Applied Biosystems Amino Acid Analysis for Hydrolysate Samples

aTRAQ™ Reagents Application Kit for Use with LC/MS/MS Systems

Protocol

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Preface

This preface covers:

Safety

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 MSDSs provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About MSDSs" on page viii.)
- 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, a 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 the link for **Support**, then click the link for **MSDS Search**.
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 hazards

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

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

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

Chemical waste safety guidelines

To minimize the hazards of chemical waste:

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

Waste disposal

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

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

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

Biological hazard safety

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

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

Additional information about biohazard guidelines is available at:

http://www.cdc.gov

How to obtain more information

Related documentation

- *Applied Biosystems iTRAQ® Reagents Amine-Modifying Labeling Reagents for Multiplexed Relative and Absolute Protein Quantification: Chemistry Reference Guide* (PN 4351918)
- *Applied Biosystems Amino Acid Analysis for Physiological Samples Quick Reference Card* (PN 4445543)
- Technical and Application Notes

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

To order a hard copy of the chemistry reference guide, go to **http://store.appliedbiosystems.com**, log in, then enter the part number (4351918) in the Search field.

For technical and application notes, see "How to obtain support" on page xiii.

Obtaining information using online help

The Analyst® Software and Cliquid® Software for Routine Amino Acid Analysis have Help systems that describe how to use each feature of the user interface. Access the Help system by doing one of the following:

- Click in the toolbar or user interface of the software window
- Select the **Help** tab
- Press **F1** (not applicable to Cliquid Software)

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

IMPORTANT! The e-mail address above is for submitting comments and suggestions relating only to documentation. To order documents, download PDF files, or for help with a technical question, go to **http://www.appliedbiosystems.com**, then click the link for **Support**. (See "How to obtain support" on page xiii.)

How to obtain support

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

At the Support page, you can:

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

Introduction to aTRAQ™ Reagents Chemistry

1

This chapter covers:

Overview

The aTRAQ™ Kits for amino acid analysis of hydrolysate samples enable identification and quantitation of amino acids from hydrolyzed peptide, protein, feed, and other samples. The kits provide aTRAQ™ Reagent Δ8 for labeling samples and a mixture of Δ0 -labeled amino acids as an internal standard.

Product capabilities

With Cliquid® Software for Routine Amino Acid Analysis, the Applied Biosystems/MDS Analytical Technologies LC/MS/MS Systems allow users with minimal mass spectrometry (MS) experience to obtain data for relative and absolute quantitation of amino acids (Figure 1).

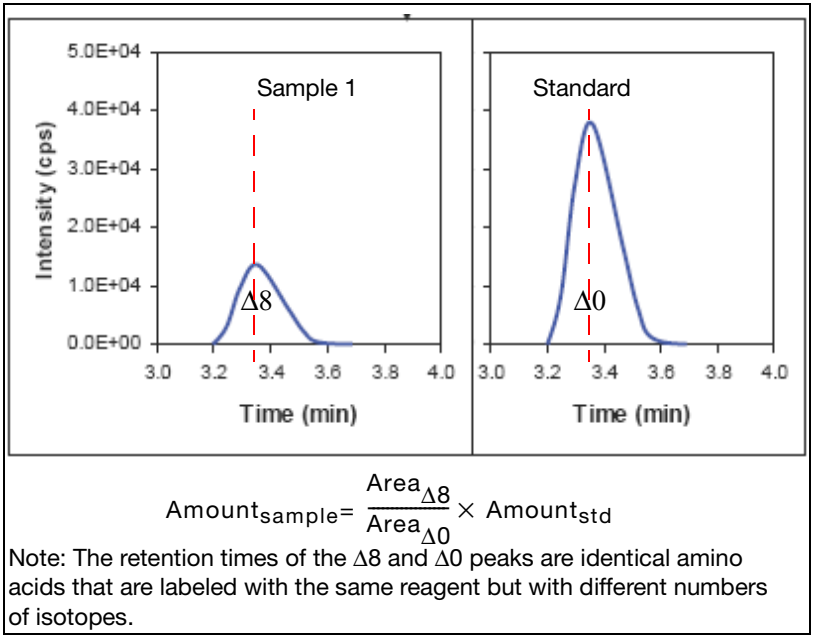

Figure 1 Representation of LC/MS/MS data showing peaks and the calculation for absolute quantitation

Available kits and materials

To order kits and materials (Table 1), go to
www.store.appliedbiosystems.com

Table 1 Kits and materials

Item	Description
aTRAQ™ Kits Hydrolysates	
Starter Kit (includes the 50-assay reagent kit, aTRAQ™ Standards Set Hydrolysates, this protocol, and the Quick Reference Card)	Provides sufficient material to run 50 aTRAQ™ Reagent Δ8-labeled samples (each containing up to 10 nmole total amino acid) with the AA Internal Standard.
50-Assay or 200-Assay Kit (includes the reagent kit, AA Internal Standard, and the Quick Reference Card)	Provides sufficient material to run 50 or 200 aTRAQ™ Reagent Δ8-labeled samples (each containing up to 10 nmole total amino acid) with the AA Internal Standard.
Standards and Controls	
aTRAQ™ Standards Set Hydrolysates	• AA Internal Standard (see page 34). • AA Unlabeled Standard contains the same amino acids as the internal standard, except norvaline and norleucine. Norvaline is incorporated during labeling. To monitor the recovery, norleucine can be added before the hydrolysis. Standard Diluent is used to dilute the AA Internal Standard. The amount of Standard Diluent to use is indicated on the Certificate of Analysis and the AA Internal Standard vial label.

Table 1 Kits and materials *(continued)*

Item	Description
Standards and Controls (continued)	
AA Internal Standard	Provides AA Internal Standard and Standard Diluent.
AA Unlabeled Standard	Provides the AA Unlabeled Standard. Contains the same amino acids as the internal standard, except norvaline and norleucine. Norvaline is incorporated during labeling. To monitor the recovery, norleucine can be added before the hydrolysis.
Column	
Applied Biosystems Amino Acid Analyzer (AAA) C18 Column	C18 reversed-phase column, 5 µm, 4.6 mm ×150 mm.

Contents of the starter kit

The aTRAQ™ Starter Kit Hydrolysates includes aTRAQ™ Reagent-Δ8, the standards set, reagents, and this document (see Table 2 on page 5). For recommendations on using the standards set, see "Quality assurance" on page 39. Order the Applied Biosystems Amino Acid Analyzer (AAA) C18 Column separately.

IMPORTANT! When you receive the shipping container, immediately store the Reagent Kit and aTRAQ™ Standards Set Hydrolysates at –15 °C or below.

IMPORTANT! Be aware that, during shipment, small volumes of material may become trapped in the cap of the product vial. Dislodge the trapped material as described in "Handling tips to ensure accurate concentrations and volumes" on page 38.

Table 2 Contents of the aTRAQ™ Starter Kit Hydrolysates

Item	Quantity	Contents
Store at –15 °C or below		
Reagent Kit (one 50-Assay Kit)		
• aTRAQ™ Reagent Δ8	4 vials, 1unit/vial	Amine-modifying labeling reagent. One unit (one vial) of reagent yields approximately 15 assays.
• Sulfosalicyclic Acid‡	1 vial, 1.8 mL	Use with the preparation of physiological samples for free amino acid analysis.
• Labeling Buffer‡	2 vials, 1.8 mL/vial	Borate buffer, pH 8.5. Also contains norvaline (20 µM).
• Hydroxylamine‡	1 vial, 1.8 mL	1.2% hydroxylamine solution. Reverses partial labeling of the phenolic hydroxyl group of tyrosine and quenches any unreacted aTRAQ™ reagent.
• Mobile Phase Modifier A‡	2 vials, 1.8 mL/vial	100% formic acid for mobile phase A and mobile phase B preparation.
• Mobile Phase Modifier B‡	2 vials, 200 µL/vial	100% heptafluorobutyric acid for mobile phase A and mobile phase B preparation.
• Isopropanol‡	1 vial, 1.8 mL	Isopropanol, absolute, for diluting aTRAQ™ Reagent.
aTRAQ™ Standards Set Hydrolysates	1	• 1 vial AA Internal Standard • 1 vial AA Unlabeled Standard • 1 vial Standard Diluent§ - 2% formic acid for reconstituting the vials of AA Internal Standard • Certificate of Analysis,. Specifies the precise amount of diluent for reconstituting this lot of standard.

Table 2 Contents of the aTRAQ™ Starter Kit Hydrolysates *(continued)*

Item	Quantity	Contents
Documentation		
Applied Biosystems Amino Acid Analysis for Hydrolysate Samples Protocol	1	This document.
Applied Biosystems Amino Acid Analysis for Hydrolysate Samples Quick Reference Card	1	A laminated card that briefly describes the steps in the labeling protocol.

‡ Can also be stored refrigerated.

§ The amount of Standard Diluent to use when diluting the AA Internal Standard is indicated on the Certificate of Analysis and the AA Internal Standard vial label.

Contents of the 50-assay and 200-assay kits

IMPORTANT! When you receive the shipping container, immediately store the Reagent Kit and the AA Internal Standard bag at –15 °C or below.

IMPORTANT! Be aware that, during shipment, small volumes of material may become trapped in the cap of the product vial. Dislodge the trapped material as described in "Handling tips to ensure accurate concentrations and volumes" on page 38.

See Table 3 on page 7 for materials contained in each kit.

Table 3 Contents of the aTRAQ™ Kit Hydrolysates 50 Assay and 200 Assay Kits

Item	Quantity in 50-Assay Kit	Quantity in 200-Assay Kit	Contents
Store at −15 °C or below			
Reagent Kit (50-Assay Kit or 200-Assay Kit) 1 shipping container with the following items:			
• aTRAQ™ Reagent Δ8	4 vials, 1 unit/vial	14 vials, 1 unit/vial	Amine-modifying labeling reagent. One unit (one vial) of reagent yields approximately 15 assays.
• Sulfosalicyclic Acid‡	1 vial, 1.8 mL	2 vials, 1.8 mL/vial	Use with the preparation of physiological samples for free amino acid analysis.
• Labeling Buffer‡	2 vials, 1.8 mL/vial	5 vials, 1.8 mL/vial	Borate buffer, pH 8.5. Also contains norvaline (20 µM).
• Hydroxylamine‡	1 vial, 1.8 mL	1 vial, 1.8 mL	1.2% hydroxylamine solution. Reverses partial labeling of the phenolic hydroxyl group of tyrosine and quenches any unreacted aTRAQ™ reagent.
• Mobile Phase Modifier A‡	2 vials, 1.8 mL/vial	6 vials, 1.8 mL/vial	100% formic acid for mobile phase A and mobile phase B preparation.
• Mobile Phase Modifier B‡	2 vials, 200 µL/vial	6 vials, 200 µL/vial	100% heptafluorobutyric acid for mobile phase A and mobile phase B preparation.
• Isopropanol‡	1 vial, 1.8 mL	1 vial, 1.8 mL	Isopropanol, absolute, for diluting aTRAQ™ Reagent.

Table 3 Contents of the aTRAQ™ Kit Hydrolysates 50 Assay and 200 Assay Kits *(continued)*

Item	Quantity in 50-Assay Kit	Quantity in 200-Assay Kit	Contents
AA Internal Standard	1 bag	4 bags	In one bag: • 1 vial of AA Internal Standard • 1 vial of Internal Standard Diluent§ - 2% formic acid for reconstituting the vial of AA Internal Standard • Certificate of Analysis. Specifies the precise amount of diluent for reconstituting this lot of standard.
Documentation			
Applied Biosystems Amino Acid Analysis for Hydrolysate Samples Quick Reference Card	1	1	A laminated card that briefly describes the steps in the labeling protocol.

‡ Can also be stored refrigerated.

§ The amount of Standard Diluent to use when diluting the AA Internal Standard is indicated on the Certificate of Analysis and the AA Internal Standard vial label.

User-supplied materials

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Table 4 User-supplied materials

Item	Quantity per Assay
Disposable gloves	As needed
Hydrolysate samples, at least 1 µg of hydrolyzed material in each sample	As needed
Pipetting accessories (pipettors and tips) suitable for 5-µL to 1-mL volumes, such as P10, P100, P1000 pipettes	As needed
Milli-Q® water or equivalent (minimum 18.2 MOhms water, conductivity maximum 0.05 µS/0.05 µMho) for mobile phase A	As needed
Methanol, HPLC-grade for mobile phase B	As needed
Bench-top centrifuge or microcentrifuge (RCF # >10,000)	1
Vortexer	1
Centrifugal vacuum concentrator	1
Standard Eppendorf Tubes™, polypropylene, 0.5-mL and 1.5-mL	As needed
Measuring cylinder, glass, 1000-mL	As needed
HPLC bottles, glass, 1000-mL	2
Autosampler vials and inserts, conical, 220-µL and 1000-µL	As needed
Applied Biosystems Amino Acid Analyzer (AAA) C18 Column (5 µm, 4.6 × 150 mm)	1
Cliquid® Software for Routine Amino Acid Analysis	—
LC/MS/MS System with a TurboIonSpray® source and required gases (see “Required MS systems and software” on page 20)	—
PEEK™ tubing, 0.005-in. ID (red)	As needed

Label Samples

2

This chapter covers:

Amino acid labeling workflow

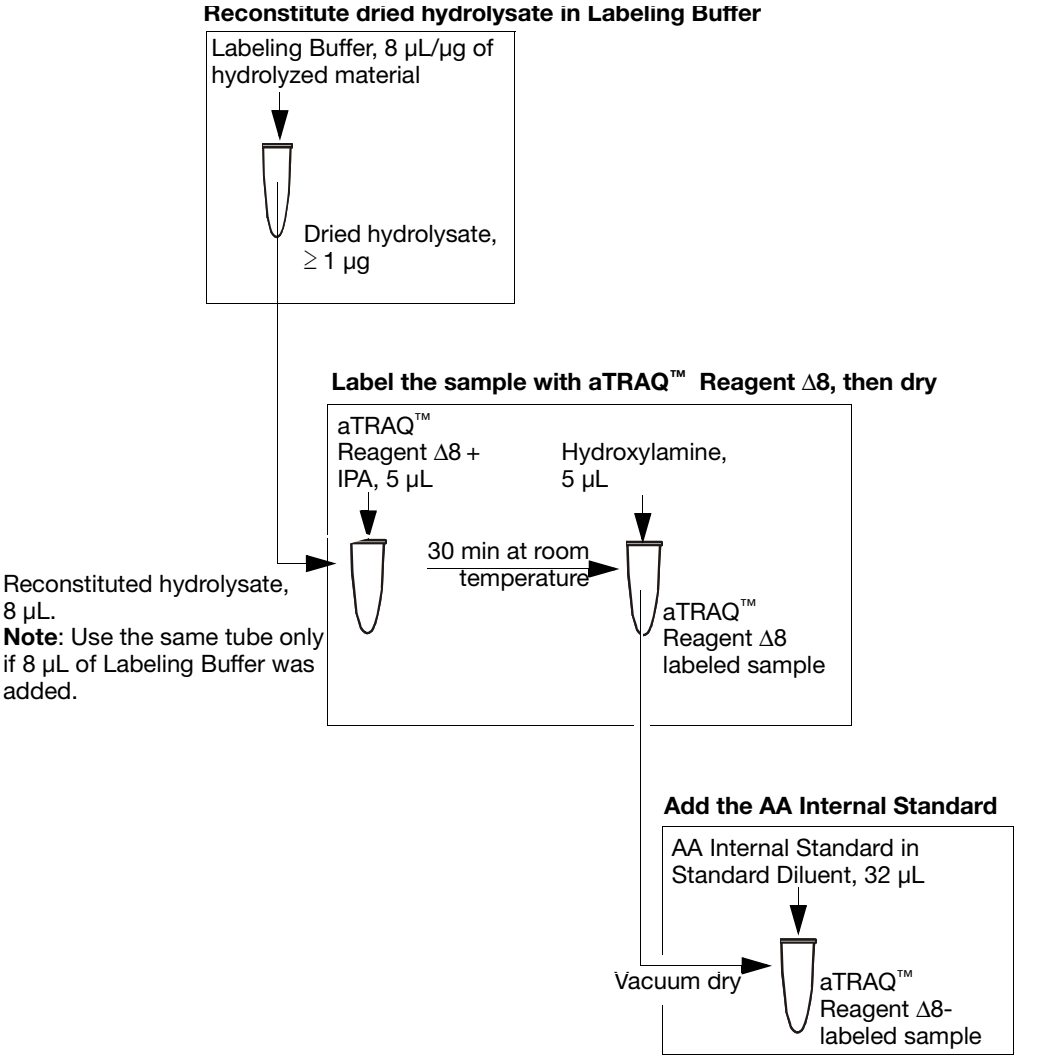

Figure 2 Labeling workflow for one hydrolysate sample

Before you begin

Review the safety warnings in "Safety" on page vii. For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer and observe all relevant precautions.

Test the labeling protocol

If you are running the protocol for the first time, Applied Biosystems recommends that you practice performing the labeling protocol as described in Appendix B, Quality Assurance, "Using Unlabeled Standard" on page 39. Analyze the practice sample by LC/MS/MS to verify the proficiency of sample handling, and efficiency of the labeling protocol for each amino acid.

IMPORTANT! When performing the labeling protocol, you pipette volumes as small as 5- and 10-µL. Slight variability in the accuracy of pipetting such small volumes can cause large variability in reagent concentrations and, consequently, analytical result. To optimize accurate pipetting, see "Handling tips to ensure accurate concentrations and volumes" on page 38.

When testing the labeling protocol, you may determine that alternative steps are required for your sample. If so, modify the procedures on pages 14 through page 16.

Review Appendix A, "Amino Acid Amounts," for information on:

- The amino acids in the internal standard that are labeled with Δ0 reagent and their amounts.
- Incorporating norvaline and norleucine standards and amounts.

Prepare the vials of reagent

Immediately before use:

- Determine the number of sample assays you need to perform, then calculate the number of vials of aTRAQ™ Reagent required to label that number of samples. One vial of aTRAQ™ Reagent Δ8 labels 15 sample assays.
- Allow the reagents and each required vial of aTRAQ™ Reagent Δ8 to reach room temperature. Return the reagents to storage at −15 °C or below within 2 hours of thawing.
- Briefly centrifuge the reagent and aTRAQ™ Reagent vials to dislodge material potentially trapped in the caps.

- Inspect the vial of Labeling Buffer. If precipitate is present, warm the vial to 37 ° C, then vortex.

Reconstitute the dried hydrolysate samples

Note: If you want to follow the hydrolysis recovery, add 160 pmole of norleucine per µg of sample.

1. If necessary, dry the hydrolysate sample.

   IMPORTANT! For optimal labeling, the hydrolysate sample must be completely dry.

2. Add Labeling Buffer (contains 20 µM L-norvaline).

   To each sample tube containing 1 µg hydrolysate:

   a. Add 8 µL Labeling Buffer.

   b. Vortex to mix, then spin.

   To each sample tube containing >1 µg hydrolysate:

   a. For every 1 µg of hydrolysate, add 8 µL of Labeling Buffer. For example, if your sample contains 5 µg of hydrolysate, add 40 µL of Labeling Buffer.

   b. Vortex to mix, then spin.

   c. Transfer an 8-µL aliquot of the hydrolysate sample/Labeling Buffer solution to a fresh tube for labeling in "Label the samples with aTRAQ™ Reagent Δ8" on page 15. If you need to repeat the aTRAQ™ Reagent labeling, refrigerate the remaining sample.

Label the samples with aTRAQ™ Reagent Δ8

Prepare the labeling reagent solution

Repeat the following procedure for each required vial of aTRAQ™ Reagent Δ8.

IMPORTANT! Throughout the procedure, cap each tube promptly to avoid evaporation.

1. Spin the vial of aTRAQ™ Reagent Δ8 (at room temperature) to bring the solution to the bottom of the vial.
2. Add 70 µL of isopropanol. Date the vial (discard after 4 weeks).
3. Vortex the solution to mix, then spin.

Label samples

Repeat the following procedure for each sample.

IMPORTANT! Throughout the procedure, cap each tube promptly to avoid evaporation.

1. To a sample from step 2 in "Reconstitute the dried hydrolysate samples" on page 14, add 5 µL of the aTRAQ™ Reagent Δ8 solution.

   IMPORTANT! Immediately store unused aTRAQ™ Reagent Δ8 solution at –15 °C or below.
2. Vortex to mix, then spin.
3. Incubate the sample at room temperature for at least 30 min.
4. Add 5 µL of Hydroxylamine.
5. Vortex to mix, then spin.
6. Dry the sample completely in a centrifugal vacuum concentrator (generally not more than 1 hour).

IMPORTANT! Unless you immediately continue to the next section (to combine the labeled sample with the internal standard), store the dried labeled samples at –15 °C or below.

Add the internal standard

The following procedure yields enough material for approximately ten 2-µL injections for each sample. See Appendix A, "Amino Acid Amounts," for the aTRAQ™ Reagent-labeled amino acids in each injection.

Prepare the internal standard solution

1. Spin a vial of AA Internal Standard to bring the lyophilized material to the bottom of the vial.
2. Prepare a 5 pmol/amino acid/µL internal standard solution by reconstituting one vial of AA Internal Standard as follows:
   a. Find the amount of Standard Diluent that is specified on the AA Internal Standard vial label (approximately 1.8 mL).
   b. Dispense 1 mL of the Standard Diluent into the AA Internal Standard vial.

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

   c. Vortex the vial in 30- to 60-second increments until all material is dissolved.
   d. Add the remaining Standard Diluent (approximately 0.8 mL).
   e. Vortex to mix.

Add the internal standard solution to the labeled samples

For each sample from step 6 on page 15:

1. Add 32 µL of AA Internal Standard solution. Store unused AA Internal Standard solution at –15 °C or below.
2. Vortex to mix, then spin.
3. Transfer the labeled sample/internal standard mixture to an autosampler vial with a low-volume insert.
4. To remove potential air trapped in the bottom of the vial, tap or spin the vial.

Continue to Chapter 3, "LC/MS/MS Analysis."

LC/MS/MS Analysis 3

This chapter covers:

Hardware overview

Required MS systems and software

- API 3200™ System
- API 4000™ System
- 3200 QTRAP® System
- 4000 QTRAP® System
- Analyst® Software 1.5 or later, using the IntelliQuant integration algorithm, and Cliquid® Software for Routine Amino Acid Analysis

Note: To update Analyst Software, see the *Cliquid® Amino Acid Software for Routine Amino Acid Analysis Installation Guide*.

Recommended HPLC autosamplers

- Agilent 1100 series, with:
  - Binary pump G1312A
  - Well-plate autosampler G1367A
  - Column oven G1316A
- Agilent 1200 series, with:
  - Binary pump G1312A
  - Well-plate autosampler G1367B
  - Column oven G1316A
- Shimadzu Prominence, with:
  - System controller CBM-20A
  - 2 Isocratic pumps LC-20AD [includes automatic purge (flush) kit and semi-micro gradient mixer SUS-20A]
  - Autosampler SIL-20AC
  - Column oven CTO-20AC

Note: During the Cliquid Software installation, acquisition and quantitation method files preconfigured for the above systems are installed.

Overview

Analyst software

Analyst Software provides a single point of control for the mass spec and HPLC devices. A user experienced in MS can customize the automated method development, data analysis, review, and reporting features.

Cliquid® software

The Cliquid® Software for Routine Amino Acid Analysis module communicates with the Analyst Software to retrieve and store information, allowing users with minimal MS experience to analyze samples by using an intuitive point-and-click interface. By selecting the corresponding option on the Home page, you can perform the Hydrolysate Sample Assay, Hydrolysate System Suitability Test, and column maintenance. Refer to the *Cliquid® Software Help System* for detailed information on the Cliquid® software.

Workflow

Figure 3 shows the workflow for analyzing the aTRAQ™ Reagent-labeled samples using the recommended MS and HPLC systems.

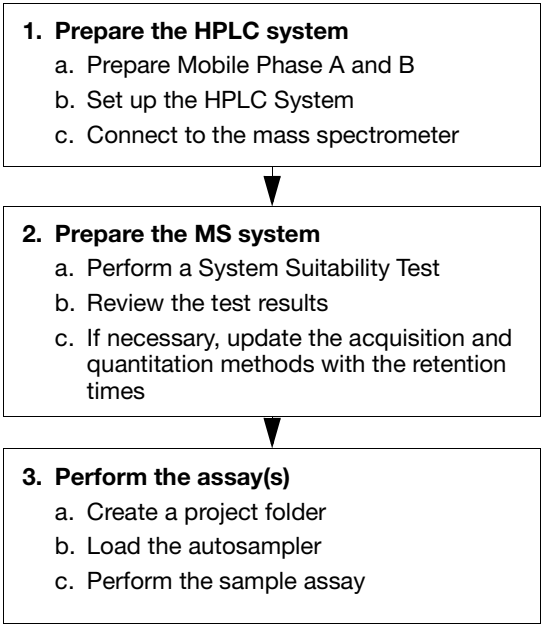

Figure 3 HPLC/MS/MS analysis workflow

Before you begin If necessary, have the Lab Manager:

- Set up the hardware profile and create customized acquisition and quantitation methods for HPLC autosamplers other than those recommended on page 20. Appendix D, "Developing an Acquisition Method," has recommended starting point values for creating the methods.
- Perform mass calibration if the MS has not been calibrated in 3 to 6 months or if the MS source has been recently cleaned. Verify the calibration by performing a system suitability test or analyzing a control sample, then update the retention times in the quantitation method.

  Note: If you use the recommended MS and HPLC systems, you can perform the system suitability test on page 25.

Prepare the HPLC system

Review the safety warnings in "Safety" on page vii. For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer and observe all relevant precautions.

Prepare the mobile phases

Note: The following procedure yields sufficient mobile phase A (1 liter) and B (500 mL) for analysis of up to 75 injections.

To prepare mobile phase A:

1. In a 1-L volumetric flask, add approximately 500 mL of Milli-Q® water or equivalent, HPLC-grade.
2. Add:
   - 1.00 mL Mobile Phase Modifier A
   - 100.0 µL Mobile Phase Modifier B
3. Swirl the flask to mix.
4. Bring to volume with Milli-Q water or equivalent, HPLC-grade, then mix.

   For optimal shelf-life, transfer the solution to an amber glass bottle. Label the bottle with the date prepared (discard unused mobile phase A after a week).

To prepare mobile phase B:

1. In a 500-mL volumetric flask, add approximately 250 mL of methanol, HPLC-grade.
2. Add:
   - 0.50 mL Mobile Phase Modifier A
   - 50.0 µL Mobile Phase Modifier B
3. Gently swirl the flask to mix.
4. Bring to volume with methanol, HPLC-grade, then mix.
5. Transfer the solution to an appropriate bottle.

Set up the HPLC system

1. Set up the HPLC system with mobile phases A and B, and connect the Amino Acid Analysis (AAA) C18 Column according to the documentation provided with your equipment.

   IMPORTANT! Review the safety information provided with your equipment and the safety warnings in "Safety" on page vii.

   IMPORTANT! Use the column only for the Applied Biosystems Amino Acid Analysis Labeling Protocol. Any other use may compromise the integrity of the column.

2. Flush the system.

If the column has been stored, see Appendix C, "Equilibrate before reuse," page 42.

Prepare the MS system

Review the safety warnings in "Safety" on page vii. For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer and observe all relevant precautions.

Perform the system suitability test

The system suitability test warms up the mass spectrometer and peripherals, and verifies that the entire system (HPLC and mass spectrometer) is working properly. The test also validates the retention times and sensitivity levels for the MS system.

Perform the system suitability test at least once a day (before running samples), using the AA Internal Standard as your sample. If necessary, flush the system before starting the test.

Repeat the system suitability test until retention times stabilize. For a system with a new column or being used for the first time after storage, perform the test at least three times; with a column that is in standby mode, perform the test at least two times. Equilibrate the column by running the system suitability test with an equilibration time of 15 min.

The system suitability test takes approximately 30 minutes to complete. To perform the system suitability test:

1. Prepare a vial of AA Internal Standard as described on page 17.
2. Transfer 100 µL of AA Internal Standard to an autosampler vial and place it in the HPLC autosampler. Note the plate code and position (if applicable), rack code, rack position, and sample position of the vial.
3. If Analyst Software is open, close it.
4. Open Cliquid Software by clicking  on the desktop.

5. Enter your log in information, then click **Get Started**. For a Lab Technician, the Home page in Figure 4 opens. (The Home page for a Lab Manager displays additional tasks.)

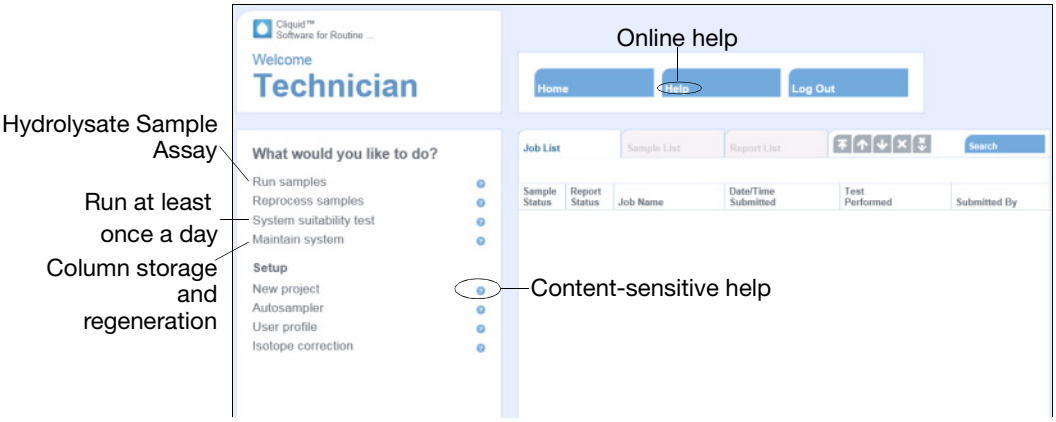

Figure 4 Features of the Home page for a Lab Technician

6. In the Home page (Figure 4), select **System suitability test**.

7. Proceed through the wizard, clicking **Next** to advance to the next page. When prompted, select or enter the following:

System Suitability Test Wizard Page	Selection or Input
Choose test	Select **Hydrolysate System Suitability**.
Position sample	For the vial of AA Internal Standard, enter the: • Rack code • Rack position • Sample position • If required for your autosampler: – Plate code – Plate position ()
Customize report	1. Select **Hydrolysate System Suitability**. 2. Select the report output format.
Submit sample	Specify an equilibration time. Recommended times for a system that is: • Running = 0 min • In standby mode = 2 min • Being started = 10 min • Has new buffers or column = 15 min

8. Click **Submit**. The Home page opens, with the system suitability test added to the sample list.

IMPORTANT! Do not add sample runs to the job list until the system suitability test is complete. You may need to update the retention times in the acquisition method.

IMPORTANT! While Cliquid Software is running and/or processing submissions, Analyst software cannot be opened. Before starting Analyst software, wait until all samples are processed, then log out of the Cliquid Software.

Review the system suitability test results

After a green check mark appears in both the Sample Status and Report Status columns next to the test name in the job list, the test and report are complete.

1. Click the test name in the job list to highlight the row, then select the **Report List** tab.
2. To open the system suitability test report, click the **View** button beside the report. The MS Word version of the report is displayed.

   Note: Although the report is created through Cliquid Software, it is saved in the Analyst Data\Projects directory. To access the report in other formats, go to Analyst Data\Projects\System suitability test\Results folder.
3. Review the report for failed items. If the:
   - Analyte retention times (RT) differ from the expected retention time by more than 0.5, have your lab manager update the retention times in the acquisition and quantitation method files.
   - Analyte peak areas are less than the expected peak areas, repeat the system suitability test. If most or all of the peak areas are below the threshold, the MS may need tuning. If only a few of the peak areas are below the threshold, then you may need a fresh AA Internal Standard sample.
4. Read the diagnosing statement on the report. For additional diagnosing information, see online Help, System Suitability Test.

Continue to troubleshoot and repeat the system suitability test until all compounds pass.

Perform the sample assay

Create a project folder

All data files are associated with a project. A project folder must exist before you use Cliquid Software to build a sample list or customize a report. Although created through Cliquid Software, the project folder is stored in [Drive]\Analyst Data\Projects.

To create a new project folder for an assay:

1. In the Cliquid Software Home page (Figure 4 on page 26), click **New project**. The New Project screen opens.
2. Enter a name for the project folder.
3. Click **Create**.
4. After "Project created successfully" is displayed, click **Done** to open to the Home page.

IMPORTANT! Refer to the documentation provided with your equipment for safety information. Review the safety warnings in "Safety" on page vii.

Load the autosampler

Place the sample, control, and, if applicable, allo-isoleucine vials in the HPLC rack. Record the corresponding plate code and position (if applicable), rack code, rack position, and sample position of the vials.

Perform the sample assay

1. In the Cliquid Software Home page (Figure 4 on page 26), select **Run samples**.
2. Proceed through the wizard, clicking **Next** to advance to the next page. When prompted, select or enter the following:

Table 5 Run samples selections and input

Sample Assay Wizard page	Selection or input
Choose test	Select **Hydrolysate Sample Assay.**
Build sample list	1. In the sample list template, select the project. 2. Import a sample list or enter sample list information as follows: a. In the Name field, enter the name of your sample. b. Press the **Tab** key or click the first autosampler-specific field that is displayed. The fields are auto-populated with the information from the default autosampler configuration set for the system. c. In the remaining fields, specify the values in each drop-down list or enter values as applicable. – For category (the reference range against which obtained sample concentrations are compared), select **Standard** or **None**. Additional categories may have been created by the Lab Managers. – For normalization value, enter a value only if you analyze a urine sample. **IMPORTANT!** For samples other than urine, leave the field blank or enter **0**. Entering a value yields an erroneous results table. – For internal standard (IS) concentration, enter the numbers on the Certificate of Analysis for the AA Internal Standard for each amino acid. d. For information about the other fields, see online Help, “Entering Sample List Information”. 3. Repeat steps a through c in step 2 for each sample. 4. After you complete entering samples, click **Next**. The software validates the field entries for proper format and flags any formatting errors. 5. Correct all formatting errors. 6. (Optional) Click  to save the sample list.

Table 5 Run samples selections and input *(continued)*

Sample Assay Wizard page	Selection or input
Customize report	Select the appropriate report-generating option. If you choose to generate reports: • After all samples are acquired or after each sample is acquired – Continue on to choose report style and select report output format • Later using the Reprocess samples task – Click **Next** to proceed Submit samples
Submit samples	1. Specify an equilibration time. Recommended times for a system that is: – Running = 0 min – In standby mode = 2 min – Being started = 10 min – Has new buffers or column = 15 min 2. Review the HPLC setup summary. 3. Review the Test, Sample List, and Report Details summary. Correct inaccuracies by navigating to the appropriate screen (by clicking the **Back** button). Alternatively, click **Cancel** to return to the Home page. **IMPORTANT!** If you return to the Home page before completing the submission, all entries in the sample list are lost.

3. After completing the Submit samples page, click **Submit**. The Home page opens, displaying the test in the job list.

Amino Acid Amounts

A

This appendix covers:

AA Internal Standard

Approximately 9.0 nmol of each of the following amino acids is labeled with aTRAQ™ Reagent Δ0. The precise amount of amino acids in a vial of AA Internal Standard is determined for each lot of standard, and is used to determine the volume of Standard Diluent required to make an approximately 5 pmol/µL solution. The exact concentration of amino acid in the reconstituted standard is reported on the Certificate of Analysis.

- L-serine
- Glycine
- L-aspartic acid
- L-alanine
- L-threonine
- L-glutamic acid
- L-histidine
- L-proline
- L-arginine
- L-methionine sulfoxide
- L-cystine
- L-lysine
- L-valine
- L-norvaline
- L-methionine
- L-tyrosine
- L-isoleucine
- L-leucine
- L-norleucine
- L-phenylalanine

Provided reagents

Labeling Buffer Labeling Buffer contains 20 µM norvaline, which is subsequently labeled with aTRAQ™ Reagent Δ8.

An assay injection

A 2-µL injection of the samples prepared according to the labeling protocol (Chapter 2) contains:

- aTRAQ™ Reagent Δ8-labeled amino acids in the sample.
- 10 pmole of aTRAQ™ Reagent Δ8-labeled norvaline and norleucine if you added 160 pmole of norleucine per µg of sample before the hydrolysis.
- Approximately 10 pmole of each Δ0-labeled amino acid in the standard, including norvaline and norleucine.The exact amounts depend on the concentrations reported on the Certificate of Analysis.

Quality Assurance

B

This appendix covers:

Handling tips to ensure accurate concentrations and volumes

Small volume handling tips

To ensure accurate concentrations throughout the labeling protocol:

- 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 at 10,000 × g for 2 minutes
- Cap each tube promptly to avoid evaporation
- Store materials at the recommended 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:
  - 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
  - Immersion depth (see the pipette manufacturer's recommendation)
- 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.

Quality assurance

The aTRAQ™ Starter Kit Hydrolysates provides two standards:

- **AA Internal Standard** – Used as an internal standard for quantitation of the labeled samples.
- **AA Unlabeled Standard** – To verify the performance of the entire methodology (see below).

Testing the labeling protocol

If you are running the protocol for the first time, Applied Biosystems recommends that you practice performing the protocol to label the vial of AA Unlabeled Standard. Analyzing the practice sample by LC/MS/MS (see Chapter 3, LC/MS/MS Analysis) provides information about the proficiency of sample handling and the efficiency of the labeling protocol for each amino acid.

Verify that peaks display at m/z 113 and 121. Most amino acids are stable in the unlabeled amino acid solution, so the calculated amount should be 80 to 120 µM. You may, however, observe lower amounts of Met and higher amounts of MOx because, while in solution, Met can oxidize to MOx. Asparagine and glutamine in the standard can convert to Glu and Asp, so you may observe higher amounts of Glu and Asp, too.

Using Unlabeled Standard

Follow the labeling protocol (Chapter 2), substituting 1.6 µL of 100-µM AA Unlabeled Standard (containing 160 pmole of each amino acid) for a hydrolysate sample.

After labeling with aTRAQ™ Reagent Δ8, the AA Unlabeled Standard contains the same amino acids as the vial of AA Internal Standard except norleucine (see page 34).

After labeling with aTRAQ™ Reagent Δ8 and adding AA Internal Standard, a 2-µL injection contains:

- Approximately 10 pmole of each Δ0-labeled amino acid
- Approximately 10 pmole of each Δ8-labeled amino acid

Workflow efficiency

The efficiency of the labeling protocol workflow can be observed by monitoring the recovery of the norleucine and norvaline that are spiked in the aTRAQ™ Reagent Δ8 labeled sample. Norvaline is introduced into the sample with the Labeling Buffer. You can also spike norleucine into your sample prior to the hydrolysis if you want to monitor the hydrolysis efficiency. Add 160 pmole of norleucine per µg of sample.

Typically, the workflow is acceptably efficient when the amount of norleucine and norvaline recovered is 100 µM ±20%. If the experimentally determined amount is unacceptable, repeat the labeling protocol with additional samples.

Column Maintenance

C

This appendix covers:

Maintaining the HPLC column

IMPORTANT! Review "Prepare the mobile phases" on page 23.

Wash the column

Before storing the Amino Acid Analysis (AAA) C18 Column, use Milli-Q water or equivalent as the sample and wash the column as follows:

1. Prepare 500 mL of 70% acetonitrile/30% Milli-Q® water or equivalent.
2. On the HPLC system, replace the Buffer B solution with the 70% acetonitrile/30% Milli-Q solution.
3. Flush the HPLC system.
4. In the Cliquid® Amino Acid Analysis Software Home page (Figure 4 on page 26), select **Maintain System**.
5. In the Choose Wizard page, select **Column Storage and Regeneration**. The system washes the column with 25 mL of 70% acetonitrile/30% solution at 1.0 mL/min for 25 min.

After completing the task, remove the column and seal the ends with two end caps. Store the column at room temperature.

Equilibrate before reuse

IMPORTANT! Use the column only for the Applied Biosystems Amino Acid Analysis Labeling Protocol. Any other use may compromise the integrity of the column.

Before using a column that is stored, use Milli-Q water or equivalent as the sample and equilibrate the column as follows:

1. Set up the HPLC system with the Amino Acid Analysis (AAA) C18 Column and the recommended Mobile phases A and B (see "Prepare the mobile phases" on page 23).
2. Flush the HPLC system.
3. Perform the system suitability test at least three times. Repeat until the retention times stabilize.

Developing an Acquisition Method

D

This appendix covers:

MRM overview

The preconfigured acquisition and quantitation method files provided with the Cliquid® Amino Acid Analysis Software define a multiple reaction monitoring (MRM) mass spectrometry experiment.

MRM allows you to set:

- The first quadropole filter to select the labeled amino acid of interest (precursor ion) for *fragmentation*

  and

- Another quadropole filter to select the cleaved aTRAQ™ Reagent label of interest (product ion) for *detection*

You also select the retention time and MS parameters for the compound of interest.

The MRM scan has one experiment using scheduled MRM. Scheduled MRM sets a window around the retention time during which specific amino acids are monitored allows for collecting more data points per peak and more accurate quantitation.

Developing an acquisition method for non-supported instruments

The values in Table 6 through Table 8 are the values used in the preconfigured acquisition and quantitation method files. These values can be used as starting points for a Lab Manager to create customized methods for non-supported autosamplers.

HPLC conditions

The recommended column temperature is 50 °C, injection volume is 2 µL, and flow rate is 0.8 mL/min. Table 6 provides the recommended LC gradient.

Table 6 Recommended LC gradient for the assay

Total Time (min)	%Mobile Phase A	%Mobile Phase B
0.0	98	2
6.0	60	40
10.0	60	40
11.0	10	90
12.0	10	90
13.0	98	2
18.0	98	2

TIS values

Table 7 shows the TurboIonSpray® (TIS) source Source/Gas and Compound values.

Table 7 Recommended TIS values

Gas or compound	LC/MS/MS systems			
	API 3200™	3200 QTRAP®	API 4000™	4000 QTRAP®
TurboIonSpray® source/gas values				
CUR	20	20	20	20
CAD	3	Medium	3	Medium
IS	1500	1500	1500	1500
TEM	600	600	600	600
GS 1	60	60	60	60
GS 2	60	60	60	60
ihe	On	On	On	On
Compound values				
DP	30	30	30	30
FP	n/a	n/a	n/a	n/a
EP	10	10	10	10
CE‡	30	30	30	30
CXP	5	5	5	5

‡ The CE value for Cys and Lys (Q1 mass >400) is 50.

MRM values

Table 8 for the Q1 (precursor ion) and Q3 (product ion) masses.

Table 8 MRM transitions for the amino acids

Amino Acid	Abbreviation	Q1/Q3 mass (amu)
L-Serine	Ser	IS 246.2/113.1 Analyte 254.2/121.1
Glycine	Gly	IS 216.1/113.1 Analyte 224.1/121.1
L-Aspartic acid	Asp	IS 274.1/113.1 Analyte 282.1/121.1
L-Alanine	Ala	IS 230.2/113.1 Analyte 238.2/121.1
L-Threonine	Thr	IS 260.2/113.1 Analyte 268.2/121.1
L-Methionine sulfoxide	MOx	IS 306.1/113.1 Analyte 314.2/121.1
L-Glutamic acid	Glu	IS 288.2/113.1 Analyte 296.2/121.1
L-Histidine	His	IS 296.2/113.1 Analyte 304.2/121.1
L-Proline	Pro	IS 256.2/113.1 Analyte 264.2/121.1
L-Arginine	Arg	IS 315.2/113.1 Analyte 323.2/121.1
L-Cystine	Cys	IS 521.2/113.1 Analyte 537.2/121.1
L-Lysine	Lys	IS 427.3/113.1 Analyte 443.3/121.1
L-Valine L-Norvaline	Val Nva	IS 258.2/113.1 Analyte 266.2/121.1

Table 8 MRM transitions for the amino acids *(continued)*

L-Methionine	Met	IS 290.2/113.1 Analyte 298.2/121.1
L-Tyrosine	Tyr	IS 322.2/113.1 Analyte 330.2/121.1
L-Isoleucine L-Leucine L-Norleucine	Ile Leu Nle	IS 272.2/113.1 Analyte 280.2/121.1
L-Phenylalanine	Phe	IS 306.2/113.1 Analyte 314.2/121.1

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