Development of a clinically-acceptable lentiviral vector for cystic fibrosis airway gene therapy

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Introduction:

Lentiviral (LV) vectors are a promising gene delivery vehicle for cystic fibrosis airway gene therapy. However, the development of a clinically-acceptable method suited to large-scale lentivirus production remains a barrier to the translation of gene therapy to the clinic. Current virus production methods that involve transient transfection of adherent cell culture have limited-scalability and often result in contamination of the vector preparation with potentially immunogenic components such as bacterially-derived plasmid DNA, and animal sera products. Accordingly, a scalable process for the production of clinically-acceptable vector is required.

Methods:

Plasmid DNA removal using DNase I

Unconcentrated lentivirus supernatants (500 µL) were treated with 2 µL deoxyribonuclease I (DNase I) (Sigma-Aldrich), 10 mM $MgCl_2$ and 2 mM EGTA [1]. 293T cells in 6-well culture plates were transduced with either the DNase I treated virus, untreated virus, or negative controls. Cultures were maintained under standard conditions for 3 weeks with cell samples collected at weekly intervals.

Concentrated lentivirus (2 µL) was mixed with 1 µL DNase, 20 mM Tris-HCl, pH 8.3, and 2 mM $MgCl_2$ (Sigma-Aldrich), in a reaction volume of 10 µL. This solution was incubated at 37°C for 1 hour and was then applied to 293T cells along with the untreated virus and negative controls. Once the cells reached confluency, cell samples were collected.

Genomic DNA was isolated from the cell pellets using the Wizard SV genomic DNA Purification System (Promega). Residual plasmid DNA in extracted genomic DNA samples was detected by PCR using primers for either a 273 bp or 210 bp fragment of the cytomegalovirus (CMV) promoter that is present in all helper plasmids. PCR products were visualised on an agarose gel.

Suspension culture virus production

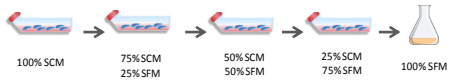

Figure 1: Sequential process of adapting adherent HEK 293FT cells in DMEM/ 10%FCS to FreeStyle™ serum-free media in suspension culture. SCM = serum-containing media, SFM = serum-free media [2].

Virus expressing luciferase was produced in a one litre culture seeded with 293FT cells at a density of 1 x 10^6 cells/mL in FreeStyle™ media. To the culture 28.6 g Fibra-cel® disks (Eppendorf) were added, and cells were incubated with shaking for one hour to allow for attachment. Cells were transfected using calcium-phosphate co-precipitation and a five-plasmid system (Table 1). The virus supernatant was purified using ion-exchange chromatography and was concentrated via ultracentrifugation. Virus pellets were resuspended in 0.9% (w/v) NaCl and 0.1% (w/v) mouse serum albumin in a final volume of 150 µL.

Virus assays

Adherent 293T cells transduced with virus were assessed by FACS to quantify the GFP positive cells and RNA titres were determined using a commercial qPCR lentivirus titration kit (abm) according to the manufacturer's instructions.

Data is expressed as mean and standard deviation.

Results:

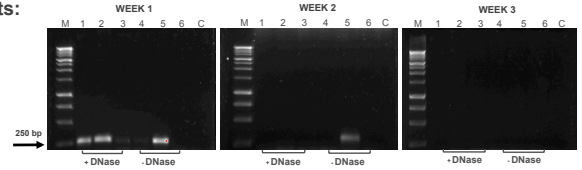

Figure 2: Unconcentrated virus: PCR products for detection of plasmid DNA in DNase I treated and untreated LV over three weeks. Presence of plasmid DNA is indicated by a 273 bp product (visible in lanes 1-5 week 1 and lane 5 week 2). Lane M: 1 kb ladder, lanes 1-3: DNase treated virus, lanes 4-6: untreated virus, lane C: negative control.

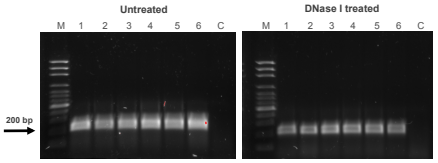

Figure 3: Concentrated virus: PCR products for detection of plasmid DNA in DNase I treated and untreated LV. The presence of plasmid DNA is indicated by a 210 bp product (visible in all lanes except control) Lane M: 100 bp ladder, lanes 2-6: DNase treated virus or untreated virus, lane C: negative control.

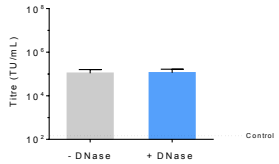

Figure 4: Titre measured by FACS revealed no difference in titre between DNase treated and untreated virus

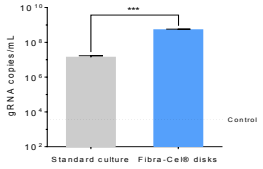

Figure 5a: RNA titres indicated higher titres using Fibra-Cel® disks when compared to standard suspension culture *** $p < 0.001$

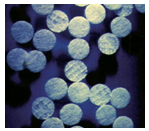

Figure 5b: Fibra-cel® disks

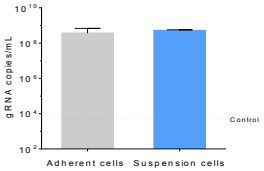

Figure 6a: RNA titres suggested comparable levels of virus production in adherent and suspension culture

Figure 6b: Suspension culture virus preparation. Titre following ~10,000-fold concentration was 1.11 x 10^9 TU.

Table 1: DNA quantities used for transfection of suspension culture

Plasmid	DNA quantity (µg)
pHIV-1SDMEF1α-Luc	2189.39
pcDNA3Tat101ml	40.70
pHCMV-Revmlwhvpre	40.70
pHCMV-gagpolmlstwhv	12.88
pHCMV-G	101.74

Discussion:

DNase I treatment

The treatment of lentivirus preparations with DNase I did not appear to compromise vector titre as has previously been reported [1,3,4] (Figure 4). However, in contrast to preceding studies, DNase I was not effective at removing residual plasmid DNA from either unconcentrated or concentrated lentiviral vector preparations (Figure 2 and Figure 3). It is notable that contaminating DNA pellets along with the virus during ultracentrifugation, therefore we theorise one possible explanation for the ineffectiveness of DNase treatment is the inadvertent encapsulation of plasmid DNA into virus-like particles, making it inaccessible to the enzyme.

Suspension culture virus production in serum-free media

The Fibra-Cel® disk system was pursued for virus production, as the titres achieved using this approach were significantly higher than those obtained using the standard suspension culture system (Figure 5a). The high virus titres achieved using the Fibra-Cel® disk system were likely due to the large surface area provided by the disks, which allowed for maximal cell densities to be attained, and subsequently, higher virus yields. The disks may have also improved cell viability by allowing uniform diffusion of nutrients to the cells, protecting the cells from mechanical shear, and preventing the formation of cell clumps.

The method described here for serum-free, suspension culture virus production, resulted in comparable RNA titres to those obtained with cells cultured adherently in the presence of serum (Figure 6a). Titres typically achieved using the standard calcium-phosphate co-precipitation of adherent 293T cells grown in DMEM/10% FCS range from 10^5 up to 10^8 TU/mL, while in present study titres of 10^8 TU/mL were achieved using serum-free suspension culture.

Conclusions:

DNase I was not effective at removing residual plasmid DNA from the vector preparations. As a result, enzymatic removal of plasmid DNA from viral preparations may not be a feasible approach for clinical applications. The protocol developed in this study for serum-free, suspension culture virus production resulted in high titres that were comparable to those routinely achieved using adherent cells. Furthermore, relative to traditional adherent virus production methods, this protocol is scalable, less labour intensive, and has less potential for contamination of the final vector preparation. However, clinical applications will require much greater volumes of concentrated vector, therefore bioreactor vessels and scalable purification and concentration methods are currently being investigated.

References:

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