved. However, the large amount of reagents and personnel time required for flask and cell factory cultures make these methods cumbersome. To manipulate hundreds of tissue culture flasks inside a biosafety cabinet without sterility issues is a huge concern. Cell factories and their open system nature also make them contamination prone during repeated manipulation. Additionally, large volumes of reagents are needed, making vector manufacturing in this system a rather wasteful process.

Our results demonstrate that vector particle titers, comparable to those attained in flask or cell factory cultures, can be achieved in the Quantum system by utilizing the large surface area of a hollow fiber bioreactor coupled with media perfusion to efficiently culture producer cells while eliminating the need for periodic enzymatic passaging. Transient transfection by calcium phosphate precipitation with plasmid ratios similar to those applied in regular flask cultures was possible and provided comparable results to flask and cell factory cultures. Thus, the potential for automated, compact, and efficient production of lentiviral vector becomes a reality with the use of this method. While pre- and post-Quantum procedures (culturing cells for seeding, reagent preparation, filling of bags, and postprocessing) still require a fair amount of open-system manipulation in a biosafety cabinet, the Quantum system itself is designed to be functionally closed. Any other procedure in the closed system bioreactor can easily be performed in a nonclassified environment with minimized footprint and oversight (a maximum of two operators at any one time was needed for an entire vector manufacturing run in our studies).

To assess the capabilities of the cell factory system compared to the hollow-fiber system, total particle titers were performed to quantify gross particle yields from both platforms. Transducing titers were not performed for this study because this work primarily focused on comparing the total number of particles achieved, and not necessarily the number of transducing units (TU) produced. One concern of ours was that transduction efficiency could be affected by the presence of cellular debris and envelope protein, so further downstream processing beyond Centricon Plus-70 units may be required to increase vector purity. We have explored the potential of tangential flow filtration as a means of concentrating large volumes of viral vector; while this method is currently not sterile, we are striving to develop an aseptic method for large-scale vector concentration. With increased vector purity, increased transduction efficiency becomes more plausible, and tangential flow filtration is just one of the tools we plan to include in our future studies. We have also experimented with polyethylenimine as an alternative transfection reagent, which has been in use since the mid-1990s,[31] and polyethylenimine could also have an effect on the resulting lentivector's transduction efficiency. We are currently planning a statistically relevant number of new vector manufacturing experiments to measure and optimize infectious titers from lentiviral vectors produced in various large-scale platforms, applying the abovementioned additional purification strategies, which will be presented in a future publication.

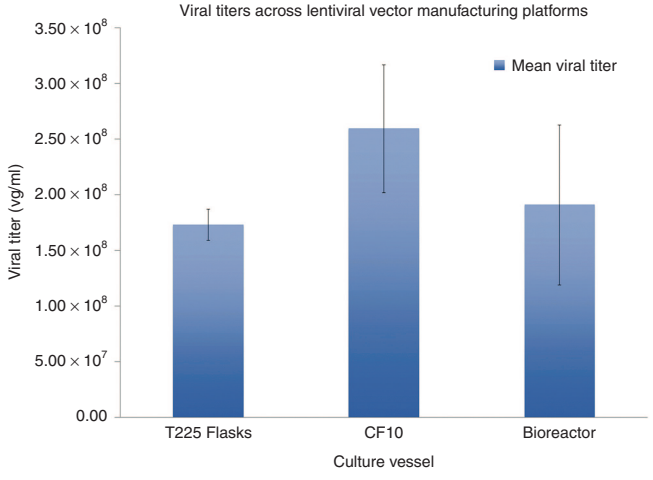

Figure 2 Viral titer comparison between lentiviral vector production methods. The results of the T225 flask and CF10 vector production runs were compared with the bioreactor production runs, yielding comparable titer values (approximately 1.7×10^8 vg/ml to 2.5×10^8 vg/ml) across all three production methods. Production in the T225 flasks ($n = 5$) yielded a standard error* of 1.41×10^7 vg/ml; CF10s ($n = 2$) yielded a standard error of 5.75×10^7 vg/ml, and the bioreactor yielded a standard error of 7.19×10^7 vg/mL. Variance was calculated by taking the square of the standard deviation. *Standard errors of the mean are expressed on the graph as error bars.

Table 3 Transfection reagent mix ratios

	Concentration	*Per T225 flask (ml)*	*Per CF10 (ml)*	*Per bioreactor (ml)*
Sterile H_2O	N/A	0.707	19.985	41.90
$CaCl_2$	2 mol/l	0.500	14.133	29.70
$MgCl_2$	2 mol/l	0.121	3.420	7.20
HeBS	2×	1.960	55.403	116.60
VSVG	0.25 mg/ml	0.020	0.565	2.4
Δ8.9	1.4 mg/ml	0.018	0.509	2.14
iPSC	0.86 mg/ml	0.029	0.820	3.49
Total volume		3.355	94.835	203.43

Vector production runs in T225 flasks and in CF10s were performed in parallel, each time using plasmids and calcium phosphate reagents in the above ratios. Plasmid and transfection excipient ratios were determined through previous in-house optimization studies. Excipient ratios for transfecting cells inside the bioreactor differ due to the restricted volume of approximately 180–200 ml inside the IC loop. While plasmid amounts were proportionally scaled up from those amounts used in T225 flasks or CF10s, precipitation reagent amounts were kept in the same ratios relative to each other but in smaller volumes due to the 200 ml volume restriction.

Table 4 Viral titer comparison between lentiviral vector production methods

Viral titer (vg/ml)	*T225 flasks*[a]	*CF10*[b]	*Bioreactor*[c]
Trial #1	1.90×10^{8}	2.02×10^{8}	1.915×10^{8}
Trial #2	2.03×10^{8}	3.17×10^{8}	2.790×10^{8}
Trial #3	1.88×10^{8}	N/A	1.025×10^{8}
Trial #4	1.24×10^{8}	N/A	N/A
Trial #5	1.60×10^{8}	N/A	N/A
Mean	1.73×10^{8}	2.60×10^{8}	1.91×10^{8}
Standard deviation	3.16×10^{7}	8.13×10^{7}	8.83×10^{7}
Variance	9.96×10^{14}	6.61×10^{15}	1.64×10^{16}

[a]Each trial performed in T225 flasks involved two T225 flasks, [b]each trial performed in CF10s involved a single CF10, and [c]each trial performed in the bioreactor involved a single hollow-fiber cartridge.

In terms of output, while our data show that a single bioreactor replaces only approximately three CF10s, the advantages of having a fully automated system, with only the need to replace media and waste bags, allows for quicker and easier operation as well as the possibility of running several Quantums simultaneously. Thus, product output from using the Quantum system is greatly increased while the required number of man-hours is only slightly increased per additional Quantum. In contrast, because they require so much manual manipulation, each additional CF10, CF40, or other static culture platform warrants a greatly increased number of man-hours to produce the same amount of vector. Additionally, in order to manually manipulate and incubate a system as large as a CF40 requires multiple personnel and a Nunc Cell Factory Incubator (Thermo Scientific, Waltham, MA) built specially for fitting CF10s and CF40s. Automatically manipulating CF40s requires a special "Nunc™ Automatic Cell Factory™ Manipulator System" (Thermo Scientific). Both items together cost six times more than a single Quantum (see Cost Analysis supplement), occupy a much larger footprint and require a large clean/nonclassified (CNC) room at minimum; if a biosafety cabinet and other large equipment (*e.g.*, centrifuge) are required, then the room will need to be even larger and ISO certified as well. Additionally, due to its large lever arms and the large torque required to manipulate items as heavy as CF40s, the Nunc Automatic Cell Factory Manipulator System may potentially run into more structural issues with repeated use, as opposed to the Quantum's small and highly stable peristaltic pumps.

While the Quantum certainly optimizes the manufacturing process and makes it more compact, it should be emphasized that more than just a few Quantum units are required to produce enough lentiviral vector for phase 2 or phase 3 clinical trials, which sometimes warrant batch sizes comprised of hundreds of liters. Furthermore, Centricon centrifugal units are nonsterile, making them unsuitable for clinical biologics manufacturing. Rather than providing ready-to-use solutions for large-scale clinical manufacturing, however, our primary aim is to provide a proof of principle and explore new manufacturing and processing methods on different culture platforms. This and subsequent experiments in various systems could potentially lead to an even more efficient method capable of providing the amount of product needed for clinical trials.

For future studies, we plan to extend the vector manufacturing capability to other gene therapy vectors that can be manufactured using plasmid transfection methods. Additionally, we are exploring sterile concentration methods suited for handling large product volumes in an aseptic environment as well as further minimizing time spent in open culture to create the possibility of generating a truly clinical-grade vector. Further exploration of the Quantum system's capabilities will also include the manufacturing of novel clinical-grade cell types such as induced pluripotent stem cells and vector transduced cells for use in clinical trials.

MATERIALS AND METHODS

Quantum hollow fiber bioreactor

The Quantum bioreactor (Terumo BCT, Lakewood, CO) is a computer controlled, closed system automated device originally engineered for culturing and large scale expansion of cellular products. It consists of a single use disposable set with built in fluidics, a hollow fiber cartridge, bags for product loading and harvesting, filtering media and waste containment. With the door closed, the unit itself is able to regulate temperature and functions as an incubator. As shown in Figure 1, the hollow fiber cartridge consists of two separate circulation loops incorporating both an intracapillary loop (IC) and an extracapillary loop (EC) that can be used to load cells or add and remove media, reagents and waste. A computerized touch screen provides the user access to the software allowing the parameters of a preprogrammed task to be defined and modified (circulation rates, washes, harvesting) and also allows for the creation of completely new custom tasks that can be saved. Required gases can be pumped into the system via external tanks and tubing that runs through integrated gas filters at the device.

Bioreactor preparation

The cell expansion set and cartridge were fluid primed, coated with fibronectin and conditioned with media initially. The day before cell seeding into the hollow fiber cartridge, a cell expansion set was loaded into the Quantum system and the "Load Cell Expansion Set" program was executed. For the priming step, 4 liters of Dulbecco's phosphate-buffered saline (GE Healthcare, Little Chalfont, UK) were transferred, using a peristaltic pump inside a biosafety cabinet into a media bag which was sterile-welded onto the bioreactor system. The "Prime Expansion Set" program task was executed and the expansion set-associated tubing and fluidics were filled with Dulbecco's phosphate-buffered saline. Next, 100 ml of 0.5 mg/ml human fibronectin (BD Biosciences, Franklin Lakes, NJ) in Dulbecco's phosphate-buffered saline were transferred into a cell inlet bag, which was welded onto the bioreactor system, loaded into the cartridge using the "Coat Bioreactor" program task and allowed to incubate for 16 hours at 37 °C. After incubation, an "IC/EC Washout" task was performed to remove excess fibronectin. To condition the cartridge, 4 liters of HEK-293T culture media were transferred into a 4 liters media bag which was sterile-welded onto the system. The "Condition Media" program task was run, while equilibrating and maintaining a gas mixture containing 5% CO_2 with culture media inside the bioreactor.

Cell culture

Previously, clinical grade HEK 293T cells were obtained from the National Gene Vector Biorepository, Indianapolis, Indiana. Cell cultures were passaged in T225 tissue culture flasks (Corning, Corning, NY) to maintain exponential growth phase in D10 media consisting of Dulbecco's modified Eagles medium/high glucose (GE Healthcare, Little Chalfont, UK) with 10% fetal bovine serum (GE Healthcare) and 200 mmol/l GlutaMAX (Life Technologies, Gibco). Four liters of culture media were prepared in a 4 l media bag for maintenance of the bioreactor run. To seed the hollow fiber bioreactor cartridge, 1×10^{9} HEK 293T cells were trypsinized and resuspended into 100 ml of culture media, loaded into a cell inlet bag and seeded into the hollow fiber bioreactor cartridge previously coated with fibronectin. Cells were allowed to attach for 24 hours before transfection by an "Attach Cells" task preprogrammed into the system. Prior to transfection in the bioreactor, the cells were incubated for 24 hours to allow for attachment. During attachment and for the rest of the run, media samples were taken from the EC sampling port

for lactate measurements using a Lactate Plus meter and test strips (Sports Resource Group, Minneapolis, MN) as an indicator of cell growth. For small-scale transfections, T225 tissue culture flasks were seeded with 2.5×10^7 cells per flask and incubated for 24 hours. Each trial conducted in T225 flasks consisted of two flasks. In addition, HEK-293T cells were also seeded into CF10s at 7×10^8 cells per stack in approximately 1.3 l of D10 media.

Plasmids

For transfections, lentiviral vectors were generated using the following plasmids: (i) pMDG-VSVG envelope encoding a vesicular stomatitis virus, G glycoprotein. (ii) pCMV-dR8.91 packaging plasmid containing the (gag/pol) structural and polymerase genes. (iii) pMNDU3-OKSM-PGK-EGFP-WPRE transgene construct containing a polycistronic, 4-factor iPSC induction cassette (OCT4, KLF4, SOX2, and c-Myc) and a PGK promoter driven EGFP green fluorescent protein. For the bioreactor, 10 mg preps of plasmid DNA were grown up in chemically competent DH10B *Escherichia coli* obtained from Life Technologies and purified using an Endotoxin-Free Giga-Prep Kit (Qiagen). Purified plasmids had endotoxin levels ≤0.5 EU/ml.

Calcium-magnesium phosphate transfection

To perform the large-scale bioreactor transfection, a triple transfection of pMDG-VSVG, pCMV-dR8.91 and pMNDU3-OKSM-PGK-EGFP-WPRE was performed. Plasmids were used at a ratio of 5:5:1 (3.0 mg of dR8.91 packaging plasmid/3.0 mg of OKSM-EGFP transfer plasmid/600 µg of VSVG). Briefly, a two-part transfection reagent mix was prepared based on plasmid concentrations (Table 3). The volume of each reagent was adjusted to a volume totaling the approximately 180–200 ml capacity of the bioreactor cartridge's IC loop. All three plasmids were mixed in one vessel containing 41.90 ml of sterile water, 29.70 ml of 2 mol/l $CaCl_2$ (Sigma-Aldrich, St. Louis, MO) and 7.20 ml of 2 mol/l $MgCl_2$ (Sigma-Aldrich, St. Louis, MO). In a separate container, 116.60 ml of 2× phosphate-containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered saline (HeBS) solution was prepared. The two mixtures were combined and quickly loaded into a cell inlet bag, precipitated and then loaded into the bioreactor cartridge using the "Add Bag Contents" preset task to transfect the cells. A 47 ml chase with complete Dulbecco's modified Eagles medium was performed with the IC waste valve closed and the EC waste valve open to ensure that the reagent mix was in close proximity with the walls of the hollow fibers and in direct contact with the cells. Subsequently, the "Attach Cells" task was run a second time to allow media to be fed to the cells via the EC loop while leaving the IC loop, containing the cells and transfection mix, undisturbed. The system was allowed to incubate at 37 °C and 5% CO_2 for approximately 15 hours. For transfections in CF10s, 700 µg of dR8.91 packaging and OKSM-EGFP transfer plasmids were used with 140 µg of VSVG envelope plasmid. Transfections performed in T225 flasks used 25 µg each of the dR8.91 and OKSM-EGFP plasmids together with 5 µg of VSVG per flask. For transfections in the bioreactor, a calcium-magnesium phosphate transfection mix was used, and all transfections were performed overnight. The following day, the transfection mixes were removed from the bioreactor, cell stacks and flasks and replaced with serum free UltraCULTURE media (Lonza, Basel, Switzerland) for harvesting vector.

Vector harvest and downstream processing

Lentiviral vector produced from HEK-293T cells grown in the bioreactor was collected over 36 hours by continuous perfusion of serum free media through the IC loop and into a 4 l collection bag, transferred to sterile 1 l media bottles, and stored at −80 °C. Similarly, the vector-containing supernatants from the CF10s were collected and pooled as a single harvest into sterile bottles, and stored at −80 °C. Vector-containing supernatants from the T225 runs were pooled together and taken directly for concentration and titer measurement.

Before treatment with Benzonase Nuclease (Sigma-Aldrich, St. Louis, MO), the supernatants were clarified through a 0.45-µm media filter (Corning, Corning, NY). Benzonase Nuclease was added to each of the viral supernatant collections in a concentration of 50 units/ml and incubated in a standard 5% CO_2, 37 °C incubator for one hour to eliminate residual plasmids from the transfections. Vector supernatants from the T225 flasks were pooled together to obtain a total volume of 60 ml prior to concentration. Vector supernatants from the CF10s and bioreactor were frozen at −80 °C after taking a 60 ml sample for testing. The 60 ml samples were concentrated by spin-filtration using Centricon Plus-70 (Millipore, Billerica, MA) centrifugal units with a 100-kDa cutoff. The spin-filtration units were centrifuged at 3,500 rpm for 30 minutes, then inverted and placed into collection cups that were centrifuged for 5 minutes at 1,100 rpm. Approximately 300 µl of concentrated vector was collected from each of the spin-filtration units.

Particle titer by qPCR

Lentiviral vector RNA was titered by qPCR using a qPCR Lentivector Titration Kit (Applied Biological Materials, Richmond, Canada) according to the manufacturers' protocol. Briefly, samples were diluted 1:10 and 1:100 in phosphate-buffered saline prior to lysis. Viral lysis was performed using 2 µl of diluted viral supernatant added to 18 µl Virus Lysis Buffer provided. The mixture was incubated for 3 minutes at room temperature to obtain the viral lysate. Reactions were set up in triplicate and a no template control was used. The qRT-PCR program was performed on an Applied Biosystems 7300-Real Time PCR system using the following qRT-PCR parameters: 1 cycle of reverse transcription was performed at 42 °C for 20 minutes, 1 cycle of enzymatic activation was performed at 95 °C for 10 minutes, 40 cycles of denaturation was performed at 95 °C for 15 seconds, and 40 cycles of annealing/extension was performed at 60 °C for 1 minute. Sample titers were calculated from C_t values using the following equation:

Titer of viral lysate = $5 \times 10^7 / 2^{3(Ctx\text{-}Ct1)/(Ct2\text{-}Ct1)} *$ (dilution factor) where C_{tx} equals the average of three C_t sample values, C_{t1} equals the average of three C_t values from Standard 1 values and C_{t2} equals the average of three C_t values from Standard 2.

Transducing titers were not performed as part of our study.

Data presentation and statistical analysis

All values presented in Figure 2 represent the mean, with the standard error of the mean represented by error bars. The number of experiments performed in each culture platform is shown in Table 4. All significant differences were evaluated using Welch's *t*-test, comparing experimental data obtained from production runs performed in CF10s and the bioreactor to data obtained using pre-established protocols in T225 flasks.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by the University of California Davis and by the James B. Pendleton Charitable Trust. We thank the UC Davis Health System Office of the Dean for their support, and Carson Rhodes at Terumo BCT, Inc. for providing Quantum system materials and for helpful discussions. Dr. Jim Beltzer is employed by the Cell Processing division at Terumo BCT, Inc.

Funding sources: These findings are the result of work supported by the University of California Davis, Sacramento, CA and by the James B. Pendleton Charitable Trust, Bellevue, WA. The views expressed in this paper are solely those of the authors.

REFERENCES

1. Reiffers, J, Bernard, P, David, B, Vezon, G, Sarrat, A, Marit, G *et al.* (1986). Successful autologous transplantation with peripheral blood hemopoietic cells in a patient with acute leukemia. *Exp Hematol* **14**: 312–315.
2. Kessinger, A, Armitage, JO, Landmark, JD, Smith, DM, Weisenburger, DD (1988). Autologous peripheral hematopoietic stem cell transplantation restores hematopoietic function following marrow ablative therapy. *Blood* **71**: 723–727.
3. Buckley, RH (1999). Hematopoietic stem-cell transplantation for the treatment of severe combined immunodeficiency. *N Eng J Med* **340**: 508–516.
4. Rosenberg, SA, Aebersold, P, Cornetta, K, Kasid, A, Morgan, RA, Moen, R *et al.* (1990). Gene transfer into humans—immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N Eng J Med* **323**: 570–578.
5. Blaese, RM, Culver, KW, Miller, AD, Carter, CS, Fleisher, T, Clerici, M *et al.* (1995). T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. *Science* **270**: 475–480.
6. Felgner, PL, Gadek, TR, Holm, M, Roman, R, Chan, HW, Wenz, M *et al.* (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA* **84**: 7413–7417.
7. Van Tendeloo, VF, Ponsaerts, P, Lardon, F, Nijs, G, Lenjou, M, Van Broeckhoven, C *et al.* (2001). Highly efficient gene delivery by mRNA electroporation in human hematopoietic

cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. *Blood* **98**: 49–56.
8. Tsurumi, Y, Takeshita, S, Chen, D, Kearney, M, Rossow, ST, Passeri, J *et al.* (1996). Direct intramuscular gene transfer of naked DNA encoding vascular endothelial growth factor augments collateral development and tissue perfusion. *Circulation* **94**: 3281–3290.
9. Edelstein, ML, Mohammad, RA and Jo, W (2007). Gene therapy clinical trials worldwide to 2007—an update. *J Gene Med* **9**: 833–842.
10. Bordignon, C, Yu, SF, Smith, CA, Hantzopoulos, P, Ungers, GE, Keever, CA *et al.* (1989). Retroviral vector-mediated high-efficiency expression of adenosine deaminase (ADA) in hematopoietic long-term cultures of ADA-deficient marrow cells. *Proc Natl Acad Sci USA* **86**: 6748–6752.
11. Kohli, M, Rago, C, Lengauer, C, Kinzler, KW and Vogelstein, B (2004). Facile methods for generating human somatic cell gene knockouts using recombinant adeno associated viruses. *Nucleic Acids Res* **32**: e3–e3.
12. Candotti, F, Shaw, KL, Muul, L, Carbonaro, D, Sokolic, R, Choi, C *et al.* (2012). Gene therapy for adenosine deaminase-deficient severe combined immune deficiency: clinical comparison of retroviral vectors and treatment plans. *Blood* **120**: 3635–3646.
13. Hacein-Bey-Abina, S, Pai, SY, Gaspar, HB, Armant, M, Berry, CC, Blanche, S *et al.* (2014). "A Modified γ-Retrovirus Vector for X-Linked Severe Combined Immunodeficiency." *N Eng J Med* **371**: 1407–1417.
14. Takahashi, K and Shinya, Y (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**: 663–676.
15. Smith, AG (1991). Culture and differentiation of embryonic stem cells. *J Tissue Culture Methods* **13**: 89–94.
16. Byrne, JA, Simonsson, S, Western, PS and Gurdon, JB (2003). Nuclei of adult mammalian somatic cells are directly reprogrammed to oct-4 stem cell gene expression by amphibian oocytes. *Current Biol* **13**: 1206–1213.
17. Chin, MH, Mason, MJ, Xie, W, Volinia, S, Singer, M, Peterson, C *et al.* (2009). Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. *Cell Stem Cell* **5**: 111–123.
18. Kim, K, Doi, A, Wen, B, Ng, K, Zhao, R, Cahan, P *et al.* (2010). Epigenetic memory in induced pluripotent stem cells. *Nature* **467**: 285–290.
19. Raya, A, Rodríguez-Pizà, I, Guenechea, G, Vassena, R, Navarro, S, Barrero, MJ et al (2009). Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. *Nature* **460**: 53–59.
20. Yusa, K, Rashid, ST, Strick-Marchand, H, Varela, I, Liu, PQ, Paschon, DE et al (2011). Targeted gene correction of [agr] 1-antitrypsin deficiency in induced pluripotent stem cells. *Nature* **478**: 391–394.
21. Zhao, J and Lever, AM (2007). Lentivirus-mediated gene expression. *Methods Mol Biol* **366**: 343–355.
22. Grieger, JC and Richard, JS (2005). "Packaging capacity of adeno-associated virus serotypes: impact of larger genomes on infectivity and postentry steps." *J Virol* **79**: 9933–9944.
23. Wu, Z, Hongyan, Y and Peter, C (2010). "Effect of genome size on AAV vector packaging." *Mol Ther* **18**: 80–86.
24. Sauer, B (1988). Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci USA* **85**: 5166–5170.
25. Sebastiano, V, Zhen, HH, Haddad, B, Bashkirova, E, Melo, SP, Wang, P et al (2014). Human COL7A1-corrected induced pluripotent stem cells for the treatment of recessive dystrophic epidermolysis bullosa. *Sci Transl Med* **6**: 264ra163.
26. Hanley, PJ, Mei, Z, Durett, AG, Cabreira-Harrison Mda, G, Klis, M, Li, W *et al.* (2014). Efficient manufacturing of therapeutic mesenchymal stromal cells with the use of the Quantum Cell Expansion System. *Cytotherapy* **16**: 1048–1058.
27. Roberts, I, Baila, S, Rice, RB, Janssens, ME, Nguyen, K, Moens, N *et al.* (2012). Scale-up of human embryonic stem cell culture using a hollow fibre bioreactor. *Biotechnol Lett* **34**: 2307–2315.
28. Hassell, T, Gleave, S and Butler, M (1991). Growth inhibition in animal cell culture. *Appl Biochem Biotechnol* **30**: 29–41.
29. Merten, OW, Charrier, S, Laroudie, N, Fauchille, S, Dugué, C, Jenny, C *et al.* (2010). Large-scale manufacture and characterization of a lentiviral vector produced for clinical ex vivo gene therapy application. *Hum Gene Ther* **22**: 343–356.
30. Ausubel, LJ, Hall, C, Sharma, A, Shakeley, R, Lopez, P, Quezada, V *et al.* (2012). Production of CGMP-grade lentiviral vectors. *BioProcess Int* **10**: 32–43.
31. Boussif, O, Lezoualc'h, F, Zanta, MA, Mergny, MD, Scherman, D, Demeneix, B *et al.* (1995). "A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine." *Proc Natl Acad Sci USA* **92**: 7297–7301.

Supplementary Information accompanies this paper on the *Molecular Therapy—Methods & Clinical Development* website (http://www.nature.com/mtm)