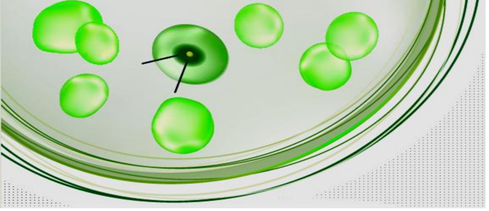


Highly Purified and Concentrated Lentiviral Vectors for *in Vitro* and *in Vivo* Validation of Candidat Genes

Hélène Vergnault, Régis Gayon, Christine Duthoit, Alexandra Iché and Pascale Bouillé

VECTALYS – Prologue Biotech rue Pierre et Marie Curie BP28262 31682 LABEGE Cedex – www.vectalys.com

vectalys
your gene transfer partner

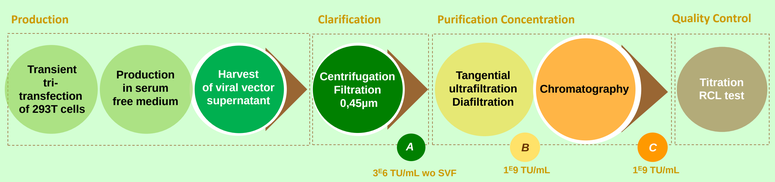
Vectalys is a gene delivery platform providing viral recombinant vectors for gene target identification and validation. With these tools, Vectalys provides an integrated and flexible service going from DNA to animals. Retroviral production and purification are one of our core activities to improve *ex vivo* and *in vivo* gene transfer. These applications require development, well-characterisation, standardization and up-scaling of production and purification methods. Crude supernatants contain contaminants that need to be removed to increase the potency and safety of the final product. Impurities usually come from serum, plasmid DNA or are released by intact or disrupted producer cells.

Vectalys has optimized a robust process for the production of high titer viral supernatant in serum free medium. Following the production step, two different processes of purification have been developed depending on the applications. First, ultrafiltration allows rapid and significant concentration/purification of large lentiviral vectors volumes with high vector recovery. This leads to 1E7 to 1E9 TU/mL purified samples and offers efficient transduction of primary and stem cells for *in vitro* or *ex vivo* strategies. Second, for *in vivo* experiments, the need in higher purity is required. So, Vectalys has included a chromatography step to get ultra-pure viral preparations. These stocks samples are injected into rat and mouse tissues in order to validate gene transfer efficiency, diffusion and toxicity.

Highly purified and concentrated lentiviral vectors

Vectalys strenght

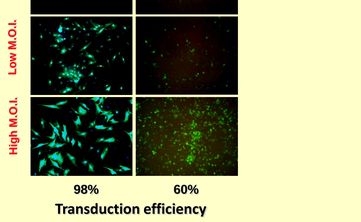
The development of **large-scale production and purification methods** for the generation of high titer preclinical grade retroviral vectors is critical to advances in gene therapy. Moreover, viral stocks contain **contaminants that need to be removed** to increase the potency and safety of the final product. Impurities usually come from **serum, plasmid DNA** or are released by intact or disrupted producer cells. Since preclinical protocols require high titer and high quality retroviral stocks (from 1^E7TU/mL for *ex vivo* trials up to 1^E9TU/mL for *in vivo* trials), this involves concentration and purification of large volumes of supernatant. The process is described below:



70% of production costs may be associated with the downstream processing and purification operations. Strategic consideration should be given to the design of appropriate separation systems in tandem with the current development of vectors and producer cell systems.

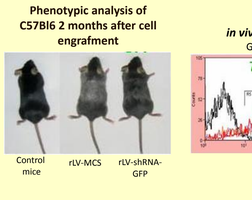
Lentiviral vector purification and concentration

Ultrafiltration allows rapid and significant concentration/purification of **large volumes of lentiviral** vectors with **high vector recovery**. Membrane fouling is circumvented by use of tangential flow techniques.



☺ : ✓ large volumes treatment
✓ scalable
✓ easy to perform
☹ : ✓ membrane fouling (formation of protein film)
⇨ tangential filtration

Tangential Cross-Flow Filtration ⇨ high flow rate



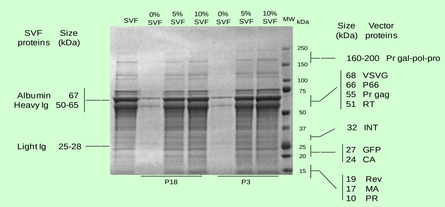
Anion exchange chromatography allows **selective purification of viral vectors** and improve vector quality at the end of the process.

Lentiviral vector production without serum

Vectalys has optimized a robust process for the production of high titer viral supernatant in **serum free medium**. Lentiviral vectors rLV-EF1-GFP were produced by transient tri-transfection in P3 and P18-293T cells, in 0, 5 and 10% SVF by using standard phosphate calcium procedures. Viral supernatant were harvested and analyzed.

N° of Passage	% SVF	Titer (1) (TU/mL)	PP/TU (2)	TU/tot prot (3) (TU/mg)
P18	10%	**$1,2^E6$**	192	**$3,9^E5$**
	5%	**8^E5**	263	**$3,9^E5$**
	0%	**$1,8^E6$**	161	**$1,9^E6$**
P3	10%	**$1,4^E6$**	229	**$4,8^E5$**
	5%	**$9,9^E5$**	273	**5^E5**
	0%	**$1,5^E6$**	120	**$1,5^E6$**

(1) Transducing units (TU) were determined by FACS. (2) Physical particles (PP) were quantified by HIV-p24 ELISA in order to determine PP/TU ratio. (3) Total proteins were quantified by spectrophotometry at 280nm.



Total proteins of viral supernatants were quantified and 15µg were separated by SDS-PAGE electrophoresis and visualized by coomassie blue staining .

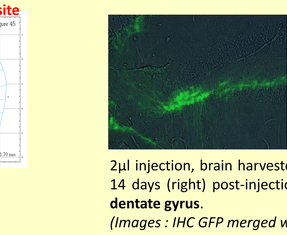
Results show that :
- **the absence of SVF improves the transduction efficiency of the viral vectors.**
- titer (TU/mL) increases while physical particles on transducing units ratio (PP/TU) decreases.
- well-controlled producer cells allow to produce high quality vectors independently on the cell passage.

High quality concentrated as well as crude viral vector batches

Lentiviral vectors rLV-EF1-GFP were produced by transient tri-transfection in 293T cells, in serum free medium. Viral supernatant were harvested **A**, purified by tangential ultrafiltration **B** and further purified and concentrated by chromatography **C**

	A	B1	B2	C
Titer	1^E6 TU/mL	1^E7 TU/mL	1^E9 TU/mL	1^E9 TU/mL
Volume	10mL few liters	10mL	1mL	1mL
purity	crude	+	++	++++
purification	-	UF	2xUF	UF + Cht**

*Transducing units (TU) were determined by FACS. Physical particles (PP) were quantitfied by HIV-p24 ELISA in order to determine PP/TU ratio. *Ultrafiltration ; **Chromatography*



Viral proteins were separated by SDS-PAGE electrophoresis and visualized by coomassie blue staining.

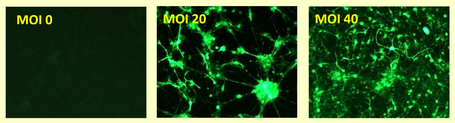
Results show :
- **Vectalys** is able to produce high volumes of crude supernatant of 3^E6 TU/mL viral vector. The crude vector batch is already high quality with **a PP/TU ratio about 150**.
Such batche transduction of immortalized cells.
- Ultrafiltration process allows to concentrate viral vectors **up to $2,5^E9$ TU/mL.** This is important to increase the efficiency of transduction for more delicate cells like primary cells.
-Chromatography step **greatly improves the purity** of the final viral vector stock. These preparations are designed **for *in vivo*** injection experiments.

Validation of candidat genes by *in vitro* or *in vivo* strategies

In vitro Transduction

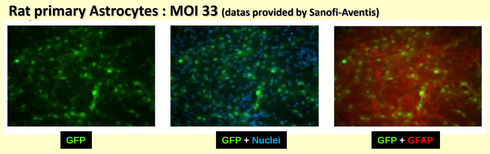
Primary CNS cell types

Primary cortical neurons from E-16 rat embryos



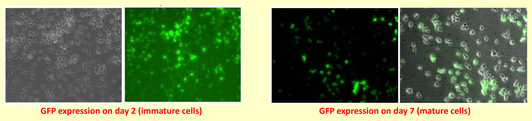
Transduction with HIV-derived vectors expressing e-GFP 5 days after extraction. Cells were maintained in culture during 17 days after transduction.

Rat primary Astrocytes : MOI 33 (datas provided by Sanofi-Aventis)



200 000 cells / well ; Stained with GFAP antibody 8 days post-transduction

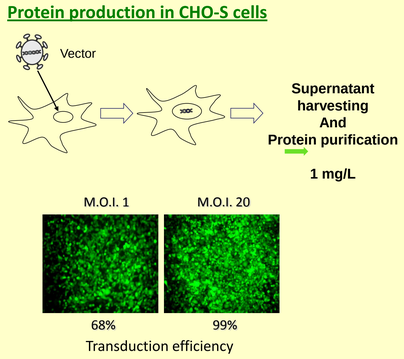
Rat primary Oligodendrocytes : MOI 20 (datas provided by Sanofi-Aventis)



Oligodendrocytes progenitors (OLP) cells obtained from newborn rat neocortex

Stem cells transduction

Protein production in CHO-S cells

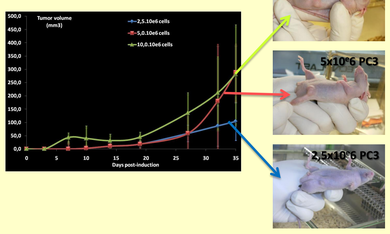


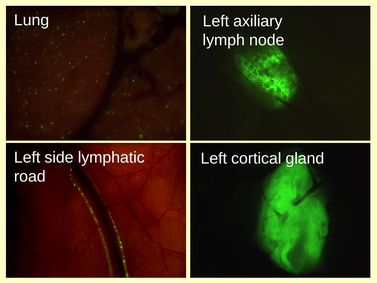
Ex vivo Strategies

Tumoral models

***In vivo* injection of human tumorigenic and metastatic cell lines (PC3, OVCAR-3) genetically modified *in vitro* by a GFP-lentivirus.**

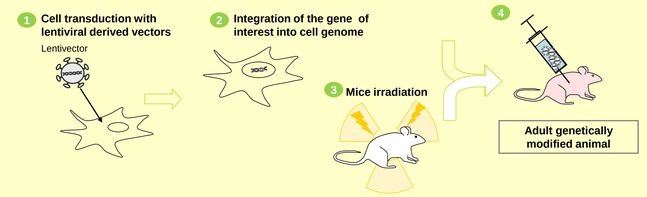
Male Nude mice injected sub-cutaneously with PC3 cells.





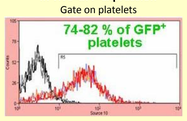
Nude male : analysis 8 weeks post-injection of **10x10^6** PC3-GFP (s.c. left injection). Tumor size : 800 mm³

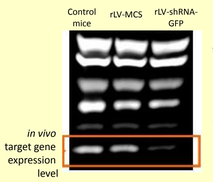
Genetically modified hematopoietic stem cells injection (datas provided by Sanofi-Avantis)

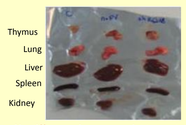


Phenotypic analysis of C57Bl6 2 months after cell engrafment

***in vivo* GFP expression**
Gate on platelets







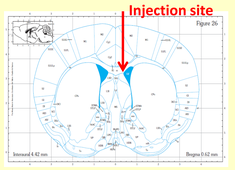
No difference in organ weight or volume

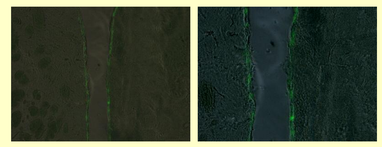
In vivo Injections

CNS Injection

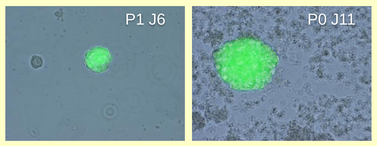
Stereotactic injection of high concentration/and high purity GFP-lentiviral suspension in adult C57BL/6 mice.
Targets: Neural Stem Cells.
Validation of the targets by NeuroSpheres Assay (NSA) and observation of GFP positive cells in brain sections

Injection in the sub-ventricular zone (SVZ)

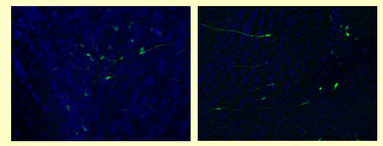




10µl injection, brain harvested and fixed 7 days post-injection, sections of the injected **ventricule**.
(Images : IHC GFP merged with brightfield)

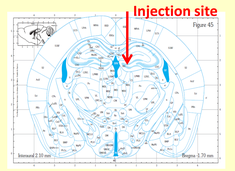


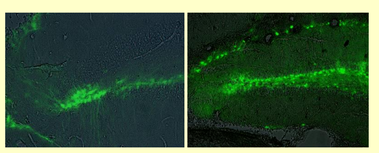
10µl injection , NSA 17 hours post-injection (left).
5µl injection, NSA 7 days post-injection (right).
(Images : GFP merged with brightfield)



10µl injection, brain harvested and fixed 7 days post-injection, sections of the **olfactory bulb** : GFP positive cells are matureneurons .
(Images : IHC GFP vs DAPI)

Injection in the subgranular zone of the Dentate Gyrus (DG)





2µl injection, brain harvested and fixed 7 days (left) or 14 days (right) post-injection, sections of the injected **dentate gyrus**.
(Images : IHC GFP merged with brightfield)