

Amino Acid Analysis for Physiological Samples

iTRAQ[®] Reagents Application Kit for Use with LC/MS/MS Systems

Protocol

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Preface

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Safety


Safety alert words Four safety alert words appear in our user documentation at points in the document where you need to be aware of relevant hazards. Each alert word—**IMPORTANT**, **CAUTION**, **WARNING**, **DANGER**—implies a particular level of observation or action, as defined below.

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 our instruments and protocols are potentially hazardous and can cause injury, illness, or death.

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the 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 You can obtain the MSDS for any chemical supplied with this kit at www.sciex.com/msds.

Note: For the MSDSs of chemicals not distributed with this kit, 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 our 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**

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

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


- The *xTRAQ Family of Amine -Modifying Labeling Reagents for Multiplexed Relative and Absolute Quantification: Chemistry Reference Guide*.
- *Amino Acid Analysis for Physiological Samples Quick Reference Card* (PN 4375241)
- 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.sciex.com>, click the link for **Support**, then click the literature link and perform a literature search.

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)

How to obtain support

We are committed to meeting the needs of your research. Please go to www.sciex.com and go to the **Support** tab for local support information.

Contacting Technical Support in North America

To contact technical support:

- By telephone: Dial 1.877.740.2129
- By fax: Dial 1.650.627.2803

Introduction to iTRAQ[®] Reagents Chemistry

1

This chapter covers:

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Overview

The Amino Acid 45/32™ Analyzer Kits - Physiological enable identification and quantitation of amino acids in plasma and serum, urine, and cerebrospinal fluid (CSF) samples. The kits provide iTRAQ® Reagent 115 for labeling samples and a mixture of iTRAQ Reagent 114-labeled amino acids as an internal standard.

Product capabilities

With Cliquid® Software for Routine Amino Acid Analysis, the AB SCIEX Amino Acid 45/32™ Analyzer system allows users with minimal mass spectrometry (MS) experience to obtain data for relative and absolute quantitation of amino acids (Figure 1).

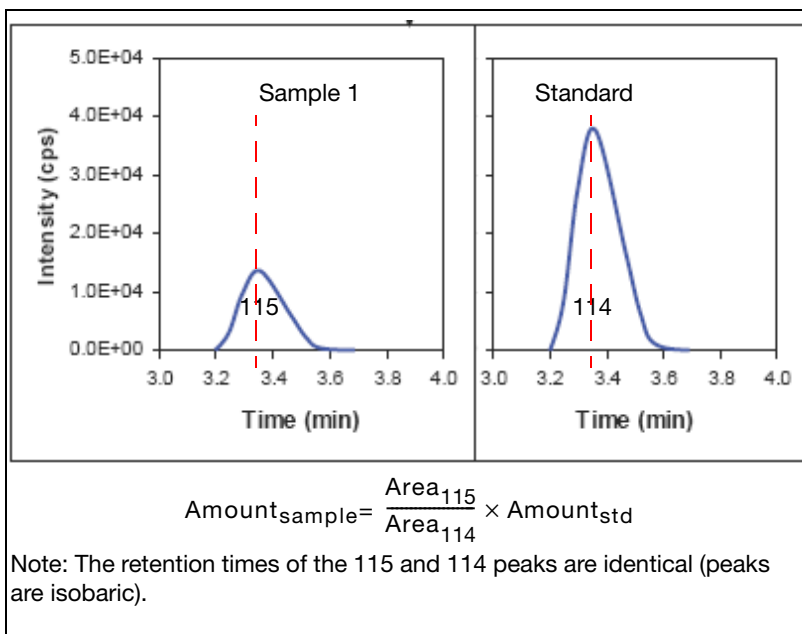


Figure 1 Representation of LC/MS/MS data showing isobaric peaks and the calculation for absolute quantitation

Available kits and materials

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

Table 1 Kits and materials

Item	Description
Amino Acid 45/32™ Analyzer Kits - Physiological	
Starter Kit (includes the 50-assay reagent kit, AA 45/32™ Phys Standards Set, this protocol, and the Quick Reference Card)	Provides sufficient material to run 50 iTRAQ Reagent 115-labeled samples (each containing up to 10 nmol total amino acid) with the iTRAQ Reagent 114-labeled amino acid internal standard.
50-Assay or 200-Assay Kit (includes the reagent kit, AA 45/32™ Phys Standard - 114 Labeled, and the Quick Reference Card)	Provides sufficient material to run 50 or 200 iTRAQ Reagent 115-labeled samples (each containing up to 10 nmol total amino acid) with the iTRAQ Reagent 114-labeled amino acid internal standard.
Standards and Controls	
AA 45/32™ Phys Standards Set	<p>Provides Phys Standard - 114 Labeled, - 115 Labeled, and Unlabeled, and AA 45/32™ Phys Standard Diluent.</p> <ul style="list-style-type: none"> • AA 45/32™ Phys Standard - 114 Labeled and - 115 Labeled contain the same amino acids (see page 36). • The AA 45/32™ Phys Standard - 115 Labeled solution is used to calculate isotope correction factors before running the samples. • AA 45/32™ Phys Standard - Unlabeled contains the same amino acids as the labeled standards, except norvaline and norleucine. Norvaline and norleucine are incorporated during labeling. <p>AA 45/32™ Phys Standard Diluent is used to dilute Phys Standard. The amount of Standard Diluent to use is indicated on the Certificate of Analysis and the Phys Standard vial label.</p> <p>Also provides allo-isoleucine and control plasma.</p>

Table 1 Kits and materials (continued)

Item	Description
Standards and Controls (continued)	
AA 45/32™ Phys Standard (includes Standard Diluent)	Provides AA 45/32™ Phys Standard (- 114 Labeled, - 115 Labeled, and Unlabeled) and Standard Diluent. See AA 45/32™ Phys Standards Set (above) for comments.
AA 45/32™ Phys Allo-isoleucine	Provides the amino acid allo-isoleucine for identification.
AA 45/32™ Phys Control Plasma	Provides a known plasma sample for quality-control purposes.
Recommended Column	
Amino Acid Analyzer (AAA) C18 Column	C18 reversed-phase column, 5 µm, 4.6 mm ∞ 150 mm

Contents of the starter kit

The Starter Kit - Physiological includes iTRAQ® Reagent-115, the standards set, reagents, and this document (see Table 2). For recommendations on using the standards set, see “Quality assurance” on page 41. Order the Amino Acid Analyzer (AAA) C18 Column separately.



WARNING CHEMICAL HAZARD. Some of the chemicals provided in your reagent kit may be hazardous. Before handling the reagents, read the MSDSs that accompany your first shipment. Always follow the safety precautions (wearing appropriate protective eyewear, clothing, gloves, and so on) presented in each MSDS.

IMPORTANT! When you receive the shipping container, immediately store the Reagent Kit and AA 45/32™ Phys Standards Set at -15 to -25 °C.

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 40.

Table 2 Contents of the Amino Acid 45/32 Analyzer Starter Kit - Physiological

Item	Quantity	Contents
Store at -15 to -25 °C		
Reagent Kit (one 50-Assay Kit)		
<ul style="list-style-type: none"> iTRAQ® Reagent 115 	4 vials, 1 unit/vial	Amine-modifying labeling reagent. One unit (one vial) of reagent yields approximately 15 assays. Includes a Certificate of Analysis providing purity information.
<ul style="list-style-type: none"> AA 45/32™ Phys Sulfosalicylic Acid 	1 vial, 1.8 mL	10% sulfosalicylic acid to precipitate proteins from the sample. Contains norleucine (400 pmol/μL).
<ul style="list-style-type: none"> AA 45/32™ Phys Labeling Buffer† 	2 vials, 1.8 mL/vial	Borate buffer, pH 8.5. Also contains norvaline (20 pmol/μL).
<ul style="list-style-type: none"> AA 45/32™ Phys Hydroxylamine 	1 vial, 1.8 mL	1.2% hydroxylamine solution. Reverses partial labeling of the phenolic hydroxyl group of tyrosine and stabilizes cysteine to prevent oxidation to cystine.
<ul style="list-style-type: none"> Mobile Phase Modifier A‡ 	2 vials, 1.8 mL/vial	100% formic acid for mobile phase A and mobile phase B preparation
<ul style="list-style-type: none"> Mobile Phase Modifier B‡ 	2 vials, 200 μL/vial	100% heptafluorobutyric acid for mobile phase A and mobile phase B preparation
<ul style="list-style-type: none"> Isopropanol‡ 	1 vial, 1.8 mL	Isopropanol, absolute, for diluting iTRAQ Reagent
AA 45/32™ Phys Standards Set	1	<ul style="list-style-type: none"> 1 vial AA 45/32™ Phys Standard - 114 Labeled 1 vial AA 45/32™ Phys Standard - 115 Labeled 1 vial AA 45/32™ Phys Standard - Unlabeled 1 vial Allo-isoleucine 1 vial AA 45/32™ Phys Control Plasma 2 vials AA 45/32™ Phys Standard Diluent§ - 0.5% formic acid for reconstituting the vials of AA 45/32™ Phys Standard - 114 Labeled, and - 115 Labeled. Certificate of Analysis

Table 2 Contents of the Amino Acid 45/32 Analyzer Starter Kit - Physiological (*continued*)

Item	Quantity	Contents
Documentation		
<i>Amino Acid Analysis for Physiological Samples Protocol</i>	1	This document.
<i>Amino Acid Analysis for Physiological Samples Quick Reference Card</i>	1	A laminated card that briefly describes the steps in the labeling protocol.

‡ Can be stored at room temperature.

§ The amount of Standard Diluent to use when diluting Phys Standard is indicated on the Certificate of Analysis and the AA 45/32™ Phys Standard - 114 Labeled and - 115 Labeled vial labels.

Contents of 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 MSDSs that accompany your first shipment. Always follow the safety precautions (wearing appropriate protective eyewear, clothing, gloves, and so on) presented in each MSDS.

IMPORTANT! When you receive the shipping container, immediately store the Amino Acid 45/32 Reagent Kit and the AA 45/32™ Phys Standard - 114 Labeled bag at -15 to -25 °C.

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 40.

See Table 3 for materials contained in each kit.

Table 3 Contents of the Amino Acid 45/32™ Analyzer Kit - Physiological 50 Assay and 200 Assay Kits

Item	Quantity in 50-Assay Kit	Quantity in 200-Assay Kit	Contents
Store at – 15 to – 25 °C			
Reagent Kit (50-Assay Kit or 200-Assay Kit) 1 shipping container with the following items:			
• iTRAQ Reagent 115	4 vials, 1 unit/vial	14 vials, 1 unit/vial	Amine-modifying labeling reagent. One unit (one vial) of reagent yields approximately 15 assays. Includes a Certificate of Analysis providing purity information.
• AA 45/32™ Phys Sulfosalicylic Acid	1 vial, 1.8 mL	2 vials, 1.8 mL/vial	10% sulfosalicylic acid to precipitate proteins from the sample. Also contains norleucine (400 pmol/μL).
• AA 45/32™ Phys Labeling Buffer [‡]	2 vials, 1.8 mL/vial	5 vials, 1.8 mL/vial	Borate buffer, pH 8.5. Also contains norvaline (20 pmol/μL).
• AA 45/32™ Phys 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.
• 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 iTRAQ Reagent

Table 3 Contents of the Amino Acid 45/32™ Analyzer Kit - Physiological 50 Assay and 200 Assay Kits (*continued*)

Item	Quantity in 50-Assay Kit	Quantity in 200-Assay Kit	Contents
AA 45/32™ Phys Standard - 114 Labeled	1 bag	4 bags	In one bag: <ul style="list-style-type: none"> • 1 vial of AA 45/32™ Phys Standard - 114 Labeled. • 1 vial of AA 45/32™ Phys Standard Diluent§ - 0.5% formic acid for reconstituting the vial of AA 45/32™ Phys Standard - 114 Labeled • Certificate of Analysis. Specifies the precise amount of diluent for reconstituting this lot of standard.
Documentation			
<i>Amino Acid Analysis for Physiological Samples Quick Reference Card</i>	1	1	A laminated card that briefly describes the steps in the labeling protocol.

‡ Can be stored at room temperature.

§ The amount of Standard Diluent to use when diluting Phys Standard is indicated on the Certificate of Analysis and the AA 45/32™ Phys Standard - 114 Labeled and - 115 Labeled vial labels.

User-supplied materials


 **WARNING CHEMICAL HAZARD.** Some of the chemicals referred to in this protocol (such as those in Table 4) may not have been provided with your kit. If the chemicals are not provided, they are not manufactured or sold by us. Obtain the MSDSs from their manufacturers.

Table 4 User-supplied materials

Item	Quantity per Assay
Disposable gloves	As needed
Physiological samples, at least 40 μ L of each	As needed
Pipetting accessories (pipettors and tips) suitable for 5- μ L 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 μ S/0.05 μ Mho) for mobile phase A	As needed
Acetonitrile, 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- μ L and 1000- μ L	As needed
Amino Acid Analyzer (AAA) C18 Column (5 μ m, 4.6 \times 150 mm)	1
Cliquid [®] Software for Routine Amino Acid Analysis	—
LC/MS/MS System with a TurbolonSpray [®] source and required gases (see “Required MS systems and software” on page 22)	—
PEEK [™] tubing, 0.005-in. ID (red)	As needed

Labeling Physiological Samples

2

This chapter covers:

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Labeling the samples with iTRAQ® Reagent 115.	16
Adding the internal standard	18

Amino acid labeling workflow

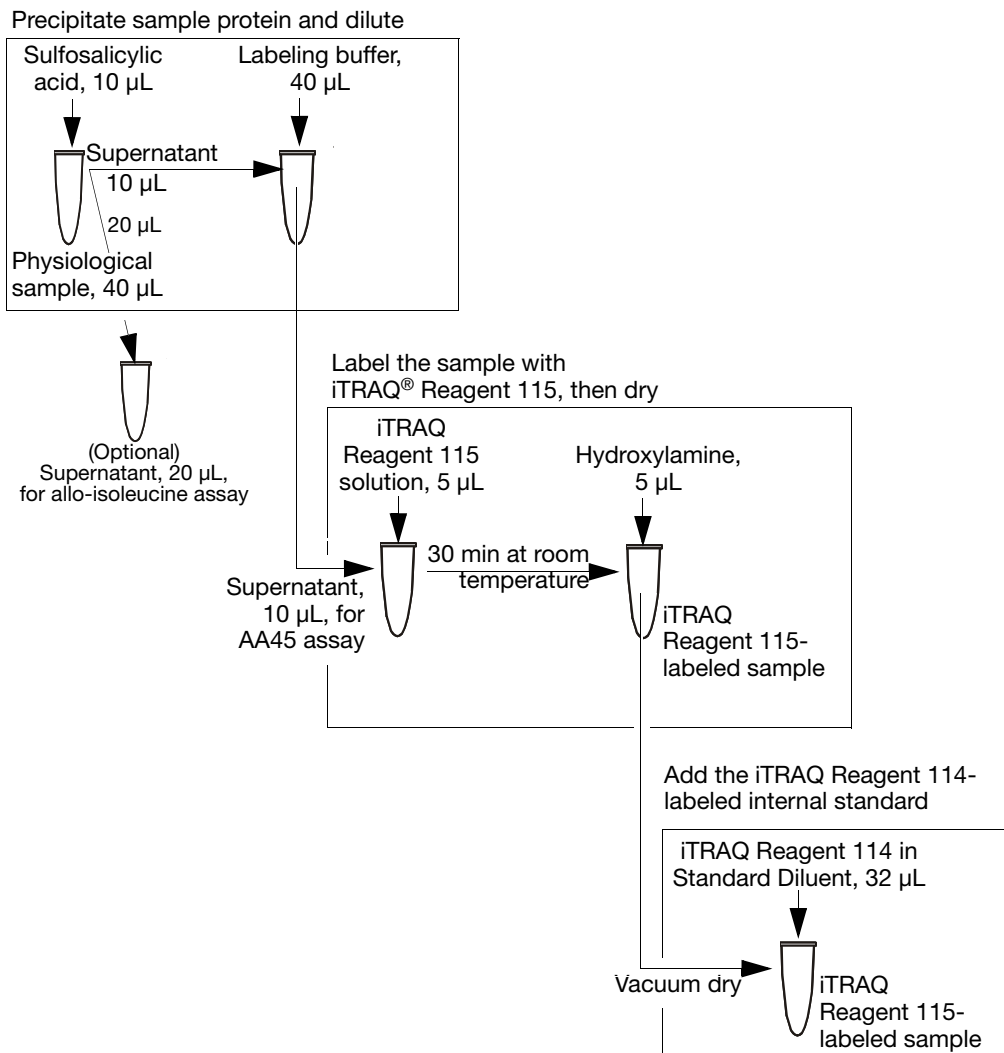


Figure 2 Labeling workflow for one physiological sample

Before you begin

Review safety warnings

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

Testing the labeling protocol

If you are running the protocol for the first time, it is strongly recommended that you practice performing the labeling protocol as described in Appendix B, Quality Assurance, “Using AA 45/32™ Phys Standard - Unlabeled” on page 42. 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- μ L. 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 40.

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 18.

Review Appendix A, “Amino Acid Amounts,” for information on:

- The amino acids in the internal standard that are labeled with iTRAQ® Reagent 114 and their amounts
- Incorporating norvaline and norleucine standards and amounts
- Using allo-isoleucine as a separate standard

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 iTRAQ Reagent required to label that number of samples. One vial of iTRAQ Reagent 115 labels 15 sample assays.
- Allow the reagents and each required vial of iTRAQ Reagent 115 to reach room temperature. Return the reagents to storage at – 15 to – 25 °C within 2 hours of thawing.
- Briefly centrifuge the reagent and iTRAQ 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.

Precipitating sample protein and diluting



WARNING BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. For more information, see “Biological hazard safety” on page xi.



WARNING 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.

AA 45/32™ Phys Sulfosalicylic Acid is harmful if swallowed. It causes eye, skin, and respiratory tract irritation.

AA 45/32™ Phys Labeling Buffer is harmful if inhaled or swallowed. It causes eye, skin, and respiratory tract irritation, and may cause central nervous system depression and damage to the liver and kidneys.

IMPORTANT! The sulfosalicylic acid that is used to precipitate proteins also supplies the norleucine standard.

Follow the procedures below for each physiological sample.

Precipitating protein

1. Transfer 40 μL of a physiological sample to a tube.
2. Add 10 μL of AA 45/32 Phys Sulfosalicylic Acid (contains 4000 pmol norleucine).
3. Vortex to mix, then spin at $10,000 \times g$ for 2 minutes.

Note: Protein precipitate may not form in urine and CSF samples.

4. Transfer 10 μL of the supernatant to a clean tube.
5. (Optional) For allo-isoleucine determination, transfer 20 μL of supernatant directly to a clean autosampler tube.

Diluting with labeling buffer

1. Add 40 μL of Labeling Buffer (contains 800 pmol norvaline) to the 10- μL aliquot of supernatant from step 4 above.

2. Vortex to mix, then spin.
3. Transfer 10 μL of the supernatant to a clean tube. This sample is labeled with iTRAQ Reagent in the next section.
4. Label and refrigerate the remaining supernatant to use if you need to repeat the iTRAQ Reagent labeling.

Labeling the samples with iTRAQ[®] Reagent 115



DANGER 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.

AA 45/32[™] Phys Hydroxylamine is harmful if swallowed. It causes eye, skin, and respiratory tract irritation, and may cause allergic reactions. Heating it may cause an explosion.

iTRAQ[®] Reagent 115 may be fatal if inhaled, absorbed through the skin, or swallowed. It causes eye, skin, and respiratory tract burns. It may cause heart, liver, and kidney damage, and central nervous system depression. It contains a chemical known to the State of California to cause cancer. It is a flammable liquid and vapor.

Isopropanol is a flammable liquid. Vapors may form explosive mixtures with air. Exposure may cause eye, skin, and upper respiratory tract irritation. It may cause central nervous system effects such as drowsiness, dizziness, and headache.

Preparing the labeling reagent 115 solution

Repeat the following procedure for each required vial of iTRAQ Reagent 115.

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

1. Spin the vial of iTRAQ Reagent 115 (at room temperature) to bring the solution to the bottom of the vial.
2. Add 70 μ L of isopropanol. Label and date the vial (discard after 4 weeks).
3. Vortex the solution to mix, then spin.

Labeling samples Repeat the following procedure for each sample.

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

1. To the sample from step 3 on page 15, add 5 μ L of the iTRAQ Reagent 115 solution.

IMPORTANT! Immediately store unused iTRAQ Reagent 115 solution at -15 to -25 $^{\circ}$ C.

2. Vortex to mix, then spin.
3. Incubate the sample at room temperature for at least 30 min.
4. Add 5 μ L of AA 45/32 Phys 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 to -25 $^{\circ}$ C.

Adding the internal standard

The following procedure yields enough material for approximately ten 2- μ L injections for each sample. See Appendix A, “Amino Acid Amounts,” for the iTRAQ Reagent-labeled amino acids in each injection.



DANGER 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.

AA 45/32™ Phys Standard - 114 Labeled is harmful if swallowed. It causes serious eye, skin, and respiratory tract irritation, and may cause allergic reactions. Heating it may cause an explosion. It contains a chemical known to the State of California to cause cancer. **AA 45/32™ Phys Standard Diluent** is harmful if swallowed. It causes eye and skin burns, and may cause allergic reactions. It is a combustible liquid and vapor.

Preparing the internal standard solution

1. Spin a vial of AA 45/32 Standard - 114 Labeled to bring the lyophilized material to the bottom of the vial.
2. Prepare a 5 pmol/amino acid/ μ L internal standard solution by reconstituting one vial of AA 45/32 Standard - 114 Labeled as follows:
 - a. Pipette the amount of AA 45/32 Phys Standard Diluent that is specified on the Phys Standard vial label (approximately 1.8 mL).
 - b. Dispense 1 mL of the Phys Standard Diluent into the AA 45/32 Standard - 114 Labeled vial.

IMPORTANT! Never lay a pipette on its side or invert a pipette with sample in its tip.
 - c. Vortex the vial in 30- to 60-second increments until all material is dissolved.
 - d. Add the remaining Phys Standard Diluent (approximately 0.8 mL).
 - e. Vortex to mix.

**Adding the
internal standard
solution to the
labeled samples**

For each sample from step 6 on page 17:

1. Add 32 μL of AA 45/32TM Phys Standard - 114 Labeled solution.
Store unused AA 45/32TM Phys Standard - 114 Labeled solution at -15 to -25 $^{\circ}\text{C}$.
2. Vortex to mix, then spin.
3. Transfer the iTRAQ Reagent 115-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.”

This chapter covers:

Hardware overview	22
Overview	23
Prepare the HPLC system	25
Prepare the MS system	27
Perform the AA45 and allo-Isoleucine assays	31

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 45 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 AA45 Sample Assay, allo-isoleucine Assay, 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 iTRAQ[®] Reagent-labeled samples using the recommended MS and HPLC systems.

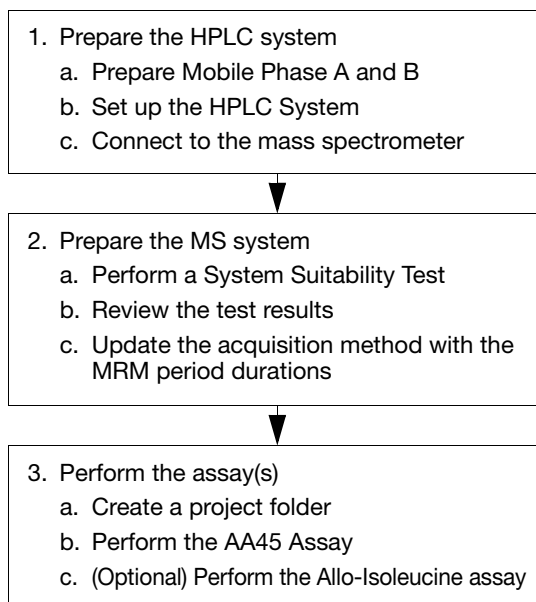


Figure 3 HPLC/MS/MS analysis workflow

Before you begin If necessary, have the Lab Manager:

- For HPLC autosamplers other than those recommended on page 22, set up the hardware profile and create customized acquisition and quantitation methods. Appendix E, “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 use the recommended MS and HPLC systems, you can perform the system suitability test on page 27.

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 us, 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.



DANGER

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
 - 100.0 μ L 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 acetonitrile, HPLC-grade.
2. Add:
 - 0.50 mL Mobile Phase Modifier A
 - 50.0 μ L 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 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 D, “Equilibrating before reuse,” page 52.

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 us, 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




DANGER CHEMICAL HAZARD. AA 45/32™ Phys Standard - 115 Labeled is a flammable liquid and vapor. Harmful if swallowed. Causes skin and respiratory tract irritation. Causes serious eye irritation. May cause sensitization by skin contact. Heating may cause an explosion.

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, and calculates and applies the isotope correction factor for the MS system.

Perform the system suitability test at least once a day (before running samples), using AA 45/32™ Phys Standard - 115 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 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 45/32 Phys Standard - 115 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. If Analyst Software is open, close it
4. Open Cliquid Software by clicking  on the desktop.
Cliquid
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.)

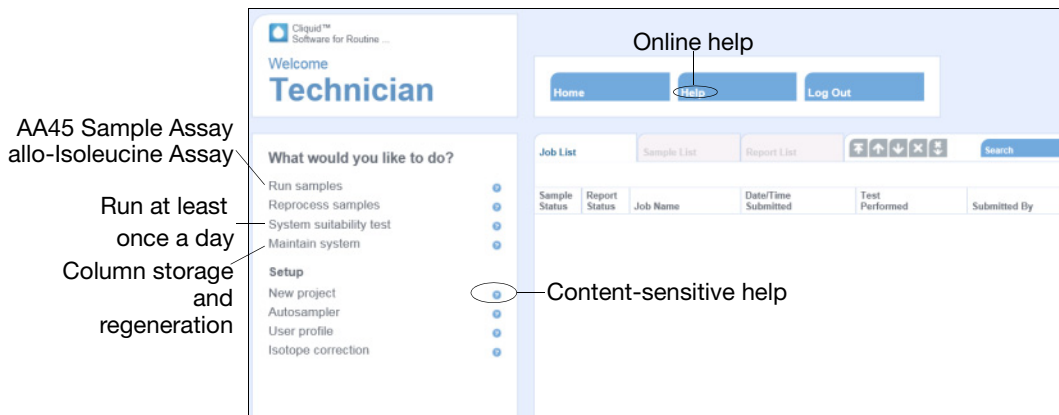


Figure 4 Features of the Home page for a Lab Technician

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 AA45 System Suitability.
Position sample	For the vial of AA 45/32™ Phys Standard - 115 Labeled, enter the: <ul style="list-style-type: none"> • Rack code • Rack position • Sample position • If required for your autosampler: <ul style="list-style-type: none"> – Plate code – Plate position ()
Customize report	1. Select AA45 System Suitability. 2. Select the report output format.
Submit sample	Specify an equilibration time. Recommended times for a system that is: <ul style="list-style-type: none"> • 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:
 - 115 isotope correction values are greater than 0.05, perform mass calibration, then repeat the System Suitability Test.
 - 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, 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 and the average correction factor is 0.05 or less.

Perform the AA45 and allo-Isoleucine assays

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 4 on page 28), 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.

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, 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.

AA45 assay

1. In the Cliquid Software Home page (Figure 4 on page 28), 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


AA45 Sample Assay Wizard page	Selection or input
Choose test	Select AA45 Sample Assay.
Build sample list	<ol style="list-style-type: none"> 1. In the sample list template, select the project 2. Import a sample list or enter sample list information as follows: <ol style="list-style-type: none"> 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. <ul style="list-style-type: none"> – 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, enter a value only if you analyze a urine sample. <p>IMPORTANT! For samples other than urine, leave the field blank or enter 0. Entering a value yields an erroneous results table.</p> <ul style="list-style-type: none"> – For internal standard (IS) concentration, enter 100 for each amino acid. d. For information about the other fields, see online Help, “Entering Sample List Information”. <ol style="list-style-type: none"> 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.

Table 5 Run samples selections and input

AA45 Sample Assay Wizard page	Selection or input
Customize report	Select the appropriate report-generating option. If you choose to generate reports: <ul style="list-style-type: none"> • 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	<ol style="list-style-type: none"> 1. Specify an equilibration time. Recommended times for a system that is: <ul style="list-style-type: none"> – 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. <p>IMPORTANT! If you return to the Home page before completing the submission, all entries in the sample list are lost.</p>

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

Allo-isoleucine assay (optional)

Using the same HPLC buffers and column as the AA45 Assay, analyze the 20- μ L aliquot of supernatant prepared in “Precipitating protein” on page 14:

1. In the Cliquid Software Home page, select **Run samples**.
2. When prompted to choose a test, click **allo-Isoleucine Assay**.
3. Proceed through the wizard, clicking **Next** to advance to the next page. When prompted, specify the same option and parameter values used for the AA45 Sample Assay (see Table 5 on page 32).

For additional information, see Appendix C, “Allo-Isoleucine Assay QA and Method Development.”

Amino Acid Amounts

A

This appendix covers:

AA 45/32 TM Phys Standard - 114 Labeled	36
Provided reagents	37
An AA45 assay injection.	37

AA 45/32™ Phys Standard - 114 Labeled

Approximately 9.0 nmol of each of the following amino acids is labeled with iTRAQ® Reagent 114. The precise amount of amino acids in a vial of AA 45/32™ Phys Standard - 114 Labeled is determined for each lot of standard, and is used to determine the volume of Standard Diluent required to make a 5 pmol/μL.

- O-phospho-L-serine
- O-phospho-ethanolamine
- Taurine
- L-asparagine
- L-serine
- hydroxy-L-proline
- Glycine
- L-glutamine
- Ethanolamine
- L-aspartic acid
- L-citrulline
- Sarcosine
- β-alanine
- L-alanine
- L-threonine
- L-glutamic acid
- L-histidine
- 3-methyl-L-histidine
- 1-methyl-L-histidine
- L-homocitrulline
- Argininosuccinic acid
- γ-amino-n-butyric acid
- D,L-β-amino-isobutyric acid
- L-α-amino-n-butyric acid
- L-α-aminoadipic acid
- L-anserine
- L-carnosine
- L-proline
- L-arginine
- δ-hydroxylysine
- L-ornithine
- Cystathionine
- L-cystine
- L-lysine
- L-valine
- L-norvaline
- L-methionine
- L-tyrosine
- L-homocystine
- L-isoleucine
- L-leucine
- L-norleucine
- L-phenylalanine
- L-tryptophan

Provided reagents

AA 45/32™ Phys Sulfosalicylic Acid



WARNING CHEMICAL HAZARD. AA 45/32™ Phys

Sulfosalicylic acid is harmful if swallowed. It causes eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

AA 45/32 Phys Sulfosalicylic Acid contains 400 pmol/μL norleucine, which is subsequently labeled with iTRAQ Reagent 115.

AA 45/32™ Phys Labeling Buffer



WARNING CHEMICAL HAZARD. AA 45/32™ Phys

Labeling Buffer causes irritation to skin, eyes, and respiratory tract. Affects central nervous system, liver, and kidneys. Harmful by inhalation and if swallowed.

AA 45/32 Phys Labeling Buffer contains 20 pmol/μL norvaline, which is subsequently labeled with iTRAQ Reagent 115.

An AA45 assay injection

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

- iTRAQ Reagent 115-labeled amino acids in the sample
- 10 pmol of iTRAQ Reagent 115-labeled norvaline and norleucine
- 10 pmol of each iTRAQ Reagent 114-labeled amino acid in the standard, including norvaline and norleucine.

Quality Assurance

B

This appendix covers:

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Quality assurance	41
Testing the labeling protocol	42
Workflow efficiency	43

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 $10,000 \times g$ 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

 **DANGER CHEMICAL HAZARD.** AA 45/32™ Phys Standard - 114 Labeled is harmful if swallowed. It causes serious eye, skin, and respiratory tract irritation, and it may cause allergic reactions. Heating it may cause an explosion. It contains a chemical known to the state of California to cause cancer. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

 **DANGER CHEMICAL HAZARD.** AA 45/32™ Phys Standard - 115 Labeled is a flammable liquid and vapor. Harmful if swallowed. Causes skin and respiratory tract irritation. Causes serious eye irritation. May cause sensitization by skin contact. Heating may cause an explosion.

 **WARNING BIOHAZARD.** AA 45/32™ Phys Control Plasma has been tested for the following infectious markers and found negative: HIV 1/2- and HCV-antibodies, hepatitis B surface antigen, HIV 1/2- and HCV-genome (PCR). However, the Control Plasma should be considered as potentially infectious and treated with appropriate care. For more information, see “Biological Hazard Safety” on page xi.

The Amino Acid 45/32 Analyzer Starter Kit - Physiological provides three standards and a control plasma:

- **AA 45/32™ Phys Standard - 114 Labeled** – Used as an internal standard for quantitation of the labeled samples.
- **Standard - 115 Labeled** – To verify the performance of the chromatographic separation and that the sensitivity is acceptable. Also used to determine the Correction Factors.
- **Amino Acid 45/32 Standard - Unlabeled** – To verify the performance of the entire methodology (see below).
- **Control Plasma** – To verify the performance of the entire methodology (see below).

Testing the labeling protocol

If you are running the protocol for the first time, it is strongly recommended that you practice performing the protocol to label the vial of Amino Acid 45/32 Standard - Unlabeled. 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 114 and 115. Most amino acids are stable in the unlabeled amino acid solution, so the calculated amount should be 80 to 120 μM . You may observe lower amounts of Gln, Asa, and Met because while in solution those amino acids degrade over time. Also, since Gln converts to Glu, you may observe higher amounts of this amino acid.

Using AA 45/32™ Phys Standard - Unlabeled

Follow the labeling protocol (Chapter 2), substituting 40 μL of 100-pmol/ μL Amino Acid 45/32 Standard - Unlabeled (containing 4 nmol of each amino acid) for a physiological sample.

After labeling with iTRAQ Reagent 115, the Amino Acid 45/32 Standard - Unlabeled contains the same amino acids as the vial of Amino Acid 45/32 Standard - 114 Labeled (see page 36).

After labeling with iTRAQ Reagent 115 and adding AA 45/32™ Phys Standard - 114 Labeled, a 2- μL injection contains:

- 10 pmol of each iTRAQ Reagent 114-labeled amino acid
- 10 pmol of each iTRAQ Reagent 115-labeled amino acid

Using control plasma

Follow the labeling protocol, substituting 40 μL of Control Plasma for a physiological sample. For the amino acids and concentrations in the Control Plasma, see the Certificate of Analysis.



WARNING BIOHAZARD. AA 45/32™ Phys Control Plasma has been tested for the following infectious markers and found negative: HIV 1/2- and HCV-antibodies, hepatitis B surface antigen, HIV 1/2- and HCV-genome (PCR). However, the Control Plasma should be considered as potentially infectious and treated with appropriate care. For more information, see “Biological Hazard Safety” on page xi.

Reconstitute the vial of control plasma with 3.0 mL of Milli-Q® water or equivalent. Over a period of approximately 15 min, vortex the vial repeatedly until all visible material is dissolved. When dissolved, the solution is cloudy, but no observable particles remain.

IMPORTANT! As shipped, the lyophilized control plasma is stable for 36 months when stored at 4 °C. The reconstituted control plasma is stable up to:

- 5 hours when stored at 25 °C
- 24 hours when stored at 4 °C
- 10 days when stored at –20 °C

To avoid repeated freeze and thaw cycles, transfer 40 μL aliquots of the reconstituted control plasma into fresh tubes.

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 iTRAQ Reagent 115 labeled sample.

Typically, the workflow is acceptably efficient when the amount of norleucine and norvaline recovered is $100 \mu\text{M} \pm 20\%$. If the experimentally determined amount is unacceptable, repeat the labeling protocol with additional samples.

Allo-Isoleucine Assay QA and Method Development

C

This appendix covers:

Quality assurance	46
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Quality assurance



WARNING CHEMICAL HAZARD. AA 45/32™ Phys Sulfosalicylic acid is harmful if swallowed. It causes eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

IMPORTANT! Review the safety warnings in “Safety” on page vii.

Each injection contains about 160 pmol of allo-iso-leucine, 160 pmol of isoleucine and leucine from the unlabeled standard, and 160 pmol of norleucine from the sulfosalicylic acid.

To verify allo-iso-leucine and isoleucine peak separation:

1. Combine 40 μL of Amino Acid 45/32 Standard - Unlabeled, 40 μL of allo-iso-leucine, and 10 μL of AA 45/32™ Phys Sulfosalicylic Acid (provides norleucine) in a tube.
2. Vortex to mix, then spin.
3. Transfer the mixture to an autosampler vial.
4. Perform the Allo-Isoleucine Assay.
5. Review the report. Optimal peak separation yields four distinct peaks (see Figure 5) with a minimum of 0.2 min (12 sec) from peak to peak.

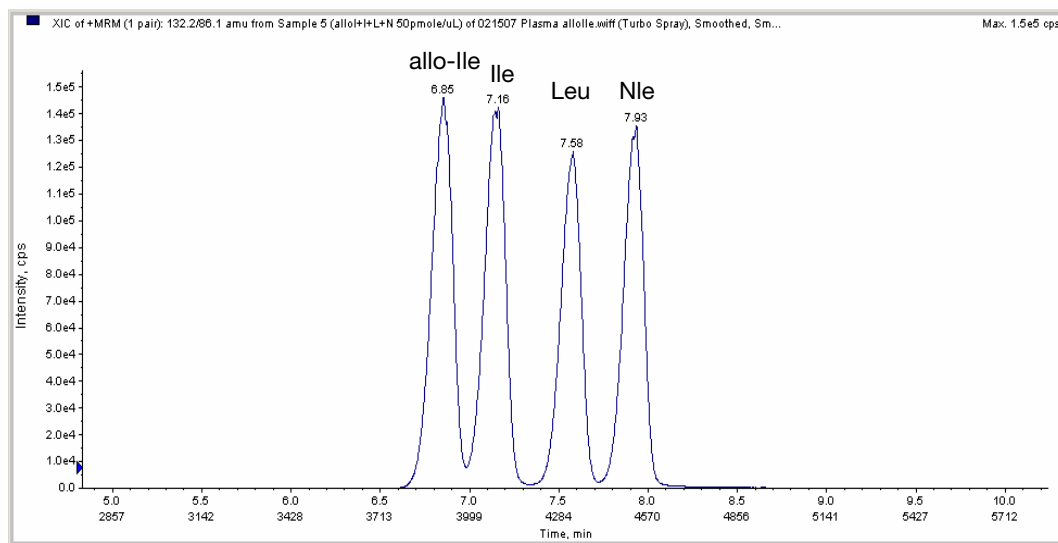


Figure 5 Representative total ion chromatograph showing optimal peak separation.

Developing an allo-iso-leucine acquisition method for non-supported autosamplers

The values in Tables 6 and 7 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 allo-iso-leucine assay uses the same mobile phases and HPLC conditions as the AA45 Assay, but the gradient is different. The gradient differs in the 10.0 min values (Mobile Phase A, 84%; Mobile Phase B, 16%).

Table 6 LC gradient for the allo-iso-leucine assay

Total time (min)	%Mobile phase A	%Mobile phase B
0.0	98.0	2.0
10.0	84.0	16.0
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 The TurboIonSpray® source Source/Gas and Compound values for the allo-isoleucine assay are the same as those of the AA45 Assay, except for DP (30) and CE (18).

Table 7 TurboIonSpray® source Source/Gas and Compound values

Gas or compound	LC/MS/MS systems			
	API 3200™ system	3200 QTRAP® system	API 4000™ system	4000 QTRAP® system
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	18	18	18	18
CXP	5	5	5	5

MRM values

The MRM Q1 value is 132; the Q3 value is 86. Dwell time is 100 msec

Column Maintenance

D

This appendix covers:

Maintaining the HPLC column 52

Maintaining the HPLC column

IMPORTANT! Review “Prepare the mobile phases” on page 25.

Washing 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 28), 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.

Equilibrating before reuse

IMPORTANT! Use the column only for the 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 25).
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

E

This appendix covers:

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Developing an AA45 assay acquisition method for non-supported autosamplers	55

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 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 AA45 assay, the MRM scan has one experiment with five periods scanned in positive polarity. Organizing the experiment into periods in which specific amino acids are monitored allows for collecting more data points per peak and more accurate quantitation.

Developing an AA45 assay acquisition method for non-supported autosamplers

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 column temperature is 50 °C, injection volume is 2 µL, and flow rate is 0.8 mL/min. Table 8 provides the recommended LC gradient.

Table 8 Recommended LC gradient for the AA45 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 Table 9 shows the TurboIonSpray® (TIS) source Source/Gas and Compound values.

Table 9 Recommended TIS values

Gas or compound	LC/MS/MS systems			
	API 3200™	3200 QTRAP®	API 4000™	4000 QTRAP®
TurbolonSpray® 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

MRM values Table 10 for the Q1 (precursor ion) and Q3 (product ion) masses.

Table 10 MRM transitions for the AA45 assay

Amino acid	Abbreviation	Q1, mass (amu) (labeled amino acid)	Q3, mass (amu) (iTRAQ™ Reagent label)
O-phospho-L-serine	PSer	330.12	114.11
		330.12	115.11
O-phosphoethanolamine	PEtN	286.13	114.11
		286.13	115.11
Taurine	Tau	270.13	114.11
		270.12	115.11
L-asparagine	Asn	277.17	114.11
		277.16	115.11
L-serine	Ser	250.16	114.11
		250.15	115.11
Hydroxy-L-proline	Hyp	276.17	114.11
		276.17	115.11
Glycine	Gly	220.15	114.11
		220.14	115.11
L-glutamine	Gln	291.18	114.11
		291.18	115.11
Ethanolamine	EtN	206.17	114.11
		206.16	115.11
L-aspartic acid	Asp	278.15	114.11
		278.14	115.11
L-citrulline	Cit	320.21	114.11
		320.20	115.11
Sarcosine	Sar	234.16	114.11
β-alanine	Bala	234.16	115.11
L-alanine	Ala		

Table 10 MRM transitions for the AA45 assay (*continued*)

Amino acid	Abbreviation	Q1, mass (amu) (labeled amino acid)	Q3, mass (amu) (iTRAQ™ Reagent label)
L-threonine	Thr	264.17	114.11
		264.17	115.11
L-glutamic acid	Glu	292.17	114.11
		292.16	115.11
L-histidine	His	300.18	114.11
		300.18	115.11
3-methyl-L-histidine	3MHis	314.20	114.11
1-methyl-L-histidine	1MHis	314.19	115.11
L-homocitrulline	Hcit	334.23	114.11
		334.22	115.11
Argininosuccinic acid	Asa	435.24	114.11
		435.23	115.11
γ -amino-n-butyric acid	GABA	248.18	114.11
D,L- β -aminoisobutyric acid	bAib	248.17	115.11
L- α -amino-n-butyric acid	Abu		
L- α -aminoadipic acid	Aad	306.18	114.11
		306.18	115.11
L-anserine	Ans	385.24	114.11
		385.23	115.11
L-carnosine	Car	371.22	114.11
		371.21	115.11
L-proline	Pro	260.18	114.11
		260.17	115.11
L-arginine	Arg	319.23	114.11
		319.22	115.11

Table 10 MRM transitions for the AA45 assay (*continued*)

Amino acid	Abbreviation	Q1, mass (amu) (labeled amino acid)	Q3, mass (amu) (iTRAQ™ Reagent label)
δ-hydroxylysine	Hyl	451.32	114.11
		451.31	115.11
L-ornithine	Orn	421.31	114.11
		421.30	115.11
Cystathionine	Cth	511.29	114.11
		511.27	115.11
L-cystine	Cys	529.24	114.11
		529.23	115.11
L-lysine	Lys	435.33	114.11
		435.31	115.11
L-valine	Val	262.19	114.11
L-norvaline	Nva	262.19	115.11
L-methionine	Met	294.16	114.11
		294.16	115.11
L-tyrosine	Tyr	326.19	114.11
		326.18	115.11
L-homocystine	Hcy	557.27	114.11
		557.26	115.11
L-isoleucine	Ile	276.21	114.11
L-leucine	Leu	276.20	115.11
L-norleucine	Nle		
L-phenylalanine	Phe	310.19	114.11
		310.19	115.11
L-tryptophan	Trp	349.20	114.11
		349.20	115.11

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