Label IT® siRNA Tracker Intracellular Localization Kits

Protocol for MIR 7212, 7213, 7214, 7215, 7216, 7217

Quick Reference Protocol, SDS and Certificate of Analysis available at mirusbio.com/7212

INTRODUCTION

When small interfering RNA (siRNA) are introduced into mammalian cells in culture, sequence-specific inhibition of target mRNA expression can be achieved without triggering an interferon response. This RNA interference effect can be long-lasting and detectable after many cell divisions, making siRNA an extremely effective tool for inhibiting target gene expression. The *Label* IT® siRNA Tracker Intracellular Localization Kit provides a straight-forward approach to covalently attach a Cy®3, Cy®5, CX-rhodamine, TM-rhodamine, fluorescein or biotin marker to siRNA, which allows tracking of functional fluorescently labeled siRNA in 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 *Label* IT® siRNA Tracker labeled siRNA.

SPECIFICATIONS

Storage	Store *Label* IT® Reagent at -20°C in both dried and reconstituted forms. Store all other kit components -20°C.
Product Guarantee	The *Label* IT® Reagent is stable at -20°C for 6 months after reconstitution. Unreconstituted *Label* IT® Reagent and all other components are guaranteed 1 year from the date of purchase, when properly stored and handled.
Kit Size	Contains sufficient material to label 50 µg (5 x 10 µg reactions) of siRNA

Cap the *Label* IT® siRNA Tracker Reagent tightly and avoid exposure to moisture and light.

Materials Supplied

Label IT® siRNA Tracker Kits are supplied in ***one*** of the following formats:

Product Name	*Label* IT® Tracker™ Reagent	Excitation Wavelength (nm)	Emission Wavelength (nm)	Product No.
Label IT® siRNA Tracker Intracellular Localization Kit	Cy®3	550	570	MIR 7212
	Cy®5	649	670	MIR 7213
	CX-Rhodamine	576	597	MIR 7214
	TM-Rhodamine	546	576	MIR 7215
	Fluorescein	492	518	MIR 7216
	Biotin	n/a	n/a	MIR 7217

The following components are included in the *Label* IT® siRNA Tracker Kits:

Kit Component	Full Size	Reagent Cap Color
Label IT® siRNA Tracker Labeling Reagent	Dried pellet	Red
Reconstitution Solution	100 µl	Brown
10X Labeling Buffer A	100 µl	Orange
siRNA Dilution Buffer	1 ml	White

Customer Notice:
The *Label* IT® Reagent cap has changed to a high-profile design with a red cap insert to improve handling and identifiability.

Materials required, but not supplied

- Molecular biology-grade water
- Microcentrifuge tubes
- Nucleic acid sample (starting material)
- Materials for EtOH purification
- Optional: Detection reagents

For Research Use Only.

BEFORE YOU START:

Important Tips for Optimal Nucleic Acid Labeling

The suggestions below generally yield strong labeling with minimal background and will maximize performance with most applications.

- **Reagent preparation.** Prior to first use, warm the vial containing the *Label* IT® Reagent to room temperature and centrifuge briefly (pulse) to collect the dried pellet. For subsequent uses, warm the vial of reconstituted *Label* IT® Reagent to room temperature before opening.
- **Reaction scalability.** The *Label* IT® labeling reactions can be scaled up or down to label different amounts of sample as required for alternate reaction conditions. When adjusting reaction volumes, maintain a 1X final concentration of Labeling Buffer A and ensure that the *Label* IT® Reagent does NOT constitute greater than 20% of the total reaction volume.
- **Labeling ratio.** The 1:1 (v:w) ratio of *Label* IT® siRNA Tracker Reagent to siRNA outlined in this protocol typically results in a labeling density of 1 label per every 15-40 base pairs. To modify the labeling density of the sample, simply increase or decrease the amount of *Label* IT® Reagent used in the reaction or adjust the reaction incubation time, as the labeling reaction is linear over the first three hours of incubation at 37°C. It is important to note that longer incubation times or labeling ratios greater than 2:1 (*Label* IT® siRNA Tracker Reagent to siRNA, vol:wt) could adversely affect the functionality of the siRNA.
- **DNase and RNase-free materials.** Wear gloves at all times when working with RNA. Use DNase-free and RNase-free reagents and plasticware.
- **Addition of *Label* IT® Reagent.** Add the *Label* IT® Reagent to the labeling reaction last.
- **Determining the Labeling Density of the Nucleic Acid Sample.** A labeling density of 1 label per every 15-40 base pairs can be expected if using a 1:1 (v:w) ratio of *Label* IT® siRNA Tracker Reagent to nucleic acid. If it is necessary to determine the exact labeling density of your sample, see instructions on our website in the *Label* IT® Frequently Asked Questions or Tips from the Bench. The relative density of labels on purified, labeled nucleic acid can be estimated by one of the following methods:

  For Fluorescent dyes:

  1. Spectrophotometric absorbance at λ_{max} of the dye. Several micrograms of sample may be required to generate significant λ_{max} absorbance readings.
  2. Fluorescent microscopy. Spot serial dilutions of purified labeled sample onto a glass slide and view with a fluorescent microscope

  For non-fluorescent dyes:

  1. Dot blot analysis. Fix dilutions of the labeled sample to a membrane, then detect with appropriate reagents.
  2. Gel shift analysis. A labeled sample may demonstrate a distinct reduction in electrophoretic mobility compared to unlabeled control sample.

A 1:1 (v:w) *Label* IT® Reagent to siRNA labeling ratio results in a labeling density suitable for most applications. Labeling ratios greater than 2:1 (vol:wt) could adversely affect the functionality of the siRNA.

LABELING PROTOCOL

The standard labeling procedure outlined below results in an approximate labeling density of one *Label* IT® siRNA Tracker label per every 15-40 base pairs of siRNA duplex which allows sensitive detection for most transfection applications. If an alternative labeling density is required, simply increase or decrease the amount of labeling reagent in the reaction or adjust the reaction incubation time. See the 'Reaction scalability' section in Before You Start (page 2) for further details.

A. Prepare and reconstitute the *Label* IT® siRNA Tracker Reagent

1. Before the first use, warm the *Label* IT® siRNA Tracker Reagent vial to room temperature and centrifuge briefly (pulse) to collect the dried pellet.
2. Warm the Reconstitution Solution to room temperature. This solution remains frozen at 4° C. Please ensure that it is completely thawed before use.
3. For the first use only, add 50 µl pre-warmed Reconstitution Solution to the *Label* IT® siRNA Tracker pellet.
4. To ensure complete reconstitution of the pellet, mix well by vortexing and centrifuge briefly (pulse) to collect the solution.

B. Label a Duplex siRNA or Single-Stranded siRNA(*) Sample

1. Prepare the labeling reaction according to the example shown below. Add the reagents in the order listed. Be sure to add the *Label* IT® siRNA Tracker Reagent last.

Duplex siRNA Labeling Reaction	
Molecular Biology-grade H_2O (DNase, RNase-free)	60 µl
10X Labeling Buffer A	10 µl
siRNA duplex (40 µM stock)*	20 µl
Label IT® siRNA Tracker Reagent	10 µl
Total Volume	**100 µl**

Increase or decrease the amount of *Label* IT® Reagent in the reaction or adjust the reaction incubation time to modify the labeling density. The *Label* IT® Reagent should not exceed 20% of the total reaction volume.

This example labels ~10 µg of siRNA duplex at a 1:1 (v:w) ratio of *Label* IT® Reagent to siRNA for a labeling density of 1 label per 15-40 bases. To modify the labeling density, increase or decrease the amount of *Label* IT® Reagent in the reaction or adjust the incubation time. The *Label* IT® Reagent should never exceed 20% of the total reaction volume.

***If using single stranded siRNA in the labeling reaction**, add 40 µl siRNA (40 µM stock) and reduce the volume of Molecular Biology-grade H_2O to 40 µl to maintain the 1:1 (v:w) ratio. Single stranded siRNAs can be annealed to generate siRNA duplexes following the labeling reaction. Each strand may be labeled with different labels, if desired.

NOTE: Single stranded RNA oligonucleotides are more easily degraded by RNase activity than siRNA duplexes, and their recovery following EtOH precipitation may be less efficient.

C. Incubate the reaction at 37°C for 1 hour.

NOTE: After 30 minutes of incubation, briefly centrifuge the reaction to minimize the effects of evaporation and maintain the appropriate concentration of the reaction components.

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D. Purification using Ethanol Precipitation

NOTE: For labeling reaction volumes <100 µl, bring the volume to 100 µl with 1X Labeling Buffer A or molecular biology-grade water before adding sodium chloride and ethanol.

1. Add 0.1 volume of 5M sodium chloride and 2 - 2.5 volumes of ice cold 100% ethanol to the reaction. Mix well and place at ≤ -20°C for at least 30 minutes.
2. Centrifuge at full speed (>14,000 x g) in a refrigerated microcentrifuge for 15-30 minutes to pellet the labeled nucleic acid. Once pelleted, gently remove the ethanol with a micropipetter; do not disturb the pellet. NOTE: Small siRNA quantities are difficult to visualize. Mark and orient the precipitate-containing tubes in the microfuge such that the pellet will form in a known place.
3. Wash the pellet once with 500 µl room temperature 70% ethanol. Centrifuge at full speed for an additional 15-30 minutes.
4. Remove all traces of ethanol with a micropipetter. DO NOT allow the sample to dry longer than 5 minutes as the pellet may become difficult to resuspend.
5. Resuspend the *Label* IT® siRNA Tracker labeled siRNA in an appropriate volume of siRNA Dilution Buffer. If the example outlined in this protocol was used, resuspend the labeled siRNA in 20 µl (*40 µl for single strand siRNA) siRNA Dilution Buffer to bring the concentration to approximately 40 µM.

   NOTE: siRNA duplexes can be annealed in 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.
6. If an exact siRNA concentration is required, quantify the purified, labeled siRNA on a spectrophotometer and dilute to the desired working concentration (e.g. 10 µM).
7. Store the purified, labeled siRNA on ice for immediate use or at -20°C for long-term storage. Protect the *Label* IT® siRNA Tracker labeled sample from light.

Ethanol purification of the *Label* IT® labeled nucleic acid is optimal if spectrophotometric quantification is required.

APPLICATION NOTES

A. *In Vitro* Tracking Experiments
Subcellular localization and target gene functionality can be monitored in the same experiment following the delivery of the *Label* IT® labeled sample into mammalian cells in culture. The *Label* IT® Tracker™ and *Label* IT® siRNA Tracker Intracellular Localization Kits are specifically tailored for effective and nondestructive Labeling of plasmid DNA or siRNA for *in vitro* nucleic acid tracking applications. To identify the ideal *Trans*IT® transfection reagent for labeled DNA/siRNA delivery to your cell type, see Related Products (page 8) or visit the Reagent Agent Transfection Database at www.mirusbio.com/ra.

Use RNase and DNase-free components.

B. *In Vivo* Tracking Experiments
Subcellular localization and reporter transgene expression can be monitored following the introduction of labeled nucleic acid into mammalian cells *in vivo*. The *Trans*IT®-QR Hydrodynamic Delivery Solution is designed specifically for safe and efficient delivery of plasmid DNA and siRNA into laboratory mice using the hydrodynamic tail vein injection procedure. Nucleic acids delivered with this kit primarily target the liver, with lower levels of expression detected in the spleen, lung, heart and kidneys.

C. Biotin Detection
A variety of commercially available secondary detection conjugates are compatible with *Label* IT® siRNA Tracker Biotin labeling. The potential for multi-color siRNA tracking is enhanced when the experimental design includes detection of a Biotin-labeled siRNA with a unique fluorophore conjugate and the direct detection of Cy®3, Cy®5, fluorescein or Rhodamine-labeled siRNA(s). The following describes a post-labeling avidin/streptavidin conjugation procedure for cells grown and transfected on Poly-D-Lysine coated coverslips in 24-well plates. If using alternative well sizes, scale volumes accordingly:

1. After an appropriate post-transfection incubation period (e.g. 48-72 hours), aspirate media from cells transfected with labeled siRNA, and wash twice with PBS.
2. Fix the cells by adding approximately 0.25 ml of 4% formaldehyde (freshly prepared in PBS) to each well. Incubate at room temperature for 20 minutes.
3. Aspirate the formaldehyde from the wells and gently wash the cells 3 times with PBS.
4. Dilute the desired avidin/streptavidin conjugate to ~20 ng/µl (or best concentration determined for the reagent of choice) in PBS.
5. Gently add ~50 µl of the diluted avidin/streptavidin conjugate to each coverslip (still within the 24-well plate).
6. Incubate at room temperature for at least 1 hour, protected from light.
7. After incubation, remove the avidin/steptavidin detection solution from the cells and wash the coverslip(s) 3 times with PBS.
8. To each well, add approximately 1 ml PBS to help with removal of the coverslips and to prevent drying.
9. Gently remove the coverslip(s) from wells with a forceps, and mount the coverslip(s) cell-side down on a glass slide containing pre-designated areas of anti-fade/mounting solution. Use capillary action to drain excess mounting solution from under coverslip using a Kimwipes™ tissue.
10. Seal all edges of the coverslip to the glass slide with nail polish or rubber cement.
11. View the slide on a fluorescent microscope using the appropriate filter sets.

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

TROUBLESHOOTING GUIDE

Problem	Solution
Suboptimal Nucleic Acid Labeling	
Poor quality of siRNA sample	Avoid siRNA degradation by using RNase-free handling procedures and plasticware. Degradation can be detected on acrylamide gels.
Incomplete labeling reaction	Incubate the reaction at 37°C for 1 hour. The reaction may be extended to 2 hours to increase the labeling density.
	A quick spin after 30 minutes will minimize the effect of evaporation.
Insufficient volume of *Label* IT® Reagent added to the reaction	Use 1 µl of *Label* IT® siRNA Tracker Reagent per 1 µg of siRNA duplex. See 'Labeling Protocol' (page 3) for proper labeling reaction setup.
Labeling reaction was not scaled properly	Keep the volume of *Label* IT® Reagent less than 20% of the total reaction volume, and ensure that the final concentration of Labeling Buffer A is 1X.
	Avoid using nucleic acid samples in high salt, as NaCl concentrations greater than 50 mM can inhibit the labeling reaction.
Improper storage of reagents	Store both reconstituted and unreconstituted *Label* IT® Reagent tightly capped at -20°C, and protect from exposure to light and moisture.
	Warm vial to room temperature and briefly spin to collect contents before opening.
Nucleic acid pellets were allowed to over-dry (after EtOH Purification)	Do not allow the labeled siRNA pellet to dry extensively after ethanol precipitation. Remove all traces of the ethanol wash and resuspend immediately in siRNA Dilution Buffer or 100 mM NaCl, 50 mM Tris, pH 7.5 in RNase-free water. **Do not** use water as this can denature the siRNA at low concentration during long-term storage.

Problem	Solution
Low Transfection Efficiency	
Suboptimal levels of siRNA transfected	Avoid siRNA degradation by using RNase-free handling procedures and plastic ware. Degradation can be detected on acrylamide gels.
	Use 25-100 nM siRNA (final concentration in the well) and recommended conditions for transfection per the transfection reagent of choice.
Cell density (% confluence) not optimal at the time of transfection	The recommended cell density for most cell types at the time of transfection is 70-90% confluence. Determine the optimal cell density for each cell type in order to maximize transfection efficiency. Maintain this density in future experiments for reproducibility.
Cells not actively dividing at the time of transfection	Divide the culture at least 18–24 hours before transfection to ensure that the cells are actively dividing and reach optimal cell density at time of transfection.
Inhibitor present during transfection	The presence of polyanions, such as dextran sulfate or heparin, can inhibit transfection. Use transfection medium that does not contain these polyanions.

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Problem	Solution
High Cellular Toxicity	
Transfection mixture and cells were not mixed thoroughly	Add transfection complexes drop-wise to cells and mix thoroughly to evenly distribute by rocking the dish back and forth and side to side.
	Do not swirl or rotate the dish, as this may result in uneven distribution.
siRNA knockdown of an essential gene	If the siRNA is directed against a gene that is essential to the cell, cytotoxicity may be observed due to knockdown of the target gene. Include a transfection control with non-targeting siRNA to compare the cytotoxic effects of the gene being knocked down.
Excessive amount of transfection reagent/siRNA complex used in transfection	Reduce the amount of transfection reagent and siRNA used in the transfection. NOTE: The pre-determined optimal reagent:siRNA ratio (µl transfection reagent to µg siRNA) should be maintained for complex formation.
Cell density was too low at time of transfection	Typically, cells should be ~80% confluent at the time of transfection. If transfections were performed at a lower confluence and cytotoxicity was observed, grow cells to a higher cell density and repeat the experiment.

Problem	Solution
Poor Visualization of Labeled siRNA in Cells	
Label IT® signal lost over time	Observe transfected cells within 48 hours of the transfection to ensure a robust *Label* IT® Tracker signal. After 48 hours, the *Label* IT® signal may become too dilute to detect.
Low labeling ratio	Increase the labeling ratio by increasing the volume of *Label* IT® siRNA Tracker Reagent to weight of siRNA (see Labeling Protocol).
Excessive exposure to light	Protect *Label* IT® siRNA Tracker Reagent and labeled samples from light.
Improper filter sets used to detect fluorescent signal	See Materials Supplied section for the appropriate Excitation/Emission wavelength for *Label* IT® siRNA Tracker fluorophores.
Sub-optimal siRNA transfection	See *Low Transfection Efficiency* section.
Improper storage of labeled DNA	*Label* IT® siRNA Tracker labeled siRNA must be stored at -20°C, protected from light.
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 visible light microscope.

Problem	Solution
Little or No Inhibition of Target Gene Expression Observed	
Labeling density is too high	Label siRNA at a lower ratio. See *Before You Start* (page 2) and *Labeling Protocol* (page 3) sections for labeling ratio recommendations and instructions for modifying labeling density.
Suboptimal siRNA concentration	Determine the optimal siRNA concentration by titrating from 10–50 nM (final concentration in the well). We recommend starting with 25 nM siRNA (final concentration in the well). In some instances, higher concentrations of siRNA up to 200 nM may be necessary to achieve sufficient knockdown of the gene of interest.
Poor transfection efficiency	See *Low Transfection Efficiency* section.
Observation time is not optimal	Perform a time course to determine the kinetics of expression for the protein of interest.

RELATED PRODUCTS

- *Label* IT® Nucleic Acid Labeling Kits
- *Label* IT® Nucleic Acid Modifying Kit, Amine
- *Label* IT® Tracker™ Intracellular Nucleic Acid Localization Kits
- *Label* IT® Plasmid Delivery Controls
- *Label* IT® RNAi Delivery Controls
- Ingenio® Electroporation Solution and Kits
- *Trans*IT-X2® Dynamic Delivery System
- *Trans*IT®-2020 Transfection Reagent
- *Trans*IT®-LT1 Transfection Reagent
- *Trans*IT® Cell Line Specific Transfection Reagents and Kits

For details on our products, visit www.mirusbio.com.

Contact Mirus Bio for additional information.

Mirus Bio LLC
5602 Research Park Blvd, Ste 210
Madison, WI 53719
Toll-free: 888.530.0801
Direct: 608.441.2852
Fax: 608.441.2849

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