iTRAQ® Reagents – 8plex

Amine-Modifying Labeling Reagents for Multiplexed Relative and Absolute Protein Quantitation

Protocol

For Research Use Only
Not for Use in Diagnostics

For Research Use Only. Not for use in diagnostic procedures.

Information in this document is subject to change without notice. AB Sciex Pte. Ltd. assumes no responsibility for any errors that may appear in this document.

AB SCIEX PTE. LTD. DISCLAIMS ALL WARRANTIES WITH RESPECT TO THIS DOCUMENT, EXPRESSED OR IMPLIED, INCLUDING BUT NOT LIMITED TO THOSE OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. IN NO EVENT SHALL AB SCIEX PTE. LTD. BE LIABLE, WHETHER IN CONTRACT, TORT, WARRANTY, OR UNDER ANY STATUTE OR ON ANY OTHER BASIS FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING BUT NOT LIMITED TO THE USE THEREOF.

NOTICE TO PURCHASER: LIMITED LICENSE

The purchase of this product includes a limited, non-transferable license to use this amount of the product to practice the labeling methods using encoded isobaric and/or isomeric tagging reagents which may be claimed in pending and planned U.S. and international patent applications exclusively licensed to AB Sciex Pte. Ltd, and patents issuing therefrom, solely for the purchaser's research activities. No use in diagnostic procedures is authorized. The license granted herein is personal to the original purchaser and may not be transferred to any other party outside the purchaser's company. No other right or license is conveyed or granted either expressly, by implication or by estoppel, to resell or repackage this product. Certain other restrictions and limitations may apply as set forth in warranty statements and other documentation provided with the purchase of iTRAQ reagent technology.Further information can be obtained by contacting the Director of Licensing, c/o AB Sciex Pte. Ltd., 110 Marsh Rd., Foster City, California 94404, USA.

Part Number 4375249 Rev. C
08/2010

Contents

Preface

Chapter 1 Introduction to iTRAQ® Reagents – 8plex Chemistry

Chapter 2 iTRAQ® Reagents – 8plex - Labeling Protocol

Chapter 3 iTRAQ® Reagents – 8plex - Sample Mixture Analysis

Preface

This preface covers:

Safety

Safety Alert Words

Four safety alert words appear in our user documentation at points 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.

Definitions

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.

Chemical Hazard Warning

WARNING **CHEMICAL HAZARD.** Some of the chemicals used with our instruments and protocols are potentially hazardous and can cause injury, illness, or death.

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 vi.)
- 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.

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

You can obtain the MSDS for any chemical supplied with this kit at **www.sciex.com/msds.**

Note: For the MSDSs of chemicals not distributed with this kit, contact the chemical manufacturer.

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 our 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 the 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

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; **http://bmbl.od.nih.gov**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **http://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.

Additional information about biohazard guidelines is available at:

http://www.cdc.gov

How to Obtain More Information

Related Documentation

- The *xTRAQ Family of Amine -Modifying Labeling Reagents for Multiplexed Relative and Absolute Quantification: Chemistry Reference Guide*.
- *iTRAQ® Reagents–8plex Chemistry Quick Reference Card* (PN 4383502)
- Technical and Application Notes

For the portable document format (PDF) versions of the chemistry reference guide, this protocol, and the quick reference card, go to **http://www.sciex.com**, then click the link for **Support**. Click the literature link and perform a literature search.

For technical and application notes, see "How to Obtain Support" on page x.

Obtaining Information from the Software Help System

The ProteinPilot™ Software has a Help system that describes how to use each feature of the user interface. Access the Help system by doing one of the following:

- Click in the toolbar of the software window
- Select the **Help** tab
- Press **F1**

How to Obtain Support

We are committed to meeting the needs of your research. Please go to **www.sciex.com** and go to the **Support** tab for local support information.

Contacting Technical Support in North America

To contact technical support:

- By telephone: Dial 1.877.740.2129
- By fax: Dial 1.650.627.2803

Introduction to iTRAQ® Reagents – 8plex Chemistry

1

This chapter covers:

Overview

iTRAQ® Reagents – 8plex are provided as a set of eight, isobaric reagents: iTRAQ® Reagents – 8plex - 113, 114, 115, 116, 117, 118, 119, 121‡.

The use of eight reagents allows multiplexing of up to eight different samples in a single LC/MS/MS experiment.

Key Features

Using iTRAQ Reagents – 8plex to label peptides allows you to:

- Analyze normal, diseased, and drug-treated states in the same experiment or time-course study.
- Run duplicate, triplicate, and quadruplet analyses of the same sample in one experiment.
- Label multiple peptides in a peptide digest (including those from proteins with post-translational modifications) in 2 hours at room temperature.
- Label multiple peptides per protein, increasing confidence in identification and quantitation.

For easy data interpretation for relative and absolute protein quantitation, analyze samples using ProteinPilot™ Software and one of the following AB SCIEX instruments:

- 5600 Triple TOF™ Systems
- QTRAP® Systems
- 5800 MALDI TOF/TOF™ Instruments

‡ There is no iTRAQ Reagent - 120. The phenylalanine immonium ion mass is 120.

For More Information

iTRAQ Reagent – 8plex Chemistry Reference Guide

Refer to the *xTRAQ Family of Amine -Modifying Labeling Reagents for Multiplexed Relative and Absolute Quantification: Chemistry Reference Guide* for supplementary information on iTRAQ Reagent chemistry.

Software Help System

The ProteinPilot™ Software has a Help system that describes how to use each feature of the user interface. See "Obtaining Information from the Software Help System" on page x.

Services And Support Information

For services and support information, see "How to Obtain Support"on page x.

Assay, iTRAQ® Reagent – 8plex, and Buffer Kits

See Table 1-1 for ordering guidelines.

Table 1-1 Kit Ordering Guidelines

Maximum Number of Sets to Label ‡	Order§ iTRAQ® Reagent – 8plex -	And the Appropriate Number of Buffer Kits
One 8plex set	One Assay Kit - Provides one set of iTRAQ Reagents – 8plex and sufficient material to run one 8plex set of iTRAQ Reagents – 8plex-labeled sample digests.	No Buffer Kit. The buffers are included in the One Assay Kit.
Five 8plex sets	Multi-Plex Kit - Provides five 1-unit tubes of each iTRAQ Reagent – 8plex	One Buffer Kit
Twenty-five 8plex sets	25 U Kit - Provides one 25-unit tube of each iTRAQ Reagent – 8plex	One Buffer Kit per ten 8plex sets
Fifty 8plex sets	50 U Kit – Provides one 50-unit tube of each iTRAQ Reagent – 8plex	One Buffer Kit per ten 8plex sets

‡ Number of sets of iTRAQ Reagents – 8plex-labeled sample digests if following the iTRAQ Reagents – 8plex labeling protocol.

§ For information about ordering trypsin and a cation-exchange cartridge system through www.sciex.com, see Table 1-5 on page 1-10.

Kit Materials Packaged with the One Assay Kit

WARNING **CHEMICAL HAZARD.** Some of the chemicals provided in your reagent kit may be hazardous. Before handling the reagents, read the material safety data sheets (MSDSs) that accompany your first shipment. Always follow the safety precautions (wearing appropriate protective eyewear, clothing, and gloves, etc.) presented in each MSDS. To receive additional copies of MSDSs at no extra cost, see "Obtaining MSDSs" on page vii.

IMPORTANT! When you receive the shipping container, immediately remove the iTRAQ Reagents – 8plex box and store items at –15 to –25 °C.

See Table 1-2 for materials contained in each kit.

Table 1-2 iTRAQ® Reagents – 8plex One Assay Kit materials

Item	Quantity in the One Assay Kit	Contents
iTRAQ® Reagents – 8plex box‡		
• iTRAQ Reagent – 8plex 113	1 vial, 1 unit/vial	Amine-modifying labeling reagent. One unit (one vial) of reagent labels 20 to 100 µg of protein digest.
• iTRAQ Reagent – 8plex 114	1 vial, 1 unit/vial	
• iTRAQ Reagent – 8plex 115	1 vial, 1 unit/vial	
• iTRAQ Reagent – 8plex 116	1 vial, 1 unit/vial	
• iTRAQ Reagent – 8plex 117	1 vial, 1 unit/vial	
• iTRAQ Reagent – 8plex 118	1 vial, 1 unit/vial	
• iTRAQ Reagent – 8plex 119	1 vial, 1 unit/vial	
• iTRAQ Reagent – 8plex 121	1 vial, 1 unit/vial	
• Certificate of Analysis	1	Provides purity information for each reagent.

Table 1-2 iTRAQ® Reagents – 8plex One Assay Kit materials *(continued)*

Item	Quantity in the One Assay Kit	Contents
Six-Protein Mix‡	4 vials	Used in testing the protocol. Contains: • Bovine serum albumin (22 µg) • α-lactalbumin (10 µg) • β-galactosidase (38 µg) • lysozyme (10 µg) • Apotransferrin (25 µg) • β-lactoglobulin (24 µg)
Dissolution Buffer (pH 8.5)	2 vials, 1.5 mL/vial	Dissolves the sample. Buffers the labeling reaction. Contains 0.5 M triethylammonium bicarbonate (TEAB).
Denaturant	1 vial, 50 µL/vial	Disrupts the hydrogen, hydrophobic, and electrostatic bonds of the proteins. Contains 2% SDS.
Reducing Reagent	1 vial, 100 µL/vial	Reduces the disulfide bonds of the proteins. Contains 50 mM tris-(2-carboxyethyl) phosphine (TCEP).
Cysteine-Blocking Reagent	1 vial, 50 µL/vial	Reversibly blocks the cysteine group. Contains 200 mM methyl methanethiosulfonate (MMTS) in isopropanol.
Isopropanol	1 vial, 1.8 mL/vial	Absolute, HPLC-grade or better. Dissolves the iTRAQ Reagents and optimizes labeling.
iTRAQ® Reagents – 8plex Protocol	1	This document.
iTRAQ® Reagents – 8plex Chemistry Quick Reference Card	1	Laminated card that provides a quick reference to the steps in the iTRAQ Reagents – 8plex protocol.

‡ Store at –15 to –25 °C or colder.

Kit Materials Packaged with the iTRAQ® Reagent - 8plex Multi-Plex, 25 U, and 50 U Kits

WARNING **CHEMICAL HAZARD.** Some of the chemicals provided in your reagent kit may be hazardous. Before handling the reagents, read the material safety data sheets (MSDSs) that accompany your first shipment. Always follow the safety precautions (wearing appropriate protective eyewear, clothing, and gloves, etc.) presented in each MSDS. To receive additional copies of MSDSs at no extra cost, see "How to Obtain Support"on page x.

IMPORTANT! When you receive the shipping container, immediately remove the iTRAQ Reagents – 8plex box and store its contents at –15 to –25 °C.

See Table 1-3 for materials contained in each kit. One unit of iTRAQ Reagent – 8plex labels 20 to 100 µg of protein digest.

Table 1-3 iTRAQ® Reagent – 8plex Multi-Plex Kit and iTRAQ® Reagents – 8plex - 25 U and - 50 U Kit materials

Item	Quantity in the Multi-Plex Kit	Quantity in the 25 U Kit	Quantity in the 50 U Kit	Contents
iTRAQ® Reagents – 8plex box‡				
• iTRAQ Reagent – 8plex 113	5 vials, 1 unit/vial	1 vial, 25 units/vial§	1 vial, 50 units/vial§	Amine-modifying labeling reagent. One unit of reagent labels 20 to 100 µg of protein digest.
• iTRAQ Reagent – 8plex 114	5 vials, 1 unit/vial	1 vial, 25 units/vial	1 vial, 50 units/vial	
• iTRAQ Reagent – 8plex 115	5 vials, 1 unit/vial	1 vial, 25 units/vial	1 vial, 50 units/vial	
• iTRAQ Reagent – 8plex 116	5 vials, 1 unit/vial	1 vial, 25 units/vial	1 vial, 50 units/vial	
• iTRAQ Reagent – 8plex 117	5 vials, 1 unit/vial	1 vial, 25 units/vial	1 vial, 50 units/vial	
• iTRAQ Reagent – 8plex 118	5 vials, 1 unit/vial	1 vial, 25 units/vial	1 vial, 50 units/vial	

Table 1-3 iTRAQ® Reagent – 8plex Multi-Plex Kit and iTRAQ® Reagents – 8plex - 25 U and - 50 U Kit materials *(continued)*

Item	Quantity in the Multi-Plex Kit	Quantity in the 25 U Kit	Quantity in the 50 U Kit	Contents
• iTRAQ Reagent – 8plex 119	5 vials, 1 unit/vial	1 vial, 25 units/vial	1 vial, 50 units/vial	
• iTRAQ Reagent – 8plex 121	5 vials, 1 unit/vial	1 vial, 25 units/vial	1 vial, 50 units/vial	
• Certificate of Analysis	1	1	1	Provides purity information for each reagent.
iTRAQ® Reagents – 8plex Protocol	1	1	1	This document.
iTRAQ® Reagents – 8plex Chemistry Quick Reference Card	1	1	1	Laminated card that provides a quick reference to the steps in the labeling protocol.
Isopropanol	3 vials, 1.8 mL/vial	0	0	Absolute, HPLC-grade or better. Dissolves the iTRAQ Reagents and optimizes labeling.
Certificate of Analysis	1	0	0	Provides purity information for each reagent.

‡ Store at –20 °C or colder.

§ Immediately after opening the 25 unit and 50 unit vial of iTRAQ Reagent – 8plex for the first time, aliquot the appropriate volume [see the certificate of analysis] required to label samples into single-use tubes and store under inert gas at –20 °C or colder.

Kit Materials Packaged with the Buffer Kit

WARNING **CHEMICAL HAZARD.** Some of the chemicals provided in your reagent kit may be hazardous. Before handling the reagents, read the material safety data sheets (MSDSs) that accompany your first shipment. Always follow the safety precautions (wearing appropriate protective eyewear, clothing, and gloves, etc.) presented in each MSDS. To receive additional copies of MSDSs at no extra cost, see "How to Obtain Support"on page x.

See Table 1-4 for materials contained in each kit.

Table 1-4 iTRAQ® Reagent – 8plex - Buffer Kit materials

Item	Quantity in the Buffer Kit	Contents
Dissolution Buffer (pH 8.5)	4 vials, 1.5 mL/vial	Dissolves the sample. Buffers the labeling reaction. Contains 0.5 M triethylammonium bicarbonate (TEAB).
Denaturant	2 vials, 50 µL/vial	Disrupts the hydrogen, hydrophobic, and electrostatic bonds of the proteins. Contains 2% SDS.
Reducing Reagent	2 vials, 100 µL/vial	Reduces the disulfide bonds of the proteins. Contains 50 mM tris-(2-carboxyethyl)-phosphine (TCEP).
Cysteine-Blocking Reagent	2 vials, 50 µL/vial	Reversibly blocks the cysteine group. Contains 200 mM methyl methane-thiosulfonate (MMTS) in isopropanol.
Isopropanol	3 vials, 1.8 mL/vial	Absolute, HPLC-grade or better. Dissolves the iTRAQ® Reagents – 8plex and optimizes labeling.
Certificate of Analysis	1	Provides purity information for each reagent.

User-Supplied Materials

WARNING **CHEMICAL HAZARD.** Some of the chemicals referred to in this protocol (such as those in Table 1-5) are not provided with your kit. When using chemicals not provided by or purchased from us, obtain the MSDS directly from the chemical manufacturer.

Table 1-5 User-supplied materials

Item	Volume or Quantity per Assay
Disposable gloves	As needed
Test samples (for example, a diseased cell state); up to seven samples	20 to 100 µg protein
Control sample (for example, a normal cell state)	20 to 100 µg protein
Trypsin with $CaCl_2$ (10 pack, P/N 4352157) or Trypsin without $CaCl_2$ (8 pack, P/N 4370285)	1 to 2 vials for a 4plex experiment 2 to 4 vials for an 8plex experiment
Pipettors and tips suitable for 1 µL to 1 mL	As needed
Fraction-collection tubes and rack • Screw or snap-cap tubes, 0.5- to 2-mL for running the iTRAQ® Reagent protocol • 1.5- and 15-mL tubes for performing cation-exchange chromatography	 3 to 6 per assay As needed
Syringe, 2.5- and 10-mL (2-inch blunt needle, 22-gauge)	1
Cation-exchange cartridge system such as the cation-exchange cartridge system (P/N 4326747)	1
If you use complex samples that require fractionation, a high-resolution cation-exchange column (for example, PolySulfoethyl A Column, 5 micron 200 Å bead, from PolyLC, Inc., 4.6 × 100 mm, P/N 104SE0502). Select a column size with the appropriate binding capacity for your sample size.	1
pH paper • pH range 2.5 to 4.5 – to test the pH of the sample before loading on the cation-exchange cartridge • pH range 7 to 10 – to test the pH of the sample after addition of label	As needed

Table 1-5 User-supplied materials *(continued)*

Item	Volume or Quantity per Assay
Milli-Q® water or equivalent (minimum 18.2 MOhms water, conductivity maximum 0.05 µS/0.05 µMho)	50 mL
Heating block, 60 °C	1
Incubator, 37 °C	1
Bench-top centrifuge	1
Vortexer	1
Centrifugal vacuum concentrator	1
Mass spectrometer with analysis software (for example, an 5800 MALDI TOF/TOF™ Instrument with ProteinPilot™ Software)	1
Capillary reversed-phase HPLC system	1
If you analyze using Nanospray® ESI source mass spectrometry, New Objective, Inc. coated fused-silica PicoTips® (coating applied to tip end, Cat. #FS360-20-10-CE). Also requires tubing fitting from Upchurch Scientific (F-230, PEEK™ sleeve).	1

Workflow

iTRAQ Reagents – 8plex labeling involves:

- Reducing, cysteine blocking, digesting, and labeling each sample in a single tube. The single-tube process avoids potential sample loss in individual samples that may cause inaccuracies in quantitation.
- Combining all iTRAQ Reagent – 8plex-labeled samples into one sample mixture for LC/MS/MS analysis. If losses occur during analysis, each sample has the same loss and the ratios are preserved.

Figure 1-1 summarizes the iTRAQ Reagent – 8plex workflow for a duplex-type experiment. Up to eight samples can be prepared and analyzed in a single experiment.

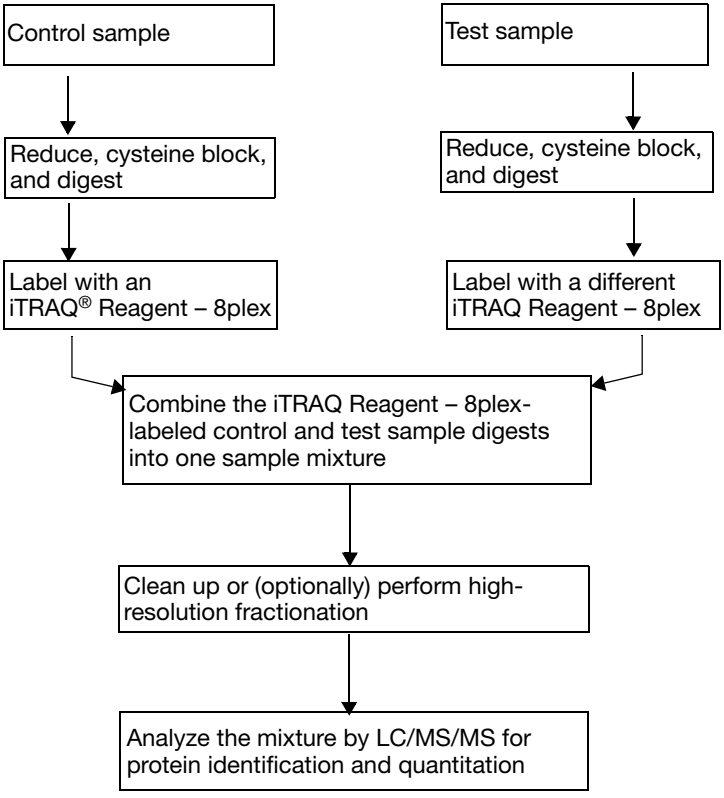

Figure 1-1 Overview of iTRAQ® Reagents – 8plex methodology

iTRAQ® Reagents – 8plex - Labeling Protocol

2

This chapter covers:

This chapter describes preparing your sample, testing and running the iTRAQ® Reagents – 8plex labeling protocol, and preparing the sample mixture for analysis using cation-exchange chromatography.

Before You Begin

Required Materials

See Chapter 1, "Kit Materials Packaged with the One Assay Kit" and "User-Supplied Materials."

Preparing Your Sample

If your sample contains a substance that may interfere with the iTRAQ Reagents – 8plex labeling protocol (Table 2-1), perform acetone precipitation (page 2-3) to clean up the sample.

Table 2-1 Substances that may interfere with the iTRAQ® Reagents – 8plex labeling protocol

Potential Interfering Substance	Potential Interference	When to Perform Acetone Precipitation
Thiols (for example, DTT and mercaptoethanol)	Interfere with cysteine blocking.	Before beginning the protocol.
High amounts of detergents and denaturants (see Table 2-2 for concentration limits of some acceptable detergents/denaturants.)	Inactivate trypsin.	If the substance is needed to solubilize your sample, precipitate after reducing the protein and blocking cysteine.
Active proteases		
Primary amines‡, for example, those in: • Ammonium acetate • Ammonium bicarbonate • Ammonium citrate • Ammonium phosphate • Ammonium tartrate • AMPD [2-amino-2-methyl-1,3-propanediol] • Aminoguanidine bicarbonate salt • AMP [2-amino-2-methyl-1-propanol] • Ethanolamine • Gly-gly • Tris buffers	React with iTRAQ® Reagents – 8plex, interfering with labeling.	Before trypsin digestion.
For information, see the *xTRAQ Family of Amine -Modifying Labeling Reagents for Multiplexed Relative and Absolute Quantification: Chemistry Reference Guide*		

‡ Non-peptide amines also interfere with labeling (see "Modifications" on page 2-6.)

Acetone Precipitation

IMPORTANT! If you perform acetone precipitation after trypsin digestion, sample can be lost.

WARNING **CHEMICAL HAZARD. Acetone** is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To clean up a sample by acetone precipitation:

1. Chill acetone to –20 °C and the sample tube containing the sample to 4 °C.
2. Add six volumes of cold acetone to the cold sample tube.
3. Invert the tube three times.
4. Incubate the tube at –20 °C until a flocculent forms (30 minutes to 4 hours).
5. Spin at 6,000 × *g* for 10 minutes.
6. Decant the acetone. Do not dry.
7. Use the precipitated pellet as your sample in "Reducing the Proteins and Blocking Cysteine," step 1, page 2-7.

Testing the Protocol

To verify that your sample preparation protocol does not interfere with the iTRAQ Reagents – 8plex labeling, it is strongly recommended that you run a control sample through the entire protocol before you run an actual experiment (see "Running the Labeling Protocol" on page 2-7).

If you lack enough control sample to test the protocol, prepare a sample using your sample conditions and the 6-Protein Mix in the 1-Assay kit. Label the 6-Protein Mix sample with one of the iTRAQ Reagents – 8plex. One vial of 6-Protein Mix (containing 129 µg of protein) is enough for one to two iTRAQ Reagents – 8plex labeling reactions.

1. Prepare 6-Protein Mix as follows:
   - To emulate samples containing up to 50 µg protein, add 50 µL Dissolution Buffer to one vial of 6-Protein Mix.
   - To emulate samples containing 100 µg protein, add 25 µL 0.5 M Dissolution Buffer to each of two vials of 6-Protein Mix.
2. Vortex to mix, then spin.
3. Transfer 20 µL to a sample tube for labeling with an iTRAQ Reagent – 8plex. Transfer a second 20 µL aliquot to another sample tube for labeling with a different iTRAQ Reagent – 8plex.
4. Follow the protocol, starting with "Reducing the Proteins and Blocking Cysteine," step 2 on page 2-7.

Sample Solubility

If your sample is insoluble after adding Dissolution Buffer and Denaturant (steps 1 and 2 on page 2-7), choose an alternative detergent/ denaturant or buffer from Table 2-2. The listed buffers are free of primary amines and can buffer at pH 8.0 to 8.5 when used at a concentration of at least 0.3 M.

IMPORTANT! During the labeling reaction, the buffer concentration must be at least 0.06 M and the pH of the sample mixture must be greater than 7.5. If the pH is less than 7.5, labeling efficiency can be significantly reduced. Be sure to check and adjust pH as stated in step 4 on page 2-9.

Table 2-2 Recommended alternative detergents/denaturants and buffers

Alternative Detergent/Denaturant	Alternative Buffer
• SDS • OG (octyl B-D-glucopyranoside) • NP®-40 • Triton® X-100 • Tween® 20 • CHAPS • Urea (6 M) **IMPORTANT!** When using urea, always use a fresh solution. When reducing a sample containing urea, incubate the tubes at 37 °C for 1 hour (step 5 on page 2-7)	• BES • BICINE • Boric acid • CHES • DIPSO • EPPS • HEPBS • HEPES • HEPPSO • MOBS • MOPS • Phosphate buffer (excluding ammonium phosphate) • PIPES • POPSO

Modifications

If the protocol test fails, modify your sample preparation or the protocol according to the guidelines in the *xTRAQ Family of Amine - Modifying Labeling Reagents for Multiplexed Relative and Absolute Quantification: Chemistry Reference Guide.*

The iTRAQ Reagents – 8plex labeling protocol provides conditions for labeling 20 to 100 µg of protein digest with one unit (one vial, 2 µmol) of iTRAQ Reagent – 8plex. If you modify the labeling protocol, keep in mind that:

- The concentration of the following components must be within the indicated ranges during the labeling reaction ("Labeling the Protein Digests with the iTRAQ® Reagents – 8plex" on page 2-9):
  - Protein digest, 0.5 to 1 mg/mL
  - Buffer (Dissolution Buffer or alternative buffer), 90 to 100 mM
  - iTRAQ® Reagent, 40 mM ± 5%
  - Total organic content concentration, greater than 65%
- Sample source and preparation affect the amount of protein that can be labeled. Some samples, such as plasma, contain non-peptide amines that are not measurable using proteins assays such as BCA and Bradford. The non-peptide amines can react with iTRAQ Reagents – 8plex, competing with peptide derivitization. It may be necessary to decrease the amount of sample accordingly. For example, although 100 µg of a yeast sample is likely to be efficiently labeled with one unit of iTRAQ Reagent – 8plex, to obtain the same labeling efficiency with a plasma sample may require decreasing the sample size.
- Each vial of iTRAQ Reagent – 8plex is filled and capped under an inert gas to protect the reagent from moisture. Preparing aliquots of the reagent for future use can result in moisture entering the vial and hydrolyzing the reagent over time, leading to poor labeling efficiency.

Running the Labeling Protocol

The iTRAQ Reagents – 8plex labeling protocol involves:

- Reducing the sample, denaturing the sample, and blocking the cysteines
- Digesting the proteins with trypsin
- Labeling the digested proteins with iTRAQ Reagents – 8plex
- Combining the labeled digests into one sample mixture

Reducing the Proteins and Blocking Cysteine

WARNING **CHEMICAL HAZARD.** Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
Denaturant (2% SDS) causes eye and skin irritation.
Reducing Reagent causes eye, skin, and respiratory tract irritation.
Cysteine Blocking Reagent is a flammable liquid and vapor. Exposure causes eye and respiratory tract irritation and central nervous system depression.

IMPORTANT! If your sample contains thiols, perform acetone precipitation (see "Preparing Your Sample" on page 2-2).

1. To each of up to eight sample tubes (each containing 20 to 100 µg of protein), add 20 µL of the Dissolution Buffer from the kit.

   IMPORTANT! For samples that may contain non-protein amines, use smaller amounts of sample. (See "Modifications" on page 2-6.)

2. Add 1 µL of the Denaturant from the kit and vortex to mix.

   IMPORTANT! If your sample is insoluble, see "Sample Solubility" on page 2-4.

3. To each sample tube, add 2 µL Reducing Reagent.
4. Vortex to mix, then spin.
5. Incubate the tubes at 60 °C for 1 hour.
6. Spin to bring the sample to the bottom of the tube.
7. To each tube, add 1.0 µL Cysteine Blocking Reagent.

8. Vortex to mix, then spin.
9. Incubate the tubes at room temperature for 10 minutes.

Digesting the Proteins with Trypsin

WARNING **CHEMICAL HAZARD. Trypsin** causes eye, skin, and respiratory tract irritation. Exposure may cause an allergic reaction. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

IMPORTANT! If necessary, perform acetone precipitation to remove contaminants that may interfere with trypsin digestion or iTRAQ Reagents – 8plex labeling (see "Preparing Your Sample" on page 2-2).

1. For a 4plex experiment, reconstitute 1 to 2 vials of trypsin with 25 µL of Milli-Q® water or equivalent. For an 8plex experiment, reconstitute 2 to 4 vials of trypsin.
2. Vortex to mix, then spin.
3. To each sample tube, add 2 to 10 µL of the trypsin solution.
4. Vortex to mix, then spin.
5. Incubate the tubes at 37 °C overnight (12 to 16 hours).
6. Spin to bring the protein digest to the bottom of the tube.

Labeling the Protein Digests with the iTRAQ® Reagents – 8plex

WARNING **CHEMICAL HAZARD.** Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Isopropanol is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry skin and cause irritation. Exposure may cause central nervous system effects such as drowsiness, dizziness, and headache.

iTRAQ Reagents - 8plex 113-119 and 121 cause eye and respiratory tract irritation. Exposure may cause blood damage.

1. Freshly prepare iTRAQ Reagent – 8plex:
   a. Allow each required vial of iTRAQ Reagent – 8plex to reach room temperature.
   b. Spin to bring the solution to the bottom of the vial.
   c. Add 50 µL of isopropanol to each room-temperature vial of iTRAQ Reagent – 8plex.
   d. Vortex each vial to mix, then spin.
2. Transfer the contents of one freshly prepared iTRAQ Reagent – 8plex vial to one sample tube.

   For example, for a duplex-type experiment, transfer the contents of the iTRAQ Reagent – 8plex 114 vial to the sample 1 protein digest tube and transfer the contents of the iTRAQ Reagent – 8plex 117 vial to the sample 2 protein digest tube.
3. Vortex each tube to mix, then spin.
4. Test the pH by placing 0.5 µL of the solution on pH paper with a pH range 7.0 to 10.0. If necessary, add up to 5 µL of Dissolution Buffer to adjust the pH to 7.5 to 8.5 (maintain organic concentration at a minimum of 60%).

   IMPORTANT! For optimal labeling efficiency, the pH must be greater than 7.5. If the pH is less than 7.5, labeling efficiency can be significantly reduced. Be sure to check and adjust pH as specified.
5. Incubate the tubes at room temperature for 2 hours.

Combining the iTRAQ® Reagents – 8plex-Labeled Samples

CHEMICAL HAZARD. **iTRAQ Reagent - 8plex 113-119 and 121** cause eye and respiratory tract irritation. Exposure may cause blood damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1. Combine the contents of each iTRAQ Reagent – 8plex-labeled sample tube into one tube.‡
2. Vortex to mix, then spin.

Note: Unless you immediately continue to the next chapter (to analyze the sample mixture), store the sample mixture at –20 °C or colder. Analyze the sample mixture within a few days.

‡ (Optional) Before combining the samples, you can analyze an aliquot of each sample by MS/MS to verify the presence of iTRAQ Reagent – 8plex-labeled peptides. Before analyzing, reduce the organic solvent concentration (see step 1 on page 3-3), then clean up the sample using a ZipTip®. In MS/MS analysis, verify that you see peaks at the m/z of the appropriate iTRAQ Reagent – 8plex reporter group. If not, relabel the protein digest.

iTRAQ® Reagents – 8plex - Sample Mixture Analysis

3

This chapter covers:

Preparing the Sample Mixture for LC/MS/MS Analysis

The following substances in an iTRAQ® Reagent – 8plex-labeled sample mixture may interfere with LC/MS/MS analysis:

- Dissolution Buffer (0.5 M TEAB)
- Organic solvent (isopropanol and acetonitrile) approximately 65%
- 1 mM Reducing Reagent (tris-(2-carboxyethyl) phosphine (TCEP)
- 0.02% SDS
- 5 mM calcium chloride
- Excess iTRAQ Reagents – 8plex

Before you perform LC/MS/MS analysis, clean up the sample mixture using cation-exchange chromatography. For a simple sample mixture, use a cation-exchange cartridge system such as our cation-exchange cartridge system (P/N 4326747). The procedure is summarized on page 3-3. For more information, see the *xTRAQ Family of Amine -Modifying Labeling Reagents for Multiplexed Relative and Absolute Quantification: Chemistry Reference Guide.*

In addition to cleanup, complex sample mixtures may require fractionation. Use a high-resolution cation-exchange column to clean up and fractionate the mixture in one process. For more information, see *xTRAQ Family of Amine -Modifying Labeling Reagents for Multiplexed Relative and Absolute Quantification: Chemistry Reference Guide.*

Performing Cation-Exchange Chromatography

The procedure below describes performing cation-exchange chromatography using the Cation-Exchange Cartridge System. For information about required materials and assembling, washing, and storing the cartridge, see *xTRAQ Family of Amine -Modifying Labeling Reagents for Multiplexed Relative and Absolute Quantification: Chemistry Reference Guide.*

CHEMICAL HAZARD. Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Cation-Exchange Buffer–Load, Cation-Exchange Buffer–Elute, Cation-Exchange Buffer–Clean, and Cation-Exchange Buffer-Storage contain acetonitrile, a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause blood damage. Keep away from heat, sparks, and flame.

- **Load** – 10 mM potassium phosphate (KH_2PO_4) in 25% acetonitrile at pH 3.0
- **Elute** – 10 mM KH_2PO_4 in 25% acetonitrile/350 mM potassium chloride (KCl) at pH 3.0
- **Clean** – 10 mM KH_2PO_4 in 25% acetonitrile/1 M KCl at pH 3.0
- **Storage** – 10 mM KH_2PO_4 in 25% acetonitrile at pH 3.0, + 0.1% sodium azide (NaN_3)

iTRAQ® Reagents – 8plex 113-119 and 121 cause eye and respiratory tract irritation. Exposure may cause blood damage.

1. Reduce the concentrations of buffer salts to less than 20 mM and reduce organics by diluting the sample mixture at least 10 fold with Cation-Exchange Buffer-Load.

   Alternatively, if you are using a volatile buffer, evaporate the sample mixture to less than 30 µL. Reconstitute the sample mixture with 4 mL Cation-Exchange Buffer-Load.

2. Vortex to mix.

3. Remove an aliquot and check that the pH is between 2.5 and 3.3. If not, adjust the pH by adding more Cation-Exchange Buffer–Load or 1 N phosphoric acid.

4. Condition the cartridge by injecting 1 mL of the Cation-Exchange Buffer–Clean. Divert to waste.
5. Inject 2 mL of the Cation Exchange Buffer–Load. Divert to waste.
6. Slowly inject (≈1 drop/second) the diluted sample mixture onto the cation-exchange cartridge and collect the flow-through in a sample tube.
7. Inject 1 mL of the Cation-Exchange Buffer–Load to wash the TCEP, SDS, calcium chloride, and excess iTRAQ Reagents from the cartridge. Collect the flow-through in a sample tube.

   IMPORTANT! Keep the flow-through until you verify by MS/MS analysis that loading on the cation-exchange cartridge was successful. If loading fails, you can repeat loading using the flow-through after you troubleshoot the cause of the loading failure.
8. To elute the peptides, slowly inject (~1 drop/second) 500 µL of the Cation-Exchange Buffer–Elute. Capture the eluate in a clean 1.5-mL tube. Collect the eluted peptides as a single fraction.
9. Wash the undigested proteins such as trypsin from the cation-exchange cartridge by injecting 1 mL of the Cation-Exchange Buffer–Clean. Divert to waste.
10. Inject 2 mL of the Cation Exchange Buffer–Load. Divert to waste.

Repeat for additional sample mixtures. After completing all sample mixtures, inject 2 mL of the Cation Exchange Buffer–Storage and store the cartridge as recommended.

Guidelines and Tutorials for LC/MS/MS Analysis

For guidelines on separating peptides by capillary or nano reversed-phase HPLC using an AB SCIEX system, see the tutorials that are provided with your system that describe conditions required for specific techniques (for example, see “Using the Agilent nanoLC System.”)

AB Sciex Pte. Ltd.
110 Marsh Road
Foster City, CA 94404 USA
+1 877.740.2129

Printed in USA, 08/2010
Part Number 4375249 Rev. C