



## Laboratory Procedure Manual

Analyte: N-terminal hemoglobin adducts of  
Acrylamide, Glycidamide, and Ethylene  
Oxide

Matrix: Red Blood Cells

Method: Liquid Chromatography Tandem Mass  
Spectrometry

Method No: 1015

Revised:

as performed by:

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### Important Information for Users

CDC periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

## Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table:

<b>File Name</b>	<b>Variable Name</b>	<b>SAS Label</b>
ETHOX_K	LBXEOA	Ethylene Oxide (pmol/g)

# 1 SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

## 1.1 Clinical and Public Health Relevance

Acrylamide (AA) has been identified as neurotoxic (1,2), mutagenic (3), and possibly genotoxic (4) to animals and humans. It is classified as a probable carcinogen to humans by the International Agency for Research on Cancer (IARC) and as a potential occupational carcinogen by the Occupational Safety and Health Administration (OSHA). People are exposed to AA through certain occupational activities that involve the production and use of AA, as well as through tobacco smoke (5-8) and dry-heated food (9-12). Actual exposure to AA in the general population and possible changes in this exposure over time are not known.

Glycidamide (GA), the primary metabolite of AA, has a higher reactivity towards nucleophilic reagents than AA. Results from animal studies suggest that genetic damage in somatic and germ cells is dependent upon the metabolism of AA to GA by CYP2E1 (13-15). To obtain comprehensive information about AA exposure and to assess potential health effects related to this exposure, it is necessary to measure both AA and GA exposure.

Ethylene oxide (EO) has been detected in exogenous sources, such as tobacco smoke (16), automobile exhaust, and some food. EO is also formed endogenously in animals and humans as a result of CYP2E1 mediated metabolic oxidation of ethylene. It is formed in vivo during normal physiological processes, such as methionine oxidation, lipid peroxidation, and via the metabolic activity of intestinal bacteria (17). EO has been classified as a Group 1 human carcinogen by the IARC (18). Information on endogenous and exogenous EO exposure in the general population is very limited.

Information on exposure to these chemicals in the general population is needed to assess the potential health effects associated with this exposure and to monitor changes in exposure over time.

## 1.2 Test Principle

This procedure describes a method to measure hemoglobin (Hb) adducts of AA, GA, and EO in human erythrocytes. Specifically, it measures the adduct of these chemicals at the N-terminal valine of the Hb protein chains (N-[2-carbamoyl-ethyl]valine, N-[2-hydroxycarbamoyl-ethyl]valine, and N-[2-hydroxyethyl]valine for AA, GA, and EO adducts, respectively).

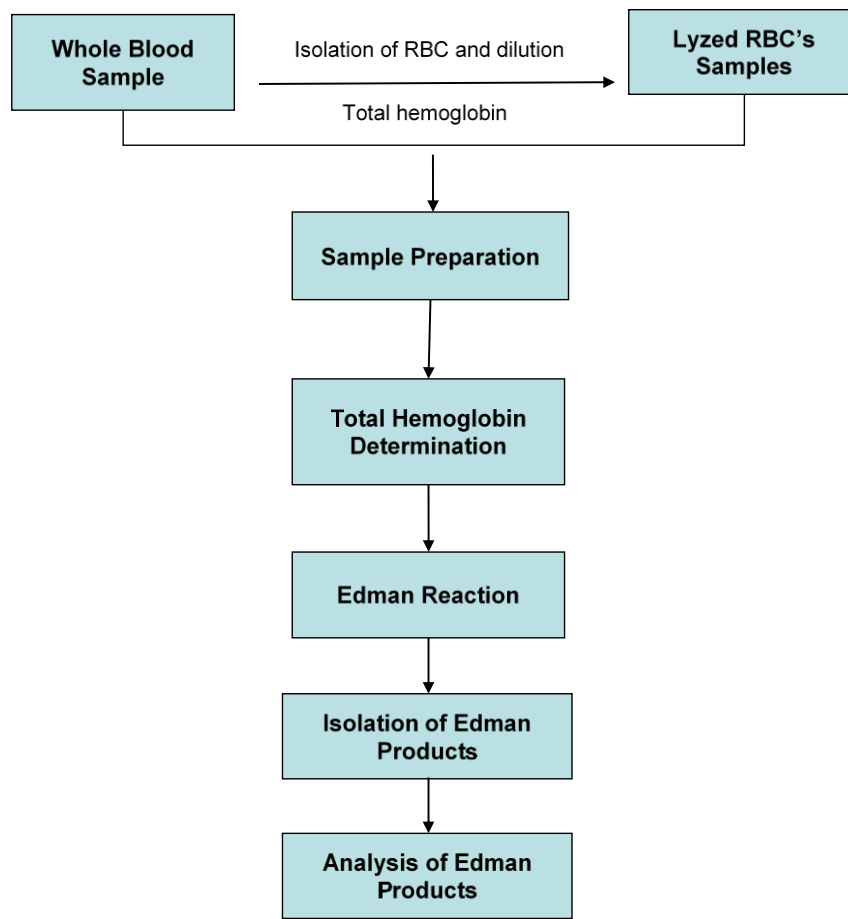
This method utilizes a modified Edman reaction, in which the N-alkylated amino acid forms Edman products in neutral or alkaline conditions, rather than changing the pH to acidic conditions required in a conventional Edman reaction (19). The reaction principle was first described for N-terminal Hb adducts of EO, propylene oxide, and styrene oxide (20), but it was later optimized to increase the yield of Edman products of the targeted adducts (21). This optimized method was then successfully applied to adducts produced from other

chemicals such as AA, GA, and acrylonitrile (ACN) (22-25). The method was further refined and modified in-house to increase sensitivity and enable automation (26,27).

The procedure described here consists of 5 parts (Scheme 1):

- Specimen preparation
- Measurement of total Hb in sample solutions
- Modified Edman reaction
- Isolation of Edman products
- Analysis of Edman products by High Performance Liquid Chromatography/Tandem Mass Spectrometry (HPLC-MS/MS)

The results of this procedure are reported in pmol of adduct per gram of total Hb present in the sample, so the amount of Hb used for the modified Edman reaction is required. Therefore, this procedure includes a measurement procedure for total Hb. Total Hb is calculated using a commercial assay kit based on a well-established procedure that is commonly used in clinical chemistry (28). Quantitation of AA, GA, and EO Hb adducts is performed using synthesized octapeptides with the same amino acid sequence as the N-terminal of the Hb beta-chain but with AA, GA, or EO attached at the valine.



**Scheme 1:** Measurement Procedure for AA, GA, and EO Adducts in Red Blood Cells

## 1.3 Scope

The procedure described in this document is intended to quantitatively measure N-terminal AA, GA, and EO Hb adducts in red blood cells (RBC). It addresses all aspects related to the process, such as specimen collection, storage, processing, analysis, and reporting.

Specific details relating to equipment maintenance and operation can be found in the manufacturers' manuals. Additional details are located in designated work instructions created and maintained by the Protein Biomarker Laboratory (PBL). This document is not intended to provide information on data interpretation.

This method is intended for obtaining information about human exposure to these chemicals. It is not intended to assess disease risk or for diagnosing certain diseases.

This method was developed for measuring Hb adducts in humans and may not be suitable for measuring Hb adducts in animals.

## 2 SAFETY PRECAUTIONS

### 2.1 General Safety

All blood specimens should be considered potentially positive for bloodborne pathogens, including, but not limited to, Human Immunodeficiency Virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV). HBV vaccination series is recommended for all analysts performing this measurement procedure.

Universal precautions must be observed by using the appropriate personal protective equipment (PPE). Proper PPE includes protective gloves, laboratory coats, and safety glasses that must be worn at all times during all this procedure. Additional information can be found in the laboratory Chemical Hygiene Plan (CHP).

Disposable bench covers must be used during sample preparation and handling, and they must be discarded after use. All work surfaces must be wiped with 10% bleach solution after work is finished.

Extra caution is required when removing external screw caps of sample cryovials. Always place a Kimwipe over the screw cap to prevent any blood exposure (a face shield may also be worn).

### 2.2 Chemical Hazards

All acids, bases, organic solvents, and other reagents used in this procedure must be handled with extreme care; they are caustic, flammable, and toxic. They must be handled in a well-ventilated area or, as required, inside a chemical fume hood.

Glacial Acetic Acid: Do not inhale vapor. Flammable liquid and its vapors are corrosive. Inhalation may cause lung and tooth damage. Liquid and mist cause severe burns to all body tissue and may be fatal if swallowed.

Ethyl Acetate: Do not inhale vapor. Flammable liquid and its vapors are corrosive. Inhalation may cause central nervous system depression and eye irritation. It may also cause skin irritation and liver and kidney damage. May cause respiratory tract irritation and may be harmful if inhaled.

Isopropyl Ether: Do not inhale vapor. Forms explosive peroxides upon prolonged storage. Keep container in well ventilated location.

Pentafluorophenyl Isothiocyanate: Do not inhale vapor. Handle only in well-ventilated areas. Do not get in eyes, on skin or on clothing. This chemical is also referred to as the Edman reagent.

Formamide: Avoid contact with skin or eyes. Use adequate ventilation. It is toxic to reproduction. Store away from acids.

Methanol: Do not inhale vapor. Flammable and toxic, avoid contact with skin or eyes. Danger of permanent damage through inhalation, eye and skin contact, and if swallowed.

Sodium Hydroxide: Avoid contact with skin or eyes. Use adequate ventilation. Exposure of the eyes may result in permanent damage and dermal contact causes irritation. May cause respiratory tract irritation. Corrosive to aluminum.

Toluene: Do not inhale vapor. Toxic and flammable liquid. Keep container in a cool, well-ventilated area. Avoid contact with skin and eyes. Keep away from heat. Store in a segregated and approved area. Keep container tightly closed and sealed until ready for use. Keep away from incompatible chemicals, such as oxidizing agents.

Hemoglobin Reagent Set: Do not inhale vapor. Use adequate ventilation. May cause irritation of eyes, respiratory system, gastrointestinal system and skin. Contains cyanide.

Nitric Acid: May be fatal if inhaled. Causes severe eye, skin, respiratory, and digestive tract burns. Contact with other materials may cause a fire. Acute pulmonary edema or chronic obstructive lung disease may occur from inhalation of the vapors. It is Corrosive to metal and is a strong oxidizer.

Safety data sheets (SDS) for these chemicals are readily accessible as hard copies in the laboratory. If needed, SDS for other chemicals can be viewed at <http://www.ilpi.com/msds/index.html>.

**CAUTION!** Glacial Acetic Acid, Ethyl Acetate, Isopropyl Ether and Toluene are volatile organic compounds. Wear gloves, safety glasses, lab coat and/or apron, and work only inside a properly operating chemical fume hood. Keep container tightly closed and sealed in the designated flammable cabinet until ready for use.

## 2.3 Radioactive Hazards

There are no radioactive hazards associated with this measurement procedure.

## 2.4 Mechanical Hazards

There are minimal mechanical hazards when performing this procedure using standard safety practices. Analysts must read and follow the manufacturers' information regarding safe operation of equipment. Avoid direct contact with the mechanical and electronic components of any analytical equipment and instrumentation unless all power is in the "off" state. Generally, mechanical and electronic maintenance and repair must only be performed by qualified technicians. Manuals are located in the sample preparation area of the Protein Biomarker Laboratory (PBL).

## 2.5 Waste Disposal

All solid waste generated during sample preparation (e.g., disposable plastic pipette tips, gloves, bench diapers, caps), including residual sample material, must be placed in the appropriate biohazard auto-clavable bags and waste pans until sealed and auto-claved.

All glass pipette tips and any sharps (e.g., broken glass) must be placed in appropriate sharps containers.

All liquid waste must be labeled and processed in accordance with Centers for Disease Control and Prevention (CDC) policies. Waste disposal must be performed in compliance with CDC policies and regulations. A hard copy of the CDC Safety Policies and Practices Manual is located in the laboratory and an electronic copy can be accessed at [http://intranet.cdc.gov/nceh-atsdr/dls/safety\\_manual/](http://intranet.cdc.gov/nceh-atsdr/dls/safety_manual/).

Uncontaminated plastic that is coded 1 through 7 can be recycled. Uncontaminated, clean plastic includes empty pipette tip boxes and inserts and clean plastic bottles from reagents, bleach, and buffers that have not come into contact with biohazardous material.

## 2.6 Training

Analysts performing this procedure must have successfully completed all requested safety trainings.

Additional training requirements include, but are not limited to, documented training on specific instrumentation outlined in this procedure.

Analysts performing this procedure must be familiar with the following:

- Exposure Control Plan
- Chemical Hygiene Plan
- Relevant Safety Data Sheets
- DLS Safety Manual
- DLS Policies and Procedures Manual

## **3 COMPUTERIZATION AND DATA-SYSTEM MANAGEMENT**

### **3.1 Software and Knowledge Requirements**

This procedure requires familiarity with software operated instruments such as Thermo Fisher Scientific LC-MS/MS (using Xcalibur 2.2 version or higher) and Tecan (using Evoware Software version 4.11.5878 or higher). Specific training is required to properly operate these software systems.

Calculations on the data obtained from the LC-MS/MS software are performed via calculation templates created in Microsoft Excel by the analysts after receiving specific training from qualified staff. The calculation results are transferred to a database created and maintained by the Division of Laboratory Sciences (DLS). Assessment of bench quality control (QC) results is performed using a program created with Statistical Analysis System Institute Inc. (SAS) software and maintained by the DLS.

The database activities and QC calculations are performed by specially trained staff.

### **3.2 Sample Information**

All samples must be labeled as described in the DLS Policies and Procedures Manual. Samples must not contain personal identifiers, and all samples are referenced to a blind-coded sample identifier.

### **3.3 Data Maintenance**

Information about samples and related analytical data are reviewed for transcription errors and overall validity prior to being entered into the database. Filing of electronic and physical files is the responsibility of designated staff. The database is maintained by DLS staff and routinely backed up by CDC Information Technology Services Office (ITSO).

### **3.4 Information Security**

Information security is managed at multiple levels. The information management system that contains final reportable results is access-restricted through user identification (ID) and password security. Access to raw and in-process data requires specific knowledge on software manipulation. Site security is provided through restricted access to individual laboratories, buildings, and offices. Participant confidentiality is protected by blind coded sample IDs.



## 4 PREPARATION FOR REAGENTS, CALIBRATION MATERIALS, CONTROL MATERIALS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

### 4.1 Equipment, Chemicals, and Consumables

The chemicals, equipment, and other materials described below or equivalents can be used in this measurement procedure.

#### 4.1.1 Equipment, Chemicals, and Consumables Used for Reagent Preparation

1. HANNA HI pH Meter with Orion Micro-Combination Electrode, pH Range -2-20, Temperature Range -20-120 °C (Hanna Instruments Inc., Woonsocket, RI)
2. 500 mL Glass Beaker (Corning Incorporated, Lowell, MA)
3. 500 mL Pyrex Graduated Glass Cylinder, Tolerance  $\pm 1.4$  mL, (Kimble Chase Life Science and Research Products LLC, Vineland, NJ)
4. 15 mL Plastic Falcon Tubes (KSE, Durham, NC)
5. 50 mL Plastic Falcon Tubes (KSE, Durham, NC)
6. 1L Glass Bottles With Screw Tops (Wheaton Industries Inc., Millville, NJ)
7. 1000  $\mu$ l Pipette (Eppendorf AG, Hamburg, Germany)
8. 200  $\mu$ l Pipette (Eppendorf AG, Hamburg, Germany)
9. Repeater Pipette (Eppendorf, Ramsey, MN)
10. Pasteur Transfer Pipettes (Samco Scientific, San Fernando, CA)
11. Disposable Pasteur Pipets, 5/8" (Fisher Scientific, Suwanee, GA)
12. Octagonal Stirring Bars, 1 Inch Length; 0.312 Inch Diameter (Fisher Scientific, Suwanee, GA)
13. Scholar™ 5 x 5 Inch PC-171 Magnetic Stirrer (Corning Incorporated, Lowell, MA)
14. Milli-Q Water, Resistivity, 18 megaOhm-cm DI Water at 25 ° C, 18.2 (Aqua Solutions, Jasper, GA)
15. Ethyl Acetate, HPLC/ACS Grade (Fisher Scientific, Suwanee, GA)
16. Toluene, HPLC/ACS Grade, (Fisher Scientific, Suwanee, GA)
17. Isopropyl Ether, Certified (Fisher Scientific, Suwanee, GA)
18. Glacial Acetic Acid, Certified ACS Grade, (Fisher Scientific, Suwanee, GA)
19. Methanol, HPLC Grade (Fisher Scientific, Suwanee, GA)
20. Acetic Acid ACS Grade (J. T. Baker, Phillipsburg, NJ)
21. Sodium Hydroxide 0.2N Solution (Fisher Scientific, Suwanee, GA)
22. Pointe Hemoglobin Control (MedTest Dx, Canton, MI)
23. Hemoglobin Reagent Set (Teco Diagnostics, Anaheim, CA)

#### 4.1.2 Equipment, Chemicals, and Consumables Used for Calibration Materials

1. Mettler Toledo XP205 Analytical Balance (Electronic, "0.0000 g", Max 220.0 g, Min 1.4 mg, Mettler Toledo, Columbus, OH)
2. Water Bath- Iso Temp 3016 Regulator Apparatus (Fisher Scientific, Suwanee, GA)
3. 500 mL Pyrex Silanized Volumetric Fasks, Tolerance  $\pm 0.08$  mL, (Kimble Chase Life Science and Research Products LLC, Vineland, NJ)
4. AA Octapeptide (AA-VHLTPEEK), (Certified Concentration with Stated Uncertainty), Peptide Content 71.0%, HPLC Purity 96%, FW=1022.5 g/mol, CAS No: 1608 (Bachem, King of Prussia, PA)
5. Labeled AA Octapeptide [AA-Val(<sup>13</sup>C<sub>5</sub>,<sup>15</sup>N)-HLTPEEK], Peptide Content 72.3%, HPLC Purity 92.5%, FW=1029.09 g/mol CAS No: 1739-B, (Bachem King of Prussia, PA)
6. GA Octapeptide (GA-VHLTPEEK), (Certified Concentration with Stated Uncertainty), Peptide Content 71.0%, HPLC Purity 90.0%, FW=1038.5 g/mol, CAS No: 1660 (Bachem, King of Prussia, PA)
7. Labeled GA Octapeptide [GA-Val(<sup>13</sup>C<sub>5</sub>,<sup>15</sup>N)-HLTPEEK], (Certified Concentration with Stated Uncertainty), Peptide Content 73.0%, HPLC Purity 93.9%, FW=1045.1 g/mol, CAS No: 1740-B (Bachem, King of Prussia, PA)
8. EO Octapeptide (EO-VHLTPEEK), (Certified Concentration with Stated Uncertainty), Peptide Content 98.5%, HPLC Purity 70.3%, FW=995.5 g/mol, CAS No: 4051266 (Bachem, King of Prussia, PA)
9. Labeled EO Octapeptide [EO-Val(<sup>13</sup>C<sub>5</sub>,<sup>15</sup>N)-HLTPEEK], (Certified Concentration with Stated Uncertainty), Peptide Content 72.0%, HPLC Purity 90%, FW=1002.1 g/mol, CAS No: 4051578 (Bachem, King of Prussia, PA)

### 4.1.3 Equipment, Chemicals, and Consumables Used for Sample Processing

1. Centrifuge 5810R, (Eppendorf, Ramsey, MN)
2. MultiPulse Vortexer (Glas-Col, Terre Haute, IN)
3. Repeater Plus Pipetter (Eppendorf, Ramsey, MN)
4. 96 well 2 mL Square Well Plates (Seahorse Labware, Chicopee, MA)
5. Robotic Reservoirs, Convuluted Bottom (Thermo Scientific, Waltham, MA)
6. ArctiSeal 96-well Square Silicone w/ PTFE Spray Coating (Arctic White LLC, Bethlehem, PA)
7. BioChromato 6-well Slit Seal (BioChromato, San Diego, CA)
8. Combitips Plus Pipet Tips, 10 mL (Eppendorf, Ramsey, MN)
9. Orbitron Rotator II, Model 26250, (Boekel Scientific, Feasterville, PA)
10. Swing-Bucket Rotor (Eppendorf, Ramsey, MN)
11. 48-well, 5 mL, Rectangular Well, Pyramid Bottom, Natural Polypropylene Plate (Fisher Scientific, Suwanee, GA)
12. 96-well, Microtiter Plate, Nonbinding Surface (Fisher Scientific, Suwanee, GA)
13. 48-well, 7.5 mL Fritted Plate, with 25 µm PE Frit, Long Drip (Fisher Scientific, Suwanee, GA).
14. 48-well, 7.5 mL, Rectangular Well, Pyramid Bottom, Natural Polypropylene (Fisher Scientific, Suwanee, GA)
15. 96-well, 1 mL, Round Well Collection Plate (Waters Corporation, Milford, MA) (
16. Bulk Isolute Sorbent HM-N (Biotage, Charlottesville, VA)
17. 1000 µl, Conductive Pipetting Tips for Tecan (Molecular Bio Products, San Diego, CA)
18. 200µl, Conductive Pipetting Tips for Tecan (Molecular Bio Products, San Diego, CA)
19. 150uL, filtered MCA96 SBS pipette tips for Tecan (Tecan, Durham, NC)
20. 2 mL Polyethylene 96-well Pattern Sealing Film (Bio Tech Solutions, Vineland, NJ)
21. Nalgene 2mL Cryovials with Ext-Tread (Fisher Scientific, Suwanee, GA)
22. HemoCue 201 Microcuvettes (HemoCue Inc, Lake Forest, CA)
23. Tecan Freedom Evo 200 (Tecan US., Research Triangle Park, NC)
24. Vortex- Genie 2 with Well Plate Adapter (Scientific Industries Inc, Bohemia, NY)
25. Precision Oven (Thelco Laboratories, Torrance, CA)
26. Eppendorf Centrifuge 5810 R V4.2 with A-4-62 Rotor (GMI, Ramsey, MN)
27. Ultravap Mistral Evaporator (Arctic White, LLC Bethlehem, PA)
28. Glas-Col Digital Pulse Mixer (Clas-Col, LLC. Inc. Terre Haute, IN)
29. GeneVac EZ-2.3 Evaporation System with Side Bridge Holders and Universal Rotor (GeneVac Inc., Valley Cottage, NY)
30. Eppendorf 8-Channel Pipette 50-1200 µl, (Eppendorf, Westbury, NY)
31. Eppendorf 1000 µl Manual Pipette (Eppendorf AG ., Hamburg, Germany)
32. Eppendorf 200 µl Manual Pipette, (Eppendorf AG., Hamburg, Germany)
33. Eppendorf 1000 µL Electronic Pipette (Eppendorf AG, Hamburg, Germany)
34. Eppendorf 200 µL Electronic Pipette (Eppendorf AG, Hamburg, Germany)
35. Eppendorf 100 µL Electronic Pipette (Eppendorf AG, Hamburg, Germany)
36. Eppendorf Repeater Pipette (Eppendorf, Ramsey, MN)
37. Transfer Pipettes (Samco Scientific, San Fernando, CA)
38. Boekel Orbitron Rotator, Model II (Fisher Scientific, Suwanee, GA)
39. Fisherbrand Octagonal Stirring Bars, 1"L x 5/16" D (Fisher Scientific, Suwanee, GA)
40. Plain Wood Applicators, 5 3/4"L x 1/12"D (Fisher scientific, Suwanee, GA)
41. Steril Cottoned wood applicators, 5 3/4"L x 1/12"D (Fisher Scientific, Suwanee, GA)
42. Formamide ACS Grade (USB, Cleveland, OH)
43. Pentafluorophenyl Isothiocyanate 96 % (Alfa Aesar, Ward Hill, MA)
44. Methanol, Optima LCMS Grade (Fisher scientific, Suwanee, GA)
45. Ethyl Acetate HPLC/ACS Grade, (Fisher Scientific, Suwanee, GA)
46. Isopropyl Ether, Certified (Fisher Scientific, Suwanee, GA)
47. Toluene, HPLC/ACS Grade, (Fisher Scientific, Suwanee, GA)

### 4.1.4 Equipment, Chemicals, and Consumables Used for Sample Measurement

1. 2 mL Polyethylene 96-well Pattern Sealing Film (BioTech Solutions, Vineland, NJ)
2. RAPID Slit Seal 96-well Sealing Tape (Rikaken USA, Inc., Bethesda, MD)
3. HPLC column: Luna C18(2), 100 x 2.0 mm, 3 µ, Phenomenex (Torrance, CA)
4. Epoch 2 Microplate Spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT)
5. TSQ Altis with Atmospheric Pressure Chemical Ionization Unit (Thermo Electron, San Jose, CA)
6. Vanquish UHPLC with Auto-Sampler and Photodiode Array Detector (Thermo Electron, San Jose, CA)
7. HemoCue Hb 201+ (HemoCue Inc, Lake Forest, CA)
8. Methanol, Optima LCMS Grade Grade (Fisher Scientific, Suwanee, GA)
9. Water, Optima LCMS Grade (Fisher Scientific, Suwanee, GA)
10. Formic Acid, HPLC Grade (Fisher Scientific, Suwanee, GA)
11. Isopropanol, Certified ACS (Fisher Scientific, Suwanee, GA)
12. Deionized Water with Resistance to at least 18 megaOhm-cm and Filter Before use, using 0.45 µm Nylon Filters

## 4.2 Preparation of Reagents and Solutions

### 4.2.1 Preparation of pH Adjusted Formamide Solution

This solution is used to dilute blood samples and adjust the pH of the sample to pH of 6.5-7.5. Add about 1 mL glacial acetic acid to a 1L bottle of formamide using a glass syringe. Mix well by inverting the bottle and measure pH. Make sure pH is between 6-7. The prepared solution can be stored in a refrigerator (5-7°C) for 4 weeks.

### 4.2.2 Preparation of Tecan Solvent Wash Solution (5% Methanol)

The pipetting system requires water to be present in the system for the liquid handling. Wash solvent is prepared by transferring 3800 mL of 18 Ohm-cm DI water to a 4L beaker and adding 200 mL of Methanol by a graduated cylinder. Mix solution well. Store the solution in Tecan Wash container and refill solution as needed.

### 4.2.3 Preparation of Hemoglobin Reagent Set (HRS) Solution

HRS is used to prepare the reagent solution for total Hb measurement. According to the manufacturer's instructions, transfer the package containing the powder into a 1 L volumetric flask. Fill the volumetric flask up to 1 L mark with LCMS grade water and invert at least 5 times for proper mixing. The solution is stored in amber glassware. If amber glassware is unavailable, cover the container completely with aluminum foil to avoid light. Store solution at room temperature (18-26°C) for no longer than 2 years.

### 4.2.4 Preparation of Solution for Liquid-Liquid Extraction

This solution is used for supported liquid-liquid extraction of the analytes. Measure 500 mL isopropyl ether, 400 mL ethyl acetate, and 100 mL toluene with clean graduated cylinders and mix them into a 1 L glass bottle, using separate graduated cylinders for each solvent. Cap the bottle and invert several times to mix thoroughly. Solution must be prepared fresh before each use.

### 4.2.5 Preparation of Extraction Plates

Fill the Extraction Plates (48-well 7.5-mL plate with 25  $\mu$ m PE frit and long drip) to the top with Isolute sorbent. Tap off excess and ensure uniform distribution of solvent across all wells. Final well volume on each plate should be filled to about 0.5 cm from the top. Extraction plates can be prepared in advance and stored in a desiccator until use.

## 4.3 Calibration Materials

### 4.3.1 Preparation of Calibrator Stock Solutions, Intermediate Stock and Working Solutions

**All glassware that is in contact with the octapeptide calibrators must be silanized.** Unsilanized glassware allows for adsorption of the peptides, resulting in inaccurate calibrator concentrations.

The calibrator working solutions are prepared from a calibrator stock solution as indicated in Table 1.

When preparing these stock solutions, the purity of the standard materials need to be taken into account. Depending on the purity of the standard material, different amounts of materials may be used to achieve the desired concentration of the stock solution. Table 1 shows an example of how current calibrator stock solutions are prepared. The example provided in Table 1 provides sufficient volume to produce 500 vials of each calibrator level if 500 mL is prepared for each level of calibrators, which is sufficient for approximately 1,000 analytical runs, assuming use of 1 set of calibrators for 2 calibration curves.

**Table 1:** Dilution scheme for AA, GA, and EO Octapeptide Calibrator Stock Solutions

Calibrator Solutions	Target Concentration of Octapeptides			Amount of peptide and diluent volume (Diluant: LCMS grade water)
	AA	GA	EO	
Stock Solution	68.52 μmol/L	66.98 μmol/L	69.83 μmol/L	2.57 mg AA Octapeptide → 25 mL 2.73 mg GA Octapeptide → 25 mL 2.51 mg EO Octapeptide → 25 mL
Intermediate	2192.77 nmol/L	2143.35 nmol/L	5327.60 nmol/L	0.80 mL of both AA and GA Octapeptide (Calibrator Stock) → 25 mL  0.75 mL EO Octapeptide (Calibrator Stock) → 10 mL

1. Preparation of Calibrator Stock Solution
  - a. Remove AA-VHLTPEEK (AA octapeptide), GA-VHLTPEEK (GA octapeptide), and EO-VHLTPEEK (EO octapeptide) material from freezer to sit in a desicator overnight at room temperture.
  - b. Calibrate the analytical balance following the manufacturer's instructions.
  - c. Weigh 2.57 (±0.001) mg of AA octapeptide on a clean aluminum foil surface and transfer it to a 25 mL volumetric flask.  
Weigh 2.73 (±0.001) mg of GA octapeptide on a clean aluminum foil surface and transfer it to a separate 25 mL volumetric flask.  
Weigh 2.51 (±0.001) mg of EO octapeptide on a clean aluminum foil surface and transfer it to a separate 25 mL volumetric flask.
  - d. Add LCMS water to the fill line of each volumetric flask and place the flask in a 20°C water bath for 2 hours.
  - e. Remove flasks from the water bath and readjust the water level in each flask to the fill line with 20°C LCMS water.

- f. Mix solution well by inversion 10 times.
  - g. Transfer each solution into separate 50 mL falcon tubes and label appropriately.
  - h. Store at 2-8°C if not to be used immediately.
2. Preparation of AA/GA and EO Calibrator Intermediate Stock Solutions
- a. Transfer 0.800 mL of both AA and GA calibrator stock solutions into a single 25 mL volumetric flask using a calibrated pipette.
  - b. Add LCMS water to the fill line of the volumetric flask and place the flask in a 20°C water bath for 2 hours.
  - c. Remove flask from the water bath and readjust the water level to the fill line with 20°C LCMS water.
  - d. Mix solution well by inversion 10 times.
  - e. Transfer solution to 50 mL falcon tubes.
  - f. Label tubes appropriately.
  - g. Store at 2-8°C if not to be used immediately.
  - h. For the EO intermediate stock solution, transfer 0.750 mL of the EO stock solution into a silanized 10 mL volumetric flask using a calibrated pipette.
  - i. Repeat steps b to h for final preparation of the EO intermediate stock solution.
3. Preparation of the different levels of calibrators

The calibrators are prepared as shown in Table 2 and 3.

**Table 2:** Dilution scheme for EO Octapeptide Calibrators

Calibrator Solution	Target Concentration of Octapeptides (nmol/L)		Dilution Scheme based on stock solutions described in Table 1 (Diluant: LCMS grade water)
	EO		
A15C01L02	5.03		480 µL EO Calibrator Intermediate Stock Solution → 500 mL
A15C02L02	10.48		1000 µL EO Calibrator Intermediate Stock Solution → 500 mL
A15C03L02	20.95		150 µL EO Calibrator Stock Solution → 500 mL
A15C04L02	41.90		300 µL EO Calibrator Stock Solution → 500 mL
A15C05L02	83.80		600 µL EO Calibrator Stock Solution → 500 mL
A15C06L02	167.60		1200 µL EO Calibrator Stock Solution → 500 mL
A15C07L02	335.21		2400 µL EO Calibrator Stock Solution → 500 mL

**Table 3:** Dilution scheme for AA and GA Octapeptide Calibrators

Calibrator Solution	Target Concentration of Octapeptides (nmol/L)		Dilution Scheme based on stock solutions described in Table 1 (Diluant: LCMS grade water)
	AA	GA	
A15C01L03	1.10	1.07	100 µL Calibrator Intermediate Stock Solution → 200 mL
A15C02L03	2.19	2.14	200 µL Calibrator Intermediate Stock Solution → 200 mL
A15C03L03	4.39	4.29	400 µL Calibrator Intermediate Stock Solution → 200 mL
A15C04L03	8.77	8.57	800 µL Calibrator Intermediate Stock Solution → 200 mL
A15C05L03	17.54	17.15	1600 µL Calibrator Intermediate Stock Solution → 200 mL
A15C06L03	34.26	33.49	100 µL Each of AA and GA Calibrator Stock Solutions → 200 mL
A15C07L03	68.52	66.98	200 µL Each of AA and GA Calibrator Stock Solutions → 200 mL

Prepare the Calibrators by Performing the Following Tasks:

1. Adjust the intermediate solutions to 20 °C using a water bath.
2. Transfer the volumes of the EO calibrator intermediate stock and calibrator stock solutions stated in Table 2 to a separate 500 mL silanized volumetric flasks, using calibrated adjustable pipettes.
3. Add LCMS water to just below the fill line of the volumetric flask and place the flask in a 20°C water bath for 2 hours.
4. Remove flask from the water bath and readjust the water level to the fill line with 20°C LCMS water.
5. Mix solution thoroughly by inverting the flask 20 times.
6. Aliquot solutions in 0.9 mL aliquots in appropriately labeled cryovials and store them at -70 °C. Each vial is sufficient for four analytical runs. Calibrator solutions cannot be reused.
7. For the preparation of the AA/GA calibrator solutions, transfer the volumes of the AA/GA calibrator intermediate stock and calibrator stock solutions as stated in Table 3 to a 200 mL silanized volumetric flasks with adjustable calibrated pipettes.
8. Repeat steps 3-6 for final preparation of AA/GA calibrator solutions.

#### 4.3.2 Preparation of Calibrator Solutions for Total Hemoglobin Measurement

Concentrations of calibration solutions are prepared from lyophilized Hb supplied by Analytical Control Systems Inc.. Prepare five levels of calibrators with following target concentration: 20, 16, 12, 8, 4, 2, 1, and 0.5 g/dL.

1. Add 2.5 mL LCMS DI to 1 bottle of lyophilized Hb linearity control.
2. Place on Hematology Mixer for about 30 minutes to mix thoroughly and label as "Hb linearity control stock".
3. Dilute the Hb linearity control with LCMS grade water, using the dilution scheme shown in Table 3.
4. Label vials appropriately.
5. Store calibrators at 2-8°C until use.

**Note:** Expiration date of Hb calibrators is seven days following preparation.

**Table 4.** Preparation of the Total Hb Calibration Curve

Calibration Curve Level	Concentration based on current lot (g/dL)	Amount of Hb linearity control stock (µL)	Amount of DI H <sub>2</sub> O (µL)	Total Volume (mL)
1	20.0	800	0	0.800
2	16.0	700	175	0.875
3	12.0	600	400	1.000
4	8.0	400	600	1.000
5	4.0	200	800	1.000
6	2.0	100	900	1.000
7	1.0	50	950	1.000
8	0.5	100 µL (of 4.0 g/L Stock)	700	0.800

### 4.3.3 Preparation of Internal Standard Solutions

**All glassware that is in contact with the octapeptide must be silanized.**

Unsilanized glassware allows for adsorption of the peptides, resulting in inaccurate calibrator concentrations.

Isotope labeled octapeptides AA-V(<sup>13</sup>C<sub>5</sub>,<sup>15</sup>N)- HLTPEEK, GA-V(<sup>13</sup>C<sub>5</sub>,<sup>15</sup>N)- HLTPEEK, and EO- V(<sup>13</sup>C<sub>5</sub>,<sup>15</sup>N)- HLTPEEK are used to prepare internal standard stock and working solutions in 18 megaOhm-cm DI water. The internal standard stock solution, is used to prepare the internal standard working solution. Table 4 shows an example of how current internal standard solutions are prepared. This procedure produces 200 vials of internal standard working solution, which is sufficient for 10,000 samples assuming use of 100 µL of internal standard working solution per sample.

**Table 5:** Desired Internal Standard Concentrations for AA, GA, and EO Octapeptide Solutions

Internal Standard Solution	Target Concentration of Isotope Labeled Octapeptides			Amount of peptide and diluent volume (Diluant is 18 megaOhm-cm DI water)
	AA	GA	EO	
Stock Solution (µmol/L)	176.77	172.50	170.07	2.72 mg AA Octapeptide IS → 10 mL 2.63 mg GA Octapeptide IS → 10 mL 2.63 mg EO Octapeptide IS → 10 mL
Working Solution (nmol/L)	44.20	64.68	127.56	1.0 mL AA Internal Standard Stock Solution 1.5 mL GA Internal Standard Stock Solution 3.0 mL EO Internal Standard Stock Solution → 2,000 mL

#### 1. Preparation of Internal Standard Stock Solution

- a. Remove AA-V(<sup>13</sup>C<sub>5</sub>,<sup>15</sup>N)- HLTPEEK, GA-V(<sup>13</sup>C<sub>5</sub>,<sup>15</sup>N)- HLTPEEK, and EO-V(<sup>13</sup>C<sub>5</sub>,<sup>15</sup>N)- HLTPEEK material from freezer and allow them to sit in a desicator for overnight under room temperature.
- b. Calibrate the analytical balance following the manufacturer's instructions.

Weigh 2.72 ( $\pm 0.001$ ) mg of AA-V( $^{13}\text{C}_5$ ,  $^{15}\text{N}$ )- HLTPEEK, 2.63 ( $\pm 0.001$ ) mg of GA-V( $^{13}\text{C}_5$ ,  $^{15}\text{N}$ )- HLTPEEK, and 2.63 ( $\pm 0.001$ ) mg of EO- V( $^{13}\text{C}_5$ ,  $^{15}\text{N}$ )- HLTPEEK on clean aluminum foil surface and transfer them to separate 10 mL volumetric flasks.

- c. Add LCMS water to the fill line of the volumetric flasks and place the flasks in a 20°C water bath for 1 hour..
- d. Remove flask from the water bath and readjust the water level to the fill line with 20°C LCMS water.
- e. Mix solution thoroughly by inverting the flask 20 times.
- f. Transfer solution into a separate 15 mL falcon tubes, label tubes appropriately and store them in the -70 °C freezer.

## 2. Preparation of Internal Standard Working Solution

- a. Adjust internal standard stock solution to 20 °C using a water bath.
- b. Transfer 1.0 mL of AA internal standard stock solution, 1.75 mL of GA internal standard stock solution, and 3.0 mL of EO internal standard stock solutions into a 2,000 mL volumetric flask using a 1 mL calibrated pipette.
- c. Add LCMS grade water to the fill line of the volumetric flask and place the flask in a 20°C water bath for 1 hour.
- d. Remove flask from the water bath and readjust the water level to the fill line with LCMS grade water.
- e. Mix solution thoroughly by inverting the flask 20 times.
- f. Aliquot 12 mL solution in 15-mL falcon tubes, label tubes appropriately and store them in the -70 °C freezer.

**Note:** This solution is stable for 10 years.

# 5 COLLECTING, STORING, AND HANDLING SPECIMENS; CRITERIA FOR SPECIMEN REJECTION

## 5.1 General Specimen Requirements

For analysis of AA, GA, and EO Hb adducts, a sample of 1.0 mL 1:1 diluted red blood cells is preferable to allow for repeat analyses. A sample volume of 500  $\mu\text{L}$  of diluted lysed red blood cells is required for analysis. Additional sample is needed if blood clots are present in the vial.

No fasting or special time of day for specimen collection is required. Specimens for AA, GA, and EO Hb adduct analysis may be fresh or frozen erythrocytes or Ethylenediaminetetracetic acid tripotassium salt dehydrate (EDTA) whole blood. This procedure was evaluated with EDTA whole blood and erythrocytes obtained from EDTA whole blood. The applicability of this method for other coagulants has not been tested.

An appropriate amount (between 1.0 mL and 2.0 mL) of red blood cells is dispensed into 2.0 mL cryogenic vials with external screw caps. These cryovials must be labeled in accordance with CDC and NCEH/DLS policies and regulations. If testing for adducts other than AA, GA, and EO are requested to be measured, then the sample may need to be



divided. In such a case, appropriate amounts of lysed red blood cells should be transferred to a cryovial labeled in accordance with CDC and NCEH/DLS policies and regulations. Refer to the DLS Policies and Procedures Manual for additional criteria (29).

## 5.2 Specimen Storage

Collected specimens can be shipped at refrigerated temperatures (2-8 °C) or frozen on dry ice. Specimens can be stored at refrigerated temperatures for up to 3 days prior to analysis. For long-term storage, samples must be stored at -70 °C. Samples stored at -70 °C are stable for 5 years. Two and three freeze-thaw cycles did not exhibit any changes. Multiple freeze-thaw cycles of diluted red blood samples may increase the formation of blood clots, which complicates the analysis of the samples. Therefore, diluted red blood samples should not be subjected to more than 3 freeze-thaw cycles.

## 5.3 Unacceptable Specimens

Specimens that have been subjected to more than 3 freeze/thaw cycles, were shipped and/or received at room temperature, or exhibit evidence of leakage and/or breakage are not accepted for testing.

# 6 PROCEDURE OPERATION INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

Abiding by the manufacturer's acceptance criteria, all instruments are checked for proper function prior to operation. Specific details related to the operating instructions, preventative maintenance (PM), and specific file names used in the execution are documented in the work instructions ( Appendix 8).

## 6.1 Specimen Storage and Handling During Testing

All vials must be labeled according to DLS Policies and Procedures Manual. Incorrectly labeled vials should not be processed.

Barcodes are scanned during sample preparation, sample transfer, and analysis to ensure individual samples can be tracked throughout the procedure.

Specimens should be at room temperature prior to sample preparation. Any unused portion of the specimen should be returned to -70 °C freezer for storage.

Samples ready for analysis by HPLC-MS/MS are stored at 2-8 °C.

## 6.2 Preparation of Samples for Analysis

All samples are processed along with 2 sets of 3 bench QC samples, 1 blank, and 1 set of calibrators (7 levels). Up to four plates (total of 136 patient samples) can be processed in parallel. Plate layout example: 1 blank, 7 calibrators, 3 quality control samples, 34 samples and 3 quality control samples.

1. Assess all samples for acceptability using the criteria described in sections 5.2 and

- 0.
2. Frozen 1:1 (v/v) diluted RBCs, QC samples, internal standard working solutions, and calibrator working solutions are allowed to reach room temperature and are homogenized by placing them on the rotator at medium speed for about 30 minutes. pH adjusted formamide solution, 0.2N sodium hydroxide solution, and Edman reagent are also allowed to reach room temperature before use.
3. Place all patient samples, QC samples, internal working solution, and calibrator working solution in the Tecan Freedom Evo 2-200 Liquid Handler in their designated locations, in a manner that allows the instrument's barcode reader to scan all barcodes correctly. Place all additional reagents in the instrument at their designated positions.

### 6.2.1 Scanning of the Barcodes

1. Scan the barcodes of all coded vials and reagents.
2. When a barcode cannot be read, the Tecan Freedom Evo 2-200 (Tecan) instrument software will prompt the analyst to manually enter the barcode information. After the scanning process is completed, an Excel file is generated by Tecan containing the barcode information and location of the particular samples, calibrators, and reagents in the instrument. The file name consists of the date and time of when the scan is performed. This file is then transferred to a defined location on the CDC network. The information is used to create a run sequence for the UHPLC-MS/MS instrument and to verify run log sheets.

## 6.3 Total Hemoglobin Measurements

The Hb measurement provides information about the Hb concentration in the samples analyzed during Edman reaction. The amount of Hb used for the Edman reaction is needed to calculate the concentration of adducts as pmol of adduct per gram of total Hb.

The measurement procedure is performed by a commercial assay called "Hemoglobin Reagent Set" (HRS). HRS solution is prepared according to section 4.2.4 of this SOP. For additional information see package insert (**Appendix 11**).

In principle, the Hb and its derivatives, except sulfhemoglobin, are oxidized to methemoglobin by ferricyanide in the presence of alkali. Cyanmethemoglobin is formed from methemoglobin by reaction with cyanide. The resulting colored complex has a peak absorption at 540 nm, the value of which is proportional to total Hb concentration.

### 6.3.1 Total Hemoglobin Analysis

Allow the tHb Calibrators and tHb QC set to reach room temperature.

A maximum of 2 plates (96 wells 2 mL) can be processed one at one time using the following steps of HRS test.

1. Tecan pipettes 50  $\mu$ L of blood samples and method 1015 QC samples from cryovials to 96-well , 2 mL plates (Dilution plate). The program will pause with a wait prompt to allow assessing of samples for blood clots. If a blood clot is observed, it is removed by the operator using a wooden applicaton stick. If the blood clot is large, the sample is removed from the cell and the same sample is pipetted manually to the plate without blood clots.
2. Tecan pipettes 50  $\mu$ L of ACS calibrators and tHb QC samples to the Dilution plate.
3. Tecan transfers 450  $\mu$ L of 0.9% saline into each well of the Dilution Plates and mixes all samples in the wells.
4. Tecan pipettes 50  $\mu$ L of the diluted samples, ACS CCs, and tHb QCs into the HRS plate.
5. Tecan transfers 950  $\mu$ L of HRS solution into the HRS plate using 150  $\mu$ L MCA tips and mixes the solutions. The analyst visually checks the mixtures for homogeneity. Manual mixing may be required.
6. Let the HRS plates sit for 3 minutes to ensure reaction completion.
7. Insert a 96-well Microtiter plate (MTP plate) into the spectrophotometer and read the blank plate at 540 nM to obtain a background reading.
8. Tecan transfers 200  $\mu$ L of the samples from the HRS plate to the MTP plate.
9. Insert the MTP plate into the spectrophotometer and read the absorbance of the samples at 540 nm.
10. After reading the absorbance of the samples, the instrument software automatically subtracts the background absorbance values from the absorbance of the plate with the sample solutions. The "Raw" results are stored as an excel file on the local hard drive.
11. Steps 7-10 are repeated with a new MTP plate.

### 6.3.2 Total Hemoglobin Calculations

1. Assess the data to ensure validity of the calibration curve. Determine if the slope, intercept, and correlation coefficient ( $R^2$ ) values are consistent with method evaluation values.
2. Assess validity of the tHb QC samples to be within the in-house established limit, check for outliers, and repeat measurement if necessary.
3. Assess sample validity of the difference between sample replicates to be within 10 percent or better.
4. Assess validity for the amount of total Hb to be within the calibration curve range.
5. The template will mark a cell "HC" if a measurement fails to pass these assesments. If this happens, the samples will be measured with the HemoCue meter and the value will be entered into the data file for the corresponding cell.
6. Each sample's absorbance is measured in duplicate and averaged.

## 7. Calculation:

$$\text{Whole blood Hemoglobin } \left( \frac{g}{dL} \right) = \frac{A_u}{A_s} \times C_s$$

A<sub>u</sub>= absorbance of unknown

A<sub>s</sub>=absorbance of standard

C<sub>s</sub>=concentration of standard g/dL

## 6.4 Edman Reaction Preparation

In this step, the N-terminal valines containing AA, GA, and EO are cleaved from the Hb protein chains by the Edman reagent. The resulting pentafluorophenyl thiohydantoin derivatives are isolated from the reaction mixture by liquid-liquid extraction on diatomaceous earth and prepared further for UHPLC-MS/MS analysis (Discussed later in sections 6.6 and 6.7, for extraction and UHPLC-MS/MS analysis respectively).

The blank, calibrators, quality control samples and patient samples are transferred to four 48-well 5 mL wellplates. These well plates are named “*Edman Plates*”. Solvents and reagents are added and the Edman reaction is performed. The following steps will take place using the Tecan.

1. Place the Edman Plates in their appropriate locations on the Tecan.
2. Tecan pipettes 350 µL of 1:1 (v/v) diluted RBC from the cryovials using 1000 µL conductive tips to the Edman Plates. If a blood clot is observed, it is removed by the operator using a wooden applicator stick. If the blood clot is too large, the sample is removed from the cell and the same sample volume is manually pipetted to the cell. Visually check that the volumes are consistent throughout the plates. If the volume is not sufficient, record samples that appear low. Samples will be flagged during data processing if the volume is insufficient, and the data reviewer will assign an error code. Samples with error codes will be repeated.
3. Tecan adds 100 µL of internal standard working solution to all the wells of the plate using 200 µL conductive tips. Visually check each well to confirm uniform addition.
4. Tecan pipets 200 µL of the calibration curve (CC) solutions and 200 µL LCMS Grade water into the assigned wells (blank samples). Visually check the volume level in each well to ensure the volume is the same. The column containing the CCs and Blank corresponds to the plate number. For example, the CCs are dispensed in the first column of the first plate, the second column of the second plate, etc.
5. Tecan adds 200 µL LCMS grade water to CC samples and blank sample using conductive tips.
6. Tecan adds 1.5 mL pH adjusted formamide to all wells.
7. Tecan adds 0.2N sodium hydroxide solution to CCs to adjust the pH.
8. Tecan will then pause and a reminder will appear about measuring pH of samples. Gently vortex the plate to mix samples thoroughly before pH measurements.
9. Before proceeding, ensure that the samples and CCs are adjusted to a pH range of 7.0 (±0.5). Measure the pH of the samples in 6 random cells including one QC sample and one CC sample.
10. Place the Edman Plates back on the instrument in their appropriate locations and uncap the Edman reagent vial.

**Note: Filling of the Edman reagent vial is performed only in the chemical fume hood.**

Tecan pipets 20  $\mu$ L of Edman reagent to all wells of the plates. When the program is finished, remove plates from the Tecan, seal with silicon plate sealers, vortex, and transfer to the shaker located in the oven.

## 6.5 Edman Reaction Performance

Incubate samples at 55 ( $\pm$ 1)  $^{\circ}$ C for 2 hours in the oven. Ensure samples are shaken during incubation time using a laboratory shaker.

## 6.6 Supported Liquid-Liquid Extraction of Edman Products

In this section of the procedure, the Extraction Plate, Collection Plate, and Reconstitution Plate are prepared. The Tecan transfers all sample solutions from the Edman Plate to the Extraction Plate by performing the following tasks:

1. Transfer the samples from the Edman Plate to the 48-well 7.5 mL Extraction Plate, filled with Isolute material. Place each Extraction Plate on top of a corresponding 48-well 7.5-mL Collection Plate.
2. Wait 20 minutes for samples to properly penetrate through the sorbent material.
3. Add 8 mL of the organic mixture containing 50/40/10 (v/v/v) of isopropyl ether, ethyl acetate, and toluene to each well.
4. Let all Extraction/Collection Plates sit for at least 20 minutes to elute the solvent. Carefully lift each Extraction Plate and check if solvent is still dripping (if so, allow the plates to sit longer, until all solvent has passed and no dripping is observed).
5. Transfer the Collection Plate containing the extraction solutions to the evaporator (Glas-Col digital pulse mixer) for evaporation over 55 minutes.
6. Wash the walls of each well in the Collection Plate with ethyl acetate to recover the analytes on the walls. Use the repeater pipette with a 10 mL tip and set the pipette dial to one, corresponding to a 200  $\mu$ L dispense volume. Wash each wall of the Collection Plate with 200  $\mu$ L of ethyl acetate. Then, add an additional 200  $\mu$ L to the wider walls of the wells for a total wash volume of 1,200  $\mu$ L per well.
7. Vortex each plate for 30 seconds.
8. Place the Collection Plate, containing ethyl acetate, and a 96-well, 2 mL "*Reconstitution Plate*" in the Tecan instrument.
9. Transfer the samples recovered from the wall wash step to the 96-well 2 mL Reconstitution Plate. Two Collection Plates can be transferred to one Reconstitution Plate.
10. Evaporate the ethyl acetate by placing the Reconstitution Plate in an Ultravap Mistral evaporator for 30 minutes.

## 6.7 Sample Preparation for HPLC-MS/MS Analysis

When all solvents have been evaporated from the Reconstitution Plate, perform the following tasks using the Tecan automated instrument:

1. Transfer the Reconstitution Plate from the Ultravap Mistral evaporator to the Tecan.
2. Tecan adds 75  $\mu\text{L}$  of methanol and 175  $\mu\text{L}$  of LCMS Grade water to each well, in that order. Vortex the Reconstitution Plate to ensure thorough mixing.
3. Transfer the Reconstitution Plate to the centrifuge. Centrifuge samples for 15 minutes at 3,700 rpm and 5  $^{\circ}\text{C}$ , then return the Reconstitution Plate to the Tecan.
4. Transfer the samples from the Reconstitution Plate to a new 96-well, 2 mL per well, plate named Final Plate.
5. Tecan pipettes 250  $\mu\text{L}$  of the samples from the Reconstitution Plate to the Final Plate.
6. Seal the Final Plate and transfer it to the sample compartment, set at 5  $^{\circ}\text{C}$ , for analysis by UHPLC-MS/MS.

## 6.8 Edman Products Analysis by UHPLC-MS/MS

All daily, weekly, and monthly preventative maintenance or cleaning are performed according to the manufacturer's requirements. The file containing the tuning and calibration is named by date. The instrument maintenance instructions and log-books are kept in the laboratory next to the instrument.

### 6.8.1 Analysis Sequence

1. A run sequence is created using the information obtained from the sample barcode readings performed by the Tecan. The run sequence is then used by the UHPLC-MS/MS software to analyze the samples. The run sequence consists of two 48-well plates combined in one 96-well plate, as well as additional samples, such as water and instrument controls, to assess carry-over and instrument function throughout the run. The sequence is created by combining the barcode ID information with the UHPLC-MS/MS instrument files and the data processing method file using a sequence template file. An example of a sequence can be found in **Appendix 12**.
2. Once the sequence is finished, check all samples for validity and re-inject samples as needed.

## 6.9 Chromatographic and Mass Spectrometric Conditions

The instrument control sample contains the analytes and internal standard. The instrument control is added to each batch to verify appropriate function of the instrument and chromatographic condition. Additionally, a water sample is added after every eighth sample. The instrument control sample and the water sample are kept in separate vials in the auto-sampler of the UHPLC-MS/MS instrument. All samples prepared in one batch are analyzed as one batch by the instrument. Typical chromatograms of a sample are shown in Appendix 1.

The UHPLC-MS/MS parameters used are listed in tables 6, 7, and 8.

### Chromatographic conditions

Syringe: 500 µL DLW  
 Injection volume: 50 µL  
 Loop size: 100 µL  
 Column: Luna 3u C18 (2) 100Å 100 mm× 2.0 mm ID, 3 µm particle size  
 Column Oven: 45 (±1)°C  
 Solvent A: Methanol (LCMS optima)  
 Solvent B: Water (LCMS optima)  
 Solvent C: not used  
 Solvent D: not used  
 Flow Rate: 550 µL/min  
 Multiplex valve: MX Series II MXT715-102 (2 position, 10-port)

**Table 6:** UHPLC Gradient conditions for Pump A

Step	Time	Flow rate (µL/min)	% A	% B
0	0.00	0.550	60.0	40.0
1	7.50	0.550	60.0	40.0

**Table 7:** UHPLC Gradient conditions for Pump B

Step	Time	Flow rate (µL/min)	% A	% B
0	0.00	0.550	60.0	40.0
1	1.00	0.600	98	2
2	3.000	0.600	98	2
3	3.50	0.550	60.0	40.0
4	7.50	0.550	60.0	40.0

### Mass spectrometric conditions

Acquisition mode: Single Reaction Monitoring (SRM)  
 Acquisition Delay: 1 min  
 Ionization: APCI in the Positive Ion Mode  
 Spray Current: 4.5 µA  
 APCI Vaporization Temperature: 450 °C  
 Capillary Temperature: 270 °C  
 Sheath Gas: Argon 45 psi

Aux Gas: Argon 5 psi  
 CID: 1.0 mTorr  
 Chrom filter: 10  
 Segment: 3  
 Width: 0.010  
 Time: 0.100 s  
 Q1 PW: 0.70  
 Q3 PW: 0.70

**Table 8:** SRM ion transitions of analytes and internal standards

Analyte	Ions	SRM (m/z)	Collision Energy (V)	Expected Retention time (min)
AA-Val-PFPTH	Quantitation	396→379	10	4.09
	Confirmation	396→337	20	
AA-Val( <sup>13</sup> C <sub>5</sub> , <sup>15</sup> N)-PFPTH	Quantitation	402→385	10	4.09
	Confirmation	402→343	20	
GA-Val-PFPTH	Quantitation	412→395	10	3.40-3.72
	Confirmation	412→367	17	
GA-Val( <sup>13</sup> C <sub>5</sub> , <sup>15</sup> N)-PFPTH	Quantitation	418→401	10	3.40-3.72
	Confirmation	418→373	17	
EO-Val-PFPTH	Quantitation	369→130	20	5.20
	Confirmation	369→116	27	
EO-Val( <sup>13</sup> C <sub>5</sub> , <sup>15</sup> N)-PFPTH	Quantitation	375→135	20	5.20
	Confirmation	375→121	27	

### UV detector conditions

Scan Wavelength: 200-600 nm  
 Scan Bandwidth: 1 nm  
 Margin for Negative Absorbance (mAU): 100  
 Scan Step: 1 nm  
 Sampling Rate: 5 Hz  
 Channel sample rate: 10 Hz  
 A Channel wavelength: 210 nm; Channel bandwidth: 9 nm  
 B Channel wavelength: 254 nm; Channel bandwidth: 9 nm  
 C Channel wavelength: 280 nm; Channel bandwidth: 9 nm

## 6.10 HPLC-MS/MS Data Processing

Transfer raw data files obtained from the UHPLC-MS/MS measurements to the corresponding folder on the network drive. Data is integrated and processed using Xcalibur or Indigo Ascent to identify relevant chromatographic peaks and to integrate the peak areas accordingly. Manual integration may be required if automatic processing fails to integrate the peak properly. These two methods of integration are comparable.

All data files are organized by date, Run ID, and operator. The Run ID represents a batch of samples that includes calibrators and controls and that is physically located in one 48-



well plate. At regular operating conditions, up to four runs are prepared per day. For analysis of samples by UHPLC-MS/MS, two 48-well plates are combined into one 96-well plate. Thus, data and results generated by the UHPLC-MS/MS system contain 2 runs per UHPLC-MS/MS analysis.

## 6.11 Data Calculations

Upon completion of the sample integration, all necessary calculations are performed in a Microsoft Excel spreadsheet. Using the quantitation settings on the Thermo Scientific Xcalibur or Indigo Ascent software, result tables containing the integrated peak areas are calculated and then transferred to the spreadsheet for further analysis. All samples are measured in singlicate. To calculate analyte concentration, the analyte and internal standard area counts ratios are used. Calibration curves are generated with the area ratios from the calibrator samples and their assigned values using unweighed linear regression for AA and GA and using  $1/x^2$  weighted linear regression for EO. We don't process further for sample batches with calibration curves not meeting DLS Policies and Procedure Manual and laboratory specific quality criteria. The analyte concentration was calculated using the area ratio calculated for the unknown sample and the regression parameters of the corresponding calibration curves. Area ratios for analytes outside the established linear range will not be further processed. Reanalyze these samples after appropriate dilution or concentration.

## 7 CALIBRATION AND CALIBRATION VERIFICATION

### 7.1 Calibration

#### 7.1.1 Calibration of Instruments and Equipment

All pipettes are calibrated annually and verified after 6 months following procedures recommended by the manufacturers.

Accuracy of other equipment are verified regularly according to the manufacturer's recommendation or using established references (e.g., commercial buffer solutions, external thermometers).

#### 7.1.2 Calibration of Measurement

Calibrators used in this measurement procedure are commercial standards. Calibrators are analyzed together with each set of samples. Calibration solutions are prepared starting with volumetric measurements. See Appendix 9 for metrological traceability according to ISO 17511.

### 7.2 Calibration Verification

Calibration and calibration verification of equipment are performed every six months and are required to substantiate the continued accuracy of the test system throughout the laboratories. Mass spectrometry instruments are regularly calibrated for mass accuracy

by following the manufacturers test system instructions. These instructions specify calibration materials and recommended frequency.

With each set of samples, calibrators (7 levels), and QCs (low, medium, and high) covering the reportable range of AA, GA, and EO adducts, are analyzed in compliance with the DLS Policies and Procedures Manual Section 10. Possible shifts in calibration are assessed by comparing bench QC material data against predefined acceptance limits using the SAS software program, maintained by DLS.

Higher order reference materials are currently not available for these analytes or measurements. The quality of calibrator materials is assessed according to DLS Policies and Procedures Manual section 6.13.

Calibration verification is carried out according to DLS Policies and Procedures Manual and CLIA regulations. Calibration verification is performed by analyzing calibration material that represents the lowest end (lowest third), the middle portion (middle third), and highest end (higher third) of the reportable range.

For calibration verification, internal quality control materials are prepared by spiking known amounts of calibration standards into matrix at concentrations that represent the low end, mid portion, and high end of the calibration curve. The calibration materials used for spiking are octapeptide standards that have been well characterized by definitive test methods (i.e., quantitative nuclear magnetic resonance, amino acid analysis, and high resolution mass spectrometry). The low, middle, and high spiked materials are analyzed as unknowns in the same manner as patient samples to confirm that the calibration of the instrument is stable throughout the laboratory's reportable range.

## 8 METHOD PERFORMANCE CHARACTERISTICS

Method performance for this method including accuracy, precision, sensitivity, specificity, and stability is provided in Appendix A of this method documentation. **The signatures of the branch chief and director of the Division of Laboratory Sciences on the first page of this procedure denote that the method performance fits for the intended use of the method.**

### 8.1 Analytical Measurement Range and Linearity Limits

Linearity of the method was determined according to Clinical and Laboratory Standard Institute (CLSI) guideline EP06 (30). The method is linear from 1.10 – 140.34 nmol/L for AA, 1.07 – 137.17/L for GA, and 5.03 – 670.51 nmol/L for EO. The total hemoglobin calibrators have a linear range from 1.3 to 20.4 g/dL.

The reportable range of the adducts is 3.9-6,169 pmol AA/g Hb, 4.9-6,029 pmol GA/g Hb, and 12.9-29,473 pmol EO/g Hb. Samples below this analytical measurement range will not be reported. Samples above this range can be diluted with saline and then reanalyzed.

## 8.2 Limit of Detection

LOD was determined in serum matrix according to Taylor's method (31) by calculating the standard deviation (SD) at different standard concentrations. The absolute values of the SD were then plotted versus concentration. The overall detection limit depends on the amount of Hb present in each sample. Detection limit in matrix for AA is 3.9 pmol/g Hb, GA is 4.9 pmol/g Hb, and EO is 12.9 pmol/g Hb adducts. See table 9.

**Table 9:** LOD calculation in matrix

	AA (pmol/g)	GA (pmol/g)	EO (pmol/g)
LOD 3x intercept	3.9	4.9	12.9

## 8.3 Analytical Specificity

A specific chemical reaction to Hb adducts occurs within the "modified Edman reaction". Only Hb adducts that can react with the Edman reagent under the conditions used in this method will form the Edman products. Thus, this method is not affected by free AA, GA, or EO in the blood sample or possible AA, GA, and EO contaminations in the laboratory. In addition, a large number of chemicals such as aldehydes react with Hb but cannot react with the Edman reagent.

Compound identification is performed by comparing the chromatographic retention time of the analyte with the structurally identical IS. The analyte is identified as such when it has the same chromatographic retention time as the IS. See Appendix 1 for IS and analyte peak identification.

Compound identification is performed by tandem MS monitoring the specific mass to charge ratio of the analyte and its analyte specific product ions.

## 8.4 Accuracy and Precision

Precision of the method was evaluated according to CLSI guideline EP5-A2 (32) and the DLS Policies and Procedure Manual by analyzing 3 levels of QC materials in duplicate on 20 different days. The evaluation includes within-run, between-run, and total precision assessments. See Table 10 and 11. Total precision using DLS method for three levels of QC material did not exceed 15% relative standard deviation (coefficient of variation, CV). Please refer to Appendix A for more details.

The precision of the method is reflected in the variance of QC samples with three different concentration levels analyzed over time. Records of the method precision are maintained in the quality assurance (QA) binder. The evaluation of within- and between-run imprecision are similar. The concentration of the QC ranges are applicable to the method.

**Table 10:** Within-run precision values evaluated according to DLS Policies and Procedures Manual

Analyte	Within Run Precision (%CV) Low	Total RSD (%) Low	Within Run Precision (%CV) Medium	Total RSD (%) Medium	Within Run Precision (%CV) High	Total RSD (%) High
AA adducts	5.75	6.39	4.93	6.43	6.43	7.73
GA adducts	6.58	9.85	6.17	7.78	7.09	9.65
EO adducts	7.78	14.77	5.33	6.51	5.81	9.14

**Table 11:** Between-run precision values evaluated according to DLS Policies and Procedures Manual

Analyte	Between Run Precision (%CV) Low	Total RSD (%) Low	Between Run Precision (%CV) Medium	Total RSD (%) Medium	Between Run Precision (%CV) High	Total RSD (%) High
AA adducts	2.80	6.39	4.12	6.43	4.29	7.73
GA adducts	7.33	9.85	4.74	7.78	6.55	9.65
EO adducts	12.56	14.77	3.75	6.51	7.06	9.14

Accuracy measurement was determined according to CLSI guideline EP15-A2 (33). When reference samples or methods are unavailable, the accuracy is assessed through the recovery of known amount of analyte spiked into a blank matrix. The deviation of the mean from the true values serves as the measure of accuracy.

Primary or matrix-based reference materials are not available for AA, GA, and EO octapeptides. The concentration of the calibrators was assessed by measuring the peptides used as calibrators directly by MS and was verified using standard addition methodology of the octapeptide standard material. The concentration of the calibrator is considered confirmed if the difference in results of our in-house method and the confirmation method is less than 12% (see Table 12).

**Table 12:** Accuracy by standard addition

Adducts	Expected pmol	Measured pmol in Low Pool	Measured pmol in High Pool	% Recovery in Low Pool	% Recovery in High Pool
AA Adducts	3.16	3.18	2.95	100.6	93.5
	4.74	4.47	4.29	94.3	90.5
	6.32	6.38	7.07	101.0	111.9
GA Adducts	2.84	2.81	2.79	98.9	98.4
	4.26	4.28	4.39	100.5	103.1
	5.68	5.65	5.89	99.4	103.6
EO Adducts	11.83	13.22	12.55	111.8	106.1
	17.74	19.25	19.32	108.5	108.9
	23.65	25.45	24.56	107.6	103.8

## 8.5 Limitations of Method, Interfering Substances and Ruggedness

No known interferences exist with this method for the detection and quantitation of AA, GA, and EO adducts in the blank sample. However, other known and unknown compounds are detected with this method at different chromatographic retention times. Poor chromatographic separation may cause interferences with these compounds, so chromatographic retention times of the analytes need to be monitored.

AA, GA, and EO adducts are formed due to exposure coming from different sources such as food, smoking and certain occupational activities related to handling AA and EO. The adduct concentrations measured with this method do not provide any information about the exposure source(s).

Ruggedness testing is performed to determine external influences such as temperature and pH that may affect the measurement result. Five critical elements within the method measurement are evaluated and tested for ruggedness. The following parameters were assessed:

1. Temperature: The temperature of the reaction as described in Section 6.7 should be within the range of 45°C to 65°C. The imprecision was highest mostly for temperatures other than 55°C.
2. Formamide volume: The target formamide volume was set to 1.5 mL. No change in method performance was observed at 1 mL and 2 mL volumes.
3. pH of acid-adjusted formamide: The pH of acid-adjusted formamide as described in Section 4.2 should be within the pH range of 5.5 to 7.5. Decrease in method performance was observed at pH 8.5.
4. Hemoglobin levels: The accuracy of the results will not be affected when the blood samples are diluted  $\leq 1:1$  with saline. Higher dilutions lead to inaccurate results.
5. Freezing and thawing: 3 Freeze-thaw cycles do not affect the total Hb measurement.

## 8.6 Stability of the Analytes

The stability of the analytes was evaluated by measuring low and high QC pools and calibrator stock solutions at time of preparation and after storage at different temperatures (-70 °C for 1 years, 20 °C for 4 hours, and 4 °C for 48 hours). The stability of the analytes in processed samples at 4 °C was evaluated at time of preparation and storage up to 20 days. Data for stability has shown in Appendix A Table 15.

## 9 QUALITY ASSESSMENT AND PROFICIENCY TESTING

### 9.1 Quality Control Procedures

#### 9.1.1 Quality Control Materials

##### Bench QC Materials

The bench QC pools used in this method are comprised of three levels of concentrations, spanning from the “low-normal” to “high-normal” ranges of the analytes of interest. The intent of bench QC is for the analyst to evaluate the performance of the analytical system. The bench QC specimens are inserted in each sample batch and processed the same as the patient specimens.

Depending on the specimen type being used for analysis, three levels of erythrocyte controls or whole blood controls are analyzed in duplicates in each run as bench QC materials.

Hb AA adducts ranges of 73 - 94 pmol/g Hb(low QC pool), 99 – 144 pmol/g Hb(medium QC pool) and 137-197 pmol/g Hb(high QC pool) are targeted within two SD.

Hb GA adducts ranges of 25 - 40 pmol/g Hb(low QC pool), 49 – 75 pmol/g Hb(medium QC pool) and 68-100 pmol/g Hb(high QC pool) are targeted within two SD.

Hb EO adducts ranges of 108 - 144 pmol/g Hb(low QC pool), 143 – 221 pmol/g Hb(medium QC pool) and 184 - 273 pmol/g Hb(high QC pool) are targeted within two SD.

The QC pools are prepared by selecting and pooling blood from non-smoking individuals and smoking individuals. Erythrocytes, or whole blood (1,000 µL), are aliquotted into 2.0 mL cryovials, capped, and frozen. The QC pools are stored at -70 °C and are stable for at least 5 years. Means plus range limit for all pools are established by analyzing duplicates for at least 20 consecutive runs to characterize the pools.

The QC materials for tHB measurements provided by the assay manufacturer are used and assessed according to the manufacturer’s recommendations. An in-house characterization is also performed to assess the manufacturer’s values. Duplicate measurements for at least 20 consecutive runs are established and means and limits are

calculated. If results vary from the manufacturer's values, the in-house characterized values are assigned as total Hb limits.

### **Blind QC Materials**

Blind QC samples are QC materials placed in vials, labeled, and processed so that they are indistinguishable from the subject samples handled by the analyst. The supervisor and/or the designated quality assurance officer decodes and reviews the results of the blind specimens without the analyst knowing of their presence in the runs. At least one low-normal concentration and one high-normal concentration QC material should be run in the laboratory for this purpose.

#### **9.1.2 Quality Control Limit Evaluation**

The results from the QC pools are assessed after each run. Acceptance criteria for values obtained with the bench QC materials "QC limits" are established according to the procedure described by Caudill et al. (35) and DLS PM Section 6.

The measurements are declared "in control" if all three QC results are within 2 Si limits.

If one of the three QC results is outside the 2 Si limit, then apply the rules below and reject if any condition is met - the run is then declared "out of control":

- (a) 1 3S Rule—Run mean is outside a 3 Si limit
- (b) 2 2S Rule—Two or more of the three run results are outside the same 2 Si limit
- (c) 10 Xbar Rule—Current and previous nine run results are on the same side of the characterization mean
- (d) R 4S Rule—two consecutive standardized\* run results differ by more than 4 Si (i.e. 95 percent range limit).

\* Standardized results are used because different SLQIDs have different means.

**Note:** Since runs have a single result per SLQCID for 3 SLQCIDs, comparison of results for the R 4S rule will be with the previous result within the current run or with the last result of the previous run.

A QC program written in SAS is available from the DLS Quality Assurance Officer and should be used to apply these rules to QC data and generate Shewhart QC charts. No results for a given analyte are to be reported from an analytical run that has been declared "out of control" for that analyte as assessed by internal (bench) QC.

The initial limits of the QC materials are established by analyzing pool material in 20 consecutive runs. When necessary, limits are updated to include more runs.

While a study is in progress, electronic copies of the QC results from each run are stored in the analyte-specific folder. Electronic copies of the tracking of the QC results over time are stored in the analyte-specific folder. A hardcopy of the QC results from each run is also kept by the person responsible for data checking.

### 9.1.3 Remedial Action if Calibration or QC Systems Fail to Meet Acceptable Criteria

If the calibration or QC systems fail to meet acceptable criteria, suspend all operations until the source or cause of failure is identified and corrected. If the source of failure is easily identifiable, (e.g., failure of the mass spectrometer or a pipetting error) the problem is immediately corrected. Otherwise, additional assessments such as evaluating function and operation of each individual instrument used with this method are performed. Before beginning another analytical run, several QC materials (in the case of QC failure) or calibration standards (in the case of calibration failure) are re-analyzed. After reestablishing calibration or quality control, analytical measurements of patient samples can resume. QC failures are reviewed with the supervisors and documented. Measures to prevent re-occurrences of the same problem are taken.

## 9.2 Proficiency Testing (PT)

No commercial proficiency testing/external quality assessment program exists for the analytes reported with this measurement procedure. Currently, CDC is the only laboratory performing this method as described. Therefore, the PT scheme for this method is administered by an in-house Proficiency Testing Coordinator according to CLSI guideline GP29-A (36). Five proficiency testing pools spanning the full range of analyte values likely to be encountered in human specimens are prepared in-house, and they are then characterized by measuring 40 separate vials from each pool in at least 20 different runs. The mean and standard deviation (SD) of each run is obtained from these measurements.

PT challenges will be performed twice per year (once every 6 months). For that, the Proficiency Testing Coordinator will randomly select 5 vials for use in a particular proficiency testing challenge.

When these PT samples have been analyzed, the results will be given to the PT Coordinator, who will check the data to see if at least 4 of the 5 results for each analyte are within the set limits (80% is considered passing as described in the CLIA regulations). The limits are determined using the characterization mean of the appropriate pool plus or minus a factor multiplied by the appropriate pool SD. The value of the factor will correspond to a 0.01 two-sided significance level adjusted for the number of analytes.

The tHB PT is performed through an external proficiency test provider.

## 10 REFERENCE RANGES (NORMAL VALUES)

Population-based reference ranges have been established. In this study, samples were analyzed from the NHANES study of 5,686 samples (men, women, and children age: 3-60+ years) and were analyzed for AA, GA adducts (37). In non-smokers, the range was found to be 32.1 to 89.6 pmol/g Hb, 28.7 to 114.0 pmol/g Hb, and 19.6 to 312.2 pmol/g Hb for AA, and GA adducts, respectively. In smokers, the range was found to be 50.7 to 277.0



pmol/g Hb, 43.7 to 235 pmol/g Hb, and 23.4 to 576.8 pmol/g Hb for AA and GA adducts, respectively.

Reference ranges for EO adducts were adapted from the NHANES 2013-2016 reported data. The values are from an NCEH dataset generated by DLS statisticians: the geometric mean concentrations for EO adducts is 27.38 pmol/g for non-smokers and is 150.93 pmol/g for smokers (range: 9.2 – 458.84 pmol/g Hb).

## **11 TEST RESULT REPORTING SYSTEM**

Results are reported to 3 significant digits based on assay sensitivity calculations. Data is reported in pmol of adduct per gram of total hemoglobin.

The test reporting system as described in the DLS Policies and Procedures Manual is used when reporting test results. The system consists of review steps at multiple levels, such as results verification by a DLS statistician and DLS management.

## **12 ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

If the analytical system fails, it is recommended that the specimens be stored at -70 °C until the analytical system is restored to functionality.

No alternate testing method exists for the measurement procedure.

## **13 PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Following successful completion of analysis, remaining samples will be retained until all results have been reported and sufficient time has passed for review of the results. After this time, samples are either returned to the contact person who requested the analysis or are treated according to DLS and CDC policy.

Standard record keeping (e.g., database, notebooks, data files) is used to track specimens. Records (including related QA/QC data) are maintained for 3 years, and duplicate records are kept off-site in electronic format. Study subject confidentiality is protected by providing personal identifiers only to the medical officer if needed or remain with the contact person who requested the analyses.

## **14 TRANSFER OR REFERRAL OF SPECIMENS**

Transfer or referral of specimens will follow the procedures outlined in the DLS Policies and Procedures Manual.

## **15 CRITICAL CALL RESULTS (PANIC VALUES); PROTOCOL FOR REPORTING CRITICAL CALLS**

Currently, no information is available linking Hb adduct data to certain health outcomes.

Test Result Reporting System; Protocol for Reporting Critical Calls Not applicable  
Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

## **16 PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTING INADEQUATELY PREPARED SLIDES**

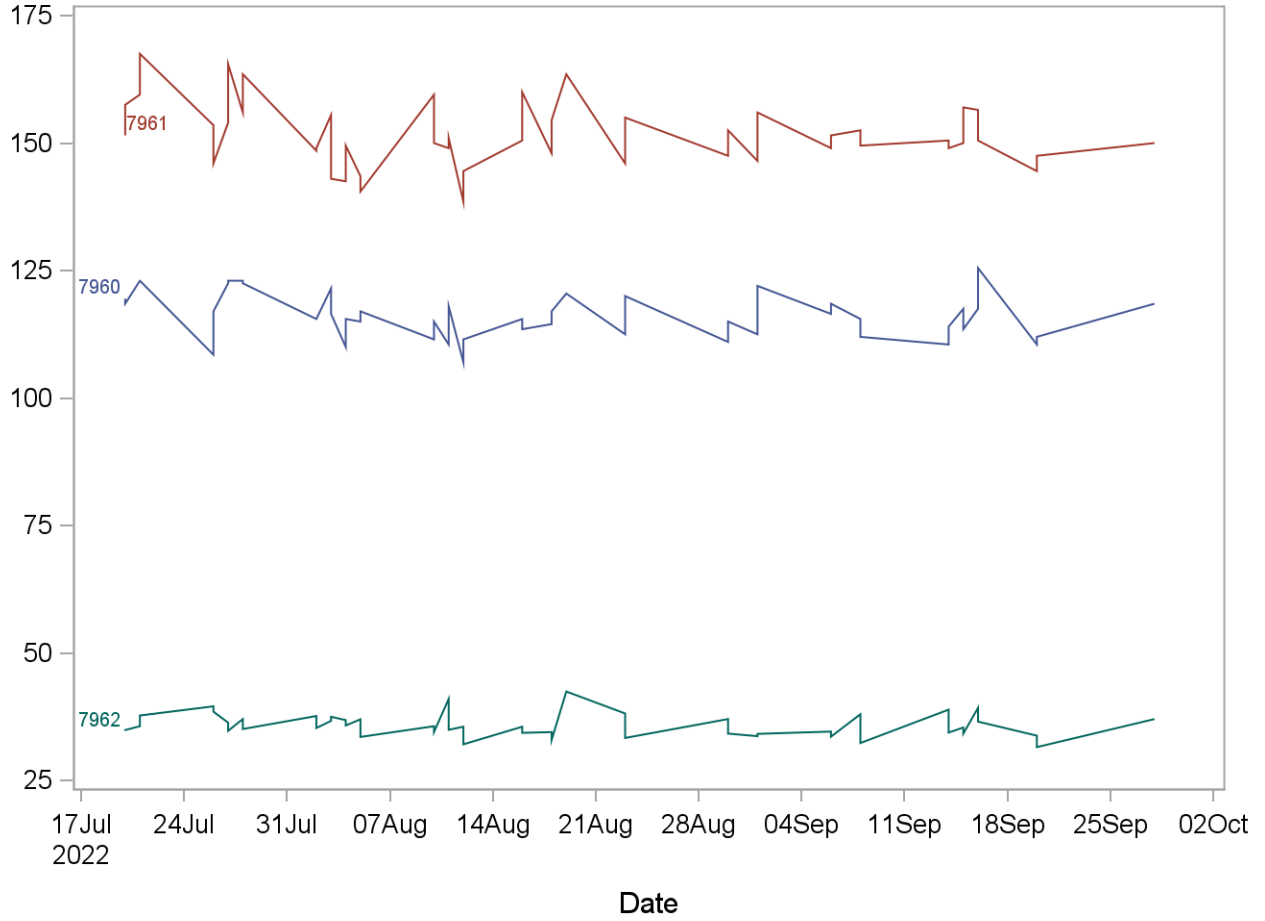
Not applicable for this procedure.

## **17 SUMMARY STATISTICS AND QC GRAPH**

Please see following page.

### 2019-2020 Summary Statistics and QC Chart LBXEOA (Ethylene Oxide (pmol/g Hb))

Lot	N	Start Date	End Date	MEAN	Standard Deviation	Coefficient of Variation
7960	48	20JUL22	28SEP22	116.177	4.433	3.8
7961	48	20JUL22	28SEP22	151.604	6.371	4.2
7962	48	20JUL22	28SEP22	35.802	2.265	6.3



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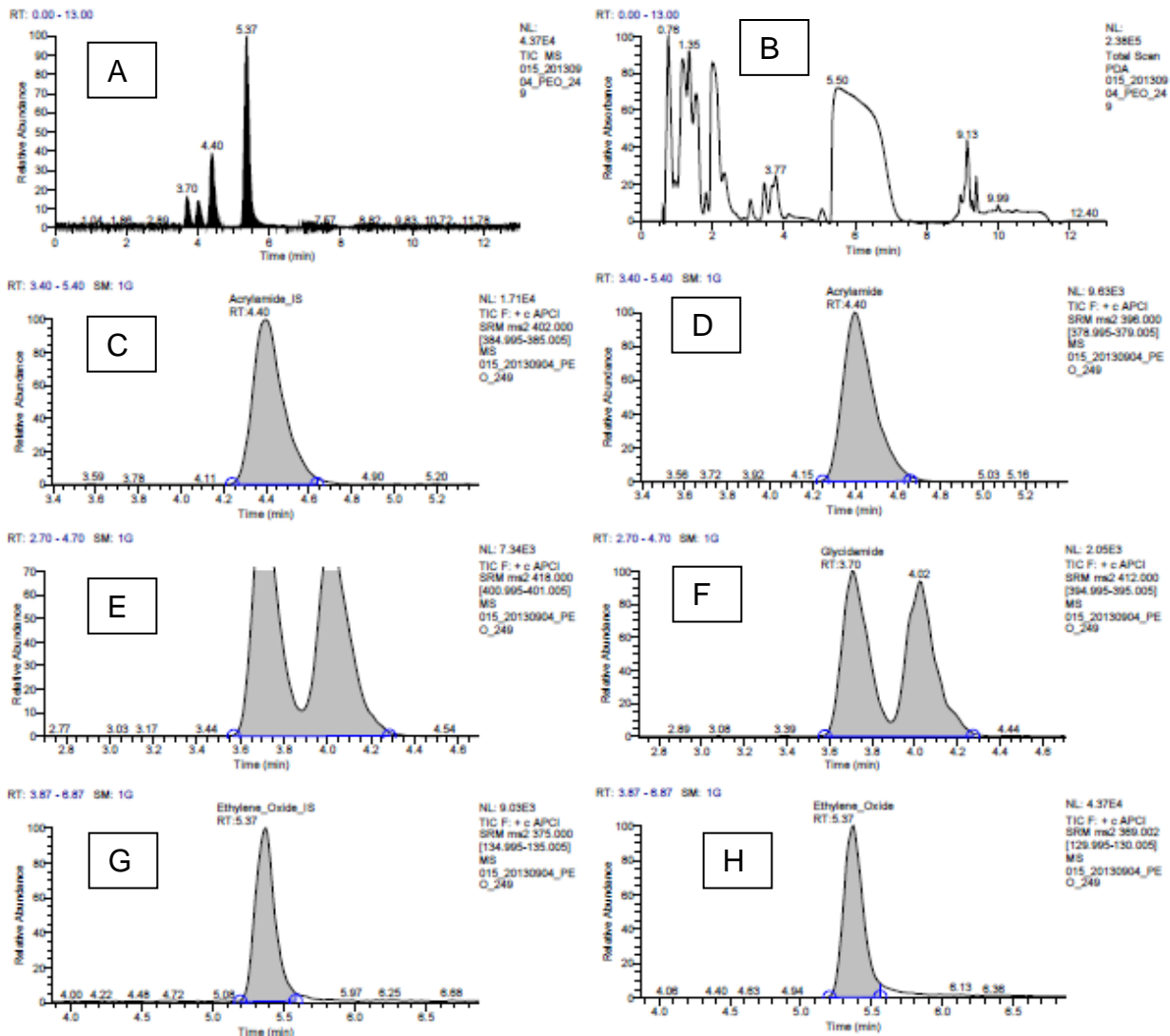
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## **19 APPENDICES**

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- Appendix 3. Symbols, Abbreviations, Terminology
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- Appendix 5. Location of Information as Required by CLIA
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## Appendix 1. AA, GA, and EO adduct chromatography



- A: Total Ion Chromatogram
- B: PDA Chromatogram
- C: AA Internal Standard Adduct Chromatogram
- D: AA Adduct Chromatogram
- E: GA Internal Standard Adduct Chromatogram
- F: GA Adduct Chromatogram
- G: EO Internal Standard Adduct Chromatogram
- H: EO Adduct Chromatogram



## Appendix 2. Related Documents

1. DLS Policies and Procedures Manual. [..\Data\CLIA\\_QA\\_PPM related\AA CLIA test\DLS Policy and Procedures Manual \DLS\\_PoliciesandProceduresManual-07.18.2012.pdf](..\Data\CLIA_QA_PPM related\AA CLIA test\DLS Policy and Procedures Manual \DLS_PoliciesandProceduresManual-07.18.2012.pdf)
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### Appendix 3. Symbols, Abbreviations, Terminology

<b><sup>13</sup>C<sub>5</sub></b>	Labeled carbon (5) isotope
<b><sup>15</sup>N<sub>1</sub></b>	Labeled nitrogen (1) isotope
<b>APCI</b>	Atmospheric Pressure Chemical Ionization
<b>CC</b>	Calibration Curve
<b>CCB</b>	Clinical Chemistry Branch
<b>CDC</b>	Centers for Disease Control and Prevention
<b>CHP</b>	Chemical Hygiene Plan
<b>CLIA</b>	Clinical Laboratory Improvement Act/Amendment
<b>CLSI</b>	Clinical and Laboratory Standards Institute
<b>CV</b>	Coefficient of Variant
<b>DI</b>	De-ionized
<b>dL</b>	Deciliter
<b>DLS</b>	Division of Laboratory Sciences
<b>DNA</b>	Deoxyribonucleic Acid
<b>EDMAN</b>	Pentafluorophenyl Isothiocyanate
<b>EDTA</b>	Ethylenediaminetetraacetic Acid
<b>FA</b>	Formamide
<b>FDA</b>	Food and Drug Administration
<b>g</b>	Grams
<b>Hb</b>	Hemoglobin
<b>HC</b>	HemoCue
<b>HIV</b>	Human Immunodeficiency Virus
<b>HPLC</b>	High Performance Liquid Chromatography
<b>HRS</b>	Hemoglobin Reagent Set
<b>Hz</b>	Hertz
<b>IARC</b>	International Agency for Research on Cancer
<b>ID</b>	Identifier
<b>IS</b>	Internal Standards
<b>ISO</b>	International Organization for Standardization
<b>ITSO</b>	Information Technology Service Office
<b>L</b>	Liter
<b>LC-MS/MS</b>	Liquid Chromatography/Tandem Mass Spectrometry
<b>LOD</b>	Limit of Detection
<b>LOQ</b>	Limit of Quantitation
<b>mg</b>	Milligram
<b>mL</b>	Milliliter
<b>mm</b>	Millimeter
<b>MSDS</b>	Material Safety Data Sheets
<b>MTP</b>	Microtiter Plate
<b>N/A</b>	Not Applicable
<b>NaOH</b>	Sodium Hydroxide
<b>NCEH</b>	National Center of Environmental Health
<b>NHANES</b>	National Health and Nutrition Examination Survey

<b>nm</b>	Nanometer
<b>OHS</b>	Occupational Health and Safety
<b>OSHA</b>	Occupational Safety and Health Administration
<b>PBL</b>	Protein Biomarker Laboratory
<b>pH</b>	Negative Logarithm of the Molar Concentration of Dissolved Hydronium Ions.
<b>PM</b>	Preventative Maintenance
<b>pmol</b>	Picomole
<b>psi</b>	Pounds Per Square Inch
<b>PT</b>	Proficiency Testing
<b>Q0-Q3</b>	Quadrupole Mass Analyzers
<b>QA</b>	Quality Assurance
<b>QC</b>	Quality Control
<b>RBC</b>	Red Blood Cells
<b>RPM</b>	Revolutions Per Minute
<b>SAS</b>	Statistical Analysis Software
<b>SD</b>	Standard Deviation
<b>SRM</b>	Selected Reaction Monitoring
<b>tHB</b>	Total Hemoglobin
<b>umol</b>	Micromole
<b>v/v</b>	Volume concentration
<b>VHLTPEEK</b>	Peptide (Valine, Histidine, Leucine, Threonine, Proline, Glutamic Acid, Glutamic Acid, Lysine)
<b>WI</b>	Work Instructions

### Terminology

The terminology defined in CLIA '88 (57 FR 7139 Subpart A Sec Sec. 493.2) is used in this document. Otherwise the terminology described in the Clinical and Laboratory Standards Institute's terminology database was used. The database can be accessed at: ([http://www.clsi.org/Content/NavigationMenu/Resources/HarmonizedTerminologyDatabase/Harmonized\\_Terminolo.htm](http://www.clsi.org/Content/NavigationMenu/Resources/HarmonizedTerminologyDatabase/Harmonized_Terminolo.htm))

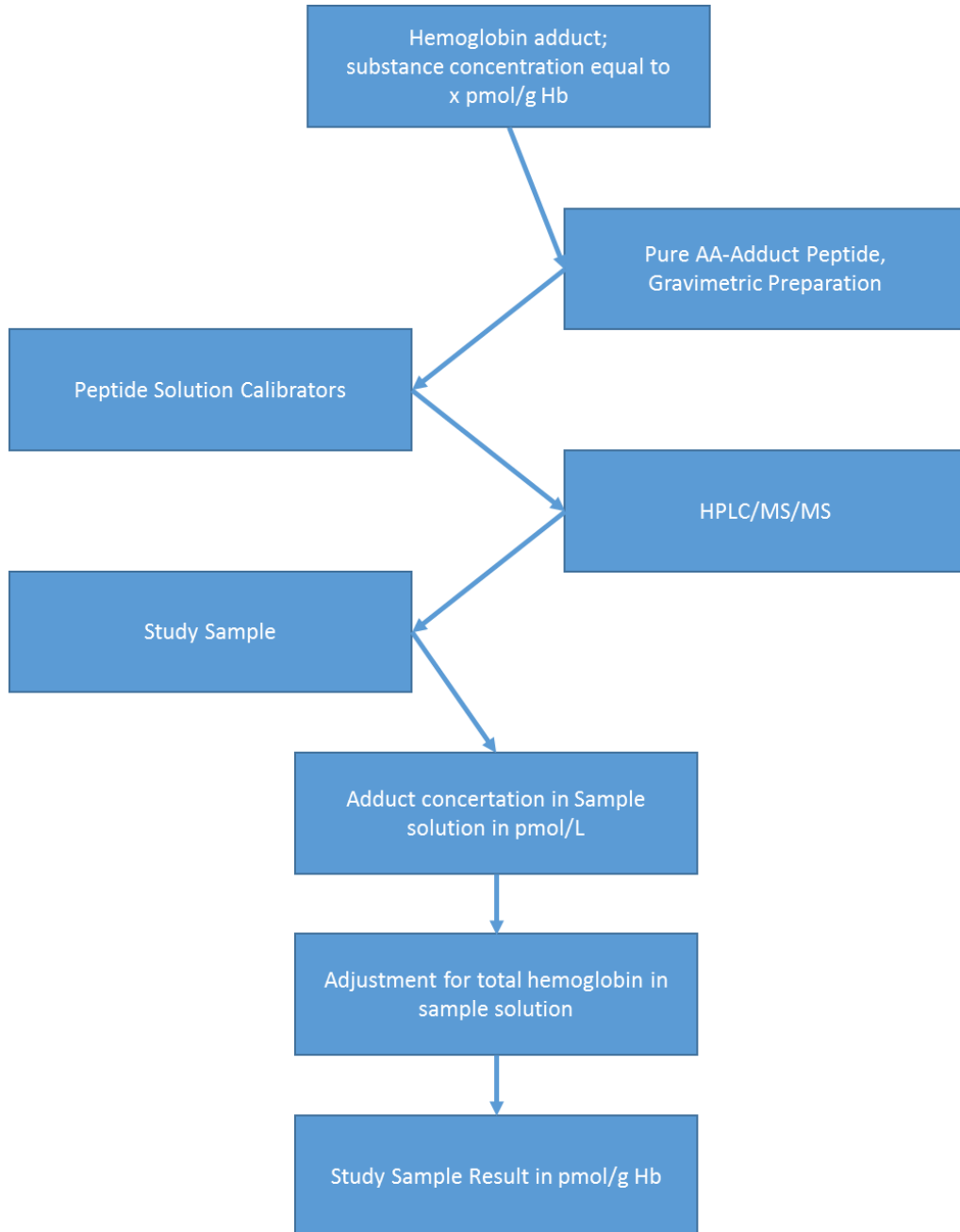
**Appendix 4.** Location of information required by the DLS P& PM.

<b>Required Section</b>	<b>Section in this Document</b>
Requirements for Specimen Collection and Processing, Including Criteria for Specimen Rejection	5
Step-by-Step Performance of the Procedure, Including Test Calculations and Interpretation of Results	0
Preparation of Reagents, Calibrators, Controls, Solutions and Other Materials Used in Testing	0
Calibration and Calibration Verification Procedures	7
Reportable Range for Patient Test Results	8.1
Quality Control Procedures, Including PT Materials and Programs/Procedures Used	8-9
Remedial Action to be Taken When Calibration or Control Results are Outside Acceptable Limits	9.1.3
Limitation in Methods, Including Interfering Substances	8.5
Reference Range (normal values)	10
Life-Threatening or "Panic Values"	15
Pertinent Literature References	17
Specimen Storage Criteria	5.2, 7.1
Protocol for Reporting Panic Values	15
Course of Action if Test System Becomes Inoperable	9.1.3, 12
Criteria for Referral of Specimens (usually not needed)	14
Safety Considerations for Performing the Method	0

## Appendix 5: PBL WORK INSTRUCTIONS FOR THE METHOD

Work Instruction Number	Document Title
PBLW11LT01	Creating Laboratory Waste Tickets
PBLW11PM01	TSQ Operation, Preventive Maintenance, and MS Tune and Calibration
PBLW11PM02	Eppendorf Centrifuge Operation and Preventive Maintenance
PBLW11PM03	Eon Spectrometer Operation and Preventive Maintenance
PBLW11PM04	GeneVac Operation and Preventive Maintenance
PBLW11PM05	Tecan General Operation and Preventive Maintenance
PBLW11PM06	Ultavap Mistral Operation and Preventive Maintenance
PBLW11PM07	Glascol Operation and Preventive Maintenance
PBLW11PM08	ROSS pH Electrode and pH meter Operation, Calibration, and Preventive Maintenance
PBLW11PM09	HemoCue Operation and Preventive Maintenance
PBLW11PM10	Thermomixer Operation and Preventive Maintenance
PBLW11PM11	Plate Sealer Operation and Preventive Maintenance
PBLW11RP01	Preparation of Tecan Solvent Wash Solution
PBLW11RP02	Silanization of Glassware
PBLW11RP03	Preparation of ACS Calibrators, tHb standards, and HRS Solution for tHb measurement
PBLW11SH01	Sample Log
PBLW11SH02	RBC Isolation Procedure
PBLW11SH03	Isolation of Globin
PBLW01DT01	Xcalibur Integration Procedure for Test Method 1015
PBLW01DT02	Indigo Ascent Integration Procedure for Test Method 1015
PBLW01DT03	Data Calculation and Results Interpretation for Method 1015
PBLW01DT04	1015 Error codes
PBLW01MS01	Tecan Usage for Method 1015
PBLW01MS02	HPLC/MS Setup for Method 1015
PBLW01RP01	MS Instrument Standard for 1015
PBLW01RP02	Preparation of Mobile Phases for LC/MS/MS for Method 1015
PBLW01RP03	Internal Standard for Method 1015
PBLW01RP04	Preparation of AAGAEO Calibrators
PBLW01RP05	pH Adjusted Formamide Solution
PBLW01RP06	Preparation of Extraction Solution and Extraction Plate
PBLW000001	Procedure for Barcode Labeling
PBLW000002	Review Export NHANES Data to Westat
PBLW000003	Network Structure
PBLW000004	Pipette Verification Procedure
PBLW000005	Sample Log in to Starlims
PBLW000006	Starlims Data upload Procedure
PBLW000007	Reporting a Run
PBLW000009	Procedure for IRB Submission
PBLW000010	Method Valid. Procedure
PBLW000011	Balance Operation
PBLP000001	Report, Policy, WI, Coding Guide within PBL and LRL
PBLP110001	Policy on Laboratory Notebooks for the Adducts Group

## Appendix 6: Metrological Traceability of Acrylamide, Glycidamide and Ethylene Oxide Measurements



## Appendix 7: List of tables

<b>Table Numbers</b>	<b>Name</b>	<b>Section of SOP</b>
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Table 3	Dilution Scheme for AA and GA Octapeptide	4.3.1
Table 4	Preparation of the Total Hb Calibration Curve	4.3.2
Table 5	Desired Internal Standard Concentrations for AA, GA and EO Octapeptide Solution Concentrations (nmol/L)	4.3.2
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Table 7	UHPLC Gradient Conditions for Pump B	6.10
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Table 14c	Summary: Accuracy using Spike Recovery	Appendix A
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Table 15b	1015 Method Precision for GA adduct	Appendix A
Table 15c	1015 Method Precision for EO adduct	Appendix A
Table 16a	AA Adduct Quality Control Sample Stability	Appendix A
Table 16b	GA Adduct Quality Control Sample Stability	Appendix A
Table 16c	EO Adduct Quality Control Sample Stability	Appendix A

## Appendix 8: HRS Solution Preparation



### TECO DIAGNOSTICS

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### HEMOGLOBIN REAGENT SET

#### INTENDED USE

Hemoglobin reagent set is used for the quantitative determination of hemoglobin in human blood.

#### INTRODUCTION

Hemoglobin is a porphyrin-iron (II) protein compound that transports oxygen from the lungs to body tissues where it is utilized for energy metabolism. Measurements of hemoglobin from venous or capillary blood aid in the detection of a variety of conditions which alter the normal hemoglobin concentration of blood, e.g. anemia or polycythemia. The determination of iron content in whole blood is the most accurate method for assessing blood hemoglobin. Of the various methods used, cyanmethemoglobin is the most widely accepted. It is this internationally adapted method that is employed in this procedure.<sup>1</sup>

#### PRINCIPLE

In the cyanmethemoglobin method, erythrocytes are lysed by a stromatolytic agent in the presence of a surfactant and release their hemoglobin into solution. Hemoglobin is oxidized to methemoglobin by ferricyanide, and the methemoglobin is converted into the stable cyanmethemoglobin by addition of KCN. The absorbance of cyanmethemoglobin is measured at 540 nm and color intensity is proportional to hemoglobin concentration.<sup>2</sup>

#### REAGENT COMPOSITION

When reconstituted as directed, the reagent for hemoglobin contains the following:

1. Hemoglobin reagent: Potassium ferricyanide 0.5 mM, potassium cyanide 0.7 mM, buffers and stabilizers included.
2. Standard: Methemoglobin (60 mg/dl) dissolved in cyanmethemoglobin reagent. This amount is equivalent to 15.0 g/dl hemoglobin. This standard has been referenced against a CAP (College of American Pathologists) certified standard to its concentration and further checked by using the known molar absorptivity of cyanmethemoglobin.

#### WARNINGS AND PRECAUTIONS

1. For *in vitro* diagnostic use only.  
**CAUTION:** *In vitro* diagnostic reagents may be hazardous. Handle in accordance with good laboratory procedures which dictate avoiding ingestion, and eye or skin contact.
2. Contains cyanide. Poison - may be fatal if swallowed. **DO NOT PIPETTE BY MOUTH.**
3. Do not mix with acids. Discarding with large volumes of water.
4. Specimens should be considered infectious and handled appropriately.
5. Use distilled or deionized water where indicated.

#### REAGENT PREPARATION

Reagent comes in a ready to use form.

#### REAGENT STORAGE AND STABILITY

Store the hemoglobin reagent and standard at room temperature (15 - 30°C).

#### REAGENT DETERIORATION

Do not use hemoglobin reagent if:

1. It has become a different color than yellow.
2. The reagent becomes turbid or a precipitation forms.

#### SPECIMEN COLLECTION

1. Use whole blood with EDTA as an anticoagulant.
2. Oxalate, citrate or heparin may also be used as anticoagulants.
3. Capillary or venous blood may be collected if used before clotting occurs.
4. Whole blood mixed well with an anticoagulant appears stable for one (1) week at room temperature (15 - 30°C).

#### INTERFERING SUBSTANCES

1. Substances that cause turbidity will falsely elevate the hemoglobin value. These include lipids, abnormal plasma proteins (macroglobulinemia) or erythrocyte stroma.
2. A review by Young *et al.* reveals the numerous drugs that exert an *in vivo* effect to decrease blood hemoglobin.<sup>3</sup>

#### MATERIALS REQUIRED BUT NOT PROVIDED

1. Accurate pipetting devices
2. Timer
3. Test tubes/rack
4. Spectrophotometer with ability to read at 540 nm

#### GENERAL INSTRUCTIONS

The reagent for Hemoglobin is intended for use either as an automated procedure on chemistry instruments or as a manual procedure on a suitable spectrophotometer.

#### AUTOMATED PROCEDURE

Refer to appropriate application manual available.

#### MANUAL PROCEDURE

1. Dispense 2.0 ml of hemoglobin reagent into test tubes labeled "blank", "control", "patient", etc.
2. Place 0.01 ml (10 µl) of sample into respective tubes. Mix.
3. Allow all tubes to stand for three (3) minutes at room temperature.
4. To a tube labeled standard, place 2.0 ml of standard.
5. Set spectrophotometer to 540 nm and zero with the reagent blank. (Wavelength range: 520 - 550 nm).
6. Read and record absorbance values of all tubes.
7. See CALCULATIONS to obtain values.

#### NOTES:

1. For spectrophotometers requiring greater volumes for proper reading, use 4.0 ml reagent and 0.02 ml (20 µl) sample. Follow above instructions.
2. Final color appears quite stable but should be read within one (1) hour to avoid evaporation.

#### LIMITATIONS

1. This procedure measures hemoglobin and its derivatives except sulfhemoglobin.
2. Specimens with values above 20.0 g/dl must be re-run using one half the sample volume. Multiply final results by two (2).

#### CALIBRATION

Use hemoglobin standard provided.



**CALCULATIONS**

Abs. = Absorbance

$\frac{\text{Abs. of unknown}}{\text{Abs. of standard}} \times \text{Conc. of Standard (g/dl)} = \text{Value (g/dl)}$

Abs. of standard

*Example:* If a 15 g/dl standard has an absorbance of 0.602 and the absorbance of the unknown is 0.480 then:

$$\frac{0.480}{0.602} \times 15.0 = 11.9 \text{ g/dl}$$

**QUALITY CONTROL**

It is recommended that controls be included in each set of assays. Commercially available control material with established hemoglobin values may be routinely used for quality control. The assigned value of the control material must be confirmed by the chosen application. Failure to obtain the proper range of values in the assay of control material may indicate either reagent deterioration, instrument malfunction, or procedural errors.

**EXPECTED VALUES<sup>4,5</sup>**

Adult Males	13.0 - 18.0 g/dl
Adult Females	11.0 - 16.0 g/dl
Children	10.0 - 14.0 g/dl
Newborns	14.0 - 23.0 g/dl

Factors such as age, race, exercise, season and altitude are reported to influence the values of normal ranges. The above range should serve only as a guideline. Each laboratory should establish its own range.

**PERFORMANCE CHARACTERISTICS**

- Linearity:** 20 g/dl.
- Sensitivity:** Based on an instrument resolution of 0.001 absorbance, the present procedure has a sensitivity of 0.03 g/dl.
- Comparison:** Studies conducted against a similar procedure yielded a coefficient of correlation of 0.98 with a regression equation of  $y = 1.03x - 0.48$  on samples with values from 7.2 to 17.9 g/dl (n= 20).
- Precision:**

*Within Run:* Two samples of human blood were assayed twenty (20) times and the following within run precision was obtained.

	<u>Mean (g/dL)</u>	<u>S.D.</u>	<u>C.V.%</u>
Normal	13.8	0.6	4.6
Abnormal	10.2	0.3	3.4

*Run-to-Run:* Two samples of human blood were assayed for five (5) consecutive days and the following run to run precision was obtained.

	<u>Mean (g/dL)</u>	<u>S.D.</u>	<u>C.V.%</u>
Normal	14.3	12.3	0.5
Abnormal	12.3	0.5	4.3

**REFERENCES**

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**H526: 11/01**

Manufactured by:



TECO DIAGNOSTICS  
1268 N. LAKEVIEW AVE.  
ANAHEIM, CA 92807  
U.S.A.



Authorized Representative:  
Emergo Europe  
P.O. Box 149  
4300 AC Zierikzee  
The Netherlands

## Appendix A: Method Performance Documentation

**Table 13:** LOD, specificity and fit for intended use

Analytes	Limit of Detection (LOD)	Interferences successfully checked in at least 50 human samples	Accuracy, precision, LOD, specificity and stability meet performance specifications for intended use
AA	3.9 pmol/g	yes	yes
GA	4.9 pmol/g	yes	yes
EO	12.9 pmol/g	yes	yes

**Table 14a: Accuracy using Spike Recovery in Low Pool**

Measured concentration of Low Pool									
AA	Replicate	Expected pmol	Day 1 pmol	Mean pmol	Recovery (%)	Day 2 pmol	Mean pmol	Recovery (%)	Mean Recovery (%)
Sample	1	0	2.77	2.73	N/A	2.54	2.58	N/A	N/A
	2		2.57			2.78			
	3		2.86			2.4			
Sample + Spike 1	1	3.16	5.96	5.86	98.83	5.81	5.81	102.33	100.6
	2		5.70			5.81			
	3		5.91			5.82			
Sample + Spike 2	1	4.74	7.76	7.62	103.04	6.5	6.64	85.63	94.3
	2		7.48			6.80			
	3		7.61			6.61			
Sample + Spike 3	1	6.32	9.10	9.27	103.53	8.07	8.80	98.49	101.0
	2		9.36			9.05			
	3		9.36			9.29			
GA	Replicate	Expected pmol	Day 1 pmol	Mean pmol	Recovery (%)	Day 2 pmol	Mean pmol	Recovery (%)	Mean Recovery (%)
Sample	1	0	1.31	1.33	N/A	1.15	1.22	N/A	N/A
	2		1.34			1.24			
	3		1.34			1.3			
Sample + Spike 1	1	2.84	4.10	4.25	102.79	3.99	3.92	95.11	98.9
	2		4.29			3.86			
	3		4.36			3.93			
Sample + Spike 2	1	4.26	5.35	5.59	99.99	5.47	5.53	101.00	100.5
	2		5.75			5.56			
	3		5.67			5.54			
Sample + Spike 3	1	5.68	7.05	7.24	104.02	6.74	6.61	94.85	99.4
	2		7.30			6.37			
	3		7.36			6.71			
EO	Replicate	Expected pmol	Day 1 pmol	Mean pmol	Recovery (%)	Day 2 pmol	Mean pmol	Recovery (%)	Mean Recovery (%)
Sample	1	0	4.92	4.70	N/A	4.28	4.35	N/A	N/A
	2		4.17			4.26			
	3		5.01			4.5			
Sample + Spike 1	1	11.83	18.61	18.44	116.21	16.45	17.04	107.36	111.8
	2		17.57			17.13			
	3		19.16			17.56			
Sample + Spike 2	1	17.74	24.65	24.95	114.13	22.6	22.61	102.92	108.5
	2		26.15			21.93			
	3		24.04			23.27			
Sample + Spike 3	1	23.65	31.05	30.89	110.74	30.22	29.06	104.47	107.6
	2		30.56			28.32			
	3		31.06			28.64			

**Table 14b:** Accuracy using Spike Recovery in High Pool

Measured concentration of High Pool									
AA	Replicate	Expected pmol	Day 1 pmol	Mean pmol	Recovery (%)	Day 2 pmol	Mean pmol	Recovery (%)	Mean Recovery (%)
Sample	1	0	6.92	7.17	N/A	6.45	6.63	N/A	N/A
	2		7.55			6.32			
	3		7.04			7.1			
Sample + Spike 1	1	3.16	10.13	10.09	92.27	10.2	9.62	94.73	93.5
	2		9.67			9.27			
	3		10.46			9.41			
Sample + Spike 2	1	4.74	11.74	11.85	98.78	10.7	10.53	82.22	90.5
	2		11.92			10.11			
	3		11.90			10.81			
Sample + Spike 3	1	6.32	14.36	14.16	110.69	14.25	13.77	113.04	111.9
	2		14.57			13.53			
	3		13.57			13.52			
GA	Replicate	Expected pmol	Day 1 pmol	Mean pmol	Recovery (%)	Day 2 pmol	Mean pmol	Recovery (%)	Mean Recovery (%)
Sample	1	0	3.45	3.54	N/A	3.29	3.33	N/A	N/A
	2		3.50			3.26			
	3		3.67			3.5			
Sample + Spike 1	1	2.84	6.51	6.47	103.28	5.9	5.99	93.49	98.4
	2		6.43			6.03			
	3		6.48			6.08			
Sample + Spike 2	1	4.26	8.45	8.28	111.26	7.2	7.38	94.99	103.1
	2		8.16			7.38			
	3		8.24			7.53			
Sample + Spike 3	1	5.68	9.75	9.67	107.85	8.69	8.98	99.37	103.6
	2		9.71			9.14			
	3		9.54			9.09			
EO	Replicate	Expected pmol	Day 1 pmol	Mean pmol	Recovery (%)	Day 2 pmol	Mean pmol	Recovery (%)	Mean Recovery (%)
Sample	1	0	9.87	9.56	N/A	8.18	9.00	N/A	N/A
	2		9.31			9.42			
	3		9.50			9.4			
Sample + Spike 1	1	11.83	22.22	21.82	103.71	21.9	21.84	108.59	106.1
	2		21.30			21.37			
	3		21.95			22.30			
Sample + Spike 2	1	17.74	29.37	29.28	111.18	27.3	27.91	106.59	108.9
	2		29.62			28.19			
	3		28.86			28.21			
Sample + Spike 3	1	23.65	34.69	34.15	103.97	32.45	33.52	103.67	103.8
	2		32.08			33.93			
	3		35.68			34.18			

**Table 14c:** Summary: Accuracy using Spike Recovery

Analyte	Total Mean recovery (%)	SD (%)
AA	98.6	7.7
GA	100.7	2.2
EO	107.0	2.7

**Table 15a: 1015 Method Precision for AA adduct**

Quality material 1						
Run	Result 1 pmol/g	Result 2 pmol/g	Mean pmol/g	SS 1	SS 2	2*mean^2
1	62.94	60.43	61.68	1.57	1.57	7609.45
2	64.09	58.35	61.22	8.24	8.24	7495.94
3	63.78	61.16	62.47	1.72	1.72	7804.12
4	50.41	59.09	54.75	18.81	18.81	5994.87
5	57.97	58.78	58.37	0.16	0.16	6815.00
6	57.69	65.50	61.59	15.25	15.25	7587.50
7	59.87	59.67	59.77	0.01	0.01	7144.52
8	65.84	61.08	63.46	5.67	5.67	8053.88
9	68.37	62.39	65.38	8.94	8.94	8549.61
10	62.37	64.89	63.63	1.59	1.59	8097.81
<b>Grand sum</b>	1224.65	<b>Grand mean</b>	61.23			
			<b>Sum squares</b>	<b>Mean Sq Error</b>	<b>Std Dev</b>	<b>Rel Std Dev (%)</b>
		<b>Within Run</b>	123.93	12.39	3.52	5.75
		<b>Between Run</b>	164.27	18.25	1.71	2.80
		<b>Total</b>	288.20		3.91	<b>6.39</b>
Quality material 2						
Run	Result 1 pmol/g	Result 2 pmol/g	Mean pmol/g	SS 1	SS 2	2*mean^2
1	125.60	133.34	129.47	14.99	14.99	33525.33
2	128.31	133.07	130.69	5.66	5.66	34160.83
3	138.86	140.13	139.50	0.40	0.40	38920.32
4	139.30	131.65	135.47	14.65	14.65	36706.59
5	132.75	136.22	134.49	3.01	3.01	36175.05
6	143.42	155.49	149.46	36.38	36.38	44674.65
7	117.48	135.01	126.24	76.79	76.79	31874.82
8	117.71	133.69	125.70	63.88	63.88	31600.61
9	140.06	139.05	139.55	0.26	0.26	38949.71
10	141.26	136.55	138.91	5.54	5.54	38591.63
<b>Grand sum</b>	2698.98	<b>Grand mean</b>	134.95			
			<b>Sum squares</b>	<b>Mean Sq Error</b>	<b>Std Dev</b>	<b>Rel Std Dev (%)</b>
		<b>Within Run</b>	443.15	44.31	6.66	4.93
		<b>Between Run</b>	956.05	106.23	5.56	4.12
		<b>Total</b>	1399.19		8.68	<b>6.43</b>
Quality material 3						
Run	Result 1 pmol/g	Result 2 pmol/g	Mean pmol/g	SS 1	SS 2	2*mean^2
1	191.34	195.34	193.34	4.00	4.00	74764.20
2	174.41	169.79	172.10	5.33	5.33	59237.59
3	176.70	196.42	186.56	97.18	97.18	69610.47
4	170.73	196.35	183.54	164.19	164.19	67373.14
5	180.66	173.90	177.28	11.44	11.44	62855.99
6	202.08	218.35	210.21	66.18	66.18	88377.54
7	182.77	199.81	191.29	72.64	72.64	73182.87
8	175.45	168.17	171.81	13.24	13.24	59035.11
9	180.42	210.51	195.46	226.35	226.35	76411.70
10	178.94	194.67	186.80	61.89	61.89	69790.48
<b>Grand sum</b>	3736.80	<b>Grand mean</b>	186.84			
			<b>Sum squares</b>	<b>Mean Sq Error</b>	<b>Std Dev</b>	<b>Rel Std Dev (%)</b>
		<b>Within Run</b>	1444.90	144.49	12.02	6.43
		<b>Between Run</b>	2456.58	272.95	8.01	4.29
		<b>Total</b>	3901.48		14.45	<b>7.73</b>

**Table 15b: 1015 Method Precision for GA adduct**

Quality material 1						
Run	Result 1 pmol/g	Result 2 pmol/g	Mean pmol/g	SS 1	SS 2	2*mean^2
1	33.97	34.98	34.48	0.25	0.25	2377.08
2	34.45	33.14	33.79	0.43	0.43	2283.76
3	32.18	30.67	31.43	0.57	0.57	1975.25
4	31.13	30.89	31.01	0.02	0.02	1923.45
5	31.16	30.02	30.59	0.33	0.33	1871.35
6	30.76	29.74	30.25	0.26	0.26	1829.95
7	27.67	29.24	28.46	0.62	0.62	1619.80
8	38.97	36.19	37.58	1.92	1.92	2824.52
9	32.56	35.69	34.13	2.45	2.45	2329.52
10	31.77	39.86	35.81	16.38	16.38	2565.40
<b>Grand sum</b>	655.05	<b>Grand mean</b>	32.75			
			<b>Sum squares</b>	<b>Mean Sq Error</b>	<b>Std Dev</b>	<b>Rel Std Dev (%)</b>
		<b>Within Run</b>	46.43	4.64	2.15	6.58
		<b>Between Run</b>	145.60	16.18	2.40	7.33
		<b>Total</b>	192.03		3.23	<b>9.85</b>
Quality material 2						
Run	Result 1 pmol/g	Result 2 pmol/g	Mean pmol/g	SS 1	SS 2	2*mean^2
1	55.31	59.90	57.60	5.27	5.27	6636.08
2	55.42	59.42	57.42	4.00	4.00	6593.64
3	55.37	53.62	54.50	0.77	0.77	5940.04
4	58.12	53.41	55.77	5.56	5.56	6219.77
5	48.75	52.93	50.84	4.36	4.36	5169.66
6	58.02	64.63	61.33	10.92	10.92	7522.38
7	48.00	55.07	51.54	12.50	12.50	5312.19
8	56.42	55.21	55.82	0.36	0.36	6230.69
9	62.04	61.32	61.68	0.13	0.13	7608.01
10	50.20	58.18	54.19	15.92	15.92	5873.74
<b>Grand sum</b>	1121.35	<b>Grand mean</b>	56.07			
			<b>Sum squares</b>	<b>Mean Sq Error</b>	<b>Std Dev</b>	<b>Rel Std Dev (%)</b>
		<b>Within Run</b>	119.60	11.96	3.46	6.17
		<b>Between Run</b>	234.58	26.06	2.66	4.74
		<b>Total</b>	354.17		4.36	<b>7.78</b>
Quality material 3						
Run	Result 1 pmol/g	Result 2 pmol/g	Mean pmol/g	SS 1	SS 2	2*mean^2
1	98.34	92.39	95.37	8.83	8.83	18189.50
2	95.20	79.02	87.11	65.46	65.46	15175.75
3	82.50	87.37	84.94	5.93	5.93	14428.55
4	87.96	95.37	91.67	13.72	13.72	16805.97
5	82.18	83.27	82.72	0.30	0.30	13686.50
6	97.89	97.98	97.94	0.00	0.00	19183.05
7	84.85	88.27	86.56	2.92	2.92	14984.83
8	81.16	72.37	76.76	19.31	19.31	11784.76
9	83.20	89.18	86.19	8.94	8.94	14858.23
10	92.00	109.14	100.57	73.48	73.48	20228.94
<b>Grand sum</b>	1779.65	<b>Grand mean</b>	88.98			
			<b>Sum squares</b>	<b>Mean Sq Error</b>	<b>Std Dev</b>	<b>Rel Std Dev (%)</b>
		<b>Within Run</b>	397.78	39.78	6.31	7.09
		<b>Between Run</b>	968.95	107.66	5.83	6.55
		<b>Total</b>	1366.73		8.59	<b>9.65</b>

**Table 15c: 1015 Method Precision for EO adduct**

Quality material 1						
Run	Result 1 pmol/g	Result 2 pmol/g	Mean pmol/g	SS 1	SS 2	2*mean^2
1	77.89	83.61	80.75	8.17	8.17	13040.59
2	78.43	77.07	77.75	0.46	0.46	12091.51
3	66.11	63.04	64.57	2.36	2.36	8339.16
4	72.68	68.97	70.82	3.43	3.43	10031.98
5	59.58	58.50	59.04	0.29	0.29	6971.55
6	65.41	78.18	71.80	40.78	40.78	10309.81
7	66.58	69.08	67.83	1.57	1.57	9201.82
8	99.09	92.83	95.96	9.79	9.79	18417.06
9	77.32	78.26	77.79	0.22	0.22	12102.58
10	68.18	88.25	78.22	100.71	100.71	12236.45
<b>Grand sum</b>	1489.07	<b>Grand mean</b>	74.45			
			<b>Sum squares</b>	<b>Mean Sq Error</b>	<b>Std Dev</b>	<b>Rel Std Dev (%)</b>
		<b>Within Run</b>	335.56	33.56	5.79	7.78
		<b>Between Run</b>	1875.42	208.38	9.35	12.56
		<b>Total</b>	2210.98		11.00	<b>14.77</b>
Quality material 2						
Run	Result 1 pmol/g	Result 2 pmol/g	Mean pmol/g	SS 1	SS 2	2*mean^2
1	175.49	162.39	168.94	42.90	42.90	57082.97
2	197.00	182.74	189.87	50.86	50.86	72099.44
3	179.74	178.90	179.32	0.18	0.18	64310.25
4	188.78	178.35	183.56	27.23	27.23	67391.40
5	163.17	171.45	167.31	17.14	17.14	55985.75
6	178.68	212.49	195.59	285.83	285.83	76508.39
7	167.78	168.98	168.38	0.36	0.36	56705.34
8	186.88	182.05	184.47	5.83	5.83	68055.66
9	181.36	190.75	186.06	22.02	22.02	69233.41
10	184.28	177.98	181.13	9.91	9.91	65617.27
<b>Grand sum</b>	3609.25	<b>Grand mean</b>	180.46			
			<b>Sum squares</b>	<b>Mean Sq Error</b>	<b>Std Dev</b>	<b>Rel Std Dev (%)</b>
		<b>Within Run</b>	924.53	92.45	9.62	5.33
		<b>Between Run</b>	1655.00	183.89	6.76	3.75
		<b>Total</b>	2579.54		11.75	<b>6.51</b>
Quality material 3						
Run	Result 1 pmol/g	Result 2 pmol/g	Mean pmol/g	SS 1	SS 2	2*mean^2
1	227.55	245.56	236.56	81.16	81.16	111916.76
2	247.61	213.86	230.73	284.77	284.77	106474.83
3	194.09	206.98	200.53	41.54	41.54	80427.22
4	228.46	233.07	230.77	5.30	5.30	106506.56
5	193.45	197.25	195.35	3.62	3.62	76323.16
6	215.95	231.55	223.75	60.82	60.82	100130.65
7	197.99	204.58	201.29	10.85	10.85	81034.18
8	190.01	181.54	185.77	17.90	17.90	69024.46
9	214.90	207.31	211.11	14.40	14.40	89132.97
10	207.43	239.24	223.34	252.95	252.95	99757.48
<b>Grand sum</b>	4278.39	<b>Grand mean</b>	213.92			
			<b>Sum squares</b>	<b>Mean Sq Error</b>	<b>Std Dev</b>	<b>Rel Std Dev (%)</b>
		<b>Within Run</b>	1546.64	154.66	12.44	5.81
		<b>Between Run</b>	5495.66	610.63	15.10	7.06
		<b>Total</b>	7042.30		19.56	<b>9.14</b>

**Table 16a: AA Adduct Quality Control Sample Stability**

AA adduct pmol/g tHb						
Quality Material 1	A17QC1L02	Initial measurement	Bench-top stability	Three freeze-thaw cycles	Short-Term Stability	Long-Term Stability
Replicate 1		73.62	74.21	77.84	69.59	80.17
Replicate 2		80.52	76.27	72.87	79.05	79.27
Replicate 3		80.12	71.66	80.04	76.50	79.51
QC Char Fixed Mean	69.374	78.09	74.05	76.92	75.05	79.65
<b>% difference from QC Char Fixed Mean</b>		<b>12.6%</b>	<b>6.7%</b>	<b>10.9%</b>	<b>8.2%</b>	<b>14.8%</b>
<b>% difference from the initial measurement</b>			<b>5.2%</b>	<b>1.5%</b>	<b>3.9%</b>	<b>2.0%</b>
Quality Material 2	A17QC2L02	Initial measurement	Bench-top stability	Three freeze-thaw cycles	Short-Term Stability	Long-Term Stability
Replicate 1		123.76	121.86	114.23	124.01	111.73
Replicate 2		125.50	131.31	108.06	131.73	116.95
Replicate 3		135.06	116.46	114.76	127.51	118.70
QC Char Fixed Mean	111.585	128.11	123.21	112.35	127.75	115.79
<b>% difference from QC Char Fixed Mean</b>		<b>14.8%</b>	<b>10.4%</b>	<b>0.7%</b>	<b>14.5%</b>	<b>3.8%</b>
<b>% difference from the initial measurement</b>			<b>3.8%</b>	<b>12.3%</b>	<b>0.3%</b>	<b>9.6%</b>
Quality Material 3	A17QC3L02	Initial measurement	Bench-top stability	Three freeze-thaw cycles	Short-Term Stability	Long-Term Stability
Replicate 1		170.48	164.66	151.33	155.25	171.77
Replicate 2		170.62	176.14	145.78	171.42	151.89
Replicate 3		172.70	180.52	165.63	159.79	157.84
QC Char Fixed Mean	156.471	171.27	173.77	154.25	162.15	160.50
<b>% difference from QC Char Fixed Mean</b>		<b>9.5%</b>	<b>11.1%</b>	<b>1.4%</b>	<b>3.6%</b>	<b>2.6%</b>
<b>% difference from the initial measurement</b>			<b>1.5%</b>	<b>9.9%</b>	<b>5.3%</b>	<b>6.3%</b>
AA adduct pmol/g tHb (Processed Sample)						
Quality Material 1	A17QC1L02	Initial measurement	Processed sample stability			
Replicate 1		81.73	69.40			
Replicate 2		67.87	79.48			
Replicate 3		68.12	70.16			
QC Char Fixed Mean	69.37	72.57	73.01			
<b>% difference from QC Char Fixed Mean</b>		<b>4.6%</b>	<b>5.2%</b>			
<b>% difference from the initial measurement</b>			<b>0.6%</b>			
Quality Material 2	A17QC2L02	Initial measurement	Processed sample stability			
Replicate 1		126.18	109.60			
Replicate 2		104.33	106.99			
Replicate 3		111.28	109.53			
QC Char Fixed Mean	111.58	113.93	108.71			
<b>% difference from QC Char Fixed Mean</b>		<b>2.1%</b>	<b>2.6%</b>			
<b>% difference from the initial measurement</b>			<b>4.6%</b>			
Quality Material 3	A17QC3L02	Initial measurement	Processed sample stability			
Replicate 1		198.89	176.28			
Replicate 2		168.36	194.78			
Replicate 3		143.45	141.82			
QC Char Fixed Mean	156.47	170.23	170.96			
<b>% difference from QC Char Fixed Mean</b>		<b>8.8%</b>	<b>9.3%</b>			
<b>% difference from the initial measurement</b>			<b>0.4%</b>			



**Table 16b: GA Adduct Quality Control Sample Stability**

GA adduct pmol/g tHb						
Quality Material 1	A17QC1L02	Initial measurement	Bench-top stability	Three freeze-thaw cycles	Short-Term Stability	Long-Term Stability
Replicate 1		36.93	35.74	34.48	31.76	33.86
Replicate 2		35.38	30.76	27.82	33.07	37.13
Replicate 3		35.38	31.47	38.68	31.21	32.46
Mean	32.60	35.90	32.66	33.66	32.01	34.48
<b>% difference from QC Char Fixed Mean</b>		<b>10.1%</b>	<b>0.2%</b>	<b>3.3%</b>	<b>1.8%</b>	<b>5.8%</b>
<b>% difference from the initial measurement</b>			<b>9.0%</b>	<b>6.2%</b>	<b>10.8%</b>	<b>3.9%</b>
Quality Material 2	A17QC2L02	Initial measurement	Bench-top stability	Three freeze-thaw cycles	Short-Term Stability	Long-Term Stability
Replicate 1		57.47	60.40	62.54	61.00	60.57
Replicate 2		54.56	63.81	57.12	64.96	63.94
Replicate 3		66.31	60.30	62.65	58.92	57.45
Mean	60.00	59.45	61.50	60.77	61.63	60.65
<b>% difference from QC Char Fixed Mean</b>		<b>0.9%</b>	<b>2.5%</b>	<b>1.3%</b>	<b>2.7%</b>	<b>1.1%</b>
<b>% difference from the initial measurement</b>			<b>3.5%</b>	<b>2.2%</b>	<b>3.7%</b>	<b>2.0%</b>
Quality Material 3	A17QC3L02	Initial measurement	Bench-top stability	Three freeze-thaw cycles	Short-Term Stability	Long-Term Stability
Replicate 1		85.53	88.46	83.35	83.51	85.87
Replicate 2		88.67	85.39	76.03	85.98	94.61
Replicate 3		102.71	85.59	84.07	83.14	84.34
Mean	80.70	92.30	86.48	81.15	84.21	88.27
<b>% difference from QC Char Fixed Mean</b>		<b>14.4%</b>	<b>7.2%</b>	<b>0.6%</b>	<b>4.4%</b>	<b>9.4%</b>
<b>% difference from the initial measurement</b>			<b>6.3%</b>	<b>12.1%</b>	<b>8.8%</b>	<b>4.4%</b>
GA adduct pmol/g tHb (Processed Sample)						
Quality Material 1	A17QC1L02	Initial measurement	Processed sample stability			
Replicate 1		34.220	29.82			
Replicate 2		29.992	31.56			
Replicate 3		29.953	28.26			
Mean	32.60	31.39	29.88			
<b>% difference from QC Char Fixed Mean</b>		<b>3.7%</b>	<b>8.3%</b>			
<b>% difference from the initial measurement</b>			<b>4.8%</b>			
Quality Material 2	A17QC2L02	Initial measurement	Processed sample stability			
Replicate 1		60.55	53.81			
Replicate 2		57.06	51.69			
Replicate 3		56.72	51.16			
Mean	60.00	58.11	52.22			
<b>% difference from QC Char Fixed Mean</b>		<b>3.1%</b>	<b>13.0%</b>			
<b>% difference from the initial measurement</b>			<b>10.1%</b>			
Quality Material 3	A17QC3L02	Initial measurement	Processed sample stability			
Replicate 1		87.32	70.16			
Replicate 2		78.99	76.97			
Replicate 3		70.78	68.61			
Mean	80.70	79.03	71.91			
<b>% difference from QC Char Fixed Mean</b>		<b>2.1%</b>	<b>10.9%</b>			
<b>% difference from the initial measurement</b>			<b>9.0%</b>			

**Table 16c: EO Adduct Quality Control Sample Stability**

EO adduct pmol/g tHb						
Quality Material 1	A17QC1L02	Initial measurement	Bench-top stability	Three freeze-thaw cycles	Short-Term Stability	Long-Term Stability
Replicate 1		115.03	115.16	114.95	124.54	119.56
Replicate 2		116.09	111.23	130.66	106.17	121.85
Replicate 3		131.51	106.67	117.86	112.44	133.06
Mean		121.033	120.88	111.02	121.15	114.39
<b>% difference from QC Char Fixed Mean</b>		<b>0.1%</b>	<b>8.3%</b>	<b>0.1%</b>	<b>5.5%</b>	<b>3.1%</b>
<b>% difference from the initial measurement</b>			<b>8.2%</b>	<b>0.2%</b>	<b>5.4%</b>	<b>3.3%</b>
Quality Material 2	A17QC2L02	Initial measurement	Bench-top stability	Three freeze-thaw cycles	Short-Term Stability	Long-Term Stability
Replicate 1		171.39	161.25	165.53	181.22	175.61
Replicate 2		158.49	157.33	147.78	193.67	177.83
Replicate 3		184.57	164.84	160.15	175.64	211.80
Mean		169.268	171.49	161.14	157.82	183.51
<b>% difference from QC Char Fixed Mean</b>		<b>1.3%</b>	<b>4.8%</b>	<b>6.8%</b>	<b>8.4%</b>	<b>11.3%</b>
<b>% difference from the initial measurement</b>			<b>6.0%</b>	<b>8.0%</b>	<b>7.0%</b>	<b>9.9%</b>
Quality Material 3	A17QC3L02	Initial measurement	Bench-top stability	Three freeze-thaw cycles	Short-Term Stability	Long-Term Stability
Replicate 1		210.99	205.82	203.94	213.72	236.24
Replicate 2		198.63	196.98	191.58	249.78	233.06
Replicate 3		231.35	219.60	193.78	247.70	246.61
Mean		216.120	213.66	207.47	196.43	237.07
<b>% difference from QC Char Fixed Mean</b>		<b>1.1%</b>	<b>4.0%</b>	<b>9.1%</b>	<b>9.7%</b>	<b>10.4%</b>
<b>% difference from the initial measurement</b>			<b>2.9%</b>	<b>8.1%</b>	<b>11.0%</b>	<b>11.7%</b>
EO adduct pmol/g tHb (Processed Sample)						
Quality Material 1	A17QC1L02	Initial measurement	Processed sample stability			
Replicate 1		130.413	122.10			
Replicate 2		110.859	128.18			
Replicate 3		101.933	110.36			
Mean		121.033	114.40	120.22		
<b>% difference from QC Char Fixed Mean</b>		<b>5.5%</b>	<b>0.7%</b>			
<b>% difference from the initial measurement</b>			<b>5.1%</b>			
Quality Material 2	A17QC2L02	Initial measurement	Processed sample stability			
Replicate 1		175.48	164.80			
Replicate 2		153.68	182.27			
Replicate 3		148.08	157.23			
Mean		169.268	159.08	168.10		
<b>% difference from QC Char Fixed Mean</b>		<b>6.0%</b>	<b>0.7%</b>			
<b>% difference from the initial measurement</b>			<b>5.7%</b>			
Quality Material 3	A17QC3L02	Initial measurement	Processed sample stability			
Replicate 1		233.24	218.15			
Replicate 2		196.09	233.36			
Replicate 3		182.06	192.77			
Mean		216.120	203.80	214.76		
<b>% difference from QC Char Fixed Mean</b>		<b>5.7%</b>	<b>0.6%</b>			
<b>% difference from the initial measurement</b>			<b>5.4%</b>			