**Adeno-associated and lentiviral vector production in 2D and 3D formats with adherent cells in chemically defined, blood-free media**

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**Abstract**

Large scale manufacturing of viral vectors or vaccines with adherent cells still relies heavily on the inclusion of fetal bovine serum for the growth and production phases. The inclusion of serum presents numerous problems with the undefined chemical makeup, the undesirable safety profile, and the constraints and limitations on the global supply. Despite these challenges, alternatives to serum for adherent cells have been limited; however, advances in large-scale production of recombinant human proteins have enabled the advancement of blood-free media that can support adherent cell growth. In order to circumvent the need for serum in adherent platforms, we developed a serum and blood-free, chemically defined medium specific for adherent human epithelial kidney cells and evaluated growth kinetics as well as viral vector production with associated adenovirus and lentivirus. We observed doubling times equal to or faster than doubling times observed in serum containing medium. We also demonstrate transfection efficiencies and viral titers that are equivalent to or higher than that of serum. Our results demonstrate that fetal bovine serum is not required for culture of adherent HEK cells, and that a serum-free, blood-free, chemically defined approach can be reliably implemented in the production of viral vectors for gene therapy.

**Key words:** Serum-free, blood-free, lentivirus, AAV

**Introduction**

Advanced technology for gene delivery in living eukaryotic cells to correct a mutated gene, normalize gene expression, or deliver novel synthetic genes has resulted in a recent surge in therapeutic strategies to manage monogenic human diseases. Pseudotyped viral vectors are one such promising technology for gene delivery in eukaryotic cells. For example, third generation, self-inactivating lentiviral vectors (LV), derived from human immunodeficiency virus (HIV), are a propitious candidate for introducing genes to hematopoietic stem cells to correct primary immunodeficiencies and hemoglobinopathies (Milone & O'Doherty, 2018; Negre et al., 2016). Furthermore, these retroviral vectors have been used to introduce genes into mature T cells to induce antitumor responses through the generation of modified T cell receptors (TCRs) and chimeric antigen receptors (CARs) (Bobisse et al., 2009; Liu et al., 2020; Milone & O'Doherty, 2018; Tang et al., 2019; Wilkins, Keeler, & Flotte, 2017). In parallel, recent successes in using adeno-associated virus (AAV) in the treatment of monogenic diseases, such as ZOLGENSMA® for spinal muscular atrophy, provides firm proof of principal of these therapeutic strategies and has only fueled the enthusiasm for viral-mediated genetic manipulation in additional untested clinical applications (Al-Zaidy et al., 2019; Hacker, Bentler, Kaniowska, Morgan, & Buning, 2020).

Methodologies utilized in the manufacturing of these viral vectors has received notable attention considering some of these recent clinical successes. Both AAV and LV are currently being evaluated for clinical applications; however, the two viruses differ greatly in the mode of packaging from the producing cells (Chazal & Gerlier, 2003). Classically, these vectors are produced by transient transfection using adherent HEK293 (293) or the SV-40-transformed HEK293T (293T) cell line in fetal bovine serum (FBS) containing media (Arena, Chou, Harms, & Wong, 2019; McCarron, Donnelley, & Parsons, 2017; Pear, Nolan, Scott, & Baltimore, 1993; Robert et al., 2017; Sena-Esteves, Tebbets, Steffens, Crombleholme, & Flake, 2004). Though both of these cell lines exhibit the ability to readily adapt to growth and vector production in suspension culture using chemically defined media, overall vector yield can sometimes be negatively affected at scale (Cameau, Pedregal, & Glover, 2019; Clement & Grieger, 2016; Iyer, Ostrove, & Vacante, 1999; Morenweiser, 2005; Segura, Garnier, Durocher, Coelho, & Kamen, 2007). However, utilization of the classical adherent format can have significant setbacks due to the lack of scalability of production vessels typically employed at small scale (Clement & Grieger, 2016). The iCELLis® fixed bed bioreactor has emerged as an enabling technology to efficiently scale adherent-based processes in a controlled and highly integrated environment. This technology has been utilized in the manufacture of LV and AAV with commercially viable yields (Lennaertz, Knowles, Drugmand, & Castillo, 2013; Powers, Piras, Clark, Lockey, & Meagher, 2016; Rajendran et al., 2014; Valkama et al., 2018).

Though the iCELLis bioreactor represents a major breakthrough in the advancement of viral vector manufacturing, utilization of this bioreactor still relies heavily on the use of FBS to provide the appropriate mitogenic and survival signaling to 293 and 293T cells in order to achieve densities appropriate for transfection (Leinonen et al., 2020; Powers et al., 2016; Valkama et al., 2018). Use of FBS in large scale clinical manufacturing applications represents a significant safety risk due to potential adventitious agent contamination, unreliable supply chain from limited global supply, and considerable variability in vector yield due to lot-to-lot composition variations (van der Valk et al., 2018). In the United States, the use of FBS for the manufacture or testing of biologics requires rigorous screening for 10 different viruses prior to approval by the USDA (Hawkes, 2015). However, many questions remain about the sensitivity of tests to determine low levels of viral contamination, and the possibility of novel viral contaminants that are not readily tested for in serum lots (Sadeghi et al., 2017). Furthermore, FBS has considerable variation in composition, with proteomics studies identifying over 1,800 proteins and 4,000 metabolites, highlighting the lack of chemical definition in serum (Gstraunthaler, Lindl, & van der Valk, 2013). Nonetheless, several major protein components have been characterized and their functional contribution in cell culture is beginning to be elucidated (Gstraunthaler, 2003). Of these components, serum albumin is the most abundant and may be the most well described as it provides multiple functions *in vitro*, including antioxidant capabilities, macromolecule delivery, and free radical scavenger functions (Anraku, Yamasaki, Maruyama, Kragh-Hansen, & Otagiri, 2001; Francis, 2010). In addition, serum transferrin delivers iron to cells via catalytic cycling (Luck & Mason, 2012). Cytokines deliver the necessary mitogenic signaling to maintain the proliferation rate and promote survival of HEK cells. Together, these components provide the required factors for successful cell culture.

To circumvent the many challenges and drawbacks associated with using serum in viral vector production media for adherent cells, we utilized recombinant human proteins to formulate a chemically defined, serum and blood-free media that is optimized specifically for production of viral vector in 293 and 293T cells in the iCELLis fixed bed bioreactor. This media does not contain serum derived or blood derived proteins (blood-free) and is free from animal components. The growth kinetics, transfection efficiency, and productivity of this chemically defined medium in both 2D flatware and the fixed bed bioreactor exhibited equivalent performance to FBS supplemented DMEM. Total production time in the iCELLis bioreactor was also reduced to 5 days, demonstrating fast and efficient production, ultimately reducing cleanroom time and cost. Overall, the data presented here provides proof of principal that poorly defined and highly variable FBS can be replaced with recombinant proteins to formulate a chemically defined, blood-free, viral vector production medium suitable for large scale clinical applications.

**Materials and Methods**

*Cell lines and culture methods*

HEK293 and HT-1080 cells were obtained from ATCC (Catalog CRL-1573 and CCL-121). HEK293T cells were a generous gift from Dr. Erik Linklater. All cell lines were maintained in high glucose DMEM with GlutaMAX (Gibco, Grand Island, NY) supplemented with 10% US-sourced FBS (Gibco, Grand Island, NY), referred to as complete DMEM in this paper. Cultures were maintained in the presence of 100 U/mL Penicillin-Streptomycin (Gibco, Grand Island, NY) and cultured at 5% CO2 in 95% humidified incubators. Transition of 293 and 293T cells to OptiPEAK HEK293t® blood-free media, referred to as OptiHEK in this paper (InVitria, Junction City, KS), consisted of direct adaptation from cells cryopreserved in complete DMEM. OptiHEK cultured cells were maintained in a 10% CO2 and 95% humidity environment on CellBIND-treated flasks (Corning, Corning, NY). Cells were considered to be media-adapted to OptiHEK after 3 passages. All OptiHEK were maintained in the presence of 10 U/mL Penicillin-Streptomycin (Gibco, Grand Island, NY). Cells were maintained by passaging two times a week and seeding at a density of 10,000 cells per cm2. Cell density and viability were determined by trypan blue exclusion using a hemocytometer.

*Transfection of 2D cultures with GFP reporter plasmid for Transfection Efficiency*

Log phase and media-adapted 293 or 293T cells were plated in T-75 flasks in triplicate at an initial density of 50,000 cells/cm2 in either complete DMEM or OptiHEK and cultured for 24 hours to allow for full cell adherence at standard growth conditions. For transfection, 0.2 µg/cm2 Monster Green® Fluorescent Protein phMGFP plasmid (Promega, Madison, WI) was diluted 40-fold in either OptiMEM (Gibco, Grand Island, NY) for serum-cultured cells or OptiHEK supplemented with 1mg/mL of Optiferrin (InVitria Junction City, Kansas) for OptiHEK cultured cells. PEIpro (Polyplus Transfection, Illkrich, France) was added at a 1:1 ratio of 1µg PEI to 1µg pDNA and was diluted separately using the same complexing medium at the same dilution factor as pDNA. Complexes were formed by addition of pDNA to PEI and formed at rest for 15 minutes at room temperature. Post complexing, additional complexing media was added to equal 0.08 mL/cm2. Conditioned media was removed from the cells and DNA-PEI complexes were added by complete media exchange for the transfection mix. Cells were incubated at standard growth conditions for 2 hours and complexes were subsequently withdrawn, and a complete media exchange was performed. The appropriate growth medium was added back to the flasks and cells were further sub-cultured for 48 hours. Cells were harvested by 1x TrypLE + 1 mM EDTA (Gibco, Grand Island, NY) and analyzed via flow cytometry (Accuri C6 plus, BD Biosciences, San Jose, CA).

*2D Viral Vector Production of AAV-2-pAAV-ZsGreen1 and Lenti-pSIH1-H1-siLuc-copGFP*

2D transfection of AAV-2-pAAV-ZsGreen1 and Lent-pSIH1-H1-siLuc-copGFP was carried out as described above. Both AAV2 and LV were produced with a GFP transgene to allow for evaluation of transduction efficiency. Plasmids used for AAV-2 vector generation included the AAVpro® Helper Free System (AAV2) combined with the pAAV-ZsGreen1 vector sourced from Takara Bio (Mountain View, CA). AAV2 virus was harvested by cell lysis through the addition of 1x of a 10x lysis buffer (20mM MgCl­2, 10% Tween-20, 500mM HEPES, pH 8.0) directly into the cell medium. The pSIH1-H1-siLuc-copGFP lentivirus plasmid was obtained from System Biosciences (Palo Alto, CA). The plasmids for the 2nd generation lentiviral vector, psPAX2 and pMD2.G, were a generous gift from Dr. Ian Cartwright. Transfection with lentivirus plasmids was performed as described above with the following modifications: 0.16 µg/cm2 total plasmid DNA was used per transfection and cells were seeded at an initial density of 75,000 cells per cm2. A ratio of 3µg transfer plasmid: 2µg VSV-G envelope: 1µg packaging plasmid was used. Following transfection, lentivirus was produced for 48 hours, and then total supernatant was harvested and immediately analyzed for functional titer.

*iCELLis Fixed Bed Bioreactor Runs for Lentivirus Production*

iCELLis bioreactors (0.53m2, Pall Corporation, Westborough MA) fitted with biomass probes (Aber, Arlington VA) and DO and pH probes were assembled and calibrated per the manufacturer’s instructions. Bioreactors were sterilized by autoclave and then batched with either 650 mL of complete DMEM or OptiHEK 24 hours prior to cell inoculation to ensure complete wetting of the cell attachment surface and normalization of biomass probe conductivity. Immediately prior to inoculation, biomass probes were set to zero and cell inoculation was considered to be Time = 0 (T0). Bioreactor parameters were monitored online via BioXpert SCADA software and spent media was collected from the bioreactors once daily to measure media metabolites, offline pH, and osmolality. Bioreactor setpoints prior to transfection were maintained at 37 °C, pH 7.25, linear speed rate of 2 cm/s, and 95% DO or 55% DO for serum conditions, unless otherwise stated. Bioreactors were transfected with LV vector plasmids by transfecting with either OptiMEM (for serum expanded cells) or OptiHEK supplemented with 1 mg/mL recombinant transferrin (for serum-free expanded cells). Plasmid ratios were maintained as mentioned in 2D production of LV. 10% of the working volume was used for complexation, then 650mL of the transfection media including transfection complexes were added to the bioreactors by a complete media exchange. Bioreactors were transfected for 2 hours while maintaining bioreactor set points. Post transfection, bioreactors underwent a complete media change into full growth media and bioreactors were run for an additional 72 hours with the following set points: pH 6.8, linear speed 2 cm/s, 55% DO, unless otherwise noted. LV vectors were harvested by collected the complete supernatant from the bioreactors, clarified by centrifugation, and immediately used for functional titering.

*Functional Titering*

Functional titering was performed with transduction using a highly permissive cell line, HT-1080, which the AAV2 serotype is well described to readily transduce (Li, Bowles, van Dyke, & Samulski, 2005). HT-1080 cells were plated in 12-well plates at 30,000 cells/well and allowed to adhere for 2 hours at normal growth conditions. For GFP transduction, AAV-2-pAAV-ZsGreen1 and Lenti-pSIH1-H1-siLuc-copGFPwere titrated in infection medium consisting of DMEM+10% FBS supplemented with 0.1% F-68 (Gibco, Grand Island, NY). AAV2 was diluted in infection media and was added at 0.078 mL/cm2 to HT-1080 cells, which were subsequently incubated at standard growth conditions for 3 hours. Cells were then overlayed with 1 mL/well of complete DMEM and HT-1080 cells were further incubated for 48 hours to allow for GFP expression. Post 48 hour incubation, cells were harvested with 1x TrypLE and analyzed by flow cytometry for GFP expression. Functional titer for AAV2 was determined by standard curve using a positive control cell lysate containing a known concentration of AAV2-GFP full capsids determined by ELISA. The linear portion of the curve was used to estimate the functional titer of the unknown samples. Transductions using the Lenti-pSIH1-H1-siLuc-copGFP were done in the presence of 10 µg/mL polybrene (Millipore Sigma, St. Louis, MO). Lentivirus vectors were titered as previously described (Sena-Esteves & Gao, 2018). At the conclusion of the subculture time, cells were harvested with 1x TrypLE + 1 mM EDTA and enzyme was quenched with 10% FBS in DPBS (Gibco, Grand Island, NY). Cells were pelleted and resuspended in DPBS supplemented with 10% FBS, and 1x F-68. GFP expression was determined by flow cytometry.

*Statistics and Imaging*

Graphing and statistics were performed with R/R-studio version 4.1.0. A Welch’s modified T-Test was used to determine statistical significance unless otherwise noted. A p value < 0.05 was considered to be statistically significant. All experiments were carried out in at least three independent experiments. Images were taken with an Olympus IX81 Inverted microscope.

**Results**

*Growth Kinetics and Transfection Efficiencies of HEK293 and HEK293T in Flatware*

To determine the performance of 293 and 293T in blood-free, chemically defined conditions, cells banked in complete DMEM were directly adapted to OptiHEK and subjected to serial passages to compare doubling times. By direct adaptation of HEK cells grown in complete DMEM, we did not observe any problems with adherence to tissue culture plastic or changes in morphology when cells were placed into OptiHEK. We did, however, observe that 293T cells proliferated much more rapidly than the parental 293 cells. In complete DMEM, we calculated a doubling time of 29.81 ± 4.74 hours for 293T versus 40.71 ± 9.63 hours observed for the parental 293 cells (**Figure 1, A**). In head-to-head comparison of doubling times, we did observe a consistent faster doubling time in OptiHEK media compared to complete DMEM; however, the differences were not statistically significant (p ≥ 0.05 as determined by a Student’s T Test). In OptiHEK, 293T and 293 doubling times were 24.35 ± 2.54 and 33.87 ± 5.59 hours, respectively (**Figure 1, A**). Cells cultured in the chemically defined media exhibited the expected morphologies in both cell lines compared to serum containing media (**Figure 1, B-E**).

We next hypothesized that 2D transfection efficiency would be equivalent in complete DMEM compared to OptiHEK since growth kinetics showed equivalent performance. To test this, we transfected cells with a single GFP plasmid reporter in T Flasks to determine PEI-mediated transfection efficiency post adaptation to each media formulation. GFP expression was evaluated 48 hours post transfection and efficiency was calculated based on percent positive single cells for GFP as determined by flow cytometry. 293 and 293T cell lines exhibited greater GFP reporter expression in OptiHEK compared to complete DMEM (**Figure 2**). Transfection efficiency for 293 cells expanded in complete DMEM was 56.2 ± 8.17% GFP positive while OptiHEK-expanded cells had a transfection efficiency of 83.4 ± 4.43% (**Figure 2,** p < 0.005). 293T cells exhibited similar trends, though 293T exhibited a slightly lower transfection efficiency versus the parental 293 line. In complete DMEM, the transfection efficiency of 293T was 42.70 ± 8.18% positive GFP versus 73.0 ± 15.49% in complete OptiHEK (**Figure 2,** p < 0.005). Taken together, these results suggest that HEK cells cultured in OptiHEK do not exhibit any growth deficiencies and show improved transfection efficiencies to cells grown in the presence of serum.

*AAV-2-pAAV-ZsGreen1 and Lenti-pSIH1-H1-siLuc-copGFP Production in 2D flatware*

Transient transfection of multiple plasmids remains the method of choice for both small scale and large-scale virus production. The equivalent growth kinetics and improved transfection efficiency we observed with OptiHEK prompted us to test both AAV and LV production in OptiHEK compared to complete DMEM. We first initiated these studies in 2D flatware to determine the feasibility of virus production in adherent, blood-free conditions. Functional AAV2 titers produced in OptiHEK compared to complete DMEM showed no significant difference in the average functional titer as determined by flow cytometry, although there was a slight increase in the mean functional titer for AAV2 produced in OptiHEK with means of 3.19e+09 ± 2.06e+09 capsids/cm2 for serum and 3.97e+09 ± 2.98e+09 capsids/cm2 for OptiHEK (**Figure 3, A**). This result suggests that OptiHEK functions comparably to complete DMEM in terms of viral vector production for a non-enveloped, ssDNA based virus.

To determine the production efficiency of OptiHEK with an enveloped retrovirus, we evaluated lentivirus production with 293T cells. The number of cells plated for lentivirus production was increased from our standard transfection protocol to account for cell loss with media changes as 293T cells are more loosely attached than 293 (Lin et al., 2014). We also decreased the total µg of plasmid DNA used for transfection, as this resulted in a significant increase in functional titers in both OptiHEK and complete DMEM (data not shown). These changes resulted in functional titers for both complete DMEM and OptiHEK greater than 1e+07 TU/mL (**Figure 3, B**). In head-to-head comparison of lentivirus production in OptiHEK and complete DMEM, we observed no significant difference in the average functional titer over three independent experiments, although the average functional titer for OptiHEK was greater than the average for serum conditions (**Figure 3, B**). The average functional TU/mL for serum was 1.79e+07 ± 1.03e+07 TU/mL and the average for OptiHEK was 3.53e+07 ± 2.81e+07 TU/mL. Together, our results demonstrate that blood-free media can achieve equivalent or higher viral vector production compared to complete DMEM.

*Scale up of Lenti-pSIH1-H1-siLuc-copGFP Production*

The success we observed in 2D flatware in chemically defined, blood-free conditions suggested that the process could be scaled up to 3D systems that represent large scale manufacturing. To demonstrate that our 2D protocol could be readily scaled up, we produced lentivirus vectors with 0.53 m2 iCELLis Nano bioreactors using OptiHEK and complete DMEM and 293T cells. We performed a total of 2 runs with 4 vessels total to assess the productivity in OptiHEK compared to complete DMEM. In the first production run comparing complete DMEM and OptiHEK, bioreactors were seeded at an initial density of 7,000 cells/cm2 and transfected at day four of growth. Cells grown in both media showed an initial lag phase, as indicated by measurements from the biomass probe, although this was more pronounced in the serum grown cells compared to blood-free (**Figure 4, A**). Following transfection, linear speed was dropped for both media conditions from 2 cm/s to 1 cm/s, with the hypothesis that LV particles are sensitive to the shear stress generated by the constant agitation. DO and pH parameters were also maintained at the same set points for both media conditions following transfection. In the first production run, glucose and lactate trends were comparable for both conditions, however lactate initially started higher for complete DMEM due to the presence of lactate in serum (**Figure 4 B, C**). Cells cultured in OptiHEK also grew slightly faster, consistent with our 2D results, reaching a higher cell density at the time of transfection compared to complete DMEM. Despite the higher cell density at the time of transfection in OptiHEK, functional titer that was evaluated at 48 and 72 hours post transfection (HPT) was 2.5 fold lower in OptiHEK compared to complete DMEM, suggesting that the chosen parameters were not optimal for LV production in blood-free conditions (Run 1, **Figure 5**). Functional titer also dropped between 48 and 72 hours post transfection in both media, indicating a loss of vector stability or a cessation in vector production. We therefore performed optimization studies in OptiHEK to determine the optimal parameters for LV production in the iCELLis Nano bioreactor using OptiHEK (**Supplemental Figure 1**). The parameters tested included transfection density, linear speed post transfection, and DO post transfection (**Supplemental Figure 1, A-C**). We also hypothesized that lowering the pH post-transfection may be required for LV VSV-G pseudotyped virus envelope stability, as suggested by others (Holic et al., 2014). Of the parameters tested, lower transfection density consistently resulted in high viral titer, suggesting that higher transfection densities do not correlate with higher functional titer in blood-free conditions (**Supplemental figure 1, C & D**). DO was also negatively correlated with functional titer, suggesting that oxygen is not heavily consumed during virus production post transfection.

With optimized production parameters for OptiHEK, we performed a second LV production run comparing serum containing DMEM to OptiHEK (Run 2, **Figure 4 & 5**). The same parameters were applied to both media conditions. The second head-to-head run was seeded at a density of 10,000 cells/cm2 and cells did not display a lag phase in either medium. By day 2 of growth, cells had reached our target transfection density of roughly 75,000 cells/cm2 (**Figure 4, A**). Lactate and glucose trends were also comparable for both medium, with total glucose levels only dropping to 1 g/L by the end of production (**Figure 4 B, C**). LV particles were harvested 48 and 72 HPT and functionally titered. The highest functional titer was achieved 72 HPT or 5 days post bioreactor inoculation for both media. OptiHEK yielded the highest functional titer, with an estimated titer of 1.2e+07 TU/mL at 72 HPT (Run 2, **Figure 5**). Complete DMEM yielded a much lower functional titer, reaching 2.5e+06 TU/mL by 72 HPT (Run 2, **Figure 5**). Optimization of the production parameters achieved high titer yields in the chemically-defined media (Run 2**, Figure 5**). Thus, our optimized production parameters and chemically-defined conditions would be supportive of clinical demands for LV vectors (Parameters listed in **Table 1**).

**Discussion**

Adherent *in vitro* culture of HEK cells has typically relied on the inclusion of FBS for the successful attachment and proliferation of these cells. However, the inclusion of serum introduces a large number of variables due in part to the lack of chemical definition, which is further exasperated by reports of fraudulent FBS that has been mixed with bovine albumin or other undefined additives (Gstraunthaler, Lindl, & van der Valk, 2014; van der Valk et al., 2018). Levels of undefined growth factors, cortisol, and lipids can vary tremendously between lots of FBS, and all of these components can have significant effects on the growth and productivity of cells. In the United States, FBS certification also relies on the rigorous testing of several viral contaminants; however, this list does not account for novel viral contaminants that can cross the placental barrier and moreover there are many questions surrounding whether or not these tests can detect low levels of viral contaminants that can then go on to be propagated in the lab. Sterility measures to eliminate contaminants from FBS, such as gamma irradiation or charcoal stripping can also have significant effects on serum components, leading to variability and un-reproducible results (Plavsic, Nims, Wintgens, & Versteegen, 2016; Sikora, Johnson, Lee, & Oesterreich, 2016). This lack of chemical definition and high variability has caused researchers to rigorously test batches of FBS, resulting in costly and time consuming performance tests that are not regulated between labs (Baker, 2016).

Despite the wide concerns over the reproducibility and efficacy of FBS use in cell culture, alternatives for adherent cells are lacking. Furthermore, the inadequacy of definition for serum-free or xeno-free has resulted in numerous products that are nevertheless dependent on bovine or human sera derived components. Given the need to overcome the dependence on FBS and blood-derived components, we developed a chemically defined, serum-free and blood-free cell culture medium that supports the adherence and proliferation of HEK cells. OptiHEK is formulated with recombinant human proteins that are not derived from animal sources or blood serum and has been optimized to support 2D and 3D growth and viral vector production with HEK cells. We demonstrate here that HEK-293 and HEK-293T cells grown in OptiHEK show doubling times that are equal to or faster than doubling times observed with complete DMEM (**Figure 1**). Improved doubling times become especially important when considering large seed-trains that are required to inoculate large-scale bioreactors for manufacturing. We have also observed that HEK cells can be immediately adapted to OptiHEK without gradually decreasing concentrations of serum, improving on concerns about the risk of moving cells from serum to blood-free conditions.

We further demonstrate that a chemically-defined and blood-free medium is also capable of supporting cells in 2D and 3D, can serve as a reliable transfection medium, and does not sacrifice functional viral titer for chemically definition. We observed both high transfection efficiency (**Figure 2**) and high AAV and LV titer (**Figure 3**) in OptiHEK compared to DMEM supplemented with FBS. LV production seemed to benefit the most from chemically defined and blood-free medium, which as a secreted, enveloped virus may be more subjected to components in the medium. Furthermore, we also showed that a 5-day total production time is possible with LV production (**Figure 4, A**), achieving high titer 72 hours after transfection, greatly reducing production costs and cleanroom time. We also identified transfection density as a key parameter affecting viral titer (**Supplemental Figure 1**), suggesting that transfecting a low cell density can still achieve high viral titer and again, cut down on time and production costs. Our results also suggest that maintaining a linear speed of 2 post transfection does not negatively impact viral titer, and may even enhance titer, as we observed viral titer to drop from 48 to 72 hours in our first production run of complete DMEM and OptiHEK (**Figure 5**). Lastly, our production parameters did not result in high titers for complete DMEM, suggesting that components within serum may inhibit transfection or virus production at scale. Taken together, our results demonstrate that recombinant proteins in a chemically defined and blood-free medium can achieve high titer virus production at scale and without the constraints and limitations of serum.

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**Author Contributions**

S.A.P and R.A wrote the manuscript and designed experiments. S.A.P, R.A, and A.P performed experiments, analyzed data, and edited the manuscript. N.H and A.L provided expertise on iCELLis bioreactor runs and helped in the design of these experiments as well as editing the manuscript. All authors approved the final manuscript.

**Conflict of Interest**

S.A.P, R.A, and A.P are employees are InVitria. N.H and A.L are employees of Pall Corporation. The authors have no other competing interests to declare.

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**Tables**

**Table 1. Set point parameters for lentivirus production with OptiHEK in the iCELLis bioreactor**

|  |  |  |
| --- | --- | --- |
| **Parameter** | **Pre-transfection** | **Post-transfection** |
| Linear speed | 2 | 2 |
| DO | 95 | 55 |
| pH | 7.25 | 6.8 |

**List of Figure Captions**

**Figure 1: Growth characteristics and morphology of adherent HEK cells in complete DMEM and chemically defined OptiHEK**. (A) Doubling time of HEK293 and HEK293T cells in complete DMEM and OptiHEK. Cells were passaged for 6 passages and doubling times were calculated at each passage. Error bars represent standard deviation of three replicates. (B, C) Brightfield images of HEK293 cells after 3 days of growth in 2D flatware cultured in complete DMEM (B) and OptiHEK (C). (D, E) Brightfield images of HEK293T cells after 3 days of growth in 2D flatware cultured in complete DMEM (D) and OptiHEK (E). Scale bar represents 100 µm.

**Figure 2: Transfection Efficiency of HEK293 and HEK293T in complete DMEM and OptiHEK.** Transfection efficiency in HEK293 cells (left) and HEK293T cells (right) cultured in complete DMEM and OptiHEK. In all cases, cells were transfected with a single plasmid GFP reporter and then harvested 48 hours later and analyzed by flow cytometry. The mean was calculated from 3 independent experiments with 2 replicates each. Error bars represent standard deviation from the mean. \*\* represents p > 0.005 as determined by Welch’s modified T test.

**Figure 3: Viral vector Production in 2D**. (A) Estimated AAV2 functional titer determined by HT-1080 transduction assay and flow cytometry for GFP positive cells. Capsids were determined using a standard curve method based on a standard of known concentration. (B) Estimated LV functional titer determined by HT-1080 transduction assay and flow cytometry for GFP positive cells. LV Functional titer was calculated as previously described (Sena-Esteves & Gao, 2018). The averages were calculated from 3 independent experiments. Error bars represent standard deviation from the mean.

**Figure 4: iCELLis bioreactor-based production of Lentivirus.** (A) Growth curves for HEK293T cells during LV production in complete DMEM and OptiHEK in the iCELLis bioreactor. Cell density per cm2 was calculated from online measurements with the Aber biomass probe. (B) Glucose consumption during LV production in complete DMEM and OptiHEK for two head-to-head production runs. (C) Lactate production during LV production in complete DMEM and OptiHEK for two head-to-head production runs.

**Figure 5: Lentivirus functional titer produced in the iCELLis Nano Bioreactor.** Functional titer of LV produced with complete DMEM or OptiHEK. Supernatant for titering was collected from the bioreactor vessels at 48 hours post transfection (HPT) or 72 HPT and titered with HT-1080 as described in the methods section. Run 1 represents first head-to-head production run (Left). Run 2 represents the second head-to-head production run after optimization studies (Right).