

Molecular Probes™
invitrogen detection technologies

**TC-FIAsh™ TC-ReAsH™ II In-
Cell Tetracysteine Tag Detection
Kits**

Revised 07-March-2011
MP 34561

Table of Contents

Kit Contents and Storage.....	v
Product Qualification	v
Introduction	1
Overview.....	1
Tetracycline Tag Detection Technology	2
Working with Arsenic Compounds	5
Methods	6
General Guidelines for Labeling.....	6
General Guidelines for Dual Labeling	9
General Guidelines for Detecting TC-Tag Fusion Proteins	10
Labeling TC-Tag Fusion Proteins	11
Using BAL Wash Buffer.....	16
Detecting TC-Tag Fusion Proteins.....	17
Appendix.....	20
Troubleshooting.....	20
Contact Information	22
Purchaser Notification.....	23
References	25

Kit Contents and Storage

Kit Components

The TC-FIAsH™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits contain the components listed below.

Kit	Component Concentration	Volume
TC-FIAsH™ II In-Cell Tetracysteine Tag Detection Kit (T34561)		
FIAsH-EDT ₂ labeling reagent	2 mM in DMSO	40 µL
BAL wash buffer	100X in ddH ₂ O (25 mM)	1.2 mL
TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit (T34562)		
ReAsH-EDT ₂ labeling reagent	2 mM in DMSO	40 µL
BAL wash buffer	100X in ddH ₂ O (25 mM)	1.2 mL

Molecular Weights of the Labeling Reagents

The table below lists the molecular weights and micrograms supplied for each labeling reagent.

Reagent	Molecular Weight	µg Supplied
FIAsH-EDT ₂ labeling reagent	664.50 g/mol	53.2
ReAsH-EDT ₂ labeling reagent	545.38 g/mol	43.6

Storage

Upon receipt, store the components of the TC-FIAsH™ and TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit as detailed below.

Component	Storage
FIAsH-EDT ₂ labeling reagent	≤-20°C, protected from light
ReAsH-EDT ₂ labeling reagent	≤-20°C, protected from light
BAL wash buffer	2-6°C, air sensitive

Product Qualification

FIAsH-EDT₂ and ReAsH-EDT₂ Labeling Reagents

The FIAsH-EDT₂ and ReAsH-EDT₂ labeling reagents are qualified as follows:

Purity: Determined by HPLC

Mass: Determined by mass spectroscopy

Functionality: To ensure performance, the FIAsH-EDT₂ and ReAsH-EDT₂ reagents are functionally tested using GripTite™ 293 MSR cells transfected with the pcDNA™6.2/nTC-Tag-p64 vector and using the protocols included in this manual.

BAL Wash Buffer

To ensure performance, BAL wash buffer is functionally tested using GripTite™ 293 MSR cells transfected with the pcDNA™6.2/nTC-Tag-p64 vector and using the protocols included in this manual.

Ordering Information

Ordering information for the TC-FIAsHTM and TC-ReAsHTM II In-Cell Tetracysteine Tag Detection Kits is provided below.

Product	Amount	Catalog no.
TC-FIAsH TM TC-ReAsH TM II In-Cell Tetracysteine Tag Detection Kit (with Mammalian Gateway [®] expression vectors)	1 kit	T34563
TC-FIAsH TM II In-Cell Tetracysteine Tag Detection Kit	1 kit	T34561
TC-ReAsH TM II In-Cell Tetracysteine Tag Detection Kit	1 kit	T34562

Introduction

Overview

Introduction

The TC-FIAsh™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits use the FIAsh ReAsH technology to facilitate site-specific fluorescent labeling of proteins in live mammalian cells. Use of the FIAsh ReAsH technology provides a sensitive method for *in vivo* detection and subcellular localization of proteins fused to the TC-Tag using fluorescence microscopy.

Advantages of the Labeling System

Using the FIAsh ReAsH technology and the TC-FIAsh™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits for fluorescent labeling of recombinant proteins provides the following advantages:

- Small size of the TC-Tag (6 amino acids, 585 Da) is less likely to interfere with the structure or biological activity of the protein of interest
 - FIAsh-EDT₂ and ReAsH-EDT₂ labeling reagents are membrane-permeable and readily cross the cell membrane, allowing labeling and detection of recombinant proteins in live mammalian cells
 - FIAsh-EDT₂ and ReAsH-EDT₂ labeling reagents bind the TC-Tag with high specificity and high affinity (nanomolar or lower dissociation constant), allowing targeted labeling of the protein of interest⁴
 - FIAsh-EDT₂ and ReAsH-EDT₂ labeling reagents become strongly fluorescent (green and red, respectively) only upon binding the TC-Tag, allowing specific detection of TC-tagged proteins
 - FIAsh-EDT₂ and ReAsH-EDT₂ labeling reagents can be applied sequentially on the same sample, allowing temporal detection of protein turnover and trafficking¹
 - ReAsH-EDT₂ labeling reagent can be used for both fluorescence-based microscopy and electron microscopy²
 - FIAsh-EDT₂ labeling reagent provides a superior alternative to yellow-fluorescent protein (YFP) when coupled with cyan-fluorescent protein (CFP) for FRET-based cellular analysis²
 - BAL wash buffer greatly increases the signal to noise ratio compared to Disperse Blue 3, the wash buffer supplied with previous TC-FIAsh™ TC-ReAsH™ products
 - BAL wash buffer is much more olfactorily friendly than EDT, another wash buffer used for background reduction
-

Tetracysteine Tag Detection Technology

Introduction

The TC-FIAsh™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit uses biarsenical labeling reagents to bind and detect proteins containing a tetracysteine motif (i.e. TC-Tag).³ The biarsenical labeling reagents are nonfluorescent until they bind the tetracysteine motif at which time they become highly fluorescent.

Components of the System

The TC-FIAsh™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits consist of two major components:

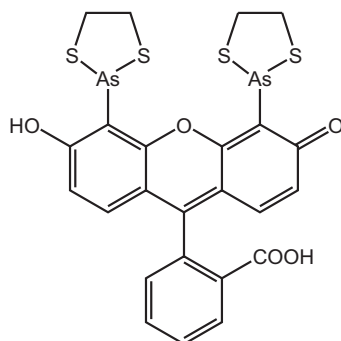
- The tetracysteine TC-Tag (Cys-Cys-Pro-Gly-Cys-Cys) in the pcDNA™6.2/TC-Tag-DEST vector. When fused to a gene of interest, the TC-Tag allows the expressed fusion protein to be specifically recognized by a biarsenical labeling reagent. For more information on the tetracysteine motif, see below.
 - A biarsenical labeling reagent, FIAsh-EDT₂ or ReAsH-EDT₂, which becomes fluorescent upon binding to recombinant proteins containing the TC-Tag. The FIAsh-EDT₂ or ReAsH-EDT₂ labeling reagents are supplied pre-complexed to the dithiol EDT (1,2-ethanedithiol) which stabilizes and solubilizes the biarsenic reagents. For information on how the FIAsh-EDT₂ or ReAsH-EDT₂ labeling reagents bind the TC-Tag and become fluorescent, see the next page.
-

Tetracysteine Motif

Both the FIAsh-EDT₂ and ReAsH-EDT₂ labeling reagents bind a tetracysteine motif consisting of Cys-Cys-Xaa-Xaa-Cys-Cys where Cys equals cysteine and Xaa equals any amino acid other than cysteine. This motif is rarely seen in naturally occurring proteins allowing specific fluorescent labeling of recombinant proteins fused to the TC-Tag. In the TC-FIAsh™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits, the optimized Cys-Cys-Pro-Gly-Cys-Cys tetracysteine motif is used as this motif has been shown to have a higher affinity for and more rapid binding to biarsenic compounds as well as enhanced stability compared to other characterized motifs.⁴

FIAsh-EDT₂ Labeling Reagent

The FIAsh-EDT₂ labeling reagent is based on the FIAsh reagent and is a nonfluorescent, biarsenical derivative of fluorescein.³ FIAsh-EDT₂ reagent is supplied pre-complexed to EDT, is membrane-permeable, and readily enters the cell. See the figure below for the structure of FIAsh-EDT₂ labeling reagent.



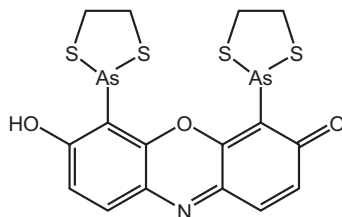
Formula: C₂₄H₁₈As₂O₅S₄
Molecular Weight: 664.50

continued on next page

Tetracysteine Tag Technology, continued

ReAsH-EDT₂ Labeling Reagent

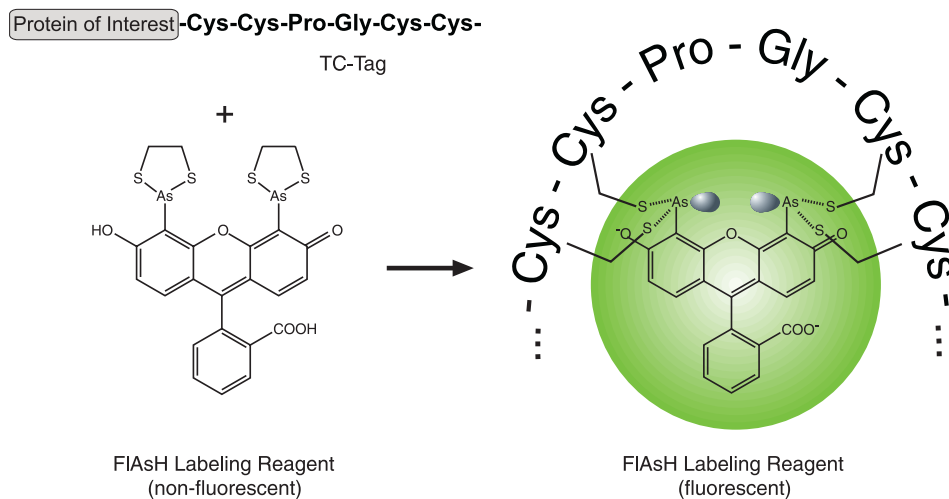
The ReAsH-EDT₂ labeling reagent is based on the ReAsH reagent and is a nonfluorescent, biarsenical derivative of the red fluorophore resorufin.² ReAsH-EDT₂ reagent is supplied pre-complexed to EDT, is membrane-permeable, and readily enters the cell. See the figure below for the structure of ReAsH-EDT₂ reagent.



Formula: C₁₆H₁₃As₂NO₃S₄
Molecular Weight: 545.38

How the FIAsH-EDT₂ and ReAsH-EDT₂ Labeling Reagents Bind the TC-Tag

Both the FIAsH-EDT₂ and ReAsH-EDT₂ labeling reagents bind the TC-Tag through four covalent bond formations—the two arsenic groups of the FIAsH-EDT₂ and ReAsH-EDT₂ reagents each bind two thiols in the TC-Tag tetracysteine sequence (see diagram below). Upon binding, the FIAsH-EDT₂ and ReAsH-EDT₂ reagents are converted to a highly fluorescent state that can be detected at the appropriate emissions peaks (see next page).

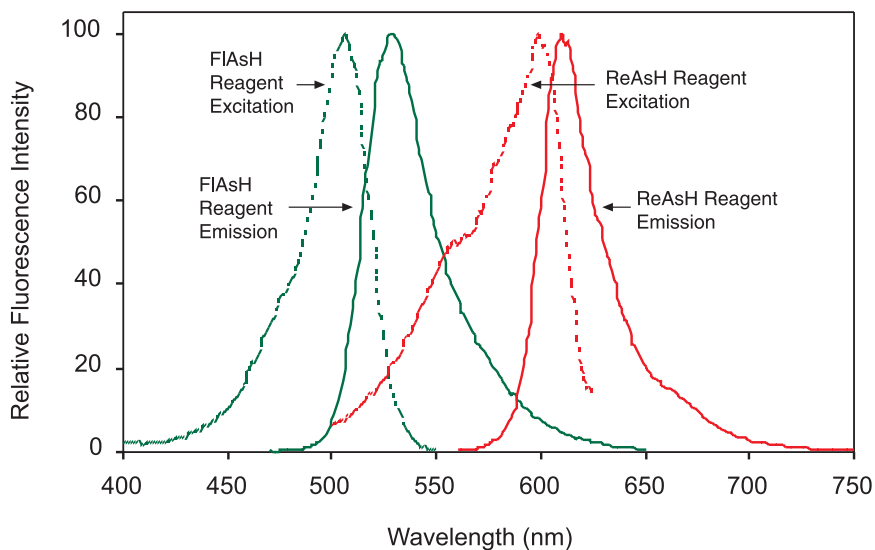


continued on next page

Tetracysteine Tag Technology, continued

Fluorescence Spectra for the FIAsh and ReAsH Reagents

Once the FIAsh-EDT₂ and ReAsH-EDT₂ labeling reagents bind the TC-Tag fused to your protein, the reagent will emit a green (FIAsh) or red (ReAsH) fluorescent signal when excited at the appropriate wavelength. The fluorescent excitation and emission spectra for the FIAsh and ReAsH reagents are provided below.



Labeling Reagent	Excitation Maximum	Emission Maximum
FIAsh reagent	508 nm	528 nm
ReAsH reagent	593 nm	608 nm

Working with Arsenic Compounds

Introduction

The FIAsH-EDT₂ and ReAsH-EDT₂ labeling reagents are biarsenical compounds and should be handled with care. See below for general guidelines.

Caution: Exercise caution when handling the FIAsH-EDT₂ and ReAsH-EDT₂ labeling reagents. We recommend the following guidelines:

- Review the Material Safety Data Sheet before handling.
 - Wear protective clothing, eyewear, and gloves suitable for use with dimethyl sulfoxide (e.g. nitrile gloves) when handling the FIAsH-EDT₂ and ReAsH-EDT₂ labeling reagents
 - Discard all excess reagents that contain or have come in contact with arsenic according to your institution's guidelines and all applicable local, state, and federal requirements.
-

Dermal Toxicity Evaluation

A dermal toxicity evaluation of the FIAsH-EDT₂ and ReAsH-EDT₂ labeling reagents was independently performed by MB Research Laboratories, Spinnerstown, PA, USA by applying a full vial of material to the mouse skin. In this study, no adverse reaction or toxicity was noted. Although arsenic compounds are toxic, this product contains <0.2% of an organic arsenic compound that shows no toxicity at a maximum dose level likely to be handled. The toxicology of this material, however, has not been fully investigated. Handle according to your chemical hygiene plan and prevent contact with this material.

Accidental Spills and Accidental Contact

Treat accidental spills of the FIAsH-EDT₂ and ReAsH-EDT₂ labeling reagents on surfaces with 10% bleach for 10 minutes and then carefully clean up. Discard arsenic-containing waste according to your institution's guidelines.

Treat accidental contact of the FIAsH-EDT₂ and ReAsH-EDT₂ labeling reagents with human skin by washing excess reagent off with soap and water as soon as possible. Do not treat arsenic skin exposure with EDT (1,2-ethanedithiol) since EDT may promote uptake of arsenic reagents into the body. Consult a physician following contact.

Methods

General Guidelines for Labeling

Introduction

The TC-FIAsh™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits facilitate site-specific labeling and detection of recombinant proteins in live mammalian cells. Using the kit allows you to monitor cellular protein expression under real-time, physiological conditions. Once recombinant proteins are labeled and detected using fluorescence microscopy, cells may be cultured further for use in additional assays or other downstream applications.

Factors to Consider When Choosing a Labeling Reagent

Molecular Probes offers the TC-FIAsh™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits for fluorescent labeling of proteins fused to the TC-Tag. Because we have noticed transient phenotypic effects using the ReAsH-EDT₂ labeling reagent (see below) and because the ReAsH-EDT₂ reagent is sensitive to photo-bleaching when exposed to continuous illumination, we generally recommend using the FIAsh-EDT₂ labeling reagent to label your recombinant protein.

The TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit offers an alternative labeling strategy that allows you to perform additional applications. We recommend using the TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit for the following conditions:

- You wish to perform pulse label experiments on the same TC-Tagged protein using both the FIAsh-EDT₂ and ReAsH-EDT₂ labeling reagents to monitor protein synthesis over time
 - You are already using a green fluorescent reporter (e.g. GFP, FITC-conjugated antibody) in your assays and require a second labeling reagent
 - You wish to detect labeled proteins by electron microscopy (see page 10 for more information)
-

Phenotypic Effects With ReAsH-EDT₂ Reagent

We have noticed changes in cell morphology approximately 24 hours after labeling proteins with ReAsH-EDT₂ reagent in HEK293 and COS-7 cell lines. Cells appear to round up and the general shape and pattern of the cells appear disrupted. This phenotypic effect is transient as cells recover 48 hours after labeling and is thought to be due to the generation of singlet oxygens when the ReAsH-EDT₂ reagent is exposed to high intensity light.⁴ We have not observed this effect on cells labeled with the FIAsh-EDT₂ labeling reagent.

If you are using the ReAsH-EDT₂ reagent to label your protein, you may observe similar morphological changes in your cells depending on your cell line, expressed recombinant protein, and application. Note that these phenotypic effects may transiently influence any downstream assays you wish to perform.

continued on next page

General Guidelines for Labeling, continued

TC-Tag Vectors

Before you use one of the TC-FIAsh™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits to label and detect your protein of interest *in vivo*, you must clone your gene of interest into a Gateway® entry vector to create an entry clone, generate an expression clone by performing an LR recombination reaction between the entry clone and a destination vector (e.g. pcDNA™6.2/cTC-Tag-DEST or pcDNA™6.2/nTC-Tag-DEST), and then express the protein of interest by transforming the appropriate host cell culture. The TC-FIAsh™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit with Mammalian Gateway® Expression Vectors (T35358) contains red- and green-fluorescent reagents needed for TC-Tag detection and the Gateway® expression vectors for N- or C-terminal tagging of the gene of interest. For more information about these vectors, see the instruction manual for T35358, visit our website (<http://probes.invitrogen.com/>), or contact Technical Services.

Expressing Your Gene of Interest with the TC-Tag

Once you have generated an expression construct containing your gene fused to the TC-Tag, you may:

- Transfect your construct into the cell line of choice for transient or stable expression of the gene of interest (Mammalian Gateway® Expression Vectors, provided in The TC-FIAsh™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit with Mammalian Gateway® Expression Vectors (T35358)),
- or
- Deliver and express your construct in either dividing or non-dividing mammalian cells via a replication-incompetent, HIV-1-based lentivirus (ViraPower™ II Lentiviral Lumio™ Gateway® Vectors)

NOTE: The TC-FIAsh™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit works best for labeling proteins that are expressed at high levels or are concentrated in a subcellular region. If you are expressing proteins at low levels (e.g. from a weak promoter) or if you are expressing a cytoplasmic protein, we recommend conducting initial labeling and detection studies using the TC-FIAsh™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits in transiently transfected cells where protein expression levels are higher. Once labeling conditions are optimized, you may analyze your protein in stable cell lines.

Labeling Cells

- We recommend designing your experiment so that cells will be at optimal density at the time of labeling. Suspension cells typically label most efficiently at a density of $1-2 \times 10^6$ cells/ml. Adherent cells label most efficiently when they are 60–90% confluent at the time of labeling.
 - For cells transduced with lentivirus, it may be useful to culture the cells in Opti-MEM® Reduced Serum Media or another reduced-serum media overnight (~16–18 hours) before labeling, to reduce the background from serum.
-

Controls

- For transfection experiments, we recommend transfecting cells with a positive expression control and control vector that does not encode the TC-Tag, to control for fluorescent artifacts or alterations in cell morphology that could affect the labeling and detection of your protein.
 - For lentivirus transduction experiments, we recommend using a positive expression control and a negative transduction control. To prepare the negative control, follow the transduction procedure in the ViraPower™ Lentiviral Expression System manual, except omit the virus. Then use the “mock-transduced” cells in the labeling procedure to determine the level of background from the FIAsh-EDT₂ or ReAsH-EDT₂ reagent.
-

continued on next page

General Guidelines for Labeling, continued

Factors Affecting Protein Labeling

A number of factors can influence the degree of protein labeling and, consequently, the success of your detection experiment. These factors include:

- Composition of labeling media
- Concentration of FIAsh-EDT₂ or ReAsH-EDT₂ reagent used to label proteins
- Length of incubation with FIAsh-EDT₂ or ReAsH-EDT₂ reagent

Each of these factors is discussed further in this section.

Labeling Media

Because the FIAsh-EDT₂ and ReAsH-EDT₂ reagents will nonspecifically bind to serum proteins, including bovine serum albumin (BSA), we recommend using labeling media that is serum-free or contains low amounts of serum (~1–2% serum). Opti-MEM[®] Reduced-Serum Medium (1X) is available separately from Invitrogen. Other buffered solutions, including Hank's Balanced Salt Solution (HBSS) and HEPES Buffer Saline (HBS), are suitable. If you are culturing adherent cells, make sure the labeling media contains calcium and magnesium to promote cell attachment.

Concentration of Reagent

For transfected cells, we recommend labeling in medium containing 2.5 μ M FIAsh-EDT₂ or ReAsH-EDT₂ labeling reagent as a starting point. For cells transduced with lentivirus, a FIAsh-EDT₂ or ReAsH-EDT₂ labeling reagent concentration of 1.25 μ M may be optimal. Depending on the levels of specific and background fluorescent signal, you can optimize the FIAsh-EDT₂ or ReAsH-EDT₂ labeling reagent concentration to better visualize your labeled protein. We recommend trying a concentration range of 1 μ M to 10 μ M FIAsh-EDT₂ or ReAsH-EDT₂ labeling reagent.

Labeling Time

As a starting point, we recommend labeling cells for 30–60 minutes. Generally, fluorescent signal is detectable 15 minutes after labeling and increases steadily for about 90 minutes. We generally do not see any increase in the intensity of the fluorescent signal after 90 minutes. Depending on the stability of your protein, fluorescent signal may be visible up to 48 hours after labeling.

If you are labeling proteins with the FIAsh-EDT₂ or ReAsH-EDT₂ labeling reagent for the first time, we recommend optimizing the labeling time for your protein and cell line. You can accomplish this by visualizing protein labeling every 15 minutes for up to 90 minutes using a fluorescence microscope (see **Detecting TC-Tag Fusion Proteins**, page 17). As fluorescent signal from the bound FIAsh-EDT₂ or ReAsH-EDT₂ labeling reagent increases, nonspecific background fluorescence will also increase. The BAL wash buffer provided in the kit reduces this background allowing you to determine the optimal signal-to-noise ratio.

General Guidelines for Dual Labeling

Introduction

The TC-FIAsh™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit allows successive labeling of the protein of interest in living cells in order to distinguish between older and newly made protein molecules. Using such pulse-chase assays to temporally distinguish pools of protein aid in the study of protein assembly, protein internalization, and protein turnover.² In addition to the guidelines provided in the previous section, refer to the following guidelines when using the FIAsh-EDT₂ and ReAsH-EDT₂ labeling reagents for dual labeling assays.

Note: Both the FIAsh-EDT₂ and ReAsH-EDT₂ labeling reagents bind the same tetracysteine motif (i.e. TC-Tag) and, therefore, can only be used to label one recombinant protein at any given time. **Do not use the FIAsh-EDT₂ and ReAsH-EDT₂ labeling reagents to separately label two different proteins.** If you wish to detect two proteins in the same cell, you will need to use two separate reporter systems.

Example: To detect two different proteins in the same cell, express one protein fused to the TC-Tag and label with ReAsH-EDT₂ labeling reagent. Express a second protein fused to GFP. Detect both proteins using fluorescence microscopy and the appropriate filter sets.

Which Reagent to Use First

To dual label your protein of interest, you will “pulse-label” your protein with the FIAsh-EDT₂ reagent followed by a “chase-label” with the ReAsH-EDT₂ reagent. You will follow the same labeling procedure for both labeling reagents (see pages 13-14). Because the ReAsH-EDT₂ reagent can cause transient phenotypic effects on cells (see page 6), we generally recommend using FIAsh-EDT₂ as the first labeling reagent.

When to Use the Second Reagent

After the first labeling procedure with FIAsh-EDT₂ is performed, you may do any of the following:

- Wash the cells two times with the BAL wash buffer per instructions for FIAsh-EDT₂ labeling protocol, followed by ReAsH-EDT₂ labeling
 - Incubate cells for desired length of time (up to 48 hours depending on the stability of your protein) before labeling with ReAsH-EDT₂
 - Visualize protein labeled with FIAsh-EDT₂ under a fluorescence microscope before labeling with ReAsH-EDT₂
-

General Guidelines for Detecting TC-Tag Fusion Proteins

Introduction

We recommend using fluorescence microscopy to detect and localize fluorescence-labeled proteins. Guidelines for performing fluorescence microscopy are provided in the section entitled **Detecting TC-Tag Fusion Proteins** (see page 17). You may also detect fluorescent signal using fluorescence polarization or, if your protein is labeled with ReAsH-EDT₂ reagent, electron microscopy (see below).

Although the TC-FIAsH™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits are primarily designed for protein detection and localization using fluorescence microscopy, it is possible to quantitatively analyze fluorescent signal using a fluorescence plate reader or perform fluorescence-activated cell sorting (FACS) using a flow cytometer. However, depending on your protein expression levels and your application, background noise may be too high to accurately obtain and quantify fluorescent signal readings using these methods.

If you will be analyzing fluorescent signal using a fluorescence plate reader or a flow cytometer, consider the following:

- Make sure the instrument is equipped with the proper optical filters to detect the FIAsH-EDT₂ and ReAsH-EDT₂ fluorescent signals.
 - Include the proper negative controls in your experiment to determine background fluorescence levels.
-

Fluorescence Polarization

Fluorescence polarization assays provide information regarding molecular orientation and mobility and are used to study multiple processes including receptor-ligand interactions, protein degradation, and membrane fluidity. In contrast to conventional fluorescent dyes which attach through rotationally mobile single bonds, the FIAsH-EDT₂ and ReAsH-EDT₂ reagents bind the TC-Tag through four rigid covalent bond formations, making the TC-FIAsH™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits ideal for fluorescence polarization assays.⁴ For information on fluorescence polarization products available from Molecular Probes, visit our website (probes.invitrogen.com) or contact Technical Service.

Electron Microscopy

If you label your protein with the ReAsH-EDT₂ reagent, you may detect the labeled protein using electron microscopy. The ReAsH-EDT₂ labeling reagent photoconverts diaminobenzidine into a highly localized precipitate that can be stained and detected under an electron microscope.^{2,4} The FIAsH-EDT₂ reagent does not catalyze this photoconversion process and, thus, is not compatible with electron microscopy.

Labeling TC-Tag Fusion Proteins

Introduction

To label recombinant proteins fused to the TC-Tag, you will need to incubate your cells with the FIAsh-EDT₂ or ReAsH-EDT₂ labeling reagent. Guidelines and instructions are provided in this section to label your recombinant protein. We recommend waiting a minimum of 24 hours post-transfection or 48 hours post-transduction before labeling to ensure adequate expression of your protein.

Important: If you plan to culture cells further after labeling with FIAsh-EDT₂ and ReAsH-EDT₂ reagents, be sure to maintain sterility throughout the experiment.

- Perform all manipulations within a tissue-culture hood
 - Prepare solutions using sterile reagents
-

Previously published articles regarding FIAsh-EDT₂ or ReAsH-EDT₂ labeling of proteins required addition of 1,2-ethanedithiol (EDT) during the labeling procedure and during subsequent washing steps to reduce nonspecific binding.³⁻⁵ Due to the odor and toxicity of EDT, however, the labeling procedures provided in this section have been specifically developed to **not** require addition of EDT.

The BAL wash buffer provided in the kit minimizes nonspecific binding without the odor associated with EDT.

Thawing and Aliquoting Reagent

We recommend aliquoting the FIAsh-EDT₂ and ReAsH-EDT₂ reagents to minimize freeze/thaw cycles. Let the frozen FIAsh-EDT₂ or ReAsH-EDT₂ reagent warm to room temperature (protected from light), and aliquot 5–10 µl into multiple tubes.

When using individual aliquots, warm to room temperature (protected from light) and remove the desired amount of reagent. Immediately recap the tube to reduce moisture uptake. Store aliquots at ≤−20°C, protected from light.

Note: Both the FIAsh-EDT₂ and ReAsH-EDT₂ reagents may change color during storage due to changes in pH. This color change is normal and does not affect the performance of the reagent.

continued on next page

Labeling TC-Tag Fusion Proteins, continued

Recommended Labeling Conditions

Use the following recommended conditions to label your protein with the FIAsh-EDT₂ and ReAsH-EDT₂ reagents. For more information, see the section entitled **General Guidelines for Labeling**, page 6.

Condition	Recommendation
Tissue culture format	<ul style="list-style-type: none"> You may plate cells in any size tissue culture plate of choice (e.g. 6-well format). Make sure that your tissue culture plate is compatible with your detection instrument.
Cell density	For optimal labeling efficiency: <ul style="list-style-type: none"> Plate adherent cells such that they will be 60–90% confluent at the time of labeling. Label suspension cells at a density of $1-2 \times 10^6$ cells/ml.
Labeling Media	For optimal efficiency, label cells in Opti-MEM [®] Reduced-Serum Medium, HBSS, or HBS. Note: If you are culturing adherent cells, make sure the labeling media contains calcium and magnesium to promote cell attachment.
Concentration of FIAsh-EDT ₂ and ReAsH-EDT ₂ reagents	<ul style="list-style-type: none"> For transfected cells, incubate in 2.5 μM FIAsh-EDT₂ or ReAsH-EDT₂ reagent. For cells transduced with lentivirus, incubate in 1.25 μM FIAsh-EDT₂ or ReAsH-EDT₂ reagent. To optimize fluorescent signal, vary the FIAsh-EDT ₂ or ReAsH-EDT ₂ reagent concentration from 1 μM to 10 μM.
Labeling time	For most applications, label proteins for 30–60 minutes.

Materials Needed

Be sure to have the following materials on hand before beginning:

- FIAsh-EDT₂ or ReAsH-EDT₂ labeling reagent (supplied with the TC-FIAsh™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit; warm to room temperature, protected from light)
- Opti-MEM[®] Reduced-Serum Medium (Catalog no. 31985-062)
- HBSS, with calcium and magnesium (for lentivirus-transduced cells) (Catalog no. 10425-092)
- BAL wash buffer (supplied), warmed to room temperature
- Mammalian cell line of interest expressing TC-Tagged recombinant protein (plated in the tissue culture format of choice or in suspension, as appropriate)

continued on next page

Labeling TC-Tag Fusion Proteins, continued

Preparing the Labeling Solution

The FIAsh-EDT₂ and ReAsH-EDT₂ reagents are provided as an 800X solution. Follow the guidelines below to make a 1X labeling solution:

- **For transfected cells**, add the appropriate amount of FIAsh-EDT₂ or ReAsH-EDT₂ reagent to Opti-MEM[®] Medium to make a 1X labeling solution and vortex to mix (1X labeling solution is 2.5 μM FIAsh-EDT₂ or ReAsH-EDT₂ reagent). Make just enough 1X labeling solution for your immediate needs.
- **For lentivirus-transduced cells**, add the appropriate amount of FIAsh-EDT₂ or ReAsH-EDT₂ reagent to HBSS to make a 0.5X labeling solution and vortex to mix (0.5X labeling solution is 1.25 μM FIAsh-EDT₂ or ReAsH-EDT₂ reagent). Make just enough 0.5X labeling solution for your immediate needs.
- Make the labeling solution just before use and keep at room temperature until required.
- Refer to the table below for recommended amounts of labeling solution to use for various tissue culture formats.

Culture Vessel	Labeling Solution per Well
6-well	1 ml
12-well	500 μl
24-well	250 μl
48-well	100 μl
96-well	50 μl

Protocol for Transfected Adherent Cells

Follow this protocol to label transfected adherent cells with FIAsh-EDT₂ or ReAsH-EDT₂ labeling solution. You may plate your cells in any tissue culture format of choice. We recommend that your cells are 60–90% confluent at the time of labeling for optimal results. We also recommend including a positive expression control and a non-TC-Tag vector control in your experiment to determine foreground and background fluorescence (see page 7).

1. Remove the growth medium from the cells and wash cells once with Opti-MEM[®] I Reduced-Serum Medium.
2. Add the appropriate amount of 1X FIAsh-EDT₂ or ReAsH-EDT₂ labeling solution prepared with Opti-MEM[®] Medium to each well (see **Preparing the Labeling Solution**, above).

Important: Appropriately discard any unused FIAsh-EDT₂ or ReAsH-EDT₂ labeling solution according to your institution's guidelines. Do not reuse the 1X FIAsh-EDT₂ or ReAsH-EDT₂ labeling solution.

3. Cover the plate to prevent the solution from evaporating.
4. Incubate the cells at room temperature for 30 minutes, protected from light.
Note: Extending the incubation time may increase the fluorescent signal, but may also increase the background.
5. If you are ready to detect your labeled protein using fluorescence microscopy, proceed to **Using BAL Wash Buffer**, page 16. If you are performing dual labeling and wish to label your protein with the second labeling reagent **before** visualizing cells, proceed to **Performing Dual Labeling**.

continued on next page

Labeling TC-Tag Fusion Proteins, continued

Protocol for Transfected Cells in Suspension

Follow this protocol to label transfected cells in suspension with FIAsh-EDT₂ or ReAsH-EDT₂ labeling solution. We recommend including a positive expression control and a non-TC-Tag vector control in your experiment to determine foreground and background fluorescence (see page 7).

1. For each sample, pellet cells by centrifugation and wash the cell pellet once with Opti-MEM[®] I Reduced-Serum Medium.
2. Resuspend the pellet with 1X FIAsh-EDT₂ or ReAsH-EDT₂ labeling solution prepared with Opti-MEM[®] Medium (see **Preparing the Labeling Solution**, previous page) to a final density of 1×10^6 cells/ml. Transfer the cells to your tissue culture format of choice.

Important: Appropriately discard any unused FIAsh-EDT₂ or ReAsH-EDT₂ labeling solution according to your institution's guidelines. Do not reuse the 1X FIAsh-EDT₂ or ReAsH-EDT₂ labeling solution.

3. Cover the plate to prevent the solution from evaporating.
4. Incubate the cells at room temperature for 30 minutes, protected from light.
Note: Extending the incubation time may increase the fluorescent signal, but may also increase the background.
5. If you are ready to detect your labeled protein using fluorescence microscopy, proceed to **Using BAL Wash Buffer**. If you are performing dual labeling and wish to label your protein with the second labeling reagent **before** visualizing cells, proceed to **Performing Dual Labeling**, below.

Protocol for Cells Transduced with Lentivirus

Follow this protocol to label cells transduced with lentivirus with FIAsh-EDT₂ labeling solution. You may plate your cells in any tissue culture format of choice. We recommend that your cells are 60–90% confluent at the time of labeling for optimal results. We also recommend including a positive expression control and a negative transduction control (i.e., cells not treated with virus) in your experiment to determine foreground and background fluorescence (see page 7).

1. Remove the growth medium from the cells and wash cells 2–3 times with HBSS.
2. Add the appropriate amount of 0.5X FIAsh-EDT₂ labeling solution prepared with HBSS to each well (see **Preparing the Labeling Solution**, previous page).

Important: Appropriately discard any unused FIAsh-EDT₂ or ReAsH-EDT₂ labeling solution according to your institution's guidelines. Do not reuse the 0.5X FIAsh-EDT₂ or ReAsH-EDT₂ labeling solution.

3. Cover the plate to prevent the solution from evaporating.
4. Incubate the cells at room temperature for 15 minutes, protected from light.
Note: Extending the incubation time may increase the fluorescent signal, but may also increase the background.
5. To detect your labeled protein using fluorescence microscopy, proceed to **Using BAL Wash Buffer**, page 16. If you are performing dual labeling and wish to label your protein with the second labeling reagent **before** visualizing cells, proceed to **Performing Dual Labeling**, next page.

continued on next page

Labeling TC-Tag Fusion Proteins, continued

Performing Dual Labeling

If you wish to label your protein with the second labeling reagent **before** visualizing cells under a microscope, we recommend you do the following:

- Prepare the second labeling solution immediately before use (see **Preparing the Labeling Solution**, page 13).
 - If you will be immediately labeling your protein with the second reagent, remove the first labeling solution and discard appropriately. Wash cells twice with BAL wash buffer and repeat the labeling procedure with the second labeling solution.
 - If you will be incubating your cells between labeling procedures, remove the first labeling solution and discard appropriately. Wash cells once with Opti-MEM[®] Medium, add complete growth medium to the cells, and incubate at 37°C for the desired amount of time. Repeat the labeling procedure with the second labeling solution.
-

Using BAL Wash Buffer

Introduction

BAL wash buffer is a nonfluorescent, hydrophobic reagent that is supplied with the TC-FIAsh™ TC-ReAsH™ II In-Cell Tetracycline Tag Detection Kits to reduce the background fluorescent signal.⁴ BAL wash buffer is much more olfactorily friendly than EDT, another wash buffer that has been used for reducing background. Follow the guidelines below to prepare and add BAL wash buffer to your cells.

Materials Needed

Be sure to have the following materials on hand before beginning:

- BAL wash buffer (supplied with the TC-FIAsh™ TC-ReAsH™ II In-Cell Tetracycline Tag Detection Kits; warm to room temperature)
 - Opti-MEM® Reduced-Serum Medium (Catalog no. 31985-062)
 - HBSS, with calcium and magnesium (for lentivirus-transduced cells) (Catalog no. 10425-092)
 - Mammalian cell line of interest (incubating with FIAsh-EDT₂ or ReAsH-EDT₂ labeling solution)
-

Aliquoting BAL Wash Buffer

Store BAL wash buffer at 2–6°C. BAL wash buffer may cause eye and skin irritation. Wear protecting clothing, eyewear, and gloves when handling. Refer to the Material Safety Data Sheet before handling.

Preparing and Adding the BAL wash buffer

BAL wash buffer is supplied as a 100X solution. Make the 1X stock solution just before use and keep at room temperature until needed. Follow the instructions below to prepare a 1X stock solution of BAL wash buffer and to add it to your cells.

1. To make a 1X stock solution, add the appropriate amount of 100X BAL wash buffer to Opti-MEM® Medium (for transfected cells) or HBSS (for transduced cells). Vortex to mix (1X solution is 250 µM BAL wash buffer). You will add twice the volume of BAL wash buffer to each well as you did the FIAsh-EDT₂ or ReAsH-EDT₂ labeling solution (see table on page 13). Keep at room temperature until needed.
Example: If you added 1 ml of FIAsh-EDT₂ or ReAsH-EDT₂ labeling solution to each well in a 6-well plate, you will need 12 ml of 1X BAL wash buffer (2 ml per well). To make the 1X stock solution, add 120 µl of BAL wash buffer to 12 ml of Opti-MEM® Medium.
 2. After the incubation with the FIAsh-EDT₂ or ReAsH-EDT₂ labeling solution, carefully remove the labeling solution and discard appropriately.
 3. Wash cells once with 1X BAL wash buffer, and discard appropriately.
 4. Repeat step 3. Add appropriate volume of Opti-MEM® Medium and proceed to **Detecting TC-Tag Fusion Proteins**, page 17.
-

Detecting TC-Tag Fusion Proteins

Introduction

After you have labeled your protein, you may detect protein expression and localization in live cells by visually observing the fluorescence of the bound FIAsh-EDT₂ or ReAsH-EDT₂ reagent. General guidelines are provided below to select the type of microscope and filter sets to optimally visualize your labeled protein using fluorescence microscopy. Fluorescent signal from FIAsh-EDT₂ or ReAsH-EDT₂ reagent may be visible for up to 48 hours depending on the stability of your protein.

Recommended Filter Sets

A standard fluorescein (FITC) filter set is suitable for visualizing proteins labeled with the FIAsh-EDT₂ labeling reagent. A standard Texas Red[®] filter set is suitable for visualizing proteins labeled with the ReAsH-EDT₂ labeling reagent. Refer to the table below for the maximum excitation and emission values for each reagent. For a diagram of the fluorescence emission spectra for the FIAsh-EDT₂ and ReAsH-EDT₂ reagents, see page 4.

Labeling Reagent	Excitation Maximum	Emission Maximum	Recommended Filter Set
FIAsh-EDT ₂ reagent	508 nm	528 nm	FITC
ReAsH-EDT ₂ reagent	593 nm	608 nm	Texas Red [®]

While the FITC and Texas Red[®] filter sets are suitable for viewing proteins labeled with the FIAsh-EDT₂ and ReAsH-EDT₂ labeling reagents, respectively, the transmission wavelengths for these filter sets do not precisely overlap with the excitation and emission maxima of these reagents. If you experience difficulty detecting your protein due to low expression levels or unusually high background, you may need to use optimized filter sets with specifications that more closely match the excitation and emission maxima of the FIAsh-EDT₂ and ReAsH-EDT₂ reagents.

Color Camera

If desired, you may use a color camera that is compatible with the microscope to photograph the cells. We recommend using a digital camera or high sensitivity film, such as 400 ASA or greater. For optimal pictures, we recommend photographing cells using a short exposure time (0.5 seconds or less) although this time may vary depending on your protein and application.

Note: If you are dual labeling your protein with the FIAsh-EDT₂ and ReAsH-EDT₂ labeling reagents, you will need to capture the images separately and superimpose them using imaging or graphics software.

As with other fluorescent dyes, avoid photo-bleaching the labeled cells. The ReAsH-EDT₂ reagent is particularly sensitive to continuous illumination through a high magnification, high numerical aperture objective with UV or any other wavelength of light that can excite the reagent.

To reduce photo-bleaching, limit exposure of cells to excitation light by analyzing fluorescent signal for a few seconds at a time. Alternatively, use a lower magnification objective or decrease the lamp intensity to reduce exposure of the substrate to light.

continued on next page

Detecting TC-Tag Fusion Proteins, continued

What You Should See

Recombinant proteins fused to the TC-Tag will appear brightly labeled and will emit a green (FIAsH-EDT₂) or a red (ReAsH-EDT₂) fluorescent signal that should be easy to detect above the background fluorescence. Note that cells will appear lightly and uniformly green or red depending on the labeling reagent used. This is normal background fluorescence caused by autofluorescence of the cells, autofluorescence of unbound reagent, and nonspecific binding of the FIAsH-EDT₂ and ReAsH-EDT₂ labeling reagents. For lentivirus-transduced cells, use the negative control (“mock-transduced”) cells to determine the level of background labeling reagent fluorescence.

If you have trouble detecting your labeled protein above the background fluorescence, see the recommendations below.

If you are experiencing a high level of background fluorescence or if your foreground fluorescent signal is low, try the following:

- Decrease the lamp intensity of the microscope.
 - Adjust the concentration of the FIAsH-EDT₂ or ReAsH-EDT₂ labeling reagent in the labeling solution and repeat the labeling procedure. Use a range of 1 to 10 μM labeling reagent.
 - If you used Opti-MEM[®] to dilute the FIAsH-EDT₂ or ReAsH-EDT₂ labeling reagent and wash your cells, use HBSS in place of Opti-MEM[®] (as described in **Protocol for Cells Transduced with Lentivirus** on page 14)
 - If you are using a cell type that tolerates overnight growth in reduced serum media, culture the cells in Opti-MEM[®] or another reduced serum media overnight (~16–18 hours) and then repeat the labeling procedure.
 - Adjust the incubation time of the cells with the FIAsH-EDT₂ or ReAsH-EDT₂ labeling solution. Visualize cells every 15 minutes up to 90 minutes to optimize the labeling time.
-

Detecting p64

If you transfected cells with the pcDNA[™] 6.2/nTC-Tag-p64 control plasmid supplied with the TC-FIAsH[™] TC-ReAsH[™] II In-Cell Tetracysteine Tag Detection Kit with Mammalian Gateway[®] Expression Vectors, you will detect p64 expression in the nucleoli. p64 will appear as discreet, brightly-labeled, punctate spots localized within the nuclei of cells.

Culturing Cells Further

If you plan to culture cells further after detecting your labeled recombinant protein, carefully remove the labeling solution from your cells and discard appropriately. Wash cells once with Opti-MEM[®] Medium, add complete growth medium, and continue to culture cells at 37°C.

Reminder: If you labeled your cells with ReAsH-EDT₂ Labeling Reagent, you may notice transient phenotypic effects approximately 24 hours after performing the labeling procedure (see page 6).

continued on next page

Detecting TC-Tag Fusion Proteins, continued

Performing Dual Labeling

If you are performing dual labeling and are ready to label your protein with the second labeling reagent, we recommend you do the following:

- Prepare the second labeling solution right before use (see **Preparing the Labeling Solution**, page 13).
 - If you will be immediately labeling your protein with the second reagent, wash cells and repeat the labeling/washing procedure with the second labeling solution.
 - If you will be incubating your cells between labeling procedures, wash cells once with Opti-MEM[®] Medium, add complete growth medium to the cells, and incubate at 37°C for the desired amount of time. Repeat the labeling procedure with the second labeling solution.
-

In-Gel Tetracysteine Tag Detection

For sensitive and specific in-gel detection of TC-Tagged fusion proteins, we recommend the Lumio™ Green Detection Kit available from Invitrogen (LC6090). The Lumio™ Green Detection Kit enables immediate visualization of TC-Tagged proteins in polyacrylamide gels using a UV transilluminator or a visible light laser-based scanner and without the need for staining or western blotting. In addition, the BenchMark™ Fluorescent Protein Standard (LC5928) allows you to easily visualize molecular weight ranges of proteins labeled with Lumio™ Green Detection Reagent.

Appendix

Troubleshooting

Introduction

This section lists potential problems and possible solutions that may help you troubleshoot your FIAsh-EDT₂/ReAsH-EDT₂ labeling experiments.

Problem	Reason	Solution
Weak or no fluorescent signal	Low expression of TC-Tagged protein	<ul style="list-style-type: none"> • Increase protein labeling time. • Increase concentration of labeling reagent in the labeling solution (up to 10 μM). • Culture cells for a longer period of time before labeling to ensure adequate protein expression. • Re-assess transfection conditions. • For lentivirus-transduced cells, transduce cells at a higher MOI (e.g., 10–100).
	Poor transfection efficiency	<ul style="list-style-type: none"> • Re-assess transfection conditions. • Use Lipofectamine™ 2000 for transfection.
	Poor transduction efficiency	See the troubleshooting section of the ViraPower™ Lentiviral Expression System manual.
	Labeling reagents have lost activity	<ul style="list-style-type: none"> • Store labeling reagents ≤–20°C, protected from light. • Aliquot labeling reagents to avoid multiple freeze/thaw cycles. • Use freshly prepared labeling solution to label proteins.
	Concentration of labeling reagent in labeling solution is too low	Increase the concentration of labeling reagent in the labeling solution (up to 10 μM).
	Labeling time too short	Increase the length of time cells are exposed to the labeling reagent.
	Incorrect filter set used	<ul style="list-style-type: none"> • Use a standard FITC filter set to detect proteins labeled with FIAsh-EDT₂ reagent. • Use a standard Texas Red® filter set to detect proteins labeled with ReAsH-EDT₂ reagent. • Enhance visualization of proteins that are difficult to detect by using optimized filter sets (see page 17).
	ReAsH-EDT ₂ reagent is photobleaching	<ul style="list-style-type: none"> • Analyze fluorescent signal for only a few seconds at a time. • Use a lower magnification objective. • Decrease the lamp intensity.

continued on next page

Troubleshooting, continued

Problem	Reason	Solution
High background fluorescence levels	Too much serum in labeling media	<ul style="list-style-type: none"> If you are using a cell type that tolerates overnight growth in reduced serum media, culture the cells in Opti-MEM[®] or another reduced serum media overnight (~16–18 hours) and then repeat the labeling procedure. Dilute the labeling reagent in HBSS and perform the wash steps in the labeling procedure with HBSS (as described in the procedures for lentivirus-transduced cells)
	Concentration of labeling reagent in labeling solution is too high	Decrease the concentration of labeling reagent in the labeling solution (to as low as 1 μM).
	Labeling time too long	Decrease the length of time cells are exposed to the labeling solution.
	Nonspecific binding of the labeling reagent	Use BAL wash buffer after labeling.
	Lamp intensity of microscope too high	Decrease lamp intensity.
Cells exhibit a green or red hazy background	Labeling reagent not removed prior to visualizing cells under the microscope	Remove labeling reagent and wash once with Opti-MEM [®] Medium.
Cells detach from the surface of the well	Cells are disturbed when reagents are added and removed	<ul style="list-style-type: none"> Gently add and remove reagents during transfection, labeling, and wash steps. Plate cells on poly-D-lysine or other type of coated plate for better attachment
	Labeling media does not contain calcium or magnesium	Use labeling media that contains calcium and magnesium to promote cell attachment (e.g. Opti-MEM [®] I Reduced-Serum Medium).
Altered cell morphology (e.g. cells appear rounded up)	Cells exposed to ReAsH-EDT ₂ reagent showing transient phenotypic effects	Phenotypic effects are transient and should disappear after approximately 48 hours (see page 6).
Photographs of cells show high background fluorescence	Exposure time too long	Take photographs using a short exposure time (0.5 seconds or less).

Contact Information

Further information on Molecular Probes products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Paisley, United Kingdom. All others should contact our Technical Service Department in Eugene, Oregon. Please visit our website—probes.invitrogen.com—for the most up-to-date information.

Molecular Probes, Inc.

29851 Willow Creek Road, Eugene, OR 97402

Phone: (541) 465-8300

Fax: (541) 335-0504

Customer Service: 6:00 am to 4:30 pm (Pacific Time)

Phone: (541) 335-0338

Fax: (541) 335-0305

probesorder@invitrogen.com

Toll-Free Ordering for USA:

Order Phone: (800) 438-2209

Order Fax: (800) 438-0228

Technical Service: 8:00 am to 4:00 pm (Pacific Time)

Phone: (541) 335-0353

Toll-Free (800) 438-2209

Fax: (541) 335-0238

probestech@invitrogen.com

Invitrogen European Headquarters

Invitrogen, Ltd.

3 Fountain Drive

Inchinnan Business Park

Paisley PA4 9RF, UK

Phone: +44 (0) 141 814 6100

Fax: +44 (0) 141 814 6260

Email: euroinfo@invitrogen.com

Technical Services:

eurotech@invitrogen.com

Molecular Probes products are high-quality reagents and materials intended for research purposes only. These products must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Please read the Material Safety Data Sheet provided for each product; other regulatory considerations may apply.

All names containing the designation ® are registered with the U.S. Patent and Trademark Office.

Copyright 2005, 2011 Molecular Probes, Inc. All rights reserved. This information is subject to change without notice.

Purchaser Notification

Introduction

Use of the TC-FIAsh™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits are covered under the licenses detailed below.

Limited Use Label License for Molecular Probes Products

For research use only. Not intended for any animal or human therapeutic or diagnostic use. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Molecular Probes, Inc., Business Development, 29851 Willow Creek Road, Eugene, OR 97402. Tel: (541) 465-8300. Fax: (541) 335-0504.

References

1. Nature Methods 2, 171 (2005);
2. Science 296, 503 (2002);
3. Science 281, 269 (1998);
4. J Am Chem Soc 124, 6063 (2002);
5. Methods Enzymol 327, 565 (2000).

