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:

Clinical Chemistry Branch
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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 AMDGYD_H Information

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

File Name	Variable Name	SAS Label
AMDGYD_H	LBXACR	Acrylamide (pmol/g Hb)
	LBXGLY	Glycidamide (pmol/g Hb)
	LBXEOA	Ethylene Oxide (pmol/g Hb)

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 probably 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 acrylamide through certain occupational acitvities that involve production and use of acrylamide, as well as tobacco smoke (5-8) and dry-heated food (9-12). The actual exposure of the general population to acrylamide 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 acrylamide exposure and to assess potential health effects related to this exposure, it is necessary to measure both AA and GA exposures.

Ethylene oxide (EO) has been detected in tobacco smoke (16), automobile exhaust, and some food. EO, a metabolite of ethylene, is formed endogenously in animals and humans as a result of CYP2E1 mediated metabolic oxidation of ethylene. It is also formed in vivo during normal physiological processes such as methionine oxidation, lipid peroxidation, and the metabolizing activity of intestinal bacteria (17). EO has been classified as a human carcinogen (Group 1) 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 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, the adduct of these chemicals at the N-terminal valine of the Hb protein chains (N-[2-carbamoylethyl]valine, N-[2-hydroxycarbamoyl-ethyl]valine, and N-[2-hydroxyethyl]valine for AA, GA, and EO adducts, respectively).

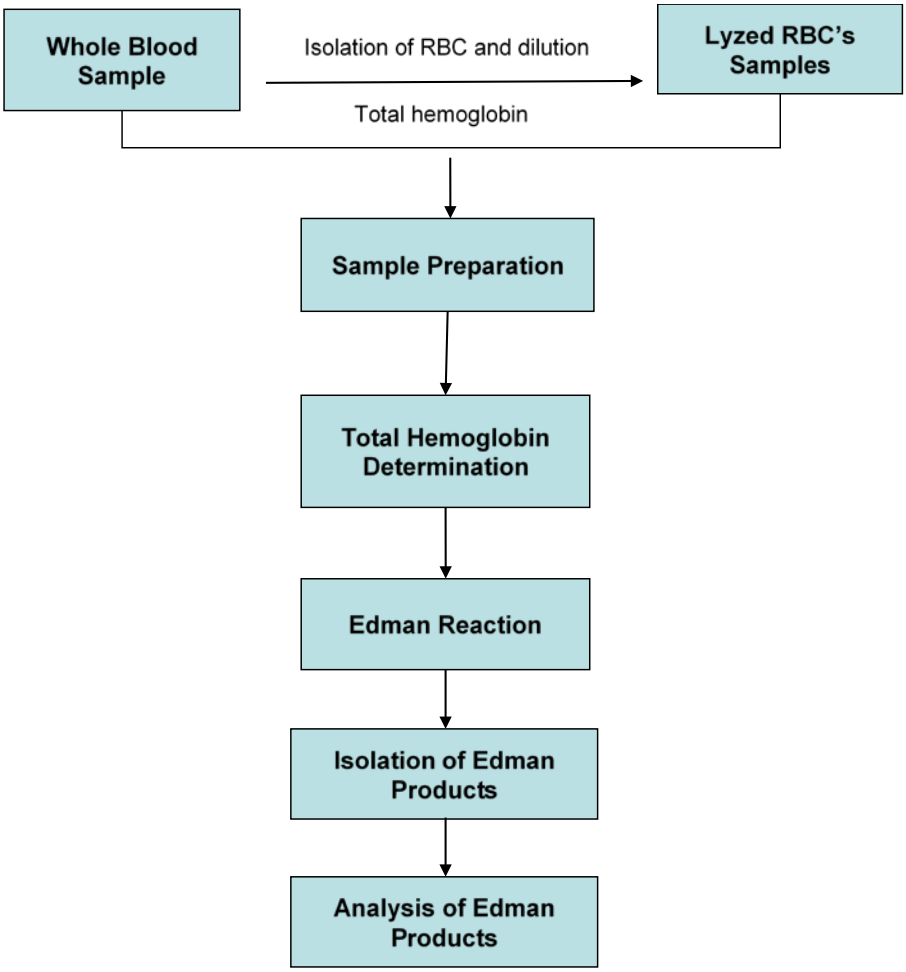
This method utilizes a modified Edman reaction, in which the N-alkylated amino acid forms Edman products in neutral or alkaline conditions without changing the pH to the acidic conditions required in conventional Edman reaction procedures (19). The reaction principle was first described for N-terminal Hb adducts of ethylene oxide, propylene oxide, and styrene oxide (20), but later optimized to increase yield of Edman products of these adducts (21). This optimized method was then successfully applied to adducts produced by 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 4 parts (Scheme 1):

- Specimen preparation
- Measuring total Hb in sample solution used for Hb adduct measurements
- Performing the modified Edman reaction in the sample solution
- Isolating Edman products
- Analysis of Edman products by High Performance Liquid Chromtagoraphy/Tandem Mass Spectrometry (HPLC-MS/MS) and processing results

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

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



1.3 Scope

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

Specific details relating to equipment maintenance and operation can be found in the manufacturers' manual. Additional details are located in designated work instructions created and maintained by the Protein Biomarker Laboratory (PBL). Further, 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.

The method was developed for measuring hemoglobin adducts (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 bloodbourne pathogens including but not limited to Human Immunodeficiency Virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV). HBV vaccination series is required for all analysts performing this measurement procedure.

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

Disposable bench covers must be used during sample preparation and sample handling and 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, other reagents, and organic solvents used in this measurement procedure must be handled with extreme care; they are caustic, flammable, and toxic. Therefore, they must be handled only in a well-ventilated area or, as required, under a chemical fume hood.

Glacial Acetic Acid: Do not breathe 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 breathe vapor. Flammable liquid and vapors. 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 breathe vapor. Forms explosive peroxides upon prolonged storage. Keep container in well ventilated location.

Pentafluoropenyl isothiocyanate: Do not breathe 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. Wear appropriate personal protective equipment (clothing, safety goggles, and gloves) because it is toxic to reproduction. Store away from acids.

Methyl Alcohol (Methanol): Do not breathe 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. Wear appropriate personal protective equipment (clothing, safety goggles, and gloves). Eye contact may result in permanent eye damage and contact with skin causes skin irritation. May cause respiratory tract irritation. Corrosive to aluminum.

Toluene: Do not breathe 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 breathe vapor. Use adequate ventilation. Irritating to eyes, respiratory system, gastrointestinal system and skin. Contains cyanide.

Nitric Acid: Danger. May be fatal if inhaled. Causes severe eye and skin burns. Causes severe respiratory and and digestive tract burns. Contact with other material may cause a fire. Acutepulmonary edema or chronic obstructive lung disease may occur from inhalation of the vapors of nitric acid. Corrosive to metal and it is a strong oxidizer.

Material safety data sheets (MSDS) for these chemicals are readily accessible as hard copies in the laboratory. If needed, MSDS 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. Follow the manufacturer's operating instructions found in the respective equipment manual. 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 (exact location) and electronic copy can be accessed at. http://intranet.cdc.gov/nceh-atsdr/dls/safety_manual/

Uncontaminated plastic that is coded 1 through 7 can be recycled by placing in the trash bin located in laboratory. Uncontamined clean plastic includes empty pipette tip boxes and inserts, and clean plastic bottles from reagents, bleach, and buffers all of which has 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 not limited to, documented training on specific instrumentation outlined in this procedure.

Analyst performing this procedure must be familiar with the following:

- Exposure Control Plan
- Chemical Hygiene Plan
- Relevant Material 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 familarity with software operated instruments such as Thermo Finnigan 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 obtained from the LC-MS/MS software are performed via calculation templates created in Microsoft Excel. The calculation results obtained with the Excel templates 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 dedicated and specially trained staff. Initial calculations using the Excel templates are performed by the analysts after receiving specific training from qualified 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 contain final reportable results are 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. PG 403-S Delta-Range Chemical Balance (Electronic “0.000 g”, Max 410.0 g, Min 0.02g, Mettler Toledo, Columbus, OH)
2. Accumet AB 15 pH Meter with Orion Micro-Combination Electrode, pH Range 0-14, Temperature Range 0-100 ºC (Fisher Scientific, Suwanee, GA)
3. 500 mL Glass Beaker (Corning Incorporated, Lowell, MA)
4. 500 mL Pyrex Graduated Glass Cylinder, Tolerance ±1.4 mL, (Kimble Chase Life Science and Research Products LLC, Vineland, NJ)
5. 1 mL, 2mL, 3mL, and 4mL Volumetric Glass Pipettes. (Fisher Scientific, Suwanee, GA)
6. 15 mL Plastic Falcon Tubes (KSE, Durham, NC)
7. 50 mL Plastic Falcon Tubes (KSE, Durham, NC)
8. 1L Glass Bottles With Screw Tops (Wheaton Industries Inc., Millville, NJ)
9. 1000 µl Pipette (Gilson Inc., Middleton, WI)
10. 200 µl Pipette, Variable (Gilson Inc., Middleton, WI)
11. Repeater Pipette (Eppendorf, Ramsey, MN)
12. Pasteur Transfer Pipettes (Samco Scientific, San Fernando, CA)
13. Disposable Pasteur Pipets, 53/4” (Fisher Scientific, Suwannee, GA)
14. Octagonal Stirring Bars, 1 Inch Length; 0.312 Inch Diameter (Fisher Scientific, Suwanee, GA)
15. Scholar™ 5 x 5 Inch PC-171 Magnetic Stirrer (Corning Incorporated, Lowell, MA)
16. Milli-Q Water, Resistivity, 18 megaOhm-cm DI Water at 25 ° C, 18.2 (Aqua Solutions, Jasper, GA)
17. Ethyl Acetate, HPLC/ACS Grade (Fisher Scientific, Suwanee, GA)
18. Toluene, HPLC/ACS Grade, (Fisher Scientific, Suwanee, GA)
19. Isopropyl Ether, Certified (Fisher Scientific, Suwanee, GA)
20. Glacial Acetic Acid, Certified ACS Grade, (Fisher Scientific, Suwanee, GA)
21. Methanol, HPLC Grade (Fisher Scientific, Suwanee, GA)
22. Acetic Acid ACS Grade (J. T. Baker, Phillipsburg, NJ)
23. Sodium Hydroxide 0.2N Solution (Fisher Scientific, Suwanee, GA)
24. Hemo Point H2, Hemoglobin Control, (Stanbio Laboratory, Boerne, TX)
25. Hemoglobin Reagent Set (Teco Diagnostics, Anaheim, CA)
26. Standard material: Lyophilized Hemoglobin, Part # HC-110LIN (Analytical Control Systems Inc., Fishers, IN)

4.1.2 Equipment, Chemicals, and Consumables Used for Calibration Materials

1. PG 403-S Delta-Range Chemical Balance (Electronic “0.000 g” , Max 410.0 g, Min 0.02g, Mettler Toledo, Columbus, OH)
2. Water Bath- Iso Temp 3016 Regulator Apparatus (Fisher Scientific, Suwanee, GA)
3. 500 mL Pyrex Silanized Volumetric Fasks, Tolerance ±0.08 mL, (Kimble Chase Life Science and Research Products LLC, Vineland, NJ)
4. AA Octapeptide (AA-VHLTPEEK), (Certified Concentration with Stated Uncertainty), Purity 71.0%, FW=1022.5 g/mol, CAS No: 1608 (Bachem, King of Prussia, PA)
5. Labeled AA Octapeptide [AA-Val($^{13}C_5$,^{15}N)-HLTPEEK], purity 80%, FW=1028.2 g/mol CAS No:1739-B, (Bachem King of Prussia, PA)
6. GA Octapeptide (GA-VHLTPEEK), (Certified Concentration with Stated Uncertainty), Purity 71.0%, FW=1038.5 g/mol, CAS No: 1660 (Bachem, King of Prussia, PA)
7. Labeled GA Octapeptide [GA-Val($^{13}C_5$,^{15}N)-HLTPEEK], (Certified Concentration with Stated Uncertainty), Purity 71.0%, FW=1045.2 g/mol, CAS No: 1740-B (Bachem, King of Prussia, PA)
8. EO Octapeptide (EO-VHLTPEEK), (Certified Concentration with Stated Uncertainty), Purity 73.0%, FW=996.1 g/mol, CAS No: 4051266 (Bachem, King of Prussia, PA)
9. Labeled EO Octapeptide [EO-Val($^{13}C_5$, ^{15}N)-HLTPEEK], (Certified Concentration with Stated Uncertainty), Purity 72.0%, 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, Convoluted Bottom (Thermo Scientific, Waltham, MA)
6. ArctiSeal 96-well Square Silicone w/ PTFE Spray Coating (Arctic White LLC, Bethlehem, PA)
7. Combitips Plus Pipet Tips, 10 mL (Eppendorf, Ramsey, MN)

8. EZ-2.3 Evaporation System with Side Bridge Holders and Universal Rotor (GeneVac Inc., Valley Cottage, NY)
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, 250 µL, Uniplate V-Well Bottom Microplate (Whatman Inc., Piscataway, NJ)
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 Polyehtylene 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. GeneVac EZ-2.3 Evaporation System with Side Bridge Holders and Universal Rotor (GeneVac Inc., Valley Cottage, NY)
28. Eppendorf 8-Channel Pipette 50-1200 µl, (Eppendorf, Westbury, NY)
29. Gilson 1000 µl Pipette (Gilson Inc., Middleton, WI)
30. Gilson 200 µl Pipette, (Gilson Inc., Middleton, WI)
31. Gilson 10 µl Pipette, (Gilson Inc., Middleton, WI)
32. Eppendorf Repeater Pipette (Eppendorf, Ramsey, MN)
33. Transfer Pipettes (Samco Scientific, San Fernando, CA)
34. Boekel Orbitron Rotator, Model II (Fisher Scientific, Suwanee, GA)
35. Fisherbrand Octagonal Stirring Bars, 1”L x 5/16” D (Fisher Scientific, Suwanee, GA)
36. Plain Wood Applicators, 5 ¾”L x 1/12”D (Fisher scientific, Suwanee, GA)
37. Steril Cottoned wood applicators, 5 ¾”L x 1/12”D (Fisher Scienctific, Suwanee, GA)
38. Formamide ACS Grade (USB, Cleveland, OH)
39. Pentafluorophenyl Isothiocyanate 96 % (Alfa Aesar, Ward Hill, MA)
40. Methanol, HPLC Grade (Fisher scientific, Suwanee, GA)
41. Ethyla Acetate HPLC/ACS Grade, (Fisher Scientific, Suwanee, GA
42. Isopropyl Ether, Certified (Fisher Scientific, Suwanee, GA)
43. 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. Power Wave Eon Spectrophotometer (Bio-Tek Instruments, Winooski, VT)
5. Finnigan TSQ Quantum Vantage with Atmospheric Pressure Chemical Ionization Unit (Thermo Electron, San Jose, CA)
6. Accela HPLC with Auto-Sampler and Photodiode Array Detector (Thermo Electron, San Jose, CA)
7. HemoCue Hb 201+ (HemoCue Inc, Lake Forest, CA)
8. Methanol, HPLC Grade (Fisher scientific, Suwanee, GA)
9. Formic Acid, HPLC Grade (Fisher Scientific, Suwanee, GA)
10. Isopropanol, Certified ACS (Fisher Scientific, Suwanee, GA)
11. 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. Using a graduated cylinder, transfer 1L formamide to a bottle and add 1 mL glacial acetic acid using a glass syringe. Mix well by inverting the bottle and measure pH. Make sure pH is between 6-6.5. 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 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 Hemoglobin measurement. According to the manufacutrer's instructions, transfer the individually package containing the powder into a 1 L volumetric flask using 18 megaOhm-cm DI water. Fill the volumetric flask up to 1 L mark and invert at least 5 times for proper mixing. The solution is stored in amber glassware. If amber glassware is unavailable, cover 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 needs to be prepared freshly before use.

4.2.5 Preparation of Extraction Plates

Fill the *"Extraction Plates*" (48-well 7.5-mL plate with 25 µm 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 or ¼ inch 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 250 vials of each calibrator level,

which is sufficient for approximately 1,100 analytical runs, assuming use of 1 set of calibrators for 4 calibration curves.

Calibrator Solutions	Target Concentration of Octapeptides			Amount of peptide and diluent volume (Diluant: 18 megaOhm-cm DI water)
	AA	GA	EO	
Stock Solution	66.92 µmol/L	142.01 µmol/L	83.81 µmol/L	2.51 mg AA Octapeptide → 25 mL 5.77 mg GA Octapeptide → 25 mL 1.16 mg EO Octapeptide → 10 mL
Intermediate	9,369.38 nmol/L	8,520.49 nmol/L	N/A	1.40 mL AA Octapeptide (Calibrator Stock) → 10 mL 0.60 mL GA Octapeptide (Calibrator Stock) → 10 mL

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

1. Preparation of Calibrator Stock Solution
    a. Remove AA-VLHTPEEK (AA octapeptide), GA-VLHTPEEK (GA octapeptide), and EO-VLHTPEEK (EO octapeptide) material from freezer to sit in a desicator over night.
    b. Calibrate the analytical balance following the manufacturer's instructions.
    c. Weigh 2.51 (±0.001) mg of AA octapeptide on a clean aluminum foil surface and transfer it to a 25 mL volumetric flask.
    Weigh 5.77 (±0.001) mg of GA octapeptide on a clean aluminum foil surface and transfer it to a separate 25 mL volumetric flask.
    Weigh 1.16 (±0.001) mg of EO octapeptide on a clean aluminum foil surface and transfer it to a separate 10 mL volumetric flask.
    d. Add 18 megaOhm-cm DI water to just below the fill line of the volumetric flasks
    e. Place flasks in a water bath for at least 15 minutes to reach 20°C.
    f. Add 18 megaOhm-cm DI water to the fill line.
    g. Mix solution well by inversion.
    h. Aliquot each solution in separate 50 mL falcon tubes and label appropriately.
    i. Store at 2-8^{o}C if not to be used immediately.
2. Preparation of Calibrator Intermediate Stock Solution
    a. Transfer 1.400 mL AA calibrator stock solution and 0.600 mL GA calibrator stock solution into a single 10 mL volumetric flask using a calibrated pipette.
    b. Add 18 megaOhm-cm DI water to just below the fill line of the volumetric flask.
    c. Place flask in the water bath for at least 15 minutes to reach 20 °C.
    d. Add 18 megaOhm-cm DI water (at 20 °C) to the fill line.
    e. Mix solution well by inversion.
    f. Transfer solution to 50 mL falcon tubes
    g. Label tubes appropriately.
    h. Store at at 2-8^{o}C if not to be use immediately.
3. Preparation of Working Solutions

The calibrator working solutions are prepared as shown in Table 2.

Calibrator Working Solution	Target Concentration of Octapeptides (nmol/L)			Dilution Scheme based on stock solutions described in Table 1 (Diluant: 18 megaOhm-cm DI water)
	AA	GA	EO	
AcrC01L00	1.22	1.11	5.03	65 µL Calibrator Intermediate Stock Solution + 30 µL EO Calibrator Stock Solution → 500 mL
AcrC02L00	2.34	2.13	10.90	125 µL Calibrator Intermediate Stock Solution + 65 µL EO Calibrator Stock Solution → 500 mL
AcrC03L00	4.68	4.26	20.95	250 µL Calibrator Intermediate Stock Solution + 125 µL EO Calibrator Stock Solution → 500 mL
AcrC04L00	9.37	8.52	41.91	500 µL Calibrator Intermediate Stock Solution + 250 µL EO Calibrator Stock Solution → 500 mL
AcrC05L00	18.74	17.04	83.81	1000 µL Calibrator Intermediate Stock Solution + 500 µL EO Calibrator Stock Solution → 500 mL
AcrC06L00	37.48	34.08	167.63	2000 µL Calibrator Intermediate Stock Solution + 1000 µL EO Calibrator Stock Solution → 500 mL
AcrC07L00	74.96	68.16	335.25	4000 µL Calibrator Intermediate Stock Solution + 2000 µL EO Calibrator Stock Solution → 500 mL

Table 2: Dilution scheme for AA, GA, and EO Octapeptide Calibrator Working Solution

Prepare the Calibrator Working Solutions by Performing the Following Tasks:

1. Adjust the calibrator stock and intermediate solutions to 20 °C using a water bath.
2. Transfer the volumes of calibrator intermediate stock solution and EO calibrator stock solution stated in Table 1 to separate 500 mL volumetric flasks, using grade A volumetric pipettes.
3. Add 18 megaOhm-cm DI water to just below the fill line of the volumetric flask.
4. Place flask in the water bath for at least 15 minutes to reach 20 ºC and add 18 megaOhm-cm DI water to the fill line.
5. 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 working solutions cannot be reused.

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: 20.4, 10.2, 5.1, 2.6, and 1.3 g/dL.

1. Add 2.5 mL 18 megaOhm-cm DI to 1 bottle of lyophilized Hb linearity control.
2. Place on Hematology Mixer for about 30 minutes to mix throughoutly and this is labeled “Hb linearity control stock”.
3. Dilute the Hb linearity control with 18 megaOhm-cm DI water, using the dilution scheme shown in Table 3.
4. Label vials appropriately
5. Place calibrators at 2-8ºC until use.

Note: Expiration date of Hb calibrators is seven days.

Calibration Curve Level	Concentration based on current lot (g/dL)	Amount of Hb linearity control stock (mL)	Amount of DI H_2O (mL)	Total Volume (mL)

1	20.4	1.20	0.00	1.2
2	10.2	0.60	0.60	1.2
3	5.1	0.30	0.90	1.2
4	2.6	0.15	1.05	1.2
5	1.3	0.0753	1.125	1.2

Table 3. Preparation of the Total Hb Calibration Curve

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($^{13}C_5$,^{15}N)- HLTPEEK, GA-V($^{13}C_5$,^{15}N)- HLTPEEK, and EO- V($^{13}C_5$,^{15}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.

Internal Standard Solution	Target Concentration of Isotope Labeled Octapeptides (µmol/L))			Amount of peptide and diluent volume (Diluant is 18 megaOhm-cm DI water)
	AA	GA	EO	
Stock Solution	77.18	128.20	75.01	0.81 mg AA Octapeptide IS → 10 mL 1.41 mg GA Octapeptide IS → 10 mL 1.16 mg EO Octapeptide IS → 10 mL
Working Solution	38.60	64.10	37.51	0.5 mL Internal Standard Stock Solution → 1,000 mL

Table 4: Desired Internal Standard AA, GA, and EO Octapeptide Solution

1. Preparation of Internal Standard Stock Solution
    a. Remove AA-V($^{13}C_5$,^{15}N)- HLTPEEK, GA-V($^{13}C_5$,^{15}N)- HLTPEEK, and EO-V($^{13}C_5$,^{15}N)- HLTPEEK material from freezer and allow them sit in a desicator for overnight.
    b. Calibrate the analytical balance following the manufacturer's instructions. Take 0.81 (±0.001) mg of AA-V($^{13}C_5$, ^{15}N)- HLTPEEK, 1.41 (±0.001) mg of GA-V($^{13}C_5$, ^{15}N)- HLTPEEK, and 1.16 (±0.001) mg of EO- V($^{13}C_5$,^{15}N)-HLTPEEK and add all to the same 10 mL volumetric flask.
    c. Add 18 megaOhm-cm DI water to the flask just below the fill line of the volumetric flask.
    d. Place flask in the water bath for at least 15 minutes to reach 20 ºC and add 18 megaOhm-cm DI water to the fill line.
    e. Mix solution.

f. Transfer solution into a 15 mL falcon tube, label tubes appropriately and store it in the -70 ºC freezer.

2. Preparation of Internal Standard Working Solution
    a. Transfer 0.5 mL of internal standard stock solution into a 1,000 mL volumetric flask using a 1 mL calibrated pipette.
    b. Add 18 megaOhm-cm DI water to the flask to the fill line of the volumetric flask.
    c. Mix solution thoroughly by inverting the flask 20 times.
    d. Aliquot 11 mL solution in 15-mL falcon tubes, label tubes appropriately and store them in the -70 ºC 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 packed red blood cells is preferable to allow for repeat analyses. A sample volume of 500 µL 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 meausured, then the sample may need to be divided. In such a case, appropriate amounts of lysed red blood cells should be transferred to a sterile 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 ºC) or frozen on dry ice. Specimens can be stored at refrigerated for up to 3 days prior to analysis. For long-term storage, samples must be stored at -70 ºC. Samples stored at -70 ºC are stable for 5 years. Two and three freeze-thaw cycles did not exhibit any changes. Multiple freeze-thaw cycles of diluted whole blood samples possibly increase the formation of blood clots, which complicates the analysis of the samples. Therefore, diluted whole 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 shown evidence of leakage and/or breakage are not to be accepted for testing.

6 PROCEDURE OPERATION INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

Abiding to 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 the process of sample preparation, sample transfer, and analysis to ensure individual samples can be tracked throughout the process.

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 together with 3 bench QC samples, 1 blank, and 1 set of calibrators (7 levels). Typically, four plates (total of 148 patient samples) can be processed in parallel. Plate layout example: 1 reagent blank, 7 calibrators and 37 samples and 3 quality control samples.

1. Assess all samples for acceptability using the criteria described in this Standard Operating Procedure (SOP) section 5.2 and 5.3.
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. Centrifuge blood samples and QC materials at 15°C and 4,000 rpm for 10 minutes. After centrifugation, visually inspect each sample tube for air bubbles; remove air bubble if present to facilitate automated pipetting. Use a Kimwipe to wipe bubble off the top of the cryovials if necessary.
4. Place all patient samples, QC samples, internal working solution, and calibrator working solution on the Tecan automated instrument in the designated locations in a manner that allows the instrument's barcode reader to read all barcodes correctly. Place all additional reagents on the instrument at the designated positions.

6.2.1 Scanning of the Barcodes and Prepare the "*Dilution Plate"*

1. Scan the barcodes of all coded vials and reagents.
2. When a barcode cannot be read, the Tecan Freedom Evo 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 containing the barcode information and location of the particular sample, calibrator, and reagents on the instrument. The file name will consist of current date and time when the scan was performed. This file is then transferred to a defined location on the CDC network and the information is used to create a run sequence for the HPLC-MS/MS instrument and to verify run log sheets. The scanned sequence file is saved on the instument computer.
3. Tecan pipets 500 µL of 1:1 (v/v) diluted RBCs from the cryovials using 1,000 µL conductive tips to 48-well 5 mL plates ("Dilution Plate"). The "*Dilution Plates"* assessed for presence of blood clots. 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 cell avoiding pipetting of blood clots.

6.3 **Total Hemoglobin Measurements**

The Hb measurement must be performed prior to the Edman procedure. The Hb measurement provides information about the Hb concentration in the "*Dilution Plate".* This sample solution is used during the modified Edman reaction. The amount of Hb used for Edman reaction is needed to calculate the adducts results 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 hemoglobin 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 peak absorption at 540 nm, the value of which is proportional to total hemoglobin concentration.

The procedure can only be continued after all total Hb (tHb) measurements have been assessed and validated.

6.3.1 Total Hemoglobin Analysis

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

A maximum of four plates can be processed one at a time using the following steps of HRS test.

1. Tecan aspirates 50 µL of blood samples, tHb QCs (low,medium, and high) and five levels of ACS calibratiors from the sample rack onto '*HRS 1.95 mL Plate'* using 200 µl conductive disposable pipette tips.
2. Tecan aspirates a total of 1.95 mL into the '*HRS 1.95 mL Plate'* and 1.60 mL of HRS solution to '*1.60mL HRS Plate'* respectively using 150µL MCA tips.
3. Tecan mixes the blood-HRS solution of the '*1.95mL HRS Plate'*. The analyst will visually check the mixture for any suspended blood. Manually mixing may be required to homogenize the mixture.
4. Tecan then aspirates 400 µL of sample mixture from the '*1.95mL HRS Plate'* onto '*1.60mL HRS Plate'* and mixes the solutions. Analyst to visually check mixture for homogeneity. Manual mixing may be required.
5. Insert Mirotiter Plate (*MTP Plate*) in the plate reader and read the absorbance of the empty plate at 540nm.
6. Tecan transfers a total of 200 µL of sample mixture from the '*1.60ml HRS Plate'* onto the 96-well *MTP Plate*.
7. Insert the 96-well MTP in the plate reader and read the absorbance of the sample solution at 540nm.
8. After reading the absorbance, the instrument software automatically subtracts the absorbance of the empty plate from the absorbance of the plate with sample solution and stores the "raw" results as a text-file on the local hard drive.

6.3.2 Total Hemoglobin Calculations

1. Assess data to ensure validity of the calibration curve by assessing at the slope, intercept, and correlation coefficient (R^2) values for consistency with method evlaution values.
2. Assess validity of the tHb QC samples to be within the in-house established limit, check for outliers, repeat measurement if necessary.
3. Assess sample validity between sample replicates to be within 10 percent or better.
4. Assess validity for the amount of total Hb to be within the calibration range 1.3 to 20.4 g/dL.
5. Template will mark a cell "HC" if a measurement fails to pass these assessments. If this happens, take sample that is marked with "HC" in the data file and measured value with HemoCue meter and then enter the value into the data file in the corresponding cell.
6. Each sample's absorbance is measured in duplicates and averaged.
7. Calculation:

$$\text{Whole blood Hemoglobin } (\frac{g}{dL}) = \frac{Au}{As}\ xCs$$

Au= absorbance of unknown
As=absorbance of standard
Cs=concentration of standard in g/dL

8. Print all tHb data files.

6.4 **Edman Reaction Preparation**

In this step the N-terminal valine containing the AA, GA, and EO is cleaved from the Hb protein chains with 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 HPLC-MS/MS analysis.

In this section of the procedure, blank, calibrators, quality control samples and patient samples are transferred to four 48-well 5 mL wellplates. This well plate is named the "*Edman Plate"*. Solvents and reagents are added and the Edman reaction is performed. Then the reaction solution undergoes liquid-liquid extraction and the extract is concentrated by evaporating the organic solvents. The following steps will take place using the Tecan.

1. Place "*Edman Plates"* in their appropriate locations on the pipetting instrument.
2. Tecan prepares the *Edman Plate* by adding 100 µL of Internal Standard to all the wells of the plate using conductive tips. Visually check each well to confirm uniform addition.
3. Tecan then transfers exactly 350 µL of blood from "*Dilution Plate"* to "*Edman Plate"*. Visually check the volume to be consistent throughout the plates. If the volume is not sufficient, record sample that appears low. Sample will be flagged if quantity is insufficient during data processing, and the data reviewer will associate an error code. Samples with error codes will be repeated.
4. Tecan pipets 200 µL calibration curves (CC) solution and 200 µL 18 megaOhm-cm DI water (as blank) into the assigned wells. Visually check the volume level in each well to be the same. The column number on the "*Edman Plate"* containing the 7 CCs and blank identifies the plate/batch number. For example, the calibration curve in the first column indicates the first plate. The calibration curve in the second column indicates the second plate, etc.
5. Tecan adds 200 µL 18 megaOhm-cm DI 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 the calibration samples 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.
9. Before proceeding, ensure that samples and calibrators 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 calibrator sample.
10. Place "*Edman Plates"* back on the instrument in their appropriate locations and uncap the Edman vial.

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

11. Tecan will pipet 20 µL of Edman reagent to all wells of the plates. When the program is finished, remove plates from the pipetting instrument, seal with silicon plate sealers, vortex, and transfer to the shaker located in the oven.

6.5 Edman Reaction Performance

Incubate samples at 55 (±1) °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 "*Edman Plate"* to the "*Extraction Plate"* by performing the following tasks:

1. Transfer the samples form the "*Edman Plate"* to 48-well 7.5 mL "*Extraction Plates"* filled with Isolute material. Place each "*Extraction Plate"* on top of the corresponding new 48-well 7.5-mL "*Collection Plate"*.
2. Wait 20 minutes for samples to properly penetrate into sorbent material.
3. Continue the method by adding 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 solvent. Carefully lift each "*Extraction Plate"* and check if solvent is still dripping (if so, let plates sit longer until all solvent has passed and no dripping is observed).
5. Remove "*Collection Plate"* containing the extraction solutions and transfer to the Glas-Col evaporator for evaporation overnight.
6. The next day wash the walls of each "*Collection Plate"* with ethyl acetate to recover the analytes on the side of the walls. Use the repeater pipette with a 10 mL tip and set the pipette dial to one. Dial one corresponds to a 200 µL dispense volume. Add 200 µL of ethyl acetate to each wall of the collection plate. Then, add an additional 200 µL to the wider walls of the wells for a total wash volume of 1,200 µL per well.
7. Vortex each plate on level 4 for 30 seconds.
8. Place "*Collection Plates"* containing ethyl acetate and "*Reconstitution Plates"* on the Tecan instrument.
9. Transfer samples recovered from the wall wash step to the 96-well 2 mL "*Reconstitution Plate"*. Two "*Collection Plat*es" are transferred to one "*Reconstitution Plate"*.
10. Remove solvents by placing the "*Reconstitution Plate"* in a GeneVac EZ-2.3.

6.7 Sample Preparation for HPLC-MS/MS Analysis

When all solvents have been dried down in the "*Reconstitution Plate",* perform the following tasks using the Tecan automated instrument:

1. Transfer the *"Reconstitution Plate"* from the GeneVac to the Tecan.
2. Tecan adds 80 µL of methanol and 120 µL of 18 megaOhm-cm DI water to each well in that order. Vortex the "*Reconstitution Plate"* for thorough mixing.
3. Transfer the "*Reconstitution Plate"* to the centrifuge. Centrifuge samples for 15 minutes at 3,700 rpm and 15°C.
4. Transfer samples from the "*Reconstitution Plate"* to a new 96-well 2 mL plate named "*Final Plate".*
5. Tecan pipet 200 µL of samples from the "*Reconstitution Plate"* to the "*Final Plate".*

6. Seal the "*Final Plate"* and transfer it to the refrigerator to await analysis by HPLC-MS/MS.

6.8 Edman Products Analysis by HPLC-MS/MS

All daily, weekly preventative maintenance or cleaning are performed according to manufacturer 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 HPLC-MS/MS software to analyze the samples in the wellplates. The run sequence consists of two 48-well plates combined in one 96-well plate, and additional samples such as water and instrument controls are added to assess carry-over and instrument function. The sequence is created by combining the barcode ID information with the LC-MS/MS instrument files and data processing method file using a sequence template file. An example of sequence can be found in Appendix 12.
2. Once 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 analyte and IS. The instrument control is added to each batch to verify appropriate function of the instrument and chromatographic condition. Additionally a water sample (Blank) is added after every eighth sample. The Instrument Control Sample and the Run Blanks are kept in a separate well-plate or vials in the auto-sampler of the HPLC-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 HPLC-MS/MS parameters used are listed in tables 5, 6, and 7.

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)^{o}C
Solvent A:	not used
Solvent B:	not used
Solvent C:	Methanol
Solvent D:	Water
Flow Rate:	550 µL/min

Multiplex valve: MX Series II MXT715-102 (2 position, 10-port)

Step	Time	%C	%D
0	0.00	60.0	40.0
1	7.50	60.0	40.0

Table 5: HPLC Gradient conditions for Pump A

Step	Time	%C	%D
0	0.00	60.0	40.0
1	0.10	60.0	40.0
2	0.11	99.0	1.00
3	4.50	99.0	1.00
4	5.00	60.0	40.0
5	7.50	60.0	40.0

Table 6: HPLC Gradient conditions for Pump B

Mass spectrometric conditions

Acquisition mode: Single Reaction Monitoring (SRM)
Acquisition Delay: 1 min
Ionization: APCI in the Positive Ion Mode
APCI Vaporization Temperature: 550 ˚C
Capillary Temperature 250 ˚C
Sheath Gas: Argon 45 psi
Aux Gas: Argon 5 psi
Q2 Gas Pressure: 1.0 mTorr
Chrom filter: 10
Segment: 1
Width: 0.010
Time: 0.100
Q1 PW: 0.30
Q3 PW: 0.70
Direct valve: not used
Ion gauge pressure: $1.22x10^{-5}$
APCI probe: B position

Analyte	SRM (m/z)	Collision Energy (V)	Expected Retention time (min)
AA-Val-PFPTH	396→379	10	4.50
AA-Val($^{13}C_5$,^{15}N)-PFPTH	402→385	10	4.50
GA-Val-PFPTH	412→395	10	3.80
GA-Val($^{13}C_5$,^{15}N)-PFPTH	418→401	10	3.80
Ethylene Oxide-Val-PFPTH	369→130	30	6.30

Ethylene Oxide-Val($^{13}C_5$,^{15}N)-PFPTH	375→135	30	6.30

Table 7: SRM mass-to-charge ratios (m/z) of analytes and internal standards

PDA 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 HPLC-MS/MS measurements to the corresponding folder on the network drive. Data is integrated and processed using the Xcalibur or Indigo Ascent. 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 four runs are prepared per day. For analysis of samples by HPLC-MS/MS, two 48-well plates are combined in one 96-well plate. Thus, data and results generated by the HPLC-MS/MS system contain 2 runs per HPLC-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 Finnigan 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 ratiois are used. Calibration curves are generated with the area ratios from the calibrator samples and their assigned values using unweighed linear regression. We don't process further for sample batches with calibration curves not meeting DLS 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 curve. 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 is performed every six months and is 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, calibration material (7 levels), and QCs (low, medium, and high) covering the reportable range of AA, GA, and EO adducts, they are analyzed in compliance within 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 a SAS software program, maintained by DLS.

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

At the end of each run, the calibration curve is reanalyzed as unknowns. The measured concentrations of these calibrators must agree with the assigned values of their set values for AA, GA, and EO adducts respectively.

Requirements for calibration verification are met by having seven calibrators and one reagent blank processed with each batch of samples.

8 METHOD PERFORMANCE CHARACTERISTICS

8.1 Analytical Measurement Range and Linearity Limits

Linearity of the method was determined according to Clinical and Laboratory Standard Institute (CLSI) guideline EP6-A (30). The method is linear from 1.22 – 140.54 nmol/L for AA, 1.11 – 127.81nmol/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 final analytical measurement range of the adducts is 3.9 -6,178 pmol AA / g Hb, 4.9 - 5618 pmol GA / g Hb, and 12.9- 29473 pmol EO / g Hb. Samples below this analytical measurement range will not be reported. Samples above this range can be diluted with 18 megaOhm-cm DI water 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 8.

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

Table 8: LOD calculation in Matrix

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 be formed. 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 ion.

8.4 Accuracy and Precision

Precision of the method was determined according to CLSI guideline EP5-A2. (32) The evaluation includes within-day and among-day assessments. See Table 9 and 10. The evaluations are carried out using QC samples at a minimum of three concentrations. The concentration of the QC range is applicable to the method.

Within-day imprecision was determined from 11 replicates of low, medium, and high QC samples. The among-day variability was assessed by measuring low, medium, and high QC samples in duplicates over 20 days. The means and SDs were calculated using the DLS SAS program for bench QC characterization.

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.

Analyte	Within-Day Precision (%CV) Low	Within-Day Precision (%CV) Medium	Within-Day Precision (%CV) High
AA adducts	7	6	5
GA adducts	6	5	6
EO adducts	4	7	7

Table 9: Within-day precision values

Analyte	Among-Day Precision (%CV) Low	Among-Day Precision (%CV) Medium	Among-Day Precision (%CV) High
AA adducts	6	8	11
GA adducts	8	8	11
EO adducts	16	9	13

Table 10: Among-day precision values

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 anlayte 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 Edman product. 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 8% (see Table 11).

Adducts	Expected pmol	Recovery in % Low pool	Recovery in % High pool
GA adducts	3.60	97.0	101.2
	7.20	96.2	100.2
AA adducts	3.84	107.7	98.5
	7.68	92.4	98.1
EO adducts	25.01	104.8	99.3
	50.03	101.6	100.3

Table 11: Accuracy by standard addition

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. 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 formamide: The pH of formamide added as described in Section 6.7 should be within the pH range of pH 5.5 to 7.0. Decrease in method performance was observed at pH 8.5.
4. Hemoglobin levels: The accuracy of the results will be not effected when the blood samples are diluted ≤ 1:1 with saline. Higher dilutions lead to inaccurate results.
5. Freezing and thawing: 2 Freeze-thaw cycles do not affect the total Hb measurement.

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 comprise 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) is aliquotted into 2.0 mL cryovials, capped, and frozen. The QC pools are stored at -70 ºC 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 manufacture's values. Duplicate measurements for at least 20 consecutive runs are established and means and plus ranges are calculated. If results vary from the manufacture'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 GP-29 P (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 30 separate vials from each pool in at least 10 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 t-HB PT is performed through an external proficienty 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 have not been established yet. An in-house assessment using a convenience sample from 127 individuals found the following adduct values: the median concentrations for EO adducts is 68.1 pmol/g for non-smokers and is 205.2 EO-Val-PFPTH pmol/g for smokers (range: 19.6- 576pmol/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, if 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

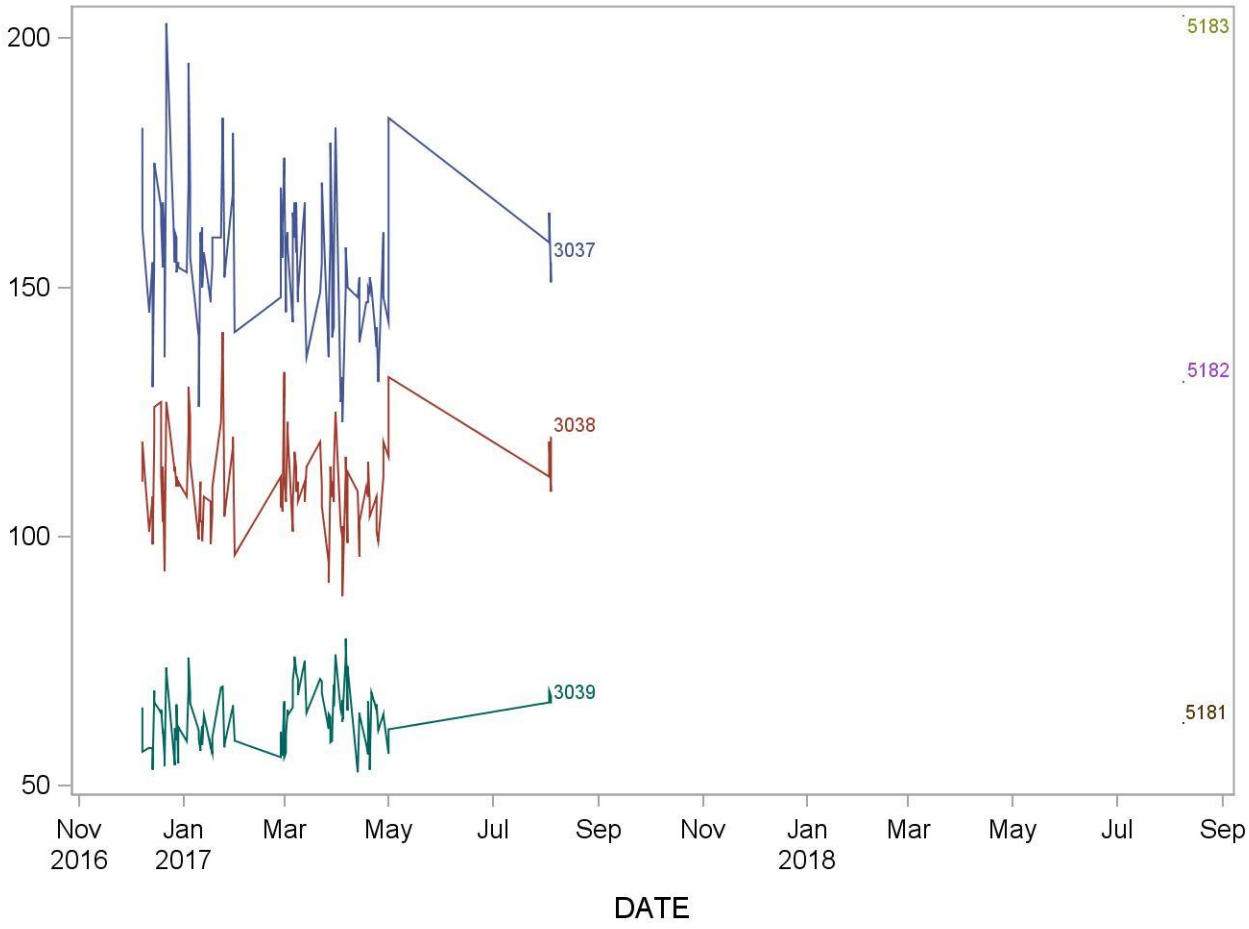
Not applicable for this procedure.

17 SUMMARY STATISTICS AND QC CHARTS

Please see the following pages.

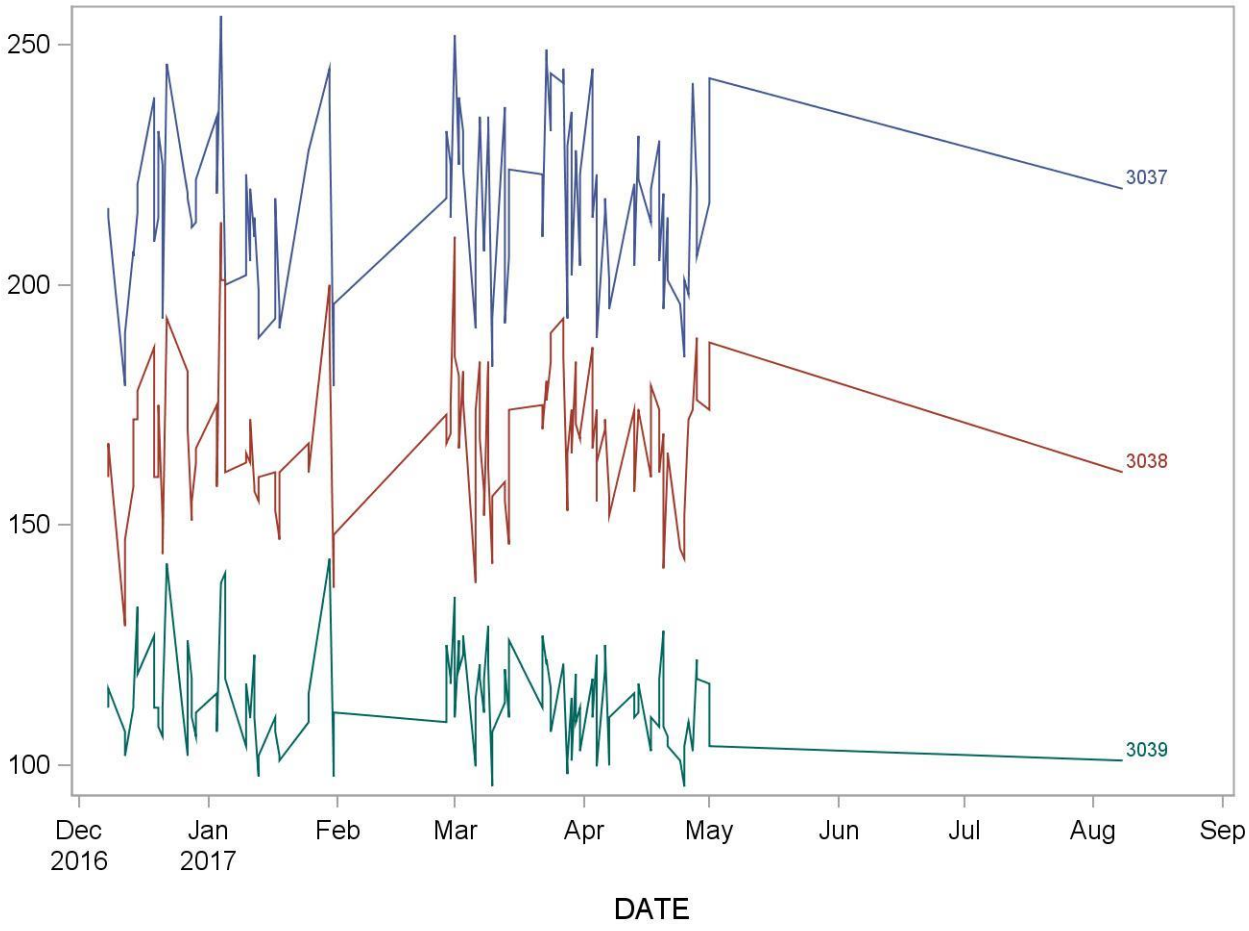
2013-2014 Summary Statistics and QC Chart for Acrylamide (pmol/g Hb)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3037	105	08DEC16	04AUG17	155.238	14.623	9.4
3038	106	08DEC16	04AUG17	110.287	9.520	8.6
3039	106	08DEC16	04AUG17	63.908	6.256	9.8



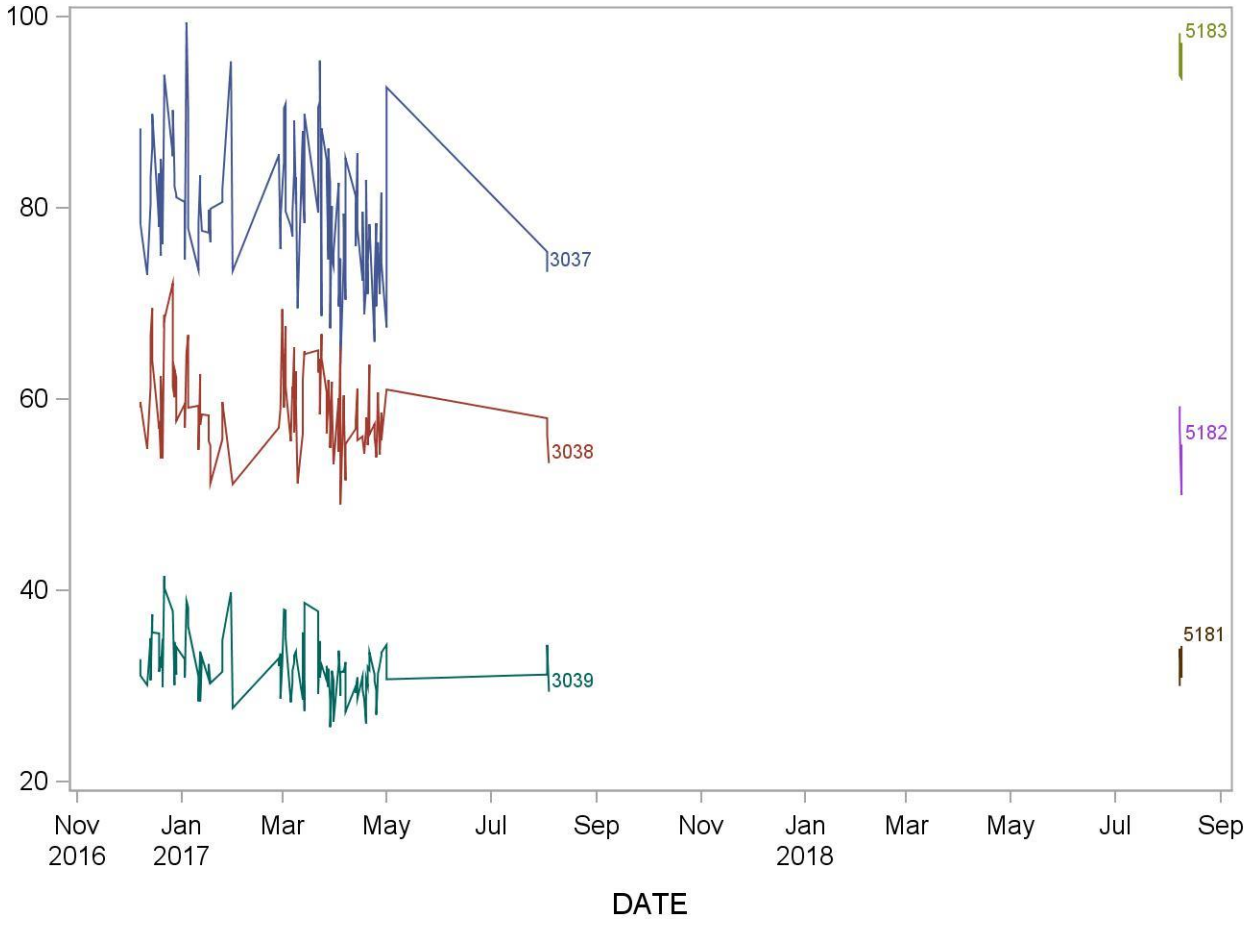
2013-2014 Summary Statistics and QC Chart for Ethylene Oxide (pmol/g Hb)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3037	115	08DEC16	08AUG17	216.678	17.926	8.3
3038	117	08DEC16	08AUG17	167.744	15.190	9.1
3039	115	08DEC16	08AUG17	113.352	10.068	8.9



2013-2014 Summary Statistics and QC Chart for Glycidamide (pmol/g Hb)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3037	110	08DEC16	03AUG17	79.969	7.078	8.9
3038	107	08DEC16	04AUG17	59.157	4.528	7.7
3039	107	08DEC16	04AUG17	32.088	3.159	9.8
5181	4	08AUG18	09AUG18	32.250	2.114	6.6
5182	4	08AUG18	09AUG18	55.300	3.901	7.1
5183	4	08AUG18	09AUG18	95.725	2.377	2.5



18 REFERENCES

1. LoPachin, R. M., Balaban, C. D., and Ross, J. F. (2003) Acrylamide axonopathy revisited. *Toxicol Appl Pharmacol* **188**, 135-153
2. Calleman, C. J., Wu, Y., He, F., Tian, G., Bergmark, E., Zhang, S., Deng, H., Wang, Y., Crofton, K. M., Fennell, T., and et al. (1994) Relationships between biomarkers of exposure and neurological effects in a group of workers exposed to acrylamide. *Toxicol Appl Pharmacol* **126**, 361-371
3. Dearfield, K. L., Douglas, G. R., Ehling, U. H., Moore, M. M., Sega, G. A., and Brusick, D. J. (1995) Acrylamide: a review of its genotoxicity and an assessment of heritable genetic risk. *Mutat Res* **330**, 71-99
4. IRAC. Some industrial chemicals. IARC Monogr Eval Carcinog Risks Hum. 1994;60:1-560
5. Calleman, C. J. (1996) The metabolism and pharmacokinetics of acrylamide: Implications for mechanisms of toxicity and human risk estimation. *Drug Metab Rev* **28**, 527-590
6. Bergmark, E. (1997) Hemoglobin adducts of acrylamide and acrylonitrile in laboratory workers, smokers and nonsmokers. *Chem Res Toxicol* **10**, 78-84
7. Perez, H. L., Cheong, H. K., Yang, J. S., and Osterman-Golkar, S. (1999) Simultaneous analysis of hemoglobin adducts of acrylamide and glycidamide by gas chromatography-mass spectrometry. *Anal Biochem* **274**, 59-68
8. Schettgen, T., Broding, H. C., Angerer, J., and Drexler, H. (2002) Hemoglobin adducts of ethylene oxide, propylene oxide, acrylonitrile and acrylamide-biomarkers in occupational and environmental medicine. *Toxicol Lett* **134**, 65-70
9. Mottram, D. S., Wedzicha, B. L., and Dodson, A. T. (2002) Acrylamide is formed in the Maillard reaction. *Nature* **419**, 448-449
10. Tareke, E., Rydberg, P., Karlsson, P., Eriksson, S., and Tornqvist, M. (2000) Acrylamide: a cooking carcinogen? *Chem Res Toxicol* **13**, 517-522
11. Tareke, E., Rydberg, P., Karlsson, P., Eriksson, S., and Tornqvist, M. (2002) Analysis of acrylamide, a carcinogen formed in heated foodstuffs. *J Agric Food Chem* **50**, 4998-5006
12. Stadler, R. H., Blank, I., Varga, N., Robert, F., Hau, J., Guy, P. A., Robert, M. C., and Riediker, S. (2002) Acrylamide from Maillard reaction products. *Nature* **419**, 449-450
13. Calleman, C. J., Bergmark, E., and Costa, L. G. (1990) Acrylamide is metabolized to glycidamide in the rat: evidence from hemoglobin adduct formation. *Chem Res Toxicol* **3**, 406-412
14. Glatt, H., Schneider, H., and Liu, Y. (2005) V79-hCYP2E1-hSULT1A1, a cell line for the sensitive detection of genotoxic effects induced by carbohydrate pyrolysis products and other food-borne chemicals. *Mutat Res* **580**, 41-52
15. Sumner, S. C., Fennell, T. R., Moore, T. A., Chanas, B., Gonzalez, F., and Ghanayem, B. I. (1999) Role of cytochrome P450 2E1 in the metabolism of acrylamide and acrylonitrile in mice. *Chem Res Toxicol* **12**, 1110-1116
16. Bono, R., Vincenti, M., Saglia, U., Pignata, C., Russo, R., and Gilli, G. (2002) Tobacco smoke and formation of N-(2-hydroxyethyl)valine in human hemoglobin. *Arch Environ Health* **57**, 416-421
17. Marsden, D. A., Jones, D. J., Lamb, J. H., Tompkins, E. M., Farmer, P. B., and Brown, K. (2007) Determination of endogenous and exogenously derived N7-(2-

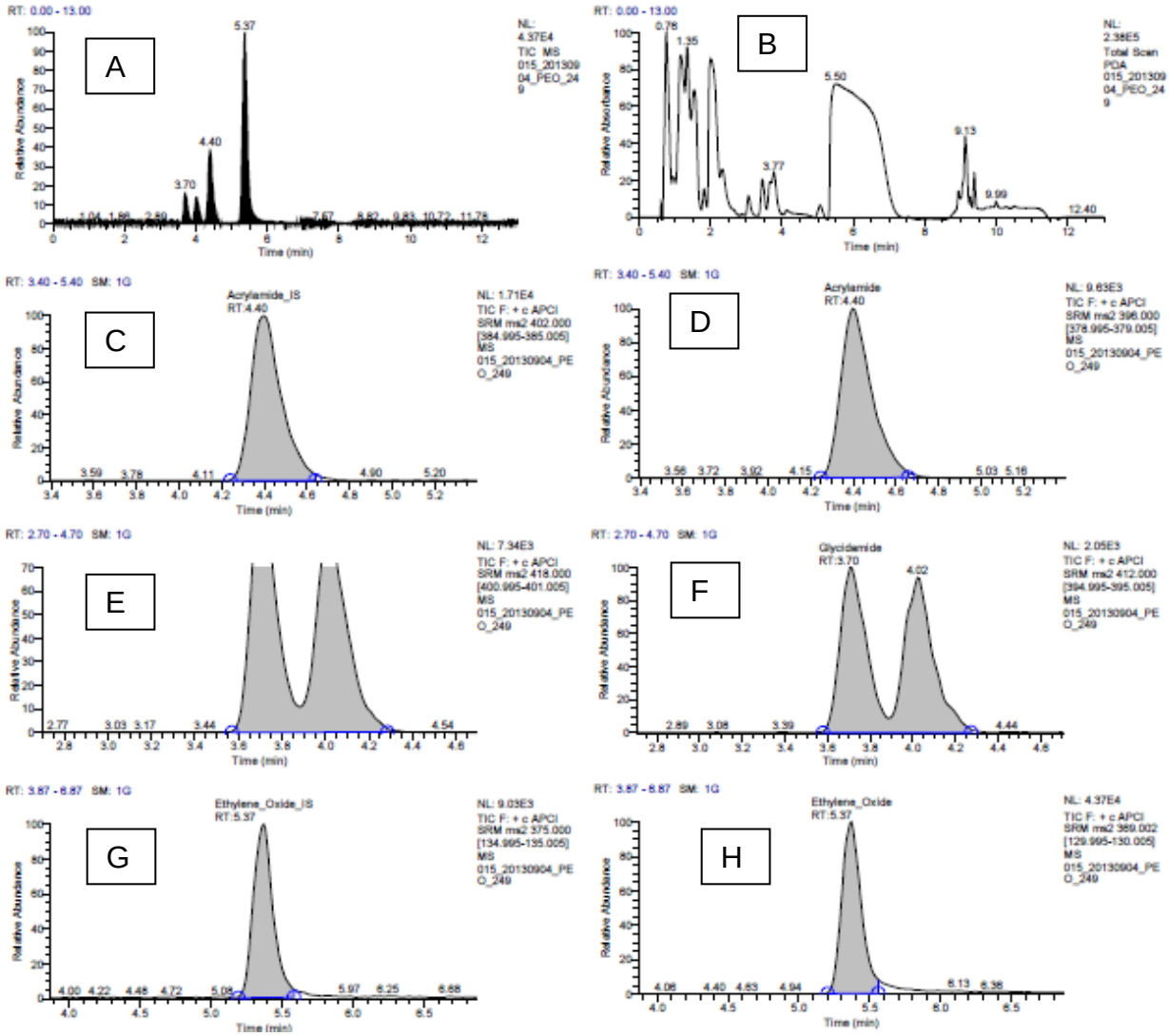
hydroxyethyl)guanine adducts in ethylene oxide-treated rats. *Chemical research in toxicology* **20**, 290-299
18. IRAC.(1999) Monographs on the Evaluation of Carcinogeneic Risks to Humans 60.
19. Tornqvist, M., Fred, C., Haglund, J., Helleberg, H., Paulsson, B., and Rydberg, P. (2002) Protein adducts: quantitative and qualitative aspects of their formation, analysis and applications. *J Chromatogr B Analyt Technol Biomed Life Sci* **778**, 279-308
20. Mowrer J, T. M., Jehseh S, Ehrenberg L. (1986) Modefied Edman Degradation Applied to Hemoglobin for Monitoring Occupational Exposure to Alkylating Agents *Toxicol. Environ. Chem.* **11:215**
21. Tornqvist, M., Mowrer, J., Jensen, S., and Ehrenberg, L. (1986) Monitoring of environmental cancer initiators through hemoglobin adducts by a modified Edman degradation method. *Anal Biochem* **154**, 255-266
22. Fennell, T. R., Sumner, S. C., Snyder, R. W., Burgess, J., Spicer, R., Bridson, W. E., and Friedman, M. A. (2005) Metabolism and hemoglobin adduct formation of acrylamide in humans. *Toxicol Sci* **85**, 447-459
23. Baum, M., Fauth, E., Fritzen, S., Herrmann, A., Mertes, P., Merz, K., Rudolphi, M., Zankl, H., and Eisenbrand, G. (2005) Acrylamide and glycidamide: genotoxic effects in V79-cells and human blood. *Mutat Res* **580**, 61-69
24. Ospina, M., Vesper, H. W., Licea-Perez, H., Meyers, T., Mi, L., and Myers, G. (2005) LC/MS/MS method for the analysis of acrylamide and glycidamide hemoglobin adducts. *Adv Exp Med Biol* **561**, 97-107
25. Paulsson, B., Athanassiadis, I., Rydberg, P., and Tornqvist, M. (2003) Hemoglobin adducts from glycidamide: acetonization of hydrophilic groups for reproducible gas chromatography/tandem mass spectrometric analysis. *Rapid Commun Mass Spectrom* **17**, 1859-1865
26. Vesper, H. W., Ospina, M., Meyers, T., Ingham, L., Smith, A., Gray, J. G., and Myers, G. L. (2006) Automated method for measuring globin adducts of acrylamide and glycidamide at optimized Edman reaction conditions. *Rapid Commun Mass Spectrom* **20**, 959-964
27. Vesper, H. W., Wang, P. M., Archibold, E., Prausnitz, M. R., and Myers, G. L. (2006) Assessment of trueness of a glucose monitor using interstitial fluid and whole blood as specimen matrix. *Diabetes Technol Ther* **8**, 76-80
28. Teco. *Hemoglobin reagent kit: Manufacturers package insert product H526. Quantitative Determination of Hemoglobin in Human Blood*
29. Policies and Procedure Manual, D. o. L. S., National Center for Environmental Health, Centers for Disease Control and Prevention, May,. (2008).
30. NCCLS document EP6-A- Evaluation of the linearity of quantitative mesurment procedures: A statistical approach. NCCLS, P., USA,. (2003).
31. Taylor JK, L. P., Chelsea, MI.(1987) Quality Assurance of Chemicla Measurements. Lewis Publishers,INC. 225 pp.78-84
32. NCCLS document EP5-A2- Evaluation of Precision Performance of Quantitative Measurment Methods; Approval Guideline Second Edition. NCLS, W., PA, USA,. (2004).
33. NCCLS document EP15-A2-User Verification of Performance for Precision and Truness; Approval Guideline Second Edition. NCLS, W., PA, USA,. (2005).

34. Meyers, T., Vesper, H. W., Scott, D., Mendez, M., and Myers, G. L. (2007) Assessing the effects of freezing and diluting specimens on total hemoglobin measurements. *Clin Chim Acta* **380**, 235-237
35. Caudill, S. P., Schleicher, R. L., and Pirkle, J. L. (2008) Multi-rule quality control for the age-related eye disease study. *Stat Med* **27**, 4094-4106
36. NCCLS document GP27-A. Using Proficiency Testing (PT) to improve the Clinical Laboratiry: Approved guidline. NCLS, W., PA, USA, . (1999).
37. Vesper HW, Caudill SP, Osterloh JD, Meyers T, Scott D, Myers GL. Exposure of the U.S. Population to Acrylamide in the National Health and Nutrition Examination Survey 2003–2004. Environmental Health Perspectives 2010;118:278–283.

19 APPENDICES

Appendix 1. AA, GA, and EO Adduct Chromatography
Appendix 2. Related Documents
Appendix 3. Symbols, Abbreviations, Terminology
Appendix 4. Document Compliance Tables
Appendix 5. Location of Information as Required by CLIA
Appendix 6. Location of Information as Required by ISO 17025
Appendix 7. Location of Information as Required by ISO 15193
Appendix 8. Work Instruction List
Appendix 9: Metrological Traceability of AA, GA, and EO Measurements
Appendix 10: List of Tables
Appendix 11: HRS Solution Preparation

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
2. CDC Safety Policies and Practices Manual. http://isp-v-ehip-asp/dlsintranet/-safety_manual/
3. Clinical Laboratory Improvement Amendments of 1988 (CLIA). 42CFR493 from February 28, 1992.
4. International Organization for Standardization (ISO). In vitro diagnostic medical devices — Measurement of quantities in biological samples — Metrological traceability of values assigned to calibrators and control materials. ISO 17511:2003(E), ISO Geneva, Switzerland. 2003.
5. International Organization for Standardization (ISO). General requirements for the competence of testing and calibration laboratories. ISO 17025:2003(E), ISO Geneva, Switzerland. 2003.
6. International Organization for Standardization (ISO). *In vitro* diagnostic medical devices —Measurement of quantities in samples of biological origin- presentation of reference measurement procedures. ISO 15193:2002(E), ISO Geneva, Switzerland. 2002.

Appendix 3. Symbols, Abbreviations, Terminology

$^{13}C_5$	Labeled carbon (5) isotope
$^{15}N_1$	Labeled nitrogen (1) isotope
APCI	Atmospheric Pressure Chemical Ionization
CC	Calibration Curve
CCB	Clinical Chemistry Branch
CDC	Centers for Disease Control and Prevention
CHP	Chemical Hygiene Plan
CLIA	Clinical Laboratory Improvement Act/Amendment
CLSI	Clinical and Laboratory Standards Institute
CV	Coefficient of Variant
DI	De-ionized
dL	Deciliter
DLS	Division of Laboratory Sciences
DNA	Deoxyribonucleic Acid
EDMAN	Pentaflurophenyl Isothiocyanate
EDTA	Ethylenediaminetetraacetic Acid
FA	Formamide
FDA	Food and Drug Administration
g	Grams
Hb	Hemoglobin
HC	HemoCue
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HRS	Hemoglobin Reagent Set
Hz	Hertz
IARC	International Agency for Research on Cancer
ID	Identifier
IS	Internal Standards
ISO	International Organization for Standardization
ITSO	Information Technology Service Office
L	Liter
LC-MS/MS	Liquid Chromatography/Tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantitation
mg	Milligram
mL	Milliliter
mm	Millimeter
MSDS	Material Safety Data Sheets
MTP	Microtiter Plate
N/A	Not Applicable
NaOH	Sodium Hydroxide
NCEH	National Center of Environmental Health
NHANES	National Health and Nutrition Examination Survey
nm	Nanometer
OHS	Occupational Health and Safety

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

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

Required Section	**Section in this Document**
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	6
Preparation of Reagents, Calibrators, Controls, Solutions and Other Materials Used in Testing	4
Calibration and Calibration Verification Procedures	7
Reportable Range for Patient Test Tesults	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	2

Appendix 5: Location of information as required by ISO 17025

Required section	**Section in this Document**
Appropriate Identification	Title Page
Scope	1
Description of the Type of Item to be Tested or Calibrated	1
Parameters or Quantities and Ranges to be Determined	1, 8.1
Apparatus and Equipment, Including Technical Performance Requirements	4
Reference Standards and Reference Materials Required	4.3, 0
Environmental Conditions Required and Any Stabilization Period needed	4, 6
Description of the Procedure, Including Affixing of Identification Marks, Handling, Transporting, Storing and Preparation of Items, Checks to be Made Before the Work is Started, Checks that the Equipment is Working Properly and, Where Required, Calibration and Adjustment of the Equipment Before Each use, the Method of Recording the Observations and Results, Any Safety Measures to be Observed	6
Criteria and/or Requirements for Approval/Rejection	5, 8
Data to be Recorded and Method of Analysis and Presentation	3, 7.8
The Uncertainty or the Procedure for Estimating Uncertainty	8.4

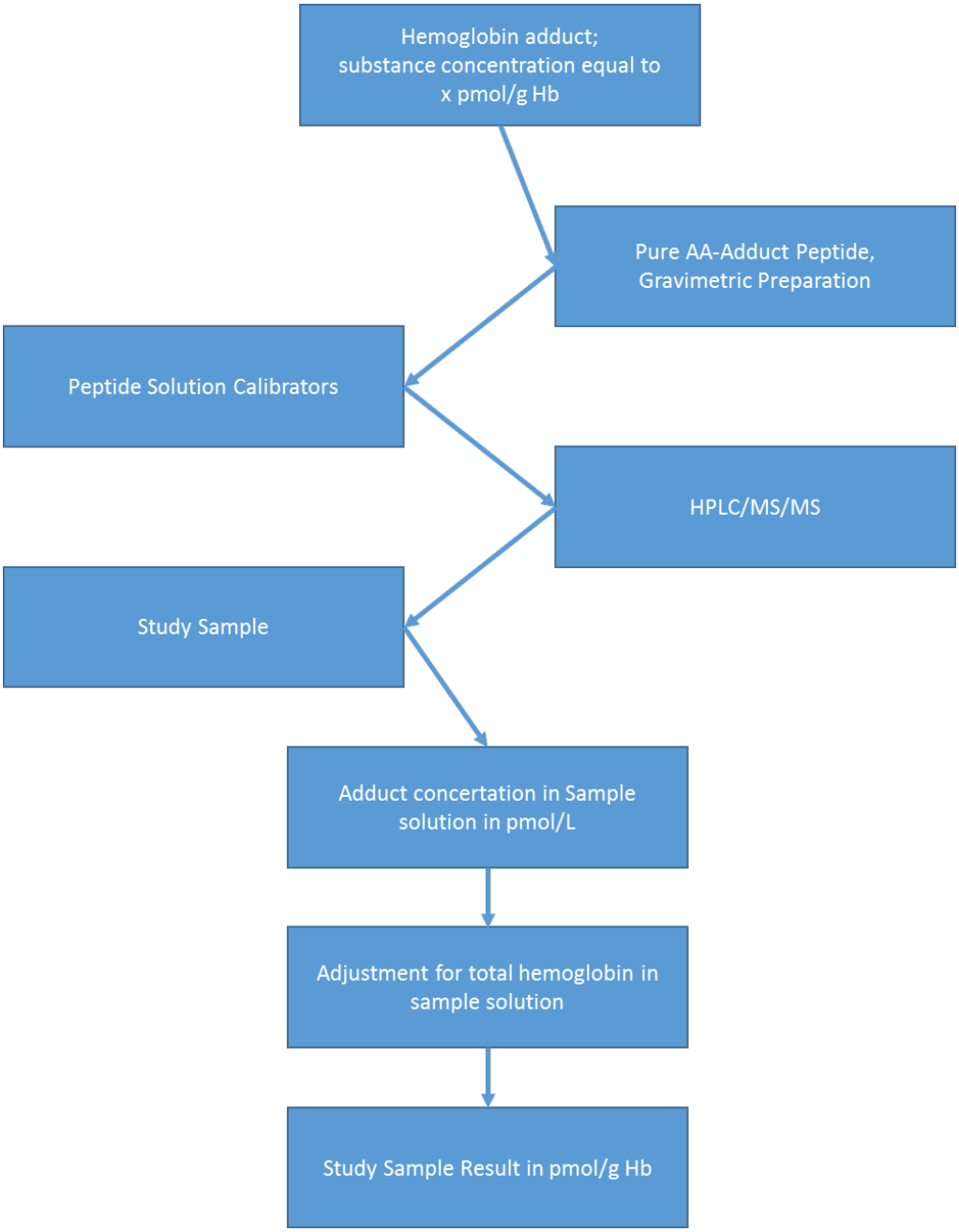
Appendix 6: Location of information as required by ISO 15193

Required section	Section in this Document
Title page	Title Page
Contents list	List of Content
Foreword	N/A
Warning and safety precautions	2
Introduction	1
Title	Title Page
Scope	1
Normative references	0
Definitions	0
Symbols and abbreviations	0
Terminology	0
Principle and method of measurement	1
Check list	
Reagents	4
Apparatus	4
Sampling and sample	5, 6.1
Preparation of measuring system and analytical portion	6
Operation of measuring system	6
Data processing	3, 7.8
Analytical reliability	8
Special cases	N/A
Validation by inter-laboratory studies	N/A
Reporting	7.8, 11
Quality assurance	8
Bibliography (Annex)	16
Dates of authorization and revision	Second page of document

Appendix 7: 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 Intrepretation 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 8:
Metrological Traceability of Acrylamide, Glycidamide and Ethylene Oxide Measurements



Appendix 9: List of tables

Appendix 10: HRS Solution Preparation

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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.[1]

PRINCIPLE
In the cyanmethemoglobin method, erythrocytes are lysed by a stromatolytic agent in the present 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.[2]

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.[3]

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
Abs. of unknown × Conc. of Standard (g/dl) = 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:

0.480 × 15.0 = 11.9 g/dl
0.602

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[4,5]

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

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

	Mean (g/dL)	S.D.	C.V.%
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.

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

REFERENCES
1. Eilers, R.J., *Am. J. Clin. Pathol.* 47:212 (1967).
2. Tietz, N.W., *Fundamentals of Clinical Chemistry*, 2nd ed., W. B. Saunders Co., Philadelphia p411 (1976).
3. Yound, D. S. *et al.*, *Clin. Chem.* 21:10, (1975).
4. Henry, R.F. *et al.*, *Principles and Techniques in Clinical Chemistry*, 2nd ed., Harper and Row, Hagerstown, MD p. 1128 - 1135 (1974).
5. Wolf, P.L., *Practical Clinical Hematology*, Johy Wiley and Sons, NY, p.144 (1973).

H526: 11/01

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