Laboratory Procedure Manual

Analyte: N-terminal hemoglobin adducts of acrylamide and glycidamide

Matrix: Red Blood Cells and Whole Blood

Method: Liquid Chromatography Tandem Mass Spectrometry

Method No: 1000

Revised:

as performed by:

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Important Information for Users

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

Data File Name	Variable Name	SAS Label
AMDGYD_D	LBXACR	Acrylamide (pmoL/G Hb)
	LBXGLY	Glycidamide (pmoL/G Hb)

Contents

1 SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

1.1 Clinical and Public Health Relevance

Acrylamide has been identified as neurotoxic (1,2), mutagenic to male germ cells (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). Research on non-occupational human exposure to acrylamide was initially driven to elucidate unexplained background biomarker concentrations in control subjects of occupational studies. These studies identified tobacco smoke (5-8) and food (9-12) as sources of acrylamide exposure in the general population. The finding that the general population is exposed to acrylamide created the need for biomonitoring of acrylamide exposure. The assessment of human exposure in the general population will define the extent of exposure and the evaluation of possible associations between exposure to acrylamide and health effects. Glycidamide, the primary metabolite of acrylamide, has a higher reactivity towards nucleophilic reagents than acrylamide. Further, results from animal studies suggest that genetic damage in somatic and germ cells is dependent upon the metabolism of acrylamide to glycidamide by CYP2E1 (13-15). The metabolism of acrylamide to glycidamide is dependent on both the amount and the mechanism of exposure and glycidamide has different toxicity than acrylamide. Therefore, it is necessary to measure both acrylamide and glycidamide adducts in order to obtain comprehensive information about acrylamide exposure and possible risks associated with this exposure.

1.2 Test Principle

This procedure describes a method to measure hemoglobin adducts of acrylamide and its primary metabolite glycidamide in human whole blood or erythrocytes. Specifically, the reaction products with the N-terminal valine of the hemoglobin protein chains (N-[2-carbamoylethyl]valine and N-[2-hydroxycarbamoyl-ethyl]valine for acrylamide and glycidamide adducts, respectively) are measured.
This method is based on a modified Edman reaction, which uses the N-alkylated amino acids ability to form Edman products in neutral or alkaline conditions without changing the pH to acidic conditions required in conventional Edman reaction procedures (16). It was first described for N-terminal hemoglobin adducts of ethylene oxide, propylene oxide and styrene oxide (17) and later optimized to increase yield of Edman products of these adducts (18). This optimized method was then successfully applied to adducts produced by other chemicals such as acrylamide, glycidamide and acrylonitrile (19-22). The method was further refined and modified in-house to increase sensitivity and enable automation (23,24).

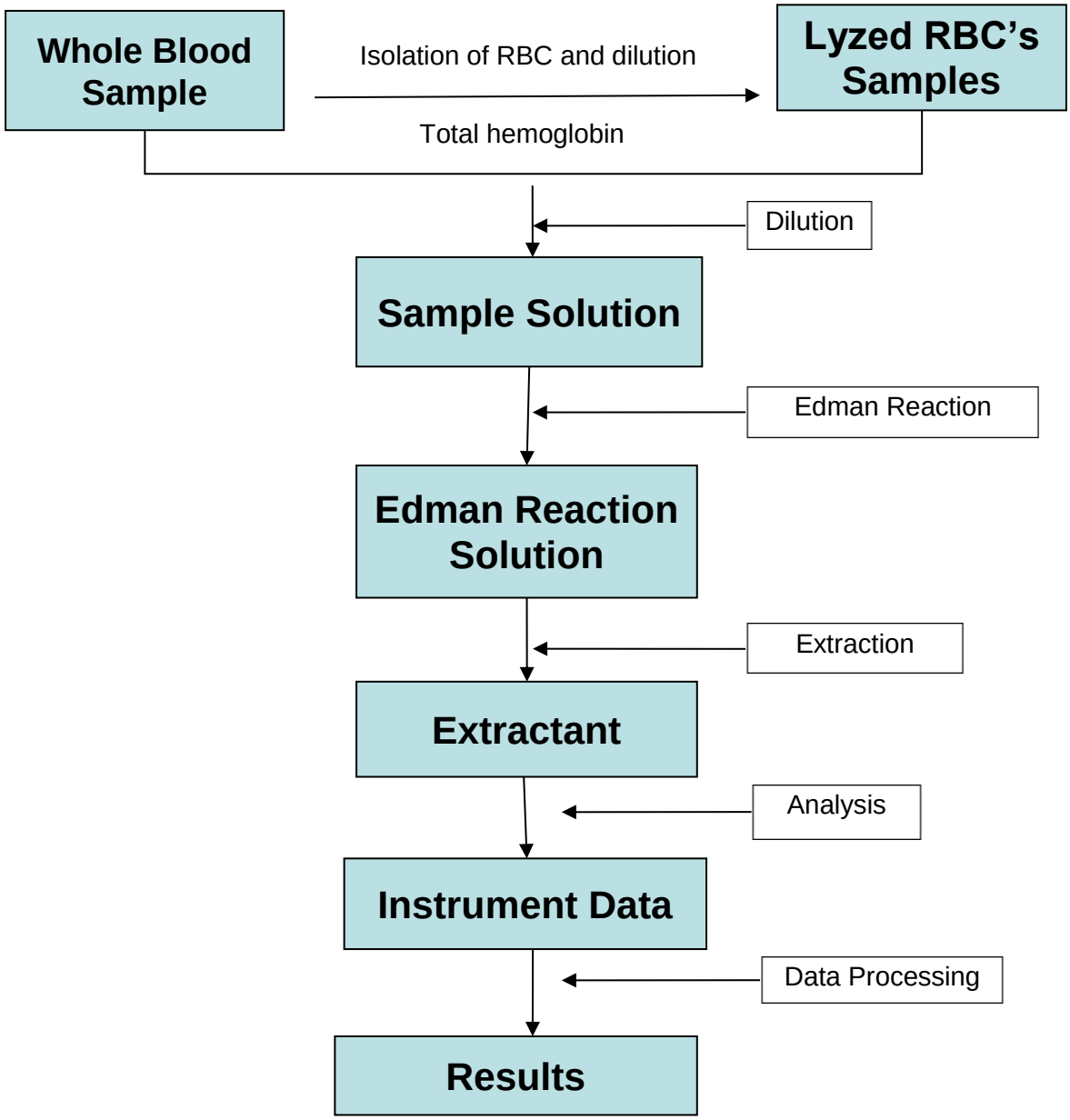
The procedure described here consists of 4 parts (Scheme 1):

- Specimen preparation for measuring hemoglobin adducts of acrylamide and glycidamide.
- Measuring total hemoglobin in sample solution for hemoglobin adduct measurements.
- Performing the modified Edman reaction in the sample solution and isolating Edman products.
- Analysis of Edman products by High Performance Liquid Chromtagoraphy/Tandem Mass Spectrometry (HPLC/MS/MS) and processing results.

Because results are reported in pmol adduct per gram of total hemoglobin present in the sample, the amount of hemoglobin 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 (25). Quantitation of the acrylamide and glycidamide hemoglobin adducts is performed using octapeptides with the same amino acid sequence as the N-terminal of the beta-chain of hemoglobin and with acrylamide and

glycidamide attached at the valine, Acrylamide-Val-His-Leu-Thr-Pro-Glu-Glu-Lys-OH and Glycidamide-Val-His-Leu-Thr-Pro-Glu-Glu-Lys-OH. The corresponding stable isotope labeled Acrylamide-Val($^{13}C_5$, $^{15}N_1$)-His-Leu-Thr-Pro-Glu-Glu-Lys-OH and Glycidamide-Val($^{13}C_5$, $^{15}N_1$)-His-Leu-Thr-Pro-Glu-Glu-Lys-OH are used as internal standards.

Scheme 1: Measurement Procedure for Acrylamide in Red Blood Cells



1.3 Scope

The measurement procedure described in this document is intended to quantitatively measure acrylamide and glycidamide adducts in red blood cells (RBC). It addresses all aspects related to the measurement process (specimen collection, storage, processing, analysis and reporting). This method was evaluated for the total hemoglobin measurements, whole blood and red blood cells.

Specific details related to equipment maintenance and operations are provided in the manufacturers' manuals and in work instructions created and maintained by the Protein Biomarker Laboratory (PBL). Further, this document is not intended to provide information on data interpretation.

2 SAFETY PRECAUTIONS

2.1 General Safety

All blood specimens must be considered potentially positive for infectious agents including Human Immunodeficiency Virus (HIV), hepatitis B virus and hepatitis C virus. Hepatitis B vaccination series is required for all analysts performing this measurement procedure.

Universal precautions must be observed: protective gloves, laboratory coats and safety glasses 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 and all other reagents and organic solvents used in this measurement procedure must be handled with extreme care; they are caustic, flammable and toxic and 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 vapor. 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.
Hexane: Do not breathe vapor. Extremely flammable liquid and vapor. It may cause flash fire. It can enter lungs and cause damage and may cause eye and skin irritation. Breathing vapors may cause drowsiness and dizziness.

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 digestive tract burns. Contact with other material may cause a fire. Acute pulmonary 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 or at http://intranet.cdc.gov/ohs.

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

2.5 Waste Disposal

All solid waste used in the sample preparation process (e.g., disposable plastic pipette tips, gloves, bench diapers, caps) as well as any residual sample material needs to 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 using the appropriate waste management and chemicals tracking systems. All waste disposals must be performed in compliance with CDC policies and regulations. The CDC Safety Policies and Practices Manual are located in the laboratory and can be accessed at http://isp-v-ehip-asp/dlsintranet/safety_manual/.
Procedure for Creating Lab Waste Tickets is found in the work instruction PBLW010004.

2.6 Training

Analysts performing this measurement procedure at a minimum must successfully complete

- Safety Courses (CDC-OHS Safety Survival Skills Parts 1 and 2, and Blood Borne Pathogens Courses)
- CDC-OHS Hazardous Chemical Waste Management Course
- Information Security Awareness Training Course
- Records Management Training

Further, the analyst must have received training on the specific instrumentation used with this measurement procedure from designated staff or the instrument manufacturer.

At a minimum, the analysts performing this measurement procedure must be familiar with the

- Exposure Control Plan
- Chemical Hygiene Plan
- Relevant Material Safety Data Sheets
- DLS Safety Manual
- DLS Policies and Procedures Manual
- DLS After-Hours Work Policy

- Policy on Confidentiality, Data Security and Release of Information
- DLS Policy on Use of Controlled Substances

3 COMPUTERIZATION AND DATA-SYSTEM MANAGEMENT

3.1 Software and Knowledge Requirements

This measurement procedure requires work with various software operated instruments such as Thermo Finnigan LC/MS/MS (using Xcalibur 2.1 version or higher) and Tecan (using Evoware Software version 4.11.5878 or higher). Specific training to operate this software is required to ensure appropriate and safe instrument function.

Further, calculations of results obtained with the HPLC/MS/MS software are performed using calculation templates created with Microsoft Excel. The calculation results obtained with the Excel templates are transferred to a database that is created and maintained by 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 dedicated laboratory staff.

3.2 Sample Information

All samples must be labeled as described in the DLS Policies and Procedures Manual. No personal identifiers are used, and all samples are referenced to a blind coded sample identifier.

3.3 Data Maintenance

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

3.4 Information Security

Information security is managed at multiple levels. The information management systems that contain the final reportable results are restricted through user identification (ID) and password security access. The computers and instrument systems that contain the raw and processed data files require specific knowledge of software manipulation techniques and physical location. Site security is provided through restricted access to the individual laboratories, buildings, and offices. Confidentiality of results is protected by referencing results to blind coded sample IDs (no names or personal identifiers).

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 as described below or equivalents can be used in this measurement procedure.

4.1.1 Equipment, Chemicals and Consumables Used for Reagent Preparation

1. Mettler Toledo PG 403-S Delta-Range Chemical Balance (Electronic "0.000 g", Max 410.0 g, Min 0.02g, Columbus, OH)
2. Fisherbrand Desiccator Cabinets, Tall (Fisher scientific, Suwanee, GA)
3. Dessicant, 6-8 Mesh 100% (EMD Chemicals, Gibbstown, NJ)
4. Accumet AB 15 pH Meter with Orion Micro-Combination Electrode, pH Range 0-14, Temperature Range 0-100 ºC (Fisher Scientific, Suwanee, GA)
5. Sato Label Maker CL612e and Label Making Software (Sato America, Charlotte, NC)
6. 500 mL Glass Beaker (Corning Incorporated, Lowell, MA)
7. 500 mL Pyrex Graduated Glass Cylinder, Tolerance ±1.4 mL, (Kimble Chase Life Science and Research Products LLC, Cat. No: 20022, Vineland, NJ)
8. Fisherbrand Octagonal Stirring Bars, 1 Inch Length; 0.312 Inch Diameter (Fisher Scientific, Cat No: 14-513-59, Suwanee, GA)
9. Gilson 1000 µl Pipette (Gilson Inc., Middleton, WI)
10. Gilson 200 µl Pipette, Variable (Gilson Inc., Middleton, WI)
11. Eppendorf Repeater Pipette (Eppendorf, Ramsey, MN)
12. 1 mL, 2mL, 3mL and 4mL Volumetric Glass Pipettes. (Fisher Scientific, Suwanee, GA)
13. Various Glass Beakers, 2L Volumetric Flask, Four 500mL and Two 100mL Graduated Cylinders, and Teflon Seal 1L and 2L Glass Bottles, Silanized Volumetric Flasks, Silanized Volumetric Pipettes, Class A Glassware (Fisher Scientific, Suwanee, GA)
14. 15 mL Plastic Falcon Tubes (KSE, Durham, NC)
15. 50 mL Plastic Falcon Tubes (KSE, Durham, NC)
16. Lab Rotators Barnstead with Flat Surface Rotor (Lab-Line, Melrose Park, IL)
17. Scholar™ 5 x 5 Inch PC-171 Magnetic Stirrer (Corning Incorporated, Lowell, MA)
18. 1L Glass Bottles With Screw Tops (Wheaton Industries Inc., Cat. No: 219440, Millville, NJ)
19. Pasteur Transfer Pipettes (Samco Scientific, Cat. No: 225, San Fernando, CA)
20. Disposable Pasteur Pipets, 53/4" (Fisher Scientific, Cat. No: 13-678-20A, Suwannee, GA)
21. Milli-Q Water, Resistivity, 18 megaOhm-cm DI Water or greater at 25 ° C, 18.2 (Aqua Solutions, Jasper, GA)
22. Ethyl Acetate, HPLC/ACS Grade (Fisher Scientific, Cat. No: E195SK-4, Suwanee, GA)
23. Toluene, HPLC/ACS Grade, (Fisher Scientific, Cat. No: T290SK-4, Suwanee, GA)
24. Isopropyl Ether, Certified (Fisher Scientific, Cat. No: E141-4, Suwanee, GA)
25. Glacial Acetic Acid, Certified ACS Grade, CAS No: 64-19-7 (Fisher Scientific, Cat. No: 64-19-7, Suwanee, GA)
26. Methanol, HPLC Grade (Fisher Scientific, Cat. No: A452SK4, Suwanee, GA)
27. Acetic Acid ACS Grade (J. T. Baker, Phillipsburg, NJ)
28. Sodium Hydroxide 0.2N Solution (Fisher Scientific, Suwanee, GA)
29. Hemo Point H2, Hemoglobin Control, 6 Vials (Stanbio Laboratory, Boerne, TX)
30. 0.9% Sodium Chloride Solution 1000 mL Bags (Hospira, Inc., Lake Forest, IL)
31. Hemoglobin Reagent Set (Teco Diagnostics, Cat. No: H526-6L, Anaheim, CA)
32. Standard material: 5 Bottles of Lyophilized Hemoglobin, Part # HC-110LIN (Analytical Control Systems Inc., Fishers, IN)

4.1.2 Equipment, Chemicals and Consumables Used for Calibration Materials

1. Mettler Toledo PG 403-S Delta-Range Chemical Balance (Electronic "0.000 g" , Max 410.0 g, Min 0.02g, 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, Cat. No: 55640, Vineland, NJ)
4. Acrylamide 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 Acrylamide 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. Glycidamide 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 Glycidamide 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)

4.1.3 Equipment, Chemicals and Consumables Used for Sample Processing

1. Eppendorf Centrifuge 5810R, (Eppendorf, Ramsey, MN)
2. Glas-Col MultiPulse Vortexer (Glas-Col, Terre Haute, IN)
3. Eppendorf Repeater Plus Pipetter (Eppendorf, Cat. No: 022260201, Ramsey, MN)
4. Sato Label Maker CL612e and Label Making Software (Sato America, Charlotte, NC)
5. 96-Well 2 mL Square Well Plates (Seahorse Labware, Cat. No: S30009, Chicopee, MA)

6. Robotic Reservoirs, Convoluted Bottom (Thermo Scientific, Cat. No: 1200-2300, Waltham, MA)
7. ArctiSeal 96 Well Square Silicone w/ PTFE Spray Coating (Arctic White LLC, Cat No: AWSM-1003SX, Bethlehem, PA)
8. Eppendorf Combitips Plus Pipet Tips, 5 mL (Eppendorf, Cat. No: 022266403, Ramsey, MN)
9. GeneVac EZ-2 Evaporation System with Side Bridge Holders and Universal Rotor (GeneVac Inc., Valley Cottage, NY)
10. Orbitron Rotator II, Model 26250, (Boekel Scientific, Feasterville, PA)
11. Eppendorf Swing-Bucket Rotor (Eppendorf, Cat. No: A-2-DWP, Ramsey, MN)
12. 48 well, 5 mL, Rectangular Well, Pyramid Bottom, Natural Polypropylene Plate (Fisher Scientific, Suwanee, GA)
13. 96 well, Microtiter Plate, Nonbinding Surface (Fisher Scientific, Suwanee, GA)
14. 48 well, 7.5 mL Fritted Plate, with 25 µm PE Frit, Long Drip (Fisher Scientific, Suwanee, GA).
15. 48 well, 7.5 mL, Rectangular Well, Pyramid Bottom, Natural Polypropylene (Fisher Scientific, Suwanee, GA)
16. 96 well, 250 µL, Uniplate V-Well Bottom Microplate (Whatman Inc., Piscataway, NJ)
17. Bulk Isolute Sorbent HM-N (Biotage, Charlottesville, VA)
18. 1000 µl, Clear Pipetting Tips for Tecan (Molecular Bio Products, San Diego, CA)
19. 1000 µl, Conductive Pipetting Tips for Tecan (Molecular Bio Products, San Diego, CA)
20. 200µl, Conductive Pipetting Tips for Tecan (Molecular Bio Products, San Diego, CA)
21. 150uL, filtered MCA96 SBS pipette tips for Tecan (Tecan, Durham, NC)
22. 2 mL PE 96-Well Pattern Sealing Film (Bio Tech Solutions, Mt. Laurel, NJ)
23. Arctic Sealer Silicone PTFE Film (Arctic White LLC, Bethlehem, PA)
24. Nalgene 2mL Cryovials with Ext-Tread (Fisher Scientific, Suwanee, GA)
25. HemoCue 201 Microcuvettes (HemoCue Inc, Lake Forest, CA)
26. CP10ST (0.5µL-10µL) M10 Pipette Tips for Microman (Gilson, Inc, Middleton, WI)
27. Aluminum Foil (Fisher Scientific, Suwanee, GA)
28. Tecan Evo (Tecan US., Research Triangle Park, NC)
29. Vortex- Genie 2 with Well Plate Adapter (Scientific Industries Inc, Bohemia, NY)
30. Precision Oven (Thelco Laboratories, Torrance, CA)
31. Eppendorf Centrifuge 5810 R V4.2 with A-4-62 Rotor (GMI, Ramsey, MN)
32. GeneVac EZ-2 Evaporation System with Side Bridge Holders and Universal Rotor (GeneVac Inc., Valley Cottage, NY)
33. Eppendorf 8-Channel Pipette 50-1200 µl, (Eppendorf, Westbury, NY)
34. Gilson 1000 µl Pipette (Gilson Inc., Middleton, WI)
35. Gilson 200 µl Pipette, (Gilson Inc., Middleton, WI)
36. Gilson 10 µl Pipette, (Gilson Inc., Middleton, WI)
37. Eppendorf Repeater Pipette (Eppendorf, Ramsey, MN)
38. Hand Held Scanner (Symbol, Technologies. Inc, Bohemia, NY)
39. Transfer Pipettes (Samco Scientific, San Fernando, CA)
40. Fisherbrand 20" Flexible-Arm Lamp with Halogen Bulb (Fisher Scientific, Suwanee, GA)
41. Boekel Orbitron Rotator, Model II (Fisher Scientific, Suwanee, GA)
42. Microman, 10µL Positive-Displacement Pipette (Gilson, Inc, Middleton, WI)
43. Fisherbrand Octagonal Stirring Bars, 1"L x 5/16" D (Fisher Scientific, Suwanee, GA)
44. Plain Wood Applicators, 5 ¾"L x 1/12"D (Fisher scientific, Suwanee, GA)
45. Fisherbrand U.S. Standard Brass Sieve No. 25, 710 µm (Fisher Scientific, Suwanee, GA)
46. Formamide ACS Grade (USB, Cleveland, OH)
47. Pentafluorophenyl Isothiocyanate 96 %(Alfa Aesar, Ward Hill, MA)
48. Methanol, HPLC Grade (Fisher scientific, Cat. No: A452SK4, Suwanee, GA)

4.1.4 Equipment, Chemicals and Consumables Used for Sample Measurement

1. 2 mL Polyethylene 96 Well Pattern Sealing Tape (BioTech Solutions, Cat. No: ZAF-PE-50, Vineland, NJ)
2. RAPID Slit Seal 96 Well Sealing Tape (Rikaken USA, Inc., Bethesda, MD)
3. HPLC column: Thermo Hypersil GOLD, 50 x 2.1 mm, 1.9 µ, (Thermo Electron, Holbrook, NY)
4. Guard column: Javelin filter for 2.1MM ID (Thermo Electron, Holbrook, NY)
5. Power Wave XS Spectrophotometer (Bio-Tek Instruments, Winooski, VT)
6. Finnigan TSQ Quantum Vantage with Atmospheric Pressure Chemical Ionization Unit (Thermo Electron, San Jose, CA)
7. Accela HPLC with Auto-Sampler and Photodiode Array Detector (Thermo Electron, San Jose, CA)
8. HemoCue Hb 201+ (HemoCue Inc, Lake Forest, CA)
9. Methanol, HPLC Grade (Fisher scientific, Cat. No: A452SK4, Suwanee, GA)
10. Formic Acid, HPLC Grade (Fisher Scientific, Cat. No: MFX04406, Suwannee, GA)
11. Isopropanol, Certified ACS (Fisher Scientific, Cat. No: A416-1, Suwannee, GA)
12. Deionized Water with Resistance to at Least 18 megaOhm-cm or greater and Filter Before use, Using 0.45 µm Nylon Filters

4.2 Preparation of Reagents Used For Sample Preparation

Preparation of reagents are described in the PBL work instructions (WI); please see specific work instructions related to the sample preparation. Work instructions are located on the network at the link below: \\cdc\project\CCEHIP_NCEH_DLS_CCB_PBL_Acrylamide\Lab\SOPs_WIs\AA Project task list\Instruction Procedures

4.2.1 PBLW010001 Preparation of pH Adjusted Formamide Solution

This solution is used to dilute blood samples and to adjust the pH of the sample to be between 6.5-7.5 Transfer formamide into the two graduated cylinders up to the 500 mL mark. If excess formamide remains, keep separate. Transfer the formamide from the two graduated cylinders back into the empty formamide bottle. To the 1 L formamide add 1 mL glacial acetic acid using the glass pipette. Mix solution. The instructions and safety information for preparing this solution can be found in the Work Instruction PBLW010001.

4.2.2 PBLW010005 Preparation of Tecan Solvent Wash Solution

The Tecan wash solvent is the liquid pipetting solvent used by the Tecan. The pipetting system requires water to be present in the system for the liquid handling. A 5% methanol solution is prepared. 5 % methanol is added to the 18 megaOhm-cm DI water or greater to prevent bacterial growth in the water. Prepare 5.0 L solution. Measure 950 mL of 18 megaOhm-cm DI water or greater using the 5 L beaker. Measure 50 mL of HPLC grade methanol using the graduated cylinder. Add the methanol directly to the 18 megaOhm-cm DI water or greater contained in the beaker. Mix content. Repeat until 5.0 L is prepared. The instructions and safety information for preparing this solution can be found in the Work Instruction PBLW010005.

4.2.3 PBLW010006 Preparation of Mobile Phase for LCMSMS

This solution is prepared for the LC/MS/MS instruments to provide suitable chromatographic conditions for the Acrylamide and Glycidamide adducts specimen separation. A 2 L 1:1 (v:v) methanol water solution is prepared. Measure 500 mL of 18 megaOhm-cm DI water or greater using the designated graduated cylinder and pour it into the LC bottle. Repeat this a second time so that the amount of water in the LC bottle equals 1000 mL. Measure 500 mL of methanol and add it to the water in the LC bottle. Repeat this a second time so that the amount of methanol in the bottle also equals 1.0 L. Close the bottle using a non-perforated top and mix the solution thoroughly. The instructions and safety information for preparing this solution can be found in the Work Instruction PBLW010006.

4.2.4 PBLW010064 Preparation of HRS Solution

This solution is used to prepare the reagent solution for the total hemoglobin measurement.
The Hemoglobin reagent Set (HRS) solution is prepared by dissolving the readily made powder in 1 L of 18 megaOhm-cm DI water or greater. Mix solution. The instructions and safety information for preparing this solution can be found in the Work Instruction PBLW010064.

4.2.5 PBLW010034 Preparation of Solution for Liquid-Liquid Extraction

This solution describes the preparation of the extraction solution used for supported liquid – liquid extraction of analytes. Add 500 mL isopropyl ether, 400 mL ethyl acetate and 100 mL toluene to a 1-L glass bottle, using separate graduated cylinders for each solvent. Pure each solvent in one container. Cap bottle and

mix thoroughly. Repeat procedure using a second 1-L bottle. The instructions and safety information for preparing this solution can be found in the Work Instruction PBLW010034.

4.2.6 PBLW010033 Preparation of Extraction Plate

This procedure describes the preparation of the extraction plates used for supported liquid – liquid extraction of the analytes. Fill the *"Extraction Plates*" (48-well 7.5-mL plate with 25 µm PE frit and long drip) with Isolute sorbent. Fill to the top and tap off excess to ensure uniform amount across all wells.
Turn plate sideways and tap several times to remove additional isolute sorbent. Fill final plate to 0.5 cm or ¼ inch from the top. Tap all sides of the plate to ensure uniform packing. Plates can be prepared the day needed and stored in a desiccator. Avoid clogging of the outlets of the filter plate when preparing the samples by placing the plate on a clean surface. The instructions and safety information for preparing this solution can be found in the Work Instruction PBLW010033.

4.3 Calibration Materials

4.3.1 Preparation of Calibrator Stock Solutions, Intermediate Stock and Working Solutions for Acrylamide and Glycidamide Octapeptide Measurement

All glassware that is in contact with the octapeptide calibrators must be silanized. See work instruction PBLW010043 Glass Silanization. Otherwise, losses due to adsorption of the peptides to the glass are observed, resulting in inaccurate calibrator concentrations.

The calibrator working solutions are prepared from calibrator stock solution. Please see PBLW010047 Preparation of the Acrylamide Stock Solution.

If different solutions are used, the preparation procedures need to be adjusted accordingly. This procedure produces 250 vials per calibrator level, which is sufficient for 1,111 analytical runs assuming use of 1 vial for four calibration curves per batch.
The following calibrator stock solutions (Table 1) are prepared:

Calibrator Stock Solution Code	Target Concentration		Dilution in 18 megaOhm-cm deionized (DI) water or greater
	Acrylamide Octapeptide	Glycidamide Octapeptide	
A stock solution	69.72 µmol/L	157.86 µmol/L	2.51 mg Acrylamide Octapeptide → 25 mL 5.77 mg Glycidamide Octapeptide → 25 mL
B intermediate	10,000 nmol/L	10,000 nmol/L	14 mL Acrylamide Octapeptide (Stock A material) → 100 mL 6.33 mL Glycidamide Octapeptide (Stock A material) → 100 mL

Table 1: Desired Acrylamide and Glycidamide Octapeptide Calibrator Stock Solution

1. Preparation of Calibrator Stock Solution "A"
    a. Remove Acrylamide-Val-His-Leu-Thr-Pro-Glu-Glu-Lys-OH (Acrylamide Octapeptide) and Glycidamide-Val-His-Leu-Thr-Pro-Glu-Glu-Lys-OH (Glycidamide Octapeptide) material from freezer and allow to reach room temperature over a period of at least 30 minutes.

b. Calibrate the analytical balance following the manufacturer's instructions.
c. Weigh out using analytical balance 2.51 (±0.001) mg of Acrylamide-Val-His-Leu-Thr-Pro-Glu-Glu-Lys-OH custom synthesized material into a clean aluminum foil surface and add it to a 25 mL volumetric flask.
   Weigh out 5.77 (±0.001) mg of Glycidamide-Val-His-Leu-Thr-Pro-Glu-Glu-Lys-OH custom synthesized material into a clean aluminum foil surface and add it a 25 mL separate volumetric flask.
d. Add 18 megaOhm-cm deionized (DI) water or greater to just below the fill line of the volumetric flasks
e. Place flasks in the 18 megaOhm-cm DI water or greater bath for at least 15 minutes to reach 20°C and add 18 megaOhm-cm DI water or greater to the fill line.
f. Mix solution.
g. Aliquot solution in 50 mL falcon tubes, each analyte separately.
h. Label tubes appropriately "Stock Solution #" and store them in the refrigerator.

2. Preparation of Calibrator Intermediate Stock Solution "B"
   a. Transfer 14 mL Acrylamide Octapeptide and 6.33 mL Glycidamide Octapeptide of calibrator stock solution "A" into a 100 mL volumetric flask using a calibrated pipette. Combine both analytes into one volumetric flask.
   b. Add 18 megaOhm-cm DI water or greater 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 and add 18 megaOhm-cm DI water or greater (at 20 °C) to the fill line.
   d. Mix solution completely.
   e. Aliquot solution in 50 mL aliquots into falcon tubes.
   f. Label tubes appropriately and store them in the refrigerator.
3. Preparation of Working Solutions
   The calibrator working solutions are prepared as shown in Table 2. The following levels of calibrator working solutions are used for measuring acrylamide and glycidamide adduct levels in human blood.

Calibrator Working Solution Code	Target Concentration		Dilution in 18 megaOhm-cm deionized (DI) water or greater
	Acrylamide Octapeptide nmol/L	Glycidamide Octapeptide nmol/L	
AcrC01L00	1.25	1.25	65 µL (Calibrator Intermediate Stock Solution) → 500 mL
AcrC02L00	2.5	2.5	125 µL (Calibrator Intermediate Stock Solution) → 500 mL
AcrC03L00	5.0	5.0	250 µL (Calibrator Intermediate Stock Solution) → 500 mL
AcrC04L00	10.0	10.0	500 µL (Calibrator Intermediate Stock Solution) → 500 mL
AcrC05L00	20.0	20.0	1000 µL (Calibrator Intermediate Stock Solution) → 500 mL
AcrC06L00	40.0	40.0	2000 µL (Calibrator Intermediate Stock Solution) → 500 mL
AcrC07L00	80.0	80.0	4000 µL (Calibrator Intermediate Stock Solution) → 500 mL

Table 2: Desired Acrylamide-Val-His-Leu-Thr-Pro-Glu-Glu-Lys-OH and Glycidamide-Val-His-Leu-Thr-Pro-Glu-Glu-Lys-OH Calibrator Working Solution

Prepare the Calibrator Working Solutions by Performing the Following Tasks:

1. Adjust the calibrator stock solution temperature to 20 °C using a water bath.
2. Transfer the volumes of calibrator stock solutions stated in Table 1 to separate volumetric flasks (500 mL) 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 least15 minutes to reach 20 ºC and add 18 megaOhm-cm DI water to the fill line.
5. Aliquot solution in 0.9 mL aliquots in appropriately labeled cryovials and store them in the -70 °C freezer. Each vial is sufficient for four analytical runs. Calibrator solution cannot be reused. This solution is stable until completely used.

4.3.2 Preparation of Calibrator Solutions for Total Hemoglobin Measurement

The following concentrations of calibration solutions are prepared from 5 bottles of the lyophilized hemoglobin linearity control, from Analytical Control Systems Inc., Fishers, IN. The hemoglobin concentration may vary based on lot number. Prepare the current lot number calibrators and the corresponding five levels 18.8, 9.4, 4.7, 2.35, and 1.18g/dl. Label the calibrator level vials with the labels that are pre created and can be found at the link below:
\\cdc\project\CCEHIP_NCEH_DLS_CCB_PBL_Acrylamide\Lab\Labels\AA Labels

1. Add 0.5 mL 18 megaOhm-cm DI water or greater to each of five vials of "Linearity Control. Place" on Hematology Mixer for mixing.
2. Combine the 5 "Linearity Control" vials into one vial after they are mixed.
3. Dilute the "Linearity Control" provided by the manufacturer with 18 megaOhm-cm DI water or greater, using the dilution scheme shown in Table 3. See work instruction PBLW010003 Preparation of ACS Total Hemoglobin Calibration Curve.
4. Label vials appropriately with date, initials, and contents. Note, that the total hemoglobin calibration material expires seven days after it is made.
5. Place calibrators in the refrigerator.

Calibration Curve Level	Dilution	Take from	Sample	dl H_2O	Concentration based on current lot	Total Volume
1	100%	Take from Stock 18.8g/dL	1.20 mL Linearity Control	0.00 mL	18.8 g/dL	1.2 mL
2	50%	Take from Stock 18.8g/dL	0.60 mL Linearity Control	0.60 mL	9.4 g/dL	1.2 mL
3	25%	Take from Stock 18.8g/dL	0.30 mL Linearity Control	0.90 mL	4.7 g/dL	1.2 mL
4	12.5%	Take from Stock 18.8g/dL	0.15 mL Linearity Control	1.05 mL	2.35 g/dL	1.2 mL
5	6.25%	Take from Stock 18.8g/dL	75.3 µL Linearity Control	1.125 mL	1.18 g/dL	1.2 mL

Table 3. Preparation of the Total Hemoglobin Calibration Curve

4.3.3 Preparation of Internal Standard Solutions

All glassware that is in contact with the octapeptide calibrators must be silanized. Otherwise, losses due to adsorption of the peptides to the glass are observed resulting in inaccurate calibrators.

The internal standard working solution is prepared from an internal standard stock solution which is prepared from custom synthesized material. If different solutions are used, the preparation procedures need to be adjusted accordingly. This procedure produces 200 vials of internal standard working solution "B", which is sufficient for 10,000 samples assuming use of 100 µL of internal standard stock working solution "B" per sample.

The Internal Standard Working Solution is prepared as follows: Acrylamide-Val($^{13}C_5$, $^{15}N_1$)-His-Leu-Thr-Pro-Glu-Glu-Lys-OH and Glycidamide-Val($^{13}C_5$, $^{15}N_1$)-His-Leu-Thr-Pro-Glu-Glu-Lys-OH are used to create internal standard stock and working solutions in 18 megaOhm-cm DI or greater water with the concentrations listed in Table 4.

Internal Standard Solution Code	Internal Standard Target Concentration		Dilution in 18 megaOhm-cm DI water or greater
	Acrylamide Octapeptide µmol/L	Glycidamide Octapeptide µmol/L	
Stock Solution A	77.18	128.2	0.81 mg Acrylamide Octapeptide → 10 mL 1.41 mg Glycidamide Octapeptide → 10 mL
Working Solution B	38.6	64.1	0.5 mL (Internal Standard Stock Solution A)→ 1,000 mL

Table 4: Desired Internal Standard Acrylamide and Glycidamide Octapeptide Solution

1. Preparation of Internal Standard Stock Solution "A"
    a. Remove Acrylamide-Val($^{13}C_5$, $^{15}N_1$)-His-Leu-Thr-Pro-Glu-Glu-Lys-OH and Glycidamide-Val($^{13}C_5$, $^{15}N_1$)-His-Leu-Thr-Pro-Glu-Glu-Lys-OH material from freezer and allow to reach room temperature over a period at least 30 minutes.
    b. Calibrate the analytical balance following the manufacturer's instructions. Weigh and transfer 0.81 (±0.001) mg of Acrylamide-Val($^{13}C_5$, $^{15}N_1$)-His-Leu-Thr-Pro-Glu-Glu-Lys-OH and 1.41 (±0.001) mg of Glycidamide-Val($^{13}C_5$, $^{15}N_1$)-His-Leu-Thr-Pro-Glu-Glu-Lys-OH to a clean 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. Aliquot solution in 10 mL aliquots (in 15-mL falcon tubes), label tubes appropriately and store them in the -70 ºC freezer.

2. Preparation of Internal Standard Working Solution "B"
    a. Transfer 0.5 mL of internal standard stock solution "A" into a 1,000 mL volumetric flask using a 1 mL calibrated pipette.
    b. Add 18 megaOhm-cm DI water or greater to the flask to the fill line of the volumetric flask.
    c. Mix solution completely.

d. Pure solution (in 15-mL falcon tubes), label tubes appropriately and store them in the -70 ºC freezer.

Note: This solution is stable until it is completely used.

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

5.1 General Specimen Requirements

For analysis of acrylamide and glycidamide hemoglobin adducts a 1.0 mL sample of whole blood or 0.5 mL sample of packed red blood cells is preferable to allow for repeat analyses. A sample volume of 350 µL of whole blood or 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 acrylamide and glycidamide hemoglobin 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 was not tested.

The appropriate amount of whole blood or red blood cells is dispensed into 2.0-mL cryogenic vials with external screw caps. Labeled vials in accordance to CDC and NCEH/DLS policies and regulations. If there are analytes other than acrylamide and glycidamide to be measured, the sample needs to be divided; the appropriate amount of blood or lysed red blood cells must be transferred into a sterile cryovial labeled in accordance to CDC and NCEH/DLS policies and regulations. Other specimen handling conditions are outlined in the DLS Policies and Procedures Manual (26).

5.2 Specimen Storage

Specimens collected in the field can be shipped refrigerated at 5 ºC or frozen on dry ice. Specimens can be kept refrigerated for 3 days. For long term storage samples are stored at -70 ºC. Samples are stable for at least 5 years if stored at -70 ˚C. Two to three freeze-thaw cycles did not show any changes in values in the in-house experiments. However, 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 must not undergo more than 3 freeze-thaw cycles.

5.3 Unacceptable Specimens

Specimens that do not meet the above mentioned criteria, were transported at room temperature or have evidence of leakage are not acceptable.

6 PROCEDURE OPERATION INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

All instruments are checked before use for proper function using the manufacturer's acceptance criteria. Specific details related to the operation instructions, preventative maintenance (PM) and specific file names used in the execution are documented in work instructions. A list of all work instructions can be found in appendix 8.

Preventative Maintenance of instruments: See work instructions relevant to instruments.

- PBLW010007 PM of ROSS pH Electrode and pH meter
- PBLW010008 PM of Eppendorf Centrifuge 5810R
- PBLW010010 PM of PowerWave
- PBLW010011 PM of GeneVac
- PBLW010012 PM of GlasCol
- PBLW010015 PM of Tecan
- PBLW010016 PM of Triple Stage Quadrupole

6.1 Specimen Storage and Handling During Testing

All vials are labeled according to DLS Policies and Procedures Manual. Barcodes are scanned during the process of sample preparation, sample transfer and analysis in order to ensure individual samples can be tracked throughout the process. The work instruction procedure for creating barcode labels is available on the network see PBLW000001.

Specimens are allowed to reach room temperature for sample preparation. The unused portion of the patient specimen is returned to the freezer and stored at -70 °C. Samples ready for analysis by HPLC/MS/MS are either stored at 5 °C in the refrigerator or at 15 °C in the HPLC/MS/MS instrument sample tray.

6.2 Preparation of Samples for Analysis

All samples are processed together with 3 bench QC samples, 1 reagent blank and 1 set of calibrators (7 levels). Typically 148 patient samples are processed in one day. Total number of samples per plate include: 1 reagent blank, 7 calibrators and 37 samples and 3 quality control samples. A total of up to four plates are prepared in one day.

When fewer than 148 patient samples are processed the plate number varies:
Plate one will consist of 37 patient samples.
Plates one and two will consist of 74 patient samples.
Plates one through three will consist of 111 patient samples.
Plates one through four will consist of 148 patient samples.

1. Assess all samples for acceptability using the criteria described in the SOP section 5.2 and 5.3.
2. Dilute whole blood samples by hand 1:0.33 (v/v) with 6 mM K_3EDTA solution in saline by combining 1 mL of sample and 300 µL of 6 mM K_3EDTA in a cryovial and homogenizing gently using a laboratory rotator. Do not dilute red blood cells 1:0.33 (v/v). If red blood cells are lyzed and viscous add 1:1 (v/v) saline solution (0.9% sodium chloride) to add back the volume that was plasma. Sample dilution is performed as soon as the samples arrive in the laboratory and before they are processed.
3. Frozen red blood cell samples, 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.

4. 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.
5. Place all patient samples, QC samples, internal working solution and calibrator working solution on the designated automated instrument in the designated locations in a manner that allows the instrument's barcode reader to read all barcodes properly. Place all additional reagents on the instrument at the designated positions. See work instruction PBLW010032 for instrument setup. Work instruction PBLW010049 lists reagent volumes needed based on the number of plates being processed.

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

6. Follow work instruction PBLW010032. This task involves scaning all sample vials and transfers 500 µL blood samples from cryovials to the "*Dilution Plate*".
7. Scan the barcodes of all coded vials and reagents. All cryovials must have a small cap placed underneath them in the instrument rack. This is necessary to ensure that the vials are properly detected and scanned by the barcode reader.
8. When a barcode cannot be read, the instrument software will prompt the analyst to manualy enter the barcode information using a manual barcode scanner. After the scanning process is successfully 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 located on the instument computer: C:\Program Files\Tecan\EVOware\output\scan
9. The instrument executes pipetting of 500 µL of blood samples from the cryovials using 1,000 µL conductive tips to 48-well 5 mL plates. Plates are named "*Dilution Plate" and numbered* one through four. The program will pause with a wait prompt to allow assessing samples in the "*Dilution Plate"* for blood clots. The "*Dilution Plates"* are held under a lamp to visibly assess whether a blood clot is present in the well plate. If a blood clot is observed, it is removed by the operator using a wooden application stick. If the blood clot is large, sample is removed from the cell and the same sample is pipetted manually to the plate without blood clots.

6.3 Total Hemoglobin Measurements

The hemoglobin measurement procedure must be performed first before continuing with the Edman procedure. The hemoglobin measurement provides information about the hemoglobin concentration in the "*Dilution Plate".* This sample solution is used later for the modified Edman reaction. The amount of hemoglobin in the Edman reaction is calculated in grams per deciliter from the sample solution and from the specific volume that is used for the modified Edman reaction. The result is later used to calculate the reported results for acrylamide and glycidamide hemoglobin adducts in picomoles of adduct per gram of total hemoglobin (t-HB). This step is necessary because the concentration of hemoglobin in the sample or sample solution is not known and it is variable.

The measurement procedure performed in this protocol is a commercial clinical assay named Hemoglobin Reagent Set (HRS). The manufacturer's package insert and instructions are found in PBLW010064 work instruction. HRS solution is prepared according to the manufacturer's instruction. The HRS solution is prepared by dissolving the readily made powder in 1 L of 18 megaOhm-cm DI water or greater. See package insert attached in 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. Specimens with values above 20.0 g/dL must be re-run using one half of the sample volume and the final result must be multiplied by 2.

6.4 Hemoglobin Analysis by Spectrophotometer

In this portion of the procedure an "HRS Plate 1.95 mL" and an" HRS Plate 1.60 mL" HRS solution and the "Microtitor Plate" (MTPs) are prepared. The hemoglobin reagent set (HRS) solution is used for measuring the concentration of total hemoglobin in blood samples. The main steps for the total hemoglobin analysis by spectrophotometer and the associated work instructions are listed below.

Prepare "*HRS Plate"* by Adding 50 µL of Blood Sample and Transfer to MTP to Measure Absorbance.

1. All of the steps below are performed for each of the four plates one plate at a time.
2. Prepare *HRS Plate 1.95 mL* and *HRS Plate 1.60 mL,* a total of eight 48-well 5 mL plates with HRS solution according to WI PBLW010064 and PBLW010032.
3. The instrument will pipette 50 µL ACS calibrators from the five levels to the "*HRS Plate 1.95 mL"*.
4. The instrument will add 50 µL of blood to 43 cells in the wells "*HRS Plate 1.95 mL"*.
5. Always vortex the hemoglobin QC vials and test HemoCue (HC) values before use. Use current QC materials, assure and verify their lot number and expiration date.
6. The instrument will add 1.60 mL of HRS solution to each well of the "*HRS Plates 1.60 mL"* and 1.95 mL of HRS solution to each well of the "*HRS Plate 1.95 mL"*.
7. The instrument will mix all wells, but checked mixing vissualy. If poor mixing is observed, mix manually by pipetting the solutions with a 1 mL pipette. Let the "*HRS Plate 1.95 mL"* sit for three minutes to complete reaction.
8. The instrument will transfer 400 µL of solution from the "*HRS Plate 1.95 mL"* to the "*HRS Plate 1.60 mL"*. Again, check for mixing and mix manually if necessary. Let sit for three minutes to complete reaction.

Read Blank *MTP* and Transfer Sample from *HRS Plate to MTP*

9. See PBLW010052 for detailed instructions on how to operate the PowerWave instrument. Open the PowerWave software (KC4) on the computer and load program named HB_reading_AA. Read the absorption of the empty 96-well *MTP*.
10. Place the 96-well *MTP* on the Tecan instrument as described in PBLW010032 and the instrument will transfer 200 µL of sample solution from the *HRS Plate* to each well of the 96-well *MTP*.

Measure Absorbance of the *MTP* Content

11. Insert the 96-well *MTP* with the 200 µL sample solutions back onto the PowerWave plate reader and select okay. Measure the absorbance of the sample solution at 540 nm. Each *MTP* is measured individually. Once the final absorbance is measured for the first *MTP*, the second, third and fourth *MTP* can be measured, one at a time. Perform all steps below for the first plate before continuing with the second, third and fourth absorbance measurement.
12. After reading the absorption, 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 C drive.

6.5 Total Hemoglobin Data Processing

Microsoft Excel files are listed below; files from various MTP's plates will be combined and will be grouped as indicated below.
Templates can be found on the network drive as designated below:
\\cdc\project\CCEHIP_NCEH_DLS_CCB_PBL_Acrylamide\Lab\Data\Calc.Templates\Templates with CC in Column,1,2,3,4
Spectrophotometer files are located on the spectrophotometer on C drive at
C:\Documents and Settings\nceh22\KC4\KC4 Data

Spectrophotometer file is the "raw 1" file named "yyyymmdd_P(NH)_Plate_1_raw.xls"
Spectrophotometer file is the "raw 2" file named "yyyymmdd_P(NH)_Plate_2_raw.xls"
t-HB template "1_2" is named "HB_HRS_Calc_P_1_2_Ham_5ptCC.xlsm"

Spectrophotometer file is the "raw 3" file named "yyyymmdd_P(NH)_Plate_3_raw.xls"
Spectrophotometer file is the "raw 4" file named "yyyymmdd_P(NH)_Plate_4_raw.xls"
t-HB template "3_4" is named "HB_HRS_Calc_P_3_4_Ham_5ptCC.xlsm"

Calculations are performed by combining the raw data files from the spectrophotometer measurement with t-HB calculation templates. All calculations can be performed using Microsoft Excel template named "1_2" and "3_4". Use the appropriate template for plates "1 and 2" or plates "3 and 4" to ensure proper sample assignment. The template assumes the samples are arranged in the plate as described in work instruction PBLW010032.

1. Open spectrophotometer "raw 1" and "raw 2" or "raw 3" and "raw 4" files and import result values into the corresponding template and excel tab named raw "1" and "2" or raw "3" and "4" on the "HB_HRS template".
2. Copy all absorption values from the spectrophotometer raw file "1" and "2", specifically from cells B4 through M11 in the template file (depending upon which plate you are working with). Use the "Paste Special Link" function to the template tab named "raw".

3. Save the template names by modifying the files under the appropriate name using the current date and corresponding run ID in the network-drive. The template file will be saved as “yyyymmdd_tHB data”.

Note: All raw absorption data information can be copied and pasted in one step for each MTP. The designated file format in the template files are set up for the data as they are produced by the PowerWave KC4 Software.

6.6 Total Hemoglobin Data Calculations

The procedure can only be continued after all total hemoglobin measurements have been assessed, found valid and any necessary corrective actions have been performed.

1. Assess validity of the calibration curve by assessing at the slope, intercept and correlation coefficient (R^2) values.
2. Assess validity of the 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 hemoglobin to be within the desired range 5 to 20.0 g/dL.
5. Assess concentration values of the tHb QCs and patient samples.
6. 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.
7. Each sample’s absorbance is measured in duplicate and averaged.
8. For more details see work instruction PBLW010021.
9. Calculation:

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

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

10. Print all tHB data files.

6.7 Edman Reaction Preparation

In this step the N-terminal valine containing the acrylamide and glycidamide is cleaved from the hemoglobin protein chains with the Edman reagent. The resulting pentafluorophenyl thiohydantoin derivatives are isolated from the reaction mixture by supported liquid-liquid extraction on diatomaceous earth and prepared further for HPLC/MS/MS analysis.

In this portion 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 supported liquid-liquid extraction on diatomaceous earth and the extract is evaporated.

1. Place "*Edman Plates"* in their appropriate locations on the pipetting instrument. See work instruction PBLW010032 for more details.
2. Pipetting instrument will prepare the *Edman Plate* by transferring exactly 350 µl volumes of the blood samples 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 not sufficient during data processing and the data reviewer will associate an error code. Samples with such codes will be repeated.
3. The pH of the pH adjusted formamide and one quality control sample must be pre-measured and recorded to ensure that patient samples will be adjusted to the proper pH. See work instruction PBLW010007 for ROSS pH Electrode and pH meter operation instructions.
4. Prepare the calibration curve samples according to the work instruction. Each plate will contain one column of 200 µL CC, 100 µL of internal standard and 200 µL 18 megaOhm-cm DI water or greater (as blank) in the assigned wells. Visually check the volume level in each well. The volume level must be the same for all calibrators. Throughout the four plates the CC is added to varying columns to identify 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.
5. Instructions for preparing the pH adjusted formamide are found in work instruction PBLW010001. The instrument will add 1.5 mL pH-adjusted formamide to all wells. Program repeats the process until 1.5 mL total volume is added.
6. If whole blood samples are used instructions for preparing the pH adjusted formamide are found in work instruction PBLW010054. In this document the calibration curve pH adjustment is also described.
7. The instrument will add 200 µL 18 megaOhm-cm DI water or greater to CC samples and 100 µL of internal standard solution to all samples except to the blank using conductive tips. To the blank sample the instrument will add a total of 400 µL 18 megaOhm-cm DI water or greater.
8. To the CC samples the instrument will add 0.2N NaOH solution to adjust the pH. The volume is variable depending on the pH of the formamide lot.
9. The instrument program will then pause and a prompt will appear to measure the pH. Mix samples throughly.
10. Before proceeding ensure that red blood 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. Values are recorded in the laboratory journal.
11. 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 hood. Press okay on the prompt to add the Edman reagent on the computer and continue pipetting. The program will pipette 20 µL of Edman reagent to all wells of the plates. When the program has finishs, remove plates from the pipetting instrument, seal with silicon plate sealers, vortex and transfer to the shaker in the oven.

6.8 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.

6.9 Supported Liquid-Liquid Extraction of Edman Products

In this portion of the procedure an Extraction Plate, Collection Plate and Reconstitution Plate are prepared. To transfer samples to the *Extraction Plate* for supported liquid-liquid extraction, arrange plates and supplies on the instrument as described in PBL010032 work instructions. This program transfers all sample solutions from *Edman Plate* to the *Extraction Plate* by performing the following task:

1. Transfer the *Edman Plate* samples to four 48-well 7.5 mL *Extraction Plates* filled with Isolute material. Preparation of the *Extraction Plate* is described in work instruction PBLW010033 . Place each *Extraction Plate* on top of the corresponding new 48-well 7.5-mL *Collection Plate*.
2. Transfer content from the *Edman Plate* to the *Extraction Plate.*
3. Wait 20 minutes for samples to properly penetrate into sorbent material.
4. 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.
5. 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).
6. Remove *Collection Plate* containing the extraction solutions and leave in a Glas-Col evaporator overnight. For instructions on operating the Glas-Col, see work instruction PBL010058. The Glas-Col instrument will automatically shut off after solvent evaporates.
7. 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 dial on 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.
8. Vortex each plate on level four for 30 seconds.
9. Arrange *Collection Plates* containing ethyl acetate and supplies on the instrument as described in work instruction PBLW010032.
10. Transfer samples recovered from the wall wash step to a new 96-well 2 mL plate named *Reconstitution Plate*. Two *Collection Plat*es are transferred to one *Reconstitution Plate*.
11. Remove solvents by placing the *Reconstitution Plate* in a GeneVac EZ-2 concentrator. Use Method 02: Low BP from the GeneVac instrument. The instrument is set to “low boiling point” and “no lamp” setting. Dry the *Reconstitution Plate* for four hours. During this time the majority of the solvent will evaporate and approximately 100 µL residual volume will remain. See work instruction PBLW010059 for more details on how to operate the GeneVac instrument.

6.10 Sample Preparation for HPLC/MS/MS Analysis

When all solvents have been dried down from the *Reconstitution Plate* perform the following task:

1. Reconstitute the samples as described in work instruction PBLW010032.
2. Program adds 80 µL of methanol and 120 µL of 18 megaOhm-cm DI water or greater to each well in that order. Operator vortexes the *Reconstitution Plate* .
3. Transfer the *Reconstitution Plate* to the centrifuge. Centrifuge samples for 15 minutes at 3,700 rpm and 15 °C.
4. To transfer samples from the *Reconstitution Plate* to a new 96 well 250 µl plate named *Final Plate* see work instruction PBLW010032 .
5. Pipette 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.

6.11 Edman Products Analysis by HPLC/MS/MS

1. Tuning and calibration is performed each time the S-lens is removed for cleaning the instrument; usually after preventative maintenance or cleaning of the Q0 unit biannually. The file containing the tuning and calibration is named by date (yyyymmdd APCI high flow). An example of the instrument settings is described in work instruction PBL010045.
2. The instrument maintenance instructions and log-books are kept in the laboratory next to the instrument. The corona discharge needle is checked daily. The sample tube is flushed with methanol daily, and the transfer line tube is cleaned.
3. Mobile phase solvent preparation is described in work instruction PBLW010006 and instrument operation is described in work instruction PBLW010041. Preventative maintenance is described in work instruction PBLW010016.

6.12 Run Sequence Setup

In this section, a run sequence is created using the information obtained from the barcodes of the sample vials. 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 instrument file and data processing method file using a sequence template file found on the network at: \\cdc\project\CCEHIP_NCEH_DLS_CCB_PBL_Acrylamide\Lab\Data\Calc. Templates\Templates with CC in Column,1,2,3,4\Seq_20120320_for Vantage38.xls

1. Load template file named “Seq_20120320_for Vantage38.xls”
2. Copy information from barcode ID scan file to excel worksheet “Sequence_template_for 4 plates.xls” into tab “Barcode Raw” using the “Paste Special Link” function. After copying the information, the sequence information is automatically compiled. Review the information in the “Sequence Sheet 1-4” worksheet to ensure that the operator ID, instrument ID, date, plate #, plate names and sample ID’s are correct.
3. Save sheet “Export Sequence 1-4” as “csv” file using the date and Plate RunIDs as the file name (e.g., 20060612_PNH_255_256). When naming the file use the date the plate was prepared.
4. Open Finnigan Xcalibur software and activate “Sequence Setup”.

5. Import the “csv” file created from the Excel file and add additional information such as injection volume, path or instrument method if not there already. See work instruction PBLW010041 for more details about the method layout.
6. Save the sequence using the same name as used for the “csv” file.
7. Perform the instrument setup and performance check. Instuctions are found in work instruction PBLW010016.
8. Ensure that all the solvent reservoirs contain enough solvent to run the sequence. Check the Argon gas pressure. It must be about 20 psi. Ensure the argon gas tank is above 20 psi.
9. Empty the waste solvent reservoir of the LC system into the LC waste 4 L bottle labeled for the acrylamide project.
10. Ramp up flow of the HPLC eluent to the described flow rate and wait until pressure is stable. Record the HPLC pressure before each sequence run in the appropriate log sheet.
11. Load the sequence.
12. Place the sample plates that correspond to the loaded sequence in the auto-sampler of the LC system.
13. Ensure cell “A1” of the plate is at the left back position and the plates are positioned on the rack corresponding to the sequence.
14. Run the sequence to analyze samples.
15. Once sequence is finished check all samples for validity.
16. Re-inject samples that are not valid.

6.13 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 sample containing Sample Reconstitution Solution (Run 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 in one batch on the same instrument. Typical chromatograms of a sample are shown in Appendix 1.

The following HPLC/MS/MS parameters are used and are listed in table 5, table 6, table 7 and table 8.

Chromatographic conditions

Syringe:	500 µL DLW
Injection volume:	50 µL
Loop size:	100 µL
Column:	Luna 3u C18 (2) 100Å 100 mm× 2.0 mm ID, 3 µm particle size
Column Oven:	45 (±1)^{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 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 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:	1.22×10^{-5}
APCI probe:	B position

Analyte	SRM (m/z)	Collision Energy (V)	Transition used for:	Expected Retention time (min)
Acrylamide-Val-PFPTH	396→379	10	Quantitation	2.45
$^{13}C_6$-Acrylamide-Val($^{13}C_5$,^{15}N)-PFPTH	402→385	10	Quantitation	2.45

Table 7: Acrylamide SRM masses (m/z)

Analyte	SRM (m/z)	Collision Energy(V)	Transition used for:	Expected Retention time (min)
Glycidamide-Val-PFPTH	412→395	10	Quantitation	2.25
$^{13}C_6$-Glycidamide-Val($^{13}C_5$,^{15}N)-PFPTH	418→401	10	Quantitation	2.25

Table 8: Glycidamide SRM masses (m/z)

Surveyor PDA Method
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

6.14 HPLC/MS/MS Data Processing

Transfer raw data files obtained from the HPLC/MS/MS measurements to the corresponding folder on the Network drive \\cdc\project\CCEHIP_NCEH_DLS_CCB_PBL_Acrylamide\Lab\Data\. To integrate and process the data using the Finnigan Xcalibur program and Microsoft Excel calculation templates, refer to the integration work instructions PBLW010044.

All files and data 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. Prior to every analytical run, create two new folders in the network to add all file from the instrument computer to the network drive.

1. Data produced by the HPLC/MS/MS system is transferred to a network drive. Data is processed at a separate data processing station.
2. Because samples are transferred from the vial to the well plate, it is important to know the sample ID of each cell in the well plate. The instrument uses a defined program for sample transfer that enables the reliable tracking of samples in the well plate. The relationship of vial positions to each cell in the well plate is described in work instruction PBLW010032.
3. For a evaluation of files and data handling see work instruction PBLW010021.
4. Using a dedicated data processing method within the Xcalibur software, relevant chromatographic peaks are identified based on their retention time and the area under the curve is integrated. Manual integration may be required if automatic processing fails to integrate the peaks properly.
5. Integrated peaks are documented as electronic files in “pdf`” format and integration results are saved as “xqn” files.
6. The integration result “RST” files are exported into a Microsoft Excel file. The files are then imported to a template where final results are calculated.
7. Integrations and integration results are reviewed by a specially trained and dedicated individual. Errors

detected are returned to the analyst for correction. Only data that passes this review process are considered for further processing.

8. As previously mentioned, two new folders in the network drive are created prior to every experiment, File names are created as follows: (PNH abbreviation is used for NHANES samples, P is abbreviated for non NHANES plates)
    - YYYYMMDD_P_1_2
    - YYYYMMDD_PNH_1_ 2
9. A copy of the template file "Seq_20120320_for Vantage38.xls"(fill out all pink fields with the correct information) add to appropriate folder.
10. Copy the total hmoglobing file to the folder and rename the file "HB_HRS_Calc_P_1_2_Ham_5ptCC.xlsm" using the appropriate date and plate number:
    - Sequence_YYYYMMDD_P(NH)_ 1_ 2_ 3_ 4
    - Hb_Calc_YYYYMMDD_P(NH)_ 1_ 2
    - Hb_Calc_YYYYMMDD_P(NH)_ 3_ 4, respectively
11. A copy of "HB_HRS_Calc_P_3_4_Ham_5ptCC.xlsm" place it in the second folder and renamed to Hb_Calc_YYYYMMDD_P(NH)_Plate#3_Plate#4."

6.15 Data Calculations

1. All necessary calculations are performed in a Microsoft Excel spreadsheet after the result tables containing integrated areas, calculated using the quantitation settings on the Finnigan Xcalibur are transferred.. All samples are measured in singlicate. The zero point calibrator has no internal standard and serves as the reagent blank.
2. For all samples, quality control and calibrators, the area ratios are calculated from the quantitation ion.
3. Area ratios for calculating analyte concentration are calculated from the analyte and IS area counts.
4. Calibration curves are generated with the area ratios from the calibrators and their assigned values using ordinary linear regression not forced through zero. The calibration curve is assessed for outliers and other problems resulting in non-linear behavior of data points. Sample batches with invalid calibration curves are not processed further.
5. The analyte concentration in red blood cell sample is calculated using the area ratio 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 used to calculate reportable results. These samples will be reanalyzed after appropriate dilution or concentration.

7 CALIBRATION AND CALIBRATION VERIFICATION

7.1 Calibration

7.1.1 Calibration of Instruments and Equipment

All manual pipettes are calibrated annually following procedures recommended by the manufacturers. See work instruction PBLW000004 for more detail. The procedure follows instructions provided by and in compliance with ISO 8655 procedures ("Gravimetric methods for the determination of measurement error")

Accuracy of other equipment such as pH-meters, oven temperatures, and thermometers 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 and are performed according to in-house purity check. See work instruction PBLW010038 for more details. 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 calibrated for mass accuracy regularly by following the manufacturers test system instructions using calibration materials specified and with at least frequency recommended by the manufacturer. See work instructions PBLW010045 MS Tune and Calibration for more detail.

With each set of samples, 7 levels of calibration material and a low, medium, and high QC material covering the reported range of acrylamide and glycidamide adducts, 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 used and maintained by DLS.

Higher order reference materials are currently not available for these analytes or measurements. Exchange of calibrators with other laboratories measuring the same analyte with different methodologies has been performed and the concentration of the calibrators has been confirmed. 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 acrylamide and glycidamide adducts respectively.

Requirements for calibration verification are met by having seven calibrators (at low, medium and high levels) 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 (27). The method consists of two parts. The first part examines whether a nonlinear polynomial fits the data better than a linear one. The second part assesses whether the difference between the best-fitting nonlinear and linear polynomial is less than the amount of allowable bias for the method. The method is linear for acrylamide and glycidamide adducts in the range of 2.5 – 100 nmol/L. The total hemoglobin method is linear from 1.18 to 18.8 g/dL. The analytical measurement range would translate to 7.6 -4,842 pmol/g hemoglobin for acrylamide and glycidamide adducts respectively. Samples outside this analytical measurement range are re-analyzed for confirmation before results are reported. Samples above this range will be diluted further with 18 megaOhm-cm DI water or greater and will be processed by using higher sample volumes for the Edman reaction.

The evaluated concentration range from 2.5 nmol/L to 100 nmol/L showed linear instrument response analyte concentration relationship. No significant higher order (polynomial) relationship was detected. External calibration range is assessed every six month by adding a 100 nM calibrator to the calibration curve and assessing linearity of the extended calibration curve using R^2 as parameter. For further instruction see work instruction PBLW010025.

8.2 Limit of Detection

The limit of detection was determined (using Taylor's method (28)) by calculating the standard deviation at different standard concentrations. The absolute values of the standard deviations were then plotted versus concentration.
The intercept of the least squares fit of this line equals S_0 (value = 0.045 pmol for acrylamide adducts, 0.064 pmol for glycidamide adducts) with $3S_0$ (0.14 pmol for acrylamide adducts, 0.19 pmol/ glycidamide adducts) being the LOD.
The lowest standard (1.25 nmol/L) is used as the method reportable limit, in place of the calculated LOD.

Acrylamide adducts:	3 pmol/g hemoglobin sample solution
Glycidamide adducts:	4 pmol/g hemoglobin sample solution

Table 9: LOD and LOQ calculation

8.3 Analytical Specificity

A specific chemical reaction to hemoglobin adducts occurs within the “modified Edman reaction”. Only hemoglobin 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 acrylamide or glycidamide in the blood sample or possible acrylamide or glycidamide contaminations in the laboratory. Further, a large number of chemicals such as aldehydes do react with hemoglobin and 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. (30) The evaluation includes within and among days assessment. See Table 10 and 11. 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 high, medium and low QC samples in duplicate each over 20 days and calculating the means and SDs 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.

Table 10: Within-day precision values

Analyte	Within-Day Precision (%CV) Low	Within-Day Precision (%CV) Medium	Within-Day Precision (%CV) High
Acrylamide adducts	9	7	9
Glycidamide adducts	12	10	9

Table 11: Among-day precision values

Analyte	Among-Day Precision (%CV) Low	Among-Day Precision (%CV) Medium	Among-Day Precision (%CV) High
Acrylamide adducts	8	9	11
Glycidamide adducts	12	15	14

Accuracy measurement was determined according to CLSI guideline EP15-A2 (31). When reference samples or methods are not available, accuracy is accessed through recovery of the addition of known amount of anlayte spiked into a blank matrix. The deviation of the mean from the true values serves the measure of accuracy.

Primary or matrix-based reference materials are not available for acrylamide and glycidamide 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. See work instruction PBLW010024 for further details and table 12 for results. 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 5 %.

Table 12: Accuracy by standard addition

Expected	Recovery in % Low pool	Recovery in % High pool
Glycidamide adducts	99.1	99.4
Acrylamide adducts	97.6	98.7

Recovery was accessed by mixing low and high known samples in various fractions and collating the expected value versus the measured value. The recovery of the calibrator is considered confirmed if the difference in results of our in-house method and the confirmation method is less than 5 %. See Table 13.

Table 13: Recovery by mixing

Average Recovery by Mixing	Glycidamide adducts	Acrylamide adducts
Avg 25% / 50% / 75%	95.9	96.0

8.5 Limitations of Method, Interfering Substances and Ruggedness

No known interferences exist with this method for the detection and quantitation of acrylamide and glycidamide 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 and needs to be assessed by monitoring the chromatographic retention time of the analytes.

Acrylamide and glycidamide adducts are formed due to exposure coming from different sources such as food, smoking and certain occupational activities related to handling of acrylamide. 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. Six critical elements within the method measurement are evaluated and tested for ruggedness.
The following parameters were assessed:

1. pH test (23): The pH of the reaction as described in Section 6.7 must be within the pH range of 7.0(±0.5).
2. Temperature test (23) :The temperature of the reaction as described in Section 6.7 must be within the range of 55°C (±1.0)°C
3. Formamide volume: The formamide volume was assessed to be optimal when adding 1.5 mL. Decrease in method performance was observed at 1 to 2 mL volume.
4. Total hemoglobin amount test: The amount of hemoglobin used in the sample was assessed and determined that the optimal range of hemoglobin to obtain good signal intensity is between 14 to 53 mg of hemoglobin in 350 µl volume.
5. Isolute fraction test: Isolute particle fractions were tested to determine the extraction efficiency. It was determined that the larger particle size (710µm or greater) results in better method performance.
6. Total hemoglobin freezing and thawing (32): Freeze thaw cycles do not affect the total hemoglobin 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 comprise three levels of concentration spanning 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 either erythrocyte controls, or whole blood controls are analyzed in duplicate in each run as bench QC materials.

Hemoglobin acrylamide adducts ranges of 53 - 86 pmol/g hemoglobin (low QC pool), 89 – 122 pmol/g hemoglobin (medium QC pool) and 118-186 pmol/g hemoglobin (high QC pool) are targeted within two SD.

Hemoglobin glycidamide adducts ranges of 40 - 63 pmol/g hemoglobin (low QC pool), 52 – 81 pmol/g hemoglobin (medium QC pool) and 67-94 pmol/g hemoglobin (high QC pool) are targeted within two SD.

The low QC pool is prepared by selecting and pooling blood from non-smoking individuals. The medium QC pool is prepared by mixing a high QC pool with a low QC pool 1:1 (v/v). The high QC pools are obtained from people actively smoking tobacco products.
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 4 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 t-HB measurements provided by the assay manufacturer are used and assessed according to the manufacturers recommendations. An in-house characterization is also performed to assess the manufactures values. Duplicate measurements for at least 20 consecutive runs is 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 hemoglobin 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 is run in the laboratory for this purpose.

9.1.2 Quality Control Limit Evaluation

Acceptance criteria for values obtained with the bench QC materials "QC limits" are established according to the procedure described by Caudill et al. (33). See also DLS PM Section 6.

The rules described in the DLS Policies and Procedures Manual section 6 together with the acceptance criteria are applied to measurement results obtained with the QC materials. Sample runs are rejected if:

- 13s: one bench QC result is outside a 3 SD limit
- R4s:current and previous bench QC results are outside the same 2 SD limit
- 10x: current and previous 9 run results are on same side of the characterization mean
- the current and the previous run results differ by more than 4 SD
- one bench QC result is beyond the characterization mean ± 4 SD

For further details, see the DLS Policies and Procedures Manual. Quality control evaluation is performed using a SAS program developed and maintained by DLS.

Assessment of runs using QC materials
The results from the QC pools are checked after each run. The measurements are declared "in control" if all three QC results are within 2s limits and therefore run is accepted. If one of the three QC results is outside the 2s limits then apply rules below and reject if any condition is met - the run is then declared "out of control".

A QC program written in SAS is available from the DLS QA Officer and are applied 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 measurement of patient samples is resumed. QC failures are reviewed with the supervisors and documented. Measures to prevent re-occurrence of the same problem are taken.

9.2 Proficiency Testing

Acrylamide and Glycidamide Proficiency Testing (PT)

Scope of PT

No commercial proficiency testing/external quality assessment program exists for the analytes reported with this measurement procedure. Currently, we are 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 (34). Five proficiency testing pools spanning the full range of analyte values likely to be encountered in human specimens are prepared in-house and characterized by measuring 30 separate vials from each pool in at least 10 different runs. The means and SD are obtained from these measurements.

An individual that is not involved in acrylamide and glycidamide adduct measurements will obtain from the Proficiency Testing Coordinator a sheet that tells which labels to place on each set of vials and labels the vials.

Frequency of PT

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.

Documentation of PT

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 times 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.

t-HB proficiency testing

The t-HB PT is performed through Medical Laboratory Evaluation proficiency testing. The test is performed three times a year. A low and a high sample is measured and submitted to the Medical Laboratory Evaluation program. The Medical Laboratory Evaluation programs notify our laboratory with pass or fail result.

10 REFERENCE RANGES (NORMAL VALUES)

Population-based reference ranges have been established. In this study, samples were analyzed from the NHANES study in 5,686 samples (age: 3– 60 +years) in man, women and children and were analyzed for acrylamide and glycidamide adducts.

In non-smokers the range was found to be 32.1 to 89.6 pmol/g Hb and 28.7 to 114.0 pmol/g Hb for acrylamide and glycidamide adducts, respectively.

In smokers the range was found to be 50.7.1 to 277.0 pmol/g Hb and 43.7 to 235 pmol/g Hb for acrylamide and glycidamide adducts, respectively (35).

11 TEST RESULT REPORTING SYSTEM

Results are reported to 3 significant digits based on assay sensitivity calculations. Data are reported in pmol/g 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 hemoglobin 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

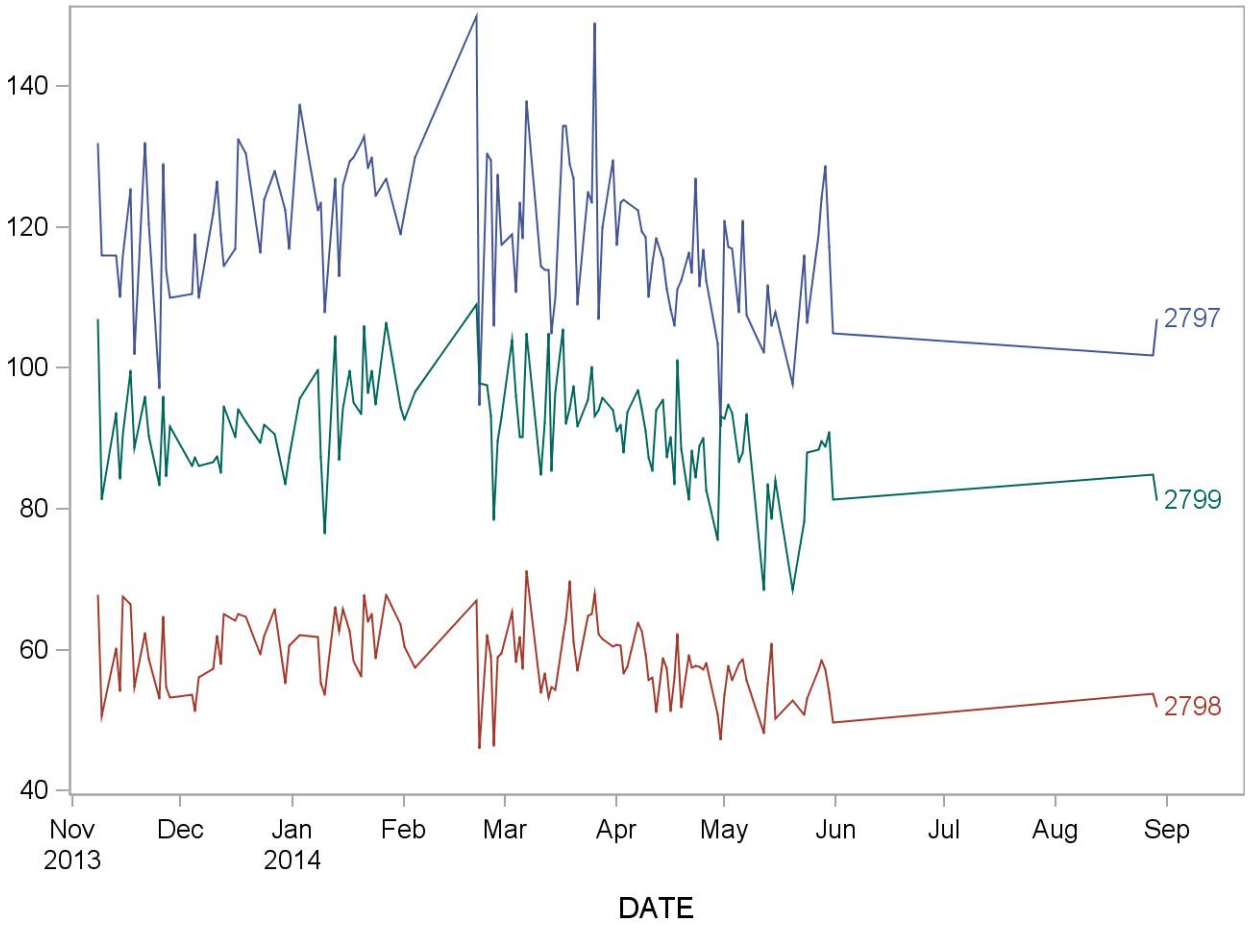
Not applicable for this procedure.

17 SUMMARY STATISTICS AND QC GRAPHS

Please see following pages.

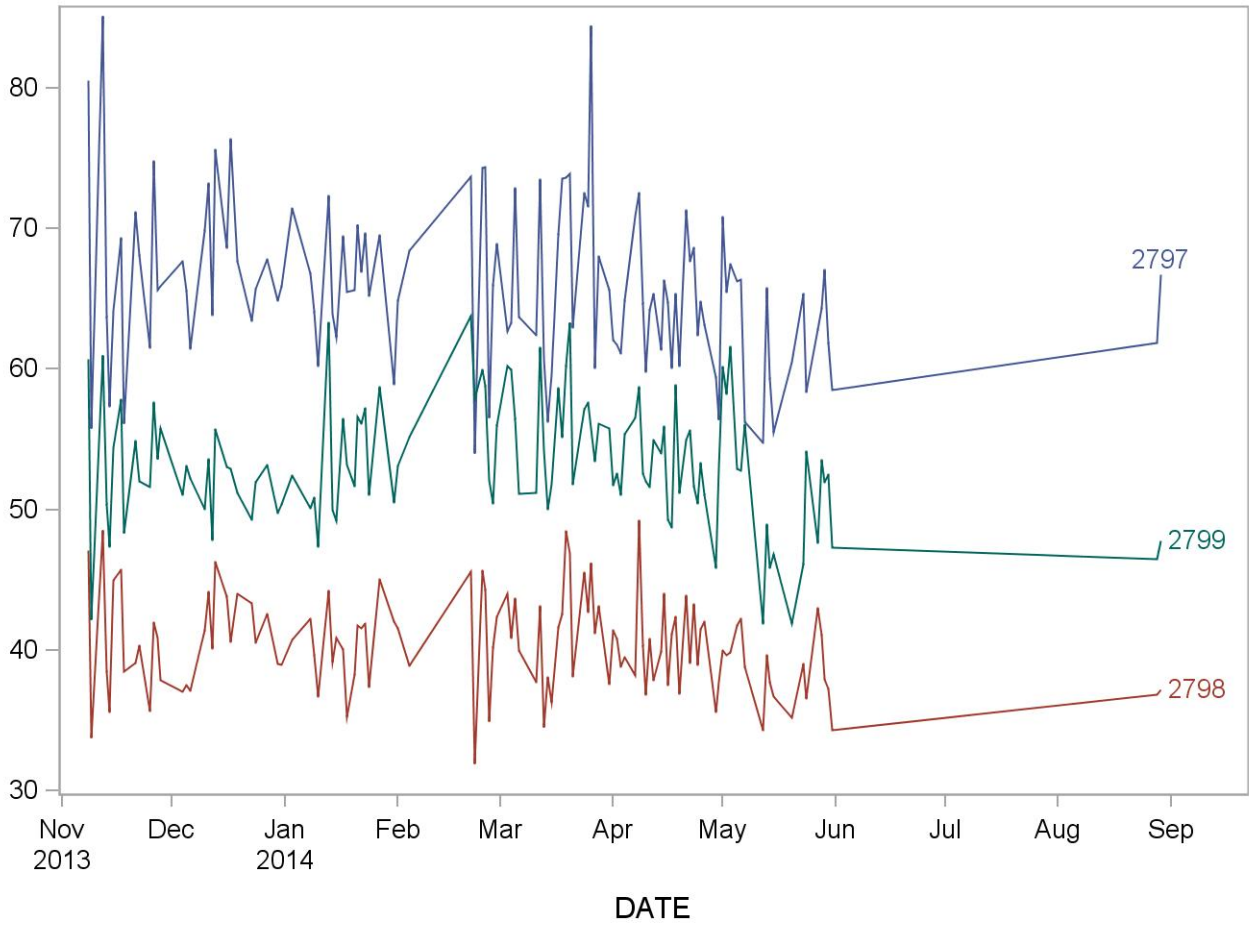
2005-2006 Summary Statistics and QC Chart for Acrylamide (pmol/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2797	242	08NOV13	29AUG14	118.378	12.644	10.7
2798	242	08NOV13	29AUG14	58.559	6.017	10.3
2799	242	08NOV13	29AUG14	90.831	8.677	9.6



2005-2006 Summary Statistics and QC Chart for Glycideamide (pmol/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2797	240	08NOV13	29AUG14	65.725	6.784	10.3
2798	240	08NOV13	29AUG14	40.485	4.070	10.1
2799	240	08NOV13	29AUG14	53.324	4.984	9.3



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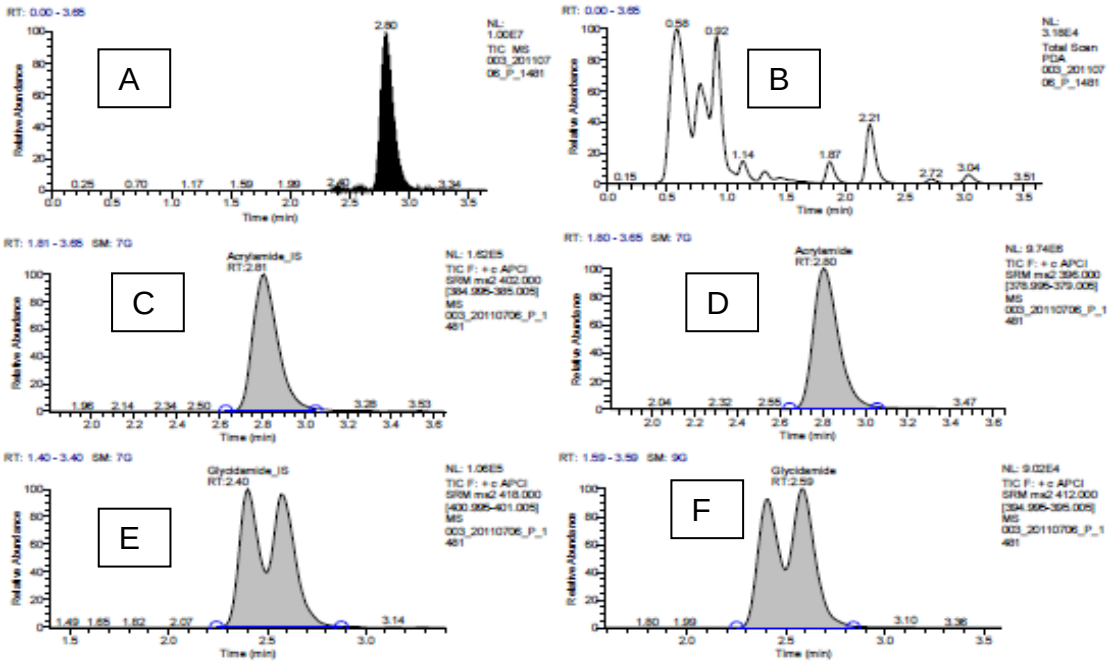
Appendix 1. Acrylamide and Glycidamide adduct chromatography



Component Name	RT	Area	S/N	Width
GA_conf	N/A	N/A	N/A	N/A
AA_conf	N/A	N/A	N/A	N/A
Glycidamide_IS	2.40	1567058.51	2994.96	0.63
Glycidamide	2.59	1353835.43	1119.64	0.59
Acrylamide	2.80	80065184.56	213683.4 0	0.41
Acrylamide_IS	2.81	1318975.86	2324.95	0.42

A: Total Ion Chromatogram
B: PDA Chromatogram
C: Acrylamide Internal Standard Adduct Chromatogram
D: Acrylamide Adduct Chromatogram
E: Glycidamide Internal Standard Adduct Chromatogram
F: Glycidamide Adduct Chromatogram

Appendix 2. Related Documents

Normative References

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

Abbreviations

$^{13}C_5$	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
the reportable range for patient test results	8.1
quality control procedures, including PT materials and programs/procedures used	8-9
remedial action to be taken when calibration or control results are outside acceptable limits	9.1.3
limitation in methods, including interfering substances	8.5
reference range (normal values)	10
life-threatening or "panic values"	15
pertinent literature references	17
specimen storage criteria	5.2, 7.1
protocol for reporting panic values	15
course of action if test system becomes inoperable	9.1.3, 12
criteria for referral of specimens (usually not needed)	14
safety considerations for performing the method	2

Appendix 5: **Location of information as required by CLIA**

Required section	Section in this Document
Requirements for patient preparation; specimen collection, labeling, storage, preservation, transportation, processing, and referral; and criteria for specimen acceptability and rejection	3.2, 5,
Microscopic examination, including the detection of inadequately prepared slides	16
Step-by-step performance of the procedure, including test calculations and interpretation of results	6
Preparation of slides, solutions, calibrators, controls, reagents, stains, and other materials used in testing	4
Calibration and calibration verification procedures	7
The reportable range for test results for the test system as established or verified	8.1
Control procedures	8
Corrective action to take when calibration or control results fail to meet the laboratory's criteria for acceptability	9.1.3
Limitations in the test methodology, including interfering Substances	8.5
Reference intervals (normal values)	10
Imminently life-threatening test results or panic or alert Values	15
Pertinent literature references	17
The laboratory's system for entering results in the patient record and reporting patient results including, when appropriate, the protocol for reporting imminent life threatening results, or panic, or alert values	3, 7.7, 13
Description of the course of action to take if a test system becomes inoperable	9.1.3, 12

Appendix 6: 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 7: **Location of information as required by ISO 15193**

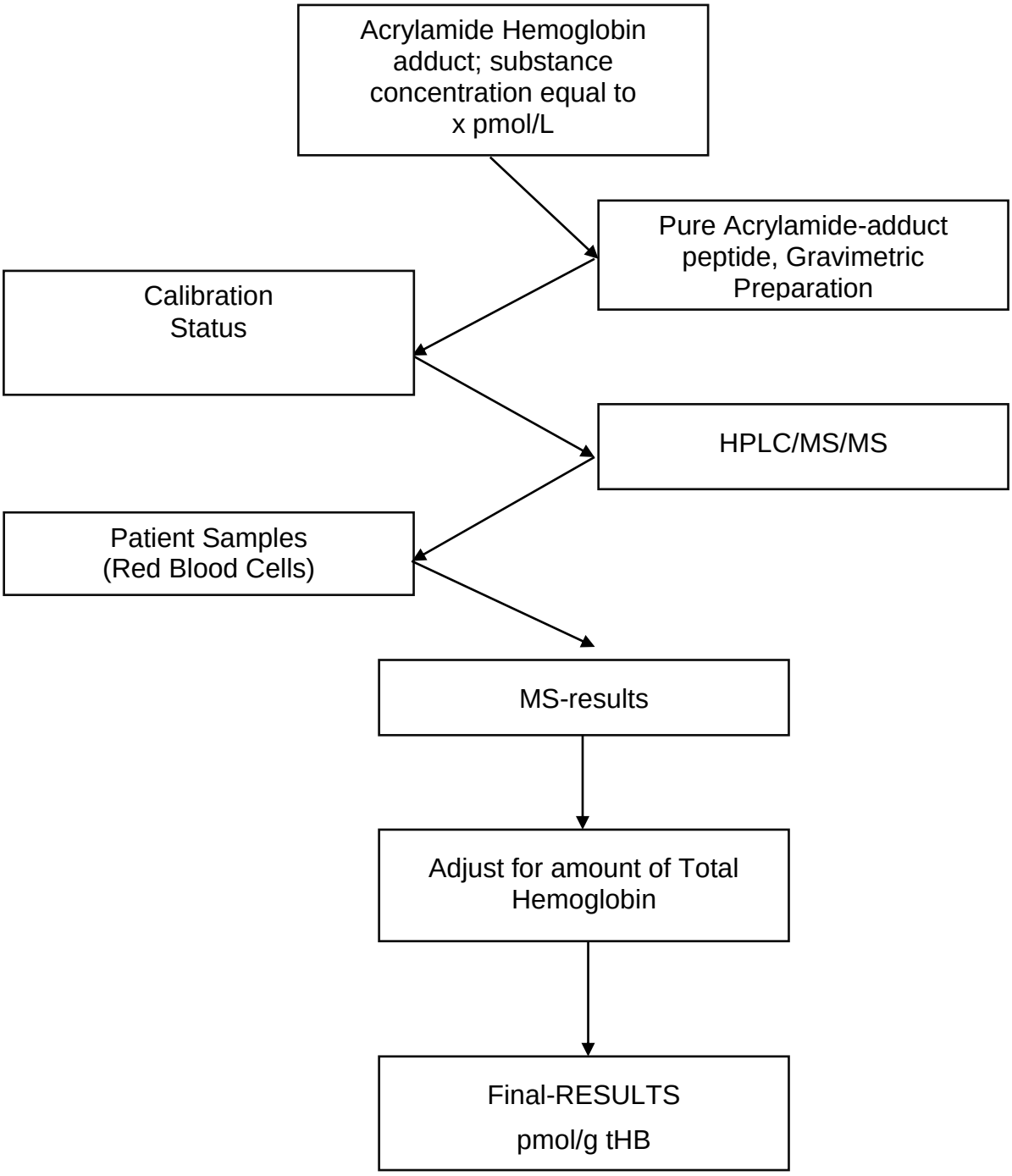
Required section	Section in this Document
Title page	Title Page
Contents list	List of Content
Foreword	N/A
Warning and safety precautions	2
Introduction	1
Title	Title Page
Scope	1
Normative references	0
Definitions	0
Symbols and abbreviations	0
Terminology	0
Principle and method of measurement	1
Check 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 8: PBL WORK INSTRUCTIONS FOR ACRYLAMIDE METHOD

Work Instruction No.	Document Name
PBLW010001	PBLW010001 Preparation of pH Adjusted Formamide Solution
PBLW010003	PBLW010003 Preparation of ACS Calibrators for tHB measurement
PBLW010004	PBLW010004 Creating Lab Waste Tickets
PBLW010005	PBLW010005 Preparation of Tecan Solvent Wash Solution
PBLW010006	PBLW010006 Preparation of Mobile Phases for LC/MS/MS
PBLW010007	PBLW010007 PM of ROSS pH Electrode and pH meter
PBLW010008	PBLW010008 PM Eppendorf Centrifuge 5810R
PBLW010009	PBLW010009 PM Matrix
PBLW010010	PBLW010010 PM Powerwave
PBLW010011	PBLW010011 PM GeneVac
PBLW010012	PBLW010012 PM GlasCol
PBLW010013	PBLW010013 N/A
PBLW010014	PBLW010014 PM Hamilton Starlet
PBLW010015	PBLW010015 PM Tecan
PBLW010016	PBLW010016 PM TSQ
PBLW010017	PBLW010017 RBC Isolation Procedure
PBLW010018	PBLW010018 Acrylamide Calibrator Lot to Lot Comparison
PBLW010019	PBLW010019 Thermometer Records
PBLW010021	PBLW010021 Procedure Data Evaluation
PBLW010022	PBLW010022 Preparation of 6mM K3EDTA Blood Dilution Solution
PBLW010023	PBLW010023 Lib Resources Search
PBLW010024	PBLW010024 CLIA AA Method Accuracy Test
PBLW010025	PBLW010025 CLIA CC Verification Test
PBLW010026	PBLW010026 CLIA PW Comparison Test
PBLW010027	PBLW010027 CLIA HC Accuracy Test
PBLW010028	PBLW010028 CLIA IS Comparison Test
PBLW010029	PBLW010029 CLIA Robotic Pipette Comparison Test
PBLW010030	PBLW010030 CLIA HC Instrument Comparison Test
PBLW010031	PBLW010031 CLIA MS Instrument Comparison Test
PBLW010032	PBLW010032 Tecan Method, Layouts and Operation
PBLW010033	PBLW010033 Preparation of Extraction Plate
PBLW010034	PBLW010034 Preparation of Extraction Solution
PBLW010035	PBLW010035 Preparation of 0.2N NaOH Solution
PBLW010036	PBLW010036 N/A
PBLW010037	PBLW010037 HemoCue Operation
PBLW010038	PBLW010038 Stock Soluion Purity Check
PBLW010039	PBLW010039 Matrix Method, Layouts and Operation
PBLW010040	PBLW010040 Hand Held Hamilton Pipettor Method and Operation
PBLW010041	PBLW010041 HPL/MS/MS Method Layouts and Operation
PBLW010042	PBLW010042 Hamilton Starlet method, Layouts and Operation
PBLW010043	PBLW010043 Glass Silanization
PBLW010044	PBLW010044 Integration
PBLW010045	PBLW010045 MS Tune and Calibration
PBLW010046	PBLW010046 Preparation of IS Stock Solution
PBLW010047	PBLW010047 Preparation of Acrylamide Stock Solution
PBLW010049	PBLW010049 Regents Needed
PBLW010052	PBLW010052 PowerWave Method, Layout and Operation
PBLW010054	PBLW010054 Preparation of pH Adjusted Formamide Solution for Whole Blood
PBLW010058	PBLW010058 Glascol Operation
PBLW010059	PBLW010059 Genevac Operation
PBLW010062	PBLW010062 Sample Log
PBLW010064	PBLW010064 Preparation of HRS solution
PBLW000001	PBLW000001 Procedure of barcode labeling
PBLW000004	PBLW000004 Procedure of Pipette Verification

Appendix 9:

Metrological Traceability of Acrylamide Measurements



Appendix 10: List of tables

Table Numbers	Name	Section of SOP
Scheme 1	Measurement Procedure for Acrylamide in Red Blood Cells	1.2
Table 1	Desired Acrylamide (AO) and Glycidamide (GO) octapeptide Calibrator Stock Solution Concentration (nmol/L)	4.31
Table 2	Desired Acrylamide (AO) and Glycidamide (GO) octapeptide Calibrator Working Solution Concentration (nmol/L)	4.31
Table 3	Dilution of Total Hemoglobin Linearity Controls	4.3.2
Table 4	Desired Internal Standard Acrylamide and Glycidamide octapeptide Solution Concentrations (nmol/L)	4.3.3
Table 5	HPLC Gradient Pump A (MS Pump)	6.13
Table 6	HPLC Gradient Pump B (MS Pump)	6.13
Table 7	AA SRM masses(m/z)	6.13
Table 8	GA SRM masses (m/z)	6.13
Table 9	LOD and LOQ calculation in serum	8.2
Table 10	Within-Day Precision Values	8.4
Table 11	Among-Day Precision Values	8.4
Table 12	Recovery by standard addition	8.4
Table 13	Recovery by mixing	8.4

Appendix 11: HRS Solution Preparation

TECO DIAGNOSTICS
1268 N. Lakeview Ave.
Anaheim, CA 92807
1-800-222-9880

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

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H526: 11/01

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

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