Amino Acid Analysis for Physiological Samples

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

Protocol
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Preface

This preface covers:

• Safety on page 5
• How to obtain more information on page 8
• How to obtain support on page 9

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

Tip! 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 6.
• 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. For the MSDSs of chemicals not distributed with this kit, contact the chemical manufacturer.

Chemical waste hazards

WARNING! 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.

Tip! 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://bmb.l.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 or institutions’ 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

• xTRAQ Family of Amine-Modifying Labeling Reagents for Multiplexed Relative and Absolute Quantification: Chemistry Reference Guide (PN 4351918)

• Amino Acid Analysis for Physiological Samples Quick Reference Card (PN 4445543)

• Technical and Application Notes

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

For technical and application notes, see How to obtain support on page 9.

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 the help icon in the toolbar or user interface of the software window

• Select the Help tab

• Press F1 (not applicable to the 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
This chapter covers:

- Overview on page 11
- Available kits and materials on page 12
- Contents of the starter kit on page 12
- Contents of the 50-assay and 200-assay kits on page 14
- User-supplied materials on page 15

**Overview**

The aTRAQ™ Kits for physiological amino acid analysis enable identification and quantitation of amino acids in plasma and serum, urine, cerebrospinal fluid (CSF) samples, and other samples containing free amino acids. The kits provide aTRAQ™ Reagent $\Delta$8 for labeling samples and a mixture of $\Delta$0-labeled amino acids as an internal standard.

**Product capabilities**

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

**Note:** The retention times of the $\Delta$8 and $\Delta$0 peaks in Figure 1-1 are identical amino acids that are labeled with the same reagent but with different numbers of isotopes.

![Figure 1-1](image)

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

\[
\text{Amount}_{\text{sample}} = \frac{\text{Area}_{\Delta 8}}{\text{Area}_{\Delta 0}} \times \text{Amount}_{\text{std}}
\]
Available kits and materials

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

Table 1-1 Kits and Materials

<table>
<thead>
<tr>
<th>Item</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>aTRAQ™ Kits Physiological</strong></td>
<td></td>
</tr>
<tr>
<td>Starter Kit</td>
<td>Provides sufficient material to run 50 aTRAQ™ Reagent-Δ8-labeled samples (each containing up to 10 nmole total amino acid) with the aTRAQ™ Internal Standard.</td>
</tr>
<tr>
<td>50-Assay or 200-Assay Kit</td>
<td>Provides sufficient material to run 50 or 200 aTRAQ™ Reagent-Δ8-labeled samples (each containing up to 10 nmole total amino acid) with the aTRAQ™ Internal Standard.</td>
</tr>
<tr>
<td><strong>Standards and Controls</strong></td>
<td></td>
</tr>
</tbody>
</table>
| aTRAQ™ Standards Set Physiological | • aTRAQ™ Internal Standard.  
|                               | • aTRAQ™ Unlabeled Standard.  
|                               | • allo-Isoleucine (can be used to test the separation of unlabeled allo-isoleucine, isoleucine, leucine, and norleucine). |
| aTRAQ™ Internal Standard      | • Standard Diluent to dilute the aTRAQ™ Internal Standard. The amount of Standard Diluent to use is indicated on the Certificate of Analysis and the aTRAQ™ Internal Standard vial label. |
| aTRAQ™ Unlabeled Standard     | Provides the aTRAQ™ Unlabeled Standard. Contains the same amino acids as the internal standard, except norvaline and norleucine. Norleucine and norvaline are incorporated during labeling. |
| Control Plasma                | Provides a known plasma sample.                                             |
| **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 aTRAQ™ Reagent-Δ8, the standards set, reagents, Control Plasma, and this document (see Table 1-2 on page 13). For recommendations on using the standards set, see Quality assurance on page 36. Order the Amino Acid Analyzer (AAA) C18 Column separately.
Caution: When you receive the shipping container, immediately store the Reagent Kit, aTRAQ™ Standards Set Physiological, and Control Plasma at -15°C or below.

**Tip!** 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 35.

### Table 1-2 Contents of the aTRAQ™ Starter Kit Physiological

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reagent Kit (one 50-Assay Kit)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• aTRAQ™ Reagent Δ8</td>
<td>4 vials, 1unit/vial</td>
<td>Amine-modifying labeling reagent. One unit (one vial) of reagent yields approximately 15 assays.</td>
</tr>
<tr>
<td>• Sulfosalicylic Acid†</td>
<td>1 vial, 1.8 mL</td>
<td>10% sulfosalicylic acid to precipitate proteins from the sample. Also contains approximately 400 µM norleucine (the exact concentration is on the Certificate of Analysis).</td>
</tr>
<tr>
<td>• Labeling Buffer†</td>
<td>2 vials, 1.8 mL/vial</td>
<td>Borate buffer, pH 8.5. Also contains approximately 20 µM norvaline (the exact concentration is on the Certificate of Analysis).</td>
</tr>
<tr>
<td>• Hydroxylamine†</td>
<td>1 vial, 1.8 mL</td>
<td>1.2% hydroxylamine solution. Reverses partial labeling of the phenolic hydroxyl group of tyrosine and quenches any unreacted aTRAQ™ Reagent.</td>
</tr>
<tr>
<td>• Mobile Phase Modifier A†</td>
<td>2 vials, 1.8 mL/vial</td>
<td>100% formic acid for mobile phase A and mobile phase B preparation.</td>
</tr>
<tr>
<td>• Mobile Phase Modifier B†</td>
<td>2 vials, 200 µL/vial</td>
<td>100% heptafluorobutyric acid for mobile phase A and mobile phase B preparation.</td>
</tr>
<tr>
<td>• Isopropanol†</td>
<td>1 vial, 1.8 mL</td>
<td>Isopropanol, absolute, for diluting aTRAQ™ Reagent.</td>
</tr>
</tbody>
</table>
| **aTRAQ™ Standards Set Physiological**    | 1                 | • 1 vial aTRAQ™ Internal Standard  
• 1 vial aTRAQ™ Unlabeled Standard  
• 1 vial Standard Diluent - 2% formic acid for reconstituting the vials of aTRAQ™ Internal Standard. The Certificate of Analysis specifies the precise amount of diluent for reconstituting this lot of standard. |
| **Control Plasma**                        | 1 vial, 3 mL      | Provides a known plasma sample.                                          |
|                                           | lyophilized       |                                                                          |
Table 1-2 Contents of the aTRAQ™ Starter Kit Physiological (Continued)

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Documentation</td>
<td>1</td>
<td>This document.</td>
</tr>
<tr>
<td>Amino Acid Analysis for Physiological Samples Protocol</td>
<td>1</td>
<td>A laminated card that briefly describes the steps in the labeling protocol.</td>
</tr>
</tbody>
</table>

† Can also be stored refrigerated.

Contents of the 50-assay and 200-assay kits

Tip! When you receive the shipping container, immediately store the Reagent Kit and the aTRAQ™ Internal Standard bag(s) at -15°C or below.

Tip! 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 35.

See Table 1-3 on page 14 for materials contained in each kit.

Table 1-3 Contents of the aTRAQ™ Kit Physiological 50 Assay and 200 Assay Kits

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity in 50-Assay Kit</th>
<th>Quantity in 200-Assay Kit</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent Kit (50-Assay Kit or 200-Assay Kit)</td>
<td>1 shipping container with the following items:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• aTRAQ™ Reagent Δ8</td>
<td>4 vials, 1 unit/vial</td>
<td>14 vials, 1 unit/vial</td>
<td>Amine-modifying labeling reagent. One unit (one vial) of reagent yields approximately 15 assays.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Sulfosalicyclic Acid†</td>
<td>1 vial, 1.8 mL</td>
<td>2 vials, 1.8 mL/vial</td>
<td>10% sulfosalicyclic acid to precipitate proteins from the sample. Also contains approximately 400 μM norleucine (the exact concentration is on the Certificate of Analysis).</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Labeling Buffer†</td>
<td>2 vials, 1.8 mL/vial</td>
<td>5 vials, 1.8 mL/vial</td>
<td>Borate buffer, pH 8.5. Also contains approximately 20 μM norvaline (the exact concentration is on the Certificate of Analysis).</td>
</tr>
</tbody>
</table>
Table 1-3 Contents of the aTRAQ™ Kit Physiological 50 Assay and 200 Assay Kits

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity in 50-Assay Kit</th>
<th>Quantity in 200-Assay Kit</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Hydroxylamine†</td>
<td>1 vial, 1.8 mL</td>
<td>1 vial, 1.8 mL</td>
<td>1.2% hydroxylamine solution. Reverses partial labeling of the phenolic hydroxyl group of tyrosine and quenches any unreacted aTRAQ™ Reagent.</td>
</tr>
<tr>
<td>• Mobile Phase Modifier A†</td>
<td>2 vials, 1.8 mL/vial</td>
<td>6 vials, 1.8 mL/vial</td>
<td>100% formic acid for mobile phase A and mobile phase B preparation.</td>
</tr>
<tr>
<td>• Mobile Phase Modifier B†</td>
<td>2 vials, 200 µL/vial</td>
<td>6 vials, 200 µL/vial</td>
<td>100% heptafluorobutyric acid for mobile phase A and mobile phase B preparation.</td>
</tr>
<tr>
<td>• Isopropanol†</td>
<td>1 vial, 1.8 mL</td>
<td>1 vial, 1.8 mL</td>
<td>Isopropanol, absolute, for diluting aTRAQ™ Reagent.</td>
</tr>
<tr>
<td>aTRAQ™ Internal Standard</td>
<td>1 bag</td>
<td>4 bags</td>
<td>• 1 vial aTRAQ™ Internal Standard</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• 1 vial aTRAQ™ Unlabeled Standard</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• 1 vial Standard Diluent - 2% formic acid for reconstituting the vials of aTRAQ™ Internal Standard</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Certificate of Analysis. Specifies the precise amount of diluent for reconstituting this lot of standard.</td>
</tr>
</tbody>
</table>

**Documentation**

| Amino Acid Analysis for Physiological Samples Quick Reference Card | 1 | 1 | A laminated card that briefly describes the steps in the labeling protocol. |

† Can also be stored refrigerated.

**User-supplied materials**

Table 1-4 User-supplied materials

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity per Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disposable gloves</td>
<td>As needed</td>
</tr>
<tr>
<td>Physiological samples, at least 40 µL of each sample</td>
<td>As needed</td>
</tr>
<tr>
<td>Pipetting accessories (pipettors and tips) suitable for 5 µL to 1 mL volumes, such as P10, P100, P1000 pipettes</td>
<td>As needed</td>
</tr>
<tr>
<td>Item</td>
<td>Quantity per Assay</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Milli-Q® water or equivalent (minimum 18.2 MOhms water, conductivity maximum 0.05 µS/0.05 µMho) for mobile phase A</td>
<td>As needed</td>
</tr>
<tr>
<td>Methanol, HPLC-grade for mobile phase B</td>
<td>As needed</td>
</tr>
<tr>
<td>Bench-top centrifuge or microcentrifuge (RCF # &gt;10,000)</td>
<td>1</td>
</tr>
<tr>
<td>Vortexer</td>
<td>1</td>
</tr>
<tr>
<td>Centrifugal vacuum concentrator or inert gas evaporator</td>
<td>1</td>
</tr>
<tr>
<td>Standard Eppendorf Tubes™, polypropylene, 0.5-mL and 1.5-mL</td>
<td>As needed</td>
</tr>
<tr>
<td>Measuring cylinder, glass, 1000-mL</td>
<td>As needed</td>
</tr>
<tr>
<td>HPLC bottles, glass, 1000-mL</td>
<td>2</td>
</tr>
<tr>
<td>Autosampler vials and inserts, conical, 220 µL and 1000 µL</td>
<td>As needed</td>
</tr>
<tr>
<td>Amino Acid Analyzer (AAA) C18 Column (5 µm, 4.6 X 150 mm)</td>
<td>1</td>
</tr>
<tr>
<td>Cliquid® Software for Routine Amino Acid Analysis</td>
<td>--</td>
</tr>
<tr>
<td>LC/MS/MS System with a TurbolonSpray® source and required gases (see Recommended MS systems and software on page 23)</td>
<td>--</td>
</tr>
<tr>
<td>PEEK™ tubing, 0.005-in. ID (red)</td>
<td>As needed</td>
</tr>
</tbody>
</table>
This chapter covers:

- Amino acid labeling workflow on page 18
- Before you begin on page 19
- Precipitate sample protein and diluting on page 20
- Label the samples with aTRAQ™ Reagent Δ8 on page 20
- Add the internal standard on page 21
Amino acid labeling workflow

Precipitate sample protein and dilute

Sulfosalicyclic acid, 10 µL  
Labeling Buffer, 40 µL

Supernatant 10 µL

Physiological sample, 40 µL

Label the sample with aTRAQ™ Reagent, then dry

aTRAQ™ Reagent Δ8 + IPA, 5 µL  
Hydroxylamine, 5 µL

30 minutes at room temperature

aTRAQ™ Reagent Δ8 labeled sample

Add the aTRAQ™ Internal Standard

aTRAQ™ Internal Standard in Standard Diluent, 32 µL

Reduce volume

aTRAQ™ Reagent Δ8 labeled sample

(Optional) Diluted supernatant, 5 µL, for allo-isoleucine assay

Diluted supernatant, 10 µL, for assay

Figure 2-2  Labeling workflow for one physiological sample
Before you begin

Review the safety warnings in Safety on page 5. 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.

Test 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 aTRAQ™ Unlabeled Standard on page 36. 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.

Tip! When performing the labeling protocol, you pipette volumes as small as 5- and 10-µL. Slight variability in the accuracy of pipetting such small volumes can cause large variability in reagent concentrations and, consequently, analytical result. To optimize accurate pipetting, see Handling tips to ensure accurate concentrations and volumes on page 35.

When testing the labeling protocol, you may determine that alternative steps are required for your sample. If so, modify the procedures between Label the samples with aTRAQ™ Reagent Δ8 on page 20 and Add the internal standard on page 21.

Review Appendix A: Amino Acid Amounts for information on:

- The amino acids in the internal standard that are labeled with Δ0 reagent and their amounts
- Incorporating norvaline and norleucine standards and amounts
- 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 aTRAQ™ Reagent required to label that number of samples. One vial of aTRAQ™ Reagent Δ8 labels 15 sample assays.
- Allow the reagents and each required vial of aTRAQ™ Reagent Δ8 to reach room temperature. Return the reagents to storage at -15°C or below within 2 hours of thawing.
- Briefly centrifuge the reagent and aTRAQ™ Reagent vials to dislodge material potentially trapped in the caps.
- Inspect the vial of Labeling Buffer. If precipitate is present, warm the vial to 37°C, then vortex.
Precipitate sample protein and diluting

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

Follow the procedures below for each physiological sample.

Precipitate protein

1. Transfer 40 µL of a physiological sample to a tube.
2. Add 10 µL of Sulfosalicylic Acid (contains approximately 4000 pmol norleucine).
3. Vortex to mix, then spin at 10,000 x g for 2 minutes.

Note: Protein precipitate may not form in all physiological samples.

4. Transfer 10 µL of the supernatant to a clean tube.

Dilute with labeling buffer

1. Add 40 µL of Labeling Buffer (contains approximately 800 pmol norvaline) to the 10-µL aliquot of supernatant from step 4.
2. Vortex to mix, then spin.
3. Transfer 10 µL of the supernatant to a clean tube. This sample is labeled with aTRAQ™ Reagent in the next section.
4. Refrigerate the remaining supernatant to use if you need to repeat the aTRAQ™ Reagent labeling or are performing the optional allo-isoleucine analysis.

Label the samples with aTRAQ™ Reagent Δ8

Prepare the labeling reagent solution

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

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

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

Repeat the following procedure for each sample.

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

1. To a sample from step 2 in Label the samples with aTRAQ™ Reagent △8 on page 20, add 5 µL of the aTRAQ™ Reagent solution.

   **Tip!** Immediately store unused aTRAQ™ Reagent solution at -15°C or below.

2. Vortex to mix, then spin.
3. Incubate the sample at room temperature for at least 30 min.
4. Add 5 µL of Hydroxylamine.
5. Vortex to mix, then spin.
6. Incubate the sample at room temperature for at least 15 minutes.
7. If you are performing the optional allo-isoleucine analysis, add 5 µL of the diluted supernatant from step 4 in Dilute with labeling buffer on page 20.

Add the internal standard

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

Prepare the internal standard solution

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

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

   iii. Vortex the vial in 30 to 60 second increments until all material is mixed.
   iv. Add the remaining Standard Diluent (approximately 0.8 mL).
   v. Vortex to mix.
Add the internal standard solution to the labeled samples

For each sample from step 6 on page 21:

1. Add 32 µL of aTRAQ™ Internal Standard solution. Store unused aTRAQ™ Internal Standard solution at -15°C or below.
2. Vortex to mix, then spin.
3. Reduce the volume of the sample to approximately 30 µL using a vacuum concentrator or inert gas evaporator. If the volume of the sample is below 15 µL, add 20 µL of water.

**Note:** For MS instruments more sensitive than the API 3200™ System or the 3200 QTRAP® System, it may be necessary to dilute the sample with water so that the detector is not saturated. If more than 300 µL of water is added, then the sample reduction step is not necessary.

4. Transfer the labeled sample/internal standard mixture to an autosampler vial with a low-volume insert.
5. 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 on page 23
- Overview on page 24
- Prepare the HPLC system on page 26
- Prepare the MS system on page 27
- Perform the sample assay on page 30

Hardware overview

Recommended MS systems and software

- API 3200™ System
- 3200 QTRAP™ System

**Note:** For MS instruments more sensitive than the API 3200™ System or the 3200 QTRAP® System, it may be necessary to dilute the sample with water so that the detector is not saturated.

- Analyst® Software 1.5 or later, using the IntelliQuant integration algorithm, and Cliquid® Software for Routine Amino Acid Analysis

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

Recommended HPLC autosamplers

Agilent 1100 series, with:

- Binary pump G1312A
- Well-plate autosampler G1367A
- Column oven G1316A

Agilent 1200 series, with:

- Binary pump G1312A
- Well-plate autosampler G1367B
- Column oven G1316A

Shimadzu Prominence, with:

- System controller CBM-20A
• 2 Isocratic pumps LC-20AD [includes automatic purge (flush) kit and semi-micro gradient mixer SUS-20A]
• Autosampler SIL-20AC
• Column oven CTO-20AC

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

### Overview

**The Analyst® software**

The 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.

**The 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 Physiological Sample Assay, Physiological System Suitability Test, and column maintenance. Refer to the Cliquid® Software Help for detailed information on the Cliquid® software.
Workflow

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

1. Prepare the HPLC system
   i. Prepare Mobile Phase A and B
   ii. Set up the HPLC System
   iii. Connect to the mass spectrometer

2. Prepare the MS system
   i. Perform a System Suitability Test
   ii. Review the test results
   iii. If necessary, update the acquisition and quantitation methods with the retention times.

3. Perform the assay(s)
   i. Create a project folder
   ii. Load the autosampler
   iii. Perform the sample assay

Figure 3-3  HPLC/MS/MS analysis workflow

HPLC/MS/MS analysis workflow

Before you begin

If necessary, have the Lab Manager:

- Set up the hardware profile and create customized acquisition and quantitation methods for HPLC autosamplers other than those recommended in Figure 3-3 on page 25. Appendix D: Developing an Acquisition Method has recommended starting point values for creating the methods.
- Perform mass calibration if the MS has not been calibrated in 3 to 6 months or if the MS source has been recently cleaned. Verify the calibration by performing a system suitability test or analyzing a control sample, then update the retention times in the quantitation method.
Prepare the HPLC system

Review the safety warnings in Safety on page 5. For the MSDS of any chemical not distributed with this kit, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer and observe all relevant precautions.

Prepare the mobile phases

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

To prepare mobile phase A:

1. In a 1-L volumetric flask, add approximately 500 mL of Milli-Q® water or equivalent, HPLC-grade.
2. Add:
   - 1.00 mL Mobile Phase Modifier A
   - 100.0 µL Mobile Phase Modifier B
3. Swirl the flask to mix.
4. Bring to volume with Milli-Q® water or equivalent, HPLC-grade, then mix.
   
   For optimal shelf-life, transfer the solution to an amber glass bottle. Label the bottle with the date prepared (discard unused mobile phase A after a week).

To prepare mobile phase B:

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

Set up the HPLC system

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

**Note:** If you use the recommended MS and HPLC systems, you can Perform the system suitability test on page 27.
2. Flush the system.

If the column has been stored, see Appendix C: Equilibrate before reuse.

Prepare the MS system

Review the safety warnings in Safety on page 5. 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

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

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

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

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

1. Prepare a vial of aTRAQ™ Internal Standard.
2. Transfer 100 µL of aTRAQ™ Internal Standard to an autosampler vial and place it in the HPLC autosampler. Note the plate code and position (if applicable), rack code, rack position, and sample position of the vial.
3. If the Analyst® Software is open, close it.
4. Open the Cliquid® Software by clicking the icon on the desktop.
5. Enter your log in information, then click Get Started. For a Lab Technician, the Home page opens (Figure 3-4). (The Home page for a Lab Manager displays additional tasks.)
6. In the Home page (Figure 3-4), select **System suitability test**.

7. Proceed through the wizard, click **Next**. When prompted, select or type the values from Table 3-5.
8. Click **Submit**. The Home page opens, with the system suitability test added to the sample list.

**Tip!** 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.

**Tip!** While the Cliquid® Software is running and/or processing submissions, the Analyst® Software cannot be opened. Before starting the 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.
3. Review the report for failed items. If the:
   • Analyte retention times (RT) differ from the expected retention time by more than 0.5, have your lab manager update the retention times in the acquisition and quantitation method files.
   • Analyte peak areas are less than the expected peak areas, repeat the system suitability test. If most or all of the peak areas are below the threshold, the MS may need tuning. If only a few of the peak areas are below the threshold, then you may need a fresh aTRAQ™ Internal Standard sample.

4. Read the diagnosing statement on the report. For additional diagnosing information, see Help, System Suitability Test.

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

**Perform the sample assay**

**Create a project folder**

All data files are associated with a project. A project folder must exist before you use the Cliquid® Software to build a sample list or customize a report. Although created through the 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 3-4), click New project.
   The New Project screen opens.
2. Type a name for the project folder.
3. Click Create.
4. After Project created successfully is displayed, click Done to open to the Home page.

**Tip!** Refer to the documentation provided with your equipment for safety information. Review the safety warnings in Safety on page 5.

**Load the autosampler**

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

1. In the Cliquid® Software Home page, select Run samples, see Figure 3-4.
2. Proceed through the wizard, click Next. When prompted, select or type from Table 3-6.

Table 3-6 Selections or Input for the Sample Assay Wizard

<table>
<thead>
<tr>
<th>System Suitability Test Wizard Page</th>
<th>Selection or Input</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choose test</td>
<td>Select Physiological Sample Assay</td>
</tr>
</tbody>
</table>
| Build sample list                   | 1. In the sample list template, select the project.  
                                        2. Import a sample list or enter sample list information as follows:  
                                           i. In the Name field, type the name of your sample.  
                                           ii. 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.  
                                           iii. In the remaining fields, specify the values in each list or enter values as applicable.  
                                               • For category (the reference range against which obtained sample concentrations are compared), select Standard or None. (Additional categories may have been created by the Lab Managers).  
                                               • For normalization value, enter a value only if you analyze a urine sample.  
| Note: For samples other than urine, leave the field blank or type 0. Typing a value yields an erroneous results table. |

- For internal standard (IS) concentration, enter the numbers on the Certificate of Analysis for the aTRAQ™ Internal Standard for each amino acid.

iv. For information about the other fields, see online Help, Entering Sample List Information.

3. Repeat step i through step iii in step 2 for each sample.
4. After you complete entering samples, click Next. The software validates the field entries for proper format and flags any formatting errors.
5. Correct all formatting errors.
6. Click the save icon to save the sample list (Optional).
Table 3-6 Selections or Input for the Sample Assay Wizard (Continued)

<table>
<thead>
<tr>
<th>System Suitability Test Wizard Page</th>
<th>Selection or Input</th>
</tr>
</thead>
</table>
| Customize report                    | Select the appropriate report-generating option. If you choose to generate reports:  
  - After all samples are acquired or after each sample is acquired - Continue on to choose report style and select report output format  
  - Later using the Reprocess samples task - Click Next to Submit samples |
| Submit sample                       | 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 (click Back). Alternatively, click Cancel to return to the Home page. |

Note: If you return to the Home page before completing the submission, all entries in the sample list are lost.

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

This appendix covers

- aTRAQ™ Internal Standard on page 33
- Provided reagents on page 33
- An assay injection on page 34

aTRAQ™ Internal Standard

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

Table A-7 Amino Acids labeled with aTRAQ Reagent Δ0

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>O-phospho-L-serine</td>
<td>L-glutamic acid</td>
<td>L-ornithine</td>
</tr>
<tr>
<td>O-phospho-ethanolamine</td>
<td>L-histidine</td>
<td></td>
</tr>
<tr>
<td>Taurine</td>
<td>3-methyl-L-histidine</td>
<td>L-cystine</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>1-methyl-L-histidine</td>
<td>L-lysine</td>
</tr>
<tr>
<td>L-serine</td>
<td>L-homocitrulline</td>
<td>L-valine</td>
</tr>
<tr>
<td>hydroxy-L-proline</td>
<td>Argininosuccinic acid</td>
<td>L-norvaline</td>
</tr>
<tr>
<td>Glycine</td>
<td>γ-amino-n-butyric acid</td>
<td>L-methionine</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>D,L-β-amino-isobutyric acid</td>
<td>L-tyrosine</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>L-α-amino-n-butyric acid</td>
<td>L-homocystine</td>
</tr>
<tr>
<td>L-aspartic acid</td>
<td>L-α-aminoadipic acid</td>
<td>L-isoleucine</td>
</tr>
<tr>
<td>L-citruiline</td>
<td>L-anserine</td>
<td>L-leucine</td>
</tr>
<tr>
<td>Sarcosine</td>
<td>L-carnosine</td>
<td>L-norleucine</td>
</tr>
<tr>
<td>β-alanine</td>
<td>L-proline</td>
<td>L-phenylalanine</td>
</tr>
<tr>
<td>L-alanine</td>
<td>L-arginine</td>
<td>L-tryptophan</td>
</tr>
<tr>
<td>L-threonine</td>
<td>δ-hydroxylysine</td>
<td></td>
</tr>
</tbody>
</table>

Provided reagents

Sulfosalicylic Acid

Sulfosalicylic Acid contains approximately 400 µM norleucine (the exact concentration is on the Certificate of Analysis), which is used as a check of the recovery and labeling efficiency. After
performing the assay, the calculated amount of norleucine should be approximately 100 µM (the exact concentration is on the Certificate of Analysis).

**Labeling Buffer**

Labeling Buffer contains approximately 20 µM norvaline (the exact concentration is on the Certificate of Analysis), which is used as a check the labeling efficiency. After performing the assay, the calculated amount of norvaline should be approximately 100 µM (the exact concentration is on the Certificate of Analysis).

**An assay injection**

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

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

This appendix covers

- Handling tips to ensure accurate concentrations and volumes on page 35
- Quality assurance on page 36
- Testing the labeling protocol on page 36
- Workflow efficiency on page 37

Handling tips to ensure accurate concentrations and volumes

**Small volume handling tips**

To ensure accurate concentrations throughout the labeling protocol:

- Have all vials of samples and reagents at room temperature
- Capture all material from the sides and cap of the vial by centrifuging (spinning) the vials at 10,000 × g for 2 minutes
- Cap each tube promptly to avoid evaporation
- Store materials at the recommended conditions

To ensure accurate pipetting:

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

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

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

The aTRAQ™ Starter Kit Physiological provides three standards and a control plasma:

- **aTRAQ™ Internal Standard** - Used as an internal standard for quantitation of the labeled samples.
- **allo-Isoleucine** - To verify the performance of the chromatographic separation and that the sensitivity is acceptable for the allo-isoleucine analysis.
- **aTRAQ™ Unlabeled Standard** - 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 aTRAQ™ Unlabeled Standard. Analyzing the practice sample by LC/MS/MS (Chapter 3: LC/MS/MS Analysis) provides information about the proficiency of sample handling and the efficiency of the labeling protocol for each amino acid.

Verify that peaks display at m/z 113 and 121. Most amino acids are stable in the unlabeled amino acid solution, so the calculated amount should be 80 to 120 µM. You may observe lower amounts of Gln, Asn, Asa, Cth, and Met because while in solution those amino acids degrade over time. Also, since Asn and Gln convert to Asp and Glu, respectively, you may observe higher amounts of these amino acids.

Using aTRAQ™ Unlabeled Standard

Follow the labeling protocol (Chapter 2), substituting 40 µL of 100-µM aTRAQ™ Unlabeled Standard (containing 4 nmole of each amino acid) for a physiological sample.

After labeling with aTRAQ™ Reagent Δ8, the aTRAQ™ Unlabeled Standard contains the same amino acids as the vial of aTRAQ™ Internal Standard.

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

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

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.
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.

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 aTRAQ™ Reagent Δ8 labeled sample.

Typically, the workflow is acceptably efficient when the amount of norleucine and norvaline recovered is within 20% of the value listed on the Certificate of Analysis. If the experimentally determined amount is unacceptable, repeat the labeling protocol with additional samples.

**allo-Isoleucine separation**

**Tip!** Review the safety warnings in Safety on page 5.

Each injection contains about 10 pmole of allo-isoleucine, 10 pmole of isoleucine and leucine from the unlabeled standard, and 10 pmole of norleucine from the sulfosalicylic acid.

To verify allo-isoleucine and isoleucine peak separation:

1. Combine 20 µL of AA Unlabeled Standard, 20 µL of allo-isoleucine, 5 µL of Sulfosalicylic Acid (provides norleucine), and 365 µL water in a tube.
2. Vortex to mix, then spin.
3. Transfer the mixture to an autosampler vial.
4. Perform the Physiological Sample Assay.
5. Review the report. Optimal peak separation yields four distinct peaks with a minimum of 0.2 min (12 sec) from peak to peak, see Figure B-5.
Figure B-5  Representative total ion chromatograph showing optimal peak separation.

<table>
<thead>
<tr>
<th>Item</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>allo-Ile</td>
</tr>
<tr>
<td>2</td>
<td>Ile</td>
</tr>
<tr>
<td>3</td>
<td>Leu</td>
</tr>
<tr>
<td>4</td>
<td>Nle</td>
</tr>
</tbody>
</table>
Column Maintenance

This appendix covers

- Maintaining the HPLC column on page 39

Maintaining the HPLC column

Tip! Review Prepare the mobile phases on page 26.

Wash the column

Before storing the Amino Acid Analyzer (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® Software Home page (Figure 3-4 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.

Equilibrate before reuse

Tip! 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 Analyzer (AAA) C18 Column and the recommended Mobile phases A and B, see Prepare the mobile phases on page 26.
2. Flush the HPLC system.
3. Perform the system suitability test at least three times. Repeat until the retention times stabilize.
This appendix covers

- MRM overview on page 41
- Developing an acquisition method for non-supported instruments on page 41

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

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

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

Developing an acquisition method for non-supported instruments

The values in Table D-8 through Table D-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 D-8 provides the recommended LC gradient.

Table D-8 Recommended LC gradient for the assay

<table>
<thead>
<tr>
<th>Total Time (min)</th>
<th>%Mobile Phase A</th>
<th>%Mobile Phase B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>98</td>
<td>2</td>
</tr>
<tr>
<td>6.0</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>10.0</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>11.0</td>
<td>10</td>
<td>90</td>
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</table>
Table D-8 Recommended LC gradient for the assay (Continued)

<table>
<thead>
<tr>
<th>Total Time (min)</th>
<th>%Mobile Phase A</th>
<th>%Mobile Phase B</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.0</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>13.0</td>
<td>98</td>
<td>2</td>
</tr>
<tr>
<td>18.0</td>
<td>98</td>
<td>2</td>
</tr>
</tbody>
</table>

**TIS values**

Table D-9 shows the TurboIonSpray® (TIS) source Source/Gas and Compound values.

Table D-9 Recommended TIS values

<table>
<thead>
<tr>
<th>Gas or compound</th>
<th>LC/MS/MS Systems</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>API 3200™</td>
</tr>
<tr>
<td>TurboIonSpray® source/gas values</td>
<td></td>
</tr>
<tr>
<td>CUR</td>
<td>20</td>
</tr>
<tr>
<td>CAD</td>
<td>3</td>
</tr>
<tr>
<td>IS</td>
<td>1500</td>
</tr>
<tr>
<td>TEM</td>
<td>600</td>
</tr>
<tr>
<td>GS 1</td>
<td>60</td>
</tr>
<tr>
<td>GS 2</td>
<td>60</td>
</tr>
<tr>
<td>ihe</td>
<td>On</td>
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</table>

<table>
<thead>
<tr>
<th>Compound values</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP</td>
</tr>
<tr>
<td>FP</td>
</tr>
<tr>
<td>EP</td>
</tr>
<tr>
<td>CE†</td>
</tr>
<tr>
<td>CXP</td>
</tr>
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</table>

†. The CE value for allo-isoleucine (Q1 mass <150) is 18 and the CE value for Asa, Hly, Orn, Cth, Cys, Lys, and Hcy (Q1 mass >400) is 50.

**MRM values**

See Table D-10 for the Q1 (precursor ion) and Q3 (product ion) masses.

Table D-10 MRM transitions for the amino acids

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Abbreviation</th>
<th>Q1/Q3 Mass (amu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-Phospho-L-serine</td>
<td>PSer</td>
<td>IS 326.1/113.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Analyte 334.1/121.1</td>
</tr>
<tr>
<td>O-Phosphoethanolamine</td>
<td>PETN</td>
<td>IS 282.1/113.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Analyte 290.1/121.1</td>
</tr>
</tbody>
</table>
Table D-10  MRM transitions for the amino acids  (Continued)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Abbreviation</th>
<th>Q1/Q3 Mass (amu)</th>
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</thead>
<tbody>
<tr>
<td>Taurine</td>
<td>Tau</td>
<td>IS 266.1/113.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Analyte 274.1/121.1</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>Asn</td>
<td>IS 273.2/113.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Analyte 281.2/121.1</td>
</tr>
<tr>
<td>L-Serine</td>
<td>Ser</td>
<td>IS 246.2/113.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Analyte 254.2/121.1</td>
</tr>
<tr>
<td>Hydroxy-L-proline</td>
<td>Hyp</td>
<td>IS 272.1/113.1</td>
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<tr>
<td></td>
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<tr>
<td>Glycine</td>
<td>Gly</td>
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</tr>
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<td>Analyte 224.1/121.1</td>
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<td>L-Glutamine</td>
<td>Gln</td>
<td>IS 287.2/113.1</td>
</tr>
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<td></td>
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<tr>
<td>Ethanolamine</td>
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<td></td>
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<td>L-Aspartic acid</td>
<td>Asp</td>
<td>IS 274.1/113.1</td>
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<td></td>
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</tr>
<tr>
<td>L-Citrulline</td>
<td>Cit</td>
<td>IS 316.2/113.1</td>
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<td></td>
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<tr>
<td>Sarcosine β-Alanine</td>
<td>Sar</td>
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<td>L-Alanine</td>
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<td>1-Methyl-L-histidine</td>
<td>1Mhis</td>
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<td>3-Methyl-L-histidine</td>
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<td>L-Homocitrulline</td>
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<td>Argininosuccinic acid</td>
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<td></td>
<td>Analyte 431.2/131.1</td>
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<tr>
<td>γ-Amino-n-butyric acid</td>
<td>GABA</td>
<td>IS 244.2/113.1</td>
</tr>
<tr>
<td>D, L-β-Aminoisobutyric acid</td>
<td>bAid</td>
<td>Analyte 252.2/121.1</td>
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<td>L-α-Amino-n-butyric acid</td>
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<td>L-Anserine</td>
<td>Ans</td>
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<td>Analyte 375.2/121.1</td>
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Table D-10 MRM transitions for the amino acids (Continued)

<table>
<thead>
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<th>Amino Acid</th>
<th>Abbreviation</th>
<th>Q1/Q3 Mass (amu)</th>
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<tbody>
<tr>
<td>L-Proline</td>
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<td>Hyl</td>
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<td>Cys</td>
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<tr>
<td>L-Leucine</td>
<td>Leu</td>
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<td>L-Norleucine</td>
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</tr>
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<tr>
<td>Unlabeled L-Isoleucine</td>
<td>uIle</td>
<td>IS (uNle) 131.2/86.1</td>
</tr>
<tr>
<td>Unlabeled L-Leucine</td>
<td>uLeu</td>
<td>IS (uNle) 131.2/86.1</td>
</tr>
<tr>
<td>Unlabeled L-Norleucine</td>
<td>uNle</td>
<td>IS (uNle) 131.2/86.1</td>
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</table>