

## **Production of Lentiviral Vectors Encoding Recombinant Factor VIII Expression in Serum-Free Suspension Cultures**

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### **ABSTRACT**

*Lentiviral vector-mediated gene transfer offers several advantages over other gene delivery vectors when considering gene and cell therapy applications. However, using these therapies in clinical applications involves large-scale vector production in an efficient and cost-effective manner. Here we describe a high yield production of a lentivirus encoding recombinant factor VIII in a scalable and GMP-compliant culture system, based on serum free suspension cultures and transient transfection with an inexpensive reagent, polyethylenimine (PEI), reaching a total viral yield of  $2.48 \times 10^8$  particles.*

**Key words:** Factor VIII, Hek293 cells, lentiviral vectors, polyethylenimine, serum-free suspension culture, transient transfection

### **INTRODUCTION**

Hemophilia A is a bleeding disorder caused by coagulation factor VIII (FVIII) deficiency. Currently, the treatment of choice for hemophilia A is replacement therapy with plasma-derived or recombinant FVIII. Although this is an efficient treatment, there are some factors that make it an inconvenient therapy, such as the short period of FVIII activity in plasma. The treatment is a large burden for patients that require a frequent number of infusions. Furthermore, Hemophilia A is an ideal candidate for gene therapy since the defect is attributable to the lack of a single gene product (FVIII) that normally circulates in low amounts in plasma (200 ng/mL). In addition, the blood concentration of factor VIII obtained is not critical, once a small increase in plasma

concentration can convert a severe case of hemophilia into a moderate case, improving the quality of life.

The most promising vectors for gene therapy concerning hemophilia A are the lentiviral and adeno-associated virus (AAV) vectors (Chuah et al. 2013). Lentivirus vectors (LV) have gained most attention over the last 20 years due to their ability to transduce non-dividing cells and large packaging capacity (Quinonez and Sutton 2002). Nevertheless, recent studies have shown that they have much lower oncogenic potential than other retroviruses because LV do not integrate with high frequency near promoters of proto-oncogenes and genes that control cell proliferation. Lentiviral gene therapy for hemophilia A has been successfully used in several pre-clinical studies (Liras et al. 2012; Chuah et al. 2013; High et al.

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2014) and is close to clinical approval. Furthermore, it has been favorably reviewed by the US Recombinant DNA Advisory Committee, as well as by the US Food and Drug Administration (FDA) (High et al. 2014).

To enable the clinical implementation of these encouraging studies, it has become necessary to develop efficient and scalable production processes for such vectors. Most studies focus on the generation of an optimized lentiviral vector encoding a high-expression of factor VIII. To the best of our knowledge, there is no study reporting the development of the production process for lentiviral vectors for hemophilia A, which is of paramount importance for the success of this therapy.

Traditionally, lentiviral vector productions for research and clinical purposes are performed by transfection of adherently growing cells, especially 293 cells, in staggered production systems. To maximize the cell culture surface, equipment such as Cell Factories (Thermo Scientific) or HYPERFlasks (Corning) are favored. The scale-up of this type of production is costly, labor-intensive and limited by the largest available scale (Schweizer and Merten 2010). One of the main obstacles for LV production is its low-titer, specially encoding a large size protein such as FVIII, and low half-life. Average titers of crude LV stocks range between  $10^5$  to  $10^7$  transducing units (tu)/mL (Mochizuki et al. 1998; Ansorge et al. 2009). These titers can be increased by centrifugation to generate  $10^9$  tu/mL. However, these concentrations remain too low for gene therapy clinical trials, where the number of functional vector particles needed is at least  $10^{11}$ - $10^{12}$  particles per patient (MacGregor 2001).

Currently, the most successful alternative for large scale LV production is based on transient transfection in serum-free suspension cultures using polyethylenimine (PEI) (Ansorge et al. 2009; Schweizer and Merten 2010). PEI-mediated transfections have been reported to be more consistent, reproducible and productive (Pham et al. 2006; Kuroda et al. 2009; Toledo et al. 2009). Moreover, PEI is chemically stable, inexpensive and has a broad pH range for transfection (Boussif et al. 1995). According to Ansorge et al. (2009), PEI-mediated transient transfection allows for the production of LV in suspension culture without the need of medium exchange prior to or after transfection, making the process scalable (Segura et al. 2007).

Here we report on the production of a third generation LV encoding recombinant factor VIII expression by PEI-mediated transfection in a serum free suspension culture using HEK293SF-3F6 cells. We show that our production process can generate a total of  $2.48 \times 10^8$  functional virus particles ( $2.48 \times 10^8$  tu in a total volume of 100 mL) on a scalable and GMP-compliant platform.

The cultures were performed using the Hek293SF-3F6 cell line (developed by the National Research Council, Canada). This cell line was adapted to a serum-free condition with high specific growth rate and cell density (Côté et al. 1998). The cells were cultured at 37°C and 5% CO<sub>2</sub> in the serum free commercial medium HyQSF4TransFx293 (Hyclone, Logan, UT), specially developed for the transfection of Hek293SF-3F6 cells, in 125 mL Erlenmeyer flasks (Corning) at 120 rpm. Medium supplementation was evaluated with 5% of Cell Boost 5 (CB5 5%, Hyclone, Logan, UT, USA), which contains nutrients such as lipids, amino acids, vitamins, and growth factors. Cell growth and viability were determined by the dye exclusion test with erythrosine B in hemocytometer.

To produce lentiviral particles, containing the FVIII gene, we used a third generation vector cPPT-C(FVIII<sub>delB</sub>)IGWS (p1054) encoding a B-domain deleted FVIII and an EGFP separated by IRES element, under control of the CMV promoter (Picanço et al. 2007). We also used the packing plasmid pCMV $\Delta$ 8.91 (encoding *gag-pol*, *rev* and *tat* genes from HIV-1) and the vector pMD2.VSVG encoding the vesicular stomatitis virus G (VSV-G). Plasmids were produced with an anion exchange purification method or with the commercially available kits QIAGEN Plasmid Giga Kit (Qiagen, Mississauga, ON).

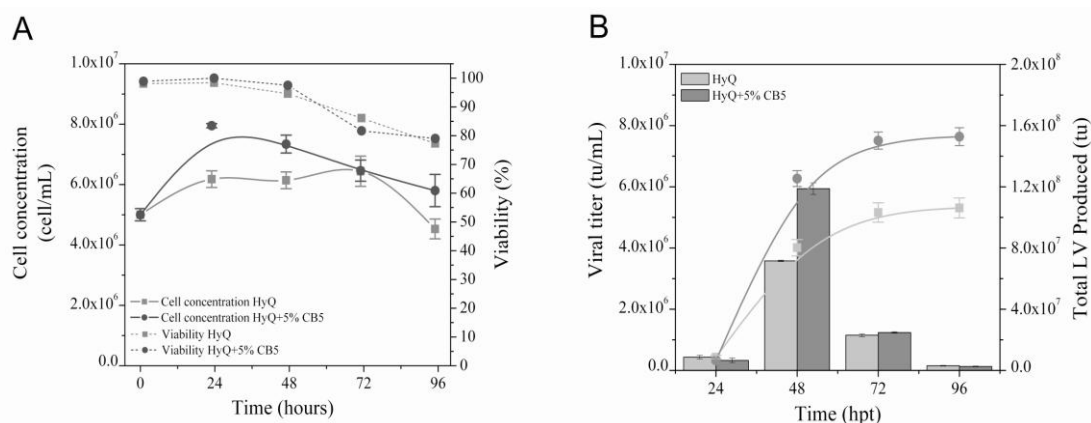
In the transient transfection protocol, we used 250 mL Erlenmeyer flasks (Corning) with 50 mL of culture medium and PEI reagent (25-kDa linear PEI, Polysciences), as previously described by Ansorge et al. (2009). The experiments for the production of lentiviral vector particles encoding FVIII expression (cPPT-C(FVIII<sub>delB</sub>)IGWS) were performed in Hek293SF-3F6 cells at the concentration of  $5 \times 10^6$  cells/mL and DNA:PEI ratio of 1:2. A plasmid mass ratio of 1:1:2 and 1:2:4 (pMD2.VSVG:pCMV $\Delta$ 8.91:p1054) was used to prepare the DNA-PEI complexes in a volume corresponding to 10% of the total culture volume. The mixture was incubated for 15 min at room temperature prior to adding the cell culture. A few hours before transfection, the cell

suspension was centrifuged (300 x g for 5 min) and resuspended at  $5 \times 10^6$  cells/mL. Sodium butyrate 5 mM, dissolved in 1M n-butyric acid (Sigma B-2503) and neutralized with 10 M NaOH, was added to the medium after the transfection in predetermined times. The medium was totally exchanged every day in order to simulate a perfusion culture and enhance the volumetric production. Every 24 h, the cell suspension was centrifuged (300 xg, 5min) and resuspended in 20 mL of fresh medium. A volume of 5 mL of metabolized supernatant was filtered in 0.45  $\mu$ m HT Tuffryn (Pall, Ann Arbor, MI) membranes, to remove cell debris, and stocked at  $-80^\circ\text{C}$  for lentiviral quantification. The culture was maintained for 4 days.

The viral titer was determined by flow cytometry-based methodology. We used Hek293E (clone 6E) cultivated in Freestyle 17 fresh medium (Invitrogen). The transduction and virus titer followed the methodology proposed by Segura et al. (2007).

### ***Effect of medium supplementation on FVIII lentiviral vector production***

Transfection efficiency is dependent on the medium formulation, the presence of nutrients and additives (Pham et al. 2003; Pham et al. 2005; Pham et al. 2006). Thus, we analyzed the lentiviral production profile using HYQSF4 (HyQ) culture medium supplemented or not with Cell Boost 5 (CB5). Although there was no difference in cell viability, cell growth was higher in the supplemented medium during the lentiviral production, with a maximum cell density ( $X_{\text{max}}$ ) of  $7.95 \times 10^6$  cells/mL (Fig. 1A). Therefore, the FVIII-LV production was also higher; reaching  $5.94 \times 10^6$  tu/mL in CB5 supplemented cultures compared to control condition  $3.58 \times 10^6$  tu/mL at 48 h post transfection (Fig. 1B). The effect of adding CB5 to the FVIII-LV production is better observed when considering the cumulative production rather than the volumetric daily production (Fig. 1B). In the CB5 supplemented medium, there was a total of  $1.528 \times 10^8$  tu after 96 h.



**Figure 1** - Effect of CB5 supplementation in cell density (A), FVIII-LV volumetric (Bars) and cumulative production (Curves) (B).

### ***Effect of DNA concentration on FVIII lentiviral vector production***

In order to enhance lentiviral production, we performed a second experiment, evaluating different DNA concentrations:  $0.4 \mu\text{g}/10^6$  cells and  $0.6 \mu\text{g}/10^6$  cells. The use of  $0.6 \mu\text{g}$  per  $10^6$  cells resulted in smaller cell densities (Fig. 2A) and cumulative LV-productivity of less than  $5 \times 10^7$  tu (Fig. 2B).

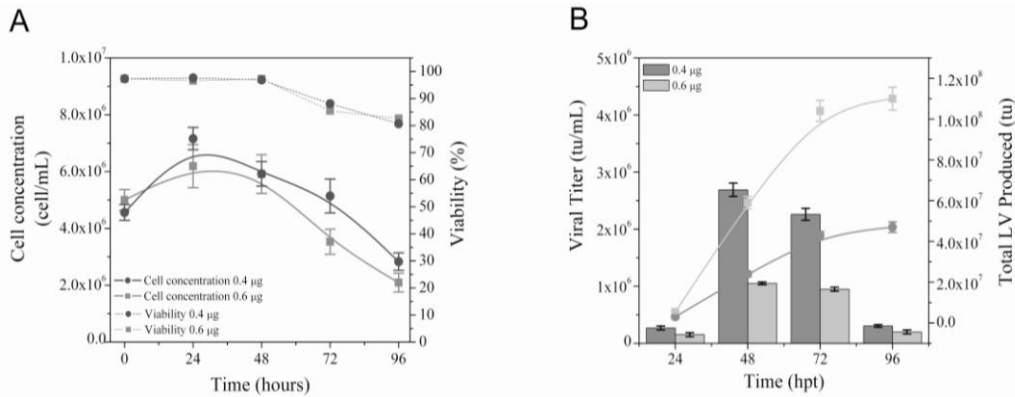
Although cell concentration also declined after 48 h in the  $0.4 \mu\text{g}$  per  $10^6$  cells protocol (Fig. 2A), the volumetric LV-production was significantly higher

at 48 and 72 h and the cumulative production reached  $1.1 \times 10^8$  transducing units after 96 h (Fig. 2B).

A number of studies have reported optimal efficiency of transfection using DNA concentration in the range of  $0.4$ - $0.6 \mu\text{g}$  per  $10^6$  cells in PEI-mediated protocols (Sun et al. 2008; Kuroda et al. 2009). Besides, PEI is known to have a toxic effect (Godbey and Mikos 2001; Kunath et al. 2003; Sun et al. 2008) and the concentration of PEI: DNA complex (polyplex) may influence the efficiency of the transient transfection. In the study

described here, the highest polyplex amount resulted in higher cell death and significantly

lower LV production.

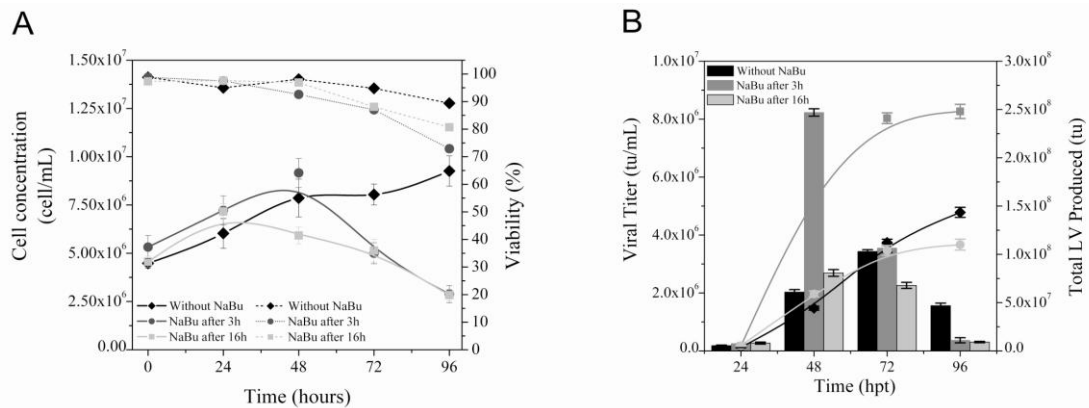


**Figure 2** - Effect of DNA concentration in cell density (A), FVIII-LV volumetric (Bars) and cumulative (Curves) production (B).

**Effect of sodium butyrate on FVIII lentiviral vector production**

Our results showed that NaBu addition inhibits cell growth. When NaBu was added 3 h post-transfection (hpt), X<sub>max</sub> was 9 × 10<sup>6</sup> cells/mL, declining after 48 h. However, when the addition of NaBu occurred after 16 h, X<sub>max</sub> was 7 × 10<sup>6</sup> cells/mL, declining only after 24 h (Fig. 3A). Although a variety of studies use sodium butyrate after 16 h post-transfection (Karolewski et al.

2003), in our study the cumulative productivity was lower in comparison with the control experiment. However, adding NaBu after 3 h resulted in significantly higher titers than both the control and the 16-h protocol. At 48 hpt, we obtained a volumetric production of 8.23 × 10<sup>6</sup> tu/mL (Fig. 3B). Moreover, the cumulative production reached 2.48 × 10<sup>8</sup> tu, compared to 1.1 × 10<sup>8</sup> tu obtained with the 16-hpt protocol (Fig. 3B).



**Figure 3**- Effect of sodium butyrate on cell density (A), FVIII-LV volumetric (Bars) and cumulative (Curves) production (B).

Sodium butyrate (NaBu) was successfully used to enhance recombinant protein (Damiani et al. 2013; Lee et al. 2014) and lentivirus and retrovirus production (Karolewski et al. 2003; Merten 2004). Transcriptional silencing of the LV-related transfected genes was demonstrated as a drawback

in LV production ( Kafri et al. 1999; Jaalouk et al. 2000; Ni et al. 2005). Therefore, the addition of sodium butyrate, at a concentration range of 2-20 mM, is reported to increase LV productivity (Soneoka et al. 1995; Sakoda et al. 1999; Jaalouk et al. 2000; Karolewski et al. 2003; Merten 2004;

Sena-Esteves et al. 2004) by enhancing transfected gene expression through histone hyperacetylation (Altenburg et al. 1976; Kruh 1982; Gloger et al. 1985).

By investigating the effect of medium supplementation, total DNA mass and different times for sodium butyrate addition, we were able to obtain high vector titers up to  $8.23 \times 10^6$  tu/mL. This production level is significantly higher than the one obtained in other studies reporting LV carrying FVIII cDNA. Radcliffe and colleagues (2008), using 293T cells in static culture to produce LV carrying a B-domain deleted FVIII cDNA, were able to reach a titer of approximately  $1 \times 10^6$  tu/mL (Radcliffe et al. 2008). The levels reported here can also be considered relevant considering the size of the FVIII insertion (9.3 kb). Yacoub and colleagues (2007) showed that the production of lentivirus with 7.2-7.5kb insert size resulted in titers of  $1-5 \cdot 10^5$  tu/mL.

In this study, we successfully described a scalable and GMP-compliant protocol for the production of lentiviral vectors encoding the coagulation factor VIII cDNA by PEI-mediated transient transfection in serum-free suspension culture. This protocol can be easily scaled-up in perfusion bioreactors to produce vectors at sufficient quantities for clinical studies. Moreover, it is cost-effective and less laborious than the traditional production platform based on adherent cells growing in cell factories.

This protocol can also be used to produce sufficient LV to transduce suspension serum-free adapted mammalian cells to enable the generation of a recombinant cell line producing FVIII. This methodology eliminates the time-consuming and laborious steps of adapting a FVIII producing cell line, which is normally generated with adherent cells growing in bovine fetal serum, and reduces the risk of decreasing or losing protein expression levels during the adaptation protocol.

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