Applied Biosystems mTRAQ™ Reagents

Amine-Modifying Labeling Reagents for Relative and Absolute Protein Quantitation

Protocol

Applied Biosystems

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Contents

Preface and Important Safety Information

This preface covers:

Safety Information

Safety Alert Words

Four safety alert words appear in Applied Biosystems 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.

– 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.

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

– 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

CHEMICAL HAZARD. Some of the chemicals used with Applied Biosystems 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

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

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

2. In the Keyword Search field, enter the chemical name, product name, MSDS part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose

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

Chemical Waste Safety 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.
- 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; **bmbl.od.nih.gov**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **www.access.gpo.gov/ nara/cfr/waisidx_01/ 29cfr1910a_01.html**).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at **www.cdc.gov**.

How to Obtain More Information

Related Documentation

The Applied Biosystems mTRAQ™ Reagents Quick Reference Card, a laminated card that provides a quick reference to the steps in the labeling protocol, is shipped with the kit.

Portable document format (PDF) versions of this guide and supporting documentation for the Applied Biosystems/MDS Analytical Technologies analytical systems and software are also available (see "How to Obtain Support" on page ix).

Send Us Your Comments

Applied Biosystems welcomes your comments and suggestions for improving its user documents. You can e-mail your comments to:

techpubs@appliedbiosystems.com

The e-mail address above is only for submitting comments and suggestions relating to documentation. To order documents, download PDF files, or for help with a technical question, see "How to Obtain Support" on page ix.

How to Obtain Support

For the latest services and support information for all locations, go to **www.appliedbiosystems.com**, then click the link for **Support**.

At the Support page, you can:

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

In addition, the Support page provides access to worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.

Product Information 1

Purpose of the mTRAQ™ Reagents

The **Applied Biosystems** mTRAQ™ Reagent**s** Kit is a triplex set of non-isobaric (differing mass) amine labeling reagents useful for performing relative quantitation experiments of targeted proteins, peptides, and post-translational modifications by LC/MS using Multiple Reaction Monitoring (MRM).

The mTRAQ Reagents are based on the same chemical structure as the iTRAQ® Reagents - 4plex. The mTRAQ Reagent Δ4 has the identical structure and chemical composition as iTRAQ® Reagent 117 - 4plex.

The mTRAQ Reagent Δ0 has the same structure but lacks the stable isotopes (C13, N15, O18) resulting in a molecular weight 4 Da lower than the Δ4 reagent.

The mTRAQ Reagent Δ8 has the same structure as Δ0 and Δ4, but with additional stable isotopes (C13, N15 and O18) resulting in a molecular weight 4 Da higher than mTRAQ Reagent Δ4 and 8 Da higher than mTRAQ Reagent Δ0. Therefore, when analyzing by MRM, the same peptide labeled with each reagent is distinguishable in MS mode (three non-isobaric parent ions) and MS/MS mode (non-isobaric sequence ions).

Product Capabilities

With mTRAQ Reagents, internal standards are easy to create, eliminating the need for synthetic peptides. Typically, the internal standard is labeled with Δ8, and the test samples are labeled with Δ0 or Δ4. Two workflows are described in this protocol:

- **Reference Internal Standard (RIS)** – Use the RIS workflow when the reference standard is well defined. For example, to study the time course of protein expression changes, label a representative Time 0 protein sample with mTRAQ Reagent Δ8. This is the reference standard. All other samples at various time points are labeled with mTRAQ Reagent Δ0 or Δ4. Compare each of the Δ0 or Δ4-labeled sample time points to the Δ8-labeled Time 0 reference internal standard.
- **Global Internal Standard (GIS)** – Use the GIS strategy for a broad range of applications with large numbers of samples. Label a representative mix of control and test samples with mTRAQ Reagent Δ8 to create the GIS. All samples are labeled with mTRAQ Reagent Δ0 or Δ4. Compare each of the Δ0 and Δ4-labeled samples to the Δ8-labeled GIS mixture.

mTRAQ™ Reagents Assay Kit Capabilities

Seven kits containing mTRAQ Reagents Δ0, Δ4, and Δ8 are available:

- mTRAQ™ Reagent**s 10 Assay Kit** (PN 4374771)

  Contains mTRAQ Reagent Δ0, Δ4 and Δ8 sufficient to perform 10 assays. Includes 10 -1 unit vials of each of the 3 reagents.
- 3 separate kits, each containing sufficient mTRAQ Reagents to perform **10** assays. Each kit contains 10 1-unit vials of the appropriate reagent:

  mTRAQ™ Reagent Δ0 **10 Assay Kit** (PN 4440014)

  mTRAQ™ Reagent Δ4 **10 Assay Kit** (PN 4427696)

  mTRAQ™ Reagent Δ8 **10 Assay Kit** (PN 4427697)

- 3 separate kits, each containing sufficient mTRAQ Reagents to perform **50** assays. Each kit contains 50 1-unit vials of the appropriate reagent:

  mTRAQ™ Reagent Δ0 **50 Unit Pack** (PN 4440015)

  mTRAQ™ Reagent Δ4 **50 Unit Pack** (PN 4427698)

  mTRAQ™ Reagent Δ8 **50 Unit Pack** (PN 4427700)

The mTRAQ Reagent Kits provide you with three non-isobaric tags that differ by either 4 or 8 Daltons. You can use them as a triplex or a duplex. The kit configurations allow you to pick the most beneficial mass difference for your duplex experiments. When a duplex workflow is required, we recommend using the combination of the mTRAQ™ Reagent Δ0 and mTRAQ™ Reagent Δ8. This combination results in a larger delta mass between the labeled peptides over the other possible combinations. This is an advantage when monitoring MRMs to larger highly charged peptides.

To perform the labeling protocol, you must also order a buffer kit. The iTRAQ® Reagent **- 8Plex Buffer Kit** (PN 4381664) contains sufficient material to perform up to 80 mTRAQ™ Reagent assays.

mTRAQ Reagents Storage: Materials Included

IMPORTANT! When you receive the shipping container of mTRAQ Reagents vials, immediately remove it and store it at –20 °C.

IMPORTANT! When visually inspecting the reagent vials, the volume of material may appear to be insufficient. During shipment, small volumes of material occasionally become trapped in the cap of the vial. To dislodge the trapped material, allow the vial of reagent to reach room temperature, then briefly centrifuge it. Return the reagents to storage at –20 °C within 2 hours of thawing.

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 vi.

Table 1 10 and 50-Unit Pack Materials and Storage Conditions

Kit	Quantity of Reagent	Contents (Store at -20 °C)
10 Assay Kit	10 1-Unit vials of Δ0 10 1-Unit vials of Δ4 10 1-Unit vials of Δ8	Amine modifying reagent. One unit of reagent labels approximately 100 µg of protein.
Each Triplex kit includes one copy of **Applied Biosystems mTRAQ Reagents Protocol** (this document) and **Applied Biosystems mTRAQ Reagents Quick Reference Card** (a laminated card that provides a quick reference to the steps in the labeling protocol.)		
10 Assay Δ0	10 1-Unit vials of Δ0	Amine modifying reagent. One unit of reagent labels approximately 100 µg of protein.
10 Assay Δ4	10 1-Unit vials of Δ4	Amine modifying reagent. One unit of reagent labels approximately 100 µg of protein.
10 Assay Δ8	10 1-Unit vials of Δ8	Amine modifying reagent. One unit of reagent labels approximately 100 µg of protein.
50 Assay Δ0	50 1-Unit vials of Δ0	Amine modifying reagent. One unit of reagent labels approximately 100 µg of protein.
50 Assay Δ4	50 1-Unit vials of Δ4	Amine modifying reagent. One unit of reagent labels approximately 100 µg of protein.

Kit	Quantity of Reagent	Contents (Store at -20 °C)
50 Assay Δ8	50 1-Unit vials of Δ8	Amine modifying reagent. One unit of reagent labels approximately 100 µg of protein.
Each kit includes a **Certificate of Analysis** which provides purity information for each reagent.		

Table 2 Applied Biosystems 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. Provides organic solvent for optimizing labeling efficiency.
Certificate of Analysis	1	Provides the pH of the Dissolution Buffer, concentration of the Reducing Reagent, and purity information for the isopropanol.

mTRAQ Reagents Storage: Materials Not Included

Software

Several useful software packages are available from Applied Biosystems/MDS Analytical Technologies (see "How to Obtain Support" on page ix):

Table 3 Useful software packages

Task	Software
Select targets	ProteinPilot™ Software
Collect the MRM data	MRMPilot™ Software
Quantitate the MRM data	MultiQuant™ Software

Materials and Equipment

WARNING **CHEMICAL HAZARD.** Some of the chemicals referred to in this protocol (such as those in Table 4) are not provided with your kit. When using chemicals not provided by or purchased from Applied Biosystems, obtain the material safety data sheet directly from the chemical manufacturer.

Table 4 User-supplied materials

Item	Volume or Quantity per Assay
Disposable gloves	As needed
Test samples	20 to 100 µg protein
Control samples (containing the same amount of protein as the test samples)	20 to 100 µg protein
Trypsin with $CaCl_2$ (Applied Biosystems, 10 pack, P/N 4352157) or Trypsin without $CaCl_2$ (Applied Biosystems, 8 pack, P/N 4370285)	As needed, one vial (25 µg) digests 250 µg of protein.
Pipettors and tips suitable for 1 µL to 1 mL	As needed
autosampler vials	As needed
tubes	As needed
Syringe, 2.5- and 10-mL (2-inch blunt needle, 22-gauge)	1

Table 4 User-supplied materials

Item	Volume or Quantity per Assay
(Optional) Acetone for sample cleanup before labeling	As needed
(Optional) Cation-exchange cartridge system with 0.2 to 1 mL/min flow rate, such as the Applied Biosystems cation-exchange cartridge system (P/N 4326747) for labeled sample cleanup before MS/MS analysis	1
Fraction-collection tubes, 1.5- and 15-mL, and rack for performing cation-exchange chromatography	As needed
(Optional, for complex samples that require fractionation) 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 the 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
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, one of the AB SCIEX QTRAP® Systems with MRMPilot™ Software and MultiQuant™ Software) and reversed-phase HPLC system.	1

Workflow Overview

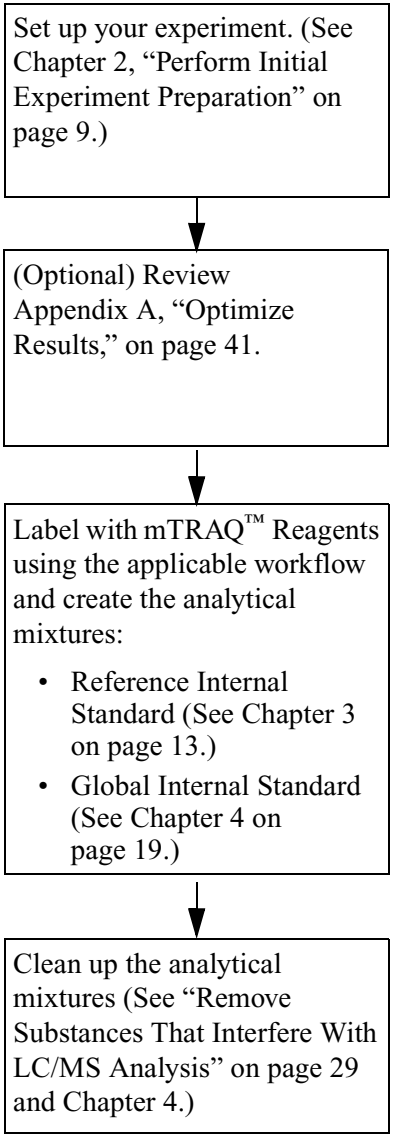

Figure 1 Workflow overview

Perform Initial Experiment Preparation 2

Practice the Labeling Protocol

If you are running the protocol for the first time, Applied Biosystems recommends that you practice performing the labeling protocol.

Using a Known Standard

Practice performing the labeling protocol using a simple digest such as β-galactosidase (described in Chapter 5 on page 29). Analyzing the labeled β-galactosidase samples by LC/MS/MS provides information about the proficiency of sample handling and the efficiency of the labeling protocol.

Using a Control Sample

Practice performing the labeling protocol using a representative internal standard and sample to determine if your samples are soluble in Dissolution Buffer and are compatible with the kit reagents. If not, alternative steps may be required (see "Optimize Labeling Efficiency" on page 43 for guidelines to modify the protocol).

If appropriate, design the experiment and determine the amounts of materials needed (page 10). Remove substances that may interfere with LC/MS analysis ("Remove Substances That Interfere With LC/MS Analysis" on page 29), if necessary, then analyze the sample according to your workflow.

To Develop an MRM Method

Using the data from your practice run and MRMPilot™ Software, iteratively develop an acquisition method. Use the acquisition method when performing the MRM quantitation analysis of the analytical sample mixtures with MultiQuant™ Software.

To Develop the Initial Method

Transfer the previously obtained discovery data to MRMPilot™ Software, then develop the initial MRM transitions (Q1, Q3, and collision energy) for each peptide of interest.

To Develop the Final Method

Perform an initial MRM analysis of the labeled samples using the initial acquisition method. Iteratively refine the MRM transitions for each peptide of interest using MRMPilot™ Software until specific, sensitive MRM transitions are obtained.

Design the Labeling Experiment

Determine the Targets

Review the previously obtained discovery data using ProteinPilot™ Software to select the proteins, peptides, and post-translational modifications to target.

Choose an Internal Standard Format

The internal-standard format determines the amount of materials needed for labeling. Two formats for internal standards are presented in this document:

- **Reference Internal Standard (RIS)** – For experiments in which a defined reference standard exists against which a set of samples will be compared. For information, see "Label with mTRAQ™ Reagents: Reference Internal Standard" on page 13.
- **Global Internal Standard (GIS)**– For experiments in which a set samples are combined into a mixture against which each individual sample will be compared. For information, see "Label with mTRAQ™ Reagents: Global Internal Standard" on page 19.

Determine Amounts of Materials

Reference Standard

Prepare 100 µg of internal standard mixture protein for each 100 µg of test sample protein. To determine the total amount of internal standard to prepare, consider the number of test samples you wish to label, and the minimum sample amount suitable for your analytical method. In most cases, use the full amount of labeled samples available. For example, in an experiment using:

- **Reference Internal Standard (RIS)** – To each of six test samples containing 20 µg protein, 20 µg of the internal standard mixture protein is added. Therefore, the minimum amount of internal standard sample to prepare for a **duplex** is 6 × 20 µg protein, for a total of 120 µg protein.

  The minimum amount of internal standard sample to prepare for a **triplex** is 3 x 20µg protein, for a total of 60µg of protein.

- **A Global Internal Standard (GIS)** – Prepare sufficient amount of each test material to divide into two replicates.

  When analyzing six samples, start with 30 µL of each sample and place 10 µLof each into a tube marked Replicate A. Replicate As will be pooled and labeled with Δ8. This is the GIS. The GIS is then aliquoted into 3 tubes containing 20 µL each.

  The remaining 20 µLof each sample is placed into tubes labeled Replicate B. Label Replicate B1, B3 and B5 with Δ0 and Replicate B2, B4 and B6 with Δ4. Pool one GIS with one Replicate B Δ0 and one Replicate B Δ4.

  – Replicate A is used to create the GIS (labeled with mTRAQ Reagent Δ8).

  – Replicate B is labeled with either mTRAQ Reagent Δ0 or mTRAQ Reagent Δ4.

  Prepare sufficient GIS to add an aliquot of GIS (1:1:1) to each sample set.

Trypsin

Determine the number of vials of trypsin to reconstitute. One 25 µg vial of trypsin, reconstituted, digests 250 µg of protein.

mTRAQ Reagent Δ0, Δ4 and Δ8

Determine the number of vials of mTRAQ Reagent Δ0, Δ4, and Δ8 to reconstitute. One unit of mTRAQ Reagent labels 100 µg of protein digest.

IMPORTANT! The procedure is written for a 1-unit vial of mTRAQ™ Reagent. When using a 50-unit vial of mTRAQ™ Reagent, aliquot and store the reagent properly to avoid hydrolytic degradation. Immediately after opening the vial, aliquot the appropriate volume (see the certificate of analysis) required to label samples into single-use tubes and store them under inert gas at –20 °C.

Label with mTRAQ™ Reagents: Reference Internal Standard

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Note: The information presented in this chapter applies to the Reference Internal Standard (RIS) workflow. If you are using the Global Internal Standard (GIS) workflow, see Chapter 4.

IMPORTANT! The procedure is written for a 1-unit vial of mTRAQ™ Reagent. When using a 50-unit vial of mTRAQ™ Reagent, aliquot and store the reagent properly to avoid hydrolytic degradation. Immediately after opening the vial, aliquot the appropriate volume (see the certificate of analysis) required to label samples into single-use tubes and store them under inert gas at –20 °C.

Workflow

To perform the labeling protocol using the Reference Internal Standard (RIS) workflow, you:

1. Prepare the samples (100 µg of protein) for labeling.
2. Prepare the digests.
3. Label the Reference Internal Standard Digest with mTRAQ Reagent Δ8.
4. Label each Sample digest 1 with mTRAQ Reagent Δ0 and Sample Digest 2 with Δ4.
5. Create and clean up the analytical sample mixtures.

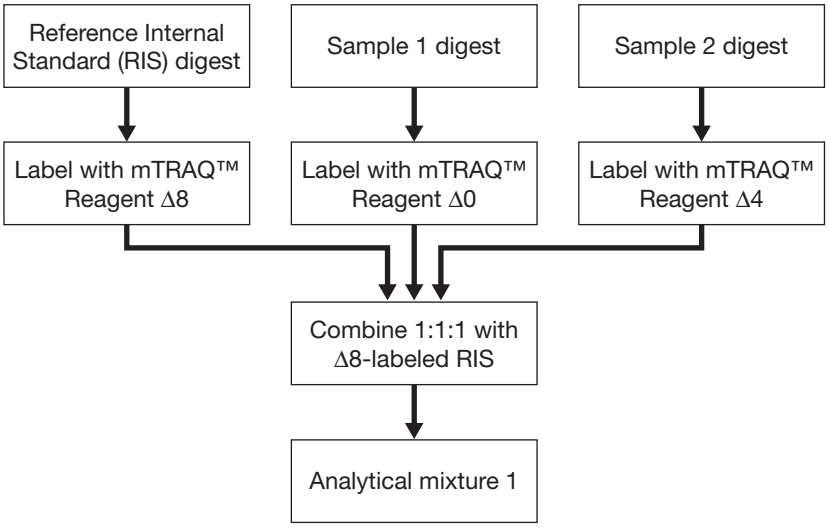

Figure 2 Triplex RIS workflow for the two samples. This workflow is extensible to many samples by using the same Δ8-labeled RIS with multiple sample pairs.

If there is a unique reference standard for each sample, modify the procedure appropriately.

Prepare the Samples for Labeling

Review Warnings and Handling Tips

Review the safety warnings in "Safety Information" on page v.

IMPORTANT! Slight pipetting variability of small volumes can cause large variability in reagent concentrations and analytical results. To optimize accurate pipetting, see "Small Volume Handling Tips" on page 41.

Remove Interfering Substances

If the samples contain substances that may interfere with the mTRAQ™ Reagent protocol, remove interfering substances by performing acetone precipitation ("Remove Substances That Interfere with Labeling" on page 43).

Practice the Protocol

Practice the protocol and perform the experiment preparation as described in Chapter 2 on page 9. When testing the protocol, alternative steps may be needed for the sample. If alternate steps are required, modify the procedures on pages 16 through 18. For information, see Chapter 2.

Prepare the Reference Standard Sample Digests

Reduce and Cysteine Block

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.
Trypsin causes eye, skin, and respiratory tract irritation. Exposure may cause an allergic reaction.

1. To the reference standard sample and each sample tube (containing 100 µg of protein), add 20 µL Dissolution Buffer.
2. To each tube, add 1 µL of the Denaturant, then vortex to mix.
3. To each tube, add 2 µL Reducing Reagent, then vortex to mix and spin.
4. Incubate the tubes at 60 °C for 1 hour.
5. Spin to bring the material to the bottom of the tube.
6. To each tube, add 1 µL Cysteine Blocking Reagent.
7. Vortex the tubes to mix, then pulse-spin.
8. Incubate the tubes at room temperature for 10 minutes.

Digest

1. For every approximately 250 µg of protein to digest, reconstitute a 25-µg vial of trypsin with 25 µL of Milli-Q® water or equivalent.
2. Vortex to mix, then pulse-spin.
3. To each tube of reduced and cysteine blocked-sample (containing 100 µg of protein), add 10 µL of the trypsin solution.
4. Vortex to mix, then pulse-spin.
5. Incubate the tubes at 37 °C overnight (12 to 16 hours).

6. Spin to bring the material to the bottom of the tube.

   Note: In order to maximize labeling efficiency, the volume of the sample digest must be less than 40 µL. If the volume of the sample digest is greater than 40 µL, completely dry the sample in a centrifugal vacuum concentrator, then reconstitute with 30 µL Dissolution Buffer.

Label the RIS Digest with mTRAQ Reagent Δ8

DANGER **CHEMICAL HAZARD. Isopropanol** is a flammable liquid. Vapors may form explosive mixtures with air. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry skin and cause irritation. It may cause central nervous system effects such as drowsiness, dizziness, and headache. Wear appropriate protective eyewear, clothing, and gloves.

WARNING **CHEMICAL HAZARD.** mTRAQ™ Reagents Δ0**,** Δ4 **and** Δ8 cause eye, skin, and respiratory tract irritation. Exposure may cause blood damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

For the reference internal standard digest:

1. Allow a 1-unit vial of mTRAQ Reagent Δ8 to reach room temperature.
2. Spin to bring the solution to the bottom of the tube.
3. Add 50 µL of isopropanol to the room-temperature mTRAQ Reagent Δ8 vial.
4. Vortex the vial to mix, then pulse-spin.
5. Transfer the contents of the mTRAQ Reagent Δ8 vial to the RIS digest tube.
6. Vortex the tube to mix, then pulse-spin.
7. Incubate the tube at room temperature for 1 hour.

Label Each Sample Digest with mTRAQ Reagent Δ0 or Δ4

For each sample digest:

1. Allow a 1-unit vial of mTRAQ Reagent Δ0 and Δ4 to reach room temperature.
2. Spin to bring the solution to the bottom of the tube.
3. Add 50 µL of isopropanol to the room-temperature mTRAQ Reagent Δ0 and Δ4 vials.
4. Vortex the vials to mix, then pulse-spin.
5. Transfer the contents of the mTRAQ Reagent Δ0 to Sample 1 and Δ4 to the Sample 2 vial.
6. Vortex the tube to mix, then pulse-spin.
7. Incubate the tube at room temperature for 1 hour.

Create and Clean up the Analytical Mixtures

For each labeled digest, combine an equivalent aliquot of labeled RIS and labeled samples in a fresh tube (Figure 2 on page 14).

IMPORTANT! Unless you immediately continue to clean up and analyze the sample mixture, store the sample mixture at –20 °C.

Clean Up the Mixture

Following the protocol as written yields analytical sample mixtures containing TCEP, SDS, calcium chloride, and excess mTRAQ™ Reagent that may interfere with LC/MS analysis. To optimize analytical results, perform sample cleanup, such as cation-exchange chromatography (“Remove Substances That Interfere With LC/MS Analysis” on page 29).

Label with mTRAQ™ Reagents: Global Internal Standard

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The information presented in this chapter applies to the Global Internal Standard (GIS) workflow. If you are using the Reference Internal Standard (RIS) workflow, see Chapter 3.

IMPORTANT! The procedure is written for a 1-unit vial of mTRAQ™ Reagent. When using a 50-unit vial of mTRAQ™ Reagent, aliquot and store the reagent properly to avoid hydrolytic degradation. Immediately after opening the vial, aliquot the appropriate volume (see the certificate of analysis) required to label samples into single-use tubes and store them under inert gas at –20 °C.

Workflow

To perform the labeling protocol using the Global Internal Standard (GIS) workflow, you:

1. Prepare the samples (each sample contains 200 µg of protein) for labeling.
2. Prepare the digests.
3. Split each digest into Replicates A and B, each containing 100 µg of protein (see Figure 3 on page 20).
4. Label each Replicate A digest with mTRAQ Reagent Δ8.
5. Pool all Δ8-labeled Replicate A digests to create the Global Internal Standard Digest Mixture (see Figure 4 on page 21).
6. Label each Replicate B digest with either mTRAQ Reagent Δ0 or Δ4.
7. Create and clean up the analytical sample mixtures.

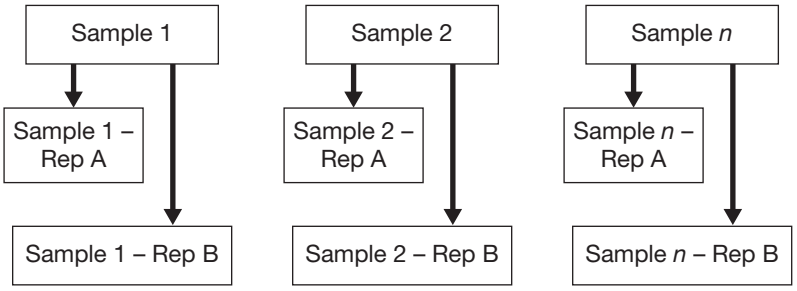

Figure 3 Replicates for the workflow for a global internal standard

In the procedure presented in this chapter, the mTRAQ Reagent Δ8-labeled global internal standard is created by labeling the individual Replicate A sample digests, then pooling the labeled samples to create the GIS. See Figure 4 on page 21.

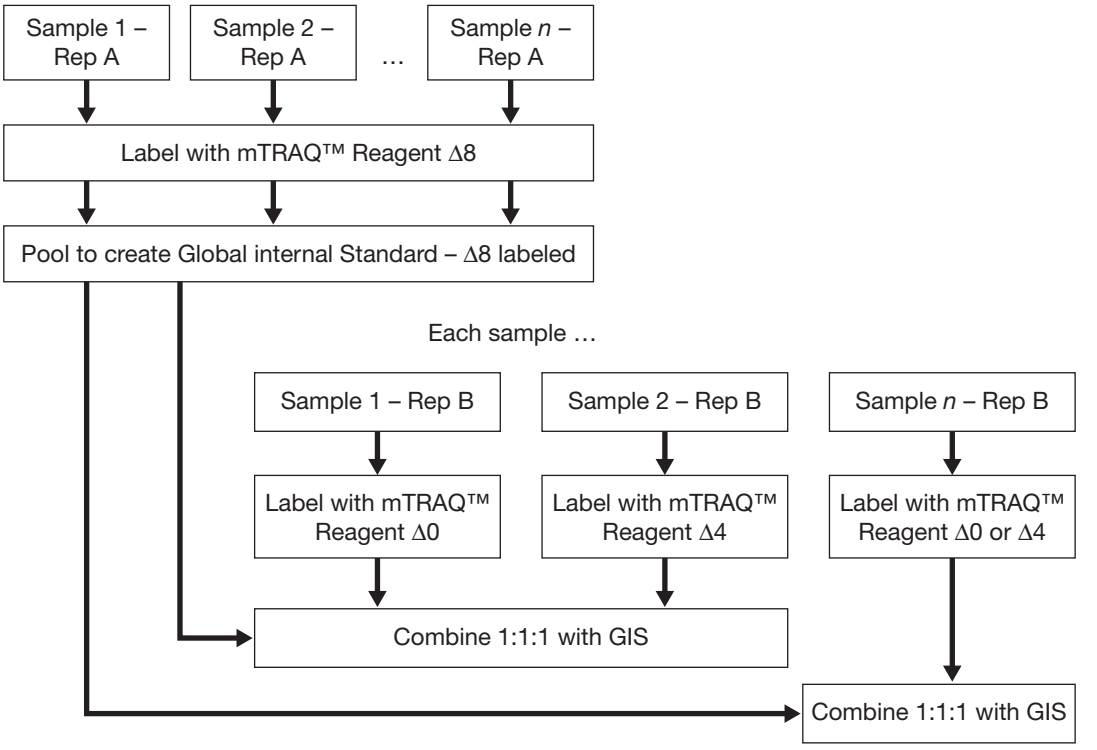

Figure 4 Triplex GIS workflow – creation of analytical mixtures with a global internal standard

Prepare the Samples for Labeling

Review Warnings and Handling Tips

Review the safety warnings in "Safety Information" on page v.

IMPORTANT! Slight pipetting variability of small volumes can cause large variability in reagent concentrations and analytical results. To optimize accurate pipetting, see "Small Volume Handling Tips" on page 41.

Remove Interfering Substances

If the samples contain substances that may interfere with the mTRAQ™ Reagents protocol, remove interfering substances by performing acetone precipitation ("Remove Substances That Interfere with Labeling" on page 43).

Practice the Protocol

Practice the protocol and perform the experiment preparation as described in Chapter 2 on page 9. When testing the protocol, alternative steps may be needed for the sample. If alternate steps are required, modify the procedures on pages 22 through 26. For information, see Chapter 2.

Prepare the Sample Digests

Reduce and Cysteine Block

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.
Trypsin causes eye, skin, and respiratory tract irritation. Exposure may cause an allergic reaction.

1. To each sample tube containing 200 µg of sample, add 40 µL Dissolution Buffer.
2. Add 2 µL of the Denaturant in the kit and vortex to mix.
3. To each tube, add 4 µL Reducing Reagent.
4. Vortex to mix, then pulse-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 2 µL Cysteine Blocking Reagent.
8. Vortex to mix, then pulse-spin.
9. Incubate the tubes at room temperature for 10 minutes.

Digest

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 mTRAQ™ Reagents labeling (see "Remove Substances That Interfere with Labeling" on page 43).

1. Reconstitute each vial of trypsin required with 25 µL of Milli-Q® water or equivalent.
2. Vortex to mix, then pulse-spin.
3. To each sample tube, add 20 µL of the trypsin solution.
4. Vortex to mix, then pulse-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 80 µL. If the volume of the sample digest is greater than 80 µL, dry the sample in a centrifugal vacuum concentrator, then reconstitute with 60 µL Dissolution Buffer.

Create the Replicates

For each sample digest, transfer an aliquot containing 100 µg of protein to a fresh tube labeled as Replicate A (for GIS, subsequently labeled with mTRAQ Reagent Δ8). Transfer another aliquot containing 100 µg of protein to a fresh tube labeled as Replicate B (subsequently labeled with mTRAQ Reagent Δ0 or Δ4 for analytical mixture).

Note: Replicates A and B can contain less than 100 µg of protein, but must contain the same amount of protein. For example, if the Replicate B aliquots are calculated to contain 80 µg of protein, then the Replicate A must contain 80 µg of protein.

Label Each Replicate A with mTRAQ Reagent Δ8 and Create the GIS

Label Each Replicate A with Δ8

DANGER **CHEMICAL HAZARD. Isopropanol** is a flammable liquid. Vapors may form explosive mixtures with air. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry skin and cause irritation. It may cause central nervous system effects such as drowsiness, dizziness, and headache. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

WARNING **CHEMICAL HAZARD.** mTRAQ™ Reagents Δ0**,** Δ4 **and** Δ8 cause eye, skin, 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. Allow each 1-unit vial of mTRAQ Reagent Δ8 required to reach room temperature.
2. Spin to bring the solution to the bottom of the tube.
3. Add 50 µL of isopropanol to each room-temperature mTRAQ Reagent Δ8 vial.
4. Vortex each vial to mix, then pulse-spin.
5. For each Replicate A, transfer the contents of one prepared mTRAQ Reagent Δ8 vial to the Replicate A tube.
6. Vortex to mix, then pulse-spin.
7. Incubate at room temperature for 1 hour.

Create the GIS

In a fresh tube, pool together the entire contents of each Replicate A tube to create the GIS. Vortex the tube to mix, then pulse-spin.

Aliquot the GIS into single-use tubes. Each tube should contain the same amount of protein as contained in the Replicate B tubes (the Δ0 and Δ4 -labeled sample).

IMPORTANT! Unless you immediately continue to clean up and analyze the sample mixture, store the sample mixture at –20 °C.

Label Each Sample with mTRAQ Reagent Δ0 or Δ4

To label the Replicate B sample digests:

1. Allow each 1-unit vial of mTRAQ Reagent Δ0 and Δ4 required to reach room temperature.
2. Spin to bring the solution to the bottom of the tube.
3. Add 50 µL of isopropanol to each room-temperature mTRAQ Reagent Δ0 and Δ4 vial.
4. Vortex each vial to mix, then pulse-spin.
5. Transfer the contents of one vial of mTRAQ Reagent Δ0 to Replicate B sample digest 1 and Δ4 to the Replicate B sample digest 2 tube containing 100 µg protein.
6. Vortex each tube to mix, then pulse-spin.
7. Incubate the tubes at room temperature for 1 hour.

   IMPORTANT! Unless you immediately continue to clean up and analyze the sample mixture, store the sample mixture at –20 °C.

Create and Clean Up the Analytical Mixtures

For each sample, combine an aliquot of mTRAQ Reagent Δ0 and Δ4-labeled Replicate B with the single-use vial of mTRAQ Reagent Δ8-labeled global internal standard.

IMPORTANT! Unless you immediately continue to analyze the sample mixture, store the sample mixture at –20 °C.

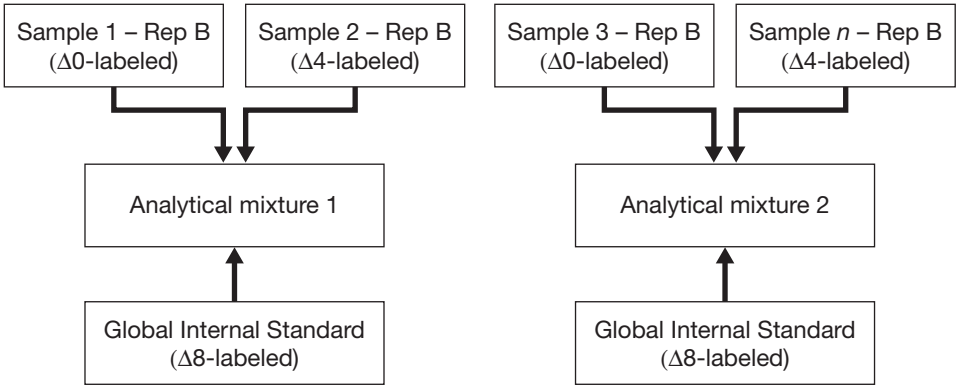

Figure 5 Triplex GIS workflow – creation of analytical mixtures with a global internal standard.

Clean Up Each Analytical Mixture

Following the protocol as written yields analytical sample mixtures containing TCEP, SDS, calcium chloride, and excess mTRAQ™ Reagent that may interfere with LC/MS analysis. To optimize analytical results, perform sample cleanup, such as cation-exchange chromatography (see “Cation-Exchange Clean Up” on page 30).

LC/MS/MS MRM Analysis 5

Before You Begin

Practice the Analysis

To practice the labeling protocol and the MRM method you developed, prepare and analyze a known simple digest, such as β-galactosidase (β-gal).

Follow the labeling protocol as presented in Chapter 3 on page 13, using 100 µg of β-gal as test sample 1, 100 µg of β-gal as test sample 2, and a third sample of 100 µg of β-gal as the reference internal standard sample. The resulting analytical sample mixture is a 1:1:1 mixture of mTRAQ Reagent Δ0-labeled peptides, mTRAQ Reagent Δ4-labelled peptides, and the same peptides labeled with mTRAQ Reagent Δ8.

Remove Substances That Interfere With LC/MS Analysis

The analytical sample mixture may contain substances that interfere with LC/MS analysis. The cleanup procedure presented in this chapter is a cation-exchange procedure using the Applied Biosystems Cation-Exchange Cartridge System.

For information about required materials and assembling, washing, and storing the cartridge, see the *Applied Biosystems iTRAQ™ Reagents Chemistry Reference Guide* (see "How to Obtain More Information" on page ix).

Phosphate vs. Formate Buffers

The cleanup procedure presented in this appendix uses phosphate buffers. Alternatively, use a volatile, non-phosphate-containing buffer system such as formate buffers. Formate buffers are compatible with the cartridge system and the suggested high-resolution cation-exchange column ("mTRAQ Reagents Storage: Materials Not Included" on page 6).

The recommended formate load and elute buffers are:

- **Cation-Exchange Buffer–Load** – 10 mM ammonium formate in 15% acetonitrile, pH adjusted to 3.0 with formic acid.
- **Cation-Exchange Buffer–Elute** – 500 mM ammonium formate in 15% acetonitrile, pH adjusted to 3.0 with formic acid.

IMPORTANT! The chromatograph may not be the same as you have observed with a phosphate buffering system. For example, the formate buffer interferes with the peptide elution profile at 214 nm. Monitor the peptide elution profile at 254/230.

Cation-Exchange Clean Up

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

Cation-Exchange Buffer–Load and Cation-Exchange Buffer–Elute (see below) 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.

- **Cation-Exchange Buffer–Load** – 10 mM potassium phosphate (KH_2PO_4) in 25% acetonitrile at pH 3.0
- **Cation-Exchange Buffer–Elute** – 10 mM KH_2PO_4 in 25% acetonitrile/350 mM potassium chloride (KCl) at pH 3.0
- **Cation-Exchange Buffer–Clean** – 10 mM KH_2PO_4 in 25% acetonitrile/1 M KCl at pH 3.0

mTRAQ Reagent Δ0**,** Δ4 **and** Δ8 cause eye, skin, 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 doing **one** of the following:
   - Dilute the analytical sample mixture at least 10-fold with Cation-Exchange Buffer-Load or a suitable volatile buffer.
   - Reduce the volume of the sample in a centrifugal vacuum concentrator to less than 30 µL. Reconstitute the sample mixture with 3 mL Cation-Exchange Buffer-Load.
2. Vortex the tube to mix.

3. Remove an aliquot of the mixture and check that the pH is between 2.5 and 3.3. If necessary, adjust the pH by adding more Cation-Exchange Buffer–Load, 1 N phosphoric acid, or formic acid, as appropriate.
4. Condition the cartridge by injecting 1 mL of the Cation-Exchange Buffer–Clean, or a suitable non-phosphate-containing buffer. Divert to waste.
5. Inject 3 mL of the Cation Exchange Buffer–Load or a suitable non-phosphate-containing buffer. 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 or a suitable non-phosphate-containing buffer to wash the TCEP, SDS, calcium chloride, and excess mTRAQ™ Reagent 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, 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 or a suitable non-phosphate-containing buffer. 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 or a suitable non-phosphate-containing buffer. Divert to waste.
10. Inject 2 mL of the Cation Exchange Buffer–Load or a suitable non-phosphate-containing buffer. Divert to waste.

Repeat for additional samples. After completing the cleanup procedure for all analytical mixtures, wash and store the column as recommended.

IMPORTANT! If you used a formate buffer system, be sure to flush the HPLC system and column thoroughly before switching to another buffer.

Suggested LC/MS/MS MRM Settings

The suggested LC/MS/MS MRM settings presented in this appendix are recommended for analyzing the β-gal practice samples with the Applied Biosystems/MDS Analytical Technologies 4000 QTRAP® System. These settings provide a starting point for developing the optimal settings for your samples and system.

To order or download PDF documents helpful when using the 4000 QTRAP® System (such as system user guides and tutorials or technical notes), see "How to Obtain Support" on page ix.

IMPORTANT! Clean up the analytical sample mixtures (see "Remove Substances That Interfere With LC/MS Analysis" on page 29) before analyzing.

LC/MS

Table 5 Suggested injection amounts

Column size	Suggested Injection Amounts
2 mm	1 pmol
75 µm	100 fmol

Table 6 Suggested mobile phases A and B

Compound	Mobile Phase A	Mobile Phase B
Milli-Q® water or equivalent	98%	2%
Acetonitrile, HPLC-grade	2%	98%
Formic acid	0.1%	0.1%

High Flow Rate LC/MS

Table 7 Suggested gradient time and percent mobile phase B for high-flow LC/MS

Time	Flow Rate (µL/min)	%B
0.1	650	6
5	650	30
6	650	90
7	650	90
8	650	5
35	650	5

Table 8 Suggested settings for the 4000 QTRAP System parameters for high flow rate LC/MS

4000 QTRAP System Parameters	Setting
CUR	25
CAD	High
IS	4500
TEM	500
GS1	50
GS2	50
ihe	ON
DP	70

Low Flow Rate LC/MS

Table 9 Suggested gradient time and percent mobile phase B for low-flow LC/MS

Time	Flow Rate (nL/min)	%B
0.1	300	5
2	300	5
17	300	30
20	300	80
23	300	80
25	300	5
45	300	5

Table 10 Suggested settings for the 4000 QTRAP System parameters for low flow rate LC/MS

4000 QTRAP System Parameters	Setting
CUR	20
CAD	High
IS	2300
GS1	5-10
GS2	0
IHT	150
DP	70

MRM

Table 11 Suggested MRM Settings for the 4000 QTRAP System‡

Peptide	Q1 Mass (amu)	Q3 Mass (amu)	CE
TPHPALTEAK (see Peak 1, Figure 6 on page 38)			
Δ8	454.3	596.3	28
Δ4	451.6	592.3	28
Δ0	449	588.3	28
Unlabeled	532.8	729.4	32
YSQQQLMETSHR (see Peak 2, Figure 6)			
Δ8	552.6	760.3	31
Δ4	551.2	760.3	31
Δ0	549.9	760.3	31
Unlabeled	503.4	760.3	25
WVGYGQDSR (see Peak 3, Figure 6)			
Δ8	608.3	881.3	36
Δ4	606.3	881.3	36
Δ0	604.3	881.3	36
Unlabeled	534.2	782.3	32
APLDNDIGVSEATR (see Peak 4, Figure 6)			
Δ8	536.9	719.4	32
Δ4	534.9	719.4	32
Δ0	532.9	719.4	32
Unlabeled	729.4	1176.6	40

Table 11 Suggested MRM Settings for the 4000 QTRAP System‡

Peptide	Q1 Mass (amu)	Q3 Mass (amu)	CE
VDEDQPFPAVPK (see Peak 5, Figure 6)			
Δ8	546.6	659.3	31
Δ4	544	655.3	31
Δ0	541.3	651.3	31
Unlabeled	671.4	755.5	35
GDFQFNISR (see Peak 6, Figure 6)			
Δ8	616.3	911.5	39
Δ4	614.3	911.5	39
Δ0	612.3	911.5	39
Unlabeled	542.3	636.4	27
IDPNAWVER (see Peak 7, Figure 6)			
Δ8	624.3	871.4	38
Δ4	622.3	871.4	38
Δ0	620.3	871.4	38
Unlabeled	550.3	871.4	31
VNWLGLGPQENYPDR (see Peak 8, Figure 6)			
Δ8	953.4	1245.6	53
Δ4	951.4	1245.6	53
Δ0	949.4	1245.6	53
Unlabeled	879.4	1075.5	44

Table 11 Suggested MRM Settings for the 4000 QTRAP System‡

Peptide	Q1 Mass (amu)	Q3 Mass (amu)	CE
LSGQTIEVTSEYLFR (see Peak 9, Figure 6)			
Δ8	946	1014.5	50
Δ4	944	1014.5	50
Δ0	942	1014.5	50
Unlabeled	872	1143.6	43
LPSEFDLSAFLR (see Peak 10, Figure 6)			
Δ8	771.9	1281.6	44
Δ4	769.9	1281.6	44
Δ0	767.9	1281.6	44
Unlabeled	697.9	1184.6	35

‡ Time is 20 msec throughout.

Representative Labeled β-gal Chromatograph

Three samples of Beta-gal are labeled individually with either Δ0, Δ4 or Δ8 (1:2:4) and then combined (Figure 6). A magnification of peak 10 shows the relative abundance of this peptide in these samples (Figure 7). The sample was then analyzed via LC-MRM as shown below (Figure 6.). Peak widths are no more than 0.2 min half-height.

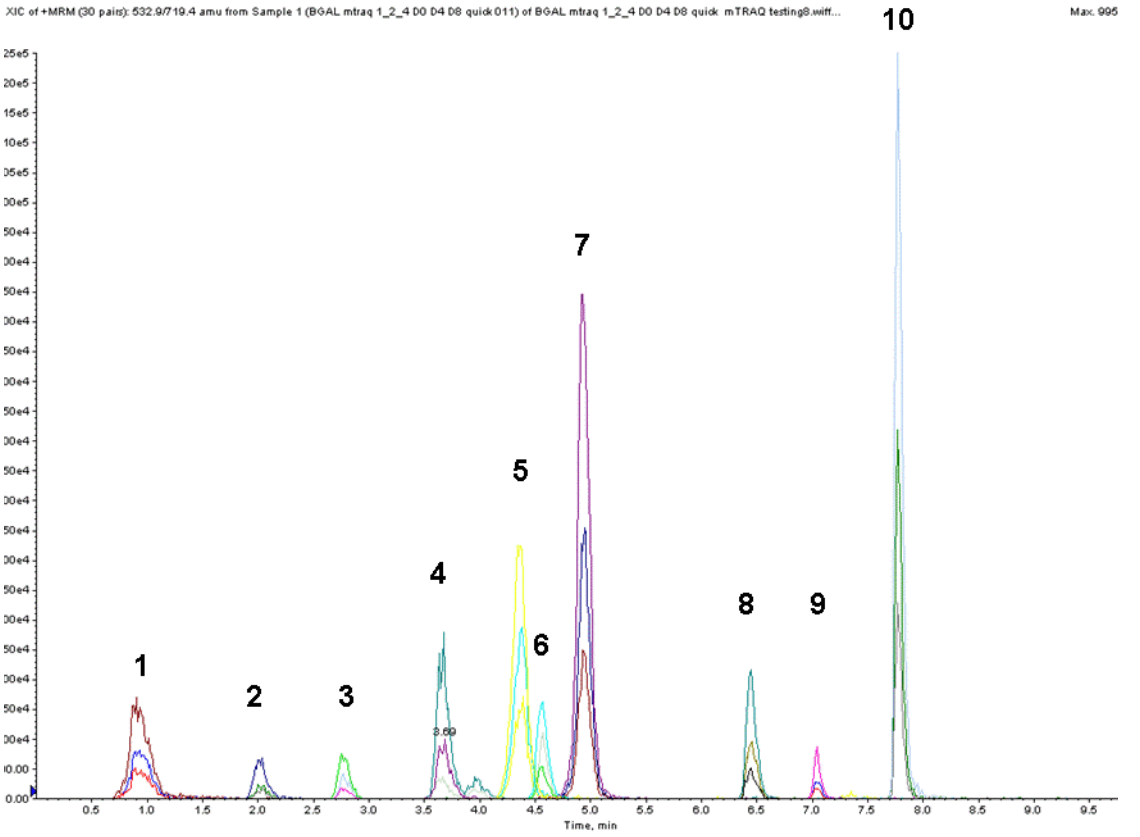

Figure 6 Representative chromatograph of mTRAQ™ Reagent-labeled β-galactosidase

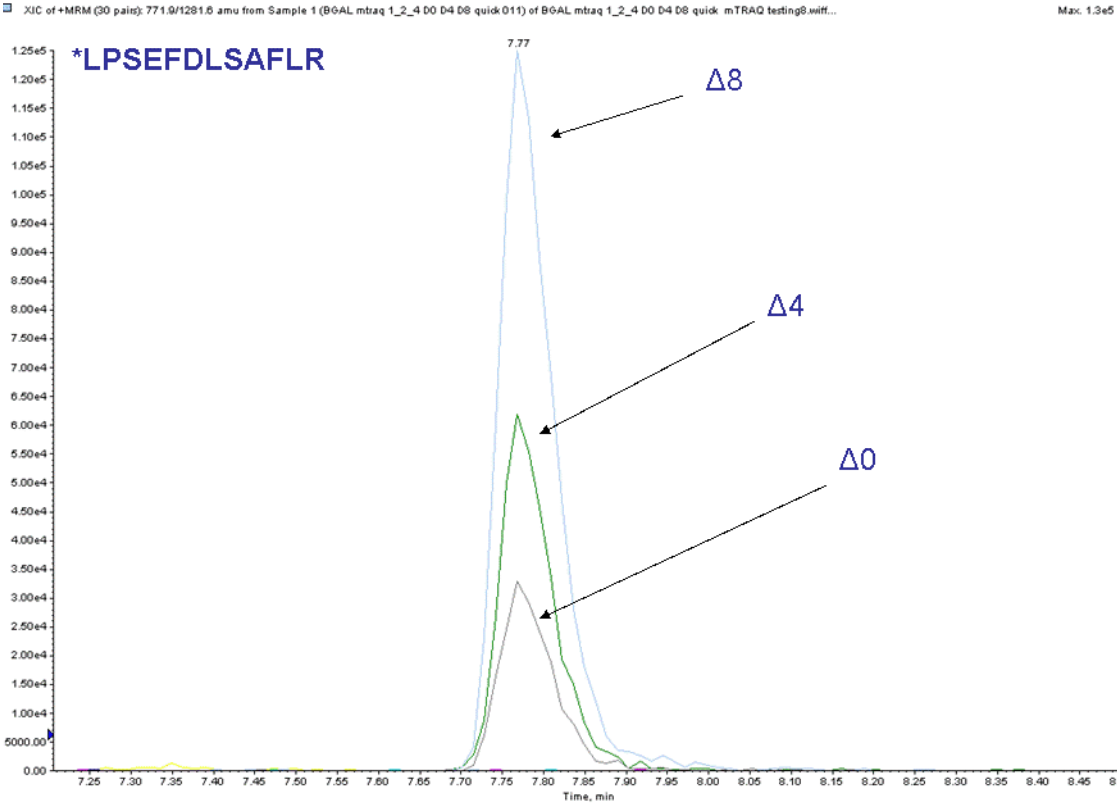

Figure 7 β-Gal labeled with mTRAQ Δ0, Δ4 Δ8, mixed at a ratio of 1:2:4

Optimize Results A

Optimize Accuracy of Volumes and Concentrations

Reagent Handling Tips

- Immediately before use, allow the reagents required to reach room temperature.
- Return the reagents to storage at –20 °C within 2 hours of thawing.
- Briefly centrifuge the reagent vials to dislodge material potentially trapped in the caps.

Small Volume Handling Tips

Throughout the labeling protocol, to ensure accurate volumes and concentrations:

- Have all vials of samples and reagents at room temperature.
- Capture all material from the sides and cap of the vial by centrifuging (spinning) the vials briefly (pulse spin).
- Store materials following the recommended temperatures and conditions.

To ensure accurate pipetting:

- Use high-quality disposable tips.
- Use a fresh tip for each pipetting step.
- For each sample draw, use the same:
  - Immersion depth (see the pipette manufacturer's recommendation).
  - Pressure on the plunger at the first stop while immersing the tip in the sample.
  - Slow and smooth technique when pressing and releasing the plunger.
- Avoid air bubbles.

  If an air bubble is trapped in the tip during filling, dispense the sample back into the tube. Pipette again using a fresh tip.

- Each time you dispense the sample:
  - Be consistent when you pause between reaching the first stop and pressing the plunger to the second stop.
  - Keep the plunger fully depressed while withdrawing the pipette from the tube, sliding the tip along the wall of the tube.

IMPORTANT! Never lay a pipette on its side or invert a pipette with sample in the tip.

Optimize Labeling Efficiency

Remove Substances That Interfere with Labeling

If the control and test samples contain a substance that may interfere with the mTRAQ™ Reagents protocol, perform acetone precipitation ("Acetone Precipitation" on page 44) to clean up the sample.

Table 12 Substances that may interfere with the mTRAQ™ 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 13 on page 46 for concentration limits of some acceptable detergents/denaturants.)	Inactivate trypsin	If the substance is needed to solubilize the sample, after reducing the protein and blocking cysteines
Active proteases		
Primary amines, for example, those in: 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	React with mTRAQ™ Reagent, interfering with labeling	Before trypsin digestion

Acetone Precipitation

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 remove specific impurities from a sample using acetone precipitation:

1. Chill acetone to –20 °C and each control and test sample-containing tube to 4 °C.
2. To each chilled tube, add six volumes of cold acetone.
3. Invert each tube three times.
4. Incubate each tube at –20 °C until a flocculent forms (30 minutes to four hours).
5. Spin each tube at 6,000 × g for 10 minutes.
6. Decant the acetone from each tube. Do not dry the material (pellet) in the tube.

The precipitated pellet contains the sample proteins to be digested and labeled.

Modify the Protocol

If when practicing the protocol, you determine that alternate steps are required to achieve optimal labeling efficiency, refer to Appendix A of the *Applied Biosystems iTRAQ® Reagents Chemistry Reference Guide* for guidelines for modifying the protocol.

Optimize Sample Solubility

For optimal labeling efficiency, the sample must be fully soluble prior to digestion. If the sample is insoluble after adding Dissolution Buffer and Denaturant (steps 1 and steps 2 on page 16, RIS; steps 1 and 2 on page 22, GIS), choose an alternative detergent/denaturant or buffer (Table 13).

For the labeling reaction to achieve optimal efficiency, the pH during labeling must be greater than 8.0 and the buffer concentration at least 0.06 M. Test the pH during labeling (step 7 on page 18, RIS; step 7 on page 26, GIS) and, if necessary, add up to 5 µL of an appropriate buffer to adjust the pH and the buffer concentration.

The following 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.

Table 13 Recommended alternative detergent/ denaturant and buffers

Alternative Detergent/Denaturant (Concentration Limit at Trypsin Digestion)	Alternative Buffer
SDS (0.05%) OG (octyl B-D-glucopyranoside) (1%) NP®-40 (1%) Triton® X-100 (1%) Tween® 20 (1%) CHAPS (1%) Urea (<1M) When using urea, always use a fresh solution. When reducing a sample containing urea, incubate the tubes at 37 °C for 1 hour (step 4 on page 16, RIS; step 5 on page 22, GIS).	BES BICINE Boric acid CHES DIPSO EPPS HEPBS HEPES HEPPSO MOBS MOPS Phosphate Buffered Saline PIPES POPSO

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