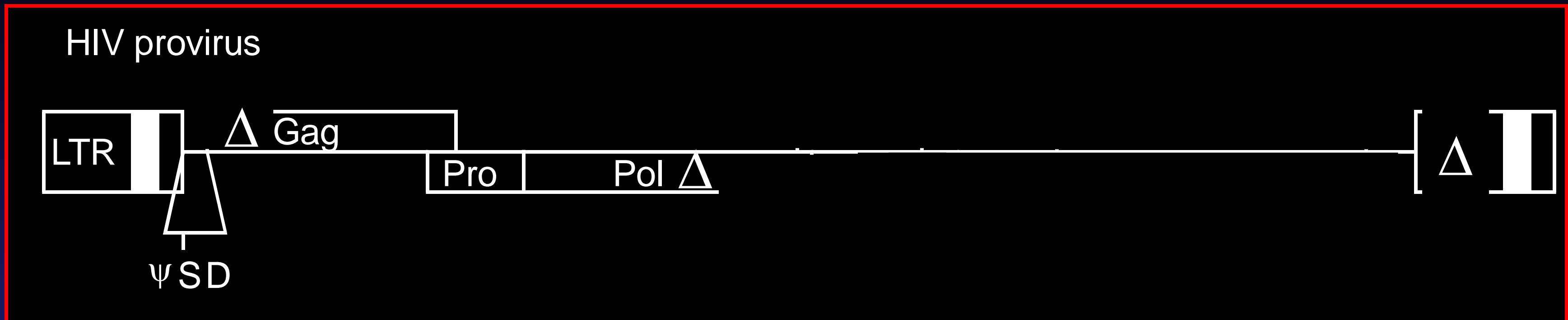
Lentiviral Vectors: design, production, and titration

ONPRC Lentiviral Vector Core
Molecular and Cellular Biology Core
Greg Dissen



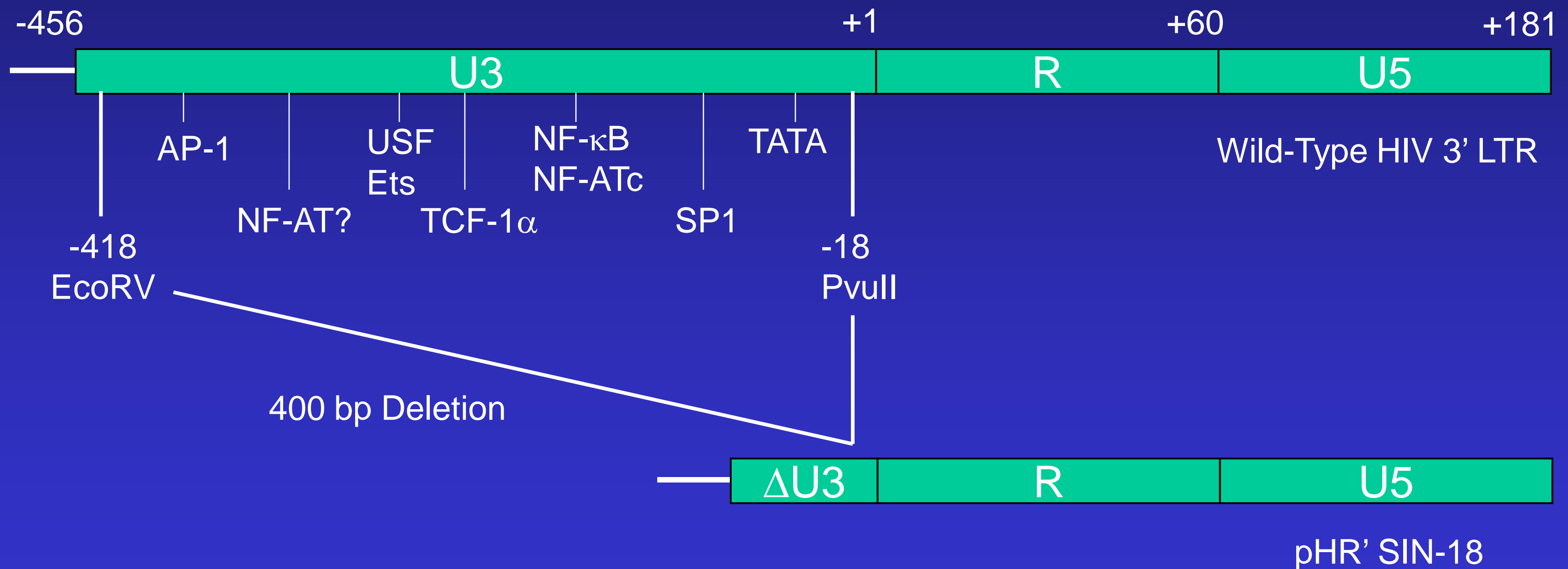
2nd and 3rd generation viral vectors

1. Viral backbone was stripped to allow room for transgenes

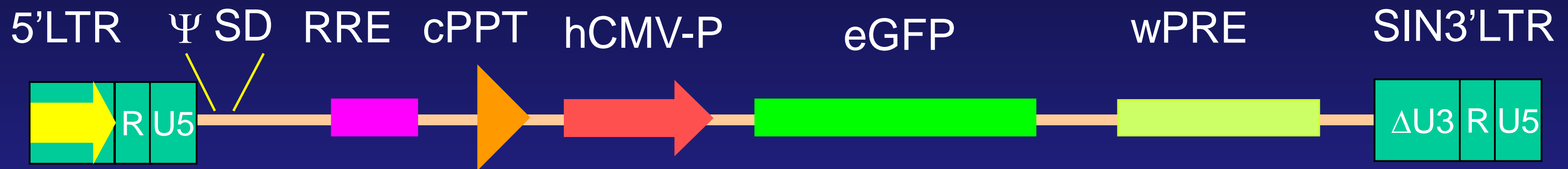
2. Development of the Self-Inactivating (SIN) vector

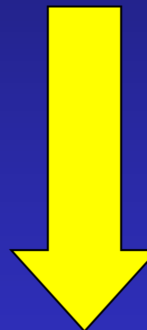
Lentiviral Vector System

2. Modification of 3'LTR "Self Inactivating"

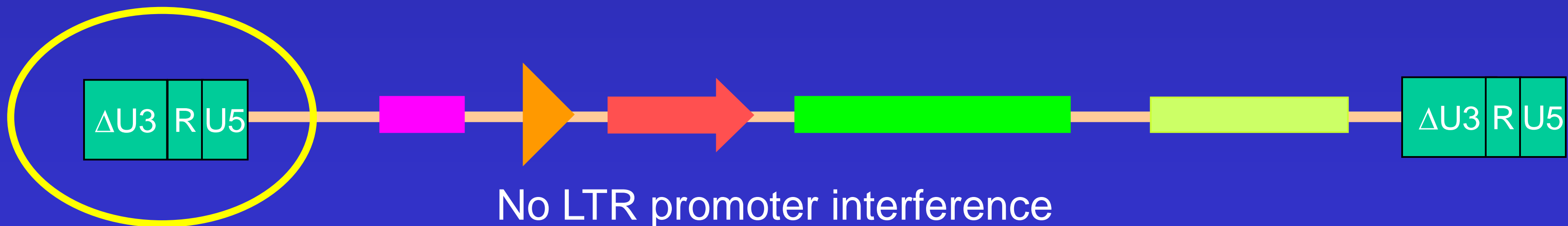


Lentiviral Vector System (3rd generation)



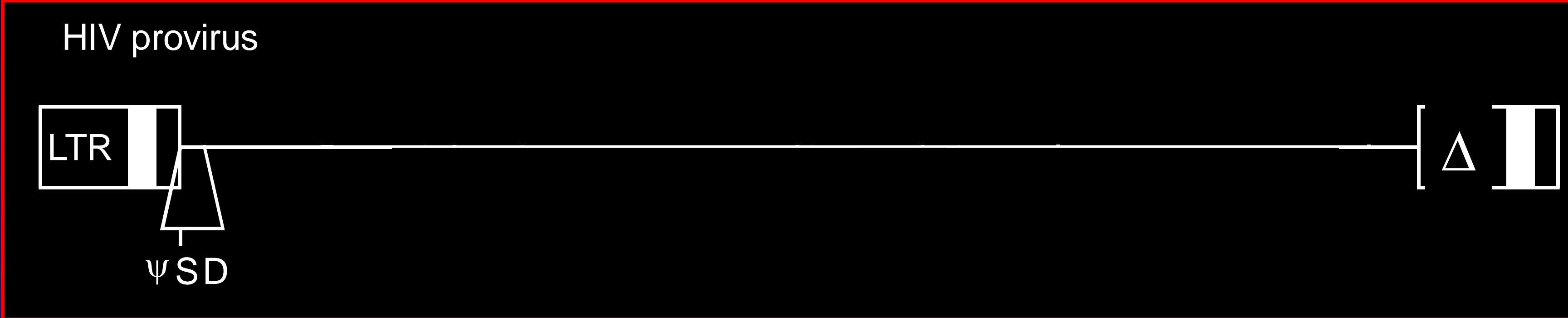


Integration into the Host Genome



No LTR promoter interference

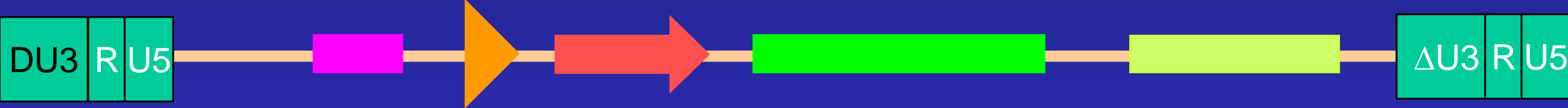
Self Inactivation



Lentiviral Vector Systems

2nd Generation vector

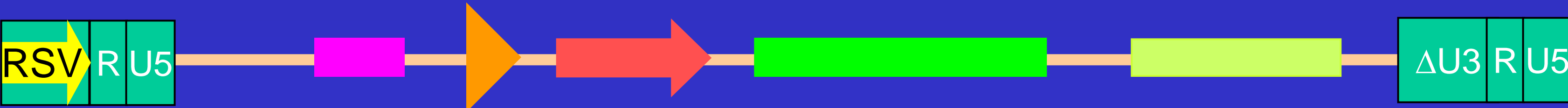
SIN



Requires Tat for production

3rd Generation vector

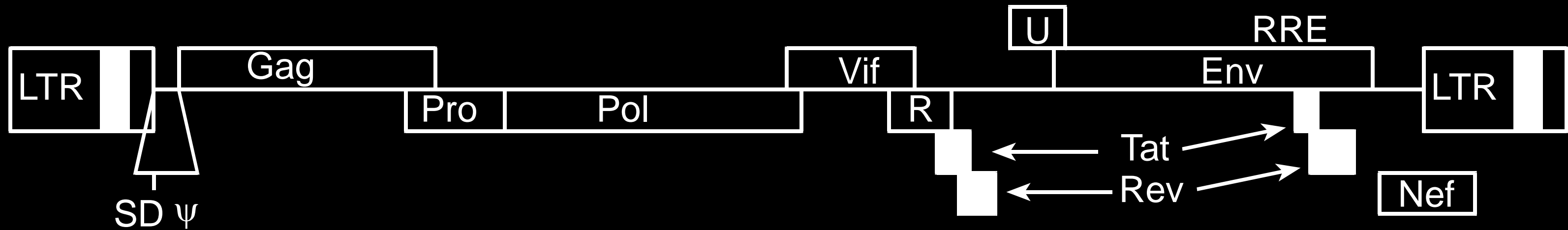
SIN



Constitutive promoter
RSV or CMV

Tat is not required

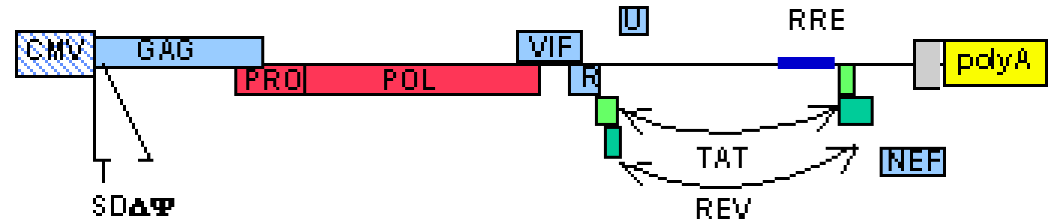
Lentiviral Vector Generations



1st Generation:

HIV-1 core proteins
Enzymes and
Accessory factors
From separate plasmid
And env plasmid

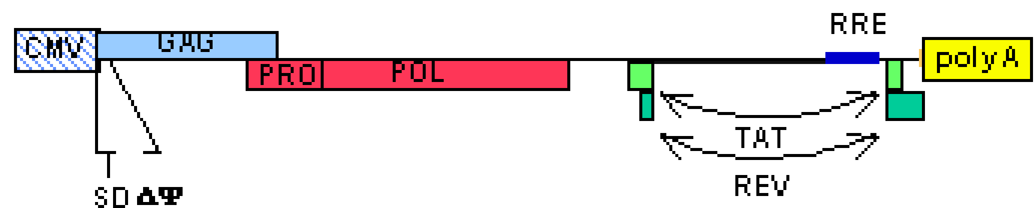
pLV
+
pMD.G



2nd Generation:

Packaging reduced
gag, pol, tat, rev
And env plasmid

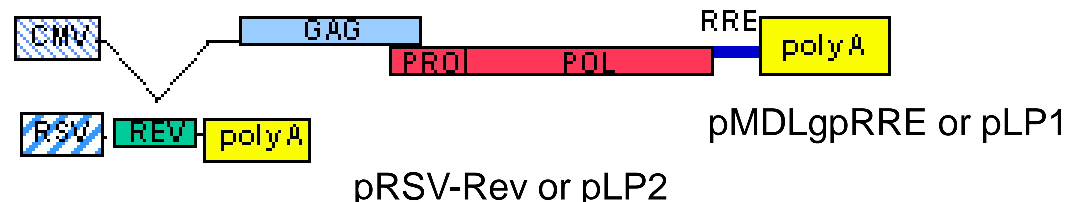
pLV
+
pMD.G



3rd Generation:

Requirement for tat
Eliminated, rev
Moved to separate
plasmid

pLV
+
pMD.G



Packaging plasmids

4th generation?

Note: The components that make up the Lenti-X family of products are designed to work together as a system. Substituting components from other manufacturers or that users have developed in-house, may affect performance and or safety. We recommend that you utilize our complete system, however, if you do decide to use components other than those developed by Clontech, please carefully consider performance and safety implications.

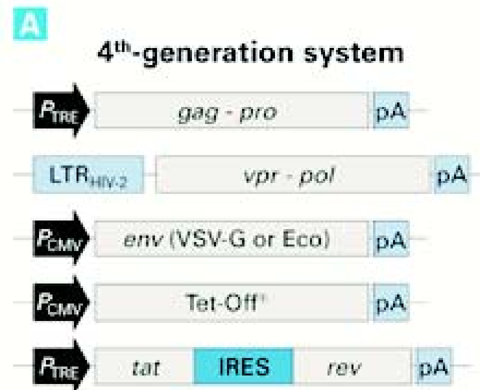
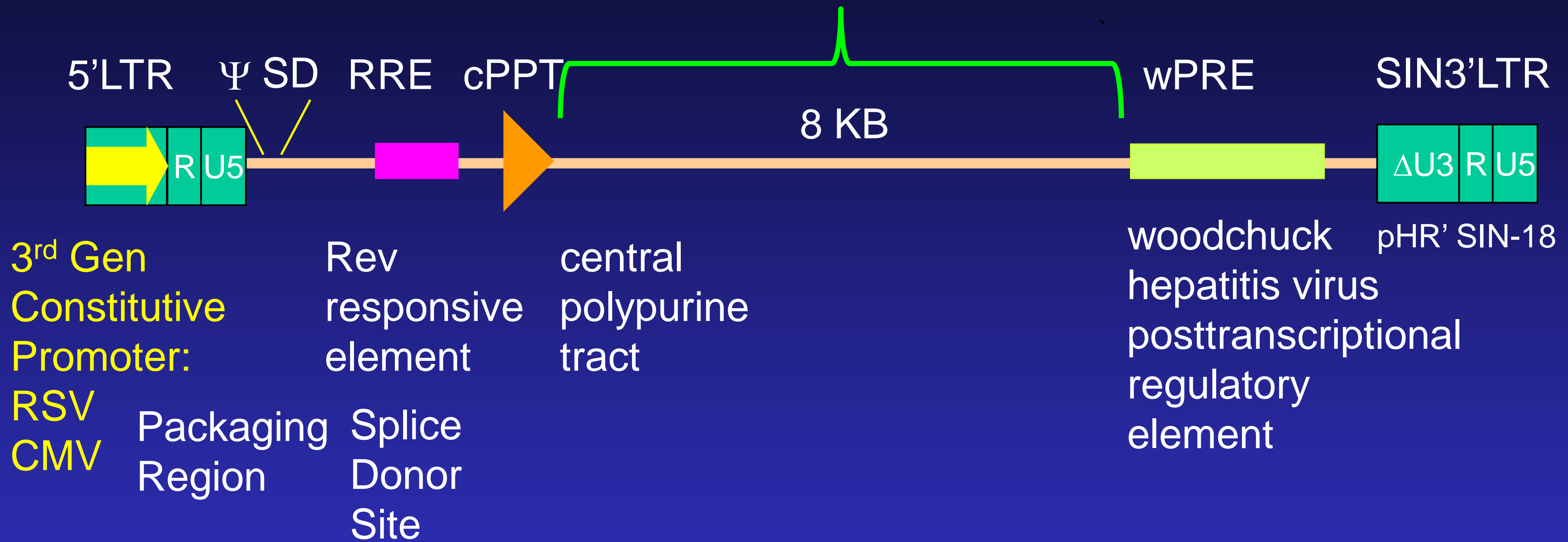


Figure 1: Clontech's Lenti-X HT Packaging System consists of 5 separate components (Panel A), mixed in proprietary proportions for optimized packaging activity. The separation of the *gag*, *pol*, and *env* genes effectively reduces the incidence of RCL (Wu *et al.* 2000). Other 3rd generation systems (**Panel B**) which do not contain separate *gag* and *pol* sequences have higher RCL-generating potential. High levels of expression of essential viral components are driven by the Tet-Off® and Tat transactivators, resulting in high titers of virus. The *pol* gene is fused to *vpr* to ensure transport of the reverse transcriptase/integrase protein into the recombinant viral particle. Not all vector elements are shown.

Clontech

Lentiviral Vector Systems

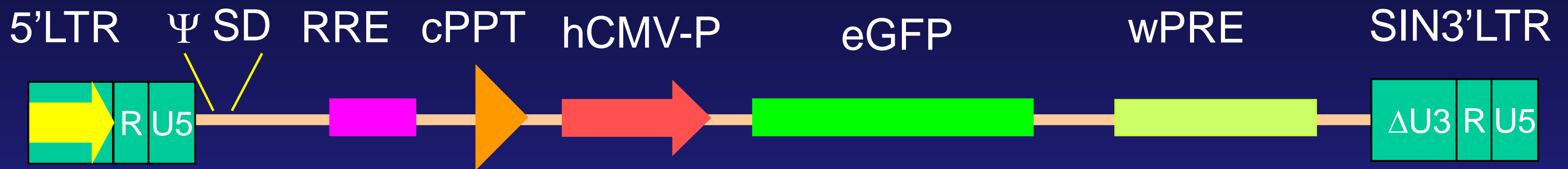


Rev is essential for viral replication
Binds mRNAs removing them from splicesome = full-length and partially spliced

Both cPPT and wPRE increase
Transduction efficiency and transgene expression

Lentiviral Vector Systems

Reporter Vector



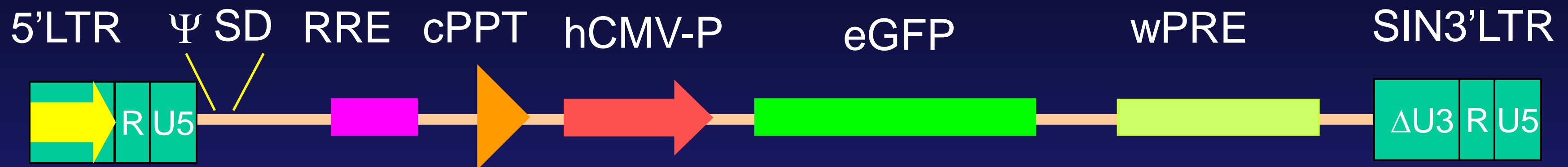
RNA Polymerase II

Reporter

Constitutive:
CMV
SV40
hEFp
PGK

Tissue Specific

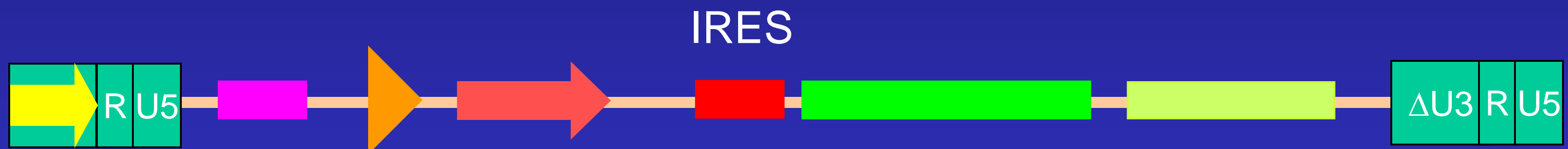
Lentiviral Vector System (3rd generation)



New components:

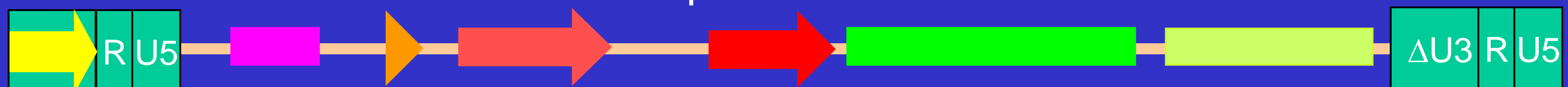
Internal Ribosome Entry Site:

IRES



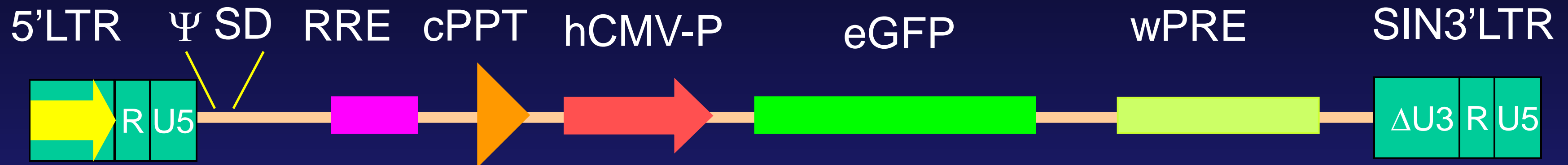
Allows the production of two proteins from one mRNA. A Bicistronic RNA.

2nd promoter

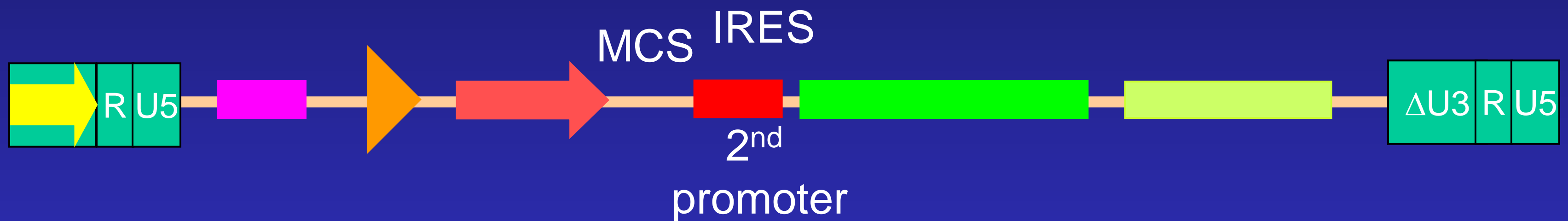


Allows the production of two mRNAs from one vector.

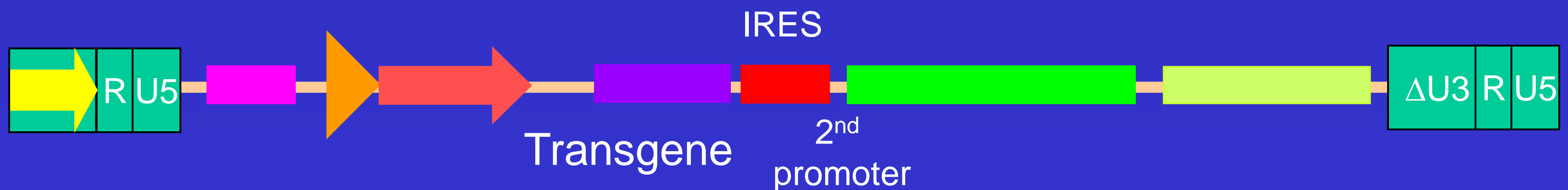
Lentiviral Vector System (3rd generation)



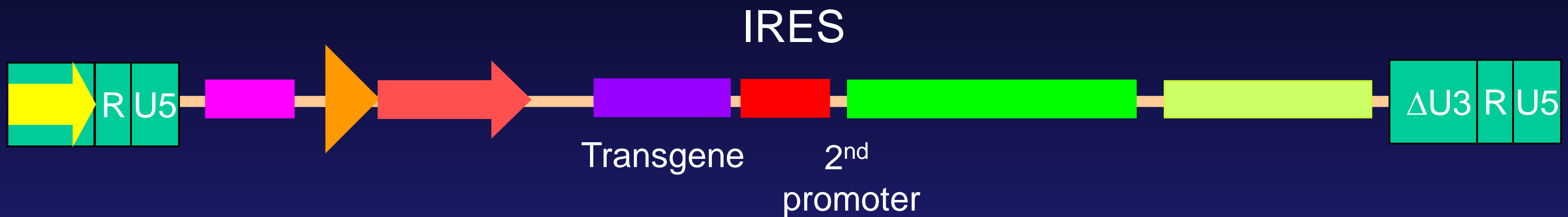
New components:



Multiple Cloning Site for transgenes to be expressed:

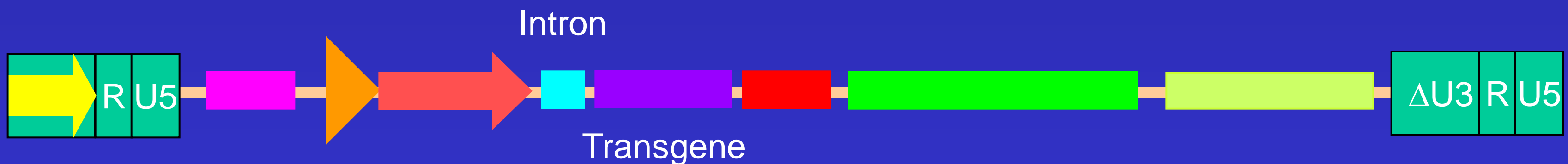


Lentiviral Vector System (3rd generation)



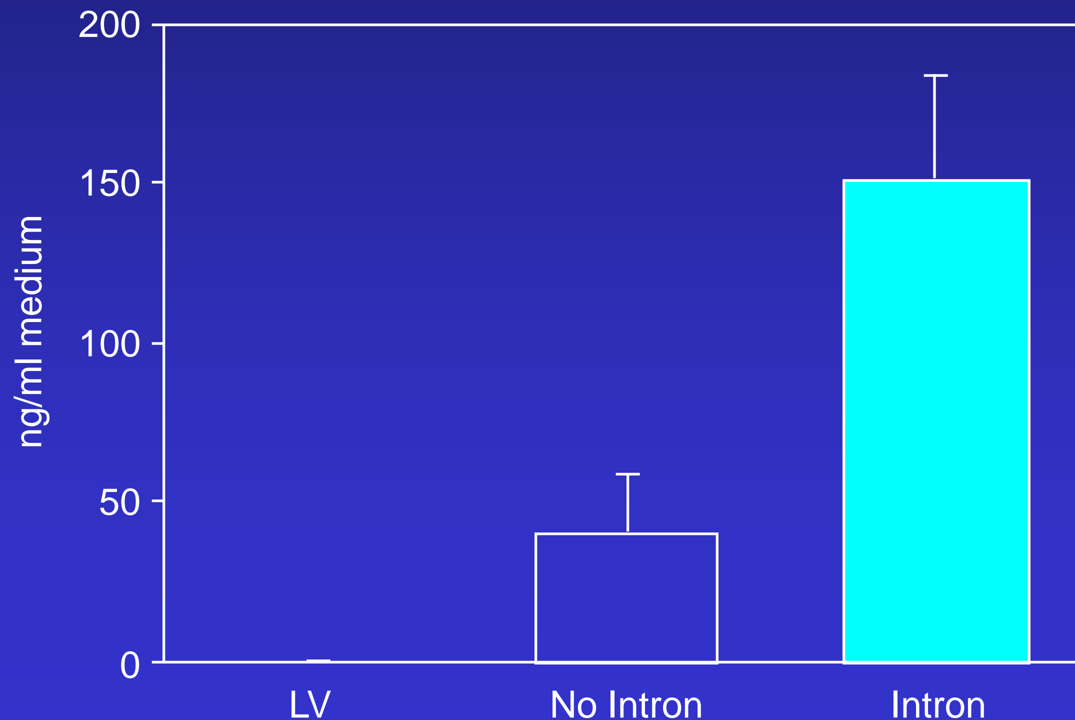
A heterologous intron had been found to increase expression of transgenes in transgenic mice.

Insertion of a heterologous Intron



Rat insulin II intron A

Lentiviral Vector System (3rd generation)



Small peptide Ligand is Produced and Expression is Enhanced From vector Containing Heterologous Intron

Lentiviral Vector System (3rd generation)

CMVp Intron Jag IRES eGFP

1 R U5 ΔU3 R U5

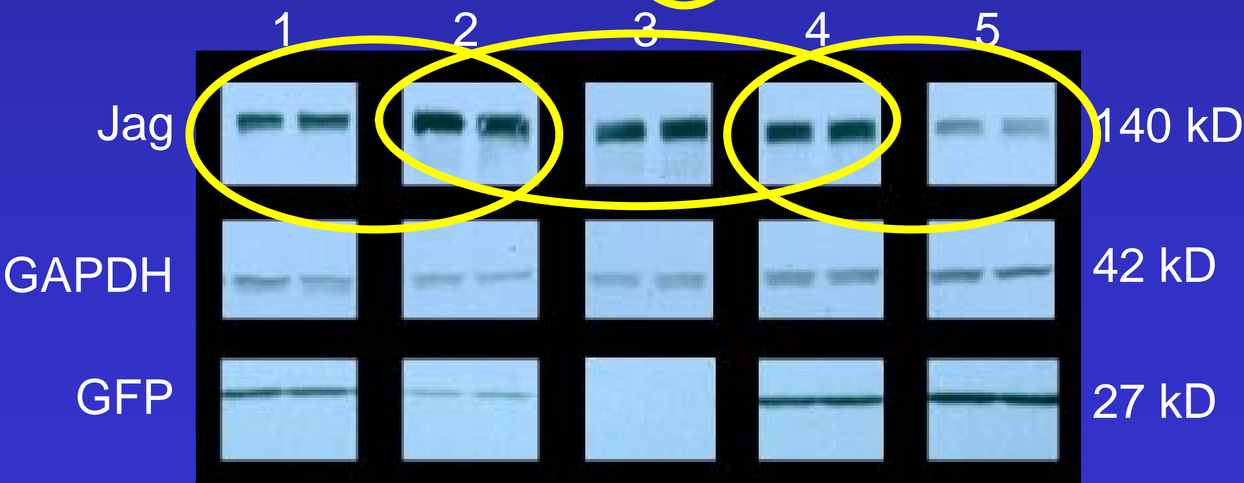
2 R U5 ΔU3 R U5

3 R U5 ΔU3 R U5

4 R U5 ΔU3 R U5

5 R U5 ΔU3 R U5

hEFp



Lentiviral Vector System (3rd generation)

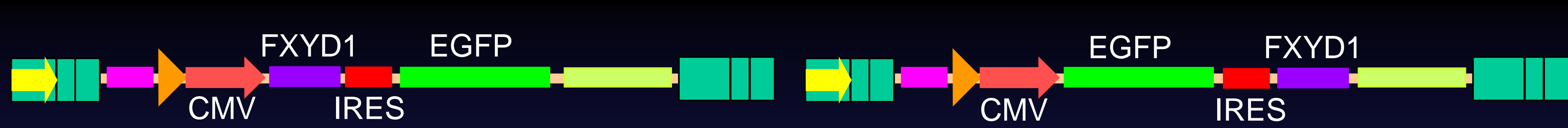
CMVp Intron Jag IRES eGFP

1 R U5 ΔU3 R U5
2 R U5 ΔU3 R U5
3 R U5 ΔU3 R U5
4 R U5 ΔU3 R U5
5 R U5 ΔU3 R U5

hEFp

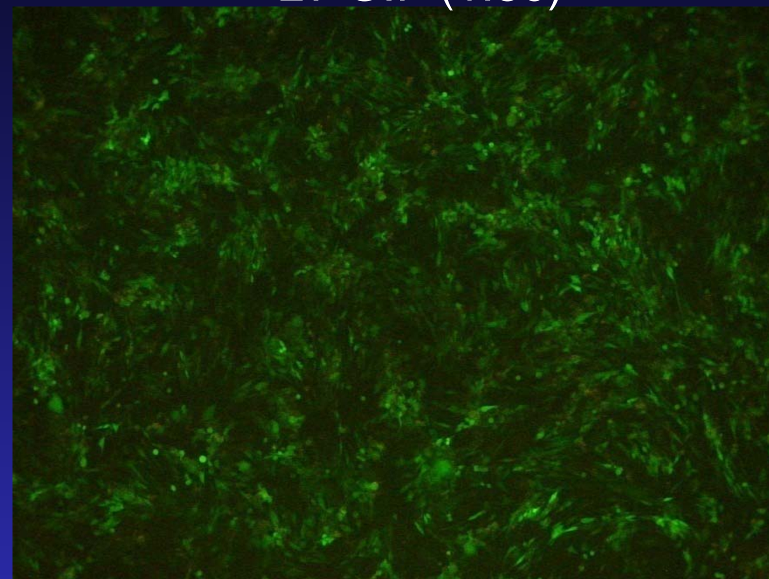
Titer Values (TU/mL)

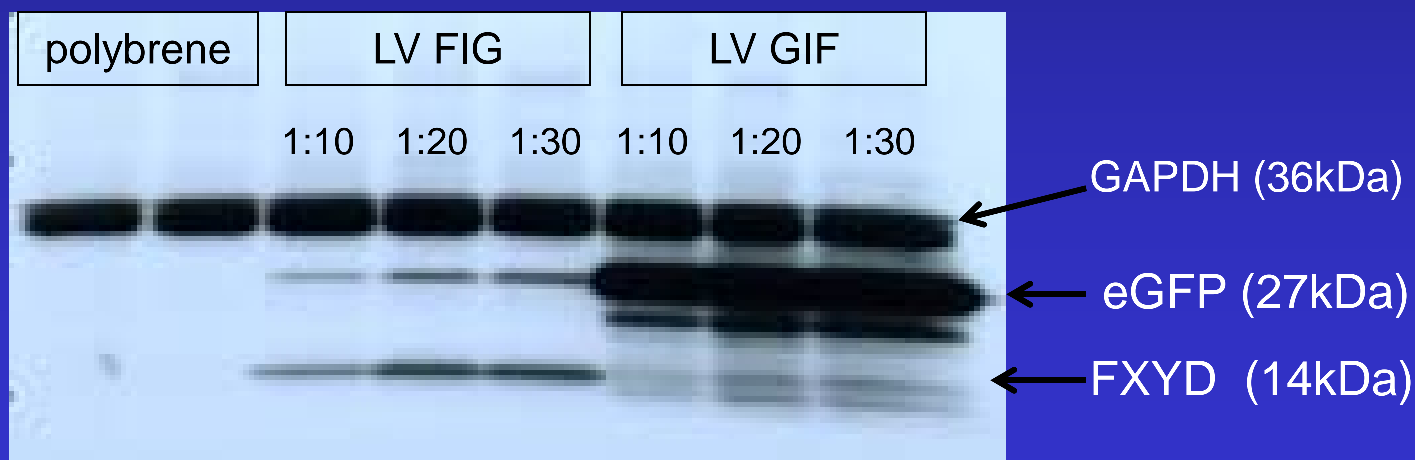
	Real-time	GFP	Mean Fluorescence
LV1	1.50E+07	7.10E+06	35.21 RFU
LV2	1.36E+07	3.34E+06	24.31 RFU
LV3	1.55E+07	bkg	bkg
LV4	1.10E+07	1.20E+07	54.39 RFU
LV5	2.26E+07	1.42E+07	66.26 RFU



LV FIG (1:30)

LV GIF (1:30)





Infection of Hib5 (6 Well plate, 200,000 cells/well)

Lentiviral Vector System: Gene Suppression

pPRIME
Potent RNA Interference using MicroRNA Expression

A

3'
5'

miR30-shRNA secondary structure

C CGU C GACGUUUGUAGG--CUGACUUUCG U A G G A GCAC C G A
GCG CUGUAAACAUCC GACUGGAAGCUGUG A
C A UC

miR-30

antisense of shRNA

C CGU N NNNNNNNNNNNNNNNNNNNNAU U A G G A CAC C G A
GCG NNNNNNNNNNNNNNNNNNNNUAGUG A
C Z

pSM2

miR30=
Retinoblastoma (Rb)
Targeting sequence:
AGCAGTTCGATATCTACTGAAA

pPRIME-CMV-GFP
miR30-shRNA

CMV R U5 RRE CMV GFP 5'miR30 3'miR30 CM R WRE ΔU3 R U5

cPPT

Xho I

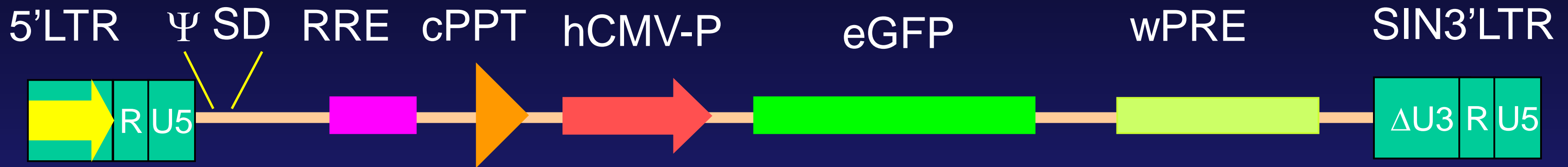
EcoRI

Pol II driven shRNA was more active than the Pol III construct

pPRIME-
TET-GFP
CMV-dsRED
CMV-Neo
T-REX-GFP

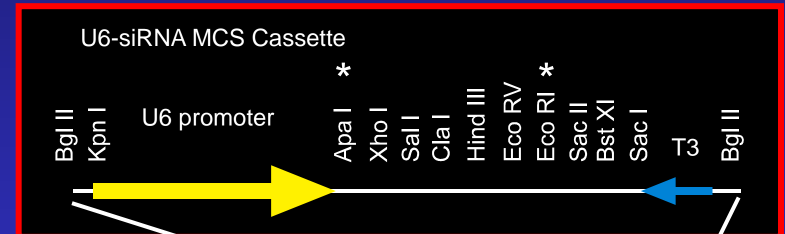
Stegmeier, 2005

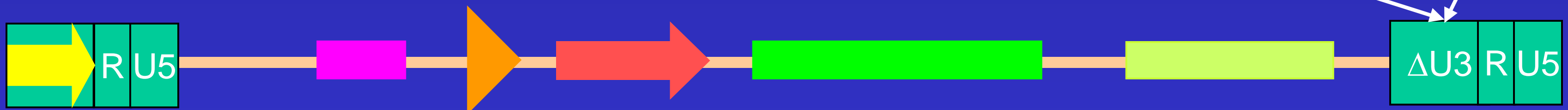
Lentiviral Vector System: Gene Suppression



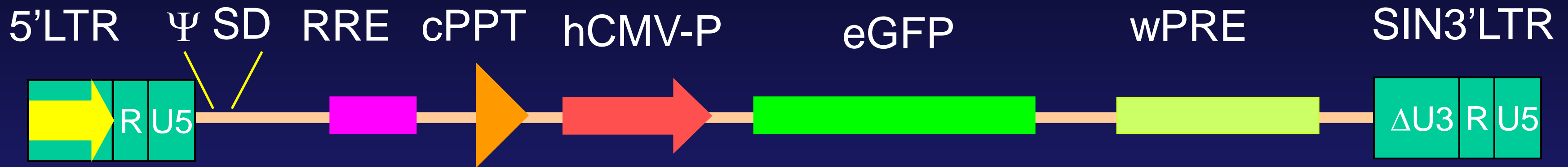
New components:

486 bp





Lentiviral Vector System: Gene Suppression



New components:

486 bp

U6-siRNA MCS Cassette

Bgl II, Kpn I, U6 promoter, Apa I *, Xho I, Sal I, Cla I, Hind III, Eco RV, Eco RI *, Sac II, Bst XI, Sac I, T3, Bgl II

U6-siRNA

ΔU3 R U5 — ΔU3 R U5

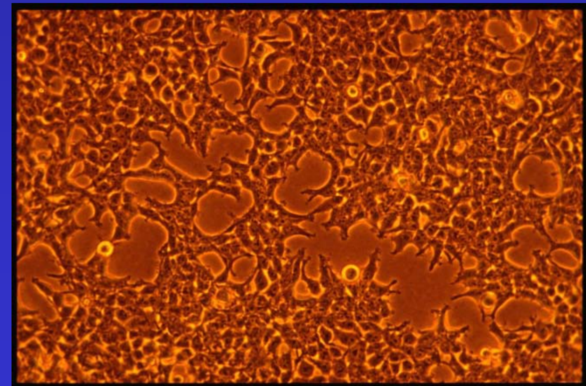
Virus Production

Day 1

1. Cells: human Embryonic Kidney 293T/17
   Cells have been transformed with temperature sensitive large T antigen
   Strain was selected specifically for its high transfectability

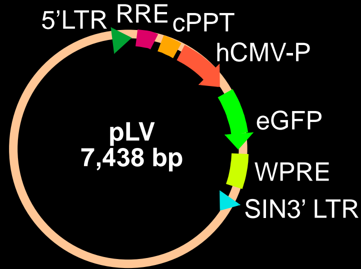
2. Cells are grown in antibiotic free conditions DMEM (1.5 g/l Na Bicarbonate), 4.5 g/l Glucose, Defined fetal bovine serum, 10% CO2
   Advantage to antibiotic free medium = immediately know when there is a problem/contamination

3. Cells are plated to achieve 70 confluency in 10 cm dishes that have been coated with poly-L-lysine (6 to 11 x 10^6 cells/dish)

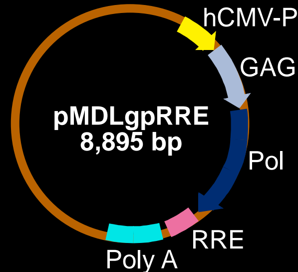


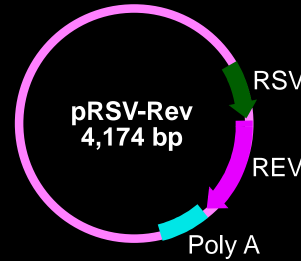
Lentiviral Vector System

Transgene

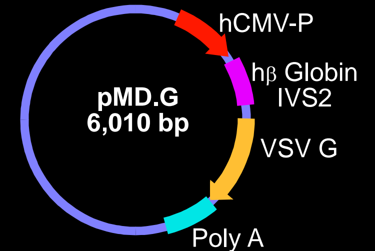


Packaging





Envelope



pMDLpg.RRE

pRSV-Rev

pMD.G

pLV

Transfection

$CaPO_4$

$CaPO_4$

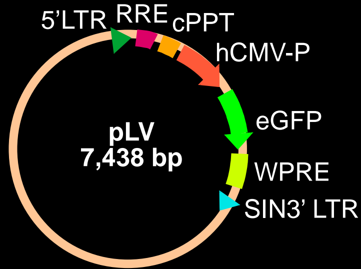
293T Cells

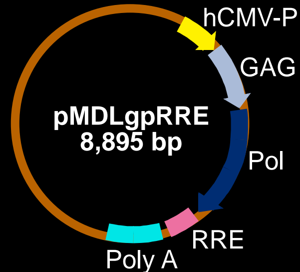
Lentiviral Vector System

Transgene

Packaging

Envelope





pMDLpg.RRE

pRSV-Rev

pLV

pMD.G

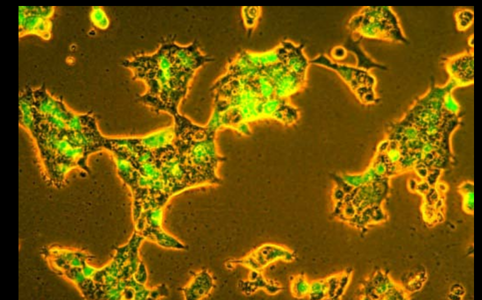
Transfection

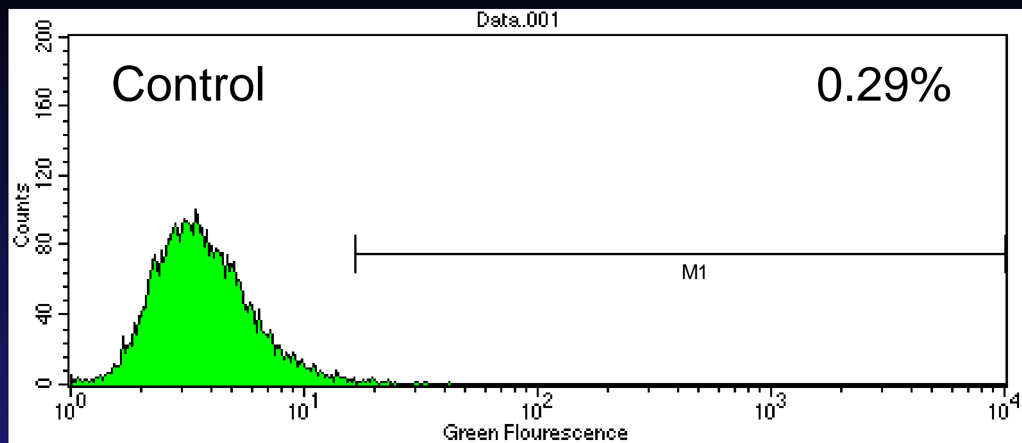
$CaPO_4$

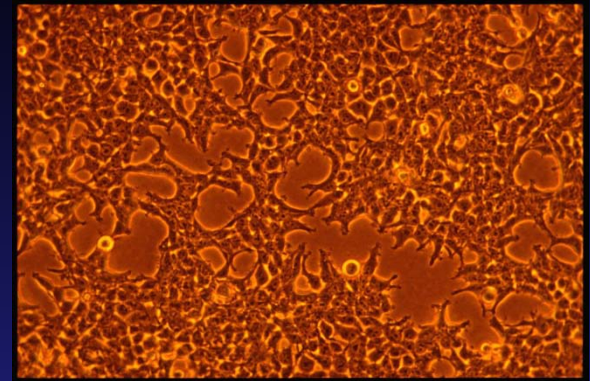
$CaPO_4$

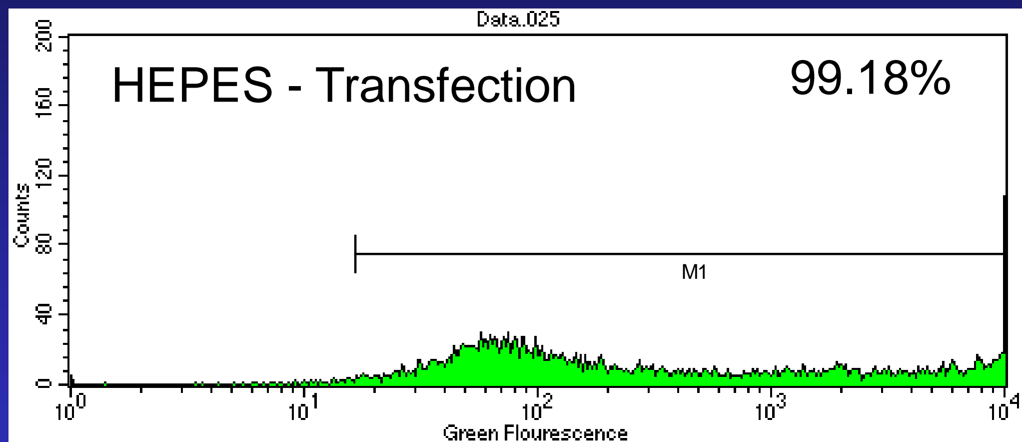
293T Cells

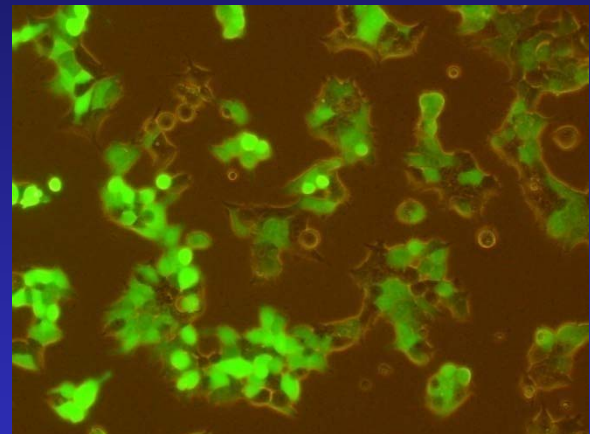
Transfected 293T cells

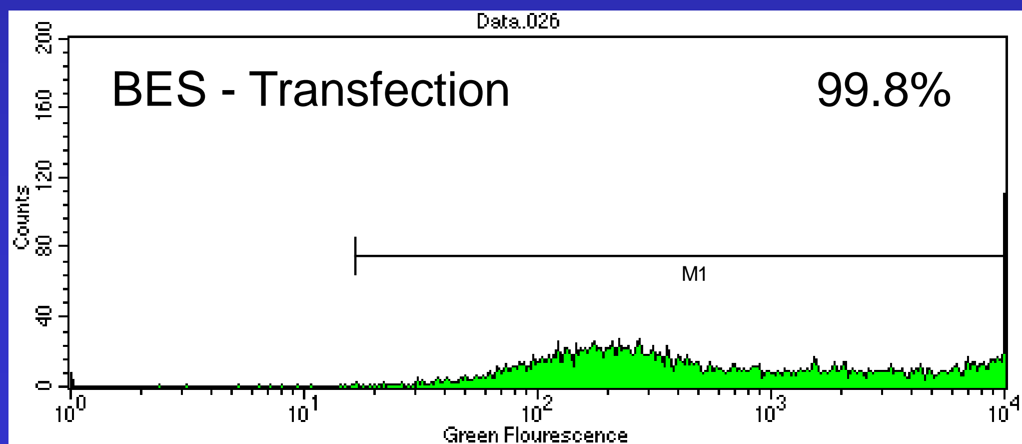


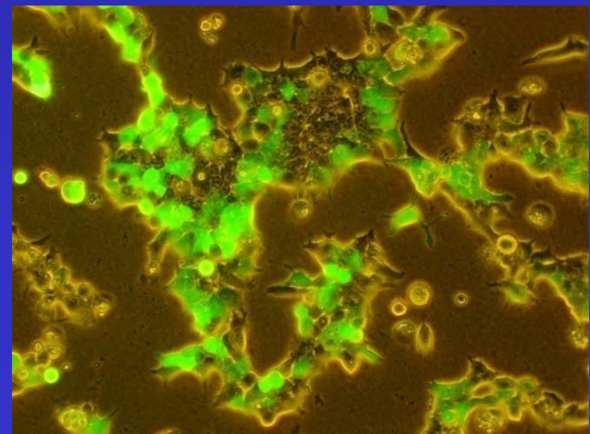

Data.001
Control
0.29%
Counts
Green Flourescence
M1




Data.025
HEPES - Transfection
99.18%
Counts
Green Flourescence
M1




Data.026
BES - Transfection
99.8%
Counts
Green Flourescence
M1



FACs Titer:

pMDLpg.RRE

pRSV-Rev

pMD.G

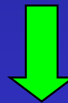
pLV

Transfection

Transfected 293T cells

293T Cells

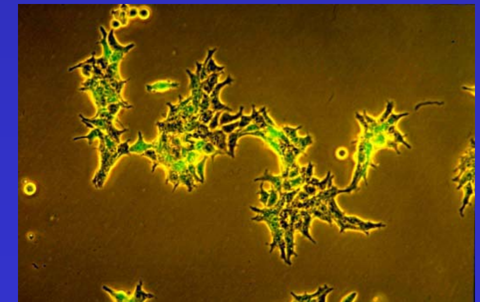
Conditioned Medium



FACs detects Infected cells, Expressed as Percentage of total

Infection

Infected 293T cells



Virus Production

Titer Analysis Possibilities:

FACS for gene expression product:

- Dependent on Promoter activity
- Constitutive promoter = useful Titers that predict infection rate
- Tissue specific promoters might not give useful titers

Real-Time PCR for integrated viral DNA in host genome

- Dependent on infection and integration into the host genome
- Real-Time PCR Titers predict infection rate

Reverse transcription Real-Time PCR for viral RNA

- Dependent only on the presence of the viral RNA
- Does not predict infection rate of the viral particles

Acknowledgements

Molecular and Cellular Biology Core
Oregon National Primate Research Center

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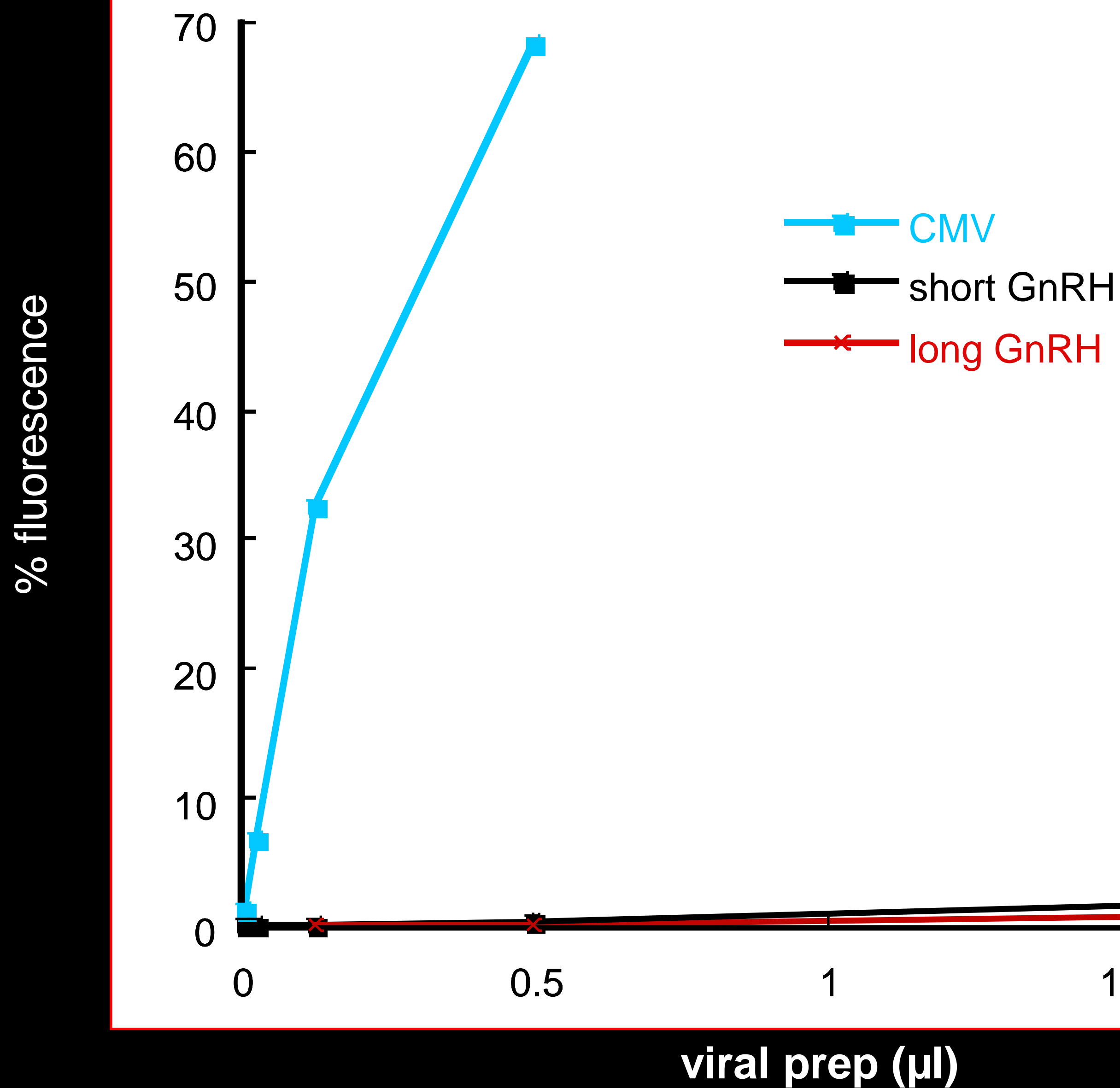
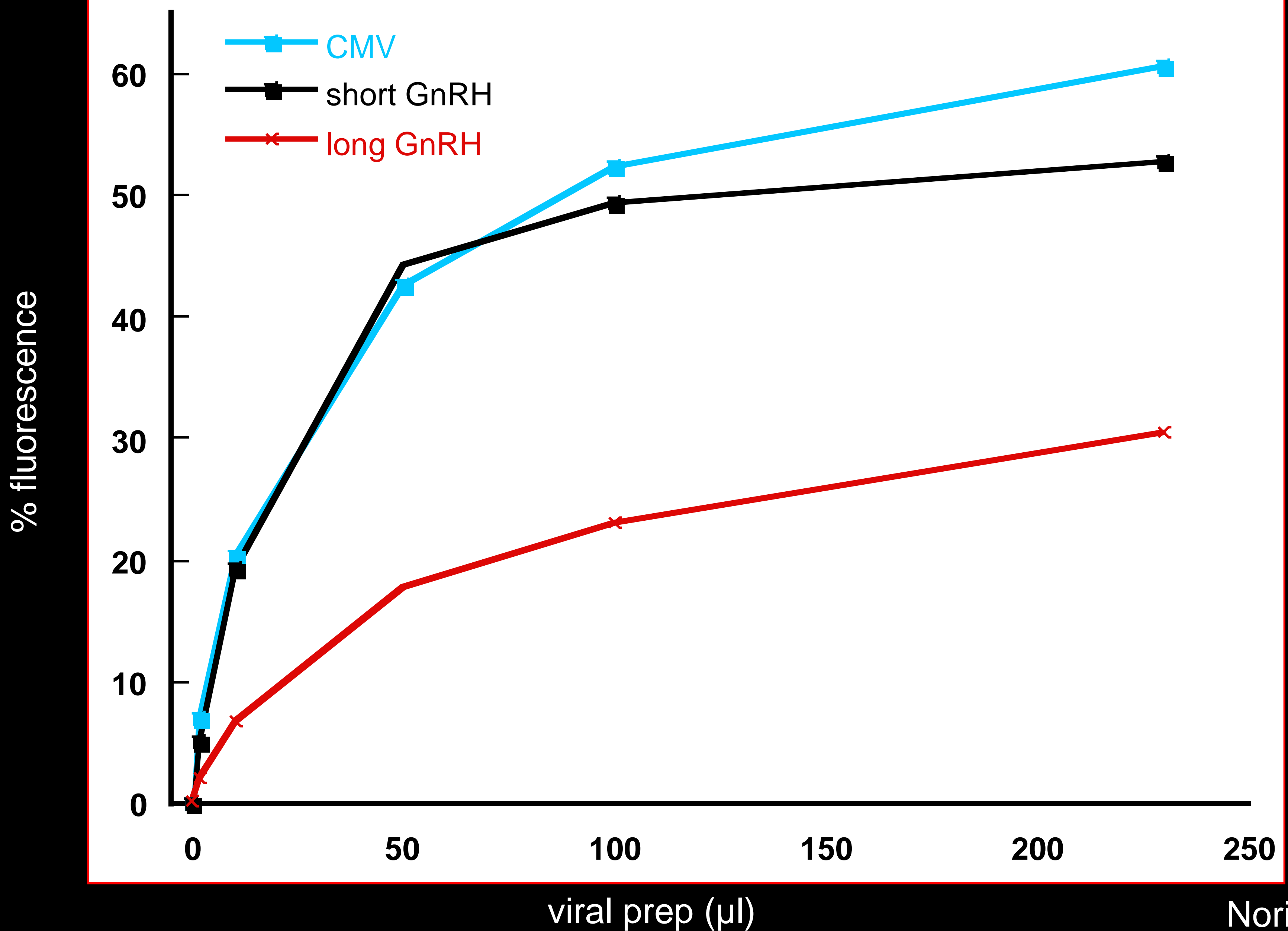
Fluorescence in 293-T embryonic kidney cells



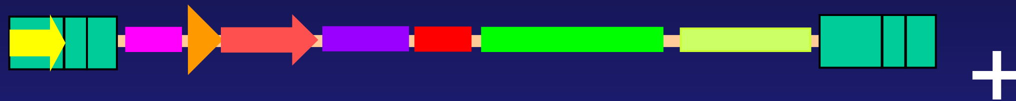
Noriega

Fluorescence in GT1-7 neuronal cells



Replication Competent Lentivirus (RCL)

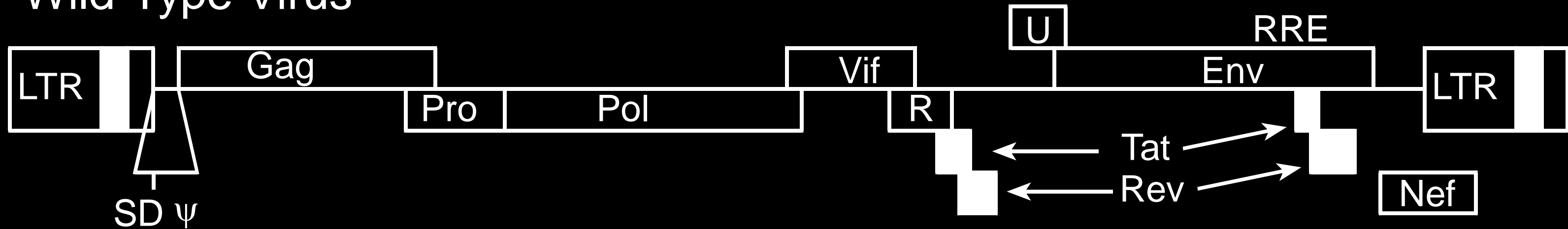
3rd Generation Lentiviral Vector



\+

Replication competent LTR
Gag,pol, rev, env, tat

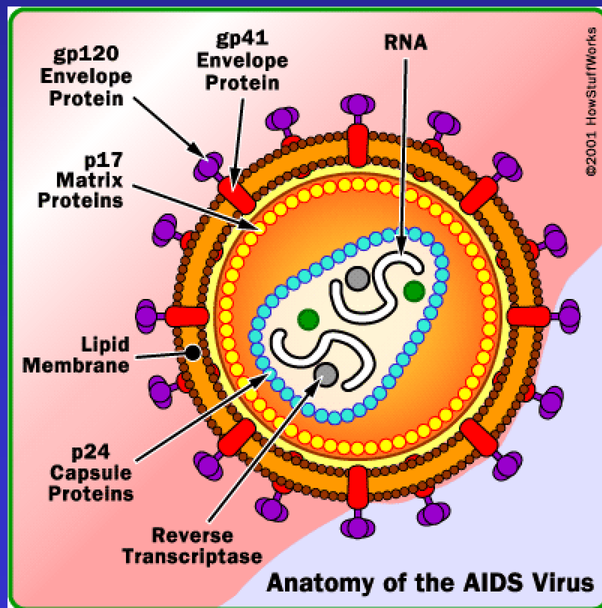
Source:
Carry over from packaging or
Envelope plasmids
Or
Endogenous viruses



Replication Competent Lentivirus (RCL)

Protocol:

1. Infect 1 million SupT-1 cells with 5 million viral TUs
2. Pass the cells 3 times over 2-3 weeks
3. Test the medium for p24 protein with ELISA kit (commercial)



Test Preps + Std