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Product Information

MISSION® Lentiviral Transduction Particles – Lentiviral MicroRNA Inhibitors

Catalog Numbers **HLTUD0001-HLTUD2235, MLTUD0001-MLTUD1405, CSTTUD, HLTUD001C, HLTUD002C**

Storage Temperature –70 °C

TECHNICAL BULLETIN

Product Description

MicroRNAs (miRNAs) are a class of genome-encoded nucleic acids that have been shown to regulate gene expression in a variety of organisms. These small, noncoding RNA molecules function by mediating transcript degradation, inhibiting translation, or a combination of these mechanisms. This type of regulation generally occurs by base pairing of the miRNA to a target sequence within the 3' UTR of a transcript. Thousands of miRNAs have been identified and classified via sequencing or bioinformatics approaches that are based on strongly conserved sequence motifs. The University of Manchester operates the publicly available *miRBase Sequence Database*, where microRNA data are managed and annotated.

Individual microRNA inhibitors are designed using a proprietary algorithm, which is based on the work of Haraguchi, T, *et al.* [1], and in collaboration with Dr. Hideo Iba, University of Tokyo. This algorithm utilizes the tough decoy (TuD) design. Each miRNA inhibitor construct has been cloned and sequence verified to ensure a match to the target miRNA.

The Lentiviral Transduction Particles are produced from sequence-verified lentiviral plasmid vectors. The Lentiviral microRNA Inhibitors are cloned into the TRC2-pLKO-puro vector (see Figure 1). Co-transfection of this vector into the appropriate cell line with compatible packaging plasmids produces viral particles that can be used to transduce mammalian cells. The vector also contains elements needed for reverse transcription of viral RNA and integration of viral DNA into the host cell genome. Additionally, the Woodchuck Hepatitis Post-Transcriptional Regulatory Element[2] (WPRE) is included, allowing for enhanced expression of transgenes delivered by lentiviral vectors.[3] This lentiviral vector also carries a puromycin resistance gene for selection of cells.

Unlike murine-based MMLV or MSCV retroviral systems, lentiviral-based particles permit efficient infection and integration of the specific miRNA inhibitor construct into differentiated and non-dividing cells, such as neurons and dendritic cells,[4] overcoming low transfection and integration difficulties when using these cell lines. Self-inactivating replication incompetent viral particles are produced in packaging cells (HEK293T) by co-transfection with compatible packaging plasmids.[5-6]

In addition, the lentiviral transduction particles are pseudotyped with an envelope G glycoprotein from Vesicular Stomatitis Virus (VSV-G), allowing transduction of a wide variety of mammalian cells including primary and embryonic stem cells.[7] The lentiviral transduction particles are titered via a p24 antigen ELISA assay and pg/ml of p24 are then converted to transducing units per ml using a conversion factor.

To use the lentiviral microRNA inhibitors one needs to first select the miRNA targets to be inhibited/ investigated. If unsure about the levels of miRNA expression in a particular cell line, it is critical that you determine those levels by conducting qRT-PCR or similar assay. We recommend using the MystiCq™ microRNA cDNA Synthesis Mix. Starting with total RNA or RNA preparations pre-enriched for microRNAs, this kit provides all the components necessary to convert mature microRNAs into cDNA templates for qPCR. Once miRNA targets have been chosen and quantified in the cell line of choice, cells can be transduced with lentiviral particles containing the miRNA inhibitors. Levels of target mRNA or protein can then be assessed using a qRT-PCR assay (optional) or Western Blotting, respectively. The lentiviral miRNA inhibitors can also be used to investigate the functional role of the miRNA being studied.

Components/Reagents
The individual constructs are provided in Dulbecco's Modified Eagle's Medium with 10% heat-inactivated fetal bovine serum and penicillin-streptomycin. There are several available options for volume.

Standard Volume:
- 0.2 mL

Custom Volumes:
- 0.1 mL
- 1.0 mL
- 2.0 mL
- 5.0 mL
- 10.0 mL

Orders of 25 or fewer lenti microRNA inhibitors are provided in individual vials. Orders of >25 lenti microRNA inhibitors are provided in a 96-well plate. 96-well plates are provided with a CD containing plate map positions.

Precautions and Disclaimer
These products are for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Though the lentiviral transduction particles produced are replication incompetent, it is highly recommended that they be treated as **Risk Group Level 2 (RGL-2)** organisms.[8] Follow all published RGL-2 guidelines for handling and waste decontamination.

Storage/Stability
All components are stable for at least six months after receipt when stored at –70 °C. Avoid repeated freeze/thaw cycles, which will severely reduce functional viral titer.

Related Products
- Hexadimethrine Bromide, Catalog Number H9268
- Puromycin dihydrochloride, Ready Made Solution, 10 mg/ml in H_2O, Catalog Number P9620
- Minimum Essential Medium containing 10% fetal calf serum or growth medium optimized for the specific cell line
- MISSION ExpressMag® 96-Well Magnetic Kit, Catalog Number SHM02
- MystiCq® microRNA cDNA Synthesis Mix, Catalog Number MIRRT
- KiCqStart® SYBR® Green qPCR ReadyMix™ Catalog Number KCQS00
- mirPremier® microRNA Isolation Kit Catalog Number SNC10, SNC50
- MISSION microRNA Mimics
- MISSION Lenti microRNA Inhibitor, ath-miR416, Negative Control 1, Sequence from *Arabidopsis thaliana* with no homology to human and mouse gene sequences, Catalog Number HLTUD001C
- MISSION Lenti microRNA Inhibitor, cel-miR-243-3p, Negative Control 2, Sequence from *Caenorhabditis elegans* with no homology to human and mouse gene sequences, Catalog Number HLTUD002C
- MISSION TRC2 pLKO.5-puro-CMV-TurboGFP™ Positive Control Transduction Particles, Catalog Number SHC203V or MISSION® TRC2 pLKO.5-puro Empty Vector Control Transduction Particles puro, Catalog Number SHC201V
- qRT-PCR Reagents, please visit http://www.sigma-aldrich.com/pcr
- Prestige Antibodies, please visit http://www.sigma-aldrich.com/prestige

Procedure for the Use of MISSION Lentiviral microRNA Inhibitor Transduction Particles

Day 1
Plate the mammalian cell line of choice in complete medium 24 hours prior to transduction. Take into account the length of time that the cells will be cultured prior to performing miRNA target inhibition analysis when determining plating density. Typically cells are transduced at 50-80% confluency.

Day 2
Thaw the lentiviral stock slowly on ice. Gently spin down material in tubes before opening. Add hexadimethrine bromide (the chemical equivalent of Polybrene) to the cells at a final concentration of 8 μg/ml.

Note: Hexadimethrine bromide enhances transduction of most cell types. However, some cells, such as primary neurons, are sensitive to hexadimethrine bromide. When using sensitive cells, do not add the hexadimethrine bromide and the cells should still be transduced.

Following addition of hexadimethrine bromide, gently swirl the plate to mix. Add the appropriate amount of viral particles at a suitable multiplicity of infection (MOI) and swirl the plate gently to mix. Incubate the cell-viral particle mixture at 37 °C overnight.

Multiplicity of Infection (MOI) is the number of transducing lentiviral particles per cell. It is highly recommended that for each new cell type to be transduced, a range of MOI be tested.

To calculate MOI: (total number of cells per well) x (desired MOI) = total transducing units needed (TU)

(total TU needed) / (TU/ml reported on C of A) = total ml of lentiviral particles to add to each well

Notes

a. When transducing a lentiviral construct into a cell line for the first time, it is recommended that a range of MOIs (0.5-20) be used to find the optimum degree of transduction efficiency.
b. When overnight incubation presents a toxicity concern, cells may be incubated for as little as 4 hours before changing the medium. Cells can be transduced in reduced medium volumes to increase transduction efficiencies.

Day 3

Remove the viral particle-containing medium and replace it with fresh, pre-warmed complete culture medium.

Day 4

If placing cells under puromycin selection, remove the medium and replace it with fresh, complete medium that contains the appropriate amount of puromycin for selection of transduced cells. Proceed to day 5.

Note: When the appropriate concentration of puromycin for a specific cell type is unknown, perform a kill curve experiment. Typically, puromycin concentrations ranging from 0.5-10 μg/ml are sufficient to kill most untransduced mammalian cell lines.

Puromycin titration (kill curve) should be performed when working with a new cell type.

1. Plate 1.6 x 10^4 cells into wells of a 96-well plate with 120 μL fresh media.
2. The next day add 0.5-10 μg/ml of puromycin to selected wells.
3. Examine viability every 2 days.
4. Culture for 3 – 14 days depending on the growth rate of the cell type and the length of time that cells would typically be under selection during a normal experimental protocol. Replace the media containing puromycin every 3 days. The minimum concentration of puromycin that causes complete cell death after the desired time should be used for that cell type and experiment.

Note: Excess puromycin can cause many undesired phenotypic responses in most cell types.

Day 5 and forward

Replace medium with fresh, puromycin-containing medium every 3-4 days until resistant colonies can be identified (generally, 10-12 days after selection). Pick a minimum of 5 puromycin-resistant colonies and expand each clone to assay for inhibition of the target miRNA.

Note: Due to the random integration of the lentivirus into the genome, varying levels of miRNA inhibition may be seen from different puromycin-resistant clones. Testing a number of puromycin-resistant clones will allow a determination of which one provides the optimal degree of inhibition.

Assessing loss of miRNA function

miRNA targets can be validated by quantitating target protein and/ or messenger RNA levels in response to miRNA downregulation. miRNA functional studies may require simultaneous analyses of both mRNA and protein expression. While qRT-PCR can be used to assess levels of target transcript, Western analysis or other validated immunoassays are used to investigate the impact on protein quantity. Reporter assays, such as a dual luciferase reporter assay, are used to study the interaction between miRNAs and their target sites. Many researchers find it is necessary to use a combination of assays to assess miRNA target inhibition.

Figure 1. TRC2 Lentiviral Plasmid Vector TRC2-pLKO-puro Features

Name	Description
hU6	U6 Promoter
cppt	Central polypurine tract
hPGK	Human phosphoglycerate kinase eukaryotic promoter
PuroR	Puromycin resistance gene for mammalian selection
WPRE	Woodchuck Hepatitis Post-Transcriptional Regulatory Element
SIN/3′ LTR	3’ self inactivating long terminal repeat
f1 ori	f1 origin of replication
AmpR	Ampicillin resistance gene for bacterial selection
pUC ori	pUC origin of replication
5’ LTR	5’ long terminal repeat
Psi	RNA packaging signal
RRE	Rev response element
miRNA inhibitor	miRNA inhibitor sequence (clone specific)

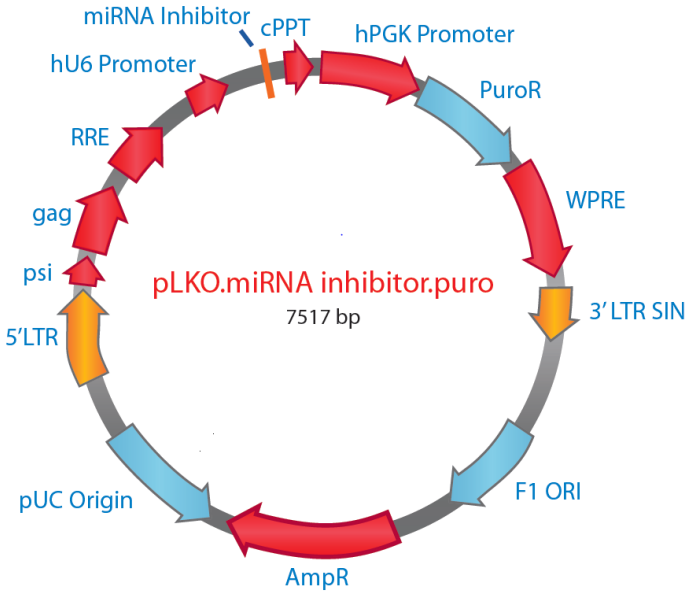
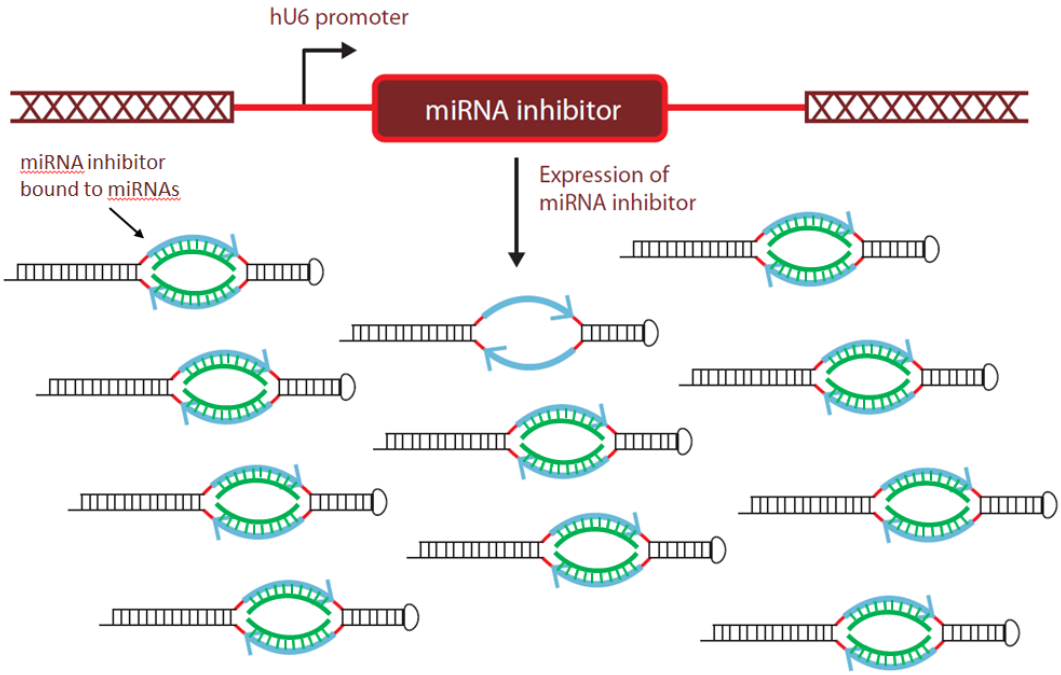


Figure 2. Lentiviral miRNA inhibitor expression regulates miRNA function

Expression of the miRNA inhibitor is driven by the U6 promoter upon genomic integration of the lentiviral transfer vector into the host cell post-transduction. miRNA inhibitors are able to competitively bind specific miRNAs and prevent them from regulating their endogenous targets[1].



Troubleshooting Guide

Problem	Cause	Solution
No Transduction of cells	Viral stock stored incorrectly	Store stocks at −70 °C. Do not freeze/thaw more than 2 times.
Low miRNA inhibition detected due to low transduction efficiency	Population of cells not transduced efficiently	Select for transduced cells with puromycin, and allow selected population to become mitotic
	Hexadimethrine bromide not included during transduction	Transduce in the presence of hexadimethrine bromide.
	Non-dividing cell type used	Transduce at a higher MOI, or evaluate transduction enhancement reagents, such as ExpressMag.
	MOI is too low	Transduce at a higher MOI, or evaluate transduction enhancement reagents, such as ExpressMag.
	It is unknown how efficiently cells can be transduced with VSV-G pseudotyped lentiviral particles	Try transducing cells with contol lentiviral particles such as MISSION® Lenti microRNA Inhibitor, ath-miR416, Negative Control 1, Catalog Number HLTUD001C, MISSION® TRC2 pLKO.5-puro-CMV-TurboGFP™ Positive Control Transduction Particles (SHC203V), or MISSION® TRC2 pLKO.5-puro Empty Vector Control Transduction Particles puro (SHC201V) to establish experimental parameters for transductions
	Cells were harvested and assayed too soon after transduction	Harvest cells 72 hours after transduction, and not earlier. Alternatively, results may be improved by placing cells under puromycin selection because untransduced cells will be killed.
Low miRNA inhibition detected due to target choice or cell line variabilities in expression of the miRNA	microRNA is not expressed at a high enough level for analysis in cell line	Evaluate miRNA levels in cell type of choice via qRT-PCR using the MystiCq™ microRNA cDNA Synthesis Mix kit. Consider alternative cell lines. Use a combination of assays to test the miRNA target, such as qRT-PCR, Western analysis, and reporter assays such as a dual luciferase assay
No signal from reporter assay (such as a dual luciferase assay)	Reporter assay might not be working correctly	Transfect cells with ready-to-use MISSION miRNA mimics along with reporter plasmid. These small, double-stranded RNA molecules, designed to mimic endogenous mature miRNA molecules when introduced into cells, will aid in assessing if the assay is working.
Cytotoxic effects observed after transduction	Target miRNA is essential for cell viability	Be sure that target miRNA is not essential for cell growth or viability.
	Hexadimethrine bromide was used during transduction	Be sure that cells are not sensitive to hexadimethrine bromide. Omit the hexadimethrine bromide during the transduction.
	Too much puromycin was used for selection	Determine the puromycin sensitivity of the cells by performing a kill curve and use the minimum concentration required to kill the untransduced cells.

Control Selection Table

Recommended Control	Objective
Negative Control: Untreated Cells	Untreated cells will provide a reference point for comparing all other samples.
Negative Control: Transduction with lentiviral particles containing a miRNA inhibitor sequence from *Arabidopsis*	MISSION Lenti microRNA Inhibitor, ath-miR416, Negative Control 1, Catalog Number HLTUD001C These viral particles contain a sequence designed to inhibit a miRNA found in *Arabidopsis* that will not target any mammalian miRNAs. Side-by-side transductions using these control particles and experimental lenti microRNA inhibitors will allow for observation of cellular effects of the transduction process, the puromycin selection process, and will provide control cells for downstream applications such as qRT-PCR and Western blot analysis.
Negative Control: Transduction with lentiviral particles containing a miRNA inhibitor sequence from *C. elegans*	MISSION Lenti microRNA Inhibitor, cel-miR-243-3p, Negative Control 2, Catalog Number HLTUD002C These viral particles contain a sequence designed to inhibit a miRNA found in *Caenorhabditis elegans* that will not target any mammalian miRNAs. Side-by-side transductions using these control particles and experimental lenti microRNA inhibitors will allow for observation of cellular effects of the transduction process, the puromycin selection process, and will provide control cells for downstream applications such as qRT-PCR and Western blot analysis.

Cell Type Table

The cell types listed below have been successfully infected by pLKO.2-puro based lentiviral particles. Optimal conditions will need to be determined for your experimental needs. For the most updated cell line list, and some guidelines for conditions, please visit:
http://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/shrna/learning-center/getting-started.html

Cell lines, human	**Cell Type**		**Cell lines, human**	**Cell Type**		**Primary cells human**	**Cell Type**
HEK293	embryonic kidney cells		A431	epidermal carcinoma		dendritic	immature dendritic
HeLa	cervical adenocarcinoma		THP1	monocytic		T-cells	lymphocytes
A549	lung adenocarcinoma		RAW264.7	macrophage		epithelial	prostate
H1299	lung carcinoma		SH-SY5Y	brain neuroblastoma		fibroblasts	primary mammary
HT29-D4	colon carcinoma		HCN-1A	brain cortical neuron		**Primary cells, other species**	**Cell Type**
HepG2	hepatocellular carcinoma		SupT1	T-cells		ECS	mouse embryonic stem cells
HCT116	colon carcinoma		BJ-TERT	diploid fibroblasts		fibroblasts	mouse embryonic fibroblasts
MCF7	breast carcinoma		**Cell lines, mouse**	**Cell Type**		MC3T3-E1	mouse bone marrow derived
MCF10A	breast carcinoma		NIH3T3	fibroblast		molar mesenchymal	mouse embryonic mesenchymal
Panc-1	pancreatic epithelioid carcinoma		**Primary cells, human**	**Cell Type**		cardiomyocytes	rat neonatal cardiomyocytes
PC3	prostate carcinoma		astrocytes	normal			
DU145	prostate carcinoma		C3H10T1/2	mesenchymal			

References

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