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How To Use Lentivirus In Mammalian Cell Lines

June 27, 2018

Presenter:

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Senior Application Scientist
GeneCopoeia, Inc.

GeneCopoeia Products and Services

Functional Genomics & Cell Biology

Clones	*Viral systems*	*Stable Cell Lines*	*Kits & reagents*	*Fluorescent detection*
ORF	Lentivirus	CRISPR-Cas9 stable cell lines	Transfection	Cell function assays
Promoter	AAV	Labeled cancer cell lines	Luciferase	Nucleic acid detection
miRNA		Cancer biomarker mutant cell lines	FISH probes	Cell structure probes
CRISPR			Indel detection	Fluorescent dyes
shRNA			Cloning	

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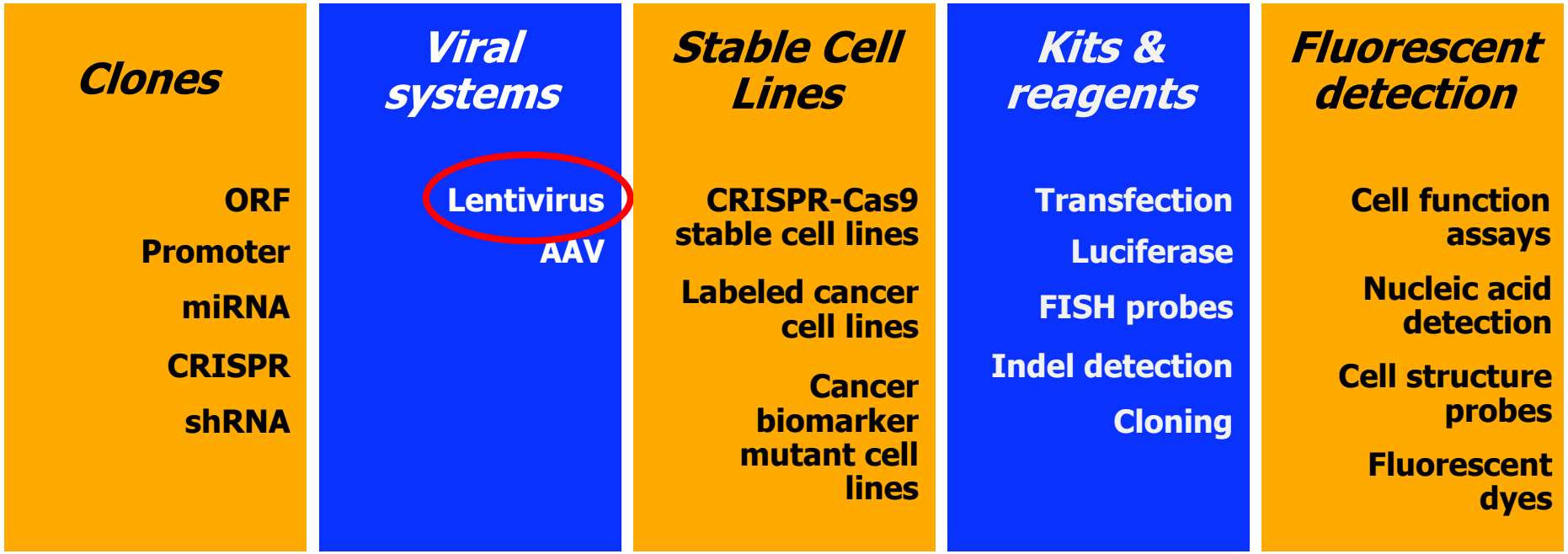
Functional Genomics & Cell Biology



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GeneCopoeia Lentiviral Products and Services

Product/service	Description
Lentiviral clones and cloning vectors	Pre-made and custom clones carrying ORFs, promoters, shRNAs, miRNA 3' UTRs, precursors, and inhibitors, sgRNAs, and more. Available with multiple promoters, tags and reporters. Vectors for do-it-yourself cloning of sequences of interest.
Lentifect™ lentiviral particles	Pre-made and custom-packaged, ready to use lentiviral particles. Produced from GeneCopoeia's extensive, genome-wide clone collections or from customer-submitted clones.
Lenti-Pac™ Lentiviral Packaging Reagents	Complete system of reagents for do-it-yourself lentiviral particle production. Includes packaging plasmids, packaging cell line, particle concentration solution, and titration kit.

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GeneCopoeia Lentiviral Products and Services

Features

- Infect nearly all mammalian cell types
- Can be used to deliver relatively large DNA sequences-up to about 5-6 kb in length
- Can be used to generate stable cell lines, or drive stable gene expression in organs and tissues *in vivo*, due to integration of the transgene at random locations in the genome

Outline

- Lentivirus: Applications
- Lentivirus: Technology overview
- Packaging lentivirus
- Transduction with lentivirus
- Things to watch out for

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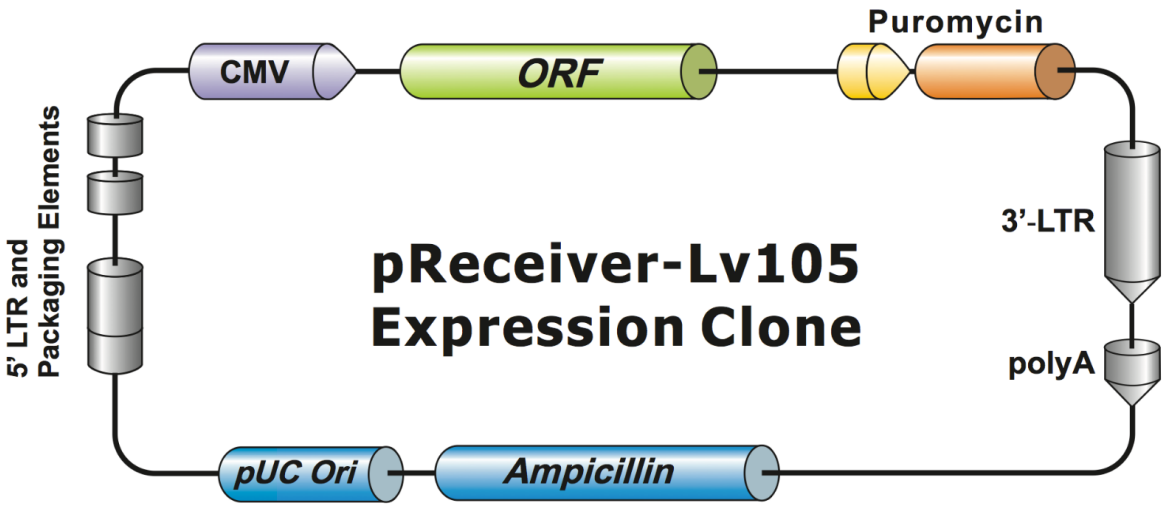
Lentivirus applications

Why use virus for DNA delivery?

- DNA transfection not always possible or practical. Some cell lines difficult or impossible to transfect.
- Necessary for *in vivo*/therapeutic applications
- Most mammalian cells support infection by engineered lentivirus

Lentivirus applications

Protein expression via open reading frame (ORF) clones



Features

- Available for most human and mouse genes
- Some rat and zebrafish genes available too!
- Only the ORF is inserted- no natural 5' or 3' UTRs
- 82 lentiviral vector types. Custom options available
- Nearly-whole genome collections available pre-made in 3 vector types

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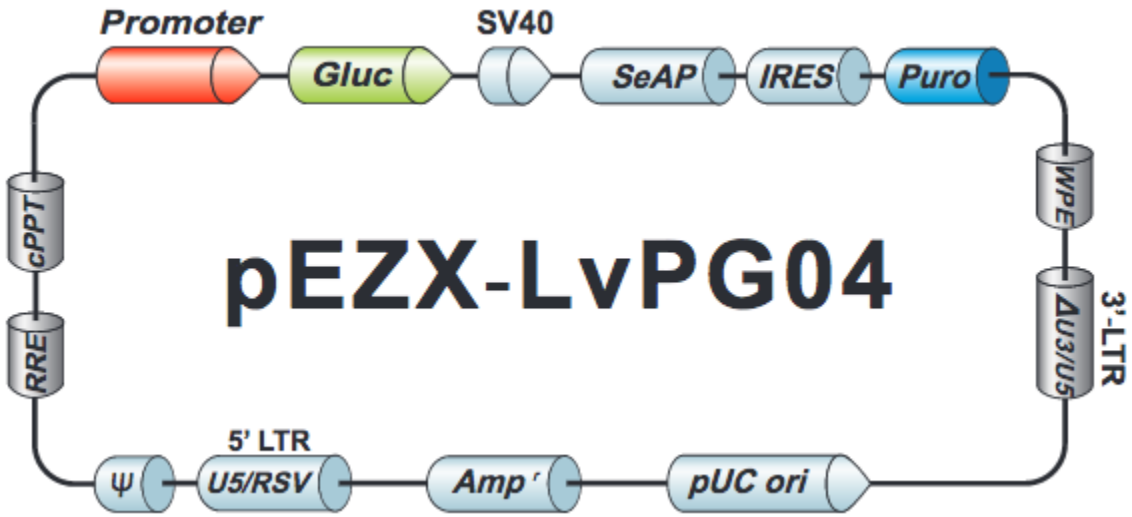
Lentivirus applications

Analyze promoter function



Features

- Available for most human and mouse genes
- Predicted promoter sequence joined to 5' end of reporter gene
- 5 lentiviral vector types. Custom options available
- Exclusively from GeneCopoeia: Secreted Gaussia luciferase + secreted alkaline phosphatase to detect activity without cell lysis!

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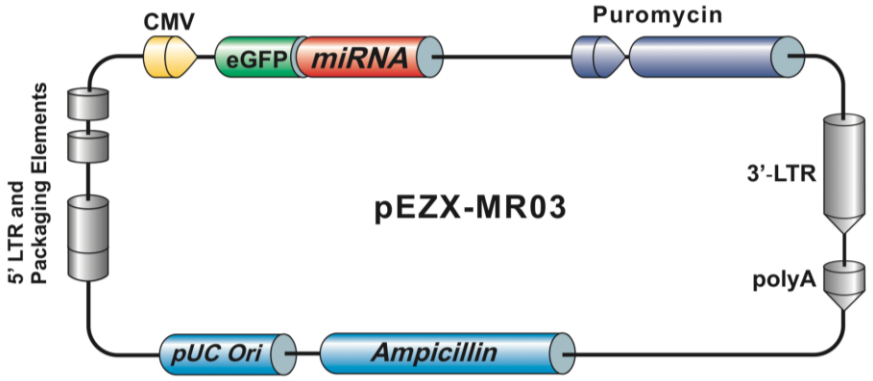
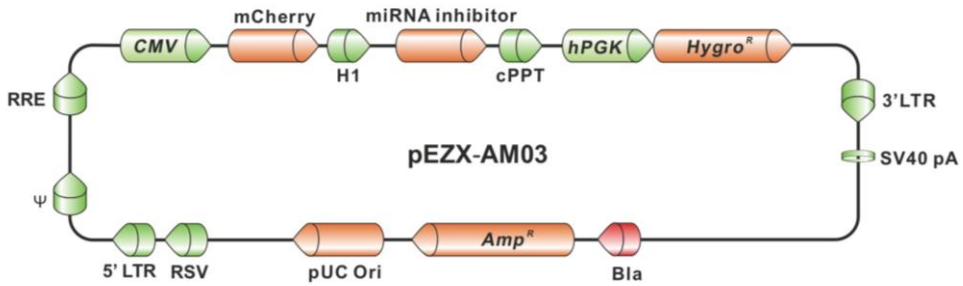
Lentivirus applications

Analyze miRNA function



GeneCopoeia miRNA precursor vector



GeneCopoeia miRNA inhibitor vector

Features

- Available for most human, mouse, and rat genes
- Expresses miRNA from polII promoter, and inhibitor from either U6 or H1 promoter
- 5 lentiviral vector types. Custom options available

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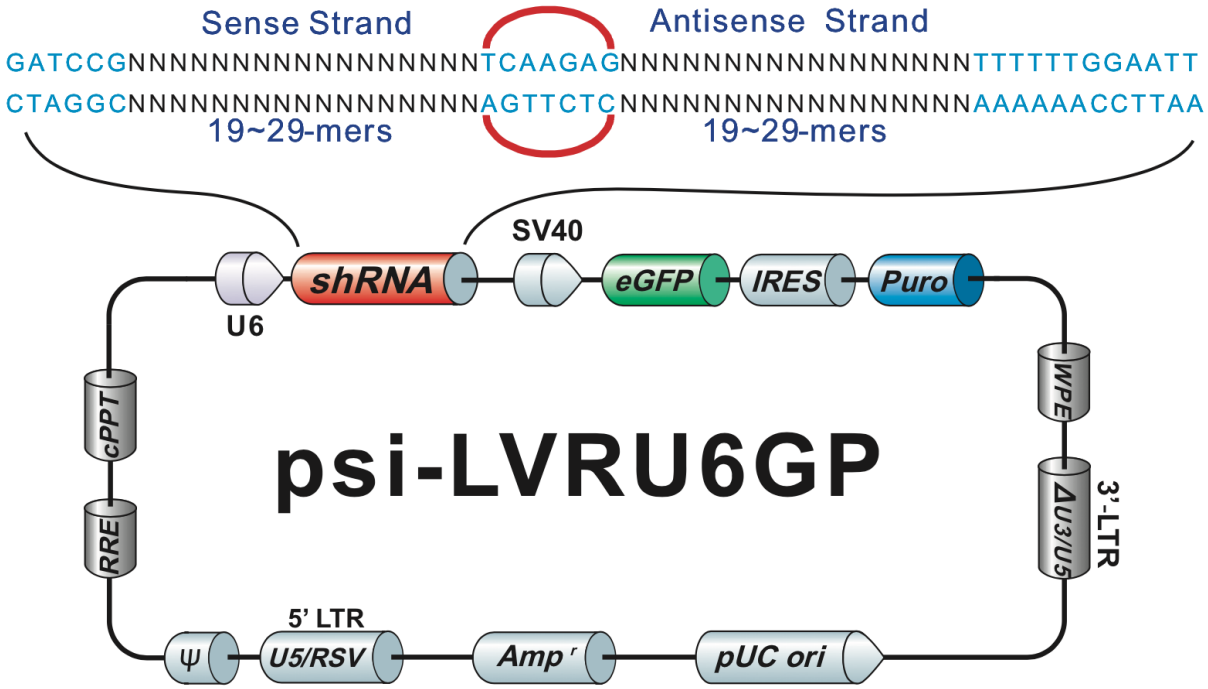
Lentivirus applications

Knock genes down using RNAi/shRNA



Features

- Available for most human, mouse, and rat genes
- Expresses shRNA from either U6 or H1 promoter
- 12 lentiviral vector types. Custom options available
- Guaranteed knockdown*

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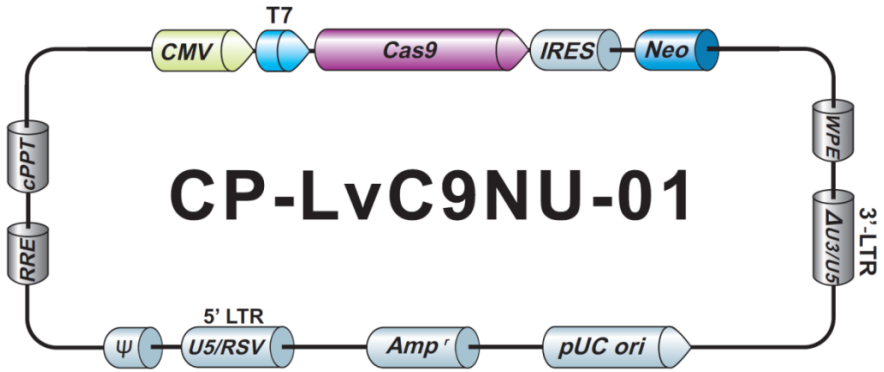
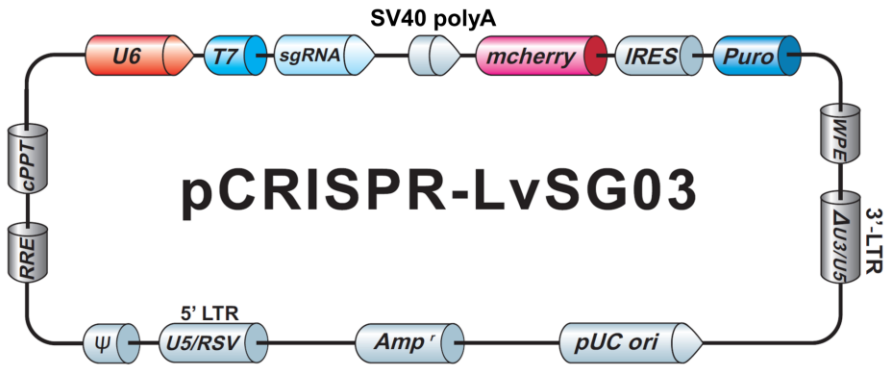
Lentivirus applications

CRISPR-Cas9





Features

- 2-component system (Cas9 lentivirus and sgRNA lentivirus)
- Expresses Cas9 from polII promoter
- Expresses sgRNA from U6 promoter
- Can be used for gene knockout, knockdown (CRISPRi), activation, base editing, and more

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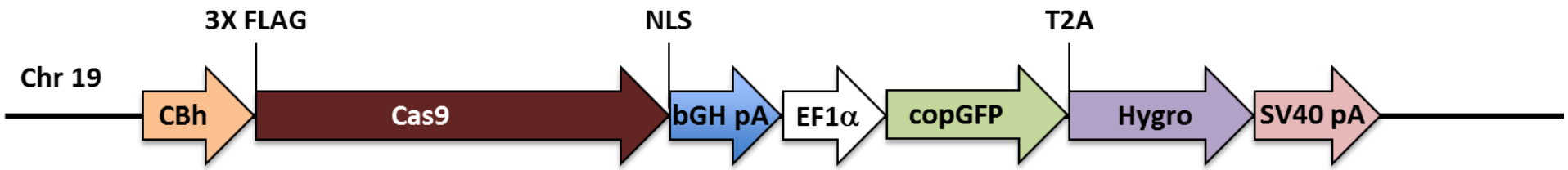
Lentiviral CRISPR

GeneCopoeia Cas9 stable cell lines



Features

- Cell lines with Cas9 stably integrated in the genome
- >70 pre-made cell lines available in human, mouse, and rat cell lines
- Functionally validated for Cas9 activity
- Ideal for lentiviral CRISPR applications

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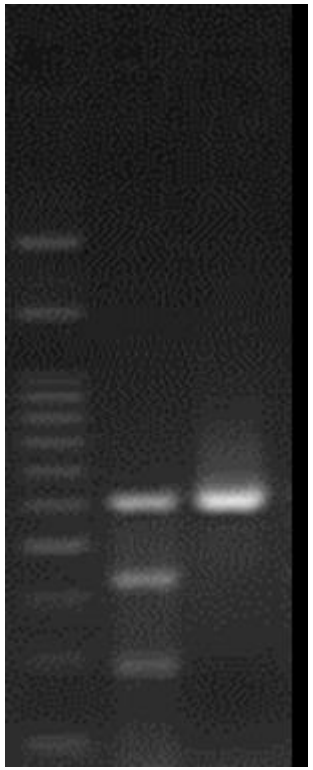
Lentiviral CRISPR

GeneCopoeia Cas9 stable cell lines



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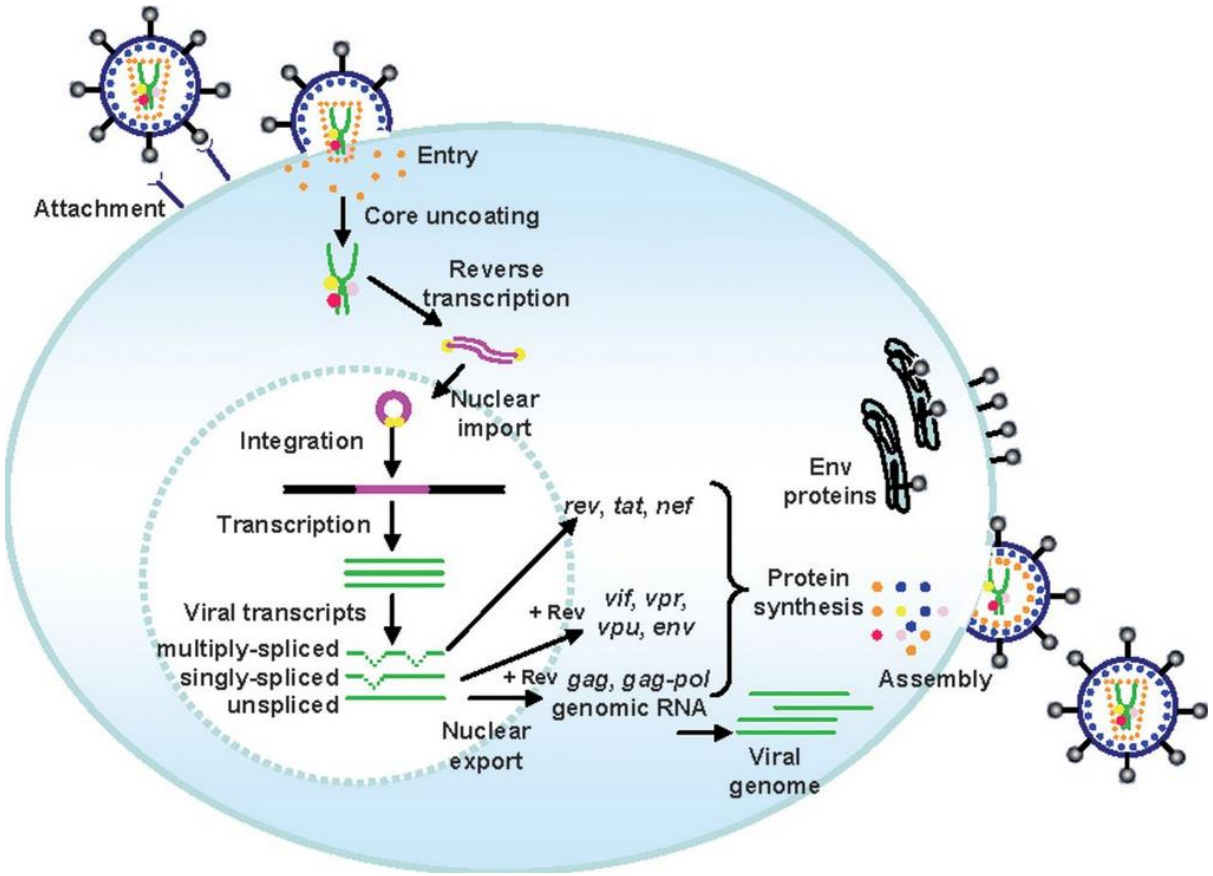
Outline

- Lentivirus: Applications
- Lentivirus: Technology overview
- Packaging lentivirus
- Transduction with lentivirus
- Things to watch out for

Lentivirus technology



Sakuma, et al. (2012). Biochem. J. 443, 603.

- Class of retroviruses that includes human immunodeficiency virus (HIV)
- Single stranded RNA genome of ~9.7 kb
- Integrates into genomic DNA
- Infect dividing & non-dividing cells

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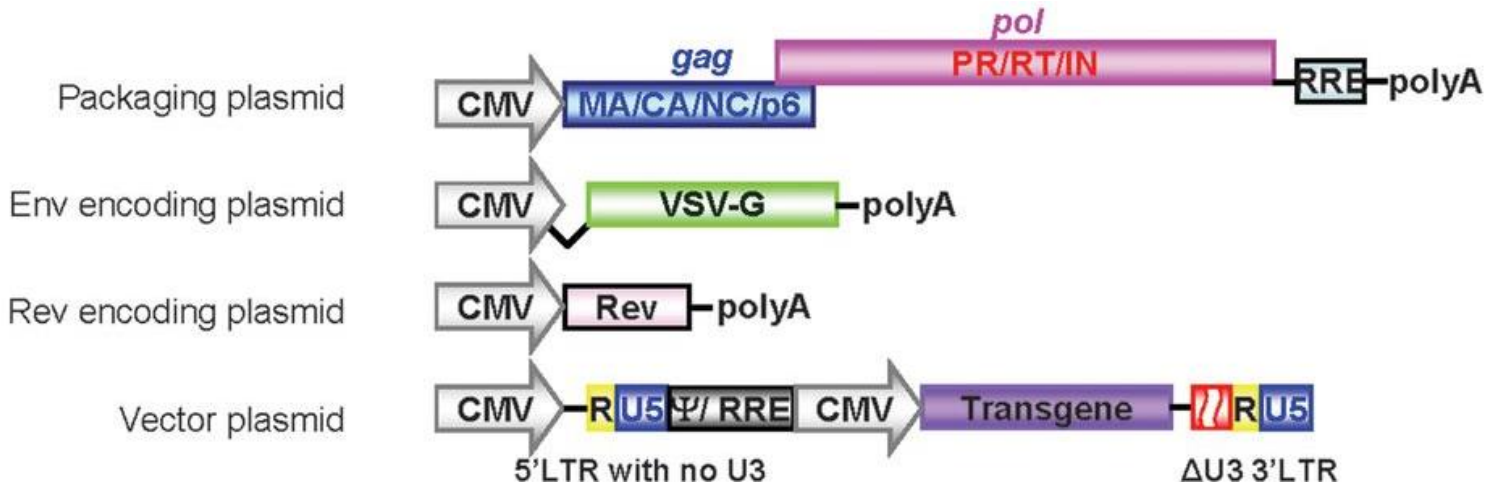
Lentivirus technology

3rd generation lentivirus



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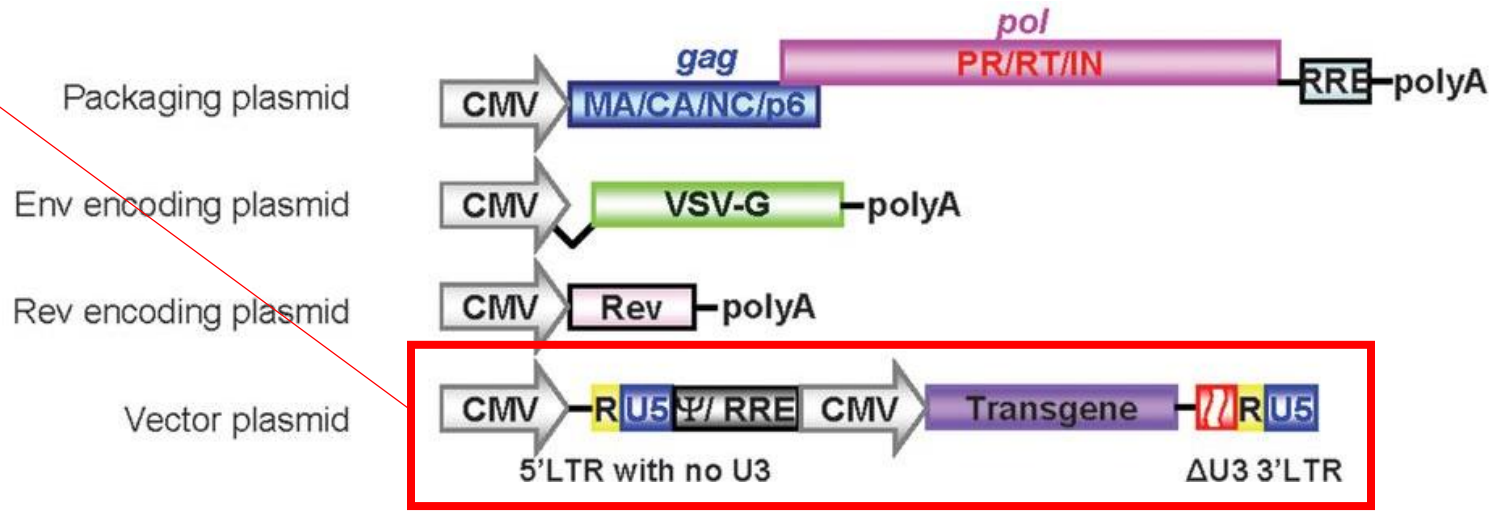
Lentivirus technology

3rd generation lentivirus

1. Remove all viral protein-coding genes from Vector/transfer plasmid



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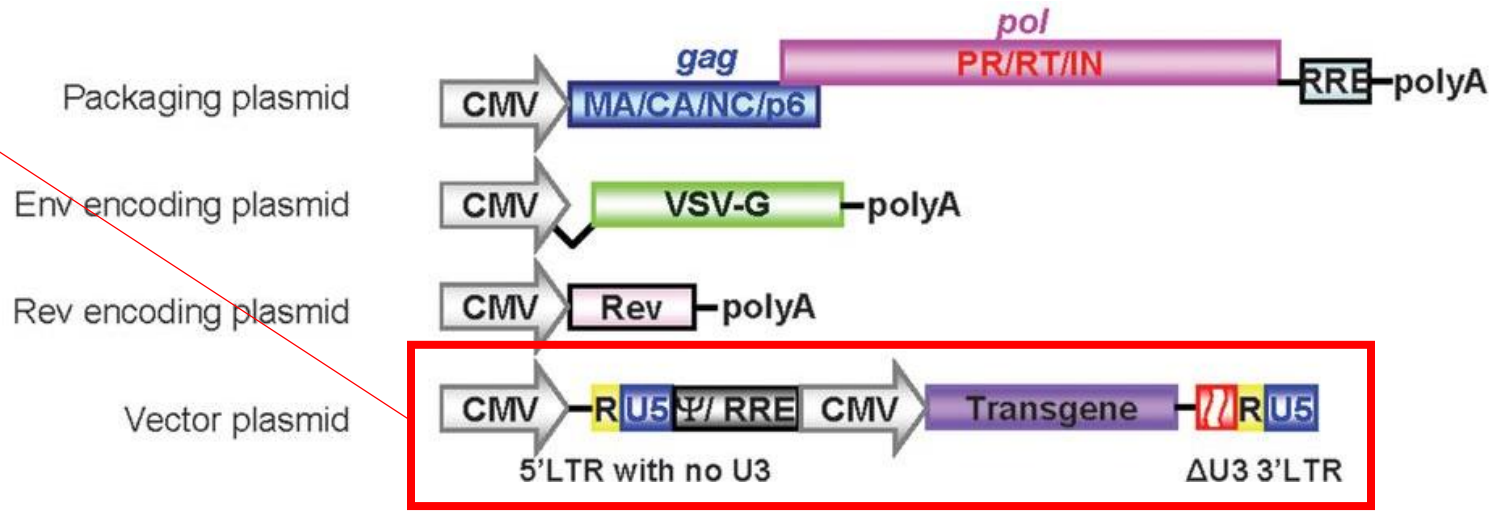
Lentivirus technology

3rd generation lentivirus

1. Remove all viral protein-coding genes from Vector/transfer plasmid

2. Eliminate viral accessory genes (nef, vif, vpu, vpr)



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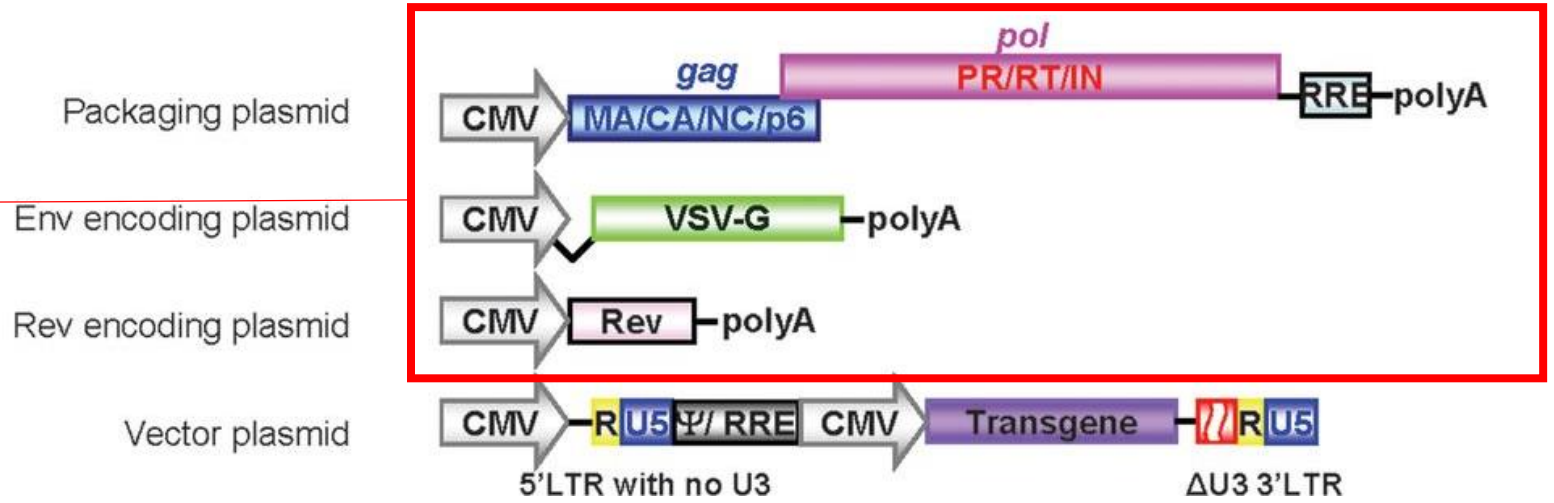
Lentivirus technology

3rd generation lentivirus

1. Remove all viral protein-coding genes from Vector/transfer plasmid

2. Eliminate viral accessory genes (nef, vif, vpu, vpr)

3. Split viral protein-coding genes onto 3 separate plasmids



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Lentivirus technology

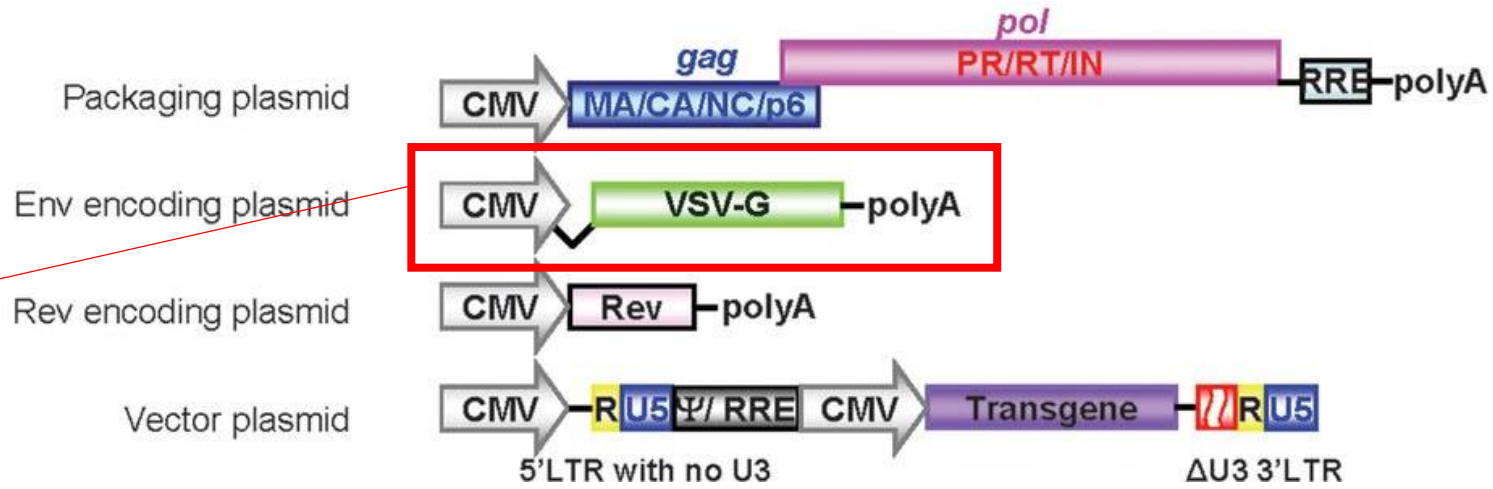
3rd generation lentivirus

1. Remove all viral protein-coding genes from Vector/transfer plasmid

2. Eliminate viral accessory genes (nef, vif, vpu, vpr)

3. Split viral protein-coding genes onto 3 separate plasmids

4. Change env to another glycoprotein (tropism)



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Lentivirus technology

3rd generation lentivirus

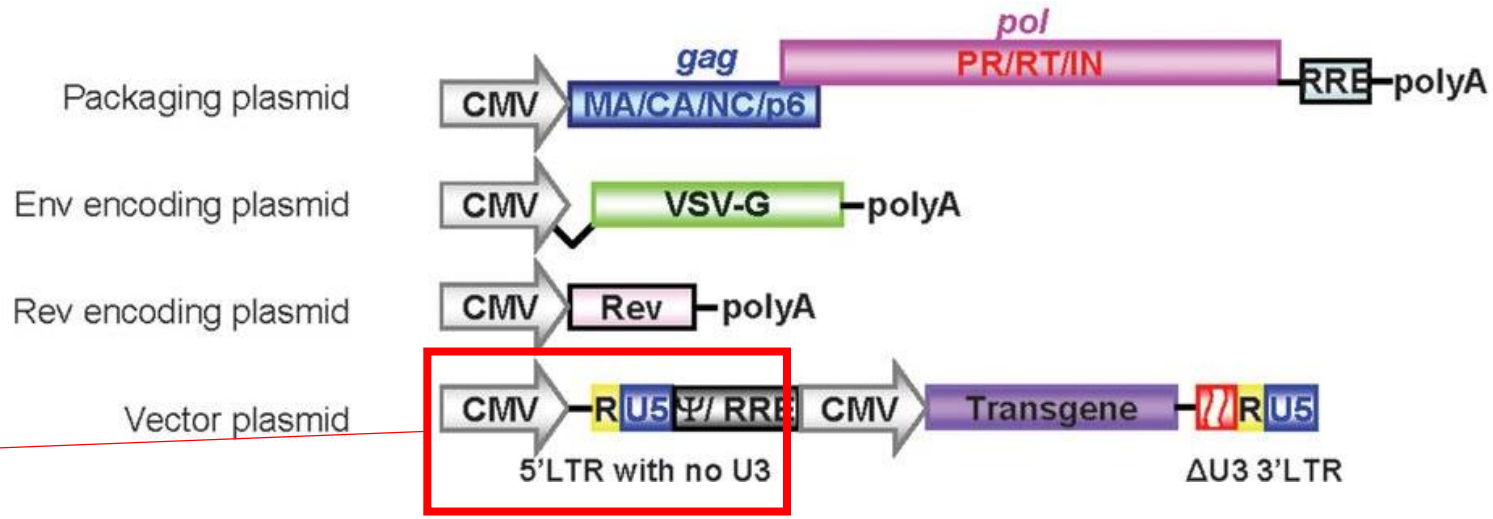
1. Remove all viral protein-coding genes from Vector/transfer plasmid

2. Eliminate viral accessory genes (nef, vif, vpu, vpr)

3. Split viral protein-coding genes onto 3 separate plasmids

4. Change env to another glycoprotein (tropism)

5. Truncate 5' LTR, replace with heterologous promoter to remove tat-dependence



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Lentivirus technology

3rd generation lentivirus

1. Remove all viral protein-coding genes from Vector/transfer plasmid

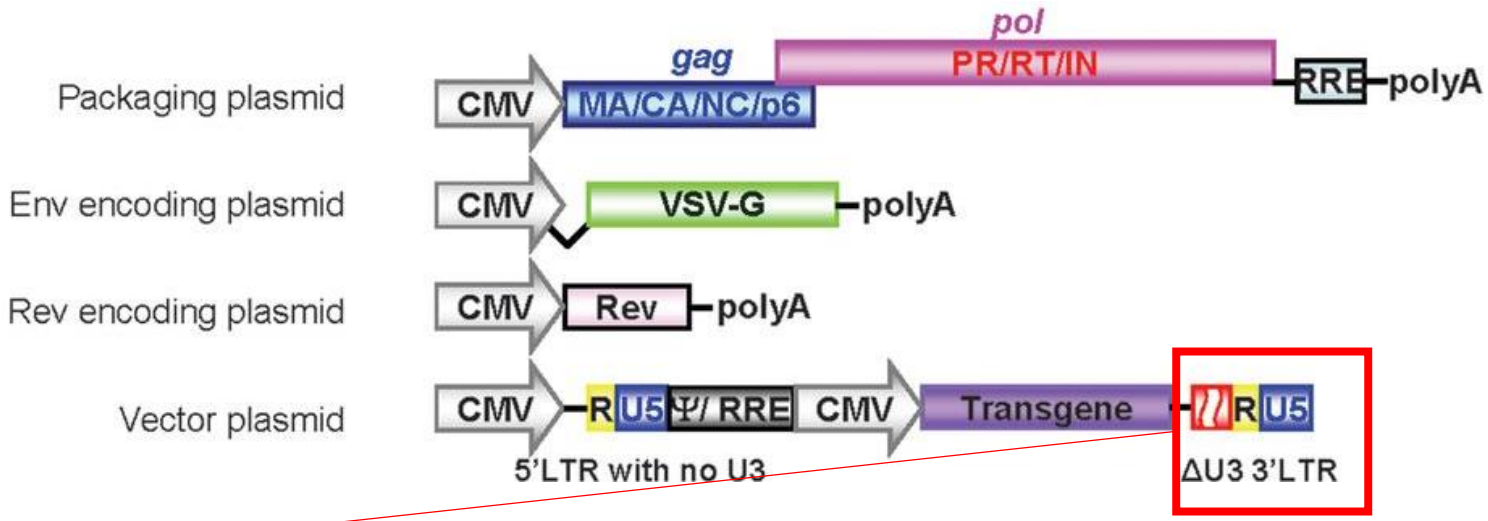
2. Eliminate viral accessory genes (nef, vif, vpu, vpr)

3. Split viral protein-coding genes onto 3 separate plasmids

4. Change env to another glycoprotein (tropism)

5. Truncate 5' LTR, replace with heterologous promoter to remove tat-dependence

6. Delete U3 region of 3' LTR (SIN)



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Lentivirus technology

Insert capacity

- HIV genome ~9.7 kb LTR-LTR
- Virus has physical limit. Can only fit up to certain length
- Titers drop off when distance LTR-LTR >9 kb, but can package up to about 15 kb

Outline

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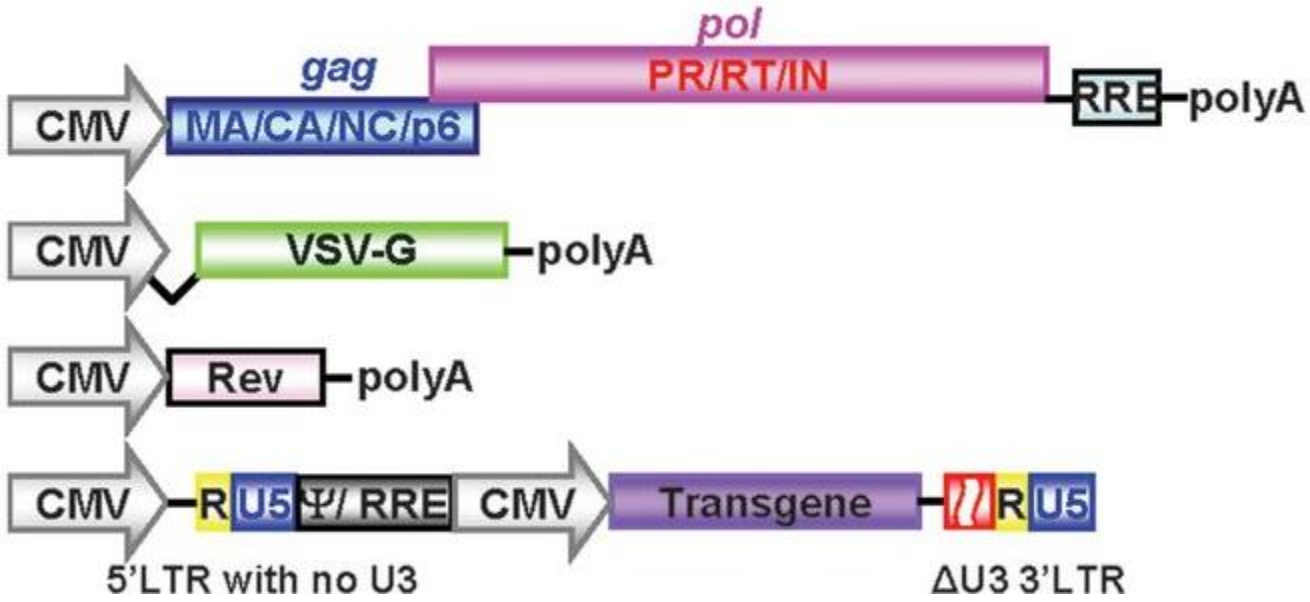
Lentivirus packaging

What do I need?

- Gag-pol packaging plasmid
- Env plasmid (VSV-G or other)
- Rev plasmid
- Transfer (vector) plasmid (contains gene you want to express)



Sakuma, et al. (2012). Biochem. J. 443, 603.s

- Packaging cell line. Most use HEK293T
- Transfection reagent

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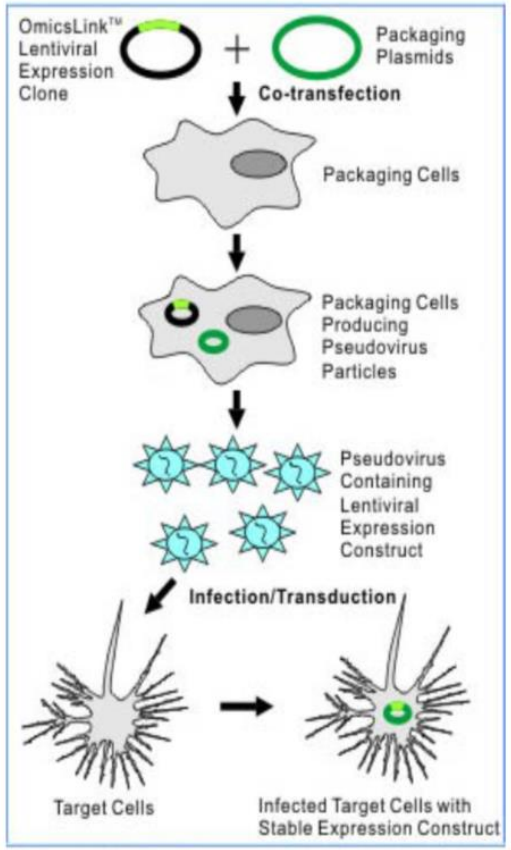
Lentivirus packaging

GeneCopoeia Lenti-Pac™ packaging reagents

- Lenti-Pac™ HIV Expression Packaging Kit: Optimized for high-titer lentivirus packaging
  - Packaging plasmid mix (packaging plasmid, VSV-G env plasmid, Rev plasmid)
  - GFP control plasmid
  - Transfection reagent (Endofectin™ Lenti)
  - TiterBoost™: Proprietary reagent that increases viral titers 5-10 fold

Lentivirus packaging

Packaging workflow



Lenti-Pac™ HIV Expression Packaging Kit

Lenti-Pac™ HEK393T packaging cells

Endofectin™ Lenti

Lenti-Pac™ Titration kit

Lenti-Pac™ Concentration solution

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Lentivirus packaging

Purification

- Purification is not required for cell culture use. Can just use medium to infect
- However, purification will increase titer and get rid of potentially unwanted cell debris and proteins from medium
- Purification is absolutely required for *in vivo* (animal) use to avoid toxicity and immunological response.

Lentivirus packaging

GeneCopoeia Lenti-Pac™ Concentration Solution

- Simple protocol: Just centrifuge or filter particles (to remove cells and debris), mix with the concentration solution and incubate, then centrifuge and resuspend in PBS
- Concentrates particles and increases titer 10-100 fold
- Also helps in removing some cellular debris and proteins
- Link to protocol: http://www.genecopoeia.com/wp-content/uploads/2017/06/Lenti-Pac_Lentivirus_Concentration_Solution_Manual.pdf

Lentivirus packaging

Why should I determine titer?

- Necessary to determine success of packaging reaction

- Also needed to determine correct volume of virus needed for infection

Lentivirus packaging

Titration methods: Physical and Functional titer

- Physical titer is expressed as Viral Particles per ml (VP/ml), but titer is most commonly expressed functionally, as Transduction Units per ml (TU/ml)
- Functional titer: Determine actual number of infectious viral particles
- Functional titer determination works best if lentivirus expresses a fluorescent reporter
- Can also use a colony-forming assay following antibiotic selection

Lentivirus packaging

Functional titer: Fluorescence

Day 1: Seed cells (recommend H1299 cells) in 24-well plates

↓

Day 2: Infect cells with different volumes of virus

↓

Day 3: Split cells and transfer to 6-well plates

↓

Day 5: Either visualize the cells under fluorescent microscope or trypsinize cells, count in hemocytometer, & FACS sort

Lentivirus packaging

Functional titer: Fluorescence

$$\text{Titer} = \text{Fraction of positive cells} \times \frac{\text{Total \# of cells}}{\text{Volume of particles (ml)}}$$

So, if 50% of 100,000 cells are fluorescent from 1 ul virus, then

$$\text{Titer} = 0.5 \times \frac{100{,}000}{0.001} = 5 \times 10E7 \text{ TU/ml}$$

Recommend doing triplicates

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Lentivirus packaging

Functional titer: Drug selection

Day 1: Seed cells (recommend H1299 cells) in 24-well plates

↓

Day 2: Infect cells with different volumes of virus

↓

Day 3: Split cells and transfer to 6-well plates

↓

Day 5: Replace medium with fresh medium containing antibiotic (such as puromycin)

↓

Day 14: Fix and stain colonies with crystal violet. Count colonies and estimate the fraction of positives based on the original # of cells plated

Lentivirus packaging

Functional titer: Drug selection

$$\text{Titer} = \text{Fraction of positive cells} \times \frac{\text{Total \# of cells}}{\text{Volume of particles (ml)}}$$

So, if 50% of 100,000 cells are drug resistant from 1 ul virus, then

$$\text{Titer} = 0.5 \times \frac{100{,}000}{0.001} = 5 \times 10E7 \text{ TU/ml}$$

Recommend doing triplicates

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Lentivirus packaging

Titration methods: Physical titer

- Determine number of copies of virus and estimate number of infectious viral particles
- Not as accurate as determining functional titer, due to detection of components that can be present in non-functional particles
- Can be determined for any lentiviral particles
- Much more convenient, universal, and faster than determining functional titer

Lentivirus packaging

Physical titer: 2 widely-used methods

- p24 method: Use ELISA to determine # of copies of p24 capsid protein in sample

- qPCR method: Use quantitative PCR to determine # of copies of viral genome-faster and more convenient than p24 ELISA method

Lentivirus packaging

Physical titer: Lenti-Pac™ HIV qRT-PCR Titration Kit

- qRT-PCR based lentiviral titration to determine the copy numbers of HIV lentiviral particles.
- Simple fast, & convenient: Can have results in as little as 2 hours
- Contains all reagents needed for RNA extraction, reverse transcription, and qPCR
- Detailed protocol at: http://www.genecopoeia.com/wp-content/uploads/2017/06/Lenti-Pac_qRT-PCR_Titration_Kit_manual.pdf

Lentivirus packaging

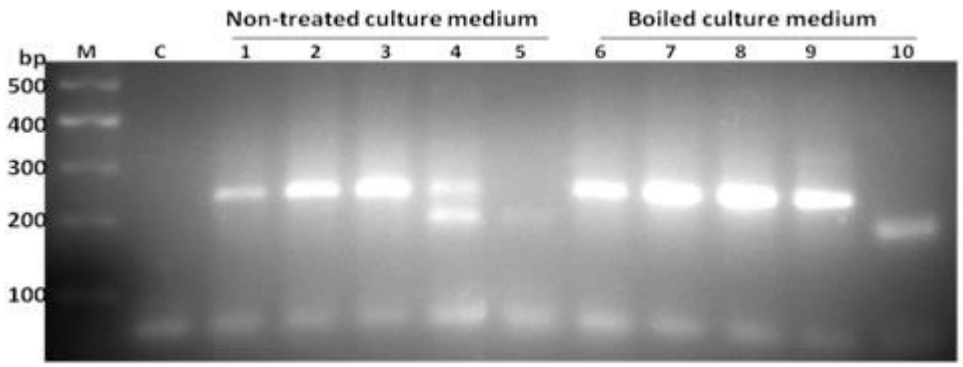
Important considerations

- Generation: The Lenti-Pac™ packaging system is 3rd generation
  - Can use for packaging GeneCopoeia lentiviral plasmids or any other 3rd generation plasmid
  - **Cannot** use for packaging 2nd generation plasmids, which are Tat-dependent
- Lentivirus must be handled in a Biosafety level 2 (BSL-2) facility. Make sure your facility has this capability
- Plasmid propagation: Transform bacteria with your plasmids. Use a stability strain of *E. coli* like GeneCopoeia's GCI-L3.
- Plasmids should be endotoxin-free. Be sure to use plasmid preparation products that remove endotoxin, which can kill cells
- Avoid freeze-thaw of lentiviral particles, which reduces particle viability.
- How much to make-MOI

Lentivirus packaging

Important considerations (cont'd)

- Mycoplasma: Cells should be mycoplasma-free. Use GeneCopoeia's Mycoguard™ mycoplasma detection kit
  - PCR-based detection kit for multiple strains of mycoplasma
  - Convenient: No need to pre-treat culture medium
  - Fast: Results in as little as 2 hours



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GeneCopoeia Lentiviral Particles

Features

- Ready-to-use. Let GeneCopoeia do the packaging, concentration, and titration for you
- High-titers-up to 1 x 10E9 TU/ml
- Available in 2 purity levels: Purified-for in vitro (cell culture), and ultra-purified (for animal models)

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Lentivirus transduction

Considerations

- MOI: Determine optimal MOI of cell line before use
- Health of cells: Need to be low passage and mycoplasma-free
- Selection or screening. How are you going to detect infection?
- Stable pool vs. single clone
- BSL-2
- Adherent cells vs. suspension cells. Suspension cells are harder to infect than adherent cells.

Lentivirus transduction

Multiplicity of infection (MOI)

- Number of infectious particles per cell
- Optimal number. If too low, don’t get enough infection. If too high, can be toxic
- Varies based on cell line
- Known for some cells. Others must be determined experimentally

Lentivirus transduction

Multiplicity of infection (MOI)

Cell line	Tissue	Cancer/cell type	Species	MOI
A431	Epithelial	Carcinoma	Human	5
A549	Lung	Carcinoma	Human	5
Astrocytes	Nervous system	Primary	Human	1
B16-F10	Epithelial	Melanoma, metastatic	Mouse	5
BMM	Bone Marrow	Primary	Human	8
BxPC-3	Pancreas, epithelial	Adenocarcinoma	Human	10
H3255	Lung	Carcinoma, NSCLC	Human	10
HCT116	Colon	Carcinoma	Human	5
HeLa	Cervix	Carcinoma, epitheloid	Human	3
HEK293T	Kidney	Tumor	Human	5
Hepa1-6	Liver	Carcinoma	Mouse	3
HMVEC	Endothelial	Endothelial, microvascular	Human	100
HT-29	Colon	Adenocarcinoma	Human	3
HUVEC	Umbilicus	Endothelial cells	Human	100

Cell line	Tissue	Cancer/cell type	Species	MOI
Jurkat	Blood	Leukemia, Acute T Cell	Human	10
LLC-1	Lung	Carcinoma	Mouse	6
LNCaP	Prostate	Carcinoma	Human	5
MM200	Skin	Melanoma	Human	5
MCF-7	Breast	Adenocarcinoma	Human	2
MDA-MB-231	Breast	Adenocarcinoma	Human	1
MM-AN	Skin	Melanoma, metastatic	Human	16
MMC	Breast	Carcinoma	Mouse	4
MRC-5	Lung, embryonic	Fibroblasts	Human	1
NB4	Blood	Leukemia, acute promyelocytic	Human	10
PC12	Adrenal gland	Pheochromocytoma	Rat	20
SKOV-3	Ovary	Adenocarcinoma	Human	15
U-2 OS	Bone	Osteosarcoma	Human	5

Lentivirus transduction

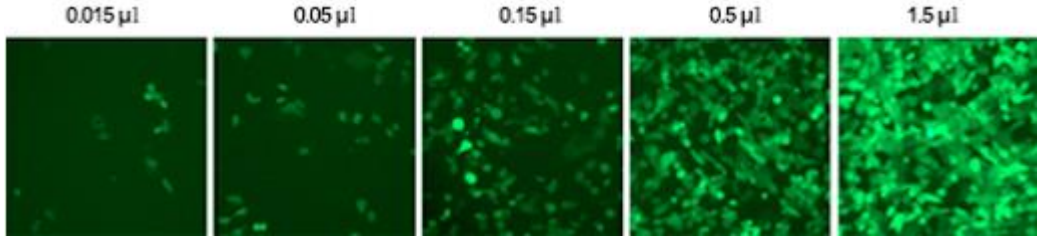
Determining MOI

- Transduce cells with varying dilutions of lentivirus
- Can be any lentivirus, but important to have good marker
- Best to use fluorescent reporter
- Detailed protocol for transducing cells: http://www.genecopoeia.com/wp-content/uploads/2018/03/Lentivirus-protocol-GeneCopoeia.pdf

Lentivirus transduction

GeneCopoeia Pre-made control particles

- Great for pre-determining optimal MOI of a cell line
- Express fluorescent reporters such as GFP, YFP, & mCherry
- Available with different selection markers, such as Puromycin and neomycin
- High titers (>1 x 10E8 TU/ml)
- Pre-made and available for next-day shipping



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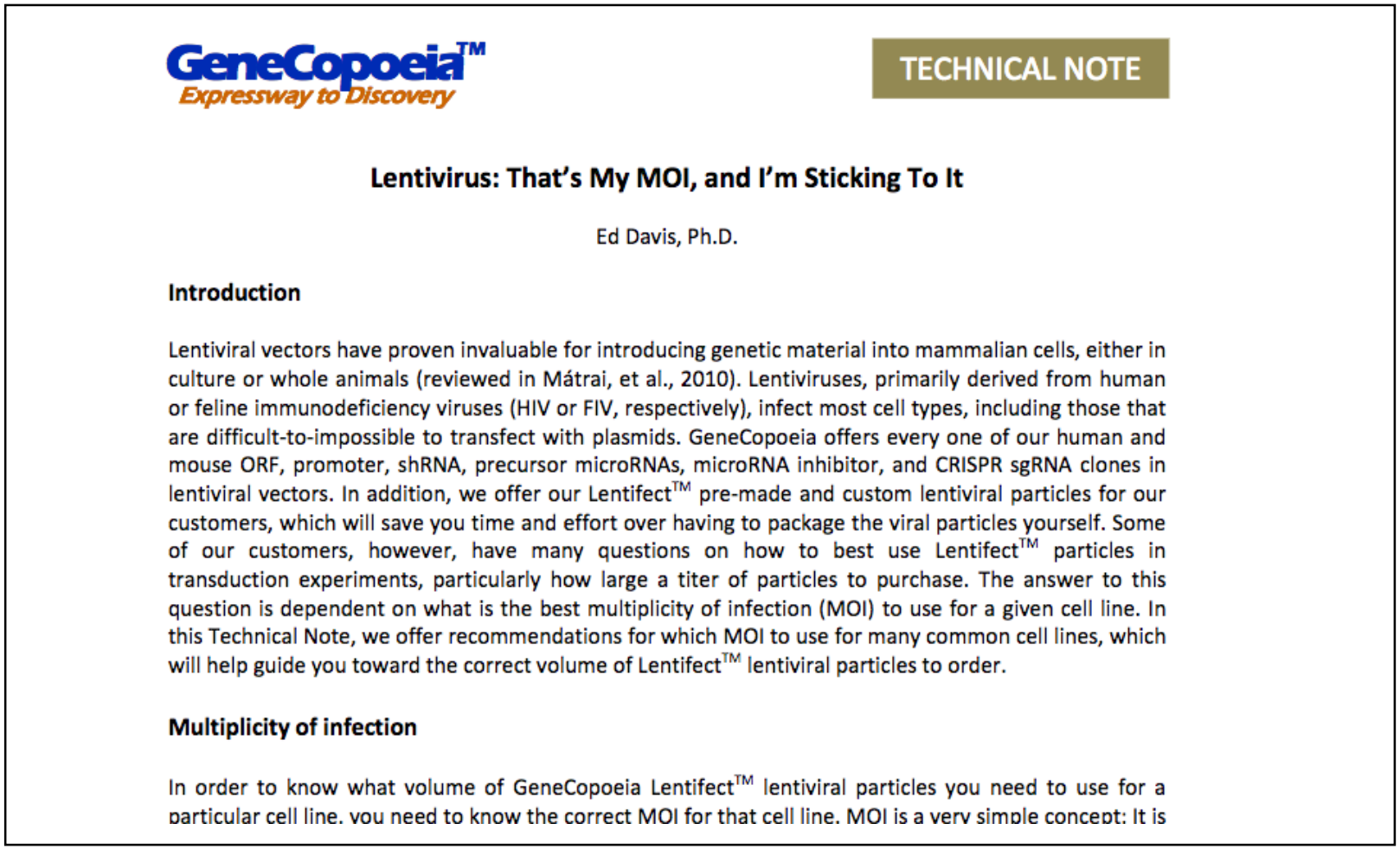
Lentivirus transduction

Multiplicity of infection (MOI)



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TECHNICAL NOTE

Lentivirus: That's My MOI, and I'm Sticking To It

Ed Davis, Ph.D.

Introduction

Lentiviral vectors have proven invaluable for introducing genetic material into mammalian cells, either in culture or whole animals (reviewed in Mátrai, et al., 2010). Lentiviruses, primarily derived from human or feline immunodeficiency viruses (HIV or FIV, respectively), infect most cell types, including those that are difficult-to-impossible to transfect with plasmids. GeneCopoeia offers every one of our human and mouse ORF, promoter, shRNA, precursor microRNAs, microRNA inhibitor, and CRISPR sgRNA clones in lentiviral vectors. In addition, we offer our Lentifect™ pre-made and custom lentiviral particles for our customers, which will save you time and effort over having to package the viral particles yourself. Some of our customers, however, have many questions on how to best use Lentifect™ particles in transduction experiments, particularly how large a titer of particles to purchase. The answer to this question is dependent on what is the best multiplicity of infection (MOI) to use for a given cell line. In this Technical Note, we offer recommendations for which MOI to use for many common cell lines, which will help guide you toward the correct volume of Lentifect™ lentiviral particles to order.

Multiplicity of infection

In order to know what volume of GeneCopoeia Lentifect™ lentiviral particles you need to use for a particular cell line, you need to know the correct MOI for that cell line. MOI is a very simple concept: It is

Download from:

http://www.genecopoeia.com/wp-content/uploads/2015/01/Technical-Note-Lentiviral-MOI-201411.pdf

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Lentivirus transduction

Generating stable cell lines

- Lentivirus stably integrates by default
- Can use antibiotic selection to create a stable pool, or use to generate single clones
- Alternatively, can use fluorescence sorting
- General protocol for infecting cells with lentivirus: http://www.genecopoeia.com/wp-content/uploads/2018/03/Lentivirus-protocol-GeneCopoeia.pdf

Lentivirus transduction

Stable pool vs. single clone

- Stable pools are much less labor intensive, and are often sufficient for most short-term studies
- However, lentivirus integration is random.
- Stable pool population will be mixed. Subpopulations will have:
  - Insertions at different locations in the genome
  - Different # of copies of insertions

Lentivirus transduction

Why should I do single clone isolation?

- Can isolate single clones with varying levels of expression
- Single clones might be more stable over time
- “Clean up” genetic background

Lentivirus transduction

Clonal isolation methods

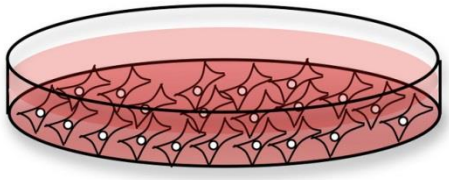
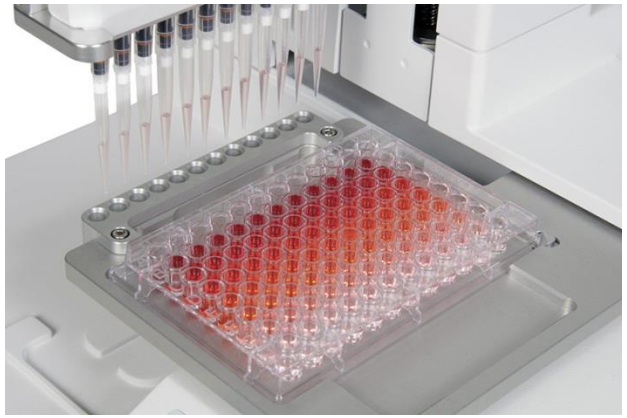


Plate for single colonies and pick off dish

OR

Fluorescence sorting

OR



Do serial dilutions in multi-well plates

- Minimizes potential effects of unwanted modifications resulting from random insertion, cell division or off-targeting

Lentivirus transduction

What do I do after infection?

- Depends on what you are expressing in the lentivirus (ORF, promoter, shRNA, CRISPR, etc.)

Type of insert	Detection methods	GeneCopoeia products
ORF	• Western blot • Fluorescence • qRT-PCR • Luciferase assays	• Labeled secondary antibodies • All-In-One™ First Strand cDNA synthesis kit • BlazeTaq™ qPCR mix • Luc-Pair™ firefly and *Renilla* luciferase assay kits
Promoter-reporter	• Dual luciferase assay • Fluorescence	• Secrete-Pair™ *Gaussia* luciferase assay kits
CRISPR (gene KO, interference, or activation)	• PCR-based mutation detection • qRT-PCR	• IndelCheck™ Insertion/deletion detection system • All-In-One™ First Strand cDNA synthesis kit • BlazeTaq™ qPCR mix
shRNA (gene knockdown)	• Western blot • qRT-PCR	• Labeled secondary antibodies • All-In-One™ First Strand cDNA synthesis kit • BlazeTaq™ qPCR mix
miRNA (precursor or inhibitor)	• qRT-PCR	• All-In-One™ First Strand cDNA synthesis kit • BlazeTaq™ qPCR mix

Outline

- Lentivirus: Applications
- Lentivirus: Technology overview
- Packaging lentivirus
- Transduction with lentivirus
- Things to watch out for

Lentivirus transduction

Things to watch out for

- Expression: Sometimes, stable cell lines lose gene expression over time. Consider single clone isolation and banking of multiple clones.
- Titer. If titer is low, could the insert be too big? Or are the cells unhealthy?
- Safety. Make sure to handle under BSL-2 conditions. Cover up all exposed skin-particles can infect you too!

Summary

- Lentiviral vectors are engineered vehicles that are highly efficient for DNA delivery to a wide variety of dividing and non-dividing cells.
- Packaging lentivirus is straightforward, but requires many components and experience to achieve good results.
- Infecting cells with lentivirus is simple, but requires much consideration of factors such as titer, MOI, the health of the cells, and whether you need stable pools or single clones.
- GeneCopoeia provides solutions for virtually every phase of the workflow for using lentivirus to establish stable cell lines, from lentiviral plasmids, packaging reagents and accessories, lentiviral particle production, qPCR reagents, & more.

Blog

Subscribe to our blog!

Stay up-to-date on the latest technologies
behind GeneCopoeia's products

Check it out here:

http://genecopoeia-2432656.hs-sites.com/blog/new-discoveries-using-lentiviral-vectors-for-screening-cancer-cells

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Thank You!

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