



## Laboratory Procedure Manual

*Analyte:*           **Perchlorate, Nitrate, and Thiocyanate**

*Matrix:*           **Urine**

*Method:*           **Ion Chromatography with Tandem Mass Spectrometry (IC-MS/MS)**

*Method No:*       **2150.03b (modification)**

*Revised:*           **1/31/2011**

*as performed by:*

Tobacco and Volatiles Branch  
Division of Laboratory Sciences  
National Center for Environmental Health

*contact:*

*Dr. David Chambers, Chief*  
Volatile Organic Compounds Laboratory  
Phone:           770-488-7894  
Fax:              770-488- 0181  
Email: [BKB3@cdc.gov](mailto:BKB3@cdc.gov)

Dr. Jim Pirkle, Director  
Division of Laboratory Sciences

### **Important Information for Users**

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

**Public Release Data Set Information**

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

<b>File Name</b>	<b>Variable Name</b>	<b>SAS Label (and SI units)</b>
PERNT_F	URXNO3	Nitrate, urine (ng/mL)
	URXUP8	Perchlorate, urine (ng/mL)
	URXSCN	Thiocyanate, urine (ng/mL)

## 1. Clinical Relevance and Summary of Test Principle

### a. Clinical Relevance

Perchlorate, nitrate and thiocyanate are polyatomic anions that can disrupt thyroid function by competitively inhibiting iodide uptake at the sodium-iodide symporter (NIS).<sup>1,2</sup> Pharmacological doses of NIS-inhibitors or iodine deficiency can significantly reduce iodide uptake. Sufficient inhibition of iodide uptake can lead to decreased thyroid hormone production, and chronically impaired thyroid function can lead to hypothyroidism<sup>3,4</sup> and impaired neurodevelopment in infants<sup>5</sup>. Linkage between health effects and environmental exposure to NIS inhibitors requires improved exposure assessment. By assessing exposure to these four toxicologically-related analytes (perchlorate, nitrate, thiocyanate and iodide) in one assay, the relative impact of each chemical on thyroid function can be estimated and thus provide useful information for assessing the potential association between exposure and health effects.

Nitrate is commonly found in physiological fluids resulting from both exogenous and endogenous sources including a variety of foods (green leafy vegetables, milk) and drinking water. Thiocyanate is commonly found in physiological fluids, primarily as a metabolite of cyanide exposure from tobacco smoke or diet<sup>6-8</sup>. Perchlorate exposure is widespread in the U.S.<sup>9</sup>. Perchlorate has been associated with decreased thyroid function in females with urinary iodine < 100µg/L<sup>10</sup>, indicating the need to assess exposure to perchlorate, other iodide uptake inhibitors and iodide.

### b. Test Principle

This method is a quantitative procedure for the measurement of perchlorate, thiocyanate, and nitrate in human urine using ion chromatography coupled with electrospray tandem mass spectrometry<sup>11</sup>. Chromatographic separation is achieved using an Ion Pac AS16 column with sodium hydroxide as the eluent. The eluent from the column is ionized using an electrospray interface to generate and transmit negative ions into the mass spectrometer. Comparison of relative response factors (ratio of native analyte to stable isotope-labeled internal standard) of unknowns with known standard concentrations yields individual analyte concentrations.

## 2. Safety Precautions

### a. Reagent toxicity or carcinogenicity

Perchlorate and other NIS inhibitors can reversibly inhibit thyroid function at doses of µg per kg body weight per day. Therefore avoid intake of perchlorate (oral or inhalational). Additionally, some perchlorate salts (e.g. ammonium perchlorate) are strong oxidizers. Take special care to prevent contact of solid ammonium perchlorate salt with combustible or oxidizable material, since this constitutes an extreme fire and explosion hazard. However, aqueous solutions of perchlorate do not present a fire or explosion hazard. Perchlorate solutions can irritate skin and mucous membranes, and thus avoid dermal exposure. Observe

Universal Precautions (wear gloves, lab coat, and safety glasses) while handling all human urine. Place disposable supplies (pipette tips, autosampler tubes, gloves, etc.) contaminated with urine in a biohazard autoclave bag. Keep autoclave bags in appropriate containers until sealed and autoclaved. Wipe down all work surfaces with a surface disinfectant/decontaminant, when work is finished.

**b. Radioactive hazards**

None.

**c. Microbiological hazards**

Follow Universal Precautions. Because of the possibility of exposure to various microbiological hazards, take appropriate measures to avoid any direct contact with the urine specimen. Wear gloves, lab coats and safety glasses while handling all human urine products. A Hepatitis B vaccination series is recommended for health care and laboratory workers who are exposed to human fluids and tissues.

**d. Mechanical hazards**

There are only minimal mechanical hazards when performing this procedure using standard safety practices. Laboratorians should read and follow the manufacturer's information regarding safe operation of the equipment. Avoid direct contact with the mechanical and electronic components of the mass spectrometer unless all power to the instrument is off. Generally, mechanical and electronic maintenance and repair should only be performed by qualified technicians. The autosampler and the mass spectrometer contain a number of areas which are hot enough to cause burns. Take precautions when working in these areas.

**e. Protective equipment**

Follow standard safety precautions when performing this procedure, including using a lab coat/disposable gown, safety glasses, appropriate gloves, and chemical fume hood. Refer to the laboratory Chemical Hygiene Plan and CDC Division of Laboratory Sciences safety policies and procedures for details related to specific activities, reagents, or agents.

**f. Training**

Formal training in the use of the ion chromatograph and mass spectrometer is necessary. Users are required to read the operation manuals and should demonstrate safe techniques in performing the method.

**g. Personal hygiene**

Follow Universal Precautions. Take care when handling chemicals or any biological specimen. Routinely use gloves and wash hands properly. Refer to the laboratory Chemical Hygiene Plan and CDC Division of Laboratory Sciences safety policies and procedures for details related to specific activities, reagents, or agents.

**h. Disposal of waste**

- 1) Dispose of waste materials in compliance with laboratory, federal, state, and local regulations.
- 2) Dispose of solvents and reagents in an appropriate container clearly marked for waste products and temporarily stored in a chemical fume hood.
- 3) Place all disposable items that come in direct contact with the biological specimens in a biohazard autoclave bag that is kept in an appropriate container until sealed and autoclaved.
- 4) Immediately place unshielded needles, pipette tips and disposable syringes into a sharps container and autoclave when this container becomes full.
- 5) Wipe down all surfaces with a surface disinfectant/decontaminant when work is finished.
- 6) Wash any non-disposable glassware or equipment that comes in contact with biological samples with bleach solution before reuse or disposal.
- 7) Wash, recycle, or dispose of any other non-disposable glassware in an appropriate manner.

**Observe Universal Precautions.** Dispose of all biological samples and diluted specimens in a biohazard autoclave bag at the end of the analysis according to CDC/DLS guidelines for disposal of hazardous waste.

**3. Computerization; Data-System Management**

**a. Software and knowledge requirements**

This method has been validated using the Dionex IC system controlled by PeakNet Software coupled with an Applied Biosystems Inc. mass spectrometer run with Analyst 1.4 software. Results are exported from Analyst software to Microsoft Excel files and entered into the ATLAS relational database. Knowledge of and experience with these software packages (or their equivalent) are required to utilize and maintain the data management structure.

**b. Sample information**

Enter information pertaining to particular specimens into the database either manually or transfer electronically. Transfer the result file electronically into the database. Use no personal identifiers; reference all samples to a blind-coded sample identifier.

**c. Data maintenance**

Check all samples and analytical data prior to being entered into the ATLAS database for transcription errors and overall validity. Routinely back up the database locally onto a computer hard drive and CDs through the standard practices of the NCEH network. Contact the local area network manager for emergency assistance.

**d. Information security**

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

**4. Procedures for Collecting, Storing, and Handling Specimens; Criteria for Specimen Rejection**

**a. Special instructions**

No special instructions such as fasting or special diets are required.

**b. Sample collection**

- 1) Collect urine specimens from subjects in polystyrene cryovials or polypropylene (PP) centrifuge tubes.
- 2) Lot screen specimen collection containers to ensure the absence of any analyte contamination.
- 3) Use sterile collectors for specimen acquisition.

**c. Sample handling**

Specimen handling conditions are outlined in the DLS protocol for urine collection and handling (copies available in the laboratory and specimen handling offices). Collection, transport, and special requirements are discussed in the division protocol.

- 1) Transport and store urine specimens at  $4\pm 3^{\circ}\text{C}$ .

- 2) Once received, freeze urine specimens at  $-20\pm 5^{\circ}\text{C}$  until time for analysis.
- 3) Refreeze portions of the sample that remain after analytical aliquots are withdrawn at  $-20\pm 5^{\circ}\text{C}$ . Samples are not compromised by repeated freeze and thaw cycles. Preliminary experiments indicate that perchlorate is stable in urine samples for  $> 9$  months when stored at temperatures  $\leq -20^{\circ}\text{C}$ .

**d. Sample quantity**

The minimum amount of specimen required for analysis is 0.50 mL, with the optimal amount being 2 mL.

**e. Unacceptable specimens**

- 1) Reject specimens if suspected of contamination due to improper collection procedures or devices. Specimen characteristics that may compromise test results include contamination of urine by contact with dust, dirt, etc. from improper handling.
- 2) Reject samples with visible microbiological growth (e.g. mold, bacteria). In all cases, a second urine specimen should be requested.
- 3) Record on the sample transfer sheet a description of reasons for each rejected sample such as low sample volume, leaking or damaged container.

**5. Procedures for Microscopic Examinations; Criteria for Rejecting Inadequately Prepared Slides**

Not applicable for this procedure.

**6. Preparation of Reagents, Calibration Materials, Control Materials, and all Other Materials; Equipment and Instrumentation**

**a. Reagents and sources**

Reagents and sources used during the development, validation, and application of this method are listed in Table 1. All chemicals and solvents are used without further purification. Reagents procured from other sources should meet or exceed these listed requirements.

Table 1. Reagents and Sources.

Reagent	Grade	Source *
Sodium Perchlorate	98%	Sigma Aldrich, St. Louis, MO

**Perchlorate, Nitrate, and Thiocyanate in Urine  
NHANES 2009-2010**

Ammonium Perchlorate	99.999%	Sigma Aldrich, St. Louis, MO
Potassium Thiocyanate		Sigma Aldrich, St. Louis, MO
Perchlorate 1000µg/mL	Certified Solution	AccuStandard, New Haven, CT
Nitrate 1000µg/mL	Certified Solution	AccuStandard, New Haven, CT
Labeled Sodium Perchlorate ( <sup>18</sup> O <sub>4</sub> )	98%	Isotec, Miamisburg, OH
Labeled Potassium Nitrate ( <sup>15</sup> N)	99%	Cambridge Isotope Lab, Andover, MA
Labeled Potassium Thiocyanate ( <sup>15</sup> N)	98%	Isotec, Miamisburg, OH
Deionized Water	18 MOhm-cm	Barnstead water purifier

\* or equivalent

**b. Preparation of Calibration Materials**

1) Stock Solutions and dilutions

a) *Stock Solution*

Stock solutions are prepared by dilution of certified solutions (1000 µg/mL) for each of the analytes into deionized (DI) water to give target concentrations of 100, 10, and 1 µg/L depending on the current needs. For example, prepare these stock solutions in volumetric flasks by diluting 10 mL into 100 mL total volume (100 µg/L), 1 mL into 100 mL total volume (10 µg/L), or 1 mL into 1000 mL total volume (1 µg/L). These stock solutions are used to prepare the working standard solutions as shown in Table 2-4.

b) *Labeled Internal Standard Solution*

1. Labeled Perchlorate

- i. Weigh approximately 2.5 mg of <sup>18</sup>O-labeled sodium perchlorate, transfer to a 25-mL volumetric flask and take to volume with DI water to produce an approximate 100-ppm concentrated stock solution.
- ii. Dilute the initial stock solution approximately 1:20 (1.25 mL of a 100-ppm stock into a 25-mL volumetric flask diluted with DI water) to produce a final concentration of 5 ppm.

2. Labeled Nitrate (<sup>15</sup>NO<sub>3</sub>)

Weigh approximately 25 mg of <sup>15</sup>NO<sub>3</sub>, transfer to a 25-mL volumetric flask and dilute to volume with DI water to produce an approximate 1000 ppm solution.

3. Labeled Thiocyanate (SC<sup>15</sup>N)

Weigh approximately 25 mg of SC<sup>15</sup>N, transfer to a 25-mL volumetric flask and dilute to volume with DI water to produce an approximate 1000 ppm solution.

4. Internal Standard solution Mix

- i. Prepare 1000 mL of the working labeled internal standard solution by adding 800  $\mu\text{L}$  of 5  $\text{ng}/\mu\text{L}$  labeled perchlorate, 10,000  $\mu\text{L}$  of 1000  $\text{ng}/\mu\text{L}$  labeled nitrate, and 1300  $\mu\text{L}$  of 1000  $\text{ng}/\mu\text{L}$  labeled thiocyanate into a 1000-mL volumetric flask.
- ii. Dilute this solution to 1000 mL with DI water and transfer to a 1-L glass bottle.
- iii. The concentration of the working solution for  $\text{Cl}^{18}\text{O}_4$ ,  $^{15}\text{NO}_3$ , and  $\text{SC}^{15}\text{N}$  is approximately 0.004, 10, and 1.3  $\text{ng}/\mu\text{L}$ , respectively, from which 500  $\mu\text{L}$  is added to the sample.

2) Working Standard Solutions

Prepare working standard solutions by aliquoting known amounts of each analyte from previously prepared stock solutions (6.b.1) and diluting to final volume with DI water in a volumetric flask. Standard solutions (1-9) are prepared as presented in Tables 2-4, which specifies stock solution to use, volume to aliquot and final volume for each standard.

Table 2. Perchlorate Calibration Standards

Standard ID	Stock Solution		Final Solution	
	Concentration $\text{ng}/\mu\text{L}$ (ppm)	Volume ( $\mu\text{L}$ )	Final Concentration in urine, $\text{ng}/\text{mL}$ (ppb)	Total Volume (mL)
SSmmyy01	1.0	12.5	0.05	25
SSmmyy02	1.0	10	0.10	10
SSmmyy03	1.0	33	0.33	10
SSmmyy04	1.0	100	1.0	10
SSmmyy05	10	33	3.3	10
SSmmyy06	10	100	10	10
SSmmyy07	100	33	33	10
SSmmyy08	100	75	75	10
SSmmyy09	100	100	100	10

\* *mmyy* represents the month and year of standard preparation.

Table 3. Nitrate Calibration Standards

Standard	Stock Solution	Final Solution
----------	----------------	----------------

<b>ID</b>	<b>Concentration ng/μL (ppm)</b>	<b>Volume (μL)</b>	<b>Final Concentration in urine, ng/mL (ppb)</b>	<b>Total Volume (mL)</b>
SSmmyy01	1000	125	500	25
SSmmyy02	1000	100	1000	10
SSmmyy03	1000	250	2500	10
SSmmyy04	1000	500	5000	10
SSmmyy05	1000	1000	10000	10
SSmmyy06	1000	2500	25000	10
SSmmyy07	1000	5000	50000	10
SSmmyy08	1000	7500	75000	10
SSmmyy09	1000	X <sup>a</sup>	100000	X <sup>a</sup>

<sup>a</sup>Nitrate in standard mix solution 9 is added separately when preparing calibration curve. See procedure below in section 8.2

Table 4. Thiocyanate Calibration Standards

<b>Standard ID</b>	<b>Stock Solution</b>		<b>Final Solution</b>	
	<b>Concentration ng/μL (ppm)</b>	<b>Volume (μL)</b>	<b>Final Concentration in urine, ng/mL (ppb)</b>	<b>Total Volume (mL)</b>
SSmmyy01	100	25	10	25
SSmmyy02	100	25	25	10
SSmmyy03	100	50	50	10
SSmmyy04	100	100	100	10
SSmmyy05	1000	25	250	10
SSmmyy06	1000	50	500	10
SSmmyy07	1000	100	1000	10
SSmmyy08	1000	250	2500	10
SSmmyy09	1000	500	5000	10

Aliquots of these solutions are store in 1.5-mL vials at -20±5°C until use. After the vial is used, store it at 4±3°C.

### **Preparation of Control Materials**

- 1) Quality Control materials

- a) Prepare quality control (QC) materials by collecting human urine.
- b) Analyze urine samples collected and pool together to create two urine pools.
- c) Fortify each urine pool with the different analytes to achieve levels within the linear range of the method: typical target levels for low QC are 3, 2000, and 45,000  $\mu\text{g/L}$  for perchlorate, thiocyanate and nitrate, respectively; and for a high QC 70, 4000, and 75000  $\mu\text{g/L}$  for perchlorate, thiocyanate and nitrate, respectively.
- d) After fortifying the urine to reach target concentrations, store the QC solutions overnight at  $4\pm 3^\circ\text{C}$  for equilibration.
- e) After overnight equilibration let QC solutions reach room temperature and aliquot into 1.2-mL cryovials.
- f) Store at  $-70\pm 5^\circ\text{C}$  until use.

2) Proficiency Testing materials

- a) Prepare proficiency testing (PT) materials from certified 1000  $\mu\text{g/L}$  reference solutions for each of the analytes (AccuStandard, New Haven, CT).
- b) Four target concentrations covering the linear range for each analyte are selected.
- c) Dilute to final concentration with water in a 25-mL volumetric flask.
- d) Blind-code aliquots and store in cryovials at  $-70\pm 5^\circ\text{C}$  until use.
- e) Analyze PT samples twice a year as well as following any major maintenance on the instrumentation
- f) Proficiency testing samples are blind coded for analysis; results are evaluated by an external quality control officer.

**Note:** Proficiency Testing materials are prepared by the team lead and blind to the analyst. Consult with the team lead when additional PT materials need to be prepared.

**c. Other materials and supplies**

Materials / supplies and sources used during the development, validation, and application of this method are listed below. Materials/supplies procured from other sources should meet or exceed these specifications. All materials that have direct contact with sample matrix were lot-screened to verify no perchlorate contamination.

- Nalgene 1.8-mL cryovials (Fisher Scientific, Fairlawn, NJ).
- Eppendorf Repeater Plus Pipette (Brinkmann Instruments Inc., Westbury, NY).
- Rainin Electronic Pipettes (100, 250, and 1000- $\mu\text{L}$ ; Rainin, California)
- Pasteur pipettes and bulbs (Kimble Glass, Inc., Vineland, NJ).
- VWR Brand Mini vortexer (The Lab Depot, Alpharetta, GA).

- 1.5-mL Vial Kit with Split Septum (Dionex, Sunnyvale, Ca)
- ASRS Ultra II, 2mm Suppressor (Dionex, Sunnyvale, Ca)
- Ion Pac ® AS 20 Column (Dionex, Sunnyvale, Ca)
- Nalgene Sterilization filter unit (Fisher Scientific, Fairlawn, NJ)
- Envirocide Surface Disinfectant/ Decontaminant Cleaner

**d. Instrumentation**

Aliquoting of urine and quality control samples was conducted using a Hamilton Microlab Star workstation (Hamilton Robotics, Inc. Reno, NV). Analyses of samples were conducted with a Dionex ion chromatography system equipped with a GP50 gradient pump, AS50 autosampler, AS50 thermal compartment and a 2-mm anion self-regenerating suppressor (ASRS Ultra II) operated in the external water mode (Dionex Corp, Sunnyvale, CA). PeakNet 6 chromatography software was used for system control. The separation was performed using an Ion Pac AS16 column (2 x 250mm, Dionex) with a 25-µL injection loop. An Applied Biosystems API4000 triple quadrupole mass spectrometer (Foster City, CA) with electrospray interface was used for the detection of perchlorate.

1) Ion chromatograph configuration

The ion chromatograph configuration is described in Table 5 below. The separation conditions were optimized to obtain resolution between perchlorate and other interferences present in urine (e.g. sulfate).

Table 5. Ion Chromatograph Configuration

<b>Parameter</b>	<b>Setting</b>
Column type	AS16 (2 x 250 mm)
Column temperature	30°C
Eluent	50 mM potassium hydroxide
Flow	0.5 mL/min
Injection Loop Volume	25 µL
Suppressor	ASRS Ultra II

2) Mass spectrometer SRM configuration

The following parameters were optimized for the ions of interest. These parameters should be re-optimized when transferring the method to another instrument. The mass spectrometer was operated under Multiple Reaction Monitoring (MRM) mode. The transitions of interest are presented in Table 6 and typical mass spectrometer parameters are presented in Tables 7 and 8.

Table 6. Perchlorate MRM Transitions

<b>Analyte</b>	<b>MRM Transition</b>
Perchlorate $\text{ClO}_4^-$	
Quantification	98.9 / 83.1
Confirmation	100.6 / 85.2
Labeled Perchlorate, $\text{Cl}^{18}\text{O}_4^-$	106.9 / 88.97
Nitrate, $\text{NO}_3$	
Quantification	62.0 / 45.8
Confirmation	62.0 / 62.0
Labeled Nitrate, $^{15}\text{NO}_3$	63.0 / 47.1
Thiocyanate, $\text{SCN}$	
Quantification	58.0 / 58.8
Confirmation	60.0 / 60.0
Labeled Thiocyanate, $\text{SC}^{15}\text{N}$	59.0 / 59.0

Table 7. Mass Spectrometer Configuration.

<b>Parameter</b>	<b>Setting</b>
Scan type	MRM
Polarity	Negative
Ion Source	Turbo Spray
Temperature	600°C
IS	-4000 V
CAD	12
CUR	10
GSI	45 psi
GS2	45 psi
Dwell Time	400 msec
Probe Y distance	2.0 mm

Table 8. Mass Spectrometer Parameters Characteristics for each Analyte

<b>Analyte</b>	<b>DP</b>	<b>EP</b>	<b>CE</b>	<b>CXP</b>
Perchlorate				
Quantification	-55	-10	-45	-1
Confirmation	-60	-10	-38	-3

Nitrate				
Quantification	-40	-10	-40	-5
Confirmation	-40	-10	-35	-6
Thiocyanate				
Quantification	-76	-6	-55	-7
Confirmation	-35	-10	-25	-3

## 7. Calibration and Calibration Verification

### a. Creation of curve

#### 1) Calibration Data

- i. Prepare fresh calibrators for each set of unknown analyses.
- ii. Analyze each set of unknowns to form the calibration curve for that set of samples.
- iii. Generate a linear calibration curve with nine standards using the ratio of the peak area of the analyte to the labeled internal standard.

#### 2) Calculation of curve statistics

Determine the slope, intercept and R-squared value for the nine-point calibration curve using a 1/x-weighted linear regression in Analyst 1.4 software.

#### 3) Evaluation of curve statistics

Evaluate the calibration curve statistics to ensure that the R-squared value of the curve is equal to or greater than 0.990, and that the linearity of the standard curve extends over the entire standard range. If the calculated value of one calibrator deviates by greater than 20% from the actual value then that one calibrator can be excluded.

#### 4) Calibration verification

Calibration is verified by analyzing a full set of calibrators with every run. In addition, an external standard blind to the analyst is analyzed at least once every 6 months and whenever the instrument is non-operational due to repairs or maintenance. This external standard blind result must agree with certified or accepted values within the 95% confidence and range intervals.

### b. Use of the calibration curve

The lowest point on the calibration curve is the lowest reportable level and the highest point is above the expected range of results. The remaining points are distributed between these two extremes, with the majority of points in the concentration range where most unknowns fall.

## 8. Procedure Operation Instructions; Calculations; Interpretation of Results

An analytical run consists of a blank, 9 calibration standards, 2 low level QCs, 2 high level QCs and up to 75 unknown urine samples.

### a. Sample preparation

#### 1) Preliminary sample preparation steps

- a) Allow frozen urine specimens, quality control materials, calibration standards and synthetic urine to reach ambient temperature.
- b) Mix samples thoroughly by inversion or vortexing.
- c) Set up and label a series of 1.5-mL autosampler vials corresponding to the number of blanks, standards, QCs and samples to be analyzed.

#### 2) Preparation of standards (1-8)

- a) Using a 100- $\mu$ L pipettor transfer 50  $\mu$ L of the appropriate standard stock solution into the appropriately marked autosampler vial.
- b) Using a 1000- $\mu$ L pipettor add 450  $\mu$ L of DI water.
- c) Using a 1000- $\mu$ L pipettor add 500  $\mu$ L of the internal standard solution to make a final volume of 1 mL.
- d) Cap the vial and mix for a few seconds using a vortex mixer.

#### 3) Preparation of standard 9

- a) Using a 100- $\mu$ L pipettor transfer 50  $\mu$ L of standard mix 9 into the appropriately marked autosampler vial.
- b) Using a 100- $\mu$ L pipettor add 50  $\mu$ L of the 1000 ppm nitrate certified stock solution.
- c) Using a 1000- $\mu$ L pipettor add 400  $\mu$ L of DI water.
- d) Using a 1000- $\mu$ L pipettor add 500  $\mu$ L of the internal standard solution to make a final volume of 1 mL.

#### 4) Preparation of the blank

- a) Using a 1000- $\mu$ L pipettor transfer 500  $\mu$ L of DI Water into the appropriately marked autosampler vial.

- b) Using a 1000- $\mu$ L pipettor add 500  $\mu$ L of the internal standard solution to make a final volume of 1 mL
- c) Cap the vial and mix for a few seconds using a vortex mixer.

5) Preparation of the low Quality Control sample

- a) Mix (either by vortexing or repetitive sample inversion) the QC sample.
- b) Aliquot 250  $\mu$ L of QC low stock solution into the autosampler vial using the Hamilton MicroLab Star (Appendix I).
- c) Using a 300- $\mu$ L pipettor add 250  $\mu$ L of DI water
- d) Using a 1000- $\mu$ L pipettor add 500  $\mu$ L of the internal standard solution to make a final volume of 1 mL.
- e) Cap the vial and mix for a few seconds using a vortex mixer.

7) Preparation of the high Quality Control sample

- a) Mix (either by vortexing or repetitive sample inversion) the QC sample.
- b) Aliquot 250  $\mu$ L of QC high stock solution into the autosampler vial using the Hamilton MicroLab Star (Appendix I).
- c) Using a 300- $\mu$ L pipettor add 250  $\mu$ L of DI water
- d) Using a 1000- $\mu$ L pipettor add 500  $\mu$ L of the internal standard solution to make a final volume of 1 mL.
- e) Cap the vial and mix for a few seconds using a vortex mixer.

7) Preparation of the unknown specimens

- a) Mix (either by vortexing or repetitive sample inversion) the unknown sample.
- b) Aliquot 250  $\mu$ L of unknown into the autosampler vial using the Hamilton MicroLab Star (Appendix I).
- c) Using a 300- $\mu$ L pipettor add 250  $\mu$ L of DI water.
- d) Using a 1000- $\mu$ L pipettor add 500  $\mu$ L of the internal standard solution to make a final volume of 1 mL.
- e) Cap the vial and mix for a few seconds using a vortex mixer.

**Note:**

- a) For the delivery of internal standard and DI water an Eppendorf Repeater Plus Pipette can be used.

**b. Instrument and software setup for the IC-MS/MS**

1) Preliminary system setup

a) Tuning and calibration of the mass spectrometer

- i. Set the y-distance of the probe to 6 mm and infuse the PPG 3000 solution at a 10  $\mu\text{L}/\text{min}$  flow rate.
- ii. Using **Manual Tuning**, load the PPG 3000 calibration file. In the tuning window make sure that the mass spectrometer is showing peaks for each ion in the calibration file. This is to make sure that the tuning solution is constantly flowing into the mass spectrometer.
- iii. Once checked, perform a **Resolution Optimization with Calibration** upon success.
- iv. Make sure that the following specified parameters are met. For peak width, the resolution is set to  $0.60 \pm 0.05$  mass units and sensitivity is met using the ion 932 m/z with an intensity of  **$2.0 \times 10^7$  minimum** (combined intensity of 10 scans).
- v. Check the tune and mass calibration of the instrument weekly.

b) IC system setup

- i. Fill the mobile phase bottles with filtered and sonicated (5-min) fresh DI water.
- ii. Ensure that the water reservoir for the suppressor is full.
- iii. Load the program file mmdyy\_INIS.pgm and start the pump.
- iv. Allow the system to equilibrate for 1 hr prior to starting a run
- v. Once the total conductivity in the system reaches a value less than 3  $\mu\text{Siemens}$ , the system is ready.

c) Performance evaluation

- i. Allow the system to equilibrate with the method to be run (both MS and IC).
- ii. To check the performance of the system, inject a standard three times to ensure equilibration of the system.
- iii. Examine the peak to ensure an acceptable signal-to-noise ratio ( $S/N > 10$  for the lowest standard).
- iv. Once these limits are met the system is ready to start a run.

2) Final setup and operation

- a) Create the run sequence

In the PeakNet software of the IC system, create a sequence for the run using the wizard. Make sure that the appropriate number of samples is loaded and the appropriate program is selected (*mmddy* – NIS.pgm; where *mmddy* is the most recent date that the program was changed and/or saved).

- b) Assign the acquisition and quantitation methods
  - i. Import the .csv file obtained from ATLAS into Analyst. The .csv file includes the sequence information of the standards, QCs, and unknowns to be analyzed.
  - ii. Select the acquisition method (*mmddy*\_INIS.dam; where *mmddy* is the most recent date that the method was changed and / or saved) and the quantitation method (*mmddy*\_INIS.qmf; where *mmddy* is the most recent date that the method was changed and / or saved).
  - iii. The letter “I” before the methods name (NIS) correspond to the first letter of the instruments name (J for Joker and M for McDreamy).
  - iv. Ensure that the icons on the right corner of the window are green indicating that the system has equilibrated and is ready to start.
- c) Submit and start batch in Analyst
  - i. Open and submit the **Equilibration** batch as well as the batch of the unknowns to be analyzed.
  - ii. Press the “Start Sample” icon on top of the window to start the run.
  - iii. The instrument waits for a sync signal from the IC to start the acquisition.
- d) Start the sequence in IC
  - i. Click **Batch** in the main menu and select edit.
  - ii. Once the window is open, select the sequence to be run starting with the equilibration sequence.
  - iii. Once the sequences are selected, press **Start**, making sure that the MS is ready to start.
  - iv. The system will immediately start by turning green on the first sequence to run.

### 3) System shutdown

After the end of an analytical run flush the system with DI water to eliminate any salt residue accumulation. After flushing the system shut down the IC instrument as well as the MS.

### c. **Processing of data**

- 1) Once the run has finished, note the final pressure as well as conductivity in the instrument maintenance book.

- 2) Quantify all raw data files using the quantitation capabilities of the Analyst software. The peaks are automatically integrated using the quantitation method created for the analysis.
- 3) Visually review the integration of each peak and manually correct when needed.
- 4) Generate a calibration curve from the calibrators; QCs, unknowns and blanks are quantified against the calibration curve.
- 5) Save the reviewed data files in a report file and export as a text file.
- 6) Open the text file in the Excel file mcrSCIEUp8.xls available on the Q drive and run the file through the macro “mcrFormatResults”.
- 7) Save the data as an Excel file and import into the ATLAS Perchlorate database for further evaluation, including the QC evaluation described in Section 10.b.2.
- 8)

## **9. Reportable Range of Results**

### **a. Linearity Limits**

The reportable range of results for perchlorate using this method is 0.05 to 100 µg/L. The lower reportable limit corresponds to the lowest standard 0.05 µg/L which is greater than the detection limit for the method. The upper reportable limit corresponds to the concentration of the highest standard 100 µg/L. In the case of nitrate and thiocyanate the lowest reportable levels are 500 and 10 µg/L respectively. The upper reportable limits are 100,000, and 5000 µg/L.

Table 9. Method Detection Limits, Lowest Reportable Values and Calibration Ranges

<b>Compound</b>	<b>Linear Range (µg/L)</b>	<b>R<sup>2</sup></b>	<b>Limit of Detection (µg/L)</b>	<b>Lowest Reportable Level (µg/L)</b>
Nitrate	500 - 100000	0.9930	143	500
Perchlorate	0.05 - 100	0.9998	0.004	0.05
Thiocyanate	10 - 5000	0.9992	0.681	10

### **b. Limit of Detection**

The limit of detection was determined (using Taylor’s method<sup>12</sup>) by calculating the standard deviation at different standard concentrations following repeated measurements of the concentration standards in urine. The absolute values of the standard deviations were then plotted versus concentration. The intercept of the least squares fit of this line equals  $S_0$ ;  $3S_0$  equals the limit of detection (LOD). Since the LOD is below the lowest standard, the lowest standard is used as the lowest reportable level.

**c. Accuracy**

The accuracy of the assay was established by analyzing certified perchlorate standards blind to the analyst (i.e. Proficiency Testing samples) and matrix spike samples. The accuracy of the method was obtained by comparing the concentration calculated from analyzing the samples to the theoretical concentration. The results of these measurements are given in Table 10.

Table 10. Method Accuracy and Precision

Analyte	Sample	Average %CV <sup>a</sup>	Average Absolute % Diff <sup>b</sup>
Nitrate	Proficiency Test (1500 - 62000 µg/L)	1.88	4.54
	Spiked Urine (5000 – 75000 µg/L)	3.98	2.39
Perchlorate	Proficiency Test (0.19 - 72.0 µg/L)	4.7	3.78
	Spiked Urine (4.0 - 75 µg/L)	2.14	3.59
Thiocyanate	Proficiency Test (100 – 4000 µg/L)	0.48	5.08
	Spiked Urine (600 - 4000 µg/L)	0.82	1.81

<sup>a</sup> Coefficient of Variation

<sup>b</sup> Average absolute value of % difference between theoretical and calculated amount

**d. Precision**

The precision of the method is reflected in the variance of quality control samples analyzed over time. The coefficient of variation (CV) of the method determined by analyzing 20 QC samples is listed in Table 11 below.

**e. Analytical specificity**

IC-MS/MS is the most selective analytical method in use for quantifying the target analytes in complex aqueous matrices. Ion chromatography produces reproducible chromatographic resolution of the target analytes, even in the most concentrated urine samples. The analyte peaks elute in well-defined regions of the chromatogram with no visible interferences and very low background. Tandem mass spectrometry provides a further degree of selectivity, by filtering out all ions except a specific transition of parent to daughter ion for each analyte. Additionally, qualifier ratios are determined by comparing the responses of the quantitation ion and the confirmation ion transitions over the standard and QC samples. The average value of this ratio  $\pm 20\%$  is used to confirm the analyte determined in unknown samples that are found at levels above the limit of detection.

**10. Quality Assessment and Proficiency Testing**

**a. Quality Assessment**

Quality assessment procedures follow standard practices<sup>13</sup>. Daily experimental checks are made on the stability of the analytical system. Blanks and standards, as well as QC materials, are added to each day's run sequence. The QC blank is analyzed at the beginning of each run to check the system for possible contamination or in the spiking solutions and/or reagents. Relative retention times are examined for the internal standard to ensure the choice of the correct chromatographic peak. A calibration curve is developed for the batch using a complete set of calibration standards. The calibration curve must be linear with an R<sup>2</sup> value of at least 0.990. The results from the analysis of a QC standards obtained using this calibration curve are compared with acceptance criteria given below to assure the proper operation of the analysis.

**b. Quality Control Procedures**

1) Establishing QC limits

Quality control limits are established by characterizing assay precision with 20 distinct analyses of each QC pool. Two different pools of quality control material are used. Different variables are included in the characterization analyses (e.g. different analysts, columns, reagents) to capture realistic assay variation over time. The mean, standard deviation, coefficient of variation, and confidence limits are calculated from this QC characterization data set. Individual quality control charts for the characterization runs are created, examined, and quality control limits are used to verify assay precision and accuracy on a daily basis. Typical QC characterization statistics are listed in Table 11. Limits are based on statistical calculation accounting for 2 QCs analyzed in each analytical run.

Table 11. NIS Quality Control Samples QC 0707

Analyte ID	QC ID	Count	Mean	$\sigma$	%C V	Mean - 3 $\sigma$	Mean - 2 $\sigma$	Mean + 2 $\sigma$	Mean + 3 $\sigma$
Nitrate	QH0707	182	95,332	1,45 2	1.5	90,657	92,225	98,438	100,006
	QL0707	182	33,459	1,05 2	3.1	30,072	31,208	35,709	36,845
Thiocyanate	QH0707	186	3,532	142. 9	4.1	3,072	3,226	3,837	3,992
	QL0707	186	1,927	73.5	3.8	1,690	1,769	2,084	2,163

<b>Perchlorate, Nitrate, and Thiocyanate in Urine</b> <b>NHANES 2009-2010</b>
--

				7					
Perchlorate	QH0707	188	72.54	2.16	3.0	65.58	67.92	77.17	79.50
	QL0707	188	3.2	0.11	3.4	2.85	2.96	3.43	3.55

$\sigma$  = standard deviation, %CV = % coefficient of variation

2) Quality Control evaluation

After the completion of a run, the calculated results from the analysis of quality control samples are compared to the established quality control limits to determine if the run is “in control”. The quality control rules apply to the average of the beginning and ending analyses of each of the QC pools. The quality control results are evaluated according to Westgard<sup>12</sup> rules:

- a) If both the low and the high QC results are within the  $2\alpha$  limits, then accept the run.
- b) If one of two QC results is outside the  $2\alpha$  limits, then apply the rules below and reject the run if any condition is met.
  - i.  **$1_{3\alpha}$**  – Average of both low QC OR average of both high QC is outside of a  $3\alpha$  limit.
  - ii.  **$2_{2\alpha}$**  – Average of both low QC AND average of both high QC is outside of  $2\alpha$  limit on the same side of the mean.
  - iii.  **$R_{4\alpha}$  sequential** – Average of both low QC AND average of both high QC is outside of  $2\alpha$  limit on opposite sides of the mean.
  - iv.  **$10_x$  sequential** – The previous 9 average QC results (for the previous 9 runs) were on the same side of the mean.

If a QC result is declared “out of control”, the results for all patient samples analyzed during that run are invalid for reporting.

c. **Proficiency Testing**

1) Scope of PT

The proficiency testing (PT) scheme for this method is administered by an in-house Proficiency Testing Coordinator. Aqueous proficiency testing materials were purchased, diluted into synthetic urine, and blind-coded by the in-house PT Coordinator. The samples are analyzed blind and the results evaluated by the in-house PT coordinator.

2) Frequency of PT

Five samples of unknown PT concentrations are analyzed twice a year using the same method described for unknown samples.

3) Documentation of PT

Analytical PT results are reviewed by the analyst and laboratory supervisor and submitted to the in-house PT Coordinator electronically. The PT results are evaluated by the PT Coordinator; the analysis passes proficiency testing if  $\geq 80\%$  of the results deviate  $\leq 20\%$  from the known value. A summary report of the PT evaluation is maintained by the laboratory supervisor. If the assay fails proficiency testing then the sample preparation and instrumentation are thoroughly examined to identify and correct the source of assay error. Unknown specimens are not analyzed until the method successfully passes proficiency testing.

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

If an analyte result for a quality control material falls outside of the  $3\sigma$  limits for mean or range it fails the QC criteria described in section 10.b.2, then the following steps are taken.

- 1) If a particular calibration standard is obviously in error, remake a new dilution of that calibration standard (see section 8.a.2), reanalyze it, and reprocess the sample analyses using this new result as part of the calibration curve.
- 2) Prepare a fresh dilution of the failing QC material (working QC standard) (see sections 8.a.4 & 5) and re-analyze it.
- 3) Prepare fresh dilutions of the calibration standards (see section 8.a.1), and re-analyze the entire calibration curve using the freshly prepared standards.

If these three steps do not result in correction of the “out of control” values for QC materials, the supervisor should be consulted for other appropriate corrective actions. Analytical results are not reported for runs that are out of statistical control.

## **12. Limitations of Method, Interfering Substances and Conditions**

The described method is highly selective. Due to excellent chromatographic and mass spectrometric resolution, we have not found any substances that have similar chromatographic and mass spectrometric characteristics. In less than 1% of urine samples the presence of an unknown compound does distort perchlorate chromatography. This problem is resolved by diluting the sample 5-fold and re-analyzing it.

## **13. Reference Ranges (Normal Values)**

Reference ranges for perchlorate, nitrate and thiocyanate are presented in Table 12, as derived from NHANES 2001-2002 data for study participants ages 6+.

Table 12. Geometric mean and selected percentiles of urinary perchlorate, nitrate and thiocyanate levels.

Analyte	Sample Size	GM	5 <sup>th</sup> Percentile	50 <sup>th</sup> Percentile	95 <sup>th</sup> Percentile
Perchlorate	2820	3.54	0.78	3.6	14
		(3.29-3.81) <sup>a</sup>	(0.68-0.91)	(3.4-3.9)	(11-17)
Nitrate	2815	45857	11000	51000	120000
		(43919-47881)	(9700-11000)	(49000-53000)	(12000-13000)
Thiocyanate	2817	1446	230	1300	9900
		(1366-1530)	(190-270)	(1300-1500)	(8500-11000)

<sup>a</sup> 95% Confidence Interval.

#### 14. Critical Call Results (“Panic Values”)

The health effects of chronic exposure to trace levels of perchlorate are unclear. Therefore a definitive panic value has not been established. The National Academy of Sciences has reviewed the toxicological literature for perchlorate and recommended 0.0007 mg/Kg-day as a reference dose. This dose correlates to a urinary perchlorate excretion rate of 35 µg per g creatinine and would be flagged as a “high exposure level”. Greer et al reported possible inhibition of thyroid hormones at a dose of 0.5 mg/Kg-day of perchlorate<sup>14</sup>. This dose correlates to a urinary perchlorate excretion rate of 24,000 µg/g creatinine, which would be set as the “Critical Call Value”.

#### 15. Specimen Storage and Handling During Testing

Specimens may reach and maintain ambient temperature during analysis. Perchlorate in urine is stable at room temperature. If the measurement is delayed until the next day, refrigerate the samples at 4±3°C.

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

Alternate validated methods have not been evaluated for measuring perchlorate in urine. If the analytical system fails, refrigerate the samples at 4±3°C until the analytical system is restored to functionality. If long-term interruption (greater than 4 weeks) is anticipated, store urine specimens at -20±5°C.

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

Results are reported to two significant digits based on assay sensitivity calculations. Study subject data is reported in both concentration units (ng/mL) and adjusted based on creatinine excretion ( $\mu\text{g/g}$  creatinine).

Once the validity of the data is established by the QC/QA system outlined above, these results are verified by a DLS statistician, and the data reported in both hard copy and electronic copy. This data, a cover letter, and a table of method specifications and reference range values will be routed through the appropriate channels for approval (i.e. supervisor, branch chief, division director). After approval at the division level, the report will be sent to the contact person who requested the analyses.

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

If greater than 1 mL of sample remains following successful completion of analysis, this material should be returned to storage at  $-20\pm 5^{\circ}\text{C}$  in case reanalysis is required. These samples shall be retained until valid results have been obtained and reported and sufficient time has passed for review of the results.

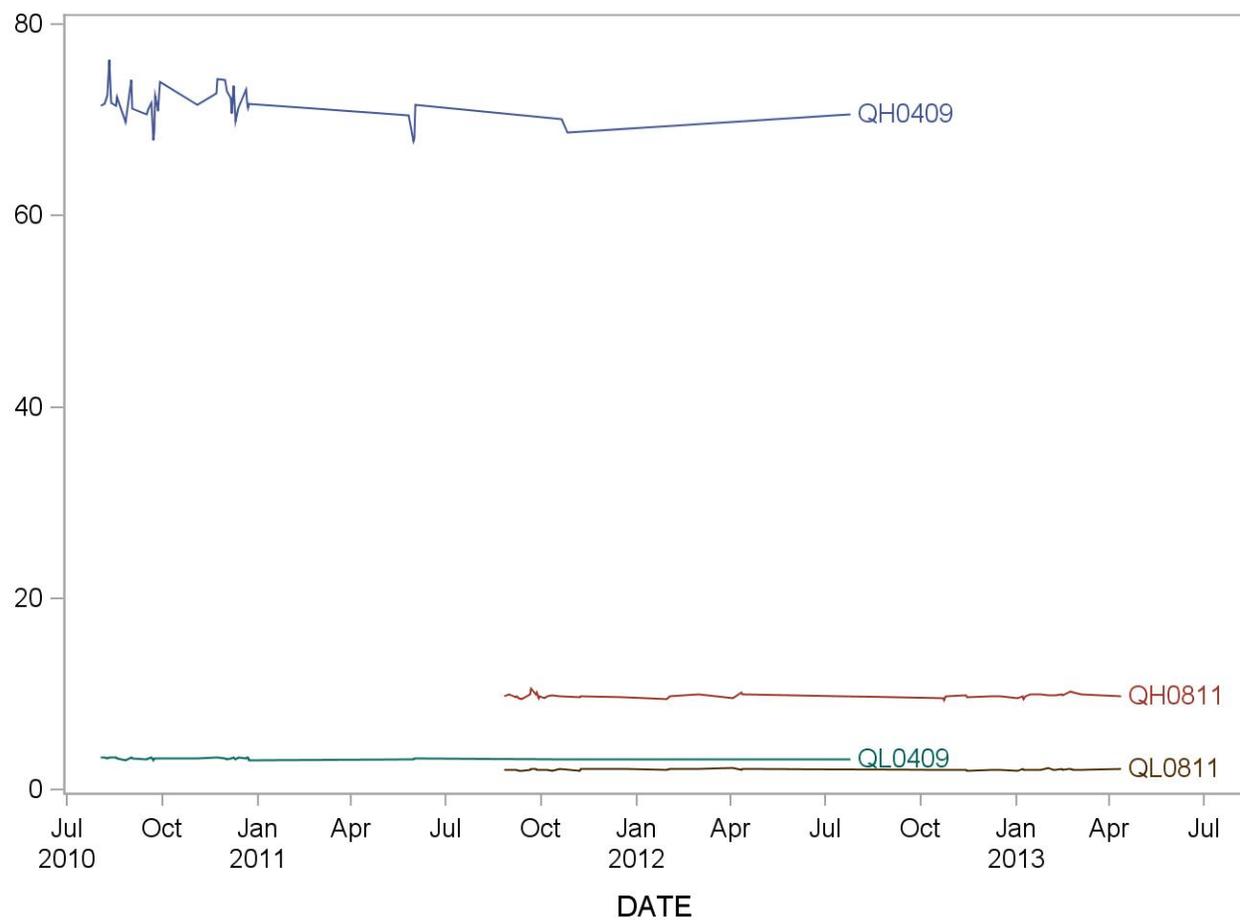
Standard record keeping (e.g., database, notebooks, data files) is used to track specimens. Records are maintained for 3 years, including related QA/QC data, and duplicate records will be kept off-site in electronic format. Study subject confidentiality is protected by providing personal identifiers only to the medical officer.

### **19. Summary Statistics and QC Graphs**

See following pages

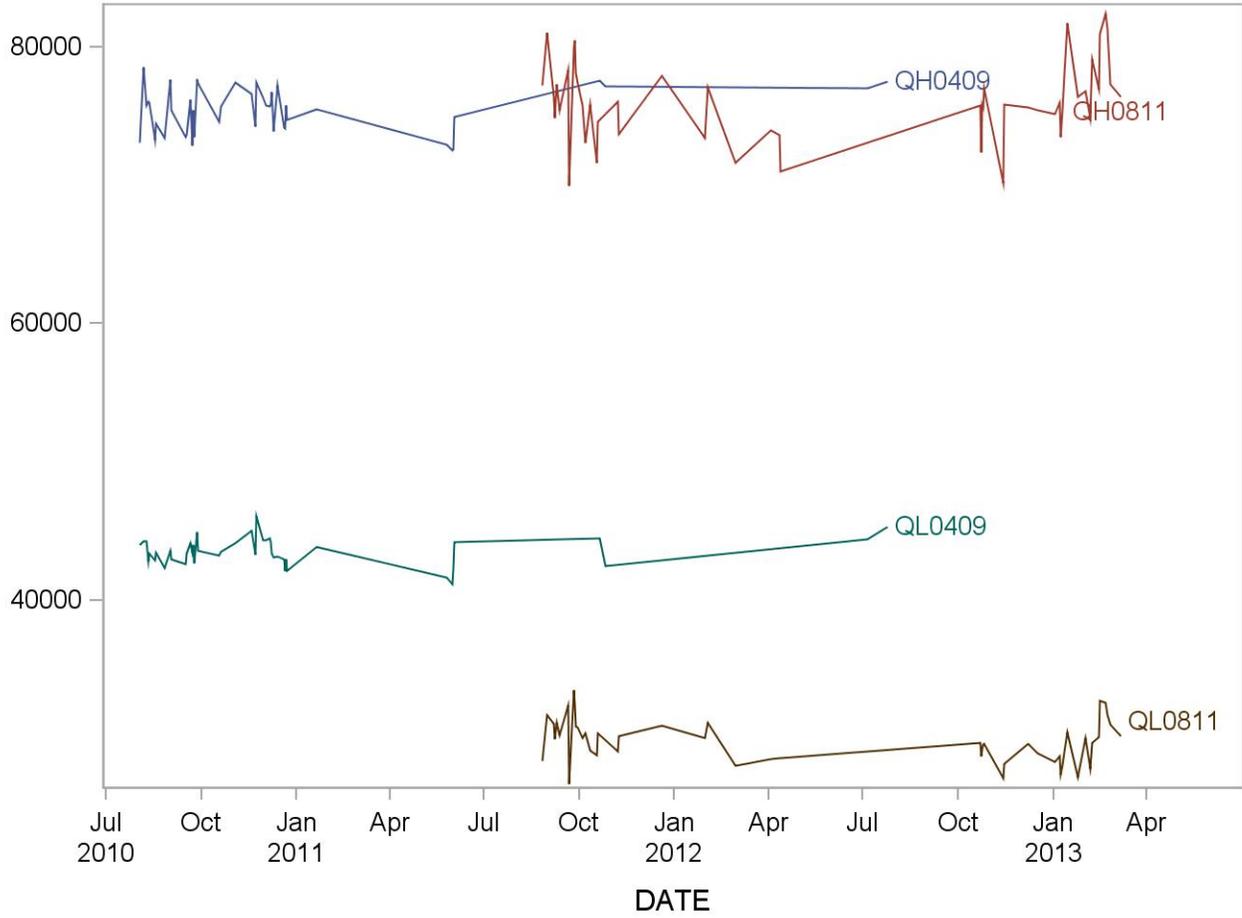
**2009-2010 Summary Statistics and QC Chart for Perchlorate, urine (ng/mL)**

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0409	40	02AUG10	25JUL12	71.54750	1.83373	2.6
QL0409	40	02AUG10	25JUL12	3.29713	0.08290	2.5
QH0811	51	26AUG11	12APR13	9.84611	0.22622	2.3
QL0811	51	26AUG11	12APR13	2.13265	0.06335	3.0



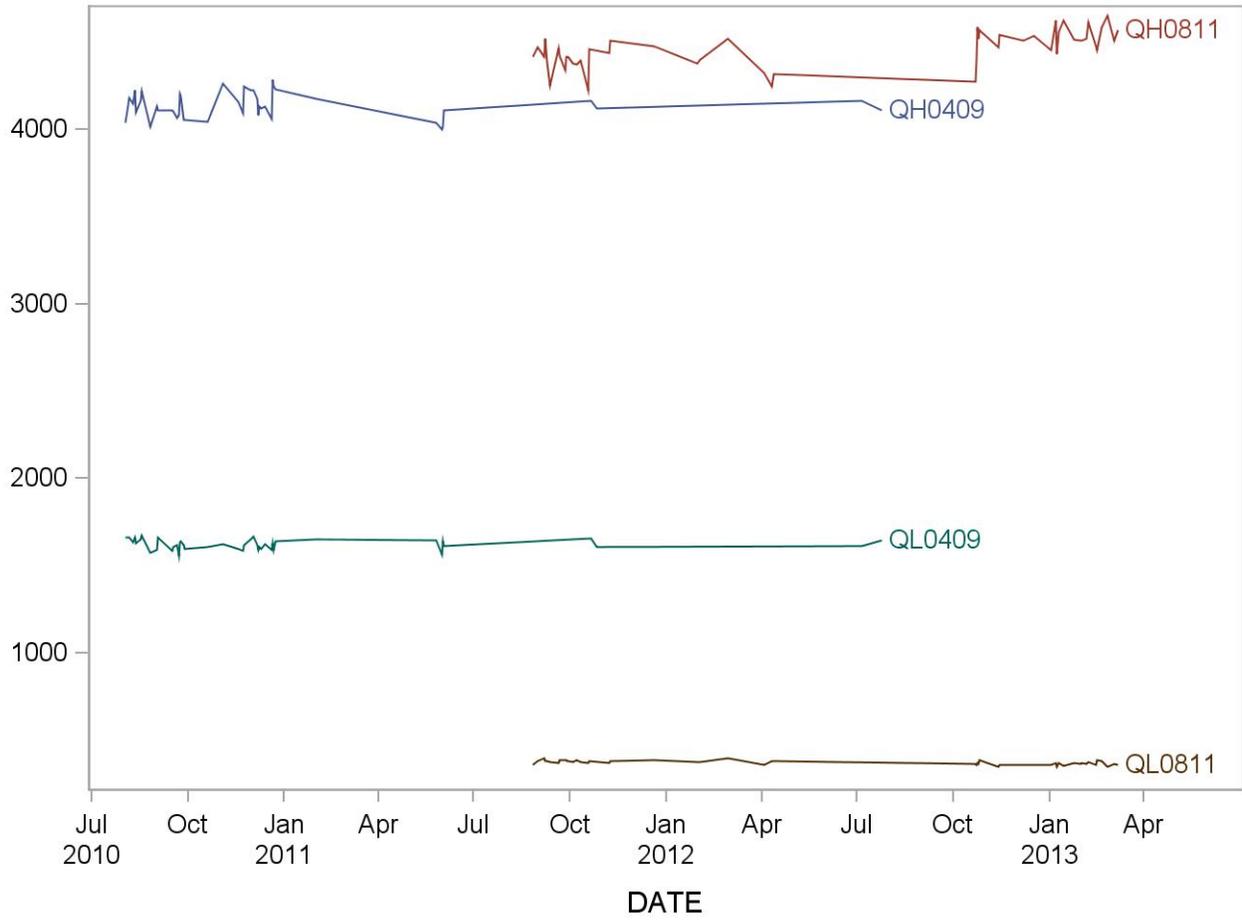
**2009-2010 Summary Statistics and QC Chart for nitrate urine**

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0409	45	02AUG10	25JUL12	75450.0	1684.8	2.2
QL0409	45	02AUG10	25JUL12	43545.6	1028.0	2.4
QH0811	51	26AUG11	07MAR13	76066.7	2869.4	3.8
QL0811	51	26AUG11	07MAR13	29783.3	1544.7	5.2



**2009-2010 Summary Statistics and QC Chart for thiocyanate urine**

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0409	44	02AUG10	25JUL12	4135.909	74.17196	1.8
QL0409	44	02AUG10	25JUL12	1621.932	29.17900	1.8
QH0811	52	26AUG11	07MAR13	4462.885	100.3052	2.2
QL0811	52	26AUG11	07MAR13	370.8654	12.50965	3.4



#### Reference List

1. Tonacchera, M. *et al.* Relative potencies and additivity of perchlorate, thiocyanate, nitrate, and iodide on the inhibition of radioactive iodide uptake by the human sodium iodide symporter. *Thyroid* **14**, 1012-1019 (2004).
2. Wyngaarden, J. B., Stanbury, J. B. & Rapp, B. The effects of iodide, perchlorate, thiocyanate and nitrate administration upon the iodide concentrating mechanism of the rat thyroid. *Endocrinology* **52**, 568-574 (1953).
3. Braverman, L. E. & Utiger, R. D. *Werner & Ingbar's The Thyroid: A fundamental and clinical text*. Braverman, L. E. & Utiger, R. D. (eds.), pp. 719-720 (Lippincott Williams & Wilkins, Philadelphia, PA, 2000).
4. Wolff, J. Perchlorate and the thyroid gland. *Pharmacol. Rev.* **50**, 89-105 (1998).
5. Hetzel, B. S., Potter, B. J. & Dulberg, E. M. The iodine deficiency disorders: nature, pathogenesis and epidemiology. *World Rev. Nutr. Diet.* **62**, 59-119 (1990).
6. Bertelsen, J. B. & Hegedus, L. Cigarette smoking and the thyroid. *Thyroid* **4**, 327-331 (1994).
7. Borgers, D. & Junge, B. Thiocyanate as an indicator of tobacco smoking. *Prev. Med.* **8**, 351-357 (1979).

8. Paula, C. A. *et al.* - Combination of cassava flour cyanide and urinary thiocyanate measurements of school children in Mozambique. - *International Journal of Food Sciences & Nutrition* **55**, 183-190 (2004).
9. Blount, B. C., Valentin-Blasini, L., Mauldin, J. P., Pirkle, J. L. & Osterloh, J. D. Perchlorate Exposure of the U.S. Population, 2001- 2002. *Journal of Exposure Science and Environmental Epidemiology* **17**, 400-407 (2007).
10. Blount, B. C., Pirkle, J. L., Osterloh, J., Valentin-Blasini, L. & Caldwell, K. L. Urinary Perchlorate and Thyroid Hormone Levels in Adolescent and Adult Men and Women Living in the United States. *Environ. Health Perspect.* **114**, 1867-1871 (2006).
11. Valentín-Blasini L, Blount BC, Delinsky A. Quantitation of iodide and sodium-iodide symporter inhibitors in human urine using ion chromatography tandem mass spectrometry. *J. Chrom. A.* 2007; 1155(1):40-46.
12. Taylor JK *Quality Assurance of Chemical Measurements*. Lewis Publishers, New York (1987).
13. Caudill, S.P., Schleicher, R.L., Pirkle, J.L. Multi-rule quality control for the age-related eye disease study. *Stat. Med.* 2008; 27(20), 4094-40106.
14. Greer, M. A., Goodman, G., Pleus, R. C. & Greer, S. E. Health effects assessment for environmental perchlorate contamination: the dose response for inhibition of thyroidal radioiodine uptake in humans. *Environ. Health Perspect.* **110**, 927-937 (2002).

*Use of trade names is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.*

---

## **APPENDIX I**

### **1. Automated Sample Aliquoting Technique**

Instrument used: [Hamilton MicroLab Star](#), (Hamilton Robotics, Inc. Reno, NV)

- a) Remove urine samples for Ultra-Freezer (-70°C), and place them in the refrigerator (0-4°C) to thaw overnight.
- b) On the morning of aliquoting, remove samples from the refrigerator and allow them to get to room temperature.
- c) Invert each box of samples 7 times, and allow samples to stand for 10 minutes.
- d) When removing caps, make sure they stay in the proper order.
- e) Place cryovial tubes containing unknown urine samples and blank Dionex vials in the Hamilton racks (The urine samples will be aliquoted into the Dionex vials).

- f) Please note: There are 32 holders in each Hamilton rack; and the holders are numbered in order from 1-32. Place the tubes in numerical order (left to right).
- g) Load tubes and dispensing tips onto the Hamilton instrument.
- h) The Hamilton mixes each sample five times (Aspirating and dispensing 1mL of sample each time).
- i) When finish mixing, the Hamilton dispenses 250  $\mu$ L of urine into its corresponding Dionex vial.
- j) Cap the Dionex vials and recap the urine samples.
- k) Store samples at  $-70^{\circ}\text{C}$  until analysis.