Applied Biosystems Amino Acid Analysis for Hydrolysate Samples

iTRAQ® Reagents Application Kit for Use with Applied Biosystems/MDS Sciex Amino Acid 20/20 Analyzer

Protocol

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Not for Use in Diagnostics

Applied Biosystems

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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.

CAUTION – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

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

DANGER – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Chemical Hazard Warning

WARNING **CHEMICAL HAZARD.** Some of the chemicals used with 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 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, 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 **https://docs.appliedbiosystems.com/msdssearch.html**
2. In the Search field of the MSDS Search page:
   a. Type in the chemical name, part number, or other information that you expect to appear in the MSDS of interest.
   b. Select the language of your choice.
   c. Click **Search**.

3. To view, download, or print the document of interest:
   a. Right-click the document title.
   b. Select:
      - **Open** – To view the document
      - **Save Target As** – To download a PDF version of the document to a destination that you choose
      - **Print Target** – To print the document
4. To have a copy of an MSDS sent by fax or e-mail, in the Search Results page:
   a. Select **Fax** or **Email** below the document title.
   b. Click **RETRIEVE DOCUMENTS** at the end of the document list.
   c. Enter the required information.
   d. Click **View/Deliver Selected Documents Now**.

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, 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 Relative and Absolute Proteomic Quantitation Using iTRAQ™ Reagent Methodology Chemistry Reference Guide*
- *Applied Biosystems/MDS Sciex Cliquid® Software for Routine Amino Acid Analysis Online Help*
- *Applied Biosystems Amino Acid Analysis Quick Reference Card for Hydrolysate Samples*
- *Applied Biosystems/MDS Sciex Cliquid® Software for Routine Amino Acid Analysis Site Planning Guide*

For the portable document format (PDF) versions of the chemistry reference guide, this protocol, and the quick reference card, go to **http://www.appliedbiosystems.com**, then click the link for **Support**. 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 (PN 4351918) in the Search field.

Note: For additional documentation, see "How to Obtain Support" on page xiii.

Obtaining Information Using Online Help

The Analyst® and Cliquid® Software 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 of the software window
- Select the **Help** tab
- Press **F1** (not applicable in 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 only for submitting comments and suggestions relating 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" below).

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:

- 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.

Introduction to iTRAQ® Reagent Chemistry

1

This chapter covers:

Overview

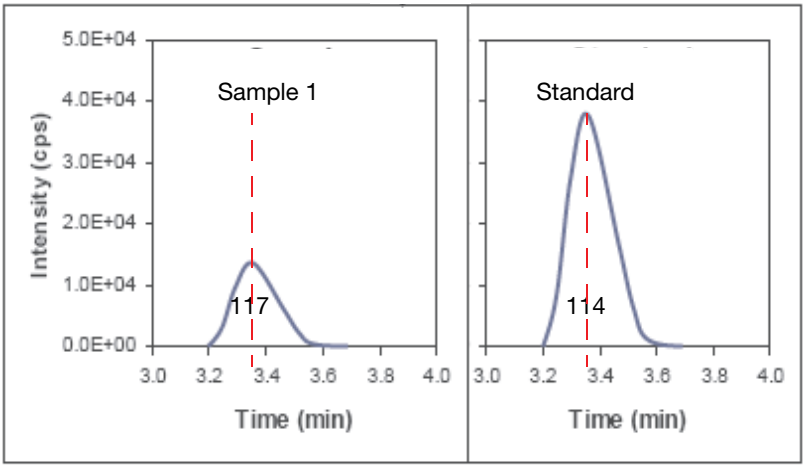
The Applied Biosystems Amino Acid Analysis for Hydrolysate Samples Protocol Protocol enables identification and quantitation of amino acids. The kit provides iTRAQ® Reagent 117 for labeling hydrolysate samples and an iTRAQ Reagent 114-labeled amino acid internal standard.

Product Capabilities

Using iTRAQ Reagent to label amino acids allows you to assay amino acid levels in:

- Peptide hydrolysates
- Protein hydrolysates
- Hydrolysates from animal feed

Analysis using the Applied Biosystems/MDS Sciex Amino Acid 20/20 Analyzer system and Cliquid® Software for Routine Amino Acid Analysis (ordered separately) provides easy data interpretation for relative and absolute amino acid quantitation (Figure 1).



$$\text{Amount}_{\text{sample}} = \frac{\text{Area}_{117}}{\text{Area}_{114}} \times \text{Amount}_{\text{std}}$$

Note: The retention times of the 117 and 114 peaks are identical (peaks are isobaric).

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

For More Information

Refer to the *Applied Biosystems Relative and Absolute Proteomic Quantitation Using iTRAQ™ Reagent Methodology Chemistry Reference Guide* for supplementary information on:

- iTRAQ Reagent chemistry, kits, and kit materials
- How to test, run, and modify the iTRAQ Reagents protocol
- Sample handling guidelines

To order or download a copy of the chemistry guide, see "How to Obtain More Information" on page xii.

Starter and Assay Kits and Accessories

To order the following kits or accessories, go to

http://store.appliedbiosystems.com

Kits

- Applied Biosystems/MDS Sciex Amino Acid 20/20 Analyzer Starter Kit - Hydrolysate – Provides sufficient material to run 50 iTRAQ Reagent 117-labeled samples with iTRAQ Reagent 114-labeled amino acid internal standard and the standards set.
- Applied Biosystems iTRAQ™ Reagent Application Kit - Amino Acid 20/20 Analyzer (50 Assay) – Provides sufficient material to run 50 iTRAQ Reagent 117-labeled samples with iTRAQ Reagent 114-labeled amino acid internal standard.
- Applied Biosystems iTRAQ™ Reagent Application Kit - Amino Acid 20/20 Analyzer (200 Assay) – Provides sufficient material to run 200 iTRAQ Reagent 117-labeled samples with iTRAQ Reagent 114-labeled amino acid internal standard.

Accessories

- Applied Biosystems Amino Acid 20/20 Standards Set - Hydrolysate (see Table 1)
- Applied Biosystems Amino Acid 20/20 Standard - 114 Labeled - Hydrolysate
- Applied Biosystems Amino Acid Analysis (AAA) C18 Column

Kit Materials Packaged with the Starter Kit

The Applied Biosystems/MDS Sciex Amino Acid 20/20 Analyzer Starter Kit - Hydrolysate includes the 50-Assay Kit, the standards set, this document, and a quick reference card (Table 1). For recommendations on using the Standards Set, see "Quality Control Tests" on page B-48.

IMPORTANT! When you receive the shipping container, immediately remove the iTRAQ® Reagent Application Kit and the standards set. Store both items at –15 to –25 °C.

Table 1 Applied Biosystems/MDS Sciex Amino Acid 20/20 Analyzer Starter Kit - Hydrolysate materials and storage conditions

Item	Description
Store at –15 to –25 °C	
iTRAQ™ Reagent Application Kit - Amino Acid 20/20 Analyzer (50 Assay)	One kit. Includes iTRAQ Reagent 117, Certificate of Analysis, buffers, solvents, hydroxylamine, and mobile phase modifiers for performing the labeling protocol. For a detailed list of the materials, see Table 2 on page 1-6.
Amino Acid 20/20 Standards Set - Hydrolysate	Dried standard of 20 amino acids (≈10 nmol/amino acid) labeled with iTRAQ Reagent 114 and iTRAQ Reagent 117, unlabeled standard, and sample diluent for quality control testing. For information, see "Quality Control Tests" on page B-48. • 1 vial Hydrolysates Standard - 114 Labeled • 1 vial Hydrolysates Standard - 117 Labeled • 1 vial Hydrolysates Standard - Unlabeled • 2 vials Sample Diluent - Amino Acid • Certificate of Analysis The vials of Hydrolysates Standard - 114 Labeled and - 117 Labeled contain the same amino acids (see page A-36). The vial of Hydrolysates Standard - Unlabeled contains the same amino acids as the labeled standards, except norvaline. Norvaline is incorporated during labeling.

Table 1 Applied Biosystems/MDS Sciex Amino Acid 20/20 Analyzer Starter Kit - Hydrolysate materials and storage conditions *(continued)*

Item	Description
Documentation	
Applied Biosystems Amino Acid Analysis for Hydrolysate Samples Protocol Protocol	This document.
Applied Biosystems Amino Acid Analysis Quick Reference Card for Hydrolysate Samples	A laminated card that provides a quick reference to the steps in the Amino Acid Analysis for Hydrolysate Samples iTRAQ™ Reagents Labeling Protocol, LC/MS/MS conditions, and the amino acids in the internal standard.

Kit Materials Packaged with the 50-Assay and 200-Assay Kits

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

IMPORTANT! When you receive the shipping container, immediately remove the iTRAQ® Reagents Application Kit and the Hydrolysates Standard - 114 Labeled bag. Store both items at –15 to –25 °C.

See Table 2 for materials contained in each kit.

Table 2 iTRAQ™ Reagents Application Kit - Amino Acid 20/20 Analyzer (50 Assay or 200 Assay) materials and storage conditions

Item	Quantity in 50-Assay Kit	Quantity in 200-Assay Kit	Contents
Store at –15 to –25 °C			
iTRAQ™ Reagents Application Kit - Amino Acid 20/20 Analyzer (50 Assay or 200 Assay)			
• iTRAQ Reagent 117	4 vials, 1 unit/vial	14 vials, 1 unit/vial	Amine-modifying labeling reagent. One unit (one vial) of reagent labels approximately 15 assays.
• Certificate of Analysis	1	1	Provides purity information for iTRAQ Reagent 117.
• Labeling Buffer - Amino Acid‡	1 vial, 1.8 mL	4 vials, 1.8 mL/vial	Borate buffer, pH 8.5. Also contains norvaline (30 pmol/µL).
• Hydroxylamine	1 vial, 250 µL	1 vial, 250 µL	6% hydroxylamine solution. Reverses partial labeling of the phenolic hydroxyl group of tyrosine.
• Sample Diluent - Amino Acid‡	1 vial, 1.8 mL	4 vials, 1.8 mL/vial	3.5% formic acid for reconstituting hydrolysates standard.
• Mobile Phase Modifier A‡	2 vials, 1.8 mL/vial	6 vials, 1.8 mL/vial	100% formic acid for mobile phase A and B preparation.
• Mobile Phase Modifier B‡	1 vial, 200 µL	3 vials, 200 µL/vial	100% heptafluorobutyric acid for mobile phase A and B preparation.
• Isopropanol‡	1 vial, 1.8 mL	1 vial, 1.8 mL	Isopropanol, absolute.

Table 2 iTRAQ™ Reagents Application Kit - Amino Acid 20/20 Analyzer (50 Assay or 200 Assay) materials and storage conditions *(continued)*

Item	Quantity in 50-Assay Kit	Quantity in 200-Assay Kit	Contents
Hydrolysates Standard - 114 Labeled	1 bag	4 bags	• 1 vial of Hydrolysates Standard - 114 Labeled. Dried standard of 20 amino acids labeled with iTRAQ Reagent 114 to use as an internal standard. For information, see "Amino Acids in Hydrolysates Standard - 114 Labeled" on page A-36. • 1 vial of Sample Diluent - Amino Acid • Certificate of Analysis. Provides the isotopic purity value and precise amount of diluent for reconstituting a vial of standard.
Documentation			
Applied Biosystems Amino Acid Analysis Quick Reference Card for Hydrolysate Samples	1	1	Laminated card that provides a quick reference to the steps in the Amino Acid Analysis for Hydrolysate Samples iTRAQ™ Reagents Labeling Protocol, LC/MS/MS conditions, and the amino acids in the standard.

‡. Can be stored at room temperature.

User-Supplied Materials

WARNING **CHEMICAL HAZARD.** Some of the chemicals referred to in this protocol (such as those in Table 3) 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 3 User-supplied materials

Item	Quantity per Assay
Disposable gloves	As needed
Hydrolysate samples, dry, at least 1 µg peptide or protein each	As needed
Pipetting accessories (gel loader tips, pipettors and tips) suitable for 1-µL to 1-mL volumes, such as P2, P10, P100, P1000 pipettes	As needed
pH paper, pH range 2.5 to 4.5 and 6.5 to 10 to test the pH of the sample when troubleshooting	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
Acetonitrile, HPLC-grade for mobile phase B	As needed
Bench-top centrifuge or microcentrifuge	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	2
HPLC bottles, glass, 1000-mL	2
Autosampler vials and inserts, conical, 220-µL and 1000-µL	As needed

Table 3 User-supplied materials *(continued)*

Item	Quantity per Assay
LC/MS/MS System with a TurboIonSpray® source and required gases. (For information, see the *Applied Biosystems/MDS Sciex* Cliquid® Software for Routine Amino Acid Analysis *Site Planning Guide, mass spectrometer configuration*.)	—
PEEK™ tubing, 0.005 in. ID (red)	As needed
Applied Biosystems Amino Acid Analysis (AAA) C18 Column, reversed-phase, 5 µm, 4.6 mm x 150 mm	—
Applied Biosystems/MDS Sciex Cliquid® Software for Routine Amino Acid Analysis	—

Workflow

In the iTRAQ Reagent labeling protocol for amino acid analysis of protein hydrolysate samples, you label your sample with iTRAQ Reagent 117, then add iTRAQ Reagent 114-labeled amino acid standard as an internal standard (Figure 2).

Next you analyze the sample/internal standard mixture using LC/MS/MS. The use of the labeled internal standard of multiple amino acids allows for correction of variations in the detection response as well as in quantitation.

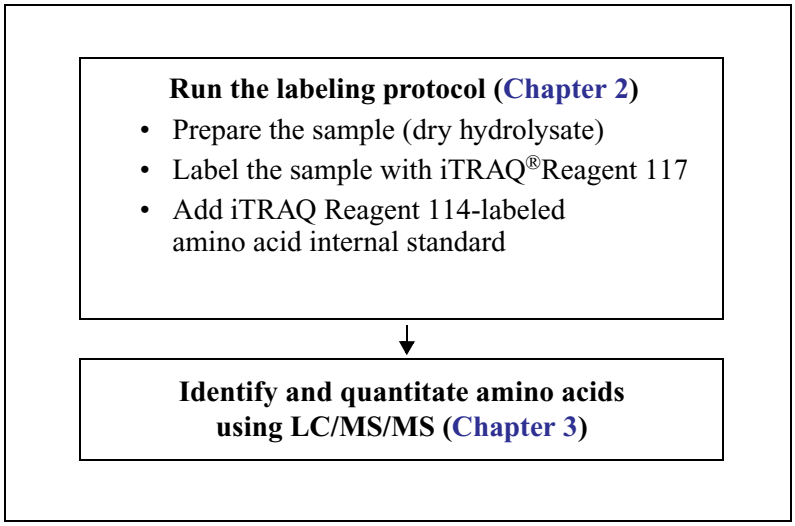


Figure 2 Workflow for amino acid analysis of hydrolysate samples using Applied Biosystems iTRAQ® Reagents

Running the Labeling Protocol 2

This chapter covers:

Overview

Hydrolysate Sample Size

The protocol labels 1 µg of hydrolysate sample (dry, approximately 10 nmol amino acid) with iTRAQ® Reagent 117. The hydrolysate sample can be a peptide hydrolysate, protein hydrolysate, or a hydrolysate from animal feed.

For samples containing more than 1 µg of hydrolysate, maintain the total amino acid amount at no more than 10 nmol and modify the protocol as specified in step 2 on page 14 and step 6 on page 15. Figure 3 summarizes the workflows according to hydrolysate sample size.

If one or more amino acid peaks exhibit saturated signal, dilute the sample with an equal volume of sample diluent. See Appendix B, "Quality Control and Troubleshooting," Table 7.

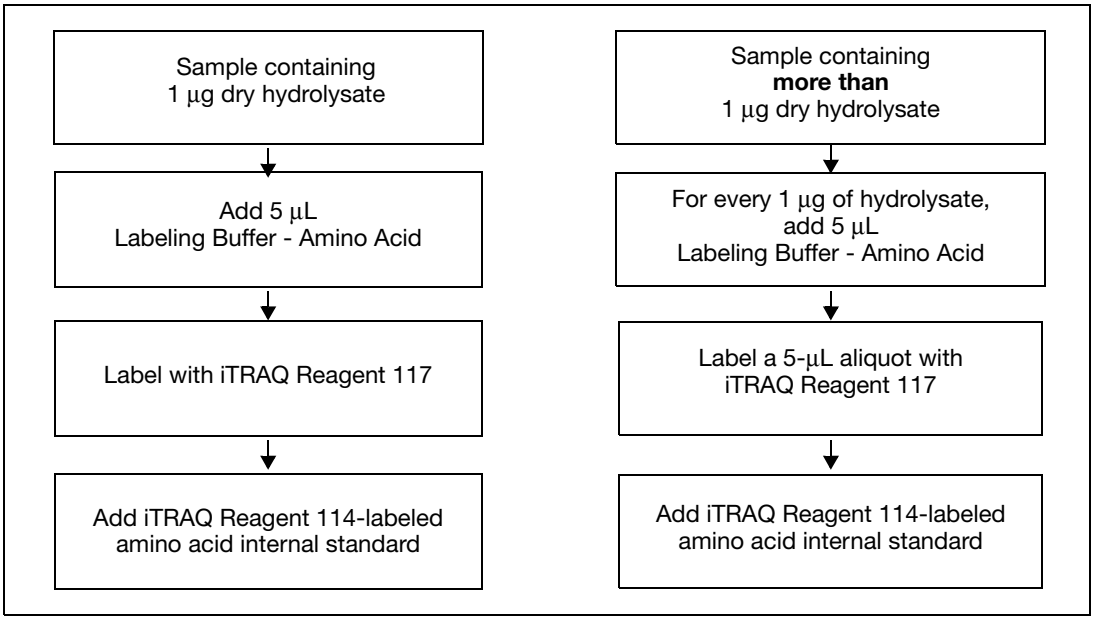


Figure 3 Workflows according to hydrolysate sample size

Assays/Vial

One vial of iTRAQ® Reagent 117 labels approximately 15 assays.

Before You Begin

- Allow the reagents and each required vial of iTRAQ Reagent 117 and Hydrolysates Standard - 114 Labeled to reach room temperature. Return the reagents to storage at –15 to –25 °C within 2 hours.
- If necessary, dry the hydrolysate sample.

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

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

Pipetting Accuracy

Pipetting accuracy is critical for the success of each assay. Review the pipetting recommendations in "Small Volume Handling Tips to Ensure Accurate Concentrations and Volumes" on page 46.

Optional Quality Control Tests

For optional quality-control tests, see "Quality Control Tests" on page 48.

Labeling the Hydrolysate Sample with iTRAQ® Reagent 117

WARNING **CHEMICAL HAZARD.** Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
Isopropanol is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry skin and cause irritation. Exposure may cause central nervous system effects such as drowsiness, dizziness, and headache.
iTRAQ® Reagent 117 is a flammable liquid and vapor. Exposure may cause eye and respiratory tract irritation and blood system damage.

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

1. If necessary, dry the hydrolysate sample.

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

2. Add Labeling Buffer - Amino Acid‡:

   To each sample tube containing 1 µg hydrolysate:

   a. Add 5 µL Labeling Buffer - Amino Acid.

   b. Vortex to mix, then spin.

   To each sample tube containing more than 1 µg hydrolysate:

   a. For every 1 µg of hydrolysate, add 5 µL of Labeling Buffer - Amino Acid. For example, if your sample contains 6 µg of hydrolysate, add 30 µL of Labeling Buffer - Amino Acid

   b. Vortex to mix, then spin.

3. Spin each required vial of iTRAQ Reagent 117 (at room temperature) to bring the solution to the bottom of the vial.

4. Add 70 µL of isopropanol. Mark the vial as “diluted.”

5. Vortex each vial to mix, then spin.

‡. Labeling Buffer - Amino Acid contains 30 pmol/µL norvaline.

6. Label each hydrolysate sample prepared in step 2 with iTRAQ Reagent 117:
   - To each sample tube containing 1 µg hydrolysate:
     a. Add 5 µL of diluted iTRAQ Reagent 117. Cap the tube promptly to avoid evaporation.
     b. Vortex to mix, then spin.
   - To each sample tube containing more than 1 µg hydrolysate:
     a. Transfer a 5-µL aliquot of the hydrolysate sample/Labeling Buffer - Amino Acid solution to a fresh tube.
     b. To the aliquot, add 5 µL of diluted iTRAQ Reagent 117. Cap the tube promptly to avoid evaporation.
     c. Vortex to mix, then spin.
7. Incubate the sample tubes at room temperature for at least 30 min.
8. Add 1 µL of hydroxylamine to each sample tube.
9. Vortex each sample tube to mix, then spin.
10. Incubate the sample tubes at room temperature for at least 5 min.
11. Dry the samples completely in a centrifugal vacuum concentrator (generally not more than an 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 to –25 °C. Store unused diluted iTRAQ Reagent 117 at –15 to –25 °C for up to 4 weeks (see Appendix B, "Material Storage.")

Adding the iTRAQ Reagent 114-Labeled Amino Acid Internal Standard

WARNING **CHEMICAL HAZARD.** Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
iTRAQ® Hydrolysates Standard - 114 Labeled causes respiratory tract and skin irritation.
iTRAQ® Reagent 117 is a flammable liquid and vapor. Exposure may cause eye and respiratory tract irritation and blood system damage.

1. Prepare a 6-pmol/µL iTRAQ Reagent 114-labeled amino acid internal standard by reconstituting one vial of Hydrolysates Standard - 114 Labeled with Sample Diluent - Amino Acid. The amount of Sample Diluent - Amino Acid to use is indicated on the reagent vial label and Certificate of Analysis (approximately 1.67 mL).
2. Vortex to mix, then spin.

   The iTRAQ Reagent 114-labeled amino acid internal standard can be stored at –15 to –25 °C.
3. Add 25 µL of the iTRAQ Reagent 114-labeled amino acid internal standard to each dried iTRAQ Reagent 117-labeled sample.
4. Vortex each tube to mix, then spin.

IMPORTANT! This procedure yields enough material for approximately three 5-µL injections for each sample. Discard any remaining material.

See Appendix A, “Standard Specifications,” for the iTRAQ Reagent-labeled amino acids in a 5-µL injection.

LC/MS/MS Analysis 3

This chapter covers:

Hardware Overview

Recommended MS System and Software

- API 2000™ System
- Analyst® Software 1.4.2 or later, using the IntelliQuan integration algorithm, and Cliquid® Software for Routine Amino Acid Analysis

Note: To update Analyst Software with hotfixes, see the instructions on the software installation CD.

Alternative MS Systems

- API 3200™ System
- API 4000™ System
- 3200 QTRAP® System
- 4000 QTRAP® System

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 AA20 Sample Assay, AA20 System Suitability Test, and Column Storage and Regeneration.

Workflow

The workflow below (Figure 4) outlines analyzing the iTRAQ® Reagent-labeled samples using the recommended MS and HPLC systems.

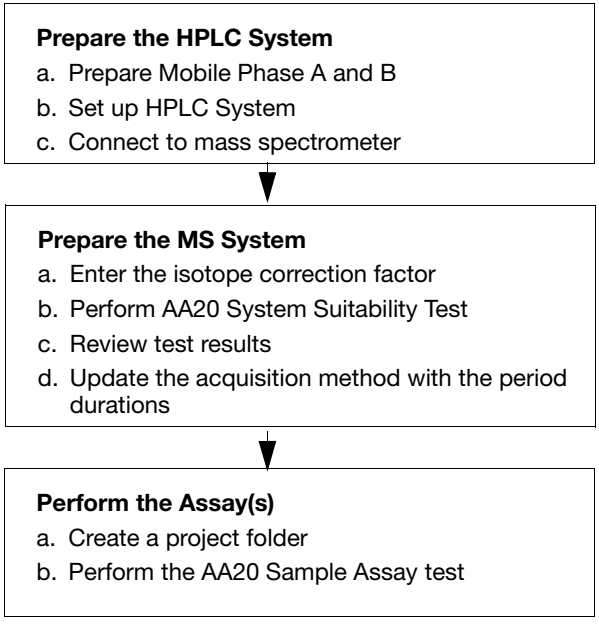


Figure 4 HPLC/MS/MS analysis workflow

Before You Begin

If necessary, have the Lab Manager perform the following tasks:

- For HPLC autosamplers other than those recommended on page 18, set up the hardware profile and create customized acquisition and quantitation methods. Appendix C, "Developing an Acquisition Method," has recommended starting point values for creating the methods.
- If the MS has not been calibrated in 3 to 6 months or if the MS source has been recently cleaned, perform mass calibration. 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 are using the recommended MS and HPLC systems, you can perform the system suitability test on page 25.

Prepare the HPLC System

Review Safety Warnings

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 L) and B (500 mL) for analysis of up to 75 injections.

CHEMICAL HAZARD. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions. Wear appropriate protective eyewear, clothing, and gloves.
Mobile Phase Modifier A is harmful if swallowed. It causes eye and skin burns, and may cause allergic reactions. It is a combustible liquid and vapor.
Mobile Phase Modifier B Causes skin and eye burns. Avoid breathing vapor. Use with adequate ventilation. Do not get in eyes or on skin.

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
   - 50.0 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 200 mL of acetonitrile, HPLC-grade.
2. Add:
   - 0.50 mL Mobile Phase Modifier A
   - 25.0 µL Mobile Phase Modifier B
3. Gently swirl the flask to mix.
4. Bring to volume with acetonitrile, 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.
3. If necessary, perform column regeneration. To perform column regeneration using Cliquid Software, select Maintain system (Figure 7 on page 25; see Help for more information).

Prepare the MS System

Review Safety Warnings

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.

Connect the Source

For the API 2000 System, connect the source using the three-way split insert as shown below (Figure 5).

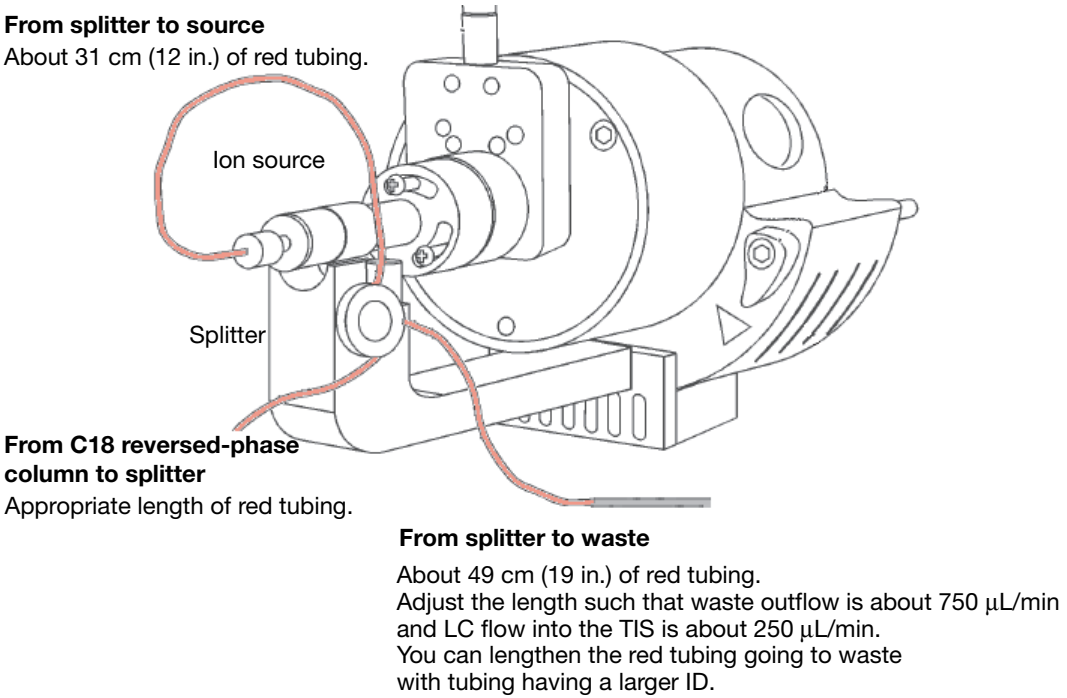
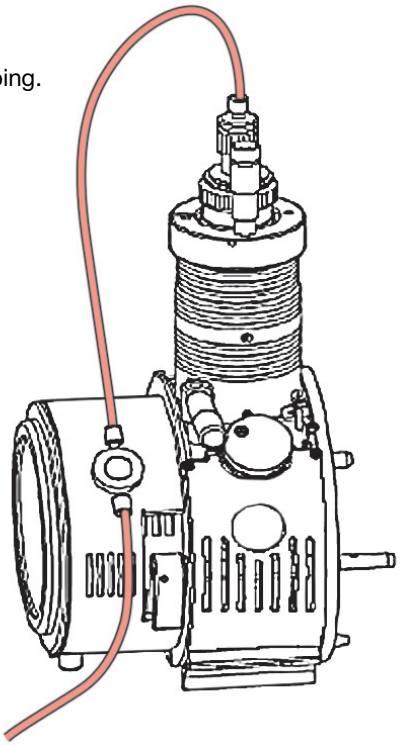


Figure 5 Source plumbing (tubing not drawn to scale) for the API 2000™ System

For the API 3200, API 4000, 3200 QTRAP, and 4000 QTRAP systems, the three-way split insert is not required. For these systems, connect the source as shown below (Figure 6).

From grounding plug to source
About 30 to 31 cm (12 in.) of red tubing.



From C18 reversed-phase column to source grounding plug
Appropriate length of red tubing, about 31 cm (12 in.)

Figure 6 Source plumbing (tubing not drawn to scale) for the API 3200™ and API 4000™ systems and 3200 QTRAP® and 4000 QTRAP® systems

Open Cliquid® Software

1. If Analyst Software is open, close it
2. Open Cliquid Software by clicking 

on the desktop.
3. Enter your login information, then click **Get Started**. For a Lab Technician, the Home page in Figure 7 opens. (The Home page for a Lab Manager displays additional tasks.)

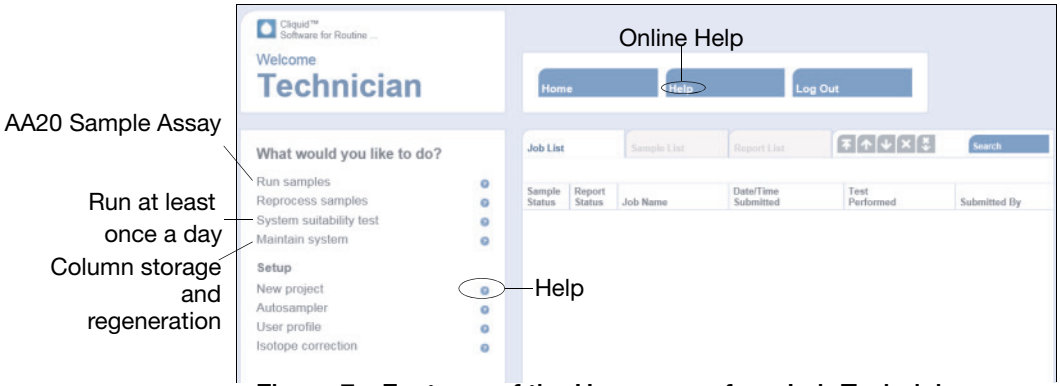


Figure 7 Features of the Home page for a Lab Technician

Perform the System Suitability Test

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

Perform the system suitability test at least once a day (before running samples), using Hydrolysates Standard - 114 Labeled 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 in use for the first time after storage, perform the test at least three times; for 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 Hydrolysates Standard - 114 Labeled as described on page 16.
2. Place the tube of standard solution in the HPLC autosampler. Note the plate code and position (if applicable), rack code, rack position, and sample position of the vial.
3. In the Home page (Figure 7 on page 25), select **System suitability test**.
4. 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 AA20 System Suitability.
Position sample	For the vial of Hydrolysates Standard - 114 Labeled, enter the: • Rack code • Rack position • Sample position • If required for your autosampler: – Plate code – Plate position
Customize report	1. Select AA20 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

5. 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 acquisition method with the period information in the System Suitability report.

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 click 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 (Figure 8).

   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.

AA20 System Suitability Test Report

Data		Sample	
Data File	2008_01_25_135314_AA20 System Suitability.wiff	Sample Type	Unknown
Date and Time	1/15/2008 4:23:55 PM	Vial	2
Project	System suitability test\new Data	Injection Volume	5 µL
Acquisition		Sample ID	
Method	AA20 SST.dam	Sample Comment	
Instrument	API 3200 AA0030403PT	Results	
Operator	API3200AAA\AAA	Result Table	2008_01_25_135314.rdb

Diagnose if:

- Analyte RT differs from the Expected RT by more than 0.5
- Analyte Peak Area is less than Expected Peak Area

Test Results

	Amino Acid	Expected RT	Analyte RT	Expected Peak Area	Analyte Peak Area
Pass	Gly	3.4	2.7	100000	265000
Pass	Ala	4.1	4.1	200000	482000
Pass	Pro	6.2	5.2	350000	803000
Pass	Lys	6.9	6.0	1000	130000
Pass	Val	7.2	7.2	250000	576000
Pass	Ile	8.7	8.7	350000	791000
Pass	Phe	10.1	9.4	300000	502000

If any compounds were not found with at least the expected Peak Area, the test should be repeated. If only a single compound fails, check the test mixture and column. If more compounds fail, check the LC system connections & mobile phases, autosampler injection performance and instrument tuning. Please contact your system manager or service representative for assistance.
Once all compounds pass, use the periods specified below to adjust the corresponding sample acquisition methods in the Analyst® software.

Diagnosing statement

Period 1	Period 2	Period 3	Period 4
4.67	1.93	3.78	14.63

Figure 8 Page 1 of the AA20 System Suitability Test Report

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 quantitation method file.
    - Analyte peak areas are less than the expected peak areas, reprocess the data. To access the report for reprocessing, go to the Report list, then click the **Rereport** button beside the report. After the peak areas are acceptable, repeat the System Suitability Test.
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.

Prepare the Analyst® Software Database

Update the Period Durations

After the system suitability test is successful and all compounds pass, the Lab Manager must update the a multiple reaction monitoring (MRM) period duration values (Figure 9) in the AA20 Sample.dam acquisition method. For an overview of the MRM experiment, see page 56. To update acquisition methods, refer to the documentation provided with the Analyst software.

System Suitability Report from Cliquid® Software

AA20 System Suitability Test Report

Data		Sample	
Data File	2008_01_25_135314_AA20 System Suitability.wiff	Sample Type	Unknown
Date and Time	1/15/2008 4:23:55 PM	Vial	2
Project	System suitability test\new Data	Injection Volume	5 µL
Acquisition		Sample ID	
Method	AA20 SST.dam	Sample Comment	
Instrument	API 3200 AA0030403PT	**Results**	
Operator	API3200AAA\AAA	Result Table	2008_01_25_135314.rdb

Test Results

	Amino Acid	Expected RT	Analyte RT	Expected Peak Area	Analyte Peak Area
Pass	Gly	3.4	2.7	100000	265000
Pass	Ala	4.1	4.1	200000	482000
Pass	Pro	6.2	5.2	350000	803000
Pass	Lys	6.9	6.0	1000	130000
Pass	Val	7.2	7.2	250000	576000
Pass	Ile	8.7	8.7	350000	791000
Pass	Phe	10.1	9.4	300000	502000

If any compounds were not found with at least the expected Peak Area, the test should be repeated. If only a single compound fails, check the test mixture and column. If more compounds fail, check the LC system connections & mobile phases, autosampler injection performance and instrument tuning. Please contact your system manager or service representative for assistance.
Once all compounds pass, use the periods specified below to adjust the corresponding sample acquisition methods in the Analyst® software.

Period 1	Period 2	Period 3	Period 4
4.67	1.93	3.78	14.63

Analyst® Software Window

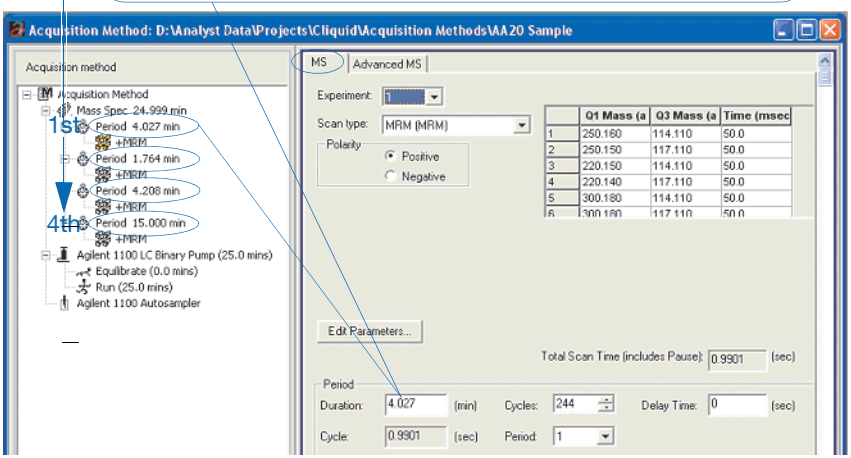


Figure 9 Updating the period durations

Enter Isotope Correction

The isotopic purity of the 114-labeled amino acids is used to adjust the calculated concentration. This value appears on the Certificate of Analysis provided in your Applied Biosystems Amino Acid 20/20 Analyzer Starter kit.

To specify the isotope correction factor:

1. In the Cliquid Software Home page (Figure 7 on page 25), select **Isotope correction**. The Isotope page opens.
2. In the 114 Isotope correction factor field, enter the value from the Certificate of Analysis as a whole number.
3. Click **Update**. The message “Update successful” appears.
4. Click **Done** to return to the Home page.

Perform the Amino Acid 20/20™ Sample Assay

Before You Begin

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 7 on page 25), click **New project** to open the New Project screen.
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.

Review the Safety Information

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 and control vials in the HPLC rack, noting the corresponding plate code and position (if applicable), rack code, rack position, and sample position of the vials.

AA20 Sample Assay

1. In the Cliquid Software Home page (Figure 7 on page 25), select **Run samples**.
2. Proceed through the wizard, clicking **Next** to advance to the next page. When prompted, select or enter the following:

Table 4 Run samples selections and input

AA20 Sample Assay Wizard Page	Selection or Input
Choose test	Select **AA20 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 inside the first autosampler-specific field displayed to auto-populate the fields with the information from the default autosampler configuration set for the system. c. In the following fields, specify the values from each drop-down list or enter values as applicable: • For category (the reference range against which obtained sample concentrations are compared), select Standard, None, or Control. Additional categories may have been created by the Lab Managers. • For normalization value, leave the field blank or enter 0. Entering a value yields an erroneous results table. • For internal standard (IS) amount, enter 30 for each amino acid. d. For the remaining fields, leave the field blank or enter 0. 3. Repeat steps a through c 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.
Customize report	Select the appropriate report-generating option. If you choose to generate: • 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 to Submit samples

Table 4 Run samples selections and input

AA20 Sample Assay Wizard Page	Selection or Input
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.

Standard Specifications A

Overview

This appendix covers:

Amino Acids in Hydrolysates Standard - 114 Labeled

A vial of Hydrolysates Standard - 114 Labeled contains approximately 10 nmol of each of the following amino acids labeled with iTRAQ® Reagent 114‡. The precise amount of amino acids in a vial is determined for each lot of standard, and is used to determine the volume of Sample Diluent required to make a 6 pmol/µL solution.

- Alanine
- Arginine
- Aspartic acid
- Cysteine
- Glutamic acid
- Glycine
- Histidine
- Isoleucine
- Leucine
- Lysine
- Methionine
- Methionine sulfoxide
- Norleucine
- Norvaline§
- Phenylalanine
- Proline
- Serine
- Threonine
- Tyrosine
- Valine

‡ Hydrolysates Standard - 114 Labeled also contains iTRAQ Reagent 114-labeled ammonium chloride, cysteic acid, taurine, and tryptophan, which are not supported in the Cliquid® Software for Routine Amino Acid Analysis.

§ Also present in Labeling Buffer - Amino Acid (30 pmol/µL).

iTRAQ® Reagent-Labeled Amino Acids in a 5-µL Injection

A 5-µL aliquot prepared following the labeling protocol (Chapter 2) contains:

- iTRAQ Reagent 117-labeled amino acids in the sample
- 30 pmol of iTRAQ Reagent 117-labeled norvaline
- 30 pmol of each iTRAQ Reagent 114-labeled amino acid in the standard, including norvaline

iTRAQ Reagent 114-labeled norleucine can be used to monitor recovery/losses during hydrolysis. iTRAQ Reagent 117-labeled norvaline provides an indication of the labeling efficiency.

Each amino acid has one label except for L-lysine, which has two labels.

Representative Report of the Separation of Amino Acids

The Cliquid® Software for Routine Amino Acid Analysis allows you to summarize the data of interest using the report feature. For information, access the online Help while in the Cliquid® Software by selecting the **Help** tab (Figure 5). For additional information, see "How to Obtain Support" on page xiii.

Figure 10 shows a representative chromatogram of amino acid internal standards, with peak identification added. For the Ala, Glu, Arg and Val, Met, Nva peaks, see the report for retention time to identify the individual peaks.

Figures 11 and 12 are a representative report of the analysis of a 5-µL injection of labeled standard analyzed using the conditions recommended in Chapter 3, "LC/MS/MS Analysis."

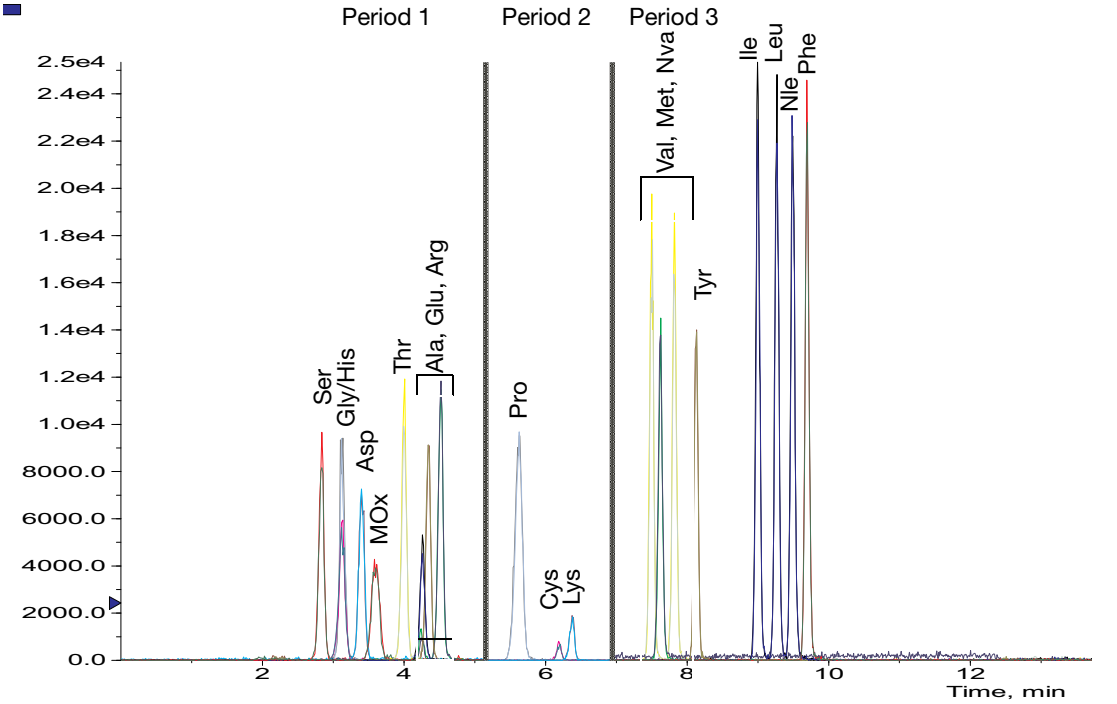


Figure 10 Representative chromatogram of the amino acids in the internal standard (see Figure 11 on page 39 for retention times)

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Created with Analyst Reporter
Printed: 7/5/2006 3:42 PM

Sample Name: name1 **Vial #:** 2

Data File	2006_06_29_115915_Protein Hydrolysate_0001.wiff		
Result Table	2006_06_29_115915_Protein Hydrolysate_0001.rdb		
Acquisition Date	6/29/2006 12:17:55 PM	Injection Volume	5.0
Acquisition Method	proteinAAA.dam	Algorithm Used	MQL
Instrument Name	API 2000	Sample Type	Unknown
Project	AAA_PAL validation	114 Isotope Purity	92.00

Results Summary

#	Amino Acid	RT (min)	Area	IS Area	IS Amount (pmol)	Calculated Amount (pmol)	% of Total
1.	Ser	2.84	4.86e+04	4.53e+04	30.0	29.6	5.0
2.	Gly	3.12	5.00e+04	4.45e+04	30.0	31.0	5.3
3.	His	3.12	3.53e+04	3.53e+04	30.0	27.6	4.7
4.	Asp	3.40	4.25e+04	4.17e+04	30.0	28.1	4.8
5.	MOx	3.62	3.46e+04	3.26e+04	30.0	29.3	5.0
6.	Thr	3.99	5.90e+04	5.13e+04	30.0	31.7	5.4
7.	Ala	4.52	5.91e+04	5.98e+04	30.0	27.3	4.6
8.	Glu	4.34	4.55e+04	4.69e+04	30.0	26.8	4.6
9.	Arg	4.26	2.61e+04	2.25e+04	30.0	32.0	5.4
10.	Pro	5.63	7.28e+04	6.83e+04	30.0	29.4	5.0
11.	Cys	6.18	3.74e+03	2.93e+03	30.0	35.2	6.0
12.	Lys	6.38	9.46e+03	8.64e+03	30.0	30.2	5.1
13.	Val	7.49	7.68e+04	7.29e+04	30.0	29.1	4.9
14.	Nva	7.82	7.64e+04	7.01e+04	30.0	30.1	5.1
15.	Met	7.63	5.63e+04	6.04e+04	30.0	25.7	4.4
16.	Tyr	8.12	5.88e+04	5.66e+04	30.0	28.7	4.9
17.	Ile	9.00	9.96e+04	9.06e+04	30.0	30.3	5.2
18.	Leu	9.26	9.61e+04	9.21e+04	30.0	28.8	4.9
19.	Nle	9.49	9.67e+04	9.13e+04	30.0	29.2	5.0
20.	Phe	9.69	9.44e+04	9.23e+04	30.0	28.2	4.8
						588.5	100%

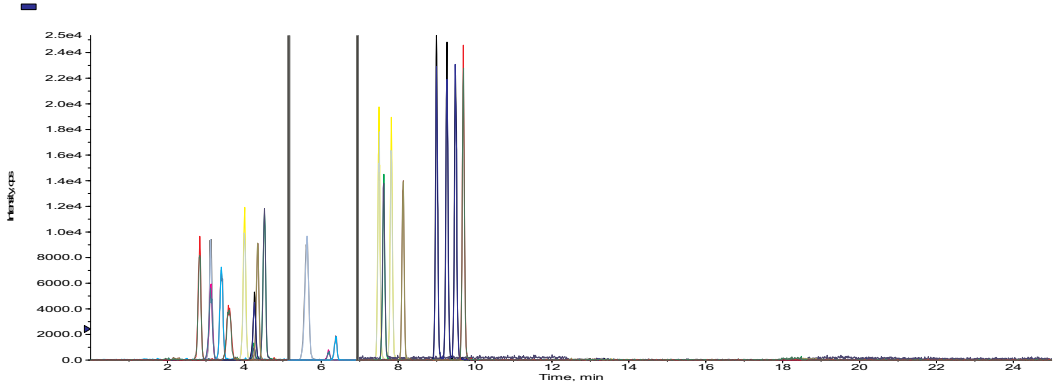


Figure 11 Representative report (page 1, results summary and full chromatogram) of the amino acids in the standard

Applied Biosystems | MDS SCIEX

Created with Analyst Reporter
Printed: 7/5/2006 3:42 PM

Sample Name: name1 **Vial #:** 2

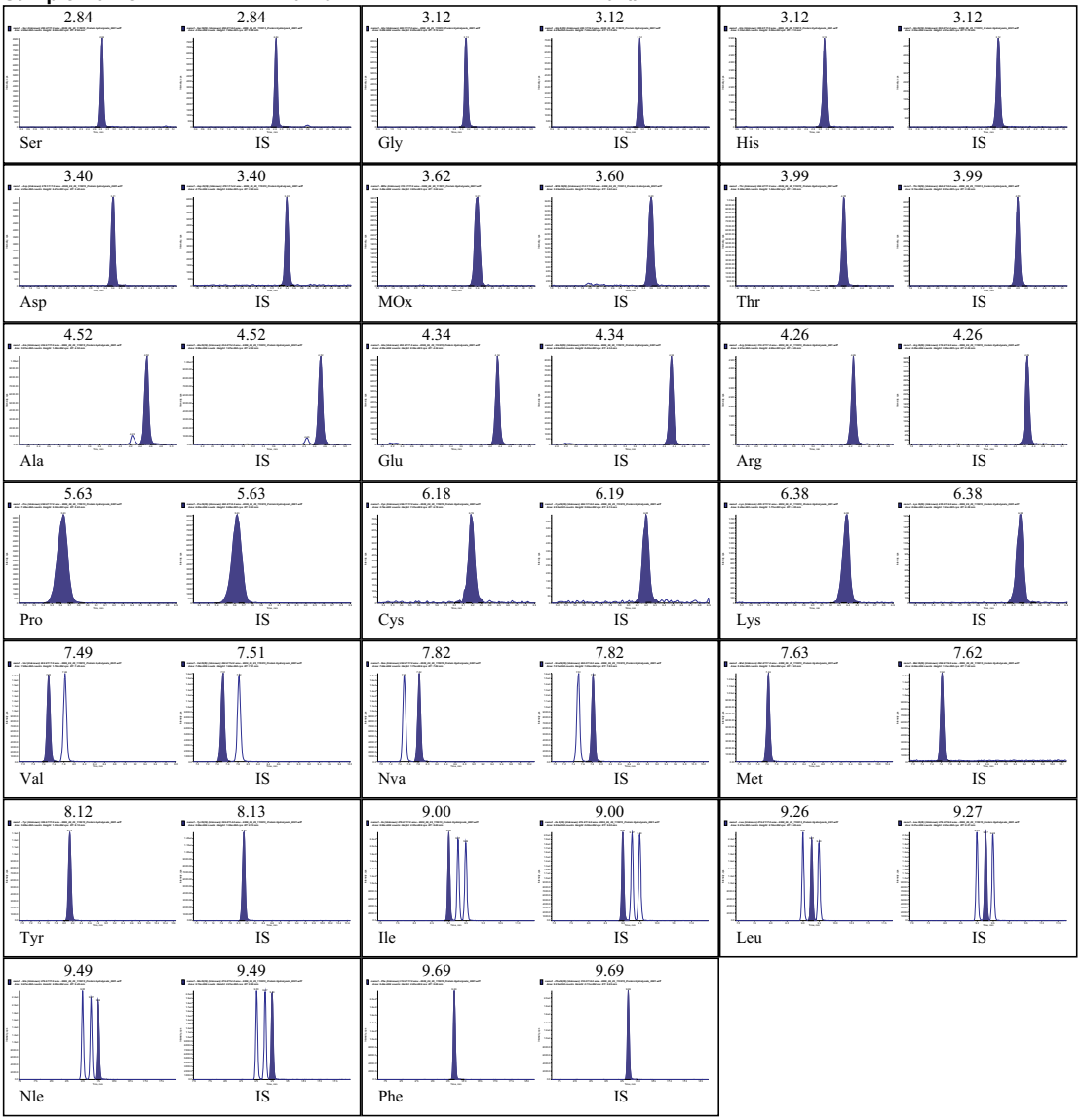
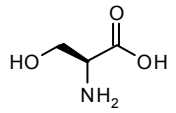
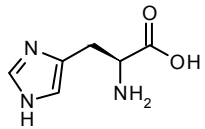
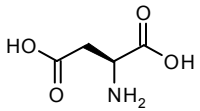
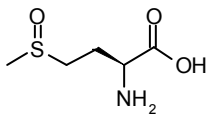
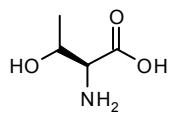
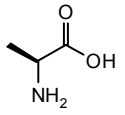
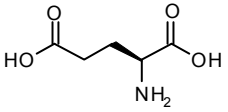
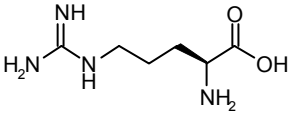
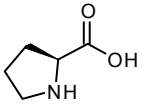
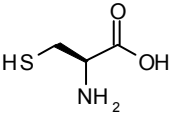
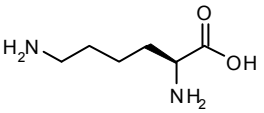
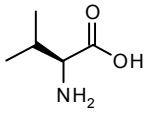
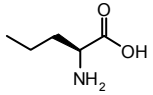
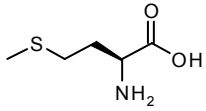
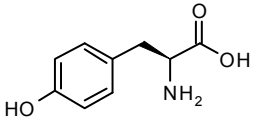
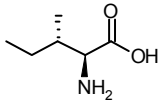
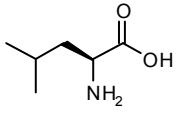


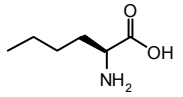
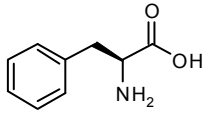
Figure 12 Representative report (page 2, individual amino acid chromatographs) of the amino acids in the standard.

Amino Acid Specifications

Structure	Name	Abbreviations (Three-letter, One-letter)	Formula	MH+	
				Un-labeled	Labeled
	L-serine	Ser, S	$C_3H_7NO_3$	106.1	250.2
	glycine	Gly, G	$C_2H_5NO_2$	76.0	220.1
	L-histidine	His, H	$C_6H_9N_3O_2$	156.1	300.2
	L-aspartic acid	Asp, D	$C_4H_7NO_4$	134.0	278.2
	L-methionine sulfoxide	MOx —	$C_5H_{11}NO_3S$	166.1	310.2
	L-threonine	Thr T	$C_4H_9NO_3$	120.1	264.2

Structure	Name	Abbreviations (Three-letter, One-letter)	Formula	MH+	
				Un-labeled	Labeled
	L-alanine	Ala, A	$C_3H_7NO_2$	90.1	234.2
	L-glutamic acid	Glu, E	$C_5H_9NO_4$	148.1	292.2
	L-arginine	Arg, R	$C_6H_{14}N_4O_2$	175.1	319.2
	L-proline	Pro, P	$C_5H_9NO_2$	116.1	260.2
	L-cysteine	Cys, C	$C_3H_7NO_2S$	122.0	266.1
	L-lysine (2 tags)	Lys, K	$C_6H_{14}N_2O_2$	147.1	435.3

Structure	Name	Abbreviations (Three-letter, One-letter)	Formula	MH+	
				Un-labeled	Labeled
	L-valine	Val, V	$C_5H_{11}NO_2$	118.1	262.2
	L-norvaline	Nva —	$C_5H_{11}NO_2$	118.1	262.2
	L-methionine	Met, M	$C_5H_{11}NO_2S$	150.1	294.2
	L-tyrosine	Tyr, Y	$C_9H_{11}NO_3$	182.1	326.2
	L-isoleucine	Ile, I	$C_6H_{13}NO_2$	132.1	276.2
	L-leucine	Leu, L	$C_6H_{13}NO_2$	132.1	276.2

Structure	Name	Abbreviations (Three-letter, One-letter)	Formula	MH+	
				Un-labeled	Labeled
	L-norleucine	Nle —	$C_6H_{13}NO_2$	132.1	276.2
	L-phenyl-alanine	Phe, F	$C_9H_{11}NO_2$	166.1	310.2

Quality Control and Troubleshooting

B

Small Volume Handling Tips to Ensure Accurate Concentrations and Volumes

Throughout the labeling protocol, to ensure accurate 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 at 10,000xg for 2 minutes
- Cap each tube promptly to avoid evaporation
- 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:
  - 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.

Material Storage

Hydroxylamine, iTRAQ® Reagent, and iTRAQ Reagent-labeled materials must be stored at –15 to –25 °C (Table 5). Improperly stored materials may result in inaccurate assays.

Table 5 Recommended storage conditions

Store at –15 to –25 °C
Hydrolysates Standard - 114 Labeled, as shipped
Hydrolysates Standard - 114 Labeled, reconstituted (iTRAQ Reagent 114-labeled amino acid internal standard)
iTRAQ Reagent 117, as shipped
Diluted iTRAQ Reagent 117 (isopropanol solution, for up to 4 weeks)
Dried iTRAQ Reagent 117-labeled sample
iTRAQ Reagent 117-labeled sample, reconstituted For optimal results, reconstitute the labeled sample at the time of assay. Some reconstituted samples can be stored at 4 °C for up to 1 week.
Hydrolysates Standard - 117 Labeled, as shipped
Hydrolysates Standard - Unlabeled, as shipped
Hydroxylamine, as shipped
May store at room temperature‡
Isopropanol, as shipped
Labeling Buffer - Amino Acid, as shipped
Mobile Phase Modifier A, as shipped
Mobile Phase Modifier B, as shipped
Sample Diluent - Amino Acid, as shipped

‡ These materials can be kept in the iTRAQ Reagent Application Kit and stored at –15 to –25 °C, or they can be stored at room temperature.

Quality Control Tests

The Amino Acid 20/20 Analyzer Starter Kit - Hydrolysate provides three standards:

- **Hydrolysates Standard - 114 Labeled** – To verify the performance of the chromatographic separation and that the sensitivity is acceptable. In conjunction with Hydrolysates Standard - 117 Labeled, to verify the quantitation performance of the system.
- **Hydrolysates Standard - 117 Labeled** – In conjunction with Hydrolysates Standard - 114 Labeled, to test the chromatographic and quantitation performance of the system.
- **Hydrolysates Standard - Unlabeled** – To verify the performance of the entire methodology (labeling protocol, separation, and quantitation).

Verifying Chromatographic Performance

To verify chromatographic performance, run the System Suitability test using iTRAQ Reagent 114-Labeled Standard. The System suitability test detects potential shifts in the peak retention times. The software then adjusts the MRM experiment period windows to accommodate the shifts.

The System suitability test is performed by:

- An Applied Biosystems field service engineer at installation
- An Applied Biosystems field application specialist while troubleshooting, if necessary
- The system owner or operator
  - After configuring the system for another use (changing the source and plumbing), then re-configuring with the TurboIonSpray® source as recommended in "Set Up the HPLC System" on page 22.
  - To correct for the changes in retention times due to the column aging and variations in batches of mobile phase.
  - Before any new batch of samples.

Testing Quantitation Performance

WARNING CHEMICAL HAZARD. iTRAQ Reagents (114, 117) are flammable liquids and vapors. Exposure may cause eye and respiratory tract irritation and blood system damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To test the quantitation performance of the system, substitute iTRAQ Reagent 117 -labeled amino acid standard for your labeled sample as follows:

1. Prepare a 6-pmol/µL iTRAQ Reagent 117-labeled amino acid standard solution by reconstituting one vial of Hydrolysates Standard - 117 Labeled with Sample Diluent - Amino Acid. The amount of Sample Diluent - Amino Acid to use is indicated on the reagent vial label and the Certificate of Analysis (approximately 1.67 mL).
2. Prepare a 6-pmol/µL iTRAQ Reagent 114 -labeled amino acid internal standard by reconstituting one vial of Hydrolysates Standard - 114 Labeled with Sample Diluent - Amino Acid. The amount of Sample Diluent - Amino Acid to use is indicated on the vial label or the Certificate of Analysis (approximately 1.67 mL).
3. Transfer a 12-µL aliquot of the iTRAQ Reagent 117-labeled amino acid internal standard solution to a fresh tube
4. To the aliquot, add 12 µL of iTRAQ Reagent 114-labeled amino acid internal standard. Cap the tube promptly to avoid evaporation.
5. Vortex to mix, then spin.

Analyzing the Samples

A 5-µL injection contains 15 pmol of each iTRAQ Reagent 114-labeled amino acid and 15 pmol of each iTRAQ Reagent 117-labeled amino acid.

Analyze the sample by LC/MS/MS (see Chapter 3, LC/MS/MS Analysis). In the Cliquid® Software, step 2, Build sample list, change the amount of internal standard to 15 pmole for each amino acid.

For most amino acids in the standards, verify that the calculated amount (pmol) of iTRAQ Reagent 117-labeled amino acid is within 20% of the internal standard amount (15 pmol).

Testing the Protocol

If you are running the protocol for the first time, Applied Biosystems recommends that you perform the protocol to label the vial of Hydrolysates Standard - Unlabeled. Analyzing the practice samples provides information about the proficiency of sample handling, and efficiency of the labeling protocol for each amino acid.

Follow the labeling protocol, substituting 1 µL of Hydrolysates Standard - Unlabeled for a hydrolysate sample containing 1 µg of hydrolysate.

The 1 µL aliquot of Hydrolysates Standard - Unlabeled contains 500 pmol of each amino acid, excluding norvaline. Norvaline is incorporated when diluting the sample with Labeling Buffer - Amino Acid (30 pmol/µL).

Analyzing the Samples

After labeling with iTRAQ Reagent 117, the Hydrolysates Standard - Unlabeled contains the same amino acids as the vial of Hydrolysates Standard - 114 Labeled (see page 36).

After labeling with iTRAQ Reagent 117 and adding iTRAQ Reagent 114-labeled amino acid internal standard, a 5-µL injection contains:

- 30 pmol of each iTRAQ Reagent 114-labeled amino acid
- 100 pmol of each iTRAQ Reagent 117-labeled amino acid (except norvaline, 30 pmol).

Analyze the sample by LC/MS/MS (see Chapter 3, "LC/MS/MS Analysis." Verify that peaks display at m/z 114 and 117. For most amino acids in the standard, verify that the calculated amount (pmol) is within 20% of the expected amount (100 pmol for each amino acid except norvaline, 30 pmol for norvaline).

Recovery Issues

Table 6 Recovery issues

Symptom	Possible Cause	Action
Low recovery of amino acids	Too large a sample amount	Prepare a fresh sample, maintaining the total amino acid at no more than 10 nmol. For samples larger than 1 µg of hydrolysate, modify the protocol as stated in step 2 on page 14 and step 6 on page 15.
	Hydrolysate contains moisture	Completely dry the hydrolysate and sample tube before adding Labeling Buffer - Amino Acid.
	iTRAQ® Reagent 117 concentration too low	Precisely dilute a fresh vial of iTRAQ Reagent 117 with 70 µL of isopropanol.
	Incomplete mixing of diluted iTRAQ Reagent 117	Vortex the tube.
	If the tube was open too long, evaporation occurred and the iTRAQ Reagent 114-labeled amino acid internal standard became concentrated.	• Dilute a fresh vial of Hydrolysates Standard - 114 Labeled. Rerun the protocol. • If you know the concentration of each amino acid, enter it in the Cliquid® Software sample list and resubmit.
High recovery of amino acids	Low concentration of iTRAQ Reagent 114-labeled amino acid internal standard due to inaccurate dilution	• Precisely dilute a fresh vial of Hydrolysates Standard - 114 Labeled. Rerun the protocol. • If you know the concentration of each amino acid, enter it in the Cliquid® Software sample list, then resubmit.
Inaccurate recovery of any or all amino acids	Pipette tip contamination	Use a fresh tip for each pipetting step.

Table 6 Recovery issues *(continued)*

Symptom	Possible Cause	Action
Inaccurate recovery of:		
Alanine or arginine	Aged iTRAQ Reagent 114-labeled amino acid internal standard yielded an artifact peak. If an artifact peak elutes slightly before the true peak, the software integrates on the artifact peak.	Manually integrate using the true peak.
Cysteine	Cysteine oxidized to its dimeric form, cystine.	The accuracy of cysteine quantitation may be lower than the other amino acids because of the instability of the monomeric form. Verify that sufficient hydroxylamine was added in step 8 on page 15. The hydroxylamine stabilizes monomeric cysteine.
Proline	Too large a sample amount	Prepare a fresh sample, maintaining the total amino acid at no more than 10 nmol. For samples larger than 1 µg of hydrolysate, modify the protocol as described in step 2 on page 14 and step 6 on page 15.
Serine	Excessive loss during hydrolysis	Hydrolyze a fresh sample using the appropriate conditions
Lysine	• Autosampler injected too small a volume of sample. • MS detector sensitivity decreased. • Column contaminated or leaking.	• Adjust autosampler injection volume. • Clean the source and sprayer tip. • Wash column and stop leak.
Tyrosine	Partial labeling of the phenolic hydroxyl group	Check that sufficient hydroxylamine was added in step 8 on page 15. The hydroxylamine reverses partial labeling of the phenolic hydroxyl group.

Resolution and Retention Time Issues

Table 7 Recovery Issues

Symptom	Possible Cause	Action
Loss of resolution between Val/NorVal and Ileu/Leu/NorLeu An acceptable chromatogram has the following resolution of the peak pairs: • Val/NorVal: 90% • Ileu/Leu/NorLeu: 75%	• Column not conditioned • Starting mobile phase condition has excess Mobile phase B • Column has exceeded its useful life span	• Condition the column by making at least two injections of iTRAQ Reagent 114- or 117-labeled Standard before running samples. • Correct the percentage of Mobile phase B in the starting phase. • Discard the column and replace it with a fresh column.
General increase in retention times	• Incorrect mobile phase concentration • Excess Mobile Phase Modifier B concentration	Carefully prepare fresh mobile phase (see "Prepare the Mobile Phases" on page 21).
General decrease in retention times	• Incorrect mobile phase concentration • Insufficient Mobile Phase Modifier B concentration	Carefully prepare fresh mobile phase (see "Prepare the Mobile Phases" on page 21).
The first few samples of a batch exhibit a shift in retention time and unstable chromatography	Column not conditioned	Condition the column by making at least two injections of iTRAQ Reagent 114- or 117-labeled Standard before running samples.
Certain amino acids with masses close to the period borders are not detected	Retention times have drifted out of the periods	Run the System Suitability test using iTRAQ Reagent 114-labeled amino acid internal standard (page 48). The System Suitability test automatically adjusts the periods.

Table 7 Recovery Issues *(continued)*

Symptom	Possible Cause	Action
One or more amino acid peaks exhibit a saturated signal (an intensity or peak height in cps greater than 1.5e6 cps).	Sample concentration too high.	Dilute the sample with an equal volume of Sample Diluent - Amino Acid. Analyze the diluted sample, then in the Cliquid® Software, step 2, Build sample list, enter the amount of internal standard as 15 pmole for each amino acid.

Developing an Acquisition Method

C

This appendix covers:

MRM Overview

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

Using MRM allows you to set the first quadropole filter to select the labeled amino acid of interest (precursor ion) for *fragmentation* and to set another quadropole filter to select the cleaved iTRAQ® Reagent label of interest (product ion) for *detection*. You also select the amount of time (dwell) that the mass spectrometer continues to detect the iTRAQ Reagent label of interest.

For an AA20 sample assay, the MRM scan has one experiment with four periods scanned in positive polarity. Organizing the experiment into three periods in which specific amino acids are monitored allows for collecting more data points per peak and more accurate quantitation. The fourth period is an equilibration period.

Developing a Customized Amino Acid 20/20™ Sample Assay Acquisition Method

The values in Tables 8 through 10 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 flow rate is 1.0 mL/min, split in the source to 200 to 250 µL/min. Table 8 provides the recommended LC gradient.

Table 8 Recommended LC gradient for the AA20 Sample Assay

Total Time (min)	%Mobile Phase A	%Mobile Phase B
0.0	98.0	2.0
10.0	72.0	28.0
10.1	0.0	100.0
16.0	0.0	100.0
16.1	98.0	2.0
25.0	98.0	2.0

TIS Values

See Table 9 for the TurboIonSpray® (TIS) source Source/Gas and Compound values.

Table 9 Recommended TIS values

Gas or Compound	LC/MS/MS systems		
	API 2000™	API 3200™ or API 4000™	3200 or 4000 Q TRAP® system
TurboIonSpray® source/gas values			
CUR	25	20	20
CAD	6	3	Medium
IS (Positive)	5500	1500	1500
TEM	375	600	600
GS 1	35	60	60
GS 2	45	60	60
ihe	On	On	On
Compound values			
DP (Positive)	45	30	30
FP	400	n/a	n/a
EP	10	10	10
CE	30	30	30
CXP	4	5	5

MRM Values

See Table 10 for the Q1 (precursor ion) and Q3 (product ion) masses and dwell values (msec).

Table 10 Periods for Experiment 1

Amino Acid	Q1 Mass (amu) (labeled amino acid)	Q3 Mass (amu) (iTRAQ™ Reagent label)
Period 1, Experiment 1 Dwell = 50		
L-serine L-serine internal standard	250.15 250.16	117.11 114.11
glycine glycine internal standard	220.14 220.15	117.11 114.11
L-histidine L-histidine internal standard	300.18 300.18	117.11 114.11
L-aspartic acid L-aspartic acid internal standard	278.15 278.15	117.11 114.11
L-methionine sulfoxide L-methionine sulfoxide internal standard	310.16 310.16	117.11 114.11
L-threonine L-threonine internal standard	264.17 264.17	117.11 114.11
L-alanine L-alanine internal standard	234.16 234.16	117.11 114.11
L-glutamic acid L-glutamic acid internal standard	292.16 292.17	117.11 114.11
L-arginine L-arginine internal standard	319.22 319.23	117.11 114.11
Period 2, Experiment 1 Dwell = 100		
L-proline L-proline internal standard	260.17 260.18	117.11 114.11
L-cysteine L-cysteine internal standard	265.12 265.13	117.11 114.11

Table 10 Periods for Experiment 1

Amino Acid	Q1 Mass (amu) (labeled amino acid)	Q3 Mass (amu) (iTRAQ™ Reagent label)
L-lysine L-lysine internal standard (2 tags)	435.32 435.33	117.11 114.11
Period 3, Experiment 1 Dwell = 50		
L-valine L-valine internal standard L-norvaline L-norvaline internal standard	262.19 262.19 262.19 262.19	117.11 114.11 117.11 114.11
L-methionine L-methionine internal standard	294.16 294.16	117.11 114.11
L-tyrosine L-tyrosine internal standard	326.18 326.19	117.11 114.11
L-isoleucine L-isoleucine internal standard L-leucine L-leucine internal standard L-norleucine L-norleucine internal standard	276.20 276.21 276.20 276.21 276.20 276.21	117.11 114.11 117.11 114.11 117.11 114.11
L-phenylalanine L-phenylalanine internal standard	310.19 310.19	117.11 114.11
Period 4, Experiment 1 Dwell = 100	310.19	114.11

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