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Technical Report

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Intracellular Localization and Expression of Labeled Plasmid DNA using *Label* IT® Tracker™ Nucleic Acid Labeling Kits

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Introduction

The transfection of plasmid DNA into mammalian cells has become a routine procedure in cellular and molecular biology experiments, yet the mechanism by which the plasmid DNA reaches its nuclear destination remains poorly understood. To increase our understanding of the transfection process, new and innovative molecular tools are needed that allow for the tracking of DNA as it enters and traverses the intracellular environment. To address this need, Mirus has developed a line of nucleic acid labeling products (***Label* IT® Tracker™ Intracellular Nucleic Acid Localization Kits**) that allow for the covalent attachment of compounds directly onto the DNA backbone via a one-step chemical reaction. These easy to use kits are available in a variety of labels (Cy™3, Cy™5, CX-Rhodamine, TM-Rhodamine, Fluorescein, and Biotin) and are supplied with Mirus' popular ***Trans*IT®-LT1 Transfection Reagent**, which provides maximum transfection efficiency and cellular viability in many mammalian cell lines. These kits represent an ideal tool for the investigation of cytoplasmic delivery and nuclear uptake of exogenous DNA during the transfection process. The *Label* IT® Tracker™ Kits are based on Mirus' proprietary nucleic acid labeling technology which facilitates the alkylation of nucleic acids primarily at the N^7 position of guanine residues. In this report we demonstrate the utility of this straightforward method to directly label and deliver plasmid DNA, of any design, for tracking experiments. Furthermore, due to the non-destructive nature of this labeling methodology (i.e. expression plasmids remain intact following labeling), we demonstrate that subcellular localization and reporter transgene expression can be monitored simultaneously both *in vitro* (cells in culture) and *in vivo* (tissues in mice).

Methods

Labeling Reaction

Plasmid DNA was labeled with the indicated *Label* IT® Tracker™ Reagent at a ratio of 1 µl reagent per 1 µg of DNA and incubated for 1 hour at 37ºC, as described in the *Label* IT® Tracker™ protocol. The sample was purified by ethanol precipitation, and the labeled DNA pellet was resuspended in sterile, molecular-biology grade water and quantified. Labeled DNA was stored, protected from light, at -20°C.

Transfection

The indicated cell line was plated on poly-D-lysine treated glass coverslips, in 6-well plates, such that their confluency would be approximately 60% the following day. At this time, transfection complexes were prepared by adding 3 µl *Trans*IT®-LT1 Transfection Reagent (provided in the kit) per 1 µg labeled plasmid DNA; 2 µg of labeled plasmid DNA were transfected per well. The complex mixture was added dropwise to the cells in their complete media. The cells were incubated at 37°C for the indicated time, and fixed on the coverslips with 4% formaldehyde in PBS for 20 minutes at room temperature. After fixing, the cells were rinsed three times with PBS and mounted for confocal microscope analysis.

Microinjection

HeLa cells were plated onto gridded coverslips and microinjected into either the cytoplasm or the nucleus using an Eppendorf 5246 Transjector and 5171 Micromanipulator. Eighteen hours post-injection, the cells were washed, fixed in formaldehyde, and mounted for microscopic evaluation.

Tail Vein Injection

Systemic delivery of plasmid DNA into adult laboratory mice was performed using Mirus' *Trans*IT®-*In Vivo* Gene Delivery System (as described in the protocol). Briefly, the DNA was complexed with the proprietary polymer and delivered via a high-pressure tail vein injection. At the indicated time post-injection, the animal was euthanized and the targeted organ harvested, cryosectioned and prepared for microscopic evaluation.

Results and Discussion

Labeling of plasmid DNA

The *Label* IT® Tracker™ labeling technology promotes the direct covalent attachment of specific tags to guanine residues of plasmid DNA. Any plasmid construct (linear or circular) can be labeled to facilitate both *in vitro* and *in vivo* tracking experiments. To demonstrate the direct, non-destructive nature of the labeling reaction, plasmid DNA was analyzed by agarose gel electrophoresis before and after labeling and purification. Plasmid DNA expressing a luciferase reporter (pCIluc) was labeled with *Label* IT® Tracker™ Fluorescein and analyzed following electrophoresis on a 1% agarose gel (Figure 1). After attachment to DNA, the fluorescent signal of the fluorescein can be easily detected on a standard transilluminator without staining. As illustrated in Figure 1, the DNA integrity (covalently closed circular form of the plasmid) is not altered by the attachment of the fluorescein tags. The desired density of fluorescein tags on the resultant labeled plasmid DNA can be easily titrated either by adjusting the ratio of *Label* IT® Tracker™ Reagent to DNA or by adjusting the length of the incubation at 37°C. Labeled plasmid DNA, when properly stored at -20°C or colder and protected from light, is stable for at least 1 year (data not shown).

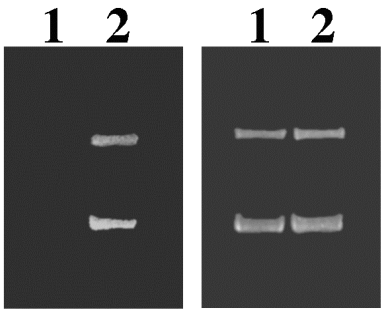

Figure 1. Covalent Labeling of Intact Plasmid DNA
pCIluc DNA was labeled with the *Label* IT® Tracker™ Fluorescein Reagent (as described in the Methods section) and resolved (lane 2), after purification, alongside unlabeled DNA (lane 1), on a 1% agarose gel. The left panel illustrates the fluorescein signal from the labeled DNA in the unstained gel (extended exposure was used to capture the fluorescent signal). The right panel illustrates the same gel after ethidium bromide staining of the DNA.

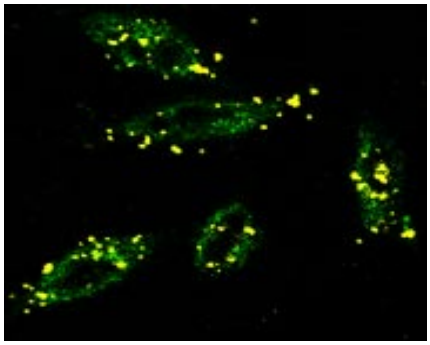

Figure 2. Tracking of Plasmid DNA in HeLa Cells
ATCC HeLa cells were transfected with *Label* IT® Tracker™ Fluorescein labeled pCIluc DNA complexed with *Trans*IT®-LT1 Transfection Reagent. The transfection was performed in complete culture media without a media change. At 24 hours post-transfection, the cells were prepared for microscopic analysis. The image shows green autofluorescence of the cells (acquired to indicate cell location) with punctate yellow fluorescent DNA signal (indicating labeled DNA).

Plasmid DNA Tracking Within Cells

Labeled plasmid DNA can be introduced into mammalian cells in culture by a variety of methods. Transfections using the broad-spectrum histone-H1 based *Trans*IT®-LT1 Transfection Reagent are characterized by low toxicity and high efficiency. As seen in Figure 2, *Label* IT® Fluorescein labeled plasmid DNA, the same preparation demonstrated in Figure 1, can be seen in 100% of cells 24 hours post-transfection. The majority of labeled DNA remains within the cytoplasm of the cell (bright localized yellow signal). Nuclear signal, if detected, is often weak and more diffuse. Nuclear localization of the transgene construct is required for its heterologous expression and appears to be the limiting step in the transfection process.

Plasmid DNA Tracking and Expression

In a similar experiment, a time course was performed using a *Label* IT® Tracker™ Cy™5 labeled vector, which expresses nuclear localizing yellow fluorescent protein (pEYFP-Nuc expression vector) (Figure 3). Reporter transgene expression can be detected as early as 4 hours post-transfection. During the time course, both the amount of intracellular labeled plasmid DNA and percentage of cells expressing YFP increase dramatically. The ability to simultaneously track the localization and expression of the same construct is an important tool for the investigation of the transfection process.

The direct visualization of fluorescently labeled DNA minimizes the number of processing steps required. Multi-color tracking experiments are also possible using spectrally distinct fluorophores. The use of *Label* IT® Tracker™ Biotin labeled plasmid DNA, and its subsequent detection using any of a variety of fluorophore conjugated anti-biotin or avidin-

based reagents can also expand the sophistication of tracking experiments.

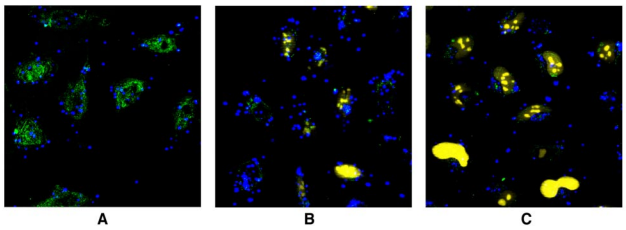

Figure 3. Tracking of Plasmid Localization and Expression
COS-7 cells transfected with *Label* IT® Tracker™ Cy™5 labeled pEYFP-Nuc and *Trans*IT®-LT1 Transfection Reagent in complete media. The green represents the autofluorescence of the cells, the blue represents the Cy™5 fluorescence, and the yellow represents pEYFP-Nuc (Clontech) expression. Images were acquired at 3 (Panel A), 8 (Panel B) and 20 hours (Panel C) post-transfection on a Zeiss LSM 510 Confocal Microscope.

Microinjection Studies

Cellular microinjection of labeled plasmid DNA allows for specific introduction into either the nuclear or cytoplasmic compartment of individual cells. Microinjection is a particularly attractive technique when the intended cells are recalcitrant to standard transfection methodologies or when individual cells need to be monitored. The data shown here (Figure 4) illustrates the tracking of Cy™5 labeled plasmid DNA. The rhodamine-dextran is used as a marker to indicate successful microinjection into the cytoplasmic compartment. Note that the labeled DNA (blue) and dextran (red) do not colocalize throughout the cytoplasm. Time course and tracking/expression experiments can also be performed using microinjection (data not shown).

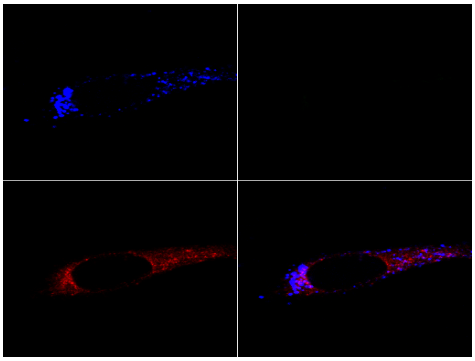

Figure 4. Cytoplasmic Microinjection of Labeled Plasmid DNA
A mixture containing 0.05 mg/ml *Label* IT® Cy™5-labeled plasmid DNA and 0.4 mg/ml Lissamine™ rhodamine labeled dextran (500 kDa size) was microinjected into the cytoplasm of cultured HeLa cells. The blue color represents cytoplasmic localization of the DNA, and the red color represents LRh-dextran. In this study, both DNA and dextran are excluded from the nucleus.

In Vivo Tracking Studies

Plasmid DNA was labeled with *Label* IT® Tracker™ Cy™3 and injected into the tail vein of mice using Mirus' *Trans*IT®-*In Vivo* Gene Delivery System (as described in the protocol). As demonstrated in Figure 5, fluorescently labeled plasmid DNA can be readily detected in hepatocytes of an injected animal within 2 hours after the injection process.

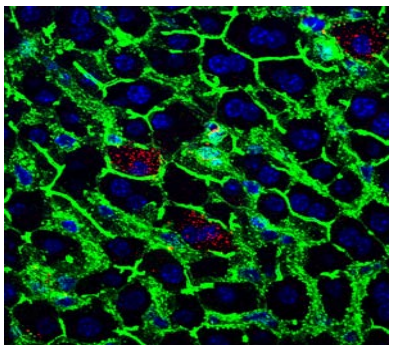

Figure 5. *In Vivo* Tracking of Plasmid DNA
Plasmid DNA (pCIluc) was labeled using the *Label* IT® Tracker™ Cy™3 Reagent. Post purification, 20 µg Cy™3 labeled DNA was complexed with the *Trans*IT®-*In Vivo* Polymer Solution (according to the *Trans*IT®-*In Vivo* Gene Delivery System protocol) and delivered into ICR (CD-1) mice (Harlan) via high pressure tail vein injection. Two hours post injection, the livers were harvested, cryosectioned, and stained (according to the manufacturer's specifications) using ToPro-3® (blue nuclear stain) (Molecular Probes) and Alexa-488 conjugated anti-F-actin antibody (green stain) (Molecular Probes) to visualize the tissue architecture. Confocal microscopy using a Zeiss LSM 510 generated compiled z-stack images. A large amount of labeled plasmid DNA is detected as distinct pink punctate staining in a small proportion of the binuclear hepatocytes with less signal detected in the larger subset of cells.

Summary

Label IT® Tracker™ Intracellular Nucleic Acid Localization Kits facilitate the direct covalent attachment of labels to intact plasmids (supercoiled or relaxed) or large linear DNA without appreciable degradation or change in conformational integrity; furthermore, the 'expression-competence' of the construct is generally maintained. The ability to custom label and transfect any plasmid construct allows for the systematic investigation of exogenous DNA biology within both *in vitro* and *in vivo* systems in a convenient and reproducible manner.

General References: *In Vivo* Delivery

1. V. Budker, T. Budker, G. Zhang, V. Subbotin, A. Loomis, and J.A. Wolff. 2000. Hypothesis: naked plasmid DNA is taken up by *cells in vivo* by a receptor-mediated process. *J. Gene Med.* 2(2): 76-88.
2. G. Zhang, V. Budker, and J.A. Wolff. 1999. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum. Gene Ther.* 10(10):1735-1737.
3. F. Liu, Y. Song, and D. Liu. 1999. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* 6(7): 1258-1266.

Product Protocols

- *Label* IT® Tracker™ Intracellular Nucleic Acid Localization Kits (Protocol #ML018)
- *Trans*IT®-*In Vivo* Gene Delivery System (Protocol #ML005)

Technical Reports

- *Trans*IT-TKO® Short Interfering RNA (siRNA) Transfection Reagent (Technical Report #MTR001)
- Nucleic Acid Labeling and Delivery Tools for Neurobiology (Technical Report #MTR003)

Mirus Product References are available at www.genetransfer.com.

Ordering Information

Label IT® Tracker™ Intracellular Nucleic Acid Localization Kits*

Product Name	Quantity	Cat. No.
Label IT® Tracker™ Cy™3	*	MIR 7010
Label IT® Tracker™ Cy™5	*	MIR 7011
Label IT® Tracker™ CX-Rhodamine	*	MIR 7012
Label IT® Tracker™ TM-Rhodamine	*	MIR 7013
Label IT® Tracker™ Fluorescein	*	MIR 7015
Label IT® Tracker™ Biotin	*	MIR 7014

*Each kit contains *Label* IT® Tracker™ Reagent, Tracker Reconstitution Solution, 10X Labeling Buffer A , and *Trans*IT®-LT1 Transfection Reagent. Each kit provides sufficient reagents to label 50 to 200 µg of plasmid DNA and perform at least 50 transfections (1 µg/well) in 35 mm wells.

*Trans*IT®-*In Vivo* Gene Delivery System

Product Name	Quantity	Cat. No.
*Trans*IT®-*In Vivo* Trial Size Kit	25 injections	MIR 5125
*Trans*IT®-*In Vivo* Full Size Kit	100 injections	MIR 5100

Each kit contains sufficient Polymer Solution and Delivery Solution to perform 25 (Trial Size Kit) or 100 (Full Size Kit) injections in 30 g mice.

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