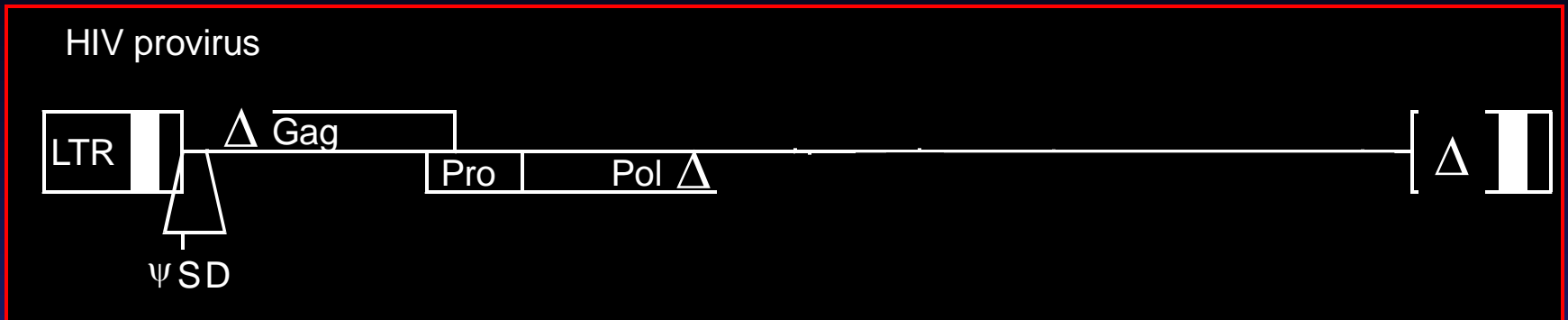


Lentiviral Vectors: design, production, and titration

ONPRC Lentiviral Vector Core
Molecular and Cellular Biology Core
Greg Dissen

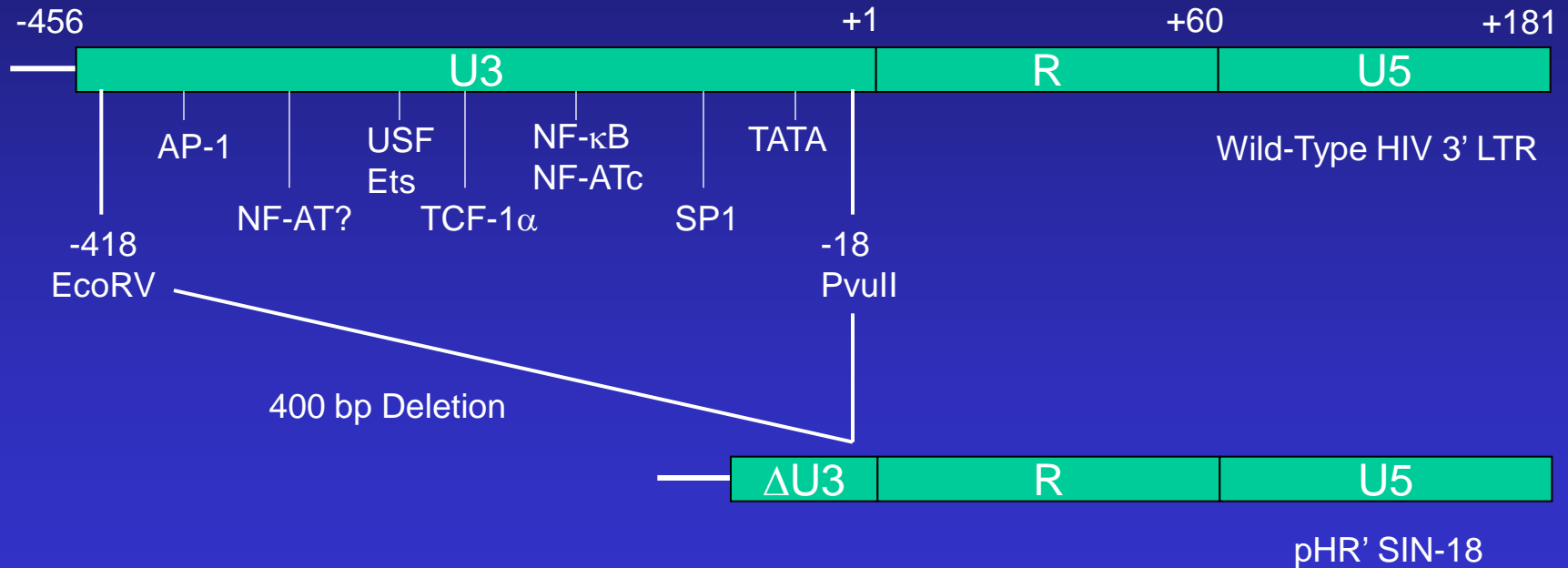


2nd and 3rd generation viral vectors

1. Viral backbone was stripped to allow room for transgenes
2. Development of the Self-Inactivating (SIN) vector

Lentiviral Vector System

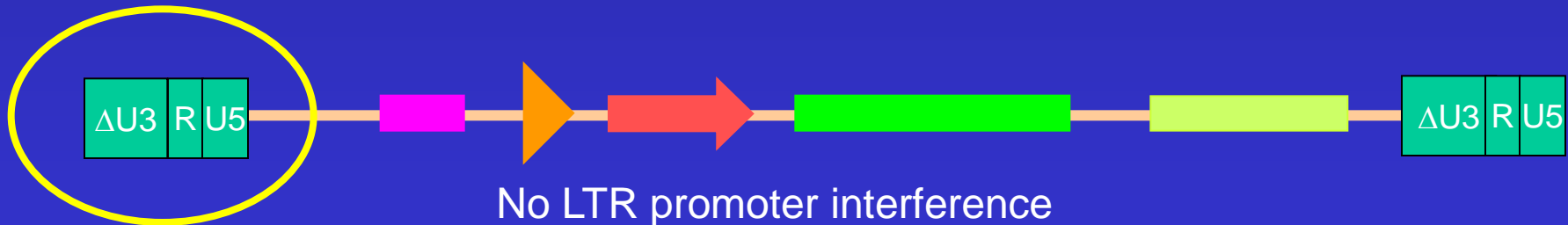
2. Modification of 3'LTR "Self Inactivating"



Lentiviral Vector System (3rd generation)

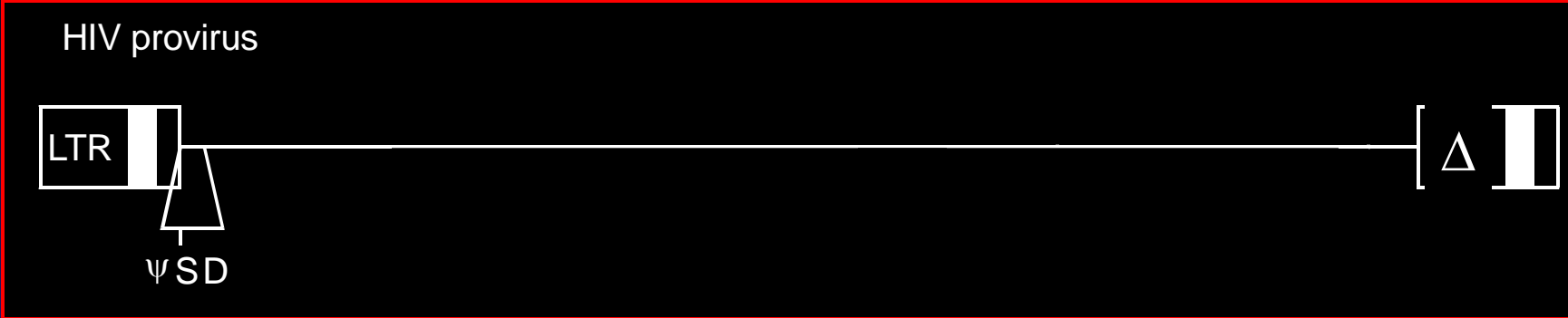


Integration into the Host Genome



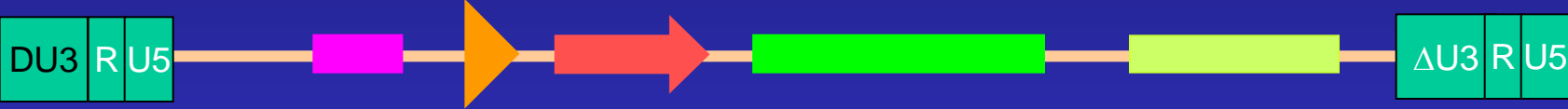
No LTR promoter interference

Self Inactivation



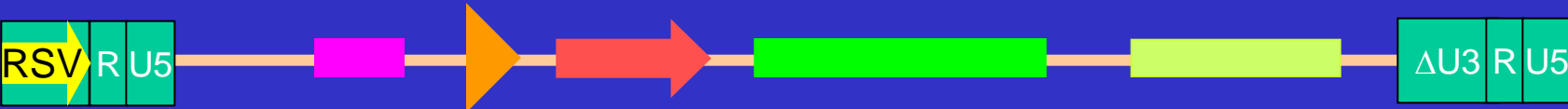
Lentiviral Vector Systems

2nd Generation vector



Requires Tat for production

3rd Generation vector

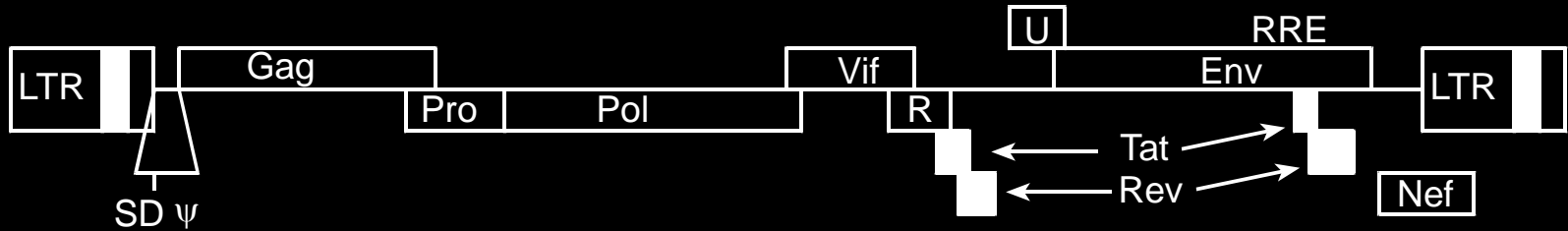


Constitutive promoter
RSV or CMV

Tat is not required

Lentiviral Vector Generations

HIV provirus:



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

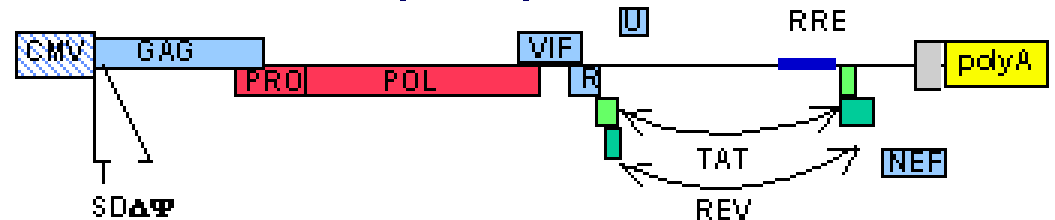
pLV
+
pMD.G

3rd Generation:

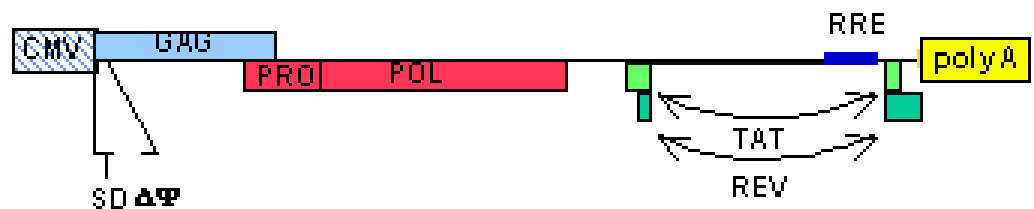
Requirement for tat
Eliminated, rev
Moved to separate
plasmid

pLV
+
pMD.G

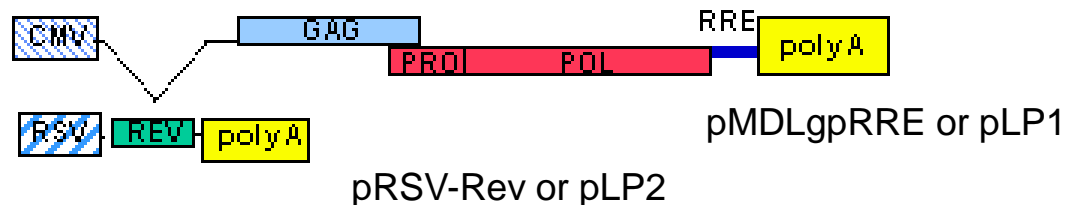
FIRST GENERATION (1996)



SECOND GENERATION (1997)



THIRD GENERATION (1998)



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.

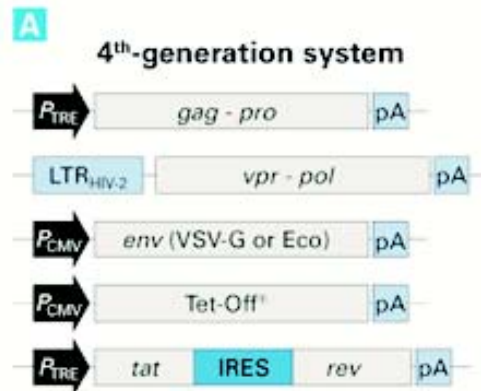
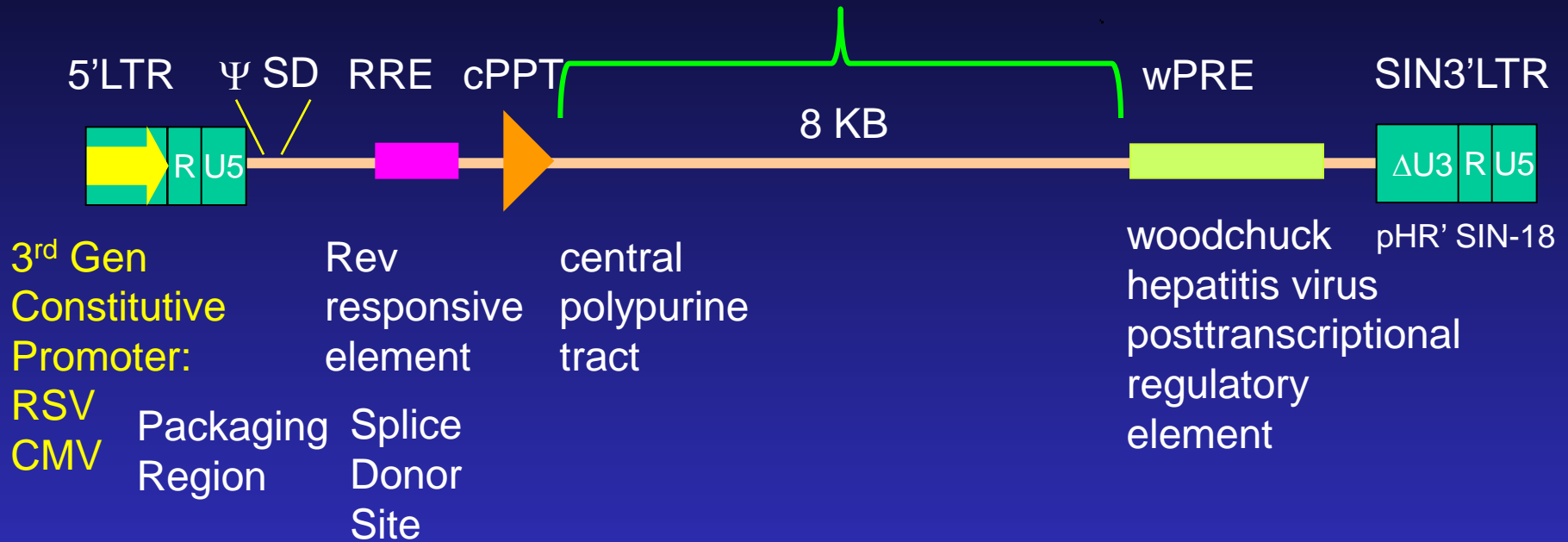


Figure 1: Clontech's Lenti-X HT Packaging System consists of 5 separate components (Panel A), mixed in proprietary proportions for optimized packaging activity. The separation of the *gag*, *pol*, and *env* genes effectively reduces the incidence of RCL (Wu et al. 2000). Other 3rd generation systems (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.

Lentiviral Vector Systems



Rev is essential for viral replication

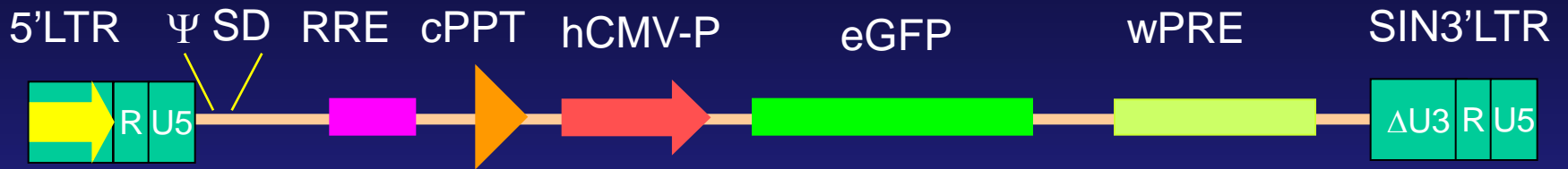
Binds mRNAs removing them from splicesome = full-length and partially spliced

Both cPPT and wPRE increase

Transduction efficiency and transgene expression

Lentiviral Vector Systems

Reporter Vector



RNA Polymerase II

Reporter

Constitutive:

CMV

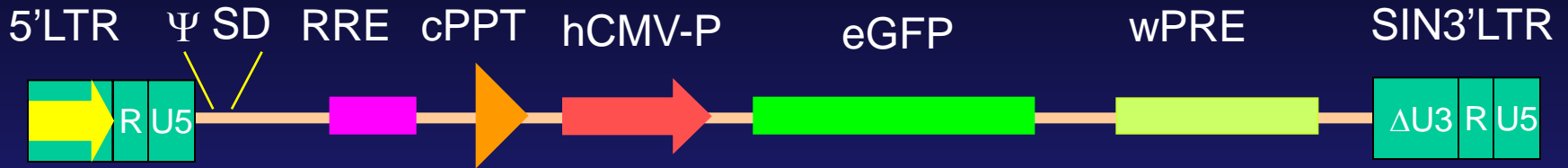
SV40

hEFp

PGK

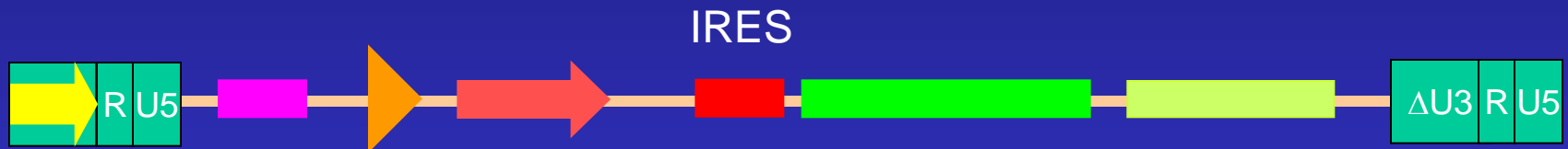
Tissue Specific

Lentiviral Vector System (3rd generation)



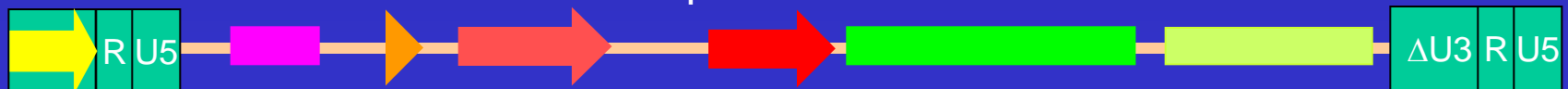
New components:

Internal Ribosome Entry Site:



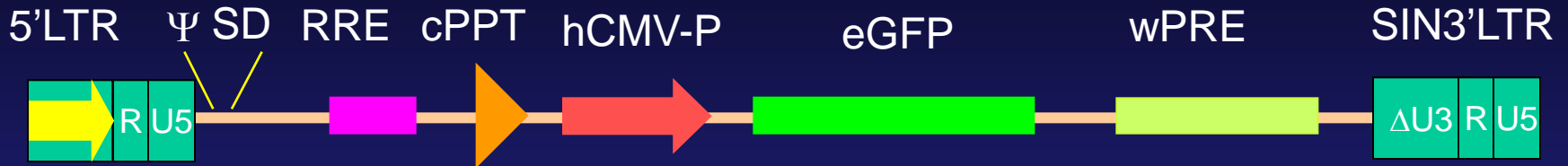
Allows the production of two proteins from one mRNA. A Bicistronic RNA.

2nd
promoter



Allows the production of two mRNAs from one vector.

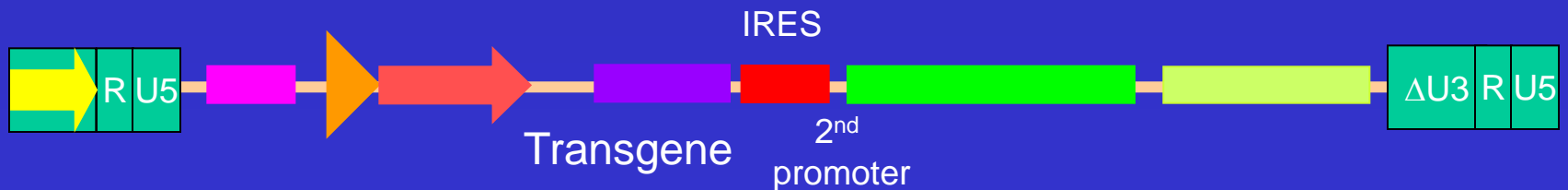
Lentiviral Vector System (3rd generation)



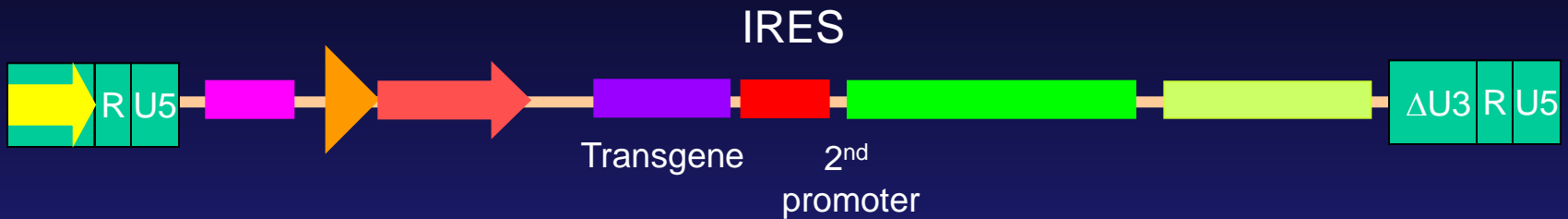
New components:



Multiple Cloning Site for transgenes to be expressed:

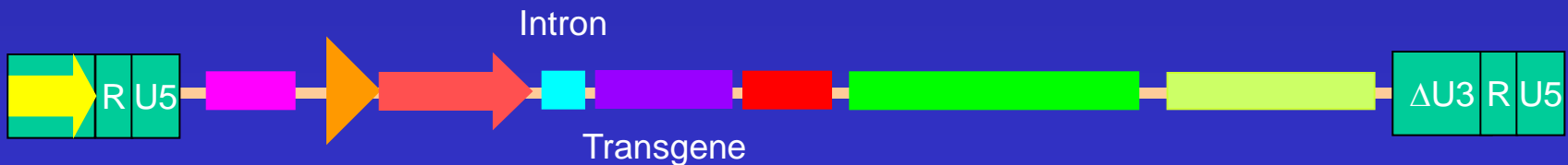


Lentiviral Vector System (3rd generation)



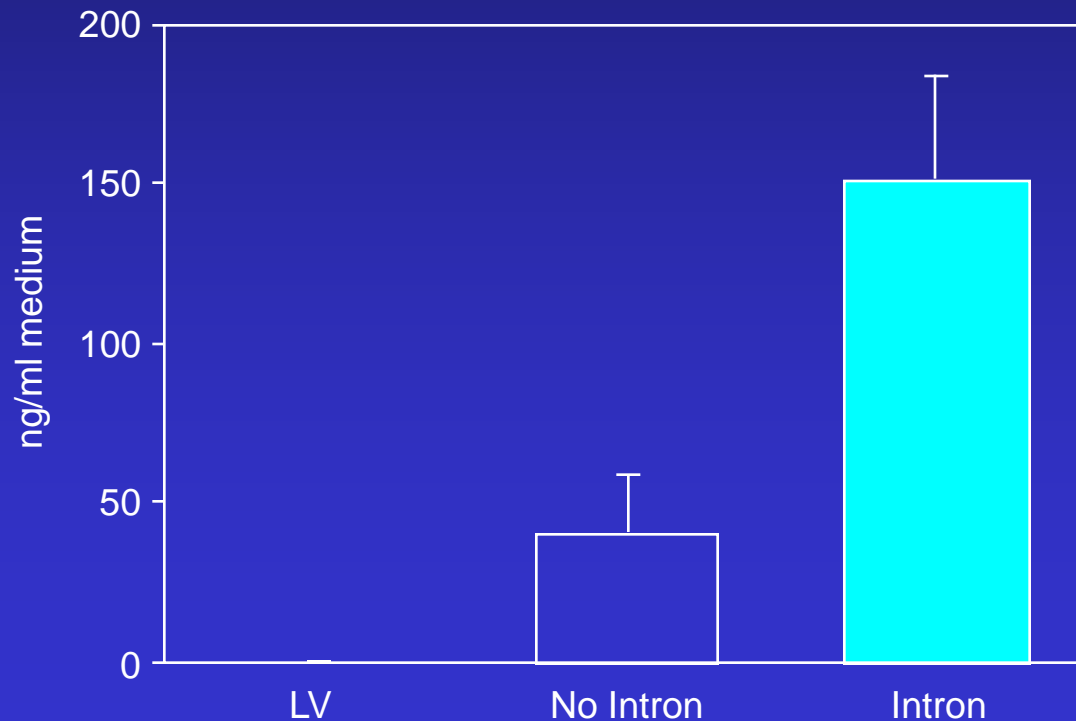
A heterologous intron had been found to increase expression of transgenes in transgenic mice.

Insertion of a heterologous Intron



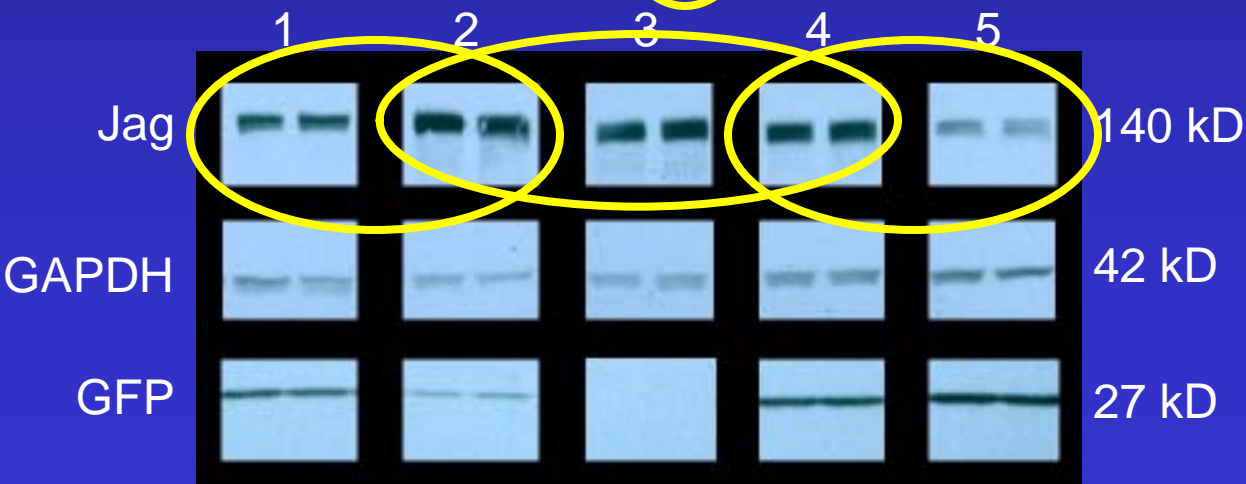
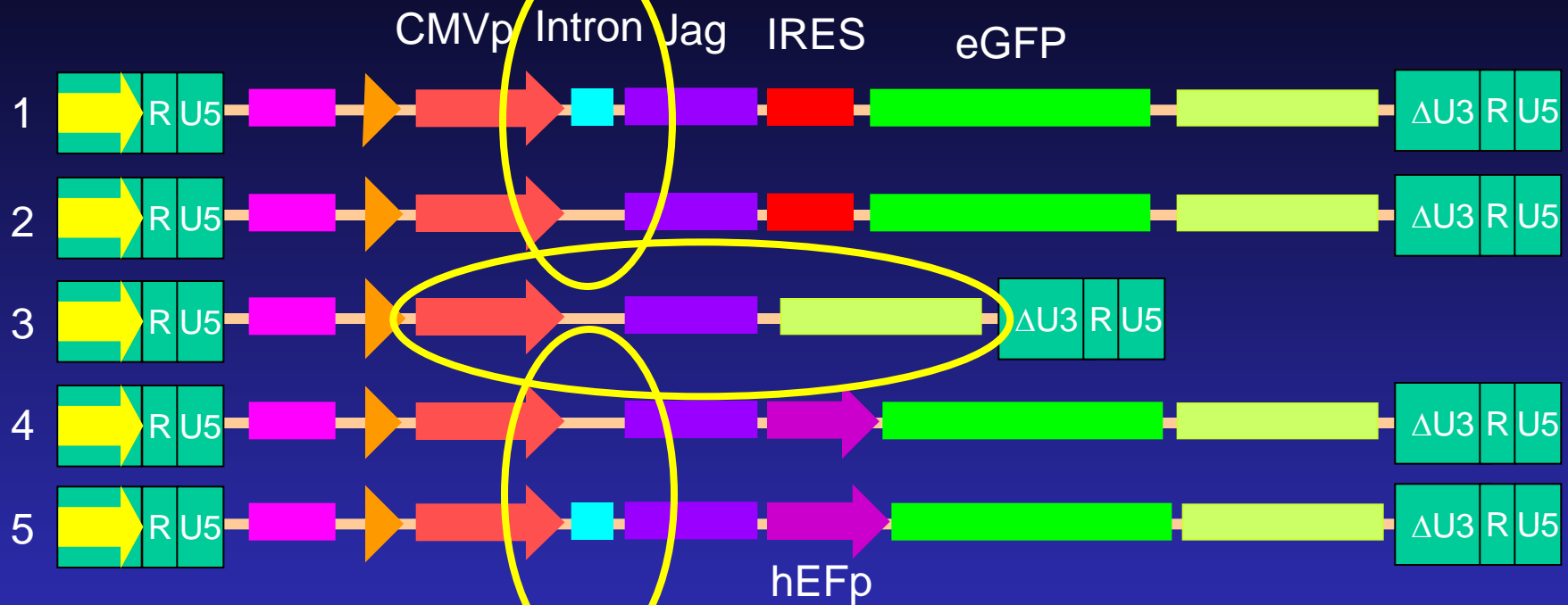
Rat insulin II intron A

Lentiviral Vector System (3rd generation)

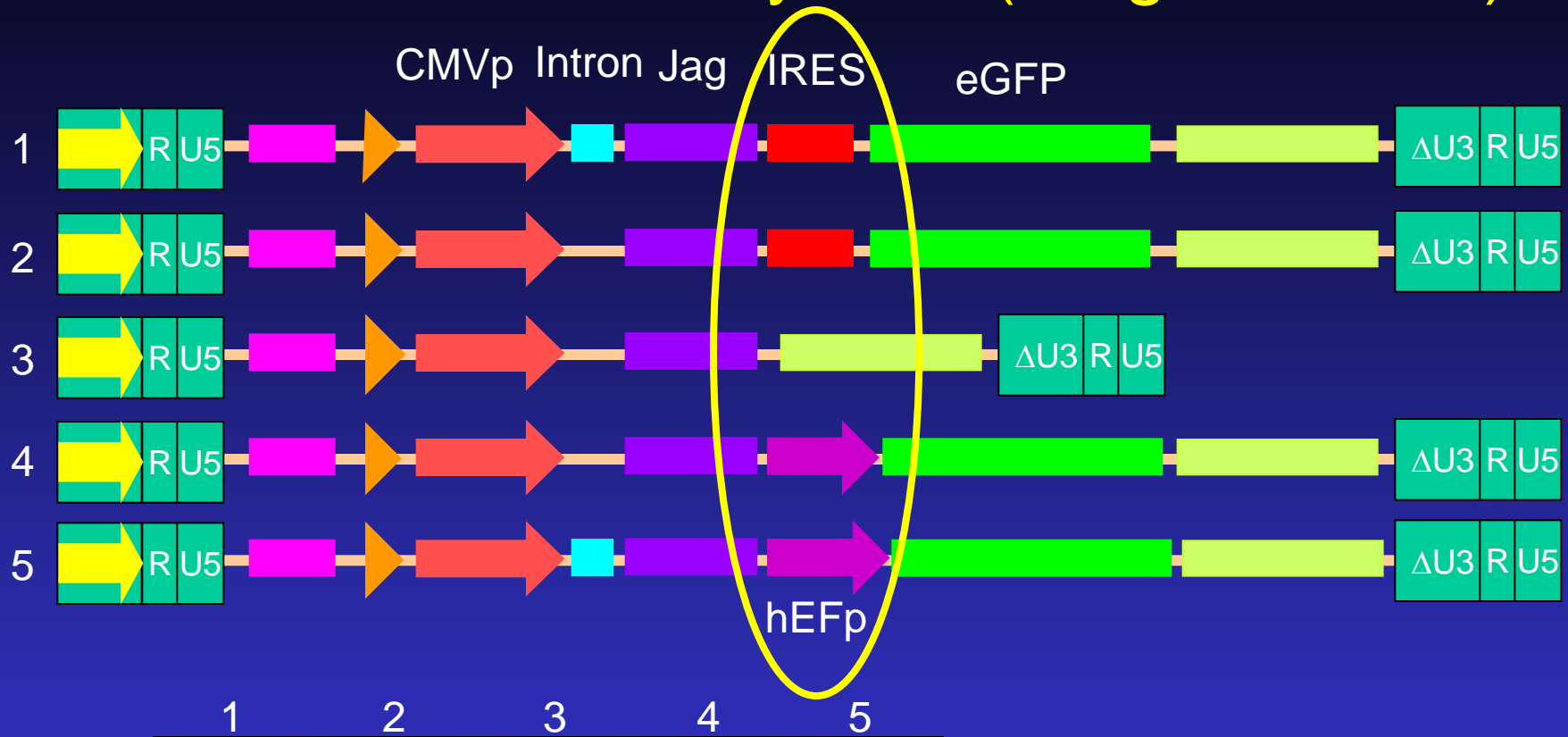


Small peptide Ligand is Produced and Expression is Enhanced From vector Containing Heterologous Intron

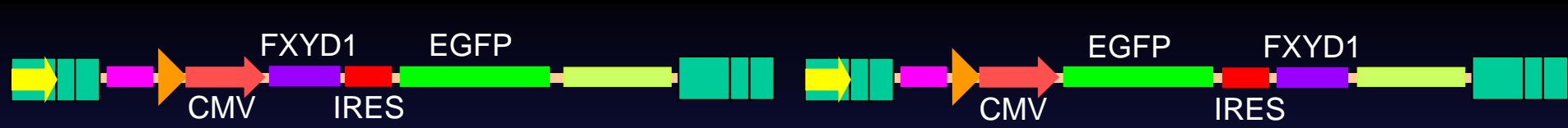
Lentiviral Vector System (3rd generation)



Lentiviral Vector System (3rd generation)

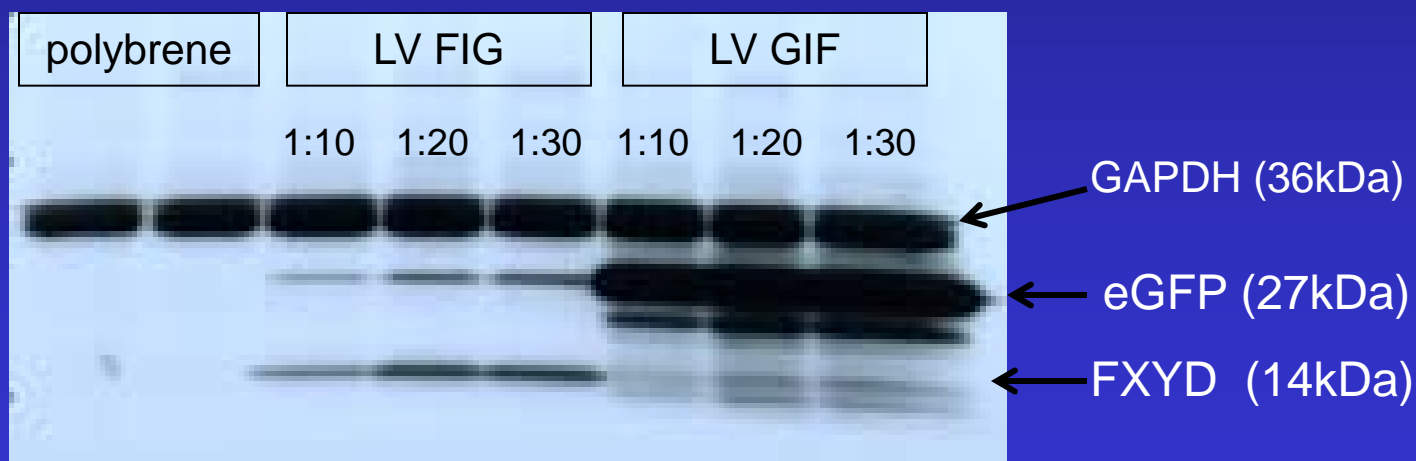
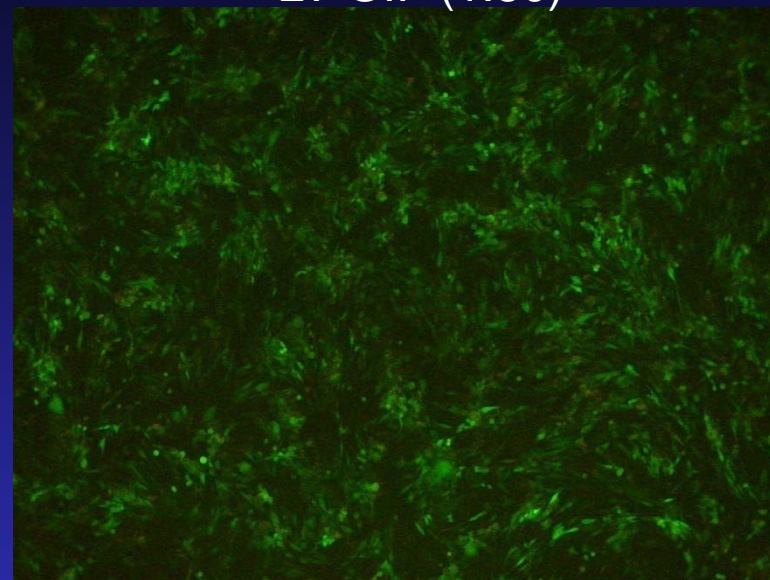


	Titer Values (TU/mL)		
	Real-time	GFP	Mean Fluorescence
LV1	1.50E+07	7.10E+06	35.21 RFU
LV2	1.36E+07	3.34E+06	24.31 RFU
LV3	1.55E+07	bkg	bkg
LV4	1.10E+07	1.20E+07	54.39 RFU
LV5	2.26E+07	1.42E+07	66.26 RFU



LV FIG (1:30)

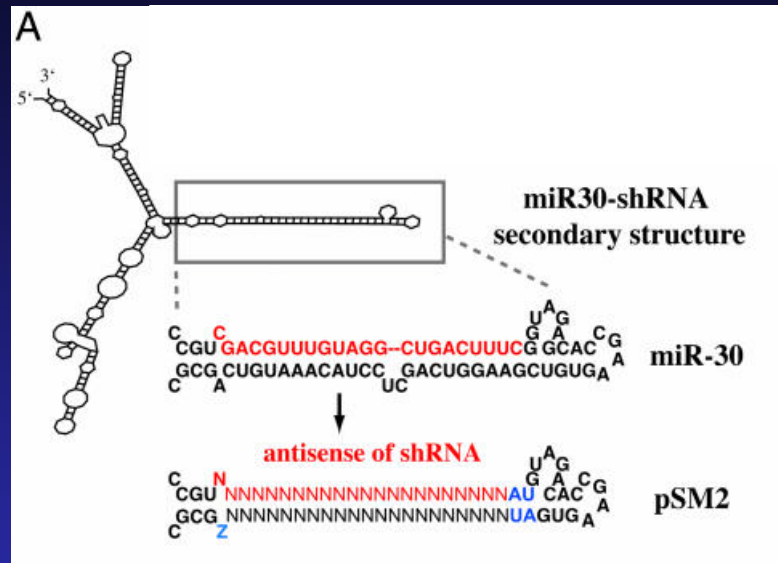
LV GIF (1:30)



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

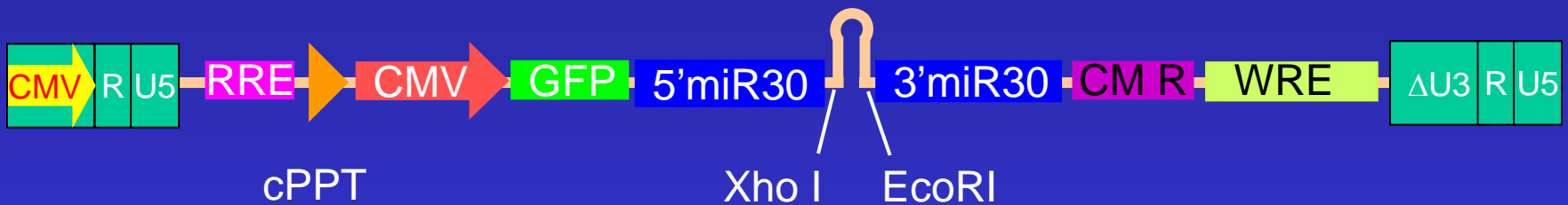
Lentiviral Vector System: Gene Suppression

pPRIME
Potent RNA Interference
using MicroRNA Expression



miR30=
Retinoblastoma (Rb)
Targeting sequence:
AGCAGTTCGATATCTACTGAAA

pPRIME-CMV-GFP
miR30-shRNA

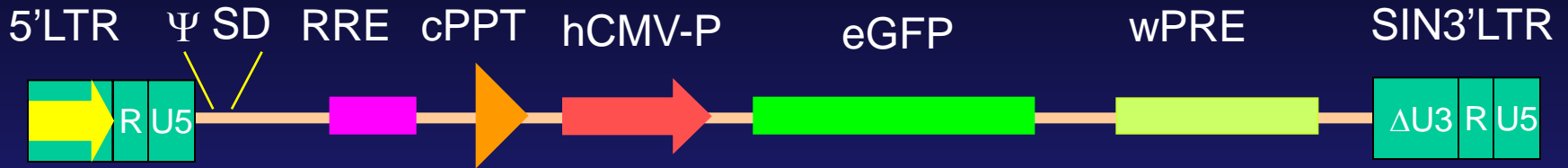


Pol II driven shRNA
was more active
than the Pol III
construct

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

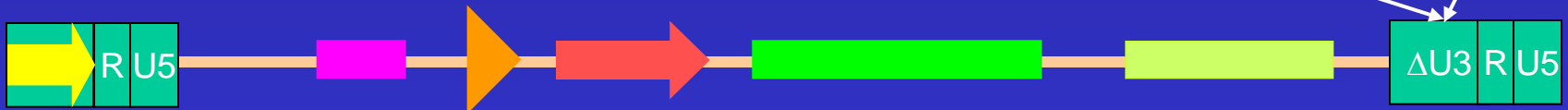
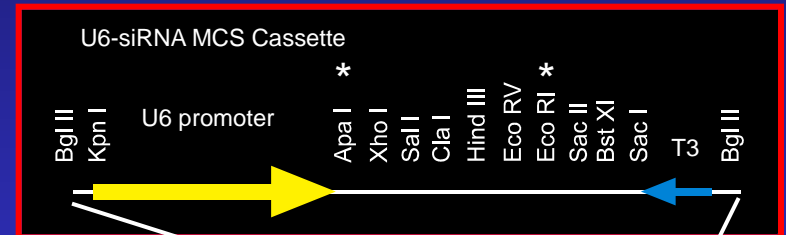
Stegmeier, 2005

Lentiviral Vector System: Gene Suppression



New components:

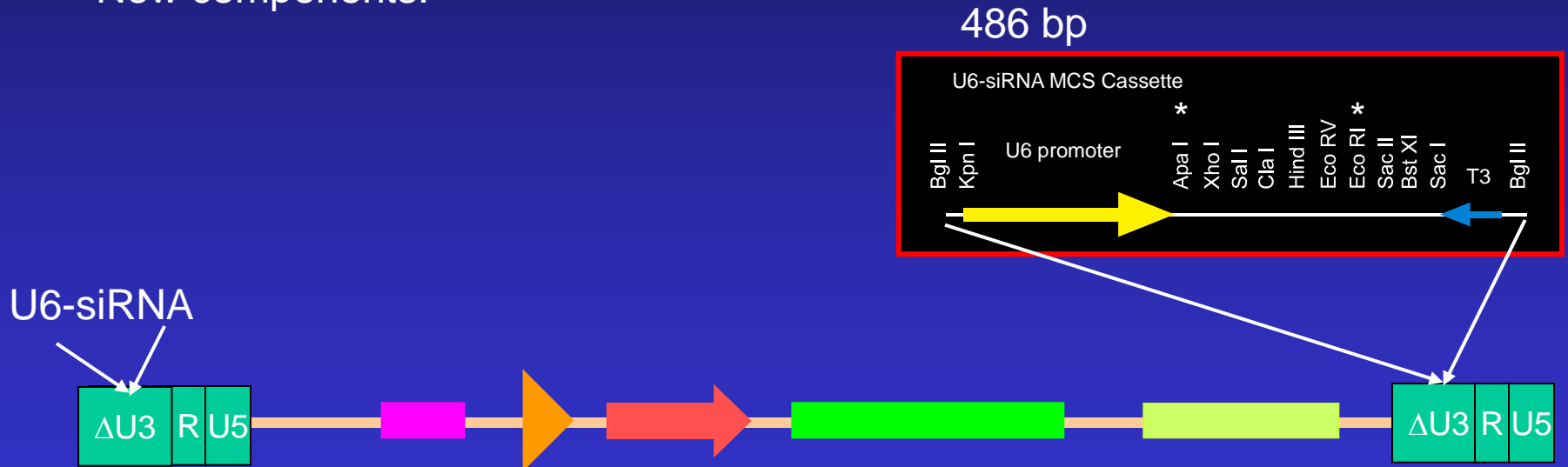
486 bp



Lentiviral Vector System: Gene Suppression



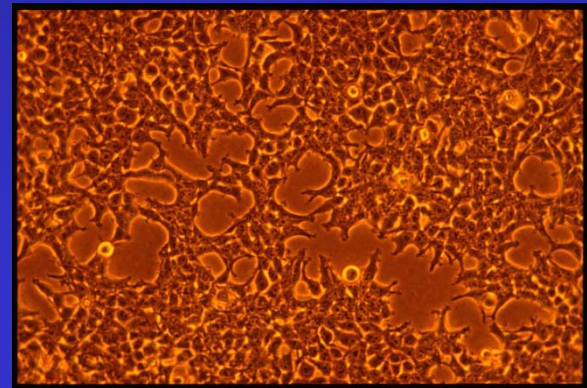
New components:



Virus Production

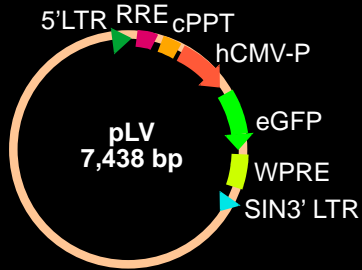
Day 1

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 10⁶ cells/dish)

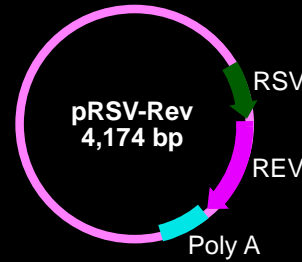
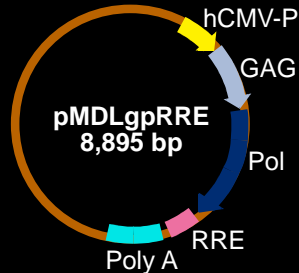


Lentiviral Vector System

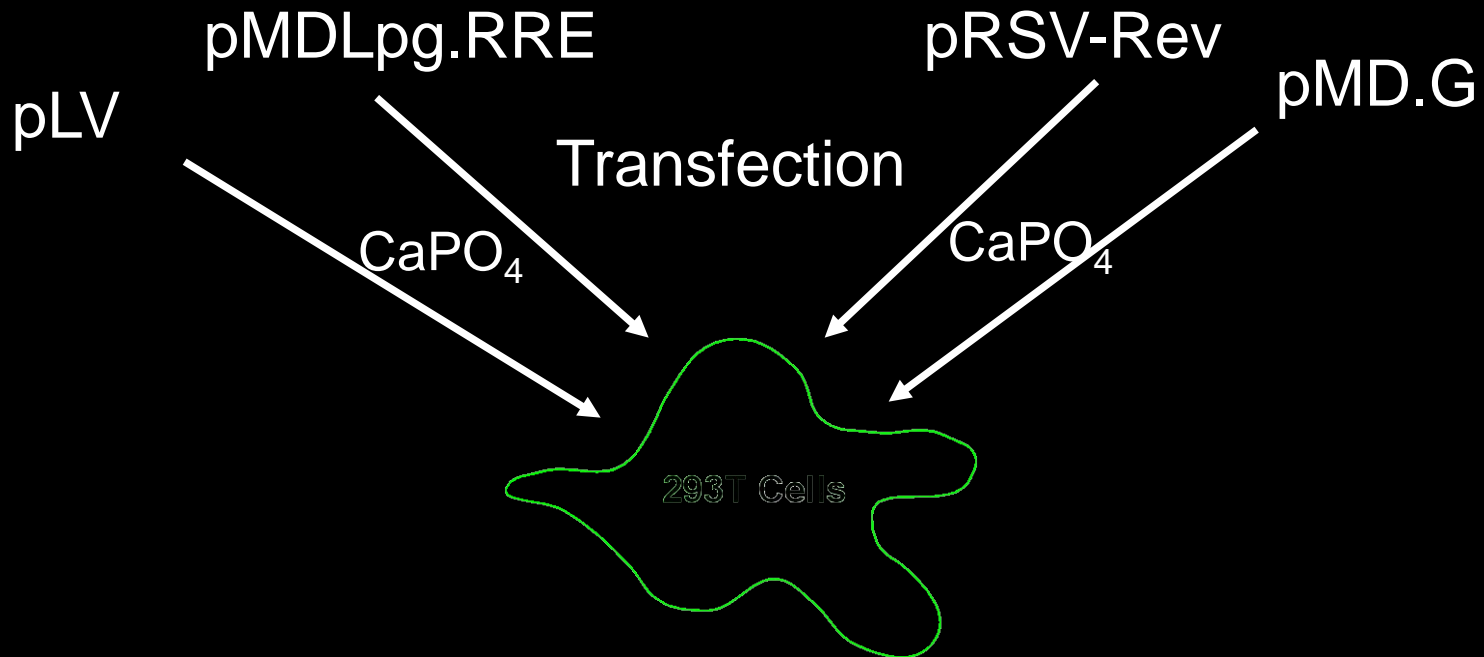
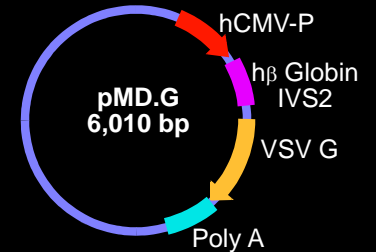
Transgene



Packaging

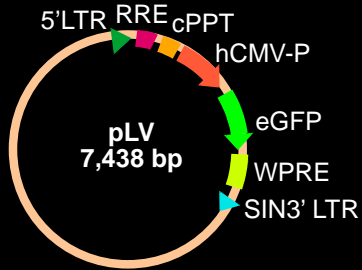


Envelope

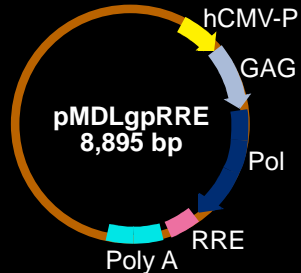


Lentiviral Vector System

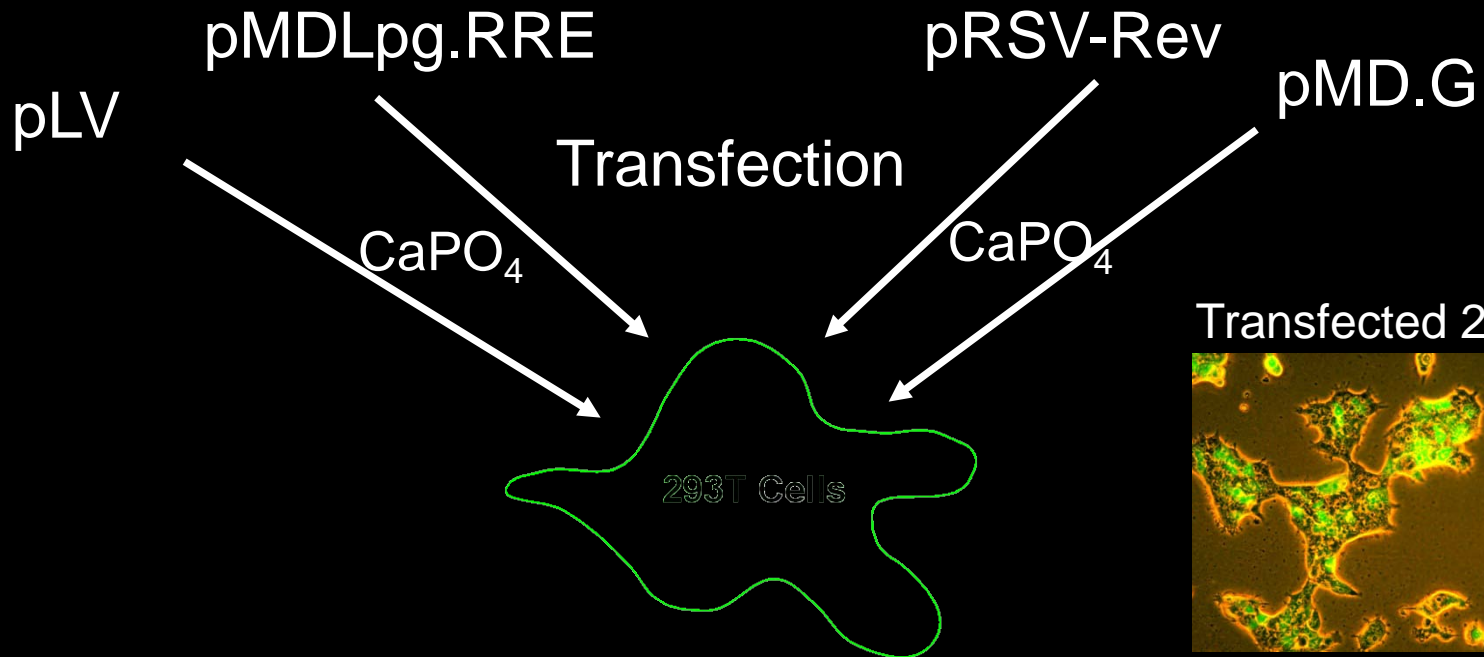
Transgene

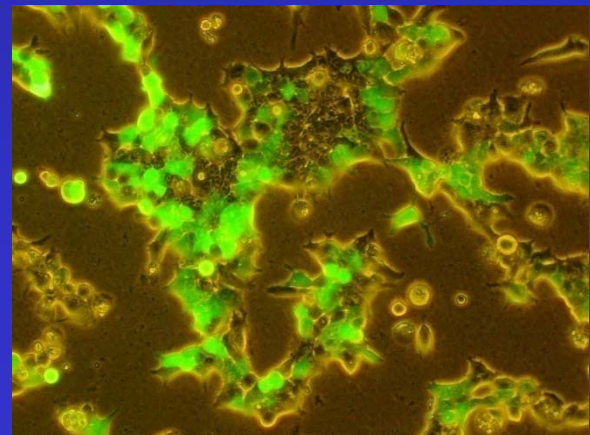
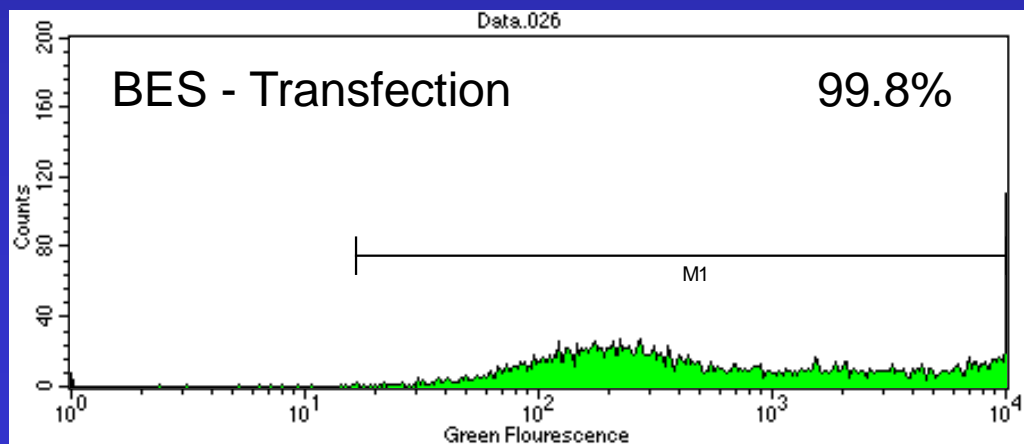
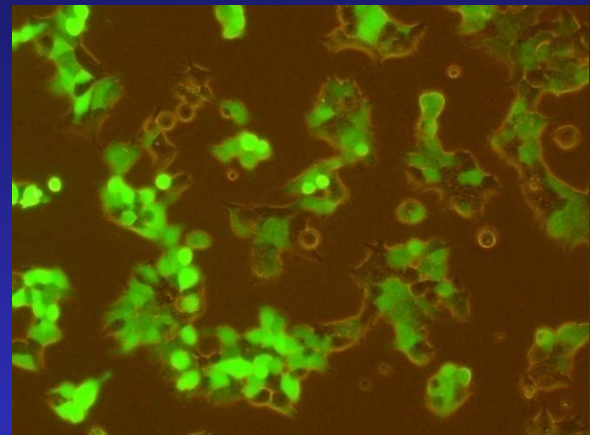
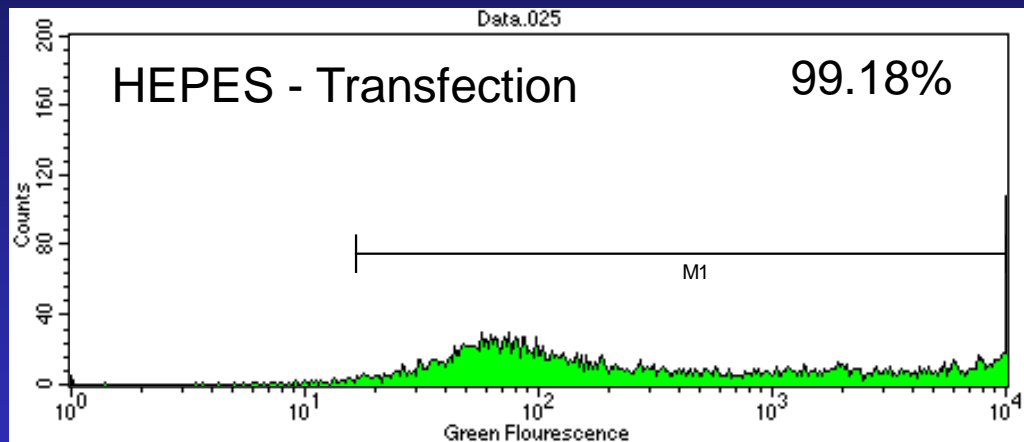
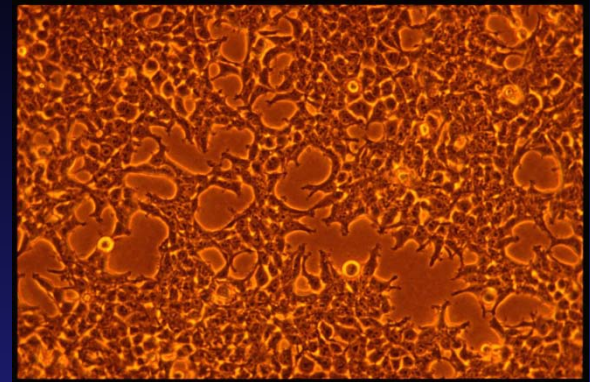
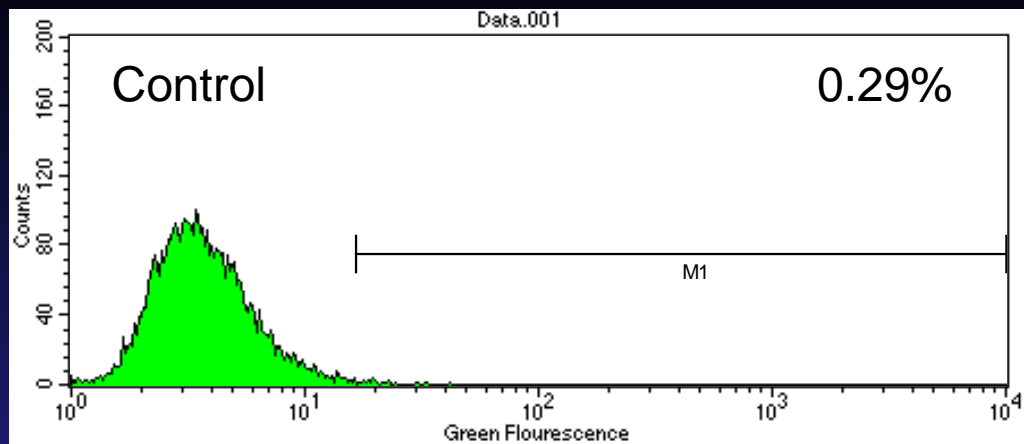


Packaging

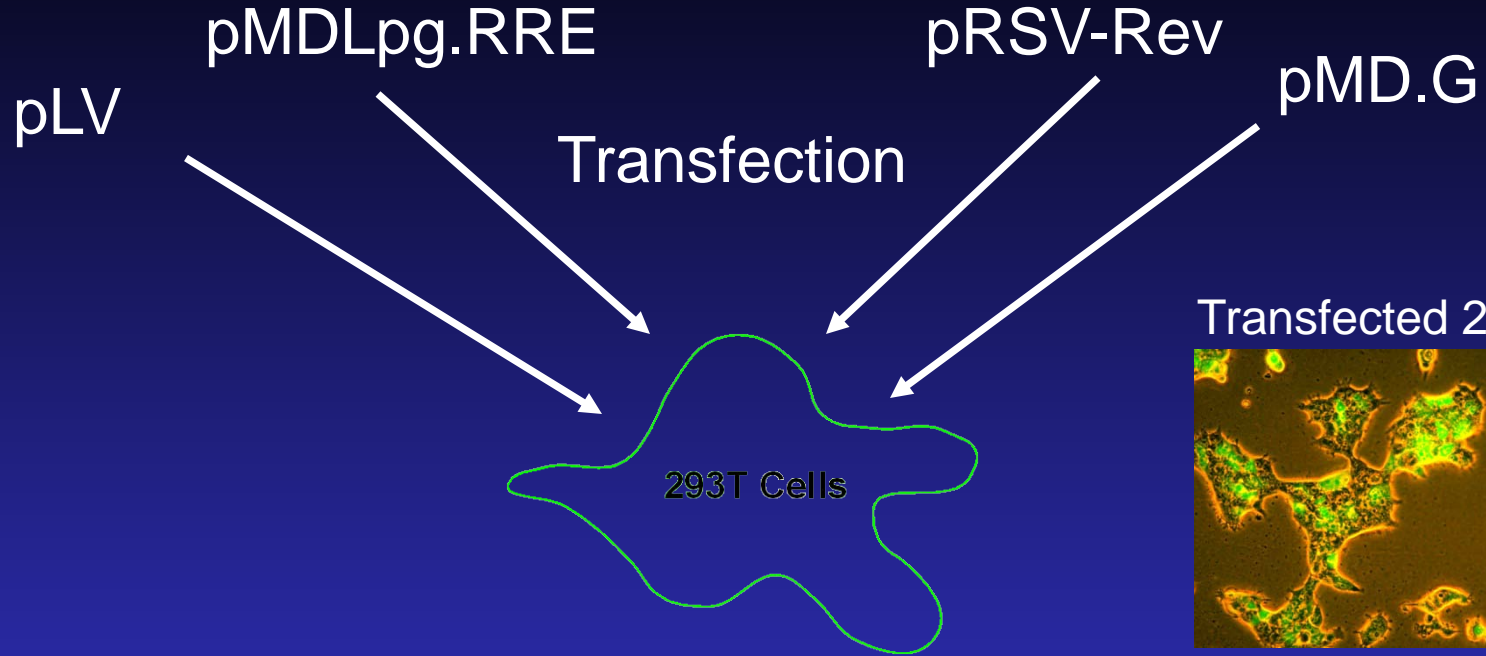


Envelope

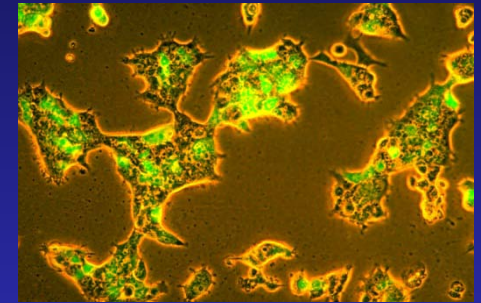




FACs Titer:



Transfected 293T cells



Conditioned Medium

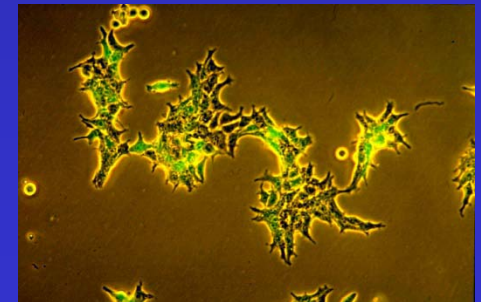


FACs detects
Infected cells,
Expressed as
Percentage of
total

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

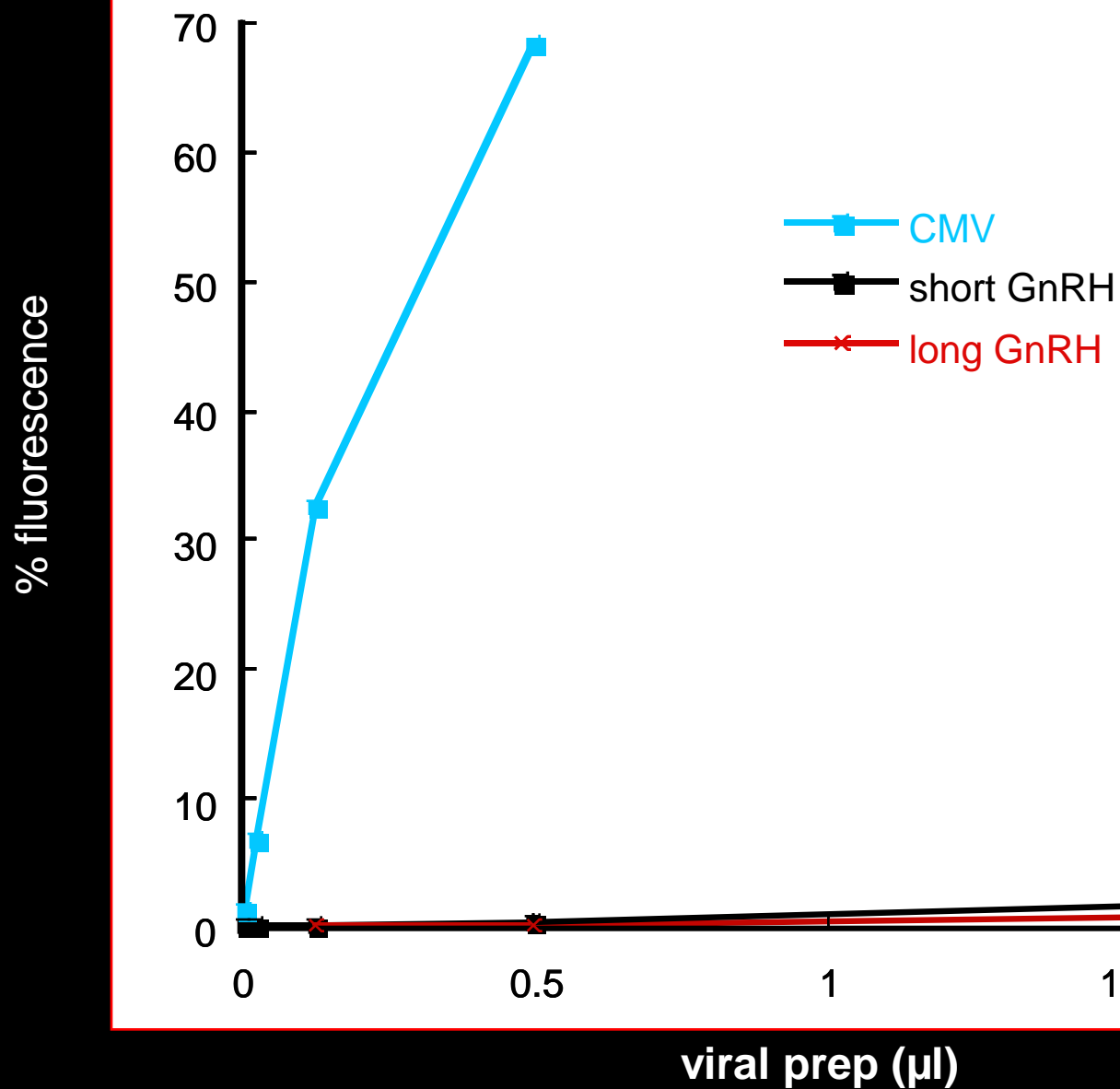
Eliot Spindel, MD., Ph.D.
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spindele@ohsu.edu

Greg Dissen, Ph.D.
Director, Lentiviral Services
disseng@ohsu.edu

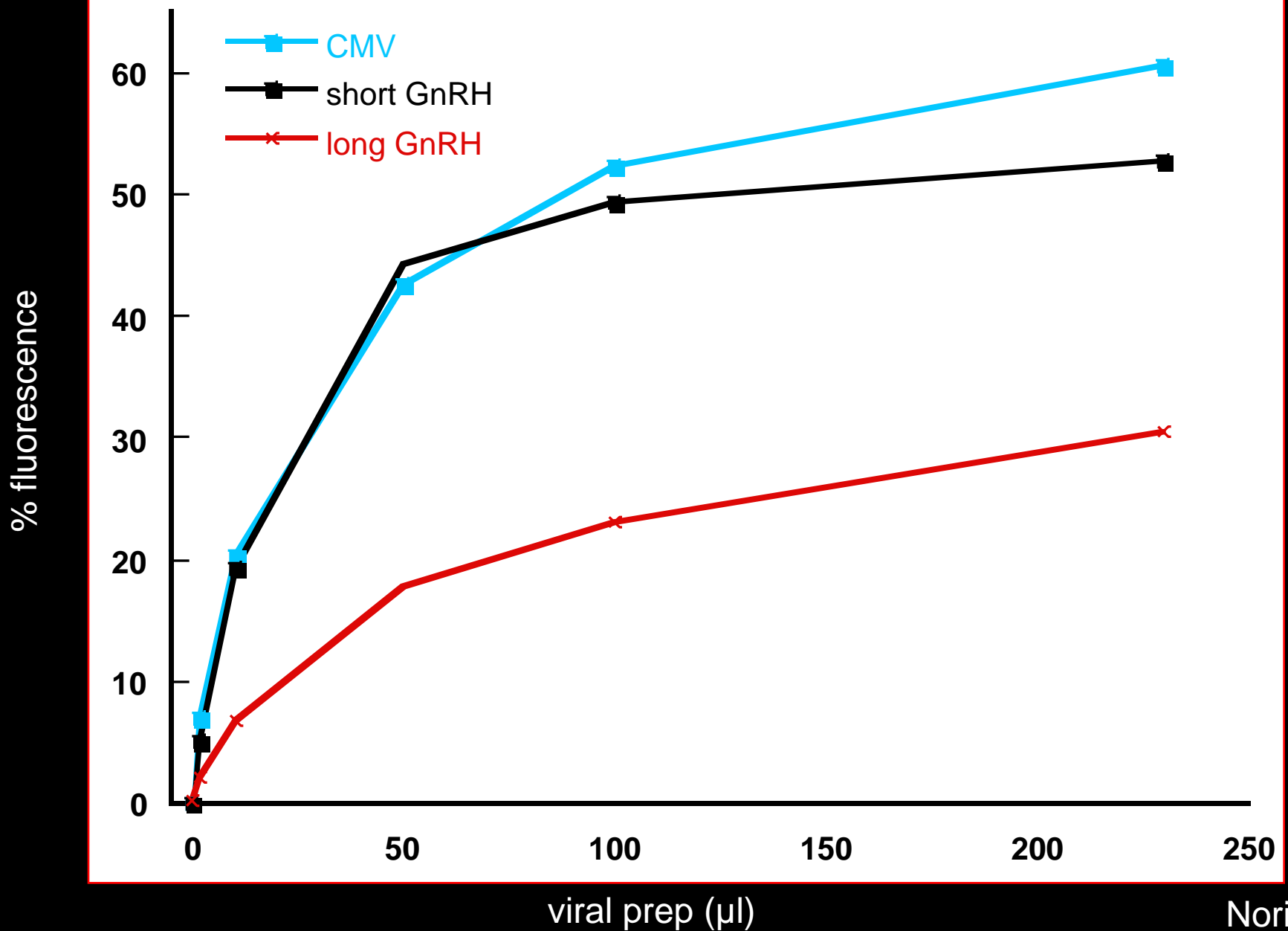
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DNA Sequencing, Realtime PCR & Robotics
jiay@ohsu.edu

CoreyAyne Singleton, M.S.
Cell culture, Genomic DNA preparation, Lentivirus production
singletc@ohsu.edu

Fluorescence in 293-T embryonic kidney cells

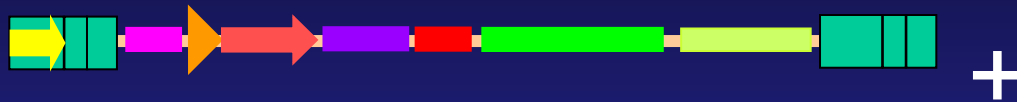


Fluorescence in GT1-7 neuronal cells



Replication Competent Lentivirus (RCL)

3rd Generation Lentiviral Vector



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

Source:

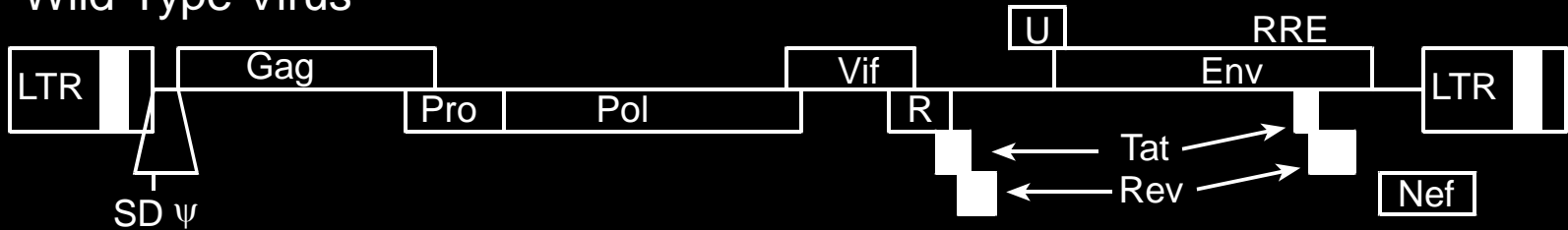
Carry over from packaging or
Envelope plasmids

Or

Endogenous viruses



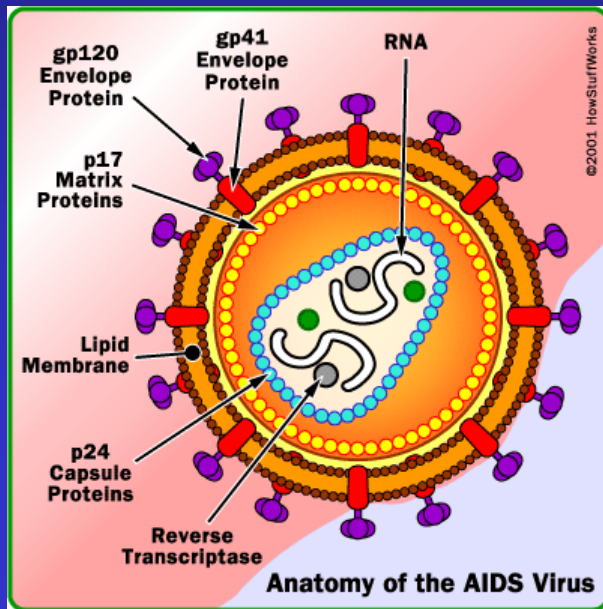
Wild-Type Virus



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

