**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

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**INTRODUCTION**

The *Label IT®* Tracker™ Intracellular Nucleic Acid Localization Kit provides a straightforward, 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**

<table>
<thead>
<tr>
<th>Storage</th>
<th>Store <em>Label IT®</em> Reagent at -20°C in both dried and reconstituted forms. Store Reconstitution Solution and 10X Labeling Buffer A at -20°C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product Guarantee</td>
<td>The <em>Label IT®</em> Reagent is stable at -20°C for 6 months after reconstitution. Unreconstituted <em>Label IT®</em> Reagent and all other reagents are guaranteed 1 year from the date of purchase, when properly stored and handled.</td>
</tr>
<tr>
<td>Kit Size</td>
<td>Contains sufficient material to label 50 to 200 µg of plasmid DNA</td>
</tr>
</tbody>
</table>

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**MATERIALS**

**Materials Supplied**

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

<table>
<thead>
<tr>
<th>Product Name</th>
<th><em>Label IT®</em> Tracker™ Reagent</th>
<th>Excitation Wavelength (nm)</th>
<th>Emission Wavelength (nm)</th>
<th>Product No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Label IT®</em> Tracker™ Intracellular Nucleic Acid Localization Kit</td>
<td>Cy®3</td>
<td>550</td>
<td>570</td>
<td>MIR 7020</td>
</tr>
<tr>
<td></td>
<td>Cy®5</td>
<td>649</td>
<td>670</td>
<td>MIR 7021</td>
</tr>
<tr>
<td></td>
<td>CX-Rhodamine</td>
<td>576</td>
<td>597</td>
<td>MIR 7022</td>
</tr>
<tr>
<td></td>
<td>TM-Rhodamine</td>
<td>546</td>
<td>576</td>
<td>MIR 7023</td>
</tr>
<tr>
<td></td>
<td>Biotin</td>
<td>n/a</td>
<td>n/a</td>
<td>MIR 7024</td>
</tr>
<tr>
<td></td>
<td>Fluorescein</td>
<td>492</td>
<td>518</td>
<td>MIR 7025</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Kit Component</th>
<th>Full Size</th>
<th>Reagent Cap Color</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Label IT®</em> Tracker™ Reagent</td>
<td>Dried pellet</td>
<td>Red</td>
</tr>
<tr>
<td>Tracker Reconstitution Solution</td>
<td>100 µl</td>
<td>Brown</td>
</tr>
<tr>
<td>10X Labeling Buffer A</td>
<td>500 µl</td>
<td>Orange</td>
</tr>
</tbody>
</table>

**Materials required, but not supplied**

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

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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 $\lambda_{\text{max}}$ of the dye. Several micrograms of sample may be required to generate significant $\lambda_{\text{max}}$ 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.
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.

<table>
<thead>
<tr>
<th>Standard DNA Labeling Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase-, RNase-free (molecular biology-grade) water</td>
</tr>
<tr>
<td>10X Labeling Buffer A</td>
</tr>
<tr>
<td>1 mg/ml nucleic acid sample</td>
</tr>
<tr>
<td>Label IT® Reagent</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
</tr>
</tbody>
</table>

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.

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 micropipette; 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.
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 TransIT® 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.

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 TransIT®-EE and TransIT®-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/streptavidin 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

<table>
<thead>
<tr>
<th>Problem</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Suboptimal Nucleic Acid Labeling</strong></td>
<td></td>
</tr>
<tr>
<td>Poor quality of nucleic acid sample</td>
<td>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.</td>
</tr>
<tr>
<td>Incomplete labeling reaction</td>
<td>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.</td>
</tr>
<tr>
<td>Insufficient volume of label IT® Reagent added to the reaction</td>
<td>Use 0.5 µl of label IT® Tracker™ Reagent per 1 µg of nucleic acid. See ‘Labeling Protocol’ for proper labeling reaction setup.</td>
</tr>
<tr>
<td>Labeling reaction was not scaled properly</td>
<td>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.</td>
</tr>
<tr>
<td>Improper storage of reagents</td>
<td>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.</td>
</tr>
<tr>
<td>Nucleic acid pellets were allowed to over-dry (after EtOH Purification)</td>
<td>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.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Problem</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low Transfection Efficiency</strong></td>
<td></td>
</tr>
<tr>
<td>Poor quality of Transfected DNA</td>
<td>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.</td>
</tr>
<tr>
<td>Cell density (% confluence) not optimal at the time of transfection</td>
<td>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.</td>
</tr>
<tr>
<td>Cells not actively dividing at time of transfection</td>
<td>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.</td>
</tr>
<tr>
<td>Inhibitor present during transfection</td>
<td>The presence of polyanions, such as dextran sulfate or heparin, can inhibit transfection. Use transfection medium that does not contain these polyanions.</td>
</tr>
</tbody>
</table>
### Problem | Solution
--- | ---
**High Cellular Toxicity** | 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.

<table>
<thead>
<tr>
<th>Mini-prep DNA used for transfection</th>
<th>Transfection mixture and cells were not mixed thoroughly</th>
<th>Excessive amount of transfection reagent/DNA complex used in transfection</th>
<th>Cell density was too low at time of transfection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Add transfection complexes drop-wise to cells and mix thoroughly to evenly distribute by rocking the dish back and forth and side to side.</td>
<td>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.</td>
<td>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.</td>
</tr>
</tbody>
</table>

### Problem | Solution
--- | ---
**Poor Visualization of Labeled DNA in Cells** |  
Poor quality labeled DNA | Use purified, intact DNA in transfections.

<table>
<thead>
<tr>
<th>Low labeling ratio</th>
<th>Excessive exposure to light</th>
<th>Improper filter sets used to detect fluorescent signal</th>
<th>Sub-optimal DNA transfection</th>
<th>Improper storage of labeled DNA</th>
<th>Cells lost during fixation or mounting procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increase the labeling ratio by increasing the volume of Label IT® Tracker™ Reagent to weight of DNA (see Labeling Protocol).</td>
<td>Protect Label IT® Tracker Reagent and labeled samples from light.</td>
<td>See Materials Supplied section for the appropriate Excitation/Emission wavelength for Label IT® Tracker™ fluorophores.</td>
<td>See Low Transfection Efficiency sections.</td>
<td>Label IT® Tracker™ labeled DNA must be stored at -20°C, protected from light.</td>
<td>Perform all washing, fixing and mounting steps gently. Check for presence of cells following each step on a visible light microscope.</td>
</tr>
</tbody>
</table>

### Problem | Solution
--- | ---
**Little or No Transgene Expression Observed** |  
Labeling density is too high | Reporter protein is not expressed | Poor transfection efficiency | Observation time is not optimal |
| 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. | Ensure that the reporter protein is being expressed and detected by transfecting an unlabeled plasmid control. | See Low Transfection Efficiency sections. | 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
- TransIT-X2® Dynamic Delivery System
- TransIT®-2020 Transfection Reagent
- TransIT®-LT1 Transfection Reagent
- TransIT® Cell Line Specific Transfection Reagents and Kits
- TransIT®- EE Hydrodynamic Delivery Solution
- TransIT®-QR Hydrodynamic Delivery Solution

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

Contact Mirus Bio for additional information.

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