Applied Biosystems
35 Wiggins Avenue
Bedford, MA 01730
(800) 542-2369 or
(781) 271-0045, Press 2

ELISA-Light™ Immunoassay System

Chemiluminescent Immunoassay Detection System with
CSPD® or CDP-*Star*® Substrate

P/N T1022, T1023, T1024, T1025, T1026

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Literature Citation: When describing a procedure for publication using this product, please refer to it as the ELISA-Light® Immunoassay System.

PREFACE

Safety Information

Note: For general safety information, see this Preface and Appendix C, “Safety” on page 8. When a hazard symbol and hazard type appear by a chemical name or instrument hazard, see the “Safety” Appendix for the complete alert on the chemical or instrument.

Safety Alert Words

Four safety alert words appear in Applied Biosystems user documentation at point in the document where you need to be aware of relevant hazards. Each alert word—**IMPORTANT, CAUTION, WARNING, DANGER**—implies a particular level of observation or action, as defined below:

IMPORTANT! – Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

CAUTION! – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

WARNING! – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

DANGER! – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

MSDSs

The MSDSs for any chemicals supplied by Applied Biosystems are available to you free 24 hours a day. For instructions on obtaining MSDSs, see Obtaining MSDSs on page 9.

IMPORTANT! For the MSDSs of chemicals not distributed by Applied Biosystems contact the chemical manufacturer.

How to Obtain Support

For the latest services and support information for all locations, go to:

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- Access worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.
- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents.
- Download PDF documents.
- Obtain information about customer training.
- Download software updates and patches.

I. INTRODUCTION

The Tropix® ELISA-Light™ chemiluminescent detection system incorporates Tropix CSPD® or CDP-*Star*® 1,2-dioxetane substrates for alkaline phosphatase with Sapphire-II™ or Emerald-II™ enhancer in a system designed for rapid and ultrasensitive analyte detection in enzyme-linked immunoassays (1-7). Light emission from alkaline phosphatase-activated CSPD® or CDP-*Star*® substrate is in the form of a "glow". Maximum light emission is reached in 5 to 60 min, depending on the temperature and the substrate chosen. Enzymatic dephosphorylation of substrate occurs at a constant rate proportional to enzyme concentration; the resulting anion decomposes with a finite half-life. Light emission can be quantitated with a variety of luminometers without the need for solution injection. Enzyme-linked immunosorbent assays (ELISAs) can be formatted in several configurations on a variety of solid supports including microplate wells, tubes, polystyrene beads or ferrite particles.

The high sensitivity obtained with Tropix 1,2-dioxetane substrates is demonstrated in a sandwich immunoassay format ELISA employing a biotinylated detector antibody and streptavidin-alkaline phosphatase conjugate, for quantitation of recombinant human IL-6. The results obtained with CSPD substrate with Sapphire-II enhancer (see figure, this page) show a significant improvement in signal-to-noise performance at all concentrations of rhIL-6 and a much wider assay dynamic range compared to those obtained with the fluorescent substrate 4-methylumbelliferyl phosphate (4-MUP), and the colorimetric substrate, *p*-nitrophenyl phosphate (pNPP). This benefit can be expected when any colorimetric ELISA is converted to 1,2-dioxetane/enhancer chemiluminescence.

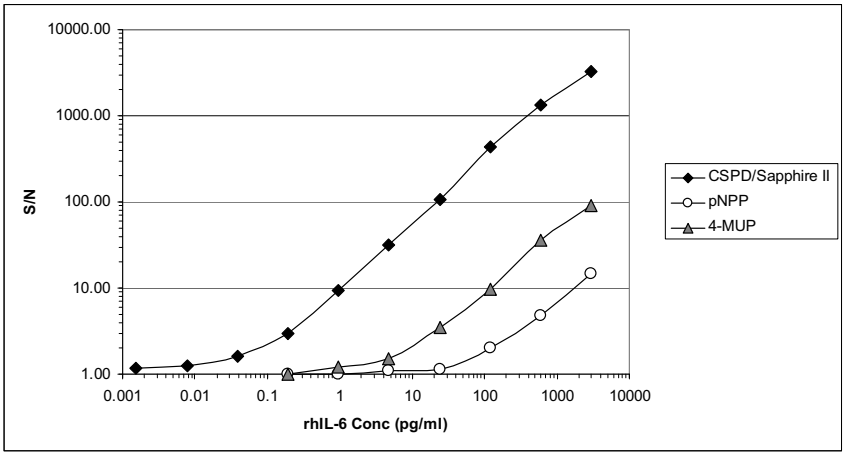

Figure. Comparison of Chemiluminescent, Fluorescent and Colorimetric Detection for ELISA Quantitation of rhIL-6.

Sandwich Immunoassay

A direct sandwich ELISA is used for the detection of large molecules with multiple antigenic sites, usually proteins. In this format, a solid support is coated with a capture antibody that immunoadsorbs the antigen from the sample; a detector antibody conjugated to alkaline phosphatase, specific for a second site on the antigen, is then added. Alternatively, an unlabeled or hapten-labeled detector antibody (not from the same species as the capture antibody) can be used, followed by a secondary antibody, anti-hapten antibody, or streptavidin-alkaline phosphatase conjugate. The enzyme-generated signal is proportional to the amount of antigen.

Sandwich immunoassay formats with 1,2-dioxetane substrates have been used for the quantitation of a variety of animal, human proteins from plasma and tissue extracts (2,3,6-13). In a related assay format, antigen-coated solid support has been used with 1,2-dioxetanes for detection of HTLV-I antibodies (14), and calculation of antibody binding constants (15). CDP-*Star* substrate with Sapphire-II enhancer or Emerald-II enhancer has become widely used for immunoassay protein detection applications such as detection of plasma proteins (16) and viral antigens in both clinical serum samples (17) and in cell culture media as a viral infection assay (18,19).

Competitive Immunoassay

Competitive ELISA formats, in which a competition for available antibody binding sites occurs between a labeled and an unlabeled antigen, are typically used for detection of small molecules. 1,2-Dioxetanes have been utilized in these assays for detection of peptides and hormones (20-22). Competitive assays generate an inverse standard curve; for increasing concentrations of antigen, a decrease in signal is observed. Because the standard curve in a competitive ELISA exhibits maximum signal at the lowest analyte concentration, it may be necessary to adjust reagent concentrations to optimize detection of low analyte concentrations. The sensitivity of chemiluminescent detection enables the use of lower concentrations of capture antibody and competing antigen to generate a standard curve which spans a much lower concentration range compared to colorimetric methods.

Whole-cell ELISA

Immunoassay detection of a surface antigen on whole cells has been demonstrated with 1,2-dioxetane chemiluminescent detection (23). β-Galactosidase enzyme conjugates can also be used with Galacton-Star® substrate with Sapphire-II enhancer for chemiluminescent immunoassay detection, particularly for whole cell ELISA applications that may exhibit high levels of cellular alkaline phosphatase (24).

Protein Detection Applications

Anti-phosphopeptide immunoassays with CSPD or CDP-*Star* substrates and Sapphire-II or Emerald-II enhancers have been developed for quantitation of several protein kinase activities, including PKA, PKC, CAM-KII, receptor interacting protein and src kinases (25), WaaP protein tyrosine kinase and sugar kinase (26) and p38 kinase (27). In addition, a receptor binding assay of a neurotrophic factor to a tyrosine kinase receptor has been demonstrated (28), as well as viral foci imaging with immunodetection (29). Quantitation of protein-protein interactions with an ELISA assay has een demonstrated with CDP-Star substrate/Sapphire-II enhancer (30), as well as quantitation of siRNA-mediated protein knockdown (31).

Nucleic Acid and Nucleic Acid-Protein Interaction Detection Applications

DNA probe hybridization assays, DNA:protein interaction assays, and DNA aptamer binding assays are often formatted in microplate wells or on other solid phases. AP-labeled probes, hapten-labeled probes or antibodies to DNA:DNA or DNA:RNA duplexes (32) can be used to detect hybridization or binding with AP-conjugated detection reagents and 1,2-dioxetane substrates. Chemiluminescent enzyme-linked oligonucleotide assay (ELONA) has been used to quantitate DNA aptamer binding to protein (33). CDP-*Star* substrate is used in microplate-based assay systems for detection of viral RNA or DNA by immunodetection (34,35), quantitative detection of labeled PCR products (36), and ELISA-PCR for mRNA quantitation (37). In addition, detection of chemical:DNA adducts in mammalian tissues has been demonstrated with CDP-*Star* substrate with Emerald-II™ enhancer (38).

II. SYSTEM COMPONENTS

All ELISA-Light™ kit components have a shelf-life of 1 yr when stored as indicated.

	T1023	T1024	T1025	T1026	T1022
Microplate assays	1,000	1,000	1,000	1,000	1,000
I-Block™ Reagent	7.5 g	7.5 g	7.5 g	7.5 g	7.5 g
10X Assay Buffer	100 mL	100 mL	100 mL	100 mL	100 mL
CSPD® Substrate/ Sapphire-II™ Enhancer RTU	100 mL	-	-	-	1 x 25 mL
CSPD® Substrate/ Emerald-II™ Enhancer RTU	-	100 mL	-	-	1 x 25 mL
CDP-*Star*® Substrate/ Sapphire-II™ Enhancer RTU	-	-	100 mL	-	1 x 25 mL
CDP-*Star*® Substrate/ Emerald-II™ Enhancer RTU	-	-	-	100 mL	1 x 25 mL

P/N T1022 is a sampler kit that contains 25 mL of each of the four substrate/enhancer combinations.

1. **I-Block™ Reagent:** Highly purified casein, dry powder. Store at room temperature.

2. **10X Assay Buffer:** 200 mM Tris (pH 9.8), 10 mM $MgCl_2$. Dilute 1:10 with water for use. Store at 4°C.

3. **Substrate/Enhancer Solution:** Ready-to-use CSPD® or CDP-*Star*® substrate (0.4 mM) containing 10% (v/v) Sapphire-II™ or Emerald-II™ enhancer. Store at 4°C.

III. IMMUNOASSAY PROTOCOLS

A. General Procedures

Ultrasensitive immunoassay detection is often limited by non-specific binding of enzyme conjugates to the plates; high quality, purified conjugates are critical (25). Detailed protocols for immunoassay formats and microplate coating are available (26). For maximal sensitivity, each assay condition should be individually optimized. Chemiluminescent ELISAs, which are read in a microplate luminometer, must be performed in opaque white plates. All steps should be performed at room temperature, unless otherwise indicated. Recommended volumes and terminology are based upon use of microplates.

Coating (for microplates): Optimal coating buffer and protein concentration (usually 0.2 to 10 μg/mL) must be empirically determined. Add 50-100 μL coating solution/well, seal plate and incubate 2 hr at 37°C or overnight at room temperature (or 4°C).

Blocking: Fill wells with Blocking Buffer. Incubate 1 hr at room temperature or overnight at 4°C.

Washing:
Automated: Use 96-well microplate washer instrumentation, filling wells to slightly below well capacity.
Manual: Wash wells by filling with buffer from a squirt bottle. Shake buffer into a sink after each wash. After the last wash, remove residual liquid by tapping plate face down on clean paper towels.

B. Direct Sandwich ELISA

Direct sandwich immunoassays are the most sensitive format for detecting soluble antigen. This format is recommended for antigens for which two distinct antibodies are available. Many variations on this format are possible, including monoclonal antibody screening of hybridoma culture supernatants.

For the following hazards, see the complete safety alert descriptions in Appendix C "Safety" on page 8:

⚠ **WARNING! CHEMICAL HAZARDS. Assay Buffer, Blocking Buffer, CSPD® Substrate/Sapphire-II™ Enhancer RTU.**

1. Coat plate with capture antibody, then wash coated plate 3X with Wash Buffer.
2. Incubate wells with Blocking Buffer, then wash blocked plate 3X with Wash Buffer.
3. Dilute antigen samples in Blocking Buffer and add 100 µL/well. Incubate 1 hr with shaking. Wash wells 3X with Wash Buffer.
4. Dilute detector antibody-alkaline phosphatase conjugate in Blocking Buffer and add 100 µL/well. Incubate for at least 1 hr with shaking. The optimal dilution must be empirically determined.
5. Wash wells 4X with Wash Buffer then twice with 1X Assay Buffer.
6. Add 100 µL/well Substrate/Enhancer solution. Incubate for 10 min and then measure at 10 min intervals until light emission has reached plateau.

C. Competitive ELISA I: Antigen-Coated Plates

This assay format is useful for detection of small antigen molecules, such as therapeutic drugs, for which a specific antibody and milligram quantities of antigen are available.

For the following hazards, see the complete safety alert descriptions in Appendix C "Safety" on page 8:

⚠ **WARNING! CHEMICAL HAZARDS. Assay Buffer, Blocking Buffer, CSPD® Substrate/Sapphire-II™ Enhancer RTU.**

1. Coat plate with antigen, then wash coated plate 3X with Wash Buffer.
2. Incubate wells with Blocking Buffer, then wash blocked plate 3X with Wash Buffer.
3. Dilute standard or test antigens separately into Blocking Buffer (to 2X final conc.).
4. Dilute antibody-alkaline phosphatase conjugate in Blocking Buffer (to 2X final conc.).
5. Add equal volumes of each to wells (50-75 µL/well). Incubate for 2 hr with shaking.
6. Wash wells 4X with Wash Buffer then once with 1X Assay Buffer
7. Add 100 µL/well Substrate/Enhancer solution. Incubate for 10 min and then measure at 10 min intervals until light emission has reached plateau.

D. Competitive ELISA II: Antibody-Coated Plates

This assay format, which is recommended for small antigens that can be modified without affecting antibody affinity, requires a biotinylated or alkaline phosphatase-conjugated antigen.

For the following hazards, see the complete safety alert descriptions in Appendix C "Safety" on page 8:

⚠ **WARNING! CHEMICAL HAZARDS. Assay Buffer, Blocking Buffer, CSPD® Substrate/Sapphire-II™ Enhancer RTU.**

1. Coat plate with capture antibody, then wash coated plate 3X with Wash Buffer.

2. Incubate wells with Blocking Buffer, then wash blocked plate 3X with Wash Buffer.

3. Dilute standard and test antigen samples separately in Blocking Buffer and add 100 µL/well. Incubate for 10 min with shaking.

4. Add 50 µL/well of biotinylated antigen or antigen-alkaline phosphatase conjugate diluted in Blocking Buffer. Incubate for 1 hr with shaking.

5. Wash wells 3X with Wash Buffer.

6. Dilute streptavidin-alkaline phosphatase conjugate in Blocking Buffer and add 100 µL/well. Incubate for 1 hr with shaking (omit this step if antigen-alkaline phosphatase conjugate was used).

7. Wash wells 4X with Wash Buffer then once with 1X Assay Buffer

8. Add 100 µL/well Substrate/Enhancer solution. Incubate for 10 min and then measure at 10 min intervals until light emission has reached plateau.

IV. TROUBLESHOOTING

The light produced from the alkaline phosphatase catalyzed decomposition of CSPD® or CDP-*Star*® substrate is a glow-type emission and the incubation time required to achieve maximum light emission is a function of alkaline phosphatase activity and temperature.

The chemiluminescent assay for alkaline phosphatase activity is subject to interference by hemoglobin and by high concentrations of protein (greater than 11.6 g/L). Bilirubin (up to 173 µmol/L) and lipid (2-100 g/L) do not interfere (1).

The optimal blocking reagents and conditions should be determined for the individual assay. Any materials which may contain contaminating alkaline phosphatase should be avoided.

V. APPENDICES

A. Solution Preparation

For the following hazards, see the complete safety alert descriptions in Appendix C "Safety" on page 8:

⚠ **WARNING! CHEMICAL HAZARDS. I-Block™ Reagent.**

All solutions should be made with deionized H_2O. 10X PBS should be kept sterile at room temperature. Blocking Buffer should be prepared fresh daily to prevent bacterial contamination. Blocking buffer may be stored at 4°C if 0.02% NaN_3 is added.

10X PBS (alternative recipes may be used)

0.58 M Na_2HPO_4	82.3 g
0.17 M NaH_2PO_4-H_2O	23.5 g
0.68 M NaCl	40.0 g
Add deionized H_2O to 1000 mL	

Wash Buffer

1X PBS or TBS	10 mL of 10X
0.05% Tween®-20 Detergent	50 µL
Add deionized H_2O to 100 mL	

Blocking Buffer

1X PBS or TBS	3 mL of 10X
0.2% I-Block™ Reagent	0.06 g
0.05% Tween®-20 Detergent	15 µL

Add 3 mL of 10X PBS to 27 mL of H_2O. Microwave for 40 sec, then add I-Block™ reagent while stirring. DO NOT BOIL. Add Tween®-20 detergent after solution has cooled. The solution will remain opaque, but particles will be dissolved. Cool to room temperature before use.

B. Use of Luminometers

We recommend using a single-mode luminometer or a multi-mode detection instrument set for luminescence measurement to measure light emission from 96- or 384-well microplates. Measure light emission for 1 sec/well, or as appropriate for the instrument. Contact Applied Biosystems Technical Support group for additional questions.

C. Safety

1. General chemical safety

Chemical hazard warning

WARNING! CHEMICAL HAZARD. Before handling any chemicals, refer to the Material Safety Data Sheet (MSDS) provided by the manufacturer, and observe all relevant precautions.

WARNING! CHEMICAL HAZARD. All chemicals in the instrument, including liquid in the lines, are potentially hazardous. Always determine what chemicals have been used in the instrument before changing reagents or instrument components. Wear appropriate eyewear, protective clothing, and gloves when working on the instrument.

WARNING! CHEMICAL HAZARD. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

WARNING! CHEMICAL STORAGE HAZARD. Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See “About MSDSs” on page 9.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer’s cleanup procedures as recommended in the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

MSDSs

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining MSDSs

The MSDS for any chemical supplied by Applied Biosystems is available to you free 24 hours a day. To obtain MSDSs:

1. Go to www.appliedbiosystems.com, click **Support**, then select **MSDS**.

2. In the Keyword Search field, enter the chemical name, product name, MSDS part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click Search.

3. Find the document of interest, right-click the document title, then select any of the following:
    - **Open** – To view the document
    - **Print Target** – To print the document
    - **Save Target As** – To download a PDF version of the document to a destination that you choose

Note: For the MSDSs of chemicals not distributed by Applied Biosystems, contact the chemical manufacturer.

Chemical waste safety

Chemical waste hazards

CAUTION! HAZARDOUS WASTE. Refer to Material Safety Data Sheets and local regulations for handling and disposal.

WARNING! CHEMICAL WASTE HAZARD. Wastes produced by Applied Biosystems instruments are potentially hazardous and can cause injury, illness, or death.

WARNING! CHEMICAL STORAGE HAZARD. Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

Chemical waste safety guidelines

To minimize the hazards of chemical waste:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Provide primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Handle chemical wastes in a fume hood.
- After emptying a waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Waste disposal

If potentially hazardous waste is generated when you operate the instrument, you must:

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure the health and safety of all personnel in your laboratory.
- Ensure that the instrument waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

  IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

General biohazard

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories (stock no. 017-040-00547-4; bmbl.od.nih.gov)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

IMPORTANT! Additional information about biohazard guidelines is available at: www.cdc.gov

Chemical alerts

For the definitions of the alert words **IMPORTANT**, **CAUTION**, **WARNING**, and **DANGER**, see "Safety alert words" on page 1.

General alerts for all chemicals

EXAMPLE: Avoid contact with (skin, eyes, and/or clothing). Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Specific chemical alerts

WARNING! CHEMICAL HAZARD. 10X Assay Buffer may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

WARNING! CHEMICAL HAZARD. CSPD® Substrate/Sapphire-II™ Enhancer RTU may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

WARNING! CHEMICAL HAZARD. I-Block™ Reagent may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

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