Label IT® siRNA Tracker Intracellular Localization Kit

Product Name	*Label* IT® siRNA Tracker Reagent	Product No.
Label IT® siRNA Tracker Intracellular Localization Kit*	Cy™3	MIR 7200
	Cy™5	MIR 7201
	CX-Rhodamine	MIR 7202
	TM-Rhodamine	MIR 7203
	Biotin	MIR 7204
	Fluorescein	MIR 7205

* Each Kit contains *Label* IT® siRNA Tracker Reagent, *Label* IT® Reconstitution Solution, 10X Labeling Buffer A, siRNA Dilution Buffer, and *Trans*IT-TKO® siRNA Transfection Reagent.

1.0 INTENDED USE

This method easily tracks functional fluorescently labeled siRNA in mammalian cells and enhances the ability to visually assess siRNA transfection efficiency. The *Label* IT® siRNA Tracker Intracellular Localization Kit provides a straight-forward approach to directly label and deliver siRNA, of any sequence containing guanine residues, in an efficient and non-destructive manner for tracking experiments. Both subcellular localization and functional inhibition of target gene expression can be monitored in the same experiment following introduction of the labeled siRNA into mammalian cells. This kit provides sufficient reagents to label 50 µg of siRNA, and sufficient *Trans*IT-TKO® Transfection Reagent to perform up to 500 transfections in 24-well plates.

2.0 DESCRIPTION

2.1 General Information

It has been shown that when short RNA duplexes are introduced into mammalian cells in culture, sequence-specific inhibition of target mRNA can be realized without triggering an interferon response. These short dsRNAs, referred to as small interfering RNAs (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 siRNA extremely effective at inhibiting target gene expression once introduced into the cell. [1,2,3]

The *Label* IT® siRNA Tracker Kits are based on Mirus' proprietary nucleic acid labeling technology. The *Label* IT® Reagents were initially developed by Mirus' scientists to monitor the subcellular localization of plasmid DNA in cells following gene delivery. These reagents covalently attach marker molecules to intact nucleic acids in a simple one-step chemical reaction. The simplicity of the highly efficient and non-destructive labeling reaction, and the availability of a variety of labels make this the ideal kit to generate labeled siRNA for *in vitro* and *in vivo* tracking experiments. The *Label* IT® siRNA Tracker Kits allow custom labeling of any single strand or duplex siRNA (provided guanine residues are present), achieving fluorescently labeled siRNA for use in tracking applications. Furthermore, the labeling density can be easily controlled, if desired. Using the non-enzymatic *Label* IT® siRNA Tracker Kits, siRNA can be custom labeled before introduction into mammalian cells. This protocol will generate labeled siRNA without hindering its inherent ability to silence targeted genes. This allows for the ideal situation in which silencing-competent fluorescently labeled siRNA can be visually monitored after the transfection process.

The *Trans*IT-TKO® Transfection Reagent, also provided in the kit, is specifically formulated for siRNA delivery to cells in culture. This reagent enables highly efficient siRNA transfection and significantly reduced levels of cell damage when compared to cationic liposome-based transfection reagents. Transfections are most effective when carried out in complete growth media, with no media change or serum addition required. siRNA, when complexed with *Trans*IT-TKO® Reagent, knocks down target gene expression in a variety of cell lines. These unique features make *Trans*IT-TKO® Transfection Reagent ideal for all siRNA-mediated gene silencing studies.

2.2 Materials Supplied

Component	Volume	Reagent Cap Color
Label IT® siRNA Tracker Labeling Reagent	dried pellet	varies with label
Label IT® Reconstitution Solution*	60 µl	clear
10X Labeling Buffer A*	60 µl	lilac
siRNA Dilution Buffer	1 ml	white
*Trans*IT-TKO® Transfection Reagent	0.5 ml	red

*Excess reagent is supplied with each kit to allow for slight variations in pipetting.

2.3 Storage and Stability

Store the *Label* IT® Reconstitution Solution, 10X Labeling Buffer A, siRNA Dilution Buffer, and *Trans*IT-TKO® Transfection Reagent at 4°C. Store the *Label* IT® siRNA Tracker Reagent at –20°C in both its dried pellet and reconstituted form. The reconstituted *Label* IT® siRNA Tracker Reagent is stable for 6 months. Unreconstituted *Label* IT® siRNA Tracker Reagent, *Trans*IT-TKO® Transfection Reagent and all other reagents are stable for 1 year from the date of purchase. Warm the *Label* IT® siRNA Tracker Reagent to room temperature and quick spin before each use. Warm the *Trans*IT-TKO® Reagent to room temperature and vortex before each use.

3.0 PROCEDURE

3.1.1 Labeling Duplex siRNA

1. Warm the tube containing the *Label* IT® siRNA Tracker Reagent to room temperature and quick spin to collect the pellet at the bottom of the tube. Add 50 µl of *Label* IT® Reconstitution Solution to the pellet in the tube. To ensure complete reconstitution of the pellet, mix well by gentle pipetting.
2. Prepare the labeling reaction according to the example shown below. The example is for labeling 10 µg siRNA duplex. The kit provides sufficient reagents to label 50 µg of siRNA (5 reactions of 10 µg each). Use molecular biology-grade (i.e. DNase and RNase-free) water. Add the *Label* IT® siRNA Tracker Reagent last. Protect the labeling reaction from light.

   Labeling Reaction Example:*

molecular biology-grade H_2O	60 µl
10X Labeling Buffer A	10 µl
siRNA duplex (~10 µg)	20 µl of a 40 µM stock
Label IT® siRNA Tracker Reagent	10 µl
Total volume:	**100 µl**

   * This example uses a 1:1 (v:w) ratio of *Label* IT® siRNA Tracker Reagent to siRNA, the optimized labeling ratio for siRNA tracking experiments. If an adjustment of the labeling ratio is needed, we recommend using a range of 1 to 2 µl of *Label* IT® siRNA Tracker Reagent per µg of siRNA (i.e. 1:1 to 2:1 (v:w)). The labeling reaction may be scaled up or down, depending on the amount of siRNA to be labeled. The volume of *Label* IT® siRNA Tracker Reagent should be less than 20% of the total reaction volume.
3. Incubate reaction at 37°C for 1 hour.
   NOTE: During the labeling reaction, perform a quick spin after 30 minutes of incubation to minimize the effect of evaporation and keep the concentration of the reaction components at the appropriate levels.
4. Remove unreacted *Label* IT® siRNA Tracker Reagent from the labeled siRNA by ethanol precipitation. Add 0.1 volume of 5 M sodium chloride and 2.5 volumes of ice cold 100% ethanol to the reaction. Mix well and place in a –20°C (or colder) freezer for at least 30 minutes.
5. Centrifuge at full speed in a refrigerated microcentrifuge for 15 minutes to pellet the labeled siRNA. Gently remove the ethanol with a pipet; do not disturb the pellet.
   NOTE: Orient the precipitate-containing tubes in the microcentrifuge in such a way that it is known where the pellet forms. Small siRNA quantities can be invisible to the naked eye.
6. Wash the pellet once with 500 µl room temperature 70% ethanol. After an additional refrigerated centrifugation at full speed for 15 minutes, remove all traces of ethanol with a micropipetter. **Do not allow the sample to air dry more than 5 minutes**, as the pellet may become extremely difficult to resuspend.
7. Resuspend the labeled siRNA in the appropriate volume of siRNA Dilution Buffer. If example in Step 2 was used, resuspend labeled siRNA in 20 µl of siRNA Dilution Buffer to bring the concentration to approximately 40 µM.
8. Quantify the concentration of the purified, labeled siRNA on a spectrophotometer if an exact concentration is required. Dilute to a suitable working concentration (10 µM), if necessary.
9. Store the purified, labeled siRNA at –20°C, protected from light. Store on ice if needed for immediate use.

3.1.2 Labeling Single Strand siRNA

NOTE: This procedure allows independent labeling of individual siRNA strands which can generate, after annealing, siRNA duplexes with one or both strands labeled. Each strand may be labeled with different labels, if desired. It is important to note that single stranded RNA oliognucleotides are more easily degraded by RNase activity than siRNA duplexes. Also, recovery following ethanol precipitation may be less efficient than that of siRNA duplexes.

1. Bring the tube containing the *Label* IT® siRNA Tracker Reagent to room temperature and quick spin to collect the pellet at the bottom of the tube. Add 50 µl of *Label* IT® Reconstitution Solution to the pellet in the tube. To ensure complete reconstitution of the pellet, mix well by gentle pipetting.
2. Prepare the labeling reaction according to the example shown below. The example is for labeling 10 µg of single strand siRNA. The kit provides sufficient reagents to label 50 µg of siRNA (5 reactions of 10 µg each). Use molecular biology-grade (i.e. DNase and RNase-free) water. Add the *Label* IT® siRNA Tracker Reagent last. Protect the labeling reaction from light.

   Labeling Reaction Example:*

molecular biology-grade H_2O	40 µl
10X Labeling Buffer A	10 µl
siRNA (~10 µg)	40 µl of a 40 µM stock
Label IT® siRNA Tracker Reagent	10 µl
Total volume:	**100 µl**

   * This example uses a 1:1 (v:w) ratio of *Label* IT® siRNA Tracker Reagent to siRNA, the optimized labeling ratio for siRNA tracking experiments. If an adjustment of the labeling ratio is needed, we recommend using a range of 1 to 2 µl of *Label* IT® siRNA Tracker Reagent per µg of siRNA (i.e. 1:1 to 2:1 (v:w)). The labeling reaction may be scaled up or down, depending on the amount of siRNA to be labeled. The volume of *Label* IT® siRNA Tracker Reagent should be less than 20% of the total reaction volume.
3. Incubate reaction at 37°C for 1 hour.
   NOTE: During the labeling reaction, perform a quick spin after 30 minutes of incubation to minimize the effect of evaporation and keep the concentration of the reaction components at the appropriate levels.
4. Remove unreacted *Label* IT® siRNA Tracker Reagent from the labeled siRNA by ethanol precipitation. Add 0.1 volume of 5 M sodium chloride and 2.5 volumes of ice cold 100% ethanol to the reaction. Mix well and place in a –20°C (or colder) freezer for at least 30 minutes.
5. Centrifuge at full speed in a refrigerated microcentrifuge for 15 minutes to pellet the labeled siRNA. Gently remove the ethanol with a pipet; do not disturb the pellet.
   NOTE: Orient the precipitate-containing tubes in the microcentrifuge in such a way that it is known where the pellet forms. Small siRNA quantities can be invisible to the naked eye.
6. Wash the pellet once with 500 µl room temperature 70% ethanol. After an additional refrigerated centrifugation at full speed for 15 minutes, remove all traces of ethanol with a micropipetter. **Do not allow the sample to air dry more than 5 minutes**, as the pellet may become extremely difficult to resuspend.
7. Resuspend the labeled siRNA in appropriate volume of siRNA Dilution Buffer. If example in Step 2 was used, resuspend labeled siRNA in 40 µl of siRNA Dilution Buffer to bring the concentration to approximately 40 µM .
8. Quantify the concentration of the purified, labeled siRNA on a spectrophotometer if an exact concentration is required.
9. Anneal siRNA strands per siRNA manufacturer's instructions. Alternatively, siRNA duplexes can be annealed in the provided siRNA Dilution Buffer by combining equal molar amounts of each strand, heating at 90°C for 1 minute, and then incubating at 37°C for 1 hour.
10. Store the purified, labeled siRNA at –20°C, protected from light. Store on ice if needed for immediate use.

3.2 Transfection Optimization

The key to successful transfection is careful optimization of reaction conditions for each individual cell type. The transfection protocols described in Sections 3.3-3.4 should result in efficient transfection of most cell types; however, to ensure optimal results the following variables should be considered:

A. Media conditions – The *Trans*IT-TKO® Reagent yields improved transfection efficiencies when transfections are performed in complete growth medium (instead of serum-free medium) without a post-transfection media change. Use serum-free medium when forming the *Trans*IT-TKO® Reagent/siRNA complexes.

B. Cell density (% confluence) at the time of transfection – The recommended confluence at the time of transfection for most cell types is 60-80% (3×10^4 to 1.2×10^5 cells per well of a 24-well plate, depending on cell size and characteristics). If this confluence does not produce optimal results, test cell densities outside of the recommended range.

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 *Trans*IT-TKO® Reagent and siRNA are titrated accordingly. Alternatively, trypsinize and re-plate cells 24 hours post-transfection at a lower density to accommodate longer incubation times.

Determine the optimal cell density for each cell type in order to maximize knockdown efficiency. Maintain this density in future experiments for reproducibility.

C. siRNA concentration – siRNA used for transfection should be highly pure, sterile, and the correct sequence. For high quality siRNA design and manufacture, Mirus recommends Dharmacon Research, Inc. (dharmacon.com). The optimal final siRNA concentration for siRNA tracking should be within the range of 25 to 100 nM in the well. As a starting point, we recommend using 50 nM. In some cases, it may be necessary to use up to 100 nM of siRNA to see maximum signal.

D. *Trans*IT-TKO® Reagent – The optimal *Trans*IT-TKO® Reagent volume can be determined by titrating the reagent within the ranges listed in Table 1. As a starting point, test three levels of *Trans*IT-TKO® Reagent, such as 1, 2, and 3 µl per well of a 24-well plate, using 50 nM siRNA (final concentration in the well). The volume of reagent that achieves the highest knockdown efficiency with the lowest cellular toxicity should be used for future transfections.

E. Transfection incubation time – The optimal incubation time can be determined empirically by testing a range of incubation times from 4-72 hours. The delivery of labeled siRNA can be visualized as soon as 4 hours post-transfection, with optimal observation at 12-24 hours post-transfection. Maximum inhibition of target gene expression is often obtained at 24-72 hours post-transfection.

F. Proper controls – Consider the following transfection controls to properly assess tracking and knockdown efficiencies.

Tracking efficiency:
1. Unlabeled siRNA
2. Labeled siRNA

Knockdown efficiency:
1. Serum-free media alone
2. Serum-free media + *Trans*IT-TKO® Reagent
3. Serum-free media + *Trans*IT-TKO® Reagent + a non-specific siRNA

Table 1. Recommended titration ranges for *Trans*IT-TKO® Transfection Reagent transfections

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
*Trans*IT-TKO® Transfection Reagent	0.18-0.74 µl	0.5-2.1 µl	1-4 µl	2-8 µl	5-20 µl	30-120 µl
10 µM stock labeled siRNA duplex (25-100 nM final concentration in well)	0.13-0.53 µl	0.38-1.5 µl	0.75-3 µl	1.5-6 µl	3.75-15 µl	22.5-90 µl
Complete Growth Media	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.

NOTE: All volumes in Table 1 are per well of the indicated plate 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 with the *Trans*IT-TKO® Transfection Reagent in 24-well plates. When performing transfections in different sized plates, the amounts of siRNA, *Trans*IT-TKO® Reagent, and culture medium should be scaled up or down in proportion to the surface area of the well. Note that the total volume of culture medium is important to ensure optimal *Trans*IT-TKO® Reagent levels and final siRNA concentrations.

3.3 siRNA Transfection of Adherent Cells in 24-well plates

A. Cell Plating

NOTE: For siRNA tracking studies, since cells tend to adhere poorly to untreated glass coverslips, we recommend plating the cells on poly-D-lysine (PDL) coated coverslips, which can be mounted for microscopic observation. Autofluorescence from tissue culture plastic can interfere with optimal direct microscopic viewing of transfected cells.

a. Place one sterile coverslip in each well.
b. Cover each coverslip with 0.25 ml of 0.1 mg/ml PDL (high molecular weight, generally greater than 3000K, e.g. Sigma Cat#P7280) prepared in sterile water.
c. Incubate at room temperature for 20 minutes.
d. Aspirate the PDL solution with a pipet.
e. Wash the coverslips with sterile water or PBS three times (free polymer may be cytotoxic) and allow to dry completely in a sterile culture hood.

1. Approximately 24 hours prior to transfection, plate cells at a cell density to obtain ~60-80% confluent the following day, in 500 µl of complete grow media per well of a 24-well plate.[a]
2. Incubate the cells overnight.[b]

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

1. Add 50 µl of serum-free[c] media to a sterile plastic tube.
2. Add the *Trans*IT-TKO® Transfection Reagent (1 to 4 µl; see Table 1) directly into the serum-free medium[c]. Mix thoroughly by pipetting.
3. Incubate at room temperature for 5-20 minutes.
4. Add labeled siRNA from Section 3.1.1 or 3.1.2 (25-100 nM final concentration in the well; see Table 1) to the diluted *Trans*IT-TKO® Reagent. Mix by gentle pipetting.
5. Incubate at room temperature for 5-20 minutes.

C. Cell Preparation for Transfections in Complete Growth Medium

NOTE: The *Trans*IT-TKO® Reagent yields improved transfection efficiencies when the transfections are performed in complete growth medium (instead of serum-free medium) without a media change following transfection.

1. If necessary, adjust the volume in the well to 250 µl of complete growth media. (see Table 1)
2. Add the *Trans*IT-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 4-24 hours[b] for siRNA tracking, and 24-72 hours for knockdown of target gene expression.
   NOTE: The above incubation is designed for transfections performed with no media change. If you wish to perform a media change 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,d] Alternatively, add complete media 4-24 hours post-transfection.
4. Mount and view cells as in Section 3.5 or 3.6, or assay for inhibition of target gene expression.

3.4 siRNA Transfection of Suspension Cells in 24-well plates

1. Seed cells in a 24-well plate at a density of 4×10^5 cells per ml of fresh complete growth medium (250 µl per well) if seeding and transfecting the same day. Alternatively, seed cells in a 24-well plate at a density of 2×10^5 cells per ml of fresh complete growth medium (250 µl per well) if seeding the cells 24 hours before transfection. See Section 3.3A for plating cells on coated coverslips.
2. Prepare the *Trans*IT-TKO® Reagent/siRNA complex mixture as described in Section 3.3, Part B.
3. Add the *Trans*IT-TKO® Reagent/siRNA complex mixture to the cell suspension and gently rock the dish back and forth and from side to side to distribute the complexes evenly.
4. Incubate for 4-24 hours[b] for siRNA tracking, and 24-72 hours to assay for knockdown of target gene expression.
   NOTE: The above incubation is designed for transfections performed with no media change. If you wish to perform a media change 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,d]
5. Mount and view cells as in Section 3.5 or 3.6, or assay for inhibition of target gene expression.

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[a] Since the optimal cell density (confluence) for efficient transfection can vary between cell types, it should be determined independently 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% CO_2. Other cell types, such as insect cells, require different temperatures and CO_2 concentrations. Use conditions appropriate for the cell type of interest.

[c] The *Trans*IT-TKO® 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.

[d] The optimal incubation time should be determined empirically by testing a range of incubation times from 4-72 hr.

3.5 Detection of *Label* IT® siRNA Tracker Cy™3, Cy™5, CX-Rhodamine, TM-Rhodamine, and Fluorescein labeled siRNA

NOTES: See Section 3.6 for Detection of Label IT® siRNA Tracker Biotin labeled siRNA.
See Table 2 for fluorescent Excitation and Emission spectra of labeled siRNA. Use appropriate microscope filters.
These suggestions are for 24-well plates. If using a larger well size, scale volumes up accordingly.
Keep exposure of the cells to light at a minimum to prevent loss of fluorescent signal.

1. Dilute formaldehyde in PBS to a final concentration of 4% (vol:vol) and store at 4°C until ready to use.
2. In a sterile culture hood, aspirate media from transfected cells, wash twice with PBS, and add approximately 0.25 ml 4% formaldehyde to each well.
3. Incubate cells at room temperature for 20 minutes.
4. Aspirate formaldehyde and gently wash wells 3 times with PBS.
5. Add approximately 0.25-0.5 ml PBS to each well to help with removal of the coverslips and to prevent drying.
6. Mount 2 coverslips per glass slide:
   a. Using a small tip pap pen (Electron Microscopy Sciences) 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. Two circles, with coverslips, will fit on a standard glass slide.
   b. Place a small drop of mounting solution in the center of each marked circle. Antifade mounting solutions may be useful when tracking with Fluorescein.
   c. Remove coverslips from wells with forceps and gently wipe off underside (non-cell side) of glass with a Kimwipe tissue.
   d. Mount carefully, cell-side down onto mounting solution.
   e. Use capillary action to drain excess mounting solution from under coverslip using a Kimwipe tissue.
   f. Seal all edges of coverslip to glass slide with nail polish or rubber cement.
7. View on a fluorescent microscope using the appropriate filter sets (Table 2).

For suspension cells, fix and wash cells in solution. Spin and collect cells between washes. To visualize suspension cells by microscopy, apply cells to mounting area on a poly-lysine charged slide to aid in the adherence of the cells to the surface. Apply coverslip over cells, and seal as above.

3.6 Detection of *Label* IT® siRNA Tracker Biotin labeled siRNA

NOTE: A variety of commercially available secondary detection fluorescent conjugates are compatible with siRNA labeled using the *Label* IT® siRNA Tracker Biotin Reagent. Furthermore, the potential for multi-color tracking experiments is enhanced when the experimental design includes detection of Biotin-labeled siRNA with a fluorophore conjugate and the direct detection of Cy™3, Cy™5, Fluorescein or Rhodamine-labeled siRNA(s). These suggestions are for 24-well plates. If using a larger well size, scale volumes up accordingly.

1. Dilute formaldehyde in PBS to a final concentration of 4% (vol:vol) and store at 4°C until ready to use.
2. In a sterile culture hood, aspirate media from transfected cells, wash twice with PBS, and add approximately 0.25 ml 4% formaldehyde to each well.
3. Incubate cells at room temperature for 20 minutes.
4. Aspirate formaldehyde and gently wash wells 3 times with PBS.
5. Dilute desired streptavidin or anti-biotin antibody conjugate in PBS to ~20 ng/µl (or the best concentration determined for the reagent of choice)
6. Gently add approximately 50 µl of the diluted Streptavidin or anti-biotin antibody conjugate to each coverslip (in a 24-well plate).
7. Incubate at room temperature, shielded from light, for at least 1 hour.
8. After incubation, remove detection solution and wash 3 times with PBS.
9. To each well, add approximately 0.25-0.5 ml PBS to aid removal of the coverslips and to prevent drying.
10. Mount 2 coverslips per glass slide:
    a. Using a small tip pap pen (Electron Microscopy Sciences) 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. Two circles, with coverslips, will fit on a standard glass slide.
    b. Place a small drop of mounting solution in the center of each marked circle. Antifade mounting solutions may be useful when tracking with Fluorescein.
    c. Remove coverslips from wells with forceps and gently wipe off underside (non-cell side) of glass with a Kimwipe tissue.
    d. Mount carefully, cell-side down onto mounting solution.
    e. Use capillary action to drain excess mounting solution from under coverslip using a Kimwipe tissue.
    f. Seal all edges of coverslip to glass slide with nail polish or rubber cement.
11. View on a fluorescent microscope using the appropriate filter sets (Table 2).

For suspension cells, fix and wash cells in solution. Spin and collect cells between washes. To visualize suspension cells by microscopy, apply cells to mounting area on a poly-lysine charged slide to aid in the adherence of the cells to the surface. Apply coverslip over cells, and seal as above.

Table 2. Excitation and emission wavelengths of the *Label* IT® siRNA Tracker Reagents and labeled siRNA

Fluorophore	Excitation Wavelength (nm) of labeled siRNA	Excitation Wavelength (nm) of labeling reagent alone	Emission Wavelength (nm)
Cy™3	549	550	570
Cy™5	648	649	670
Fluorescein	495	492	518
CX-Rhodamine	587	576	597
TM-Rhodamine	559	546	576

4.0 APPLICATION NOTES

A. Adjusting the Labeling Density

The labeling protocols in Sections 3.1.1 and 3.1.2 readily allow detection of the labeled siRNA in cultured mammalian cells. If there is a need to increase or decrease the labeling density of the final product, increase or decrease the ratio of labeling reagent to nucleic acid during the labeling reaction. Also, the labeling density can be controlled by adjusting the incubation time; the labeling reaction is linear over the first three hours of incubation at 37°C. It is important to note that a labeling ratio of greater than 2:1 (*Label* IT® siRNA Tracker Reagent to siRNA, vol:wt) or increased incubation times could adversely affect the functionality of the siRNA.

B. Functionality of labeled siRNA

The *Label* IT® siRNA Tracker Reagents label siRNA in a non-destructive manner, thus allowing the visualization of functional siRNA during the transfection process. Using the recommended range of *Label* IT® siRNA Tracker Reagent to siRNA ratios will provide labeled siRNA without hindering its inherent ability to silence targeted genes.

C. *In Vivo* Tracking Experiments

The ability to track and monitor target gene knockdown in the same experiment *in vivo* is another attractive application of this technology, especially when aspects of gene silencing can be studied in their proper biological context. siRNA, labeled with *Label* IT® siRNA Tracker Reagents, can also be used to monitor siRNA delivery to tissues *in vivo*. Efficient *in vivo* nucleic acid delivery can be obtained using Mirus' *Trans*IT® *In Vivo* Gene Delivery System. This kit is designed for the efficient delivery of plasmid DNA into laboratory animals via tail vein injection, and is also a highly efficient, nonviral method for delivering siRNA *in vivo*. Following siRNA delivery using this method, the highest levels of siRNA visualization and gene silencing are seen in the liver.

5.0 Troubleshooting

5.1 Labeling Reaction - Poor Efficiency

- **Poor quality of siRNA**
  Avoid siRNA degradation by using RNase-free handling procedures and plasticware. For high quality siRNA design and manufacture, Mirus recommends Dharmacon Research, Inc. (dharmacon.com). Degradation can be detected on acrylamide gels. Ensure that siRNA sequences contain guanine residues.
- **Labeling reaction was not scaled properly**
  Keep the volume of *Label* IT® siRNA Tracker Reagent less than 20% of the total reaction volume and the Labeling Buffer A at 1X final concentration in the reaction.
- **Improper storage of reagents**
  Store both reconstituted and unreconstituted *Label* IT® siRNA Tracker Reagents tightly capped at -20°C. Protect from exposure to light and moisture.
- **NOTE: The relative density of fluorescent labels on purified, labeled siRNA can be assessed by:**
  1. Spectrophotometric absorbance at λ_{max}. Several µg of labeled siRNA may be required to generate significant λ_{max} absorbance readings.
  2. Fluorescent microscopy. Spot dilutions of labeled siRNA onto a glass slide and view with fluorescent microscope.

5.2 Transfection - Low Transfection Efficiency

- **Suboptimal *Trans*IT-TKO® Reagent**
  Determine the optimal *Trans*IT-TKO® Reagent concentration by titrating the reagent from 1 µl to 4 µl per well of a 24-well plate. See Table 1 for recommended starting concentrations.
- **Suboptimal siRNA concentration**
  Determine the optimal siRNA concentration by titrating from 25 nM up to 100 nM in a 24-well plate. See Table 1 for recommended starting concentrations.
- **Denatured siRNA**
  Use siRNA Dilution Buffer to dilute siRNA. Do not use water as this can denature the siRNA.
- **Poor quality of transfecting siRNA**
  Avoid siRNA degradation by using RNase-free handling procedures and plasticware. For high quality siRNA design and manufacture, Mirus recommends Dharmacon Research, Inc. (dharmacon.com). Degradation can be detected on acrylamide gels.
- **Fetal calf serum present during *Trans*IT-TKO® Reagent/siRNA complex formation**
  Use serum-free medium when forming the transfection complexes.
- **Suboptimal cell density (% confluence) at time of transfection**
  The recommended cell density for most cell types at the time of transfection is 60-80% confluence. Determine the optimal cell density for each cell type in order to maximize transfection efficiency. Maintain this density in future experiments for reproducibility.
- **Complexes were added to cells growing in serum-free media**
  *Trans*IT-TKO® Reagent/siRNA complexes should be added to cells growing in complete media (serum-containing media) 5-20 minutes after complex formation. Complexes added to cells growing in serum-free media may adversely affect transfection efficiency. If you must add the complexes to cells growing in serum-free media, add complete media after 4 hours to minimize toxic effects.
- **Inhibitor present during transfection**
  The presence of polyanions, such as dextran sulfate or heparin, can inhibit transfection. Use complex formation and complete growth medium that does not contain these polyanions.
- **Cell morphology has changed**
  If the passage number of the cells is too high or too low, transfection efficiency can be adversely affected. Maintain a similar passage number between experiments to ensure reproducibility.

5.3 Transfection - High Cellular Toxicity

- **Media change or addition may be necessary**
  If incubating cells for 48-72 hours post-transfection, it may be necessary to change the complete media 24 hours post-transfection. Alternatively, supplement with additional complete media 4-24 hours post-transfection.
- **Excessive amount of TransIT-TKO® Reagent/siRNA complex mixture was used in transfection**
  Reduce the amount of *Trans*IT-TKO® Reagent/siRNA complex mixture in the transfection. See Table 1 for recommended starting concentrations.
- **Suboptimal cell density at the time of transfection**
  Grow cells to a higher cell density and repeat the transfection.
- ***Trans*IT-TKO® Reagent/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 growing in serum-free media**
  *Trans*IT-TKO®/siRNA complexes should be added to cells growing in complete media (serum-containing media) 5-10 minutes after complex formation. Complexes added to cells growing in serum-free media may be more cytotoxic. If you must add the complexes to cells growing in serum-free media, add complete media after 4 hours to minimize toxic effects.
- **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.

5.4 Tracking - Poor Visualization of Labeled siRNA in Cells

- **Poor quality of transfecting siRNA**
  Avoid siRNA degradation by using RNase-free handling procedures and plasticware. For high quality siRNA design and manufacture, Mirus recommends Dharmacon Research, Inc. (dharmacon.com). Degradation can be detected on acrylamide gels. Ensure that siRNA sequences contain guanine residues.
- **Low labeling density**
  Increase the labeling ratio (volume of *Label* IT® siRNA Tracker Reagent to weight of siRNA). See Section 3.1.1 or 3.1.2.
- **Excessive exposure to light**
  Protect samples and reagents from light.
- **Trouble detecting fluorescent signal**
  Use proper filter sets for microscopic detection. See Table 2.
- **Sub-optimal transfection efficiency**
  See Section 5.2.
- **Sub-optimal levels of siRNA transfected**
  Use 25-100 nM of labeled siRNA, and 1-4 µl of *Trans*IT-TKO® per well of a 24-well plate. See Table 1.
- **Cells lost during fixation or mounting procedure**
  Perform all washing, fixing, and mounting steps gently. Check for presence of cells following each step on a light microscope.
- **Improper storage of labeled siRNA**
  Labeled siRNA must be kept at -20˚C.

5.5 Suboptimal Knockdown of Target Gene Expression Observed

- **Labeling density is too high**
  Label siRNA at a lower ratio. See Section 3.1.1 or 3.1.2 for recommendations.
- **Poor transfection efficiency**
  See Section 5.2.
- **Poor siRNA Design**
  Verify that the siRNA sequence is a correct match to the target mRNA. Different siRNA sequences may result in different levels of target gene knockdown. Test a few different sequences for a particular target gene if knockdown efficiency is not as high as desired. To verify efficient transfection and knockdown, use *Trans*IT-TKO® Reagent to deliver a validated siRNA targeted against a ubiquitous gene, such as GAPDH or Lamin A/C, followed by western blotting or target mRNA quantification.
- **Observation time is not optimal**
  Perform time course post-transfection to determine kinetics of knockdown.
- **Poor detection of gene knockdown**
  The half-life of the protein is important in protein-based assays (Western blots, ELISAs, etc) and should be a taken into consideration when determining the optimal time to assay the cells after transfection. Detection of mRNA levels (RT-qPCR, Northerns, etc) are normally uniform from cell line to cell line. Assaying 24 hours post-transfection is usually sufficient to see knockdown of expression.

For specific questions or concerns, please contact Mirus Technical Support at 888.530.0801 or techsupport@mirusbio.com.
For a list of citations using Mirus products, please visit the Technical Resources section of our website at www.mirusbio.com.

6.0 GENERAL REFERENCES: siRNA Delivery

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.

7.0 RELATED PRODUCTS

Additional transfection reagents:*

*Trans*IT-TKO® siRNA Transfection Reagent is available for reorder as a separate product:

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

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

For determination of gene expression efficiency:
Beta-Gal Staining Kit (Product # MIR 2600)

For endotoxin removal from DNA:*
MiraCLEAN® Endotoxin Removal Kit (Product #5900)

For DNA tracking studies:
Label IT® Tracker™ Intracellular Nucleic Acid Localization Kit (Product # MIR 7010, 7011, 7012, 7013, 7014, 7015)

In Vivo* Gene Delivery Kits:
*Trans*IT®-*In Vivo* Gene Delivery System (Product # MIR 5100)

*These products are available in additional sizes; please see www.mirusbio.com.