



Laboratory Procedure Manual

Analyte: Inorganic mercury, Methylmercury, Ethyl mercury

Matrix: Blood

Method: Blood mercury speciation TSID-GC-ICP-DRC-MS
(Triple Spike Isotope Dilution Gas Chromatography-
Inductively Coupled Plasma Dynamic Reaction Cell
Mass Spectrometry)

Method No: **DLS-3020.8-02**

Adopted:

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As performed by: Inorganic and Radiation Analytical Toxicology Branch
Division of Laboratory Sciences
National Center for Environmental Health

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Important Information for Users

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

Public Release Data Set Information

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

Data File Name	Variable Name	SAS Label
IHGEM_K	LBXIHG	Mercury, Inorganic ($\mu\text{g/L}$)
	LBXIHGSI	Mercury, Inorganic (nmol/L)
	LBXBEG	Mercury, ethyl ($\mu\text{g/L}$)
	LBXBEGSI	Mercury, ethyl (nmol/L)
	LBXBEM	Mercury, methyl ($\mu\text{g/L}$)
	LBXBEMSI	Mercury, methyl (nmol/L)

I. CLINICAL RELEVANCE AND TEST PRINCIPLE

A. Clinical Relevance

Mercury (Hg) is widespread in the environment and found in its elemental form (Hg^0), inorganic forms such as mercurous (Hg^+) and mercuric (Hg^{2+}), and various organic forms such as methylmercury (MeHg), ethyl mercury (EtHg), phenyl mercury (PhHg) and others. The health effects of mercury are diverse and are dependent upon the form of mercury encountered and the severity and length of exposure. The relative order of increasing toxicity is $\text{Hg}^0 < \text{Hg}^{2+} \ll \text{CH}_3\text{Hg}^+$ [1]. With large acute exposures to elemental mercury vapor, the lungs may be injured. At levels below those that can cause lung injury, low-dose or chronic inhalation may affect the nervous system. Symptoms include weakness, fatigue, loss of weight (with anorexia), gastrointestinal disturbances, salivation, tremors, and behavioral and personality changes, including depression and emotional instability [2]. Exposure to inorganic mercury usually occurs by ingestion. The most significant effect of inorganic exposure occurs on the kidneys, where mercury accumulates, leading to tubular necrosis. In addition, there may be an irritant or corrosive effect on the gastrointestinal tract involving stomatitis, ulceration, diarrhea, vomiting, and bleeding. Psychomotor and neuromuscular effects also may occur [3].

Methylmercury is more toxic than inorganic mercury. The effects of methylmercury include changes in vision, sensory disturbances in the arms and legs, cognitive disturbances, dermatitis, and muscle wasting. The critical organ for methylmercury is the brain. Methylmercury readily crosses the blood-brain barrier due to its lipid solubility and accumulates in the brain where it is slowly converted to inorganic mercury. Whether CNS damage is due to methylmercury or inorganic mercury, or both, is still controversial [4].

Ethyl mercury is another organic form of mercury. Very little is known about ethyl mercury metabolism in humans, including whether it has the same potency as a neurotoxin, whether the blood concentration is ever significant, and even whether it crosses the blood-brain barrier. However, the use of thimerosal, which metabolizes to ethyl mercury and thiosalicylate, as a vaccine preservative makes this subject very important.

In the general population, total blood mercury exposure is due mostly to the dietary intake of organic forms, particularly methylmercury, and ranges from 0.2 to 5.8 $\mu\text{g}/\text{L}$ [5]. Urinary mercury is mainly comprised of inorganic mercury due generally to dental amalgam containing elemental mercury and occupational exposures ranging from 0.2 to 10 $\mu\text{g}/\text{L}$ [5].

This hyphenated method assesses mercury exposure as defined by exposure to individual mercury species, by analyzing blood through the use of a Solid Phase Micro Extraction (SPME) fiber for delivering sample to a gas chromatograph (GC) coupled to an inductively coupled plasma-dynamic reaction cell-mass spectrometer (ICP-DRC-MS). The method provides accurate quantification of three mercury blood species: inorganic mercury (InHg), methylmercury (MeHg), and ethyl mercury (EtHg) as listed in TABLE 1-1. Whole blood was determined to be the most suitable matrix to monitor species-specific exposure because if exposed to organic and/or inorganic forms of mercury, blood will contain various organic mercury species as well as inorganic mercury while urine contains mostly inorganic mercury.

TABLE 1-1: Mercury Species Quantified with this Method

Species Name	Abbreviation	Molecular Structure
Inorganic mercury	InHg	Hg ²⁺
Methylmercury	MeHg	CH ₃ Hg ⁺
Ethyl mercury	EtHg	C ₂ H ₅ Hg ⁺

B. Test Principle

The quantification of InHg, MeHg, and EtHg in whole blood samples is performed using a triple spike isotope dilution (TSID) method employing gas chromatography (GC) to separate the species followed by introduction into an ICP-DRC-MS for detection. TSID is a specialized extension of the Isotope Dilution (ID) technique. TSID measures individual chemical species (inorganic, methyl and ethyl mercury species) in samples using ID principles. The blood sample is spiked with known amounts of each Hg species that have been enriched with isotopic variants of the target element of interest.

The first step of this method involves the addition ("spiking") of enriched isotopes (¹⁹⁹Hg²⁺, CH₃²⁰⁰Hg⁺, and C₂H₅²⁰¹Hg⁺ or C₂H₅¹⁹⁸Hg⁺, ¹⁹⁹Hg², and CH₃²⁰¹Hg⁺) to the blood sample. Each Hg species spike is labeled with an enriched Hg isotope such that its isotopic pattern is unique to the species' chemical identity (i.e. the manner of isotope spiking is "species specific"). Next, the spiked sample is digested in tetramethylammonium hydroxide (TMAH) which disassociates bound mercury species from proteins, polypeptides, and other biomolecules. The digested blood sample with freed mercury species is chemically reacted ("derivatized") with a reagent that adds 3-carbon chains (n-propyl groups) to the mercury atom of each species molecule without compromising species identity. This type of chemical derivatization results in loss of ionic charge and reduced polarity making each mercury species molecule volatile so that it can escape the liquid phase and accumulate in the gaseous phase ("headspace") directly above the sample. Derivatization is performed inside a partially filled vial sealed with a rubber septa cap that can be penetrated by a needle.

Solid Phase Microextraction (SPME) is a sampling technique that uses a thin polymer fiber with a hydrophobic coating. The method described here uses a SPME fiber with a 100 μm coating of polydimethylsiloxane (PDMS). The SPME assembly consists of the fiber inserted in a stainless steel needle. A key design feature is that the fiber can be mechanically withdrawn into the needle during vial septum penetration and then pushed out to expose the fiber to the headspace. During headspace exposure (the "extraction" step), the gaseous derivatized Hg species adsorbs onto the PDMS coating of the SPME fiber. When other factors are held constant, the adsorbed mass increases as a function of sample concentration. After a predetermined time, the SPME fiber is retracted into the injection needle, and the needle is withdrawn from the sample vial. Subsequently, the needle moves to the injector port of the programmable temperature gradient gas chromatograph, (GC) and on programmatic command, performs a programmed temperature ramp injection sequence. This action transfers the propylated inorganic, methyl and ethyl Hg species to the head of a 30 m capillary GC column which, using He as the carrier gas, ramps the column temperature to 280°C. The order of chromatographic separation of the Hg species is based on increasing molecular weight: methylpropylmercury (derivatized methyl Hg), ethylpropylmercury (derivatized ethyl Hg), followed by dipropylmercury (derivatized inorganic Hg). Hg species exiting the GC column are seen as chromatographic peaks detected using an inductively coupled argon plasma (ICP) as the ion source and a quadrupole mass spectrometer (MS) for mass specific quantification. Species identification is based on chromatographic retention time. Species-specific isotope ratios are calculated from integrated peak areas derived from m/z signals corresponding to ¹⁹⁹Hg, ²⁰⁰Hg, ²⁰¹Hg, and ²⁰²Hg isotopes or ¹⁹⁸Hg, ¹⁹⁹Hg, ²⁰¹Hg, and ²⁰²Hg isotopes. The ICP-MS is equipped with a Dynamic Reaction Cell (DRC™) for minimizing polyatomic interferences. Operating the ICP-MS in DRC mode has an added benefit of enhancing Hg signal strength through an effect known as "collisional focusing" [6,7].

II. SAFETY PRECAUTIONS

Important

Precautionary information that is important to protecting personnel and safeguarding equipment will be presented inside a box, like this one, throughout the procedure where appropriate.

Follow universal precautions when handling blood samples. Wear gloves, a lab coat, and safety glasses while handling human blood, plasma, serum, urine, or other bodily fluid or tissue. Place disposable plastic, glass, and paper (e.g., pipette tips, autosampler tubes, and gloves) that come in contact with human biological fluids, such as blood, in a biohazard autoclave bag. Keep these bags in appropriate containers until they are sealed and autoclaved. When work is finished, wipe down all work surfaces where human biological fluid was handled with a broad spectrum disinfectant (or equivalent). Operators of this method are required to take the Hazardous Chemical Waste Management for CDC Workers course upon initial hire and yearly refreshers thereafter. Dispose of all diluted biological specimens in a biohazard autoclave bag at the end of the analysis according to CDC/DLS guidelines for disposal of hazardous waste.

All packages known to contain mercury-containing chemicals should be opened inside of a chemical fume hood. Appropriate PPE is required when opening these shipments.

If a box containing any chemicals has physical damage, do not open the box. Notify either the lab chief, deputy branch chief, or branch chief to determine how to proceed.

PerkinElmer® provides safety information that must be read before operating the instrument. This information is found in the PerkinElmer® NexION® 300D ICP-DRC-MS System Safety Manual. Possible hazards include ultraviolet radiation, high voltages, radio-frequency radiation, and high temperatures.

Handle acids and bases with extreme care; they are caustic and toxic. Reagents used in this study include those listed in Section 7. Safety Data Sheets (SDS) for these chemicals are readily accessible as hard copies in the lab. If needed, SDS for other chemicals can be viewed at <http://www.ilpi.com/msds/index.html> or through the CDC's Chemical Hazard Tracking System (CHaTS).

Caution!

Exercise caution when handling and dispensing concentrated nitric and hydrochloric acid. Always remember to add acid to water. Nitric and hydrochloric acid are corrosive chemicals that are capable of severe eye and skin damage. Wear powder-free gloves, a lab coat, and safety glasses. If nitric or hydrochloric acid comes in contact with any part of the body, quickly wash the exposed area with copious quantities of water for at least 15 minutes.

III. DATA SYSTEM MANAGEMENT

A. Data Entry and Transfer

Use bar code scanners whenever possible to enter sample identifiers into the GC-ICP-DRC-MS computer software to avoid errors associated with the keyboard-entry process and to speed up sample processing. When bar code scanners cannot be used, proofread transcribed data after entry. Handle or transfer data electronically when reporting or moving data to other computerized data-handling software. In the Inorganic and Radiation Analytical Toxicology Branch (IRAT), sample analysis results generated by this method are stored on compact discs and on the CDC network shared drive. The results must include at least the analysis date, quality control (QC) results for the run, and the results of specimen analysis by specimen identifier (ID).

B. Computer Hard Drive Maintenance

Defragment the computer hard drive regularly by using software such as Microsoft Windows® Disk Defragmenter (located in Start > Programs > Accessories > System Tools) or an equivalent defragmentation program to maximize computer performance and maintain data integrity for files on the hard drive. An entry will automatically be logged in the Windows™ system event log when this process is performed providing documentation of this step.

C. Electronic Data Backup

1. Schedule of Data Backups

Sample results generated by this analytical method are stored long-term on compact discs and on the CDC network shared drive. The stored results must include at least the analysis date, quality-control (QC) results for the run, and the results of specimen analysis by specimen identifier (ID).

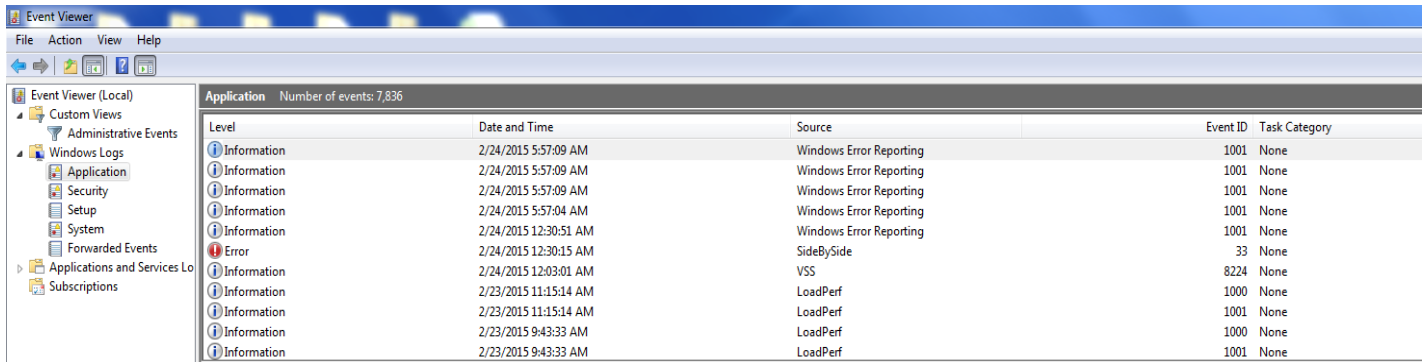
Whenever making a backup (daily or weekly) include the directories and subdirectories:

- ✓ C:\nexiondata (include all subdirectories)
 - ✓ C:\gc (must include subdirectories "data" and "methods", also include other relevant directories)
 - ✓ C:\Event Log Backup
 - ✓ Any other relevant files or folders.
- **Daily.** Full data backups onto the laboratory's Isolated Secure Network Environment (ISLE).
 - **Weekly.** Full data backups onto one or more recordable compact discs (CD-R) or digital video discs (DVD). A weekly backup is not necessary for weeks that the instrument have not been used.

2. Backup Procedures

Before making a CD or DVD backup, save the information from the Windows Event Viewer. Access the Event Viewer by either clicking on the desktop icon or by clicking on Start in Windows. The Event Viewer will appear (see **Figure 1**).

Figure 1: Event Viewer Example of the items found in the event viewer window



If the computer has a Windows 7 operating system, go to the Event Viewer and expand the “Windows Logs” folder. Right click on Application. Choose “Save All Events As...” Right click on Application. Choose “Save All Events As...” Save the file in the folder “C:/Event Log Backup”. Name the file “Application INSTRUMENT NAME MM-DD-YY” where “MM” is the month, “DD” is the day, and “YY” is the last two digits of the year. The following window will pop up.

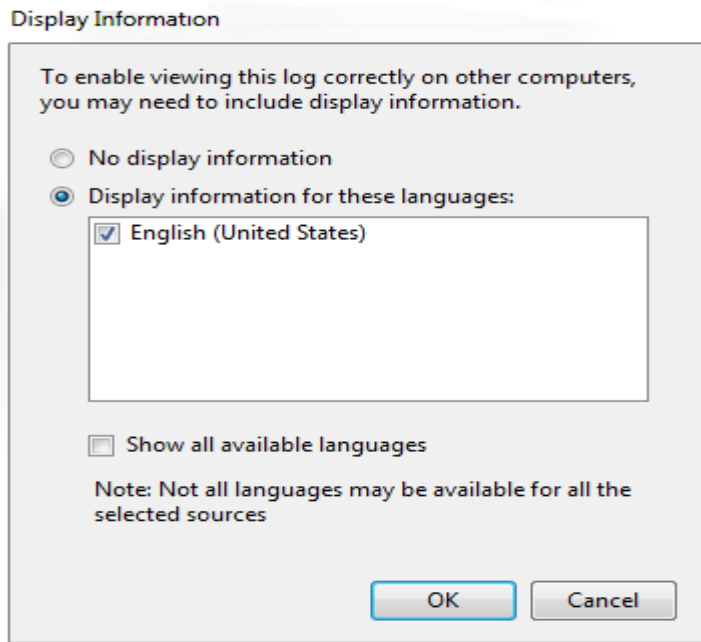


Figure 2: Display Information Screenshot of the display information window which appears when saving information to the event log backup folder.

Select English as the language. Click “Ok”. Repeat this procedure for the Security and System folders.

If the computer system is running on Windows XP, go to the Event Viewer and right click on Application. Choose “Save Log File As” and save the file as “Event Log Backup (application).evt”. Repeat this procedure for the Security and System Logs.

(a) Backups to a network

- These are done automatically at a set time on each day that a network backup is scheduled to be done. The initial set up and scheduling is done by someone in laboratory informatics support.

(b) Compact Disc Backups

- Use CD-R disks only (recordable compact disks) not CD-RW disks (rewritable compact disks) so that the compact disk cannot be over-written after creation. DVD discs are also acceptable when transferring large amounts of data or working with large databases.
- Use Roxio Creator or an equivalent software program to make CD-R/DVD backups.
- Back up the most recent data on the hard drive onto a CD or DVD. Manually select each dataset folder (subdirectories under "C:\gc" and "C:\nexiondata"), the reports folder, the event log backup, and other relevant files (i.e., optimization, tuning, and sample files) that are to be included on these backups.
- Keep the copy of the CD-R disk in a building other than the laboratory (in case of fire).

(c) Deleting Data from the Hard Drive

- If data needs to be deleted from the hard drive (i.e. for computer replacement or upgrade, to free up hard drive space, etc.) backup all of the data on the hard drive in duplicate using one of more disks.
- Verify that the data can be retrieved from the disk(s) before actually deleting the data. To verify the operation of a disk, open any file on the disk by using the appropriate computer software (ICP-DRC-MS software).
- After verifying that all backups are operational, delete the original data from the hard drive.
- Keep one copy of the disk in a building other than the laboratory (in case of fire). Keep the other copy in or near the ICP-MS laboratory for easy retrieval, if necessary.

D. Documentation of System Maintenance

1. Computer Maintenance

Record any maintenance of computer hardware or GC or ICP-DRC-MS software in the instrument logbook. Place other electronic records relating to integrity of the data and hard drive in the Windows™ event log. Back up the event log on a regular basis by saving a copy in the active "NexIONdata" directory. The event log will then be backed up along with the NexION® data when backup CD-R disks are made.

2. Instrument Maintenance

Document system maintenance in hard copies of data records (i.e., daily maintenance checklists, PerkinElmer® service records, and/or instrument logbook) as well as in electronic records relating to instrument conditions (default.dac) and tuning (default.tun). Whenever a service call is placed or a annual or biannual preventative maintenance visit occurs, store copies of any vendor-provided documents related to the service in the instrument logbook and in STARLIMS.

IV. COLLECTING, STORING, AND HANDLING SPECIMENS; CRITERIA FOR REJECTING SPECIMENS

A. Specimen Type

Specimen type is human whole blood. No special instructions for fasting or special diets are required of patient or study subjects.

B. Specimen Collection, Handling, and Storage

1. The preferred volume of blood specimen is ≥ 0.5 mL; the minimum volume is 0.4 mL.
2. Acceptable containers for specimen acquisition include pre-screened polyethylene vials and pre-screened blood collection tubes, or other suitable pre-screened equivalent. The storage containers, as well as the devices used to collect the blood, should be screened for total mercury contamination prior to use in the field and the laboratory.
3. Short term storage between 2°C and 6°C or colder (-20°C is preferred) prior to transport to the laboratory is acceptable. Upon receipt, samples must be stored at approximately -20°C or lower temperature. Long-term storage at approximately -70°C or a lower temperature is preferred.
4. Specimen handling conditions are outlined in the Division of Laboratory Sciences' Policies and Procedures Manual protocol for blood collection and handling. Collection, transport, and special requirements are discussed. If more than one blood collection tube is used to draw blood from a subject, the blood tube for trace metals tube is ideally drawn last. Draw the blood through a stainless steel needle into a pre-screened blood collection tube. Every attempt is made to ensure that blood specimens derived from outside direct CDC control are collected, stored, and transported in a manner that maintains the integrity of the sample as it was collected. This includes, but is not limited to, recognized proper sampling technique with pre-screened collection devices, storage in pre-screened containers, packaging and transport in suitably labeled containers that will maintain a temperature of approximately 4°C or less throughout transport. Once received, the specimens can be frozen at approximately -70°C or lower temperature until time for analysis. Portions of the sample that remain after analytical aliquots are withdrawn can be refrozen at approximately -70°C or lower temperature. Thawing and refreezing of samples before analysis is discouraged.

C. Criteria for an Unacceptable Specimen

The criteria for an unacceptable specimen are low volumes (< 0.4 mL), suspected contamination due to improper collection procedures or collection devices, and/or the presence of clots or microclots. Specimen contact with dust or dirt may compromise test results. In all cases mentioned above, request a second blood specimen.

V. CHEMICALS, STANDARDS, AND QUALITY CONTROL MATERIALS

A. Chemicals

1. Deionized (DI) water, ≥ 18 M Ω cm resistivity.
2. Sodium acetate anhydrous, (CAS# 127-09-3), (C₂H₃NaO₂--MW 82.04), (Millipore/Sigma, Milwaukee, WI), Item # 59929-100G-F, or equivalent.
3. Glacial acetic acid, (CAS# 64-19-7), (CH₃COOH--MW 60.05), reagent ACS grade (GFS Chemicals, Powell, OH), Item # 624, or equivalent.
4. Double-distilled Hydrochloric Acid, (CAS# 7647-01-0), (HCl-MW 36.461--37.0%--12.1M) (GFS Chemicals Inc.), Item # 2180, or equivalent.
5. Double-distilled Nitric Acid (CAS# 7697-37-2), (HNO₃-MW 63.013-70.0%--15.8M), (GFS Chemicals Inc.), Item # 621, or equivalent.
6. Methanol HPLC Grade (CAS#67-56-1), (CH₃OH-MW 32.04), (GFS Chemicals Inc.), Item #2483, or equivalent.
7. Sulfuric Acid, (CAS# 7664-93-9), (H₂SO₄-MW 98.08), (Fisher Scientific, Suwanee, GA), Trace Metal grade, item # A510-P500, or equivalent.
8. Hydrogen Peroxide, 30% solution, stabilized (CAS# 7722-84-1), (H₂O₂, MW 34.0), (EMD Millipore, Germany), item #386790, or equivalent.
9. Tetramethylammonium hydroxide, 25% w/w in water (CAS# 75-59-2), (C₄H₁₃NO-MW 91.15), (Alfa Aesar, Ward Hill, MA), Item # 20932, or equivalent.
10. Sodium tetra(n-propyl)borate, (CAS# 45067-99-0), (NaPr₄B -MW 206.16), (ABCR, Germany), Item # AB135075-0005.00-GRM or (VHG Labs, Manchester, NH) Item # DE-MS-03410-5), or equivalent.
11. Broad spectrum disinfectant, Fisher Scientific, Item # 19-130-5977, or equivalent disinfecting agent.
12. Certified pH 4 and pH 7 buffer solutions, (Fisher Scientific), Item # SB115-500 and SB107-500, or equivalent.
13. Base whole blood, Tennessee Blood Services, equivalent vendor, or donated.
14. NIST SRM 955c - Toxic Metals in Caprine Blood (NIST, Gaithersburg, MD), or equivalent.
15. Xenon gas, 100ppm (Airgas South), Item # X02HE99C350002, or equivalent.

B. Isotope Dilution Standards

1. ¹⁹⁹Hg-Isotopically-Enriched Inorganic Mercury (¹⁹⁹HgCl₂), (Applied Isotope Technologies, Inc. or AIT), Item # PN-30023 or equivalent
2. ²⁰⁰Hg-Isotopically-Enriched Methylmercury (CH₃²⁰⁰HgCl), (Applied Isotope Technologies, Inc.), Item # PN-30025 or equivalent
3. ²⁰¹Hg-Isotopically-Enriched Ethylmercury (CH₃CH₂²⁰¹HgCl), (Applied Isotope Technologies, Inc.), Item # PN-30026 or equivalent
4. ¹⁹⁸Hg-Enriched Ethylmercury (CH₃CH₂¹⁹⁸Hg), (Innovative Solutions in Chemistry S.L. or ISC

Science), Item # IES-EtHg198 or equivalent

5. Hg standard solution enriched in ^{199}Hg (^{199}Hg), (Innovative Solutions in Chemistry S.L.), Item # IES-Hg199 or equivalent
6. ^{201}Hg -enriched Monomethylmercury ($\text{CH}_3^{201}\text{Hg}$), (Innovative Solutions in Chemistry S.L.), Item # IES-MMHg201 or equivalent
7. Inorganic mercury standard, natural abundance, (Applied Isotope Technologies, Inc.), Item # PN-30021
8. Methylmercury standard, natural abundance, (Applied Isotope Technologies, Inc.), Item # PN-30024
9. Ethyl mercury standard, natural abundance, (Applied Isotope Technologies, Inc.), Item # PN-30028

C. Quality Control Materials

Quality control (QC) materials are prepared from pools of whole blood obtained from a donor source or purchased commercially. See the Preparation of Quality Control Material section for details of preparation. The control "base" blood and two QC blood pools intended for the mercury speciation assay are designated as listed in TABLE 5-1 below.

TABLE 5-1: List of Prepared Quality Control Materials

QC Level	QC Designation ID
Base pool	BB-yy###
Low pool	LB-yy###
High pool	HB-yy###

where substitutions are: yy = the last two digits of production year and ### = assigned pool identification number. QC materials intended for bench quality control purposes need to be "characterized" as described in the section "*Establish QC limits for each QC pool*".

VI. INSTRUMENTATION, EQUIPMENT, SOFTWARE, AND SUPPLIES

A. Instrumentation

1. ICP-DRC-MS system

- 1) Inductively-coupled plasma mass spectrometer, specifically, the NexION® 300D ICP-Mass Spectrometer with Universal Cell Technology (UCT™) and Dynamic Reaction Cell (DRC™) capability, PerkinElmer® LAS or equivalent
- 2) NexION® 300D ICP-MS Detector, Item # N8145000 or equivalent
- 3) Chiller 1 HP 230V/60HZ TP Quiet, PolyScience, Item # N0772046 or equivalent

2. Gas chromatography system

- 1) Gas Chromatograph, PerkinElmer® Clarus 500 or 580 or equivalent system

- 2) GC capillary column, PerkinElmer® Elite-5 30m, 0.25 mm i.d., 0.25 mm film thickness, Item # N9316076 or equivalent
- 3) GC-ICP-MS Heated Transfer Line accessory, Redshift®, Item # N0777440 for 115V or Item # N40777361 for 230V or equivalent
- 4) Solid Phase Micro Extraction (SPME) Fiber Assembly, specifically, Supelco Analytical 100µm Polydimethylsiloxane (PDMS) Coating (Red - Pack of 3), Supelco Analytical, Bellefonte, PA, Item # 57301 or equivalent
- 5) "T" union to interface GC column to transfer line and make-up gas, Redshift® Item # TL00402 or equivalent
- 6) Thermogreen® LB-2 Septa, Supelco, Item # 20654 or equivalent
- 7) Glass inlet liner, Supelco, Item # 2631405 or equivalent
- 8) Graphite ferrule, 1/16 in x 0.5 mm for use with 0.32 i.d. transfer line, pkg. of 10, PerkinElmer®, Shelton, CT, Item # 09903700 or equivalent

3. Replacement and periodic maintenance of key components

Part numbers listed below are PerkinElmer® part numbers from their 2012/2013 Consumables Catalog and 2012 NexION® 300 ICP-MS Consumables and Supplies Reference Guide unless indicated. Equivalent high quality parts from other suppliers may be used as noted.

- 1) Peristaltic pump tubing for sample and internal standard (0.76 mm i.d.), Item # N8145202, and for waste (3.16 mm i.d.), Item #N8122012 or equivalent
- 2) Autosampler probe assembly, Item # B3000161 or equivalent
- 3) NexION cyclonic spray chamber, Item # N8145013 or equivalent
- 4) Demountable quartz torch, Item # N8122006 or equivalent
- 5) Torch o-ring kit, Item # N8120100 or equivalent
- 6) Torch alignment tool, Item# WE015554 or equivalent RF load coil, Item # WE021816, or equivalent
- 7) Pump oil for the roughing pump, Item # N8122004 or equivalent
- 8) Quartz injector 1.2 mm ID, Item # N0681631 or equivalent
- 9) Quartz ball injector 2.0 mm i.d., Item # WE023948 or equivalent
- 10) Demountable quartz torch, Item # N8122006 or equivalent
- 11) Torch O-Ring Kit, package of four, Item # N8120100 or equivalent
- 12) Cone removal tool, Item # W1034694 or equivalent
- 13) NexION® platinum sampler cone, Item # W1033614 or equivalent
- 14) NexION® platinum skimmer cone, Item # W1026907 or equivalent
- 15) NexION® aluminum hyper skimmer cone, Item # W1033995 or equivalent
- 16) NexION® Sampler gasket, Item #, W1040148 or equivalent
- 17) NexION® Hyper Skimmer o-ring, Item # 09902123 or equivalent
- 18) NexION® Hyper Skimmer Screw, Item # WE027484 or equivalent
- 19) Fombulin®GV80 pump oil for the roughing pump, Item # N1845003 or equivalent
- 20) PE Sciex Coolant, Item # WE016558A or equivalent

- 21) NexION® Trap Coaxial Foreline, Item # W1036511 or equivalent
- 22) NexION® Exhaust Filter Kit SV40BI, Item # N8145005 or equivalent
- 23) TQ+ Quartz nebulizer, Meinhard, Item # TQP-50-A0.5 or equivalent
- 24) Nebulizer connection kit, Meinhard, Item # F1-C16 or equivalent
- 25) UltraCLAVE Metal O-ring ,Milestone, Shelton, CT, Item # WZ00046 or equivalent
- 26) UltraCLAVE Flat O-ring, Milestone, Shelton, CT, Item # OR0001 or equivalent

4. Robotic sample processing station

Robotic Liquid Sample Processing Workstation: LEAP® Technologies CombiPAL® twin head system, or equivalent system (Figure 3).

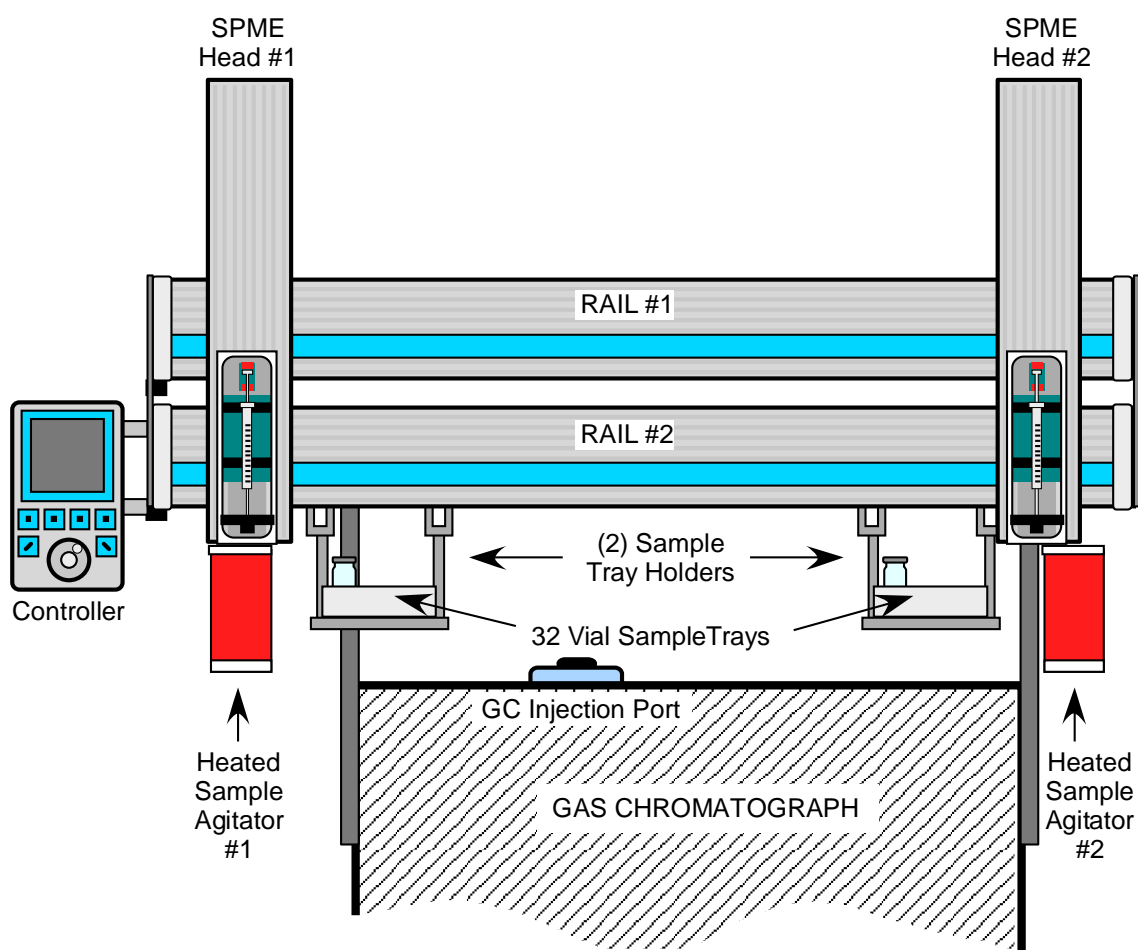


Figure 3: Twin Head SPME Processing Workstation (LEAP Technologies, Inc.)

The Twin Head SPME CombiPAL® 4 (LEAP Technology, Inc.) sample processing workstation, mounted on top of the GC, has a unique design: features two (2) computer-controlled SPME fiber injection heads that run on a dual rail system. The two SPME Heads are independently controlled and perform SPME fiber equilibration/injection operations in tandem. This configuration allows for increased SPME fiber equilibration time up to 20 minutes per SPME head while maintaining a sample-to-sample injection cycle time of 10 minutes. The result is improved SPME efficiency and faster duty rates (6 injections per hour).

5. Equipment

1. Water purification system for providing ultrapure water with a resistivity $\geq 18 \text{ M}\Omega \text{ cm}$, Aqua Solutions, Jasper, GA, or equivalent.
2. High-precision analytical balance capable of accurately weighing milligram amounts of material to the tenth of a milligram or better, Mettler XP205 Delta Range Excellence Plus (Mettler Toledo), or equivalent
3. A pH meter with one hundredth's of a pH unit readout or better, Accumet XL-150, or equivalent.
4. Calibrated hand-held adjustable pipettes that cover the range of accurate liquid delivery from $50 \mu\text{L}$ to $5000 \mu\text{L}$. Sartorius Picus NXT, Sartorius Corporation, single channel electronic programmable pipettes, or equivalent
5. Eppendorf® Repeater Xstream Pipette, Item #13-683-506, (Fisher Scientific), or equivalent
6. Vortex mixer, VWR, Item # 945303, or equivalent
7. Gas regulators for argon, helium, and xenon (Airgas, Atlanta, GA) or equivalent
8. Mechanical convection oven (FREAS Model 605, Thermo scientific, Item # 3166188), or equivalent
9. Microwave Digestion System (UltraCLAVE, Milestone Inc, Shelton, CT) or equivalent
10. Captair pyramid glove bag (Erlab, North Andover, MA), or equivalent

6. Computer Software

1. NexION® instrument control and data handling software, version 1.5 or greater must be installed on the computer controlling the NexION®
2. Chromatography data handling software, specifically, TotalChrom™ Workstation, version 6.3.1 or later (PerkinElmer® Instruments, Shelton CT). Install PerkinElmer's TotalChrom™ Workstation package on the same computer containing the NexION® Instrument Control software*. Contact PerkinElmer for installation and configuration of TotalChrom™. Alternatively, consult the TotalChrom™ Workstation User's Guide. This method assumes that version 6.3.1 of TotalChrom™ Workstation package is installed
3. Operating software for CombiPAL autosampler, specifically, Chronos (2007-2010 Axel Semrau GmbH & Co KG)
4. ChromLink™ 3.5 software (PerkinElmer Instruments). The installation of ChromLink™ is straightforward when using the supplied installation utility
5. pdFactory Pro (FinePrint Software, LLC, www.fineprint.com) or equivalent software. This product is used for creating electronic Portable Document Files (pdf) directly from any Windows® compatible application print dialog box
6. A custom Microsoft Excel® macro procedure named "Extract TC Data"

7. Supplies

1. $0.5\text{-}200 \mu\text{L}$ LowRet pipette tips, 960 tips per case, Sartorius Corporation, Item # LH-L790200, or equivalent
2. $5\text{-}350 \mu\text{L}$ LowRet pipette tips, 960 tips per case, Sartorius Corporation, Item # LH-L790350, or equivalent
3. $10\text{-}1000 \mu\text{L}$ LowRet pipette tips, 960 tips per case, Sartorius Corporation, Item # LH-L791000, or equivalent

4. 5 mL pipette tips, 1000 tips per case, Sartorius Corporation, Item # 780308, or equivalent
5. 50 mL combitips plus, 100 tips per case (Eppendorf® catalogue # 02226660-8), or equivalent
6. 10 mL combitips plus, 25 tips per case (Eppendorf® catalogue # 02226650-1), or equivalent
7. 2.5 mL combitips plus, 100 tips per case (Eppendorf® catalogue # 02226630-6), Westbury NY), or equivalent
8. 50-mL acid-cleaned volumetric flasks for triple spike solution preparation (polypropylene or Teflon flasks preferred). To acid-wash flasks, rinse with 1.2M hydrochloric acid followed by rigorous rinsing with DI water. Repeat this process several times depending on prior use of the containers.
9. 250-mL acid-cleaned volumetric flasks (UV-resistant) for derivatizing reagent (NaPr4B) preparation (polypropylene or Teflon flasks preferred). To acid-wash flasks, rinse with 1.2M hydrochloric acid followed by rigorous rinsing with DI water. Repeat this process several times depending on prior use of the containers.
10. Acid-cleaned 1L PE bottles for buffer solution preparation. To acid-wash flasks, rinse with 1.2M hydrochloric acid followed by rigorous rinsing with DI water. Repeat this process several times depending on prior use of the containers.
11. 50 mL polypropylene tubes pre-screened for Hg (Fisher Scientific), Item # 14-432-22, or equivalent.
12. 15 mL polypropylene tubes pre-screened for Hg (VWR), Item # 82050-276, or equivalent.
13. 2 mL Safe-Lock® polypropylene (PP) microcentrifuge tubes, Eppendorf® Item # 022363352, (Fisher Scientific, Suwanee, GA), or equivalent.
14. Eppendorf® tube racks for 2 mL microcentrifuge tubes, Item # 05-405-3, (Fisher Scientific, Suwanee, GA), or equivalent.
15. Blue racks for 2mL cryovials (50-vial capacity), Item # 03-375-24L, (National Scientific), or equivalent.
16. Six or more PAL Tray20mL, Item # 65487454, (CTC Analytics), or equivalent.
17. CombiPAL autosampler vials 20 mL, glass, Item # 16-2000, (Microliter Analytical Supplies, Inc.), or equivalent.
18. Head space caps for 20mL glass vials, Item # 16-0050M, (Microliter Analytical Supplies, Inc.), or equivalent.
19. Kay-Dry™ paper towels and Kim-Wipe™ tissues, Item # 06-666-11, (Fisher Scientific, Suwanee, GA), or equivalent.
20. Teflon™-coated magnetic stir bars (4), Item # 58948-974, (VWR Scientific, Buffalo Grove, IL), or equivalent.
21. Teflon™-coated magnetic stir bars, Item # 58947-140, (VWR Scientific, Buffalo Grove, IL), or equivalent.
22. Sharps containers, 8.2 qt., pack of 12, Item # 14-826-128, (Fisher Scientific, Suwanee, GA), or equivalent.
23. Cotton swabs (Hardwood Products Co. ME), or equivalent.
24. Nitrile, powder-free examination gloves (VWR or Fisher Scientific), or equivalent.
Biohazard autoclave bags (Fisher Scientific), or equivalent.

25. Heavy wall 12x75mm borosilicate glass test tubes (Fisherbrand catalog #: 14-958-10B) or equivalent.
26. 77 position WEFLON-PTFE sample rack (Catalog #: UC85034) Milestone, Shelton, CT or equivalent.

VII. STANDARD PROCEDURE

A. Preparation of Stock Solutions

1. NaOAc Buffer Solution (0.1 M sodium acetate anhydrous)

1. Dissolve 16.41 g of sodium acetate anhydrous into approximately 2000 mL of DI water in a 2000 mL polypropylene vessel with a magnetic stir bar on a magnetic stir plate, and mix thoroughly.
2. Measure pH and adjust pH to approximately 4.75 with glacial acetic acid. Dilute to final volume with DI water. Store at room temperature. Expires one year from the date prepared.
3. Other quantities of the buffer solution (e.g. 4 L) can be made with the proper adjustment to the amount of constituents added.

2. Sodium tetra(n-propyl)borate (NaPr₄B, 2% w/v)

1. Place one unopened vial containing 5.00 g of sodium tetra(n-propyl)borate, a squirt bottle of DI water, and a clean 50 mL conical centrifuge tube filled with 10-20 mL of DI water inside a glove box or tent. Fully purge glove box or tent with 100% nitrogen for approximately 5 minutes to remove oxygen.
2. Open the vial containing sodium tetra(n-propyl)borate and add 10-20 mL of DI water with constant swirling.
3. Pour the dissolved contents into the 50 mL conical centrifuge tube. Wash the reagent vial with additional DI water to dissolve remaining solids and quantitatively transfer remaining reagent to the 50 mL tube. Cap the 50 mL tube and remove from tent. Once dissolved, the NaPr₄B solution may now be safely used in normal atmosphere.
4. Inside a chemical fume hood, quantitatively transfer reagent solution containing 5.00 g of NaPr₄B to a 250 mL UV-resistant volumetric flask. Dilute to the final volume with DI water. This makes a 2% (w/v) NaPr₄B solution. Store at approximately 4°C (range of 2-6°C). Expires three weeks from the date prepared.

B. Preparation of Working Spike Solution

Caution!

Mercury compounds are toxic! Take extra care to avoid accidental dermal contact, ingestion, or inhalation of these materials. Wear appropriate personal protective equipment. At a minimum, wear a laboratory coat and latex or nitrile gloves. Clean up any spill that might occur according to applicable hazardous material spill procedures.

Exercise the utmost care in executing all measurements precisely to obtain the best accuracy for the final concentrations.

1. Preparation of Working Triple Spike Standards Solution

Remove the vendor-supplied standard solutions of the following from cooled storage and allow to reach room temperature: In¹⁹⁹Hg, Me²⁰⁰Hg, and Et²⁰¹Hg AIT enriched isotopes or In¹⁹⁹Hg, Me²⁰¹Hg, and Et¹⁹⁸Hg ISC Science enriched isotopes. The following intermediate and working solutions are prepared gravimetrically allowing the final concentrations of "spike" material in the working solution to be determined by weight. An Excel worksheet was developed to calculate the final concentrations of working triple spike standards solution, and the template for the worksheet can be found in APPENDIX B.

a. Intermediate standard solution

Pipette 100 µL each of In¹⁹⁹Hg, Me²⁰⁰Hg, and Et²⁰¹Hg AIT standards or In¹⁹⁹Hg, Me²⁰¹Hg, and Et¹⁹⁸Hg ISC Science standards into separately labeled 2 mL microcentrifuge tubes. Add 900 µL of DI water to each and mix thoroughly. (Note that the volumes pipetted depend on the concentrations of the original stock solutions).

b. Working solution (containing a mixture of three isotope standards):

1. Prepare a solution containing 2% hydrochloric acid, 3% acetic acid and 1% methanol by filling a clean 50 mL polypropylene tube approximately half full with DI water then adding 1mL of hydrochloric acid, 1.5mL of acetic acid and 0.5mL of methanol. Fill to the 50 mL mark with DI water.
2. Fill a clean 50 mL volumetric flask approximately half full with the solution prepared in step b.1. above.
3. Pipette 100 µL of each intermediate standard solution into the flask. Discard the remaining intermediate standard solutions after the aliquots have been taken. Bring the volume to 50 mL with the solution prepared in the step b.1. above. (Note that the volumes pipetted depend on the concentrations of the original stock solutions).
4. The concentration of each isotope standard in the working solution will be within a range of 0.5–3 µg Hg/L. Calculate the exact concentration of each isotope standard contained in the Working Triple Spike Standards Solution by using the Excel worksheet for spike calculation (see example in APPENDIX B). Store at 4°C. The working solution expires three weeks from the date prepared.

C. Preparation of Quality Control Material

Order whole human blood from an appropriate vendor or use donated blood. Characterize for total Hg using this method or an equivalent method.

1. Base pool preparation

Human blood with the lowest mercury content available must be used. Distribute base blood into pre-labeled 2 mL polypropylene cryovials in approximately 1.5 mL aliquots.

2. Low and High quality control pools (QC) preparation

Analyze base blood for InHg, MeHg, and EtHg species. Add appropriate volumes of InHg, MeHg, and EtHg (not enriched) species to obtain "low" and "high" pools. While constantly stirring each pool, aliquot approximately 1.5 mL of blood into a number of pre-labeled 2 mL vials to provide QC material for at least 1000 runs. Store aliquotted QC material at approximately -70°C or lower temperature.

D. Sample preparation

Caution!

Work with open vials containing biological samples inside of a biological safety cabinet (BSC). Recap vials before removing them from BSC. Wear appropriate personal protective equipment (lab coat, safety glasses, and gloves).

1. Preparation of blood samples for digestion

- a. Digestion method 1: Convection Oven
 - 1) Pipette 100 μ L of blood samples into pre-labeled 2 mL Safe-Lock microcentrifuge tubes.
 - 2) Add 100 μ L of Triple Spike Standards Solution. Recap and vortex each tube before continuing to the next tube.
 - 3) Add 500 μ L of TMAH to all tubes. Cap and vortex.
 - 4) Place rack containing capped tubes in the convection oven set to $80 \pm 3^\circ\text{C}$ for ≥ 20 hours.
- b. Digestion method 2: UltraCLAVE Microwave Digestion
 - 1) Pipette 100 μ L of blood samples into pre-labeled 12x75mm glass test tubes.
 - 2) Add 100 μ L of Triple Spike Standards Solution. Vortex each tube before continuing to the next tube.
 - 3) Add 500 μ L of TMAH to all tubes. Vortex gently. Optionally, cover the test tubes with ventilated microwave digestion caps.
 - 4) Refer to SOP DLS 3517 (*Operation of the UltraCLAVE Microwave Digestion System for the Mercury Speciation Isotope Dilution Method*) for instructions of usage of the UltraCLAVE for digestion of the samples and quality control materials.

2. Derivatization of the digested samples:

- a. Aliquot 275 μ L of digested samples into pre-labeled 20 mL SPME headspace vials.
- b. Add 7.7 mL of 0.1M NaOAc Buffer Solution using the Eppendorf® Repeater Xstream followed by 250 μ L of NaPr₄B Derivatization Reagent. Cap the vial immediately and mix.

E. Requirements for Batch Analysis of Samples and QC Material

Process a predetermined set of samples and QC material for batch analysis. One "batch" run is defined as the analysis of a contiguous set of samples bracketed by "Bench QC" material at the beginning and end of the set. Each Bench QC level (typically, a "high" and a "low") must be analyzed at the beginning and again at the end of the batch run. QC needs to be treated like the unknown samples, i.e., each QC sample is individually prepared and goes through all of the same steps as the unknown samples. It is not appropriate to report the QC results coming from the split analysis of a single QC sample if it has already been processed (i.e., diluted, centrifuged, filtered, digested, derivatized, etc.). Note that this limitation does not apply to the duplicate use of QC material originating from the same original vial as long as they both are processed identically like unknown samples and *on the same day*.

1. Identify, gather, and thaw a predetermined number of sample tubes/vials containing the blood samples to be analyzed in a batch run.
2. For each batch of samples run, thaw one tube of low and high bench QC (often identified as "LB-yyxxx" and "HB-yyxxx"; for explanation of nomenclature, see Quality Control Material section).

3. Label the necessary number of 2 mL microcentrifuge vials or glass tubes with appropriate identification to ensure that they will be matched to their corresponding unknown samples and QC. Similarly, label an equal number of 20 mL SPME vials.
4. Use the preparation procedure outlined in VII D to prepare blood samples and QC materials.
5. Cap all autosampler vials with the proper fitting septum caps.

The use of a barcode scanning device to electronically record sample identification from barcodes printed on vial labels must be utilized if available.

F. GC Instrument Program

1. Set the GC analytical run parameters to what is listed in TABLE 7-1. Refer to the Clarus 500 or 580 User Guide for programming specifics.
2. One or more parameters specified in TABLE 7-1 may be changed, if determined necessary, to meet analytical performance goals.

TABLE 7-1: GC Settings

Parameter	Setting
GC injector Temperature	Step #1: 1 min @ 220°C Step #2: linear ramp to 280°C, hold
Carrier gas flow rate (Helium)	2 mL/min (splitless mode)
Oven Temperature	Step #1: 1 min @ 75°C Step #2: linear ramp to 250°C (at a rate of 45 °C/min)
Optimization gas flow rate (Xenon)	0.25 mL/ min
Transfer line Temperature (Aux - auxiliary zone)	250°C
(Aux - auxiliary pneumatics)	OFF (all)

G. CombiPAL Autosampler Program

1. CombiPAL is initially set up and optimized by LEAP Technology service engineers, or other trained personnel. The settings can be optimized according to analyst needs (refer to PAL system user manual). The following procedures present the key steps that are taken to set up the CombiPAL autosampler system for this method.
 - Using the PAL control terminal, start at a window displaying "JOB QUEUE". Press ESC to return to the previous menu. Press function key F1 - "MENU". Rotate the outer knob to scroll through items in a menu list. To select a highlighted item press the central knob (ENTER button). Then use the outer knob to scroll through available options for that item or to change a numeric value. Then press the inner knob again to ENTER the displayed option.
 - In the "MENU" window, select "Set-up" press "F3" then press "ENTER" scroll to "Object" press "ENTER" then select "Tray Holders", "Trays", , "Agitators", "Injectors" and enter parameters from TABLE 7-2. X, Y, and Z parameters have to be optimized for each SPME arm. The left SPME arm will be referred to as (L) and the right as (R) in the TABLE 7-2 below. After entering parameters,

always press “F1” to check the position. These parameters are only suggestions and have to be optimized for each instrumental setup.

TABLE 7-2: CombiPAL® Autosampler Settings

Parameter	Setting
Tray Holders (Agitator)	
Tray holder XYZ (R)	157.0,178.9, 166.3 mm
Agitator position (R) XY Z	360.4, 2.3, 93.2 mm
Tray holder XYZ (L)	-165.0,179.1, 165.2 mm
Agitator position (L) XYZ	-295.9, 1.6, 93.1 mm
Stand by Temp	OFF
Actual Temp	25.7°C (changes)
Tray (Agitator)	
Offset XYZ (get vial out of the agitator)	0, 0, 55.5 mm
Tray (Tray)	
Tray type	VT32-20
Offset X, Y, Z (R)	-55.3, -187.0, -2.0 mm
Offset X, Y, Z (L)	-55.3, -188.1, -2.0 mm
Injector (GCInj)	
Needle Penetration	35.0 mm
Offset X, Y, Z (L)	-22.6, 204.5, 247.1 mm
Offset X, Y, Z (R)	-153.3, 204.8, 246.3 mm

- CombiPAL is operated by Chronos software. Click on the Chronos icon on the operating computer desktop. Chronos method files used for this method can be accessed through clicking on "Method Editor" on the Main Menu of Chronos. In the right corner, click on the "Load" tab which allows the analyst to upload the needed method file. There are five method files used in this method: SPME bakeout LEFT.cam, SPME bakeout RIGHT.cam, SPME conditioning LEFT.cam, SPME conditioning RIGHT.cam, and TWIN SPME_cdc.cam. The settings can be changed by the analyst for better separation and analytical response.
 - To clean SPME fibers every day before starting a run,- SPME bakeout LEFT.cam and SPME bakeout RIGHT.cam are used. The settings are as shown in TABLE 7-3.

TABLE 7-3: Chronos Settings (Baking)

Parameter	Setting
Bakeout Time (s)	420
GC Runtime + Cooling Down (s)	120

- To condition new SPME fibers use - SPME conditioning LEFT.cam and SPME conditioning RIGHT.cam. The settings are as shown in TABLE 7-4.

TABLE 7-4: Chronos Settings (Conditioning)

Parameter	Setting
Bakeout Time (s)	1800
GC Runtime + Cooling Down (s)	120

- To perform a run, the file "TWIN SPME_cdc.cam" is used. The settings are as shown in TABLE 7-5.

TABLE 7-5: Chronos Parameter Settings (for Run)

Parameter	Setting
Source Tray	Tray1,1
Source Vial	1,1; 2,2; 3,3 etc.
Incubation Time	1 s
Enrichment Time	1200 s
Desorption Time	420 s
GC Cooling Down	180 s

H. ICP-DRC-MS Instrument Setup

To improve workflow efficiency, program the steps described in this section before the day of analysis.

1. Programming the DRC Gas Flow Delay Parameter

A special DRC™ setting, called "Flow Delay", needs to be changed from its default setting to avoid the problem of the ICP-MS software forcing a time delay of several seconds before collecting data at the start of a chromatographic run when in DRC mode. With the NexION®, this setting can only be changed by entering the NexION® software's service mode. This change only needs to be done once per software installation or upgrade, or if the setting was deliberately changed by a field service engineer. It is a good idea to inform the service engineer who intends to perform work on the instrument of the importance of returning the "Flow Delay" to the non-default value of 1.

Important!

While in Service Mode, DO NOT make changes to any setting except for the one change described below.

1. From within the NexION® software in the "Instrument Control Session" window, choose menu item Options > Configuration.
2. Click on the "set" button under the "Cell Gas Delays" section. If the "Flow Delay" (Gas changes while in DRC™ Mode) setting is a value other than "1", change the value in the field named "Flow Change" to "1". Click "Apply" then click "Close."

2. Programming the NexION® ".mth" file

1. If it is not already open, launch the NexION® program and in the "Instrument Control Session" window, click "File" > "Review Files". Click the "Load" button for "Method" (the first item on the list). Navigate to the folder "C:\NexIONdata\Method" and click on the "GC_Hg.mth" file followed by "Open".
2. If the "GC_Hg" file cannot be found, or it has been changed or corrupted in a manner that makes its use questionable, close the open file dialog box and close the Review Files window by clicking the "Done" button. Complete the following steps; otherwise, proceed to step 3:
 - Make the active method file the active window (do this by clicking on the tool bar icon that looks like a notepad with a "Cu" on it). Then click "File" > "New" on the menu bar and choose "Data Only" in the New Method window that appears. Click "OK" then maximize the window. Fill in this window with the information in TABLE 7-6.

TABLE 7-6 NexION® Timing Parameters

Parameter	Setting
Sweeps/Reading:	1
Readings/Replicate:	2725
Number of Replicates:	1
Tuning File:	C:\NexIONdata\Tuning\default.tun
Optimization File:	C:\NexIONdata\Optimization\gc_xe_drc.dac

- On the first line of the worksheet-like table, click in the cell of row 1 of the "Analyte (*)" column. Type "Hg" then press "Enter" key. The row will suddenly be filled-in with mercury's "Begin Mass (amu)" of 201.971 (or something close) and several default parameters. Right click on "Hg" and a periodic table will appear. Select five Hg isotopes from mass ~197.9670 to ~201.9710 and click "OK". All five isotopes will be seen in the "Analyte (*)" column. Tab to next cells and fill in the information shown in TABLE 7-7.

TABLE 7-7: NexION® Analyte Parameters

Parameter	Setting
Analyte:	Hg
Begin Mass (amu):	197.9670* (first row, then proceed to the 4 other isotopes)
End Mass:	<leave empty>
Scan Mode:	Peak Hopping
MCA Channels:	1
Dwell Time:	25
Integration Time:	68125 (automatically determined by software)

*Actual mass may differ by a few hundredth of amu.

- Click on the "Processing" tab and enter the following information:

TABLE 7-8: NexION® Processing Parameters

Parameter	Setting
Detector:	Dual
Measurement Unit:	Cps
Process Spectral Peak:	Average
Process Signal Profile:	Average
Apply Smoothing:	Checked
Factor:	5
Auto Lens:	Off
Isotope Ratio Mode:	Off

- Skip the "Equation" tab. Click on the "Sampling" tab and enter the following information:

TABLE 7-9: NexION® Sampling Parameters

Parameter	Setting
Peristaltic Pump Under Computer Control:	Unchecked
Sampling:	External

- Click on the "Report" tab and enter the information in TABLE 7-10.

TABLE 7-10: NexION® Report Parameters

Parameter	Setting
Report View Send to Printer:	Unchecked
Report Options Template:	<leave empty> *
Automatically Generate NetCDF File:	Checked C:\nexiondata\reportoutput\
Report to File Send to File:	Unchecked
Report Options Template:	<leave empty> *
Report File Name:	<leave empty> *
Report Format:	<leave empty> *
File Write Option	<leave empty> *

*Content of these fields is not important since Send To Printer/File is unchecked.

- Choose menu item "File" > "Save As" and navigate to the "C:\NexIONdata\Methods\" folder. Enter "GC_Hg" as the name of the method file and click the "Save" button.
3. The NexION® method "GC_Hg" is now loaded into memory.

3. Creating the NexION® Sample Table ".sam" file

1. If it is not already open, launch the NexION® program and in the window titled "Instrument Control Session", choose menu item "File" > "Review Files". Click the "New" button for "Dataset" which is the second item on the list. Navigate to the folder "C:\gc\data" and enter the file name "Hg<yymmdd>" where yy = the last 2 digits of the current year, mm = the month, and dd = the date of run. Click the "Open" button. The new dataset folder has been created and is now active. Click "DONE" in the Review Files Window.
2. Click on the tool bar icon that looks like three Erlenmeyer flasks. Choose "File" > "New" on the menu bar. A new window will appear titled "Samples - [Untitled]". Click the "Batch" tab then click on the "Sample Template" button. A dialog box titled "Sample Template Data" will appear. Enter the information as given in TABLE 7-11.

TABLE 7-11: Sample Template Data

Parameter	Setting
Sample ID:	001_ <sample name>
Measurement Action (*):	Run Sample
Method:	GC_Hg.mth
Sample type:	Sample
Wash Override (sec)	0

3. Parameters not mentioned in TABLE 7-11 can be left blank.
4. From the menu bar, choose "File" > "Save As" and save the file in the directory "C:\gc\data" using the name "Hg<yymmdd>.sam" where yy = last 2 digits of current year, mm = this month, and dd = date of run.
5. It is a good idea to save a copy of this file as a template, thereby avoiding the need to re-create it every time.

I. ICP-DRC-MS and GC Performance Checks and/or Maintenance

The following performance checks are recorded in the instrument log book.

1. Daily Performance Check

1. Each day, before samples are analyzed, aqueous blanks and a 10X dilution of Low Bench QC must be analyzed to ensure that the instrument is functioning properly. Prepare all the following using the same technique and supplies as samples, unless stated otherwise.
 - (a) **Aqueous Blank 1.** To prepare, add 275 µL of TMAH into a 20 mL vial. To each vial, add 7.7 mL of NaOAc Buffer solution and 250 µL of derivatization reagent (NaPr₄B). Cap the vial immediately and gently mix it. Aqueous blank 1 is ready to be analyzed.

- (b) **Aqueous Blank 2.** To prepare, add 7.7 mL of NaOAc Buffer solution to each vial followed by adding 250 μ L of derivatization reagent (NaPr₄B). Cap the vial immediately and gently mix it. Aqueous blank 2 is ready to be analyzed.
 - (c) **10X Diluted Low Bench QC.** To prepare, mix 150 μ L of LB QC, 1350 μ L of base blood, 1500 μ L of triple spike solution, and 7500 μ L of TMAH. This sample is digested and subsequently analyzed in the same manner as patient samples.
2. After SPME-GC-ICP-MS analysis of Aqueous Blanks and 10X Diluted Low Bench QC are complete, open the Aqueous Blanks and 10X Diluted Low Bench QC RAW files in TotalChrom™. Examine the Aqueous Blank chromatograph for possible indicators of contamination. Then look at the 10X Diluted Low Bench QC chromatogram. This chromatogram will have visible peaks for the mass 202 isotopes of MeHg, EtHg, and InHg. The integrated area under each mercury species peak has to be greater than 300 (arbitrary units), otherwise there may not be sufficient sensitivity and the instrument may need to be optimized (see weekly performance check section).

2. Weekly and Monthly Performance Check and Maintenance

1. Visual check of torch, injector, RF coil, and cones:
 - Visually check the cleanliness of these components. Notate any cleaning / replacing of consumables on the "Daily Maintenance / Performance Checklist". For details on cleaning procedures, refer to the IRAT Weekly Maintenance SOP.
 - **Injector:** If there are deposits on the inside of the injector, remove the injector and clean it with 1-5 % v/v nitric acid and a cotton swab. Alternatively, replace injector with a spare and clean the dirty one in an overnight soak in 5% v/v nitric acid (can be ultrasonicated, but is not typically necessary).
 - **Torch:** Check for melting, cracking, and cleanliness. If necessary, replace with a spare and soak overnight in 5% v/v nitric acid bath. If torch is only dirty, replacement / cleaning can be deferred to the next regular weekly maintenance day.
 - **RF coil:** Check for excessive corrosion (flaking). Replace if necessary.
 - **Sampler and skimmer cones:** Check for excessive buildup of matrix, cracking, or pitting. If necessary, replace dirty cones with clean spare cones and clean.
2. Replace GC injector septum and glass liner weekly.
3. Weekly, the optimization of both plasma and mass spectrometer parameters of the ICP-MS is achieved by using a mixture of xenon (Xe) in argon (Ar). The ICP-MS system is optimized with Xe gas because of its similarities in ionization potential to Hg.
 - Ignite the plasma and set the flow rate of the Xe gas mixture (0.01-0.1% Xe in Ar) to approximately 0.25 mL/min (depending on Xe% in Ar) on the GC main display soon after plasma ignition to achieve Xe intensity of > 350,000 cps (counts per second). In the NexION® software, upload "gc_xe_daily_drc.mth". After the gas signal stabilizes, in the "Sample" window press "Analyze Sample" in "manual" mode. Perform X-Y optimization to obtain the highest possible Xe intensity (at least >100,000 cps). Proceed with the following steps to optimize other parameters in TABLE 7-12 for maximum Xe intensity.
 - In the NexION® software upload "gc_xe_xy_drc.mth". Also in the NexION® in the window titled "Instrument Control Session", choose menu item "File" > "Review Files". Click the "Load" button for "Optimization" (the sixth item on the list). Navigate to the folder "C:\NexIONdata\Optimize" and click on "gc_xe_drc.dac" file then click the "Open" button.

- SmartTune™ is used for DRC Mode optimization. Optimization parameters can be selected from "edit list". The appropriate method file in the "method" section needs to be selected. The optimization for maximum Xe intensity needs to be performed (see IRAT Optimization SOP).
 - After recommended parameters are optimized, save the optimized file as "gc_xe_drc.dac". The current optimized values will appear automatically and will be similar to the ones in TABLE 7-12.
 - After optimization for maximum Xe intensity, navigate to the folder "C:\NexIONdata\Method" and click on the "gc_xe_daily_drc.mth" file then click the "Open" button. Record this data in the instrument logbook.
4. Replace the GC column and charcoal trap every two months, or sooner if degradation in instrument performance is seen.

TABLE 7-12: NexION® Optimization Parameters

Parameter	Setting	Parameter	Setting
Nebulizer Gas Flow (NEB):	1.5	Rpa	0
Auxiliary Gas Flow:	1.2†	Rpq	.5†
Plasma Gas Flow:	15†	Cell Gas A	0.3†
ICP RF Power:	1450	Cell Gas B	0
Analog Stage Voltage:	-1950†	DRC Mode NEB	1.5
Pulse Stage Voltage:	950†	DRC Mode QRO	- 16.90†
Quadrupole Rod Offset Std	0	DRC Mode CRO	- 1.90†
Cell Exit Voltage	-25†	DRC Cell Exit Voltage	-4.25†
Cell Entrance Voltage	-25†	DRC Cell Entrance Voltage	-4.25†
Deflector Voltage	-13†	DRC Deflector Voltage	-13†
Discriminator Threshold	17†	Axial Field Voltage	150†

†Suggested starting values only. Optimum parameters will depend on the outcome of the optimization procedure.

J. ICP-DRC-MS Warm Up

1. Launch the NexION® ICP-DRC-MS software and note whether all graphical indicators of instrument readiness are green. If not, take the appropriate actions described in the instrument's software and hardware manual.
2. Perform necessary maintenance checks as listed on the Daily Maintenance Checklist. Note the base vacuum pressure in the INSTRUMENT window of the software. (Before igniting the plasma, the vacuum is typically about 8×10^{-6} torr). Keep a record of any maintenance procedures along with the base vacuum pressure on the Daily Maintenance Checklist.
3. In the INSTRUMENT window of the NexION® software, click the "Front Panel" tab and click the plasma "Start" button to ignite the plasma. In the same window, the ignition sequence bar (blue progress bar) will start to expand from the right, indicating the approximate time before plasma ignition. The plasma may at first flicker but it will establish a more or less steady intensity after 5-10 seconds.

On a rare occasion, the plasma may ignite emitting an orange, violently flickering light,

and electrical discharge noises will be heard. In this case, immediately shut off the plasma by pressing the shutoff button on the front panel of the instrument. Wait 30 seconds then investigate the cause of the plasma misfire. A more common occurrence is that the plasma may extinguish itself a few seconds after ignition. Promptly reignite by pressing the "Start" button on the ICP-DRC-MS instrument's front control panel. Usually, the plasma will stay lit after the second try. If not, investigate the cause of the plasma instability (refer to the NexION® Hardware Guide).

4. After successful ignition and the instrument has warmed up and stabilized, perform the daily performance check.
5. Fill in the Daily Maintenance Checklist to record to the completed optimization procedures. If a tuning (mass-calibration) procedure was done, save it to the file "default.tun," and also in a separate file containing the analysis date "default_MMDDYY.tun" (where MM=month, DD=day, and YY=year).

K. GC-ICP-DRCII-MS System Startup

1. Create the daily data folder in C:\GC\Data under the name Hgyymmdd (i.e., Hg110422)
2. Launch NexION® Instrument Control program if it is not already up. Do not launch or start any other programs at this time.
3. Check that the correct NexION® method is loaded and active in the window "Instrument Control Session". If it is not correct, load the correct Method file. Check under the Sampling tab that "Peristaltic pump under computer control" is unchecked, and the pull-down menu "Sampling" indicates "External".
4. Check that the correct Sample file in the window "Instrument Control Session" is active. If it is not correct, load the correct Sample file.
5. Load created dataset file "Hgyymmdd"
6. Enter sample names into the or NexION® sample table
 - a. Click on the tool bar icon that looks like three Erlenmeyer flasks. If the current Samples window is not this run's sample file, then choose "File" > "Open" on the menu bar and navigate to and open this run's current data folder in "C:\gc\data\". Click on the file named "Hg<yymmdd>.sam" (yy = year, mm = digit month, dd = date) and open it. The Samples window will be the one created in the section creating the or NexION® Sample Table ".sam" file.
 - b. Fill in the name of each sample by double-clicking after the "_" (underscore) in the cell "sample ID". Type in the sample name and press "Enter" on the keyboard. In this manner, enter the name of blanks, quality control, and samples that will be analyzed in the run. If barcodes are used on the sample labels, use the barcode scanner attached to the ICP-DRC-MS computer to scan the sample ID from the barcode on each sample before placing it into position in CombiPAL autosampler tray.
 - c. Fill in the samples table in similar fashion to what is shown in TABLE 7-13 below.

TABLE 7-13: NexION® Sample Table

A/S Loc.	Batch ID	Sample ID	Measurement Action	Method ...	Wash Speed (+/- rpm)
1		001_ Aq.Blk	Run Sample		0
1		002_ Aq.Blk	Run Sample		0
2		003_ LB 10X	Run Sample		0
2		004_ LB 10X	Run Sample		0
3		005_ LB-yyxxx	Run Sample		0
3		006_ HB-yyxxx	Run Sample		0
4		007_ Sample	Run Sample		0
4		008_ Sample	Run Sample		0
...rows for 18 samples and SRM material were omitted for brevity					
16		031_LB-yyxxx	Run Sample		0
16		032_HB-yyxxx	Run Sample		0

In the example table above, a run of 20 samples is shown so the last vial ends up being placed in A/S Location #16 (this location corresponds to autosampler location which is defined in Chronos, since there are two autosampler trays two #16 are used - "Left" and "Right". It is not necessary to put A/S loc in NexION® - only for the analyst's benefit).

The numbers preceding the underscore correspond to the order of injection. These numbers will later help the analyst find individual chromatograms based on injection number instead looking for specific sample names during post-run data processing in TotalChrom™.

- When the sample table entries are verified to be correct choose "File" > "Save".
- Print the or NexION® sample table by choosing the "File" > "Print Setup" > "Reports". In the ensuing dialog box, select the preferred printer and click "OK". Next, choose "File" > "Print" and then click the "Print" button. Refer to the printout of the NexION® sample table for the correct vial positions when loading samples into the CombiPAL autosampler tray.

L. Starting the Run

1. Check that the GC methods are correctly programmed.
2. Check that CombiPAL® autosampler methods are correctly programmed.
3. This step offers the advantage that the NexION® data files will be converted in real time to TotalChom™.raw" files that have names containing a date-time stamp corresponding to the actual time of injection.
 - Launch TotalChrom™ Navigator. In the resulting TotalChrom™ Navigator window, choose menu item "Apps" > "ChromLink™" (alternatively, you may launch ChromLink™ from the operating system "Start" > "Programs" menu).
 - In the ChromLink™ program window, choose the menu item "Configuration" > "Mass Details" and check the Nominal Name and Mass for mercury isotopes. If it is missing or the NexION® tune ("default.tun") file was re-optimized earlier then ChromLink™ needs to be configured (see Configuration of ELAN® ChromLink™ on page 38 for details). To save time, the analyst may choose to close the TotalChom™ Navigator and ChromLink™ windows and skip step 6 in

its entirety. Data file conversion via ChromLink™ can easily be done during post-run data reprocessing.

- In the ChromLink™ program window, click on the "Browse" button just right of the "ELAN® ChromLink™ file location" field. Navigate to the current working folder, double-click on it then click the "OK" button so that ChromLink™ knows where to save its processed files.
 - Otherwise, refer to step (b) of Data Processing and Analysis on page 39 for details on proper setting of the ELAN® ChromLink™ window's parameter fields. In the ELAN® ChromLink™ window, click the button "Start Processing NexION® Data Files" to put ChromLink™ in watch mode so it will process each data for each injection in real time. A new dialog box will open and indicate it is ready to convert data and waiting for the first file.
4. Launch the Chronos program, which communicates with the CombiPAL autosampler and NexION® software.
- In the main menu, click on "Sample List" then upload analysis method for SPME fiber cleaning "baking".
 - Upload "SPME bakeout LEFT.cam" (to clean the left fiber) then select "Create Schedule". The Schedule window will appear with time intervals required for the measurement. Click "RUN" on the main menu. The run window appears. Click "run" again to proceed with the fiber cleaning.
 - Repeat step (b) to clean the right fiber and use "SPME bakeout RIGHT.cam"
 - After fiber cleaning and daily performance, check to ensure that the QC and samples are ready for analysis using SPME fibers. On the "Main Menu", select "Sample List" then click "Load List". Upload Dual SPME .csl file. This file communicates with the left and right fibers. A table similar to the one below (TABLE 7-14) will appear. The table will have the Analysis Method "C:\Chronos\Methods\TWIN_SPME_CDC_2.cam " uploaded if the agitator is not used or the Analysis Method "C:\Chronos\Methods\TWIN_SPME_out_of_tray_rev2.cam" if the agitator is used.

TABLE 7-14 Chronos Samples Table

Analysis Method	Source Vial	Incubation Time (s)	Enrichment Time (s)	Desorption Time (s)	GC cooling down (s)
C:\Chronos\Methods\TWIN SPME_cdec.CAM	1	1	1200	420	180
	1	1	1200	420	180
	2	1	1200	420	180
	2	1	1200	420	180
Rows for source vials 3 to 31 are omitted					
	32	1	1200	420	180
	32	1	1200	420	180

- "Source Vial" column shows which autosampler position will be analyzed first. There are two racks (left and right) with vial positions 1 to 32. The left rack gets

analyzed by the left SPME fiber and the right rack gets analyzed by the right SPME fiber. There are two numbers "1" in the source vial column. The first one always corresponds to left rack/left fiber.

- Chronos communicates to the autosampler through "source vial" position. The analyst has to make sure that the samples in NexION® correspond to the locations seen by Chronos. For Example, if the analyst wants to analyze slots 1-5 on both racks (total of 10 samples), the rows below 10 need to be deleted. To delete, select the rows and click "Remove Samples".
5. Check that the DRC gas is indeed flowing by making the NexION® instrument window active and clicking on the Diagnostics tab. Inspect the Cell Gas A or B whose value will be fluctuating around the current value ± 0.01 mL/min. If it is not, see section "Turning on the Reaction Cell Gas" for details on how to turn on the DRC gas.
 6. Check that all blanks, QC, and sample vials are loaded into their correct positions in the CombiPAL autosampler tray as designated by the NexION® sample window and in the positions seen by Chronos.
 7. As a reminder, before samples are analyzed, the daily performance check will be analyzed to ensure that the instrument is functioning properly.
 - Click on the NexION® "Instrument Control Session" window to make it active. Highlight the samples and click the "Analyze Batch" button. A Run Progress box will appear indicating that the NexION® software is now awaiting a signal from Chronos that indicates the occurrence of an injection.
 - In Chronos: to analyze samples, select "Create Schedule" then press "RUN" on the main menu and "RUN" again on the run window.
 - View the chromatograms in TotalChrom™ and in the real time window of NexION® to ensure that there are no problems with the analysis. After the aqueous blanks and 10X LB been analyzed, the run can be started.
 8. Open the NexION® "Instrument Control Session" Real-Time window by clicking the tool bar button that looks like a Gaussian distribution (the blue chromatographic peak). After the Real-Time window opens, click on the drop-down menu and select "Signal". Real-time data will now be displayed.
 9. The CombiPAL® autosampler will seek the first vial and make an injection. A blue bar in the NexION® progress box will now indicate that data is being collected. The system can now run unattended.
 10. Check the progress of the run after 2 or 3 injections. Note the chromatograms appearing in the NexION® Real Time window. Adjust the signal scale in the Real Time window, as necessary. Compare the positions and peak heights of each mercury species. It helps to visually compare it to a printed reference chromatogram. If abnormalities in retention time, peak height, or peak shape are readily apparent, the analyst may need to stop the autosampler, abort the run in the Chronos program, and stop the NexION®. Correct the problem(s) and restart the run.

Important

Remember to disable the NexION®'s Auto Stop feature before re-enabling it otherwise the NexION® may perform an auto shutoff prematurely.

M. Instrument Shut Down

1. The autosampler will stop after all samples have been analyzed.
2. Shut off ICP-DRC-MS plasma.
3. At the controller computer, visit the NexION® Instrument Control Session application and open the "Dataset" window. Confirm that all samples ran successfully and that the corresponding data for each sample is listed in this window.
4. Remove the QC and sample vials from the GC tray. Discard them according to CDC biohazard waste disposal guidelines.

VIII. POST-RUN DATA ANALYSIS

A. Configuration of TotalChrom™ Integration Method

The following information is presented as a starting point to help the analyst develop robust integration method parameters that will work best for most chromatography data. Many of these parameters will work just fine as presented below. However, the separation chemistry of GC columns can vary due to frequency of use, column replacement, or because of individual sample "oddities". Some parameters may need to be adjusted from time to time to maximize the ability of TotalChrom™ to properly integrate peaks and identify components with minimum operator intervention. Therefore, the analyst must pay particular attention to the chromatograms produced in every run and make necessary adjustments as warranted. The analyst must be familiar with the TotalChrom™'s frequently used integration functions, which are described in Chapter 18 of *TotalChrom™ Workstation User's Guide: Volume II*.

1. The creation of a new method file in TotalChrom™ is done the first time TotalChrom™ is setup, or it will need to be recreated if the file "Hg.mth" cannot be found or has been corrupted. In the TotalChrom™ Navigator window, choose the menu item "Build" > "Method." In the next dialog box, click the "Create a new method" radio button and click "OK." The default method will load into the method editor.
2. Click on the "Components" item in the menu bar in Method Editor. If the menu item "Delete All Components" is not grayed out, select it and click "OK" when prompted to "Delete all components, calibration levels, and calibration replicates".
3. Choose the menu Item "Process" > "Integration". Click on the "Integration" tab in the "Process" window. Enter the information shown in TABLE 8-1. These values are to be used as a starting point, but the analyst may make appropriate changes to one or more of the integration parameters as necessary.

TABLE 8-1: Integration

Basic Parameters		Advanced Parameters	
	Value		Value
Bunching Factor :	1	Peak Separation Criteria	
Noise Threshold :	115	Width ratio :	0.2
Area Threshold :	577	Valley to peak ratio :	0.01
		Exponential Skim Criteria	
		Peak height ratio :	5.000
		Adjusted height ratio :	4.000
		Valley height ratio :	3.000

- Click on the "Baseline Timed Events" tab. Enter the information shown below in TABLE 8-2. The analyst may make appropriate changes to one or more of the Baseline Timed Events as may prove necessary.

TABLE 8-2: Baseline Timed Events

Defined Events				
Time	Event	Value	Code	Level
0.000	Set Area Threshold	125	AT	
0.000	Set Noise Threshold	25	NT	
0.000	Smooth Peak Ends On	5	+SM	
2.222	Set Bunching Factor	4	BF	

- Click on the "Optional Reports" tab. Uncheck the box for "Keep temporary files".
- Click on the "Replot" tab. Enter the information shown in TABLE 8-3. The analyst may make appropriate changes to one or more of the Replot parameters as may prove necessary.

TABLE 8-3: Replot

Plots		Miscellaneous	
<i>Generate a separate replot :</i>	checked	<i>Start plot at end of delay :</i>	checked
<i>Number of pages:</i>	1	<i>Gradient overlay :</i>	not checked*
<i>Retention Labels :</i>	Top of Plot*	<i>Draw baselines :</i>	checked*
<i>Component Labels :</i>	Actual time	<i>Timed Events :</i>	checked*
<i>Scaling Type :</i>	Absolute Scaling	<i>Plot Title :</i>	Chromatogram
Scaling Parameters		<i>X axis label :</i>	Time [min]
<i>Scale Factor :</i>	1.000000*	<i>Y axis label :</i>	Response [mV]

*These parameters maybe altered to suit the analyst.

- It is unnecessary to click on the "User Programs" tab because it is not used. Close the Process window by clicking on the "OK" button.
- In the Method Editor window, choose the menu item "Components" > "Global Information." Click on the "Integration" tab in the "Process" window. Enter the information shown in TABLE 8-4:

TABLE 8-4: Global Information

Volume units :	µL	Unidentified Peak Quant.	
Quantitation units :	ng	Calibration factor :	1.000e+06
Sample Volume :	1.000	Always use calib. Factor :	selected
Void time (min) :	0.000		
Calibration		RRT Calculation	
External Standard	selected	Use first peak in run as RRT reference:	selected
Reject outliers during calibration :	not checked		
Sample Amount Options			
Correct amounts for calibration standards :	not checked		
Convert unknown samples to concentration units:	checked		

- The "LIMS Results" tab is not used. Click the "OK" button to close the window. The parameters in TABLE 8-4 are starting points. The analyst may make appropriate changes to one or more of the Global Information parameters as may prove necessary.

- In the Method Editor window, choose the menu item "Components" > "New Component." The white list box in the left portion of the window will be empty. Click in the empty field labeled "Name" and type "Hg0". Press the tab key and enter "1.614" in the field labeled "Retention time". Select the radio button labeled "Peak" if it is not already selected. Leave the other fields and check boxes unaltered. Click the "New Component" button. Enter each of the component names and parameters listed in TABLE 8-5.

TABLE 8-5: Method Editor - Components Settings

Name	Retention Time	Absolute window	Relative window	Find tallest peak in window
Hg ⁰	1.614	2	3	No
InHg	3.470	0	3	No
MeHg	2.640	0	3	Yes
EtHg	3.176	0	3	Yes

- Click the "New Component" button before starting a new component. After entering the last component, click the "OK" button. The values for Retention Time, Absolute Window, and Relative Window serve as starting points. The analyst may alter these values as actual chromatographic results may dictate.
- In the Method Editor window, choose the menu item "Components" > "Defaults." Click on the "Identification" tab". Enter the information shown in TABLE 8-6.

TABLE 8-6: Components Defaults – Identification

Parameter	Setting	Parameter	Setting
Component Type :	Peak	Reference :	blank
Absolute window :	0	Internal Standard :	blank
Relative window :	3	Find tallest peak :	Not checked

- Click on the "Calibration" tab in Components Defaults Window. Enter the information shown in TABLE 8-7.

TABLE 8-7: Components Defaults – Calibration

Parameter	Setting	Amount
Calibration Type :	Use Calibration Factor	1.0000E-6
Scaling :	None	
Weighing :	None	
Response :	Area	

- The "User Values/LIMS" tab is not used. Close the "Components Defaults" window by clicking the "OK" button.
- In the Method Editor window, Choose "File" > "Save As." A window appears inviting you to enter any information pertinent to this method, which will be saved with the method. Enter your name and the date this method was created. Click "OK" and a

"TotalChrom File-Save-As" dialog box opens. Navigate the directory tree to get to the folder C:\GC\Methods). Double-click on this folder. In the "File name:" field, enter "Hg-Template.mth". If there is already a file in that folder with the same name, highlight that file and right-click the mouse. Choose "Rename" and give the file a new name (e.g. add "backup" to the name). Now, you can click the "Save" button. Close the "Method Editor" window.

B. Configuration of ELAN® ChromLink™

ELAN® ChromLink™ will be configured after initial installation of the program or when the NexION® tune ("default.tun") file is re-optimized, and when there is available at least one recent NexION® NetCDF file (with the ".nc" extension) containing data for the mass of interest that was collected since last update of the "default.tun" file.

1. Launch TotalChrom™ Navigator. In the resulting TotalChrom™ Navigator window, choose menu item "Apps" > "ChromLink™" (alternatively, you may launch ChromLink™ from the operating system Start > Programs menu).
 - Inside the ELAN® ChromLink™ window, click on menu item "Configuration" > "Default TotalChrom Method". Click on the "Browse..." button and navigate to the directory C:\gc\methods\. Select "Hg-TC.mth," and click the "Open" button. "C:\gc\methods\Hg-TC.mth" will now be the ChromLink™ default method. Click "OK" to close the "Default TotalChrom Method" window.
 - Inside the ELAN® ChromLink™ window, click on the "Set" button. A window titled "Operating Mode" will open. Inside the ELAN® ChromLink™ window, click on the "Set" button. A window titled "Operating Mode" will open. Click on the "Automatic - process all NexION® NetCDF files in specified location" radio button. The lower radio buttons will gray out. Click the "OK" button to close the window.
 - Click on the "Browse" button by the NexION® NetCDF file - location/file to be converted" space. Choose the path "C:\NexIONdata\reportOutput". The location/file to be converted will now read "C:\NexIONdata\reportOutput*.nc".
 - Click on the "Browse..." button for " NexION®NetCDF ChromLink™ file location (sequence and raw files generated by ChromLink™)" field. An open file dialog box will open; choose the file location to which all the data will be directed (usually the data file that was created that day).
 - Click on the "Start Processing NexION® Data Files" button. A window titled "Processing NexION® Data" will appear.
2. At this time, ChromLink™ may be closed by selecting "File" > "Exit." Click "OK" at the dialog box asking if you want to quit ChromLink™.
3. In addition to configuring ChromLink™ itself, it is necessary to alter one value in the "seed" method file that ChromLink™ uses to set a select number of parameters to certain default values. This step only needs to be done once following the installation of ChromLink™™.
 - In the TotalChrom™ Navigator window, choose the menu item "Build" > "Sequence" and a dialog box called "Startup" will appear. Click on the radio button labeled "Load sequence stored on disk" then click the "OK" button. Navigate to the folder on the C drive that contains the ChromLink™ program file (usually in C:\PenExe\ChromLink™ but if it is not there, check under the C:\Program Files directory). Click on the sequence file "seed.seq" to highlight it (if this file is missing, reinstall ChromLink™). Click the "Open" button. A spreadsheet style sequence table will present itself in a window called

"Sequence Information - Channel A". There will be a minimized window for channel B data; ignore this window.

- Choose menu item "File" > "Save." Close the Sequence Editor window by choosing "File" > "Exit" from the menu bar.

C. Data Processing and Analysis

Refer to Figure 2 "Post-Run Data Processing Work Flow Diagram" (page 44) for a summary representation of the important aspects of post-run data processing.

1. Open Microsoft Windows® File Explorer and open the current working GC data directory (e.g., C:\GC\Data\- 2. If it is not already open, launch TotalChrom™.
- 3. If ChromLink™ was not run in real-time data collection mode during the run then do the following:
 - In the TotalChrom™ Navigator window, choose menu item "App" > "ChromLink™." Choose the menu item "Configuration" > "Mass Details" and check the Nominal Name and Mass for mercury. If it is missing or altered then ChromLink™ needs to be configured (see Configuration of ELAN® ChromLink™ on page 38 for details).
 - Check that the Mode field indicates "Automatic - Process all NetCDF files in specified location". If it does not, click the Set button to the right of this field and in the resulting "Operating Mode" dialog box, click the "Automatic - process all NexION® NetCDF files in specified location" radio button, then click the OK button. Next, check that the Field labeled " NexION® NetCDF file - location/file to be converted" indicates the correct data folder. This will be "C:\Nexiondata\Reportoutput*.nc". If it is not, click "Browse" to the right of it and navigate to that folder. Double-click on that folder then click "OK" to close the front most dialog box. Last, click "Browse" to the right of the field labeled "ELAN® ChromLink™ file location..." In the dialog box "Select TotalChrom Data Location", navigate to the folder containing the run data and double-click on it. Click "OK" to close that dialog box. In the ELAN® ChromLink™ window, click the button "Start Processing NexION® Data Files" to start processing of the run data. A new dialog box will open and provide current information on the status of the data conversion.
 - When data conversion by ChromLink™ is completed within a minute or two, a message in the step field will indicate "Successfully Finished". Click "Close." At this point, you may close the ELAN® ChromLink™ application by choosing "File" > "Exit" or clicking on the window "x" box. In the resulting "OK to quit?" confirmation dialog box, click "OK."
- 4. In the TotalChrom™ Navigator window, choose the menu item "Build" > "Method." Click the "Load method stored on disk" radio button and click "OK." In the TotalChrom™ File-Open" dialog box, find C:\GC\Methods folder and open the "GC_Hg.mth" file. The template method file will now be loaded.
- 5. If instead of loading the method file, an error message appears stating that the file is unavailable because it is in use and asks if you would like to open it in Read-Only mode, click the "No" button. Cancel the Open-File dialog box. Exit the Graphic Method Editor. In the Navigator window, choose menu item "Admin" > "CAM Administrator." A window will appear with two panes. In the left pane, click on the "+" sign in front of "TotalChrom Servers" to expand it. Click on the computer icon on the next line that just appeared to highlight it. In the right pane, under the heading "Resource/Instrument",

select the first item. If there is more than one item, select every item by shift-clicking on each item. Every item will now be highlighted. Choose "Edit" > "Remove Locks" (or press the Delete key on the keyboard). Next, click on the "+" sign in front of "Users" to expand it. Click to highlight your TotalChrom™ user name that appeared. In the right pane, under the heading "Resource/Instrument", select every item and Choose "Edit" > "Remove Locks." This action serves to unlock files and make them available for editing. If in the future, TotalChrom™ gives an error message that files cannot be edited because they are locked, use CAM Administrator to unlock them. Choose "File" > "Exit" to quit CAM Administrator. Start again at the beginning of this step to open the Method Editor.

6. Choose "File" > "Save As." At the next window, you will be prompted to enter information about the method. You may enter pertinent information but this is optional. Click the "OK" button and a TotalChrom™ "File-Save-As" dialog box opens. Navigate the directory tree to get to the folder that contains the NexION® data files for this run (typically in the folder C:\GC\Data\). Double-click on this folder. In the "File name:" field, enter the same name as it exactly appears for the folder that will contain it (i.e. Hg<yyymmdd> convention where yy=2-digit year, mm=2-digit month, dd=2-digit date). Click the "Save" button then close the "Method Editor" window.
7. In the TotalChrom™ Navigator window, choose the menu item "Build" > "Graphic Edit." A TotalChrom™ "File-Open" dialog box appears, but click on cancel to close it. On the Graphic Method Editor's menu bar, choose "File" > "Open" and navigate the file-open dialog box to the folder containing the method file created in the preceding step. Click on that file and click the "Open" button. Return to the Graphic Method Editor's menu bar and choose "File" > "New Data File." Navigate to C:\GC\Data\ and double-click on the folder containing the run data. Find and click on a data file (indicated by the ".raw" extension) that corresponds to the "-LB0972". When this file appears in the File Name field, click the "Open" button. If a message box appears with the warning "Unable to open this file: default.mth", click "OK" to clear the message (you do not have to go to CAM Administration to unlock it). Do the same if another message warning box appears (i.e. click "OK" again to clear it). You will be in the "Graphic Method Editor - <path to method file>" window and see a chromatogram.
8. Under the menu item "Calibration" > "Show Windows" there will be a check mark beside "Show Windows." Retention window bars (looks like "H" style error bars) will be present when it is checked. Each retention time window bar will be located above the chromatographic baseline and contain an identified peak within its bounds. If there are any bars at the bottom of the chromatogram located below the baseline, choose menu item "Calibration" > "Edit Components." Click on the first mercury species peak that falls outside its retention time window to select it. In the group of data fields located on the right side of the window, click on the "Name" dropdown arrow (located on the right side of the data entry field) and choose the appropriate species by name. It is usually not necessary to alter the retention time window's "Absolute" and "Relative" window parameters, but you may do so if experience dictates that a change will be beneficial. Click the "Next" or "Prev" button. Repeat these steps for each mercury species peak that was not properly identified because it was outside its retention time window. When the editing of peak retention time windows is complete, click on the menu bar item "Return". Next, choose "File" > "Save" followed by "File > Exit".
9. Launch Microsoft® Excel and choose menu item GCICPMS > Create TC Sequence File (the Excel macro "Extract TC Data.xls" must be installed in Excel's Startup folder). In the open file dialog box, navigate to the current working GC data folder. Click on a RAW file then click the "Open" button. Wait about 30 seconds until a "Done" message box appears. You may leave Excel open. Skip the following step unless, for some reason, the Excel menu item GCICPMS > Create TC Sequence File cannot be run:

- Navigate to the folder containing the run data and click on the sequence file (ends with ".seq") corresponding to the run (named "Hgyymmdd.seq" where yy=2-digit year, mm=2-digit month, dd=2-digit date). Click the "Open" button. A spreadsheet style sequence table will present itself in a window called "Sequence Information - Channel A". There will be a minimized window for channel B data, ignore this window. Look for the "Method" column and click on the first cell in row 1 in this column. Right click the mouse and a contextual menu will appear; choose "Browse". In the resulting File-Select dialog box, navigate and choose the method file (ending in ".mth") created earlier. Click on the Select button. The path and name of the new method file will replace the default information in this cell. Right click this cell again and choose Fill Down. The new file name information will fill down to every cell in the "Method" column. Look for the "Rpt Fmt File" column and follow the same process that was followed with the "Method" column. Instead of choosing the method file (ending in ".mth"), choose the report file (ending in ".rpt"). If the report file is not listed, click on the "default.rpt" file. In the bar that contains the file's path, change the "default" to "Hgyymmdd." Right click this cell again and choose Fill Down. The new file name information will fill down to every cell in the "Rpt Fmt File" column.
10. In the TotalChrom™ Navigator window, choose the menu item "Reprocess" > "Batch." A new window appears titled "Batch Reprocessing". Choose menu item "File" > "Sequence" and another window appears titled "From Sequence". Locate the top field labeled "Sequence file" and look for a button with an open folder icon immediately to the right of the field. Click this button and navigate, if necessary, to the folder containing the run's sequence files. Click on the sequence file and click the "Open button." Upon return to the previous window, set Start Analysis to "Peak Detection" and End Analysis to "Quantitation". Set Batch Printer to "pdfFactory Pro". Change Batch Execution to "ndlb-168462" or anything other than "Interactive". Check "overwrite existing result files" and select "Update existing raw file header with new sequence". All other parameters will remain unchanged. The parameters are shown in TABLE 8-8.
 11. Click the "OK" button. Reprocessing of the chromatographic raw data will commence. The bottom panel in the window will update with each file's name as it is processed. When processing is done, this panel will be clear of files. Close this window.

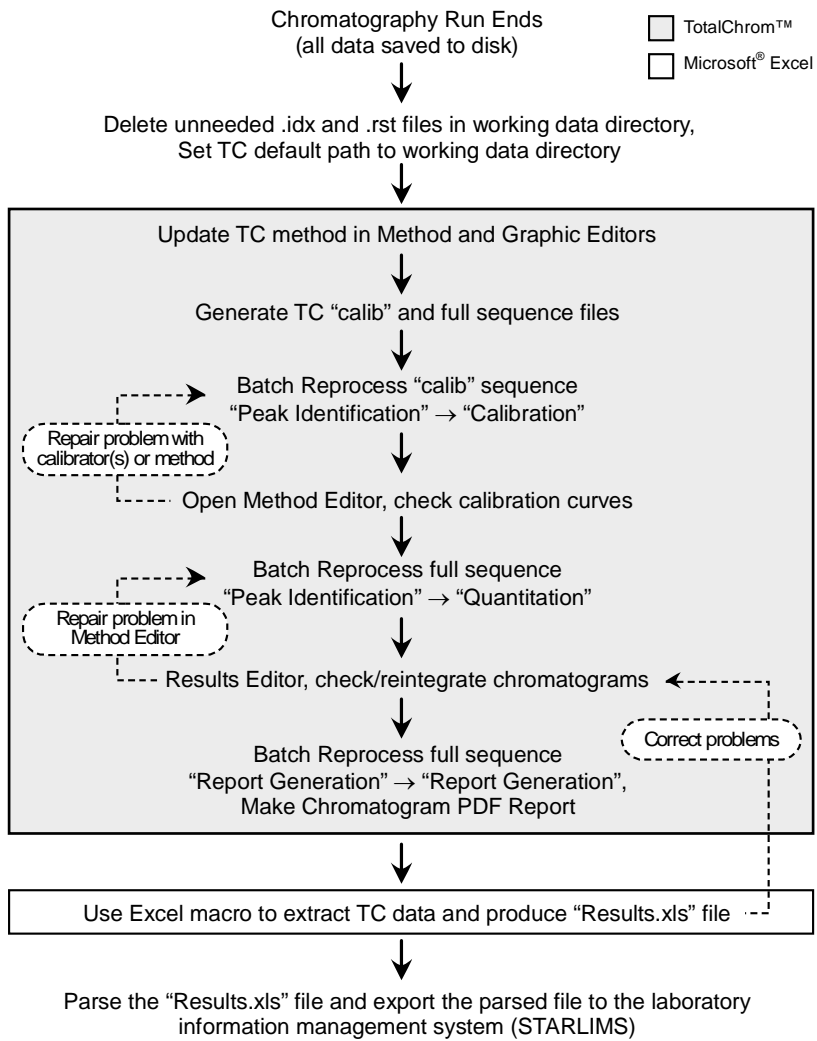


Figure 4: Post-Run Data Processing Work Flow Diagram

TABLE 8-8: TotalChrom™ Navigator - Reprocess Batch

Parameter Name	Parameter Setting
Starting Row	: 1
Ending Row	: <number of rows in sequence file>
Channel A	: Checked
Channel B	: Not Checked
Start Analysis	: Peak Detection
End Analysis	: Quantitation
Batch Execution	: "ndlab-168462" or equivalent
Batch Printer	: pdfFactoryPro
Batch Plotter	: None
Enable Optional Reports in Method	: Not Checked Grayed Out
Use Method is Result File	
Overwrite Existing Result Files	: Checked
Raw File Treatment	: Update existing raw file header with new sequence

12. In the TotalChrom™ Navigator window, choose the menu item "Reprocess" > "Results." A new window will open called "Reprocess Results". If you get an error message telling you that you can only open this in read-only mode, then unlock the files (follow the procedure described in step 4 of this section). Select from the menu "File" > "Open." In the open file dialog box, click on the "Files of type:" dropdown menu and select "IDX files (*.idx)". Navigate to the folder containing this run's data and click on the newest file (in the format of "Hg<yymmdd>-<today's date>-<time of reprocessing>"). Click "Open." In the Reprocess Results window will be presented a chromatogram for the first sample in the sequence. Carefully inspect the chromatogram one peak at a time for correct peak identification and accurate baseline. If you are satisfied that there are no integration problems, proceed to the next sample's chromatogram by selecting "File" > "Next File" from the menu bar or pressing "F3." Examine all chromatograms in this manner and make corrections in peak identity and integration as necessary. Make notes concerning issues encountered with individual chromatograms and changes that were made. If a chromatogram is changed or edited in any way, be sure to select "File" > "Save" to save your changes. See the chapter titled "Developing Processing Parameters in the Method" in the PerkinElmer TotalChrom™ Workstation Users Guide for a detailed explanation on how to use integration events to optimize the integration of a chromatogram. After review of each chromatogram, select "File" > "Exit" from the Reprocess Results menu bar.
13. Repeat step 9 except set both Start and End Analysis to "Report Generation". Set Batch Printer to "pdfFactory Pro". A new window will open. When reprocessing has completed, click the "Save" button on the pdfFactory Pro window. In the Save As dialog box that appears, navigate to the run's data folder and create a new pdf file named "Hg<yymmdd> report". Be sure to include a space between "report" and the first word of the new file. Click "Save." This pdf file is to be kept and backed up, for archival purposes, in the same folder with all the other chromatographic data files for this run. Click "Close" to close pdfFactory Pro window.

14. Open Microsoft Excel and choose "GCICPMS" > "Extract TC Data" from the menu. In the open file dialog box, navigate to the folder containing the run's data files. A box titled "Choose a Sequence File" will appear. Navigate to the folder that contains the sequence file that you are working with. Double click on the sequence file. Immediately a macro will run that will extract the data and put it into a format that is easily exported into the Laboratory Information Management System. Just before the macro finishes, a "Save As" dialog box will open giving you the opportunity to save the file as an Excel workbook. Give the file a name as follows: "Hg<yymmdd> results". The multi-tabbed Excel workbook can be parsed to obtain a worksheet that is suitable for data exportation into DLS STARLIMS.
15. Calculated "spike" concentrations for enriched InHg, MeHg, and EtHg in triple spike standards solution will be entered in the column "ppb Spike" in Microsoft Excel. On the Microsoft Excel toolbar, choose "GCICPMS" > "Deconvolute" from the menu. Sample concentration values will be calculated and appear in the column "ppb Result".
16. The data processing portion on the instrument controller computer is now complete. At this point you may close Microsoft Excel® and TotalChrom™ Navigator.
17. Throughout the sample analysis it is important to ensure that three mercury species peaks (InHg, MeHg, and EtHg) are well resolved. Thus, the following criterion is followed to accept/reject the sample analysis. The Microsoft Excel document containing concentration results displays retention times for each mercury species in the column labeled "RT". Mercury species elute from a chromatographic column in the following order MeHg, EtHg, InHg with relative retention times t_{r1} , t_{r2} , t_{r3} , respectfully. For the purpose of establishing relevant criterion, relative retention time for InHg (t_{r3}) is set to 1. The ratio $(t_{r2}/t_{r1}) = 0.91 \pm 0.13$ and $(t_{r3}/t_{r1}) = 0.77 \pm 0.12$ (\pm represents three times standard deviation). If the relative retention time ratios fall outside the allowable error, the sample analysis must be repeated.

IX. RECORDING OF SAMPLE AND QC DATA

A. Transferring the Data to the Laboratory Information Management System

1. Transfer the "Hg<yymmdd> results".xls file via recordable media (e.g., encrypted USB flash drive) to the appropriate subdirectory on the network drive where exported data are stored. (Note that directories are named according to instrument/year/month/ and study name or ID, for example, \\cdc\project\CCEHIP_NCEH_DLS_IRATB_COMMON\Nutritional\Instruments\NexION_R\2011/04/Hg110423 Results - Study 2006-09").
2. Import the instrument file into the laboratory information management system (LIMS) with appropriate documentation (e.g. instrument ID, analyst ID, column number and or other applicable run or sample specific comments).
3. Save all instrument data for that day on the shared network drive.

B. QC Data

Store the results of the QC samples analyzed in each run in the LIMS along with the patient sample data. The LIMS allows for the printing of QC reports using SAS.

X. FINAL DATA REVIEW

A. Run Data

Instead of printing the data onto paper, it should be stored as a PDF that can be reviewed. If the samples analyzed are part of a study that has an associated study folder, indicate the shared drive location of the electronically stored data.

B. Plotting QC Results

Importing data into DLS STARLIMS allows for the capture of quality control data that can be monitored through the generation of statistical quality control plots using SAS. The analyst must monitor these plots regularly for any trends in the bench QC results. If trends are observed, contact the laboratory supervisor, and investigate the cause of the trend(s). The QC results for a particular method must also be reviewed periodically by the team leader or lab chief to verify that trends in the data do not exist.

C. Supervisor Review

DLS STARLIMS allow a person with supervisory authority to review the QC and sample results in the system (electronically). After the supervisor reviews the data, he or she may release the data for reporting.

XI. LIMITS OF DETECTION

The limits of detection (LOD) for mercury species in blood specimens were calculated in accordance to the guidelines established by the Division of Laboratory Sciences. The method used to calculate the LODs is based on the recommendations of the Clinical and Laboratory Standards Institute (CLSI) and take both Type I and Type II errors into consideration. According to the guidelines established by our division, at least four concentration levels are to be analyzed in each run with one level having analyte concentrations below the projected LOD. For this method, the matrix-matched calibration blank was used to satisfy the criterion of having a level below the LOD while the first three calibrators of the standard calibration curve were used for the other three levels. All samples used for LOD calculations were human whole blood specimens. Multiple analysts were used for the measurements which covered over 60 runs over at least 60 days.

According to division protocol, for linear relationships the following equation is used to calculate the LOD: $conc_{LOD} = [\text{mean}_b + 1.645 \cdot (s_b + B)] / (1 - 1.645 \cdot A)$ where mean_b is the mean of the blank, s_b is the standard deviation of the blank, B is the y-intercept, and A is the slope. For quadratic relationships ($y = Ax^2 + Bx + C$), the following equation is used to calculate the LOD: $conc_{LOD} = [-b \pm \sqrt{b^2 - 4ac}] / 2a$ where $a = 1.645 \cdot A$, $b = (1.645 \cdot B) - 1$, and $c = \text{mean}_b + 1.645 \cdot (s_b + C)$. With both linear and quadratic relationships, the resulting calculated LOD has a 5% false negative rate. TABLE 11-1 lists the LODs calculated for this method.

As stated in the DLS Policies and Procedures Manual “uncertainty increases as values approach the limit of detection.” Division policy prohibits reporting results lower than the lowest calibration curve point. Report results below the detection limit as “< LOD” (where “LOD” is the calculated lowest detection limit). The LOD calculation is reevaluated whenever major changes to the method occur.

TABLE 11-1: Limits of Detection (LOD)*

Species Chemical Name	Abbreviated Name	Limit of Detection, µg/L
Inorganic Mercury	BHGI	0.21
Methylmercury	BHGM	0.26
Ethyl Mercury	BHGE	0.064

*The LOD's are current as of the date this version of the method was updated.

XII. REPORTABLE RANGE OF RESULTS

The analytical concentration range for each analyte in method 3020 is from the LOD to 30 µg/L. Any sample that has an analytical result above 10 µg/L (i.e. 2UB) but below 30 µg/L for any analyte must be repeated and confirmed for that analyte. In most cases, the total mercury concentration values produced by method DLS 3016 will be available prior to the analysis of blood samples by this method (DLS 3020). When the total mercury concentrations are available prior to sample analysis and each analyte is below 30 µg/L, the analyst can analyze the sample in duplicate in the same analytical run. In instances where total mercury results are either unavailable (they were not requested or the total mercury aliquot has not been analyzed yet) or should not be referenced (i.e. proficiency testing samples), the original sample must be re-digested, prepared, and analyzed to produce the confirmatory result.

Only results within the analytical concentration range may be reported without dilution of the sample. Samples having results greater than the upper limit of the analytical range must be diluted to bring the analyte within the analytical concentration range and reanalyzed using this method. The final result is the diluted sample result arithmetically corrected for the dilution. In instances where total mercury results are either unavailable (they were not requested or the total mercury aliquot has not been analyzed yet) or should not be referenced (i.e. proficiency testing samples), the original sample must be diluted, re-digested, prepared, and analyzed to produce the confirmatory result for the first dilution. Report the first analytically valid result, as long as the confirmation is within 10%. Continue repeat preparation and analysis until a concentration can be confirmed.

XIII. SPECIAL PROCEDURE NOTES - CDC MODIFICATIONS

None applicable for this operation.

XIV. QUALITY CONTROL PROCEDURES

The Inorganic and Radiation Analytical Toxicology Branch uses the method described in this protocol for environmental and occupational health screening studies. The analyst inserts bench QC specimens two times in each analytical run (a set of consecutive assays performed without interruption) so that judgments may be made on the day of analysis. Taking these samples through the complete analytical process assesses all levels of the analyte concentrations. The data from these materials are then used

to estimate methodological imprecision and to assess the magnitude of any time-associated trends. The bench QC pools used in this method comprise two levels of concentration spanning the "low-normal" and "high-normal" ranges for each mercury species. Both of these pools are analyzed before any patient samples are analyzed to ensure that all systems are functioning properly. These bench QCs must be analyzed again at the end of the run.

A. Establish QC limits for each QC pool.

A run to assess the homogeneity of the pools is performed after the pools are aliquotted into individual vials. Vials are randomly chosen and randomly analyzed, and the first and last vials dispensed are always included in the homogeneity study. Unlike the characterization of the QC, the homogeneity study can be completed in a single run. Once analysis is complete, the data is evaluated in terms of QC recovery to determine whether or not trends exist in QC during the dispensing of the pool. If the pool does not vary from beginning to end or problem vials can be identified and eliminated, the characterization of the QC is the next step. If problems do exist, the source(s) of the problem has to be identified and the pool has to be re-made and dispensed again.

To complete the characterization that will allow you to assess limits for each pool, analyze a minimum of twenty samples of each pool (low and high) on 20 different days, preferably among all of the instruments that will be used to analyze this method. During the 20 characterization runs, analyze samples from previously characterized QCs or pools with target values assigned by outside laboratories to evaluate each run's QC. Once analysis is complete, calculate the mean and standard deviation for each pool from the concentration results. These values will be used to establish the limits for each pool.

B. Precision and Accuracy

QC Results Evaluation. After completing a run, consult the QC limits to determine whether the run is "in control." The QC rules apply to the average of the beginning and ending analyses of each of the bench QC pools. The QC rules are as follows:

1. If both QC run means are within the $2S_m$ limits and individual results are within $2S_i$ limits, then accept the run.
2. If one of the two QC run means is outside a $2S_m$ limit – reject the run if:
 - **Extreme Outlier** – run mean is beyond characterization mean $\pm 4S_m$
 - **3S Rule** – run mean is outside a $3S_m$ limit
 - **2S Rule** – both run means are outside the same $2S_m$ limit
 - **10 X-bar Rule** – current and previous 9 run means are on same side of the characterization mean
3. If one of the four individual results is outside a $2S_i$ limit – reject run if:
 - **Extreme Outlier** – one individual result is beyond the characterization mean $\pm 4S_i$
 - **R 4S Rule** – within-run ranges for all pools in the same run exceed $4S_w$ (i.e., 95% range limit).
Note: Since runs have multiple results per pool for 2 pools, the R 4S rule is applied within runs only.

If the run is declared "out of control," the analysis results for all patient samples analyzed during that run are invalid for reporting.

C. Remedial Action If Calibration or QC Systems Fail to Meet Acceptable Criteria

If an analyte result for a QC material falls outside of the limits for mean or range, the following steps must be taken, if possible:

- Check the chromatograms for each blank, QC, and sample for proper peak integration and identification. Change integration parameters or manually reintegrate peaks, if necessary, and reprocess the run in TotalChom™.
- Setup a new run for the reanalysis of the patient samples affected by the previous failed run. Be sure to use freshly prepared QC material.

If these steps do not result in correction of the out-of-control values for QC materials, consult the supervisor for other appropriate corrective actions. No analytical results will be reported for runs that are not in statistical control.

XV. REFERENCE RANGES

The reference range for each mercury species (see TABLE 15-1) is based on the most recent data publicly available from the measure of the species in NHANES. Where data is absent or scant, references ranges are based on the scientific literature, if available.

TABLE 15-1: Reference ranges for Mercury Species

Species Chemical Name	Reference Range¹, µg/L
Inorganic Hg	0.410 (90 th percentile), 0.530 (95 th percentile)
Methyl Hg	0.420 (50 th percentile), 1.09 (75 th percentile), 2.62 (90 th percentile), 4.28 (95 th percentile), 0.434 (geometric mean)
Ethyl Hg ²	

¹ Above ranges are taken from NHANES population data for the 2013 to 2014 survey years as provided in the *Fourth National Report on Human Exposure to Environmental Chemicals, Updated Tables, January 2017 (Volume One)*. Sample sizes were n=5175 for inorganic mercury and methylmercury. (<http://www.cdc.gov/exposurereport/>).

² There are no established reference ranges for EtHg.

XVI. ACTION-LEVEL RESULTS

The analyst will report any patient results confirmed to be greater than the second upper boundary (defined in the laboratory information management system as the "2UB" and currently 10 µg/L) to the QC reviewer as an "elevated result". The protocol for supervisors reporting elevated results to medical personnel is defined according to the study protocol. Levels of concern for mercury in blood are >100 µg/L for children (6 years and younger) and >200 µg/L for adults. These values are based on total Hg results.

XVII. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens may reach and maintain ambient temperature during analysis. Take stringent precautions to avoid external contamination. After the samples are analyzed, return them to approximately ≤ -20°C freezer storage as soon as possible.

XVIII. ALTERNATE METHODS FOR PERFORMING TEST AND STORING SPECIMENS IF TEST SYSTEM FAILS

If the analytical system fails, freezer storage at a minimum of approximately $\leq -20^{\circ}\text{C}$ is recommended until functionality is restored.

XIX. TEST-RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

For critical calls, the supervisor must notify the supervising physician or principal investigator as soon as possible. The most expeditious means must be used (e.g., telephone or E-mail).

XX. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

The person who receives specimens or samples delivered to the Inorganic and Radiation Analytical Toxicology Branch sets up a "Specimen Folder." Fill out a tracking form and place it in the folder to be given to the analyst performing the analysis. The form tracks location, status, and final disposition of the specimens.

Use standard electronic record keeping means (e.g., compact disk backup and storage to shared drive) to track specimens. Maintain records, including related quality assurance (QA) and QC data, for two years or longer. Keep duplicate records (off site, if sensitive or critical) in electronic or hardcopy format. Use only numerical identifiers (e.g., case ID numbers); all personal identifiers are available only to the medical supervisor or project coordinator to safeguard confidentiality.

XXI. METHOD PERFORMANCE DOCUMENTATION

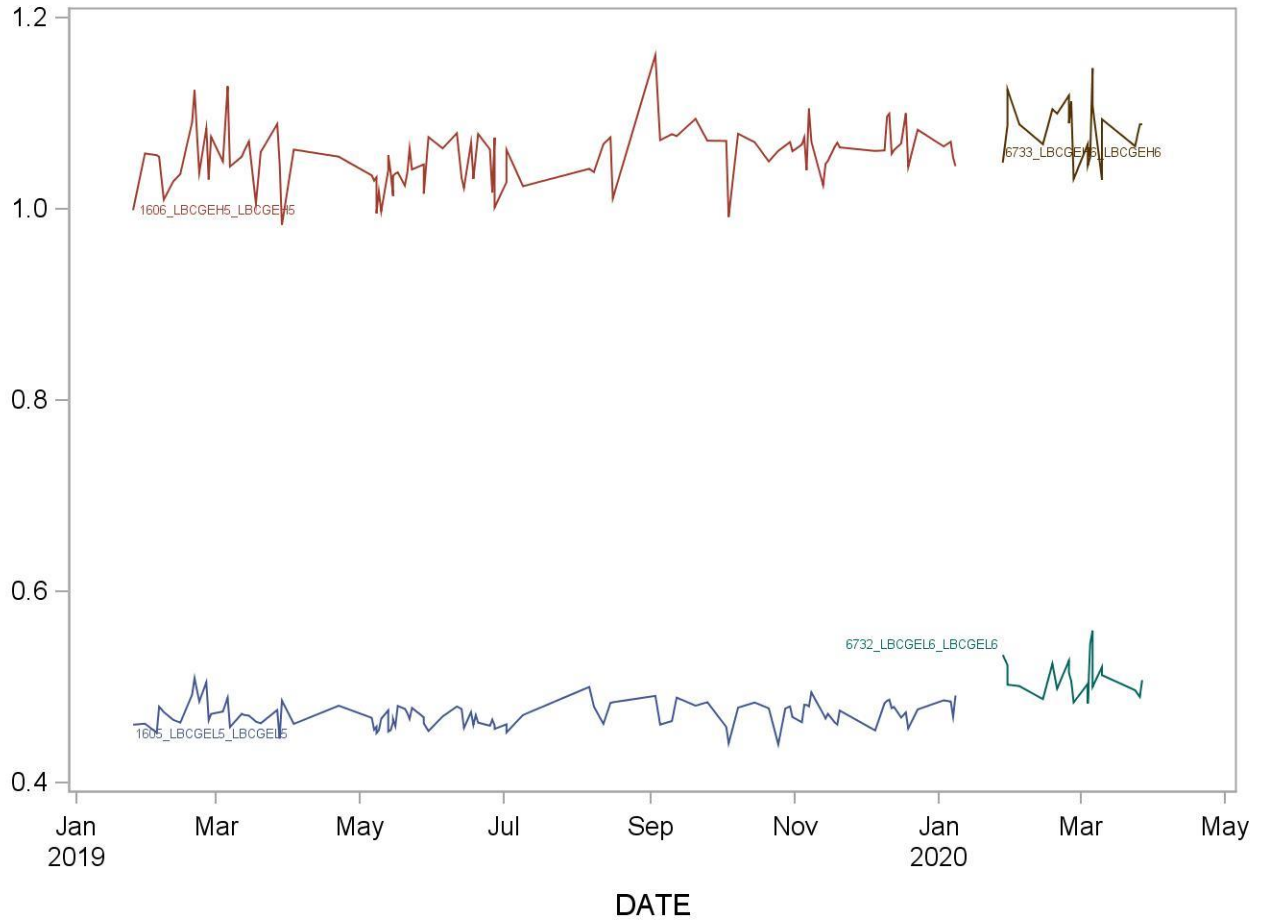
Method performance documentation for this method including accuracy, precision, sensitivity, specificity and stability is provided in Appendix A of this method documentation. The signatures of the Branch Chief and Director of the Division of Laboratory Sciences on the first page of this procedure denote that the method performance is fit for the intended use of the method.

XXII. SUMMARY STATISTICS AND QC GRAPHS

Please see following pages.

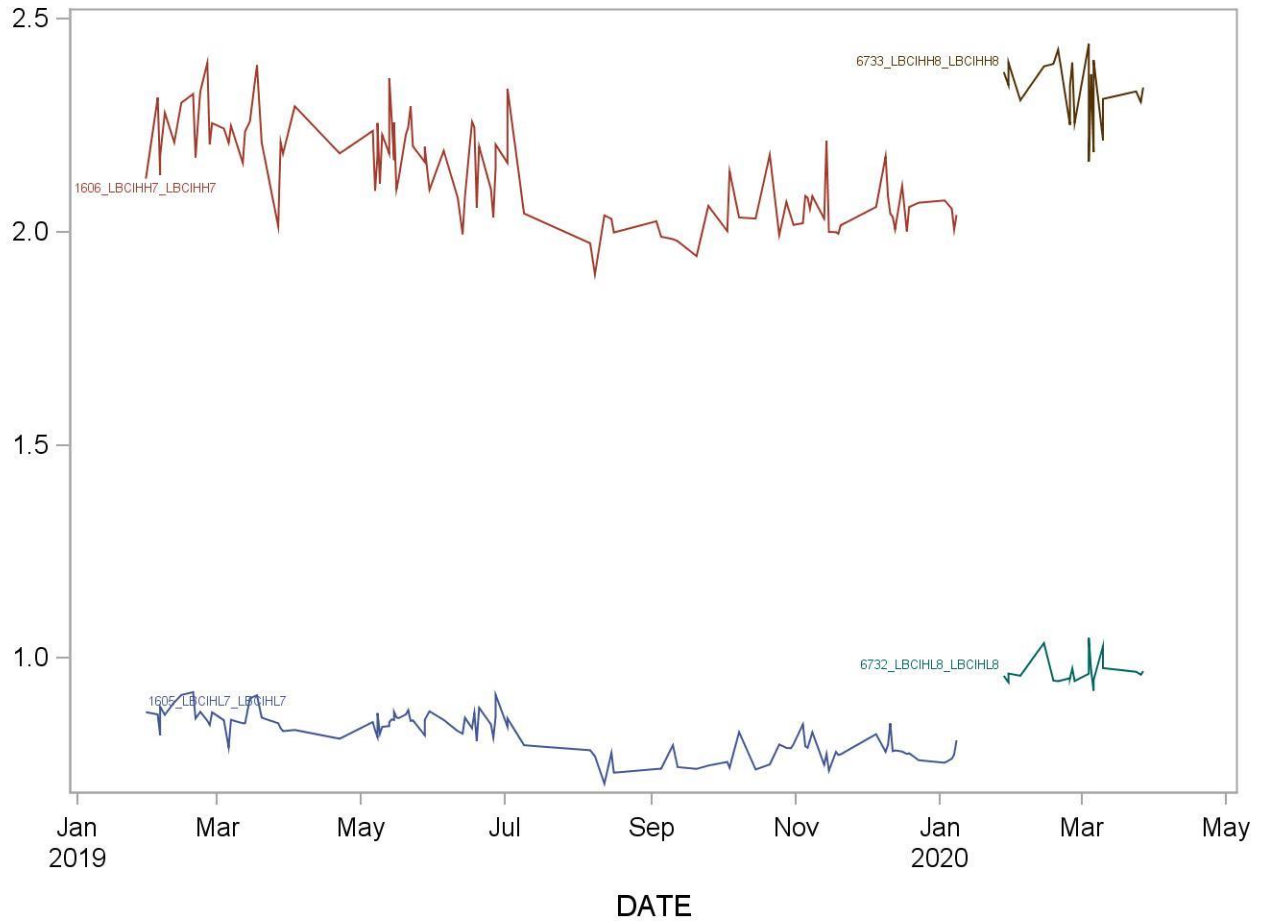
**2019-2020 Summary Statistics and QC Chart
LBXBGE (Mercury, ethyl (ug/L))**

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
1606_LBCGEH5_LBCGEH5	106	25JAN19	08JAN20	1.0538	0.0294	2.8
1605_LBCGEL5_LBCGEL5	106	25JAN19	08JAN20	0.4710	0.0130	2.8
6733_LBCGEH6_LBCGEH6	21	28JAN20	27MAR20	1.0838	0.0310	2.9
6732_LBCGEL6_LBCGEL6	21	28JAN20	27MAR20	0.5105	0.0200	3.9



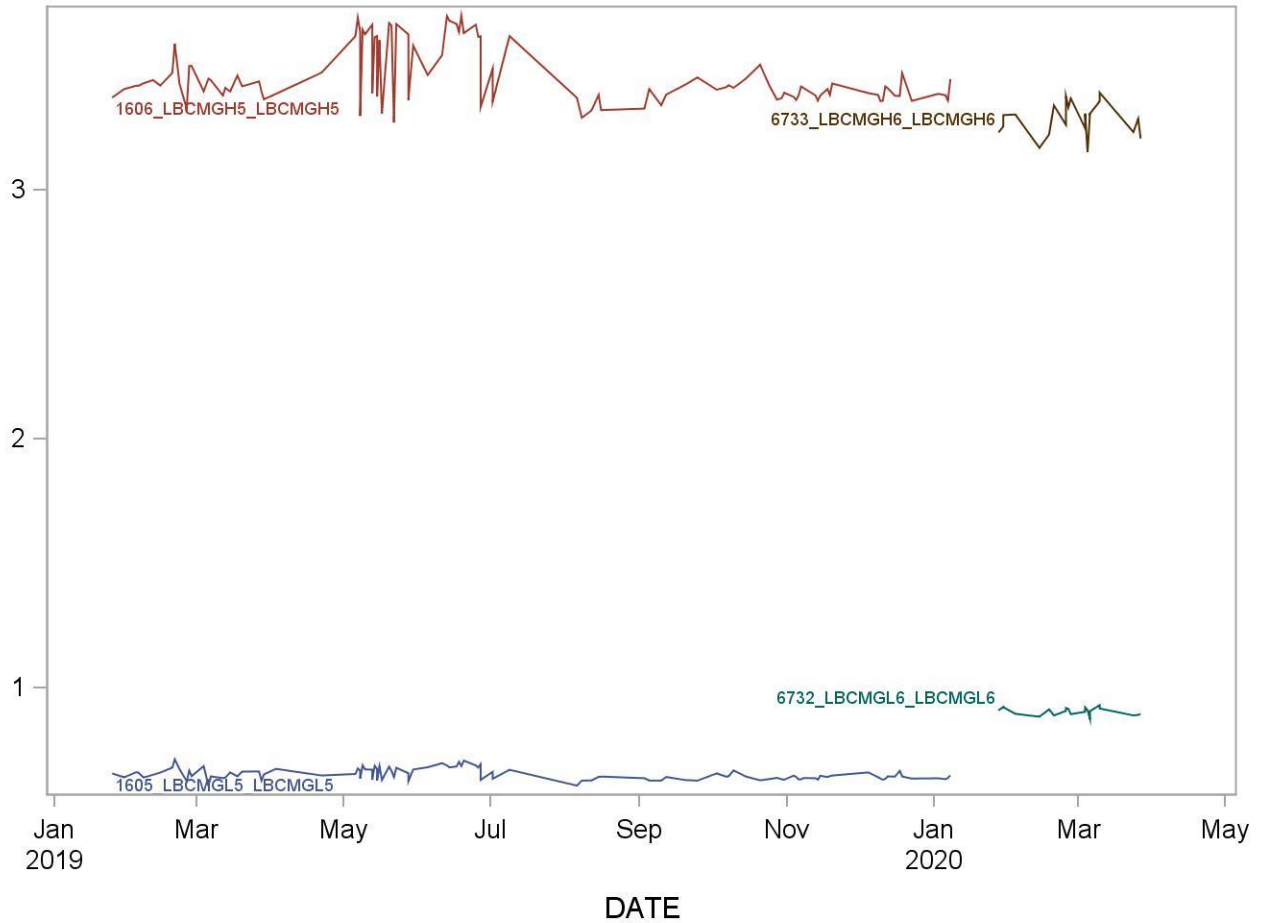
**2019-2020 Summary Statistics and QC Chart
LBXIHG (Mercury, inorganic (ug/L))**

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
1606_LBCIHH7_LBCIHH7	106	30JAN19	08JAN20	2.132	0.111	5.2
1605_LBCIHL7_LBCIHL7	106	30JAN19	08JAN20	0.821	0.048	5.9
6733_LBCIHH8_LBCIHH8	21	28JAN20	2.330	0.078	3.4	
6732_LBCIHL8_LBCIHL8	21	28JAN20	27MAR20	0.968	0.031	3.2



2019-2020 Summary Statistics and QC Chart LBXBGM (Mercury, methyl (ug/L))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
1606_LBCM5GH5_LBCM5GH5	107	25JAN19	08JAN20	3.453	0.114	3.3
1605_LBCM5GL5_LBCM5GL5	107	25JAN19	08JAN20	0.653	0.022	3.4
6733_LBCM6GH6_LBCM6GH6	21	28JAN20	27MAR20	3.282	0.066	2.0
6732_LBCM6GL6_LBCM6GL6	21	28JAN20	27MAR20	0.905	0.015	1.7



XXIII. REFERENCES

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7. Tanner S, Baranov VI, Vollkopf U. A dynamic reaction cell for inductively coupled plasma mass spectroscopy (ICP-DRC-MS). Part III. Optimization and analytical performance. *J. Anal. At. Spectrom.* 2000;15:1261-1269.

XXIV.APPENDIX

A. Appendix A. Method Performance Documentation

Accuracy using Spike Recovery

Recovery = (final concentration – initial concentration)/added concentration

Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name: Mercury Speciation in blood

Method #: 3020

Matrix: Blood

Units: µg/L

Analyte: Inorganic Mercury (IHG)

Replicate	Sample 1					Sample 2					Mean recovery (%)	SD (%)
	Spike concentration	Measured concentration			Recovery (%)	Spike concentration	Measured concentration			Recovery (%)		
		Day 1	Day 2	Mean			Day 1	Day 2	Mean			
Sample 1	0.0	0.340	0.110	0.1		0.0	0.950	0.890	0.9		97.2	3.4
2		0.110	0.110			0.870	0.880					
3		0.092	0.100			0.910	0.870					
Sample + Spike 1	5.0	5.47	5.41	5.3	103.1	5.0	5.75	5.38	5.7	95.9		
2		5.16	5.17			5.90	5.73					
3		5.30	5.29			5.37	6.01					
Sample + Spike 2	10.0	10.1	10.7	10.1	99.2	10.0	10.6	10.0	10.3	94.2		
2		9.55	10.2			11.5	10.3					
3		9.56	10.4			10.4	9.07					
Sample + Spike 3	15.0	15.2	12.6	14	94.5	15.0	15.5	15.4	15	96.2		
2		15.5	13.5			14.7	15.4					
3		15.7	13.5			15.4	15.7					

Accuracy using Spike Recovery

Recovery = (final concentration – initial concentration)/added concentration

Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name: Mercury Speciation in blood

Method #: 3020

Matrix: Blood

Units: µg/L

Analyte: Methylmercury (BHGM)

Replicate	Sample 1				Sample 2				Mean recovery (%)	SD (%)	
	Spike concentration	Measured concentration		Recovery (%)	Spike concentration	Measured concentration		Recovery (%)			
		BB	Mean			LB	Mean				
Sample	1	0.0	0.120	0.117	0.1	0.0	0.660	0.616	0.7	89.9	2.0
	2		0.082	0.192			0.670	0.725			
	3		0.130	0.220			0.670	0.679			
Sample + Spike 1	1	3.97	3.75	3.96	3.9	3.97	4.19	4.33	4.2	90.1	
	2		3.76	3.96			4.46	4.34			
	3		3.87	3.87			3.89	4.28			
Sample + Spike 2	1	7.93	7.05	7.27	7.2	7.93	7.86	8.42	7.8	90.0	
	2		7.17	7.78			7.62	7.61			
	3		6.96	7.01			7.56	7.80			
Sample + Spike 3	1	11.9	10.7	10.4	11	11.9	11.4	11.1	11	88.3	
	2		10.8	10.6			11.4	11.2			
	3		10.9	10.6			11.1	11.0			

Accuracy using Spike Recovery

Recovery = (final concentration – initial concentration)/added concentration

Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name: Mercury Speciation in blood

Method #: 3020

Matrix: Blood

Units: µg/L

Analyte: Ethyl Mercury (BHGE)

Replicate	Sample 1					Sample 2					Mean recovery (%)	SD (%)
	Spike concentration	Measured concentration			Recovery (%)	Spike concentration	Measured concentration			Recovery (%)		
		Day 1	Day 2	Mean			Day 1	Day 2	Mean			
Sample 1	0.0	0.00	0.00	0.0		0.0	0.51	0.47	0.5		91.2	4.4
Sample + Spike 1	4.34	4.08	4.34	4.2	95.9	4.34	4.57	4.67	4.7	96.6		
Sample + Spike 2	8.69	7.68	8.06	7.8	90.1	8.69	8.63	8.19	8.4	91.4		
Sample + Spike 3	13.03	10.6	11.5	11	85.7	13.03	12.0	11.7	12	87.4		

Precision

Total relative standard deviation should be $\leq 15\%$ (CV $\leq 15\%$)

Method name: Mercury Speciation in blood
Method #: 3020
Matrix: Blood
Units: $\mu\text{g/L}$
Analyte: Inorganic Mercury (IHG)

Quality material 1

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean ²
1	0.824	0.770	0.80	0.000729	0.000729	1.270418
2	0.759	0.767	0.76	1.64025E-05	1.64025E-05	1.164185405
3	0.759	0.769	0.76	2.65225E-05	2.65225E-05	1.167544805
4	0.829	0.786	0.81	0.000455822	0.000455822	1.303628045
5	0.850	0.884	0.87	0.000283923	0.000283923	1.503551405
6	0.755	0.752	0.75	2.56E-06	2.56E-06	1.13431922
7	0.864	0.982	0.92	0.003522423	0.003522423	1.704042605
8	0.796	0.808	0.80	3.364E-05	3.364E-05	1.28544578
9	0.808	0.793	0.80	5.70025E-05	5.70025E-05	1.282721445
10	0.945	1.00	0.97	0.00072361	0.00072361	1.89073458

Grand sum 16.4988 Grand mean 0.82494

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.01170181	0.001170181	0.034207908	4.15
Between Run	0.096071218	0.01067458	0.068936198	8.36
Total	0.107773028		0.076957004	9.33

Quality material 2

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean ²
1	2.06	1.97	2.01	0.00196249	0.00196249	8.08904642
2	2.00	1.97	1.98	0.000297563	0.000297563	7.862595125
3	1.96	2.11	2.04	0.00555025	0.00555025	8.29629378
4	2.06	1.95	2.00	0.002704	0.002704	8.03123042
5	2.17	2.31	2.24	0.004907003	0.004907003	10.06164941
6	2.15	1.99	2.07	0.006715802	0.006715802	8.580153125
7	2.40	2.45	2.42	0.00061504	0.00061504	11.75252162
8	1.95	2.03	1.99	0.001432623	0.001432623	7.925374845
9	2.24	2.09	2.16	0.005076563	0.005076563	9.374017005
10	2.36	2.54	2.45	0.007788062	0.007788062	11.98002301

Grand sum 42.7516 Grand mean 2.13758

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	0.07409879	0.007409879	0.086080654	4.03
Between Run	0.567939622	0.063104402	0.166874988	7.81
Total	0.642038412		0.187768849	8.78

Precision

Total relative standard deviation should be $\leq 15\%$ ($CV \leq 15\%$)

Method name: Mercury Speciation in blood
Method #: 3020
Matrix: Blood
Units: $\mu\text{g/L}$
Analyte: Methylmercury (BHGM)

Quality material 1

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean ²
1	0.656	0.667	0.66	2.97025E-05	2.97025E-05	0.875826125
2	0.688	0.685	0.69	3.4225E-06	3.4225E-06	0.942701805
3	0.689	0.705	0.70	6.4E-05	6.4E-05	0.9702245
4	0.690	0.663	0.68	0.00018769	0.00018769	0.91476338
5	0.701	0.664	0.68	0.000344103	0.000344102	0.932568245
6	0.706	0.687	0.70	9.025E-05	9.025E-05	0.97106048
7	0.698	0.643	0.67	0.000764522	0.000764523	0.898202045
8	0.658	0.686	0.67	0.000196	0.000196	0.90370568
9	0.654	0.683	0.67	0.000205922	0.000205923	0.894185645
10	0.652	0.643	0.65	2.07025E-05	2.07025E-05	0.838901045
Grand sum	13.5188	Grand mean	0.67594			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.00381263	0.000381263	0.019525957	2.89
Between Run	0.004241278	0.000471253	0.006707835	0.99
Total	0.008053908		0.020646018	3.05

Quality material 2

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean ²
1	3.70	3.61	3.66	0.001993622	0.001993623	26.75680705
2	3.44	3.55	3.49	0.00303601	0.00303601	24.3951125
3	3.63	3.43	3.53	0.00978121	0.00978121	24.91332872
4	3.54	3.48	3.51	0.001053002	0.001053003	24.65915765
5	3.40	3.50	3.45	0.002495003	0.002495002	23.86230445
6	3.56	3.58	3.57	0.00012321	0.00012321	25.44555122
7	3.57	3.46	3.52	0.002986623	0.002986623	24.75475885
8	3.62	3.47	3.54	0.00514089	0.00514089	25.11136712
9	3.61	3.70	3.65	0.00232324	0.00232324	26.69758592
10	3.22	3.73	3.48	0.06579225	0.06579225	24.15959072
Grand sum	70.8054	Grand mean	3.54027			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	0.18945012	0.018945012	0.137640881	3.89
Between Run	0.085330722	0.009481191	0	0.00
Total	0.274780842		0.137640881	3.89

Precision

Total relative standard deviation should be $\leq 15\%$ (CV $\leq 15\%$)

Method name: Mercury Speciation in blood
Method #: 3020
Matrix: Blood
Units: $\mu\text{g/L}$
Analyte: Ethyl Mercury (BHGE)

Quality material 1

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	0.44	0.46	0.45	5.476E-05	5.476E-05	0.40446018
2	0.44	0.46	0.45	8.19025E-05	8.19025E-05	0.401497605
3	0.45	0.46	0.45	1.6E-05	0.000016	0.41023682
4	0.43	0.45	0.44	7.921E-05	7.921E-05	0.3863205
5	0.52	0.48	0.50	0.000438903	0.000438903	0.505716245
6	0.43	0.46	0.45	0.00014161	0.00014161	0.39711872
7	0.49	0.47	0.48	0.00016384	0.00016384	0.4617605
8	0.49	0.44	0.46	0.000683823	0.000683822	0.428645405
9	0.52	0.47	0.50	0.00049729	0.00049729	0.49282592
10	0.46	0.44	0.45	0.000109202	0.000109203	0.401856125
Grand sum	9.2534	Grand mean	0.46267			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.00453308	0.000453308	0.021291031	4.60
Between Run	0.009167442	0.001018605	0.016812148	3.63
Total	0.013700522		0.027128515	5.86

Quality material 2

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	0.95	1.0	0.99	0.001751423	0.001751423	1.979055125
2	0.99	1.0	1.01	0.00028224	0.00028224	2.02930658
3	1.0	0.94	0.99	0.00239121	0.00239121	1.9463645
4	1.0	1.0	1.03	3.136E-05	3.136E-05	2.10699392
5	1.0	1.1	1.05	0.000531303	0.000531302	2.189278125
6	1.1	1.1	1.06	1.21E-06	1.21E-06	2.24423298
7	1.0	1.0	1.01	5.0625E-06	5.0625E-06	2.034749645
8	1.0	0.96	0.98	0.00046225	0.00046225	1.92001608
9	1.1	1.0	1.06	0.00015625	0.00015625	2.23534368
10	0.98	1.1	1.04	0.00285156	0.00285156	2.14700642
Grand sum	20.4045	Grand mean	1.020225			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	0.016927735	0.001692774	0.041143329	4.03
Between Run	0.015166042	0.001685116	0	0.00
Total	0.032093777		0.041143329	4.03

Stability

The initial measurement can be from the same day for all stability experiments.

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions

Describe condition: samples frozen three times at -80°C and then thawed (3 freeze-thaw cycles)

Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)

Describe condition: original samples (not yet digested or prepared for instrument analysis) stored at room temperature for 1 day before digestion

Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler

Describe condition: processed samples (already digested) stored at room temperature for 1 day before derivatization and instrument analysis

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis

Describe condition: samples stored at -80°C for 5 y ears

All stability sample results should be within ±15% of nominal concentration

Method name: Mercury Speciation in blood

Method #: 3020

Matrix: Blood

Units: µg/L

Analyte: Inorganic Mercury (IHG)

Quality material 1								
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	0.790	0.791	0.790	0.761	0.790	0.803	0.750	0.803
Replicate 2	0.791	0.781	0.791	0.800	0.791	0.788	0.775	0.788
Replicate 3	0.814	0.825	0.814	0.802	0.814	0.827	0.817	0.827
Mean	0.798045333	0.799107333	0.798045333	0.8	0.798045333	0.805898667	0.7808	0.8
% difference from initial measurement	--	0.1	--	-1.3	--	1.0	--	3.2

Quality material 2								
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	2.06	1.92	2.06	1.96	2.06	1.92	1.78	1.92
Replicate 2	1.98	1.92	1.98	1.95	1.98	2.03	2.26	2.03
Replicate 3	2.03	2.06	2.03	2.05	2.03	1.98	2.43	1.98
Mean	2.020284667	2.0	2.020284667	2.0	2.020284667	1.973469667	2.157141441	2.0
% difference from initial measurement	--	-2.6	--	-1.7	--	-2.3	--	-8.5

Stability

The initial measurement can be from the same day for all stability experiments.

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions

Describe condition: samples frozen three times at -80°C and then thawed (3 freeze-thaw cycles)

Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)

Describe condition: original samples (not yet digested or prepared for instrument analysis) stored at room temperature for 1 day before digestion

Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler

Describe condition: processed samples (already digested) stored at room temperature for 1 day before derivatization and instrument analysis

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis

Describe condition: samples stored at -80°C for 5 years

All stability sample results should be within ±15% of nominal concentration

Method name: Mercury Speciation in blood

Method #: 3020

Matrix: Blood

Units: µg/L

Analyte: Methylmercury (BHGM)

Quality material 1								
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	0.697	0.689	0.697	0.679	0.697	0.688	0.755	0.688
Replicate 2	0.667	0.701	0.667	0.678	0.667	0.697	0.741	0.697
Replicate 3	0.678	0.670	0.678	0.675	0.678	0.705	0.737	0.705
Mean	0.680514333	0.686867667	0.680514333	0.7	0.680514333	0.696444667	0.744533333	0.7
% difference from initial measurement	--	0.9	--	-0.5	--	2.3	--	-6.5

Quality material 2								
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	3.62	3.60	3.62	3.59	3.62	3.65	3.54	3.65
Replicate 2	3.65	3.50	3.65	3.59	3.65	3.62	3.70	3.62
Replicate 3	3.63	3.70	3.63	3.66	3.63	3.57	3.90	3.57
Mean	3.633373667	3.6	3.633373667	3.6	3.633373667	3.610190667	3.712652379	3.6
% difference from initial measurement	--	-0.9	--	-0.5	--	-0.6	--	-2.8

Stability

The initial measurement can be from the same day for all stability experiments.

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions

Describe condition: samples frozen three times at -80°C and then thawed (3 freeze-thaw cycles)

Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)

Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 1 day before digestion

Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler

Describe condition: processed samples (already digested) stored at room temperature for 1 day before derivatization and instrument analysis

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis

Describe condition: samples stored at -80°C for 5 years

All stability sample results should be within ±15% of nominal concentration

Method name: Mercury Speciation in blood

Method #: 3020

Matrix: Blood

Units: µg/L

Analyte: Ethyl Mercury (BHGE)

Quality material 1								
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	0.458	0.464	0.458	0.454	0.458	0.486	0.608	0.486
Replicate 2	0.460	0.500	0.460	0.461	0.460	0.484	0.515	0.484
Replicate 3	0.459	0.473	0.459	0.490	0.459	0.471	0.559	0.471
Mean	0.458870667	0.479011333	0.458870667	0.5	0.458870667	0.480262	0.560533333	0.5
% difference from initial measurement	--	4.4	--	2.1	--	4.7	--	-14.3

Quality material 2								
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	1.05	1.10	1.05	1.08	1.05	1.05	1.33	1.05
Replicate 2	1.06	1.09	1.06	1.10	1.06	1.08	1.00	1.08
Replicate 3	1.03	1.11	1.03	1.07	1.03	1.07	1.30	1.07
Mean	1.045439	1.1	1.045439	1.1	1.045439	1.066627333	1.211155043	1.1
% difference from initial measurement	--	5.1	--	3.5	--	2.0	--	-11.9

LOD, specificity and fit for intended use

Method name: Mercury Speciation in blood
Method #: 3020
Matrix: Blood
Units: µg/L

Analytes	Limit of Detection (LOD)	Interferences successfully checked in at least 50 human samples	Accuracy, precision, LOD, specificity and stability meet performance specifications for intended use
Inorganic Mercury	0.21	yes	yes
Methylmercury	0.26	yes	yes
Ethyl Mercury	0.064	yes	yes

B. Appendix B: Critical Parameters Testing Results

1. Critical Parameter Test #1

Evaluate the significance of the GC injector temperature

1. Test Details (to be repeated for each temperature):
 1. Set up the analysis in accordance with the prescribed method.
 2. Set the injector temperature to 200°C (reduced temperature).
 3. Digest blood QC according to the prescribed method.
 4. Analyze all digested samples together in one run.
 5. Repeat the analysis using a column oven temperature of 220°C (the method prescribed temperature).
 6. Repeat the analysis using a column oven temperature of 230°C (increased temperature).

Critical Parameter Test 1 Results. Temperature of the GC injector. One quality control pool was tested. Tests performed 8/5/10 by Mark Fresquez. Results below are the averages of observed results at each condition along with the 95 th percent confidence intervals (in parentheses).				
QC Pool ID	GC injector Temperature (°C)	InHg (µg/L)	MeHg (µg/L)	EtHg (µg/L)
QC Pool LB07292	200°C (Reduced)	1.27 (1.17, 1.38)	1.04 (0.99, 1.09)	1.22 (1.14, 1.30)
	220°C (Per Method)	1.28 (1.00, 1.55)	1.08 (1.00, 1.16)	1.32 (1.27, 1.36)
	230°C (Increased)	1.39 (1.27, 1.51)	1.09 (1.02, 1.17)	1.20 (1.06, 1.35)
Expected Range (± 2 SD)*		(0.71-1.76)	(.71-1.37)	(0.84-1.64)

*Expected range is a determined range calculated from the mean ± 2 SD of a 22-run characterization.

Conclusion: All of the analytes fell within 2SD of the mean at all temperatures; however, it was confirmed during method development that the GC injector temperature of 220 °C was the optimal choice.

2. Critical Parameter Test #2

Evaluate the significance of the CombiPAL®-agitator equilibration/extraction temperature

1. Test Details (to be repeated for each temperature):
 1. Set up the analysis in accordance with the prescribed method.
 2. Set the agitator equilibration/extraction temperature to 40°C (elevated temperature).
 3. Digest blood QC according to the prescribed method.
 4. Analyze all digested samples together in one run.
 5. Repeat the analysis using an equilibration/extraction temperature at room temperatures ~ 25°C (the method prescribed temperature).
 6. Repeat the analysis using an equilibration/extraction temperature of 50°C (increased temperature).

Critical Parameter Test 2 Results. Temperature of the CombiPAL® – agitator equilibration/extraction. One quality control pool was tested. Tests performed 8/17/10 by Mark Fresquez. Results below are the averages of observed results at each condition along with the 95 th percent confidence intervals (in parentheses).				
QC Pool ID	Equilibration/Extraction Temperature (°C)	InHg (µg/L)	MeHg (µg/L)	EtHg (µg/L)
QC Pool LB07292	~ 25°C (Per Method)	1.42 (1.20, 1.65)	1.05 (1.03, 1.07)	1.17 (1.07, 1.27)
	40°C (Increased)	1.33 (1.27, 1.39)	1.05 (1.01, 1.09)	1.17 (1.11, 1.23)
	50°C (Increased)	1.25 (1.08, 1.42)	1.07 (1.05, 1.10)	1.19 (1.06, 1.32)
Expected Range (± 2 SD)*		(0.71-1.76)	(.71-1.37)	(0.84-1.64)

Conclusion: All of the analytes fell within 2SD of the mean at all temperatures; however, it was confirmed during method development that an agitator equilibration/extraction temperature of 220 °C was the optimal choice.

3. Critical Parameter Test #3

Evaluate the significance of the length of extraction of sample from SPME fiber in GC injector

1. Test Details:

1. Set up the analysis in accordance with the prescribed method.
2. Set the extraction time to 10 minutes (reduced time).
3. Digest blood QC according to the prescribed method.
4. Analyze all digested samples together in one run.
5. Repeat the analysis using an extraction time of 15 minutes (reduced time).
6. Repeat the analysis using an extraction time of 20 minutes (the method prescribed time).

Critical Parameter Test 3. Length of extraction time. Test performed 8/18/10 by Mark Fresquez. Results below are the averages of observed results at each condition along with the 95 th percent confidence intervals (in parentheses).				
QC Pool ID	Sample extraction time (minutes)	InHg (µg/L)	MeHg (µg/L)	EtHg (µg/L)
QC Pool LB07292	10 min. (Reduced)	1.69 (1.58, 1.80)	1.02 (0.97, 1.08)	1.16 (1.09, 1.22)
	15 min. (Reduced)	1.34 (1.32, 1.38)	1.04 (1.02, 1.05)	1.20 (1.18, 1.22)
	20 min. (Per Method)	1.33 (1.32, 1.37)	1.05 (1.05, 1.09)	1.17 (1.14, 1.18)
Expected Range (± 2 SD)*		(0.71-1.76)	(.71-1.37)	(0.84-1.64)

Conclusion: All of the analytes fell within 2SD of the mean at all test values; however, it was confirmed during method development that 20 minutes was the optimal extraction time.

4. Critical Parameter Test #4

Evaluate the significance of changing GC split ratio.

1. Test Details:

1. Set up the analysis in accordance with the prescribed method.
2. Set the split ratio to 20:1 (reduced ratio).
3. Digest blood QC according to the prescribed method.
4. Analyze all digested samples together in one run.
5. Repeat the analysis using split ratio of 28:1 (the method prescribed).
6. Repeat the analysis using split ratio of 35:1 (increased ratio).

Critical Parameter Test 4. GC split ratio. Test performed 8/24/10 by Mark Fresquez. Results below are the averages of observed results at each condition along with the 95 th percent confidence intervals (in parentheses).				
QC Pool ID	GC split ratio	InHg (µg/L)	MeHg (µg/L)	EtHg (µg/L)
QC Pool LB07292	20:1 (Reduced)	1.31 (1.26, 1.36)	1.06 (1.02, 1.10)	1.17 (1.14, 1.19)
	28:1 (Per Method)	1.34 (1.29, 1.39)	1.04 (1.03, 1.05)	1.15 (1.13, 1.19)
	35:1 (Increased)	1.34 (1.27, 1.41)	1.06 (1.04, 1.08)	1.17 (1.15, 1.20)
Expected Range (± 2 SD)*		(0.71-1.76)	(.71-1.37)	(0.84-1.64)

Conclusion: All of the analytes fell within 2SD of the mean at all split ratios; however, it was confirmed during method development that GC split ratio of 28:1 was the optimal choice.

5. Critical Parameter Test #5

Evaluate the significance of changing GC carrier gas flow rate.

1. Test Details:
 1. Set up the analysis in accordance with the prescribed method.
 2. Set the flow rate 1.5 mL/min (reduced).
 3. Digest blood QC according to the prescribed method.
 4. Analyze all digested samples together in one run.
 5. Repeat the analysis using a flow rate of 2.0 mL/min (the method prescribed).
 6. Repeat the analysis using a flow rate of 2.5 mL/min (increased).

Critical Parameter Test 5. GC carrier gas flow rate. Test performed 8/24/10 by Mark Fresquez. Results below are the averages of observed results at each condition along with the 95 th percent confidence intervals (in parentheses).				
QC Pool ID	GC carrier gas flow rate (mL/min)	InHg (µg/L)	MeHg (µg/L)	EtHg (µg/L)
QC Pool LB07292	1.5 mL/min (Reduced)	1.36 (1.32, 1.39)	1.07 (1.04, 1.10)	1.16 (1.14, 1.18)
	2.0 mL/min (Per Method)	1.31 (1.25, 1.37)	1.07 (1.05, 1.08)	1.16 (1.13, 1.19)
	2.5 mL/min (Increased)	1.35 (1.31, 1.39)	1.05 (1.03, 1.07)	1.17 (1.12, 1.21)
Expected Range (± 2 SD)*		(0.71-1.76)	(.71-1.37)	(0.84-1.64)

Conclusion: All of the analytes fell within 2SD of the mean at all carrier gas flows; however, it was confirmed during method development that a carrier gas flow of 2.0 mL/min was the optimal choice.

C. Appendix B. Template for Working Triple Spike Standards Solution

1. Excel template used for calculations using AIT standards.

Triple-Spike Spiking Solution Preparation						
Experiment:			Date:			
1. Starting Stock Standard Solutions:			Units			
Vendor: AIT, Inc.			In[199]Hg	Me[200]Hg	Et[201]Hg	
Description: Isotopically enriched isotopes			($\mu\text{g/g}$)	5.1358	4.9937	5.0570
Lot#, Date:						
2. Pipet isotope stock standard sol. into 3 separate tared 1.5 mL microcentrifuge tubes. Cap tubes. Obtain net weights.						
			In[199]Hg	Me[200]Hg	Et[201]Hg	
	2a.	Volume	(μL)	50.0	50.0	50.0
	2b.	Wt with added Isotopes	(g)			
	2c.	Tube Tare wt.	(g)			
	2d.	Isotope Stock Sol., Net wt., calc	(g)	0.00000	0.00000	0.00000
3. Add water to above tubes using volumes indicated below. Cap tubes and weigh.						
			In[199]Hg	Me[200]Hg	Et[201]Hg	
	3a.	Volume of water/diluent added	(μL)	400.0	400.0	400.0
	3b.	Gross wt after water/diluent addition	(g)			
	3c.	Tube tare wt. from (line c) above	(g)			
	3d.	Isotope solution, dilution #1, net wt.	(g)	0.00000	0.00000	0.00000
		Dil. #1 dilution factor (line 3a \div line 2d)				
	3e.	New concentration of isotopes (dilution #1)*	($\mu\text{g/g}$)			
		<i>* If diluted, otherwise concentration remains unchanged from original stock standard solution.</i>				
4. Weight a 50 mL tube or volumetric flask then fill with approximately 0 mL of water (exact amount is determined below).						
	4a.	Determine tare wt of tube or volumetric flask.	(g)			
5. Quantitatively transfer the indicated volumes of each isotope to the 50 mL tube/volumetric flask containing water.						
			In[199]Hg	Me[200]Hg	Et[201]Hg	
	5a.	Vol dil #1 isotope std transferred to 50 mL tube/flask	(μL)	100.0	100.0	100.0
		If isotope standard was not diluted, quantitatively transfer entire amount, with 3x washings, to 50 mL tube/flask.				
		Rinse pipet tips in receiving tube's own solution. Recap the 1.5 mL tubes containing isotopes dilution #1.				
6. Re-weigh each 1.5 mL tube containing remaining dilution #1 of each isotope standard solution.						
			In[199]Hg	Me[200]Hg	Et[201]Hg	
	6a.	Gross wt of 1.5 mL tube + remaining dil #1 solution	(g)			
	6b.	Gross wt of 1.5 mL tube + original dil #1 sol (line 3b)	(g)			
	6c.	Wt. of dilution #1 isotope used to make dilution #2	(g)			
		<i>(for isotopes not diluted in Step 3, copy value from line 2d to line 6c)</i>				
7. Weigh the 50 mL tube containing all isotope standard additions.						
	7a.	50mL tube/flask tare wt + final solution wt	(g)			
6. Calculate final working triple-spike concentrations.						
			In[199]Hg	Me[200]Hg	Et[201]Hg	
	6a.	Concentration of dil #2 isotope solutions (line 3e)	($\mu\text{g/g}$)			
	6b.	Dil. #2 dilution factor ([line 7a - line 4a] \div line 6c)				
	6c.	Final Diluted Working Spike Conc (line 3e \div line 6b)	($\mu\text{g/g}$)			
7. Add the following volume of working triple-spike solution to each sample.						
			In[199]Hg	Me[200]Hg	Et[201]Hg	
	7a.	Vol working triple-spike solution to add per sample	(μL)	100.0	100.0	100.0
	7b.	Final Weight of each isotope added per sample	(ng)			