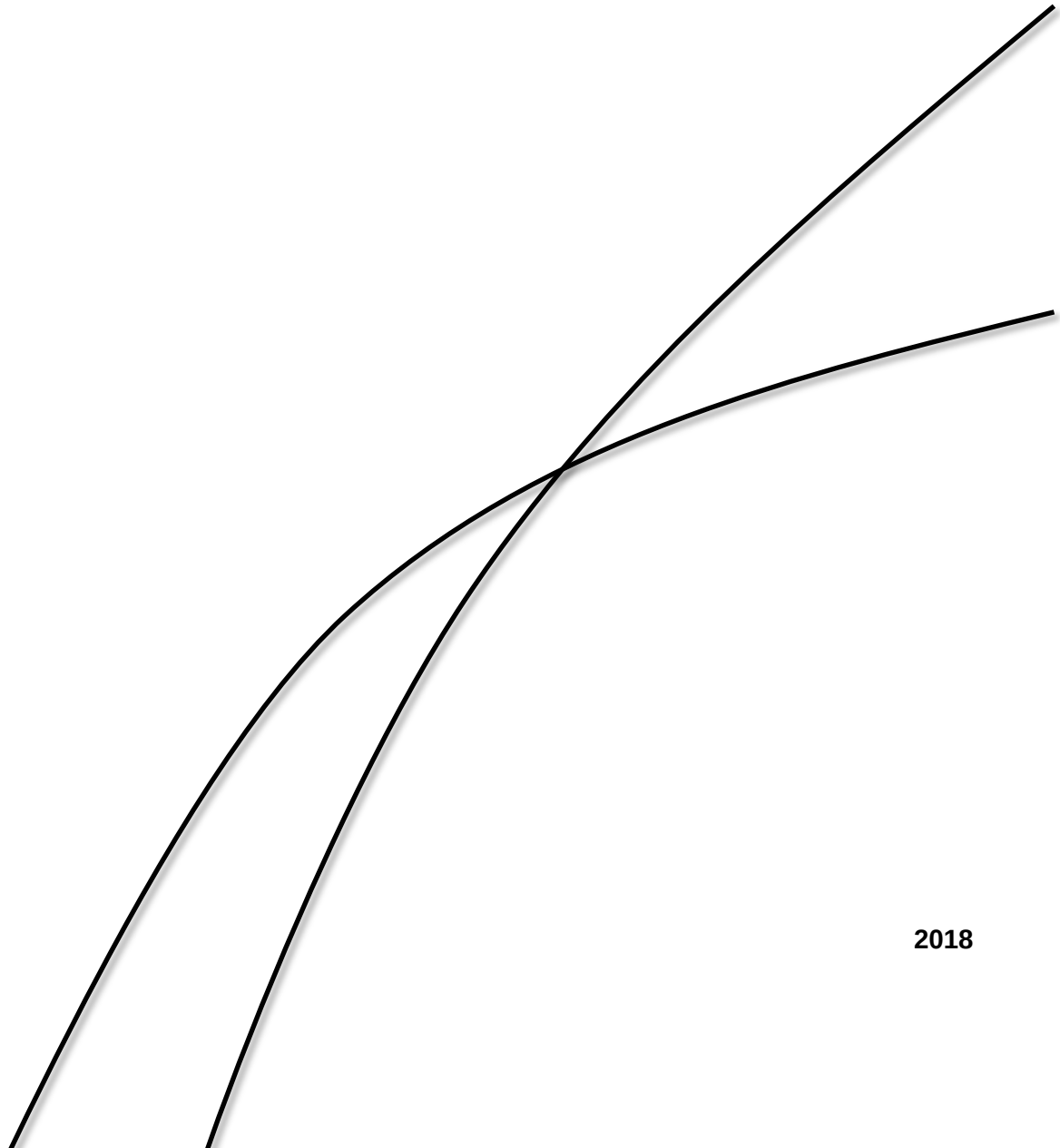
World Health Organization
REGIONAL OFFICE FOR Europe

Standard operating procedure

for assessment of mercury in cord blood
(*sampling, analysis of total mercury, interpretation of results*)



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Abstract
This standard operating procedure describes the process of assessing exposure to mercury through human biomonitoring using cord blood as a biological matrix. Sampling of cord blood, analysis of total mercury and interpretation of results are detailed in this document.

Key words
Mercury - analysis
Methylmercury Compounds - analysis
Biomarkers - analysis
Fetal Blood - chemistry
Umbilical Cord - chemistry
Maternal Exposure
Maternal-Fetal Exchange
Infant, Newborn
Environmental Exposure

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Contents

Abbreviations

Hg	mercury
ID	identity
LOD	limit of detection
LOQ	limit of quantification
SOP	standard operating procedure

Introduction: cord blood as a matrix for mercury human biomonitoring

Mercury (Hg) is a toxic and persistent pollutant that bioaccumulates and biomagnifies through food webs *(1,2)*. People are exposed to methylmercury mainly through their diet, especially through the consumption of freshwater and marine fish *(3)*. They may also be exposed to elemental or inorganic mercury through inhalation during occupational activities and via dental amalgams *(4)*. Exposure to elemental or inorganic mercury can also occur due to the use of some skin lightening creams and soaps, the presence of mercury in some traditional medicines, the use of mercury in cultural practices, and accidental mercury spills in homes, schools or other locations *(5)*.

Although the general population is exposed to only low levels of mercury, the occurrence and severity of its adverse health effects depend on its chemical form, dose, the age or developmental stage of those exposed (the fetus is considered to be the most susceptible), and the duration and route of exposure *(1,6)*.

The primary targets for mercury toxicity are the nervous system, kidneys and cardiovascular system, with developing organ systems (such as the fetal nervous system) being the most sensitive to its toxic effects. Nervous system effects are the most sensitive toxicological endpoint observed following exposure to elemental mercury and methylmercury, whereas kidney damage is the key endpoint in exposure to inorganic mercury compounds *(1)*.

The selection of biological media to assess human exposure depends on mercury compounds, exposure patterns (e.g. chronic, acute) and time of sampling after the exposure *(7)*. The presence of mercury in blood represents short-term exposure to organic and inorganic mercury, and does not provide information on long-term exposure and its variations *(7–9)*. Mercury levels in cord blood and hair are suitable biomarkers of prenatal low-level methylmercury exposure due to its selective transfer through biological barriers such as blood or hair and placenta, while inorganic mercury does not have this property. Levels in cord blood are proportional to maternal blood, but with slightly higher levels *(10,11)*. As a biomarker of prenatal exposure, mercury in cord blood is preferable, as it provides information on both the exposure of mothers and prenatal exposures of their children *(12)*.

Mercury in cord blood shows a better association with mercury-related neurobehavioural deficit in the child compared to mercury determined in maternal hair *(13)*. Hair mercury concentrations can be affected by several factors, including hair colour and variable growth rates, which limit its usefulness as an indicator of mercury concentrations in the body *(14)*. Cord blood is a non-invasive matrix, but should be collected by a nurse after birth.

1. Sampling of cord blood

1.1. Scope of the method

Collection of cord blood should be done immediately after delivery in a delivery room. Two basic methods can be used for collecting cord blood.

- Collection of cord blood after the baby is born, but before delivery of the placenta; this is referred to as “in-utero collection”, and is usually performed by a physician or a midwife.
- Collection of cord blood after the placenta is delivered and the umbilical cord is clamped; this is referred as “ex-utero collection". This method can take place in a separate area and can be performed by nursing and/or research staff.

WHO recommends that only ex-utero methods be used for cord blood sampling to prevent any negative effects on the mother and child.

1.2. Safety precautions

All precautionary measures necessary for working with blood specimens are applicable for sampling of cord blood.

- Use products that are specifically designed for cord blood collection; if needle and syringe are used, use a safe needle that can be separated from the syringe barrel.
- Gloves should be worn at all times when sampling cord blood.
- If gloves become punctured or grossly contaminated during use, they should be removed and disposed of, hands should be washed and clean gloves put on.
- On completion of handling samples, gloves should always be removed and discarded, and hands should be washed.
- Disinfectants should be used if necessary.

1.3. Materials required

The materials required for sampling cord blood are as follows:

- registration sheets for samples;
- sampling materials:
  - needles and syringes;
  - tube B1: polypropylene tube 50 mL with 0.5 mL ethylenediaminetetraacetic acid (EDTA);
  - tube B2: 10 mL polypropylene metal-free tube.

The maternity hospital should get prepared boxes with labelled collection tubes from a research laboratory in advance.

Instructions should be provided for contacting research staff and for collection, storage and transport.

1.4. Preparation/pre-treatment of the sampling material

All tubes should be washed with 10% nitric acid in purified water solution to eliminate background contamination. The details of this procedure are described below.

1. Prepare a 10% nitric acid solution from nitric acid (65% extra pure) and purified water.
2. Put the solution in a tank.
3. Open the tubes and put the tubes and lids into the tank. Ensure that all items are completely immersed.
4. Tubes should be immersed in this tank for at least three hours (preferably overnight).
5. Take out the tubes from the acid tank and put them in a tank with purified water. Shake them for 2–3 minutes. Then, move the tubes and lids to a second tank of purified water. Shake them again for 2–3 minutes.
6. Take out the tubes and lids and put them face down on clean filter paper to dry them.
7. Once the drying is finished, screw on the lids of the resulting nitric acid pre-treated tubes. Make a mark on tube B1 indicating the minimum amount of cord blood required (10 mL). Put the pre-treated vessel into a zip-lock plastic bag.

The acid solution can be re-used up to one month after its preparation. All of the procedures must be done in a chemical fume hood, using suitable personal protective equipment.

To check for contaminants, after the cleaning procedure, 5% of all tubes should be randomly selected and analysed for the mercury contamination. For this purpose, the tubes should be filled with purified water and shaken for 10 minutes. An aliquot should be analysed for the biomarkers in question (total mercury).

1.5. Sampling procedure

The cord blood collection procedure (ex-utero) is as follows.

1. Following the clamping of the umbilical cord and separation of the infant and placenta, wipe any maternal blood off the umbilical cord using gauze soaked in alcohol or an iodine-based antiseptic liquid for at least 30 seconds at the

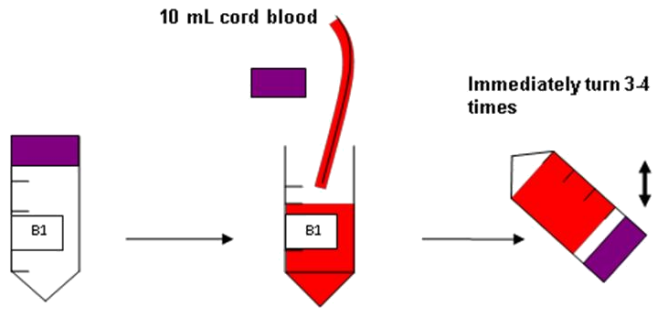
venipuncture site (vein). Sterilization of the cord at the puncture site is very important as it will prevent any contamination of the cord blood.

2. Allow the venipuncture site to dry.
3. Remove the needle covers and keep them nearby as you will need to recap the needles at the end of the collection procedure.
4. Puncture the vein of the umbilical cord at the sterilized site and allow the blood to flow out into the syringe.
5. When the syringe is full, change to a narrower needle and insert it into the vacutainer stopper so that the blood drains into the tube.
6. If blood flow stops, please proceed to the sterilization of another site closer to the placenta and use a second needle for further blood collection.
7. At the end of the collection procedure, the needles must be recapped using the saved covers to prevent needle accidents.
8. After collection, wait for 10 minutes. Then the tube should be gently turned to thoroughly mix the blood sample.

The cord blood sample should be collected and labelled as follows (see Fig. 1).

1. Collect the cord blood in tube B1 (minimum 10 mL).
2. Tube B1: turn three to four times for the blood to mix with the EDTA.
3. Put tube B1 into the zip lock bag and bring it to the laboratory.
4. Fill out the sampling form (Annex 2).

Fig. 1. Collecting the cord blood



Register the participant's information on the registration form (Annex 1). The following details should be documented:

- participant's name
- sample identity (ID) code
- date and time of child birth
- start and end times of the collection of cord blood
- collected volume of cord blood.

The form should be submitted to the survey coordination centre or the survey coordinator.

1.6. Labelling

The plastic bag, questionnaires and all collection tubes should be labelled with the identification code of the participant.

1.7. Transport and conservation of the sample

The samples must be sent to the local hospital laboratory or another special storage place in the hospital within two hours of sampling. Samples should be kept in a refrigerator or in a cold box during transportation, at below 4 °C.

1.8. Sample reception

The criteria for accepting or rejecting a sample should be defined in advance and applied during sample reception. These criteria should focus on transportation conditions, attached documentation, integrity of the packaging, correct identification, and the amount of the sample (sufficient for analysis and biobanking if samples will be stored for other research purposes).

The following points should be checked upon receipt of the cord blood samples.

- Amount and quality of the samples: an unacceptable specimen is of low volume (< 0.25 mL).
- Suspect contamination: due to improper collection procedures or collection devices.
- Integrity of the packaging: must be correctly sealed and must not have been manipulated (Note: a security seal can be placed on the package at the sampling site).
- Attached documents: all samples listed in the registry of collected samples should be contained in the package; they must be accompanied by the corresponding documents (questionnaires, etc.).
- Correct identification: samples and documents received must be properly identified with the corresponding ID code.

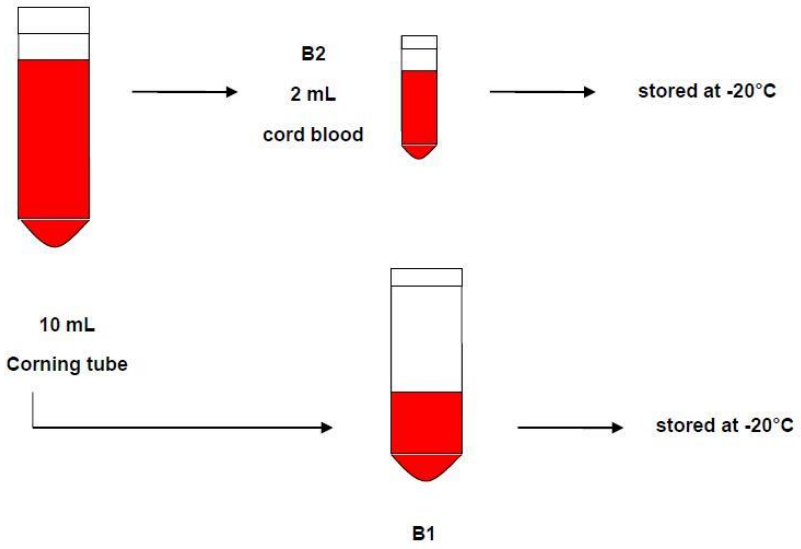
1.9. Sample aliquoting and preparation

Aliquoting can be done in a hospital laboratory in a mercury free atmosphere. The hospital laboratory should prepare in advance boxes with labelled aliquoting tubes. For mercury measurements the optimal amount of specimen is 1–2 mL (the minimum is

0.5 mL). If larger quantities are intended for storage, it is advisable to store aliquots of 1–2 mL in separate vials, rather than larger volumes in one sampling vial. Frequent thawing of blood may result in mercury losses.

Fig. 2 illustrates the process of aliquoting and storage. In Tube B2, an aliquot of 2 mL of cord blood is stored at -20 °C for analysis of mercury. A corning tube (B1) with the remaining blood is stored for possible duplicate analysis of mercury later on or analysis of other pollutants (if samples are planned to be stored for other research purposes).

Fig. 2. Aliquoting and storage of the sample



1.10. Storage and conservation

All aliquoted samples have to be stored in a freezer at below -20 ^{o}C until analysis. Specimen stability has been demonstrated for several months at -20 °C, or for several years at -70 °C.

1.11. Quality control: traceability

Traceability of the sample throughout the study is crucial, therefore this aspect should be guaranteed. The cord blood sample must be labelled with the ID code. As noted above, correct labelling of the samples and related documents is essential, but it is also necessary to be able to link the sample with the information provided by the volunteer. To this end, all documents related to the samples (questionnaires, registries, etc.) must be labelled with the same sample ID code immediately.

2. Analysis of total mercury in cord blood

The determination of total mercury in cord blood requires sensitive analytical methods performed under good quality-control conditions. Numerous analytical methods are available for analysis of total mercury in human blood, some of which are automated. In principle, two approaches exist: (1) methods based on acid digestion followed by cold vapour atomic absorption spectroscopy (CVAAS), cold vapour atomic fluorescence spectroscopy (CVAFS) and/or inductively coupled plasma mass spectrometry (ICPMS); and (2) methods based on thermal decomposition and CVAAS. The method described in this standard operating procedure (SOP) is based on the second principle combining combustion, gold amalgamation of mercury and detection by atomic absorption spectroscopy. It permits the reliable and accurate determination of total mercury in blood samples at the typical concentration ranges for environmental and occupational exposure. In order to perform these measurements a dedicated instrument needs to be provided as described later in the procedure. When such an instrument is not available, laboratories can use a procedure described in the SOP for assessment of total mercury in urine or similar *(15)*.

Numerous laboratories also use a technique proposed in the guidelines prepared by the National Institute for Minamata Disease, Japan *(15)*. This method is proposed in the SOP for assessment of total mercury in urine and can also be used for blood. The method is simple, sensitive, efficient, and most of all low cost as it requires simple equipment with atmospheric air as a carrier gas.

The method described in this SOP does not require any sample pre-treatment or extraction, very little chemical waste is expected and the likelihood of contamination is minimal.

If laboratories have other equipment for the detection of mercury in acid digested samples, it is advisable to follow the instructions provided by the instrument producers. The instructions for sampling and sample handling provided in this SOP are fit for purpose regardless of the instrumentation used for mercury detection. The limit of detection (LOD) and limit of quantification (LOQ) should be checked to assess whether they are suitable for blood samples.

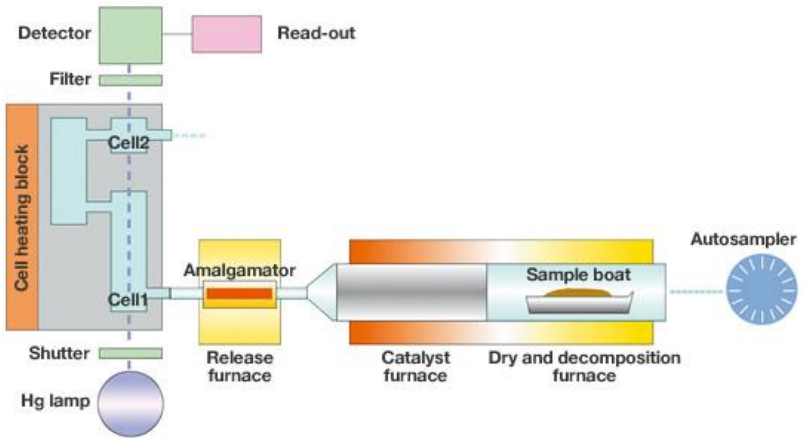
2.1. Scope of the method

The method described in this SOP is intended for the determination of total mercury in whole cord blood. For a sample of 200 mg the LOQ is 0.2 ng/mL. Concentrations of total mercury in cord blood of non-fish eaters are normally in the range of 0.5–5.0 ng/mL. In cases of higher fish consumption, values higher than 10 ng/mL frequently occur. The method described in this SOP can cover all the ranges normally reported.

2.2. Technical principle

In this SOP, mercury in blood is determined by thermal decomposition-gold amalgamation-atomic absorption spectroscopy, a very sensitive and selective analytical technique that is highly suitable for trace level analysis. Blood samples are weighed and introduced into the sample boat without any pre-treatment. The sample is then inserted into the direct mercury analyser (Fig. 3), where it is initially dried and then thermally decomposed in a continuous flow of oxygen. The combustion products are carried to and further decomposed over a hot catalyst bed. Mercury vapours are trapped on a gold amalgamator and subsequently heated, which releases all mercury vapours to the absorption cell of the atomic absorption spectrophotometer. Mercury content is determined using atomic absorption spectrometry at 253.7 nm.

Fig. 3. Direct mercury analyser



Hg = mercury.
Note: The standard version of the Milestone DMA-80 (illustrated here) is equipped with two measuring cells, a mercury lamp and mercury detector.
Source: Milestone *(16)*.

The quantitative determination of mercury is achieved using a calibration curve obtained with mercury standard solutions. The direct mercury analyser can be configured in various different ways depending on the type of model used. For the procedure described here, a measuring cell covering a working range of 0–20 ng mercury (low range) was used.

2.3. Safety precautions

The following safety precautions should be taken when analysing total mercury in cord blood.

- Place disposable plastic, glass and paper (e.g. pipette tips, auto sampler tubes and gloves) that come in contact with human biological fluids, such as blood, in a biohazard autoclave bag. Keep these bags in appropriate containers until they are sealed and autoclaved.
- Gloves, a laboratory coat and safety glasses should be worn when handling all solutions.
- Special care should be taken with concentrated hydrochloric acid since it is a caustic chemical that can cause severe eye and skin damage.
- The possible hazards of equipment use include exposure to ultraviolet radiation, high voltages and high temperatures.
- When work is finished, wipe down all work surfaces where human biological fluid was handled, with a 10% sodium hypochlorite solution or equivalent. Dispose of all biological samples and diluted specimens in a biohazard autoclave bag at the end of the analysis, according to guidelines for disposal of hazardous waste.

2.4. Equipment, materials and solutions

2.4.1. Equipment

The following equipment is required for analysing total mercury in cord blood:

- direct mercury analyser (Milestone DMA-80).

2.4.2. Materials

The following materials are required for analysing total mercury in cord blood:

- analytical balance (readability: 0.1 mg)
- microlitre pipette for 100 µL
- microlitre pipette, adjustable between 20 and 200 µL
- microlitre pipette, adjustable between 100 and 1000 µL
- tube for aliquots of blood samples – Cryovial; 2 mL
- laboratory vortex shaker
- quartz boats (1.5 mL)
- sample tray conveyor
- talc-free gloves.

2.4.3. Reagents, chemicals and gases

The following reagents, chemicals and gases are required for analysing total mercury in cord blood:

- oxygen gas (99.995% purity)

- 70% ethanol (pro analysis)
- 37% hydrochloric acid (pro analysis)
- purified water.

2.4.4 Standard solutions

Stock standard solution

A primary standard solution of mercury (stock solution) with a concentration of 1 mg/mL is prepared by weighing 0.2500 g of elemental liquid mercury (Hg^o) in a 250 mL Pyrex glass flask. To this, 2 mL of nitric acid is added, which is diluted with bidistilled water to 250 mL. The solution must be stored in a refrigerator.

Intermediate standard solution

An intermediate standard solution of mercury with a concentration of 5 µg/mL is prepared in 5% nitric acid by appropriate dilution with bidistilled water. Calibration standards are preferably prepared in glass flasks and are stable for one year if kept in a refrigerator. Before dilutions of working standard solutions are prepared the intermediate standard solutions should reach room temperature.

Working standard solutions

Working standard solutions of mercury, at two different concentrations (2 ng/mL and 10 ng/mL) are prepared in 5% hydrochloric acid by appropriate dilution. These are preferably kept in glass flasks (Teflon is suitable as well). Working standard solutions should be stored in a refrigerator when not in use. Working standard solutions should be removed from the refrigerator about two hours before use so they can reach room temperature. Working standard solutions are prepared weekly, but users are advised to check the stability under their laboratory conditions.

2.4.5 Reference materials

Reference materials certified for total mercury in blood should be used. For example, Seronorm Trace Elements Whole Blood L-1, with a reference value of 2.2 ng/g (2.0–2.4 ng/g), has been used for the validation and regular quality control of this SOP. Table 1 lists other reference materials which are available for measuring total mercury in human blood.

Table 1. Reference materials for total mercury in blood

Reference material	Reference values (ng/mL)
NIST 955c Lead in caprine blood	17.8 ± 1.6
NIST-966 Toxic metals in blood	31.4 ± 1.7
SERO210105 Trace elements in whole blood, level 1	1.97 ± 0.2
SERO210205 Trace elements in whole blood, level 2	15.2 ± 1.6
SERO210305 Trace elements in whole blood, level 3	31.4 ± 3.4

Note: new reference materials are continuously produced and replace the obsolete ones, therefore users are advised to regularly check on the availability of appropriate reference materials.

2.5. Calibration

Calibration is performed using working standard solutions described in 2.4.4.

In order to cover the appropriate measurement range, a known volume of calibration standard is pipetted. Normally a range of 0–20 ng needs to be covered (typical amounts covered are 0.05, 0.1, 0.2, 0.4, 0.5, 1.0, 2.0, 5.0, 10.0 ng). The volume of the calibration standard should not exceed 0.2 mL. The new calibration curve is done weekly or even less frequently, because calibrations are very stable. The instrument software allows for automated readings from calibration graphs stored in the system. However, working calibration standards covering the range of mercury concentrations in the sample (typically 0.2, 1.0 and 10.0 ng) should be used daily to verify the correctness of the calibration graphs stored in the system.

At the beginning of the measurement run it is necessary to check the adequacy of the calibration curve. Standard solutions 2 and 10 ng/mL are used. If the standards of mercury are not within the required range then it is necessary to repeat the calibration curve.

2.6. Procedure

2.6.1. Analytical equipment conditioning

Technical data

The technical data for the analytical equipment is as follows:

- principle: atomic absorption spectrometry;
- mercury detection system: single-beam spectrophotometer with sequential flow through two measurement cells;
- light source: low pressure mercury vapour lamp;
- wavelength: 253.65 nm;
- interference filter: 254 nm, 9 nm bandwidth;
- detector: silicon ultraviolet photodetector;

- working ranges:
  - low range: 0–7.5 ng (absorbance limit of cell 1: 0.45);
  - LOD: 0.005 ng;
- autosampler: built-in, 40 positions;
- carrier gas: oxygen, inlet gas 4 bar (60 psi), flow rate approximately 200 mL/min.

The technical data listed here were established during configuration of the instrument used in this case.

Step 1. Preparation of the direct mercury analyser

The following operations should be carried out in accordance with the user manual: opening the oxygen supply, direct mercury analyser start-up and data file creation.

Step 2. System cleaning

Before the measurements are taken, the system needs to be cleaned. Detergent is first pipetted into two quartz boats and then the empty position. An empty position should be measured with the appropriate programme. An optimum is suggested below, but users are advised to check it in their own configuration:

- drying time: 0 s (with using detergent the time is prolonged to 60 s)
- drying temperature: 200 °C
- decomposition time: 150 s
- decomposition temperature: 650 °C
- purge time: 60 s.

Step 3. System background check

Three empty quartz combustion boats should be analysed using the previous method to check that the absorbance (measured in terms of peak height) of the final samples is less than 0.0030. The acceptable system background should be established by the laboratory in accordance with the manufacturer's instructions. If the absorbance is above 0.0030, further quartz combustion boats should be analysed until the target value is obtained. If the desired background level is not attained after five quartz combustion boats have been analysed, the system should be cleaned by analysing a solution of detergent in a quartz combustion boat, followed by the procedure described above.

Step 4. Control of working standard solution

Two replicates of each standard solution should be measured (2 ng/mL and 10 ng/mL) containing approximately 0.2 ng and 1 ng mercury (100 µL of working standard solution) in order to check cell 1 (low range). The standard mercury solution is measured as follows:

- drying temperature: 200 °C
- drying time: 60 s

- decomposition temperature: 650 °C
- decomposition time: 150 s
- purge time: 60 s.

The concentration determined for the mercury working standard solution is compared to the one established by the calibration curve. If the targeted value differs more than 10%, the measurement should be repeated until a value within the targeted range is obtained. If such a value is not obtained after five attempts, a fresh standard should be prepared. If it still does not achieve the desired value, the system should be recalibrated with a set of newly prepared working calibration standards.

Step 5. Pre-measurement quality control

Two samples of certified reference material (e.g. Seronorm Whole Blood L-1) containing approximately 0.2 ng mercury (approximately 100 mg of material) should be measured in order to check cell 1 (low range). The reference material is measured as follows:

- drying temperature: 200 °C
- drying time: 120 s
- decomposition temperature: 650 °C
- decomposition time: 180 s
- purge time: 60 s.

The concentration determined for the reference material sample should be within the uncertainty range of the certified value. If this is not the case, the measurement should be repeated until a value within this range is obtained. If such a value is not obtained after five attempts, the system should be recalibrated.

Once the previous five steps have been successfully completed, the direct mercury analyser is ready for sample analysis.

2.6.2. Analytical determination

Sample weighing

Both the combustion boats and the support used to weigh the blood samples should be handled using tweezers.

Place the combustion boat support on the balance. Place a quartz combustion boat on top of the support and set the balance to zero.

Open the flask containing the sample, measure out around 200 µL of blood into the combustion boat and weigh the sample. Place the combustion boat containing the sample onto the sample tray and note the sample code, weight and tray position in the weighing log. Two replicates should be prepared for each sample. Between each sample

it is necessary to measure one blank. Special pipette tips with a filter must be used for each different sample.

Sample analysis

The quartz combustion boats containing samples and quality controls should be placed in the direct mercury analyser autosampler in the order in which they were weighed.

The samples should then be programmed by entering their code and weight, and selecting the method. The parameters of the method are as follows:

- drying temperature: 200 °C
- drying time: 200 s
- decomposition temperature: 650 °C
- decomposition time: 180 s
- purge time: 60 s.

Note. Guidance parameters must be optimized for each instrument in accordance with the manufacturer's instructions.

An example of sample sequences is provided below:

- detergent
- detergent
- blank (8x)
- working standard 2 ng/mL
- working standard 2 ng/mL
- blank
- working standard 10 ng/mL
- working standard 10 ng/mL
- blank
- reference material
- reference material
- blank
- blank
- sample 1
- sample 1
- blank
- sample 2
- sample 2
- blank
- working standard 2 ng/mL
- working standard 10 ng/mL
- blank
- sample 5

- sample 5
- blank
- sample 8
- sample 8
- working standard 2 ng/mL
- working standard 10 ng/mL
- blank.

2.6.3. Calculation of the analytical results

Data are reported directly by the equipment in terms of nanograms of mercury per gram of blood by interpolation of the measurement on the calibration curve.

The final value reported corresponds to the average of two independent measurements. If the values differ by more than 10%, the sample is to be re-measured, and the mean values of two similar results are reported.

2.6.4. Reportable results range

Mercury values are reportable when the results are obtained from a range defined by a calibration curve. If the amount of mercury obtained in the sample is outside this range, the sample should be retested as follows.

- If the value is below the lowest concentration of mercury included in the calibration, the necessary amount of blood for two new replicates should be weighed in order to obtain a new determination within the calibration range. The maximum amount of blood taken for analysis should not exceed 250 mg.
- If the value is above the highest calibration point, lower amounts of the sample should be weighed in order to obtain readings within the calibration range.

2.7. Quality control

The precision and accuracy of biomarker analyses carried out by toxicological laboratories must be continuously checked by means of quality assurance measures.

In general, quality assurance in laboratories comprises internal and external quality control (see also the *Quality control programme for mercury human biomonitoring*), as described below.

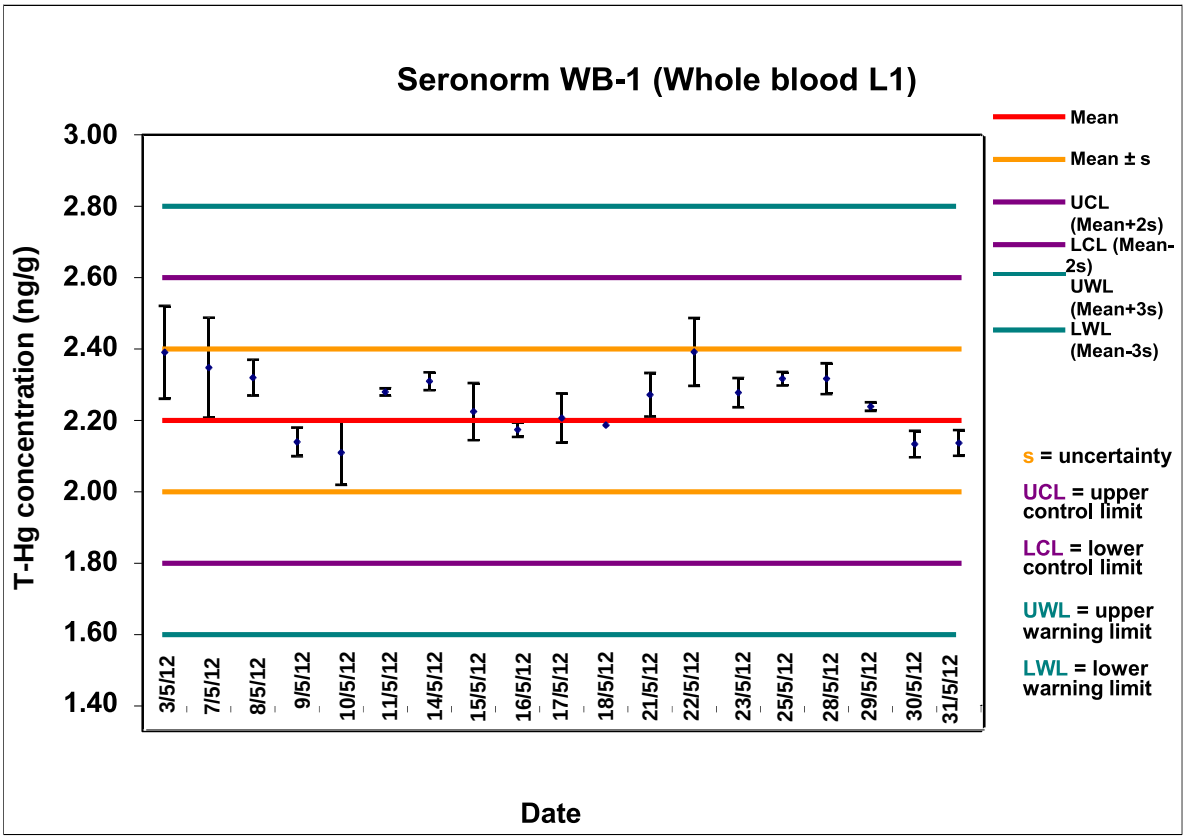
- Internal quality control is a set of procedures used by the staff of a laboratory to continuously assess results as they are produced in order to determine whether they are reliable enough to be released.

- External quality assessment is a system for objectively checking laboratory performance using an external quality control system. External quality control can be achieved by participation in suitable inter-laboratory comparisons, if available.

In this SOP, quality control materials are used to evaluate accuracy and precision. Fig. 4 provides an example of a quality control chart for blood reference material. Seronorm Whole Blood L-1 (2.2±0.2 ng/mL) has been used for quality control in this SOP.

Laboratories are advised to carefully control the performance of the analytical method on a regular basis as described in International Organization for Standardization/ International Electrotechnical Commission (ISO/IEC) 17025:2005 *(17)*.

Fig. 4. An example quality control chart



T-Hg = total mercury.

2.8. Evaluation of the method

Each laboratory should comply with the standard "ISO/IEC 17025:2005 General requirements for the competence of testing and calibration laboratories" *(17)*. The method should be validated for its performance criteria (sensitivity, linearity, recovery, robustness, precision, accuracy, LOD, etc.) and should be accompanied by

measurement uncertainty estimation, as the latter is a fundamental property of a result and a requirement of the standard ISO/IEC 17025:2005. It is advisable to consult freely available guides from EURACHEM *(18)*, particularly those dealing with validation protocols and uncertainty assessment. The concentration levels of mercury in the blood can be very low and the LOQ should be below 0.1 ng/mL to be able to measure concentrations in the general population. Those using the methodology outlined in this SOP are highly recommended to follow the glossary available in *Terminology in analytical measurement: Introduction to VIM 3 (19)*.

For the method described in this SOP, the performance criteria and measurement uncertainty estimation are specified below.

2.8.1. Limit of detection and limit of quantification

The LOQ was determined from the lowest point of the calibration curve, which was 0.05 ng. Taking into account the mass of a sample measured (0.2 g), the LOQ was 0.25 ng/g.

The LOD was determined as the LOQ/3, which was 0.8 ng/g.

2.8.2. Precision

As a measure of the degree of reproducibility of the described analytical method, routine analysis of cord blood samples over the course of a longer time period (e.g. one year) is used. For the purpose of demonstration, the results of one measurement series (n=15) of total mercury in cord blood are shown in Table 2. Each sample was analysed in two replicates.

Table 2. Results of duplicate measurements of total mercury in cord blood samples and their relative differences

Sample	Result D1 (ng/g)	Result D2 (ng/g)	Mean value (D1+D2)/2	Difference (D1-D2)	Relative difference (D1-D2/mean)
Cord blood 1	0.87	0.86	0.87	0.01	0.012
Cord blood 2	3.24	3.25	3.25	-0.01	-0.003
Cord blood 3	5.45	5.68	5.57	-0.23	-0.041
Cord blood 4	1.22	1.22	1.22	0.00	0.000
Cord blood 5	1.28	1.40	1.34	-0.12	-0.090
Cord blood 6	4.67	4.55	4.61	0.12	0.026
Cord blood 7	1.34	1.32	1.33	0.02	0.015
Cord blood 8	2.92	2.92	2.92	0.00	0.000
Cord blood 9	1.16	1.21	1.19	-0.05	-0.042
Cord blood 10	1.85	1.58	1.72	0.27	0.157
Cord blood 11	3.67	3.73	3.70	-0.06	-0.016
Cord blood 12	1.42	1.36	1.39	0.06	0.043
Cord blood 13	2.83	2.81	2.82	0.02	0.007
Cord blood 14	1.94	1.98	2.00	-0.04	-0.020
Cord blood 15	1.19	1.24	1.22	-0.05	-0.041

D1 = measurement 1; D2 = measurement 2.

To assess reproducibility or repeatability, standard deviation of replicate measurements is calculated using the following equation.

$$RSD_d = \frac{S_d}{\sqrt{n}}$$

RSD_d – relative standard deviation of duplicate measurements
S_d – standard deviation of relative differences ((*D1-D2)/mean*)
n – number of replicates (n=2)

The repeatability calculated for the given set of measurements was 3.9%.

2.8.3. Trueness

The trueness of our results was estimated using the reference material Seronorm WB-1 (Whole blood L1). As a measure of trueness of our results, recovery (*R*) was calculated based on measurements of the reference material over a course of one month. The observed levels were compared against the reference value using the following equation.

$$R = \frac{observed\ value}{reference\ value}$$

R – recovery

An example of measurements of total mercury in the reference material is given in Table 3.

Table 3. Measurements of total mercury in Seronorm WB-1 (Whole blood L1)

Measurement	Measured value (ng/g)	True value (ng/g)	Recovery (%)
Day 1	2.39	2.2	109
Day 2	2.35	2.2	107
Day 3	2.32	2.2	105
Day 4	2.14	2.2	97
Day 5	2.11	2.2	96
Day 6	2.28	2.2	104
Day 7	2.31	2.2	105
Day 8	2.23	2.2	101
Day 9	2.17	2.2	99
Day 10	2.21	2.2	100
Day 11	2.19	2.2	99
Day 12	2.27	2.2	103
Day 13	2.39	2.2	109
Day 14	2.28	2.2	104
Day 15	2.32	2.2	105
Day 16	2.32	2.2	105
Day 17	2.24	2.2	102
Day 18	2.13	2.2	97
Day 19	2.14	2.2	97

Based on the measurements given in Table 3, the recovery calculated was 102%.

2.8.4. Measurement uncertainty

Measurement uncertainty for total mercury in cord blood by thermal desorption and CVAAS was estimated based on ISO 21748:2010 “Guidance for the use of repeatability, reproducibility and trueness estimates in measurement uncertainty estimation”. For this purpose, reproducibility (repeatability) and recovery data from our validation study was used.

Uncertainty of repeatability (u_{rep}) was 3.9% (Section 2.8.2), while uncertainty of recovery ($u(R_m)$ or u_{rec}) was 1.7 % and was calculated using the following equation.

$$u(\overline{R}_m) = \overline{R}_m \times \sqrt{\left(\frac{s_{obs}^2}{n \cdot \overline{C}_{obs}^2}\right) + \left(\frac{u(C_{ref})}{C_{ref}}\right)^2}$$

R_m – recovery
s_{obs} – standard deviation of the observed data
C_{obs} – mean value of the observed data
C_{ref} – reference value
$u(C_{ref})$ – uncertainty of reference value

In the final step, Step 4, combined uncertainty was calculated. Before combination, all uncertainty contributions must be expressed as standard uncertainties (standard deviations). The combined uncertainty (u_c) was calculated using the following equation.

$$u_c = \sqrt{u_{rep}^2 + u_{rec}^2}$$

u_c – combined uncertainty
u_{rep} – error due to reproducibility
u_{rec} – error due to recovery

Expanded uncertainty (U) was expressed by multiplying u_c with the factor k. The choice of the factor k is based on the level of confidence desired. For an approximate level of confidence of 95%, k is 2.

The estimated measurement uncertainty for the determination of total mercury in cord blood by thermal decomposition and CVAAS is 4.3%, expanded uncertainty (k=2) is 8.4%. The estimation is valid for a "normal" exposure range, that is below 5.8 ng/g.

3. Interpretation of results

Blood mercury levels reflect exposure through ingestion of contaminated fish or drinking water, inhalation of elemental mercury vapour in ambient air, and exposure through dental amalgams and medical treatments. The presence of mercury in blood indicates recent or current exposure to mercury. There is a direct relationship between mercury concentrations in human blood and consumption of fish contaminated with methylmercury. Usually blood methylmercury concentration reaches a maximum within 4–14 hours and undergoes clearance from the blood to other body tissues after 20–30 hours *(6)*.

At the initial stage of data analysis (descriptive statistics), basic statistical values are calculated for each biomarker: minimum and maximum values, percentage of subjects having the biomarker value above the LOQ or above the LOD, and geometric mean. Percentile values, the values of a variable below which a certain percentage of observations fall, may also be calculated: 50th percentile (P50; median), 90th percentile (P90) and 95th percentile (P95). Percentages of results exceeding reference values or health-based values may also be reported *(20)*.

Human biomonitoring data can be interpreted via comparing the measured biomarker levels to health-relevant biomonitoring reference values. In this context, the German Human Biomonitoring Commission has derived reference values for several compounds *(21)*. These values have been determined based on either exposure-effect relationships (e.g. for cadmium, lead, mercury and pentachlorophenol) or derived from tolerable daily intake values *(20)*.

The blood mercury geometric means in most national surveys in Europe were below or around 1 µg/L. However, in some subpopulations exposure levels exceeded the health-based value of 5 µg/L *(20)*.

WHO considers the normal mean concentration of total mercury in blood to be 5–10 µg/L in individuals with no consumption of contaminated fish *(6)*. The United States National Research Committee identifies 2 µg/L as the normal mean concentration for populations with little or no fish consumption in the United States *(22)*.

Estimating exposure through biomonitoring cord blood levels of about 5–6 µg/L and blood mercury concentrations of about 4–5 µg/L. This relationship is generally directly proportional.

Table 4 provides an example of blood concentrations in populations in different provinces of Canada, from an Arctic Monitoring and Assessment Programme (AMAP) study (2003) *(23)*.

Table 4. Summary of data from Canada on levels of mercury and methylmercury in maternal blood

Country/ethnic group/region	Number of individuals sampled	Total mercury mean (µg/L)	Total mercury range (µg/L)	Methylmercury mean (µg/L)	Methylmercury range (µg/L)
Canada Caucasian 1 (1994–99)	134	0.9	nd–4.2	0.69	nd–3.6
Metis/Dene (1994–1995)	92	1.4	nd–6.0	0.8	nd–4.0
Other (1995)	13	1.3	0.2–3.4	1.2	nd–3.0
Baffin 1(1996)	31	6.7	nd–34	6.0	nd–29
Inuvik 1 (1998–1999)	31	2.1	0.6–24	1.8	nd–21
Kitikmeot 1 (1994–1995)	63	3.4	nd–13	2.9	nd–11
Kivalliq 1 (1996–1997)	17	3.7	0.6–12	2.7	0.4–9.7
Nunavik 2 (1995–2000)	162	9.8	1.6–44	na	na

nd = not detected; na = not available.
Source: AMAP 2003 *(23)*.

The average ratios between intake (µg/kg day) and blood levels (µg/L) among a population, overtime, are expected to be generally consistent. The quantitative relationship between mercury levels in blood and daily average dose (or intake) levels of mercury (especially methylmercury) are fairly well understood.

Therefore, such dose conversions can often be made with reasonable confidence, if enough information is known about the various mercury forms and other factors. Population variability should, however, be noted in dose conversion *(24)*. For example, a daily average methylmercury intake of 0.1 µg per kilogram of body weight (0.1 µg/kg per day) by an adult woman is estimated to result in a cord blood level of about 5–6 µg/L *(24)*.

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Annex 1. Registration of cord blood samples

To be filled in by the midwife

Name of hospital:_____ **Name of midwife collecting samples:**_____

Patient name	**Sample ID**	**Childbirth information**	**Collection of cord blood**	**Sample information Tube 1**	**Sample information Tube 2**
		Date : _____ Time: _____	Time start collection Time end collection	**Tube B1 with EDTA**: volume=_____ mL (**min 10 mL**) stored at -20 ^{0}C	**Tube B2** : volume=_____mL (**min 2 mL**) stored at -20 ^{0}C
		Date : _____ Time: _____	Time start collection Time end collection	**Tube B1 with EDTA**: volume= _____ mL (**min 10 mL**) stored at -20 ^{0}C	**Tube B2** : volume=_____mL (**min 2 mL**) stored at -20 ^{0}C
		Date : _____ Time: _____	Time start collection Time end collection	**Tube B1 with EDTA**: volume=_____ mL (**min 10 mL**) stored at -20 ^{0}C	**Tube B2** : volume=_____mL (**min 2 mL**) stored at -20 ^{0}C

EDTA = Ethylenediaminetetraacetic acid.

Annex 2. Cord blood sample collection form

Name of mother		
Medical record number		
Study ID of mother		
Medical worker	Signature: _____ Printed name: _____	
1.	Date and time of sample collection	------/-----/-------/ (day/month/year) Start: -------/------ (hour/min)
2.	How many hours prior to the sample collection was your last meal?	_ _ hours
3.	Volume of collected blood (approximately)	_ _ _ mL

Note. The procedures described in this standard operating procedure (SOP) are only suitable for the pollutants concerned in this document. Other pollutants or biomarkers might be preferably measured in plasma or blood cells, for which a separate SOP should be used.