*Trans*IT® siRNA Transfection Reagents for High Efficiency siRNA Delivery *In Vitro*

Robert Brazas, Shyla Millard, Tracy Kawleski, and James Hagstrom
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Introduction

The ability to efficiently knockdown target gene expression plays a critical role in the study of molecular and cellular processes, and recently a new phenomenon, RNA interference, has emerged as a powerful tool for such studies. RNA interference (RNAi) is the sequence-specific degradation of target mRNA that is triggered in a cell by double stranded RNA containing one strand that is complementary to the target mRNA.

In exciting research first reported by Elbashir and Tuschl[1], it has been shown that when short RNA duplexes, as opposed to long dsRNA, are introduced into mammalian cells in culture, RNAi sequence-specific destruction of target mRNA can be achieved without triggering a non-specific interferon response. These short dsRNAs, referred to as short interfering RNAs (siRNA), act in concert with cellular proteins to cleave greater than 95% of the target mRNA in a cell. The RNAi effect can be long lasting and may be detectable after many cell divisions. These properties make siRNA extremely effective at inhibiting target gene expression and a powerful tool for analyzing the loss of function phenotype for specific cellular targets.[1,2,3,4]

In response to these significant findings, Mirus Bio Corporation has developed two siRNA-specific delivery reagents, the *Trans*IT-TKO® and *Trans*IT®-siQUEST™ Transfection Reagents. Both of these novel reagents enable highly efficient siRNA transfections with minimal levels of cellular damage as compared to cationic-liposome based transfection reagents. Transfections are most effective when carried out in complete growth media, eliminating the need for media changes or serum addition. The *Trans*IT-TKO® and *Trans*IT®-siQUEST™ Transfection Reagents are highly effective, allowing the use of extremely low levels of siRNA to successfully knockdown both transient and endogenous gene expression in a wide variety of mammalian cell lines. Due to their unique formulations, each transfection reagent has a distinct transfection profile allowing the user to identify the optimal siRNA transfection reagent for a particular cell line. These features make the *Trans*IT® siRNA Transfection Reagents ideal for all siRNA-mediated gene-silencing studies.

Results and Discussion

Mirus Bio's siRNA transfection reagents are specifically designed to efficiently deliver siRNA to cells *in vitro*. The *Trans*IT-TKO® and *Trans*IT®-siQUEST™ Transfection Reagents, combined with a target-specific siRNA, can efficiently silence endogenous, stable, or transient gene expression.

Optimization of siRNA delivery using *Trans*IT-TKO® or *Trans*IT®-siQUEST™ Transfection Reagents.

In order to achieve the highest efficiency siRNA transfection and corresponding effective knockdown of target gene expression, there are six key factors that should be optimized:

- siRNA design
- Cell density at the time of transfection
- Cell passage number
- Volume of siRNA transfection reagent used
- Concentration of siRNA
- Post-transfection incubation time

For additional information on the optimization of siRNA transfections, see the *Trans*IT-TKO® or *Trans*IT®-siQUEST™ Transfection Reagent protocols.

In vitro subcellular localization of siRNA after delivery using *Trans*IT-TKO® and *Trans*IT®-siQUEST™ Transfection Reagents.

In order to visualize the subcellular localization and measure the delivery efficiency of siRNA after transfection with the *Trans*IT® siRNA Transfection Reagents, *Label* IT® siRNA Tracker-labeled siRNA duplexes were transfected into HeLa cells using *Trans*IT-TKO® or *Trans*IT®-siQUEST™ Transfection Reagents then visualized by confocal microscopy (Figure 1). The majority of the siRNA is cytoplasmic. Both diffuse and punctate signal was observed, indicating that there may be two populations of delivered siRNA molecules; one which is free in the cytoplasm (diffuse) and the other which is contained within endosomes (punctate). Each cell contains the labeled siRNA, demonstrating high efficiency delivery by the *Trans*IT® siRNA Transfection Reagents (Figure 1 and data not shown).

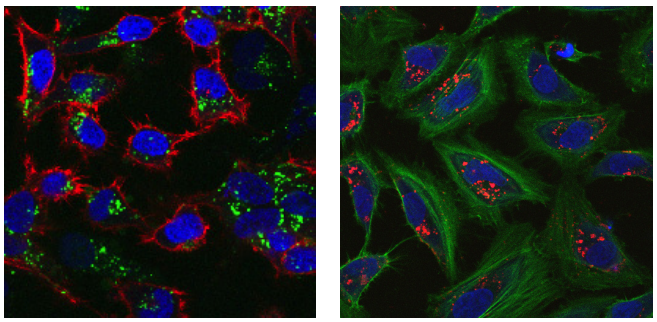

Figure 1. Delivery of Fluorescently-Labeled siRNA using *Trans*IT-TKO® and *Trans*IT®-siQUEST™ Transfection Reagents. (Left Panel) HeLa (70% confluence) cells in 12-well plates were transfected with *Trans*IT-TKO® Transfection Reagent (3 µl) and *Label* IT® siRNA Tracker Fluorescein-labeled siRNA duplexes (GREEN, 50 nM final concentration in the well). The cells were incubated 24 hours post-transfection then fixed and counterstained with TO-PRO®-3 (nuclei, BLUE) (Invitrogen) and Alexa Fluor® 546 Phalloidin (actin, RED) (Invitrogen). Confocal images were acquired on a Zeiss LSM 510 Confocal Microscope. **(Right Panel)** HeLa cells were transfected as described above except that 3 µl of *Trans*IT®-siQUEST™ Transfection Reagent and *Label* IT® siRNA Tracker Cy™3-labeled siRNA duplexes (25 nM final concentration in the well) (RED) were used. Actin was counterstained with Alexa Fluor® 488 Phalloidin (GREEN) (Invitrogen).

Sequence specific knockdown of a transgene after delivery of siRNA using *Trans*IT-TKO® or *Trans*IT®-siQUEST™ Transfection Reagents.

siRNA complexed with either *Trans*IT-TKO® or *Trans*IT®-siQUEST™ Transfection Reagent has the ability to selectively knockdown target gene expression without affecting the expression of non-targeted genes. To illustrate this ability and the broad spectrum transfection capability of each transfection reagent, firefly luciferase and sea pansy luciferase expression vectors were co-transfected into a variety of cell lines using either *Trans*IT®-LT1 Transfection Reagent or a *Trans*IT® cell-line specific plasmid transfection reagent. Four hours later, *Trans*IT-TKO® Reagent or *Trans*IT®-siQUEST™ Reagent/anti-firefly luciferase siRNA complexes were added to the cells such that the final concentration of siRNA in the well was 25 nM. The level of firefly and sea pansy luciferase expression was measured 24 hours post-transfection and the level of firefly luciferase expression was normalized to sea pansy luciferase expression. The percent firefly luciferase expression was determined by comparing the normalized expression levels to the normalized reagent alone transfected control. Using 25 nM anti-firefly luciferase siRNA, there was no decrease in the level of sea pansy luciferase expression compared to the reagent alone control (data not shown), demonstrating the specificity of both the anti-firefly luciferase siRNA and the RNAi pathway. However, there were dramatic decreases in firefly luciferase expression due to the RNAi effect. Each uniquely formulated siRNA transfection reagent has a distinct transfection profile. For example, while both reagents are highly efficient in a broad range of cell lines, we find *Trans*IT-TKO® Reagent is optimal in HEK 293, COS-7 and RAW264.7 cells while *Trans*IT®-siQUEST™ Reagent is the recommended reagent for A549, CHO-K1, HepG2, MCF-7 and Vero cells (Table 1).

Cell Line (Source)	Percent Firefly Luciferase Knockdown	
	*Trans*IT-TKO® Reagent	*Trans*IT®-siQUEST™ Reagent
A549 (human lung)	80%	91%
BNL CL.2 (mouse liver)	81%	*
C2Cl2 (mouse fibroblast)	93%	*
C6 (rat brain)	95%	*
CHO-K1 (hamster ovary)	96%	97%
COS-7 (monkey kidney)	94%	90%
Daoy (human brain)	94%	*
DB-TRG-05MG (human brain)	87%	*
DI-TNC1 (rat brain)	93%	*
DU-145 (human prostate)	91%	*
HEK 293 (human kidney)	86%	82%
HeLa (human cervix)	86%	85%
Hepa1c1c7 (mouse liver)	97%	*
HepG2 (human liver)	86%	92%
Jurkat (human T lymphocyte)	86%	*
MCF-7 (human breast)	83%	91%
Neuro-2a (mouse brain)	91%	*
NIH 3T3 (mouse fibroblast)	80%	79%
NIKS (human keratinocytes)	96%	*
PC-3 (human prostate)	80%	*
Primary Rat Hepatocytes	85%	*
RAW264.7 (mouse monocyte)	84%	78%
Secondary Human Astrocytes	94%	*
SK-N-MC (human brain)	92%	*
SVGp12 (human brain)	92%	*
THP-1 (human monocyte)	91%	*
Vero (monkey kidney)	90%	93%

* Not tested

Table 1. RNA Interference using *Trans*IT-TKO® and *Trans*IT®-siQUEST™ Reagent/Anti-Firefly Luciferase siRNA Complexes. Reporter plasmids expressing firefly and sea pansy luciferase were co-transfected into a variety of cell lines using the appropriate *Trans*IT® plasmid transfection reagent. Firefly luciferase expression was knocked down using anti-firefly luciferase siRNA (25 nM final concentration in well) complexed and delivered with either *Trans*IT-TKO® or *Trans*IT®-siQUEST™ Transfection Reagent. Twenty-four hours post-transfection, firefly luciferase expression was determined and normalized to sea pansy luciferase expression in each sample to control for plasmid transfection efficiency. No reduction in sea pansy luciferase expression was observed. The percent decrease of firefly luciferase expression was determined by comparing the normalized firefly luciferase expression to the reagent alone control.

Knockdown of endogenous genes after delivery of siRNA using *Trans*IT-TKO® and *Trans*IT®-siQUEST™ Transfection Reagents.

To test the ability of the *Trans*IT® siRNA Transfection Reagents to deliver siRNA targeted against an endogenous gene, both reagents were used to deliver anti-firefly luciferase siRNA to a panel of cell lines stably expressing the firefly luciferase gene. These cell lines and the stably expressed luciferase gene are excellent models for targeting an endogenous gene because, like normal cellular genes, the firefly luciferase gene in these cells is continually expressed at steady state levels. Optimal levels of each reagent were used to deliver anti-firefly luciferase siRNA to each well, and firefly luciferase activity was assayed (Table 2). Highly effective knockdown of the stably integrated luciferase gene expression was observed after delivery using either siRNA transfection reagents. These results demonstrate the effectiveness of the *Trans*IT-TKO® and *Trans*IT®-siQUEST™ Reagents to deliver siRNA.

Cell Line (Source)	Knockdown Efficiency of Stably Integrated Firefly Luciferase	
	*Trans*IT-TKO® Reagent	*Trans*IT®-siQUEST™ Reagent
A549-Luc (human lung)**	77%	82%
CHO-K1-Luc (hamster ovary)*	86%	91%
HEK 293-Lux (human kidney)*	83%	77%
HeLa-Luc (human cervix)*	84%	82%
Hepa1c1c7-Luc (mouse liver)**	90%	92%
NIH 3T3-Lux (mouse fibroblast)*	85%	89%

Table 2. Knockdown of Stably Expressed Firefly Luciferase using *Trans*IT-TKO® and *Trans*IT®-siQUEST™ Transfection Reagents to Deliver Anti-Firefly Luciferase siRNA. The indicated cells lines stably expressing either the pGL2 (Promega) version of firefly luciferase (Lux) or the humanized pGL3 (Promega) version (Luc) were seeded in 24-well plates and incubated overnight in complete growth media. Optimal amounts of *Trans*IT-TKO® or *Trans*IT®-siQUEST™ Transfection Reagent were complexed with anti-firefly luciferase siRNA (Luc- or Lux-specific; 25 nM final concentration in the well). These complexes were then added to approximately 60% confluent cells in their complete serum-containing media. Twenty-four (*) or 48 hours (**) post-transfection, cell lysates were assayed for luciferase expression on an EG&G Berthold Lumat luminometer, using a standard luciferase assay. Percent knockdown was calculated by comparing expression levels post-siRNA transfection to the reagent alone control transfected samples.

To further demonstrate the ability of *Trans*IT-TKO® and *Trans*IT®-siQUEST™ Reagents to knockdown expression of endogenous targets, siRNAs targeted against an array of endogenous mRNAs were delivered to a variety of cells and cell lines. Twenty-four hours post-transfection, target mRNA levels were measured using quantitative real-time PCR. As illustrated in Table 3 and Figures 2 and 3, each *Trans*IT® siRNA Transfection Reagent is highly capable of delivering siRNA to a variety of cells including primary mouse hepatocytes and producing knockdown efficiencies ranging from 70-85% only 24 hours post-transfection.

Cell Line (Source)	Endogenous Target Transcript	Target Knockdown Efficiency	
		*Trans*IT-TKO® Reagent	*Trans*IT®-siQUEST™ Reagent
BNL CL.2 (mouse liver)	MAPK1	80%	*
	MAPK3	83%	*
HeLa (human cervix)	Lamin A/C	80%	*
	GAPDH	80%	*
Hepa1c1c7 (mouse liver)	MAPK1	80%	*
	MAPK3	75%	*
	MEK1	75%	*
	PTEN	80%	*
HepG2 (human liver)	MAPK1	80%	*
NIH 3T3-L1	MAPK1	70%	*
(mouse fibroblast/adipose)	MAPK3	70%	*
Secondary Human Astrocytes	Lamin A/C	80%	*
Primary Mouse Hepatocytes	ABC A1	70%	*
	Lamin A/C	81%	*
	PPARα	*	81%
Primary Rat Hepatocytes	MAPK3	85%	*

* Not tested

Table 3. RNA Interference on Endogenous Targets using *Trans*IT-TKO® and *Trans*IT®-siQUEST™ Reagents to Deliver the Various siRNAs. The cell lines indicated in the table were seeded in 12-well plates one day prior to transfection. The cells were transfected at ~60% confluence using either *Trans*IT-TKO® or *Trans*IT®-siQUEST™ Transfection Reagent and 25 nM (final concentration per well) of the indicated siRNAs. Each transfection was performed in triplicate and the results represent an average of the three samples. Cells transfected with reagent alone or non-specific siRNA served as controls for normal levels of target gene expression. The cells were harvested 24 hours post-transfection and total RNA was isolated using TRI REAGENT (Molecular Research Center). Relative target gene levels were measured by quantitative RT-PCR using target-specific primer pairs and SYBR Green detection. Expression levels between samples were normalized to total RNA or GAPDH mRNA levels measured by quantitative RT-PCR.

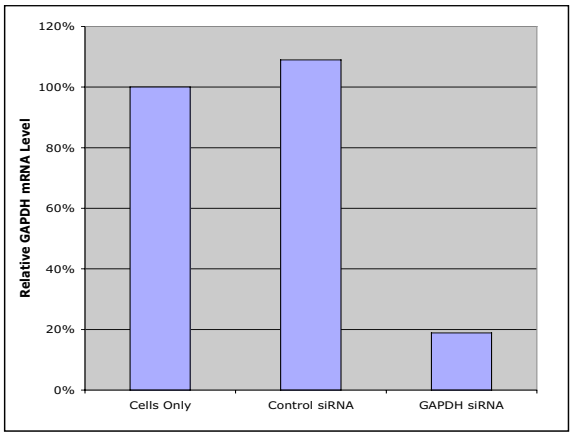

Figure 2. Knockdown of Endogenous GAPDH in HeLa Cells using *Trans*IT-TKO® Reagent to Deliver the siRNA. HeLa cells were seeded in 12-well plates one day prior to transfection. Once the cells reached 50% confluency, they were transfected using *Trans*IT-TKO® Reagent with an siRNA targeting GAPDH (25 nM final concentration in the well). Untransfected cells and cells transfected with an siRNA designed to target GFP served as controls. The cells were harvested 24 hours post-transfection and total RNA isolated using TRI REAGENT (Molecular Research Center). Total RNA concentration was measured using RiboGreen (Invitrogen). Relative GAPDH levels were measured by QPCR using a GAPDH-specific primer pair and SYBR Green detection.

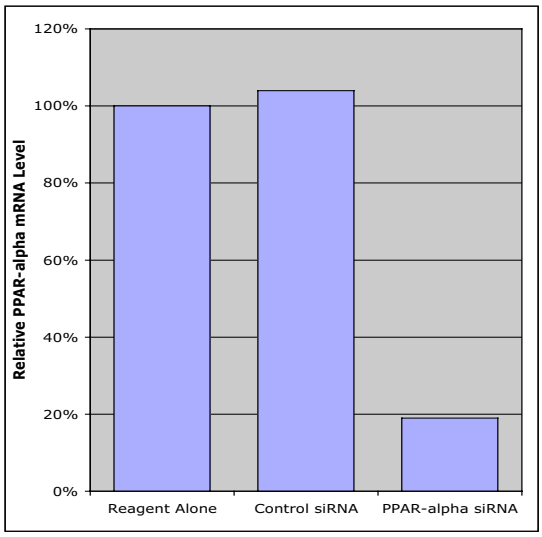

Figure 3. Knockdown of Endogenous PPAR-alpha in Primary Mouse Hepatocyes using *Trans*IT®-siQUEST™ Reagent to Deliver the siRNA. Primary mouse hepatocytes were seeded in 6-well plates one day prior to transfection at 50% confluency, The next day, the cells were transfected using *Trans*IT®-siQUEST™ Reagent and 25 nM anti-PPAR-alpha siRNA. Cells transfected with reagent alone and an siRNA designed to target secreted alkaline phosphatase served as controls. The cells were harvested 24 hours post-transfection and total RNA was isolated using TRI REAGENT (Molecular Research Center). Relative PPAR-alpha levels were measured by QPCR using a PPAR-alpha-specific primer pair and SYBR Green detection and normalized to GAPDH mRNA levels in the same samples.

Summary

This report demonstrates that both *Trans*IT-TKO® and *Trans*IT®-siQUEST™ Reagents effectively deliver siRNAs targeted against transiently transfected, stably integrated, and endogeneous targets. These novel delivery technologies enable the use of siRNA to inhibit expression of endogenous cellular genes in order to study a wide variety of biological phenomena.

- Gene function can be investigated by assessing the biological effects of siRNA-mediated knockdown of expression of that particular gene.
- Knockdown of a gene known to be required in a particular signal transduction pathway enables identification of the role that pathway plays in cellular physiology and/or differentiation.
- The ability to transfect an expression plasmid concurrently with siRNA allows the study of a wide variety of biological events that require both activation and repression of particular genes.
- Concurrent transfection can also be used in gene replacement strategies involving introduction of a plasmid containing a modified version of a gene while simultaneously knocking down the endogenous copy in the same cell.

The *Trans*IT-TKO® Transfection Reagent was the first product developed specifically for transfection of siRNA. Mirus Bio's second siRNA-specific *Trans*IT®-siQUEST™ Transfection Reagent expands and enhances the *Trans*IT® line of siRNA delivery reagents. The combination of high siRNA transfection efficiency in a wide variety of cell lines, low cellular toxicity, and ease of use make *Trans*IT-TKO® and *Trans*IT®-siQUEST™ Transfection Reagents ideal for siRNA-mediated gene knockdown studies.

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Ordering Information:

*Trans*IT-TKO® Transfection Reagent

Product #	Quantity
MIR 2150	1 ml
MIR 2154	0.4 ml
MIR 2155	5 x 1 ml
MIR 2156	10 x 1 ml

*Trans*IT®-siQUEST™ Transfection Reagent

Product #	Quantity
MIR 2110	1 ml
MIR 2114	0.4 ml
MIR 2115	5 x 1 ml
MIR 2116	10 x 1 ml

Related Products:

Label IT® siRNA Tracker Kits (Product # MIR 7200, 7201, 7202, 7203, 7204, 7205, 7206, 7207, 7208, 7209, 7210, 7211, 7212, 7213, 7214, 7215, 7216, 7217)

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