

**Label IT<sup>®</sup> Tracker<sup>™</sup>**  
**Intracellular Nucleic Acid Localization Kit**

Product Name	Label IT <sup>®</sup> Tracker <sup>™</sup> Reagent	Product No.
<i>Label IT<sup>®</sup> Tracker<sup>™</sup></i> Intracellular Nucleic Acid Localization Kit*	Cy <sup>™</sup> 3	MIR 7020
	Cy <sup>™</sup> 5	MIR 7021
	CX-Rhodamine	MIR 7022
	TM-Rhodamine	MIR 7023
	Biotin	MIR 7024
	Fluorescein	MIR 7025

\* Each Kit contains *Label IT<sup>®</sup> Tracker<sup>™</sup>* Reagent, Tracker Reconstitution Solution, and 10X Labeling Buffer A.

## 1.0 INTENDED USE

The ability to easily track fluorescently labeled DNA in the cell has the potential to greatly enhance the understanding of exogenous DNA biology. This is particularly important in the non-viral gene delivery field where tremendous efforts have focused on improving cytoplasmic delivery and nuclear uptake of DNA delivery vectors. The *Label IT<sup>®</sup> Tracker<sup>™</sup>* Intracellular Nucleic Acid Localization Kit provides a straight-forward approach to directly label and deliver plasmid DNA, of any design, in an efficient yet non-destructive manner, for tracking experiments. Both subcellular localization and reporter transgene expression can be monitored simultaneously following introduction of the labeled plasmid into mammalian cells. This kit provides sufficient reagents to label 50 to 200 µg of plasmid DNA.

## 2.0 DESCRIPTION

### 2.1 General Information

The *Label IT<sup>®</sup> Tracker<sup>™</sup>* Kits are based on Mirus Bio's proprietary nucleic acid labeling technology. The *Label IT<sup>®</sup>* 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 one-step chemical reaction. The simplicity of the highly efficient and non-destructive labeling reaction, and the availability of a variety of labels, including the very sensitive and highly popular Cy<sup>™</sup> dyes, makes this the ideal kit to generate labeled DNA for *in vitro* tracking experiments.

Traditional non-radioactive labeling methods (random priming, nick translation) are enzyme mediated and thus inherently difficult to control. In addition, these types of reactions generate labeled products that are not representative of the starting DNA, but rather consist of a series of labeled species over a variable size range. Using the non-enzymatic *Label IT<sup>®</sup> Tracker<sup>™</sup>* Kits, any plasmid (linear or circular) can be custom labeled before introduction into mammalian cells. This allows for the ideal situation in which expression-competent fluorescently labeled DNA can be visually monitored during the transfection process.

**Table 1. Excitation and emission wavelengths of the *Label IT*<sup>®</sup> Tracker<sup>™</sup> Reagents**

Labeling Reagent	Excitation Wavelength (nm)	Emission Wavelength (nm)
Fluorescein	492	518
5-carboxy-X-rhodamine	576	597
Tetramethyl-rhodamine	546	576
Cy <sup>™</sup> 3	550	570
Cy <sup>™</sup> 5	649	670

## 2.2 Materials Supplied

Component	Volume	Reagent Cap Color
<i>Label IT</i> <sup>®</sup> Tracker <sup>™</sup> Reagent	dried pellet	varies with label
Tracker Reconstitution Solution	60 µl	brown
10X Labeling Buffer A	500 µl	orange

## 2.3 Storage and Stability

The *Label IT*<sup>®</sup> Tracker<sup>™</sup> Kit should be stored at  $-20^{\circ}\text{C}$ . The *Label IT*<sup>®</sup> Tracker<sup>™</sup> Reagent should be stored at  $-20^{\circ}\text{C}$  in both its dried pellet and reconstituted form. The *Label IT*<sup>®</sup> Tracker<sup>™</sup> Reagent is stable for 6 months after reconstitution. Unreconstituted *Label IT*<sup>®</sup> Tracker<sup>™</sup> Reagent and all other reagents are stable for 1 year from the date of purchase. Warm the *Label IT*<sup>®</sup> Tracker<sup>™</sup> Reagent to room temperature and quick spin before each use.

## 3.0 PROCEDURE

### 3.1 Plasmid Labeling Reaction

1. Bring the tube containing the *Label IT*<sup>®</sup> Tracker<sup>™</sup> Reagent to room temperature and quick spin to collect the pellet since it may have become dislodged during shipping. Add 50 µl of Tracker Reconstitution Solution to the pellet in the tube. To ensure complete reconstitution of the pellet, mix well.
2. Prepare the labeling reaction according to the example shown below. Use molecular biology-grade (i.e. DNase and RNase-free) water. Add the *Label IT*<sup>®</sup> Tracker<sup>™</sup> Reagent last.

Example:\*

sterile H <sub>2</sub> O	37.5 µl
10X Labeling Buffer A	5 µl
1 mg/ml plasmid DNA	5 µl
<i>Label IT</i> <sup>®</sup> Tracker <sup>™</sup> Reagent	<u>2.5 µl</u>

Total volume: 50 µl

\* This example uses a 0.5:1 (v:w) ratio of *Label IT*<sup>®</sup> Tracker Reagent to DNA. The final concentration of DNA in the reaction is 0.1 mg/ml. For DNA tracking applications, we recommend a range of 0.25 to 1 µl of *Label IT*<sup>®</sup> Tracker<sup>™</sup> Reagent per µg of DNA (i.e. 0.25:1 to 1:1 (v:w)).

\* The labeling reaction may be scaled up or down, depending on the amount of DNA to be labeled. The amount of *Label IT*<sup>®</sup> Tracker<sup>™</sup> Reagent should be less than 20% of the total reaction volume.

3. Incubate reaction at  $37^{\circ}\text{C}$  for 1 hour.  
**NOTE:** When performing 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*<sup>®</sup> Tracker<sup>™</sup> Reagent from the labeled plasmid by ethanol precipitation. Add 0.1 volume of 5 M sodium chloride and 2 volumes of ice cold 100% ethanol to the reaction. Mix well and place in a  $-20^{\circ}\text{C}$  (or colder) freezer for at least 30 minutes.

**NOTE:** For smaller reaction volumes (<100  $\mu$ l), bring the volume up to 200  $\mu$ l with 1X Labeling Buffer A or sterile H<sub>2</sub>O before adding the sodium chloride and ethanol.

5. Centrifuge at full speed in a refrigerated microcentrifuge for 10 minutes to pellet the labeled DNA. Gently remove the ethanol with a pipet; do not disturb the pellet.

**NOTE:** Orient the precipitate-containing tubes in the microcentrifuge such that the pellet will form in a predetermined, marked place. Small DNA quantities can be invisible to the naked eye.

6. Wash the pellet once with 500  $\mu$ l room temperature 70% ethanol. After an additional centrifugation at full speed for 10 minutes, remove all traces of ethanol with a micropipetter. Do not allow the sample to air dry extensively, as the pellet may become extremely difficult to resuspend.
7. Resuspend the labeled probe in 10  $\mu$ l 1X Labeling Buffer A or sterile water.
8. Quantify the concentration of the purified, labeled DNA on a spectrophotometer. Dilute to a suitable working concentration, if necessary.
9. Store the purified, labeled DNA at  $-20^{\circ}\text{C}$ . Store on ice if needed for immediate use. Protect from light.

### 3.2 Mammalian Cell Transfection

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Mirus Bio's *TransIT*<sup>®</sup>-LT1 (Low Toxicity) Polyamine Transfection Reagent (MIR 2300) offers clear advantages for delivering DNA into cells via transfection, including high efficiency gene transfer, minimal cellular toxicity, ease of use, and transfection reproducibility. This product provides state-of-the-art transfection efficiencies with significantly reduced levels of cell damage compared to other leading transfection reagents. It is a broad-spectrum reagent based on a proprietary protein/polyamine formulation. In addition, transfections using the *TransIT*<sup>®</sup>-LT1 Reagent do not require media changes and should be carried out in serum-containing media. This unique combination makes this reagent ideal for all gene expression studies where the post-transfection state of the cell is important. Please consult Mirus' entire product line for cell-line specific and other transfection reagents. (see Section 7.0)

### 3.3 Biotin Detection

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The tracking of Biotin-labeled DNA allows the use of a wide variety of commercially available detection reagents. Furthermore, the potential for multi-color tracking experiments is enhanced when the experimental design includes detection of a Biotin-labeled plasmid with a unique fluor conjugate and the direct detection of Cy<sup>™</sup>3, Cy<sup>™</sup>5 or Rhodamine-labeled plasmid(s).

1. After a post-transfection incubation period of 12-72 hours, aspirate the media.
2. Wash cells once with PBS.
3. Fix cells as described in Section 3.4, steps 1-4 only.
4. Dilute desired Streptavidin conjugate to  $\sim 20$  ng/ $\mu$ l, or the best concentration determined for the reagent of choice, in PBS.
5. Gently add approximately 200  $\mu$ l Streptavidin conjugate dilution onto each coverslip.
6. Incubate at room temperature, shielded from light, for at least 1 hour.
7. After incubation, remove detection solution and wash 3 times with PBS.
8. To each well, add approximately 1 ml PBS to help with removal of the coverslips.
9. Mount 2 coverslips per glass slide as described in Section 3.4, step 6.

### 3.4 Cell Fixation and Mounting for Microscopy

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If desired, fix cells in 4% formaldehyde and mount coverslips on glass slides.

**NOTE:** It is best to keep exposure of the cells to light at a minimum to prevent loss of fluorescent signal.

1. Make a fresh dilution of 4% formaldehyde in PBS 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 1 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 1 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 height and width of the coverslip that will cover it. Two circles, with coverslips, will fit on a standard glass slide.
  - b. Place a small drop of antifade/mounting solution in the center of each marked circle.
  - c. Remove coverslips from wells with forceps and gently wipe off underside (non-cell side) of glass with a Kimwipe tissue.
  - d. Mount slowly, cell-side down onto antifade/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 (see Section 2.1, Table 1).

For suspension cells, fix and wash cells in solution. Centrifuge 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.

### 3.5 Microinjection

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Microinjection involves the direct transfer of a dissolved substance into a living cell. It is a powerful technique that requires specialized equipment and technical expertise. The procedure is well tolerated, and can be successfully applied to a variety of cells in culture. It is an attractive strategy for studying primary cultures or cells which are recalcitrant to even the best transfection procedures. Since cells are processed one by one, a limited number of cells can be studied. Specific recommendations are difficult to provide since there are a variety of micromanipulation systems available, ranging from manual to fully automated.

#### Microinjection Tips to Consider:

- Cells should be plated on coverslips. Gridded coverslips (for example, Eppendorf's CELLocate coverslips) allow easy location of the microinjected cells.
- Cells should be cultured in a buffered medium to maintain the pH during prolonged exposure to ambient conditions during the microinjection process. For example, culture medium supplemented with 25 mM HEPES, pH 7.2 is suitable. Also, it is recommended that the microinjection procedure for each coverslip be restricted to less than 30 minutes. If it is anticipated that the microinjection procedure will take significantly longer than 30 minutes per coverslip, replace the media with PBS and use a microscope with a stage warmer, if possible.
- The labeled DNA sample can be ethanol precipitated twice to ensure quantitative removal of any remaining *Label IT*<sup>®</sup> Tracker™ Reagent. Suitable injection buffers for labeled DNA samples include TE (10 mM Tris, pH 7.2; 0.25 mM EDTA), PBS, and 25 mM HEPES, pH 7.2. Microinjection of a sample without labeled DNA (i.e. a buffer control) is recommended to assess its effect on the cells. Depending on the intended experiment, the DNA concentration in the sample can range from 0.1 to 2 µg/µl; for example, direct detection of labeled DNA (tracking experiments) would require higher concentrations, whereas tracking and expression experiments may be optimal at lower DNA concentrations. The microinjection sample must be centrifuged for 15 minutes at 4°C at a minimum of at least 10,000 x g immediately before loading the needle.
- Fluorescent injection markers, at a concentration of 0.05-0.1%, should be added to the microinjection sample to identify successfully injected cells (i.e. fluorescently labeled dextrans, antibodies, bovine serum albumin).

- After microinjection, replace the culture media and return the cells to the incubator. After the intended incubation time, fix and mount the coverslips as described in Sections 3.3 and 3.4.

#### 4.0 APPLICATION NOTES

##### A. Adjusting the Labeling Level

The labeling protocol in Section 3.1 readily allows detection of the labeled DNA in cultured mammalian cells. If there is a need to increase or decrease the number of labels in the final product, increase or decrease the ratio of labeling reagent to nucleic acid during the labeling reaction. Also, the labeling density can be controlled by adjusting the incubation time; the labeling reaction is linear over the first three hours of incubation at 37°C.

##### B. Maintaining Intact DNA during Labeling

By using the recommended reagent to nucleic acid ratio of 0.25:1 to 1:1 (v:w) during labeling, one can obtain labeled DNA in which the vast majority (>95%) remains intact. Using labeling reagent to DNA ratios significantly above 1:1 (v:w) may result in increased nicking of the DNA strands. To ensure stability of the labeled DNA, store at -20°C.

##### C. Transgene Expression using labeled DNA

The *Label IT*<sup>®</sup> Tracker™ Reagents label intact plasmids (supercoiled or relaxed), linearized plasmid, or large linear DNA segments in a non-destructive manner, thus allowing the visualization of expression-competent DNA during the transfection process. Using the recommended range of *Label IT*<sup>®</sup> Tracker™ Reagent to DNA ratios will provide labeled plasmid DNA in which the expression of the transgene can be monitored simultaneously with plasmid localization. Reporter gene expression (for example, green fluorescent protein, β-galactosidase, luciferase, etc.), under the control of a strong CMV promoter, can be detected as early as 4 hours after transfection. Labeling of plasmid DNA at ratios significantly higher than the recommended range may result in decreased transgene expression.

##### D. *In Vivo* Tracking Experiments

The ability to simultaneously track and monitor transgene expression *in vivo* is another attractive application of this technology; especially when aspects of gene expression can be studied in their proper biological context. Plasmid DNA, labeled with *Label IT*<sup>®</sup> Tracker™ (as described in Section C above) can also be used to monitor transgene expression and plasmid localization after delivery to tissues *in vivo*. Efficient *in vivo* gene delivery can be obtained using Mirus' *TransIT*<sup>®</sup> In Vivo Gene Delivery System. This kit is specifically designed for the efficient delivery of plasmid DNA into laboratory animals via tail vein injection. The plasmid is delivered using a highly efficient, nonviral method. Following gene delivery, the highest levels of transgene expression are found in the liver, with lower levels of expression found in many other organs including the spleen, kidneys, lungs and heart.

#### 5.0 TROUBLESHOOTING

##### 5.1 Labeling Reaction - Poor Efficiency

- **Poor quality DNA**

Use clean, intact DNA in labeling reactions.

- **Labeling reaction was not scaled properly**

Perform reaction in minimal volume, keeping the amount of *Label IT*<sup>®</sup> 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*<sup>®</sup> Tracker™ Reagents tightly capped at -20°C. Protect from exposure to light and moisture.

**NOTE:** The relative density of fluorescent labels on purified, labeled DNA can be assessed by:

1. Agarose gel electrophoresis without ethidium bromide. The DNA will appear faint under UV illumination because the transilluminator emits at approximately 300 nm, which is not optimal for the fluorescent labels (see Table 1).
2. Spectrophotometric absorbance at  $\lambda_{\max}$ . Several  $\mu\text{g}$  of labeled DNA may be required to generate significant  $\lambda_{\max}$  absorbance readings.
3. Fluorescent microscopy. Spot dilutions of labeled DNA onto a glass slide and view with fluorescent microscope.
4. *In Vitro* Tracking. Transfect labeled DNA into a common cell line, such as COS-7, HeLa, or NIH 3T3 to ascertain detection efficiency and optimal parameters.

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### 5.2 Transfection - Low Transfection Efficiency

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- **Poor quality of transfecting DNA (DNA may be partially degraded or an inhibitor, such as an 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 Mirus Bio's MiraCLEAN™ Endotoxin Removal Kit (Product # MIR 5900).
- **Cell density (% confluence) not optimal at time of transfection**  
The recommended cell density for most cell types at the time of transfection is 40-70% confluence. Determine the optimal cell density for each cell type in order to maximize transfection efficiency. Maintain this density in future experiments for reproducibility.
- **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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### 5.3 Transfection - High Cellular Toxicity

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- **Transfection mixture and cells were not mixed thoroughly after adding the complexes**  
Mix thoroughly to evenly distribute the complexes to all cells. Rocking the dish back and forth and from side to side is recommended. Do not swirl or rotate the dish, as this may result in uneven distribution.
- **Excessive amount of transfection reagent/DNA complex mixture was used in transfection**  
Reduce the amount of reagent/DNA complex mixture in the transfection.
- **Cell density was too low at time of transfection**  
Grow cells to a higher cell density and repeat the experiment.

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### 5.4 Tracking - Poor Visualization of Labeled DNA in Cells

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- **Poor quality labeled DNA**  
Use purified, intact DNA in transfections.
- **Low labeling ratio**  
Increase the labeling ratio (volume of Label IT® Tracker™ Reagent to weight of DNA). See Section 3.1.
- **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**  
See Section 5.2.
- **Sub-optimal levels of DNA transfected**  
Use more DNA.
- **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.
- **Improper storage of labeled DNA**  
Labeled DNA must be kept at -20°C.

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### 5.5 Little or No Transgene Expression Observed

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- **Labeling density is too high**  
Label DNA at a lower ratio. See Section 3.1 for recommendations.
- **Reporter protein is not expressed**  
Ensure that reporter protein is being expressed and detected by transfecting an unlabeled plasmid control.
- **Poor transfection efficiency**  
See Low Transfection Efficiency section of Troubleshooting section.
- **Observation time is not optimal**  
Perform time course to determine kinetics of expression.

For specific questions or concerns, please contact Mirus Bio Technical Support at 888.530.0801 (U.S.), 608.441.2852 (international) or techsupport@mirusbio.com  
For a list of citations using Mirus Bio products, please visit the Technical Resources section of our website.  
(www.mirusbio.com)

## 6.0 GENERAL REFERENCES

### **In Vitro References:**

1. Budker, V. et al. (1996) Nature Biotechnology Vol. 14: 760-764.
2. Hagstrom, J. et al. (1996) Biochim. Biophys. Acta 1284: 47-55.
3. Fritz, J. D. et al. (1996) Human Gene Therapy 7: 1395-1404.

### **In Vivo References:**

1. Budker, V. et al. (2000) J. Gene Med. 2: 76-88.
2. Budker, V. et al. (1998) Gene Therapy 5: 272-6.
3. Lui, F. et al. (1999) Gene Therapy 6: 1258-1266.
4. Zhang, G. et al. (1999) Human Gene Therapy 10: 1735-7.
5. Zhang, G. et al. (1997) Human Gene Therapy 8: 1763-72.

## 7.0 RELATED PRODUCTS

### **Plasmid transfection reagents:\***

*TransIT*<sup>®</sup>-LT1 Transfection Reagent (Product # MIR 2300)  
*TransIT*<sup>®</sup>-LT2 Transfection Reagent (Product # MIR 2400)  
*TransIT*<sup>®</sup>-Express Transfection Reagent (Product # MIR 2000)  
*TransIT*<sup>®</sup>-HeLaMONSTER<sup>®</sup> Transfection Kit (Product # MIR 2900)  
*TransIT*<sup>®</sup>-Keratinocyte Transfection Reagent (Product # MIR 2800)  
*TransIT*<sup>®</sup>-CHO Transfection Kit (Product # MIR 2170)  
*TransIT*<sup>®</sup>-3T3 Transfection Kit (Product # MIR 2180)  
*TransIT*<sup>®</sup>-293 Transfection Kit (Product # MIR 2700)  
*TransIT*<sup>®</sup>-COS Transfection Kit (Product # MIR 2190)  
*TransIT*<sup>®</sup>-Insecta Transfection Reagent (Product # MIR 2200)  
*TransIT*<sup>®</sup>-Jurkat Transfection Reagent (Product # MIR 2120)  
*TransIT*<sup>®</sup>-Prostate Transfection Kit (Product # MIR 2130)  
*TransIT*-Neural<sup>®</sup> Transfection Reagent (Product # MIR 2140)  
*TransIT*-TKO<sup>®</sup> siRNA Transfection Reagent (Product # MIR 2150)  
*TransIT*<sup>®</sup>-siQUEST<sup>™</sup> siRNA Transfection Reagent (Product # MIR 2110)  
*TransIT*<sup>®</sup>-Oligo Transfection Reagent (Product # MIR 2160)

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