Laboratory Procedure Manual

Analyte: **Inorganic, ethyl-, and methyl- mercury (Hg)Inorganic, ethyl-, and methyl- mercury (Hg)**

Matrix: **Whole blood Whole blood**

Method: **Triple-spike isotope dilution solid phase extraction gas chromatography inductively coupled plasma mass spectrometry (TSID-SPE-GC-ICPMS)Triple-spike isotope dilution solid phase extraction gas chromatography inductively coupled plasma mass spectrometry (TSID-SPE-GC-ICPMS)**

Method No: **3020.08-05**

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as performed by: Inorganic and Radiation Analytical Toxicology (IRAT) Branch
Division of Laboratory Sciences (DLS)
National Center for Environmental Health (NCEH)

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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.

Images are included in this document as visual aids for certain topics. They are intended to be representative images only and should not be construed as absolute references. Discrepancies between the images in this document and the actual application design are not a cause for revisions to this document.

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_L	LBXIHG	Mercury, Inorganic (µg/L)
	LBXIHGSI	Mercury, Inorganic (nmol/L)
	LBXBEG	Mercury, ethyl (µg/L)
	LBXBEGSI	Mercury, ethyl (nmol/L)
	LBXBEM	Mercury, methyl (µg/L)
	LBXBEMSI	Mercury, methyl (nmol/L)

1. Summary of Clinical Relevance and 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 $Hg^0 < Hg^{2+} << CH_3Hg^+$ [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 µg/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 µg/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 **Error! Reference source not found.**. 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 Mercury Species Quantified with this Method

Species Name	Abbreviation	Molecular Structure
Inorganic mercury	InHg	Hg^{2+}
Methylmercury	MeHg	CH_3Hg^+
Ethyl mercury	EtHg	$C_2H_5Hg^+$

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 ($^{199}Hg^{2+}$, $CH_3{}^{200}Hg^+$, and $C_2H_5{}^{201}Hg^+$ or $C_2H_5{}^{198}Hg^+$, $^{199}Hg^{2+}$, and $CH_3{}^{201}Hg^+$) 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 ^{199}Hg, ^{200}Hg, ^{201}Hg, and ^{202}Hg isotopes or ^{198}Hg, ^{199}Hg, ^{201}Hg, and ^{202}Hg isotopes. We operate the ICP-MS in a Dynamic Reaction Cell (DRC™) mode to enhance Hg signal strength through an effect known as "collisional focusing" [6, 7].

2. 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.

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. Open and transfer acids in the fume hood or BSC, otherwise face shield should we worn. 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.

A. Observe all safety regulations as detailed in the Division Laboratory Safety Manual [8] and the laboratory Chemical Hygiene Plan.

B. Observe universal precautions when handling biological specimens. Wear gloves, a lab coat, and safety glasses while handling human blood, plasma, serum, urine or other bodily fluid or tissue. Bloodborne pathogens training is required.

C. Work with open vials containing biological samples inside of a biological safety cabinet (BSC). Recap vials before removing them from BSC.

D. Handle acids and bases with extreme care; they are caustic and toxic. Reagents used in this study include those listed in Section 6. Safety Data Sheet documents (SDS) for these chemicals are readily accessible as hard copies in the lab. If needed, electronic versions of SDS for other chemicals can be viewed at http://www.ilpi.com/msds/index.html or internally in the Chemical Hazard and Tracking System (CHaTS).

E. Operators of this method must take the Hazardous Chemical Waste Management for CDC Workers course upon initial hire and yearly refreshers thereafter.

F. Place disposable items (e.g., pipette tips, autosampler tubes, and gloves) that come in contact with human biological fluids, such as urine, in a biohazard autoclave bag. Keep these bags in appropriate containers until they are sealed and autoclaved.

G. 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.

H. When work is finished, spray all work surfaces where human biological fluids were handled with a broad-spectrum disinfectant or other appropriate disinfectant.

I. Review safety information for all instruments and equipment used with this method. Safety hazards from equipment and instruments in the laboratory may include ultraviolet radiation, high voltages, radio-frequency radiation, high temperatures and compressed gases.

Caution!

All packages known to contain mercury-containing chemicals should be opened inside of a chemical fume hood. Chemical Fume Hood training should be taken every three years. 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.

3. Computerization; Data System Management

A. During sample preparation and analysis, samples are identified by their sample ID. The sample ID is a number that is unique to each sample that links the laboratory information to demographic data recorded by those who collected the sample. Sample IDs are entered into the ICP-MS instrument software using a barcode scanner, whenever possible. If hand entry of a sample IDs is necessary, proofread transcribed data after entry.

B. The software used to control the instrument system (e.g., HPLC and ICP-MS), and raw data files are stored on the instrument control computer. The directories storing analytical run data are backed up nightly to the Isolated Secure Lab Environment (ISLE), which is then backed up weekly to the CDC network through a process setup by the Division's Laboratory Informatics Support team. Verify network backups as part of monthly computer maintenance (see Section 8.B(5)). Request Helpdesk support for data backup issues through the Laboratory Informatics Support Tool (LIST) in the Division of Laboratory Sciences (DLS) STARLIMS app.

C. The electronic files containing the data from the analysis are stored on the CDC network and imported into a STARLIMS database for review of the patient data, statistical evaluation of the QC data, and approval of the results. See Error! Reference source not found. for a step-by-step description of how the analyst transfers data from the ICP-MS instrument computer to the laboratory information system (STARLIMS), reviews and documents the results in STARLIMS, and submits the results for review and approval. Request Helpdesk support for DLS STARLIMS issues using the link on the dashboard of DLS STARLIMS.

D. Occasionally electronic files need to be deleted from the hard drive (i.e., for computer replacement or upgrade, to free up hard drive space, etc.). Verify the files have been transferred to the ISLE before deleting. Record action in the instrument logbook.

4. Specimen Collection, Storage, and Handling Procedures; Criteria for Specimen Rejection

Specimen handling conditions are outlined in the Division of Laboratory Sciences (DLS) Policies and Procedures Manual. The protocol discusses collection and transport of specimens and any special equipment required. If a specimen requires more than one test and needs to be divided, the appropriate amount of blood should be transferred into an appropriate cryovial labeled with the participant's ID; avoid cross-contamination.

A. Specimen type for this method is human whole blood.

B. No special instructions such as fasting, special diets, etc. are required prior to specimen collection.

C. All specimen collection devices and sample storage containers should be screened for total mercury contamination prior to use in the field and the laboratory.

D. Collect whole blood specimens in pre-screened blood collection tubes.

E. When focus of the study is metals, collect blood tubes for metals analysis first. Draw the blood through a stainless-steel needle into a pre-screened blood collection tube.

F. Glass blood tubes cannot be frozen. Store blood specimens collected in glass tubes in a refrigerator (2°C to 8°C, setpoint 4°C). Specimens collected in the field are kept refrigerated (2°C to 8 °C, setpoint 4°C) for up to 1 week and then shipped by overnight carrier.

G. Once received by DLS, they should be kept refrigerated during 'in-processing', which is typically completed within less than 4 hours and then stored in deep frozen conditions (range -50°C to -90°C, set point typically -70°C) for up to 15 business days until they are transferred to the testing laboratory.

   - During 'in-processing' specimens are aliquoted into screened cryovials so they can be frozen. Long-term storage should be in deep frozen conditions (range -50°C to -90°C, set point typically -70°C).

H. Once received by the testing laboratory sample aliquots can remain frozen until time for analysis. Portions of the sample that remain after analytical aliquots are withdrawn can be returned to deep frozen conditions (range -50°C to - 90°C, set point typically -70°C).

I. Stability testing has been conducted (See **Appendix A:** Stability). Samples were stable for 24 hours at ambient temperature (15°C to 30°C). Up to three freeze thaw cycles did not affect analyte recovery.

J. 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.

5. Procedures for Microscopic Examinations; Criteria for Rejection of Inadequately Prepared Slides

Not applicable to this method.

6. Preparation of Reagents, Calibration (Standards), Controls, and All Other Materials; Equipment and Instrumentation

A. Reagent Preparation

Prepare all reagents with deionized water (DI H_2O) with a resistance ≥ 18 MΩ·cm. Though each reagent preparation specifies a total volume of reagent prepared, these directions may be scaled up or down to prepare larger or smaller quantities if desired.

Label all reagent preparations in accordance with the Division Laboratory Safety Manual [15] and/or the DLS Joint Labeling Requirements. Specific storage conditions and expiration dates for reagents are listed below. To facilitate tracking of solvents and other chemicals transferred from the original manufacturer containment into a secondary container (e.g., solvent bottle), include the expiration date provided by the manufacturer or the lot number on the secondary container.

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

a) 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.

b) 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.

(2) Sodium tetra(n-propyl)borate ($NaPr_4B$, 2% w/v)

a) 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.

b) Open the vial containing sodium tetra(n-propyl)borate and add 10-20 mL of DI water with constant swirling.

c) 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.

d) Inside a chemical fume hood, quantitatively transfer reagent solution containing 5.00 g of $NaPr_4B$ to a 250 mL UV-resistant amber glass volumetric flask. Dilute to the final volume with DI water. This makes a 2% (w/v) $NaPr_4B$ solution. Store in refrigerator (2°C to 8°C, set point typically 4°C). Expires three weeks from the date prepared.

(3) 2% hydrochloric acid, 3% acetic acid and 1% methanol; used to prepare the working spike solution

a) Fill a clean 50 mL polypropylene tube approximately half full with DI water

b) Add 1mL of hydrochloric acid, 1.5mL of acetic acid and 0.5mL of methanol.

c) Fill to the 50 mL mark with DI water.

d) This solution will be discarded once the working solution is prepared.

(4) Base blood pool screened for mercury

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.

B. Standards Preparation

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

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, safety glasses, and latex or nitrile gloves. Clean up any spill that might occur according to applicable hazardous material spill procedures.

Remove the vendor-supplied standard solutions of the following from cooled storage and allow to reach room temperature: $Me^{201}Hg$, and $Et^{198}Hg$ 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 **0** Template for Working Triple Spike Standards Solution.

(1) Intermediate standard solution

a) Pipette 100 µL each of $In^{199}Hg$, $Me^{201}Hg$, and $Et^{198}Hg$ ISC Science standards into separately labeled 2 mL microcentrifuge tubes.

b) 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.)

c) These solutions will be discarded once the working solution is prepared.

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

a) Fill a clean 50 mL volumetric flask approximately half full with the 2% hydrochloric acid, 3% acetic acid and 1% methanol solution.

b) Pipette 100 µL of each intermediate standard solution into the flask.

c) Discard the remaining intermediate standard solutions after the aliquots have been taken.

d) 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).

e) The concentration of each mercury isotope standard in the working solution will be within a range of 0.5–3 µg/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 **0** Template for Working Triple Spike Standards Solution).

f) Store in refrigerator (2°C to 8°C, set point typically 4°C). The working solution expires three weeks from the date prepared.

C. Preparation of Quality Control Materials

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, safety glasses, and latex or nitrile gloves. Clean up any spill that might occur according to applicable hazardous material spill procedures.

Quality control (QC) materials are prepared from pools of whole blood obtained from a donor source or purchased commercially. The control "base" blood and two QC blood pools intended for the mercury speciation assay are designated as listed in Table 2 below 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”.

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

Table 2 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”.

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

(1) Preparation of bench low and high quality control pools

Analyze purchased base blood for InHg, MeHg, and EtHg species. Assign blood bags into a “low” pool or to a “high” pool according to the concentration ranges that are needed. Add additional volumes of InHg, MeHg, and EtHg (not enriched) species to achieve the desired concentrations of each "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. Prepare a quantity of vials that can be used for analytical runs over an extended period of time (at least 2 years but up to five years or longer if proven stable). Stability studies assessing long-term storage conditions of laboratory prepared quality control materials for this analytical method have been completed (see **Appendix A:** Stability). Store aliquoted QC material in deep frozen conditions (-50°C to - 90°C, set point typically -70°C).

(2) QC pool homogeneity

When a pooled QC material is prepared, an analytical run to assess the homogeneity of the pools is performed after the quality control pools have been aliquoted into individual vials. Vials are randomly chosen and randomly analyzed. The first and last vials dispensed are always included in the homogeneity study. Consult with a statistician about the appropriate design of your homogeneity study prior to dispensing the pool to ensure that sufficient statistical power is obtained.

Unlike the characterization of the QC, the homogeneity study should be completed in a single analytical run. Once sample analysis is complete, the data is statistically evaluated to determine homogeneity. If the concentration of Hg species in each pool does not vary significantly from beginning to end or if problem vials can be identified and eliminated, the characterization of the QC is the next step. If problems exist, the source(s) of the problem has to be identified and remedied before pool characterization can begin.

(3) QC pool characterization

Characterization of each QC pool establishes statistical control limits for each pool. At least 20 analytical runs are needed for a QC characterization. The preference for QC characterizations is that the characterization runs are distributed among all instruments that will be used to analyze the method. Ideally, all analysts that will run patient samples using the analytical method should complete some of the characterization runs. When available, previously characterized QC samples or pools with target values assigned by outside laboratories are also analyzed in QC characterization runs to evaluate each run. Once analysis of all characterization runs is complete, SAS is used to calculate the characterization statistics that will be used for the statistical evaluation of all future analytical runs.

D. Other Materials

Reagents and sources used with this method are listed below. Reagents used with implementation of the methodology must be equivalent to those listed below if procured from other sources. All chemicals and solvents are used without further purification.

(1) Chemicals

a) Deionized (DI) water, ≥18 MΩ cm resistivity.

b) Sodium acetate anhydrous, (CAS# 127-09-3), ($C_2H_3NaO_2$--MW 82.04), (Millipore/Sigma, Milwaukee, WI), Item # 59929-100G-F, or equivalent.

c) Glacial acetic acid, (CAS# 64-19-7), (CH_3COOH--MW 60.05), reagent ACS grade (GFS Chemicals, Powell, OH), Item # 624, or equivalent.

d) Double-distilled Hydrochloric Acid, (CAS# 7647-01-0), (HCl-MW 36.461--37.0%--12.1M) (GFS Chemicals Inc.), Item # 2180, or equivalent.

e) Double-distilled Nitric Acid (CAS# 7697-37-2), (HNO_3-MW 63.013-70.0%--15.8M), (GFS Chemicals Inc.), Item # 621, or equivalent.

f) Methanol HPLC Grade (CAS#67-56-1), (CH_3OH-MW 32.04), (GFS Chemicals Inc.), Item #2483, or equivalent.

g) Sulfuric Acid, (CAS# 7664-93-9), (H_2SO_4-MW 98.08), (Fisher Scientific, Suwanee, GA), Trace Metal grade, item # A510-P500, or equivalent.

h) Hydrogen Peroxide, 30% solution, stabilized (CAS# 7722-84-1), (H_2O_2, MW 34.0), (EMD Millipore, Germany), item #386790, or equivalent.

i) Tetramethylammonium hydroxide, 25% w/w in water (CAS# 75-59-2), (C4H13NO-MW 91.15), (Alfa Aesar, Ward Hill, MA), Item # 20932, or equivalent.

j) Sodium tetra(n-propyl)borate, (CAS# 45067-99-0), ($NaPr_4B$ -MW 206.16), (ABCR, Germany), Item # AB135075-0005.00-GRM or (VHG Labs, Manchester, NH) Item # DE-MS-03410-5), or equivalent.

k) Broad spectrum disinfectant, Fisher Scientific, Item # 19-130-5977, or equivalent disinfecting agent.

l) Certified pH 4 and pH 7 buffer solutions, (Fisher Scientific), Item # SB115-500 and SB107-500, or equivalent.

m) Base whole blood with EDTA, Tennessee Blood Services, equivalent vendor, or donated.

n) NIST SRM 955d - Toxic Metals and Metabolites in Frozen Human Blood (NIST, Gaithersburg, MD), or equivalent.

o) Xenon gas, 100ppm (Airgas South), Item # X02HE99C350002, or equivalent.

p) Nitrogen gas, Ultra High Purity (UHP) (Airgas), Product # NI UHP300, or equivalent.

q) Helium gas, Research grade (Airgas), Product # HE R300, or equivalent.

(2) Isotope Dilution Standards

a) ^{198}Hg-Enriched Ethyl mercury ($CH_3CH_2{}^{198}Hg$), (Innovative Solutions in Chemistry S.L. or ISC Science), Item # IES-EtHg198 or equivalent

b) Hg standard solution enriched in ^{199}Hg (^{199}Hg), (Innovative Solutions in Chemistry S.L.), Item # IES-Hg199 or equivalent

c) ^{201}Hg-enriched Monomethylmercury ($CH_3{}^{201}Hg$), (Innovative Solutions in Chemistry S.L.), Item # IES-MMHg201 or equivalent

(3) Lab Supplies

a) Gas regulators for argon, helium, xenon, and nitrogen (Airgas, Atlanta, GA) or equivalent

b) Captair pyramid glove bag (Model 2200 A, Erlab, North Andover, MA, item # EW-33666-50), size XL or equivalent

c) 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

d) Fomblin®GV80 pump fluid for the roughing pump, Item # N1845003 or equivalent

e) NexION® Trap Coaxial Foreline, Item # W1036511 or equivalent

f) PE Sciex Coolant, Item # WE016558A or equivalent

g) 0.5-200 µL LowRet pipette tips, 960 tips per case, Sartorius Corporation, Item # LH-L790200, or equivalent

h) 5-350 µL LowRet pipette tips, 960 tips per case, Sartorius Corporation, Item # LH-L790350, or equivalent

i) 10-1000 µL LowRet pipette tips, 960 tips per case, Sartorius Corporation, Item # LH-L791000, or equivalent

j) 5 mL pipette tips, 1000 tips per case, Sartorius Corporation, Item # 780308, or equivalent

k) 50 mL combitips plus, 100 tips per case (Eppendorf® catalogue # 02226660-8), or equivalent

l) 10 mL combitips plus, 25 tips per case (Eppendorf® catalogue # 02226650-1), or equivalent

m) 2.5 mL combitips plus, 100 tips per case (Eppendorf® catalogue # 02226630-6), Westbury NY), or equivalent

n) 50-mL acid-cleaned volumetric flasks for triple spike solution preparation (polypropylene or Teflon™ flasks preferred). To acid-wash flasks, rinse with 1.2 M hydrochloric acid followed by rigorous rinsing with DI water. Repeat this process several times depending on prior use of the containers.

o) 250-mL acid-cleaned volumetric flasks (UV-resistant) for derivatizing reagent ($NaPr_4B$) preparation (polypropylene or Teflon™ flasks preferred). To acid-wash flasks, rinse with 1.2 M hydrochloric acid followed by rigorous rinsing with DI water. Repeat this process several times depending on prior use of the containers.

p) Acid-cleaned 1 L PE bottles for buffer solution preparation. To acid-wash flasks, rinse with 1.2 M hydrochloric acid followed by rigorous rinsing with DI water. Repeat this process several times depending on prior use of the containers.

q) 50 mL polypropylene tubes pre-screened for Hg (Fisher Scientific), Item # 14-432-22, or equivalent.

r) 15 mL polypropylene tubes pre-screened for Hg (VWR), Item # 82050-276, or equivalent.

s) 2 mL Safe-Lock® polypropylene (PP) microcentrifuge tubes, Eppendorf® Item # 022363352, (Fisher Scientific, Suwanee, GA), or equivalent.

t) Eppendorf® tube racks for 2 mL microcentrifuge tubes, Item # 05-405-3, (Fisher Scientific, Suwanee, GA), or equivalent.

u) Blue racks for 2 mL cryovials (50-vial capacity), Item # 03-375-24L, (National Scientific), or equivalent.

v) Four or more PAL3 sample rack for 60 vials of 20 mL, Item #PAL3-Rack-R60, (CTC Analytics), or equivalent.

w) PAL DHR autosampler vials 20 mL, glass, Item # 16-2000, (Microliter Analytical Supplies, Inc.), or equivalent.

x) Head space caps for 20 mL glass vials, Item # 16-0050M, (Microliter Analytical Supplies, Inc.), or equivalent.

y) Kay-Dry™ paper towels and Kim-Wipe™ tissues, Item # 06-666-11, (Fisher Scientific, Suwanee, GA), or equivalent.

z) Teflon™-coated magnetic stirs bars (4), Item # 58948-974, (VWR Scientific, Buffalo Grove, IL), or equivalent.

aa) Teflon™-coated magnetic stirs bars, Item # 58947-140, (VWR Scientific, Buffalo Grove, IL), or equivalent.

bb) Sharps containers, 8.2 qt., pack of 12, Item # 14-826-128, (Fisher Scientific, Suwanee, GA), or equivalent.

cc) Cotton swabs (Hardwood Products Co. ME), or equivalent.

dd) Nitrile, powder-free examination gloves (VWR or Fisher Scientific), or equivalent.

ee) Biohazard autoclave bags (Fisher Scientific), or equivalent.

ff) Heavy wall 12x75 mm borosilicate glass test tubes (Fisherbrand catalog #: 14-958-10B) or equivalent.

gg) Position WEFLON-PTFE sample rack (Catalog #: UC85034) Milestone, Shelton, CT or equivalent.

hh) 5-350 µL LowRet pipette tips, 960 tips per case, Sartorius Corporation, Item # LH-L790350, or equivalent

ii) 10-1000 µL LowRet pipette tips, 960 tips per case, Sartorius Corporation, Item # LH-L791000, or equivalent

jj) 5 mL pipette tips, 1000 tips per case, Sartorius Corporation, Item # 780308, or equivalent

kk) 50 mL combitips plus, 100 tips per case (Eppendorf® catalogue # 02226660-8), or equivalent

ll) 10 mL combitips plus, 25 tips per case (Eppendorf® catalogue # 02226650-1), or equivalent

mm) 2.5 mL combitips plus, 100 tips per case (Eppendorf® catalogue # 02226630-6), Westbury NY), or equivalent

nn) 50-mL acid-cleaned volumetric flasks for triple spike solution preparation (polypropylene or Teflon™ flasks preferred). To acid-wash flasks, rinse with 1.2 M hydrochloric acid followed by rigorous rinsing with DI water. Repeat this process several times depending on prior use of the containers.

oo) 250-mL acid-cleaned volumetric flasks (UV-resistant) for derivatizing reagent (NaPr4BNaPr¬4B) preparation (polypropylene or Teflon™ flasks preferred). To acid-wash flasks, rinse with 1.2 M hydrochloric acid followed by rigorous rinsing with DI water. Repeat this process several times depending on prior use of the containers.

pp) Acid-cleaned 1 L PE bottles for buffer solution preparation. To acid-wash flasks, rinse with 1.2 M hydrochloric acid followed by rigorous rinsing with DI water. Repeat this process several times depending on prior use of the containers.

qq) 50 mL polypropylene tubes pre-screened for Hg (Fisher Scientific), Item # 14-432-22, or equivalent.

rr) 15 mL polypropylene tubes pre-screened for Hg (VWR), Item # 82050-276, or equivalent.

ss) 2 mL Safe-Lock® polypropylene (PP) microcentrifuge tubes, Eppendorf® Item # 022363352, (Fisher Scientific, Suwanee, GA), or equivalent.

tt) Eppendorf® tube racks for 2 mL microcentrifuge tubes, Item # 05-405-3, (Fisher Scientific, Suwanee, GA), or equivalent.

Blue racks for 2 mL cryovials (50-vial capacity), Item # 03-375-24L, (National Scientific), or equivalent.

uu) Head space caps for 20 mL glass vials, Item # 16-0050M, (Microliter Analytical Supplies, Inc.), or equivalent.

vv) Kay-Dry™ paper towels and Kim-Wipe™ tissues, Item # 06-666-11, (Fisher Scientific, Suwanee, GA), or equivalent.

ww) Teflon™-coated magnetic stirs bars (4), Item # 58948-974, (VWR Scientific, Buffalo Grove, IL), or equivalent.

xx) Teflon™-coated magnetic stirs bars, Item # 58947-140, (VWR Scientific, Buffalo Grove, IL), or equivalent.

yy) Sharps containers, 8.2 qt., pack of 12, Item # 14-826-128, (Fisher Scientific, Suwanee, GA), or equivalent.

zz) Cotton swabs (Hardwood Products Co. ME), or equivalent.

aaa) Nitrile, powder-free examination gloves (VWR or Fisher Scientific), or equivalent.

bbb) Biohazard autoclave bags (Fisher Scientific), or equivalent.

ccc) Heavy wall 12x75 mm borosilicate glass test tubes (Fisherbrand catalog #: 14-958-10B) or equivalent.

ddd) Position WEFLON-PTFE sample rack (Catalog #: UC85034) Milestone, Shelton, CT or equivalent.

E. Instrumentation

Analytical instruments used with this method are listed below. Before a product substitution is made, equivalent performance of the substitute instrumentation (e.g., GC, ICP-MS) must be demonstrated experimentally in accordance with DLS Policies and Procedures. Equivalent performance must also be demonstrated in accordance with DLS policies and procedures when multiple analysis systems are used in parallel, even if they are of the exact same type, however currently only one analytical system is used in the lab.

(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

(4) Gas Chromatograph, PerkinElmer® Clarus 500 or 580 or equivalent system

(5) GC capillary column, PerkinElmer® Elite-5 30 m, 0.25 mm i.d., 0.25 mm film thickness, Item # N9316076 or equivalent

(6) GC-ICP-MS Heated Transfer Line accessory, Redshift®, Item # N0777440 for 115V or Item # N40777361 for 230V or equivalent

(7) 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

(8) "T" union to interface GC column to transfer line and make-up gas, Redshift® Item # TL00402 or equivalent

(9) Thermogreen® LB-2 Septa, Supelco, Item # 20654 or equivalent

(10) Glass inlet liner, Supelco, Item # 2631405 or equivalent

(11) 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

(12) Robotic Liquid Sample Processing Workstation: PAL® DHR dual head system, Trajan Scientific Americas, Inc. Item# DHR419084/DHE419085, or equivalent system Figure 1. The Dual Head SPME PAL® 3 Series II sample processing workstation, mounted on top of the GC 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.

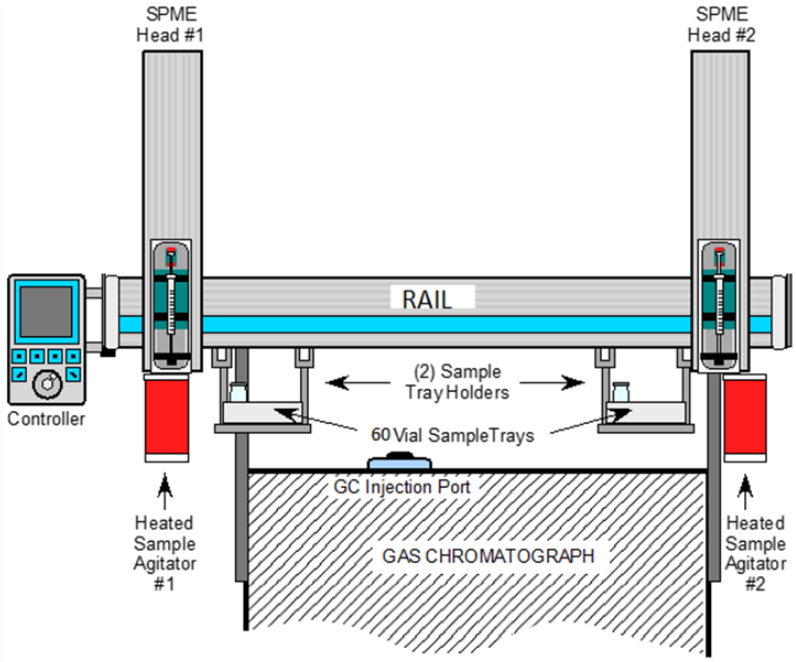


Figure 1 Dual Head SPME Processing Workstation

The Dual Head SPME PAL® 3 Series II sample processing workstation, mounted on top of the GC 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.

F. Equipment

Materials and supplies for use with this method are listed below. Items can be obtained from the following sources, or any equivalent source. In the case of simple laboratory instrumentation (e.g., pipettes, vortex mixer, analytical balance, etc.) a product listed herein may be substituted with an equivalent product from a different manufacturer provided if it meets or exceeds the specifications of the product listed.

(1) Water purification system for providing ultrapure water with a resistivity ≥18 MΩ 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 µL to 5000 µL. 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) Mechanical convection oven (FREAS Model 605, Thermo scientific, Item # 3166188), or equivalent

(8) Microwave Digestion System (UltraCLAVE, Milestone Inc, Shelton, CT, item # 70540-UC4) or equivalent

(9) Class II Biological Safety Cabinet (SterilGARD® III Advance, The Baker Company), Serial # 82774, or equivalent

(10) Chemical Fume Hood (Mott Manufacturing Ltd, Brantford, ON, Canada), Model # 7421000, Serial # 21126, or equivalent

(11) Autosampler probe assembly, Item # B3000161 or equivalent

(12) NexION cyclonic spray chamber, Item # N8145013 or equivalent

(13) Demountable quartz torch, Item # N8122006 or equivalent

(14) Torch o-ring kit, Item # N8120100 or equivalent

(15) Torch alignment tool, Item# WE015554 or equivalent RF load coil, Item # WE021816, or equivalent

(16) Quartz injector 1.2 mm ID, Item # N0681631 or equivalent

(17) Quartz ball injector 2.0 mm ID, Item # WE023948 or equivalent

(18) Cone removal tool, Item # W1034694 or equivalent

(19) NexION® platinum sampler cone, Item # W1033614 or equivalent

(20) NexION® platinum skimmer cone, Item # W1026907 or equivalent

(21) NexION® aluminum hyper skimmer cone, Item # W1033995 or equivalent

(22) NexION® Sampler gasket, Item # W1040148 or equivalent

(23) NexION® Hyper Skimmer o-ring, Item # 09902123 or equivalent

(24) NexION® Hyper Skimmer Screw, Item # WE027484 or equivalent

(25) NexION® Exhaust Filter Kit SV40BI, Item # N8145005 or equivalent

(26) TQ+ Quartz nebulizer, Meinhard, Item # TQP-50-A0.5 or equivalent

(27) Nebulizer connection kit, Meinhard, Item # F1-C16 or equivalent

(28) UltraCLAVE Metal O-ring, Milestone, Shelton, CT, Item # WZ00046 or equivalent

(29) UltraCLAVE Flat O-ring, Milestone, Shelton, CT, Item # OR0001 or equivalent

(30) Four or more PAL3 sample rack for 60 vials of 20 mL, Item #PAL3-Rack-R60, (CTC Analytics), or equivalent.

(31) PAL DHR autosampler vials 20 mL, glass, Item # 16-2000, (Microliter Analytical Supplies, Inc.), or equivalent.

G. 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

(3) 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

(4) Operating software for PAL DHR autosampler, specifically, Chronos (CTC Analytics AG, 2010 - 2017)

(5) ChromLink™ 3.5 software (PerkinElmer Instruments). The installation of ChromLink™ is straightforward when using the supplied installation utility

(6) 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

(7) A custom Microsoft Excel® macro procedure named "Extract TC Data"

7. Calibration and Calibration Verification Procedures

A. Method Calibration

(1) Triple spike isotope dilution (TSID) is a particular useful technique that quantifies species transformations, allowing the analyst to calculate the concentrations of each species without the use of an external calibration curve [9]. Central to TSID design is a “spike” solution containing a mixture of three mercury species standards, each synthesized to be enriched with different isotopes of mercury ($^{199}HgCl_2$, $CH_3{}^{200}HgCl$ and $C_2H_5{}^{201}HgCl$), and must be added to each patient sample and equilibrated before mercury transformation reactions occur. Since the spike is chemically identical to the native form of mercury species contained in the sample, chemical transformations can be measured and mathematically corrected [10, 11]. The TSID methodology offers major improvements in accuracy, precision and LOD over standard calibration curves.

(2) ICP-MS intensity signals of the three mercury species (iHg, MeHg and EtHg) are measured at m/z 198, 199, 200, 201 and 202. The $^{198}Hg/^{202}Hg$, $^{199}Hg/^{202}Hg$, $^{200}Hg/^{202}Hg$, and $^{201}Hg/^{202}Hg$ peak area ratios are calculated and exported into a Microsoft Excel spreadsheet originally developed by Ouerdane and colleagues [12-14] where a deconvolution algorithm mathematically corrects for mercury species interconversions. Mercury species concentrations are calculated using these isotope dilution equations. Isotopic impurities associated with isotopically enriched mercury standards, provided in ISC certificate of analysis, are included in calculating final mercury concentrations.

(3) Calibration verification is not performed with this method because it uses the isotope dilution method that does not use an external calibration.

(4) We participate in an internal proficiency testing (PT) program for Hg speciation for all three Hg species because no external PT program exists. Five samples are analyzed two times per year and submitted to an internal QA department. Additional details about the administration of internal PT programs can be found in the Internal Proficiency Testing SOP (IRAT-OC-QA.01.01).

- Records of PT performance are signed by a Technical Supervisor and stored in the LIMS.

B. Instrument Calibration

(1) ICP-MS - The calibration of the ICP-MS is performed annually as part of a preventative maintenance program and performed by a qualified service engineer.

- Preventative maintenance records are stored in the LIMS.

(2) GC - The GC system is serviced annually as part of a preventative maintenance program and performed by a qualified service engineer.

- Preventative maintenance records are stored in the LIMS.

(3) Handheld pipettes - Pipette calibration verification is performed every six months using a certified volume verification system. If a calibration verification fails, the pipette is sent to the vendor for service and calibration.

- Calibration and calibration verification records are stored in the LIMS.

(4) Diluter (e.g., Hamilton Microlab 625) – Diluter calibration verification is performed annually by the vendor or qualified service engineer. If a calibration verification fails, the diluter is sent to the vendor for service and calibration.

- Calibration and calibration verification records are stored in the LIMS.

(5) pH meter - pH meters are shipped to the vendor every year for calibration. Calibration verification is performed by the analyst prior to use with commercial calibration solutions.

- Vendor calibration records are stored in the LIMS.
- pH calibration verification records are stored in the equipment notebook.

(6) Balance - Balances are calibrated annually by the vendor or qualified service engineer. Calibration verification is performed by the analyst prior to use with certified weights.

- Vendor calibration records are stored in the LIMS.
- Balance calibration verification records are stored in the equipment notebook.

(7) UltraCLAVE Microwave Digestion - The Microwave Digestion system is serviced annually as part of a preventative maintenance program and performed by a qualified service engineer.

- Preventative maintenance records are stored in the LIMS.

(8) Convection Oven - Convection Oven system is serviced annually as part of a preventative maintenance program and performed by a qualified service engineer.

- Preventative maintenance records are stored in the LIMS.

8. Procedure Operating Instructions; Calculations; Interpretation of Results

A. Preliminaries

(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 instrument function checks as listed on the electronic instrument performance log sheet. 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 in the electronic instrument performance log sheet.

(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 stabilize 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) Set the GC analytical run parameters to what is listed in **Error! Reference source not found.**. Refer to the Clarus 580 User Guide for programming specifics. One or more parameters specified in **Error! Reference source not found.** may be changed, if determined necessary, to meet analytical performance goals.

Table 3 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
Total flow ratio	28:1 @ 0.25 min
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)

(5) After successful ignition and the instrument has warmed up and stabilized, perform the optimization of both plasma and mass spectrometer parameters of the ICP-MS 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.

a) Set the flow rate of the Xe gas mixture (100 ppm Xe in Helium) to approximately 0.25 mL/min on the GC main display soon after plasma ignition to achieve Xe intensity of > 100,000 cps (counts per second). In the NexION® software, upload "Xe Torch Alignment.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 **Error! Reference source not found.** for maximum Xe intensity.

b) If X-Y optimization does not achieve Xe intensity of > 100,000 cps, advanced instrument optimizations can be performed, e.g., axial field voltage or deflector voltage.

c) Save the optimized file as "Default.dac". The current optimized values will appear automatically and will be similar to the ones in **Error! Reference source not found.**.

Table 4 NexION® Optimization Parameters

Parameter	Setting	Parameter	Setting
Nebulizer Gas Flow (NEB):	1.5	RPa	0
Auxiliary Gas Flow:	1.2†	RPq	0.3†
Plasma Gas Flow:	15†	Cell Gas A	0.5†
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.			

(6) PAL DHR is initially set up and optimized by 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 PAL DHR autosampler system for this method.

a) In the PAL control terminal, parameters for X, Y, Z positions of reference point, vial # 1, 10, 60, and GC injection portal can be viewed under “User Level” (default mode). 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.

b) To calibrate the PALtool head alignment, using the PAL control terminal, press left and right “—” button at the same time to access “Extended User Level”. Under the main menu, select the SPME that needs to be calibrated then press ”ENTER”; open Options menu, select “Calibrate PALtool” press “ENTER”. Go to “Next”, the screen will direct user to move the PALtool manually to the reference point and press “Save” to save the X, Y, Z positions. In the main menu, scroll to the Tray Holder that need to be calibrated, press “ENTER”; select “Slot 1”, “Rack 1”, and then open Option menu, select “Teach PALmodule”; confirm the Robert Arm (Left for Tray 1, Right for Tray 2), press “Run”. The control panel will direct user to move the head to the teach point (vial position 1, 10,and 60). Press “Save” after finishing each step to save the X, Y, Z positions.

c) To calibrate the X, Y, Z positions of GC injection portal, scroll to “Injector 1” in the main menu press “ENTER”; then open Options menu, select “Teach PALmodule”; select Robot

Arm (Left or Right), press “Run”. The control panel will direct user to move the head to the teach point (i.e., GC injection portal) and press “SAVE” to save the X, Y, Z positions.to optimize X, Y, Z position.

(7) PAL DHR 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. Click on the "Load" tab on the left which allows the analyst to upload the needed method file. There are three method files used in this method: PAL3_DHR_Bakeout.cam, PAL3_DHR_Conditioning.cam and PAL3_DHR.cam. The settings can be changed by the analyst for better separation and analytical response.

a) Cleaning the SPME fiber is done at least every ten runs (see Section 8.B on Ten run maintenance) To clean SPME fibers PAL3_DHR_Bakeout.cam is used. Use sample lists SPME Bakeout LEFT.csl and SPME Bakeout RIGHT.csl for left and right fibers respectively. The settings are as shown in Table 5.

- Record SPME fiber cleaning in electronic instrument log sheet

Table 5 Chronos Settings (Baking)

Parameter	Setting
Bakeout Time	420 s
GC Runtime + Cooling Down	120 s
Needle Penetration inlet	48 mm

b) If a new SPME fiber is installed it will need to be conditioned; use - PAL3_DHR_Conditioning.cam and sample lists SPME Conditioning LEFT.csl and SPME Conditioning RIGHT.csl for left and right fibers respectively. The settings are as shown in Table 6.

- Record SPME fiber replacement in electronic instrument log sheet

Table 6 Chronos Settings (Conditioning)

Parameter	Setting
Bakeout Time	1800 s
GC Runtime + Cooling Down	120 s
Needle Penetration inlet	48 mm

c) To perform a run, the file “PAL3_DHR.cam” is used. The corresponding sample list is “PAL3_DHR.csl”. The settings are as shown in Table 7.

Table 7 Chronos Parameter Settings (for Run)

Parameter	Setting
Source Tray	Tray Holder 1:Slot1
Vial	1,1; 2,2; 3,3 etc.
Enrichment Time	1200 s
Desorption Time	420 s
GC Cooling Down	180 s
Needle Penetration vial	38 mm
Fiber Exposure	12 mm
Needle Penetration inlet	48 mm

(8) After optimization for maximum Xe intensity, navigate to the folder "C:\NexIONdata\Method" and click on the "Xe DRC Daily.mth" file then click the "Open" button. Record this data in the instrument logbook.

(9) Complete the performance check before starting analytical run.

(10) Fill in the performance log /maintenance log to record to the completed any 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).

(11) Samples and QC Material

a) Identify, gather, and thaw a predetermined number of sample tubes/vials containing the blood samples to be analyzed in a batch run.

b) 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).

c) 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.

d) Use the preparation procedure outlined in VII D to prepare blood samples and QC materials.

e) Cap all autosampler vials with the proper fitting septum caps.

(12) Creating the NexION® Sample Table ".sam" file

a) 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.

b) 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 8.

Table 8 Sample Template Data

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

c) Parameters not mentioned in Table 8 can be left blank.

d) 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.

e) 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.

B. Instrument Maintenance

(1) Complete “10 run Maintenance” following completion of 10 analytical runs on the instrument (See **Appendix E:** Electronic Instrument Performance log sheets)

a) Replace GC injector septum and glass liner.

b) Examine SPME fibers for discoloration and missing parts of polymer layer. If not in good condition replace the SPME

(2) If instrument is not passing Performance Check, perform visual check of torch, injector, RF coil, cones, column, and charcoal trap, replace if needed:

a) Visually check the cleanliness of these components. Notate any cleaning / replacing of consumables in the maintenance logbook.

b) 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).

c) 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.

d) RF coil: Check for excessive corrosion (flaking). Replace if necessary.

e) Sampler and skimmer cones: Check for excessive buildup of matrix, cracking, or pitting. If necessary, replace dirty cones with clean spare cones and clean.

f) Column edges: Check for jagged or burned edges. Perform capillary cutting if needed.

(3) Replace the charcoal trap if degradation in instrument performance is seen.

(4) Replace the GC column if chromatography is not performing well, peak degradation is apparent.

(5) The instrument computer should be rebooted at least monthly. Verify ISLE and MUST drive backups monthly.

- Record in the instrument electronic logbook on the Instrument Maintenance worksheet.

(6) Record all maintenance of computer hardware or instrument control software in the instrument logbook. This includes disk defragmentation, software upgrades, software patches, etc.

C. Preparing a Run

(1) Each day, before samples are analyzed, aqueous blanks and a dilution of Low Bench QC must be analyzed to ensure that the instrument is functioning properly. Aqueous blanks are analyzed to ensure there is no contamination present in the reagents used to prepare patient samples. Dilute Low Bench QC sample to bring mercury species concentration levels in the QC close to the LOD values for mercury species of the analytical method. Then analyze the diluted Low Bench QC to test the sensitivity of the instrument. 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 ($NaPr_4B$). 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 µL of derivatization reagent (NaPr4B). Cap the vial immediately and gently mix it. Aqueous blank 2 is ready to be analyzed.

c) Diluted Low Bench QC. Depending on which concentration level of LB QC is available, this QC sample is diluted with base blood to achieve similar concentrations of mercury species as the method's LOD values. Diluted LB QC sample is prepared, digested, and analyzed in the same manner as patient samples.

(2) After SPME-GC-ICP-MS analysis of Aqueous Blanks and Diluted Low Bench QC are complete, open the Aqueous Blanks and Diluted Low Bench QC RAW files in TotalChrom™. Examine the Aqueous Blank chromatograph for possible indicators of contamination. Then

look at the 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).

- Update electronic instrument performance log sheet to indicate instrument sensitivity was sufficient for sample analysis.

(3) Preparation of bench QC and unknown blood 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).

a) Digestion method 1: Convection Oven

1. Pipette 100 µL of blood samples into pre-labeled 2 mL Safe-Lock microcentrifuge tubes.
2. Add 100 µL of Triple Spike Standards Solution. Recap and vortex each tube before continuing to the next tube.
3. Add 500 µL of TMAH to all tubes. Cap and vortex.
4. Place rack containing capped tubes in the convection oven set to 80 ± 3°C for ≥ 20 hours.

b) Digestion method 2: UltraCLAVE Microwave Digestion

1. Pipette 100 µL of blood samples into pre-labeled 12x75 mm glass test tubes.
2. Add 100 µL of Triple Spike Standards Solution. Vortex each tube before continuing to the next tube.
3. Add 500 µL of TMAH to all tubes. Vortex gently. Optionally, cover the test tubes with ventilated microwave digestion caps.
4. Refer to Appendix F: Operation of the UltraCLAVE Microwave Digestion System for the Mercury Speciation Analytical Method for instructions of usage of the UltraCLAVE for digestion of the samples and quality control materials

c) Derivatization of the digested samples:

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

D. Initiating a Run

(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 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 PAL DHR autosampler tray.

c) Fill in the samples table in similar fashion to what is shown in

d) Table 9 below.

Table 9 NexION® Sample Table

A/S Loc.	Batch ID	Sample ID	Measurement Action	Method	…	Sample Type
1		001_ Aq.Blk	Run Sample	hg drc.mth		Sample
1		002_ Aq.Blk	Run Sample	hg drc.mth		Sample
2		003_ Diluted LB	Run Sample	hg drc.mth		Sample
2		004_ Diluted LB	Run Sample	hg drc.mth		Sample
3		005_ LB-yyxxx	Run Sample	hg drc.mth		Sample
3		006_ HB-yyxxx	Run Sample	hg drc.mth		Sample
4		007_ Sample	Run Sample	hg drc.mth		Sample
4		008_ Sample	Run Sample	hg drc.mth		Sample
…rows for 18 samples and SRM material were omitted for brevity						
14		027_LB-yyxxx	Run Sample	hg drc.mth		Sample
14		028_HB-yyxxx	Run Sample	hg drc.mth		Sample

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 #14 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™.

1. When the sample table entries are verified to be correct, choose "File" > "Save".
2. Print the 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 PAL DHR autosampler tray.

(7) Check that the GC methods are correctly programmed.

(8) Check that PAL® DHR autosampler methods are correctly programmed.

(9) 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.

a) 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).

b) 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.

c) 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.

d) 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.

(10) Launch the Chronos program, which communicates with the PAL DHR autosampler and NexION® software.

a) In the main menu, click on "Sample list" then upload analysis method for SPME fiber cleaning "baking".

b) Upload "SPME Bakeout LEFT.csl" (to clean the left fiber) then select "Create" button in the Schedule section. The “Schedule and run control” window will appear with time intervals required for the measurement. Click "Start queue" on the top of the window to proceed with the fiber cleaning.

c) Repeat step above to clean the right fiber and use "SPME Bakeout RIGHT.csl"

d) 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" tab in the List submenu on the left. UploadPAL3_DHR.csl file. This file communicates with the left and right fibers. A table similar to the one below (Table 10) will appear. The table will have the Analysis Method ”C:\Users\NexION-M\Documents\CDC PAL DHR419085\Methods\PAL3_DHR.cam" (NexION-M) and “C:\User\NexION-R\Documents\CDC PAL DHR419084\Methods\PAL3_DHR.cam” (NexION-R) uploaded.

e) "Source Vial" column shows which autosampler position will be analyzed first. There are two racks (left and right) with vial positions 1 to 60. 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.

f) 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), highlight Row 1 through 10 in the sample list and click “Create” button in the Schedule section with “overlapped” option checked. The run schedule will appear in “Schedules and run control” on the Main Menu.

Table 10 Chronos Samples Table

Analysis Method	Source Vial	Enrichment Time (s)	Desorption Time (s)	GC cooling down (s)
C:\Users\NexION-M\Documents\CDC PAL DHR419085\Methods\PAL3_DHR.cam*	1	1200	420	180
	1	1200	420	180
	2	1200	420	180
	2	1200	420	180
Rows for source vials 3 to 59 are omitted				
	60	1200	420	180
	60	1200	420	180

(11) 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.

(12) Check that all blanks, QC, and sample vials are loaded into their correct positions in the PAL DHR autosampler trays as designated by the NexION® sample window and in the positions seen by Chronos.

(13) As a reminder, before samples are analyzed, the daily performance check will be analyzed to ensure that the instrument is functioning properly.

a) 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.

b) In Chronos: to analyze samples, select "Create" button in the Schedule section, then click "Start queue" on the Schedule and run control window.

c) 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 diluted LB been analyzed, the run can be started.

(14) 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.

(15)The PAL® DHR 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.

(16)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.

E. Processing and Reporting a Run

Refer to Figure 2 "Post-Run Data Processing Work Flow Diagram" 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\<current working folder>). Select all files ending with the .rst and .idx and "delete" them to the Microsoft Windows® Recycling Bin.

(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:

a) 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 Appendix H:Error! Reference source not found.B Configuration of ELAN® ChromLink™ for details).

b) 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 " ELAN® NetCDF file - location/file to be converted" indicates the correct data folder. This will be "C:\Users\Public\Documents\PerkinElmer Syngistix\ICPMS\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 ELAN® 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.

c) 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 "HgSpecGC1.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 theNexION® 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<yymmdd> 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 "-LB19009". 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 “Hg SPECIATION” > “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 “Hg SPECIATION” > “Create TC Sequence File” cannot be run:

a) 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.

b) 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.

(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." A chromatogram for the first sample in the sequence will appear in the “Reprocess Results” window. 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.

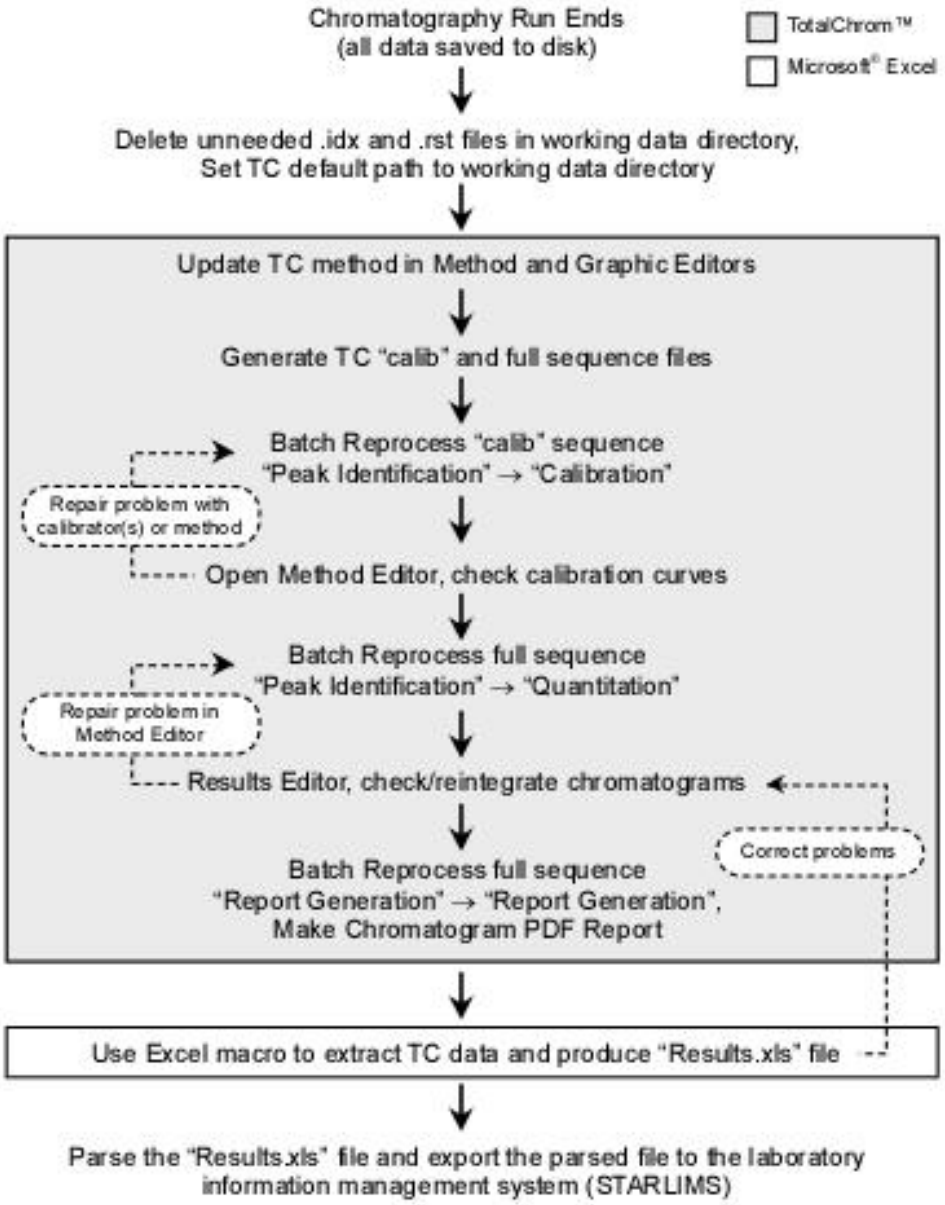


Figure 2 Post-Run Data Processing Workflow Diagram

Table 11 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	:	”Interactive” or “nexionr-pc” or equivalent
Batch Printer	:	pdfFactory Pro
Batch Plotter	:	None
Enable Optional Report(s) in Method	:	Not Checked
Use Method is Result File		Grayed Out
Overwrite Existing Result Files	:	Checked
Raw File Treatment	:	Update existing raw file header with new sequence

(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 pdFactory 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 pdFactory Pro window.

(14) Open Microsoft Excel and choose "Hg SPECIATION" > "Import TC Files" 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 "Hg SPECIATION" > "Deconvolute All Samples" 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 tr1, tr2, tr3, respectfully. For the purpose of establishing relevant criterion, relative retention time for InHg (tr3) is set to 1. The ratio (tr2/tr1) =0.91 ± 0.13 and (tr3/tr1)=0.77 ± 0.12 (± represents three times standard deviation). If the relative retention time ratios fall outside the allowable error, the sample analysis must be repeated.

(18)Transfer data to the laboratory LIMS; see **Appendix C:** General instructions for uploading data to Starlims for instructions.

(19)After an analytical run has been uploaded to the LIMS system, the analyst should note which, if any, samples are noted as anything other than "Pass" or "<LOD" in the "Status Message" column.

a) Any sample that has an analytical result above 10 µg/L (i.e., 2UB) but below 30 µg/L for any analyte must be redigested, repeated, and confirmed for that analyte. 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.

b) Any sample that has an analytical result that saturates the instrument detector (typically above 30 µg/L) for any analyte must be redigested, repeated, and confirmed for that analyte, see Section 9.

c) If the run QC fails, the following steps must be taken, if possible:

1. 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 TotalChrom™.

2. 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.

3. 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.

F. Special Method Notes

(1) Instrument Shut Down

a) The autosampler will stop after all samples have been analyzed.

b) Shut off ICP-DRC-MS plasma.

c) 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.

(2) Waste Disposal

a) Chemical waste generated during sample preparation must be disposed of through the CDC Online Waste Ticketing System (OWTS)

b) Remove the QC and sample vials from the GC tray. Discard them according to CDC biohazard waste disposal guidelines.

G. Calculations

(1) We programmed the CombiPAL® autosampler using Chronos™ software (LEAP Technologies Inc., Carrboro, NC, USA) which controls the timings for SPME fiber exposure to the sample (adsorption) and fiber injection into the GC (desorption). Our laboratory used ELANSyngistix® software (PerkinElmer Life Sciences, Shelton, CT, USA) to process ICP-MS intensity signals of three mercury species (inorganic, methyl, and ethyl) measured at m/z 198, 199, 200, 201 and 202.

(2) TotalChrom™ version 6.3 (PerkinElmer Life Sciences, Shelton, CT, USA) handled chromatography data manipulations. The ChromLink™ feature of TotalChrom™ interfaces ICP-MS and GC data. Using an in-house written macro, we imported the integrated peak areas for InHg, MeHg and EtHg from TotalChrom™ into a Microsoft Excel spreadsheet where the $^{198}Hg/^{202}Hg$, $^{199}Hg/^{202}Hg$, $^{200}Hg/^{202}Hg$, and $^{201}Hg/^{202}Hg$ ratios were calculated and exported into a second spreadsheet containing multiple spiking species-specific isotope dilution mathematical equations developed by Ouerdene and colleagues [12-14]. The Excel macro is stored in the DLS Informatics Systems and Tools Tracker in STARLIMS (Record #19).

(3) Several published methods exist for correcting isotopic ratio error attributed to the mass bias effect [10, 15-20]. We tried a variety of mass bias correction techniques during the development of our method [16, 18, 21, 22], but none improved our accuracy or precision. Conversely, we observed an among-run precision degradation as a result of using mass bias correction. Therefore, we monitor mass bias error by checking measurements of isotopic ratios in unspiked blood samples against the expected ratios for natural abundance (IUPAC abundances) mercury, the difference in abundance ratios should be less than 10% to ensure mass bias error contribution is not significant.

H. CDC Modifications

None

9. Reportable Range of Results (AMR – Analytical Measurement Range)

The reportable range of results for Hg species are listed in Table 5. The lower reportable limit is the limit of detection (LOD). The upper reportable limit is not limited by a calibration standard because there are no calibration standards used with this method. Instead, the upper reportable limit is based on a concentration that causes the instrument signal to saturate the detector. This typically occurs around 30 µg/L. We check mercury species chromatographic peak shape to determine if the detector is saturated. When signal saturation occurs, the tops of chromatographic peaks look flat. Samples that are initially measured with a Hg species concentration that saturates the detector can be diluted. Extra sample dilutions (with base blood) are allowed to bring a Hg species concentration to a level below 30 µg/L. Use the lowest dilution factor possible. Discuss reporting results from an extra sample dilution with the Technical Supervisor.

Table 12 Reportable range of Hg species

Hg species	LOD	Lower reportable limit*	Upper reportable limit**
MeHg, EtHg, IHg	See **Appendix A:**	Method LOD	30 µg/L
***Lower reportable limit is the method LOD.** ****The upper reportable limit is the concentration (before extra dilution) that typically saturates the instrument detector.**			

10. Quality Control (QC) Procedures

A. Blind Quality Controls

No blind QC samples are used with this method.

B. Bench Quality Controls

Bench QC samples are prepared from a minimum of two pools that represent low and high levels of Hg species. This assay typically uses two blood pools that represent low and high levels. Samples from these pools are prepared in the same manner as patient samples and analyzed in duplicate as part of each run (placed at the beginning and end of each run).

The results from these pools are checked after each run using a multi-rule quality control system [23] based on their characterization data, namely: the pool mean; the pooled within-run standard deviation associated with individual QC results measured in the same run (Sw); the standard deviation associated with individual QC results (Si); and the standard deviation associated with run mean QC results (Sm). QC rules have been designed to accommodate the use of 1–3 different QC pools during a run, the use of 1–2 measurements of each pool per run, and as many instruments as needed. These QC rules are described in the DLS Policies and Procedures Manual and a relevant selection applicable to this assay is shown below. The system is declared “in control” if all individual QC results are within 2S limits; the run is accepted. If not, then the rules shown below are applied and the run is rejected if any condition is met; the run is declared “out of control”:

(1) If 1 of the 3 QC run means is outside a 2Sm limit - reject run if:

a) Extreme Outlier – Run mean is beyond the characterization mean +/- 4Sm

b) 3S Rule - Run mean is outside a 3Sm limit

c) 2S Rule - 2 or more of the 3-run means are outside the same 2Sm limit

d) 10 X-bar Rule – Current and previous 9 run means are on same side of the characterization mean

(2) If one of the QC individual results is outside a 2Si limit - reject run if:

a) Extreme Outlier – One individual result is beyond the characterization mean +/- 4Si

b) R 4S Rule - 2 or more of the within-run ranges in the same run exceed 4Sw (i.e., 95% range limit). Note: Since runs have multiple results per pool for 3 pools, the R 4S rule is applied within runs only. Within both the low-and the high-QC results are within the 2s limits, the run will be accepted.

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

The initial limits are established by analyzing pool material in at least 20 analytical runs. The SAS QC program is used to monitor the QC performance over time for potential shifts, trending, or changes in assay precision. For assays performed routinely, quarterly statistics (mean, SD, CV) are calculated for each pool and compared to the characterization target values. For assays performed infrequently, statistics are calculated at least annually. As more QC data become available (covering multiple lots of reagents, multiple analysts, etc.), the initial QC limits can be reevaluated and updated. QC limits can also be reevaluated and updated as a result of a non-conforming event when the assay shows a higher than expected out of control rate and the root cause investigation does not reveal a correctable course of action to bring the assay back into control. This needs to be documented by a CAPA in STARLIMS.

While a study is in progress, QC results are stored in the STARLIMS database. For runs that are not imported into the database (e.g., R&D, troubleshooting, research-type runs), QC results are stored electronically on the instrument control computer. At the conclusion of studies, complete QC records are prepared and submitted as a study QC report in STARLIMS for review by the deputy branch chief or laboratory chief, branch chief, and a DLS statistician.

C. Sample QC Criteria

Sample QC is a set of criteria used to evaluate the quality of individual test results within a run, and to evaluate the quality of the calibrators associated with the run. In addition to the sample QC criteria set forth in the DLS Policies and Procedures Manual that pertain to the reportable range of concentration results and calibration curves, sample QC criteria are also established for method-specific concentration and non-concentration data associated with an individual result.

The method-specific concentration and non-concentration parameters identified for sample QC evaluation, along with their associated thresholds and flagging protocols (‘Pass’, ‘Check’, ‘Warn’, ‘Fail’, ‘Incmp’) are maintained and updated in the LIMS database, and sample QC assessment is performed and documented as part of run review process. No numerical results should be reported for a sample with sample QC result flagged as ‘fail’. A sample QC result flagged ‘Warn’ or ‘Check’ should be reviewed both by the analyst and supervisor to determine if the quality of

the result is suitable for reporting. Results that are flagged during sample QC evaluation may also be assigned one of a series of standard comment codes available in the LIMS database to identify the nature of the sample QC flag.

At a minimum, the following parameters are subject to sample QC evaluation in this method. Additional parameters may also be included as needed:

(1) Hg species retention times and relative retention times are defined in the TotalChrom software (see **Error! Reference source not found.**). Analysts confirm the correct peaks are integrated prior to processing data and uploading to LIMS.

(2) Samples may require repeat analysis for confirmation or an extra sample dilution (see Section 8.E(19)).

(3) No numeric analyte results < LOD should be reported.

(4) No numeric analyte results should be reported for samples that were not analyzed, e.g., missing vial, sample disqualification, QNS.

11. Remedial Action if Calibration or QC Systems Fail to Meet Acceptable Criteria

If the calibration or QC systems fail, all operations are suspended until the source or cause of failure is identified and corrected. Analytical results are not reported for runs not in statistical control. Before beginning another analytical run, several QC materials (in the case of QC failure) or calibration verification samples (in the case of calibration failure) must be analyzed. Once calibration and/or quality control have been reestablished, analytical runs may be resumed.

12. Limitations of Method; Interfering Substances and Conditions

Care is required to prevent contamination of QC materials, calibrators, and samples. The chromatograms may show other mercury species due to the nature of the matrix analyzed in this procedure.

13. Reference Ranges (Normal Values)

The reference range for each mercury species (see Table 13) 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 13 Reference range for mercury species

Species Chemical Name	**Reference Range[1], µg/L**
Inorganic Hg	0.350 (90th percentile), 0.480 (95th percentile)
Methyl Hg	0.380 (50th percentile), 1.02 (75th percentile), 2.30 (90th percentile), 3.92 (95th percentile), 0.413 (geometric mean)
Ethyl Hg[2]	< LOD
[1] Above ranges are taken from NHANES population data for the 2015 to 2016 survey years as provided in the Fourth National Report on Human Exposure to Environmental Chemicals, Updated Tables, January 2019 (Volume One). Sample sizes were n=4938 for inorganic mercury and methylmercury. (http://www.cdc.gov/exposurereport/). [2] There are no established reference ranges for EtHg.	

14. Critical Call Results ("Panic Values")

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") to the QC reviewer as an "elevated result". The concentration assigned to the 2UB for a mercury is determined by study protocol, but the default concentration is currently 10 µg/L. The protocol for supervisors reporting elevated results to medical personnel is defined according to the study protocol. The American Conference of Governmental Industrial Hygienists has a biological exposure index (BEI) of 15 µg/L for inorganic mercury in blood (end of shift at end of work week) [24].

15. Specimen Storage and Handling during Testing

Specimens may reach and maintain ambient temperature (15°C to 30°C) during analysis. Take stringent precautions to avoid external contamination. After the samples are analyzed, return them to deep-frozen conditions (-50°C to -90°C, set point typically –70°C) as soon as possible.

16. Alternate Methods for Performing Test of Storing Specimens if Test System Fails

No alternate methods are available for measuring Hg species in blood. If an analytical system fails, prepared samples can be analyzed within 24 hours (see **Appendix A:** Stability). Store remaining sample aliquots according to the storage requirements described in Section 4 of this document, until the analytical system can be restored to functionality.

17. Test Result Reporting System; Protocol for Reporting Critical Calls (If Applicable)

Test results that are approved for reporting are exported as an electronic file from the LIMS database. The data may be combined in a reporting packet that could include items e.g., a cover letter, a table of method specifications and reference range values, a QC memo, and QC. After approval at the division level, the report will be sent to the designated contact person for the reporting of the results.

18. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

This protocol does not involve referral of specimens for testing the analytes of this method at another laboratory.

DLS uses a unique barcoded specimen ID number affixed to the sample to identify and track individual samples, and which links the laboratory information to demographic data recorded by those who collected the sample. To safeguard confidentiality, only the person outside of DLS requesting the laboratory analysis will maintain information which links the specimen ID number to the patient's name. Personal identifiers (e.g., names) are not included on samples. Barcoded sample IDs are indexed in STARLIMS to both a CDC specimen identifier (CSID) and a CDC unique identifier (CUID).

Retention of samples is determined by specifications in each study protocol. A copy of each study protocol is kept in DLS STARLIMS.

Location, status, and final disposition of specimens are tracked in DLS STARLIMS according to the IRAT specimen tracing SOP [document number TBD]. Records are maintained for a minimum of 3 years.

19. Method Performance Documentation

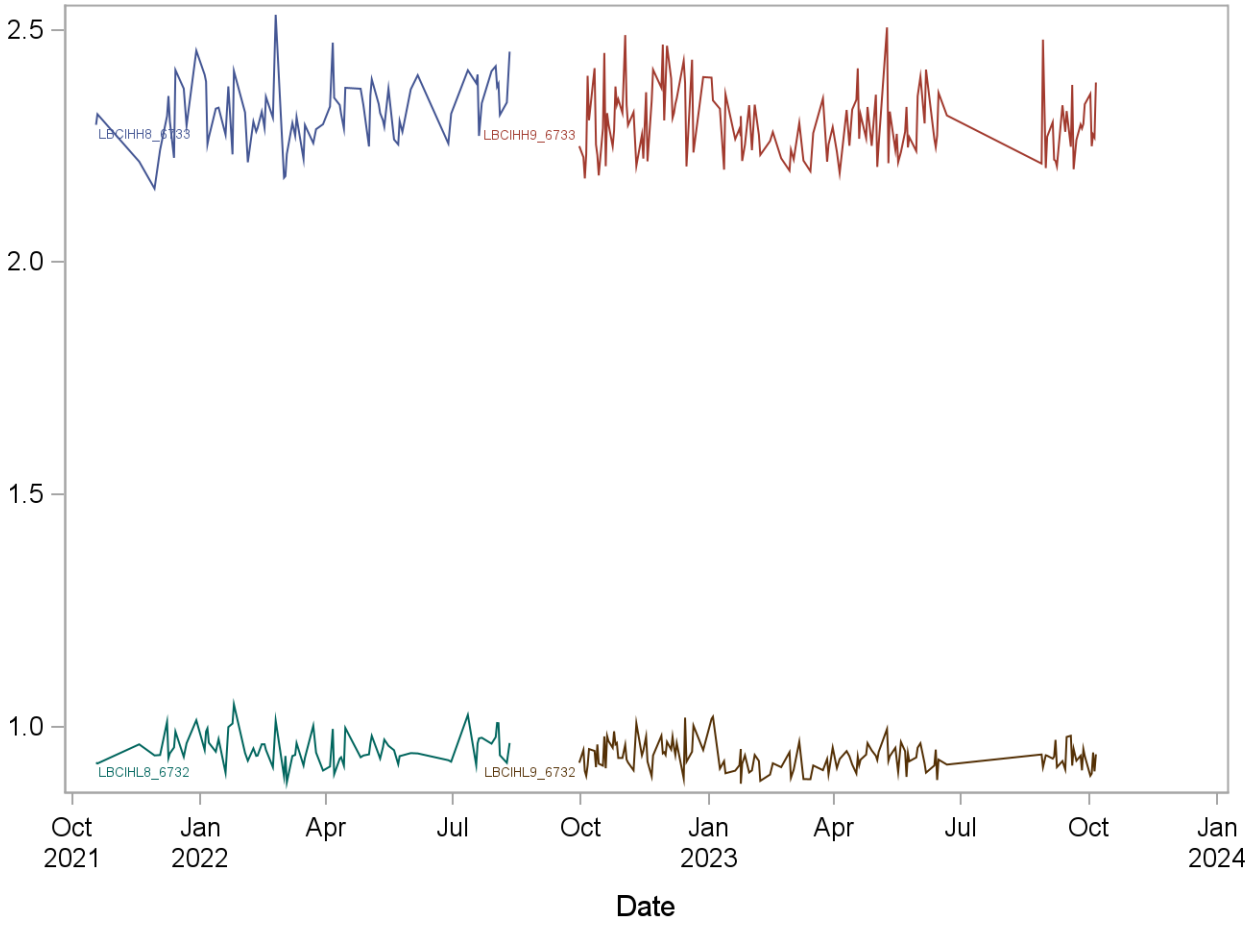
Method performance documentation for this method including accuracy, precision, sensitivity, specificity, and stability is provided in **Appendix A** of this method documentation. The approval of this procedure by the Branch Chief and CLIA Director denote that the method performance is fit for the intended use of the method.

20. Summary Statistics and QC Graphs

Please see following pages

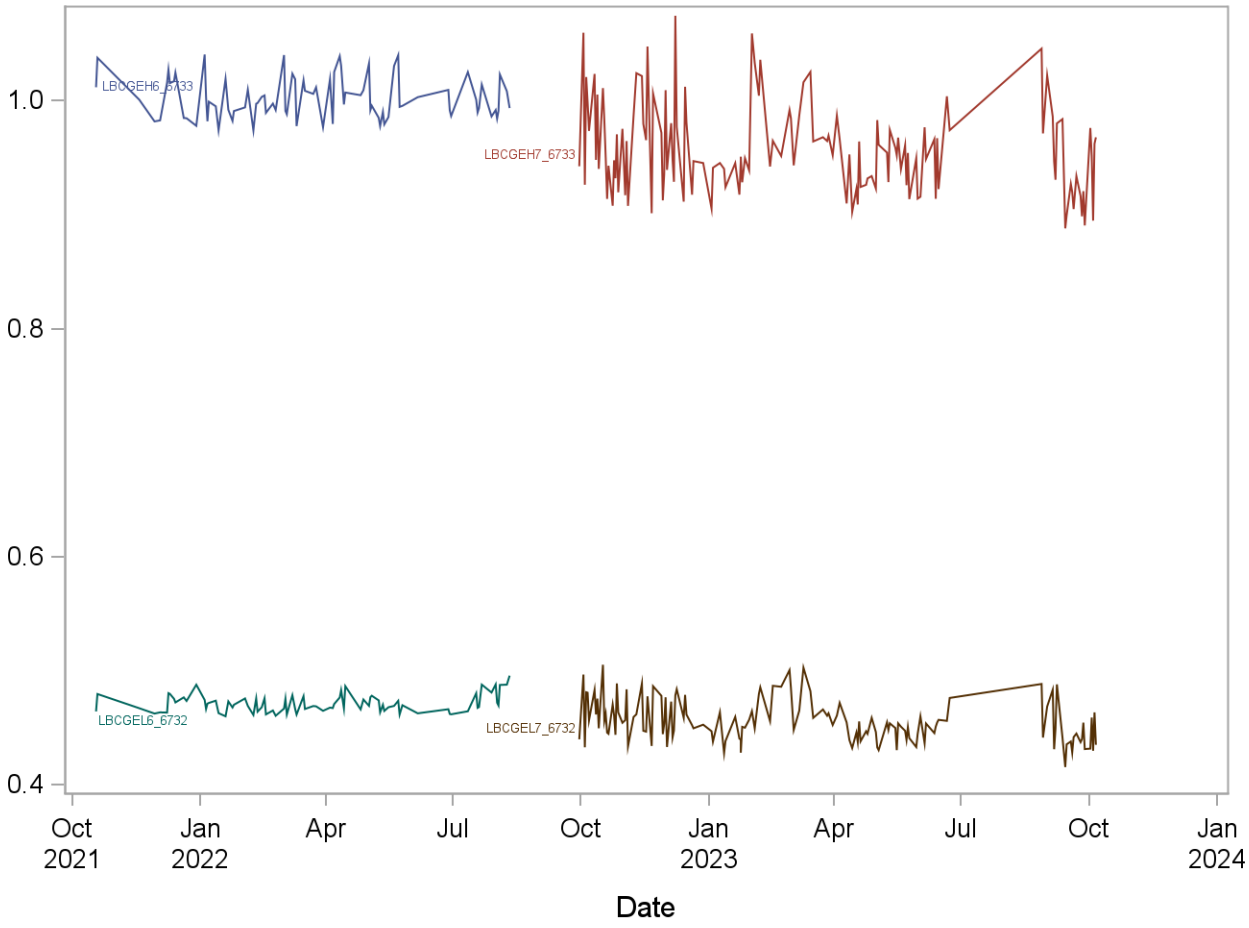
August 2021-August 2023 Summary Statistics and QC Chart
LBXIHG (Mercury, inorganic (ug/L))

Lot	N	Start Date	End Date	MEAN	Standard Deviation	Coefficient of Variation
LBCIHH8_6733	81	18OCT21	11AUG22	2.322	0.070	3.0
LBCIHH9_6733	138	30SEP22	06OCT23	2.299	0.073	3.2
LBCIHL8_6732	81	18OCT21	11AUG22	0.953	0.033	3.5
LBCIHL9_6732	138	30SEP22	06OCT23	0.935	0.029	3.2



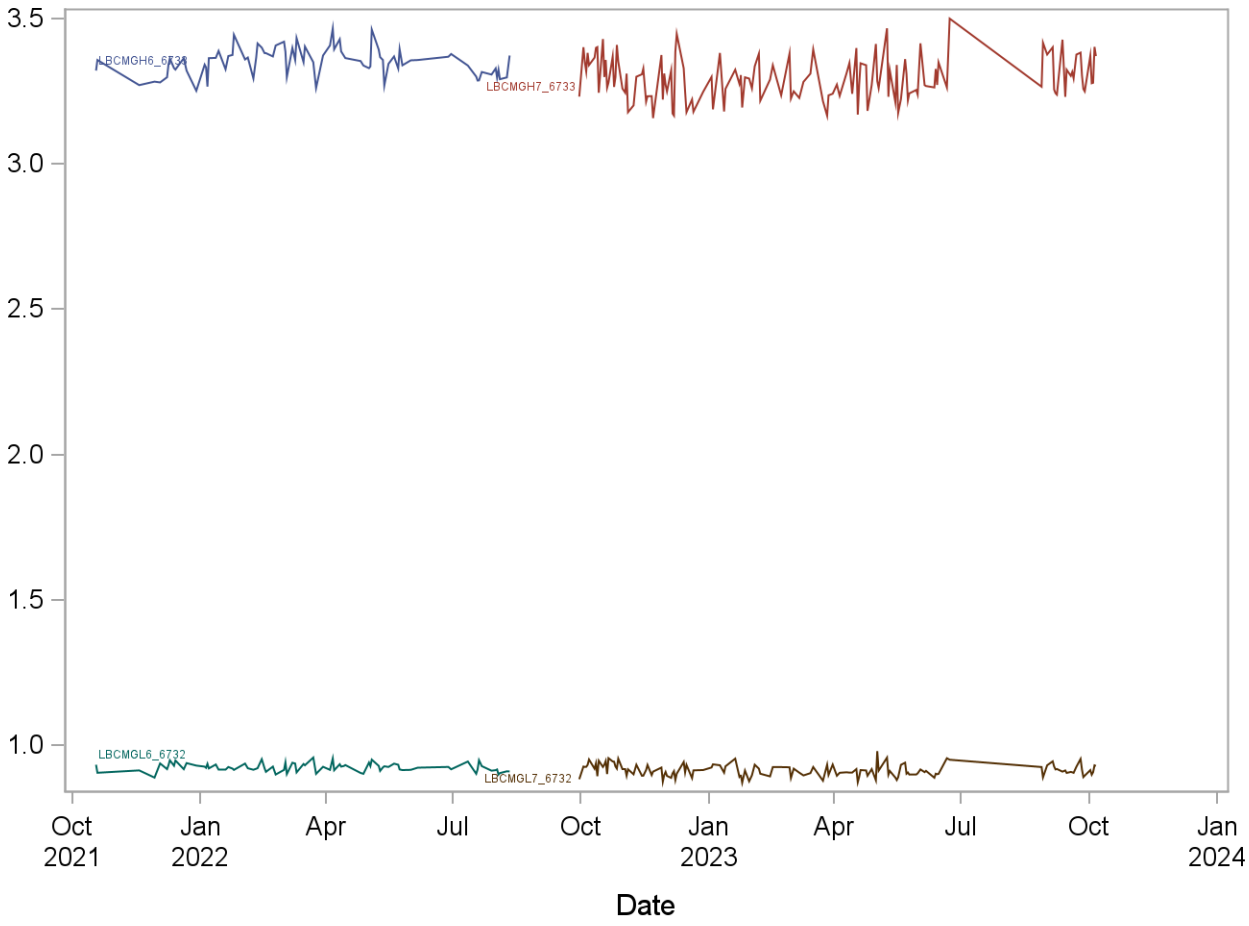
August 2021-August 2023 Summary Statistics and QC Chart
LBXBGE (Mercury, ethyl (ug/L))

Lot	N	Start Date	End Date	MEAN	Standard Deviation	Coefficient of Variation
LBCGEH6_6733	81	18OCT21	11AUG22	1.0020	0.0179	1.8
LBCGEH7_6733	138	30SEP22	06OCT23	0.9572	0.0391	4.1
LBCGEL6_6732	81	18OCT21	11AUG22	0.4712	0.0079	1.7
LBCGEL7_6732	138	30SEP22	06OCT23	0.4556	0.0185	4.1



August 2021-August 2023 Summary Statistics and QC Chart
LBXBGM (Mercury, methyl (ug/L))

Lot	N	Start Date	End Date	MEAN	Standard Deviation	Coefficient of Variation
LBCMGH6_6733	81	18OCT21	11AUG22	3.351	0.047	1.4
LBCMGH7_6733	138	30SEP22	06OCT23	3.294	0.073	2.2
LBCMGL6_6732	81	18OCT21	11AUG22	0.923	0.014	1.6
LBCMGL7_6732	138	30SEP22	06OCT23	0.913	0.021	2.3



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Acknowledgements

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)**

		Sample 1					Sample 2					Mean recovery (%)	SD (%)
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)		
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	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	1	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	1	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				

Figure 3 Accuracy data of the method evaluated using spike recovery for inorganic mercury

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)**

		Sample 1					Sample 2						
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
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	93.7	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	89.1	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	88.4	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				

Figure 4 Accuracy data of the method evaluated using spike recovery for methylmercury

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)**

		Sample 1					Sample 2					Mean recovery (%)	SD (%)
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)		
Sample	1	0.0	0.00	0.00			0.0	0.51	0.47				
	2		0.020	0.01	0.0			0.50	0.48	0.5		91.2	4.4
	3		0.012	0.00				0.47	0.48				
Sample + Spike 1	1	4.34	4.08	4.34			4.34	4.57	4.67				
	2		4.00	4.14	4.2	95.9		4.95	4.64	4.7	96.6		
	3		4.20	4.26				4.73	4.5				
Sample + Spike 2	1	8.69	7.68	8.06			8.69	8.63	8.19				
	2		7.43	7.92	7.8	90.1		8.26	8.50	8.4	91.4		
	3		7.95	8.00				8.49	8.51				
Sample + Spike 3	1	13.03	10.6	11.5			13.03	12.0	11.7				
	2		11.6	11.6	11	85.7		12.7	11.9	12	87.4		
	3		10.2	11.5				11.7	11.2				

Figure 5 Accuracy data of the method evaluated using spike recovery for ethyl mercury

Precision

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

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

Figure 6 Precision of the method evaluated using total relative standard deviation for inorganic mercury

Precision

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

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

Figure 7 Precision of the method evaluated using total relative standard deviation for methylmercury

Precision

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

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

Figure 8 Precision of the method evaluated using total relative standard deviation for ethyl mercury

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**

Figure 9 Assessing stability of inorganic mercury in whole blood samples.

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:	**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**

Figure 10 Assessing stability of methylmercury in whole blood samples.

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**

Figure 11 Assessing stability of ethyl mercury in whole blood samples

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

Figure 12 LOD, specificity and fit for this method

Appendix B: Ruggedness Testing

A. Critical Parameter Test #1

Evaluate the significance of the GC injector temperature

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 an injector temperature of 220°C (the method prescribed temperature).

(6) Repeat the analysis using an injector temperature of 230°C (increased temperature).

Table 14 Critical Parameter Test 1 Results: Temperature of the GC injector

Critical Parameter Test 1 Results. Temperature of the GC injector. One quality control pool was tested. Results below are the averages of observed results at each condition along with the 95th 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)**	**(0.71-1.37)**	**(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.

A. Critical Parameter Test #2

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

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 (RT) ~ 25°C (the method prescribed temperature).

(6) Repeat the analysis using an equilibration/extraction temperature of 50°C (increased temperature).

Table 15 Critical Parameter Test 2 Results. Temperature of the CombiPAL

Critical Parameter Test 2 Results. Temperature of the CombiPAL® – agitator equilibration/extraction. One quality control pool was tested. Results below are the averages of observed results at each condition along with the 95th 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 (RT) (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)**	**(0.71-1.37)**	**(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 at RT ~ 25°C was the optimal choice.

B. Critical Parameter Test #3

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

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).

Table 16 Critical Parameter Test 3. Length of extraction time.

Critical Parameter Test 3. Length of extraction time. Results below are the averages of observed results at each condition along with the 95th 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)**	**(0.71-1.37)**	**(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.

C. Critical Parameter Test #4

Evaluate the significance of changing GC split ratio.

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).

Table 17 Critical Parameter Test 4. GC split ratio.

Critical Parameter Test 4. GC split ratio. Results below are the averages of observed results at each condition along with the 95th 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)**	**(0.71-1.37)**	**(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.

D. Critical Parameter Test #5

Evaluate the significance of changing GC carrier gas flow rate.

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).

Table 18 Critical Parameter Test 5. GC carrier gas flow rate

Critical Parameter Test 5. GC carrier gas flow rate. Results below are the averages of observed results at each condition along with the 95th 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)**	**(0.71-1.37)**	**(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.

Appendix C: General instructions for uploading data to Starlims

(1) Open the STARLIMS application and select “Pending Runs Assigned to My Labs”.

(2) Select the method from the Test drop down menu.

(3) Click “(+)Add.” Select the instrument system used with the run and click “OK.”

(4) Click the “All Results (S)” tab. If required, click “[0] Run Instrument Macro.” Once Excel opens, select “Enable Content” and follow the prompts on screen to select method, upload instrument data file (.csv). Save the file in the folder along with the instrument data file and then close it.

(5) Click “[1] Upload Instrument File”. Use the browser to navigate to the location of the Excel file to be imported. Select your Excel Workbook file, review the data, and click “Upload”. Only recognized sample IDs will be uploaded into STARLIMS. Click "Continue".

- If STARLIMS warns that there is a run date and time stamp conflict between this run and any previous run, ensure you selected the correct file for upload before proceeding. In the ‘add samples to run’ window ensure sample IDs for the run are recognized as eligible before clicking continue. On the ‘results not imported’ window ensure that only sample IDs not intended for upload are listed before closing the window. Click "Continue".

(6) Document ‘not tested’ samples, if necessary (e.g., blood clot, missing or empty vial, quantity not sufficient) using one of the options below.

a) Click on the Run Sheet Details tab. Click on the highlighted ‘Click to Search’ text. Use the search criteria to populate the samples pending assignment list. Select the sample IDs to add to the run and click the “>” arrow button. Further sample information will be added in later steps as the entire run is processed.

b) Click on the Run Sheet Details tab. Click on “Add Unknowns by Barcode” link. Enter the sample ID by typing or barcode scanning. Click ‘Ok’ to directly add the ID to the run list. Further sample information will be added in later steps as the entire run is processed.

(7) Select “[2] Mark Null Results”. This will change any null values in NUMRES (i.e., observed result) to a pipe tab (|) (e.g., ‘not tested’ samples or analytes not tested for any samples).

(8) Select “[3] Evaluate Sample QC.” This will execute the STARLIMS spec schema for the method and assign each sample a Results Status message (e.g., Pass, Fail, Warn) and a Status Message (e.g., Pass, > 1UB, < LOD, etc.).

- Review these Results Status and Status Message columns and if necessary, manually override the automated entry in “Sample QC Passed” and enter an analyst comment. See Table 19 for examples of reasons to override automated “Sample QC Passed”. If you are unsure, discuss with your supervisor.

(9) Select “[4] Evaluate Run QC”. In the pop-up window, the QC pools from the run should automatically appear. In “For the following QC results” section, select the radio button “The

prior N* results within this date range," and enter N=40. In "From the following instruments" section, select "All instruments."

(10) Choose "Proceed to next step" on the QC Evaluation box. If there are not enough QC records within the selected date range, select "Cancel" and expand the search date range. If the number of records is adequate, select "Start the SASQC Wizard" to begin evaluating the QC criteria.

(11) Select "Save SAS Input File" as "STARLIMS_SAS_data". The default location for this file is C:\Temp, change the network location to save the SAS file in the same /Nutritional/Instrument folder file that the data is stored in. Click "Save". Select "OK" when export is complete.

(12) Click "Send to SAS Server". Check that your run number has passed and that your SAS file is referencing at least 20 runs. Save a copy of this Report as a .pdf in the data network folder. Select "Finished".

(13) Click "[5] Set Run QC Statuses". Select "Proceed" if the QC passes; otherwise change the status to fail before proceeding.

(14) Click "[6] Attach SAS QC File". Select the PDF version of the SAS output previously saved (from Step 12).

(15) Using the field filtering tool located in the top row of the data field, select "N/A" in the QC Type column.

(16) Click "Set Final Wizard." Click "Proceed." Check "Require Sample QC passed" and "Require Run QC Passed" in "Step 1: Choose the Set Final selection criteria," and select "Pass" for "Allowable Result Statuses for Set Final." Click "Proceed." Verify that the correct number of results have been set final. Click "OK." If the number of results is incorrect (due to incorrect sample IDs used or other issues), the run will have to be deleted and reimported.

- If multiple results for a sample/analyte have been uploaded, the "Set Final Wizard" will prompt you to select which result to set final. Set final the first acceptable/repeatable result and enter appropriate comment code (common examples: 32 – "dilution required," 33 – "repeated and confirmed.")

(17) Before Finishing Results, verify all sample and run QC passes are set correctly. Attach the required files to the run, e.g., Instrument file (.xlsx), SAS QC plot (.pdf), Instrument performance (.pdf), Calibration curves (.pdf), Report/chromatograms (.pdf).

(18) Fill in Run Comments and required User Fields. See Table 20 for more information.

(19) Once you choose "Finish Results", you will not be able to make revisions. When you are ready to report the results, click "Finish Results". Select the names of the individuals that you want to send your run to (the branch QA/QC officers, lab chiefs, etc.) and include your run comments in the comments section.

(20) Notify the appropriate personnel via email that the run is ready to be reviewed.

Table 19 Examples of reasons to manually override the automated "Sample QC Passed" in Starlims.

Event	Comment code
Blood clot	11 (small clot) or 12 (clotted sample)
Result > High calibrator	103
Missing vial	18
Quantity not sufficient (QNS)	21

Table 20 User fields in Starlims

Field	Recommendations	Required	Examples
Run Comments	Enter brief information related to the batch outcome, e.g.,: ▪ QC failures ▪ Confirmations ▪ Elevated results(>2UB) ▪ Confirmation run only, if no results were set final ▪ Dilutions performed ▪ For LOD runs, Instrument comparison, Monthly SRM, Analyst evaluations runs please indicate.	Y	▪ UPB failed QC. ▪ Confirmations for BPB and BMN ▪ Elevated results (>2UB) ▪ Confirmation run only, no results set final ▪ Dilutions performed ▪ LOD run ▪ Ext. Calibrator run ▪ Elevated results, dilutions performed, confirmations for USR and UPB. ▪ BMN failed QC, confirmations for BPB.
1	Other general comments about the analytical run: ▪ If calibrator is dropped from curve, please specify. ▪ If a different blank was used, please indicate QA metric information, if applicable*	Y	▪ Dropped Std.2 for UPB calibration curve. ▪ Reprocessed with aq. blank ▪ Reprocessed with 2nd cal. Curve Sample diluted and reanalyzed
2	Calibrator lot number or ID, or Spike solution ID	Y	▪2022_2208_jyy3
3	DLS Study number of samples in the run: ▪ DLS study ▪ PT name ▪ Note: R&D, LOD determination, Analyst comparison, if applicable	Y	▪ 2012-0036, NH99 ▪ NH99 ▪ CTQ QMEQAS, NH99 ▪ Analyst evaluation ▪ Monthly SRM
4	Sample Group #	Y	▪ SpcAs-1752 ▪ ICS22L01
5	Corrective action	Y (if performed)	▪ changed column ▪ new spike solution was prepared and samples re-spiked and redigested

Field	Recommendations	Required	Examples
6	Column ID	Y (chromatographic method)	▪ Column Serial Number 308
7	Lab Chief / Team Lead check	N (only used for R&D runs)	▪ DMJ
8	Hamilton Identifier	Y	▪ ML600FG9017

Appendix D: Template for Working Triple Spike Standards Solution

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		*(µg/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 addtion	*(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 ÷ line 2d)					
		3e.	*New concentration of isotopes (dilution #1)**	*(µg/g)*				
			** If diluted, otherwise concentration remains unchanged from original stock standard solution.*					
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)*				
			(for isotopes not diluted in Step 3, copy value from line 2d to line 6c)					
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)	*(µg/g)*				
		6b.	Dil. #2 dilution factor ([line 7a - line 4a] ÷ line 6c)					
		6c.	*Final Diluted Working Spike Conc (line 3e ÷ line 6b)*	*(µg/kg)*				
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)*				

Figure 13 Excel template used for calculations using ISC standards.

Appendix E: Electronic Instrument Performance log sheets

Instrument name:												
Information in the table below is only required on days the instrument was used												
After 10 days of instrument use: copy a new table from the Performance Temp worksheet, and paste below the current table												
If instrument sensitivity is low (e.g., peak areas are not > 300 cps) perform instrument maintenance on Instrument Maintenance worksheet.												
											"10 run" maintenance (complete after 10 days of use)	
Date of instrument use (mm/dd/yy)>>											**Date (mm/dd/yy)>>**	
Operating Analyst (User ID)											**Operating Analyst (User ID)**	
Instrument Performance Records											**I = inspect; R = replace**	
Vacuum Press Before plasma ignition (enter NA if plasma was already on)											Glass Liner	
Vacuum Press. After plasma ignition											GC Injector Septum	
Detector Voltage (Pulse Voltage value)											Left SPME Fiber	
MeHg Peak Area-LB QC diluted 10X (>300 cps) (enter √ = pass, X = fail)											Right SPME Fiber	
EtHg Peak Area-LB QC diluted 10X (>300 cps) (enter √ = pass, X = fail)											Other Notes	
IHg Peak Area-LB QC diluted 10X (>300 cps) (enter √ = pass, X = fail)												
Type of Analysis Work/Group Numbers Analyzed (enter NA if samples were not analyzed)												
Triple Spike ID (enter NA if samples were not analyzed)												
Notes												

Figure 14 Electronic instrument performance log sheet

Instrument service: record date of service below for quick reference; see paper instrument service log in the instrument logbook for additional details and resolutions													
Computer maintenance: record date of maintenance below; complete montly													
Instrument maintenance: performed when instrument sensitivity is unacceptable; use the Notes section to indicate additonal reasons for maintenance. Can involve cleaning and/or replacing items listed below, however inspection is allowed													
Xe optimization (X-Y): is performed everytime the plasma is turned ON													
Do not leave empty cells in the tables below. When all columns of a section are filled, copy a new section from the Maintenance Temp worksheet and paste it below; do not add colums.													
Instrument service - record date service occurred													
ICP-MS service from vendor (PMV or other service)													
PAL autosmapler service from vendor (PMV or other service)													
GC service from vendor (PMV or other service)													
Computer maintenance - record date of maintenance													
Computer reboot													
Verify ISLE and MUST drive backups													
Instrument maintenance if instrument perfomance is poor I=inspected R=replaced													
Date of maintenance (mm/dd/yy)>>													
Analyst performing maintenance (User ID)													
Injector													
Torch													
RF Coil													
Sampler cone (Sa)													
Skimmer Cone (Sk)													
Xe optimization (X-Y) (Y= yes, N= no)													
Xe (cps) (enter NA if not performed)													
Xe % RSD (enter NA if not performed)													
GC Injector													
GC Capillary Column													
Capillary Column Cut (enter "I" for inspected, or "cut")													
Notes													
Xe optimization (X-Y) only after plasma ignition													
Date (mm/dd/yy)>>													
Operating Analyst													
Xe (cps)													
Xe % RSD													

Figure 15 Electronic instrument maintenance log sheet

Appendix F: Operation of the UltraCLAVE Microwave Digestion System for the Mercury Speciation Analytical Method

A. Background

During the preparation of samples for the mercury speciation isotope dilution method, due to the amount of protein and fat in blood, it is necessary to use a strong acid or base to release metallic species. Tetramethylammonium hydroxide (TMAH) is used in method DLS 3020 to solubilize protein and release bound mercury species. TMAH was chosen because it produces precipitate-free digested samples, therefore eliminating the need for a centrifugation step in sample preparation. At room temperature, approximately 72 hours is required after TMAH addition before the mercury species can be quantified using this analytical method. Placing blood samples in a convection oven for approximately 20 hours at 80°C accelerated the digestion process and became the chosen digestion route. This digestion method works well; however, 20 hours is not practical in situations where expedited analyses are required. To reduce the digestion time and make the method more suitable for emergency response type analyses, we developed a new digestion method using the Milestone UltraCLAVE microwave digestion system which takes less than one hour. In terms of advantages, the UltraCLAVE microwave is easy to use, requires minimal maintenance, and allows for high sample throughput. Additionally, this system allowed us to reduce the digestion time without making any additional modifications to our sample preparation procedure.

B. Safety Precautions

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

Follow universal precautions. 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 urine, 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 10% (v/v) sodium hypochlorite solution. Dispose of all biological samples that are slated for disposal and diluted specimens in a biohazard autoclave bag at the end of the analysis according to CDC/DLS guidelines for disposal of hazardous waste.

C. Objective

This appendix will describe the steps needed to operate the UltraCLAVE Microwave Digestion System for the speciated mercury method (DLS 3020 Blood mercury speciation performed by TSID-GC-ICP-DRC-MS). The steps used for the microwave digestion method were programmed during the set-up of the station and do not have to be modified unless there is a change in the process. Please refer to the UltraCLAVE User Manual for the detailed description of the UltraCLAVE system, safety rules, operations, service/maintenance, and easyCONTROL software.

D. Consumables and System Components

(1) Consumables

a) UltraCLAVE Metal O-ring (Catalog #: WZ00046) Milestone, Shelton, CT or equivalent.
b) UltraCLAVE Flat O-ring (Catalog #: OR0001) Milestone, Shelton, CT or equivalent.
c) Heavy wall 12x75mm borosilicate glass test tubes (Fisher brand catalog #: 14-958-10B) or equivalent.

(2) System Components

a) Microwave Digestion System (UltraCLAVE, Milestone Inc., Shelton, CT) or equivalent.
b) 3.5L PTFE base load vessel (Catalog #85260, or equivalent, Milestone UltraCLAVE)
c) 77 position WEFLON-PTFE sample rack (Catalog #: UC85034) Milestone, Shelton, CT or equivalent.
d) Chiller: Lab Tech SMART H150-1000 or equivalent.
e) Compressor: L&W Compressors LW-100-E1 or equivalent.
f) Exhaust/ventilation
g) System computer (TERMINAL 1640, easyCONTROL software)

(3) Chemicals and Solutions

a) Water, high purity ≥ 18 MW×cm resistivity (Aqua Solutions, Jasper, GA)
b) Sulfuric Acid, (CAS# 7664-93-9), (H_2SO_4-MW 98.08), (Fisher Scientific, Norcross, GA), TraceMetal grade, item # A510-P500, or equivalent.
c) Hydrogen Peroxide, 30% solution, stabilized (CAS# 7722-84-1), (H_2O_2, MW 34.0), (EMD Millipore, Germany), item #386790, or equivalent.
d) Nitrogen gas, high purity (item #NI HP300CBA, Airgas, Atlanta, GA)
e) Bleach (1:10 dilution of a standard sodium hypochlorite solution) from any vendor.

Note: To prepare base load for digestion, 2mL of sulfuric acid and 30 mL of hydrogen peroxide are added to 320mL of DI water (Detailed preparation instructions are found on Pg. 17 “Digestion Method 2” of Section D “Sample Preparation”).

Figure 16 How to prepare base load for digestion

E. Procedure

(1) Start-up

a) Turn the UltraCLAVE main switch ON (located on the bottom, right side of the system).

b) The UltraCLAVE is ready to start. The terminal is automatically turned on, and the program is initialized.
c) On the instrument controller (laptop-like item), click on the UltraCLAVE icon (top right corner), select Administrator and put in the password “123456” or User with the password “123” to open the program that controls the microwave.
d) After Login, the start page of the easyCONTROL-640 (software operating the UltraCLAVE) is opened.

The *ultraCLAVE* program is divided into four sections:

Method	To create, control, save and load methods.
Sample	To gather all sample data in sample report/table. Here user can save, load and print out sample reports/tables.
Run	Here are graphically represented in real time the curves/profiles of temperature, pressure and power. Process runs can be saved, loaded and printed out.
System	Here it is possible to follow the status of measurement during process run. In case of stop of the automatic run, the system is operated directly from here.

Figure 17 Instrument controller window

e) The status bar (as shown below) which is found below the four sections above shows the current parameters.

A	t = Time elapsed from microwave program start	**D**	T2 = External temperature of reactor wall
B	P = Internal pressure of pressure reactor	**E**	E = Microwave power
C	T1 = Internal temperature of pressure reactor		

Figure 18 Status bar showing current parameters

f) Refer to the subsection “Digestion Method 2” in Section 8.B(3) for instructions on the preparation of samples for microwave digestion.

g) Place the sample rack with pre-labeled 12x75mm glass test tubes containing blood samples on the hanger in the vessel area (see the following figure taken directly from the UltraCLAVE users’ manual).

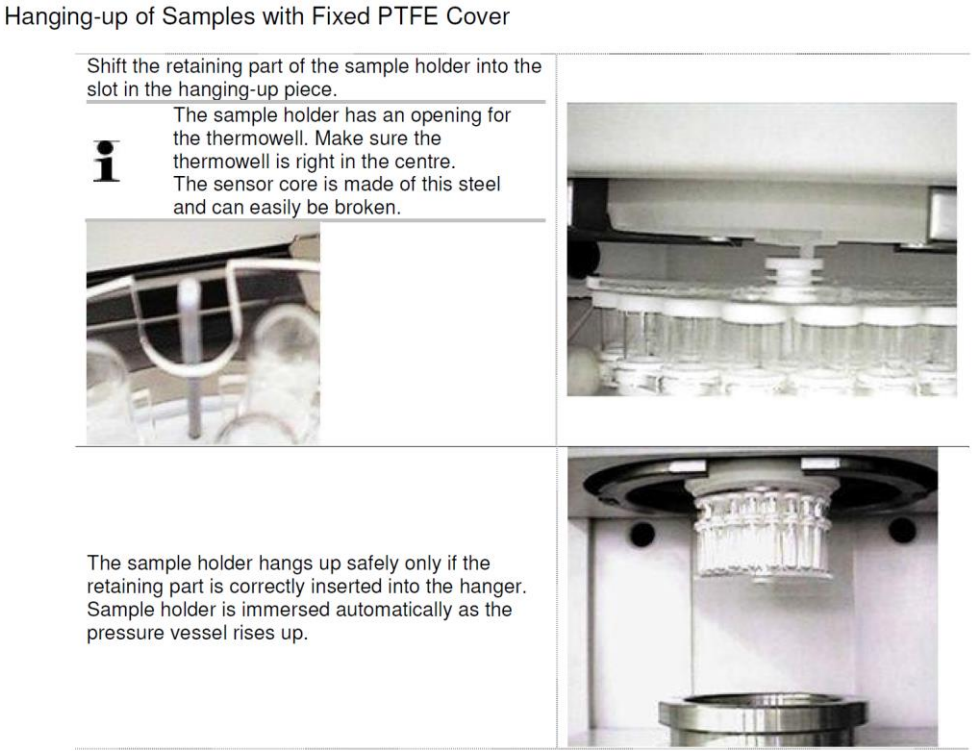


Figure 19 Hanging-up samples with fixed PTFE cover

h) Pull the black knob on the side of the UltraCLAVE cover, and close the Plexiglas shield.

i) Close and lock the doors of the UltraCLAVE enclosure.

j) Open the nitrogen cylinder valve. The pressure should be set at 80 psi.

k) Turn on the chiller, which should be set at 8°C.

(2) Starting the Run

a) Click on the “Method” tab.

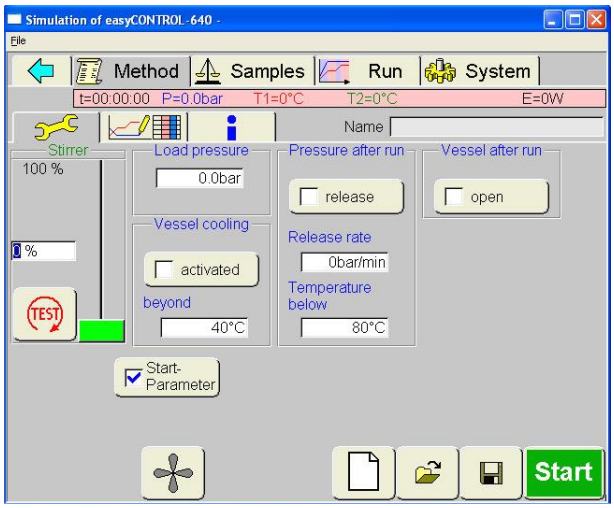


Figure 20 Method tab

b) The  tab is where parameters controlling the UltraCLAVE microwave's initial and ending operations can be filled in.

c) The parameters used for this method are listed in **Error! Reference source not found.**:

Table 21 easyCONTROL parameters

Parameter	Setting or Status
Load pressure	40 bar
Vessel cooling	✓
Pressure after run	✓
Vessel after run	✓
Release rate	10 bar/min
Beyond	50°C
Temperature below	80°C

d) On the next  tab, parameters (Time, Temperature, Power, and Pressure) for sample digestion are inputted. **Error! Reference source not found.** lists the parameters used for this method.

e) The "Info" tab -  is where additional information about rack and reactor vessels can be added (such as any notes specific to that digestion).

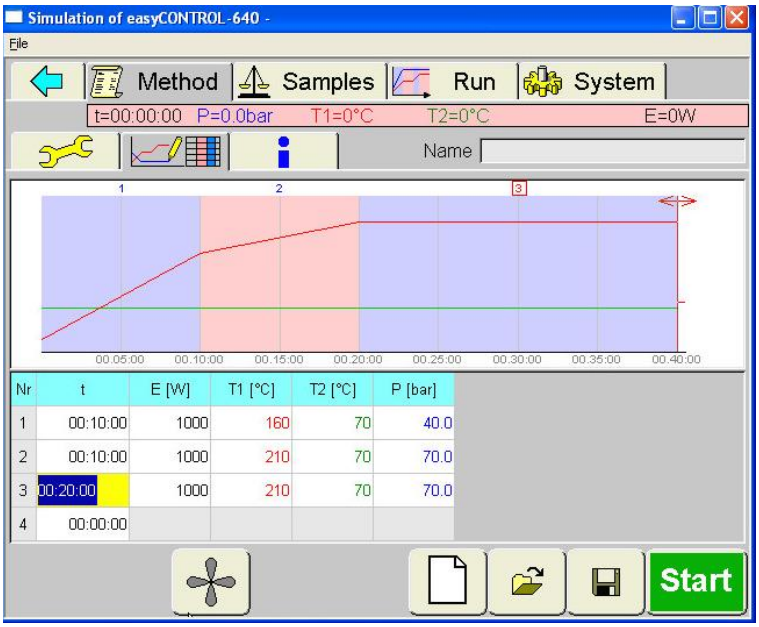


Figure 21 Method tab displays time, temperature, power, and pressure

Table 22 UltraCLAVE Digestion Settings

Time (h:m:s)	Energy (W)	T1 (°C)	T2 (°C)	P (bar)
00:10:00	1000	150	80	100
00:04:30	1000	150	80	100

f) When all the parameters are filled in, press the green “START” button. This method is now active, and sample processing will commence.
g) The entire digestion time is approximately 40 minutes including cooling of the system.
h) While the microwave program is running, the software monitors all the controlled parameters data. The profiles of temperature, pressure and microwave energy are plotted in real time in the “Graph” section (same as “RUN” tab on some versions of the software).

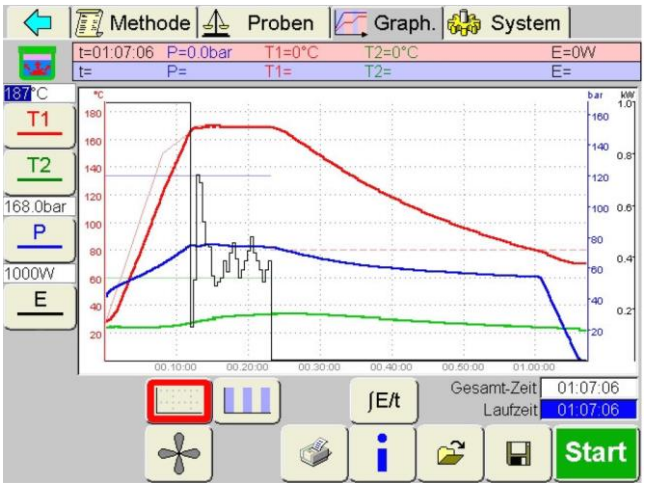
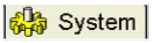


Figure 22 Graph displaying time, temperature, power, and pressure during the digestion run

i) At any point during the run, the UltraCLAVE can be stopped with the emergency STOP-Button 

. While the system is in operation, the "Stop" button shows up instead of "Start" in the picture above. Additionally, there is a large red emergency STOP button on the control panel of the UltraCLAVE instrument  that can be pressed.

j) By selecting 

, it is possible to follow the status of each UltraCLAVE mechanism during the process of a run. In case you need to stop the automatic run, the system is operated directly from here.

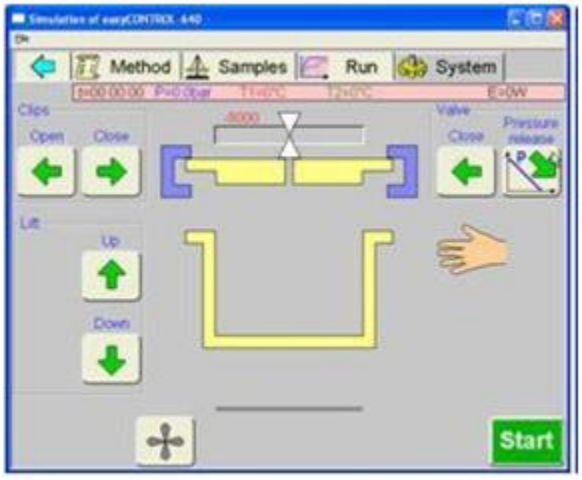


Figure 23 System tab displaying status of UltraCLAVE mechanism during the process of a run

k) After the run is complete, save the data by pressing tab in the "RUN" window. Name the method "date_temperature_sample(group)name".

(3) Cleaning the microwave digestion system

a) Using a deionized water squirt bottle, rinse the three channels (measuring block, pressure inlet, and pressure drain valve).

Figure 24 Cleaning procedure, rinsing three channels

b) Wipe and dry the PTFE cover, the steel cover laterally, and the clamps with paper towels to remove the condensate.

Figure 25 Drying procedure of the microwave digestion vessel

c) After drying, spray a small amount of "The Dry Lube" (B'Laster Corporation, Cleveland, OH or equivalent) on a clean cloth, and rub it on the clamps to protect them from corrosion.

d) Both the O-ring for pressure sealing (green colored pressure sealing O-rings) (A) and the metal high frequency ring (B) must be intact. If either is damaged, replace right away. The O-ring should be lubricated with Silicon Grease (RS Components Ltd., UK or equivalent) upon installation and after every three or four digestions.

e) Take out the PTFE -insert vessel (picture below), and discard the base load solution. Rinse and then dry the PTFE vessel and the inside of the metal vessel. After drying, spray a small amount of "The Dry Lube" onto a clean cloth and wipe the sides of the metal vessel where the clamps close.

Figure 26 Inserting digestion vessel into pressure chamber

F. Documentation of System Maintenance

Computer Maintenance: Record any maintenance of computer hardware in the instrument logbook. When changes are made to methods, back up the methods to CD. Make two copies – place one in the laboratory and one in the fireproof CD storage location. Also, place the backup file for the methods on the shared network drive under the instrument folder for the UltraCLAVE.

Instrument Maintenance: Document system maintenance in the instrument logbook and assigned laboratory notebook. Record any maintenance performed by service engineers.

G. Troubleshooting

This section will serve as a troubleshooting guide for the issues experienced by this laboratory during operation of the microwave. This section will be amended as needed as problems are encountered. Any issue not listed in this section will have to be discussed with a service engineer.

Clamp overload: If during the beginning or end of the microwave operation an error window appears that gives a message “left or right clamp overload”, this means that the vessel cannot be opened/closed and the clamps are stuck. Go to the “System” tab and manually open the clamps by pressing the green arrows (see picture below highlighted by red box). Then lower the vessel by pressing the “down” green arrow (selected by black box). Lubricate clamps before next digestion. If the problem persists, contact a service engineer.

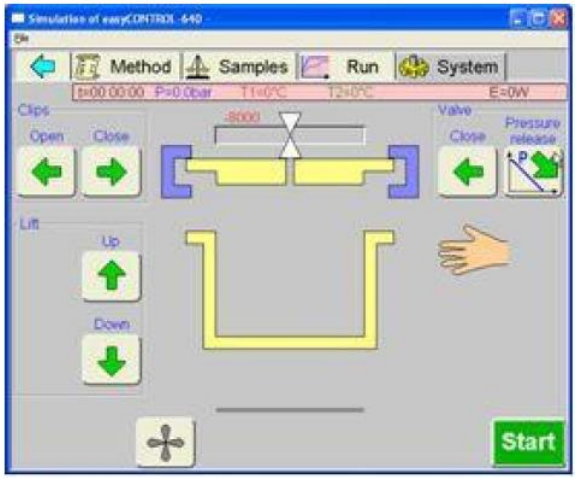


Figure 27 System tab showing the status of UltraCLAVE mechanism during the process of a run

Compressor trips a breaker: L&W Compressors LW-100-E1 trips the breaker as it goes over allowed power usage (>20 Amps). This sometimes happens if the nitrogen tank is too low. The nitrogen is set to 80 psi, but it has been observed that when the cylinder gets close to 500 psi, the compressor trips the breaker. If the breaker is tripped and the nitrogen cylinder is not 100% full, replace it. If the problem persists, contact a service engineer.

Appendix G: Programming NexION® Method

A. 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.”

B. 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:\Users\Public\Documents\PerkinElmer Syngistix\ICPMS\Method” and click on the "Hg DRC.mth" file followed by "Open".

(2) If the "Hg DRC" 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:

(a) 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 **Error! Reference source not found.**.

Table 23 NexION® Timing Parameters

Parameter	Setting
Sweeps/Reading:	1
Readings/Replicate:	2272
Number of Replicates:	1
Tuning File:	C:\Users\Public\Documents\PerkinElmer Syngistix\ICPMS\MassCal\Default.tun
Optimization File:	C:\Users\Public\Documents\PerkinElmer Syngistix\ICPMS\Conditions\Default.dac

(b) 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 **Error! Reference source not found.**.

Table 24 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 per AMU (ms):	25
Integration Time (ms):	56800 (automatically determined by software)
Profile:	Argon DRC
Argon Flow:	0.5†
RPa:	0
RPq:	0.3†

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

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

(c) Click on the "Processing" tab and enter the following information ():

Table 25 NexION® Processing Parameters

Parameter	Setting
Detector:	Dual
Blank Subtraction:	After Internal Std.
Measurement Unit:	Cps
Process Spectral Peak:	Average
Process Signal Profile:	Average
Baseline Readings:	0
Apply Smoothing:	Unchecked
QID:	Off
Isotope Ratio Mode:	Off

(d) Skip the "Equation" tab. Click on the "Sampling" tab and enter the following information (**Error! Reference source not found.**):

Table 26 NexION® Sampling Parameters

Parameter	Setting
Peristaltic Pump Under Computer Control:	Checked
Sampling Device:	External

(e) Click on the "Report" tab and enter the information in **Error! Reference source not found.**.

Table 27 NexION® Report Parameters

Parameter	Setting
Report View | Send to Printer:	Unchecked
Report Options Template:	<leave empty> *
Automatically Generate NetCDF File:	Checked C:\Users\Public\Documents\PerkinElmer Syngistix\ICPMS\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	Append *
Use International Character Set	Unchecked

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

(f) Choose menu item "File" > "Save As" and navigate to the "C:\ Users\Public\Documents\PerkinElmer Syngistix\ICPMS\Methods\" folder. Enter "Hg DRC" as the name of the method file and click the "Save" button.

(g) The NexION® method "Hg DRC" is now loaded into memory.

Appendix H: Setting up TotalChrom Integration Method and Chromlink

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**Error! Reference source not found.**. 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.

(4) Click on the "Baseline Timed Events" tab. Enter the information shown below in **Error! Reference source not found.**. The analyst may make appropriate changes to one or more of the Baseline Timed Events as may prove necessary.

(5) Click on the "Optional Reports" tab. Uncheck the box for "Keep temporary files".

Table 28 Integration

Basic Parameters		**Advanced Parameters**	
	Value		Value
Bunching Factor (pts):	1	Peak Separation Criteria	
Noise Threshold (µV):	73	Width ratio :	0.200
Area Threshold (µV):	368.00	Valley-to-peak ratio :	0.010
		Exponential Skim Criteria	
		Peak height ratio :	5.000
		Adjusted height ratio :	4.000
		Valley height ratio :	3.000

Table 29 Baseline Timed Events

Defined Events				
Time	Event	Value	Code	Level
0.000	Set Area Threshold	100.00	AT	
0.000	Set Noise Threshold	25	NT	
0.000	Smooth Peak Ends On	5	+SM	
0.000	Set Bunching Factor	3	BF	

(6) Click on the "Replot" tab. Enter the information shown in **Error! Reference source not found.**. The analyst may make appropriate changes to one or more of the Replot parameters as may prove necessary.

(7) 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.

(8) 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 **Error! Reference source not found.**.

(9) The "LIMS Results" tab is not used. Click the "OK" button to close the window. The parameters in **Error! Reference source not found.** are starting points. The analyst may make appropriate changes to one or more of the Global Information parameters as may prove necessary.

Table 30 Replot

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

Table 31 Global Information

Volume units :	µL	Unidentified Peak Quantitation	
Quantitation units :	ng	Calibration factor :	1.000000e+06
Sample Volume (µl) :	1.000	Always use calibration 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		

(10) 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 **Error! Reference source not found.**.

Table 32 Method Editor -- Components Settings

Name	Retention Time	Absolute window (s)	Relative window (%)	Find tallest peak in window
Hg^0	1.203	2.00	3.00	Not checked
InHg	3.122	0.00	3.00	Not checked
MeHg	2.312	0.00	3.00	Checked
EtHg	2.833	0.00	3.00	Not checked

(11)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.

(12)In the Method Editor window, choose the menu item "Components" > "Defaults." Click on the "Identification" tab". Enter the information shown in **Error! Reference source not found.**.

Table 33 Components Defaults – Identification

Parameter	Setting	Parameter	Setting
Component Type :	Peak	Reference :	blank
Absolute window (s):	0.00	Internal Standard :	blank
Relative window (%):	3.00	Find tallest peak :	Not checked

(13)Click on the "Calibration" tab in Components Defaults Window. Enter the information shown in **Error! Reference source not found.**

Table 34 Components Defaults – Calibration

Parameter	Setting	Amount (µV·s/ng)
Calibration Type :	Use Calibration Factor	1.000000e-6
Scaling :	None	
Weighing :	None	
Response :	Area	

(14)The "User Values/LIMS" tab is not used. Close the "Components Defaults" window by clicking the "OK" button.

(15) 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).

a) 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 "HgSpecGC1.mth," and click the "Open" button. "C:\GC\Methods\HgSpecGC1.mth" will now be the ChromLink™ default method. Click "OK" to close the "Default TotalChrom Method" window.

b) 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 NetCDF files in specified location" radio button. The lower radio buttons will gray out. Click the "OK" button to close the window.

c) Click on the "Browse" button by the ”ELAN® NetCDF file - location/file to be converted" space. Choose the path "C:\Users\Public\Documents\PerkinElmer Syngistix\ICPMS\ReportOutput". The location/file to be converted will now read "C:\Users\Public\Documents\PerkinElmer Syngistix\ICPMS\ReportOutput*.nc".

d) Click on the "Browse..." button for "ELAN® 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).

e) Click on the "Start Processing ELAN® Data Files" button. A window titled "Processing ELAN® 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™.

a) 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.

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