Journal of Virological Methods 325 (2024) 114884

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Protocols

Optimised protocols to generate high titre lentiviral vectors using a novel transfection agent enabling extended HEK293T culture following transient transfection and suspension culture

Saqlain Suleman [a,1], Serena Fawaz [a,1], Terry Roberts [a], Stuart Ellison [b], Brian Bigger [b], Michael Themis [a,c,d,*]

[a] Department of Life Sciences, College of Health, Medicine & Life Sciences, Brunel University London, Uxbridge, UK
[b] Division of Cell Matrix Biology & Regenerative Medicine, School of Biological Sciences, Faculty of Biology Medicine and Health, University of Manchester, Manchester, UK
[c] Division of Ecology and Evolution, Department of Life Sciences, Imperial College London, London, UK
[d] Testavec Ltd, Queensgate House, Maidenhead, UK

ARTICLE INFO

Keywords:
Lentivirus
Transient transfection
Gene therapy
Suspension culture
HEK293T

ABSTRACT

HIV-1 based lentiviral viruses are considered powerful and versatile gene therapy vectors to deliver therapeutic genes to patients with hereditary or acquired diseases. These vectors can efficiently transduce a variety of cell types when dividing or non-dividing to provide permanent delivery and long-term gene expression. Demand for scalable manufacturing protocols able to generate enough high titre vector for widespread use of this technology is increasing and considerable efforts to improve vector production cost-effectively, is ongoing. Current methods for LV production mainly use transient transfection of producer cell lines. Cells can be grown at scale, either in 2D relying on culturing producer cells in multi-tray flask cell culture factories or in roller bottles or can be adapted to grow in 3D suspensions in large batch fermenters. This suits rapid production and testing of new vector constructs pre-clinically for their efficacy, particle titre and safety. In this study, we sought to improve lentiviral titre over time by testing two alternative commercially available transfection reagents Fugene® 6 and Genejuice® with the commonly used polycation, polyethyleneimine. Our aim was to identify less cytotoxic transfection reagents that could be used to generate LV particles at high titre past the often used 72 h period when vector is usually collected before producer cell death is caused due to post transfection cytotoxicity. We show that LV could be produced in extended culture using Genejuice® and even by transfected cells in glass flasks in suspension. Because this delivery agent is less toxic to 293 T producer cells, following optimisation of transfection we found that LV can be harvested for more than 10 days at high titre. Using our protocol, titres of 10^9 TU/ml and 10^8 TU/ml were routinely reached via traditional monolayer conditions or suspension cultures, respectively. We propose, this simple change in vector production enables large volumes of high titre vector to be produced, cost effectively for non-clinical in vivo and in vitro applications or for more stringent downstream clinical grade vector purification.

1. Introduction

Gene therapy using retrovirus (RV) and lentivirus vectors (LV) has been used successfully to treat several monogenic diseases such as β-thalassaemia (Magrin et al., 2019), sickle cell anaemia (Urbinati et al., 2018), Wiskott–Aldrich syndrome (Aiuti et al., 2013) and more recently

Abbreviations: RV, Retrovirus; LV, Lentivirus; ADA-SCID, Adenosine deaminase severe combined immunodeficiency disease; TU, Transducing units; HIV-1, Human immunodeficiency virus 1; LTR, Long terminal repeat; VSV-G, Vesicular stomatitis virus glycoprotein; FIV, Feline immunodeficiency virus; EIAV, Equine infectious anaemia virus; HEK293T, Human embryonic kidney 293 T cells; PEI, Polyethyleneimine; DMEM, Dulbecco's modified eagle medium; PDM, Plasmid DNA medium; pDNA, Plasmid DNA; GFP, Green fluorescence protein; RCL, Replication competent virus.

* Corresponding author at: Department of Life Sciences, College of Health, Medicine & Life Sciences, Brunel University London, Uxbridge, UK.
E-mail address: michael.themis@brunel.ac.uk (M. Themis).

[1] Authors contributed equally to this work

https://doi.org/10.1016/j.jviromet.2024.114884
Received 6 June 2023; Received in revised form 2 January 2024; Accepted 8 January 2024
Available online 11 January 2024
0166-0934/© 2024 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

for CAR-T therapy of cancer of the blood and solid tumours (Nobles et al., 2020). These vectors have been shown as attractive gene delivery vehicles because they offer permanent integration of a transgene into the host genome and long-term gene expression (Lewis et al., 1992). Recently approved gene therapy vectors based on γRV include Strimvelis (GlaxoSmithKline) to treat adenosine-deaminase-deficient severe combined immune deficiency (ADA-SCID) and Yescarta (Gilead) for the treatment of large B-cell lymphoma (FiercePharma, 2020).

Over 5000 gene therapy clinical trials have so far been initiated of which several used RV and LV (https://a873679.fmphost.com/fmi/webd/GTCT) vectors. Recombinant γRV have in the past been found to produce replication competent particles capable of continued production and infection(Schwarzer et al., 2021). This problem was overcome by using three rather than 2 plasmid systems to carry cis and trans vector components, with a plasmid carrying the RV genome containing a virus psi (ψ) packaging signal in addition to a therapeutic gene cassette and two discrete plasmids devoid of the ψ site carrying the remaining virus structural genes, respectively, for vector packaging. Traditionally, RV were generated by permanent producer cell clones isolated in steps following transfection of each plasmid component with vector titres from optimised cell lines reaching titres around 10^5-10^6 TU/ml (Chong et al., 1998). Unfortunately, γRV have also been shown to cause genotoxic effects in the clinic resulting in patient leukaemia (Hacein-Bey-Abina et al., 2008, 2003). LV have replaced RV as the vector of choice with the ability to transduce both dividing and non-dividing cells, in contrast to RV that are restricted to infect dividing cells only. LV, mainly based on HIV are used for gene transfer and to generate these vectors, 3 or 4 plasmids with separated cis and trans virus components have been engineered to prevent the emergence of replication competent virus (Zufferey et al., 1997; Fouchier et al., 1996; Dull et al., 1998). In addition, the vector long terminal repeat on the cis plasmid, found at the 5′ and 3′ ends of the vector backbone that acts as a promoter and enhancer has been modified to self-inactivating configuration to prevent promoter activation of neighbouring genes following virus integration into the host genome, thereby reducing the likelihood of insertional mutagenesis (Cornetta et al., 2011; Zufferey et al., 1998).

Currently, transient transfection is used to generate LV for a number of reasons. It enables rapid production of vectors to test their efficacy for gene expression, titre and cell targeting. The plasmid used specifically to produce a glycoprotein, carried by LV particles for infection can be modified with alternative genes for switching of surface glycoproteins for cell specific pseudotyping to reach cells most suitable for gene therapy. Most commonly, though the Vesicular stomatitis virus glycoprotein (VSV-G) is used, which broadens infection to a wide range of cell types, improves LV stability and enables good vector yields following virus concentration by ultracentrifugation. However, because VSV-G envelope is highly cytotoxic, by causing syncytial formation of virus producer cells, this makes permanent production from these cells difficult. Transient transfection, therefore, circumvents VSV-G cytotoxicity by generating particles at high titre over a 72 h period when LV is harvested before cell death occurs. Typically, transient transfection yields LV at titres of 10^5 to 10^7 TU/ml that can be concentrated to 10^9 TU/ml (Coil and Miller, 2004; Estepa et al., 2001).

Currently, transient transfection of LV plasmids in 2D often use human embryonic kidney cells with expression to SV40 T antigen (HEK293T) for production, however, for the clinic, the T antigen is avoided (Tan et al., 2021). As an alternative to calcium phosphate as the most commonly used transfection reagent (Lesch et al., 2011), the cationic polymer polyethylenimine (PEI), offers transfection in serum-free conditions to adherent and suspension culture cells (Segura et al., 2013) and HEK293T cells have been adapted to grow in serum free suspension conditions (Ansorge et al., 2009) with scalability of up to 3 litres to generate high titre vector. Alternative commercially available transfection reagents include cationic lipid-based compounds such as lipofectamine™ (ThermoFisher Scientific), Fugene® 6 (Promega) and polyamines such as Genejuice® (Merck Millipore). These use a similar mechanism to PEI for intracellular delivery of DNA for successful generation of LV (Segura et al., 2013; Zhang and Vinogradov, 2010). Transfection reagents, however, often cause cytotoxicity that occurs approximately 72 hrs post transfection that limits virus production time before producer cell death(Moghimi et al., 2005). Production is also hampered by expression of the VSV-G envelope protein used to pseudotype virus particles for efficient infection of target cells(Cronin et al., 2005). To circumvent this, cells have been engineered for controlled LV production using inducible VSV-G expression that enables long term producer cell growth in bioreactors (Ikeda et al., 2003; Merten et al., 2016; Manceur et al., 2017). Inducible VSV-G expression also enables stable cell lines to be created, however, this is still time-consuming and costly. As this is not suitable to test newly developed novel LV constructs for high titre, and efficacy of gene transfer and expression of reporter or therapeutic genes, transient transfection is the preferred choice for pre-clinical vector production.

Generation of LV during the short 72 hr time frame post transfection wastes expensive transfection reagents and plasticware, used to grow cells (Tan et al., 2021). This time limitation to vector harvest is understood to be due to transfection reagent toxicity and to the toxic nature of VSV-G expression, which causes cell death by syncytial formation between cells in close proximity as monolayer cultures (Eslahi et al., 2001). In this study, we examined ways to improve LV production. Firstly, we tested an alternative transfection reagent Genejuice® next to Fugene® 6 and the less expensive, but cytotoxic PEI polycation, that each are commonly used for high titre LV production. To avoid cell toxicity, we also investigated whether post transfected cells could be rapidly converted to grow in suspension with the hypothesis that this may avoid syncytial formation by cell-to-cell contact. Finally, having found cells can be converted to suspension cultures, to avoid the use of expensive commercially available transfection agents, we optimised the low-cost polycation PEI for transfection of HEK293T cell monolayers at a concentration that reduced cytotoxicity whilst providing high level plasmid transfer. We then showed this agent could be used to transfect cell cultures directly in suspension in glass bottles to avoid the use of expensive plasticware. These simple changes to currently used protocols offer significant reduction in the cost of LV production at high titre suitable to non-clinical vector testing and further downstream processing.

2. Materials and methods

2.1. Cell culture

HEK293T cells were cultured in Gibco™ DMEM, high glucose, GlutaMAX™ Supplement, with pyruvate (Fisher Scientific, Hemel Hempstead) supplemented with 10% (v/v) foetal bovine serum (Gibco) and 1% Penicillin Streptomycin (Gibco) at 37 °C with 5% CO^2. Cells were serially passaged when 70–80% confluent. Cells were passaged regularly when confluent using TrypLE™ Express (Gibco), according to manufacturer's instructions. Cell viability and survival following recovery from liquid nitrogen and treatment was measured using trypan blue exclusion assay, measured via Countess IL Automated Cell Counter according to manufacturer's instructions (Fisher Scientific). Cells were also routinely tested for the absence of mycoplasma during this study.

Images of cells under brightfield or green fluorescence conditions were captured on the EVOS FLoid Imaging System (Thermofisher Scientific), at 20% and 30% intensities for brightfield and green fluorescence light respectively.

2.2. LV production in monolayer conditions

Second generation self-inactivating LV expressing the green fluorescence protein GFP (pHR LV) was produced using triple plasmid co-transfection of HEK293T cells, as previously described (Suleman et al., 2022; Khonsari et al., 2016; Tijani et al., 2018). Plasmids

(pHR'SINcPPT-SFFV-eGFP-WPRE (pHR), pMD.G2 and pCMVΔR8.74) were generated through growth of transformed DH5α or Stbl3 E. coli cells in plasmid DNA medium (PDM) (Danquah and Forde, 2007). Plasmids were harvested using the Invitrogen™ PureLink™ Expi Endotoxin-Free Mega Plasmid Purification Kit (Fisher Scientific), according to the manufacturer's instructions. Plasmid integrity were verified through restriction enzyme digests, as per manufacturer's instructions (all enzymes were purchased from New England Biolabs, Buckinghamshire). These plasmids were used for transfection and LV generation.

While PEI and Fugene® 6 transfection methods have been published widely and optimised, the use of Genejuice® has not been published widely. Therefore, optimisation of this reagent was performed transfection of HEK293T cells using 8 µg in total of pHR, pCMVR8.74 and pMD2. G plasmids in a 4:3:1 ratio, with varying ratios of pDNA to Genejuice® (1:2, 1:3, 1:6 and 1:8). Medium was replaced 24 h post transfection and collected every 24 h for 72 h post medium change. Following collection viral supernatants were cleared of cell debris by filtering through a 0.45-µm filter and stored at 4°C for further use.

For general LV generation. HEK293T cells were seeded at a density of 1.5×10^7 per T175 flask and incubated at 37 °C, 5% CO2 overnight as above. 80% confluent cells were transfected using pHR, pMD.G2 and pCMVΔR8.74 in serum free medium (Opti-MEM™, Fisher Scientific). The various parameters using different transfection reagents, including optimised conditions for Genejuice® are shown in Fig. 1A. Medium was replaced after 24 h transfection with complete medium (Gibco™ DMEM, high glucose, GlutaMAX™ Supplement, with pyruvate, supplemented with 10% (v/v) foetal bovine serum and 1% Penicillin Streptomycin). Conditioned medium collected and replaced every 24 h for a further 72 h. Following collection viral supernatants were cleared of cell debris by filtering through a 0.45-µm filter and stored at 4°C for further use.

2.3. LV ultracentrifugation

Filtered viral conditioned medium totalling approximately 35 ml was centrifuged at 23,000 rpm for 2.5 h, at 4 °C in a SW32Ti swing out rotor using an Optima XPN-80 ultracentrifuge (Beckman Coulter, High Wycombe). This is to ensure stability of virus and storage of high titre virus for downstream experiments. Following centrifugation, the viral pellet was resuspended in 200 µl of Opti-MEM™ (concentration factor of 175x) and frozen at − 80 °C for long term storage.

2.4. Optimisation of LV production in suspension conditions

Adherent HEK293T cells were grown in suspension using one of two methods: static and rotating. Initially cells were seeded on poly(2-hydroxyethyl methacrylate) (Sigma Aldrich, Dorset) coated plates. For transfection in static suspension, HEK293T cells were grown as a monolayer on adherent tissue culture grade plasticware as described above (Section 2.2). Cells were detached as previously described and 1.5×10^7 cells were resuspended in 12 ml Opti-MEM™ containing either PEI or Genejuice® transfection reagents and plasmids. Cells were seeded onto the coated plates and incubated at 37 °C, 5% CO_2. Medium was replaced 24 h post transfection and viral harvests collected every 24 h three times, for a total of 72 h post initial medium change. A similar protocol was used for transfection of cells grown in rotating suspension cultures. Cells were seeded into a glass Erlenmeyer flask and rotated at 100 rpm.

General maintenance of untransfected suspension cultures involved medium changes 3 times a week as well as dissociating spheroid like structures for viability, as previously described (Grasser et al., 2018).

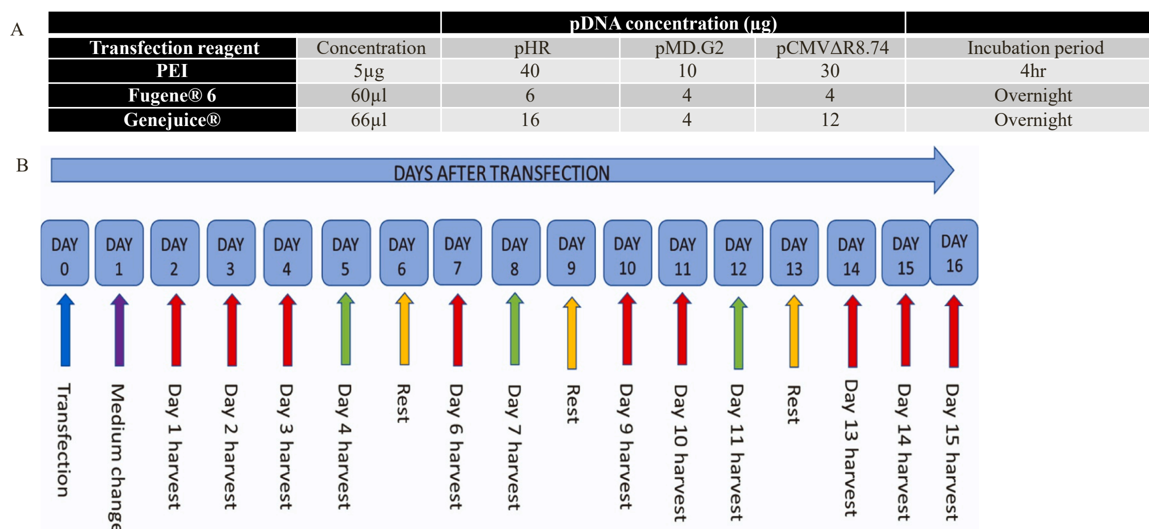
2.5. Lentiviral titration

Virus titres representing positive GFP expression as a result of LV infection (independent of integration site choice) were obtained via flow cytometry, as previously described (Suleman et al., 2022; Gay et al., 2012). Briefly, 2×10^5 HEK293T cells were seeded in a 12 well plate format overnight. Cells were then transfected with serial viral dilutions that had been complexed with 5 µg/ml polybrene (Sigma Aldrich) for 20 min at room temperature. Medium was replaced 24 h post transduction and incubated for a total of 72 h before end point analysis via flow cytometry analysis of GFP expression via ACEA Novocyte Flow Cytometer (Agilent Technologies, Cheshire). Data was analysed using NovoExpress (v1.2.1) software.

p24 titration of LV particles were calculated using Lenti-X™ p24 Rapid Titre Kit (Takara Bio, France) according to manufacturer's instructions. Samples were analysed using Elx808 absorbance reader (BioTek, Swindon) and data analysed using Gen5 (v2.06.10) software.

2.6. Gene rescue assay

HEK293T cells were transduced with pHR LV at a MOI of 10 for 24 h



A

		pDNA concentration (µg)			
Transfection reagent	Concentration	pHR	pMD.G2	pCMVΔR8.74	Incubation period
PEI	5µg	40	10	30	4hr
Fugene® 6	60µl	6	4	4	Overnight
Genejuice®	66µl	16	4	12	Overnight

Fig. 1. - pHR LV transfection parameters. A- Conditions of LV production used for transfection using PEI, Fugene® 6 and Genejuice® in monolayer transfection of cells. Various volumes and incubation times used as standard practise to compare pHR LV production between reagents. B- Schematic of LV production in a scalable fashion using Genejuice® transfection reagent. Conditioned medium from transfected cells were harvested over 15 days for virus production and titrated to optimise LV production.

before replacing medium. Cells were serially passaged over 14 days and conditioned medium diluted with fresh complete medium at a 50% (v/v) ratio and complexed with 5 μg/ml polybrene incubated at room temperature for 20 min. Conditioned medium was added to fresh uninfected HEK293T cells. Cells were incubated for 72 h before flow cytometry to detect GFP expression.

2.7. Cell viability analysis

Analysis of cell viability was performed using a trypan blue exclusion assay, using a Countess automated cell counter (Thermofisher Scientific) as per manufacturer's instructions.

2.8. Statistical analysis

Standard error of the mean is presented with numerical data where appropriate. Mann-Whitney U tests were performed between data sets to determine significance ($P < 0.05$).

3. Results

3.1. Genejuice® transfection reagent optimisation for LV production

To explore the efficiency of Genejuice® next to PEI and Fugene® 6 reagents that have been previously used for 3 plasmid transfection to produce LV from HEK293T cells, we firstly optimised Genejuice® performance by a series of reagent/plasmid ratios using 1×10^6 cells per treatment. Initially, HEK293T viability was measured using Genejuice®/plasmid DNA (pDNA) ratios of between 1:2, 1:3 (manufacturers recommendations for single plasmid transfection), 1:6 and 1:8 (Fig. 2A). Then, at these ratios, cell supernatants containing virus particles were used to infect fresh HEK293T cells for LV titration. Genejuice®/plasmid ratios of 1:2, 1:3, 1:6 and 1:8 provided unconcentrated vector titres of 3.19×10^6 TU/ml (+/- 2.56×10^6), 1.23×10^7 TU/ml (+/- 6.79×10^6), 5×10^5 TU/ml (+/- 1.27×10^5) and 2.77×10^5 TU/ml (+/- 1.11×10^5), respectively (Fig. 2B). Although the optimised ratio of 1:2 gave a titre only around 2-fold less than the manufacturers recommendation of 1:3, the 1:2 ratio was chosen for further titre optimisation as this provided higher cell survival for virus production for examination of LV production past the 72 h commonly used for virus harvest (Section 3.3) and later investigation of transfection of suspension culture cells (Section 3.4).

3.2. Superior cell viability and LV vector titre using Genejuice® versus PEI and Fugene® 6 reagent transfected HEK293T monolayers

Using optimised transfection protocols, we next compared Genejuice® to PEI and Fugene® 6 for HEK293T viability at 24, 48 and 72 hr time points (Fig. 3 A) using 1.5×10^7 cells. Once again, optimal cell survival was observed by Genejuice®, however, cell viability was greater than small scale transfection reaching up to only 5% below that of untreated cells. Next, we compared LV titre for each reagent at 72 h after HEK293T transient transfection. Genejuice® provided superior pHR LV titre production (shown as concentrated particles) than the other reagents at 2.06×10^9 (+/- 5.13×10^8) compared to 1.28×10^8 (+/- 8.44×10^7) and 1.57×10^8 (+/- 4.06×10^7), for PEI and Fugene® 6, respectively (Fig. 3 B and C). Using p24 titration gag assay, virus titre was also confirmed to be superior using Genejuice® at 2.62×10^{13} LP/ml versus 2.8×10^{12} by PEI and 1.37×10^{12} by Fugene® 6.

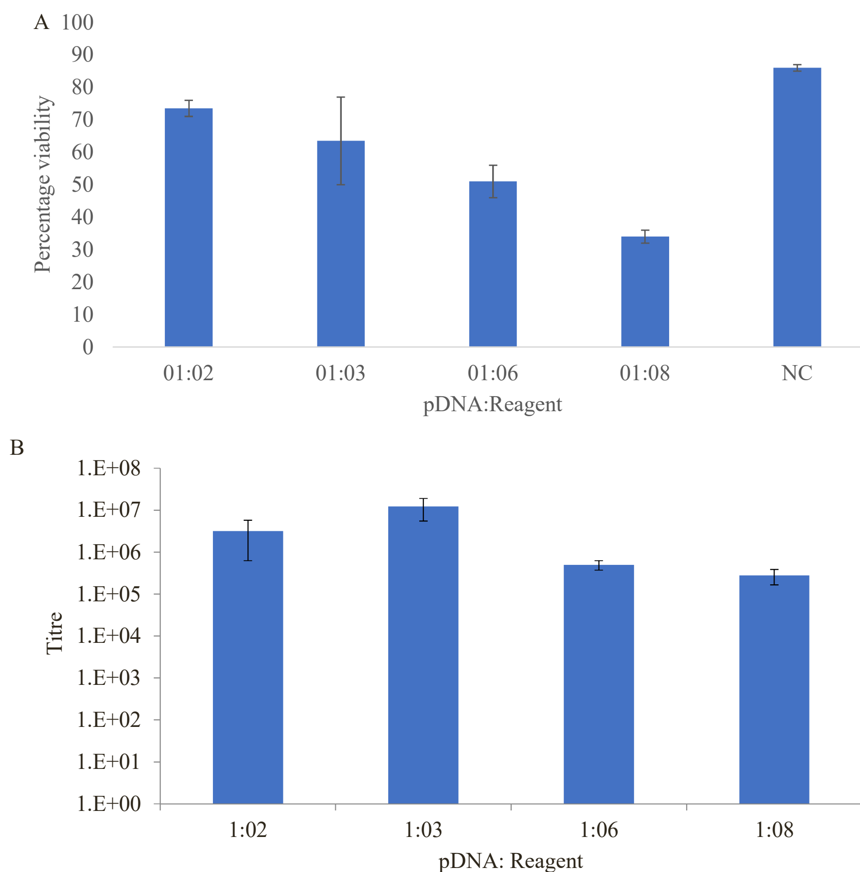


Fig. 2. - Optimisation of Genejuice® transfection. A- Viability of HEK293T cells transfected for pHR LV production using various ratios of pDNA:reagent used for transfection of 1×10^6 HEK293T cells for LV production compared to untreated cells ($n = 4$, $P < 0.05$). B- Titre of unconcentrated pHR LV generated (TU/ml), as determined through GFP expression using flow cytometry 72hrs post transduction to determine titre ($n = 4$).

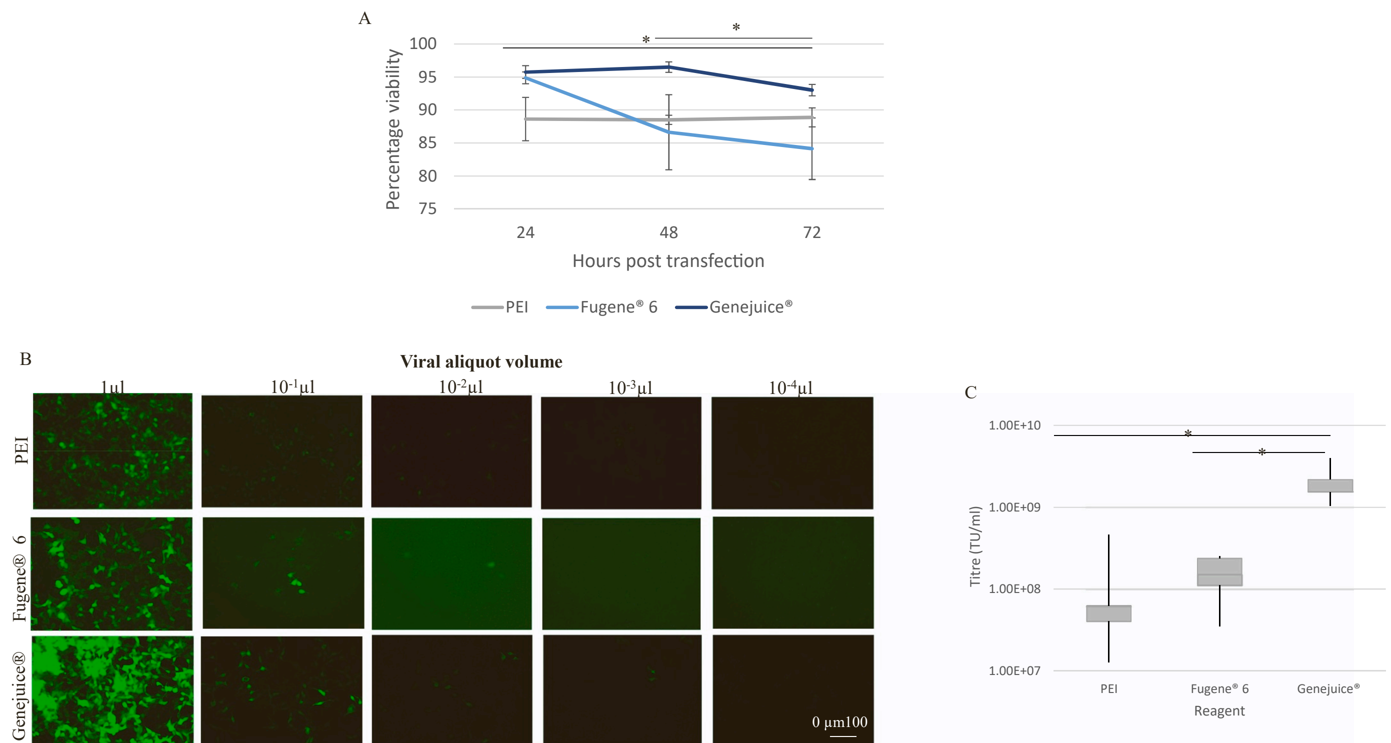


Fig. 3. - pHR LV production comparison. Comparative optimised transfection protocols were used to generate pHR LV using PEI, Fugene® 6 or Genejuice® transfection reagents.
A- Viability of HEK293T cells transfected for pHR LV production over 72 h virus collection period using a trypan blue exclusion assay. Cells transfected using PEI were found to be statistically significant in comparison to untreated cells. Cells transfected using Fugene® 6 were found to be statistically significant 48 h post transfection. These results indicate the success of Genejuice® reagent in LV production ($n = 4$, $P < 0.05$). B- Fluorescent cell microscopy of HEK293T cells transduced with LV using a limiting dilution assay. Titres were determined using flow cytometry (TU/ml). Cells shown under green fluorescence light 72 h post transduction, x10 magnification. C- LV titres generated through transient transfection of 1.5×10^7 HEK293T cells using different transfection reagents after concentration. The concentrated titres generated using Genejuice® were found to be significantly higher in comparison to titres from PEI or Fugene® 6 transfection ($n = 5$, $P < 0.05$).

3.3. Genejuice® enables extension of producer cell culture and LV production

As we showed Genejuice®/plasmid ratio of 1:2 provided optimal LV titre over PEI and Fugene® reagents with highest HEK293T survival 72 h post transfection, we sought to determine whether this protocol allows LV production by prolonged cell culture. HEK293T cells were transfected and maintained past 72 h to 16 days for LV production (Fig. 1B), during which time cells and virus supernatants were harvested. Cell viability at days 4, 7, 11 and 15 was 96.50 ± 0.50%, 95.17 ± 1.17%, 96.17 ± 1.17% and 86.17 ± 3.61%, respectively (Fig. 4 C). As we and several other laboratories have experienced, cell viability reduces drastically 72 h after PEI or Fugene® 6 transfection, however, no significant reduction was observed in Genejuice® transfected cells up to day 11 post transfection, whilst exhibiting strong GFP expression (Fig. 4 A) enabling vector harvest up to day 15. LV titre ranged from as high as 2.42×10^{11} TU/ml (day 2) and remained high between days 3 slowly reducing to 4.25×10^7 TU/ml at day 10 after which no LV titre could be found (Fig. 4 A and B). Over this time course, RCL assay showed absence of replication competent virus particles.

3.4. Optimisation of LV production using transfection of HEK293T suspension cell culture cells

We next explored alternative ways to generate LV from HEK293T cells to improve vector titre and compared these to the method described above ($n = 3$). Firstly, cells were transfected as 2D monolayers then converted to 3D suspension followed by supernatant harvest with the rationale that in suspension HEK293T cells may offer a larger cell surface area for particle budding. Second, we transfected cells in suspension with the aim of improving transfection efficiency to increase LV titre. Our rational for these approaches was also that suspension culture increases the concentration of viral particles generated per volume in cell supernatants and offers the potential for production scale up whilst eliminating ultracentrifugation of labile LV particles. This approach is benefitted by the fact that HEK293T cells can be converted to 3D suspension growth by transfer and maintenance on poly-2-hydroxyethyl methacrylate coated dishes that prevents cell adhesion.

Each method was performed, initially on a small scale using 1×10^6 HEK293T cells and supernatants were harvested at 24, 48 and 72 h post Genejuice® transfection. For cells transfected as monolayers then grown as 3D suspension cultures, a titre 7.12×10^7 was obtained collectively for these harvests, which was, on average 3-fold greater than using the equivalent cell number to generate vector by conventional monolayer transfection (Fig. 5). Transfection of 1.5×10^7 cells by this method routinely generated LV titres around 3×10^8 TU/ml.

We next used Genejuice® for transfection of 1.5×10^7 HEK293T cells in suspension and harvested virus up to 72 h post transfection, maintained on poly-2-hydroxyethyl methacrylate coated culture plastic. Levels of transfection in suspension appeared equivalent to when cells were transfected as monolayers. This method also achieved vector titre averaging 3.0×10^8 ($+/-6.91\times10^7$) TU/ml without the need for particle concentration (Fig. 5).

3.5. PEI optimisation for suspension transfection

Thus far, we showed Genejuice® offers advantages over PEI or Fugene® reagents to generate high titre LV particles with low cytotoxicity and efficient transfection of HEK293T cells in suspension. We also showed cells could be transfected as monolayer then converted to 3D

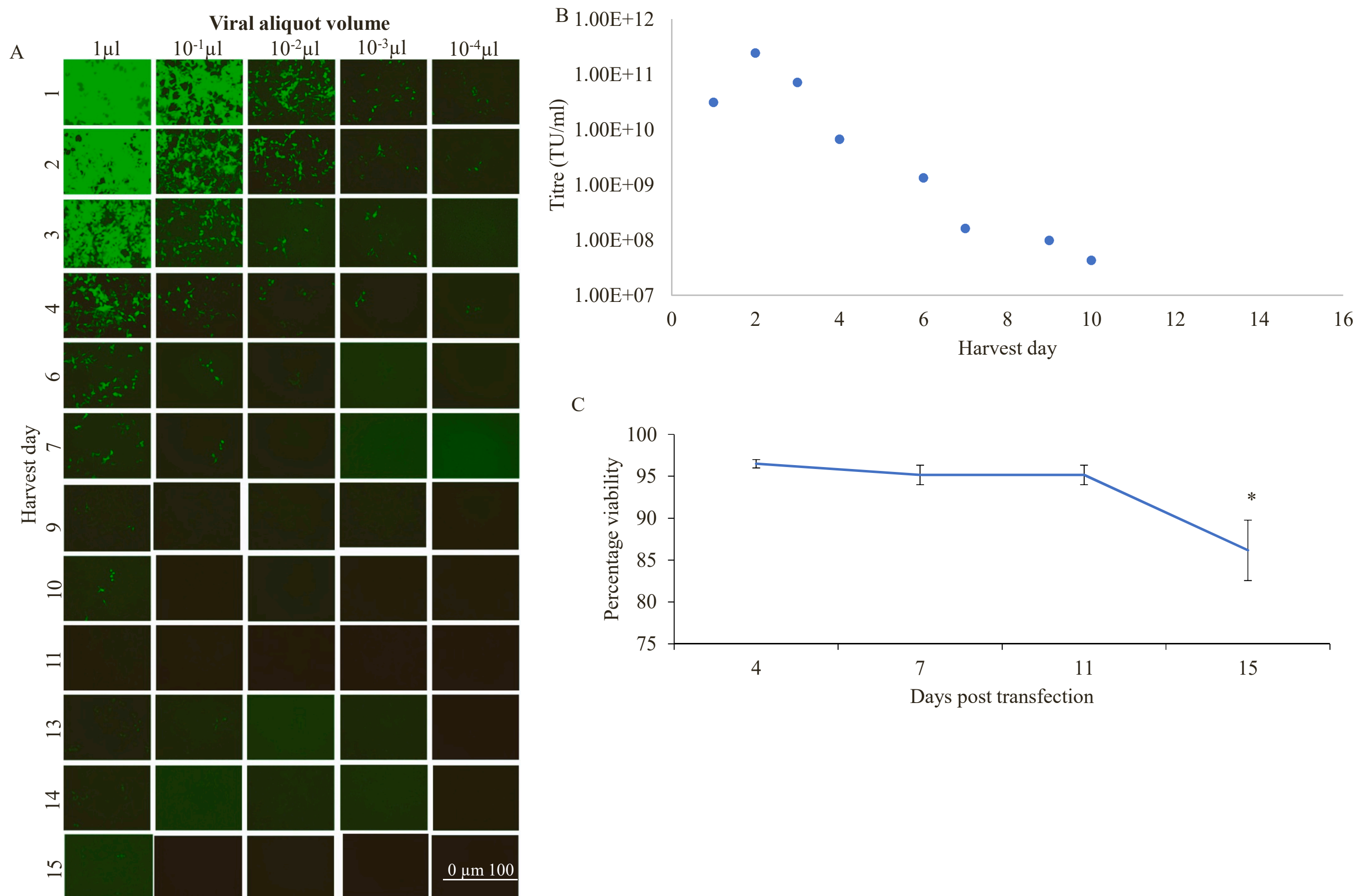


Fig. 4. - Scalable production of pHR LV using Genejuice® transfection reagent. A- Fluorescent microscopy images of HEK293T cells transduced with limiting serial dilutions of pHR LV virus daily harvests. Cells imaged under green fluorescent light 72hrs post transduction, x10 magnification. B- Infectious titre of daily harvests of pHR LV harvested. Titre was calculated using flow cytometry. A fall drop in titre over 10 days but with high titre LV produced throughout the incubation period. While conditioned medium was harvested for 15 days post transfection, no virus was quantified in harvests post 10 days. C- Viability of HEK293T cells transfected to produce LV, on a scalable methodology. Transfected cells were serially passaged over 16 days with viability measured using a trypan blue exclusion assay. A significant drop in cell viability was only seen at day 15 harvests (n = 3, $P < 0.05$, SEM shown).

suspensions and that cells could be directly transfected with high efficiency in suspension culture. However, we decided to investigate alternative ways to reduce the cost of LV production because we considered this would be prohibitive to using Genejuice® in scale-up production. Hence, we decided to explore optimisation of transfection of producer cells for LV production in monolayer using the low-cost PEI reagent. PEI has been previously used at 1µl/ml (10Mm stock solution), however, at this level PEI was observed as toxic to HEK293T cells, restricting its exposed to 4 h compared to 24 h used by other reagents such as Fugene® 6 and in this work by Genejuice® for efficient transfection. We transfected cells initially with PEI at levels of between 1–0.0001 µl/ml and found very little transfection below 0.01 µ/ml. PEI at 1 µl/ml once again providing highly reduced cell survival. At the 0.1 µl/ml dilution for 4 h 73% of cells were positive for GFP expression with 55% cell viability compared to only 32% positive GFP cells when transfection was extended for 24 h with 23% viability. We then tested PEI levels of between 0.1–0.02µl/ml for 4 and 24 h and examined cell toxicity and transfection efficiency. This resulted in successful HEK293T GFP transfection for each dilution for each transfection time, with PEI at 0.02µl/ml being the most effective for GFP expression after 24 h with levels of transfection reproducibly reaching over 90% using flow cytometry analysis and with cell viabilities close to those of untreated cells (Fig. 6).

3.6. LV production using optimised PEI transfection of producer cells as suspension cultures grown in glass vessels

We showed that low-cost PEI optimisation enabled cells to be transfected efficiently as monolayers with high viability and previously that transfection efficiency of cells in monolayers matched that of transfection in suspension using GeneJuice® as the transfection agent. We reasoned that suspension culture transfection may not require expensive plasticware for LV production and, therefore, to reduce cost of vector production further, we transfected HEK-293 T cells in suspension in reusable, autoclavable glass Erlenmeyer flasks with gentle shaking post treatment. We tested cell transfection in suspension in the presence or absence of Poly (2-hydroxyethyl methacrylate) coated vessels and assessed cell viability over 12 days. Over the 12 days in culture, Poly (2-hydroxyethyl methacrylate) coated and non-coated shaking cultures showed no difference in live cell number as well as viability. 1×10^6 cells were then transfected using GeneJuice® and the optimised PEI concentration (0.002µl/ml) in suspension in either coated or non-coated glass flasks, shaking, then crude LV was titrated using flow cytometry. Cells transfected using PEI generated higher viral titres than those using GeneJuice®, with coated vessels generating 1.7×10^6 (+/−1.8×10^5) TU/ml and non-coated vessel at 1.7×10^6 (+/−1.2×10^5). In contrast, in coated and non-coated vessels GeneJuice® generated 2.0×10^4 (+/−3.9×10^3) and 5.1×10^4 (+/−8.7×10^3) TU/ml, respectively. As no significant difference in titre was found ($p > 0.05$) when coating or non-

 Journal of Virological Methods 325 (2024) 114884

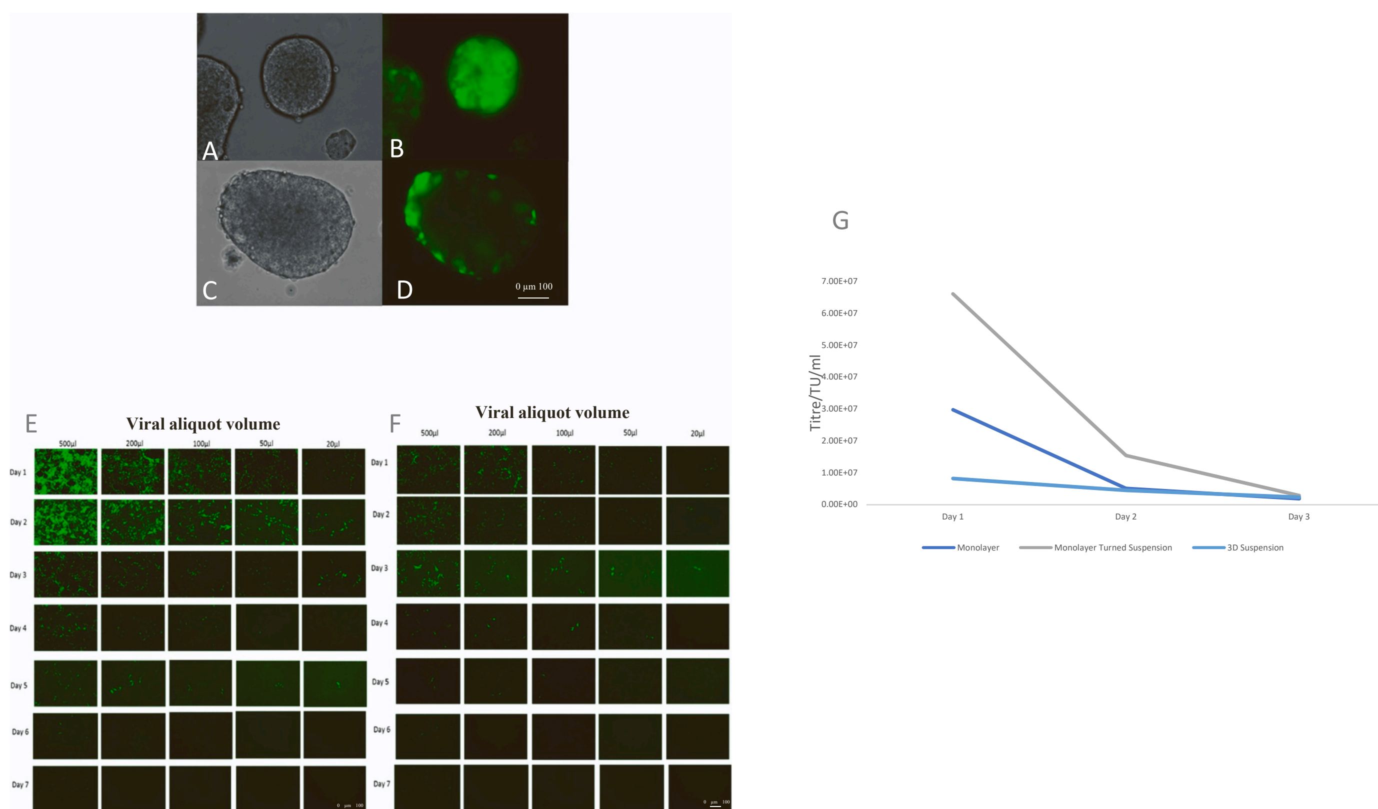


Fig. 5. Optimisation of transfection protocols for suspension LV production. 1×10^6 adherent HEK293T cells were transfected as a monolayer then transformed into suspension. Cells shown in suspension under (A) white and (B) green fluorescent light. HEK293T cells were also transfected as spheroids with images shown of cells in suspension under (C) white and (D) green fluorescent light. (E) Green fluorescent light images of LV transduced HEK293T cells with limiting dilution of unconcentrated viral supernatant generated when transfecting as a monolayer then turned suspension. (F) Green fluorescent light images of LV transduced HEK293T cells with unconcentrated viral supernatant generated when transfecting cell aggregated in suspension. Line graph showing a comparison of titres of lentiviral vectors produced through various transfection methods.

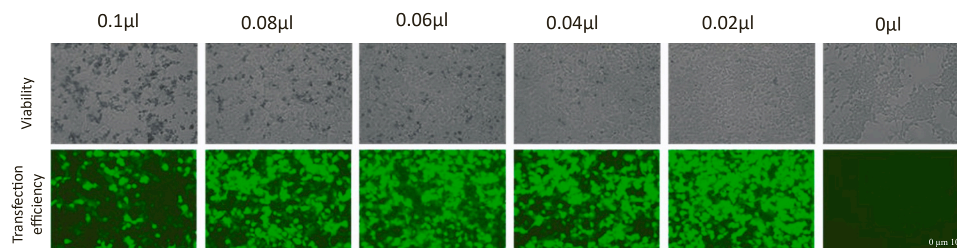


Fig. 6. Optimisation of 10 mM PEI concentrations (0.1–0.02 µl) for overnight suspension transfection. Brightfield and green fluorescent light images of cells transfected with GFP (for 24 h) using a range of PEI concentrations.

coating glass vessels, coating was no longer used. We then generated LV in larger batches without coating in glass vessels on a shaking platform for transfection with PEI or Genejuice® using 1.5×10^7 HEK293T cells (Fig. 7). When harvesting at 72 h post transfection PEI transfected cultures yielded titres of 1.6×10^8 ($+/-1.5\times10^6$) TU/ml compared to 8.1×10^4 (+/- 3.4×10^3) TU/ml using GeneJuice (n = 3).

4. Discussion

Lentivirus vectors (LV) vectors have become a highly valuable for gene therapy of rare diseases and for the generation of cells for immunotherapy. Traditionally, producer cells grown as monolayers have been used to produce LV supernatants that are then subjected to ultracentrifugation to concentrate LV particles (Ichim and Wells, 2011). However, with cell and gene therapy clinical trials having increased over the past 10 years from 1800 to 5000 listed in the National Institute of Health with several using these vectors, scalable and efficient methods using cell factories or HYPERFlasks and anion exchange chromatography (Kutner et al., 2009a,b) with refined purification technologies such as affinity chromatography (Martinez-Molina et al., 2020) have been used to generate large batches of quality assured vector.

However, important to the testing of novel constructs carrying therapeutic genes, transient transfection offers the most rapid method to generate LV for non-clinical testing before industrial protocols are used for clinical application. Finding new reagents and methods suitable to reduce the cost of transient LV production was, therefore, the focus of this report. As little information exists on the use of Genejuice® for 3 plasmid transfections of HEK293T cells, we optimised the performance of Genejuice® by a series of reagent/plasmid titrations and compared vector titre against that obtained using commonly used PEI and Fugene® 6. We found that Genejuice® reagent enables highly effective plasmid transfection that yielded LV titre at more than 10^{10} TU/ml after

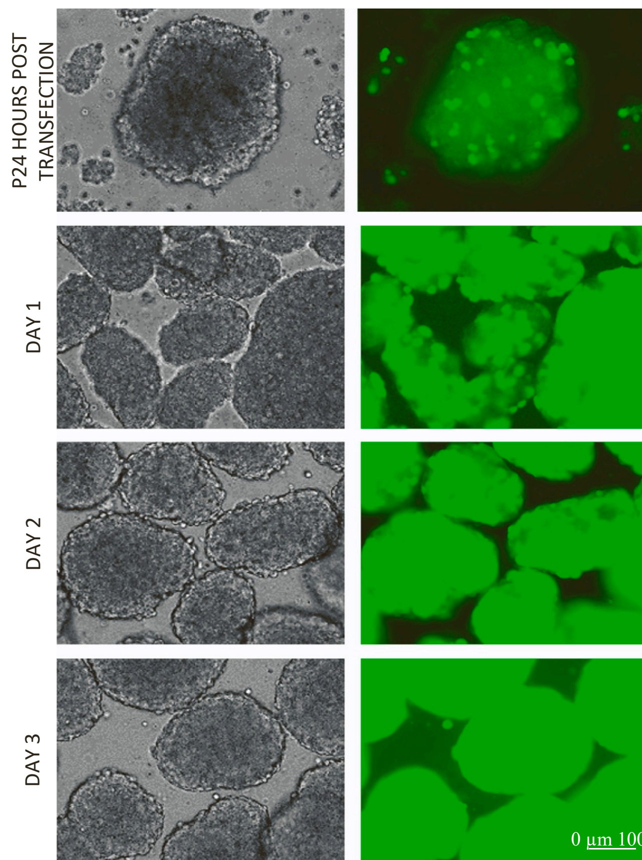


Fig. 7. Transfection of HEK293T cells in suspension on a rotating platform. Transfected HEK293T cells were transfected in suspension for LV production using an optimised protocol with PEI. White light and green fluorescent light images of transfected cells shown 72hrs post transfection. 10^8 TU/ml viral titre was generated using this protocol.

ultracentrifugation of particle supernatants harvested from monolayer cultures. Interestingly, rather than observing cell death at 72 hrs post transfection that is often observed with the other transfection reagents, Genejuice® showed low level cell toxicity, similar to that of untreated cells. This also enabled cells to survive long term in culture whilst generating LV at high titre for up to 10 days by producer cells rather than the traditional 72 hrs post transfection. This finding conflicts with our current understanding that LV harvest is only possible short-term following transient transfection of HEK293T cells, which is believed mainly due to the toxicological effects of cell surface VSV-G envelope glycoprotein expressed on transfected cells that causes syncytial formation and rapid cell death (Cronin et al., 2005). An alternative possibility, not explored in this study, could be that the plasmid ratio used to generate high titre LV did not produce excess levels of unwanted cell surface VSV-G expression. Another possibility could be that low level HEK293T cytotoxicity may be due to reduced cell-to-cell contact by Genejuice® not afforded by Fugene® 6, thereby preventing VSV-G mediated syncytial formation.

Rapid transfer of cells to suspension culture after Genejuice® transfection also generated LV at high titre. Unfortunately, whether this was restricted to Genejuice® was not shown by using PEI and Fugene® 6 in this study. This was made possible by using Poly-2-hydroxyethyl methacrylate to coat culture plasticware that enabled rapid conversion of HEK293T cells to grow in suspension with high viability. This enabled vector titre to reach approximately 3×10^8 TU/ml in small volumes as unconcentrated supernatants, which was more than 10-fold that of 2D transfection of monolayers by Genejuice®.

Although Genejuice® proved to be highly effective at transfection of HEK293T cells, scale-up would be considered costly. Hence, we explored further whether low-cost PEI could be optimised for transfection of cells in suspension. Firstly, we returned to monolayer transfection using a range of low lew PEI titrations on HEK293T cells to identify a concentration of PEI that provided high level cell viability and survival and LV titre. We also wanted to extend the period of transfection of 4 h used for PEI transfection to match that used for Genejuice® and Fugene® 6 of 24 h. By identifying 24-hour transfection with high level cell survival and GFP expression was achieved with PEI as low as 0.02 µl/ml this allowed us to determine whether this level of PEI would provide efficient transfection of HEK293T in suspension. Also, because transfection in suspension did not necessarily require cells to be grown in culture plasticware, with cost in mind, we chose to transfect cells in suspension grown in standard reusable, autoclavable glass Erlenmeyer flasks with gentle shaking post transfection. Using this protocol, we found high virus titre production was possible in crude unconcentrated supernatant. While standard monolayer transfection of cells produced titres on average around 10^9 TU/ml in 200 µl stocks, we found that suspension shaken cultures could produce vector inexpensively and routinely at 10^8 TU/ml in 3.5 ml, a volume nearly 20-fold greater, before ultracentrifugation.

As a caveat to the work described here, although treatments were always repeated and untreated cells regularly were submitted to rigorous tests for survival and viability and for the absence of mycoplasma in culture, we cannot rule out variation that inevitably exists when passaging cells either as monolayers or as suspension cultures during our investigation.

Further, our investigation does not determine whether HEK293T transduction during titration was enhanced differently by residual Genejuice®, Fugene® 6 or PEI transfection agents. Although we used polybrene in all titration experiments to attempt to standardise infection by virus batches, this may not have prevented residual transfection agent influence on gene transfer.

In conclusion, we present improved ways to generate LV particles at high titre either by monolayer or suspension conditions. Our suspension culture protocols are amenable to scale-up and require simple, readily available reagents for vector production and can even enable vector production without ultracentrifugation and at low cost. Vector stocks produced in this way may also be subjected to further downstream purification using established methods such as affinity chromatography. LV, produced by the methods described in this report can be used non-clinical where novel LV design requires in vitro or animal studies of vector efficacy and distribution and safety. Finally, we believe these optimised protocols should be easily transferable to any laboratory interested in LV gene transfer.

CRediT authorship contribution statement

Roberts Terry: Conceptualization, Resources. **Bigger Brian:** Supervision. **Ellison Stuart:** Data curation. **Themis Michael:** Supervision, Writing – review & editing. **Fawaz Serena:** Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. **Suleman Saqlain:** Formal analysis, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability statement

The data presented in this paper is available from the corresponding author upon reasonable request.

References

Aiuti, A., Biasco, L., Scaramuzza, S., Ferrua, F., Cicalese, M.P., Baricordi, C., Dionisio, F., Calabria, A., Giannelli, S., Castiello, M.C., Bosticardo, M., Evangelio, C., Assanelli, A., Casiraghi, M., Di Nunzio, S., Callegaro, L., Benati, C., Rizzardi, P., Pellin, D., Di Serio, C., Schmidt, M., Von Kalle, C., Gardner, J., Mehta, N., Neduva, V., Dow, D.J., Galy, A., Miniero, R., Finocchi, A., Metin, A., Banerjee, P.P., Orange, J.S., Galimberti, S., Valsecchi, M.G., Biffi, A., Montini, E., Villa, A., Ciceri, F., Roncarolo, M.G., Naldini, L., 2013. 'Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome'. Science 341 (6148), 1233151. https://doi.org/10.1126/science.1233151.

Ansorge, S., Lanthier, S., Transfiguracion, J., Durocher, Y., Henry, O., Kamen, A., 2009. 'Development of a scalable process for high-yield lentiviral vector production by transient transfection of HEK293 suspension cultures'. J. Gene Med. 11 (10), 868–876. https://doi.org/10.1002/jgm.1370.

Chong, H., Starkey, W., Vile, R.G., 1998. 'A replication-competent retrovirus arising from a split-function packaging cell line was generated by recombination events between the vector, one of the packaging constructs, and endogenous retroviral sequences'. J. Virol. 72 (4), 2663–2670. https://doi.org/10.1128/JVI.72.4.2663-2670.1998.

Coil, D.A., Miller, A.D., 2004. 'Phosphatidylserine is not the cell surface receptor for vesicular stomatitis virus'. J. Virol. 78 (20), 10920–10926. https://doi.org/10.1128/JVI.78.20.10920-10926.2004.

Cornetta, K., Yao, J., Jasti, A., Koop, S., Douglas, M., Hsu, D., Couture, L.A., Hawkins, T., Duffy, L., 2011. 'Replication-competent lentivirus analysis of clinical grade vector products'. Mol. Ther.: J. Am. Soc. Gene Ther. 19 (3), 557–566. https://doi.org/10.1038/mt.2010.278.

Cronin, J., Zhang, X.Y., Reiser, J., 2005. 'Altering the tropism of lentiviral vectors through pseudotyping'. Curr. Gene Ther. 5 (4), 387–398.

Danquah, M.K., Forde, G.M., 2007. 'Growth medium selection and its economic impact on plasmid DNA production'. J. Biosci. Bioeng. 104 (6), 490–497. https://doi.org/10.1263/jbb.104.490.

Dull, T., Zufferey, R., Kelly, M., Mandel, R.J., Nguyen, M., Trono, D., Naldini, L., 1998. 'A third-generation lentivirus vector with a conditional packaging system'. J. Virol. 72 (11), 8463–8471.

Eslahi, N.K., Muller, S., Nguyen, L., Wilson, E., Thull, N., Rolland, A., Pericle, F., 2001. 'Fusogenic activity of vesicular stomatitis virus glycoprotein plasmid in tumors as an enhancer of IL-12 gene therapy'. Cancer Gene Ther. 8 (1), 55–62. https://doi.org/10.1038/sj.cgt.7700270.

Estepa, A.M., Rocha, A.I., Mas, V., Perez, L., Encinar, J.A., Nunez, E., Fernandez, A., Gonzalez Ros, J.M., Gavilanes, F., Coll, J.M., 2001. 'A protein G fragment from the salmonid viral hemorrhagic septicemia rhabdovirus induces cell-to-cell fusion and membrane phosphatidylserine translocation at low pH'. J. Biol. Chem. 276 (49), 46268–46275. https://doi.org/10.1074/jbc.M108682200.

FiercePharma , 2020. Orchard Therapeutics' gene therapy Strimvelis linked to a leukemia case. Available at: ⟨https://www.fiercepharma.com/pharma/orchard-s-rare-disease-gene-therapy-strimvelis-linked-to-a-leukemia-case⟩ (Accessed: December/31 2020).

Fouchier, R.A., Simon, J.H., Jaffe, A.B., Malim, M.H., 1996. 'Human immunodeficiency virus type 1 Vif does not influence expression or virion incorporation of gag-, pol-, and env-encoded proteins'. J. Virol. 70 (12), 8263–8269. https://doi.org/10.1128/JVI.70.12.8263-8269.1996.

Gay, V., Moreau, K., Hong, S.S., Ronfort, C., 2012. 'Quantification of HIV-based lentiviral vectors: influence of several cell type parameters on vector infectivity'. Arch. Virol. 157 (2), 217–223. https://doi.org/10.1007/s00705-011-1150-5.

Grasser, U., Bubel, M., Sossong, D., Oberringer, M., Pohlemann, T., Metzger, W., 2018. 'Dissociation of mono- and co-culture spheroids into single cells for subsequent flow cytometric analysis'. Ann. Anat. = Anat. Anz.: Off. Organ Anat. Ges. 216, 1–8 doi: S0940-9602(17)30141-3 [pii].

Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M.P., Wulffraat, N., Leboulch, P., Lim, A., Osborne, C.S., Pawliuk, R., Morillon, E., Sorensen, R., Forster, A., Fraser, P., Cohen, J.I., de Saint Basile, G., Alexander, I., Wintergerst, U., Frebourg, T., Aurias, A., Stoppa-Lyonnet, D., Romana, S., Radford-Weiss, I., Gross, F., Valensi, F., Delabesse, E., Macintyre, E., Sigaux, F., Soulier, J., Leiva, L.E., Wissler, M., Prinz, C., Rabbitts, T.H., Le Deist, F., Fischer, A., Cavazzana-Calvo, M., 2003. 'LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1'. Science 302 (5644), 415–419. https://doi.org/10.1126/science.1088547.

Hacein-Bey-Abina, S., Garrigue, A., Wang, G.P., Soulier, J., Lim, A., Morillon, E., Clappier, E., Caccavelli, L., Delabesse, E., Beldjord, K., Asnafi, V., MacIntyre, E., Dal Cortivo, L., Radford, I., Brousse, N., Sigaux, F., Moshous, D., Hauer, J., Borkhardt, A., Belohradsky, B.H., Wintergerst, U., Velez, M.C., Leiva, L., Sorensen, R., Wulffraat, N., Blanche, S., Bushman, F.D., Fischer, A., Cavazzana-Calvo, M., 2008. 'Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1'. J. Clin. Investig. 118 (9), 3132–3142. https://doi.org/10.1172/JCI35700.

Ichim, C.V., Wells, R.A., 2011. 'Generation of high-titer viral preparations by concentration using successive rounds of ultracentrifugation'. J. Transl. Med. 9 https://doi.org/10.1186/1479-5876-9-137.

Ikeda, Y., Takeuchi, Y., Martin, F., Cosset, F.L., Mitrophanous, K., Collins, M., 2003. 'Continuous high-titer HIV-1 vector production'. Nat. Biotechnol. 21 (5), 569–572. https://doi.org/10.1038/nbt815.

Khonsari, H., Schneider, M., Al-Mahdawi, S., Chianea, Y.G., Themis, M., Parris, C., Pook, M.A., Themis, M., 2016. 'Lentivirus-meditated frataxin gene delivery reverses genome instability in Friedreich ataxia patient and mouse model fibroblasts'. Gene Ther. 23 (12), 846–856. https://doi.org/10.1038/gt.2016.61.

Kutner, R.H., Puthli, S., Marino, M.P., Reiser, J., 2009a. 'Simplified production and concentration of HIV-1-based lentiviral vectors using HYPERFlask vessels and anion exchange membrane chromatography'. BMC Biotechnol. 9 https://doi.org/10.1186/1472-6750-9-10.

Kutner, R.H., Zhang, X.Y., Reiser, J., 2009b. 'Production, concentration and titration of pseudotyped HIV-1-based lentiviral vectors'. Nat. Protoc. 4 (4), 495–505. https://doi.org/10.1038/nprot.2009.22.

Lesch, H.P., Laitinen, A., Peixoto, C., Vicente, T., Makkonen, K.E., Laitinen, L., Pikkarainen, J.T., Samaranayake, H., Alves, P.M., Carrondo, M.J., Yla-Herttuala, S., Airenne, K.J., 2011. 'Production and purification of lentiviral vectors generated in 293T suspension cells with baculoviral vectors'. Gene Ther. 18 (6), 531–538. https://doi.org/10.1038/gt.2010.162.

Lewis, P., Hensel, M., Emerman, M., 1992. 'Human immunodeficiency virus infection of cells arrested in the cell cycle. EMBO J. 11 (8), 3053–3058.

Magrin, E., Miccio, A., Cavazzana, M., 2019. 'Lentiviral and genome-editing strategies for the treatment of beta-hemoglobinopathies'. Blood 134 (15), 1203–1213. https://doi.org/10.1182/blood.2019000949.

Manceur, A.P., Kim, H., Misic, V., Andreev, N., Dorion-Thibaudeau, J., Lanthier, S., Bernier, A., Tremblay, S., Gelinas, A.M., Broussau, S., Gilbert, R., Ansorge, S., 2017. 'Scalable lentiviral vector production using stable HEK293SF producer cell lines'. Hum. Gene Ther. Methods 28 (6), 330–339. https://doi.org/10.1089/hgtb.2017.086.

Martinez-Molina, E., Chocarro-Wrona, C., Martinez-Moreno, D., Marchal, J.A., Boulaiz, H., 2020. 'Large-scale production of lentiviral vectors: current perspectives and challenges'. Pharmaceutics 12 (11), 1051. https://doi.org/10.3390/pharmaceutics12111051 doi: 10.3390/pharmaceutics12111051.

Merten, O.W., Hebben, M., Bovolenta, C., 2016. 'Production of lentiviral vectors'. Mol. Ther. Methods Clin. Dev. 3, 16017. https://doi.org/10.1038/mtm.2016.17.

Moghimi, S.M., Symonds, P., Murray, J.C., Hunter, A.C., Debska, G., Szewczyk, A., 2005. 'A two-stage poly(ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy'. Mol. Ther.: J. Am. Soc. Gene Ther. 11 (6), 990–995. https://doi.org/10.1016/j.ymthe.2005.02.010.

Nobles, C.L., Sherrill-Mix, S., Everett, J.K., Reddy, S., Fraietta, J.A., Porter, D.L., Frey, N., Gill, S.I., Grupp, S.A., Maude, S.L., Siegel, D.L., Levine, B.L., June, C.H., Lacey, S.F., Melenhorst, J.J., Bushman, F.D., 2020. 'CD19-targeting CAR T cell immunotherapy outcomes correlate with genomic modification by vector integration'. J. Clin. Investig. 130 (2), 673–685. https://doi.org/10.1172/JCI130144.

Schwarzer, A., Talbot, S.R., Selich, A., Morgan, M., Schott, J.W., Dittrich-Breiholz, O., Bastone, A.L., Weigel, B., Ha, T.C., Dziadek, V., Gijsbers, R., Thrasher, A.J., Staal, F. J.T., Gaspar, H.B., Modlich, U., Schambach, A., Rothe, M., 2021. 'Predicting genotoxicity of viral vectors for stem cell gene therapy using gene expression-based machine learning'. Mol. Ther.: J. Am. Soc. Gene Ther. 29 (12), 3383–3397. https://doi.org/10.1016/j.ymthe.2021.06.017.

Segura, M.M., Mangion, M., Gaillet, B., Garnier, A., 2013. 'New developments in lentiviral vector design, production and purification'. Expert Opin. Biol. Ther. 13 (7), 987–1011. https://doi.org/10.1517/14712598.2013.779249.

Suleman, S., Payne, A., Bowden, J., Al Haque, S., Zahn, M., Fawaz, S., Khalifa, M.S., Jobling, S., Hay, D., Franco, M., Fronza, R., Wang, W., Strobel-Freidekind, O., Deichmann, A., Takeuchi, Y., Waddington, S.N., Gil-Farina, I., Schmidt, M., Themis, M., 2022. 'HIV- 1 lentivirus tethering to the genome is associated with transcription factor binding sites found in genes that favour virus survival'. Gene Ther.

Tan, E., Chin, C.S.H., Lim, Z.F.S., Ng, S.K., 2021. 'HEK293 cell line as a platform to produce recombinant proteins and viral vectors'. Front. Bioeng. Biotechnol. 9, 796991 https://doi.org/10.3389/fbioe.2021.796991.

Tijani, M., Munis, A.M., Perry, C., Sanber, K., Ferraresso, M., Mukhopadhyay, T., Themis, M., Nisoli, I., Mattiuzzo, G., Collins, M.K., Takeuchi, Y., 2018. 'Lentivector producer cell lines with stably expressed vesiculovirus envelopes'. Mol. Ther. Methods Clin. Dev. 10, 303–312. https://doi.org/10.1016/j.omtm.2018.07.013.

Urbinati, F., Campo Fernandez, B., Masiuk, K.E., Poletti, V., Hollis, R.P., Koziol, C., Kaufman, M.L., Brown, D., Mavilio, F., Kohn, D.B., 2018. 'Gene therapy for sickle cell disease: a lentiviral vector comparison study'. Hum. Gene Ther. 29 (10), 1153–1166. https://doi.org/10.1089/hum.2018.061.

Zhang, H., Vinogradov, S.V., 2010. 'Short biodegradable polyamines for gene delivery and transfection of brain capillary endothelial cells'. J. Control. Release: Off. J. Control. Release Soc. 143 (3), 359–366. https://doi.org/10.1016/j.jconrel.2010.01.020.

Zufferey, R., Nagy, D., Mandel, R.J., Naldini, L., Trono, D., 1997. 'Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo'. Nat. Biotechnol. 15 (9), 871–875.

Zufferey, R., Dull, T., Mandel, R.J., Bukovsky, A., Quiroz, D., Naldini, L., Trono, D., 1998. 'Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery'. J. Virol. 72 (12), 9873–9880.