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Effects of Storage Conditions on the Morphology and Titer of Lentiviral Vectors

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Abstract

Lentiviral vectors are commonly used in laboratory experiments to stably integrate transgenes into host genomes. It has long been observed that storage of virus stocks leads to a decrease in viral titer, but the mechanisms driving this decrease have yet to be identified. To that end, lentiviral vector stocks were generated and stored as follows: room temperature for less than one hour, -80ºC for 24 hours, 4ºC for three days and 4ºC for 7 days. These stocks were subsequently evaluated with regard to their transducing ability and their morphology, specifically particle diameter. The vector that was stored at room temperature served as the control with viral morphology similar to other VSV-G pseudotyped viruses. These stocks were able to transduce ~100% of HEK 293T cells. Particles were unstable under the storage conditions tested, as evidenced by the fact that all stocks stored at -80°C and 4°C required concentration with an ultracentrifuge to generate a preparation suitable for visualization with TEM. The vector stored at -80°C for 24 hours exhibited some morphological changes, but only a slight decrease in titer. The morphology of vectors stored at 4°C for 3 and 7 days was not significantly different from the room temperature control, although titer was reduced to 60% and 30-40%, respectively. Thus, the decrease in titer observed in the lentiviral stocks generated and stored during this investigation appears to be the result of viral particle instability rather than morphological changes to individual particles.

Introduction

Lentiviral vectors are a common tool for many scientists who are interested in manipulating the genomic content in a laboratory setting. Lentiviruses are widely known for their ability to infect both dividing and non-dividing cells which allows them to target a wide range of cells, including neurons, hepatocytes, monocytes, macrophages and hematopoietic stem cells (Segura *et al*., 2006). Transgenes expressed from lentiviruses are not silenced during development; thus, they are often used to make transgenic animals by directly infecting early stage embryos or through cloning transgenic cells (Rubinson *et al*., 2003). Lentiviral vectors can stably integrate up to 10 Kilobases of foreign DNA into the host genome without transferring viral genes (Zuffery *et al*., 1998), allowing for long term expression of the transgene which can lead to long term therapeutic effects (Segura *et al*., 2006). They are self-inactivating which renders them safer for clinical use. These vectors are very useful to those investigating treatments for genetic disorders, cancer, cardiovascular, neurological and ocular diseases (Segura *et al*., 2006).

Lentiviral vectors are constructed in a tissue culture setting by simultaneously transfecting three expression constructs into Human Embryonic Kidney (HEK) 293T cells. Lentivirus particles are produced when all three constructs are simultaneously transfected into a single HEK 293T cell. The transfected cell produces viral particles and releases them into the culture media. This media is then collected and used to transduce other cell lines.

The production of lentiviral vectors is extensively time consuming, labor intensive and costly. It requires a minimum of five days to generate a single vector stock for transferring a specific gene. In addition, because every transfection may not be successful, HEK 293T cells must be maintained in culture throughout the process and repetition of the transfection experiments requires an additional investment of time and resources.

Viral titer refers to the concentration of vector particles capable of transducing target cells (Sastry *et al*., 2002). In a clinical or laboratory research setting it would be advantageous to have a stock of lentiviral vector already produced, with a known titer, to use in experiments. Thus it is necessary to evaluate viral stocks maintained under various storage conditions and determine the cause of any subsequent loss of titer. This will allow future studies to evaluate protocols for storing these

lentiviral vectors, with a minimal loss of titer. Effective storage protocols could save time, labor and money. It has long been observed that freshly collected virus-containing media has the highest titer and that storage of the media leads to a decreased titer, but the mechanisms driving this decrease have yet to be identified (Burns *et al.*, 1993; Segura *et al.*, 2006). It has been hypothesized that storage of the vector leads to morphological changes which cause the decrease in transduction ability. Alternatively, particle instability of these very labile virions may explain the decline in titer observed when stocks are stored (Segura *et al.*, 2006).

To investigate this phenomenon, VSV-G pseudotyped lentiviral vector stocks were generated using HEK 293T cells, subdivided and stored under the following conditions: room temperature for less than one hour, -80°C for 24 hours, 4°C for 3 days and 4°C for 7 days. These stocks were then used to transduce HEK293T cell lines to assess functional viral titer. This cell line is known for high rates of transduction (Pear *et al.*, 1993; Sastry *et al.*, 2002) and is thus appropriate for assessing viral stocks that are potentially very low in titer. The vector transferred a reporter gene sequence for green fluorescence protein (GFP) to the transduced cells. Therefore, the presence of GFP indicated successful integration of the transgene into the host genome, allowing the transducing ability of each viral stock to be evaluated using fluorescence microscopy. Transmission electron microscopy (TEM) was used to characterize viral morphology in each of these stocks.

Materials and Methods

Lentivirus Production:
To produce lentivirus, Human Embryonic Kidney (HEK) 293T (HEK 293T ATCC # CRL-11268) cells were simultaneously transfected with three plasmids: a transfer construct, a packaging construct, and an envelope construct. The transfer construct harbors several important genetic components, such as a directional cloning site, a puromycin resistance gene and a Green Fluorescent Protein (GFP) gene which allowed for the visualization of transfected cells using fluorescence microscopy. The envelop construct (VSV-G), codes for the Vesicular Stomatitis Virus G glycoprotein, which is capable of infecting a large range of host cells. The packaging construct (delta) carries the *gag* and *pol* genes which produce the enzymes necessary for a successful viral infection. Any individual cell that took up all three constructs was expected to produce viral particles that were expelled into the media. The HEK 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM/F12) containing 10% Fetal Bovine Serum (FBS), and supplemented with gentamicin (0.05 mg/ml) and penicillin, streptomycin, and fungizone (this media will be referred to as complete media) and were incubated at 37°C in 5% CO_2. The HEK 293T cells were plated in 100 mm tissue culture plates to reach ~80% confluency at the time of transfection. Cells were also plated in 35 mm plates containing a glass cover slip to allow for visual confirmation of transfection success. The media was refreshed 1-2 hours prior to transfection as follows: old media was aspirated then 2 mL of fresh media was added to each 35 mm plate; 8 mL of media was added to each 100 mm plate.

A calcium chloride transfection protocol was followed to simultaneously co-transfect the cells with the three plasmids described above. For the 35 mm plates, 6.3 µL of 2.0 M $CaCl_2$, 0.17 µg of VSV-G, 1.67 µg of delta, and 1.67 µg of transfer construct DNA were added to a sterile microcentrifuge tube. Sterile water was used to bring the total volume to 50 µL. For the 100mm plates, 36.0 µL of 2.0 M $CaCl_2$, 1.0 µg of VSV-G, 10.0 µg of delta, and 10.0 µg of transfer construct DNA were added to a sterile microcentrifuge tube. Sterile water was used to bring the total volume to 300 µL. To each DNA/$CaCl_2$ solution, an equal volume of 2X HEPES Buffered Saline was added. Then 2.5 µL and 15 µL of 12% dextran were added to the tubes for the 35 mm plates and 100 mm plates, respectively. The transfection solution was vortexed for 10 seconds to mix and then was added in a drop wise fashion to the media over the cells and mixed gently. The cells were incubated at 37 °C overnight in 5% CO_2. After ~18 hours post-transfection, the media was replaced with complete media supplemented with sodium butyrate (10 mM), 1 mL per 35 mm plate and 6 mL per 100 mm plate.

At 72 hours post-transfection the cover slip from each 35mm plates was placed on a slide and fixed in place with clear enamel. These slides were observed under ultraviolet epiillumination (excitation 330-385 nm, mirror 400 nm, barrier 420 nm; Olympus) to determine the transfection efficiency as cells that had taken up the transfer construct expressed GFP. Cultures with at least 80% of the cells expressing GFP were considered successful transfections.

Virus was harvested from cultures with successful transfections as follows. First, the media was collected from the 100 mm plates, put into 15 mL conical tubes and centrifuged at 300 × g for 3 minutes to precipitate cellular debris. The supernatant was then filtered through a pre-wet 0.45 micron filter into a fresh 15 mL conical tube.

Lentivirus Storage Conditions:
Upon collection, the viral media was distributed into 2 mL aliquots and these were stored at the following conditions: (1) room temperature for less than one hour, (2) -80ºC for 24 hours, (3) 4ºC for 3 days and (4) 4ºC for 7 days. These stocks were subsequently evaluated with regard to their transducing ability and morphology.

Transduction of HEK 293T Cells:
HEK 293T cells were grown in complete media in 35 mm plates with glass cover slips prior to transduction with the lentiviral vectors. With the cells at ~80% confluency, the lentiviral-containing media collected previously was prepared for use by the addition of polybrene to a final concentration of 0.2 mM. To each 35 mm plate, the complete media was replaced with 0.5 mL of viral media with polybrene. The cells were incubated overnight at 37°C and 5% CO_2. The same procedure was followed for viral vector stocks at each of the storage conditions and each experiment was run in triplicate.

Lentivirus Titer Estimate by Fluorescence Microscopy:
At 48 hours post-transduction, cover slips were recovered from each well, fixed on a glass slide with enamel, and observed under ultraviolet epiillumination as described previously. The percentage of cells which expressed Green Fluorescent Protein (GFP) was estimated for each storage condition.

Observation of Morphology by Transmission Electron Microscopy:
Negative staining with uranyl acetate was used to visualize the virus following previously published protocols (Vale *et al*., 2010; Goldsmith *et al*., 2009). A 5 µL droplet of virus in complete media was placed on a Formvar-coated, carbon stabilized 200 mesh copper grid, and after five minutes the excess was wicked away with filter paper. Then 5 µl of a 4% (w/v) aqueous uranyl acetate solution was placed on the Formvar grid and wicked away immediately. The grid was examined with a Hitachi H-7000 TEM operating at 100 KeV. Images were obtained and diameter measured of at least 10 virus particles for each of the storage conditions with the exception of the stocks stored at 4°C for 7 days, of which only 7 viral particles were photographed and measured. The micrographs were digitized using an Epson Perfection 4870 Photo flatbed scanner (16 bit grayscale; 600 dpi).

Concentration of Lentiviral Stocks with Ultracentrifugation
In order to observe virus particles which had been stored at -80°C or 4°C, ultracentrifugation to concentrate the samples was required. A volume of 1000 µL of each of the three stocks stored at these conditions was placed into thin-walled centrifuge tubes with a 15% sucrose cushion. Samples were centrifuged in a Beckman L8 80M ultracentrifuge at 19,000 × g for 90 minutes. The supernatant was removed and the virus pellet was resuspended in 30 µL of complete media for a 33 fold increase in concentration of each sample.

Statistical Analysis
A permutation procedure was used to test the null hypothesis that the mean particle diameter of viruses stored at the various conditions did not differ. A total of 9999 permutations of the data were generated and the absolute difference between means for each permutation trial was calculated. In order to refute the null hypothesis, differences between means in the original data must be greater than that seen in the reshuffled data ($p < 0.01$).

Results:

Lentivirus Titer Estimation by Fluorescence Microscopy:
The virus stored at room temperature for less than one hour exhibited the highest functional titer and was able to transfect ~100% of the HEK 293T cells (Table 1). Titer was reduced under all of the storage conditions tested, declining to ~80% when stocks were stored at -80º C for 24 hours, and to ~60% and ~30%-40% when virus was stored at 4º C for three and seven days, respectively (Table 1).

Observation of Particle Morphology Using TEM:
Negatively stained viral particles were circular to ellipsoidal in shape, frequently clustered, and varied in size from 80 to 410 nm across all samples (Figures 1 & 2; Table 2). For virus that was stored at room temperature, the concentration of particles was sufficient to observe without ultracentrifugation (Figure 1). Mean particle size for the room temperature stock was 208 nm (Table 2).

Viral stocks stored at -80°C and 4°C required concentration with an ultracentrifuge to generate a preparation suitable for visualization with TEM. In stocks that were stored at -80ºC, particles ranged in diameter from 80-200 nm with a mean diameter of 128 nm (Table 2). Mean diameter of particles stored at 4ºC for three days was 188 nm (Figure 2; Table 2). Particles stored at 4ºC for seven days had the highest mean diameter and the largest range in size, 277 and 140 – 410 nm, respectively (Table 2).

Statistical Analysis
The mean particle size of virus stored at -80º C was significantly lower than that of the other treatment groups, but all other comparisons were not statistically significant (Table 3).

Discussion

Vector expression in transduced cells using transgenes such as GFP is a technically simple and accurate method to assess functional titer (Sastry *et al*. 2002), and is less likely than other methods (ELISA assays, PCR analysis) to overestimate titer (Logan, *et al*., 2004). As expected, the fresh lentiviral vector stock had the highest titer. Negative staining revealed that particles in the fresh preparation were consistent with the general description of an enveloped virus provided by Goldsmith and Miller (2009). These viruses may take any shape (i.e. are pleomorphic), depending on how they land on the grid and the surface tension of drying forces. The short, fragile surface projections of retroviruses, of which lentiviruses are one type, are rarely seen in negatively stained preparations, and the nucleocapsid is morphologically nondescript (Goldsmith and Miller, 2009). The variation in particle size observed in this investigation is supported by the work of Fuller *et al*. (1997), who used cryo-electron microscopy to examine HIV-1 virus-like particles and found intact particles to be heterogeneous in size, varying in diameter from 120 - 260 nm. Vogt and Simon (1999) observed populations of retrovirus virions to be non-homogeneous in mass, with up to two-thirds of the Rous sarcoma virus (RSV) particles that they analyzed deviating from the mean mass by more than 10%.

The unstable nature of the virus under all storage conditions is evidence by the fact that ultracentrifugation was required to concentrate the samples so that TEM examination could be conducted. Segura *et al*. (2006) reported that retrovirus instability translates into low overall recoveries of infective viral particles. Ultracentrifugation is a common method to concentrate viruses in fluid samples to a level that they can be characterized microscopically (Goldsmith and Miller, 2009). Viruses pseudotyped with the VSV-G envelope usually withstand the force of ultracentrifugation better than most other viral envelopes (Burns *et al*., 1993; Logan *et al*., 2004), thus it is unlikely that concentration using this method affected viral morphology.

The virus stored at -80º C for 24 hours had the second highest functional titer. Although these particles were significantly smaller than the other three stocks, they retained their ability to transduce HEK 293T cells. Storage at 4ºC had a more negative effect on titer, with particles stored for 7 days exhibiting the lowest titer of all conditions tested. While the decreases in viral titer observed here may seem minimal, the highly transfectable nature of 293T cells should be noted (Pear *et al*.,

1993; Sastry *et al*., 2002). Even the minimal decrease in titer of stocks stored at -80°C would eliminate the ability of this preparation to effectively transduce a primary cell line (Ichim and Wells, 2011).

These results are consistent with previously published studies including the work Ichim and Wells (2011), who observed a decrease in viral titer of nearly ten-fold in magnitude when VSV-G pseudotyped retrovirus was stored at 4°C, concluding that this type of vector is highly unstable under these conditions. Kutner *et al*. (2009) reported that the low titers of lentivector preparations bearing glycoproteins other than VSV-G is the result of poor survival of these pseudotypes upon freezing. Xu *et al.* (2005) investigated the effect of storage conditions on the transduction efficiency of adeno-associated virus (AAV), a non-pathogenic defective parvovirus with a broad host range that is known for its extreme resistance to environmental extremes. The virus stored at -80°C remained stable and retained high transduction efficiency throughout a one-month monitoring period as assayed by measuring luciferase activity in transduced 293 T cells. Transduction efficiency of virus stored at -20°C, 4°C, room temperature and 37°C decreased continuously over time. This drop was most precipitous with the room temperature and 37°C treatments, with a sharp fall observed at day 1. At 20°C, transduction efficiency remained relatively high for the first 5 days, whereas at 4°C sharp drops were observed at days 1 and 7 and efficiency had declined to 55% by the end of one month of storage.

Morphological difference between 4ºC and room temperature particles were not detectable with the techniques employed in this investigation, and thus could not be associated with the differences in titer between the two preparations. Such was not the case in other studies, including the work by Cheslock *et al*. (2003). These researchers compared wild-type virions of murine leukemia virus to noninfectious mutant virions in which 33 amino acid residues of their capsid were deleted. Particle sizes of the wild type virions were tightly clustered within a 90-130 nm range of diameters. Mutant particles exhibited a much wider range in size, with some measuring more than 400 nm in diameter. Zhao *et al*. (2008) observed differences between infectious and empty particles using size exclusion chromatography and electron microscopy. Complete particles ranged in size from 40-100 nm, with empty particles being larger in diameter (up to 180 nm). Yeager *et al*. (1998) compared the size and morphology of mature, wild type and immature, protease-deficient retroviral particles using electron cryo-microscopy. Although the lipid bilayer envelope, pleomorphic shape, and diameter (approximately 120 nm) were similar for both types of particles, they varied in the shape of their electron dense central core.

Conclusion

This study indicates that it is a decrease in the number of virus particles, not a change in morphology, which is responsible for the decrease in functional titer of lentiviral vectors pseudotyped with VSV-G. Although a decrease in titer did occur, storage at -80ºC was superior to 4ºC. Future research will focus on the development of more effective storage protocols in order to reduce the effects on viral titer observed in this investigation. Improved storage methods will allow for large quantities of these vectors to be generated and used repeatedly rather than making fresh stocks for every experiment.

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Storage condition	**Transduced HEK293T cells (%)**
Room Temperature	100 %
-80° C	80 %
4° C, 3 days	60 %
4° C, 7 days	30 – 40 %

Table 1. Assessment of functional titer of viral stocks at each of the storage conditions, as measured by the percentage of cells which expressed Green Fluorescent Protein (GFP).

Storage condition	Range in Particle Diameter (nm)	Mean Particle Diameter (nm)	Standard Deviation
Room Temperature	160 – 240	208	24
-80° C	80 – 200	128	34
4° C, 3 days	160 – 220	188	16
4° C, 7 days	140 – 410	277	98

Table 2. Viral particle diameter at each of the storage conditions. n=10 for all treatments with the exception of 4º C, 7 days for which n=7.

	Room Temp.	-80° C	4° C, 3 days	4° C, 7 days
Room Temp.	x	**0.0002**	0.0845	0.0689
-80° C	x	x	**0.0008**	**0.0006**
4° C, 3 days	x	x	x	0.0166
4° C, 7 days	x	x	x	x

Table 3. p values for comparisons of the mean particle size of viral stocks maintained at different storage conditions. Statistically significant differences are shown in bold.

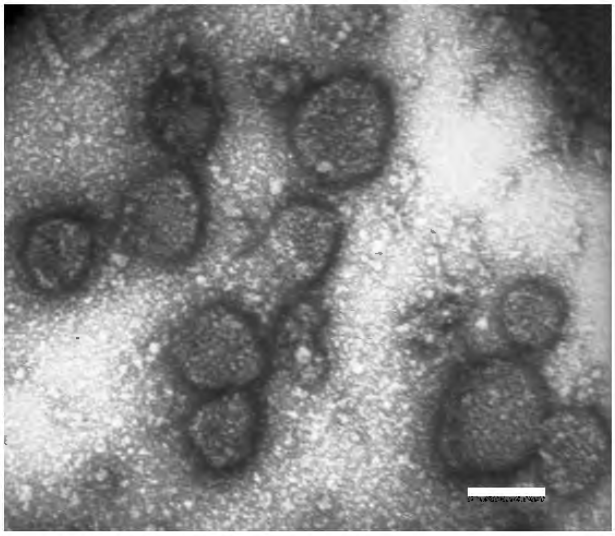

Figure 1. Transmission electron micrograph of negatively stained viral particles stored at room temperature for less than one hour. Final magnification 50,000X; Scale bar = 200 nm.

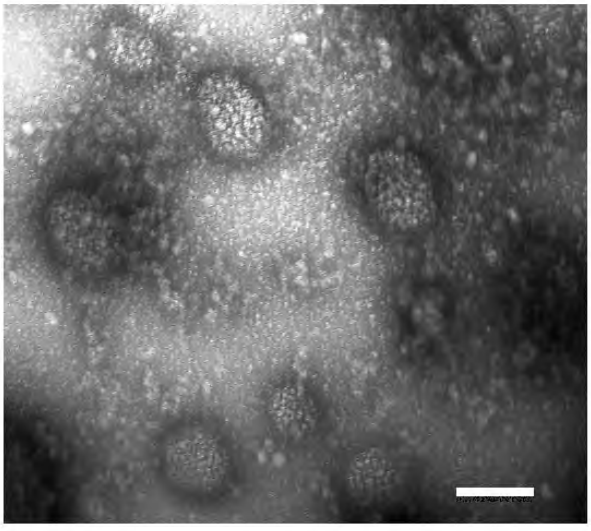

Figure 2. Transmission electron micrograph of negatively stained viral particles stored at 4º C for three days. Final magnification 50,000X; Scale bar = 200 nm.

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