

Table 3. Preparation of High-Sensitivity Amino Acid Standard Solutions. Prepare the three high-sensitivity standards by mixing together stock solutions in the volumes shown.

	Concentration of Final AA Solutions (pmol/ μ L)		
	90	22.5	9
Take 5 mL 1.8 nmol EAA	5 mL	5 mL	5 mL
Dilute with 0.1 N HCl	--	15 mL	45 mL
Diluted EAA mix	5 mL	20 mL	50 mL
Take 5 mL diluted EAA mix	5 mL	5 mL	5 mL
Add 1 nmol ISTD solution	5 mL	5 mL	5 mL
EAA-ISTD mix	10 mL	10 mL	10 mL
Take 100 μ L EAA-ISTD mix	100 μ L	100 μ L	100 μ L
Add 100 pmol AA standard	900 μ L	--	--
Add 25 pmol AA standard	--	900 μ L	--
Add 10 pmol AA standard	--	--	900 μ L
Final AA Solution with EAA and 50 pmol/μL ISTD	1 mL	1 mL	1 mL

Additional Support

For additional technical information, Eclipse AAA Technical Note (Publication Number 5980-1193) is available on the Web at www.agilent.com/chem, or from Agilent technical support at 1-800-227-9770 (U.S. and Canada, or from your local Agilent support organization).

TYPICAL SEPARATIONS

High-Resolution Separation:

(4.6 mm \times 150 mm, 5 μ m or 3.5 μ m column, PN 993400-902, 963400-902)

When more resolution is desired than is available from the high-throughput separation on a 75-mm column (Figure 1), a 150-mm column length should be used. Figure 4 shows a typical routine-sensitivity, high-resolution separation obtained from two different ZORBAX Eclipse AAA 150-mm length columns – one with 3.5- μ m particles and the other with 5- μ m particles. The chromatograms show the 338-nm UV signal that detects the OPA-derivatized primary amino acids. These separations are quite similar in appearance, but the chromatogram for the 3.5- μ m column demonstrates higher resolution resulting from higher efficiency. Back pressure on the column containing the smaller, 3.5- μ m particles is 240 to 300 bar (3,530 to 4,410 psi), while the back pressure of the column having 5 μ m particles is 160 to 210 bar (2,350 to 3,090 psi). Thus, if only primary amino acids are of interest and the 15-cm column length is used, the larger 5- μ m packing is a better choice because of less system back pressure.

Figure 5B shows the analysis using fluorescence detection. Note the difference in resolution between peaks #21 (lysine) and #22 (hydroxyproline) in Figures 5 and 6. The increase in resolution between these peaks, when using the longer column with smaller particles (150 mm, 3.5 μ m),

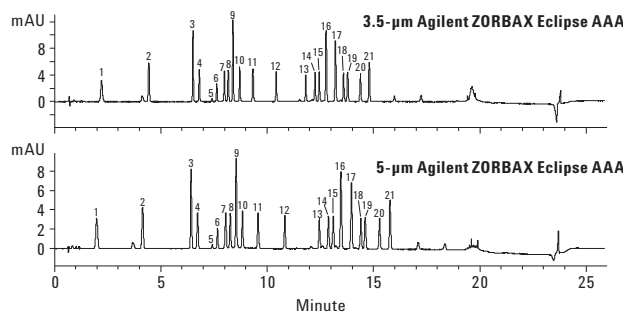


Figure 4. High-resolution analysis of 21 amino acids on the 5- μ m and 3.5- μ m ZORBAX Eclipse AAA column. Column dimensions are 4.6 mm \times 150 mm. See Table 1 for peak identification. Detection: 338 nm (OPA amino acids).

provides a longer time window that facilitates wavelength switching of the DAD or FLD between peaks #21 and #22.

When monitoring at 262 nm (Fig. 5B), a small baseline "hump" elutes between 7 and 10 minutes due to derivatization by-products. Since only the primary AAs are monitored (338 nm) during this time, the "hump" has no impact on their detection or resolution. It is best to monitor at two wavelengths for detection of secondary amino acids such as hydroxyproline. If this is not desirable, wavelength switching can be used.

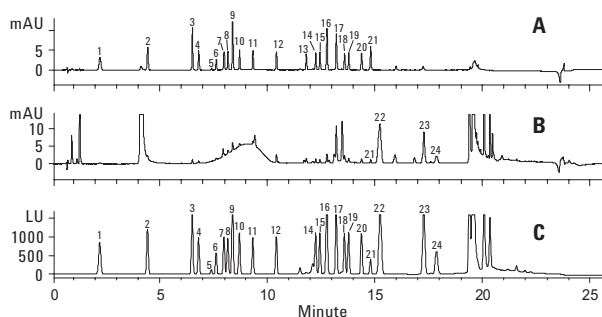


Figure 5. High-sensitivity, high-resolution analysis of amino acids using different detection modes and the Agilent ZORBAX Eclipse AAA protocol. The column dimensions are 4.6 mm \times 150 mm, 3.5 μ m. See Table 1 for peak identification. Detection: A. 338 nm (OPA amino acids), B. 262 nm (FMOC amino acids), C. fluorescence (See Experimental Conditions)

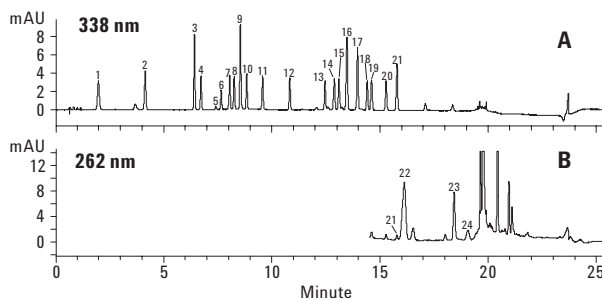


Figure 6. Routine analysis, high resolution of 24 amino acids using the Agilent ZORBAX Eclipse AAA Protocol. The column dimensions are 4.6 mm \times 150 mm, 5 μ m. See Table 1 for peak identification. Detection: A. 338 nm (OPA amino acids), B. 262 nm (FMOC amino acids).

The specific time to switch fluorescence (or UV) wavelengths may differ due to minor variations in temperature, mobile phase, etc. In Figure 5C the FLD signal monitored at 450 nm (Ex = 340 nm), is programmed to change to 305 nm (Ex = 266 nm) after peak #21 (lysine) elutes but before peak #22 (hydroxyproline) elutes. In this case, the switch was programmed to occur at exactly 15 minutes.

Lysine-Hydroxyproline Separation and Wavelength Switching:

Analysis of lysine and hydroxyproline has a major impact on the choice of detection parameters and column configuration, as well as the resulting run time. Amino acids eluting prior to hydroxyproline (up to and including lysine) are derivatized with OPA and are detected at 338 nm. Hydroxyproline elutes immediately after lysine and is the first FMOC-derivatized amino acid to elute: detection must be at 262 nm. The simplest solution is continuous collection of 338-nm and 262-nm data in two separate signals using the Agilent 1100/1200 DAD or MWD.

If the DAD and MWD are not available, the wavelength collected in a single channel can be switched under carefully chosen conditions, for detection of both OPA- and FMOC-derivatized amino acids. Collection of data in a single channel may be necessary, for instance, when using the Agilent 1100/1200 VWD. Increased resolution between lysine and hydroxyproline is possible when using more-complex gradient profiles.

When hydroxyproline is not of interest in the sample (for example, in analysis of protein hydrolysates), it is possible to use any of the column configurations and switch wavelengths at a time between lysine elution and elution of the first FMOC amino acid (sarcosine or proline). In this scenario, the 4.6 mm \times 75 mm column size is adequate and has the advantage of half the analysis time.

ORDERING INFORMATION

Eclipse AAA HPLC Columns

Description	Size (mm)	Particle Size (μ m)	Part Number
Analytical routine sensitivity, high resolution	4.6 \times 150	5	993400-902
Analytical high-sensitivity, high-resolution work using the FLD	4.6 \times 150	3.5	963400-902
Analytical, routine sensitivity, high throughput	4.6 \times 75	3.5	966400-902
Analytical high-sensitivity, high-resolution work – DAD or FLD	3.0 \times 150	3.5	961400-302
Guard (4/pk)	4.6 \times 12.5	5	820950-931
Guard hardware kit			820888-901

Derivatization Reagents

Description	Part Number
Borate buffer, 0.4 M in water, pH 10.2, 100 mL	5061-3339
FMOC reagent, 2.5 mg/mL in ACN, 1 mL	5061-3337
OPA reagent, 10 mg/mL in 0.4 M borate buffer and 3 mercaptoproprionic acid, 6 \times 1 mL ampoules	5061-3335
DTDPA reagent for analysis of cysteine, 5g	5062-2479

Vials

Description	Part Number
100- μ L conical insert with polymer feet, 100/pk	5181-1270
Amber, wide-opening, write-on, screw-top vial, 2 mL, 100/pk	5182-0716
Green screw cap, PTFE/silicone septum, 100/pk	5182-0721

Standards

Description	Part Number
Amino acid standard in 0.1 N HCl, 10 \times 1 mL ampoules	5061-3330
1 nmol/ μ L	5061-3331
250 pmol/ μ L	5061-3332
100 pmol/ μ L	5061-3333
25 pmol/ μ L	5061-3334
10 pmol/ μ L	5062-2478
Supplemental amino acids, NVA, SAR, ASN, GLN, TRP, and HYP, 1 g each	

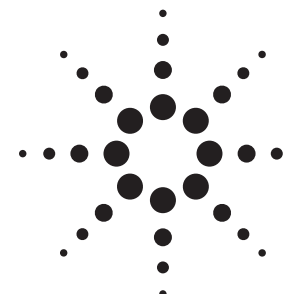
For more information on our products and services, visit our Web site at www.agilent.com/chem.

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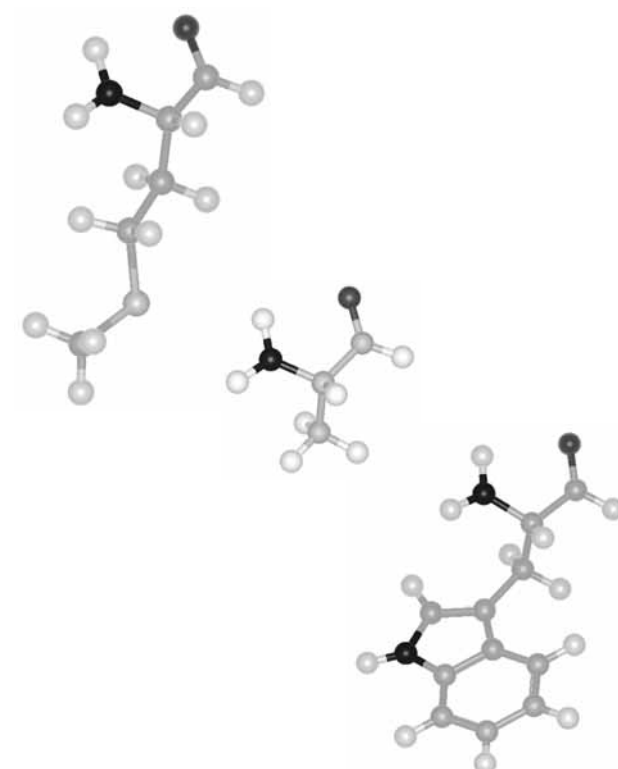
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Agilent ZORBAX Eclipse AAA Instructions for Use



INTRODUCTION

An ideal, quantitative amino acid analysis combines speed and sensitivity with reliability of both the derivatization reaction and the analytical technique. These goals are achieved with automated, online derivatization using o-phthalaldehyde (OPA) for primary amino acids and 9-fluorenylmethyl chloroformate (FMOC) for secondary amino acids; the automated derivatization is then integrated with rugged HPLC analysis using the Agilent 1100/1200 HPLC.

The Agilent ZORBAX Eclipse AAA column contains batch-qualified reversed-phase material. When used according to the protocol described above, the column enables the user to separate the amino acids commonly found in protein/peptide hydrolysates.

The A and B mobile-phase components are easy to prepare, and the gradient consists of linear segments. This combination contributes to a rugged protocol that can be accomplished on the Agilent 1100/1200 HPLC using either binary or quaternary solvent-delivery systems.

High-Throughput Routine Analysis: (4.6 mm × 75 mm, 3.5 µm column, PN 966400-902)
The chromatogram in Figure 1 illustrates typical routine sensitivity in high-throughput applications that can be obtained on the Agilent 1100/1200 HPLC binary system using the 1100/1200 DAD. A single run can be completed in 14 minutes (including re-equilibration) with adequate resolution. The primary amino acids (OPA-derivatized), shown in Figure 1A, are monitored at 338 nm while the secondary amino acids (FMOC-derivatized), shown in Figure 1B, are monitored at 262 nm. The amount injected of each amino acid was 125 pmoles in 0.5µL. See chromatograms in Figures 4 to 6 for high-resolution separations and details of lysine/hydroxyproline separation and wavelength switching.

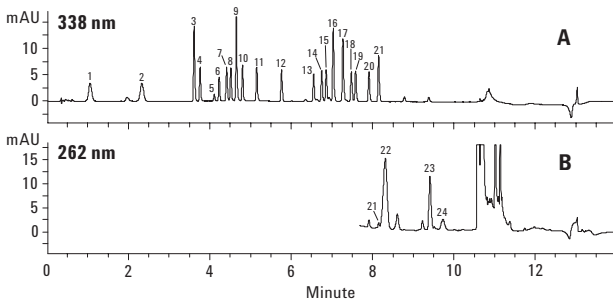


Figure 1. Routine analysis, high-throughput separation of 24 amino acids using the Agilent ZORBAX Eclipse AAA Protocol. The column dimensions are 4.6 mm × 75 mm, 3.5 µm. See Table 1 for peak identification. Detection: A. 338 nm (OPA amino acids), B. 262 nm (FMOC-amino acids).

Table 1. Amino Acid Elution Order Using Eclipse AAA Protocol

Peak No.	Amino Acid	Abbr.	Peak No.	Amino Acid	Abbr.
1	Aspartate	ASP	13	Cystine	CY2
2	Glutamate	GLU	14	Valine	VAL
3	Asparagine	ASN	15	Methionine	MET
4	Serine	SER	16	Norvaline	NVA
5	Glutamine	GLN	17	Tryptophan	TRP
6	Histidine	HIS	18	Phenylalanine	PHE
7	Glycine	GLY	19	Isoleucine	ILE
8	Threonine	THR	20	Leucine	LEU
9	Citrulline	CIT	21	Lysine	LYS
10	Arginine	ARG	22	Hydroxyproline	HYP
11	Alanine	ALA	23	Sarcosine	SAR
12	Tyrosine	TYR	24	Proline	PRO

EXPERIMENTAL CONDITIONS

Instrument

The recommended chromatographic system is the Agilent 1100 HPLC: G1312A binary pump with G1315A Diode Array Detector (DAD), 6-mm or 10-mm flow cell, and/or G1315A Fluorescence Detector (FLD). While the results shown here were obtained with the binary pump, this procedure has also been used with the Agilent 1100 quaternary pump (G1311A). The same procedure can be used on the Agilent 1200 HPLC with only minor adjustments. Using the Agilent 1200 RRLC may require more adjustments.

ZORBAX Eclipse AAA	ZORBAX Eclipse AAA	ZORBAX Eclipse AAA
4.6 mm × 75 mm, 3.5 µm	4.6 mm × 150 mm, 3.5 µm	3.0 mm × 150 mm, 3.5 µm
Agilent PN 966400-902	Agilent PN 963400-902	Agilent PN 961400-302

Optional guard column	ZORBAX Eclipse AAA
ZORBAX Eclipse AAA	4.6 mm × 150 mm, 5 µm
4.6 mm × 12.5 mm, 5 µm, 4/PK	Agilent PN 993400-902
Agilent PN 820950-931	
For use with all 4.6 mm ID columns	

Mobile Phase

A: 40 mM NaH₂PO₄ pH 7.8 [5.5 g NaH₂PO₄ monohydrate + 1 liter water, adjust to pH 7.8 with NaOH solution (10 N)]

B: ACN: MeOH: water (45:45:10, v/v/v)

It is convenient to make Mobile Phase A as a 10X stock solution with no pH adjustment; the buffer should be filtered and the concentrated solution refrigerated. The solution can be kept for several weeks and can be diluted and titrated to pH 7.8, as needed. All mobile-phase solvents should be HPLC grade.

Pump Settings

Flow:	2 mL/min
Stop time:	14 min (75-mm column) or 26 min (150-mm column)
Post time:	Off

Auxiliary Pump Settings:

Max. flow ramp:	100 mL/min
Compressibility A:	50 × 10 ⁻⁶
Minimal Stroke A:	20 µL
Compressibility B:	115 × 10 ⁻⁶
Minimal Stroke B:	Auto

Gradients:

For 75 mm column length		For 150 mm column length	
Time (min)	% B	Time (min)	% B
0	0	0	0
1	0	1.9	0
9.8	57	18.1	57
10	100	18.6	100
12	100	22.3	100
12.5	0	23.2	0
14	0	26	0

Note: To extend column life, flush column with 10 column volumes of 100% B when column will not be used for periods of overnight or longer.

Detector Settings

DAD:

Required Lamps:	
UV lamp:	Yes
Vis. lamp:	No
UV:	338 nm, 10 nm bandwidth (bw), reference: 390 nm, 20 nm bw (for OPA-amino acids) 262 nm, 16 nm bw, reference: 324 nm, 8 nm bw (for FMOC-amino acids)
Peak width:	> 0.03 min (0.5 sec)
Slit:	4 nm

FLD:

For 75 mm column			For 150 mm column		
Time (min)	Ex/Em (nm)	PMT Gain	Time (min)	Ex/Em (nm)	PMT Gain
0	340/450	10	0	340/450	10
8.5*	266/305	9	15*	266/305	9

*The specific time to switch fluorescence wavelengths may differ due to variations in temperature, mobile phase, etc.

Peak width: > 0.5 min

Autosampler:

See vial positioning (Figure 2) of a G1313A. Place vials in a similar arrangement for the G1367 or G1329 depending on the installed tray.

Injector program:

Draw 2.5 µL from vial 1 (borate buffer)
Draw 0.5 µL from sample (for example, choose vial position #11 for amino acid sample), mix "in washport" if using G1367A/C autosampler
Mix 3 µL "in air," max speed, 2x, mix "in washport" if using G1367A/C autosampler
Wait 0.5 min
Draw 0 µL from vial 2 (needle wash using water in uncapped vial)
Draw 0.5 µL from vial 3 (OPA)
Mix 3.5 µL "in air," max speed, 6x, mix "in washport" if using G1367A/C autosampler
Draw 0 µL from vial 2 (needle wash using water in uncapped vial)
Draw 0.5 µL from vial 4 (FMOC)
Mix 4 µL "in air," max speed, 6x, mix "in washport" if using G1367A/C autosampler [Optional needle rinse for high-sensitivity use: Draw 0.0 µL from vial 6 (ACN)]
Draw 32 µL from vial 5 (water)
Mix 18 µL "in air," max speed, 2x, mix "in washport" if using G1367A/C autosampler
Inject

Auxiliary:

Draw speed:	200 µL/min
Eject speed:	600 µL/min
Draw position:	0.0 mm

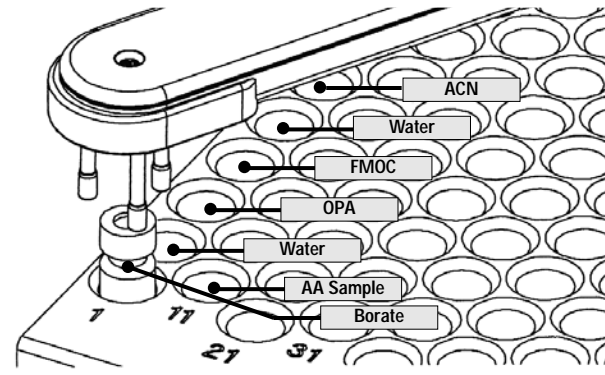


Figure 2. Position of reagent vials in the Agilent 1313A autosampler. This positioning of vials is designed for the listed injector program.

HPLC Columns

Vials:

Conical vial inserts with polymer feet (Fig. 3A) are required to hold the OPA and FMOC reagents because of the limited volumes involved. The inserts are compatible with wide-opening screw-top (Figs. 3B and 3C) or crimp-top vials. For this procedure snap-cap vials should not be used because an airtight seal is needed for both FMOC, because it is highly volatile, and OPA, because it slowly degrades in the presence of oxygen. Be careful not to use vials or caps designed for other instruments, as these may damage the Agilent G1313A autoinjector.

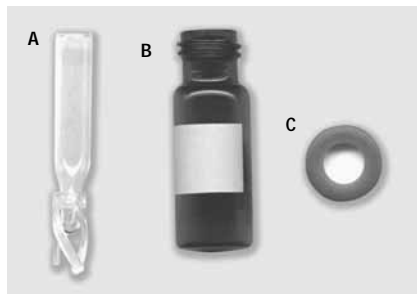


Figure 3. Insert, vial, and cap. Photo of conical insert A (Agilent PN 5181-1270), amber wide-opening vial B (Agilent PN 5182-0716), and screw cap C (Agilent PN 5182-0721), for amino acid analysis using the Agilent 1100 autosampler.

Column Compartment:

Temperature:

40 °C (left and right side)

Enable analysis: When temperature is within setpoint ± 0.8 °C

Derivatization Reagents

Borate Buffer:

Agilent PN 5061-3339

Solution of 0.4 N in water, pH 10.2. Refrigerate (4 °C) and dispense as necessary.

FMOC Reagent:

Agilent PN 5061-3337

Pipette 100-µL aliquots of the 1-mL FMOC reagent into conical inserts, cap immediately, and refrigerate (4 °C); solution is useable for 7 to 10 days, maximum after dispensing.

OPA Reagent:

Agilent PN 5061-3335

Pipette 100-µL aliquots of the 1-mL OPA reagent into conical inserts, cap immediately and refrigerate (4 °C); solution is useable for 7 to 10 days, maximum, after dispensing.

Water: Deionized, HPLC grade

See Ordering Information for descriptions and part numbers.

SAMPLE PREPARATION

Note: Each reagent vial should be replaced every day. Each 1 mL ampoule of reagent can last about 10 days (100 µL per day).

Amino Acid Mix for Chromatographic Comparisons

For chromatographic analyses, 17 amino acids from the 250 pmol/µL standard mix (PN 5061-3331), plus citrulline and the six supplemental amino acids were combined at a concentration of approximately 250 pmol/µL. The mixture was prepared by combining the two stock solutions described below. Add 1 µL of the supplemental amino acid stock solution to a fresh aliquot of the 250-pmol standard (100 µL) in the conical vial insert. Mix using a vortex mixer to complete the 24-component standard ready for injection (250 pmol/µL in water/MeOH).

250-pmol standard:

Divide 1-mL ampoule of 250 pmol/µL amino acids (PN 5061-3331) into 100-µL portions in conical vial inserts, cap, and refrigerate aliquots at 4 °C.

Supplemental amino acid stock solution:

Weigh about 0.25 mmoles of each auxiliary amino acid (GLN, ASN, TRP, NVA, HYP, and SAR) from kit (PN 5062-2478) into a 20-mL vial. Add 5 mL deionized water and ultrasonicate in a hot water bath until dissolved. Add another 5 mL water to complete dilution. Store in refrigerator (4 °C). Citrulline (Sigma-Aldrich Co., St. Louis, MO) was added in this mix at the same concentration.

For storage, do not combine supplemental amino acids with amino acid standards. Some of these supplemental amino acids degrade in HCl (especially glutamine, and to a lesser extent, asparagine).

Amino Acid Mix for Calibration Curves

For the construction of calibration curves, 17 amino acids, plus the four extended amino acids, are combined at various concentrations with fixed amounts of internal standards (ISTDs). The ISTDs (norvaline and sarcosine) are part of the supplemental amino acid kit (PN 5062-2478). The remaining amino acids in this kit (GLN, ASN, TRP, and HYP) form the extended amino acids (EAA). To make the appropriate solutions, refer to Tables 2 and 3 for low- and high-sensitivity standards, respectively.

Amino acid standards (10 pmol/µL to 1 nmol/µL):

Divide each 1-mL ampoule of standards PN 5061-3334 through 5061-3334 into 100-µL portions in conical vial inserts, cap, and refrigerate aliquots at 4 °C. Calibration curves may be made using from two to five standards depending on experimental need.

Extended amino acid (EAA) stock solution:

This solution is made using four of the six amino acids in the supplemental amino acid kit (PN 5062-2478). For use with low-sensitivity standards (Table 2), make a 25-mL solution containing 18 nmol/µL of glutamine, asparagine, tryptophan, and 4-hydroxy-proline in deionized water. Sonicate the solution until dissolved. Store the solution refrigerated at 4 °C. For use with high-sensitivity standards (Table 3), make a 1.8-nmol/µL solution by diluting 5 mL of the 18-nmol/µL standard with 45 mL deionized H₂O.

Internal standards (ISTD) stock solution:

These solutions are made using two of the six amino acids in the supplemental amino acid kit (PN 5062-2478). For use with low-sensitivity standards (Table 2), make a 25-mL solution containing 10 nmol/µL of norvaline and sarcosine in deionized water. Sonicate the solution until dissolved. Store in refrigerator (4 °C). For use with high-sensitivity standards (Table 3), make a 1-nmol/µL solution by diluting 5 mL of the 10-nmol/µL standard with 45 mL deionized H₂O. Store in refrigerator (4 °C).

Table 2. Preparation of Low-Sensitivity Amino Acid Standard Solutions. Prepare the three low-sensitivity standards by mixing together stock solutions in the volumes shown.

	Concentration of Final AA Solutions (pmol/µL)		
	900	225	90
Take 5 mL 18 nmol EAA	5 mL	5 mL	5 mL
Dilute with 0.1 N HCl	--	15 mL	45 mL
Diluted EAA mix	5 mL	20 mL	50 mL
Take 5 mL diluted EAA mix	5 mL	5 mL	5 mL
Add 1 nmol ISTD solution	5 mL	5 mL	5 mL
EAA-ISTD mix	10 mL	10 mL	10 mL
Take 100 µL EAA-ISTD mix	100 µL	100 µL	100 µL
Add 1000 pmol AA standard	900 µL	--	--
Add 250 pmol AA standard	--	900 µL	--
Add 100 pmol AA standard	--	--	900 µL
Final AA Solution with EAA and 500 pmol/µL ISTD	1 mL	1 mL	1 mL