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 7212
	Cy™5	MIR 7213
	CX-Rhodamine	MIR 7214
	TM-Rhodamine	MIR 7215
	Biotin	MIR 7217
	Fluorescein	MIR 7216

* Each Kit contains *Label* IT® siRNA Tracker Reagent, Reconstitution Solution, 10X Labeling Buffer A, and siRNA Dilution Buffer. An siRNA transfection reagent is not supplied (see Related Products Section).

1.0 INTENDED USE

The *Label* IT® siRNA Tracker Intracellular Localization Kit provides a straight-forward approach to directly label 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 the introduction of the labeled siRNA into mammalian cells. This kit provides sufficient reagents to label 50 µg (5 x 10 µg reactions) of siRNA.

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* and in vivo 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.

For optimal tracking experiments, labeled siRNA must be efficiently delivered to the cells. Mirus Bio recommends the *Trans*IT-TKO® and *Trans*IT-siQUEST® Transfection Reagents, which are specifically formulated for siRNA delivery in vitro (see Related Products Section). These reagents enable 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 either of these reagents, siRNA knocks down target gene expression in a variety of cell lines. These unique features make *Trans*IT-TKO® and *Trans*IT-siQUEST® Transfection Reagents ideal for the delivery of labeled siRNA and 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*	100 µl	brown
10X Labeling Buffer A*	100 µl	lilac
siRNA Dilution Buffer	1 ml	white

*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, and siRNA Dilution Buffer 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 and all other reagents are stable for 1 year from the date of purchase. Warm the *Label* IT® siRNA Tracker Reagent and Reconstitution Solution to room temperature and quick spin 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. The Reconstitution Solution freezes at 4°C; ensure it is completely thawed before use to obtain the required volume. 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® 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 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 the 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. Their 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. The Reconstitution Solution freezes at 4°C; ensure it is completely thawed before use to obtain the required volume. 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® 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 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 the example in Step 2 was used, resuspend the 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 Post-labeling Suggestions

For delivery and detection of labeled siRNA in vitro, see Sections 3.3, 3.4, and 3.5.
For delivery and detection of labeled siRNA in vivo, see Section 4.0, Part C.

3.3 *In Vitro* Transfection Optimization

For optimal transfection efficiency, use the *Trans*IT-TKO® or *Trans*IT-siQUEST® Transfection Reagents to deliver labeled siRNA. The key to successful transfection is careful optimization of reaction conditions for each individual cell type. Please refer to the *Trans*IT-TKO® or *Trans*IT-siQUEST® Transfection Reagent protocols for detailed instructions. If using another transfection reagent, follow the manufacturer's protocol.

We recommend transfecting 50 nM siRNA (final concentration in the well), and plating the cells on Poly-D-Lysine coated coverslips to aid microscopic evaluation.

3.4 Detection of *Label* IT® siRNA Tracker fluorescently labeled siRNA on mounted coverslips (fixed cells)

NOTE: See Section 3.5 for detection of *Label* IT® siRNA Tracker Biotin labeled siRNA. See Table 1 for fluorescent excitation and emission wavelengths of labeled siRNA. Use appropriate microscope filters. These suggestions are for the analysis of cells grown and transfected on Poly-D-Lysine coated coverslips in 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 to fix the cells.
3. Incubate cells at room temperature for 20 minutes.
4. Aspirate formaldehyde and gently wash wells 3 times with PBS to remove excess fixative.
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 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 1).

3.5 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 the analysis of cells grown and transfected on Poly-D-Lysine coated coverslips 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 to fix the cells.
3. Incubate cells at room temperature for 20 minutes.
4. Aspirate formaldehyde and gently wash wells 3 times with PBS to remove excess fixative.
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. Add approximately 0.25-0.5 ml PBS to each well 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 1).

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 1. 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 for tracking experiments. 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 after transfection. Using the recommended range of *Label* IT® siRNA Tracker Reagent to siRNA ratios will provide a labeled siRNA sample 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. 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 procedure[7].

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 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.
- **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 a fluorescent microscope.

5.2 Transfection - Low Transfection Efficiency or High Cellular Toxicity

If using *Trans*IT-TKO® or *Trans*IT-siQUEST® Transfection Reagents, see the relevant protocols for troubleshooting advice. If using another transfection reagent, follow manufacturer's recommendations and troubleshooting guide.

5.3 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, and Section 5.1.
- **Excessive exposure to light**
  Protect samples and reagents from light.
- **Trouble detecting fluorescent signal**
  Use proper filter sets for microscopic detection. See Table 1.
- **Sub-optimal transfection efficiency**
  See Section 5.2.
- **Sub-optimal levels of siRNA transfected**
  Use 25-100 nM of labeled siRNA (final concentration in the well) and recommended conditions for transfection.
- **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.

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 REFERENCES

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7.0 RELATED PRODUCTS

RNA Interference Products:*

*Trans*IT-TKO® siRNA Transfection Reagent (Product # MIR 2150)
*Trans*IT-siQUEST® siRNA Transfection Reagent (Product # MIR 2110)

Additional transfection reagents:*

*Trans*IT®-Oligo Transfection Reagent (Product # MIR 2160)
*Trans*IT®-LT1 Transfection Reagent (Product # MIR 2300)
*Trans*IT®-Express Transfection Reagent (Product # MIR 2000)
*Trans*IT®-293 Transfection Reagent (Product # MIR 2700)
*Trans*IT®-CHO Transfection Kit (Product # MIR 2170)
*Trans*IT®-HeLaMONSTER® Transfection Kit (Product # MIR 2900)
*Trans*IT®-Jurkat Transfection Reagent (Product # MIR 2120)
*Trans*IT®-Keratinocyte Transfection Reagent (Product # MIR 2800)
*Trans*IT-Neural® Transfection Reagent (Product # MIR 2140)

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)

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

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