

Frequently Asked Questions (MISSION® shRNA)

General Information about TRC vectors and Clones

Disclaimer

The Functional Genomics Facility is a distributor of TRC library collections for RNA interference. These clone collections were purchased from Sigma-Aldrich and were generated at The Broad Institute. Thus the quality of the collections is largely dependent upon what the Functional Genomics Facility received from these groups. Specific clone information and plate coordinates were provided to the Functional Genomics Facility by the suppliers of these clone collections. These collections and individual clones are distributed "as is" with no additional product validation or guarantees.

This FAQ section is partially borrowed from Sigma-Aldrich.

The Functional Genomics Facility (A University of Colorado Cancer Center Shared Resource) has gradually acquired the entire TRC MISSION® shRNA library from Sigma-Aldrich. The MISSION® shRNA clones were designed and developed by The RNAi Consortium (TRC) at the Broad Institute of MIT and Harvard. It includes hairpin sequences comprised of a 21 base stem and a 6 base loop. The hairpin sequences are each cloned into the pLKO.1 vector and sequence-verified. A minimum of 3-5 shRNA constructs are created for each target gene to provide varying levels of knockdown and to target different regions of mRNA transcript. Sigma-Aldrich guarantees that upon purchase of the entire clone set on the shRNA detail page for your gene of interest (this varies, but is typically 5 clones), at least one of those clones should yield greater than 70% knockdown at the mRNA level. For a given RefSeq, there is often a shRNA clone targeting the 3'UTR for use in phenotypic rescue studies using cDNA expression constructs.

More information on the creation of this library can be found in the following publication: [J. Moffat et.al. A Lentiviral RNAi Library for Human and Mouse Genes Applied to an Arrayed Viral High-Content Screen. Cell. 2006 Mar 24;124\(6\):1283-98.](#)

TRC1/1.5 Vector: pLKO.1-puro Vector and Use

TRC1.5 clones are in the exact same vector backbone as the TRC1 clones. Features of the pLKO.1-puro vector allow for transient or stable transfection of the shRNA as well as production of lentiviral particles. Stable gene silencing is selected using the puromycin-selectable marker while self-inactivating replication incompetent viral particles can be produced in packaging cells (HEK293T) by co-transfection with compatible packaging plasmids. TRC1.5 is exclusive to Sigma-Aldrich Life Science and contains almost 200,000 clones including more than 49,000 validated clones. It combines all of the content from TRC1 plus an additional 39,212 clones targeting 2,661 new human genes and 2,395 mouse genes. Unlike adenovirus or murine-based MMLV or MSCV retroviral systems, lentiviral-based particles permit efficient infection and integration of the specific shRNA construct into differentiated and non-dividing cells, such as neurons and dendritic cells, overcoming low transfection and integration difficulties when using these cell lines. Compared to siRNA and other vector-based systems, pLKO.1-puro provides solutions for long-term knockdown and phenotypic observation, transduction of difficult or sensitive cell lines (non-dividing cells or primary cells), and is an economical renewable resource.

With an shRNA insert, the length of the pLKO.1-puro plasmid is 7,086 bp, as indicated in the vector map (Figure 1). Without an shRNA insert, pLKO.1-puro vector has a length of 7,052 bp.

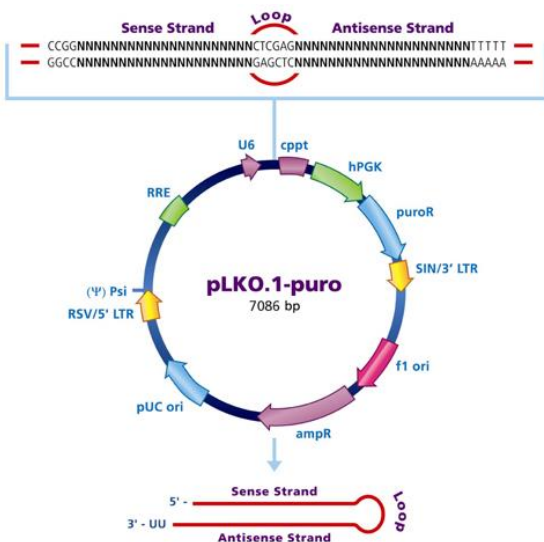


Figure 1: TRC1/1.5 Vector Map

Table 1: TRC1.5 Vector Description and Features

| Name | Description |
|---------|--|
| cppt | Central polypurine tract |
| hPGK | Human phosphoglycerate kinase eukaryotic promoter |
| puroR | Puromycin resistance gene for mammalian selection |
| SIN/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 |

TRC2 Vector: TRC2-pLKO-puro Vector and Use

The TRC2-pLKO-puro vector also allows for transient or stable transfection of the shRNA as well as production of lentiviral particles. The only change between the TRC1.5 vector and the TRC2 vector is the addition of the WPRE (or the Woodchuck Hepatitis Post-Transcriptional Regulatory Element). This allows for enhanced expression of transgenes and shRNAs delivered by a lentivirus. As with TRC1.5, stable gene silencing is achieved by using the puromycin selectable marker. Self-inactivating replication incompetent viral particles can be produced in packaging cells (HEK293T) by co-transfection with compatible packaging plasmids. TRC2-pLKO-puro provides solutions for stable long-term knockdown and phenotypic observation, transduction of difficult or sensitive cell lines (non-dividing cells or primary cells), and enhanced expression of transgenes. With an shRNA insert, the length of the TRC2-pLKO-puro plasmid is 7,518 bp, as indicated in the vector map (Figure 2). Without an shRNA insert, the TRC2-pLKO-puro vector has a length of 7,484 bp.

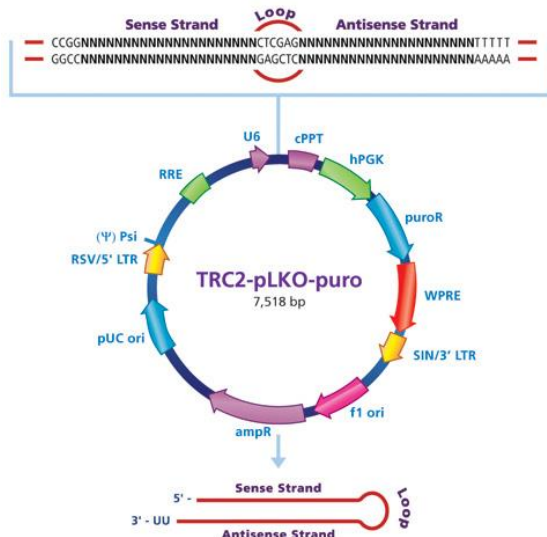


Figure 3: TRC2 Vector Map

Table 2: TRC2 Vector Description and Features

| Name | Description |
|---------|---|
| 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/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 |

What is shRNA?

shRNA stands for short-hairpin RNA. In pLKO plasmids, the short-hairpin sequence is located within the LTR regions of the lentiviral construct and its expression is driven by the U6 promoter. Upon integration of the LTR to LTR area into the host genome, the shRNA is produced by the cell and shuttled out into the cytoplasm where it is cleaved by the enzyme complex Dicer. Once this cleavage is complete, one strand of this double-stranded molecule is loaded into the RISC (RNA Induced Silencing Complex), where it seeks complementary mRNA targets for degradation.

Why is the U6 promoter used?

The human U6 promoter (a pol III promoter) is used to drive expression of the shRNA hairpin. Expression using pol III promoters is optimal for producing shRNAs due to precise initiation and termination of transcription. TRC chose U6 based on experimental data showing excellent efficiency when compared to another efficient pol III promoter (H1).

What restriction sites were used for cloning the shRNA sequences?

The shRNA was cloned into the AgeI and EcoRI sites.

Can I cut out the shRNA from one of the TRC constructs and clone it into another vectors using AgeI and EcoRI?

No. One of the restriction sites was destroyed while cloning in the shRNA constructs during library development.

Does pLKO.1 contain WPRE?

No, the TRC1 vector, pLKO.1-puro, does not contain WPRE (Woodchuck Posttranscriptional Regulatory Element). It is contained in the TRC2 vector, TRC2-pLKO-puro. WPRE is known for its ability to enhance transcription of transgenes.

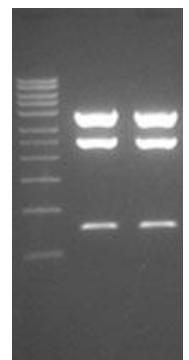


Figure 3: Band sizes following digestion with PvuII

What restriction enzymes are recommended for diagnostic digests of the clone DNA?

We recommend *Pvu* II. Restriction digest with *Pvu* II results in three bands; however, clones with an additional *Pvu* II site in the hairpin insert will produce four bands: 2513 bp, 2325 bp, 1478 bp and 776 bp.

The band sizes following digestion with *Pvu* II of the shRNA vectors are 3803 bp, 2513 bp, and 776 bp (Figure 3).

How large of an insert can pLKO.1 handle?

The larger the insert put into pLKO.1 vector, the lower the functional titer of the produced lentivirus. We generally suggest the total size of pLKO.1 be less than 9 kb.

If I order TRC1.5 clones and controls, will they work with TRC1 content?

Yes. TRC 1.5 has the same vector backbone as TRC1.

I have always ordered TRC1 products. Now, I notice that only TRC1.5 products are available. Am I getting the same products that I ordered previously?

Yes, TRC1 and TRC1.5 share the same vector backbone. TRC1.5 has all the content you love with the vector backbone you trust, now with even more coverage. TRC1.5 is largely an extended version of the old TRC1 library. All TRC1.5 controls will work with TRC1 clones.

How are TRC1, TRC1.5 and TRC2 clones different?

They are not different. TRC 1.5 has the same vector backbone as TRC1. TRC1.5 has all the content you love with the vector backbone you trust, now with even more coverage. There is no difference between TRC1 and TRC1.5, except for the extended coverage. The only change between the TRC1.5 vector and the TRC2 vector is the addition of the WPRE (or the Woodchuck Hepatitis Post-Transcriptional Regulatory Element). This allows for enhanced expression of transgenes delivered by lentivirus.

Ordering shRNA clones

In what formats can the shRNA clones be ordered?

The TRC1 library currently contains more than 159,000 pre-cloned shRNA constructs targeting 16,000 annotated human genes and 15,950 annotated mouse genes. The TRC2 library contains more than 150,000 pre-cloned shRNA constructs. Most genes have at least 3 individual clones from which to choose. Three formats are available: bacterial glycerol stock, purified plasmid DNA, and lentiviral particles.

How do I look up the shRNA clones?

Please use Sigma-Aldrich's website to identify the shRNA clones you wish to use. More information on how to find clones on Sigma-Aldrich's can be found at [Lookup tool shRNA](#).

What is a TRC Number?

A unique number developed by The RNAi Consortium to identify the TRC Library. A TRC number begins with "TRCN" followed by 10 digits. (e.g. MISSION® shRNA TRCN0000030720).

What bacterial strain is used in TRC library and what is the stability of the shRNA plasmids in these cells?

The *E. coli* strain is in DH5alphaT1R. Sigma-Aldrich sells the identical strain (i.e. identical genotype) called GC5. TRC has published several studies on plasmid stability of the library in that strain (see "[Genome-scale loss-of-function screening with a lentiviral RNAi library](#)" and "[A Lentiviral RNAi Library for Human and Mouse Genes Applied to an Arrayed Viral High-Content Screen](#)"). An early criteria when designing the plasmid vector was improved stability of the lentiviral sequences in *E. coli* over past retroviral/lentiviral libraries. That said, be aware that *E. coli* containing lentiviral plasmids do grow significantly slower when compared to bacterial strains containing standard cloning plasmids such as pUC18, pET vectors or pBR322. **We routinely grow for 20–22 hours in Terrific Broth containing 100 µg/ml of carbenicillin (rather than ampicillin) as we see better plasmid stability using that antibiotic than we do with ampicillin.** Harvest and process plasmid DNA immediately post-growth (do not let them incubate at 4 °C for hours or even days, as that will result in lower quality pDNA). According to Sigma-Aldrich's preliminary studies shRNA clones passaged in such strains such as STBL-3 tend to grow more consistently and produce higher plasmid yields than DH5aT1R.

How should I store my bacterial cultures?

Glycerol stocks should be maintained at -80 °C. Cultures should be maintained fresh, avoiding prolonged storage at 4 °C.

Why would I use the purified plasmid DNA format?

The DNA format (1 µg) is ideal for doing small transfections in 96-well plates (with replicates) or for small-scale packaging co-transfections. Plasmid purification is a costly and time-consuming step that is eliminated with this product format.

What is the expected pDNA yield from glycerol stocks?

When clones are grown up under suggested conditions, the average pDNA concentration achieved is around 55 ng/µl with a range of concentrations from 20 to 120 ng/µl (50 µl elution). The Functional Genomics Facility routinely uses QIAGEN kits for plasmid purification.

What should I do if I have trouble obtaining high yields of plasmid DNA?

Generally, high yields of DNA can be achieved with much of the collection; however, viral-based vectors are known to be somewhat difficult to prepare. If particular clones are troublesome, we recommend streaking the bacterial stocks on LB/carbenicillin plates to isolate a single colony and using DNA purification prep kits.

If I want to make my own viral particles, which format should I purchase?

The Functional Genomics Facility offers ready-to-use viral suspensions to eliminate these steps for you. However, if you choose to make your own viral particles, we recommend the purified plasmid format for packaging protocols that require less than 1 µg of plasmid DNA (i.e. typical protocols for 60 mm dishes utilize 1 µg of transfer vector and yield 4-5ml of viral particles). For large-scale production of viral particles, we recommend the bacterial glycerol stocks. The bacterial format allows for propagation of the shRNA transfer vector and facilitates larger-scale DNA purification required for scaled up packaging co-transfections. A detailed protocol to package TRC clones can be found at [Transfection and Transduction TRC shRNA](#).

Which packaging systems are compatible with pLKO.1 shRNA vectors?

The MISSION® TRC shRNAs are cloned into the pLKO.1 transfer vector that is compatible with standard 2 plasmid (packaging vector with rev gene and envelope vector) or 3 plasmid (packaging vector without rev gene, envelope vector, and rev expression vector) packaging systems.

Do you offer any cells for packaging lentivirus?

Yes, The Functional Genomics Facility does offer HEK293FT cells at \$100 per vial. It is imperative that the cells you use be healthy, never grown to more than 80% confluence, and a maximum of 20 passage number. Following these recommendations will guarantee the best viral titers.

What is the titer for the lentiviral transduction particles?

The standard offering from The Functional Genomics Facility provides each clone as 2 mL lentiviral suspension which titers in the range of 10^5 Relative Infectious Units/mL (via relative viral titering using a cell viability assay).

How many cells can I infect with the amount of lentivirus provided?

The amount of cells that can be infected depends upon the cell line being used. For HCT116, SJSA, RKO, and H460 cells we use the entire 2 ml lentiviral suspension (as little as 500 ul appears to give comparable results) for each well containing 3×10^5 cells in a 6-well plate. Primary or other difficult-to-transduce cells may require more lentiviral supernatant. We suggest decreasing the number of cells plated to increase the multiplicity of infection (MOI) if necessary. Also, performing a limiting dilution titer on your cell line will determine the optimal amount of viral particles needed for each assay.

How does the cell viability assay using limiting dilutions of lentiviral suspension compare to other methods for determining viral titer?

The Functional Genomics Facility routinely uses limiting dilutions of lentiviral suspensions and a cell viability assay to measure viral titer. In contrast to our method, Sigma-Aldrich measures viral titer using the p24 assay from [ZeptoMetrix](#), which is an ELISA against the p24 viral capsid protein. For a fluorescently tagged shRNA construct the viral titer can be measured using flow cytometry. We compared

these three different methods to calculate viral titer: p24 ELISA, Flow Cytometry and Cell viability assay. Each method gave a significantly different value for viral titer (Figure 4 A-B). Figure 4C highlights the differences in viral titers of various control shRNA from TRC1 library. The variation appears to be dependent on individual constructs.

How do you determine functional viral titer (TU/ml) using the p24 assay?

There are approximately 2000 molecules of p24 per physical particle (PP) of lentivirus:

$(2 \times 10^3) \times (24 \times 10^3 \text{ Da of p24 per PP}), 48 \times 10^6 / \text{Avogadro's \#} = (48 \times 10^6) / (6 \times 10^{23}) = 8 \times 10^{-17} \text{ g of p24 per PP}$, approximately 1 PP per $1 \times 10^{-16} \text{ g of p24}$, $1 \times 10^4 \text{ PP per pg of p24}$

A reasonably well packaged, VSV-G pseudotyped lentiviral vector will have an infectivity index in the range of 1 TU per 1000 physical particles (PP) to 1 TU per 100 PP (or less). Thus, the range is approximately 10 to 100 TU/pg of p24. It is through this conversion that TU/ml is obtained. Our current viral packaging protocol has given us highly reproducible titers.

Is a lentiviral titer of 10^6 as determined by p24 ELISA too low?

We have optimized our viral production and this titer gives excellent integration efficiency in cultured cells. You may be more familiar with adenovirus, which titers at a range around 10^9 . Lentivirus, however, has many advantages over adenovirus, such as stable integration and the ability to

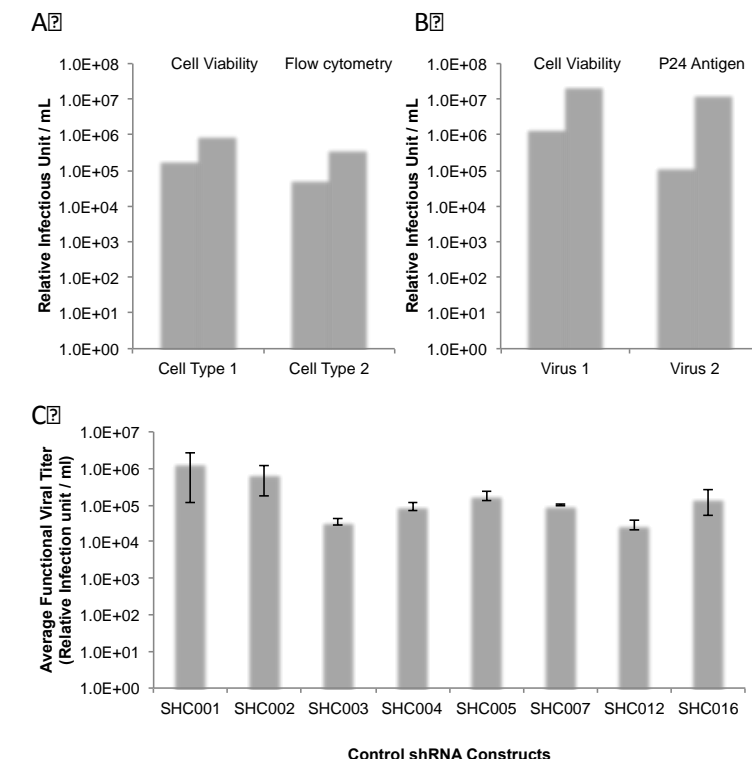


Figure 4: Comparing methods to determine viral titer

transduce primary and non-dividing cells, etc. If there are applications that require higher titer virus, the highly stable VSV-G envelope allows concentration of lentiviral particle via ultracentrifugation.

Can I extract the vector directly from the lentiviral particle?

No, this is not possible. The vector is used to make the particles in the packaging or producer cells. The viral genome contains only the RNA version of the region found between the 5' and 3' LTRs of pLKO.1 (promoter, hairpin sequence, puromycin resistance gene, etc.)

Biosafety concerns with using lentivirus

Is pLKO.1 vector a HIV-based vector and, if so, are there any biosafety issues?

The pLKO.1 vector is a lentiviral (HIV)-based plasmid. The vector is regarded as a biosafety level 2 material and safe to use due to its modified features (deletion of a number of accessory genes implicated in the virulence of HIV, minimal genome of the viral particles, non-replicating and self-inactivation features), making it incapable of producing virus once infected into the host cell. Please consult with your institution's biosafety officer on specific requirements.

How serious are lentiviral biosafety concerns? Is there a realistic risk of HIV infection?

Viral vector systems have been developed with enhanced safety features. It is recommended to use a third generation lentiviral vector system. It is a three-plasmid system consisting of:

1. The packaging vector, which contains the minimal set of lentiviral genes required to generate the virion structural proteins and packaging functions.
2. The vesicular stomatitis virus G-protein (pCMV-VSV-G) envelope vector, which provides the heterologous envelope for pseudotyping.
3. The shRNA transfer vector, which contains the sequence of interest as well as the *cis* acting sequences necessary for RNA production and packaging. The multi-plasmid approach results in no single plasmid containing all the genes necessary to produce packaged lentivirus.

Resulting particles are replication-incompetent and deletion in the U3 portion of the 3' LTR eliminates the promoter-enhancer region, further negating the possibility of viral replication. The system has also removed virulence genes which are not necessary for shRNA packaging. These features combined have improved biosafety and handling. It is recommended to use a third generation lentiviral system for its enhanced biosafety features. Though there are no known incidents of third generation systems producing replication competent virus, it is important to monitor for replication competency when performing routine lentiviral packaging. NIH guidelines recommend replication-incompetent lentiviral particles be handled as Risk Group-Level 2 (RGL2). Additional precautions may be required based upon local, state, or country regulations.

What are some successful approaches designed to minimize issues related to large scale manufacturability of viral vectors and its impact on environmental and regulatory risks?

One safety question regarding the large-scale manufacturability of viral vectors is the generation of replication-competent helper viruses during the packaging of the vector, which can occur by homologous recombination. Good laboratory practices are necessary to prevent any forms of contamination that may result in an opportunity for a replication-competent virus to form. Replication-competent virus testing is necessary. Because viral structural genes have been placed on different genetic units, multiple recombination events must occur before a replication competent helper virus is generated. Furthermore, areas of homology among the units expressing the helper virus proteins have been minimized. Also, heterologous promoters for the helper virus proteins are used. CDC-directed guidelines for BSL-2 + practices should dictate the handling of large amounts of virus and of concentrated virus.

Can the MISSION® lentiviral particles be further propagated in the lab?

No, the viral particles cannot be propagated for biosafety reasons. The MISSION® TRC lentiviral particles are replication incompetent. The particles are made using features of 2nd and 3rd generation lentiviral packaging systems. Genes for replication and structural proteins are absent in the packaged viral genome since these genes are supplied by other plasmids in the packaging cells. The viral genome contains only the region between the 5' and 3' LTRs of pLKO.1. In addition, the lentiviral vector contains a self-inactivating 3' LTR that renders it unable to produce infectious virus once it integrates into the host chromosome.

How do the features of the viral system work?

The library is lentiviral based. This allows for the production of viral particles in an appropriate packaging cell line. Upon infection with the resulting lentiviral particles, the shRNA sequence is integrated into a chromosome for stable expression of the short hairpin RNA. The VSV-G envelope is pantropic and allows delivery to virtually any cell. In addition, lentivirus does not require a mitotic event for integration into the host cell genome.

Can lentivirus pass the blood brain barrier (BBB)?

Given the size of the particles and the current VSV-G pseudotype, it is not expected that lentivirus will pass through the BBB (although we have no data to support this claim). Multiple researchers targeting areas of the brain have shown that direct cranial injection is optimal.

Do I need to modify my lab for work with lentivirus?

The NIH recommends preparing your lab for BSL-2 standards when using lentivirus. Please consult with your institution's biosafety officer on specific requirements.

How can we use the purified plasmid DNA format with the lentiviral packaging mix (pD8.9 and pVSVG) to produce lentiviral particles?

The MISSION® Lentiviral Packaging Mix can be used if you wish to produce more virus in your own lab since the viral particles we

produce for purchase are replication-incompetent. Our packaging mix is an optimized formulation of two plasmids (**pD8.9 and pVSVG**) consisting of a packaging vector containing the minimal set of lentiviral genes required to generate the virion structural proteins and packaging functions, and an envelope plasmid containing the vesicular stomatitis virus (VSV) G-protein. The exact contents and nature of the formulation are proprietary. The packaging plasmids are designed to be co-transfected along with a compatible lentiviral transfer vector into HEK293T cells in order to create high-titer pseudo-typed lentiviral particles used for downstream transduction applications.

Where does the shRNA insert into the genome when delivered with lentivirus?

It is commonly believed that lentivirus integrates randomly within the genome. However, some literature suggests that lentivirus inserts into active genes.

In vivo usage of lentiviral particles

Can MISSION® lentivirus be utilized *in vivo*?

Yes! MISSION®-produced lentivirus has been demonstrated to function *in vivo* (1, 5). According to Sigma-Aldrich, the MISSION® lentivirus has been successfully delivered via a variety of methodologies including: Intravenous (IV), Intraperitoneal (IP), Intramuscular (IM), Intratumoral, *ex-vivo*.

Is it beneficial to exchange Sigma-Aldrich buffer for PBS?

According to the feedback that Sigma-Aldrich received, virus suspended in PBS may lose some of its functionality relative to our traditional diluent (DMEM + 10% serum). However,

Where does the virus go when I inject it?

Experiments conducted by Sigma-Aldrich suggest that the virus is able to transduce a variety of organs/tissues when delivered systemically, including: muscle, liver, kidneys and lungs.

How should I track my *in vivo* experiments?

The simplest method for tracking *in vivo* experiments is via fluorescence. The facility offers alternate backbones with fluorescent markers; shRNA can easily be cloned into these vectors. Contact us for assistance with cloning shRNA into alternate vector backbone.

Can I deliver plasmid DNA *in vivo*?

Yes, plasmid DNA can be delivered *in vivo*. However, with plasmid delivery, the vast majority of cells will only be transiently transfected with the plasmid and silencing will only be transient.

What controls do I need for an *in vivo* experiment?

The empty vector SHC001 and SHC201 are critical for demonstrating that your new *in vivo* phenotype is not due to delivery methodology and viral integration, engagement of the RNAi machinery, or a potential immune response. In addition, in order to verify your phenotype it is often necessary to silence with an independent shRNA clone.

What are the pros/cons of *in vivo* vs. *ex vivo* delivery?

There are benefits of both delivery routes. Depending on your application and research goals, one may be better than another. Delivery *ex vivo* allows for complete selection of a pure population of transduced cells, and subsequent transplantation. However, direct injection of lentivirus into a discrete area may be required for certain applications.

How long does shRNA-mediated knockdown last *in vivo*?

According to Sigma-Aldrich, there is not an expected difference in knockdown duration between *in vitro* and *in vivo* studies.

Does lentivirus exhibit the same toxicity as adenovirus *in vivo*?

Lentiviruses transduce cells with higher efficiency and toxicity is lowered by the presence of a VSV-G-pseudotyped envelope. However, toxicity will vary on a case-by-case basis. Another advantage of Lentiviral vectors is that they are able to mediate persistent *in vivo* expression in a host of different cell types. The disadvantages of adenoviral vectors is that they can mediate robust expression in some cells and that expression is transient, due in part to the inflammation and cell-mediated immune response directed against the transduced cells.

References

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