Lentiviral miRNA Expression

pLV-miRNA Expression Vector System

Catalog number: mir-p###
Size: 1 ml of bacterial glycerol stock
Storage Temperature: -70°C

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Product Description

Human microRNA (hsa-miRNA) precursors and approximately 100-bp upstream and downstream flanking genomic sequences were PCR amplified and cloned into a self-inactivated (SIN) lentiviral vector to generate pLV-miRNA vectors (Fig. 1). The cloning site of pre-miRNA genomic fragments is within the intron of human EF1α promoter region. The miRNA of interest can be delivered into cells by transient transfection of the pLV-miRNA plasmid or lentiviral transduction while the miRNA lentiviral stock is prepared from cotransfecting HEK 293T cells with the pLV-miRNA plasmid and lentiviral packaging vector mix.

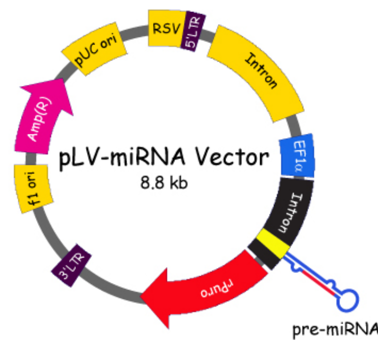


Figure 1. Lentiviral vector for miRNA expression.

Advantages of the Product

1. The pLV-miRNA plasmid is an optimized vector system for miRNA expression. Unlike other available miRNA expression vectors, the miRNA precursor is cloned into the intron of human housekeeping gene EF1α promoter region. Therefore, the process of miRNA in intron minimizes impact on expression of selection marker. Also the miRNA precursor and the selection marker are in the same transcription unit, the miRNA transcriptional activity can be easily monitored.

2. Lentiviral transduction is one of the most effective delivery system to express miRNA, shRNA, cDNA, etc. Unlike the retroviral system, the lentiviral integration is cell cycle independent. The genetic materials encoded by the lentivirus can be efficiently delivered into both dividing and non-dividing cells.

3. The lenti-miRNA viral genome is integrated into the host chromosome, thus the miRNA is stably expressed in transduced cell lines.

4. The human EF1α promoter it is unlikely to be silenced in cells. It has been reported that the activities of some viral promoters such as CMV and SV40 are potentially silenced by DNA methylation after a period of time. The human EF1α promoter, used to express miRNA precursors and the puromycin selection marker, is a housekeeping gene promoter. Therefore, it is unlikely to be silenced by methylation *in vitro* and *in vivo*.

5. The rPuro gene product, expressed from the EF1α promoter, is the red fluorescent puromycin-N-acetyl-transferase. The model cells transduced by lenti-miRNA can not only be selected in the presence of puromycin in the medium, but also display red fluorescence at excitation/emission wavelengths of 587/610 nm (Fig. 2).

6. Each miRNA precursor in the lentiviral vector was cloned from its native context, including the stem-loop precursor sequence and approximately 100-bp upstream and downstream flanking sequences. This ensured that the miRNA was properly expressed and processed, and would function similarly to its endogenous form.

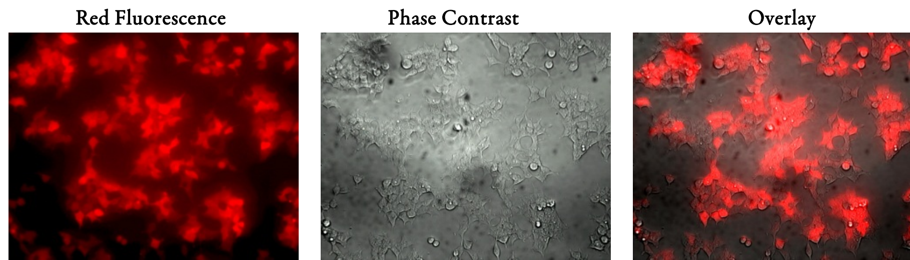


Figure 2. Microscopy images of 293T cells transduced with hsa-mir-24-1 lentivirus.

Protocol

Plasmid DNA Preparation

Day 0: Take a sterilized inoculating loop to scrape off a little of the bacteria from the frozen glycerol stock and streak at the LB agar plate with 100 μg/ml of Ampicillin. Leave plate in a 37°C incubator for overnight.

Day 1:
For miniprep: Inoculate one colony of bacteria in 6 ml of LB medium with 100 μg/ml of ampicillin in a 15-ml tube.
For maxiprep: Inoculate one colony of bacteria in 5 ml of LB medium with 100 μg/ml of ampicillin in a 15-ml tube in the morning (e.g. 9-10am) and transfer the 5-ml culture to 250 ml LB medium with 100 μg/ml of Ampicillin in the afternoon (e.g. 5-6pm).

Grow bacterial culture at 37°C for 20 hours in a floor shaker with 300 rpm.

Day 2:
For miniprep: Spin down whole 6-ml bacterial culture in a desktop centrifuge (e.g. Sorvall RT6000) at 4,000 rpm for 10 min.
For maxiprep: Spin down whole 250 ml bacterial culture in a floor centrifuge (e.g. Sorvall RC5C plus) at 4,000 rpm for 15-20 min.

Discard the supernatant and start plasmid DNA purification.

> Note: We have tested that growing bacteria in 6 ml of LB medium and shaking in a floor shaker with 300 rpm at 37°C for 20 hours gives a better yield of plasmid DNA. We recommend using Favorgen nucleic acid purification kits (http://biosettia.com/php/products-nucleic-acid-purification) to prepare high-yield and good-quality plasmid DNA for future experiments such as transfection and lentiviral production. In general, the DNA yield is around 30-50 μg and 1.0-2.0 mg for each miniprep and maxiprep, respectively. The yield and quality of miniprep plasmid DNA purified by using Favorgen plasmid DNA extraction mini kit (Catalog # FAPDE 001) is sufficient for generating 30 ml of lentivirus.

Production of miRNA lentivirus from pLV-miRNA plasmid

Production of lenti-miRNA viral stocks requires packaging of the lentiviral genomic RNA transcribed from pLV-miRNA vector with HIV-1 *gag*, *pol*, and *rev* gene products and vesicular stomatitis virus G (VSV-G) protein encoded by helper plasmids. In general, at least two helper plasmids are required, with one plasmid expressing the Gag-Pol polyprotein and an accessory protein Rev and the other expressing VSV-G as envelop protein to increase cell tropism. Nevertheless, Gag-Pol and Rev proteins can be expressed from separated plasmids as well. Therefore, a three-plasmid system or a four-plasmid system can be used to generate miRNA lentiviruses, depending on the source and nature of the helper plasmids. In the three-plasmid system, pLV-miRNA is cotransfected with two helper plasmids (Gag-Pol + Rev and VSV-G) into cells, while in the four-plasmid system, pLV-miRNA is cotransfected with three helper plasmids (Gag-Pol, Rev and VSV-G). It is generally considered to be safer to

produce the lentiviral stocks with more helper plasmids, due to the reduced chance of recombination among all vectors that generates replication-competent viruses.

The manufacturers of transfection reagents, the suppliers of lentiviral packaging constructs, and many academic laboratories have provided protocols for producing lentiviral stocks. The following procedure is provided as an example only. We produce miRNA lentiviral stocks in 293T cells using the transfection conditions summarized in a table below.

	10-cm plate		6-well plate	
	3-plasmid system	4-plasmid system	3-plasmid system	4-plasmid system
pLV-miRNA vector	9 μg		1.5 μg	
Gag-Pol + Rev expression vector[1]	6 μg		1.0 μg	
Gag-Pol expression vector[2]		4.5 μg		0.75 μg
Rev expression vector[3]		1.8 μg		0.3 μg
VSV-G expression vector[4]	3 μg	2.7 μg	0.5 μg	0.45 μg
Total plasmid DNA	18 μg		3.0 μg	
Lipofectamine™ 2000	45 μl		7.5 μl	
Total Opti-MEM	3 ml		0.5 ml	
293T cells / vol. of medium	1.0×10^7/5ml		1.7×10^6/1ml	

Below we have listed the commonly used vectors for lentiviral packaging.

[1] For example: pCMV-deltaR8.91 (TRC), psPAX2 (Addgene)

[2] For example: pMDLg/pRRE (Addgene), pLP1 (Invitrogen), pPACKH1-GAG (SBI)

[3] For example: pRSV-REV (Addgene), pLP2 (Invitrogen), pPACKH1-REV (SBI)

[4] For example: pMD.G (TRC), pMD2.G (Addgene), pCMV-VSV-G (Addgene), pVSV-G (SBI), pLP/VSVG (Invitrogen)

> Note: The transfection reagent Lipofectamine™ 2000 (LF2000, Invitrogen) is preferred for transfection. The average lentiviral titers in our preparations are around 5×10^6 - 5×10^7 infection units per ml (IU/ml) when titered with 293T cells.
> Suggestion: We recommend using Biosettia Pre-mixed Lentiviral Packaging System (Catalog # pLV-PACK-500) for optimal lentiviral production.

Day 0: Seed **6.0×10^6** (1.0×10^6) 293T cells in a **10-cm plate** (6-well plate), so that the cell density will be around 1.0×10^7 (1.7×10^6) at the time of transduction.

Day 1: Gently mix **45.0** (7.5) μl LF2000 and **1.5** (0.25) ml Opti-MEM medium and incubate at room temperature for 5 minutes. Meanwhile, gently mix **18.0** (3.0) μg in total of pLV-miRNA vector and helper plasmids mixture into 1.5 (0.25) ml Opti-MEM medium (Invitrogen).

Gently mix DNA and LF2000, incubate at room temperature for 20 minutes to allow DNA and lipid to form complexes. In the meantime, replace the overnight culture medium with **5.0** (1.0) ml DMEM + 10% FBS without antibiotics. Add the **3.0** (0.5) ml DNA-LF2000 complexes to 293T cells.

> Note: We have noticed that fetal bovine serum purchased from different manufacturers may affect the attachment of 293T cells to the bottom of tissue culture plates, resulting in variation in the efficiency of lentiviral production. We recommend switching to a different brand of FBS if 293T cells disattach from plates during lentiviral production.

Day 2: Replace the media containing the DNA-LF2000 complexes with 10.0 (2.0) ml complete medium at 12-16 hours post-transfection.

Day4: Collect supernatants at 48 hours post-transfection and transfer media to a polypropylene storage tube. Spin the virus-containing media at 1300 rpm for 5 minutes to pellet any 293T cells that were carried over during collection. Carefully transfer the supernatant to a sterile polypropylene storage tube.

> Note: Lentiviral stock may be stored at 4 °C for up to 5 days, but should be aliquoted and frozen at -80 °C for long-term storage.
>
> Suggestion: To reduce the number of freeze and thaw cycles, aliquot lentiviral stock to smaller tubes before storage at -80 °C.

Titering the lentiviral stocks

It is important to titer the lentiviral stocks in the cell line of interest to produce consistent results using the equivalent multiplicity of infection (MOI) in experiments. Knowing MOI will help you to control the copy number of lenti-miRNA integrated into the chromosomes of the cells of interest. While titering virus with drug selection, the titering procedure includes selection of stably transduced cells with puromycin and counting the puromycin-resistant cell colonies. Alternatively, a flow cytometry can be used to determine the viral titer by measuring the number of red fluorescent cells. If you are using both methods to titer your lentiviruses, please keep in mind that the titers may be different due to sensitivities of FACS machine and cell lines resistant to drug selection.

A. Determination of lentiviral titers by antibiotics selection.

Day 0: Seed the cells of your choice in a 6-well plate so that the cell density that will be ~25-50% confluent at the time of transduction.

Day 1: Thaw lentiviral stock, gently mix virus and then prepare 2 ml 10-fold serial dilutions ranging from 10^{-3} to 10^{-7} in complete medium containing 5-8 μg/ml polybrene.

Remove the medium from previous day and add 2 ml fresh dilutions into each well.

> Suggestion: Leave one well uninfected for mock control. Spin transduction in a desktop centrifuge (e.g. Sorvall RT6000) at 1,000 × g for 30-60 min at room temperature may be necessary if titering virus in cell lines other than 293T.

Day 2: Replace the medium containing virus and polybrene with 2 ml of complete medium.

Day 3-4: Replace medium with fresh medium containing puromycin to select for stably transduced cells.

> Note: At least 48 hours of transduction allows miRNA lentivirus to integrate into the host genome. The optimal puromycin concentration (range 0.5-5 μg/ml) varies from cell line to cell line.
>
> Suggestion: A pilot experiment should be performed to determine the minimum concentration of antibiotics required to kill the untransduced cells before this experiment.

Day 5-6: Replace medium with 2 ml fresh medium containing puromycin every 2 days.

Day 7-8: Allow puromycin-resistant colonies to form in dilution wells. No live cells should be growing in the mock control well.

> Note: The number of days required for the formation of visible colonies may vary among different cell lines.

Wash wells twice with 2 ml PBS

Stain cells with 1 ml 0.5% crystal violet solution in 20% ethanol and incubate for 30 minutes at room temperature.

Wash wells with distilled water by submerging the plate in a tray full of water, and repeat the wash one more time.

Dry the plate and count the number of blue-stained colonies.

The titer should be the average colony number times the dilution factor.

> Note: The transduction efficiency varies from cell line to cell line. The lentiviral titer in the cell line of your interest may be lower (sometimes more than 10-fold) or higher than the virus titer in commonly used cell lines such as 293T cells.

B. Determination of lentiviral titers by flow cytometry.

Day 0: Seed the cell of your choice in a 6-well plate so that the cell density that will be ~25-50% confluent at the time of transduction.

> Note: The number of cells seeded in the well is required to calculate lentiviral titer later.

Day 1: Thaw lentiviral stock, gently mix virus and then prepare 2 ml 10-fold serial dilutions ranging from 10^{-1} to 10^{-4} in complete medium containing 5-8 μg/ml polybrene.

Remove the medium from previous day and add 2 ml fresh dilutions into each well.

> Suggestion: Leave one well uninfected for mock control. Spin transduction in a desktop centrifuge (e.g. Sorvall RT6000) at 1,000 × g for 30-60 min at room temperature may be necessary if titering virus in cell lines other than 293T.

Day 2: Replace the medium containing virus and polybrene with 2 ml of complete medium.

Day 3-4: Follow your lab protocol to collect and resuspend cells for flow cytometry to determine the percentage of red fluorescent cells.

> Note: At least 48 hours of transduction allows lenti-miRNA to integrate into the host genome.

The titer should be the average number of live fluorescent cells times the dilution factor.

> Note: The transduction efficiency varies from cell line to cell line. The lentiviral titer in the cell line of your interest may be lower (sometimes more than 10-fold) or higher than the virus titer in commonly used cell lines such as 293T cells.

Example: 1×10^5 cells were seeded on Day 0 and the cell doubling time is around 24 hours. The FACS data showed 5% and 40% of red fluorescent cells in the 10^{-3} and 10^{-2} dilution wells, respectively. The lentiviral titer is calculated by multiplying the fraction of transduced cells by 2×10^5 (the expected number of cells in the well on Day 1, the time of transduction), and by the dilution factor.

$0.05 \times (2 \times 10^5) \times 10^3 = 1 \times 10^7$

$0.4 \times (2 \times 10^5) \times 10^2 = 8 \times 10^6$

The average lentiviral titer is 9×10^6 IU/ml

Lentiviral Transduction for Functional Analysis.

Day 0: Seed cells at appropriate density.

> Suggestion: Plate cells so that cell density will be ~10-25% confluent at the time of transduction.

Day 1: Transduction. Remove the medium from the tissue culture plate by aspiration and replace it with fresh complete medium containing 5-8 μg/ml polybrene. Gently mix lentivirus with pipette tip, and add appropriate amount of virus to each well.

> Note: (1) Polybrene may be toxic to some cell lines. (2) The non-concentrated and non-purified lentiviral stock collected from 293T supernatant may contain substances affecting the target cell growth, especially when a large volume of low-titer lentiviral stock is added. In case the lentiviral preparation inhibits cell growth, concentration and purification of the viruses may be required. (See below for a protocol of virus concentration) Alternatively, incubation time for transduction can be shortened to hours. For example, the virus-containing medium may be replaced with fresh medium after one hour of transduction.
>
> Suggestion: Transduce cells at multiplicity of infection (MOI) = 1, 5, 10, 25, and 50 to determine the optimal miRNA expression. Spin transduction in a desktop centrifuge (e.g. Sorvall RT6000) at 1,000 × g for 30-60 min at room temperature helps increase of transduction efficiency.

Day 2: Replace the transduction medium with fresh complete medium to remove lentivirus and polybrene.

Day 3-4: Select transduced cells (>50% confluence is recommended) with medium containing puromycin.

> Note: The optimal puromycin concentration varies from cell line to cell line (ranging from 0.5-5 μg/ml).
>
> Suggestion: A pilot experiment should be performed to determine the minimum concentration of puromycin required to kill the untransduced cells before this experiment.

Day 6+: Assay transduced cells.

> Suggestion: Expand the culture of cell lines stably expressing miRNA and store the cell line stocks in liquid nitrogen before analyzing the cells.

Note: We have noticed that the level of rPuro gene expression may vary among different cell lines. Therefore, please keep in mind that the red fluorescence may provide a general idea whether the model cells are transduced, but may not quantitatively reflect the percentage of cells transduced by Lenti-miRNA. It is highly recommended to determine the transduction efficiency by puromycin selection, since in some cells, the expression level of red fluorescent puromycin-N-acetyl-transferase is enough to render the cells resistant to puromycin, but not enough for the cells to display detectable red fluorescence.

Please be aware that one miRNA might suppress multiple genes at the same time, some phenotypes may come up or disappear during long-term suppression.

Optional: Concentration of lentivirus by ultracentrifugation

1. Filter lentivirus through a 0.45 μm filter.

2. Centrifuge at 25,000 rpm for 90 minutes in a SW-28 or SW-41 rotor.

3. Discard the supernatant and use a Pasteur pipette with an attached P100 tip to carefully remove the remaining medium.

4. Gently resuspend viral pellet in 1/100 volume of DMEM. Let the virus suspension sit for overnight at 4° C.

5. On the following day, mix gently, aliquot and freeze virus.

Safety Guidelines for Working with Lenti-miRNA.

The recombinant lentiviruses have been designated as Level 2 organisms by NIH and CDC. A Biosafety Level 2 (BSL-2) facility is required in order to work with lentiviruses. The information of Biosafety in Microbiological and Biomedical Laboratories (BMBL) can be downloaded from the following link:
http://www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf

Please be aware that you are working with media containing lentiviral particles which could transduce human cells.

Examples of miRNA Expression

To validate the expression of miRNAs from the precursor and flanking sequences inserted in the EF1α intron in the Lenti-miRNA expression system, a variety of analyses such as RT-PCR, Northern Blot, functional analysis and reporter assays have been carried out. The results are shown below:

RT-PCR

HEK 293 cells were transduced with Lenti-miRNA viruses expressing let-7a-1, let-7a-2, and let-7a-3 (catalog# mir-LV001-003; Fig. 3A), or LV-[hsa-mir-ctrl] (catalog# mir-LV000; Fig. 3B) in which no DNA sequence was inserted into the EF1α intron. The total RNAs were extracted 72 hours post-transduction, followed by RT-PCR to detect the transcripts of let-7a precursors. The forward primers used in PCR are let-7a-specific (let-7a-1, gccttcctgtggtgctcaactgtg; let-7a-2, taacttgtaatttccctgcttaag; let-7a-3, gtccccaggaggtgcctctggaag), while the reverse primers are specific for the EF1α intron (cgtactccaaaagctcgagctagc and ctcagtgtggggaaactccatcgc for primer sets 1 and 2, respectively) (Fig. 3).

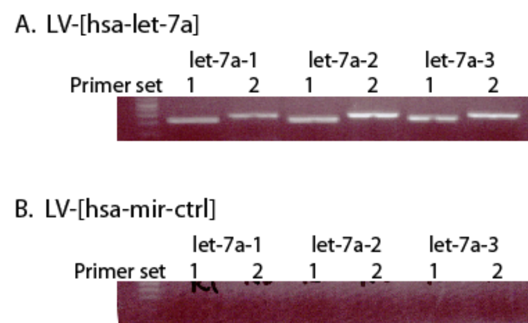


Figure 3. Detection of the expression of let-7a transcripts from the Biosettia LV-[hsa-let-7a] lentiviral transductions by RT-PCR.

Real-Time PCR

The human foreskin (BJ) cells were transduced with LV-[hsa-mir-ctrl] (catalog# mir-LV000) or LV-[hsa-mir-17_18a _19a_20a_19b-1 _92a-1] (catalog# mir-LVc02) viruses. The total RNA were extracted 72 hours post-transduction, followed by TaqMan miRNA assays (Applied Biosystems). The relative level of individual miRNA expression was determined by comparing with the negative control, LV-[hsa-mir-ctrl] (Fig. 4).

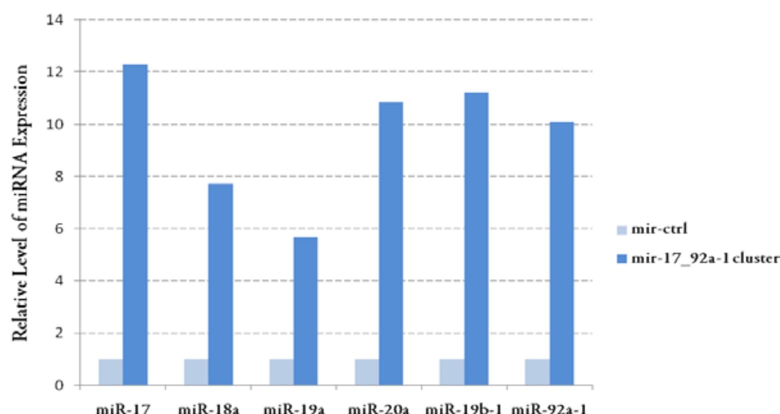


Figure 4. Detection of Lenti-miRNA by real-time PCR.

Northern Blot

HEK 293 cells were transduced with LV-[hsa-mir-ctrl] (catalog# mir-LV000) or LV-[hsa-let-7g] (catalog# mir-LV010) viruses. The total RNA were extracted 72 hours post-transduction and subjected to Northern Blotting analysis as described [1]. The total RNA from Hela cells with high expression levels of let-7g was used as positive control (Fig. 5, lane 1). Co-transduction of Lin28 with LV-[hsa-let-7g] reduced the expression of let-7g (Fig. 5, lane 5), confirming a reported finding that the let-7g biogenesis is suppressed by Lin28 [2, 3]

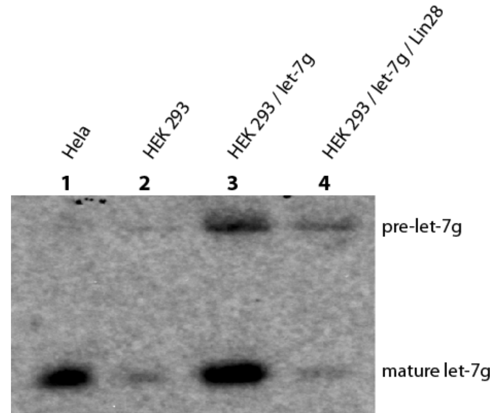
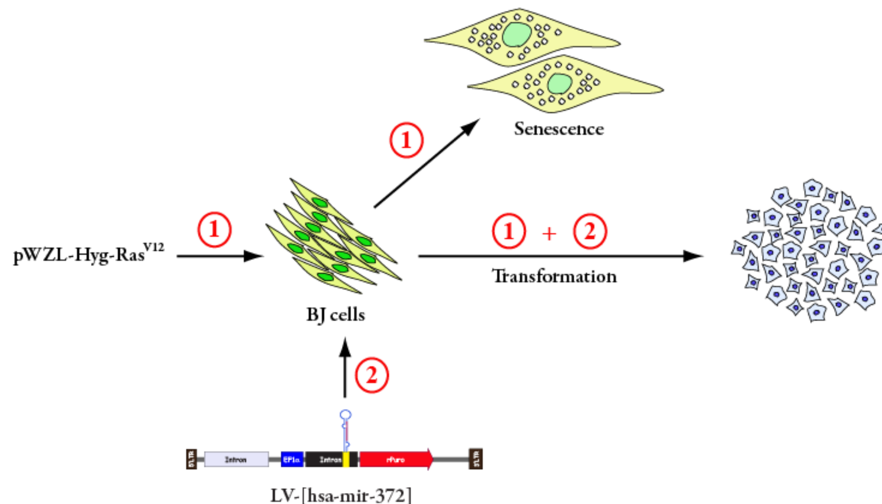


Figure 5. Detection of pre-let-7g and mature let-7g expressed from the Biosettia LV-[hsa-let-7g] lentiviral transductions by Northern Blotting.

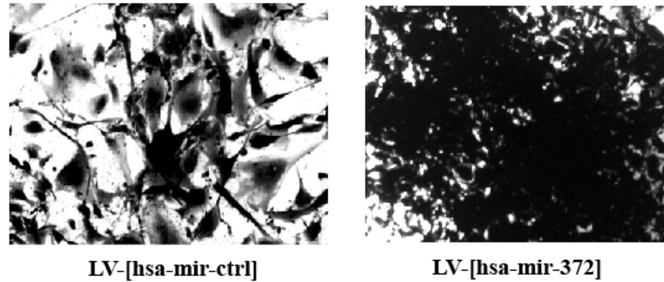
Functional Analysis

It has been shown that the presence of microRNAs mir-372 and mir-373 in human primary fibroblast cells allows the cells to bypass H-Ras^{V12} mediated growth arrest [4]. We have transduced BJ cells with retrovirus pWZL-Hyg-Ras^{V12}. After selection with hygromycin, these cells were transduced with LV-[hsa-mir-ctrl], LV-[hsa-mir-372], or LV-[hsa-mir-373] (catalog# mir-LV000, 219, and 220) 4 days after pWZL-Hyg-Ras^{V12} transduction. The BJ cells were grown for another 14 days before microscopic analysis and crystal violet staining (Fig. 6B). Our data showed that mir-372 and mir-373 abolished H-Ras^{V12}-dependent senescence, consistent with the results reported by Voorhoeve *et al.* [4].

A.



B.



C.

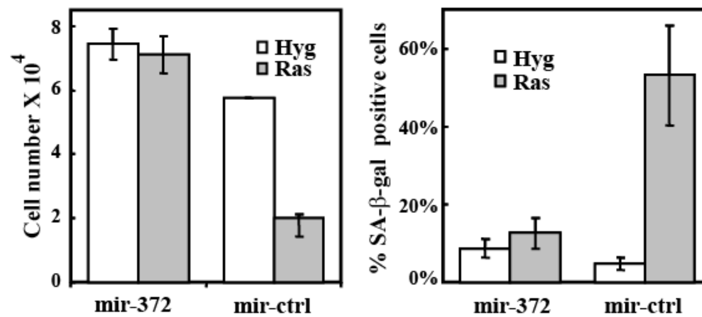
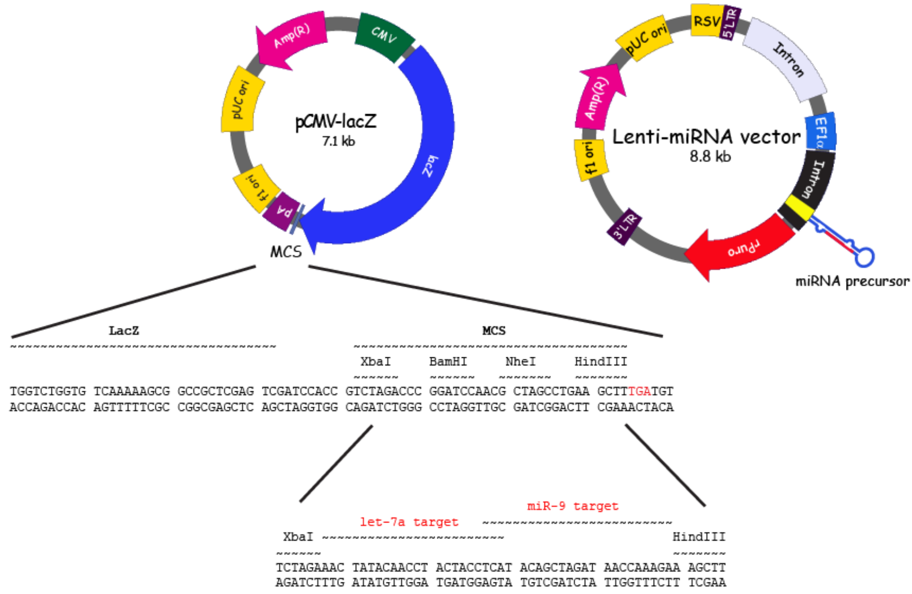


Figure 6. Functional analysis of mir-372 expressed from the Biosettia LV-[hsa-mir-372] lentiviral transductions. (A) Collaboration between oncogenic RAS and mir-372 in the transformation of primary cells. (B) Expression of mir-372 suppresses RAS-mediated senescence and transforms BJ cells. Early passage BJ cells were transduced with pWZL-Hyg-RasV12 (Ras), and then with LV-[hsa-miR-372] or control LV-[hsa-mir-ctrl] from the Biosettia Lenti-miRNA collection. Cells were stained with crystal violet for microscopic analysis at day 14 after RAS transduction. (C) Cell proliferation analysis and senescence SA-beta-gal assay. Early passage BJ cells were transduced with pWZL-Hyg-RasV12 (Ras) or pWZL-Hyg control (Hyg), and then with LV-[hsa-miR-372] or control LV-[hsa-mir-ctrl] from the Biosettia Lenti-miRNA collection. Cells were counted (left panel) and stained for SA-beta-gal (right panel) 8 days after RAS transduction.

Reporter Assay

It has been reported that miRNA is able to cleave mRNA if the mature miRNA sequence is fully complementary to the mRNA target [5]. A 42-bp DNA oligonucleotide (aactatacaacctactacctcatacagctagataaccaaaga) containing sequences complementary to both mature hsa-let-7a (tgaggtagtaggttgtatagtt) and hsa-mir-9 (tctttggttatctagctgtatga) were inserted into the 3' end of the β-galactosidase gene to serve as miRNA targets (Fig. 7A). The β-galactosidase activity is suppressed when either mature let-7a or mir-9 miRNAs is present and targeted to the β-galactosidase fusion mRNA. The β-galactosidase activity is not suppressed by the other let-7 miRNAs that do not perfectly match the inserted target sequence (Fig. 7B). The mir-ctrl, a lentiviral vector without miRNA insertion, is used as negative control.

A.



B.

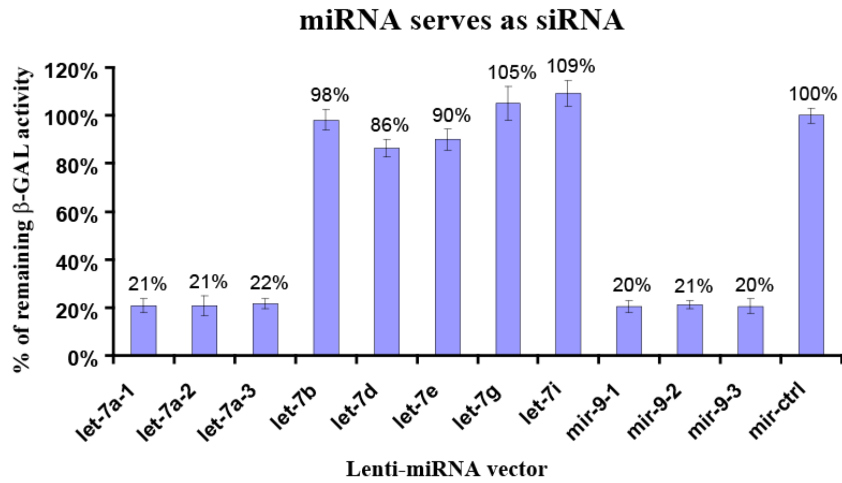


Figure 7. The lacZ reporter assay. The pCMV-lacZ and Lenti-miRNA vectors in a molar ratio of 1:10 were co-transfected into 293T cell. The β–galactosidase activity was determined 24 hours post-transfection.

References

1. Bagga, S., et al., *Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation.* Cell, 2005. **122**(4): p. 553-63.
2. Heo, I., et al., *Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA.* Mol Cell, 2008. **32**(2): p. 276-84.
3. Piskounova, E., et al., *Determinants of microRNA processing inhibition by the developmentally regulated RNA-binding protein Lin28.* J Biol Chem, 2008. **283**(31): p. 21310-4.
4. Voorhoeve, P.M., et al., *A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors.* Cell, 2006. **124**(6): p. 1169-81.
5. Zeng, Y., R. Yi, and B.R. Cullen, *MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms.* Proc Natl Acad Sci U S A, 2003. **100**(17): p. 9779-84.

Related Products

Lentiviral Packaging Mix.

Pre-mixed Plasmids for Lentiviral Packaging	Catalog #
Lentiviral Packaging Mix. 500 µg	pLV-PACK-500
Lentiviral Packaging Mix. 1000 µg	pLV-PACK-1000

Nucleic Acid Purification Kits

Plasmid DNA Purification Kits	Catalog #
Plasmid DNA Extraction Mini Kit (100)	FAPDE 001
Plasmid DNA Extraction Mini Kit (300)	FAPDE 001-1
Plasmid DNA Extraction Midi Kit (25)	FAPDE 002
Plasmid DNA Extraction Midi Kit (50)	FAPDE 002-1
Plasmid DNA Extraction Maxi Kit (10)	FAPDE 003
Plasmid DNA Extraction Maxi Kit (25)	FAPDE 003-1
EndoToxinFree Plasmid DNA Extraction Maxi Kit (10)	FAPDE 003-EF

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