

**Label IT[®] siRNA Tracker Intracellular Localization Kit
with *TransIT*-siQUEST[®] Transfection Reagent**

Product Name	Label IT [®] siRNA Tracker Reagent	Product No.
<i>Label IT[®] siRNA Tracker Intracellular Localization Kit with <i>TransIT</i>-siQUEST[®] Transfection Reagent*</i>	Cy [™] 3	MIR 7206
	Cy [™] 5	MIR 7207
	CX-Rhodamine	MIR 7208
	TM-Rhodamine	MIR 7209
	Biotin	MIR 7211
	Fluorescein	MIR 7210

* Each Kit contains *Label IT[®] siRNA Tracker Reagent*, *Reconstitution Solution*, *10X Labeling Buffer A*, *siRNA Dilution Buffer*, and *TransIT- siQUEST[®] Transfection Reagent*.

1.0 INTENDED USE

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. This kit allows tracking of functional fluorescently labeled siRNA in mammalian cells and enhances the ability to visually assess siRNA transfection efficiency. Cellular uptake, 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 *TransIT*-siQUEST[®] 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 expression 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 Bio's proprietary nucleic acid labeling technology. The *Label IT[®] Reagents* were initially developed by Mirus Bio 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, non-enzymatic, 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* tracking experiments. The *Label IT[®] siRNA Tracker Kits* allow custom labeling of any single strand or duplex siRNA. Furthermore, the labeling density can be easily controlled, if desired. This protocol will generate a labeled siRNA sample without hindering its inherent ability to silence targeted genes; silencing-competent fluorescently labeled siRNA can be visually monitored after the transfection process.

Labeled siRNA must be efficiently delivered to the cell in tracking experiments. The *TransIT*-siQUEST[®] Transfection Reagent, also provided in the kit, is specifically formulated for siRNA delivery *in vitro*. 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. When complexed with *TransIT*-siQUEST[®] Reagent, siRNA knocks down target

gene expression in a variety of cell lines. These unique features make *TransIT-siQUEST*[®] Transfection Reagent ideal for the delivery of labeled siRNA and siRNA-mediated gene silencing studies.

2.2 Materials Supplied

Component	Volume	Reagent Cap Color
<i>Label IT</i> [®] siRNA Tracker Labeling Reagent	dried pellet	varies with label
<i>Label IT</i> [®] Reconstitution Solution*	60 µl	brown
10X Labeling Buffer A*	60 µl	lilac
siRNA Dilution Buffer	1 ml	white
<i>TransIT-siQUEST</i> [®] Transfection Reagent	0.5 ml	purple

*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 *TransIT-siQUEST*[®] 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 and the *TransIT-siQUEST*[®] Transfection Reagent are stable for 6 months. 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 *TransIT-siQUEST*[®] Reagent to room temperature and vortex before each use. Warm the Reconstitution Solution to room temperature and quick spin before use.

3.0 PROCEDURE

3.1.1 Labeling Duplex siRNA

1. Warm the vials containing the *Label IT*[®] siRNA Tracker Reagent and Reconstitution Solution to room temperature and quick spin to collect the reagents at the bottom of the vials. The Reconstitution Solution freezes at 4°C; ensure it is completely thawed before use to obtain the required volume. Add 50 µl of 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 using molecular biology-grade (i.e. DNase and RNase-free) water. The example is for labeling 10 µg of siRNA duplex. The kit provides sufficient reagents to label 50 µg of siRNA (5 reactions of 10 µg each). Add the *Label IT*[®] siRNA Tracker Reagent last. Protect the labeling reaction from light.

Labeling Reaction Example:*

molecular biology-grade H ₂ O	60 µl
10X Labeling Buffer A	10 µl
siRNA duplex (~10 µg)	20 µl of a 40 µM stock
<i>Label IT</i> [®] siRNA Tracker Reagent	<u>10 µl</u>

Total volume: 100 µl

*This example uses a 1:1 (v:w) ratio of *Label IT*[®] Reagent to nucleic acid. This ratio will result in labeling efficiencies that are appropriate for siRNA tracking experiments. If there is a need to increase or decrease the density of labels in the final product, simply modify the ratio of labeling reagent to nucleic acid during the labeling reaction or adjust the incubation time of the labeling reaction. In addition, the labeling reaction may be scaled up or down, depending on the amount/volume of nucleic acid to be labeled. When scaling the labeling reaction, the amount of *Label IT*[®] Reagent should never constitute more than 20% of the total reaction volume. Ensure that the final concentration of Labeling Buffer A is 1X.

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, then 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. Gently wash the pellet once with 500 μl room temperature 70% ethanol (molecular biology grade). 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 the 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. If desired, each strand may be labeled with different labels. It is important to note that single stranded RNA oligonucleotides 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. Warm the vials containing the *Label IT*[®] siRNA Tracker Reagent and Reconstitution Solution to room temperature and quick spin to collect the reagents at the bottom of the vials. The Reconstitution Solution freezes at 4°C ; ensure it is completely thawed before use to obtain the required volume. Add 50 μl of 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
<i>Label IT</i> [®] siRNA Tracker Reagent	<u>10 μl</u>
Total volume:	100 μl

*This example uses a 1:1 (v:w) ratio of *Label IT*[®] Reagent to nucleic acid. This ratio will result in labeling efficiencies that are appropriate for siRNA tracking experiments. If there is a need to increase or decrease the density of labels in the final product, simply modify the ratio of labeling reagent to nucleic acid during the labeling reaction or adjust the incubation time of the labeling reaction. In addition, the labeling reaction may be scaled up or down, depending on the amount/volume of nucleic acid to be labeled. When scaling the labeling reaction, the amount of *Label IT*[®] Reagent should never constitute more than 20% of the total reaction volume. Ensure that the final concentration of Labeling Buffer A is 1X.

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 μ l 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 the example in Step 2 was used, resuspend labeled siRNA in 40 μ l of siRNA Dilution Buffer to bring the concentration to approximately 40 μ M.
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 *TransIT*-siQUEST[®] 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 *TransIT*-siQUEST[®] 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 *TransIT*-siQUEST[®] 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 Bio recommends Dharmacon Research, Inc. (dharmacon.com). The optimal final siRNA concentration for most siRNA tracking applications 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. *TransIT*-siQUEST[®] Reagent** – The optimal *TransIT*-siQUEST[®] Reagent volume can be determined by titrating the reagent within the ranges listed in Table 1. As a starting point, test three levels of *TransIT*-siQUEST[®] Reagent, such as 0.5, 1.5, and 3 μ l 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 + *TransIT-siQUEST*[®] Reagent
3. Serum-free media + *TransIT-siQUEST*[®] Reagent + a non-specific siRNA

Table 1. Recommended titration ranges for *TransIT-siQUEST*[®] 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
<i>TransIT-siQUEST</i> [®] 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
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, the siRNA Dilution Buffer provided. 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 *TransIT-siQUEST*[®] Transfection Reagent in 24-well plates. When performing transfections in different sized plates, the amount of siRNA, *TransIT-siQUEST*[®] 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 *TransIT-siQUEST*[®] Reagent levels and final siRNA concentrations.

3.3 siRNA Transfection of Adherent Cells in 24-well plates

A. Cell Plating

NOTE: 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 media to a sterile plastic tube^c.
2. Add the *TransIT-siQUEST*[®] Transfection Reagent (0.5 to 3 µl; see Table 1) directly into the serum-free medium. 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 *TransIT-siQUEST*[®] 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 *TransIT-siQUEST*[®] 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 μ l of complete growth media (see Table 1).
2. Add the *TransIT-siQUEST*[®] 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 μ l 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 μ l per well) if seeding the cells 24 hours before transfection. See Section 3.3A for plating cells on coated coverslips.
2. Prepare the *TransIT-siQUEST*[®] Reagent/siRNA complex mixture as described in Section 3.3, Part B.
3. Add the *TransIT-siQUEST*[®] 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.

^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% CO₂. Other cell types, such as insect cells, require different temperatures and CO₂ concentrations. Use conditions appropriate for the cell type being transfected.

^c The *TransIT-siQUEST*[®] 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 fluorescently labeled siRNA on mounted coverslips (fixed cells)

NOTE: See Section 3.6 for detection of *Label IT*[®] siRNA Tracker Biotin labeled siRNA.

See Table 2 for fluorescent excitation and emission wavelengths 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 (37%,wt:vol) in PBS to a final concentration of 4% (wt: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. At least 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).

3.6 Detection of *Label IT*[®] siRNA Tracker Biotin labeled siRNA on mounted coverslips (fixed cells)

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 (37%, wt:vol) in PBS to a final concentration of 4% (wt: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 at least 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. At least 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	Emission Wavelength (nm)
Cy [™] 3	549	570
Cy [™] 5	648	670
Fluorescein	495	518
CX-Rhodamine	587	597
TM-Rhodamine	559	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. The labeling density can also 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 samples without hindering their 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*. In mice, efficient *in vivo* delivery to select tissues can be obtained using a hydrodynamic injection protocol. In this procedure, siRNAs (in physiological saline) are rapidly injected into the tail vein of mice resulting in highly efficient delivery to liver hepatocytes^{4,5,6} Efficient siRNA delivery to limb skeletal muscle can also be achieved using an intravenous delivery injection procedure⁷.

5.0 TROUBLESHOOTING

5.1 Labeling Reaction – Suboptimal Labeling

- **Poor quality of siRNA**
Avoid siRNA degradation by using RNase-free handling procedures and plasticware. For high quality siRNA design and manufacture, Mirus Bio 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. Warm vials to room temperature before each use.
- **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 *TransIT*-siQUEST[®] Reagent**
Determine the optimal *TransIT*-siQUEST[®] Reagent concentration by titrating the reagent from 0.5 μl to 3 μl per well of a 24-well plate. It may be necessary to titrate outside of this range depending on the cell type.
- **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 Bio recommends Dharmacon Research, Inc. (dharmacon.com). Degradation can be detected on acrylamide gels.
- **Fetal calf serum present during *TransIT*-siQUEST[®] 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**
TransIT-siQUEST[®] 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-siQUEST*[®] Reagent/siRNA complex mixture was used in transfection**
Reduce the amount of *TransIT-siQUEST*[®] Reagent/siRNA complex mixture in the transfection. See Table 1 for recommended starting concentrations. Use the volume of reagent that gives the highest knockdown efficiency with the lowest cellular toxicity for future transfections.
- **Suboptimal cell density at the time of transfection**
Grow cells to a higher cell density and repeat the transfection.
- ***TransIT-siQUEST*[®] 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**
TransIT-siQUEST[®] 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 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 Bio 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 0.5-3 μ l of *TransIT-siQUEST*[®] Reagent 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, protected from light.

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 *TransIT-siQUEST*[®] 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 taken into consideration when determining the optimal time to assay the cells after transfection. Detection of mRNA levels (RT-qPCR, Northern, 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 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 at www.mirusbio.com.

6.0 GENERAL REFERENCES

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5. Zhang et al (1999) *Human Gene Therapy* **10**: 1735-1737.
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7.0 RELATED PRODUCTS

TransIT-siQUEST[®] siRNA Transfection Reagent is available for reorder as a separate product:

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

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*[®] siRNA Transfection Reagent (Product # MIR 7200,7201,7202,7203,7204,7205)
- Label IT*[®] siRNA Tracker Intracellular Localization Kit (Product # MIR 7212,7213,7214,7215,7216,7217)

Additional transfection reagents:*

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

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:*

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

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

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Mirus Bio Transfection 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 this product is guaranteed for one year from the date of purchase if stored and handled properly.

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