iTRAQ® Reagents Application Kit - Plasma
Amine-Modifying Labeling Reagents for Plasma Sample Applications

Protocol

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Preface

This preface contains:

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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:

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 (MSDS) 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 on 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.

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 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

**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; [http://bmbl.od.nih.gov](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](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](http://www.cdc.gov)
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.

To contact technical support:

- By telephone: Dial 1.877.740.2129
- By fax: Dial 1.650.627.2803
This chapter covers:

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Workflow ............................................................ 1-3
User-Supplied and Kit Materials .............................. 1-5
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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

AB SCIEX mass spectrometry systems provide ProteinPilot™ software features designed for easy iTRAQ® Reagent applications data interpretation for relative and absolute quantitation.

Kit Description

- iTRAQ® Reagents Application Kit - Plasma – Contains sufficient iTRAQ® Reagents (114, 115, 116, and 117) for 8 duplex or 4 four-plex experiments, based on 50 μg of protein per sample.
Workflow

In the iTRAQ® Reagents labeling protocol for plasma applications, you prepare the sample (remove lipids (if necessary), remove abundant proteins, desalt), then reduce, block cysteine residues, 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 - plasma methodology
# User-Supplied and Kit Materials

## Table 1-1 User-supplied materials

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity per Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disposable gloves</td>
<td>As needed</td>
</tr>
<tr>
<td>Test samples (for example, treated plasma; up to three samples)</td>
<td>50 µg protein each (or multiples of 50 µg protein)</td>
</tr>
<tr>
<td>Control sample (for example, a normal plasma)</td>
<td>50 µg protein (or multiples of 50 µg protein)</td>
</tr>
<tr>
<td>Pipettors and tips suitable for 1 µL to 1 mL</td>
<td>As needed</td>
</tr>
<tr>
<td>Fraction-collection tubes and rack</td>
<td>3 to 6 per assay</td>
</tr>
<tr>
<td>• Screw or snap-cap tubes, 0.5 to 2 mL for running the iTRAQ&lt;sup&gt;®&lt;/sup&gt; Reagent protocol</td>
<td></td>
</tr>
<tr>
<td>• Fraction-collection tubes</td>
<td>As needed</td>
</tr>
<tr>
<td>Materials needed for delipidation (if necessary)</td>
<td>As needed</td>
</tr>
<tr>
<td>Devices and/or columns for removing abundant proteins. For example:</td>
<td>1</td>
</tr>
<tr>
<td>• Agilent Multiple Affinity Removal System</td>
<td></td>
</tr>
<tr>
<td>• GenWay Seppro™ MIXED 12 LC column</td>
<td></td>
</tr>
<tr>
<td>Filter or column for desalting</td>
<td>As needed</td>
</tr>
<tr>
<td>For example:</td>
<td></td>
</tr>
<tr>
<td>• Spin filters</td>
<td></td>
</tr>
<tr>
<td>• Reversed-phase column (for example, POROS® 50 R1 column (see Appendix A))</td>
<td></td>
</tr>
<tr>
<td>Iodoacetamide (see “Reducing the Proteins and Blocking Cysteine Residues” on page 2-7)</td>
<td>20 mg per day of testing</td>
</tr>
<tr>
<td><strong>IMPORTANT!</strong> Follow the manufacturer’s instructions for storage and shelf life.</td>
<td></td>
</tr>
<tr>
<td>Amber tubes or foil to wrap tubes (see “Reducing the Proteins and Blocking Cysteine Residues” on page 2-7)</td>
<td>As needed</td>
</tr>
</tbody>
</table>
Chapter 1  

iTRAQ® Reagents for Plasma Sample Applications

Table 1-1  User-supplied materials (continued)

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity per Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin, TPCK (Trypsin treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) to inactivate any remaining chymotryptic activity (for example, P/N 4370285).)</td>
<td>1 package (25 µg trypsin per vial, 8 vials per kit)</td>
</tr>
<tr>
<td>High-resolution cation-exchange column</td>
<td>1</td>
</tr>
<tr>
<td>For example: PolySulfoethyl A Column, 5 micron 200 Å bead, from PolyLC, Inc., 4.6 x 100 mm, P/N 104SE0502. Select a column size with the appropriate binding capacity for your sample size.)</td>
<td>As needed</td>
</tr>
<tr>
<td>Suggested buffers (in Milli-Q® water or equivalent):</td>
<td>Note: Buffer A can also used for loading.</td>
</tr>
<tr>
<td>• Buffer A – 10 mM potassium phosphate (KH$_2$PO$_4$), 25% acetonitrile, pH adjusted to less than 3.0. Store at 2 to 8 °C.</td>
<td></td>
</tr>
<tr>
<td>• Buffer B – 10 mM potassium phosphate (KH$_2$PO$_4$), 1 M potassium chloride (KCl), 25% acetonitrile, pH adjusted to less than 3.0. Store at 2 to 8 °C</td>
<td></td>
</tr>
<tr>
<td>pH paper:</td>
<td>As needed</td>
</tr>
<tr>
<td>• pH range 6.5 to 10 (to test the pH of the sample when labeling)</td>
<td></td>
</tr>
<tr>
<td>• pH paper with pH range 2.5 to 4.5 (to test the pH of the sample before loading on the cation-exchange cartridge)</td>
<td></td>
</tr>
<tr>
<td>Milli-Q® water or equivalent (minimum 18.2 MOhms water, conductivity maximum 0.05 µS/0.05 µMho)</td>
<td>50 mL</td>
</tr>
<tr>
<td>Heating block or oven, 60 °C</td>
<td>1</td>
</tr>
<tr>
<td>Incubator, 37 °C</td>
<td>1</td>
</tr>
<tr>
<td>Bench-top centrifuge</td>
<td>1</td>
</tr>
<tr>
<td>Vortexer</td>
<td>1</td>
</tr>
<tr>
<td>Centrifugal vacuum concentrator</td>
<td>1</td>
</tr>
<tr>
<td>Mass spectrometer with analysis software</td>
<td>1</td>
</tr>
<tr>
<td>For example: AB SCIEX 5800 MALDI TOF/TOF™ Instrument with ProteinPilot™ Software.</td>
<td></td>
</tr>
<tr>
<td>Capillary reversed-phase HPLC system</td>
<td>1</td>
</tr>
</tbody>
</table>
User-Supplied and Kit Materials

Table 1-1 User-supplied materials (continued)

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity per Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>If you analyze using Nanospray® ESI source mass spectrometry, either of the following tips:</td>
<td></td>
</tr>
<tr>
<td>• New Objective, Inc. coated fused-silica PicoTips® (coating applied to tip end, Cat. #FS360-20-10-CE-20. Also requires tubing fitting from LC Packings, Cat. #TF-250/350.</td>
<td></td>
</tr>
<tr>
<td>• New Objective, Inc. distal coated fused-silica PicoTips, Cat. #FS360-20-10-D-20.</td>
<td></td>
</tr>
</tbody>
</table>

xTRAQ Family of Amine -Modifying Labeling Reagents for Multiplexed Relative and Absolute Quantification: Chemistry Reference Guide. | NA |

Kit Materials

IMPORTANT! When you receive the shipping container, immediately remove the reagent box from the container and store it at –15 to –25 °C.

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

Table 1-2 iTRAQ® Reagent kit materials and storage conditions

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Store at –15 to –25 °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iTRAQ® Reagent Reagent 114</td>
<td>4 vials, 1 unit/vial</td>
<td>Amine-modifying labeling reagent. One unit (one vial) of reagent labels 50 µg of protein.a</td>
</tr>
<tr>
<td>iTRAQ® Reagent Reagent 115</td>
<td>4 vials, 1 unit/vial</td>
<td></td>
</tr>
<tr>
<td>iTRAQ® Reagent Reagent 116</td>
<td>4 vials, 1 unit/vial</td>
<td></td>
</tr>
<tr>
<td>iTRAQ® Reagent Reagent 117</td>
<td>4 vials, 1 unit/vial</td>
<td></td>
</tr>
<tr>
<td>Sample Diluent (PBS Buffer, pH 7.4)</td>
<td>3 vials, 1.8 mL/vial</td>
<td>Phosphate Buffered Saline. Contains phosphate 10 mM, NaCl 138 mM, KCl 2.7 mM, pH 7.4 at 25 °C</td>
</tr>
<tr>
<td>Sample Buffer - Plasma</td>
<td>1 vial, 1.5 mL/vial</td>
<td>Dissolves the sample and buffers the reaction. Contains 1.0 M triethylammonium bicarbonate.</td>
</tr>
</tbody>
</table>
### Table 1-2  iTRAQ® Reagent kit materials and storage conditions (continued)

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reducing Reagent</td>
<td>1 vial, 100 µL/vial</td>
<td>Reduces the disulfide bonds of the proteins. Contains 50 mM tris-(2-carboxyethyl)phosphine (TCEP).</td>
</tr>
<tr>
<td>Denaturant (2% SDS Solution)</td>
<td>1 vial, 50 µL/vial</td>
<td>Disrupts the hydrogen, hydrophobic, and electrostatic bonds of the proteins. Contains 2% SDS.</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1 vial, 1.8 mL</td>
<td>Absolute, HPLC-grade or better. Used to dissolve the iTRAQ® Reagents and optimize labeling.</td>
</tr>
<tr>
<td><strong>iTRAQ® Reagents Application Kit - Plasma Protocol</strong></td>
<td>—</td>
<td>Describes how to label plasma samples with iTRAQ® Reagents.</td>
</tr>
<tr>
<td>Certificate of Analysis</td>
<td>1</td>
<td>Provides iTRAQ® Reagents isotopic purity.</td>
</tr>
</tbody>
</table>

a. To scale up, increase the sample size in multiples of 50 µg. Then multiply the number of vials of iTRAQ® Reagent and reagent amounts correspondingly. For example, for a sample containing 100 µg protein, use two vials of the iTRAQ® Reagent to label that sample, and double the reagent amounts throughout the protocol.
Related Documentation

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

- iTRAQ® Reagents chemistry
- iTRAQ® Reagents kits and kit materials
- How to test, run, and modify the iTRAQ® Reagents protocol
- Sample handling guidelines
- Suggested LC/MS/MS conditions
- Software tools available for performing quantification

To obtain PDF versions of the chemistry reference guide and this protocol go to [http://www.sciex.com](http://www.sciex.com), then click the link for Support.
This chapter covers:

Preparing the Samples .......................... 2-2
Testing the Protocol ............................ 2-6
Running the Protocol ............................ 2-7
Performing LC/MS/MS Analysis ............... 2-11
This chapter describes preparing your sample, running the iTRAQ® Reagents protocol, and analyzing the sample mixture for using cation-exchange chromatography and LC/MS/MS.

The xTRAQ Family of Amine -Modifying Labeling Reagents for Multiplexed Relative and Absolute Quantification: Chemistry Reference Guide provides additional information about the iTRAQ® Reagent chemistry, protocol, and analysis process.

Preparing the Samples

Preparing the test and control samples involves:

• Removing the lipids (if necessary)
• Removing (depleting) the abundant proteins
• Desalting (if necessary)
• Testing the protocol (optional)
• Determining sample amount and removing solvents

Perform lipid removal before depleting of abundant proteins. High lipid concentrations in your samples may reduce the overall performance of removing abundant protein/desalting by mixed-bed antibody and/or reversed-phase column(s). High lipid concentration may also interfere with the efficiency of the peptide labeling.

⚠️ 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.
Preparing the Samples

Removing the Lipids

To optimize sample preparation, delipidate the samples before depleting and desalting. To have an adequate protein concentration in your sample after delipidation, depletion, and desalting, start with approximately 50 µL of plasma sample. Approximately 50 µL of plasma sample, prepared as described in this section, yields a sufficient amount of protein to determine the protein content and to run the labeling protocol.

For an LC/MS analysis with a 150 × 0.18 mm (3 micron) column, a 50 µL plasma sample yields sufficient material to enable approximately 2-3 sets of injections. For a nano LC analysis from 10-12 SCX fractions, a 50 µL plasma sample provides approximately 5-10 sets of injections.

Removing the Abundant Proteins

Of the total protein mass in plasma, the most abundant proteins comprise greater than 90%. The general success and reproducibility of this protocol depends upon the consistent removal of the most abundant proteins in the sample. Examples of devices and/or columns for removing abundant proteins include:

- Agilent Multiple Affinity Removal System product
- GenWay Seppro™ MIXED 12 LC column

Column Pressure Limit/Capacity

IMPORTANT! Follow the manufacturer’s instructions for using the columns. Do not exceed the column manufacturer’s suggested pressure limit or column capacity. If necessary, perform multiple runs.
Buffers to Avoid

The manufacturer’s recommended buffers may contain free amines that may interfere with iTRAQ® Reagent labeling. Buffers to avoid include:

- 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 buffers

If the recommended buffer contains free amines, try using a different buffer (see Appendix A). If tris buffered saline is recommended, try substituting phosphate buffered saline. For information, see the xTRAQ Family of Amine -Modifying Labeling Reagents for Multiplexed Relative and Absolute Quantification: Chemistry Reference Guide.

Desalting a Buffer Containing Free Amines

IMPORTANT! If using a buffer containing free amines that may interfere with the iTRAQ® Reagents protocol, you must desalt using either:

- **Spin column/filters** – Available from GenWay or Agilent.
- **Reversed-phase column** – Capture the depleted proteins on a reversed-phase column then wash to remove buffer salts and elute. See Appendix A, “Alternative Buffers and Desalting Techniques.”
- **Acetone precipitation** – See Appendix A, “Alternative Buffers and Desalting Techniques.”
PREPARING THE SAMPLES

Determining Sample Amount and Removing Solvents

**IMPORTANT!** In general, 1 tube of iTRAQ® Reagent labels approximately 100 μg of digested protein. During sample labeling, the free primary amine components potentially present in plasma can react with iTRAQ® Reagent. To optimize iTRAQ® Reagent-labeling of the proteins of interest, we recommend a sample amount containing approximately 50 μg of protein.

1. Determine protein content by performing a quantitative protein assay (for example, bicinchoninic acid [BCA] assay).

2. Transfer a volume of the sample containing approximately 50 μg of protein to a new sample tube.
   
   To scale up, increase the sample size in multiples of 50 μg. Then multiply the number of vials of iTRAQ® Reagent and reagent amounts correspondingly. For example, for a sample containing 100 μg protein, use two vials of the iTRAQ® Reagent to label the sample, and double the reagent amounts throughout the protocol.

3. Dry the sample in a centrifugal vacuum concentrator.
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.

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 according to the xTRAQ Family of Amine -Modifying Labeling Reagents for Multiplexed Relative and Absolute Quantification: Chemistry Reference Guide.
Running the Protocol

Running the iTRAQ® Reagents protocol for plasma applications involves:

- Reducing the proteins and blocking the cysteine residues
- Digesting the proteins with TPCK-treated trypsin
- Labeling the digested proteins with iTRAQ® Reagents
- Combining the labeled digests into one sample mixture

Reducing the Proteins and Blocking Cysteine Residues

⚠️ 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.

Note: This procedure includes a iodoacetamide reaction that must be incubated in the dark. Use amber tubes or wrap the tubes in foil.

1. To each of up to four sample tubes containing 50 µg of sample protein (from step 3 on page 2-5), add in sequence:
   - 25 µL Sample Buffer - Plasma.
   - 1 µL of the Denaturant (2% SDS)(in the kit), vortex to mix.
   - 2 µL Reducing Reagent.

2. Vortex to mix, then spin.

3. Incubate the tubes at 60 °C for 1 hour.

4. Spin to bring the solution to the bottom of the tube.

5. To each tube, add 1 µL of a freshly prepared 84 mM solution of iodoacetamide solution.

**IMPORTANT!** The 84 mM iodoacetamide solution must be freshly prepared to avoid the presence of the degradation by-product iodine that may cause your sample to oxidize.

Prepare the 84 mM iodoacetamide solution by dissolving 15.5 mg of iodoacetamide in 1 mL Milli-Q water or equivalent. (If you have the resources to accurately weigh smaller amounts, you can prepare a smaller volume.)
6. Vortex to mix, then spin.

7. Incubate the tubes in the dark at room temperature (for example, in a drawer) for 30 minutes. If necessary, wrap the tubes in foil.

**Digesting the Proteins with TPCK-Treated Trypsin**

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

1. For each sample, reconstitute 25 mg trypsin (one vial of Trypsin, TPCK, P/N 4370285) with 25 µL of Milli-Q® water or equivalent.

2. Vortex to mix, then spin.

3. To each sample tube, add 10 µL of freshly prepared Trypsin, TPCK solution.

4. Vortex to mix, then spin.

5. Incubate the tubes at 37 °C overnight (14 to 18 hours).

6. Spin to bring the sample digest solution to the bottom of the tube.
Labeling the Protein Digests with the iTRAQ® Reagents

**WARNING** CHEMICAL HAZARD. Read the MSDS 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.

**IMPORTANT!** To scale up, increase the sample size in multiples of 50 μg. Then multiply the number of vials of iTRAQ® Reagent and reagent amounts correspondingly.

For example, for a sample containing 100 μg protein, use two vials of the iTRAQ® Reagent to label the sample, and double the reagent amounts throughout the protocol.

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

For example, for a duplex-type experiment with a 50 μg protein sample, transfer the contents of an iTRAQ® Reagent 114 vial to the sample 1 protein digest tube and transfer the contents of a iTRAQ® Reagent 117 vial to the sample 2 protein digest tube.
6. Vortex each tube to mix, then spin.
7. Test the pH by placing 1 μL of the solution on pH paper with a pH range 8.0 to 10.0. If necessary, add up to 10 μL of Sample Buffer - Plasma to adjust the pH to greater than 8.

**IMPORTANT!** For the labeling reaction to achieve optimal efficiency, the pH must be greater than 8.
8. Incubate the tubes at room temperature for 1 hour.
9. To quench the reaction, add 100 µL of Milli-Q® water or equivalent.

10. Incubate the tubes at room temperature for 30 min.

11. Dry the samples in a centrifugal vacuum concentrator.

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

1. To each of the sample tubes, add 250 µL of your loading buffer (for example, 10 mM potassium phosphate (KH₂PO₄) in 25% acetonitrile at pH 3.0).

2. Vortex to mix, then spin.

3. Combine the contents of each iTRAQ® Reagent-labeled sample tube into one tube.¹

4. Vortex to mix, then spin.

5. Test the pH by placing 1 µl of the solution on pH paper with a pH range 2.5 to 4.5. If necessary, add more cation exchange loading buffer or 1.0 N phosphoric acid to adjust the pH to between 2.5 and 3.3.

**IMPORTANT!** For the labeled peptides to be retained on the cation-exchange column, the pH must be between 2.5 and 3.3. The salt concentration must be less than 15 mM.

¹. *(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.
Performing LC/MS/MS Analysis

1. Clean up and fractionate the pooled iTRAQ reagent-labeled peptides by injecting the appropriate volume of the mixture on a high-resolution cation-exchange column.
   
   For information, see the xTRAQ Family of Amine-Modifying Labeling Reagents for Multiplexed Relative and Absolute Quantification: Chemistry Reference Guide.

2. Collect a minimum of 10 fractions.

3. Analyze each fraction by LC/MS/MS.
   
   For information about analyzing with an AB SCIEX mass spectrometer, see the instrument or software user guide at www.absciex.com.
Alternative Buffers and Desalting Techniques

This chapter covers:

Alternative Buffers .................................................. A-2
Desalting Techniques ............................................... A-3
Alternative Buffers

Buffers listed in “Removing the Abundant Proteins” on page 2-3 contain free amines that may interfere with iTRAQ® Reagent labeling. If possible, substitute one of the following recommended alternative buffers:

- BES
- BICINE
- Boric acid
- CHES
- DIPSO
- EPPS
- HEPBS
- HEPES
- HEPPSO
- MOBS
- MOPS
- Phosphate Buffer
- PIPES
- POPSO

For the labeling reaction to achieve optimal efficiency, the pH must be greater than 8 and the buffer concentration must be at a level of at least 0.06 M. These 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.

Test the pH during labeling (step 7 on page 2-9), and, if necessary, add up to 10 μL of Sample Buffer - Plasma to adjust the pH to greater than 8.
Desalting Techniques

After desalting, be sure to determine protein content by performing a quantitative protein assay (for example, bicinchoninic acid [BCA] assay).

Reversed-Phase Column Chromatography

Reversed-Phase Column
POROS® 50 R1 column, 4.5mm × 50mm, self pack (follow the operating instructions carefully)

Mobile Phase and Cleaning Solutions

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

Formic Acid is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract burns. It is harmful if inhaled, and may cause allergic reactions. Keep away from heat, sparks, and flame.

Trifluoroacetic acid (TFA) causes eye, skin, and respiratory tract burns. It is harmful if inhaled.

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

Acetonitrile (ACN) is a flammable liquid and vapor. Exposure may cause eye and respiratory tract irritation and blood system damage.

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.

Suggested mobile phase and cleaning solutions are:

- **Mobile phase A (Load and Wash)** – 5% acetonitrile, 0.1% TFA in Milli-Q® water or equivalent
- **Mobile phase B (Elute)** – 95% acetonitrile, 0.1% TFA, in Milli-Q® water or equivalent
- **Cleaning Solution** — 70% formic acid, 30% isopropanol
Performing Reversed-Phase Chromatography

1. Load the flow-through from the depletion cartridge directly on to the POROS 50 R1 column.

2. Wash the POROS 50 R1 column with 6 column volumes of Mobile phase A.

3. Elute the desalted depletion sample from the POROS 50 R1 column with 5% Mobile phase A/95% Mobile phase B and collect a 2-column volume fraction.

4. With the 5% Mobile phase A/95% Mobile phase B flowing, clean the column by injecting 350 μL of Cleaning Solution three times.

5. Re-equilibrate the POROS 50 R1 column with Mobile phase A.

6. Dry the sample in a centrifugal vacuum concentrator.

7. Reconstitute the sample in Sample Buffer - Plasma.

Alternatively, you can use the following multi-dimensional liquid chromatography (MDLC) procedure.

1. Load sample on the depletion cartridge while set in tandem with the POROS 50 R1 column. Use Sample Diluent to transfer the flow-through on to the POROS 50 R1 column.

2. Set the POROS 50 R1 column off line.

3. Prior to collecting the desalted sample, elute and regenerate the depletion cartridge following the manufacturer’s instructions.

4. Wash the POROS 50 R1 column with 6 column volumes of Mobile phase A.

5. Elute the desalted depletion sample from the POROS 50 R1 column with 5% Mobile phase A/95% Mobile phase B and collect a 2-column volume fraction.

6. With the 5% Mobile phase A/95% Mobile phase B flowing, clean the column by injecting 350 μL of Cleaning Solution three times.

7. Re-equilibrate the POROS 50 R1 column with Mobile phase A.

8. Dry the sample in a centrifugal vacuum concentrator.

9. Reconstitute the sample in Sample Buffer - Plasma.
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 four 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 Residues,” step 1, page 2-7.