Large Scale Transient Transfection for the Production of Lentiviral Vectors using the Corning® HYPERStack® Cell Culture Vessel

Application Note

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Introduction

Lentiviral vectors (LVs) have been successfully used in clinical treatments where they have been demonstrated to be safe and effective for gene transfer in cell and gene therapy applications. More than 100 ongoing clinical trials in the US, China, EU and Canada are employing lentiviral vectors for *ex vivo* modification of cells or *in vivo* therapy.[1] Overall, the market for LV production is predicted to grow to $800 million by 2026[2] as a result of its popularity in clinical trials and the market approval of recent CAR-T therapies Kymriah and Yescarta. LVs can be employed to treat hereditary diseases such as ß-thalassemia,[3] or Parkinson's disease.[4] T cells that are designed to express chimeric antigen receptors or epitope-specific transgenic T cell receptors are currently being investigated in multiple cancer immunotherapy clinical trials worldwide. As the use of LVs in new therapies has increased, so has the need for technologies that can help scale up their manufacture.

The large-scale production of LVs is currently generally achieved by directly amplifying small-scale production methods, for example by expanding the cultivation surface area by adding supplementary cultivation/production units or "scaling out".

In this LV production study, we employed the Corning HYPERStack cell culture vessel, which features Corning's proprietary High Yield PERformance (HYPER) technology to reduce the air space within the vessel and achieve higher yield per volume of space compared to traditional stacked layer cell culture vessels. The Corning HYPERStack vessel was compared with traditional culture vessels, revealing that the HYPERStack vessel can produce a superior LV titer.

Materials and Methods

Cell Culture

293T cells (ATCC CRL-3216) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Corning 10-013-CV) with 10% Fetal Bovine Serum of New Zealand origin (FBS; Corning 35-108-CV), and 1% Antibiotic-Antimycotic (100X, Gibco 15240-062).

The day before transfection, cells were seeded into a T-25 Corning CellBIND surface flask (Corning 3289), a 2-layer Corning CellBIND CellSTACK culture chamber (Corning 3310), and a Corning HYPERStack 12-layer cell culture vessel (Corning 20012) at 7 x 10^4 cells/cm^2. Cells were then grown overnight at 37°C (5% CO_2, >80% relative humidity) for a target density of 50% confluence on the day of transfection.

Deoxyribonucleic Acid (DNA) Preparation

A three-plasmid system was used: psPAX2 (Addgene 12260), pMD2.G (Addgene 12259), and pHBLVU6-MSC-CMVZsGreen (HANBIO HBLV-1002). We confirmed that the endotoxin unit (EU) value was <50 EU/µg, 260/280 >1.8 for each plasmid. A 0.22 µm filter membrane was used for sterile filtration.

Optimization of Transfection Conditions

The DNA concentration, DNA:PEIpro ratio, and transfected cell density are considered to be the main drivers of transfection efficiency. The Design of Experiments, a mathematical method widely used in bioprocess development to plan, conduct, analyze, and interpret controlled tests of multivariable processes, was used to identify the best combinations of factors to maximize the output and optimize the process.[5] Optimization was performed with the ZsGreen plasmid (DNA conc. = 1 to 2 µg per 10^6 cells), DNA:PEIpro (Ratio = 1:1 to 1:3), and 293T cells (density = 50% to 90% on the day of transfection) in a T-25 flask. The design had three levels for each factor, allowing for the interpretation of linear and quadratic effects.

Production and Harvest of LVs

The transfection complex mix was prepared for a total surface area of 7,400 cm^2 to account for the primary and comparison vessels: Corning HYPERStack 12-layer vessel, Corning CellSTACK® 2-chamber, Corning CellBIND® 25 cm^2 T-flask, plus 103 cm^2 buffer. The DNA mixture was prepared in a bottle (A) containing 70 to 80 mL of DMEM with a total of 2 µg DNA/10^6 cells with pHBLV-U6-MSC-CMV-ZsGreen, psPAX2, and pMD2.G at a 4:2:1 mass ratio. To a second bottle (B) containing 70 to 80 mL DMEM, PEIpro® (Polyplus 115-100) was added at 1:3 ratio of DNA to PEIpro. Bottle B was poured into bottle A and then mixed by turning the capped bottle upside down. The mixture was then left to stand for 10 to 20 min. at room temperature for DNA-PEI complex formation.

The fresh medium (5 mL per 25 cm^2 of DMEM containing 3% FBS + 1% Antibiotic-Antimycotic [100X]) was mixed well with the DNA-PEI complex mixture. **NOTE: The transfection mixture cannot be prepared in advance; must be used within 30 minutes after mixing.** Next, the culture media in the vessels were discarded and the abovementioned fresh transfection mixture was added back to each vessel. Vessels were then incubated at 37°C (5% CO_2, >80% relative humidity).

After 48 hours, the supernatants containing LVs were harvested and replaced with fresh medium. At 72 hours after transfection, supernatants were harvested again, and then combined with previous ones. The above method describes the optimal conditions for producing and harvesting LVs.

Titration of LVs

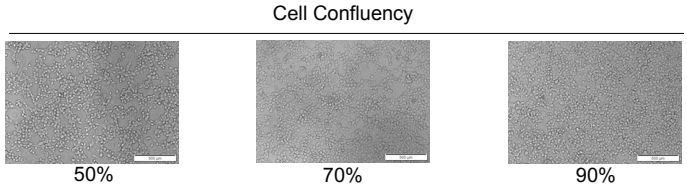
A growing population of 293T cells (1 x 10^5 cells/mL) were seeded onto 12-well plates, with 1 mL added per well; they were then incubated overnight. These cells were then supplemented with fresh medium and the same volume of the LV-containing supernatant at 1:1, 1:10, and 1:100 ratios, to a final volume of 1 mL with 8 µg/mL polybrene. After 48 hours of incubation, LV titers were quantified via fluorescence-activated cell sorting analysis of the ZsGreen reporter gene.

Results and Discussion

Optimal Transfection Conditions

Regarding ZsGreen plasmid transfection, the plasmid transfection efficiency was highest when the cell confluency was 70%, the DNA concentration was 2 µg per 10^6 cells, and DNA:PEIpro = 1:3 (Figure 1).

A



B

Flask No.	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12	F13
Cell confluency	50%	50%	50%	70%	70%	70%	90%	90%	90%	70%	90%	50%	90%
DNA (µg/10^6) (ZsGreen plasmid)	1	1.5	2	1	1.5	2	1	1.5	2	1	1	1	1
DNA:PEIpro (µg:µg)	1:1	1:2	1:3	1:2	1:3	1:1	1:3	1:1	1:2	1:1	1:1	1:2	1:2
293T cells after transfection 3 days													
Fluorescence intensity (%)	14.3	90.7	95.9	75.3	92.7	84.4	79.6	57.4	86.2	12.4	12	91.4	84.3

C

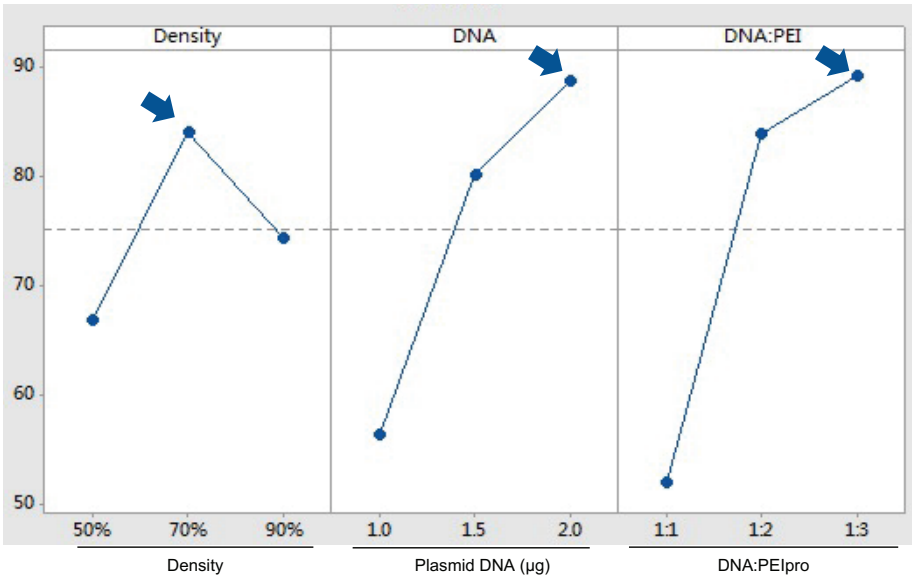


Figure 1. (A) Representative images of cell confluence at the time of transfection. (B) transfection conditions and transfection efficiency, (C) design of experiments analysis data.

Optimal Packaging Plasmid Systems

Previous research has revealed that that the demand for the pMD2.G plasmid is relatively low; thus, pMD2.G had the lowest proportion.[6] Here, various plasmid ratio tests were performed (Figure 2); a previously determined plasmid ratio was also added for comparative purposes.[6] As shown in Figure 3, the optimal ratio of plasmids was determined to be 4:2:1 (pHBLV-U6-MSC-CMV-ZsGreen:psPAX2:pMD2.G), for which the titer per milliliter was 2.35 x 10^7 TU/mL.

Flask No.	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10
pHBLV-U6-MSC-CMV-ZsGreen	2	3	2	3	1	1	2	3	1	4
psPAX2	2	1	3	3	2	3	1	2	1	2
pMD2.G	1	1	1	1	1	1	1	1	1	1

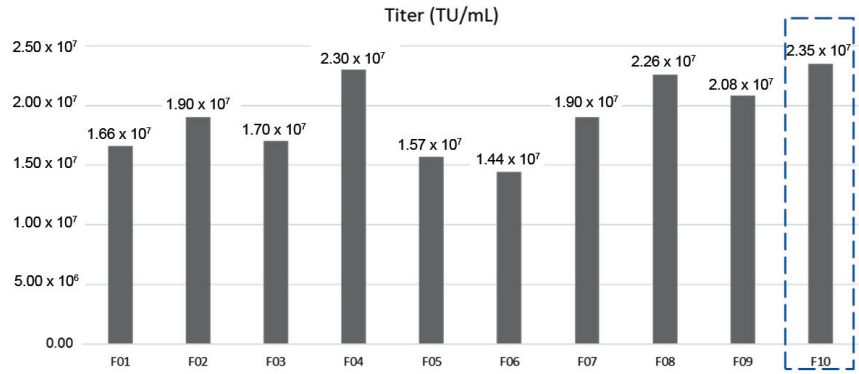


Figure 2. Packaging plasmid ratio parameters and infection titers (TU/mL).

Optimal Cell Transfection Density

In the previous result, the efficiency of plasmid transfection for a 50% cell transfection density was >95% (Figure 1). Considering the advantages of transfection at lower cell density (i.e., easier and lower cost seed train and decreased risk of cell detachment), LV production at 50% confluence on the day of transfection was compared to LV production at 70% confluence. As shown in Table 1, there was no significant difference in the LV titers between the 50% and 70% cell densities. Therefore, a 50% cell density was used to produce LVs in the Corning HYPERStack vessel.

Table 1. Titer comparison between 50% and 70% transfection densities.

Transfection Density	DNA (µg/10⁶)	DNA: PEIpro	Harvest (hours)	Titer (TU/mL)	Supernatant Volume (mL)	Total Titer (TU)
50%	2	1:3	48, 72h	1.8×10^7	10	1.8×10^8
70%	2	1:3	48, 72h	1.77×10^7	10	1.77×10^8

Production of LVs in the Corning HYPERStack Vessel

In the HYPERStack 12-layer vessel, the T-25 flask, and the 2-layer CellSTACK culture chamber with the CellBIND surface, transfection was carried out when the cell confluence reached 50%. The transfection conditions were 2 µg DNA per 10^6 cells at a DNA:PEIpro ratio of 1:3. The process for producing LVs and conducting titrations in different vessels is shown in Figure 3.

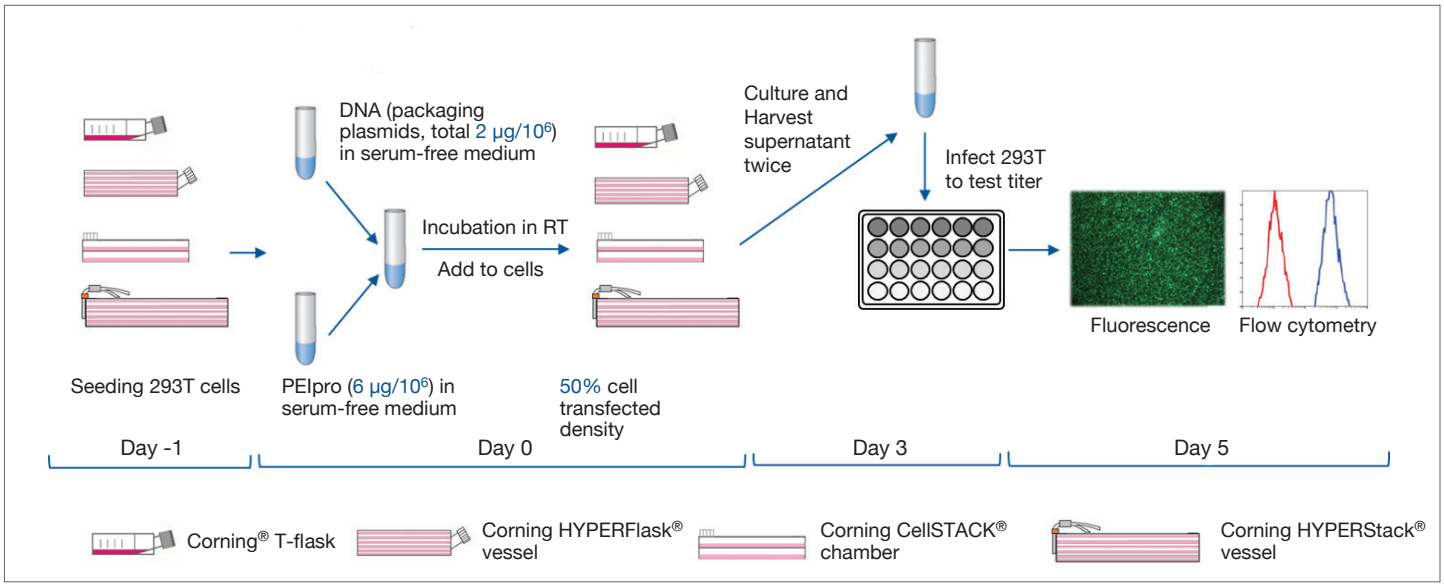


Figure 3. Comparison of LV production procedures in different vessels.

After production and titration, the HYPERStack 12-layer vessel achieved yields of 2.9 x 10^7 TU/mL and 1.2 x 10^7 TU/cm^2, in which the total titer reached 7.5 x 10^{10} TU. The unit volume and area yields of the 1-layer CellSTACK and T-25 flask were lower (Figure 4).

A

Vessels	Density	DNA (µg/10^6)	DNA:PEIpro	Harvest (hours)	Titer (TU/mL)	Total Volume (mL)	Total Titer (TU)	Titer (TU/10^6 cell)
Corning® T-25 flask	50%	2	1:3	48, 72	2.3 x 10^7	10	2.3 x 10^8	9.2 x 10^7
Corning CellSTACK® chamber	50%	2	1:3	48, 72	2 x 10^7	600	1.2 x 10^{10}	9.4 x 10^7
Corning HYPERStack® vessel	50%	2	1:3	48, 72	2.9 x 10^7	2600	7.5 x 10^{10}	1.2 x 10^8

B

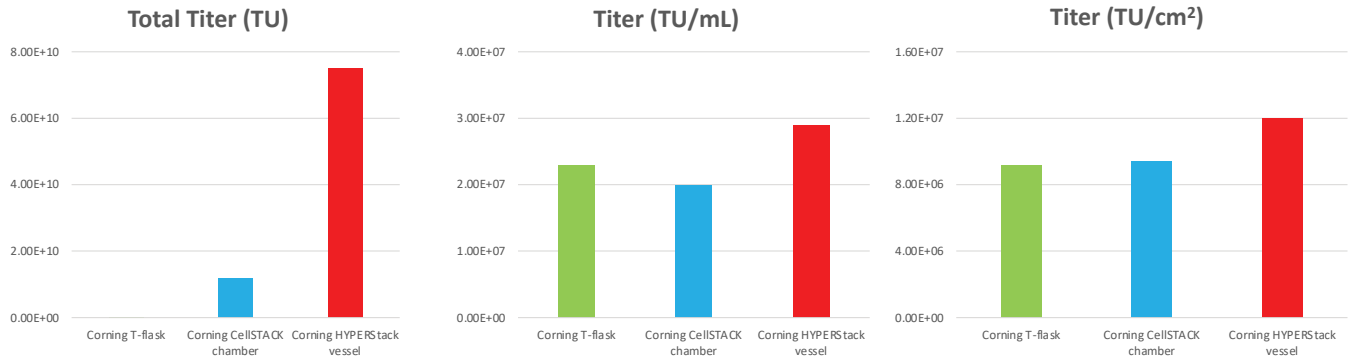


Figure 4. (A) Parameters of each experiment and harvest data, (B) including yield of virus per milliliter, total virus production, and virus yield per cm^2 of cells.

Conclusions

Comprehensive analysis revealed that the best packaging conditions comprised a transfection density of 50%, a DNA concentration of 2 µg per 10^6 cells, and a DNA:PEIpro ratio of 1:3. The optimal packaging plasmid system ratio was 4:2:1 (pHBLV-U6-MSC-CMV-:psPAX2:pMD2.G).

The HYPERStack vessel demonstrated several advantages over other vessels for LV production. The Corning HYPERStack vessel produced a higher LV titer per cm^2 than those of the T-25 flask and Corning CellSTACK culture chamber. The HYPERStack vessel also produced the highest total amount of LVs per single batch, thus rendering this process more suitable for scaled-up production.

In addition, the fully enclosed system of the HYPERStack vessel can more effectively reduce the risk of contamination than the other vessels included in this study. Furthermore, the HYPERStack vessel features more cell growth surface area for the same volume of space.

References

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