Laboratory Procedure Manual

Analyte: **Total Thyroxine, Total T4**

Matrix: **Serum**

Method: **Access 2 (Beckman Coulter)**

Method No:

Revised:

as performed by:

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Important Information for Users

University of Washington 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.

Total Thyroxine, (Total T4) in Serum
NHANES 2009-2010

Public Release Data Set Information

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

File Name	Variable Name	SAS Label
THYROD_F	LBXTT4	Total Thyroxine (mcg/dL)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

The Access Total T4 assay is a competitive binding immunoenzymatic assay. A sample is added to a reaction vessel with anti-thyroxine antibody, thyroxine-alkaline phosphatase conjugate, and paramagnetic particles coated with goat anti-mouse capture antibody and a stripping agent to dissociate all T4 from binding proteins. Thyroxine in the sample competes with the thyroxine-alkaline phosphatase conjugate for binding sites on a limited amount of specific anti-thyroxine antibody. Resulting antigen: antibody complexes bind to the capture antibody on the solid phase. After incubation in a reaction vessel, materials bound to the solid phase are held in a magnetic field while unbound materials are washed away. Then, the chemiluminescent substrate Lumi-Phos™ 530 is added to the vessel and light generated by the reaction is measured with a luminometer. The light production is inversely proportional to the concentration of thyroxine in the sample. The amount of analyte in the sample is determined from a stored, multi-point calibration curve.

The thyroxine gland secretes two hormones, L-thyroxine (T4) and L-triiodothyronine (T3) in response to thyroid stimulating hormone (TSH) produced by the pituitary gland. Given normal levels of thyroid hormone-binding proteins, hyperthyroidism is characterized by increased levels of circulating T4, hypothyroidism by decreased levels. Exceptions to this are found in patients with abnormal levels of thyroid hormone-binding proteins. Elevated levels of thyroid binding globulin (TBG) can be found in patients with the following conditions: pregnancy, acute intermittent porphyria, hyperproteinemia, and in patients undergoing estrogen therapy or taking oral contraceptives. Total T4 levels may be depressed when TBG levels are low, as in nephrotic, hepatic, gastrointestinal and neoplastic disorders, in acromegaly, hypoproteinemia, and in patients undergoing androgen, testosterone or anabolic steroid therapy. Monitoring of thyroid hormone levels after therapy is usually performed to assist in adjusting drug dosages and to confirm the clinically observable results of therapy.

Serum T4 is used in conjunction with other thyroid hormones as an indicator of thyroid function. The biological effects of the thyroid hormones are profound. They influence the rate of oxygen consumption and heat production in virtually all tissues. The mechanism of this effect may involve uncoupling of oxidation and phosphorylation. The hormones are also indispensable for growth, development, and sexual maturation.

2. SAFETY PRECAUTIONS

Consider all samples received for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe universal precautions. Wear gloves, lab coat, and safety glasses when handling all human blood products and infectious viruses. Place disposable plastic, glass, paper, and gloves that contact blood in a biohazard bag or discard pan to be autoclaved. Disinfect all work surfaces with Vephene solution. Dispose of all biological samples and diluted specimens in a biohazard bag at the end of the analysis.

Do not pipette by mouth. Do not eat, drink or smoke in designated work areas. Wash hands thoroughly after removal or personal protective devices used in handling specimens and kit reagents.

Material safety data sheets for all reagents used in the performance of this assay are kept in the Immunology Division, University of Washington Medical Center (UWMC).

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

A. Each shipment of specimens received from the NHANES mobile unit arrives with a corresponding transmittal sheet and an electronic version of the shipping/resulting file. The file structure is determined by NHANES and is described in the National Health and Nutrition Examination Survey (NHANES) Contract Laboratory Manual.

B. After the testing is completed results from the Access 2 are transferred to the laboratory server system, which is backed up daily. This instrument file contains the following information for each sample, control and calibrator tested.

Patient ID
Sample ID
Rack
Verify
Test Name
Interpretation
Result
Units
Comp. Time
Flags
LIS
Instrument
RLU
Pipettor
Sample Type
Sample Priority
Test ID
Reagent Pack Lot #
Reagent Pack Serial #
Dilution
Calibrator level
Comments
Load Date/Time

C. QC results are transferred to an Excel file using laboratory-developed software. This file calculates the QC statistics, plots Levey-Jennings charts, displays relevant instrument flags, tracks reagent lots and recent calibrations. QC results are reviewed prior to resulting samples.

D. Sample results are transferred to an Excel file using laboratory-developed software that enters results after matching sample identifiers from the instrument file with those provided in the NHANES shipping/resulting file. This Excel file is formatted to match the NHANES shipping/resulting file and the program uses the conventions outlined in the NHANES Contract Laboratory Manual.

E. Data entry is checked for errors.

F. After the total T4 testing has also been completed, resulted, and checked, the result

file is transmitted electronically to NHANES WESTAT. Electronic and hard copies of the files are kept in the laboratory.

G. Technical support for this system is provided by Westat, Rockville, MD (1-301-294-2036)

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

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

B. Serum is the preferred specimen type. Heparin plasma is acceptable. If testing is to be done within 48 hours, samples can be refrigerated at 2 to 8°C. Freeze at -20ºC or colder for longer storage.

C. Blood should be collected aseptically and the serum separated by standard laboratory techniques. Specimens may be collected by using regular or serum-separator Vacutainers. Serum should be separated from the cells within 2 hours of collection.

D. The requested sample volume for the assay is 1.0 mL, and the minimum sample volume is 0.3 mL.

E. Specimens may be stored in glass or plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.

F. Turbid samples or those with particulate matter should be centrifuged prior to assay.

G. More than one freeze-thaw cycles is not recommended.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

6. PREPARATION OF REAGENTS, CALIBRATORS (STANDARDS), CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

A. Instrumentation

1. Beckman Access or Access II Immunoassay System (Beckman Coulter, Fullerton, CA.)

   The Beckman Access is a fully automated, random access, instrument that features on-board storage of reagent packs in a refrigerated compartment; an ultrasonic probe tip for level sense detection, sample and reagent delivery, mixing, and probe cleaning to minimize carryover; barcode identification of specimens and reagent packs; temperature controlled reaction reactions; and measurement and analysis of the light signal generated by the chemiluminescent reaction (RLU) using a four parameter logistic curve math model.

The total T4 assay parameter settings for the instrument are as follows:

Parameter	Setting
Sample Volume Requirements Minimum sample volume Sample volume used for testing	 250 ul 30 ul
No. of Standard Points	6
Calibration curve calculation	Four parameter logistic curve math model
Standard Curve Measuring Range (At initial dilution; approximate values, range is dependent upon standard value)	0 – 30.0 ug/dL (mcg/dL)

2. Hewlett Packard DeskJet printer (Hewlett Packard, Boise, ID)

3. Computers (Dell Computer Corporation, Round Rock, Texas).

4. Centrifuge (Jouan Inc., Winchester, VA)

B. Equipment

1. Reaction Vessels (Beckman Coulter, Fullerton, CA)

2. Sample Cups (Fisher Scientific, Pittsburgh, PA)

3. Latex gloves, disposable (Any manufacturer).

4. Pipettes and tips (Rainin, Emeryville, CA)

C. Reagents

All reagents are purchased from Beckman Coulter, Fullerton, CA.

1. <u>R1: Access Total T4 Reagent Pack</u>

Cat. No. 33800: 100 determinations, 2 packs, 50 tests/pack.

Provided ready to use. Store upright and refrigerate at 2 to 10°C. Refrigerate at 2 to 10°C for a minimum of two hours before use on the instrument. Stable until the expiration date stated on the label when stored at 2 to 10°C. Stable at 2 to 10°C for 14 days after initial use. Signs of possible deterioration are a broken elastomeric layer on the pack or control values out of range. If the reagent pack is damaged (i.e., broken elastomer), discard the pack. All antisera are polyclonal unless otherwise indicated.

R1a: Paramagnetic particles coated with goat anti-mouse IgG suspended in TRIS buffered saline, with surfactant, bovine serum albumin (BSA), 8-anilino-1-naphthalenesulfonic acid (ANS), < 0.1% sodium azide, and 0.1% ProClin™ 300

R1b: Mouse monoclonal antibody to thyroxine diluted in TRIS buffered saline, with surfactant, protein (aves, murine, goat), < 0.1% sodium azide, and 0.1% ProClin™ 300.

R1c: Thyroxine-alkaline phosphatase (bovine) conjugate diluted in TRIS buffered saline, with surfactant, protein (aves), < 0.1% sodium azide, and

0.1% ProClin™ 300.

2. Access Substrate Cat. No. 81906: 4 x 130 ml

   Lumi-Phos*530 (buffered solution containing dioxetane), Lumigen* PPD, fluorescer, and surfactant. Bring to room temperature (15 – 30 ºC) at least 18 hours before use. Stable for 14 days at room temperature or after bottle has been opened.

3. Access Wash Buffer II: Cat # A16792
   Provided ready to use. Store at room temperature (15 – 30 ºC), stable until expiration date on label.

D. Standards/Calibration Preparation

Access Total T4 Calibrators
Cat. No. 33805: S0–S5, 2.5 mL/vial
Provided ready to use. Store upright and refrigerate at 2 to 10°C. Mix contents by gently inverting before use. Avoid bubble formation. Stable until the expiration date stated on the label when stored at 2 to 10°C. Vial is stable at 2 to 10°C for four months after initial use. Signs of possible deterioration are control values out of range. Refer to calibration card and or vial labels for exact concentrations.
S0: Human serum with < 0.1% sodium azide, and 0.5% ProClin™ 300. Contains 0.0 mcg/dL (nmol/L) thyroxine.
S1–S5: Thyroxine in human serum at levels of approximately 2.0, 4.0, 8.0, 16.0, and 30.0 mcg/dL (26, 51, 103, 206, and 386 nmol/L), respectively, with < 0.1% sodium azide, and 0.5% ProClin™ 300
Calibration Card: 1

E. Preparation of Quality Control Materials

Two different levels of serum controls are run with each run. The controls are purchased from BioRad Laboratories (Hercules, CA) or prepared in-house. Commercial controls are stored and used according to the manufacturer's recommendations. In house controls are stored frozen (-20 ºC or colder). Once thawed, the controls are stored at 2-8 ºC. All controls are used within their stated expiration dates.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

A. Calibration Curve

Total T4 concentrations are calculated by using a calibration curve. This method utilizes a four parameter logistic curve with an inverse relationship of measured light produced (RLU) to concentration of total T4 in the serum sample. Serum results are expressed as ug/dL.

Calibrators are traceable to USP Levothyroxine reference material. The assigned values were established using representative samples from this lot of calibrator and are specific to the assay methodologies of the Access reagents. Values assigned by other methodologies may be different.

An active calibration curve is required for all tests. For the Access total T4 assay, calibration is required every 21 days or whenever new lot numbers of reagents are placed into use. Refer to the Operator's Guide and Reference Manual for complete instructions on calibration procedures.

B. Verification

1. Two levels of control are run for each test series. If, within a testing series, these controls do not conform to specifications as defined in the quality control manual, the entire series is invalidated.

2. New lot numbers of calibrator are verified by running 100 or more samples tested on the previous lot number. The correlation is analyzed using one or more linear regression formulas.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Preliminaries

1. Bring all controls and patient specimens to room temperature before use. Mix any specimens or controls that have been frozen. Centrifuge samples with particulate matter prior to testing.

2. Prime system: pipettor - 1 time, and substrate - 4 times

3. Check reagent, substrate, wash buffer, and reaction vessel status. Load any needed supplies onto the instrument. Mix reagent pack contents by gently inverting pack several times before loading on the instrument. Do not invert open (punctured) packs — mix reagents by swirling gently.

B. Instrument Operation (see operator's manual for details).

1. Check sample volume to make sure that there is sufficient volume to perform testing. Gently mix, uncap and load specimens into specimen racks, with the barcode in the open slot. Make sure there are no bubbles. Alternately, use the

barcode wand to identify the specimens. If the barcode is not reading properly, sample IDs can be entered manually. Load the racks onto the instrument.

2. Select the T4 test. Note: if other thyroid testing is also ordered, the entire 8 test panel can be ordered as a group. Testing is done in singlicate. Select the sample(s) to be used for the random repeat testing.

3. The instrument automatically calculates all results. After testing is completed, results are printed and review by the technologist.

4. Remove specimens and controls soon after the instrument finishes pipetting from the sample. Return controls to the refrigerator and refreeze specimens.

5. Perform scheduled instrument maintenance (daily, weekly, and monthly) as outlined on the maintenance log. See the operator's manual for specific instructions.

C. Recording of Data

1. Using a lab developed program, specimen results are transferred from the instrument data file into the assay specific results table created from the send file corresponding to the specific sample box. The file format is Excel (Microsoft Corporation, Redmond WA). A copy of this file is printed out and checked for accuracy of data entry.

2. Control results are entered to the Assay Specific QC/Levy-Jennings Table using the Excel program. Compliance with the Westgard rules is evaluated. A copy of this table is printed out and checked for accuracy of data entry.

D. Replacement and Periodic Maintenance of Key Components1.
Daily Maintenance:

1. **Start-up:**
   Inspect fluidics module.
   Check system supplies and replace as needed.
   Clean exterior of substrate, dispense, and aspirate probes.
   Prime pipettor – 1X and substrate - 4X.
   Verify temperature.
   Shut-down:
   Check waste containers, empty if needed
   Perform clean

2. Weekly Maintenance:
   Change probes and clean them
   Clean exterior of the analyzer
   Clean upper portion of the main pipettor with alcohol wipe
   Inspect waste filter bottle for fluid
   Run system check

3. Periodic Maintenance to be performed by the manufacturer's service engineer.

E. Calculations

Patient test results are determined automatically by the system software. The

amount of analyte in a sample is determined from the measured light production by means of a stored nonlinear calibration curve. Patient test results can be reviewed using the Sample Results screen. Refer to the Operator's Guide for complete instructions on reviewing results.

9. REPORTABLE RANGE OF TEST RESULTS

Results are reported to the nearest tenth (0.1). The lowest reportable total T4 result is 0.5 ug/dL. Results above the top standard (generally near 30 ng/dL) are repeated diluted 1:2 in zero calibrator and corrected for the dilution factor prior to reporting. Estimates of imprecision can be generated from long-term quality control pool results.

10. QUALITY CONTROL (QC) PROCEDURES

A. Bench quality controls are used in this analytical method. Bench quality control specimens are tested with each analytical run (a set of consecutive assays performed without interruption) so that judgments may be made on the day of analysis. The data from these materials are then used in estimating methodological imprecision and in assessing the magnitude of any time-associated trends.

B. The bench controls are purchased in sufficient quantity to provide serum samples for all the assays for approximately 1 year. Ranges are established after 20 parallel runs with previously established controls. The quality control pools comprise two levels of concentration spanning the low and high ranges for total T4.

C. Bench quality controls are placed at the beginning of each analytical run. After analysis, the long-term quality control charts (Levey-Jennings) for each control material are consulted to determine if the system is "in control." The Levey Jennings chart plots the quality control material observations on the y-axis and the date of the observation on the x-axis. Quality control material observations are compared with the 95% and 99% confidence limits as well as with the center line (the overall mean of the characterization runs) prior to reporting any results. The system is out of control if any of the following events occur for any one of the quality control materials:

   The observation from a single pool falls outside the 99% confidence limits.
   The observations from two pools fall either both above or both below the 95% confidence limits.
   The observations from eight successive runs for one pool fall either all above or all below the center-line and the current result is above or below the 95% confidence limits.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If the run is declared "out of control", the system (instrument, calibration standards, etc.) is investigated to determine the root of the problem before any results are released. Consult with the supervisor for appropriate actions.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

A. The lowest reportable value is approximately 0.5 ug/dL. According to the manufacturer, this is the lowest detectable level of total T4 distinguishable from zero

with 95% confidence.

B. The upper limit of the reportable values is approximately 60 ug/dL, twice the value assigned to the top standard (high samples are diluted 1:2 and repeated).

C. The total T4 results should be interpreted in light of the total clinical presentation of the patient, including: symptoms, clinical history, data from additional tests, and other appropriate information.

D. According to the manufacturer, thyroid status should not depend upon results from a single thyroxine test. Complete thyroid status evaluation should include additional thyroid function tests, evaluation of thyroid autoantibodies, and physician clinical evaluation.

E. According to the manufacturer the following substances do not interfere with the assay:

Hemoglobin up to 1000 mg/dL
Bilirubin up to 10 mg/dL
Triglycerides up to 1800 mg/dl

The manufacturer performed testing to determine the cross reactivity of the assay to these substances:

Cross-Reactant	Analyte Added (ug/mL)	Cross Reactivity (%)
l-Thyroxine	5	100.0
d-Thyroxine	10	72.5
l-Triiodothyronine	500	1.24
d-Triiodothyronine	500	0.81
Tetraiodothyroacetic Acid	25	5.16
l-Diiodothyronine	5000	0.08
d-Tyrosine	5000	<0.01
l-Tyrosine	5000	<0.01
Reverse T3	100	19.95

F. For assay employing antibodies, the possibility exists for interference by heterophile antibodies in the patient sample. Patients that have been regularly exposed to animals or have received immunotherapy or diagnostics procedures utilizing immunoglobulins or immunoglobulin fragments may produce antibodies, e.g. HAMA, that interferes with immunoassays. Additionally, other heterophile antibodies such as human anti-goat antibodies may also be present in patient samples. Such interfering antibodies may cause erroneous results. Carefully evaluate the results of patients suspected of having these antibodies.

13. REFERENCE RANGES (NORMAL VALUES)

6.1 – 12.2 ug/dL

Based on manufacturer's studies using non-parametric analysis of the results measured in 533 human serum samples from apparently healthy subjects.

14. CRITICAL CALL RESULTS ("PANIC VALUES")

Not applicable to this procedure.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens should be maintained at 20-25 ^{o}C during testing. After testing, the samples are stored at -70 ^{o}C or colder.

16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

There are no acceptable alternative methods of analysis. Specimens may be stored at 4-8 ^{o}C for no longer than 2 days. Otherwise, specimens should be stored -70 ^{o}C or colder until the system is returned to functionality.

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

Not applicable to this procedure. However, e-mail notification is sent to NHANES for results that are above 18.0 ug/dL.

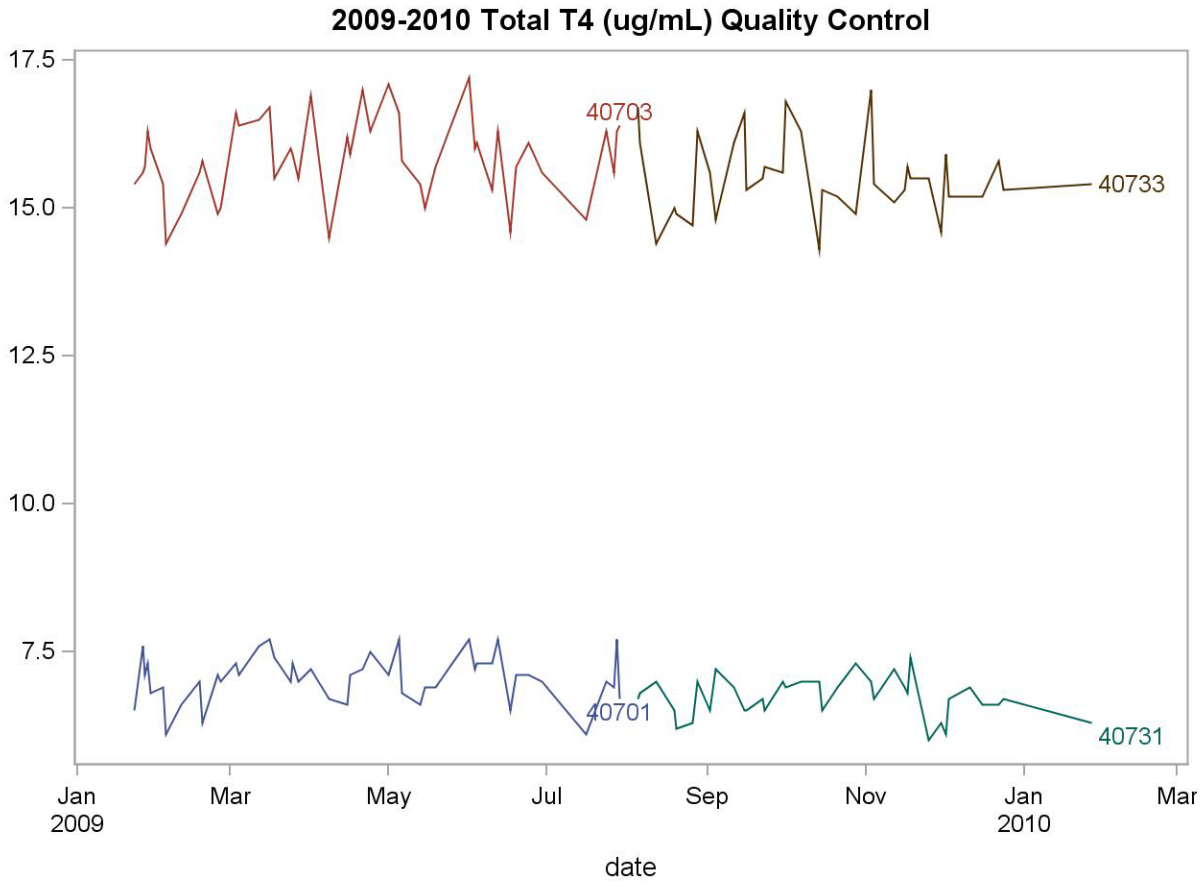
18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

Standard record keeping should be used for tracking specimens. Samples are inspected upon arrival and new boxes are added to an Excel worksheet (sample log) used to track boxes. This sample log is used to track the status of testing and resulting.

The residual serum is stored at $\leq$ -70 ^{o} C for 6 months after analysis, then it is returned to the NHANES Repository for long-term storage.

19. Summary Statistics and QC Graphs

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
40703	46	23JAN09	29JUL09	15.8435	0.6885	4.3
40701	46	23JAN09	29JUL09	7.0500	0.4081	5.8
40733	36	05AUG09	27JAN10	15.5056	0.6590	4.2
40731	36	05AUG09	27JAN10	6.7250	0.3350	5.0



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