

iTRAQ® Reagents

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

Protocol

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Safety Information


Safety Alert Words

Four safety alert words appear in 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:


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 Safety Data Sheets (SDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See “About SDSs” 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 SDS.
- 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 SDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer’s cleanup procedures as recommended on the SDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About SDSs

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

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

Obtaining SDSs

You can obtain the SDS for any chemical supplied with this kit at www.sciex.com.

Chemical Waste Hazard



WARNING CHEMICAL WASTE HAZARD. Some wastes produced by the operation of the instrument or system are potentially hazardous and can cause injury, illness, or death.

Chemical Waste Safety Guidelines

To minimize the hazards of chemical waste:

- Read and understand the Safety Data Sheets (SDSs) 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 SDS.
 - 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 SDS.
 - 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



WARNING

BIOHAZARD. Biological samples such as tissues, body fluids, 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 eyewear, clothing, and gloves. Read and follow the guidelines in these publications:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; <http://bmb1.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).

Additional information about biohazard guidelines is available at: <http://www.cdc.gov>

Introduction to iTRAQ[®] Reagents Chemistry

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This chapter covers:

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Overview

iTRAQ® Reagents are provided as a set of four, isobaric (same mass) reagents:

- iTRAQ® Reagent 114
- iTRAQ® Reagent 115
- iTRAQ® Reagent 116
- iTRAQ® Reagent 117

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

Key Features

Using iTRAQ® Reagents to label peptides allows you to:

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

ProteinPilot™ Software for the AB SCIEX instruments provides data interpretation for relative and absolute quantitation.

Available iTRAQ® Reagents Kits

- **iTRAQ® Reagents Methods Development Kit** – Contains iTRAQ® Reagents 114 and 117 and sufficient material to develop a robust protocol and verify that your sample preparation protocol does not interfere with labeling and digestion. First orders also contain cation-exchange buffers, cartridge, and hardware.
- **iTRAQ® Reagents Multiplex Kit** – Contains sufficient iTRAQ® Reagents (114, 115, 116, and 117) for 10 duplex, 6 three-plex, or 5 four-plex experiments.

Workflow

In the iTRAQ[®] Reagents labeling protocol, you reduce, cysteine block, digest, and label each sample in a single tube. The single-tube process eliminates potential sample loss in individual samples that may cause inaccuracies in quantitation.

Then you combine all iTRAQ[®] Reagent-labeled samples into one sample mixture for LC-MS/MS analysis. If losses occur during analysis, each sample experiences the same loss and the ratios are preserved.

Figure 1-1 summarizes the iTRAQ[®] Reagents workflow for a duplex-type experiment. Up to four samples can be prepared and analyzed in a single experiment.

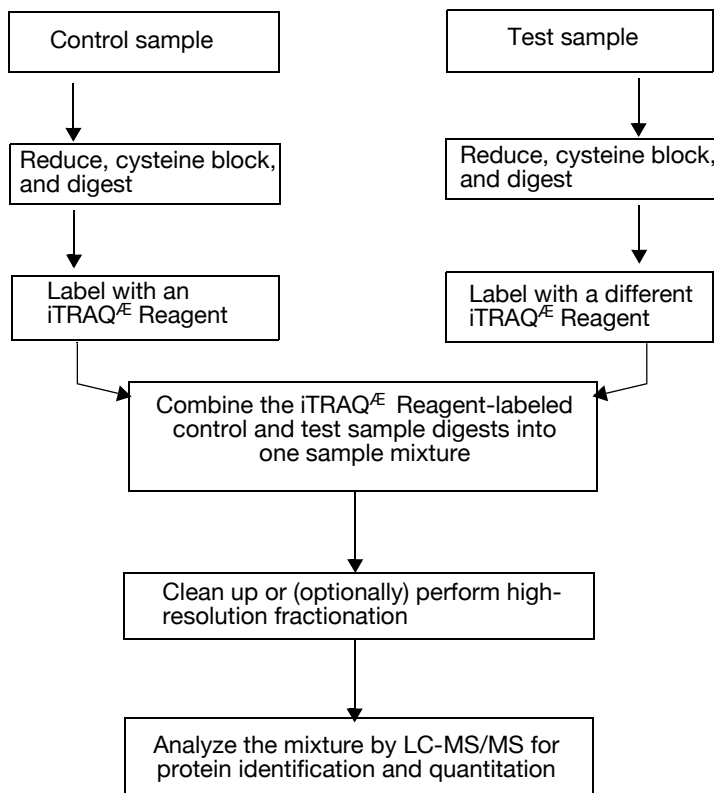


Figure 1-1 Overview of iTRAQ[®] Reagents methodology

This chapter covers:

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

Before You Begin

Preparing Your Sample If your sample contains a substance that may interfere with the iTRAQ® Reagents protocol (Table 2-1), perform acetone precipitation to clean up the sample.

Table 2-1 Substances that may interfere with the iTRAQ® Reagents 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, after reducing the protein and blocking cysteine.
Active proteases		
Primary amines (for example, those in: <ul style="list-style-type: none"> • Ammonium acetate • Ammonium bicarbonate • Ammonium citrate • Ammonium tartrate • AMPD [2-amino-2-methyl-1,3-propanediol] • Aminoguanidine bicarbonate salt • AMP [2-amino-2-methyl-1-propanol] • Ethanolamine, gly-gly, tris) 	React with iTRAQ® Reagents, interfering with labeling.	Before trypsin digestion.

If your sample is insoluble after adding Dissolution Buffer and Denaturant (steps 1 and 2 on page 2-5), choose an alternative detergent/denaturant or buffer (Table 2-2).

Table 2-2 Recommended alternative detergent/denaturant and buffers

Alternative Detergent/Denaturant (Concentration Limit at Trypsin Digestion)	Alternative Buffer
SDS (0.05%) OG (octyl B-D-glucopyranoside) (0.1%) NP [®] -40 (0.1%) Triton [®] X-100 (0.1%) Tween [®] 20 (0.1%) CHAPS (0.1%) Urea (<1M) Note: 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 2-5).	BES BICINE Boric acid CHES DIPSO EPPS HEPBS HEPES HEPPSO MOBS MOPS Phosphate Buffered Saline PIPES POPSO

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 SDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To clean up a sample by acetone precipitation:

1. Chill acetone 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 precipitate forms (30 minutes to four hours).
5. Decant the acetone. Do not dry.
6. Use the precipitated pellet as your sample in “Reducing the Proteins and Blocking Cysteine,” step 1, page 2-5.

Testing the Protocol

If you are running the protocol for the first time, it is strongly recommended that you run your Control sample through the entire protocol before you run an actual experiment. If you lack enough control sample to test the protocol, prepare a sample using your sample conditions and the Six-Protein Mix provided in the Methods Development Kit. Label the sample with one of the iTRAQ® Reagents.

Successful analysis of the iTRAQ® Reagent-labeled Control sample verifies that your sample preparation protocol does not interfere with digestion and iTRAQ® Reagents labeling. If the analysis fails, modify your sample preparation or the protocol.

Running the Protocol

Running the iTRAQ[®] Reagents protocol involves:

- Reducing the sample, denaturing the sample, and blocking the cysteines
- Digesting the proteins with trypsin
- Labeling the digested proteins with iTRAQ[®] Reagents
- Combining the labeled digests into one sample mixture

Reducing the Proteins and Blocking Cysteine



WARNING CHEMICAL HAZARD. Read the SDS, 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 four sample tubes containing 5 to 100 μg of sample (or the precipitated pellet from acetone precipitation), add 20 μL Dissolution Buffer.
2. Add 1 μL of the Denaturant in the kit and vortex to mix.
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 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 SDS, 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 labeling (see “Preparing Your Sample” on page 2-2).

1. Reconstitute a vial of trypsin with 25 μL of Milli-Q® water or equivalent. (If preparing three to four samples, reconstitute two vials.)
2. Vortex to mix, then spin.
3. To each sample tube, add 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 sample digest to the bottom of the tube.

Note: In order to maximize labeling efficiency, the volume of the sample digest must be less than 50 μL . If the volume of the sample digest is greater than 50 μL , dry the sample in a centrifugal vacuum concentrator, then reconstitute with 30 μL Dissolution Buffer.

Labeling the Protein Digests with the iTRAQ[®] Reagents



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

Ethanol is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause central nervous system depression and liver damage.

iTRAQ[®] Reagents 114-117 cause eye and respiratory tract irritation. Exposure may cause blood damage.

1. Allow each vial of iTRAQ[®] Reagent required to reach room temperature.
2. Spin to bring the solution to the bottom of the tube.
3. Add 70 μ L of ethanol to each room-temperature iTRAQ[®] Reagent vial.
4. Vortex each vial to mix, then spin.
5. Transfer the contents of one iTRAQ[®] Reagent vial to one sample tube.

For example, for a duplex-type experiment, transfer the contents of the iTRAQ[®] Reagent 114 vial to the sample 1 protein digest tube and transfer the contents of the iTRAQ[®] Reagent 117 vial to the sample 2 protein digest tube.

6. Vortex each tube to mix, then spin.
7. Incubate the tubes at room temperature for 1 hour.

Combining the iTRAQ[®] Reagent-Labeled Digest Samples



WARNING **CHEMICAL HAZARD.** iTRAQ[®] Reagents 114-117 cause eye and respiratory tract irritation. Exposure may cause blood damage. Read the SDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1. Combine the contents of each iTRAQ[®] Reagent-labeled sample tube into one tube.^a
2. Vortex to mix, then spin.

a. (*Optional*) Before combining the samples, you can analyze an aliquot of each sample by MS/MS to confirm the presence of iTRAQ[®] Reagent-labeled peptides. Before analyzing, reduce the organic concentration, 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 reporter group. If not, relabel the protein digest.

Preparing the Sample Mixture for LC-MS/MS Analysis

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

- Dissolution Buffer
- 75% organic solvent (ethanol and acetonitrile)
- 1 mM Reducing Reagent (tris-(2-carboxyethyl) phosphine [TCEP])
- 0.02% SDS
- 5 mM calcium chloride
- Excess iTRAQ[®] Reagents

Before you perform LC-MS/MS analysis, clean up the sample mixture using cation exchange chromatography. For a simple sample mixture, use the cation exchange system provided in the Methods Development Kit (first order only). See the procedure below. For a complex mixture, use high-resolution cation exchange chromatography.

Preparing a Simple Sample Mixture for LC-MS/MS Analysis

This section describes using the cation exchange system in the Methods Development Kit (first order only).



WARNING CHEMICAL HAZARD. Read the SDS, 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. iTRAQ® Reagents **114-117** cause eye and respiratory tract irritation. Exposure may cause blood damage. Read the SDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Performing Cation-Exchange Chromatography

1. Reduce the concentrations of buffer salts and organics by diluting the sample mixture at least 10 fold with Cation Exchange Buffer–Load.
2. Vortex to mix.
3. Check the pH using pH paper. If the pH is not between 2.5 and 3.3, adjust by adding more Cation Exchange Buffer–Load.
4. To condition the cartridge, inject 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 (\approx 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 the same sample tube used in step 6.

(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 μ L of the Cation Exchange Buffer–Elute. Capture the eluate in a fresh 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. If you have additional sample mixtures, repeat steps 5 through 8. If you do not have additional sample mixtures, store the cartridge as described below.

Storing the Cation-Exchange Cartridge

1. After cleaning the cartridge (step 9 above), inject 2 mL of the Cation Exchange Buffer–Storage.
2. Remove the cartridge, then seal the ends of the cartridge with the two end caps.
3. Record the number of times the cartridge has been used.
4. Store the cartridge at 2 to 8 °C.
5. Clean the needle-port adapter, outlet connector, and syringe with water.

NOTES

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