

TransIT®-siPAK Plus Trial Kit

Product	Components	Component No.	Quantity	Product No.
TransIT®-siPAK Plus Trial Kit	TransIT®-siQUEST™ Reagent*	2111	0.1 ml	MIR 2270
	TransIT-TKO® Reagent*	2152	0.1 ml	
	Label IT® RNAi Delivery Control Fluorescein*	7902	10 µg (~0.75 nmol)	
	10X RNAi Dilution Buffer	328	75 µl	

*To re-order components separately, see Related Products Section 5.0.

Please read the entire protocol carefully and proceed with the section(s) appropriate for the desired experiment.

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1.0 DESCRIPTION
1.1 General Information

Cellular uptake of long double stranded RNA (dsRNA) has been shown to induce RNA interference in a diverse group of organisms as well as insect cells in culture. RNA interference leads to the inhibition of protein expression through a sequence-specific, dsRNA-mediated destruction of the target messenger RNA (mRNA). Attempts to induce RNA interference using long dsRNA in mammalian cell lines have been met with limited success, due in part to the induction of the interferon response, which results in a general inhibition of protein synthesis. It has been shown that when short RNA duplexes (< 30 base pairs) are introduced into mammalian cells in culture, sequence-specific destruction of target mRNA can be achieved without inducing an interferon response. These short dsRNA, referred to as small interfering RNA (siRNA), act catalytically at sub-molar ratios to cleave greater than 95% of the target mRNA in the cell. The RNA interference effect can be long-lasting and may be detectable after many cell divisions. These properties make RNA interference, induced by the introduction of siRNA, extremely effective at inhibiting target gene expression.^{1,2,3}

Mirus Bio Corporation has developed the *TransIT*[®]-siPAK Trial Kit which contains samples of both the *TransIT*[®]-siQUEST[™] and *TransIT*-TKO[®] siRNA Transfection Reagents, along with a trial size aliquot of our *Label IT*[®] RNAi Delivery Control Fluorescein. Each transfection reagent contains a unique formulation that provides a distinct transfection profile for high efficiency, broad-spectrum siRNA delivery into the cell type of interest. The *Label IT*[®] RNAi Delivery Control consists of a fluorescein-labeled double-stranded RNA duplex that has the same length, charge, and configuration as standard siRNA used in RNAi studies. The RNAi sequence is chosen such that it is not homologous to any known gene and is not known to affect any cellular events.

The *TransIT*[®]-siPAK Plus Trial Kit contains 0.1 ml of each siRNA delivery reagent and 10 µg of the RNAi Delivery Control Fluorescein, which are sufficient quantities to perform up to 100 transfections and/or visualizations in 24-well plates depending on the specific cell type. Full size aliquots of these reagents are also available separately. (see Related Products, Section 5.0).

1.2 Cell Lines Successfully Tested by Mirus Bio Corporation

Using *TransIT*[®]-siQUEST[™] Reagent:

A549, BHK-21, CHO-K1, COS-7, HEK 293, HeLa, Hepa1c1c7, HepG2, MCF-7, NIH 3T3, primary mouse hepatocytes, RAW 264.7, and Vero cells.

Using *TransIT*-TKO[®] Reagent:

A549, BHK-21, BNL CL.2, C2C12, C6, CHO-K1, COS-7, Daoy, DB-TRG-05MG, DI-TNC1, DU 145, HEK 293, HeLa, Hepa1c1c7, HepG2, human astrocytes, Jurkat, keratinocytes (NIKS), MCF-7, Neuro-2a, NIH 3T3, PC-3, primary mouse hepatocytes, primary rat hepatocytes, RAW 264.7, SK-N-MC, THP-1, and Vero cells.

1.3 Specifications

TransIT[®]-siQUEST[™]

Reagent

Concentration: 3.0 mg/ml in 80% ethanol

Storage: Tightly capped at 4°C; DO NOT FREEZE

TransIT-TKO[®] Reagent

Concentration: 2.5 mg/ml in 100% ethanol

Storage: Tightly capped at 4°C; DO NOT FREEZE

Label IT[®] RNAi Delivery Control

Storage: Store the *Label IT*[®] RNAi Delivery Control at -20°C, protected from exposure to light. Store the 10X RNAi Dilution Buffer at 4°C.

Concentration: 10 µM (~0.75 nmol)

2.0 PROCEDURE

2.1 Transfection Optimization

The key to successful transfection is careful optimization of reaction conditions for each individual cell type. The transfection protocols described in Sections 2.2 to 2.5 should result in efficient transfection of most cell types; however, to ensure optimal results, consider the following variables:

- A. Media conditions** — The *TransIT*[®]-siQUEST[™] and *TransIT*-TKO[®] Reagents yield improved transfection efficiencies when transfections are performed in complete growth medium (instead of serum-free medium) without a post-transfection media change.
- B. Cell density (% confluence) at time of transfection**— The recommended cell density for most cell types is 60-80% confluence at the time of transfection. If this confluence does not produce optimal results, test cell densities outside of the recommended range. Determine the optimal cell density for each cell type in order to maximize knockdown efficiency. Maintain this density in future experiments for reproducibility.

Lower cell densities may be necessary for post-transfection incubation times greater than 48 hours. If lower cell densities are plated, ensure that the levels of transfection reagent(s) and siRNA are titrated accordingly. Alternatively, trypsinize and replate cells 24 hours post-transfection to accommodate longer incubation times.

- C. siRNA concentration**— siRNA used for transfection should be pure, sterile, and the correct sequence. As a starting point, use 25 nM siRNA (final concentration in well). Depending on the type of experiment, the optimal final siRNA concentration for transfection is typically within the range of 10 to 50 nM.
- D. Transfection reagent**—As a starting point, test three levels each of the *TransIT*[®]-*siQUEST*[™] (0.5 µl, 1.5 µl, and 3 µl) and *TransIT*-TKO[®] (1 µl, 2 µl, and 4 µl) Reagents per well of a 24-well plate using 25 nM siRNA (final concentration in the well). The optimal transfection reagent volume can be determined by titrating each reagent within the ranges listed in Table 1. The volume of reagent that achieves the highest knockdown efficiency with the lowest cellular toxicity should be used for future transfections.
- E. Post-transfection incubation time**— The optimal incubation time can be determined empirically by testing a range from 24-72 hours post-transfection depending on the stability of the target mRNA and its encoded protein. When quantifying knockdown efficiencies at the mRNA level, assaying at 24 hours post-transfection is often sufficient. When quantifying knockdown efficiencies at the protein level, longer post-transfection incubations may be necessary if the target protein has a long cellular half-life and therefore degrades slowly.
- F. Proper controls**— We recommend using a negative control containing serum-free media, *TransIT*[®] siRNA Delivery Reagent, and a non-specific siRNA such as Dharmacon's siCONTROL Non-Targeting siRNA #1 (Cat.# D-001210-01-05). Other controls to consider are a cells alone control and a reagent alone control. Perform these controls in parallel to the serum-free media/transfection reagent/specific siRNA transfections using the same volumes and transfection parameters.

Table 1. Recommended titration ranges for *TransIT*[®]-*siQUEST*[™] and *TransIT*-TKO[®] Transfection Reagents

Culture Vessel	96-well plate	48-well plate	24-well plate	12-well plate	6-well plate	10 cm dish
Surface Area*	0.35 cm ²	1.0 cm ²	1.9 cm ²	3.8 cm ²	9.6 cm ²	59 cm ²
Serum-Free Media	9 µl	25 µl	50 µl	100 µl	250 µl	1500 µl
<i>TransIT</i> [®] - <i>siQUEST</i> [™] Transfection Reagent or <i>TransIT</i> -TKO [®] Transfection Reagent**	0.09-0.6 µl	0.26-1.6 µl	0.5-3 µl	1-6 µl	2.5-15 µl	15-90 µl
	0.18-0.74 µl	0.5-2 µl	1-4 µl	2-8 µl	5-20 µl	30-120 µl
1 µM stock siRNA (10-50 nM final concentration in well)	0.5-2.6 µl	1.5-7.5 µl	3-15 µl	6-30 µl	15-75 µl	90-450 µl
Complete Growth Media (Adjusted Prior to Transfection)	44 µl	125 µl	250 µl	500 µl	1250 µl	7500 µl

*Surface areas are based on Greiner tissue culture plates and Falcon 10 cm dishes.

**Test both reagents in separately to perform a true side-by-side comparison for proper product evaluation and selection.

NOTE: All volumes in Table 1 are per one well of the indicated size. To dilute siRNA, use the manufacturer's recommended buffer. Alternatively, use 100 mM NaCl in 50 mM Tris, pH 7.5, made with RNase-free water. Do not use water alone to dilute siRNA, as this may result in denaturation of the siRNA duplex.

The following protocols are recommended for performing transfections in 24-well plates. When performing transfections in different sized wells, the amount of siRNA, serum-free media, Transfection Reagent, and culture media should be scaled up or down in proportion to the surface area of the dish (see Table 1).

2.2 siRNA Transfection in 24-well Plates (To Knockdown Expression of Endogenous Target Gene)

A1. Cell Plating of Adherent Cells

1. Approximately 24 hours prior to transfection, plate cells at an appropriate cell density to obtain ~60-80% confluence the following day (6×10^4 to 2.4×10^5 cells per ml, depending on cell size and characteristics)^a. Plate adherent cells in 500 μ l of complete growth media per well.
2. Incubate the cells overnight^b.

A2. Cell Plating of Suspension Cells

1. Immediately prior to transfection, plate cells in 250 μ l of complete growth media per well of a 24 well-plate, at an appropriate density (such as 6×10^5 to 1×10^6 cells per ml). Alternatively, plate 250 μ l of cells the day prior to transfection at an appropriate density (such as $3\text{-}5 \times 10^5$ cells per ml).

B. Complex Formation (perform this procedure immediately prior to transfection)

1. In a sterile, plastic tube, add 50 μ l (see Table 1) of serum-free media^c.
2. Add 0.5 to 4 μ l of reagent (0.5 μ l, 1.5 μ l, and 3 μ l for *TransIT*[®]-*siQUEST*[™] Reagent or 1 μ l, 2 μ l, and 4 μ l for *TransIT*-TKO[®] Reagent) directly into the serum-free media. Mix thoroughly by pipetting. For details see Table 1.
3. Add 10-50 nM siRNA (the starting recommendation is 25 nM final concentration in the well) to the diluted transfection reagent. Mix by gentle pipetting.
4. Incubate at room temperature for 5-20 minutes.

C. Cell Preparation for Transfections in Complete Growth Media

NOTE: These transfection reagents yield improved transfection efficiencies when the transfections are performed in complete growth media (instead of serum-free media).

1. For adherent cell types, adjust the volume in the well to 250 μ l of complete growth media by removing ~250 μ l (half) of the original plating media to conserve siRNA. This media reduction requires the use of less siRNA to obtain the recommended 25 nM (final concentration in the well).
2. Add the transfection reagent/siRNA complex mixture prepared in step B dropwise to the cells. Gently rock the plate back and forth and from side to side to distribute the complexes evenly. Do not swirl the plate.
3. Incubate for 24-72 hours^b.

NOTE: The above incubation is designed for transfections performed with no media change. If a media change is necessary to remove the transfection complexes, incubate the cells for 24 hours, replace the original media with fresh complete growth media, and incubate for an additional 24-48 hours^b.

4. Assay for knockdown of target gene expression.

2.3 Sequential Transfection of Plasmid DNA in a T-75 Flask and siRNA in 24-well Plates (For Adherent Cells)

NOTE: Do not transfect plasmid DNA (with a plasmid DNA transfection reagent) and siRNA (with *TransIT*[®]-*siQUEST*[™] Reagent) simultaneously. The *TransIT*[®]-*siQUEST*[™] Reagent may inhibit DNA delivery.

Simultaneous transfections using the *TransIT*[®]-LT1 Reagent (for DNA delivery) and *TransIT*-TKO[®] Reagent (for siRNA delivery) can be performed since the *TransIT*-TKO[®] Reagent does not inhibit DNA delivery. For simultaneous transfections using the *TransIT*[®]-LT1 and *TransIT*-TKO[®] Reagents, see section 2.5.

The following recommendations are for plasmid DNA transfection of adherent cells in a T-75 flask. The plasmid transfected cells are then re-plated in a 24-well plate and subsequently transfected with siRNA. Allot for one T-75 flask per 24-well plate to be transfected.

A. Cell Plating of Adherent Cells (in a T-75 Flask):

1. Approximately 24 hours prior to transfection, plate cells in a T-75 flask, at an appropriate cell density to obtain ~60-80% confluence the following day (6×10^4 to 2.4×10^5 cells per ml, adding 19 mls/flask, depending on cell size and characteristics)^a.
2. Incubate the cells overnight^b.

B. Complex Formation: *TransIT*[®]-LT1 Reagent and Plasmid DNA (pDNA)

NOTE: Mirus Bio offers several reagents for plasmid transfection, including cell line specific reagents. To choose the appropriate reagent, please see Related Products, our website (www.mirusbio.com), or contact Technical Support at techsupport@mirusbio.com or 888.530.0801. The following protocol recommends *TransIT*[®]-LT1 Transfection Reagent, a broad spectrum DNA transfection reagent, which efficiently delivers DNA into many cell types.

1. In a sterile plastic tube, add 500 μ l of serum-free media^c.
2. Add 45 μ l *TransIT*[®]-LT1 Transfection Reagent directly to the serum-free media. Mix thoroughly by pipetting.
3. Add plasmid 15 μ g DNA (titration of pDNA may be necessary) to the diluted *TransIT*[®]-LT1 Reagent. Mix by gentle pipetting.
NOTE: If transfecting more than one plasmid, mix the plasmids together in a microcentrifuge tube and incubate for 5 minutes at room temperature before adding to the diluted *TransIT*[®]-LT1 Reagent.
4. Incubate at room temperature for 15-30 minutes.

C. Plasmid Transfection

1. Add the *TransIT*[®]-LT1 Reagent/DNA complex mixture from Step B dropwise to the cells in complete growth medium. Gently rock the flask back and forth and from side to side to distribute the complexes evenly. Do not swirl the flask.
2. Incubate for 2-4 hours^b.

D. Transfer of Cells from T-75 flask(s) to a 24-well Plate(s)

1. Trypsinize the T-75 flask according to standard procedures. Transfection reagents can cause cells to adhere, therefore, ensure cells are completely removed from the bottom of the flask.
2. Add ~13-14 ml of complete growth media to the T-75 flask. Mix cells thoroughly. Plate cells (500 μ l per well of a 24-well plate) at an appropriate cell density (1.2×10^4 to 4.8×10^5 cells per ml) to obtain ~60-80% confluence at transfection.
3. Incubate for 2-3 hours to allow cells to adhere to the plate^b.

E. Complex Formation: Transfection Reagent and siRNA

1. In a sterile, plastic tube, add 50 μ l of serum-free media^c.
2. Add 0.5 to 4 μ l of reagent (0.5 μ l, 1.5 μ l, and 3 μ l for *TransIT*[®]-siQUEST[™] Reagent or 1 μ l, 2 μ l, and 4 μ l for *TransIT*-TKO[®] Reagent) directly into the serum-free media. Mix thoroughly by pipetting. For details see Table 1.
3. Add 10-50 nM siRNA (starting recommendation is 25 nM final concentration in the well) to the diluted transfection reagent. Mix by gentle pipetting.
4. Incubate at room temperature for 5-20 minutes.

F. siRNA Transfection

1. Adjust the volume in the well to 250 μ l of complete growth media by removing ~250 μ l (half) of the original plating media to conserve siRNA. This media reduction requires the use of less siRNA to obtain the recommended 25 nM (final concentration in the well).
2. Add the transfection reagent/siRNA complex mixture prepared in step E dropwise to the cells. Gently rock the plate back and forth and from side to side to distribute the complexes evenly. Do not swirl the plate.
3. Incubate for 24-72 hours^b.
NOTE: The above incubation is designed for transfections performed with no media change. If a media change is necessary to remove the transfection complexes, incubate the cells for 24 hours, replace the original medium with fresh complete growth medium, and incubate for an additional 24-48 hours^b.
4. Assay for inhibition of target gene expression.

2.4 Sequential Transfection of Plasmid DNA and siRNA in 24 well-Plates (For Suspension Cells)

NOTE: Do not transfect plasmid DNA (with a plasmid DNA transfection reagent) and siRNA (with *TransIT*[®]-siQUEST[™] Reagent) simultaneously. The *TransIT*[®]-siQUEST[™] Reagent may inhibit DNA delivery.

Simultaneous transfections using the *TransIT*[®]-LT1 Reagent (for DNA delivery) and *TransIT*-TKO[®] Reagent (for siRNA delivery) can be performed since the *TransIT*-TKO[®] Reagent does not inhibit DNA delivery. For simultaneous transfections using the *TransIT*[®]-LT1 and *TransIT*-TKO[®] Reagents, see section 2.5.

A. Cell Plating of Suspension Cells

Immediately prior to transfection, plate cells in 250 μ l of complete growth media per well of a 24 well-plate, at an appropriate density (such as 6×10^5 to 1×10^6 cells per ml). Alternatively, plate 250 μ l of cells the day prior to transfection at an appropriate density (such as $3\text{-}5 \times 10^5$ cells per ml).

B. Complex Formation: *TransIT*[®]-LT1 Reagent and Plasmid DNA (pDNA)

NOTE: Mirus Bio offers several reagents for plasmid transfection, including cell line specific reagents. To choose the appropriate reagent, please see Related Products, our website (www.mirusbio.com), or contact Technical Support at techsupport@mirusbio.com or 888.530.0801. The following protocol recommends *TransIT*[®]-LT1 Transfection Reagent, a broad spectrum DNA transfection reagent, which efficiently delivers DNA into many cell types.

1. In a sterile plastic tube, add 50 μ l of serum-free media^c.
2. Add *TransIT*[®]-LT1 Transfection Reagent (1-2 μ l per well for 24-well plates; scale up or down for different size plates) directly to the serum-free media. Mix thoroughly by pipetting or vortexing.
3. Add plasmid DNA (0.5 μ g per well for 24-well plates; titration of pDNA may be necessary) to the diluted *TransIT*[®]-LT1 Reagent. Mix by gentle pipetting.

NOTE: If transfecting more than one plasmid, mix the plasmids together in a microcentrifuge tube and incubate for 5 minutes at room temperature before adding to the diluted *TransIT*[®]-LT1 Reagent.

4. Incubate at room temperature for 15-30 minutes.

C. Plasmid Transfection in Suspension Cells

1. Add the *TransIT*[®]-LT1 Reagent/DNA complex mixture from Step B dropwise to the cells in complete growth medium. Gently rock the plate back and forth and from side to side to distribute the complexes evenly. Do not swirl the plate.
2. Incubate for 2-4 hours^b.

D. Complex Formation: *TransIT*[®]-siQUEST[™] and *TransIT*-TKO[®] Reagent and siRNA

1. In a sterile, plastic tube, add 50 μ l of serum-free media^c.
2. Add 0.5 to 4 μ l of reagent (0.5 μ l, 1.5 μ l, and 3 μ l for *TransIT*[®]-siQUEST[™] Reagent and 1 μ l, 2 μ l, and 4 μ l for *TransIT*-TKO[®] Reagent) directly into the serum-free media. Mix thoroughly by pipetting. For details see Table 1.
3. Add 10-50 nM siRNA (starting recommendation is 25 nM final concentration in the well) to the diluted transfection reagent. Mix by gentle pipetting.
4. Incubate at room temperature for 5-20 minutes.

E. siRNA Transfection in Suspension Cells

1. Add the transfection reagent/siRNA complex mixture prepared in step D dropwise to the cells. Gently rock the plate back and forth and from side to side to distribute the complexes evenly. Do not swirl the plate.
2. Incubate for 24-72 hours^b.

NOTE: The above incubation is designed for transfections performed without a media change. If a media change is necessary to remove the transfection complexes, incubate the cells for 24 hours, replace the original medium with fresh complete growth medium, and incubate for an additional 24-48 hours^b.

3. Assay for inhibition of target gene expression.

2.5 Simultaneous Transfection of DNA and siRNA using the *TransIT*-TKO[®] Reagent in 24 well-Plates

NOTE: Do not use the *TransIT*[®]-siQUEST[™] Reagent simultaneously with a DNA delivery reagent as it may interfere with DNA delivery. Simultaneous transfections using the *TransIT*-TKO[®] Reagent (for siRNA delivery) and *TransIT*[®]-LT1 Reagent (for DNA delivery) can be performed since the *TransIT*-TKO[®] Reagent does not inhibit DNA delivery.

A1. Cell Plating of Adherent Cells

1. Approximately 24 hours prior to transfection, plate 500 μ l of cells at an appropriate cell density to obtain ~60-80% confluence the following day (6×10^4 to 2.4×10^5 cells per ml, depending on cell size and characteristics)^a.
2. Incubate the cells overnight^b.

A2. Cell Plating of Suspension Cells

Immediately prior to transfection, plate cells in 250 µl of complete growth media per well of a 24 well-plate, at an appropriate density (such as 6×10^5 to 1×10^6 cells per ml). Alternatively, plate 250 µl cells the day prior to transfection at an appropriate density ($3\text{-}5 \times 10^5$ cells per ml).

B. Complex Formation

NOTE: Mirus offers several reagents for plasmid transfection, including cell line specific reagents. To choose the appropriate reagent, please see Related Products, our website (www.mirusbio.com) or contact Technical Support at techsupport@mirusbio.com or 888-530-0801. The following protocol recommends *TransIT*[®]-LT1 Transfection Reagent, Mirus Bio's broad spectrum transfection reagent, which efficiently delivers DNA into many cell types.

1. In a sterile, plastic tube, add 50 µl of serum free media^c.
2. Add *TransIT*[®]-LT1 Transfection Reagent (1-2 µl per well for 24 well plates; scale up or down for different size plates)
3. Add plasmid DNA (0.5 µg per well for 24-well plates; titration may be necessary) to the diluted *TransIT*[®]-LT1 Reagent. Mix by gentle pipetting.
NOTE: If transfecting more than one plasmid, mix the plasmids together in a microcentrifuge tube and incubate for 5 minutes at room temperature before adding to the diluted *TransIT*[®]-LT1 Reagent.
4. Incubate at room temperature for 15-30 minutes.
5. In the same tube, add the *TransIT*-TKO[®] Reagent (1-4 µl per well for 24-well plates). Mix by gentle pipetting.
6. Add siRNA (10-50 nM final concentration in the well) to the diluted complex mixture. Mix by gentle pipetting.
7. Incubate at room temperature for 5-20 minutes.

C. Dual Transfection

1. For adherent cell types, adjust the volume in the well to 250 µl of complete growth media by removing ~250 µl (half) of the original plating media to conserve siRNA. This media reduction requires the use of less siRNA to obtain the recommended 25 nM (final concentration in the well).
2. Add *TransIT*[®]-LT1 Reagent/pDNA/*TransIT*-TKO[®] Reagent/siRNA complex mixture prepared in Step B dropwise to the cells. Gently rock the plate back and forth and from side to side to distribute the complexes evenly. Do not swirl the plate.
3. Incubate for 24-72 hours^b.
NOTE: The above incubation is designed for transfections performed with no media change. If a media change is necessary to remove the transfection complexes, incubate the cells for 24 hours, replace the original medium with fresh complete growth medium, and incubate for an additional 24-48 hours^b.
4. Assay for inhibition of target gene expression.

^a Since the optimal cell density (confluence) for efficient transfection can vary between cell types, this should be determined for each cell type. Maintain the optimal seeding protocol for each cell type between experiments.

^b Standard incubation conditions for mammalian cells are 37°C in 5% CO₂. Other cell types, such as insect cells, require different temperatures and CO₂ concentrations. Use conditions appropriate for the cell type of interest.

^c The transfection reagent/siRNA complex may not form properly if the complex formation medium contains serum, resulting in poor transfection efficiencies. Any serum-free media can be used for complex formation, provided it does not contain polyanions such as dextran sulfate and heparin.

2.6 *Label IT*[®] RNAi Delivery Control Sample Preparation

1. Immediately prior to use, thaw the vial of the *Label IT*[®] RNAi Delivery Control on ice.
2. Dilute the 10 µM stock *Label IT*[®] RNAi Delivery Control 10-fold using the 10X RNAi Dilution Buffer (provided) to make a 1 µM working solution (For example, use 1 part stock *Label IT*[®] RNAi Delivery Control, 1 part 10X RNAi Dilution Buffer and 8 parts water). Dilute only as much of the stock *Label IT*[®] RNAi Delivery Control as required for the immediate experiment(s) and discard any remaining diluted Delivery Control.

NOTE: For optimal visualization, a final concentration of 25 nM per well is recommended. Plate the cells that will be used for tracking purposes on poly-D-lysine coated coverslips (for a detailed description, please refer to the *Label IT*[®]

siRNA Tracker Kit protocol, available at www.mirusbio.com). After use, return the stock solution to -20°C for storage.

3. Transfect cells on poly-d-lysine coated coverslips with *Label IT*[®] RNAi Delivery Control using procedures in Section 2.2 to 2.5.

2.7 Detection of *Label IT*[®] RNAi Delivery Control in Transfected Cells (on mounted coverslips)

NOTE: For suspension cells, fix and wash cells in a microfuge tube. Pellet cells by gentle centrifugation between washes. To visualize suspension cells by microscopy, apply cells to a Poly-d-lysine (PDL) coated slide to aid in the adherence of the cells to the surface. Apply a non-PDL treated coverslip over cells and seal as described below.

A. Detection Optimization

Assess the distribution of the fluorescent signal of the *Label IT*[®] RNAi Delivery Control in the transfected cells 4 to 24 hours post-transfection. The strength of the fluorescent signal may depend on several factors including transfection efficiency, amount of labeled control used, growth rate of the cells, and incubation time post-transfection. To obtain a strong fluorescent signal, it may be necessary to vary the final concentration of the *Label IT*[®] RNAi Delivery Control used in the transfection from 10 to 100 nM, depending on the cell line and transfection reagent used.

B. Cell Fixation (For 24-well Plates)

NOTE: Protect cells from light to prevent loss of fluorescent signal. These recommendations are for 24-well plates. If using a different well size, scale all volumes and amounts according to the surface area of the well.

1. Make fresh 4% (wt:vol) formaldehyde in PBS (commercial stocks are usually 37% (wt:vol)) and store at 4°C until ready to use.
2. Wash the transfected cells twice with PBS.
3. Fix cells in 0.25 ml per well 4% formaldehyde/PBS at room temperature for 20 minutes.
4. Aspirate formaldehyde and gently wash cells 3 times with PBS.
5. Add 0.25 ml PBS to each well.
6. For each well, mount the coverslip onto a glass slide (see Step C).

C. Slide Preparation

1. Using a small tip pap pen or nail polish, draw a complete circle on the glass slide. The diameter of the circle must be less than the diameter of the coverslip that will cover it.
2. Place a small drop of mounting solution in the center of each marked circle. Mirus Bio recommends antifade mounting solutions when using the fluorescein-labeled *Label IT*[®] RNAi Delivery Control.
3. Remove a coverslip with forceps and gently wipe off the underside (non-cell side) with a Kimwipe[®] tissue.
4. Carefully mount the coverslip, cell-side down, onto the mounting solution.
5. Use capillary action to drain excess mounting solution from under the coverslip using a Kimwipe[®] tissue.
6. Seal all edges of the coverslip to the glass slide with nail polish or rubber cement.

D. Cell Visualization

View mounted coverslips on a fluorescent microscope using the appropriate filter sets. See Table 2 for fluorescent excitation and emission wavelengths for the *Label IT*[®] RNAi Delivery Control.

Table 2. Excitation and emission wavelengths of *Label IT*[®] RNAi Delivery Control

Fluorophore	Excitation Wavelength (nm)	Emission Wavelength (nm)
Fluorescein	495	518

3.0 TROUBLESHOOTING

Low Knockdown Efficiency

- **Suboptimal volume of transfection reagent**
Determine the optimal volume by titrating each reagent using the recommended volumes in Table 1, Section 2.1. It may be necessary to titrate outside of this range depending on the cell type. Use the volume of reagent that gives the highest knockdown efficiency with the lowest cellular toxicity for future transfections.
- **Suboptimal siRNA concentration**
Determine the optimal siRNA concentration by titrating from 10 to 50 nM (final concentration in the well). We recommend starting with 25 nM of siRNA (final concentration in the well). In some instances higher concentrations of siRNA such as 100 or 200 nM may be necessary to achieve sufficient knockdown of the gene of interest.
- **Low transfection efficiency**
Follow transfection protocol steps carefully. To assess transfection efficiency of siRNA use Mirus Bio's *Label IT*[®] siRNA Tracker Kits or Labeled Delivery Controls (see Related Products Section 5.0).
- **Denatured siRNA**
To dilute siRNA, use the manufacturer's recommended buffer, or 100 mM NaCl, 50 mM Tris, pH 7.5 in RNase-free water. Do not use water as this can denature the siRNA duplex.
- **Incorrect siRNA sequence**
Ensure that the sequence of siRNA is correct for the gene of interest. More than one sequence may need to be tested for optimal knockdown efficiency.
- **Poor quality of transfecting siRNA**
Avoid siRNA degradation by using RNase-free handling procedures and plasticware. Degradation of siRNA can be detected on acrylamide gels.
- **Fetal calf serum present during complex formation**
Use serum-free medium during complex formation steps.
- **Cell density (% confluence) not optimal at time of siRNA transfection**
The recommended cell density for most cell types at the time of siRNA transfection is 60-80% confluence. Determine the optimal cell density, which may be outside the recommended range, for each cell type in order to maximize transfection efficiency. Maintain the optimal density in future experiments for reproducibility.
- **Inhibitor present during transfection**
The presence of polyanions, such as dextran sulfate or heparin, can inhibit transfection. Use transfection medium that does not contain these polyanions. If polyanions are necessary for the health of the cells, the transfection media can be replaced with the optimal cell growth media 24 hours post-transfection.
- **Proper controls were not included**
To ensure assay validation, include the following controls: cells only (for visual comparisons), transfection reagent alone, and transfection reagent with a non-specific siRNA, such as Dharmacon's siCONTROL Non-Targeting siRNA #1 (Cat.# D-001210-01-05). To verify efficient transfection and knockdown, deliver a siRNA targeted against an endogenous gene, such as GAPDH or Lamin A/C, followed by western blotting or target mRNA quantification.

High Cellular Toxicity

- **Media change or addition may be necessary**
If incubating for 48-72 hours, it may be necessary to change the complete media 24 hours post-transfection. Alternatively, add additional complete media 4-24 hours post-transfection.
- **Excessive amount of transfection reagent**

Reduce the amount of transfection reagent in the transfection. See Table 1 for recommended starting conditions. Use the volume of reagent that gives the highest knockdown efficiency with the lowest cellular toxicity for future transfections.

- **Cell density (% confluence) too low at time of transfection**
Grow cells to a higher cell density and repeat the transfection.
- **siRNA complex mixture and cells were not mixed thoroughly**
Mix thoroughly to evenly distribute the complexes to all of the cells. Rocking the plate back and forth and from side to side is recommended. Do not swirl or rotate the plate, as this may result in uneven distribution.
- **Complexes were added to cells in serum-free media**
Transfection reagent/siRNA complexes should be added to cells in complete media (serum-containing media) 5-20 minutes after complex formation. If these complexes are added to cells in serum-free media, cytotoxicity may be observed. If you must add the complexes to cells in serum-free media, add complete media after 4 hours to minimize toxic effects. Do not perform a complete media change.
- **Cell morphology has changed**
If the passage number of the cells is too high or too low they may be more sensitive to cellular toxicity. Maintain a similar passage number between experiments to ensure reproducibility.
- **Knockdown of an essential gene**
If the siRNA is directed against a gene that is essential to the cell, cytotoxicity may be observed.

Tracking - Poor Visualization of the Label IT[®] RNAi Delivery Control in Cells

- **Improper storage of Label IT[®] RNAi Delivery Control**
Store at -20°C, protected from light.
- **Compromised quality of Label IT[®] RNAi Delivery Control**
Avoid RNA degradation by using RNase-free handling procedures and plasticware.
- **Excessive exposure to light**
Protect samples and reagents from light.
- **Trouble detecting fluorescent signal**
Use proper filter sets for microscopic detection. See Table 2. Confocal microscopy may distinguish signal that is inside the cells from that adhering to the outside of the cells.
- **Suboptimal transfection efficiency**
See Section 2.1.
- **Suboptimal levels of Label IT[®] RNAi Delivery Control used**
For *in vitro* transfection, use up to 100 nM (final concentration per well).
- **Cells lost during fixation or mounting procedure**
Perform all washing, fixing, and mounting steps gently. Check for presence of cells following each step using a light microscope.

For specific questions or concerns, please contact Mirus Bio Technical Support at 888.530.0801 or techsupport@mirusbio.com

For a list of citations using Mirus Bio products, please visit the Technical Resources section of our website (www.mirusbio.com).

4.0 REFERENCES

1. Elbashir, S.M. et al. (2001) *Nature* **411**: 494-498.
2. Caplen, N.J. et al (2001) *Prot. Natl. Acad. Sci.* **98**: 9742-9747.
3. Sharp, P.A. (2001) *Genes and Development* **15**: 485-490.

5.0 RELATED PRODUCTS

Additional RNA Interference Products:

- TransIT*-TKO[®] siRNA Transfection Reagent (Product # MIR 2150)
- TransIT*[®]-siQUEST[™] siRNA Transfection Reagent (Product # MIR 2110)
- siXpress[®] PCR Vector Systems (Product # MIR 7300, 7301, 7302)
- Label IT*[®] siRNA Tracker Intracellular Localization Kit with *TransIT*-TKO[®] Transfection Reagent (Product # MIR 7200,7201,7202,7203,7204,7205)
- Label IT*[®] siRNA Tracker Intracellular Localization Kit with *TransIT*[®]-siQUEST[™] Transfection Reagent (Product # MIR 7206,7207,7208,7209,7210,7211)
- Label IT*[®] siRNA Tracker Intracellular Localization Kit (Product # MIR 7212,7213,7214,7215,7216,7217)
- Labeled RNAi Delivery Control (MIR 7900, 7903)

For determination of gene expression efficiency:

- Beta-Gal Staining Kit (Product # MIR 2600)

Transfection reagents:

- TransIT*[®]-LT1 Transfection Reagent (Product # MIR 2300)
- TransIT*[®]-LT2 Transfection Reagent (Product # MIR 2400)
- TransIT*[®]-Express Transfection Reagent (Product # MIR 2000)
- TransIT*[®]-HeLaMONSTER[®] Transfection Kit (Product # MIR 2900)
- TransIT*[®]-Keratinocyte Transfection Reagent (Product # MIR 2800)
- TransIT*[®]-CHO Transfection Kit (Product # MIR 2170)
- TransIT*[®]-3T3 Transfection Kit (Product # MIR 2180)
- TransIT*[®]-293 Transfection Kit (Product # MIR 2700)
- TransIT*[®]-COS Transfection Kit (Product # MIR 2190)
- TransIT*[®]-Insecta Transfection Reagent (Product # MIR 2200)
- TransIT*[®]-Jurkat Transfection Reagent (Product # MIR 2120)
- TransIT*[®]-Prostate Transfection Kit (Product # MIR 2130)
- TransIT*-Neural[®] Transfection Reagent (Product # MIR 2140)
- TransIT*[®]-mRNA Transfection Reagent (Product # MIR 2250)
- TransIT*-TKO[®] siRNA Transfection Reagent (Product # MIR 2150)
- TransIT*[®]-siQUEST[™] siRNA Transfection Reagent (Product # MIR 2110)
- TransIT*[®]-Oligo Transfection Reagent (Product # MIR 2160)

In Vivo Delivery Kits:

In Vivo Enhanced Expression Delivery Kit:

- TransIT*[®]-EE Hydrodynamic Delivery Solution (Product # MIR 5340)
- TransIT*[®]-EE Hydrodynamic Delivery Starter Kit (Product # MIR 5310)

In Vivo Quick Recovery Delivery Solution and Kit:

- TransIT*[®]-QR Hydrodynamic Delivery Solution (Product # MIR 5240)
- TransIT*[®]-QR Hydrodynamic Delivery Starter Kit (Product # MIR 5210)

In Vivo Gene Delivery Kits with Polymer Solution:

- TransIT*[®]-In Vivo Gene Delivery System (Product # MIR 5100, 5125)

Mirus Bio Reagents are covered by United States Patent No. 5,744,335; 5,965,434; 6,180,784; 6,383,811; 6,593,465 and patents pending. The performance of *TransIT*-TKO[®] is guaranteed for 1 year from the date of purchase if stored and handled properly. The performance of *TransIT*- siQUEST[™] is guaranteed for 6 months from the date of purchase if stored and handled properly. The *Label IT*[®] RNAi



Delivery Controls and the 10X RNAi Dilution Buffer are stable for 6 months from the date of purchase, if used and stored properly. This product is sold to the Buyer with a limited license to use this product for research only. This product, or parts from this product, may not be re-packaged or re-sold without written permission from Mirus Bio Corporation.

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