Lentiviral miRNA Inhibitor

pLV-miR-Locker Plasmid

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

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www.biosettia.com
info@biosettia.com

Product Description

In general, miRNA inhibitors can be divided into two categories: (1) chemically synthetic single-stranded oligonucleotide-based molecules such as 2'-O-methylated antisense RNA and locked nucleic acids (LNA); (2) multiple miRNA target sites encoded in a RNA transcript expressed from DNA vectors or viral genomes. Previous studies have shown that the function of miRNA in repressing endogenous gene expression can be effectively inhibited by overexpressing artificial miRNA binding sites from plasmids or viruses in the target cells [1-4].

Biosettia miRNA inhibitor called miR-Locker is a sequence containing two copies of miRNA binding sites perfectly complementary in the 5' seed region and the 3' region with a bulge at the center of a particular miRNA. To demonstrate the specific mature miRNA can effectively bind to the designed miR-Locker sequence, we have established a reporter assay by inserting the miR-Locker sequence into the 3' UTR of the LacZ gene to generate pLacZ-miR-Locker plasmid. The pLacZ-miR-Locker plasmid and its corresponding pLV-miRNA expression clone (Biosettia catalog number mir-p###) were co-transfected into 293T cells followed by β-galactosidase reporter assay (Fig. 1A). Here we show the reporter assay for miR-Lockers against hsa-mir-1, 7, 9, 17, 30a, 30c, 125b, 146a, and let-7a. The reduction of β-galactosidase activity indicate that the mature miRNA expressed from the pLV-miRNA expression vector binds to the miR-Locker sequence located in the 3' UTR of lacZ gene and further represses the translation of β-galactosidase (Fig. 1B).

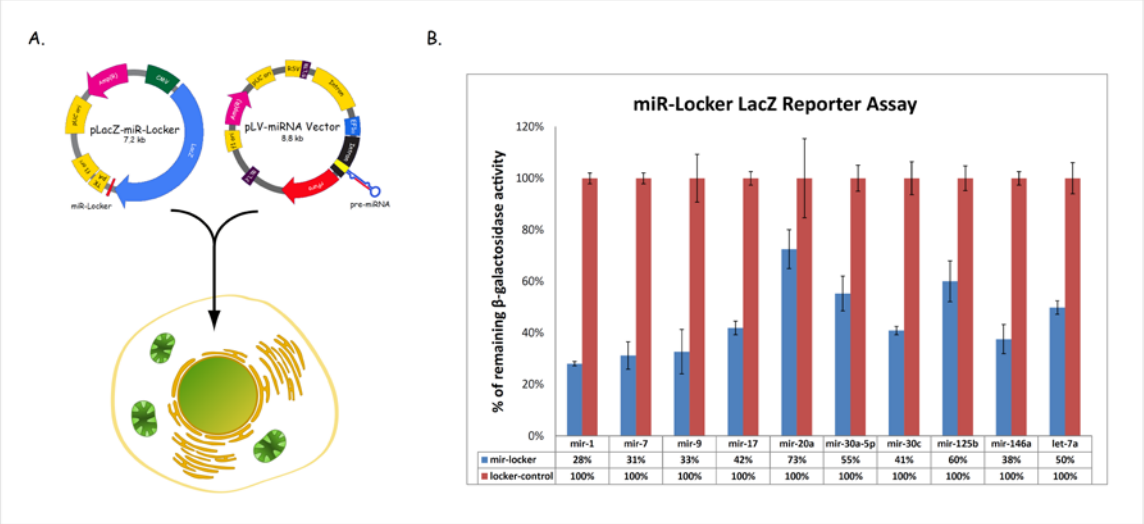

Figure 1. Determination of miR-Locker efficacy by reporter assay. (A) pLacZ-miR-Locker plasmid and its corresponding pLV-miRNA expression vector are co-transfected in a molar ratio of 1 to 10 into 293T cells. (B) The β–galactosidase activity was determined at 24 hours post-transfection. The decrease of β-galactosidase activity reveals the miR-Locker sequence cloned in the 3'UTR of lacZ gene can be recognized by the mature miRNA expressed from the pLV-miRNA vector.

The lacZ reporter assay reveals that the miR-Locker sequence can be a useful tool to serve as the miRNA pseudo-target to attenuate the miRNA-mediated translational repression on the endogenous genes. The lentiviral vector offers advantages to effectively deliver the miR-Locker sequence, to integrate into the host genome and continuously overexpress the miRNA competitive inhibitor for long-term miRNA inhibition. So, how can we overexpress miR-Locker from the lentiviral vector? One can insert the miR-Locker sequence into the 3' UTR of a reporter gene driven by a pol II promoter. Alternatively one can clone the miR-Locker into a pol III expression cassette to produce miRNA binding sites in a short RNA transcript. In Biosettia we have constructed a lentiviral vector pLV-miR-Locker in which the mouse U6 (mU6) promoter and the miR-Locker sequence are inserted into the 3'UTR of the GFP-Bsd fusion gene driven by the EF1a promoter (Fig. 2A). The miR-Locker sequence on the lentiviral genome is expressed by the pol II and the pol III promoters simutaneously (Fig. 2B). By comparing with the miR-Locker plasmid lacking the mU6 promoter inserted in the 3'UTR of GFP-Bsd fusion gene (plasmid map not shown), we have found the presence of miR-Locker on both of the pol II and the pol III transcripts are more effective in depletion of mature miRNAs (Fig. 2C).

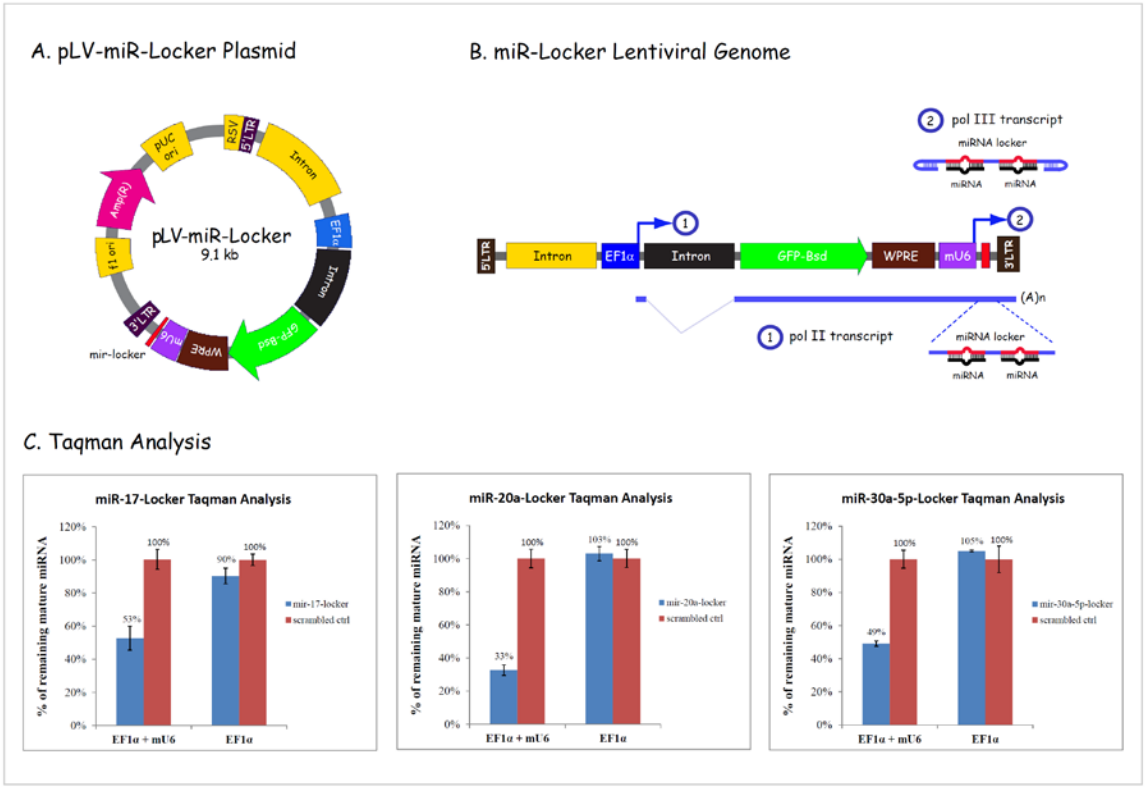

Figure 2. The pLV-miR-Locker vector design. (A) The pLV-miR-Locker-Locker lentiviral vector. The mouse U6 promoter (mU6) and miR-Locker sequence (two copies of miRNA binding sites) are cloned into the 3'UTR of GFP-Bsd reporter gene, downstream of the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), to generate the pLV-miR-Locker plasmid. (B) The miR-Locker sequence is present on both transcripts expressed by the EF1a and mouse U6 promoters and designated as 1 and 2 in the figure, respectively. (C) Efficacy of miRNA inhibition by pLV-miR-Locker. To compare the efficacy of inhibiting miRNAs by expressing miR-Locker from both EF1a and mU6 promoters on pLV-miR-Locker vector to that of only miR-Locker sequence is produced by EF1a promoter alone, the real-time PCR results showed that the mature miRNAs are depleted more effectively by the miR-Locker expressed from pol II and pol III promoters simultaneously.

Furthermore, we have generated miR-Locker lentiviruses and transduced the human foreskin BJ cells to target the endogenous mir-17, 20a, 30a-5p, 30b, and 30c mature miRNAs followed by Taqman analysis to determine the efficacy of miRNA inhibition (Fig. 3). The results are consistent with what we observed in the reporter assay described above.

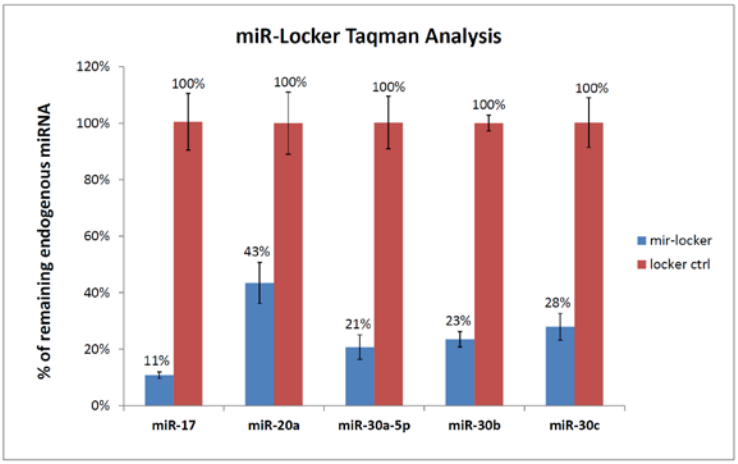

Figure 3. Determination of miR-Locker efficacy by Taqman analysis.

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 # FAPDE001) is sufficient for generating 30 ml of lentivirus.

Production of lentiviral miRNA inhibitors from pLV-miR-Locker plasmids

Production of lentiviral stocks requires packaging of the lentiviral genomic RNA transcribed from pLV-miR-Locker 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 miR-Locker lentiviruses, depending on the source and nature of the helper plasmids. In the three-plasmid system, pLV-miR-Locker is cotransfected with two helper plasmids (Gag-Pol + Rev and VSV-G) into cells, while in the four-plasmid system, pLV-miR-Locker 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-miR-Locker 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 x 10^6 - 5 x 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-miR-Locker 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 lentivirus 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 blasticidin and counting the blasticidin-resistant cell colonies. Alternatively, a flow cytometry can be used to determine the viral titer by measuring the number of green 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 blasticidin to select for stably transduced cells.

Note: At least 48 hours of transduction allows miR-Locker lentivirus to integrate into the host genome. The optimal blasticidin concentration (range 2-50 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 blasticidin every 2 days.

Day 7-8: Allow blasticidin-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 green fluorescent cells.

Note: At least 48 hours of transduction allows miR-Locker lentivirus 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 green fluorescence 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 blasticidin.
Note: The optimal blasticidin concentration varies from cell line to cell line (ranging from 2-50 μg/ml).
Suggestion: A pilot experiment should be performed to determine the minimum concentration of blasticidin 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 GFP-Bsd gene expression may vary among different cell lines. Therefore, please keep in mind that the green fluorescence may provide a general idea whether the model cells are transduced, but may not quantitatively reflect the percentage of cells transduced by miR-Locker lentivirus. It is highly recommended to determine the transduction efficiency by blasticidin selection, since in some cells, the expression level of green fluorescent blasticidin deaminase is enough to render the cells resistant to blasticidin, but not enough for the cells to display detectable green fluorescence.

Please be aware that one miR-Locker might inhibit multiple miRNAs in the same family 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 MiR-Locker Lentivirus.

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.

References

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3. Ebert, M.S., J.R. Neilson, and A.P. Sharp, MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. Nat Methods. 2007 4(9): p. 721-26.

4. Scherr, M., et al., Lentivirus-mediated antagomir expression for specific inhibition of miRNA function. Nucleic Acids Res. 2007 35(22): p. 1-9

5. Vermeulen, A., et al., Double-stranded regions are essential design components of potent inhibitors of RISC function. RNA, 2007 13(5): p. 723-30.

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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