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Running Head: Lentiviral vectors in HD

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Summary / Abstract

We describe here the potential of viral-mediated gene transfer for the modeling and treatment of Huntington's disease, focusing in particular on strategies for the tissue-specific targeting of various CNS cells. The protocols described here cover the design of lentiviral vectors, strategies for modifying their tropism, including the use of various envelopes and tissue-specific promoters, and the potential of miRNA to regulate transgene expression.

Keywords: Lentiviral vectors, tissue-specific promoters, pseudotyping, miRNA gene regulation, CNS, Huntington’s disease

1. Introduction

1.1 - Huntington's disease

Huntington's disease (HD) is a disabling, fatal neurodegenerative disorder that occurs in adulthood. Its principal symptoms are cognitive deficits and chorea, characterized by involuntary movements of large amplitude (***1***). Only symptomatic treatments, such as neuroleptics, antidepressants and anxiolytics drugs, are currently available (***2-4***). HD is caused by an abnormal CAG expansion in the huntingtin (Htt) gene, leading to a selective loss of neurons in the striatum and, at more advanced stages, in other brain areas (***5-7***). The most severely affected brain area, the striatum, consists of 95% gamma-amino butyric acid (GABAergic) medium spiny neurons (MSNs) and 5% interneurons. Both these cell types produce the mutant Htt (mHtt), but the disease has been shown to preferentially affect MSNs, with little effect on interneurons (***8-9***). Moreover, striatopallidal neurons (indirect pathway) have been shown to be more sensitive to neurodegeneration than striatonigral neurons (direct pathway; (***10-11***). There is also growing evidence to suggest that mHtt interferes with the normal functions of glial cells (***12-15***). In particular, mHtt production by astrocytes decreases the expression of glutamate transporters, leading to an accumulation of synaptic glutamate, which is highly toxic to neurons. The contribution of cell-cell interactions to the disease process remains unclear, but is likely to have a major impact on the development of treatments. Efforts are currently focused on understanding pathological mechanisms and developing therapeutic strategies based on small molecules, cell transplantation and gene therapy (***16-18***).

1.2 - Lentiviral-mediated gene transfer

Lentiviral vectors (LVs) are particularly suitable for central nervous system (CNS) gene transfer, due to their large cloning capacity and high transduction efficiency. Numerous functional and preclinical studies have demonstrated the potential of such systems to mediate the overexpression or silencing of specific genes. Most LVs are derived from the type 1 human immunodeficiency virus, but some are derived from other lentiviruses. Unlike other vectors derived from oncoretroviruses (***19-20***), *see* chapter 2 on lentiviral vectors), they efficiently transduce mitotic or post-mitotic cells. In the original seminal paper by Naldini and coworkers, high transduction frequencies in the CNS were obtained by broadening the original tropism of HIV-1, through the use of a heterologous envelope protein, the G glycoprotein of the vesicular stomatitis virus (VSV-G). In addition to its effect on tropism, VSV-G renders the viral particles highly stable, making it possible to concentrate them by ultracentrifugation, which is essential for CNS applications. Neither the receptor of the VSV-G protein nor specific host factors have been identified. However, in the 1980's, Schlegel and coworkers suggested that at least

some of the VSV-G might bind to plasma membrane phosphatidylserine (***21***). It was subsequently shown that phosphatidylserine was not the cellular receptor for VSV-G, but that it played a potential role in the post-binding step of virus entry (***22***). It was suggested that an interaction between the VSV-G protein and an unknown receptor at the cell membrane induces receptor-mediated endocytosis. The VSV-G protein may then interact with the phosphatidylserine in the endosome, allowing fusion to occur. The endoplasmic reticulum chaperone gp96 has recently been shown to be essential for the occurrence of functional VSV-G receptors at the cell surface (***23***). These findings provide evidence for a broad host range of LV, with high transduction efficiency in various organs (muscle, retina, liver, brain).

In the CNS, VSV-G-pseudotyped LVs expressing reporter genes under the control of ubiquitous promoters mostly transduce neurons (***20, 24-31***). Following the injection of LV into the parenchyma, the transgene is expressed throughout the cell bodies and axons, in the absence of retrograde or anterograde transport of viral particles. Sustained transgene expression over long periods of time has been achieved in rodent and primate brains (***24, 26, 32***).

1.3 - Altering the tropism of lentiviral vectors

Various strategies have been developed for modifying the neuronal tropism of LVs and investigating the role of other cell populations in HD. They make use of the various steps in the process of viral gene delivery: i) to modify the envelopes for pseudotyping, ii) to introduce tissue-specific promoters, and iii) to integrate miRNA targeting sequences blocking transgene expression into cells expressing the corresponding sequence (Figure 1).

1.4 - Lentiviral vectors as a tool for studying neurodegenerative diseases

LVs overexpressing or silencing specific genes have been extensively used for functional studies in the CNS. In the context of HD, gene transfer with lentiviral vectors has been successfully used i) to develop relevant models of HD by producing mutant Htt, ii) to investigate the molecular mechanisms underlying neuronal death and iii) to administer candidate treatments to the central nervous system (CNS).

1.4.1 - Lentiviral vectors for modeling HD

Since 1993 and the cloning of the Htt gene, numerous *in vitro* and *in vivo* genetic models have been produced and these models have greatly contributed to the unraveling of pathogenic pathways in HD. As a complementary approach, viral vectors have been used to model CNS neurodegeneration through the overproduction of disease-

causing proteins. This original way of creating genotypic models in animals overcomes some of the limitations of studies in transgenic animals, including the mild neuropathology observed. A growing number of publications have demonstrated the potential value of this versatile, highly flexible experimental paradigm (***18***).

The first-generation model of HD was based on VSV-G-pseudotyped LVs. de Almeida and coworkers developed a rat model based on the production of a short mutant Htt fragment in the striatum (***33***). This model is characterized by the rapid development of striatal disease reproducing the typical characteristics of HD, including the formation of Htt inclusions, neuronal dysfunction, astrogliosis and the neurodegeneration of GABAergic neurons at three months. The mutant Htt gene was strongly overexpressed (by a factor of up to 25; (***34***)), leading to the rapid appearance of typical HD functional abnormalities closely mimicking the clinical expression profile observed in HD patients (***35***). The injection of mHtt-encoding LVs into rodents is currently used to dissect the mechanisms underlying neuronal degeneration.

New models of neurodegenerative diseases have recently been developed for investigating the contribution of astrocytes to the disease (***36***). Colin and coworkers used a LV specifically targeting the astrocytes. This LV was pseudotyped with the G glycoprotein of a lyssavirus, the MOK-G envelope, and contained an miR124T detargeting sequence to inhibit transgene expression in neurons (***15, 37***). Astrocytes expressing the mHtt gene progressively develop a reactive phenotype, with decreases in glutamate transporter expression and glutamate uptake, as observed in patients with HD. This model suggests that astrocyte activation may contribute to striatal dysfunction in HD. It constitutes a new approach to understanding the disease and developing therapeutic approaches.

Another major advantage of LVs is the possibility they offer of establishing models in different species, including large animals such as non human primates (NHPs), which closely resemble humans in terms of neuroanatomy, motor behavior and cognitive characteristics. We have developed a *Macaca fascicularis* model of HD involving the injection of mHtt into multiple sites in the putamen (***38***). LVs expressing *mHtt* delivered to the putamen have been shown to reproduce the motor deficits observed in HD patients. Following bilateral injections, animals display spontaneous chorea from 16 to 30 weeks and typical deficits, such as head dyskinesia and leg dystonia. Thus, LVs provide a flexible, highly efficient and reliable system for localized and controlled production of mutant Htt in the CNS, at low cost and over very short periods of time. These rodent and primate HD models will greatly facilitate the evaluation of therapeutic strategies.

1.4.2 - Lentiviral vector gene therapy

The second application of LV gene transfer is gene therapy. Many studies have shown that LVs can be used for the efficient local and continuous delivery of a therapeutic gene (secreted or intracellular molecule) in the CNS. LV approaches lead to long-term, robust transgene expression in the brain (***18-19***). In the context of HD, various disease-modifying treatments have been investigated, including neuroprotective strategies based on ciliary neurotrophic factor (CNTF) (***39-41***), chaperones (***42***) or the c-jun-N terminal kinase pathway (***43***). However, reducing the level of mHtt mRNA itself would be the ultimate, most direct strategy for blocking polyQ pathogenesis. The possibility of designing and integrating small interfering RNAs (siRNAs) into expression vectors makes this therapeutic approach particularly attractive. Several groups, including ours, have recently demonstrated the potential of LVs for gene silencing in the rodent model of HD (***44-47***).

2. Materials

2.1 - Cloning of transfer plasmids

--Sterile water

--Easy cloning kits:

--pENTR/D-TOPO or pCR2.1 cloning kit (Invitrogen)

--pENTR4 cloning kit (Invitrogen)

--LR clonase enzyme mix (Invitrogen)

--Agarose (Invitrogen)

--Purification columns (Macherey-Nagel)

--Restriction enzymes and associated buffers (Invitrogen or New England Biolabs)

--Antarctic phosphatase / Calf intestinal phosphatase / Shrimp alkaline phosphatase (New England Biolabs)

--T4 DNA polymerase (Invitrogen)

--T4 DNA ligase (Invitrogen)

--PCR: *Pfx*, *PfuUltra II*, or *Taq* kit / Deoxynucleotides (dNTPs) kit (Invitrogen)

--Antibiotics: ampicillin (75 µg/ml) / kanamycin (50 µg/ml) / spectinomycin (100 µg/ml) (Sigma)

--Tris-EDTA (Sigma)

--Luria Broth medium / agar (Invitrogen)

--SOC medium, provided with competent cells (Invitrogen)

--Petri dishes for microbiological cultures (CML)

--Competent cells: DH10B, TOP10 (Invitrogen)

--Incubator

2.2 - 293T cells and LV production

Culture

--Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen)

--5% trypsin (Invitrogen)

--Penicillin (10,000 units/ml) / streptomycin (10,000 µg/ml) (Invitrogen)

--Phosphate-buffered saline (PBS) (Invitrogen)

--Fetal bovine serum (FBS) for cell culture (Sigma)

--Cell culture equipment (CO_2 incubator, laminar flow hood)

--10 cm Petri dishes (Falcon)

--T150 cm^2 flask (Corning)

--Cellstack (Corning)

Production

-- 0.5 M $CaCl_2$ (Sigma)

--2 x Hepes-buffered saline (Sigma)

--Stericup filter (0.45 µM pores; Millipore)

--Ultracentrifuge equipment (rotors 32Ti and SW55) (Beckman)

--1% bovine serum albumin (BSA) (Sigma)

--Low-retention tubes (Axigen)

2.3 - LV titration

2.3.1 - p24

--HIV-1 p24 ELISA kit –RETROtek, (Gentaur)

2.3.2 - qPCR

--DNase I, amplification grade (Invitrogen)

--RNase A (Invitrogen)

--RNasin (Invitrogen)

--Ethylenediaminetetraacetic acid (EDTA), (Sigma)

--Express Superscript Mix (Invitrogen)

--Express SyberGreenER Qpcr Super Mix Univ (Invitrogen)

--PureLink viral RNA/DNA kit (Invitrogen)

--DNase- and RNase-free water (Gibco)

2.3.3 - FACS

--24-well plate (Sigma)

--37% formaldehyde 37% (Sigma)

--5 ml tube for flow cytometry (Falcon)

2.4 - Primary striatal cultures

2.4.1 - Cultures of primary neurons and astrocytes

--PBS: Dilute PB to 0.1 M and add 9 g/l NaCl (Gibco)

--Poly-D lysine (30-70 kDa; Sigma): 5 mg in 100 ml sterile water

--1 M HEPES (Sigma) (100 x)

--100 mM sodium pyruvate (Sigma) (100 x)

--HBSS without Ca^{2+} and Mg^{2+}(Hank Balance Salt Solution) (Gibco)

--HBSSm: 1 x HEPES, 1 x sodium pyruvate in HBSS

--6-well plates, 24-well plates, large Petri dishes (Falcon), pair of forceps for dissection, microscissors

--Isoflurane (Abbott), pentobarbital (Sanofi)

--DNase I (3 mg/ml) (Sigma): use at 300 µg/ml in HBSSm

--Neuronal culture medium: Neurobasal (Gibco) supplemented with 2% B27 (Invitrogen)

--Glutamine (200 mM, 400 x) (Gibco)

--Penicillin (10,000 U/ml) / streptomycin (10,000 µg/ml) (100x) mixture (Invitrogen)

--Astrocyte culture medium: 10 % FBS (Gibco), 0.5 mM glutamine, 0.1% penicillin / streptomycin in DMEM (Gibco)

--Needles: 0.9 x 50 mm (20 gauge), 0.8 x 40 mm and 0.8 x 300 mm (21 gauge)

2.4.2 – Immunostaining for mutant Htt

--4 % paraformaldehyde (w/v) (Sigma), highly toxic

--Normal goat serum (NGS) (Gibco)

--Triton X-100 (Sigma)

--Primary antibody 2B4 (gift from Yvon Trottier, CNRS/INSERM, Illkirch, France) (***48***)

--Secondary antibody: coupled to fluorophores (Jackson Immunoresearch Laboratory) or biotin (Vector Laboratories)

--Vectastatin Elite ABC detection kit (Vector Laboratories)

2.5 - LV rodent model for HD

2.5.1 - Stereotaxic injections

--Animal health drugs:

- --Domitor (Covéto)
- --Ketamine (Covéto)
- --Xylazine (Covéto)
- --Isoflurane (CSP)

--Scalpels

--Stereotaxic frame

--Electric razor

--Binocular microscope

--For the cannulas:

- --34-gauge blunt-tip (Phymep)
- --Hamilton syringe (Hamilton)
- --Polyethylene catheter (Phymep)

2.5.2 – Immunostaining for tropism

--NeuN antibody (Millipore, MAB377)

--S100β antibody (Sigma, S2532)

--Gelatin from pig skin, type A (Sigma)

--Alexa Fluor (Invitrogen), store in the dark

--Basic MOM (mouse on mouse) kit (Jackson Immunoresearch Laboratory)

--Fluorsave reagent (Invitrogen)

3. Methods

3.1 – 3rd-generation HIV-1-based lentiviral vectors

Over the years, the biosafety of LVs has been greatly improved. The third generation of self-inactivating (SIN) LVs is currently in use ((***49-50***); *see* chapter 2 on lentiviral vectors). We use tat-dependent, SIN LVs split into four plasmids (***51***). This system contains a deletion in the U3 region of the LTR (Figure 2), to reduce the risks of recombination and of the formation of replicative LVs and to decrease interference between the LTR enhancer and the internal promoter driving transgene expression.

3.2 - Pseudotyping with various envelopes

The first option for modifying the tropism of LVs is to exchange the envelope glycoprotein (Env), a process known as "pseudotyping" (Figure 1). By replacing the original Env protein with other viral glycoproteins, the host range of the vector can be altered and its transduction efficiency in specific cell types increased. Glycoproteins homologous to the VSV-G protein have been proposed for CNS applications. Various G proteins from the rabies (RV-G) and Mokola (MOK-G) lyssaviruses have been proposed for this function (***28, 31, 37, 52-57***). Following the injection of RV-G-pseudotyped LV (EIAV, RabERA strain) into rat brain, 80% of the cells transduced were neurons and 20% were glial cells (***58***). Retrograde transport of the viral particles was observed in projecting areas (***58***) (*see* **Note 1**). However, β-Gal staining was weaker with the RV-G vector than with the VSV-G vector, a limitation of lyssavirus pseudotyping that has also been reported for the MOK-G envelope (viral vector production and transduction efficiency lower than for the wild-type envelope, by a factor of two to six; (***28, 37***)). Following the intrastriatal injection of MOK-G (MOKZIM strain)-pseudotyped LV (HIV-1) into rats, 71% of the transduced cells were neurons (***28***). However, the proportions of neurons transduced may differ slightly between brain areas and species. In mouse striatum infected with a MOK-G-pseudotyped LV, the percentage of β-galactosidase S100β-positive cells (glial cells) was 68%, whereas only 9% of cells were positive following transduction with VSV-G LV (***37***). Species differences (rats vs mice), the strain of MOK-G used and variability of the lentiviral vector (EIAV vs HIV-1) may account for these discrepancies.

As an alternative LV pseudotyping strategy, the use of envelopes from neurotropic viruses has been proposed. In evaluations of the tropism of LVs pseudotyped with glycoproteins from Ross River Virus (RRV, an alphavirus), which replicates in neurons and glial cells (***54***), (***59***), 56% of β-galactosidase-positive cells were astrocytes and 27% were oligodendrocytes (***59***). Finally, the lymphocytic choriomeningitis virus (LCMV, an arenavirus), which is taken up by cells after binding to α-dystroglycan, shows a strong preference for astrocytes in the neonatal rat brain (***60-61***), despite the presence of α-dystroglycan on both neurons and astrocytes.

3.3 - Design of lentiviral transfer plasmids containing the transgene expression cassette

The lentiviral transfer plasmid is a complex DNA molecule containing the various elements required for transgene expression (Figure 3). We use tat-dependent SIN transfer vectors (***62***) containing a central polypurine tract (cPPT; (***63***)). The expression cassette consists of a promoter (ubiquitous or specific to a subpopulation of cells), a transgene (*see* **Note 2**) and, in most cases, the woodchuck posttranscriptional regulatory element (WPRE), which increases transgene expression (***64***). Finally, an miR-target (miRT) can be inserted after the WPRE sequence, to restrict transgene expression.

3.4 - Tissue-specific promoters

One strategy for overcoming the lack of specificity of LVs is based on the use of tissue-specific promoters to modulate the transcription of transgenes. However, cellular promoters often have weak transcriptional activity, limiting their potential use in LVs. Furthermore, only a small number of promoters have been characterized. However, this may change in the future, as large consortia are currently making use of transcriptome databases for astrocytes, neurons and oligodendrocytes (***65-66***), to characterize functional elements in the human genome (ENCODE project, for encyclopedia of DNA elements) and to develop mini-promoters (Pleiade Promoter Project; (***67-69***)). A comprehensive analysis of the transcriptional promoter structure of 1% of the human genome (400 promoters) has revealed a strong correlation between promoter activity and the corresponding endogenous transcript levels, providing the first experimental, quantitative estimate of the contribution of the promoter to gene regulation. (***70-72***). These projects should provide new regulatory sequences to drive gene expression in a region-specific manner in the brain. In the meantime, a limited number of neuronal promoters (***73-76***) and glial promoters (***77-80***) for limiting transgene expression in the brain are currently available.

Promoters could be inserted into transfer plasmids by classical molecular biology methods or with Gateway® technology. We describe the principal cloning steps here. For further detail, please refer to specialized laboratory manuals (***81***).

3.4.1. - Molecular cloning of the promoter in a lentiviral transfer plasmid

This approach makes use of unique restriction sites at the 5' and 3' ends of each element to facilitate cloning (Figure 3). The LV transfer plasmid (10 µg) and the plasmid containing the promoter (the insert plasmid) are digested with the same restriction enzymes (REs), in a final volume of 50 µl, for 2 h (the buffer and temperature depend on the REs used). If a compatible double digestion is not possible, an additional blunting step may be required (with the T4 DNA polymerase, for example). The corresponding DNA fragments are separated by electrophoresis in an agarose gel and purified on commercially available columns. The destination plasmid is dephosphorylated to prevent self-ligation (*see* **Note 3**). Ligation is performed with T4 DNA ligase, by incubation for 1 h at room temperature or for 24 h at 14 °C for blunt-ended fragments. Competent *Escherichia coli* cells (DH10B electrocompetent, or TOP10 chemically competent strains) are transformed the next day, with 2 µl of ligation mixture. The bacteria are resuspended in 450 µl of SOC medium, spread on selective plates (250 µl/plate) and incubated overnight at 37°C. Finally, clones are analyzed and insertion of the transgene is validated by a control RE digestion and sequencing.

3.4.2 - Cloning of promoters with Gateway® technology, in lentiviral transfer plasmids

Gateway® cloning technology facilitates the insertion of large DNA fragments into lentiviral transfer plasmids (> 7-10 kb). The Gateway® system is based on homologous recombination between the *attL* and *attR* sites from the bacteriophage λ. A two-step procedure is used: 1) insertion of the promoter of interest into an entry plasmid containing *attL* sites; 2) production of a destination LV transfer plasmid containing a conversion cassette, to integrate the promoter upstream from the gene of interest. This conversion cassette contains *attR* sites, a suicide gene (ccdB, *see* **Note 4**) and the chloramphenicol resistance gene for screening of the clones (Figure 4).
Entry plasmids containing multiple cloning sites (MCS) (for example, pENTR4 (Invitrogen)) or plasmids suitable for PCR products (e.g., plasmids predigested to allow oriented cloning such as example, pENTR-D-TOPO (Invitrogen); see 3.4.2) should be used for the generation of entry plasmids with promoters.

3.4.3 - Insertion of PCR products into entry plasmids

The choice of the plasmid depends on the polymerase used for PCR. pENTR-D-TOPO® is designed for the cloning of blunt-ended DNA fragments compatible with the Pfx or *PfuUltra II* enzymes (*see* **Note 5**), whereas pCR2.1-TOPO-TA® has 3'-T overhangs for the direct ligation of *Taq*-amplified PCR products.

3.5 - miRNA detargeting strategy

Naldini and coworkers integrate microRNA (miRNA) regulatory sequences in LVs to restrict transgene expression (***82-83***). This approach is based on the insertion, at the 3' of the transgene, of copies of a microRNA (miR) target sequence (miRT) specific for a lineage. In a proof-of-principle study for the CNS, we showed that the integration of the miR124-target (miR124T) sequence into LVs inhibits transgene expression in neurons and, together with MOK-G pseudotyping, provides an effective strategy for the selective expression of transgenes in astrocytes. The brain is enriched in miR124, which is selectively expressed in neurons and not in astrocytes (***84-86***). The integration of the miR124T element leads to posttranscriptional transgene silencing in neurons but not in astrocytes (Figure 5). The choice of miRT sequence depends on several factors: the developmental stage considered, the area of the brain studied and the subpopulation of cells targeted (***87-90***). It is possible to integrate several miRTs to achieve a synergistic effect ((***83***); *see* **Note 6**). The miRT sequence may be a natural target of the endogenous miRNA, which is partly complementary to the miRT (***37***), or a sequence fully homologous to the miR (***83***). miRNA-mediated gene silencing is based on translational or posttranscriptional mechanisms (***91-93***).

3.6 - LV production

A detailed description of LV production is available in a previous issue of this book collection and other recent protocols (***62, 94-95***). In this chapter, we will describe the production of LVs pseudotyped with the VSV-G and MOK-G envelopes (*see* **Note 7**). We will provide information for the scaling up of production and the use of LVs in preclinical experiments (***95-98***).

LVs are produced over a period of four days (Figure 6). In most cases, a batch of lentiviral vectors is produced by the transient cotransfection of 293T cells with various plasmids (Figure 2). Another way of producing LVs involves the use of a packaging cell line that stably expresses the packaging, envelope and rev genes. A single transfection with the transfer plasmid is sufficient to produce recombinant vector. The disadvantage of this approach is that yields are low and the stable production of VSV-G protein in mammalian cells has been shown

to be toxic (***99-100***). Inducible packaging cell lines have recently been developed for large-scale production (***101-105***).

Day 1: Seeding of 293T cells

293T cells (ATTC–CRL-11268) are used for LV production because of their high transfection efficiency (***106-107***). These human embryonic kidney cells are immortalized with adenoviral E1A/E1B proteins and express the SV40 large T antigen, facilitating replication of the episomal plasmid. They grow in DMEM medium supplemented with 10% FBS (*see* **Note 8**) and 1% streptavidin/streptomycin. They are maintained in a humidified incubator at 37°C, under an atmosphere containing 5% CO_2. These cells are subcultured every three to four days, at a dilution of 1/5 (*see* **Note 8**).

For regular production, $5x10^6$ cells should be plated in a 10 cm Petri dish, whereas for large-scale production, 5.8×10^7 cells should be plated in a stack, as described below (Table 1; *see* **Note 9**).

Day 2: Transient transfection in the presence of calcium phosphate

The day after plating, the cells should have reached 50 to 70% confluence: smaller numbers of cells result in lower production yields. The calcium-phosphate transfection method is used (transfection efficiency of up to 95% in 293T cells). The $CaPO_2$ precipitate is prepared as shown below (Table 2) and all the reagents are incubated at room temperature before transfection. For the preparation of 1 ml of precipitate for a 10 cm Petri dish, the various plasmids are mixed in a 1.5 ml tube, with 250 µl of deionized water and then 250 µl of 0.5 M $CaCl_2$. The DNA-$CaPO_2$ solution should be added drop-by-drop to a second tube containing 500 µl of 2 x HBS, with continual vortexing. Commercial HBS should be favored to guarantee pH stability. A fine, opalescent precipitate is obtained after a few minutes. The precipitate should be added in a drop-wise manner to the cells 5 to 20 minutes later; the precipitate should not be added more than 30 min later (*see* **Note 12**). When the volume of precipitate is large, we recommend generating bubbles in the HBS with a 2 ml pipette and adding the DNA-$CaCl_2$ complex drop by drop. The medium should be replaced 5 h after transfection, and care is required to ensure that the cells are not lost, because they adhere only weakly to the plate.

Day 4: Harvesting and concentration of lentiviral vector

The supernatant is harvested and concentrated 48 h after transfection (*see* **Note 13**). An aliquot of supernatant is first removed for p24 determination in the non concentrated supernatant, to monitor production efficiency. The

supernatant is then passed through a Stericup filter with 0.45 µm pores and centrifuged at 19,000 rpm (Beckman Coulter SW 32Ti rotor) for 1.5 h at 4°C. The supernatant is removed and the pellets are pooled and resuspended in 1 ml of 1% BSA in PBS. This viral suspension can be used for *in vitro* experiments. For *in vivo* experiments, a second concentration step is required (19,000 rpm, Beckman Coulter SW 48Ti rotor) (*see* **Note 14**). The final pellet, containing the LV, is resuspended in 120 µl of 1% BSA in PBS and frozen at -80°C until use. When scaling up production, we resuspend the pellet in 300 to 400 µl of buffer per five stacks. For LVs pseudotyped with the MOK-G envelope or containing a miRT sequence with a lower production yield, the pellet is resuspended in a smaller volume (e.g. ~60 µl).

3.7 - LV titration

Several methods can be used to determine functional or physical lentiviral titers, as shown in Figure 7. The advantage of the physical titer is that it can be used for batches regardless of the promoter used and independently of the presence or absence of miRT or the envelope used for pseudotyping. It provides a quantitative estimate of the total number of physical particles. This method is particularly suitable for adjusting LV titer, as described here. By contrast, the functional titer provides a measurement of the transduction efficiency of LVs in the cells used for the test. However, permissiveness depends on LV pseudotyping and the transcriptional activity of tissue-specific promoters strongly affects outcome.

3.7.1 - Determination of the physical titer by ELISA

This ELISA determines the concentration (ng p24/µl) of p24 capsid in the samples. It is performed on non concentrated and concentrated LVs. During LV production, 5 µl aliquots of supernatant are taken and diluted in 45 µl of the lysis buffer provided in the kit. For concentrated LV-VSV-G, serial dilutions, from $1/10^6$ to $1/1.2 \times 10^7$, are prepared. For LVs pseudotyped with the MOK-G envelope or containing an miRT sequence (lower yield, *see* **Note 15**), the samples are diluted to $1/6 \times 10^6$. Once diluted in lysis buffer, LV samples can be transferred to a BSL1 (biosafety level 1) laboratory. p24 antigen determinations are carried out in accordance with the manufacturer's protocol (*see* **Note 16**).

3.7.2 - Determination of the physical titer by RT-qPCR

This technique is used to determine the quantify lentiviral mRNA copies following stable transduction in cell culture (***95, 108***). All steps up to RNA extraction are performed in BSL2 or BSL3 laboratories. The concentrated

(1000 times) viral suspension (2 µl) is added to a 1.5 ml tube containing 353 µl DNase- and RNase-free water, 5 µl of DNaseI and 50 pg of RNaseA. The contents of the tube are mixed by vortexing and the tubes are then briefly centrifuged. The mixture is incubated for 10 min at room temperature and 20 µl of RNasin is then added. The mixture is incubated for 10 minutes at 37°C and 10 µl of 25 mM EDTA for inactivation of the enzyme by heating at 70°C for 10 minutes. The tubes are then centrifuged at 2,000 x *g* for 10 s. RNA is extracted with a Pure link RNA miniKit (Ambion). For lysis, 300 µl of the lysis buffer provided in the kit is added. At the end of the procedure, the purified RNA is eluted in 30 µl of the kit solution (in RNase-free tubes). The purified RNA (5 µl) is added to a 96-well plate for reverse transcription with the Express Superscript mix and Express SYBR green ER supermix (Invitrogen). As a negative control, the RNA is added to the well without reverse transcriptase (***95***). Finally, PCR is carried out as follows: for 5 minutes at 50°C, 2 minutes at 95 °C (denaturation), and 40 cycles of 15 seconds at 95°C (denaturation), and one minute at 60 °C (amplification).

The primers used, the sequences of which are given below, recognize the LTR of HIV-1 and can be used for the titration of all LVs.

-- Sense: TGTGTGCCCGTCTGTTGTGT

-- Antisense: GAGTCCTGCGTCGAGAGAGC

The number of RNA copies is obtained from a standard curve for known numbers of copies of DNA plasmid treated in the same way as the samples.

The relative titer/ml is calculated by applying the following formula:

(number of RNA copies x dilution of vector preparation) / volume in ml.

3.7.3 – Determination of the function titer by flow cytometry

293T and HeLa cells are used for titer determination by flow cytometry (fluorescence-activated cell sorting, FACS), but this approach is suitable only for LVs expressing reporter genes encoding fluorescent proteins (GFP, for example) under the control of a promoter active in these cells. Each well of a 24-well plate are seeded with $1x10^5$ 293T cells. On the next day, the cells are infected with serial dilutions of LVs, from 1/10 to $1/10^7$, prepared in DMEM. Either 48 h or 72 (for LV-MOK-G) after transduction, the cells are washed with PBS and dissociated by treatment with 150 µl of 1x Trypsin. DMEM (150 µl) is then added to inactivate the trypsin. The cells are then fixed by incubation with 300 µl of 2% formaldehyde in PBS and transferred to FACS tubes (*see*

Note 17). Flow cytometry analysis is performed to determine the percentage of cells positive for GFP (*see* **Note 18**).

Titer is calculated as follows:

% of positive cells x number of cells on the day of infection x dilution factor (*see* **Note 19**)

3.8 - Culture and transduction of primary striatal neurons

Primary cultures are set up in a laminar flow biohazard hood in a BSL2 laboratory. The adhesion of the cells to plastic is enhanced by coating the multiwell plates with 5% poly-D lysine (400 µl/well or 1.5 ml/well for a 24- or a 6-well plate, respectively) the day before culture and incubating the plates overnight in a humidified incubator at 37°C, under an atmosphere containing 5% CO_2. The next day, the wells are rinsed with sterile water and dried under the hood.

3.8.1 – Cocultures of primary striatal neurons

Cocultures of primary striatal neurons are prepared from E15 rats (Sprague Dawley rats). A pregnant rat is anesthetized first with isoflurane and then with pentobarbital (injection below the first breast). The skin is disinfected with alcohol, the abdominal cavity opened and the uterine horns are withdrawn. Embryos are placed in HBSSm (see Materials for composition) and their heads are cut off. From this point onwards, all the material must be kept on ice. Brains are retrieved under a dissecting microscope (*see* **Note 20**) and placed on a large Petri dish containing HBSSm. The striata are dissected out (*see* **Note 21**) and collected in 5 ml of HBSSm in a Petri dish. They are then thinly sliced with microscissors. HBSSm (4 ml) and 1 ml of DNase I (3 mg/ml) are added and the mixture is incubated for 15 min at 37°C. The cells are vigorously dissociated with a 1 ml tip, to give a turbid suspension (*see* **Note 22**). The mixture is allowed to settle for 3 min on ice and 9 ml is collected in a second tube. HBSSm (1 ml) is then added to the rest of the mixture for complete dissociation of the cells. This mixture is allowed to settle for three minutes and about 2 ml is then added to the second tube (at this stage, there is approximately 11 ml of dissociated cell suspension). The dissociated cells are centrifuged at 200g (10 min, at room temperature). The supernatant is discarded and the pellet is resuspended in 5 ml of HBSSm by uptake into and release from fire-polished Pasteur pipettes. The resuspended pellet is allowed to settle on ice for three minutes and about 4 ml is transferred to a new tube. We added 5 ml of HBSSm and centrifuged again for10 min at 200g (room temperature). The supernatant is discarded and the pellet is resuspended in 5 ml of neuronal

medium culture (see Materials for composition) with a 5 ml pipette. Cell density is determined and the cells are plated to give a density of 2 x 10^5 cells/cm^2 (Table 3). Striatal primary cultures (mixture of neuronal and astroglial cells) can be kept for up to eight weeks, with the replacement of half the medium each week.

3.8.2 - Transduction and *in vivo* modeling of HD in cocultures of primary striatal neurons

Striatal cultures are transduced with LV-VSV-G at 1 division (DIV), with 10 ng of p24 antigen/10^5 cells (i.e. 180-200 ng of p24 antigen from the virus/well for a six-well plate and 35-40 ng of p24 antigen from the virus/well for a 24-well plate). With a GFP virus, fluorescence is detected in the soma and processes seven days after transduction. Fluorescence is maximal three weeks after transduction. Under these conditions, 90 to 95% of neuronal cells are infected and express reporter genes or genes of interest (***32***). When LVs expressing wild-type or mutant Htt (Htt171-18Q/82Q respectively) were used to develop an *in vitro* model of HD, mHtt resulted in a slowly progressing disease characterized by the appearance, after one month, of neuritic aggregates, followed by intranuclear inclusions, neuronal dysfunction and, finally, cell death at six to eight weeks (***32***). Immunohistochemical analysis is carried out on fixed cells, to detect human Htt aggregates (Figure 8). The primary cultures are washed with cold PBS and fixed by incubation in 4% paraformaldehyde for 10 minutes at 4°C. They are then washed with PBS and incubated in a blocking solution of PBS supplemented with 10% normal goat serum and 0.03% Triton X-100. The cells are then incubated overnight at 4°C in blocking solution containing the 2B4 antibody recognizing the N-terminal part of Htt at a dilution 1/10^3 and for 2 h with the secondary antibody coupled to a fluorophore or biotin (*see* **Note 23**).

3.9 - Culture and transduction of primary cultures of cortical astrocytes

3.9.1 - Primary culture of cortical astrocytes

Primary cultures of cortical astrocytes are generated from P1/P2 newborn C57/Bl6 mice. Forebrains are removed aseptically from the skull and placed in ice-cold PBS. The meninges are carefully removed and the neocortex is dissected free from the brainstem, thalamus, striatum and hippocampus. Cells are then dissociated with a 5 ml pipette and passed through needles of decreasing gauge, from 20 to 21 gauge. Cells are used to seed DMEM supplemented with 10% FBS, 0.5 mM glutamine and 0.1% streptomycin/penicillin (10 µg/ml and 10 U/ml, respectively) in six-well plates. The medium is replaced completely every two days. Primary astrocytes are maintained in culture for up to three weeks. They reach maximum confluence after 12 to 15 days.

3.9.2 - Transduction of primary cortical astrocytes

The equivalent of 10 ng of p24 antigen/10^5 cells (i.e. 180-200 ng of p24 antigen of virus/well for a 6-well plate and 35-40 ng of p24 antigen of virus/well for a 24-well plate) of LV-MOK-G-miRT is used to transduce cortical astrocytes. Transduction should be carried out after 10 to 11 DIV to ensure that cell confluence is optimal. Transgene expression is visible two days after transduction and expression is maximal 11 days after transduction (i.e. around 21 DIV).

4.0 – *In vivo* transduction: HD rodent model and LV tropism

4.0.1 - Stereotaxic injection: standard protocol and HD model *in vivo*

LV tropism can be investigated in primary cultures *in vitro*. However, one important limitation is that the tropism observed may differ from that observed *in vivo*. Indeed, whereas MOK-G confers an astrocytic tropism on LVs *in vivo;* LVs pseudotyped with MOK-G transduce both astrocytes and neurons *in vitro*, (data not shown). It is therefore important to check the tropism of LVs by stereotaxic injection in rodent brain.

Adult rats weighing 200-250 g and adult mice weighing 20-25 g are anesthetized with a ketamine/xylazine solution: 75 mg/kg-10 mg/kg and 150 mg/kg-10 mg/kg, respectively. The top of the head is shaved and the animals are placed in a stereotactic frame. The skin is opened to reveal the skull and the bregma is localized. Striatum coordinates are determined on the basis of a stereotaxic atlas (***109-110***). With the aid of a binocular microscope, holes are drilled at the following coordinates: i) for rat striatum: tooth bar, -3.3 mm; anteroposterior, +1 mm from bregma ; lateral, ±3 mm; ventral, -4.5 mm and ii) for mouse striatum: tooth bar, 0 mm; anteroposterior, +1 mm from bregma; lateral, ±2 mm; ventral, -2.5 mm. Before stereotaxic injection, concentrated lentiviral vector stocks are thawed and resuspended by vortexing and repeated pipetting. LV concentration is adapted according to the envelope used for pseudotyping and the species. Two to four times more MOK-G- than VSV-G-pseudotyped LV should be injected (***37***). We currently use up to 1,000 ng of LV-MOK-G and 600 ng of LV-VSV-G for rat striatum and up to 800 ng of LV-MOK-G and 200 ng of LV-VSV-G for mouse striatum. A total injection volume of 2-8 µl is acceptable for rat striatum and of 2-3 µl for mouse striatum. These volumes are adapted as a function of the structure targeted. The injection is performed with a 10-µl syringe (34-gauge blunt-tip needle attached to a polyethylene catheter), at a flow rate of 0.25 µl/min for rats and 0.2 µl/min for mice. When the injection is completed, cannulas are left in place for 5 minutes to ensure effective diffusion of the LVs and to prevent reflux. The needles are then slowly removed and the skin is sutured. Finally, the animals are kept in a recovery box maintained at 37 °C.

A rodent model of chronic HD has been developed, based on LVs encoding mutant Htt (Htt171-82Q as in the *in vitro* model). Stereotaxic injections are carried out with the VSV-G-pseudotyped LV/SIN-PGK-Htt171-82Q-WPRE, with a total of 800 ng of p24 antigen in two 4 µl aliquots delivered to the rat striatum (***33***). In this model, cell death is observed over a period of six months and mHtt protein production induces the formation of Htt aggregates as little as two weeks after viral injection (Figure 9).

4.0.2 - Immunostaining studies of tropism

Tropism is checked by immunostaining with neuronal and astrocyte markers. The protocol below concerns fluorescence immunostaining with the neuronal and astrocyte markers NeuN and S100β, respectively.
At the appropriate time-point, the animals are sacrificed for collection of brain samples. Fixed slices (4% PFA) are washed with PBS and incubated in a blocking solution of PBS supplemented with 3% NGS, 0.2% gelatin and 0.3% Triton X-100 for 1 h at room temperature. The slices are then incubated overnight at 4°C with the primary antibody at an appropriate dilution (1/200 for NeuN and 1/500 for S100β) in PBS supplemented with 3% NGS, 0.2% gelatin and 0.1% Triton X-100. The next day, the slices are washed three times in PBS. They are then incubated for 1 h at room temperature with the secondary antibody (Alexa Fluor-labeled and diluted 1/500) in PBS. Finally, slices are washed three times in PBS and mounted in a special medium for fluorescence.

4. Notes

Note 1: Retrograde transport is improved by the use of a fusion envelope glycoprotein containing the cytoplasmic domain of VSV-G fused to RV-G (***111-112***).

Note 2: The transgene may be a reporter gene (β-galactosidase or fluorescent proteins), a disease gene (mutant Htt), a therapeutic gene or a small interfering RNA expressed under the control of the polymerase III promoter (U6 or H1) or the polymerase II promoter with miR-embedded siRNA.

Note 3: Several phosphatases are available. Heat-inactivated enzymes should be favored, because ligation reaction products can be processed directly, without the need for further vector DNA purification.

Note 4: For the amplification of plasmids containing the ccdB gene, a specific bacterial strain (2T1R strain) is required.

Note 5: The *PfuUltra II* enzyme can be used for long inserts (> 10 kb).

Note 6: miRT efficacy depends on endogenous miRNA levels (***83***).

Note 7: As recommended by the French "Commission de Génie Génetique", all work with LV vectors should be carried out in a biosafety level 2-3 laboratory.

Note 8: 293T cells maintained in culture for more than 30 passages should not be used because the cells begin to detach and LV production decreases. Moreover, regular testing for mycoplasmas is recommended, to prevent contamination that might decrease cell performance. Batches of serum should be tested carefully, as they have a major impact on LV yield.

Note 9: 293T cells adhere poorly to plastic, so several flasks should be tested to determine the optimal culture conditions. We use Falcon Petri dishes and Corning flasks.

Note 10: The reproducibility of LV production can be increased by generating large batches of plasmids, checking their quality and then storing them in aliquots (1 mg/ml) to prevent the need for repeat freezing and thawing. One option is to subcontract plasmid production (packaging, VSV-G and MOK-G envelopes, Rev).

Note 11: The relative proportions of the various plasmids are optimized and must be respected to achieve high production yields. For LVs pseudotyped with the MOK-G envelope, the best results were obtained by doubling the quantity of the envelope plasmid during transfection.

Note 12: $CaCl_2$ aliquots are freshly prepared and not refrozen. The mixture should be opalescent. If a coarse precipitate formed, it should be broken down by vigorous shaking. Production yields are higher when the DNA-$CaCl_2$ complex is deposited on HBS rather than the reverse.

Note 13: Harvest is possible 24 h, 48 h or 72 h posttransfection, but the best yields are obtained 48 h posttransfection.

Note 14: Rotor and ultracentrifuge are cooled at 4°C before use.

Note 15: p24 values concentrations of 50 to 300 ng/µl are usually obtained for LV-MOK-G (supernatant from 293T cells, non concentrated LV) and of 100 to 1000 ng/µl for LV-VSV-G.

Note 16: A relative transducing unit can be estimated from the p24 titer. This conversion factor, established by D. Trono for LV-VSV-G, is based on the fact that there are approximately 10^4 physical particles per pictogram of p24. The estimated TU is thus of the order of 10^5 TU/ng p24.

Note 17: Increasing formaldehyde concentration causes the cells to autofluoresce.

Note 18: Cells are maintained in the dark at 4°C until FACS analysis.

Note 19: For calculation, only dilutions yielding from 1% to 20% positive cells should be considered.

Note 20: Use Dumont forceps (numbers 4 and 5) to withdraw the meninges and extract the brain. Place the brain in a large Petri dish on ice.

Note 21: Place the dorsal face of the brain towards you: cut out the cortex (striatum visible from above), withdraw the thalamus (the white ball at the center of the striatum) and separate the hemisphere. Then, place the external face of the brain towards you and remove the dura mater. Take off as much cortex as possible.

Note 22: To complete the dissociation: allow the mixture to stand for 3 min at room temperature. Then collect 9 ml in another tube and add 1 ml of HBSSm to the rest and repeat the dissociation.

Note 23: Cells incubated with biotin-coupled secondary antibody require further processing with the Vectastatin Elite ABC detection kit.

Figure legends

Figure 1: Schematic representation of a lentiviral vector and strategies for modifying tropism. In most cases, LVs are pseudotyped with a VSV-G envelope, which confers a neuronal tropism. The viral particle contains the enzymes essential for replication and a copy of the single-strand RNA genome. Three strategies have been proposed for modifying the tropism of LVs: changing the envelope glycoprotein, using a tissue-specific promoter or adding an miRNA-target sequence to restrict transgene expression.

Figure 2: Schematic diagram of the third-generation packaging system. Four plasmids encode the genetic materials of the lentiviral vector: i) the transfer plasmid contains the transgene of interest; ii) the packaging plasmid contains the protein capsid, protease, integrase and reverse transcriptase; iii) a plasmid expressing the rev protein; and iv) the envelope plasmid expressing the VSV or MOK glycoprotein. Cotransfected cells produce lentiviral vectors with a genome consisting exclusively of the sequences present in the transfer plasmid.

Figure 3: Schematic diagram of an SIN transfer plasmid. Dark gray elements represent the expression cassette; the bright gray element is optional and its presence depends on biological need. Abbreviations: psi, HIV-1 packaging signal; RRE, rev-responsive element; cPPT, central polypurine tract; WPRE, woodchuck posttranscriptional regulatory element; miRT, miRNA-target sequence; A, *Asp718*; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; N, *Not*I; S, *Sac*I; S', *Sal*I.

Figure 4: Gateway® technology for cloning in an LV transfer plasmid. The promoter is inserted into an entry plasmid containing the *attL1* and *attL2* recombination sites. LV destination vectors are then produced by

integrating a conversion cassette (attR1-ccdB- Cm^R-attR2) upstream from the transgene. The LR reaction is then performed.

Figure 5: Schematic diagram of the strategies for targeting specific cell types. VSV-G-pseudotyped LVs have a high neuronal tropism, whereas MOK-G-pseudotyped LVs more frequently target astrocytes. The addition of the miR124T sequence inhibits transgene expression in neurons, leading to an astrocytic pattern of transgene expression. Combination with a tissue-specific promoter should not change LV tropism but should improve biosafety.

Figure 6: Schematic diagram of the various steps in lentivirus production.

Figure 7: Schematic diagram showing the various ways to titer lentiviral vectors. Physical titers can be obtained by ELISA and the determination of p24 antigen or by RT-qPCR on viral suspensions. Functional titers can be determined by cytometry or DNA titer determinations on infected cells (***108***).

Figure 8: Expression of the mutant Htt gene in primary cultures of striatal neurons. Detection of nuclear (indicated by V) and neuritic mutant Htt inclusions 8 weeks post infection, with LV-VSV-G encoding Htt171-82Q. (***32***)

Figure 9: Expression of mutant *Htt in vivo*. After the injection of LV-VSV-G encoding Htt171-82Q, aggregates can be detected as soon as one week later and for up to 12 weeks. EM48 polyclonal antibody (not commercially available) was used. (***33***)

Tables

Table 1: 293T cell seeding conditions for LV production.

Viral batch for	***In vitro* experiments**	***In vivo* experiments in rodents**	***In vivo* experiments in large animals (non human primates)**
Number of Petri dishes	6	20	

(10cm)	
Cell stack chambers	5 stacks

Table 2: Preparation of $CaPO_2$ precipitate for LV production.

Plasmids (*see* **Note 10** and **Note 11**)	Quantity for one Petri dish (µg)	Quantity for one stack chamber (µg)
Packaging plasmid	13	150
Rev plasmid	3	35
Envelope plasmid		
VSV-G	3.75	43
MOK-G	7.25	79
Transfer plasmid	13	150

Table 3: Cell density required for striatal primary culture.

Plate	**Area of 1 well**	**Neuronal medium volume/well**	**Cell density/well**	**Total number of cells required**
6-well	8 cm^2	2.5 ml	2 x 10^6	≅ 12 x 10^6

24-well	1.9 cm^2	600 µl	0.38 x 10^6	≅10 x 10^6

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Figure 1

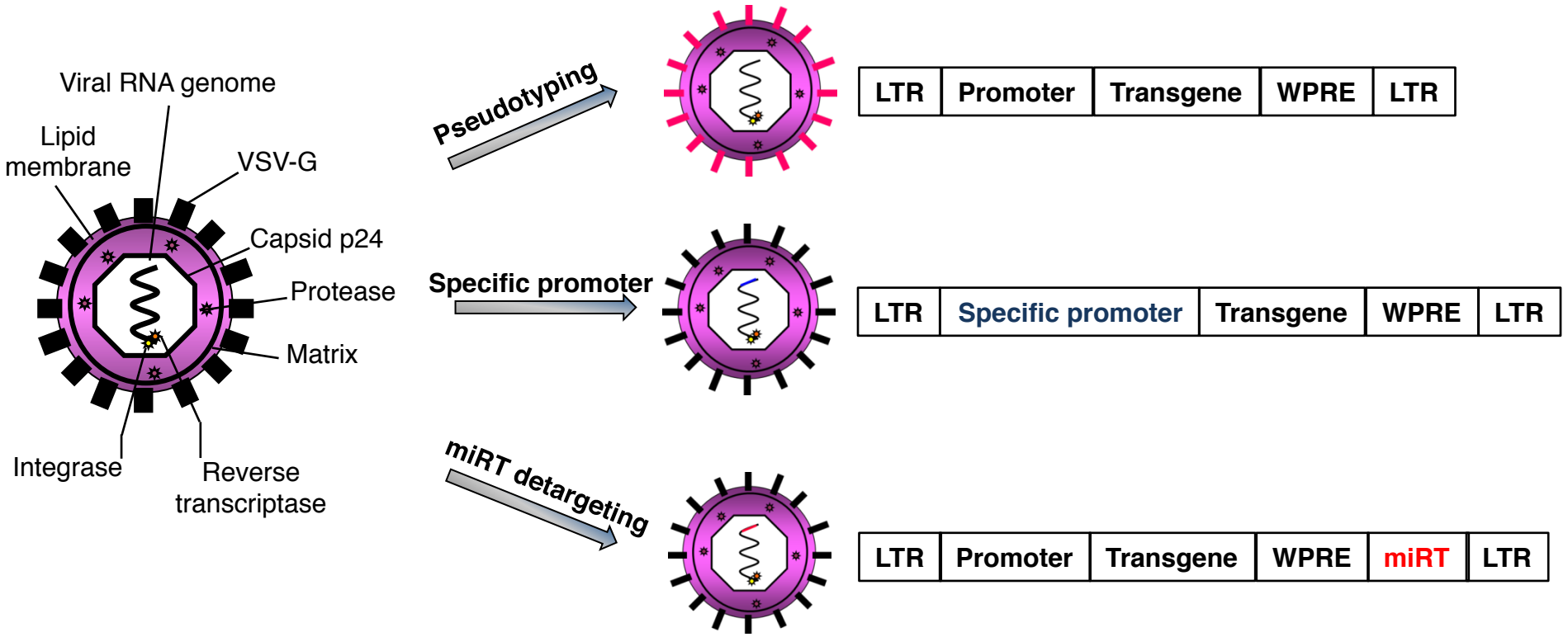
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Figure 2

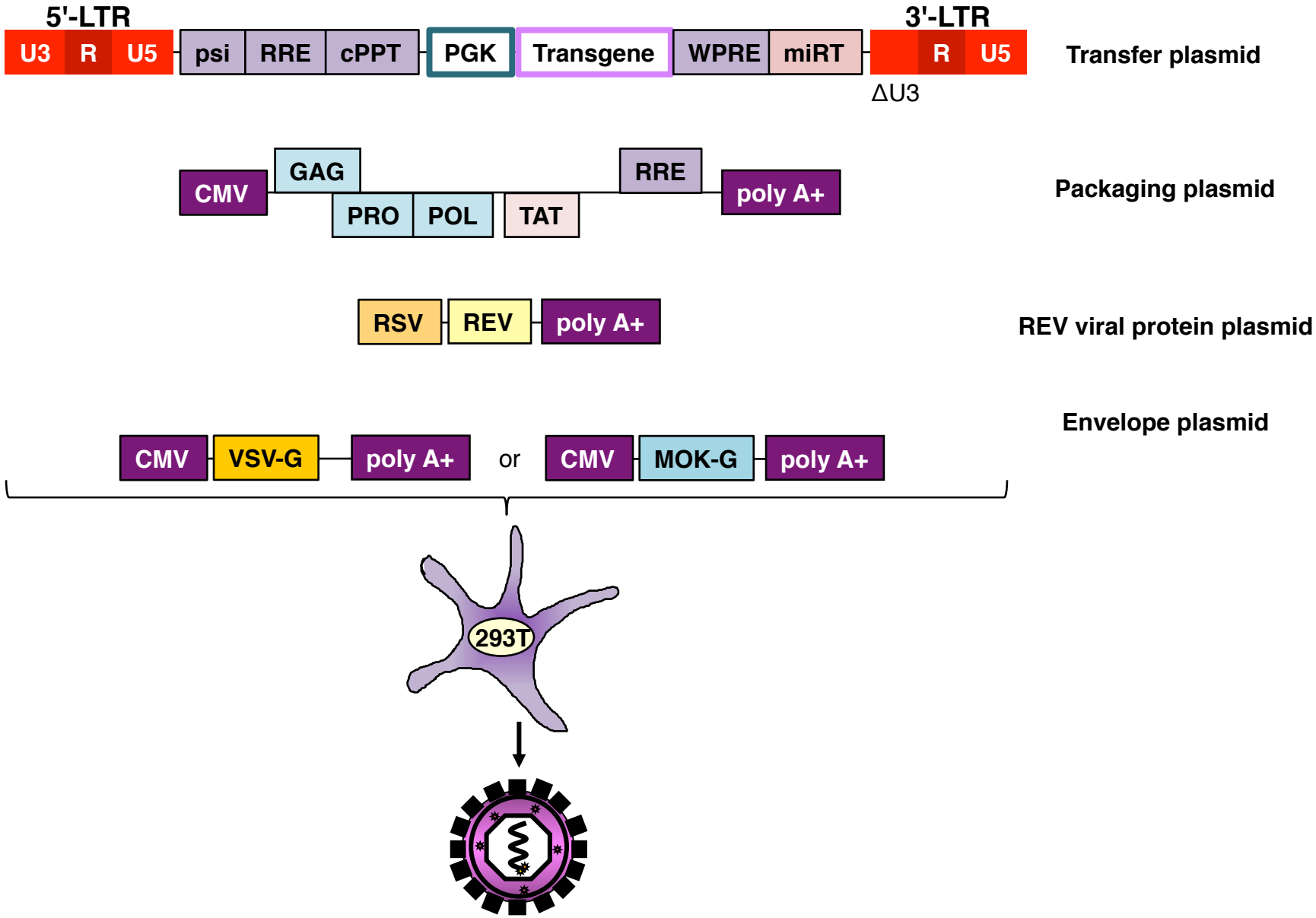
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Figure 3

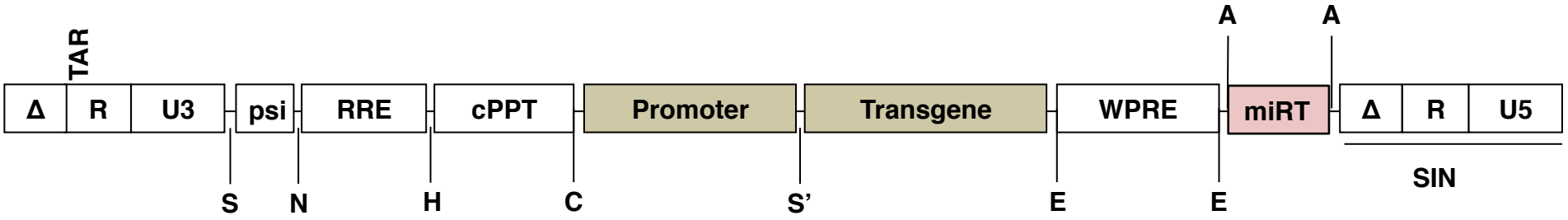

TAR
A
A
Δ
R
U3
psi
RRE
cPPT
Promoter
Transgene
WPRE
miRT
Δ
R
U5
SIN
S
N
H
C
S'
E
E

Figure 4

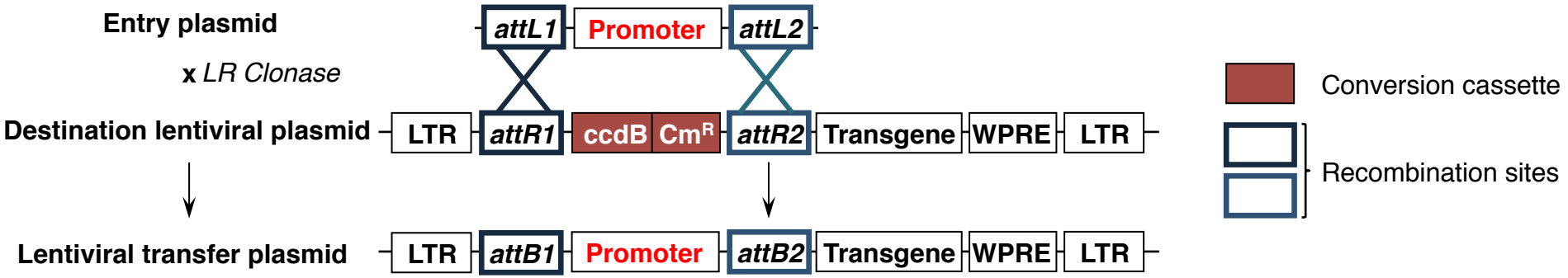

Entry plasmid
x LR Clonase
Destination lentiviral plasmid
Lentiviral transfer plasmid
attL1
Promoter
attL2
LTR
attR1
ccdB
CmR
attR2
Transgene
WPRE
LTR
LTR
attB1
Promoter
attB2
Transgene
WPRE
LTR
Conversion cassette
Recombination sites

Figure 5

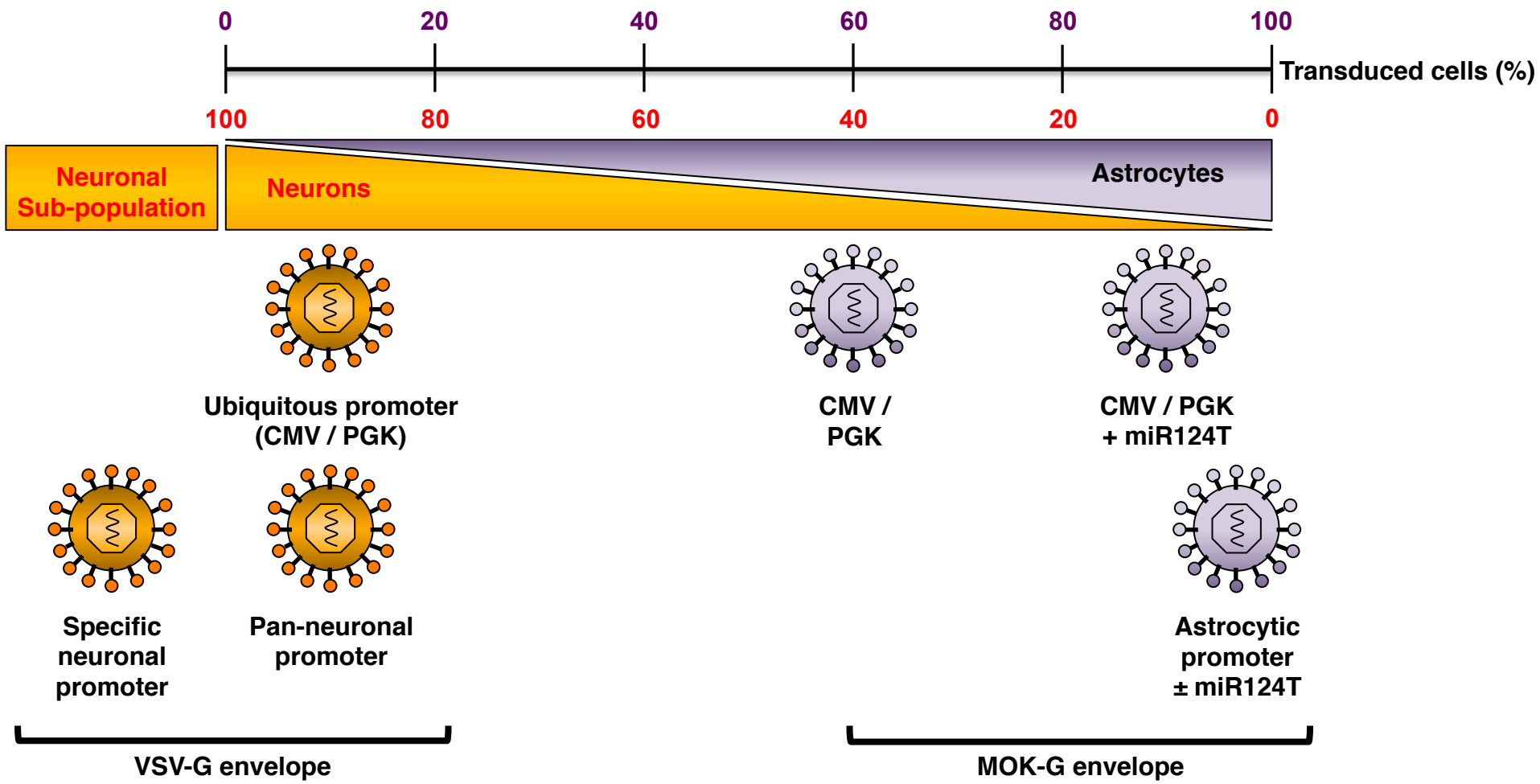
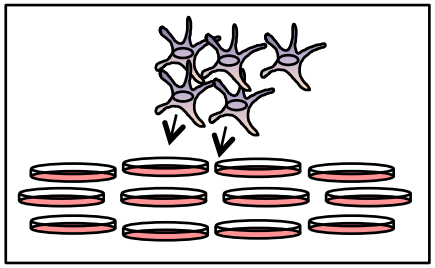

0
20
40
60
80
100
Transduced cells (%)
100
80
60
40
20
0
Neuronal Sub-population
Neurons
Astrocytes
Ubiquitous promoter (CMV / PGK)
CMV / PGK
CMV / PGK + miR124T
Specific neuronal promoter
Pan-neuronal promoter
Astrocytic promoter ± miR124T
VSV-G envelope
MOK-G envelope

Figure 6

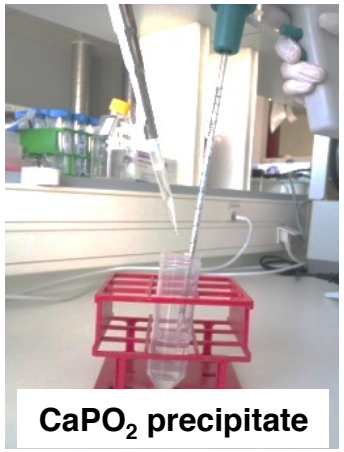
Day 1

Seeding of 293T cells



Day 2

Transient transfection by calcium phosphate



$CaPO_2$ precipitate

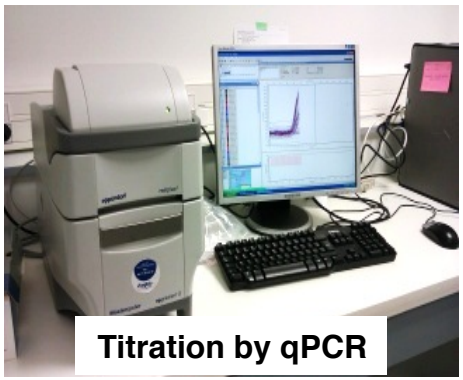
Day 4

Harvest and concentration of lentiviral vectors by ultracentrifugation

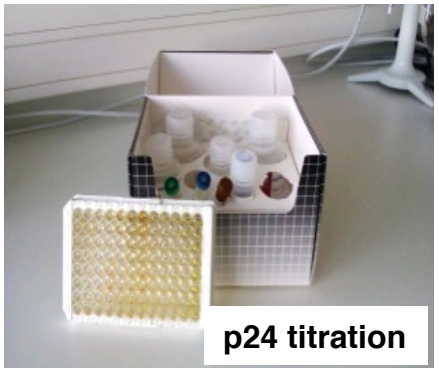
Ultracentrifuge

Day 5

Titration



Titration by qPCR



p24 titration

Figure 7

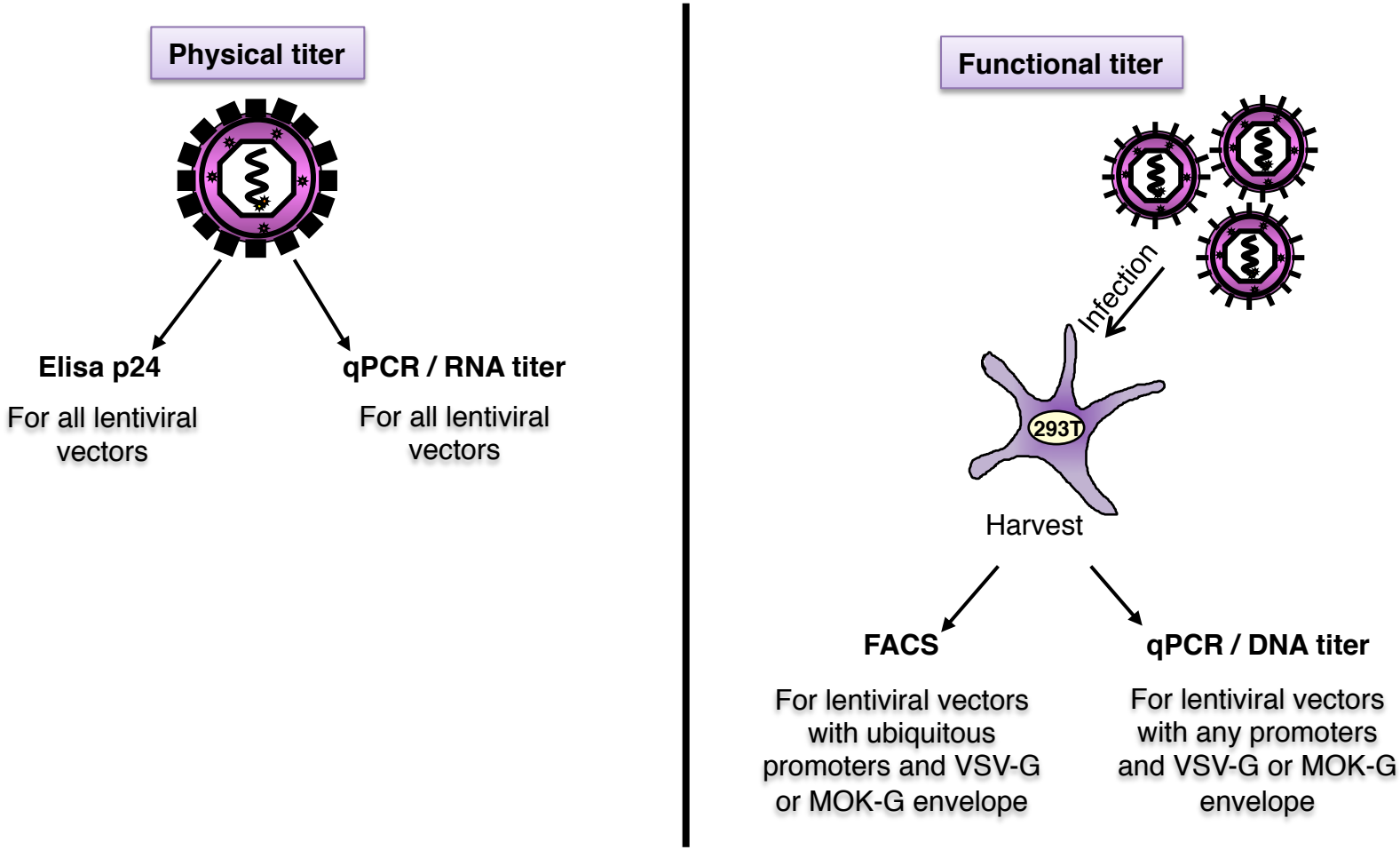

Physical titer
Elisa p24
For all lentiviral vectors
qPCR / RNA titer
For all lentiviral vectors
Functional titer
Infection
293T
Harvest
FACS
For lentiviral vectors with ubiquitous promoters and VSV-G or MOK-G envelope
qPCR / DNA titer
For lentiviral vectors with any promoters and VSV-G or MOK-G envelope

Figure 8

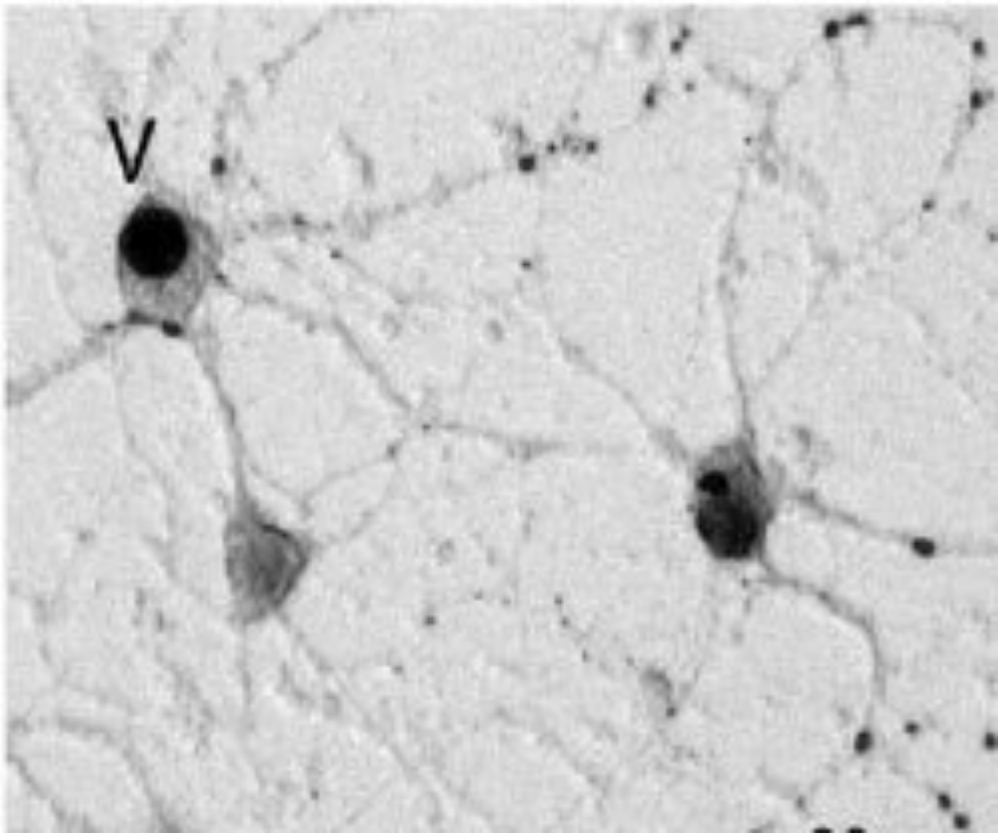


Figure 9