Enhancing Lentiviral Transduction Efficiency

Methods for Enhancing Lentiviral Transduction Efficiency

Andrea Spencer
Research and Development,
Biotechnology Division,
Sigma-Aldrich

Abstract

An experiment to directly compare three methods of lentiviral transduction of Jurkat cells was conducted in order to determine the method that yields the greatest transduction efficiency. Spinoculation was carried out in parallel with an overnight incubation of virus with cells in the presence of polybrene (hexadimethrine bromide), and with transductions conducted on fibronection-coated plates. Spinoculation was shown to be the most successful method of transduction for Jurkat cells.

Introduction

RNAi is a useful tool for functional analysis of genes and developing a potential therapeutic strategy for various diseases. Unlike murine-based MMLV or MSCV retroviral systems, lentiviral-based particles permit efficient transduction and integration of a specific shRNA construct into differentiated and non-dividing cells.[1] While incubation of lentivirus pseudotyped with G glycoprotein from vesicular stomatitis virus (VSV-G) and cells in the presence of polybrene can efficiently serve as the method of transduction for many cell types, some cells are more difficult to transduce. Therefore, modifications have been made to transduction protocols for hard-to-transduce lines, such as T-cells, to facilitate binding of viral envelope protein to cells. Some of these modifications include spinoculation,[2] magnetic transduction (see MISSION® ExpressMag® product line), transduction of cells on fibronectin-coated plates,[3] and incubation of cells with concentrated viral particles[4] (greater than or equal to 10^8 TU/ mL). The following is a spinoculation protocol that was successful in the transduction of VSV-G pseudo-typed lentivirus in Jurkat cells. The protocol was carried out in parallel with an overnight incubation of virus with cells in the presence of polybrene and with transductions conducted on fibronection-coated plates.

Materials and Methods

Multiplicity of infections (MOIs) of 0.1, 0.5, 1.0, 5.0, and 10.0 using MISSION® TurboGFP™ Control Transduction Particles **(SHC003V)** were added to 2 × 10^5 Jurkat cells in 2.0 mL of complete medium (RPMI 1640, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 10% fetal bovine serum) for the three methods of transduction tested. Cells were centrifuged at 800 × g for 30 minutes at 32 °C. Virus-containing medium was aspirated, and pellets were dissociated by pipetting in 2.0 mL of complete medium for transductions via spinoculation. Cells were then plated in 6 well plates and incubated in a 37 °C, 5% CO_2 incubator overnight. Superfibronectin **(S5171)** stock solution was diluted in sterile PBS for a final concentration of 5 µg/mL, and 3.0 mL per well was added to each well of tissue culture treated 6 well plates. Plates were then incubated at 37 °C for 1.5 hours. Each well was rinsed 2× with 2.0 mL complete medium before adding cells. Cells and viral particles were then plated in 6 well plates and incubated in a 37 °C, 5% CO_2 incubator overnight. Transductions conducted by the incubation of cells and virus were carried out by adding viral particles to complete medium containing 8 µg/mL polybrene and cells. Each sample was mixed gently by pipetting. Cells and viral particles were plated in 6 well plates and incubated in a 37 °C, 5% CO_2 incubator overnight. After approximately 18 hours of incubation, all transduced cells were centrifuged, and medium was changed to fresh complete medium. Three days post-transduction, all samples were pelleted as described above and resuspended in fresh complete medium containing 2 µg/mL puromycin. Along with the transduced cultures, a non-transduced culture was seeded in puromycin-containing medium to serve as a control for judging when the transduced cultures emerged from selection. Cells were incubated overnight as described above. All samples were centrifuged as before and medium was changed with complete medium that contained 2 µg/mL puromycin. Cells were then incubated for an additional 48 hours until they completely emerged from selection as noted by cell death due to puromycin being no longer visible, and by the non-transduced well being completely dead. An aliquot of cells was added to an equal volume of trypan blue (0.4%) and cells were counted. Two counts were made for each sample and numbers were averaged.

Results and Discussion

In all three methods of transduction tested, cell viability as determined by trypan blue dye exclusion was greater than or equal to 98%. Furthermore, for all three methods of transduction tested, the number of cells surviving puromycin selection increased in a linear fashion as MOI was increased from 1 to 10. Spinoculation was the most effective method for transducing Jurkat cells **(Figure 1)**, resulting in greater than half of the original plating density on day seven post-transduction. At lower MOIs, only minimal differences between the varied transduction methods can be observed. At higher MOIs, a more dramatic effect is seen with fibronection producing an approximately 50% increase in surviving cells over polybrene, while spinoculation generated approximately a 5-fold increase in surviving cells over polybrene.

Enhancing Lentiviral Transduction Efficiency

Figure 1. Jurkat Transductions by Various Methods

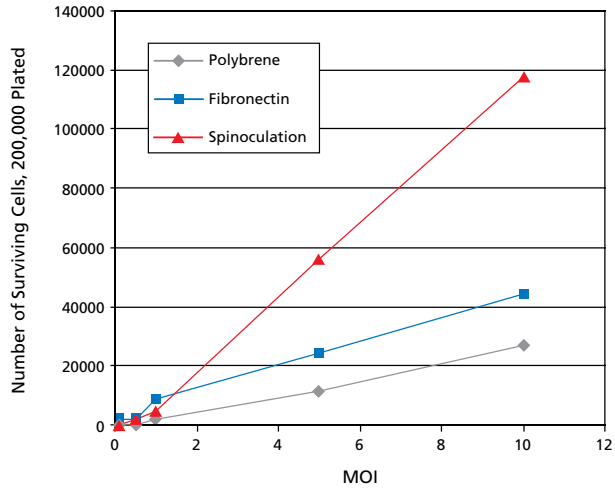
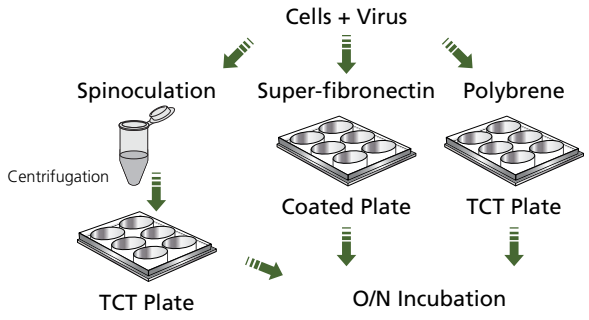


Figure 1. The graph shows viable Jurkat cell numbers resulting from the comparison of three methods of lentiviral transduction and subsequent puromycin selection. Cells were counted on day seven post-transduction.

Conclusions

While spinoculation was shown to be the most efficient method of transduction for Jurkat cells, it is important to note that the method of transduction chosen by researchers will be cell-type dependent, and that one method may work well for a particular cell type but may be suboptimal for another. It should be noted that for the spinoculation method of transduction, the time of centrifugation and the g force used may be increased to potentially increase transduction efficiency[2] in some cell types. To determine how easily a particular cell type is to transduce, one should begin with a range of MOIs using a control virus such as MISSION® Non-Target shRNA Control Transduction Particles **(SHC002V)**, and incubate cells and virus in the presence of polybrene overnight. If transduction efficiency is low using this method, the transduction protocol modifications suggested above should be performed. All of the above methodologies facilitate the binding of viral envelope proteins to cells, the first step in transduction. Jurkat are a commonly used suspension cell line, and improved transduction protocols in this line will likely be expandable to other cell types.

Figure 2. Lentiviral Transduction



References

1. Stewart, S.A. et al. 2003. Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA*, 9, 493-501.
2. O'Dahetty, U. et al. 2000. Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding. *J Virol.* 74, 10074-10080.
3. Bajaj, B. et al. 2002. Retroviral gene transfer to human epidermal keratinocytes correlates with integrin expression and is significantly enhanced on fibronectin. *Hum Gene Ther.* Oct 10;13(15), 1821-1831.
4. Burns, J.C. et al. 1993. Vesicular Stomatitis Virus G Glycoprotein Pseudotyped Retroviral Vectors: Concentration to a Very High Titer and Efficient Gene Transfer into Mammalian and Nonmammalian Cells. *Proc. Natl. Acad. Sci. USA,* 90, 8033-8037.

Ordering Information

Cat. No.	Description
SHC002V	MISSION® Non-Target shRNA Transduction Particles
SHC003V	MISSION TurboGFP Control Transduction Particles
R0883	RPMI 1640 Medium
59202C	L-Glutamine Solution 200 mM
G8644	D-(+)-Glucose solution
H0887	HEPES solution
S8636	Sodium pyruvate solution
F6178	Fetal Bovine Serum
S5171	Superfibronectin
P5493	Phosphate buffered saline
H9268	Hexadimethrine bromide
P9620	Puromycin dihydrochloride
T8154	Trypan blue solution (0.4%)

siRNA Design, Quality, and Delivery

Key Drivers of Performance in the siRNA Workflow

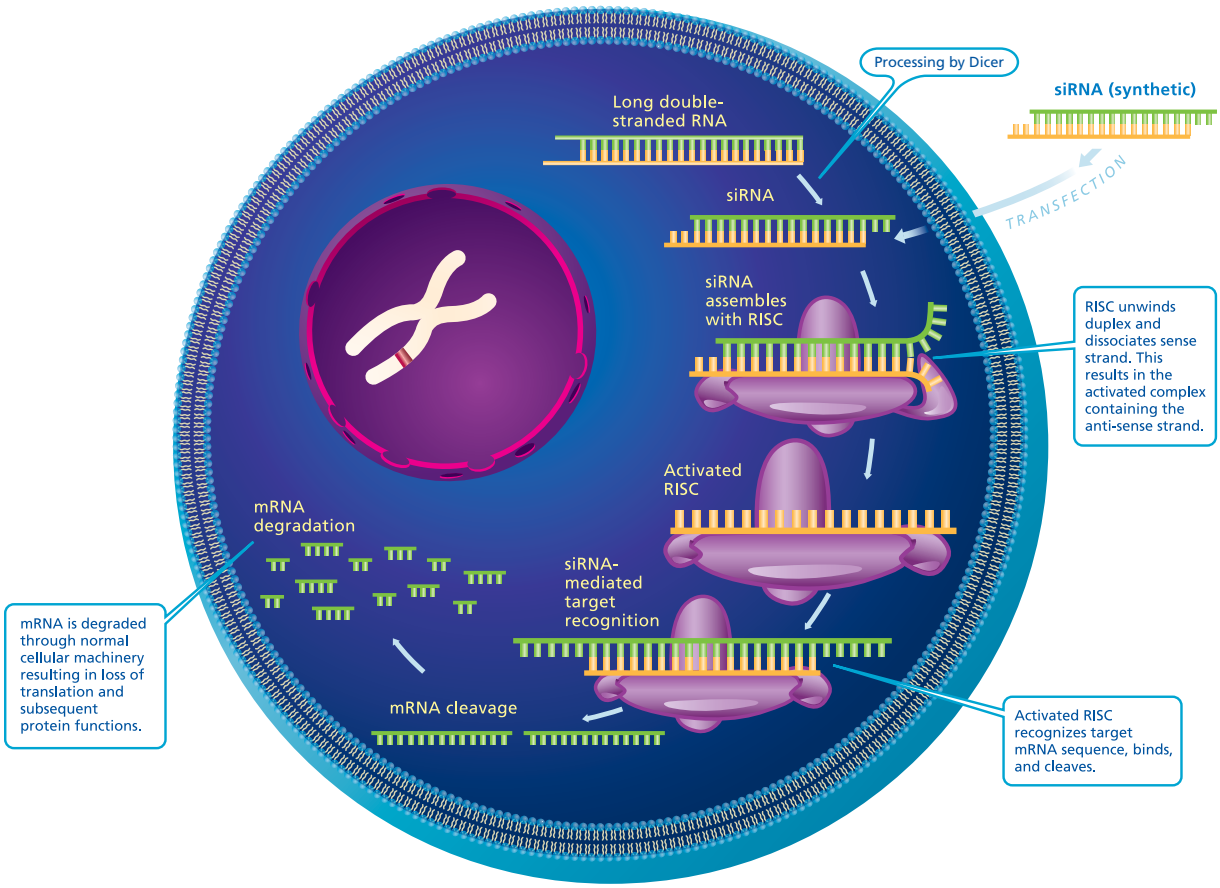
Steven Suchyta
Product Management,
Biotechnology Division,
Sigma-Aldrich

Overview of siRNAs and RNA Interference

RNA interference (RNAi) is a natural biological mechanism wherein short inhibitory RNA (siRNA) duplexes induce potent inhibition of gene expression. These siRNA duplexes are produced naturally when an enzyme, *Dicer,* cleaves long double-stranded RNA (dsRNA) into smaller fragments. The resulting 21-23 nucleotide dsRNA fragments, termed siRNAs, then associate with an RNase-containing complex to form the *RNA-induced silencing complex* (RISC). The RISC unwinds the duplex and releases the sense strand. The RISC-bound antisense strand then serves as a guide for targeting the activated complex to complementary mRNA sequences. This results in subsequent mRNA cleavage and degradation. In effect, only catalytic amounts of siRNA are required for destruction of mRNA, resulting in the knockdown or silencing of the target gene and diminished protein expression.

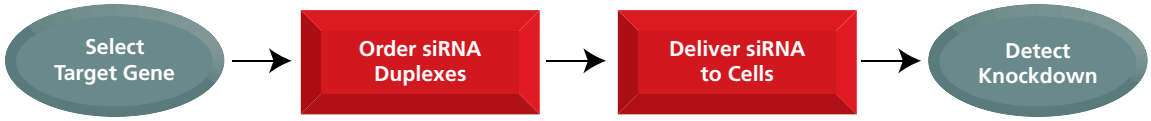
This elegant RNAi mechanism has been quickly adopted by the research community as a method for targeted gene expression knockdown. Gene expression silencing has become a very important strategy in functional genomics. Optimized siRNA reagent kits and protocols have now made RNAi experiments fast and convenient. More importantly, the availability of extensive siRNA libraries, high-throughput screening (HTS) platforms, and bioinformatics software has enabled comprehensive identification and investigation of gene functions in various metabolic pathways and disease processes. Therefore, RNA interference is a promising technology that is revolutionizing research in functional genomics and drug discovery.

RNA Interference with MISSION siRNA



siRNA Design, Quality, and Delivery

Sigma siRNA Workflow



Pre-designed MISSION® siRNA
MISSION siRNA Libraries
Custom siRNA Synthesis

N-TER™ siRNA Transfection System

GenElute™ Mammalian Total RNA Miniprep Kits
Quantitative RT-PCR
Sigma Antibodies

The siRNA Experimental Workflow

A typical siRNA experiment starts with the selection of a gene target and ends in the determination of knockdown efficiency, which is interpreted with respect to the objectives of the experiment. For studies that are focused on individual gene targets, there may be a variety of decision-making steps involved, as well as process optimization requirements. For the purpose of this article, we will take a high-level look at some of the important factors to consider when designing and executing your siRNA experiment. We will also describe the ways in which Sigma has been able to overcome some of the more challenging aspects of siRNA experimentation, and to make its knowledge and expertise available to customers.

Step 1: Design of siRNA

Current studies suggest that the design of an siRNA is an extremely important, if not the most important, factor for a successful RNAi experiment. It is for this reason that Sigma-Aldrich entered into an exclusive partnership with Rosetta Inpharmatics, a wholly owned subsidiary of Merck & Co., to use their best-in-class, proprietary siRNA design algorithm to design its MISSION® line of siRNAs. MISSION siRNAs provide RNAi researchers with cutting-edge technology for enhanced performance through improved target specificity and sensitivity in gene silencing applications.

Sigma's Partnership with Rosetta Provides Customers Access to the Following:

- *Best-in-Class siRNA Design Algorithm* – MISSION siRNA designs are based on a unique and proprietary design algorithm developed by key Rosetta scientists and bioinformaticians. This tool has been optimized and upgraded based on more than three years of extensive experimental validation and testing.
- *Comprehensive Gene Coverage* – MISSION siRNA has over 775,000 pre-designed siRNA, with multiple siRNA targeting every human, mouse, and rat gene. Finding MISSION siRNA is easy with Sigma's "Your Favorite Gene" search engine, *sigma.com/yfg*.
- *siRNAs of high specificity* – The potential for off-target effects is reduced by minimizing homology of the MISSION siRNA seed region (nucleotide residues 2-8 in the antisense strand) to the 3'UTR region of non-target mRNAs.
- *siRNAs of high potency* – MISSION siRNA sequences are designed to efficiently knock down both high and low abundance messages.
- *Guaranteed* – Sigma-Aldrich guarantees that two out of three siRNA duplexes per target gene will achieve knockdown efficiencies of greater than or equal to 75%.

Step 2: Quality siRNA Synthesis

Well-designed siRNAs will be less efficient if not accompanied by subsequent high-quality siRNA manufacturing. In most cases, high quality is assured by implementation of extensive process controls throughout the manufacturing supply chain.

For manufacture of MISSION siRNAs, Sigma utilizes its subsidiary, Custom Products, to produce world-class siRNA oligos. The most critical reagents in the synthesis of siRNA, the amidites, are manufactured under the highest quality specifications of Sigma Fine Chemicals. Sigma Custom Products manufactures MISSION siRNA using patented technologies for higher quality and faster turnaround times. The authenticity of the siRNA oligos is systematically verified by mass spectrometry, while precise siRNA concentrations are determined by UV spectrophotometry. The integrity of the duplex is confirmed by gel-shift assays. Finally, protocols are standardized at our worldwide manufacturing sites to ensure timely delivery of high-quality MISSION siRNAs or custom siRNAs anywhere in the world. Sigma Custom Products has the capability to manufacture up to 3 million oligonucleotides per year for rapid completion of large projects. For animal studies, synthesis scales of up to 100 mg and larger with purity levels up to *in vivo* quality are available.

Step 3: Delivery of siRNA to the Cell

Once an siRNA has been designed and synthesized, the researcher's next challenge lies in finding a way to deliver siRNAs into the cell. For transient knockdown of gene expression, Sigma has demonstrated efficient siRNA transfection and knockdown using the N-TER Nanoparticle siRNA Transfection System. This transfection system utilizes a cell-penetrating peptide that binds to siRNA non-covalently to form a nanoparticle. The nanoparticle then interacts directly with lipids on the surface of the plasma membrane, allowing diffusion across the cell membrane and delivery of the siRNA directly to the cytoplasm.

Features and Benefits of the N-TER Nanoparticle Transfection System

- ***Flexible*** – with N-TER you can use your choice of cell culture media, with or without serum.
- ***Rapid transfection*** – N-TER nanoparticles complexed with nuclei acids quickly cross the cell membranes and induce rapid knockdown of the target transcript, sometimes as early as 5 hours post-transfection.
- ***Efficient*** – The effective transport of the nanoparticle minimizes the amount of siRNA needed for gene knockdown and minimizes off-target and cytotoxic effects.
- ***Validated*** – The N-TER nanoparticles have been experimentally validated in a wide variety of cell types, including primary cells, neuronal cells, differentiated cells, and non-dividing cells. However, for particular applications the conditions may need to be optimized.

Step 4: Assay for Knockdown Efficiency

Experimentally, complete knockdown of a gene is difficult to achieve and often some residual expression is observed. The efficacy of gene silencing can be measured in a variety of ways, but it is very important to verify that the effect being measured is a result from the knockdown of the targeted gene and not due to off-target effects. Assays of the gene-knockdown effect can be at the transcriptional (mRNA) level, the translational (protein) level, or the phenotypic level. In general, most mRNA assays and protein assays are performed 24 to 72 hours post-transfection. However, optimal time points may need to be assessed for particular target genes or experimental conditions.

The recommended assays listed below are not intended to be comprehensive, but provide a glimpse of the different alternatives for the researcher to explore.

1. mRNA Quantitation to Monitor Transcript
   a. Measuring remaining mRNA levels for the gene of interest is a direct method for monitoring knockdown efficiency. Residual mRNA may be quantitated by a variety of methods, the most common of which is quantitative RT-PCR.

2. Quantitation to Monitor Protein Production

There are a variety of options available to assay protein production levels, from the more traditional methods such as Western blotting, ELISA, and immunofluorescence, to the recent mass spectrometric techniques such as the AQUA and the ^{18}O labeling technologies.

a. ***Western Blotting*** – This assay is analogous to Southern blotting for DNA and Northern blotting for RNA. The proteins are separated by electrophoresis, and transferred to a membrane. The protein bands are then probed with labeled antibodies. Sigma-Aldrich offers over 2,000 primary antibodies against both human and mouse proteins along with a comprehensive set of labeled anti-species secondary antibodies. Optimized protocols are widely available.

b. ***Protein-AQUA™ Labeling Technology*** – This is a mass spectrometry-based assay for the **A**bsolute **QUA**ntitation of proteins. An AQUA Peptide™ sequence is selected from the sequence of the target protein and then chemically synthesized with a stable isotope label, such as ^{13}C and ^{15}N. The target protein is enzymatically digested and a known amount of the AQUA Peptide is added to the digest as an internal standard or calibrant. The masses of the native peptide and the AQUA Peptide usually differ by 8-12 amu, depending on the peptide sequence used. The concentration of the target protein is then calculated based on the relative MS signals of the target peptide and the isotope-labeled AQUA Peptide.

c. ***^{18}O Labeling Technology*** – This mass spectrometry-based protein assay is somewhat analogous to the AQUA strategy, except that this is designed for relative protein quantitation. Proteins from two different treatment conditions are digested with trypsin and then one of the samples is labeled with ^{18}O. The masses of the two peptides differ by 4 amu and are clearly resolved by MS. The relative ion intensities of the two peptides are quantitative indicators of their relative abundance.

Conclusions

Various factors contribute to optimizing siRNA used in RNA interference studies. Design, Quality and Delivery represent three of the most important elements that allow for accurate analysis of gene knockdown. Through our ongoing commitment to the study of gene function, Sigma has forged partnerships and made investments in this area that bring these elements together to create our best-in-class pre-designed MISSION® siRNA and MISSION siRNA Libraries. The development of these products is a result of partnerships with world class bioinformatics leader Rosetta Inpharmatics for the latest design rules, the acquisition of key intellectual property, and worldwide siRNA manufacturing capabilities for unsurpassed capacity and large-project flexibility. The continuous search for new technologies and advancements by our dedicated staff of R&D scientists pull together the entire workflow and create unique solutions that enable the study of RNA interference.

To learn more about Sigma's products for RNAi and to contact Sigma's RNAi experts, please visit us online at *sigma.com/rnai*.

Ordering Information

Cat. No.	Description	Quantity
N2913	N-TER Nanoparticle siRNA Transfection System	120 µL 400 µL 1 mL
SI00100	MISSION siRNA Human Druggable Genome	1 set
SI20100	MISSION siRNA Rat Druggable Genome	1 set
SI42050	MISSION siRNA Mouse Kinase Library	1 set