Label IT® Tracker™ Intracellular Nucleic Acid Localization Kits

Protocol for MIR 7020, 7021, 7022, 7023, 7024, 7025

Quick Reference Protocol, SDS and Certificate of Analysis available at mirusbio.com/7020

INTRODUCTION

The *Label* IT® Tracker™ Intracellular Nucleic Acid Localization Kit provides a straight-forward, scalable approach to directly label and deliver plasmid DNA for intracellular tracking experiments. Unlike other non-radioactive labeling technologies (e.g. random priming, nick translation), the *Label* IT® Tracker™ Intracellular Nucleic Acid Localization Kits will covalently attach a Cy®3, Cy®5, CX-rhodamine, TM-rhodamine, biotin or fluorescein marker to nucleic acid bases without dramatically altering the starting DNA. With *Label* IT® Tracker™, both subcellular localization and reporter transgene expression can be monitored simultaneously following introduction of the labeled plasmid into mammalian cells.

SPECIFICATIONS

Storage	Store *Label* IT® Reagent at -20°C in both dried and reconstituted forms. Store Reconstitution Solution and 10X Labeling Buffer A at -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 reagents are guaranteed 1 year from the date of purchase, when properly stored and handled.
Kit Size	Contains sufficient material to label 50 to 200 µg of plasmid DNA

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

MATERIALS

Materials Supplied

Label IT® 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® Tracker™ Intracellular Nucleic Acid Localization Kit	Cy®3	550	570	MIR 7020
	Cy®5	649	670	MIR 7021
	CX-Rhodamine	576	597	MIR 7022
	TM-Rhodamine	546	576	MIR 7023
	Biotin	n/a	n/a	MIR 7024
	Fluorescein	492	518	MIR 7025

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

Kit Component	Full Size	Reagent Cap Color
Label IT® Tracker™ Reagent	dried pellet	varies with reagent
Tracker Reconstitution Solution	100 µl	brown
10X Labeling Buffer A	500 µl	orange

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 0.5:1 (v:w) ratio of *Label* IT® Tracker™ Reagent to DNA outlined in this protocol typically results in a labeling density of 1 label per every 60-140 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. For DNA tracking applications *in vitro*, we recommend a range of 0.25 to 1 µl of *Label* IT® Tracker™ Reagent per µg of DNA (i.e. 0.25:1 to 1:1 (v:w)). Lower labeling densities may be required for applications in which the labeled DNA will be used for gene expression studies *in vivo*.
- **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 60-140 base pairs can be expected if using a 0.5:1 (w:v) ratio of *Label* IT® Tracker™ Reagent to nucleic acid. If it is necessary to determine the exact labeling density of your sample, see instructions 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 nucleic acid labeling ratio results in a labeling density suitable for most applications. Lower labeling densities may be required for some applications.

LABELING PROTOCOL

The standard labeling procedure outlined below will yield an approximate labeling density of one *Label* IT® Tracker™ label per every 60-140 base pairs of double-stranded DNA. This labeling density is sufficient to allow 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 *Label* IT® Tracker™ Nucleic Acid Labeling Reagent

1. Before the first use, warm the *Label* IT® Tracker™ Reagent vial to room temperature and centrifuge briefly (pulse) to collect the dried pellet.
2. Warm the Tracker 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 Tracker Reconstitution Solution to the *Label* IT® Tracker™ pellet.
4. To ensure complete reconstitution of the pellet, mix well by vortexing and centrifuge briefly (pulse) to collect the solution.

B. Label Nucleic Acid Sample

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

Standard DNA Labeling Reaction	
DNase-, RNase-free (molecular biology-grade) water	37.5 µl
10X Labeling Buffer A	5 µl
1 mg/ml nucleic acid sample	5 µl
Label IT® Reagent	2.5 µl
Total Volume	**50 µl**

NOTE: This example labels 5 µg of DNA at a 0.5:1 (v:w) ratio of *Label* IT® Tracker™ Reagent to nucleic acid, resulting in a labeling efficiency appropriate for most applications. Increase or decrease the amount of *Label* IT® Reagent in the reaction or adjust the reaction incubation time to modify the labeling density.

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.

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.

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 nucleic acid quantities can be difficult to visualize. Mark and orient the precipitate-containing tubes in the microfuge such that the pellet will form in a predetermined 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® Tracker™ labeled nucleic acid in an appropriate volume of 1X Labeling Buffer A or sterile water.
6. If an exact nucleic acid concentration is required, quantify the purified, labeled nucleic acid on a spectrophotometer and dilute to the desired working concentration.
7. Store the purified, labeled nucleic acid on ice for immediate use or at -20°C for long-term storage. Protect the *Label* IT® 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 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 the Related Products Section (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®-EE and *Trans*IT®-QR Hydrodynamic Delivery Solutions are designed specifically for the safe and efficient delivery of nucleic acids into laboratory mice using the hydrodynamic tail vein injection procedure. Nucleic acids delivered with these kits primarily target the liver, with lower levels of expression detected in the spleen, lung, heart and kidneys.

C. Biotin Detection

Tracking Biotin-labeled DNA allows the use of a wide variety of commercially available detection reagents. The potential for multi-color tracking experiments is enhanced when the experimental design includes detection of a Biotin-labeled plasmid with a unique fluorophore conjugate and the direct detection of Cy®3, Cy®5, Fluorescein or Rhodamine-labeled plasmid(s). The following describes a post-labeling avidin/streptavidin conjugation procedure for cells grown on coverslips:

1. After a post-transfection incubation period of 12-72 hours, aspirate media from cells grown on coverslip(s) and transfected with *Label* IT® Tracker™ labeled DNA.
2. Wash cells twice with PBS.
3. Fix the cells by adding sufficient 4% formaldehyde (freshly prepared in PBS) to cover the cells. Incubate at room temperature for 20 minutes.
4. Aspirate the formaldehyde and gently wash the cells 3 times with PBS.
5. Dilute the desired avidin/streptavidin conjugate to ~20 ng/µl, or the best concentration determined for the reagent of choice, in PBS.
6. Gently load wells with ~200 µl of the conjugate dilution onto cells.
7. Incubate at room temperature for at least 1 hour, protected from light.
8. After incubation, remove the avidin/steptavidin detection solution from the cells and wash the coverslip(s) 3 times with PBS.
9. To each well, add approximately 1 ml PBS to help with removal of the coverslips and to prevent drying.
10. 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 Kimwipe tissue.
11. Seal all edges of the coverslip to the glass slide with nail polish or rubber cement.
12. 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-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 nucleic acid sample	Use purified nucleic acid (A_{260}/A_{280} between 1.8 and 2.2) that is free from proteins, carbohydrates, etc.
	Avoid nucleic acid degradation by using DNase- and RNase-free handling procedures and plasticware.
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 0.5 µl of *Label* IT® Tracker™ Reagent per 1 µg of nucleic acid. See 'Labeling Protocol' 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 nucleic acid pellet to dry extensively after ethanol precipitation. Remove all traces of the ethanol wash and resuspend immediately in 1X Buffer A or a low salt buffer of choice.
Problem	**Solution**
Low Transfection Efficiency	
Poor quality of Transfected DNA	DNA may be partially degraded or an inhibitor, such as endotoxin, may be present in the preparation. Use double-stranded, cesium chloride-purified DNA if commercial methods have not worked satisfactorily. Remove any traces of endotoxin (LPS) using the Mirus Bio MiraCLEAN® Endotoxin Removal Kit (MIR 5900).
	Use purified DNA (A_{260}/A_{280} between 1.8 and 2.2) that is free from proteins, carbohydrates, etc. Avoid nucleic acid degradation by using DNase- and RNase-free handling procedures and plastic ware.
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 time of transfection	Divide the culture at least 18-24 hours before transfection to ensure that cells are actively dividing and reach optimal cell density (70-90% confluence) at the 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.

Problem	Solution
High Cellular Toxicity	
Mini-prep DNA used for transfection	Mini-prep DNA may contain endotoxins (LPS) which are harmful to mammalian cells. Remove any traces of endotoxin using the Mirus Bio MiraCLEAN® Endotoxin Removal Kit (MIR 5900) or prep DNA using an endotoxin-free Maxi-prep kit.
	Avoid nucleic acid degradation by using DNase- and RNase-free handling procedures and plasticware.
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.
Excessive amount of transfection reagent/DNA complex used in transfection	Reduce the amount of transfection reagent and DNA used in the transfection. NOTE: The pre-determined optimal reagent:DNA ratio (µl transfection reagent to µg DNA) should be maintained for complex formation.
Cell density was too low at time of transfection	Typically, cells should be approximately 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 DNA in Cells	
Poor quality labeled DNA	Use purified, intact DNA in transfections.
Low labeling ratio	Increase the labeling ratio by increasing the volume of *Label* IT® Tracker™ Reagent to weight of DNA (see Labeling Protocol).
Excessive exposure to light	Protect *Label* IT® 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® Tracker™ fluorophores.
Sub-optimal DNA transfection	See *Low Transfection Efficiency* sections.
Improper storage of labeled DNA	*Label* IT® Tracker™ labeled DNA 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 Transgene Expression Observed	
Labeling density is too high	Label DNA 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.
Reporter protein is not expressed	Ensure that the reporter protein is being expressed and detected by transfecting an unlabeled plasmid control.
Poor transfection efficiency	See *Low Transfection Efficiency* sections.
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 siRNA Intracellular 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
- *Trans*IT®- EE Hydrodynamic Delivery Solution and Starter Kit
- *Trans*IT®-QR Hydrodynamic Delivery Solution and Starter Kit

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

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