
Product Manual

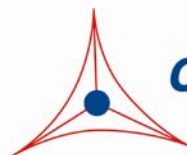
ViraSafe™ miRNA Lentiviral Expression System, Pantropic

Catalog Number

VPK-220-PAN

1 kit

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures



CELL BIOLABS, INC.
Creating Solutions for Life Science Research

Introduction

MicroRNAs (miRNAs) are 18–24 nucleotide RNA molecules that regulate the stability or translational efficiency of target mRNAs. These regulatory RNAs function by acting as sequence-specific guides which recruit a large protein complex known as the RNA-induced silencing complex (RISC) to target mRNAs which are subsequently silenced. Diverse functions have been attributed to miRNAs including the regulation of cellular differentiation, proliferation, and apoptosis. Moreover, significant evidence has accumulated implicating a fundamental role for miRNAs in the development of cancer. miRNAs are initially transcribed as long precursor transcripts known as primary microRNAs (pri-miRNAs). Within these transcripts, the mature miRNA sequences are found in ~60–80 nucleotide hairpin structures. Mature miRNAs are generated from pri-miRNAs by sequential processing (Figure 1). Pri-miRNAs are initially recognized in the nucleus by the microprocessor complex which includes as core components the RNase-III enzyme Drosha and its obligate partner DGCR8. This complex excises the hairpin structure containing the mature miRNA sequence. The liberated hairpins, referred to as precursor miRNAs (pre-miRNAs), are recognized by the nuclear export factor exportin 5 which transports them to the cytoplasm. There, the RNase-III enzyme Dicer performs a second cleavage to generate a double-stranded 18–24 nucleotide RNA molecule. The RISC then associates with this RNA duplex and unwinds it. Generally, only one strand is stably incorporated into the RISC; the other is discarded and rapidly degraded. miRNAs guide the RISC to target messages that are subsequently cleaved or translationally silenced.

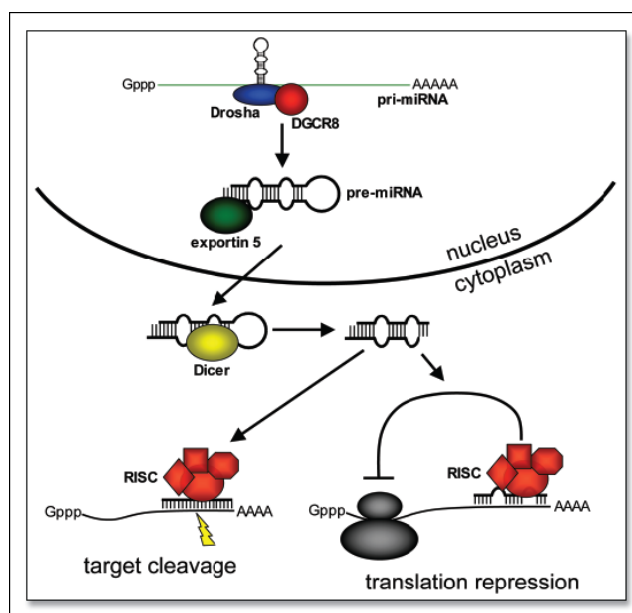


Figure 1. miRNA Biogenesis and function

Lentivirus vector based on the human immunodeficiency virus-1 (HIV-1) has become a promising vector for gene transfer studies. The advantageous feature of lentivirus vector is the ability of gene transfer and integration into dividing and non-dividing cells. The pseudotyped envelope with vesicular stomatitis virus envelope G (VSV-G) protein broadens the target cell range. Lentiviral vectors have been shown to deliver genes to neurons, lymphocytes and macrophages, cell types that previous retrovirus vectors could not be used. Lentiviral vectors have also proven to be effective in transducing brain, liver, muscle, and retina *in vivo* without toxicity or immune responses. Recently, the lentivirus

system is widely used to integrate siRNA efficiently in a wide variety of cell lines and primary cells both *in vitro* and *in vivo*.

Lentivirus particles are produced from 293T cells through transient transfection of plasmids that encode for the components of the virion (Figure 2). Due to safety concerns regarding the infectious nature of HIV-1, recent lentiviral packaging systems have separated the viral components into 3 or 4 plasmids. However, these systems still present a small chance of generating replication-competent lentivirus upon recombination. In addition, most commercial lentiviral packaging systems provide plasmids containing the viral structure proteins in a premixed formulation, making it nearly impossible to optimize the ratio of the various plasmids for your particular experiment and host cell. Also, most commercial lentivirus transfer vectors contain promoters, antibiotic selection markers and/or reporter genes which may not be optimal or even suitable for your particular expression studies.

Cell Biolabs' ViraSafe™ Lentiviral Expression System provides a much safer method to package lentivirus, while still providing high viral titers. The sequence homology with native HIV-1 has been reduced by 80-90% even compared with other commercial third-generation packaging systems. In addition, each plasmid is provided separately rather than in a packaging mixture. This allows you the flexibility to amplify individual plasmids and optimize the ratio of plasmids for your experiment.

Cell Biolabs' pSMPUW-miR-GFP/Puro Lentiviral Expression Vector is designed to clone and express an individual miRNA precursor in its native context while preserving putative hairpin structures to ensure biologically relevant interactions with endogenous processing machinery and regulatory partners, leading to properly cleaved microRNAs. Individual miRNA precursor from any species can be cloned into PshAI site (Figure 3).

The pSMPUW-miR-GFP/Puro lentiviral expression vector contains the following features:

- **miRNA Processing** – miRNA stem loop precursor in its native context is cloned in PshAI site. To preserve the putative hairpin structure and proper endogenous processing, miRNA stem loop sequence is flanked by its native intron sequence.
- **EF-1 α Promoter** - ensures a high level of expression in mammalian cells
- **GFP-Puro Fusion Marker** - to monitor cells positive for expression and stable selection with either GFP or puromycin resistance.
- **pUC Origin** - for high copy replication and maintenance of the plasmid in *E. coli*
- **Kanamycin Resistance Gene** - for selection in *E. coli*

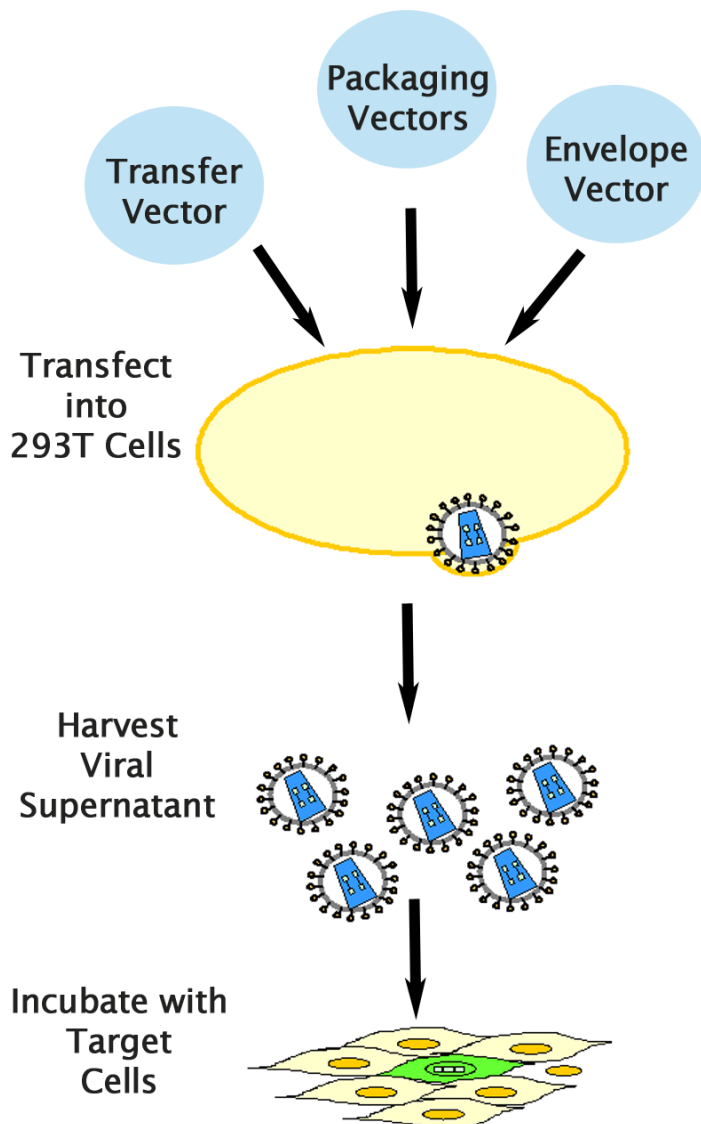
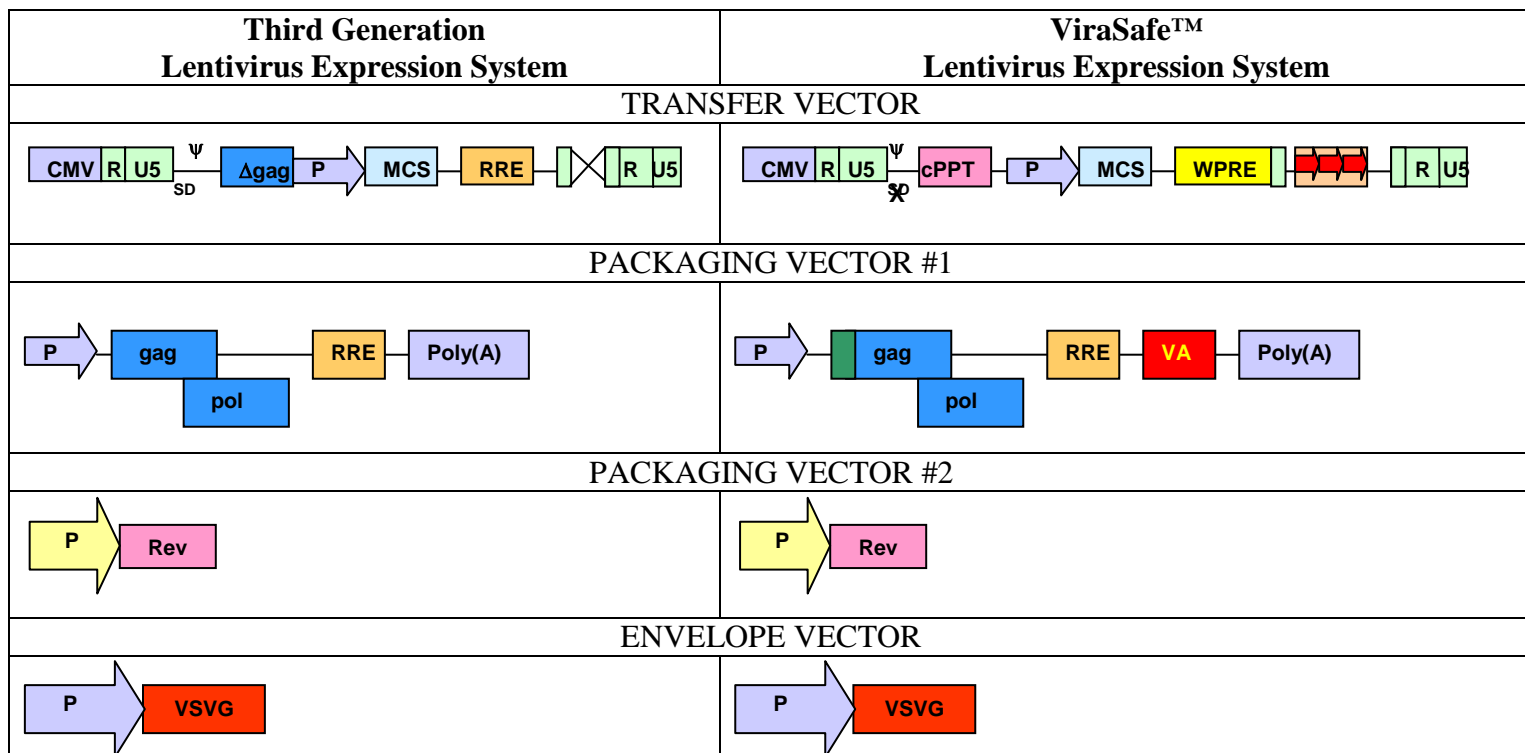


Figure 2. Lentivirus Production in 293T Cells

Related Products

1. LTV-100: 293LTV Cell Line
2. LTV-200: ViraDuctin™ Lentivirus Transduction Kit
3. LTV-300: GFP Lentivirus Control
4. VPK-090: ViraBind™ Lentivirus Concentration and Purification Kit
5. VPK-107: QuickTiter™ Lentivirus Titer Kit (Lentivirus-Associated HIV p24)
6. VPK-108-H: QuickTiter™ Lentivirus Quantitation Kit (HIV p24 ELISA)
7. VPK-205: ViraSafe™ Lentivirus Packaging System, Ecotropic
8. VPK-211: pSMPUW Universal Lentiviral Expression Vector (Promoterless)

Unique Elements of the ViraSafe™ Lentivirus Expression System



Vector Name	Element	Name	Benefits compared to other 3 rd Generation Systems
ELEMENTS ADDED			
Transfer Vector		Central Polypurine Tract	<ul style="list-style-type: none"> Increased gene expression levels
		Hybrid 3' LTR Poly(A)	<ul style="list-style-type: none"> Increased safety: prevents read-through transcription Increased viral titer: vector transcript more stable in packaging cells
		WPRE	<ul style="list-style-type: none"> Increased viral titer
Packaging Vector #1		Codon Wobble	<ul style="list-style-type: none"> Increased safety: reduces sequence homology
		Adenovirus VA	<ul style="list-style-type: none"> Increased viral titer
ELEMENTS REMOVED			
Transfer Vector		Gag sequence	<ul style="list-style-type: none"> Increased safety: reduces sequence homology
		Rev-Responsive Element	<ul style="list-style-type: none"> Increased safety: reduces sequence homology

Kit Components

1. pSMPUW-miR-GFP/Puro Lentiviral Cloning and Expression Vector (Part No. VPK-220): One 40 μ L vial at 0.25 mg/mL. The plasmid is kanamycin resistant.
Note: Please see Figure 2 for important instructions on bacterial culture of this plasmid.
2. pRSV-Rev Packaging Vector (Part No. 320022): One 40 μ L vial at 0.25 mg/mL.
3. pCMV-VSV-G Envelope Vector (Part No. 320023): One 40 μ L vial at 0.25 mg/mL.
4. pCgpV Packaging Vector (Part No. 320024): One 40 μ L vial at 0.25 mg/mL.
5. pSMPUW-LacZ Control Vector (Part No. 320025): One 40 μ L vial at 0.25 mg/mL containing a nuclear localized LacZ driven by MND retroviral LTR promoter. The plasmid is kanamycin resistant.
Note: Please see Figure 2 for important instructions on bacterial culture of this plasmid.

Materials Not Supplied

1. 293T cells: we recommend 293LTV Cell Line (Cat.# LTV-100) for high titer production of lentivirus.
2. Cell Culture Medium
3. Transfection Reagents

Storage

Upon receipt, store all other kit components at -20°C until their expiration dates.

Safety Considerations

Remember that you will be working with samples containing infectious virus. Follow the recommended NIH guidelines for all materials containing BSL-2 organisms. The ViraSafe™ Lentiviral Expression System is designed to minimize the chance of generating replication-competent lentivirus, but precautions should still be taken to avoid direct contact with viral supernatants.

pSMPUW-miR-GFP/Puro Lentiviral Expression Vector

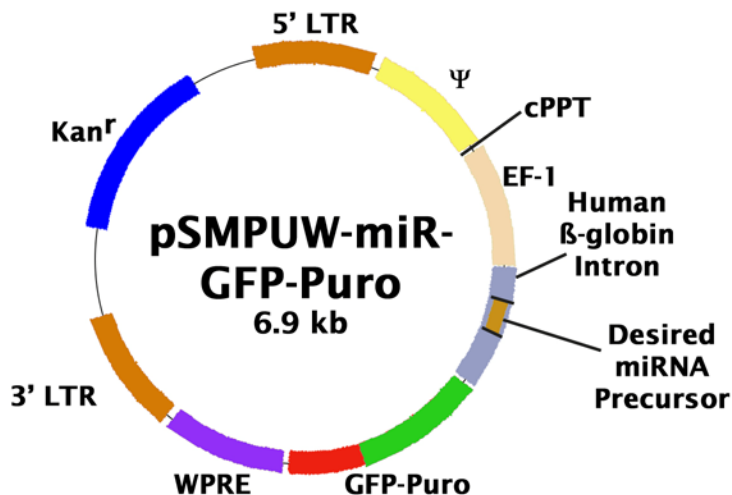


Figure 3. pSMPUW-miR-GFP/Puro Lentiviral Cloning and Expression Vector (6891 bp, **Kanamycin**-resistant). Hind III Digestion: 1331 bp, 1982 bp, 3578 bp.

Note: Bacterial culture of pSMPUW vectors should be done in medium containing 10 µg/mL Kanamycin. For maximal plasmid yield and quality, we recommend Stbl3 endoA1+ competent cells (Invitrogen) and treatment with alkaline proteinase (Promega #A1441 or Sigma #P8038) for 4-5 min using 10 units of proteinase per mL of bacterial lysate before adding neutralization solution.

miRNA precursor cloning site (PshAI): GATTAGTTCTCGAGGATCCGACTG/AAGTCGCTAGCTCGAGCTTTTGGG
PshAI

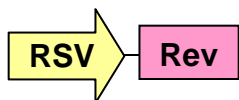


Figure 4: pRSV-Rev Packaging Vector (4180 bp, **Ampicillin**-resistant). EcoRI Digestion: 300 bp + 3880 bp

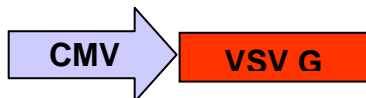


Figure 5: pCMV-VSV-G Envelop Vector (6051 bp, **Ampicillin**-resistant). EcoRI Digestion: 787 bp + 1668 bp + 3596 bp.

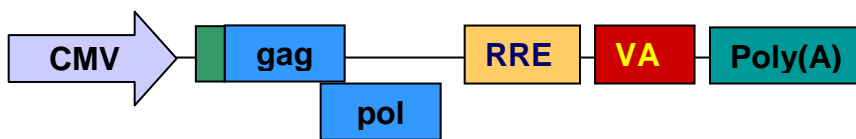


Figure 6: pCgpV Packaging Vector (9118 bp, **Ampicillin-resistant**). Pst I Digestion: 927 bp + 1424 bp + 6767 bp.

miRNA Precursor Cloning

All of our premade human and mouse miRNA precursor clones in pEP-miR and pEGP-miR vectors are based on the following design, and the resulting overexpression of the mature miRNA is confirmed by Northern blot or Real Time PCR. Here we use human let-7a-2 miRNA as an example:

1. Download desired miRNA stem loop sequence from Sanger's miRNA database:

<http://microrna.sanger.ac.uk/sequences/>

Homo sapiens let-7a-2 stem-loop structure

```

uu  g  u                uagaa ua a
agg gag uag agguuguauaguu    u c u
||| ||| ||| ||||| ||||| ||||| | | c
ucc uuc auc uccgacaugucaa    a g a
  -u  g  c                --uag gg a

```

Homo sapiens let-7a-2 stem-loop sequence

AGGUUGAGGUAGUAGGUUGUUAUAGUUUAGAAUUAUCAUCAAGGGAGAUAAACUGUACAGCCUCCUAGCUUUCCU

2. Blast search miRNA stem loop sequence: <http://blast.ncbi.nlm.nih.gov/Blast.cgi>

> [ref|NT_033899.7|Hs11_34054](#) Homo sapiens chromosome 11 genomic contig,
reference assembly
Length=38509590

```

Query 1          AGGTTGAGGTAGTAGGTTGTATAGTTTAGAATTACATCAAGGGAGATAACTGTACAGCCT 60
                |||
Sbjct 25579717   AGGTTGAGGTAGTAGGTTGTATAGTTTAGAATTACATCAAGGGAGATAACTGTACAGCCT
25579658
Query 61         CCTAGCTTTCCT 72
                |||
Sbjct 25579657   CCTAGCTTTCCT 25579646

```

3. PCR and Cloning:

- 1) Add 100 base native flank sequence to both upstream and downstream of the miRNA stem loop.

Human let-7a-2 miRNA precursor sequence including the 100 base flank sequences on both ends of the stem loop: let-7a-2 stem-loop sequence is underlined.

```
GCCCAAATAGGTGACAGCACGATGAATCATTATAAGACTAACTTGTAATTTCCCTGCTTAAGAA
ATGGTAGTTTTCCAGCCATTGTGACTGCATGCTCCCAGGTTGAGGTAGTAGGTTGTATAGTTTA
GAATTACATCAAGGGAGATAACTGTACAGCCTCCTAGCTTTCTTGGGTCTTGCACTAAACAAC
ATGGTGAGAACGATCATGATTCTCCAGGCCTTTTCTCCCTATGAAAGGTAAGATTGGGTACGA
TTATTTTATGGTATTT
```

2) Design PCR primer.

For human let-7a-2 miRNA precursor:

Forward PCR Primer: GCCCAAATAGGTGACAGCACG

Reverse PCR Primer: AAATACCATAAAAATAATCGTA

3) PCR the miRNA precursor from genomic DNA and clone the blunt-end PCR fragment into the PshAI site of the expression vector.

4) Validate the insert by DNA sequencing.

Forward Sequencing Primer: TTTGCACCATTCTAAAGAAT

Reverse Sequencing Primer: AAACCTCTTACATCAGTTAC

Lentivirus Production

1. One day before transfection, plate sufficient 293T cells or 293LTV cells (Cat. # LTV-100) to achieve 70-80% confluence on the day of transfection.
2. Transfect cells by Calcium Phosphate or other transfection reagents.

Note: We suggest transfecting cells with FuGENE® Transfection Reagent (Roche Applied Science) or Lipofectamine™ Plus (Invitrogen). We recommend the ratio of vectors at 3:1:1:1 (pSMPUW: pCMV-VSV-G:pRSV-REV:pCgpV).

3. Harvest lentiviral supernatant 36-72 hours after transfection. Supernatant can be harvested 2 or 3 times, every 12 hours. Keep it at 4°C over the collecting period.
4. Pool the collected supernatants, centrifuge 5 minutes at 1500 rpm to remove cell debris and filtrate on 0.22 µm.
5. Supernatants can be used directly or purified/concentrated if needed. For long term storage, store supernatant at -80°C in aliquots.

Post-Packaging Considerations

Packaging your lentivirus is only the first step to ensuring successful expression of your gene. The following steps should be considered prior to infection of your host cell:

1. **Concentration and purification of your lentivirus:** Because of the latent nature of lentivirus, it is imperative that your virus be highly concentrated before infecting your host cell. Also, impurities

from your viral supernatant can decrease the efficiency of infection. We recommend using Cell Biolabs' ViraBind™ Lentivirus Concentration and Purification Kit (Catalog # VPK-090).

- 2. Measure the titer of your lentivirus:** This is an important step to ensure consistent viral transduction into your host cell. However, QPCR or stable clone counting can take as much as 1-2 weeks to perform. Traditional p24 ELISA kits can greatly overestimate your lentiviral titer. Our advanced p24 ELISA, QuickTiter™ Lentivirus Titer Kit (Catalog # VPK-107), uses exclusive technology that eliminates free p24 from your supernatant, giving you much more accurate lentiviral titers. Results are obtained in 6-18 hours.
- 3. Use transduction reagents to increase infection efficiency:** Many cells are difficult to infect with lentivirus, and without supplemental reagents transduction efficiencies can be low. Reagents such as Polybrene® can help, but are often insufficient. Cell Biolabs' proprietary reagents in our ViraDuctin™ Lentivirus Transduction Kit (Catalog # LTV-200) form a super-complex with your virus to increase transduction efficiencies by promoting virus and cell interaction.

Example of Results

The following figure demonstrates typical results seen with Cell Biolabs ViraSafe™ Lentiviral Expression System. One should use the data below for reference only.

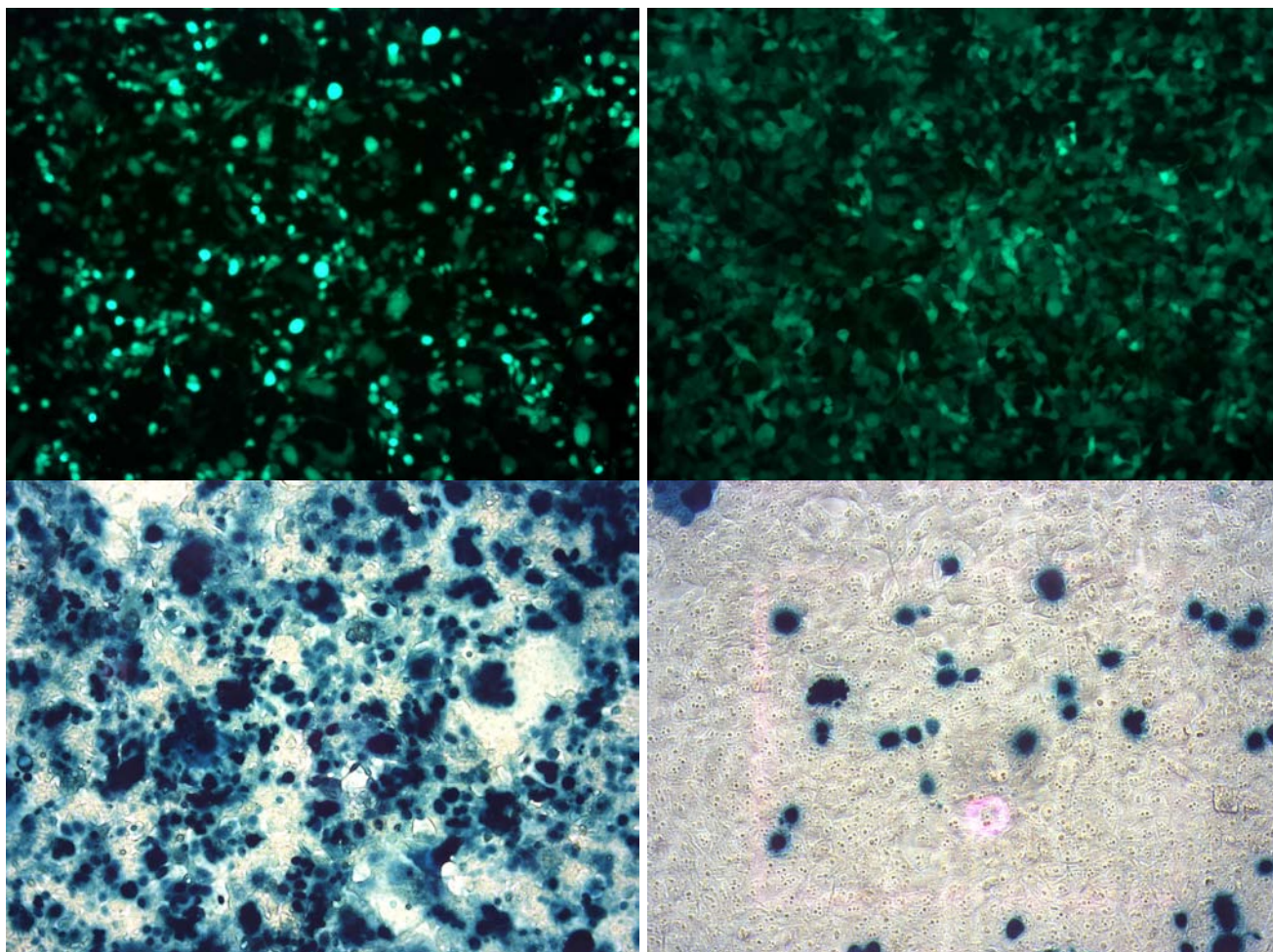


Figure 8: GFP and nLacZ Lentivirus Production and Transduction: Lentiviral supernatant is produced by cotransfecting 293T cells (Cat.# LTV-100) with pSMPUW-miR-GFP/Puro (Cat.# VPK-220) or pSMPUW-MNDnLacZ (Cat.# LTV-402) and ViraSafe™ Lentiviral Packaging System (Cat. # VPK-206). 293AD cells (Cat.# AD-100) are seeded at 100,000 cells/well in a 6-well plate overnight. Cells were infected with GFP or nLacZ lentivirus in the presence of 8 $\mu\text{g}/\text{mL}$ Polybrene for 72 hrs. **Left:** 293LTV Transfection; **Right:** 293AD Transduction.

References

1. microRNA sequences listed in Sanger's miRBase (<http://microrna.sanger.ac.uk/sequences/>).
2. John, B., C. Sander and D. S. Marks (2006) *Methods Mol Biol* **342**: 101-13.
3. Chen, M. et al. (2002). *Nature Genetics* **32(4)**: 670-675.

4. Naldini, L., U. Blomer, P. Gally, D. Ory, R. Mulligan, F. H. Gage, I. M. Verma, and D. Trono (1996) *Science* **272**:263-267.
5. Verma, I. M., and N. Somia (1997) *Nature* **389**:239-242.
6. Kahl C. A., Marsh J., Fyffe J., Sanders D. A., and K. Cornetta (2004) *J Virol.* **78**:1421-30.
7. White S. M., Renda M., Nam N. Y., Klimatcheva E., Zhu Y., Fisk J., Halterman M., Rimel B. J., Federoff H., Pandya S., Rosenblatt J. D., and V. Planelles (1999) *J Virol.* **73**:2832-40.
8. Kafri T., van Praag H., Ouyang L., Gage F. H., and I. M. Verma (1999) *J Virol.* **73**:576-84.

Appendix

Vector Features:

- 1084 ~ 1479: EF-1 α Promoter
- 1512 ~ 2002: human β -globin intron
- 1734 ~ 1743: PshAI
- 2030 ~ 3352: GFP-Puro Fusion (GFP: 2307 ~ 3023; Puro: 3030 ~ 3629)
- 5941 ~ 6756: Kanamycin Resistance Gene (complementary strand)

Plasmid Sequence:

ACTAGTCGGGGTCATTAGTTCATAGCCCATATATGGAGTTCGGCGTTACATAACTTACGGTAAATGGCCCGCTGGCTGACCGCCCAACGACCCCGCCCAT
GACGTC AATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCATTGACGTC AATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACAT
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CGGATAAAATGCTTGATGGTGGGAAGAGGCATAAATTCGCTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCATTGCAACGCTACCTTTGCCATGTT
TCAGAAACACTCTGGCGCATCGGGCTTCCCATACAATCGATAGATTGTGCGACCTGATTGCCGACATTATCGCGAGCCATTATACCCATATAAATCAGC
ATCCATGTTGGAATTTAATCCGCGCCTCGAGCAAGCAGTTTTCCCGTTGAATATGGCTCATTAACACCCTTGATTACTGTTTATGTAAGCAGACAGTTTTATT
GTTTCATGATGATATATTTTTATCTTGTGCAATGTAACATCAGAGATTTTGAGACACAACGTGGTTTAAACAAATAGTCAAAAGCCTCCGGCG

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