# Laboratory Procedure Manual

Analyte: Total Testosterone

Matrix: Serum

Method: Analysis of total testosterone in serum by ID/HPLC/MS/MS

Method No:

Revised:

as performed by:

Clinical Chemistry Branch
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**Important Information for Users**

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

**Public Release Data Set Information**

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

| File Name | Variable Name | SAS Label |
|---|---|---|
| TST_G | LBXTST | Testosterone, total (ng/dL) |

# 1 SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

## 1.1 Clinical and Public Health Relevance

Clinical guidelines and position statements recommend testing for testosterone to aid in diagnosing diseases and disorders or monitoring treatments [1-6]. Testosterone measurements are used in patient care for the diagnosis of hypogonadism in men [1] and androgen excess in women with polycystic ovary syndrome being one of the conditions causing androgen excess [7,8]. Research found that testosterone levels are associated with various diseases and conditions, such as metabolic syndrome [9], diabetes [10], cardiovascular disease [11,12], fractures [13,14], neurodegenerative disorder [15,16], and higher mortality in men with lower testosterone levels [17,18]. There is a need for population data to better define reference ranges and to further investigate associations between testosterone levels and chronic diseases.

## 1.2 Test Principle

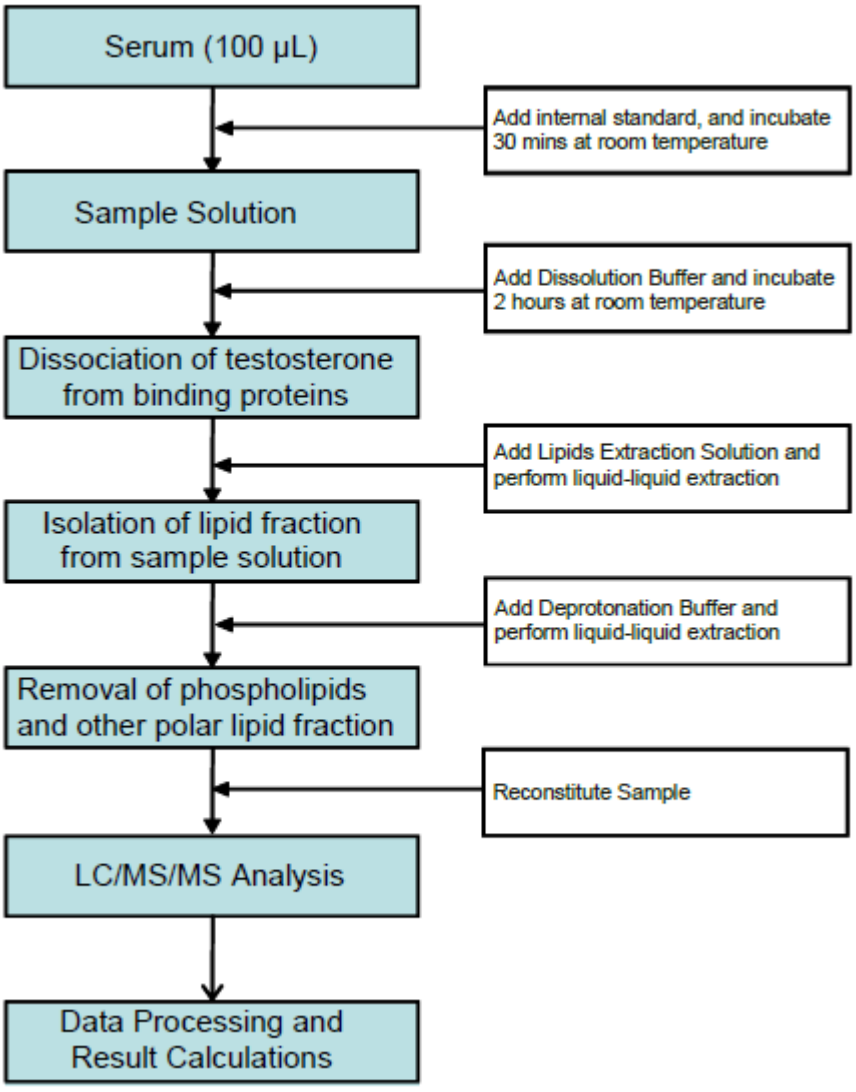
This measurement procedure describes the measurement of total testosterone (free and protein bound testosterone) in human serum. The ISO/IUPAC definition of the quantity measured with this method is 'total testosterone', the measurment is 'serum total testosterone; amount of substance concentration equal to x nmol/L'. To facilitate the clinical use of these measurements, results are converted into ng/dL. The three principle steps in this measurement procedure are: Dissociation of the analyte from binding proteins, extraction of the analyte form the sample matrix and quantitation of the analyte by isotope dilution high performance liquid chromatography tandem mass spectrometry (ID-HPLC/MS/MS) using stable isotope labeled internal standards and calibrators.

Isolation of the analyte is achieved using liquid-liquid extraction. ID-HPLC/MS/MS is performed with a triple quadrupole mass spectrometer using electrospray ionization in positive ion mode. Testosterone is indentified based on chromatographic retention time and on specific mass to charge ratio transitions using selected reaction monitoring (SRM). A $^{13}C$ isotope-labeled testosterone is used as an internal standard.

The measurement procedure described in this document has 6 tasks (Scheme 1):

1. Preparation of samples solution
2. Dissociation of testosterone from binding proteins
3. Isolation of lipids fraction from samples using liquid-liquid extraction
4. Removal of phospholipids and other polar lipids from lipid fraction using liquid-liquid extraction
5. Analysis of total testosterone by ID-HPLC/MS/MS
6. Data processing and result calculations

Scheme 1: Measurement Procedure for Total Testosterone in Serum



## 1.3 Scope

The measurement procedure described in this document is intended for quantitatively measuring all unconjugated testosterone (free and protein-bound) in human serum. Measurement of conjugated testosterone requires different methodologies. It addresses all aspects related to the measurement process (specimen collection, storage, processing, analysis and reporting). This method was evaluated for the serum and may not be suitable for other sample matrices such as total testosterone in plasma or other sample matrices.

Specific details related to equipment maintenance and operation are provided in the manufacturers' manuals and in work instructions created and maintained by the Protein Biomarker Laboratory. Further, this document is not indented to provide information on data interpretation.

# 2 SAFETY PRECAUTIONS

## 2.1 General Safety

All serum specimens should be considered potentially positive for infectious agents including HIV and the hepatitis B virus. Hepatitis B vaccination series are required for all analysts performing this measurement procedure.

Universal precautions should be observed: protective gloves, laboratory coats, and safety glasses must be worn at all times during all tasks of this measurement procedure.

Disposable bench covers must be used during sample preparation and sample handling and must be discarded after use. All work surfaces must be wiped with 10% bleach solution after work is finished.

## 2.2 Chemical Hazards

All acids, bases and all the other reagents and organic solvents used in this measurement procedure must be handled with extreme care; they are caustic, flammable and toxic and they must be handled only in a well-ventilated area or, as required, under a chemical fume hood.

Glacial Acetic Acid: Flammable liquid and vapor. Corrosive. Liquid and Mist cause severe burns to all body tissue. Maybe fatal if swallowed. Harmful if inhaled. Inhalation may cause lung and tooth damage.

Ammonium carbonate: Causes eye, skin, and respiratory tract irritation. Air sensitive. Light sensitive. Hygroscopic (absorbs moisture from the air). Unstable in air, being converted (decomposed) into ammonium bicarbonate with loss of ammonia and carbon dioxide. Also decomposes in hot water yielding ammonia and carbon dioxide.

Formic Acid: Causes eye and skin burns. Combustible liquid and vapor. Causes digestive and respiratory tract burns. Corrosive to metal. Strong reducing agent.

Ethyl acetate: Flammable liquid and vapor. May cause respiratory tract irritation. May be harmful if inhaled. May cause central nervous system depression. Causes eye irritation. May cause skin irritation. May cause liver and kidney damage.

Acetonitrile: Flammable liquid and vapor. Causes eye irritation. May be harmful if swallowed, inhaled, or absorbed through the skin. May cause skin and respiratory tract irritation. Metabolized to cyanide in the body, which may cause headache, dizziness, weakness, unconsciousness, convulsions, coma and possible death.

Hexane: Extremely flammable liquid and vapor. Vapor may cause flash fire. Breathing vapors may cause drowsiness and dizziness. Aspiration hazard if swallowed. Can enter lungs and cause damage. May cause eye and skin irritation.

Material safety data sheets (MSDSs) for these chemicals are readily accessible as hard copies in the laboratory. If needed, MSDS for other chemicals can be viewed at http://www.ilpi.com/msds/index.html or at http://intranet.cdc.gov/ohs.

CAUTION! Acetonitrile, Glacial Acetic Acid, Hexane, Ethyl Acetate are volatile organic compounds. Wear gloves, safety glasses, lab coat and/or apron, and work only inside a properly operating chemical fume hood. Keep container tightly closed and sealed in the designated flammable cabinet until ready for use.

## 2.3 Radioactive Hazards

There are no radioactive hazards associated with this measurement procedure.

## 2.4 Mechanical Hazards

There are only minimal mechanical hazards when performing this procedure using standard safety practices. Analysts must read and follow the manufacturer's information regarding safe operation of the equipment. Avoid direct contact with the mechanical and electronic components of analytical equipment and instrumentation unless all power is 'off'. Generally, mechanical and electronic maintenance and repair must only be performed by qualified technicians. Follow the manufacturer's operating instructions located in the Hormone Project area of the Protein Biomarker Laboratory.

## 2.5 Waste Disposal

All solid waste used in the sample preparation process (i.e., disposable plastic pipette tips, gloves, bench diapers, caps etc.) as well as any residual sample material needs to be placed into the appropriate biohazard autoclavable bags and waste pans until sealed and autoclaved.

All glass pipette tips and any sharps (i.e., broken glass) must be placed in appropriate Sharps Containers.

All liquid waste must be labeled and processed in accordance with CDC policies using the appropriate waste management and chemicals tracking systems. All waste disposals must be performed in compliance with CDC policies and regulations. The CDC Safety Policies and Practices Manual are located in the laboratory and can be accessed at http://isp-v-ehip-asp/dlsintranet/safety_manual/.

## 2.6 Training

Analysts performing this measurement procedure at a minimum must successfully complete

- Safety courses (CDC-OHS Safety Survival Skills Parts 1 and 2, Bloodborne Pathogens courses)
- CDC-OHS Hazardous Chemical Waste Management course
- Computer Security Awareness course
- Records Management training

Further, the analyst must have received training on the specific instrumentation used with this measurement procedure from designated staff or the instrument manufacturer.

At a minimum, the analysts performing this measurement procedure must be familiar with the

- Exposure Control Plan
- Chemical Hygiene Plan
- Relevant MSDS
- DLS Safety Manual
- DLS Policies and Procedures Manual
- DLS After-Hours Work Policy
- Policy on confidentiality, data security and release of information
- DLS Policy on Use of Controlled Substances

# 3 COMPUTERIZATION AND DATA-SYSTEM MANAGEMENT

## 3.1 Software and Knowledge Requirements

This measurement procedure requires work with different software operated instruments such as AB/Sciex MS/MS (using Analyst 1.4 & 1.5 Software version or higher) and Hamilton Starlet pipette (using Microlab Vector Software version 4.11.5878 or higher). Specific training to operate this software is required to ensure appropriate and safe instrument function.

Further, calculations of results obtained with theHPLC/MS/MS software are performed using calculation templates created with Microsoft Excel. The calculation results obtained with the Excel templates are transferred to a Database that is created and

maintained by DLS. Assessment of bench QC results is performed using a program created with SAS software and maintained by the DLS.

The database activities and QC calculations are performed by dedicated and special trained staff. Initial calculations using the Excel templates are performed by the analysts after receiving specific training from dedicated laboratory staff.

## 3.2 Sample Information

All samples must be labeled as described in the latest version of the DLS Policies and Procedures Manual. No personal identifiers are used, and all samples are referenced to a blind coded sample identifier. To facilitate specimen handling and initial interpretation of data, information about the sender of the donor is desirable.

## 3.3 Data Maintenance

Information about samples and related analytical data are checked prior to being entered into the database for transcription errors and overall validity. Filing of electronic and physical files and their maintenance is the responsibility of designated staff in the Protein Biomarker Laboratory and is described in work instruction documents. The database is maintained by DLS staff and is routinely backed up by CDC Information Technology Services Office (ITSO). ITSO must be contacted for emergency assistance.

## 3.4 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 through restricted access to the individual laboratories, buildings, and offices. Confidentiality of results is protected by referencing results to blind coded sample IDs (no names or personal identifiers).

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

## 4.1 General Specimen Requirements

For analysis of total testosterone using the measurement procedure, a minimum of 150 µL of fresh or frozen serum is needed. A sample volume of 100 µL is used for analysis. A sample volume of 0.5 mL is preferred to allow for repeat analyses.

Red cell enzymes can convert androstenedione to testosterone and will significantly increase testosterone concentrations. The increase may be 50% after 24 hours at room

temperature and should be avoided [19]. Serum should be separated from red cells within 6 hours of collection, if blood is kept at room temperature or within 24 hours if blood is stored at 4$^{o}$C. Morning fasting samples (i.e., samples collected in the morning after overnight fast) are recommended to minimize biological variability. The specimen should be transported in 2.0-mL cryogenic vial with external screw-caps. These cryovials should be labeled in accordance to CDC and DLS policies and regulations. Other specimen handling conditions are outlined in the Policies and Procedures Manual of the Division of Laboratory Sciences (DLS) [20].

## 4.2 Specimen Storage

The serum specimens can be shipped frozen on dry ice. Specimens can be kept refrigerated for 3 days. For long-term storage, samples are stored at -70 ºC. Freeze/thaw of a stored sample seems to have a minimal effect on total testosterone concentrations [21]. Studies have shown that storage of serum at -25$^{o}$C resulted in negligible changes in concentration over 40 years [22].

## 4.3Unacceptable Specimens

Specimens that do not meet the above mentioned criteria, were transported at room temperature, or have evidence of leakage are not acceptable.

# 5. Procedures for Microscopic Examinations

Not applicable for this procedure.

# 6. PREPARATION FOR REAGENTS, CALIBRATION MATERIALS, CONTROL MATERIALS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION.

## 6.1 Equipment, Chemicals and Consumables

The chemicals, equipment and other materials as described below or equivalents can be used in this measurement procedure.

### 6.1.1 Equipment, Chemicals and Consumables Used for Reagent Preparation

1. Mettler Toledo PG 403-S Delta-Range Chemical Balance (Electronic “0.000 g” , Max 410.0 g, Min 0.02g, Columbus, OH 43240).
2. Hanna HI4222 pH/ISE Dual Channel Bench Meter (Hanna Instruments USA, Woonsocket, RI) with Orion Micro-Combination pH electrode, pH range 0-14, temperature range 0-100 ºC. (Thermo Electron Corp., Bellefonte, PA).
3. Sato Label Maker CL612e and Label Making Software (Sato America, Charlotte, NC)
4. 500 ml glass beaker (Corning Incorporated, Lowell, MA)

5. 250 mL Pyrex graduated glass cylinder (tolerance ±1.4 ml, Kimble Chase Life Science and Research Products LLC, Cat. No: 20022, Vineland, NJ)
6. Fisherbrand Octagonal stirring bars, 1 inch length; 0.312 inch diameter (Fisher Scientific, Cat No: 14-513-59, Suwanee, GA).
7. Scholar™ 5 x 5 Inch PC-171 Magnetic Stirrer (Corning Incorporated, Lowell, MA)
8. 1L glass bottles with screw tops (Wheaton Industries Inc., Cat. No: 219440, Millville, NJ)
9. Pasteur Transfer pipettes (Samco scientific, Cat. No: 225, San Fernando, CA).
10. Disposable Pasteur Pipets, 53/4" (Fisher Scientific, Cat. No: 13-678-20A, Suwannee, GA
11. Milli-Q Water, Resistivity, 18.1 MΩ·cm at 25 ° C, 18.2 (Aqua Solutions, Jasper, GA).
12. Sodium Acetate, Anhydrous, ACS grade, CAS NO: 127-09-3 (Fisher Scientific, Cat. No: 127-09-3, Suwanee, GA).
13. Ammonium carbonate, ACS grade, CAS NO: 506-87-6 (Fisher, Cat. No: A656-500, Suwanee, GA).
14. Ethyl Acetate, HPLC/ACS grade (Fisher scientific, Cat. No: E195SK-4, Suwanee, GA)
15. Hexane, HPLC/ACS grade, CAS No: 110-54-3 (Fisher Scientific, Cat. No: H302-4, Suwanee, GA)
16. Sodium Hydroxide, Pellets/Certified ACS grade, CAS NO: 1310-73-2 (Fisher Scientific, Cat. No: S318-1, Suwanee, GA)
17. Acetonitrile with 0.1% formic acid, HPLC grade (Fischer Scientific, Cat No: HB9823-4, Suwannee, GA, Acetonitrile CAS NO: 75-08-8, formic acid CAS No: 64-18-6).
18. Water with 0.1% Formic Acid, HPLC grade (Fisher Scientific, Cat. No: HB523-4, Suwannee, GA, Water CAS No: 7732-18-5, Formic Acid CAS No: 64-18-6)
19. Glacial Acetic Acid, Certified ACS grade, CAS No: 64-19-7 (Fisher Scientific, Cat. No: 64-19-7, Suwanee, GA).

### 6.1.2 Equipment, Chemicals and Consumables Used for Calibration Materials

1. Mettler Toledo AX205 (Electronic "0.000 g" , Max 220.0 g, d 0.01 mg, Columbus, OH).
2. Water Bath- IsoTemp 3016 Regulator Apparatus (Fisher Scientific, Suwanee, GA)
3. 100 mL Pyrex volumetric flasks (tolerance ±0.08 ml, Kimble Chase Life Science and Research Products LLC, Cat. No: 55640, Vineland, NJ)
4. 15 mL Pyrex brand glass tubes (Corning Inc., Cat. No: 99447-16, Lowell, MA).
5. 10 mL glass volumetric pipette (Fisher Scientific, Cat. No: 13-650-2L, Suwanee, GA)
6. 7 mL Pyrex brand glass tubes (Corning Inc., Cat. No: 9826-16 , Lowell, MA).
7. 250 µL positive displacement pipette (Gilson, Inc., Cat. No: F148505, Middleton, WI)
8. 1 mL positive displacement pipette (Gilson, Inc., Cat. No: F148506, Middleton, WI)
9. Ethanol, 200 proof, CAS NO: 64-17-5 (Sigma-Aldrich, Cat. No: E7023, St. Louis, MO)
10. Testosterone in acetonitrile 1 mg/mL (certified concentration with stated uncertainty), purity 99.4%, testosterone CAS No: 58-22-0, Acetonitile CAS NO: 75-08-8 (Cerillant, Round Rock, TX)
11. [2,3,4-$^{13}C_3$]-testosterone, purity 98%, CAS No: 58-22-0 (IsoSciences, Cat. No: 6066, King of Prussia, PA)

### 6.1.3 Equipment, Chemicals and Consumables Used for Sample Processing

1. Eppendorf Centrifuge 5810R,(Eppendorf, Ramsey, MN).

2. Hamilton Microlab STARLet Liquid Handler with 8-chanel and 96-chanel pipettors (using Microlab Vector Software version 4.11.5878 (Hamilton Company, Reno, NV)
3. Water Bath- IsoTemp 3016 Regulator Apparatus (Fisher Scientific, Suwanee, GA)
4. Glas-Col MultiPulse Vortexer (Glas-Col, Terre Haute, IN).
5. Eppendorf Repeater Plus Pipetter (Eppendorf, Cat. No: 022260201, Ramsey, MN).
6. Sato Label Maker CL612e and Label Making Software (Sato America, Charlotte, NC).
7. 100 µL Positive displacement pipette (Gilson, Inc., Cat. No: F148504, Middleton, WI)
8. 96-Well 2 ml square well plates (Seahorse Labware, Cat. No: S30009, Chicopee, MA)
9. Robotic Reservoirs, Convoluted bottom (Thermo Scientific, Cat. No: 1200-2300, Waltham, MA)
10. ArctiSeal 96 Well Square Silicone w/ PTFE Spray Coating (Arctic White LLC, Cat No: AWSM-1003SX, Bethlehem, PA)
11. Eppendorf Combitips plus Pipet tips, 5 ml (Eppendorf, Cat. No: 022266403, Ramsey, MN)
12. GeneVac EZ-2 Evaporation System with side bridge holders and universal rotor (GeneVac Inc., Valley Cottage, NY).
13. Co-RE Tips, 480 standard volume tips (300 µL) with Filters (Hamilton Company, Reno, NV).
14. Orbitron Rotator II, Model 26250, (Boekel Scientific, Feasterville, PA).
15. Eppendorf Swing-bucket Rotor (Eppendorf, Cat. No: A-2-DWP, Ramsey, MN).
16. Hexane, HPLC/ACS grade, CAS No: 110-54-3 (Fisher Scientific, Cat. No: H302-4, Suwanee, GA)

#### 6.1.4 Equipment, Chemicals and Consumables Used for Sample Measurement

1. AB/Sciex API 5000 QqQ Mass Spectrometer with ESI source (AB/Sciex, Foster City, CA)
2. Agilent 1100 Series LC System (Agilent Technologies, Santa Clara, CA)
3. Column Oven Thermasphere TS-130 (Phenomenex, Cat. No: EH0-7058, Torrance,CA)
4. HTS/CTC-PAL Autosampler System (Leap Technologies, Carrboro, NC)
5. Autosampler X-Type Syringe, 100 µL, 22sg, pt 3 (Microliter Analytical Supplies, Inc., Cat. No: XT-G10022S-3, Suwanee, GA)
6. Thermo Fisher Hypersil Gold C18 Column 50 × 3 mm, 3µm (Thermo Fisher, Cat. No: 25003-053030, Waltham, MA)
7. Thermo Fisher Guard Column 10 × 3 mm drop (3 um) in cartridge (Thermo Fisher, Cat. No: 25003-013001, Waltham, MA)
8. 2 ml Polyethylene 96 Well Pattern Sealing Tape (BioTech Solutions, Cat. No: ZAF-PE-50, Vineland, NJ)

## 6.2 Preparation of Reagents Used For Sample Preparation

### 6.2.1 Dissolution Buffer (0.5 mol/L Sodium Acetate, pH 5.5)

This solution is used to dissociate total testosterone from binding globulins.

Preparation of 250 mL of Dissociation Buffer which is sufficient for a maximum of 300 serum samples. If more samples are to be processed volumes can be adjusted accordingly

1. Weigh out 10.25 g sodium acetate and transfer into a 500 mL beaker with scaling
2. Add 200 mL of DI water using a 250 mL graduated cylinder
3. Add stir bar to beaker and mix until completely dissolved
4. Measure pH of solvent with a freshly calibrated pH meter
5. Adjust pH to 5.5 (±0.1) with diluted Glacial Acetic Acid (100 ml glacial acetic acid + 100 ml water) using a transfer pipette, and record the volume of diluted glacial acetic acid used.
6. Add remainder of DI water to bring the total volume to 250 mL
7. Note in laboratory notebook starting pH, and final pH (to 1 digit)
8. Transfer to a glass bottle with plastic screw cap and appropriately label the bottle.

Solution is stable for at least 1 week – remeasure the pH prior to use to ensure stability.

### 6.2.2 Lipids Extraction Solution (Ethyl Acetate: Hexane, 300 ml + 200 ml).

This solution is used to isolate the lipid fraction from the protein fraction in the sample solution

Preparation of 1 L of Lipids Extraction Solution is sufficient for 1,000 samples. If more samples are to be processed, volumes need to be adjusted.

1. Using a 250 mL graduated cylinder measure out 600 mL of ethyl acetate and transfer to 1 L bottle with screw top
2. Using a 250 mL graduated cylinder measure out 400 mL of hexane and transfer to 1 L container
3. Close glass bottle and mix thoroughly
4. Appropriately label the bottle- including safety precautions and hazard information.

Solution is stable for at least 1-2 months and needs to be stored in the flammable cabinet

### 6.2.3 Deprotonation Buffer (0.2 mol/L Ammonium Carbonate, pH 9.8)

This solution is used to deprotonate phospholipids and to enable them to be separated from non-polar lipids.

The following procedure is written to create 250 mL of Deprotonation Buffer, which is sufficient for 300 samples. If more samples are to be processed, volumes need be adjusted accordingly.

1. Weigh out 4.80 g ammonium carbonate and transfer into a 500 mL beaker.
2. Add 200 mL of DI water using a 250 mL graduated cylinder
3. Add stir bar and mix until completely dissolved
4. Measure pH of solvent with a freshly calibrated pH meter
5. Adjust pH to 9.8 (±0.4) with 6 mol/L NaOH using a burette, and record the volume of 6 mol/L NaOH solution used.

6. Add remainder of DI water to bring the total volume to 250 mL
7. Note in laboratory notebook the starting pH, final pH (to 1 digit), and amount of NaOH volume needed to adjust the pH of the buffer
8. Transfer to a glass bottle with plastic screw cap and appropriately label the bottle, including safety precautions and hazard information

Solution is stable for at least 1 week. Measure the pH prior to each use and discard if pH is outside target range of pH 9.8 ± 0.4.

### 6.2.4 Sample Reconstitution Solution (0.1% Formic Acid in Water: 0.1% Formic Acid in Acetonitrile, 90 mL + 10 mL)

This solution is used to reconstitute samples prior to injection on HPLC/MS/MS

Preparation of 1 L of Sample Reconstitution Solution which is sufficient for 1,000 samples. If more samples are to be processed, volumes need to be adjusted.

1. Using a 250 mL graduated cylinder measure out 900 mL of water with 0.1% formic acid and transfer to 1 L bottle with screw top
2. Using a 250 mL graduated cylinder measure out 100 mL of acetonitrile with 0.1% formic acid and transfer to 1 L bottle with screw top
3. Appropriately label the bottle- including safety precautions and hazard information

Solution is stable for at least 6 months and stored in the flammable cabinet

## 6.3 Calibration Materials

### 6.3.1 Preparation of Calibrator Working Solutions

The Calibrator Working Solutions are prepared from Calibrator Stock Solution which prepared from certified, commercial solution with an assigned concentration of 1 mg/mL (see section 4.1.2). If different solutions are used, the preparation procedures need to be adjusted accordingly. This procedure produces 66 vials per calibrator level, which is sufficient for 5200 samples assuming use of 1 vial with one calibration curve per sample batch.

The following calibrator stock solutions (Table 1) are prepared:

**Table 1:** Desired Testosterone Calibrator Stock Solution Concentration (µg/mL and nmol/L)

| Calibrator Stock Solution Code | Testosterone Target Concentration | | Dilution |
|---|---|---|---|
| | µg/mL | nmol/L | |
| A | 1.00 | 3,467 | 100 µL (certified, commercial solution) → 100 mL |
| B | 0.10 | 346.7 | 10 mL (Calibrator Stock Solution A) → 100 mL |
| C | 0.01 | 34.67 | 1 mL (Calibrator Stock solution A) → 100 mL |

1. Preparation of Calibrator Stock Solution A
    a. Transfer 100 µL of a 1 mg/mL certified testosterone solution into a 100 mL volumetric flask using a positive displacement pipette.
    b. Add Ethanol to just below the fill line of the volumetric flask
    c. Place flask in the water bath for 15 minutes to reach 20°C and add ethanol (at 20°C) to the fill line.
    d. Aliquote solution in 15 mL aliquots in 6 15-mL pyrex glass tubes
    e. Label tubes appropriately and store them in the refrigerator. Tubes are for single use only.
2. Preparation of Calibrator Stock Solution B
    a. Transfer 10 mL of Calibrator Stock Solution A into a 100 mL volumetric flask using a volumetric glass pipette.
    b. Add Ethanol to just below the fill line of the volumetric flask
    c. Place flask in the water bath for 15 minutes to reach 20 °C and add ethanol (at 20 °C) to the fill line.
    d. Aliquote solution in 7 mL aliquots in 14 7-mL pyrex glass tubes
    e. Label tubes appropriately and store them in the refrigerator. Tubes are for single use only.
3. Preparation of Calibrator Stock Solution C
    a. Transfer 1 mL of Calibrator Stock Solution A into a 100 mL volumetric flask using a 1 mL positive displacement pipette.
    b. Add Ethanol to just below the fill line of the volumetric flask
    c. Place flask in the water bath for 15 minutes to reach 20 °C and add ethanol (at 20 °C) to the fill line.
    d. Aliquote solution in 7 mL aliquots in 14 pyrex glass tubes
    e. Label bottles appropriately and store them in the refrigerator.

Note: All calibrator stock solution tubes are for single use only. Do not reuse tubes as ethanol may evaporate and change the concentration of the stock solution. This solution is stable for 6 months.

The Calibrator Working Solutions (Table 2) are prepared using the Calibrator Stock Solutions. The following levels of Calibrator Working Solutions used for measuring testosterone levels in humans:

**Table 2:** Desired Testosterone Calibrator Working Solution Concentration (ng/dL and nmol/L)

| Calibrator Working Solution Code | Testosterone Target Concentration | | Dilution |
|---|---|---|---|
| | ng/dL | nmol/L | |
| CC01 | 1000 | 34.67 | 1 mL (Calibrator Stock Solution A) → 100 mL |
| CC02 | 750 | 26.00 | 750 µL (Calibrator Stock Solution A) → 100 mL |
| CC03 | 500 | 17.34 | 500 µL (Calibrator Stock Solution A) → 100 mL |
| CC04 | 250 | 8.67 | 250 µL (Calibrator Stock Solution A) → 100 mL |
| CC05 | 100 | 3.47 | 1 mL (Calibrator Stock Solution B) → 100 mL |
| CC06 | 75 | 2.60 | 750 µL (Calibrator Stock Solution B) → 100 mL |

| | | | |
|---|---|---|---|
| CC07 | 50 | 1.73 | 500 µL (Calibrator Stock Solution B) → 100 mL |
| CC08 | 25 | 0.87 | 250 µL (Calibrator Stock Solution B) → 100 mL |
| CC09 | 10 | 0.35 | 100 µL (Calibrator Stock Solution B) → 100 mL |
| CC10 | 7.5 | 0.26 | 750 µL (Calibrator Stock Solution C) → 100 mL |
| CC11 | 5.0 | 0.17 | 500 µL (Calibrator Stock Solution C) → 100 mL |
| CC12 | 2.5 | 0.09 | 250 µL (Calibrator Stock Solution C) → 100 mL |

Prepare the calibrator working solutions by performing the following tasks:

1. Adjust the Calibrator Stock Solution temperature to 20 °C using a water bath.
2. Transfer the volumes of Calibrator Stock Solutions stated in Table 1 to separate volumetric flasks (100 mL) using positive displacement pipettes.
3. Add Ethanol to just below the fill line of the volumetric flask
4. Place flask in the water bath for 15 minutes to reach 20 ºC and add ethanol (at 20 °C) to the fill line
5. Aliquote solution in 1.5 mL aliquots in appropriately labeled cryovials and store them in the refrigerator. Vials are for single use only.

Note: Do not reuse vials as ethanol may evaporate and may change the concentration of the solution. This solution is stable for 6 months.

### 6.3.2 Preparation of Internal Standard Solutions

The Internal Standard Working Solution is prepared from an Internal Standard Stock Solution which is prepared from pure compound material (see section 4.1.2). If different solutions as used, the preparation procedures need to be adjusted accordingly. This procedure produces 66 vials of Internal Standard Solution B, which is sufficient for 5200 samples assuming use of 1 mL of Internal Standard Stock Solution B per sample batch (per 96-well plate).

The Internal Standard Working Solution is prepared as followed:

The following calibrator stock solutions are prepared:
A $^{13}C_3$ labeled testosterone is used to create internal standard stock and working solutions in ethanol with the concentrations listed in Table 3.

**Table 3:** Desired Internal Standard Testosterone Solution Concentration (ng/mL and nmol/L)

| Internal Standard Solution Code | Testosterone Internal Standard Target Concentration | | Dilution |
|---|---|---|---|
| | ng/mL | nmol/L | |
| Stock Solution A | 10,000 | 3,4671 | 1 mg ($^{13}C_3$ labeled Testosterone)→ 100 mL |
| Stock Solution B | 100 | 346.71 | 1 mL (Internal Standard Stock Solution A)→ 100 mL |
| Working Solution | 1 | 3.47 | 1 mL (Internal Standard Stock Solution B)→ 100 mL |

1. Preparation of Internal Standard Stock Solution A
    a. Clean one glass weighing funnel with Ethanol and allow to dry
    b. Remove $^{13}C_3$-labeled Testosterone from refrigerator and allow to reach room temperature for a period of 30 minutes.
    c. Calibrate the analytical balance following the manufacturer's instructions
    d. Weigh and transfer 1 mg (±0.001 mg) of $^{13}C_3$-labeled Testosterone to a clean 100 mL volumetric flask.
    e. Add Ethanol to the flask just below the fill line of the volumetric flask
    f. Place flask in the water bath for 15 minutes to reach 20 $^{o}$C and add ethanol (at 20 $^{o}$C) to the fill line.
    g. Aliquote solution in 15 mL aliquots in 6 15-mL pyrex glass tubes, label tubes appropriately and store them in the -70 °C freezer.

2. Preparation of Internal Standard Stock Solution B
    a. Transfer 1 mL of Internal Standard Stock Solution A (at 20 $^{o}$C) into a 100 mL volumetric flask using a 1 mL positive displacement pipette
    b. Add Ethanol to the flask just below the fill line of the volumetric flask
    c. Place flask in the water bath for 15 minutes to reach 20 $^{o}$C and add ethanol (at 20 $^{o}$C) to the fill line.
    d. Aliquote solution in 1.5 mL aliquots in 66 1.5-mL cryovials
    e. Label cryovials appropriately and store them in the -70 °C freezer.
    Vials are for single use only. Do not reuse vials as ethanol may evaporate and change the concentration of the stock solution. This solution is stable for 6 months.
3. Preparation of Internal Standard Working Solution
    a. Transfer 1 mL of Internal Standard Stock Solution B into a 100 mL volumetric flask using a 1 mL positive displacement pipette
    b. Add Ethanol to the flask to the fill line of the volumetric flask

Note 1: Tubes and vials are for single use only. Do not reuse vials as ethanol may evaporate and change the concentration of the stock solution. This solution is stable for 6 months.

Note 2: Pure $^{13}C_3$ labeled testosterone is a controlled substance and handling of such materials must comply with DEA regulations and CDC policies for use of controlled substances. Use of pure compound testosterone requires approval and oversight by the designated custodian.

# 7. CALIBRATION AND CALIBRATION VERIFICATION

## 7.1 Calibration

### 7.1.1 Calibration of instruments and equipment

All volumetric pipettes are calibrated annually following procedures recommended by the manufacturers. Mass spectrometry instruments are calibrated for mass accuracy regularly as recommended by the manufacturer and following the manufacturer's procedures. Accuracy of other equipment such as pH-meters and oven temperatures are verified

regularly according to the manufacturer's recommendation or using established references (i.e., commercial buffer solutions, external thermometers).

### 7.1.2 Calibration of measurement

Calibrators used in this measurement procedure are traceable to commercial standard solution with certified concentration from Cerriliant. Calibration solutions are prepared starting with volumetric measurements. For Metrological traceability according to ISO 17511 [23] see Appendix 1. Calibrators are analyzed together with each set of samples.

## 7.2 Calibration Verification

Calibration verification of equipment is performed 6 months after calibration was performed or earlier when recommended by the manufacturer or as indicated in CLIA '88 (§493.1255(b)).

With each set of samples 12 levels of calibration material and a low, mid, and high quality control material covering the clinical range of reported total testosterone are analyzed. Possible shifts in calibration are assessed by comparing bench QC material data against predefined acceptance limits using a SAS software program developed and maintained by DLS (see also Section 8).

Calibration is further verified by analyzing serum material with assigned reference values for total testosterone every 6 months and comparing the results obtained against predefined acceptance limit, which is ± 6.4 % from the target value.

# 8. PROCEDURE OPERATION INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

All instruments are checked before use for correct function using the manufacturer's acceptance criteria. Specific details related to the operation instructions such as specific file names used in the execution are documented in work instructions.

## 8.1 Specimen Storage and Handling during Testing

All vials are labeled according to DLS Policies and Procedures Manual and are scanned during the process of sample preparation, sample transfer and analysis in order to ensure that individual samples can be tracked throughout the process.

Specimens are allowed to reach room temperature for sample preparation. The unused portion of the patient specimen is returned to the freezer and stored at -70 $^{0}$C. Samples ready for analysis by HPLC/MS/MS are either stored at 5 $^{o}$C in the refrigerator or at 5 °C in the HPLC/MS/MS instrument sample tray.

## 8.2 Preparation of Samples for Analysis

All samples are processed together with 3 bench QC samples, 1 reagent blank (saline), and 1 set of calibrators (12 levels). Typically 80 patient samples are processed in one batch (total number of samples per batch: 96 including 1 reagent blank, 12 calibrators, 80 samples)

1. Assess all samples for acceptability using the criteria described in section 5.2 and 5.3.
2. Thaw all samples to room temperature: Frozen serum samples, QC samples, Internal Standard Working Solutions and Calibrator Working Solutions are allowed to reach room temperature and are homogenized by placing them on the rotator at medium speed for about 30 minutes.
3. Place all patient samples, QC samples and Calibrator Working Solution on the Hamilton Microlab STARLet Liquid Handler instrument in the designated locations in a manner that allows the instrument's barcode reader to read all barcodes properly. Place all additional reagents on the instrument at the designated positions.
4. Scan the barcodes of all coded vials and reagents.
5. When a barcode cannot be read, the instrument software will prompt and will allow manual entering of the barcode information. After the scanning process is successfully completed, an MS Excel file containing the barcode information, the location of the particular sample, calibrator and reagent on the Hamilton instrument and the current date and time is automatically created on the Hamilton's computer. This file is transferred to a defined location on the CDC network and this information is used to create a run sequence for the HPLC/MS/MS instrument and to verify run log sheets.
6. Transfer 100 µL of each Calibrator Working Solution (CC01-CC12) into appropriate wells of a 96 2.0-mL deep wellplate.
7. Transfer 100 µL patient samples and QCs to a 96-2.0 mL deep wellplate ("Sample Plate") containing the Calibrator Working Solutions.
8. Transfer 100 µL of Internal Standard Working Solution to all patient samples, QCs, and calibrators.

   Note: Since the Internal Standard Working Solution contains organic solvent, protein precipitation may occur with the addition this solution.
9. Cover Sample Plate with ArctiSeal and allow serum and Internal Standard Working Solution to equilibrate using a multivortexer for approximately 30 minutes at room temperature at a setting of 45+ pulse.
10. Centrifuge the Sample Plate for 2 minutes at room temperature and 1000 rpm.
11. Recap sample vials and store remaining samples at dedicated place in -70 °C freezer.

## 8.3 Dissociation of testosterone from binding proteins

1. Place Sample Plate (from step 7.1.2), Dissociation Buffer and pipette tips on the Hamilton Microlab STARLet Liquid Handler instrument in the designated locations.
2. Add 100 µL of Dissociation Buffer to all samples

Note: If Dissociation Buffer was not prepared the same day, test and note its pH before use. Discard the buffer solution and prepare a new one, if pH is not within desired range or integrity of buffer is in doubt.

3. Cover Sample Plate with ArctiSeal and equilibrate sample solutions using a multi vortexer for approximately 2 hours at room temperature at a setting of 45 plus pulse.
4. Centrifuge the Sample Plate for 2 minutes at room temperature and 1000 rpm.

## 8.4 Isolation of lipids fraction from sample using liquid-liquid extraction

1. Place Sample Plate, Lipid Extraction Solution and pipette tips on the Hamilton Microlab STARLet Liquid Handler instrument in the designated locations.
2. Add 400 µL of Lipid Extraction Solution to all samples
3. Cover Sample Plate with ArctiSeal and place the well plate on a multi vortexer for 2 minutes at a setting of 45+ pulse.
4. Centrifuge the Sample Plate for 5 minutes at 5 $^{o}$C and 3700 rpm.
5. Place Sample Palte and a new 96-2.0 mL wellplate ("Lipid Fraction Plate") and pipette tips on the Hamilton Microlab STARLet Liquid Handler instrument in the designated locations.
6. Transfer the organic layer (top layer) into Lipid Fraction Plate using the Hamilton Microlab Star.
7. Repeat steps 5-6 and combine organic layers in the same Lipid Fraction Plate.

   Note: Assure that Sample Plate always has the correct orientation on the Hamilton Microlab Star instrument otherwise samples will get cross-contaminated. In such case, the whole sample sets needs to be discarded and repeated.
8. Evaporate organic solvent in the Lipid Extraction Plate to dryness using the the Genevac Concentrator at 'Low BP' setting (this should take approximately 2.5 hours).

## 8.5 Removal of phospholipids and other polar lipids from lipid fraction using liquid-liquid extraction

1. Place Lipid Fraction Plate, Deprotonation Buffer, hexane and pipette tips on the Hamilton Microlab STARLet Liquid Handler instrument in the designated locations.
2. Add 150 µl of Deprotonation Buffer to each sample using the Hamilton Microlab Starlet.
   Note: If Deprotonation Buffer was not prepared the same day, test and note its pH before use. Discard the buffer solution and prepare a new one, if pH is not within desired range or integrity of buffer is in doubt.
3. Add 300 µl of hexane to all samples using the Hamilton Microlab Starlet.

4. Cover Lipid Fraction Plate with seal and vortex solution for 10 min at 45+ pulse to assure all residues are dissolved.
5. Centrifuge Lipid Fraction Plate for 5 minutes at 5 $^{o}$C and 3700 rpm.
6. Transfer organic layer (top Layer) into a new 96-2.0 mL deep wellplate ("Sample Analysis Plate") using the Hamilton Microlab Starlet.
7. Repeat steps 3-6 (total of 2 liquid-liduid exctration steps) and combine all organic solutions in the same Sample Analysis Plate.
8. Evaporate organic solvent in the Sample Analysis Plate to dryness using the Genevac Concentrator at 'Low BP' setting (this should take approximately 1 hour).
9. Reconstitute sample in 200 µL of Sample Reconstitution Solution using the Hamilton Microlab Starlet. Let stand for 10 minutes at room temperature and vortex thoroughly on a multi vortexer for 60 minutes at a setting of 50 plus pulse.
10. Centrifuge the Sample Analysis Plate for 2 minutes at room temperature and 1000 rpm.

## 8.6 Analysis of Total Testosterone by HPLC/MS/MS

All samples prepared in one batch are analyzed in one batch on the same instrument. An Instrument Control Sample containing the analyte and internal standard is added to each batch to verify appropriate function of the instrument and chromatographic condition. Additionally a sample containing Sample Reconstitution Solution ("Run Blank") is added after every 8$^{th}$ samples. The Instrument Control Sample and the Run Blanks are kept in a separate wellplate or vials in the autosampler of the HPLC/MS/MS instrument.

1. An analytical run sequence file is created by importing the file containing the sample barcode information from the Hamilton instrument (section 6.2) to an Excel Worksheet ("AnaSequence Template"). This template combines the sample ID information with additional information required by DLS policy and CLIA regulation to analyze the samples on the HPLC/MS/MS system such as sample ID, Sample location on the 96-well plate, and instrument method name. The AnaSequence worksheet creates the appropriate data file names for the individual sample data.
2. The AnaSequence Excel worksheet is saved as a text file, and imported onto the HPLC/MS/MS Instrument software where the final run sequence file is saved as an Analyst software sequence file.
   The first sample in a sequence except Run Blanks is always an Instrument Control Sample (see Appendix 2 for an example of an analytical sequence).
3. The Sample Analysis Plate is loaded in the autosampler on theHPLC/MS/MS instrument as stated in the sequence file and positions of plates in the autosampler are verified against the information in the sequence file.
   Basic instrument function and settings are checked according to the HPLC/MS/MS manufacturer's instructions. It is assured that the correct instrument method is loaded and all method parameters are stable.
4. The instrument run sequence is started using Analyst software.

5. Using the Instrument Control Sample, the performance of theHPLC/MS/MS system is assessed by inspecting retention times, peak intensities, peak shapes and general chromatographic parameters. Retention times and peak intensities need to be within 15% of the expected values. When instrument malfunction is indicated, the sequence is aborted, samples are stored in the refrigerator and the problem is addressed.
6. Upon completion of the HPLC/MS/MS analysis, a new seal is applied on the Sample Analysis Plate, and the plate is stored in the designated space in the freezer at -70 $^{0}$C.

The following HPLC/MS/MS parameters are used.

**Chromatographic conditions**

| | |
|---|---|
| Injector: | CTC PAL ALS |
| Syringe: | 100 µL |
| Injection volume: | 100 µL |
| Loop size: | 250 µL |
| Column: | Hypersil Gold 50 mm× 3 mm ID, 3 µm particle size |
| Guard Column: | 10 mm×3 mm ID, 3 µm particl size |
| Column Oven: | 40 (±5)$^{o}$C |
| Solvents: | |
| Buffer A: | 0.1% Formic Acid in Water |
| Buffer B: | 0.1% Formic Acid in Acetonitrile |
| Flow Rate: | 500 µL/min |

HPLC Gradient:

| Step | Time | %A | %B |
|---|---|---|---|
| 1 | 0 | 95 | 5 |
| 2 | 16 | 5 | 95 |
| 3 | 20 | 5 | 95 |
| 4 | 20.2 | 95 | 5 |
| 5 | 23.2 | 95 | 5 |

**Mass spectrometric conditions**

| | |
|---|---|
| Acquisition mode: | SRM |
| Acquisition Delay: | 5 min |
| Ionization: | ESI in the Positive Ion Mode |
| | ESI Voltage: 5500V |
| Turbo Gas Temperature: | 700 $^{0}$C |
| Curtain Gas: | Nitrogen 22 psi |
| Collision Gas: | Nitrogen 5 psi |
| Ion Source Gas 1: | Air 50 psi |
| Ion Source Gas 2: | Air 70 psi |
| Declustering Potential: | 96 V |
| Entrance Potential: | 10 V |

Collision Energy: 29 V
Collision Exit Potential: 12 V

SRM masses (m/z):

| Analyte | SRM (m/z) | Transition used for: | Expected Retention time (min) |
|---|---|---|---|
| Testosterone | 289.4>97 | Quantitation | 9.6 |
| | 289.4>109 | Confirmation | 9.6 |
| $^{13}C_3$-Testosterone | 292.2>100 | Quantitation | 9.6 |
| | 292.2>112 | Confirmation | 9.6 |

**Diode Array Detector Conditions**
Operation Mode: Signal Data
Wavelength: 254 nm
Bandwidth: 16 nm
Margin for Negative Absorbance (mAU): 100
Slit Width: 4 mm
Sampling Rate: 10 Hz

Typical chromatograms of QC samples Low and High are shown in Appendix 3:

## 8.7 Data Processing

1. Data files generated by the HPLC/MS/MS system are transferred to the dedicated place on the CDC network.
2. Using a dedicated data processing method within the Analyst software, relevant chromatographic peaks are identified based on their retention time and the area under the curve is integrated. Manual integration maybe required if automatic processing fails to integrate the peaks properly.
3. Integrated peaks are documented as electronic files (in “pdf” format”) and integration results are saved as “rdb” and text files.
4. The integration results text file is imported in an MS Excel template where final results are calculated.
5. Integrations and integration results are reviewed by a specially trained and dedicated individual. Errors detected will be returned to the analyst for correction. Only data that passed this review process will be considered for further processing.

## 8.8 Data Calculations

1. For quality control, area ratios are calculated from the quantitation ion and the confirmation ion (“Confirmation Ion Ratio”). Only analytes that show a Confirmation Ion Ratio ±20% of the target value will be considered for further processing.

2. Area ratios for calculating analyte concentration are calculated from the analyte and internal standard area counts.
3. Calibration curves are generated with the area ratios from the calibrators and their assigned values using ordinary linear regression.
   The calibration curve is assessed for outliers and other problems resulting in non-linear behavior of data points. Sample batches with invalid calibration curves are not processed further.
4. The analyte concentration in serum is calculated using the area ratio calculated for the unknown sample and the regression parameters of the corresponding calibration curve. Area ratios for analytes outside the established linear range will not be used to calculate reportable results. These samples will be reanalyzed after appropriate dilution or concentration.

# 9. REPORTABLE RANGE OF RESULTS

## 9.1 Reportable Range of Results and linearity limits

The reportable range of results is the range in which linearity was verified. The linearity for the analytes measured in this measurement procedure was determined following CLSI guideline EP6 [24]. The reportable range of results is: 2.5-1000 ng/dL or 0.09 – 34.7 nmol/L.

## 9.2 Limit of detection (LOD)

The limit of detection was determined using Taylor's method [25]. The limit of detection is: 0.36 ng/dL or 0.012 nmol/L.

## 9.3 Analytical Specificity

Analytical specificity is achieved through:

- A sample preparation that isolates the analytes of interest from other components in the sample matrix.
- High Performance Liquid Chromatography that separates the analytes of interest and allows for compound identification based on chromatographic retention time using reference compounds and stable isotope labeled internal standards
- Mass selective detection mode that only allows for detection of the mass-to-charge ratios specific to the precursor and fragment ions for testosterone

Analytical specificity was tested

1. By assessing possible chromatographic coelution and MS detection using 13 different steroid hormones (for the list of compounds used in this assessment, see Appendix 4). None of the tested compounds showed coelution with the analytes reported in this method.

2. High, medium and low QC pools were analyzed without addition of the internal standard to assess whether compounds in the QC samples coelute with the internal standards. No coelution was detected in this experiment.

## 9.4 Accuracy (Trueness and Precision)

Within-day imprecision was determined from 11 replicates of low, medium and high QC samples. The among day variability was assessed by measuring high, medium and low QC samples in duplicate each over 20 days and calculating the means and standard deviations using the DLS SAS program for bench QC characterization.

| Analyte | Within-Day Precision (%CV) Low | Within-Day Precision (%CV) Medium | Within-Day Precision (%CV) High |
|---|---|---|---|
| Total Testosterone | 2.6 | 2.2 | 2.8 |

| Analyte | Among-Day Precision (%CV) Low | Among-Day Precision (%CV) Medium | Among-Day Precision (%CV) High |
|---|---|---|---|
| Total Testosterone | 5.5 | 3.4 | 5.3 |

The accuracy was verified by analyzing 40 patient samples with reference values assigned by a recognized reference laboratory (Prof. Dr. L. Thienpont at the University of Ghent and NIST). Deming regression analysis and difference plot analysis showed no or negligible bias between this method and the reference methods.

| Deming Regression | Bias | 95% Confidence Interval |
|---|---|---|
| Constant | 0.48 | -0.91 to 1.87 |
| Proportional | 0.99 | 0.98 to 1.00 |

| Difference Plot | Bias | 95% Limit of Agreement |
|---|---|---|
| Value | -2.3% | -11.1% to 6.4% |

# 10. QUALITY ASSESSMENT AND PROFICIENCY TESTING

Quality assessment activities for this measurement procedure follow the requirements outlined in the DLS Policies and Procedures Manual.

## 10.1 Quality Control Procedures

### 10.1.1 Quality Control Materials

Bench QC materials are used in this measurement procedure which consists of three serum materials with levels of concentration spanning the low to high ranges for the total testosterone in both men and women.

The bench QC specimens are inserted in each sample batch and processed the same as the patient specimens.

### 10.1.2 Establishing QC Limits and Quality Control Evaluation

Acceptance criteria for values obtained with the bench QC materials ("QC limits") are established according to the procedure described by Caudill et al. [26].

The rules described in the most recent version of the DLS Policies and Procedures Manual together with the acceptance criteria are applied to measurement results obtained with the QC materials. Sample runs are rejected, if

- one bench QC result is beyond the characterization mean ± 4SD,
- one bench QC result is outside a 3SD limit,
- current and previous bench QC results are outside the same 2SD limit
- current and previous 9 run results are on same side of the characterization mean
- the current and the previous run results differ by more than 4SD.

For further details, see the DLS Policies and Procedures Manual. Quality control evaluation is performed using a SAS program developed and maintained by DLS.

## 10.2 Proficiency Testing

Participation in a Proficiency Testing Program such as the one offered by the College of American Pathologists is assured [27].

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

When results of control or calibration materials fail to meet the laboratory's established criteria for acceptability, all patient test results obtained in the unacceptable test run and since the last acceptable test run must be considered adversely affected and thus cannot be reported. Specimen processing and analysis is stopped and will only resume when corrective

action have been performed that ensure the reporting of accurate and reliable patient test results.

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

**Limitations of the method**

This method was tested for total testosterone analysis in human serum and may not be suitable for other specimens such as plasma, whole blood, urine, and/or saliva. The analytical performance parameters need to be reassessed and verified when other specimen matrices are used.

**Interferring Substances**

No interfering substances were identified.

**Interfering Conditions**

Analyte may be subject to oxidation from oxygen in the air under elevated temperatures. Thus, samples should not be stored dry at ambient conditions.

## 13. REFERENCE RANGES (NORMAL VALUES)

Population-based reference ranges have not been established yet for total testosterone.

An in-house assessment using a convenience sample size from 130 individuals (men and women) was performed to obtain information on concentrations that can be expected in the general population. In this study, the following values were determined:

| Analyte | Values | | | |
|---|---|---|---|---|
| | Mean (Range) | | Median ($5^{th}$-$95^{th}$ percentile) | |
| | ng/dL | nmol/L | ng/dL | nmol/L |
| Total Testosterone-Male (n=78) | 432(122-1057) | 15.0(4.23-36.6) | 396(166-625) | 13.7(5.76-21.7) |
| Total Testosterone-Female (n=52) | 21.2(8.37-48.8) | 0.735(0.290-1.69) | 21.0(2.04-39.9) | 0.728(0.0707-1.38) |
| | | | | |

## 14. CRITICAL CALL RESULTS (“PANIC VALUES”); PROTOCOL FOR REPORTING CRITICAL CALLS

Due to the high variability in available reference ranges clinical cut off are not frequently provided. However, the following have been cited:

1. Men <150 ng/dL concern for pituitary and/or hypothalamic tumors [1]

2. Women >200 ng/dL concerns for tumors. [28]

The levels should be reported by fax, phone, or email to the supervising physician and/or principle investigator.

## 15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Any specimens not analyzed on the day of arrival in the laboratory are stored in the refrigerator (4°C - 8°C). Upon completion of analysis, specimens are stored for 1 week. NHANES specimens are frozen at -70°C and discarded after 1 year.

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

If the analytical system fails, we recommend that the specimens be stored at -70 °C until the analytical system is restored to functionality.

No alternate testing method exists for the measurement procedure.

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

Results are reported to 3 significant digits based on assay sensitivity calculations. Data are reported in ng/dL.

The test reporting system as described in the most recent version of the DLS Policies and Procedures Manual is used when reporting test results. The system consists of review steps at multiple levels such as results verification by a DLS statistician, and DLS management.

## 18. PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING.

Following successful completion of analysis, remaining samples will be retained until all results have been reported and sufficient time has passed for review of the results. After this time, samples are either returned to the contact person who requested the analysis or are treated according to DLS and CDC policy.

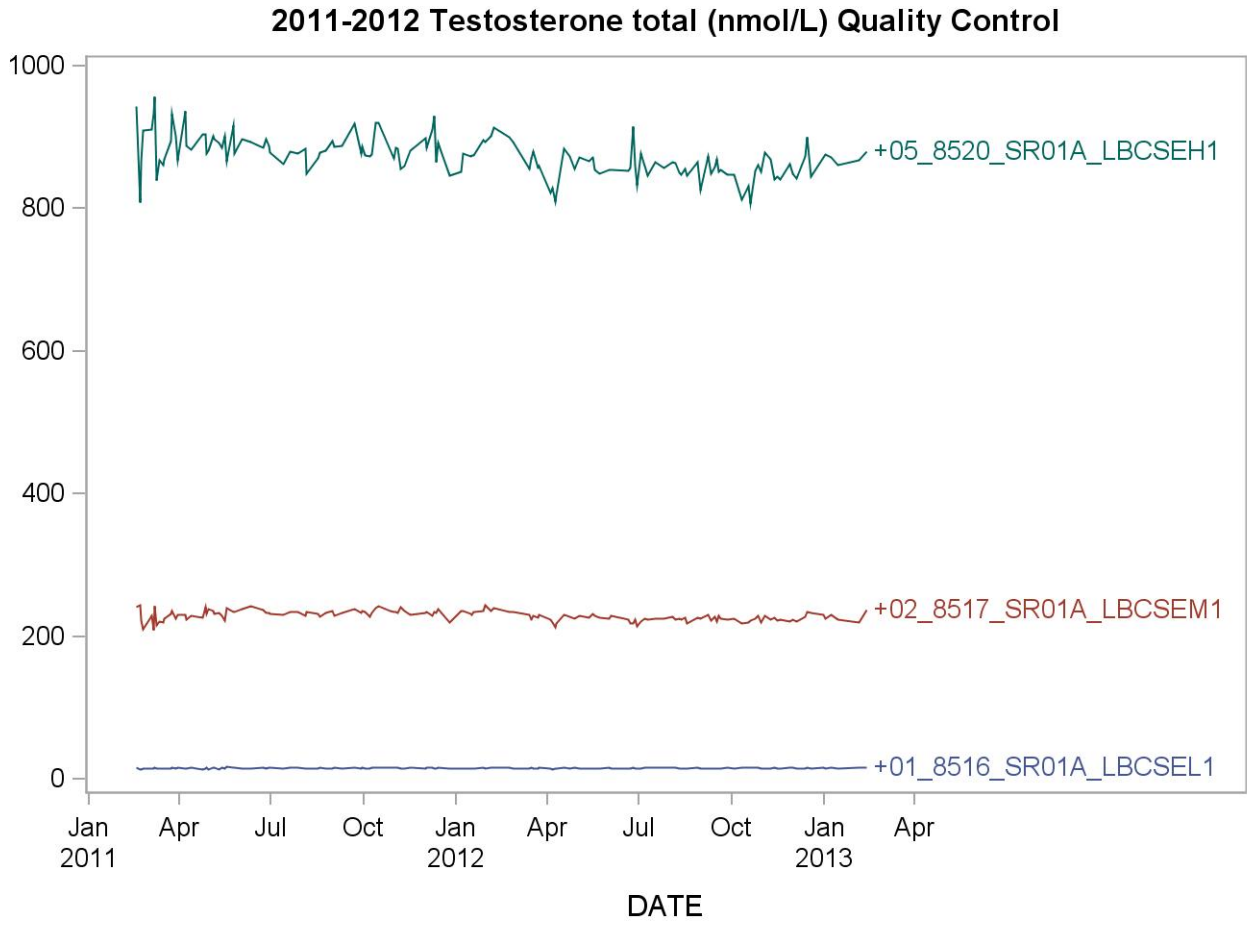
Standard record keeping (e.g., database, notebooks, data files) is used to track specimens. Records (including related QA/QC data) are maintained for 3 years, and duplicate records are kept off-site in electronic format. Study subject confidentiality is protected by providing personal identifiers only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

## 19. SUMMARY STATISTICS AND QC GRAPHS

See next page.

## Summary Statistics for Testosterone total (nmol/L)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| +05_8520_SR01A_LBCSEH1 | 161 | 17FEB11 | 12FEB13 | 873.516 | 27.135 | 3.1 |
| +01_8516_SR01A_LBCSEL1 | 161 | 17FEB11 | 12FEB13 | 15.560 | 0.586 | 3.8 |
| +02_8517_SR01A_LBCSEM1 | 161 | 17FEB11 | 12FEB13 | 229.492 | 7.050 | 3.1 |



## REFERENCES

1. S. Bhasin, G.R. Cunningham, F.J. Hayes, A.M. Matsumoto, P.J. Snyder, R.S. Swerdloff and V.M. Montori, Testosterone therapy in adult men with androgen deficiency syndromes: an endocrine society clinical practice guideline, *J. Clin. Endocrinol. Metab.* **91** (2006), pp. 1995–2010
2. American Association of Clinical Endocrinologists Polycystic Ovary Syndrome Writing Committee, American Association of Clinical Endocrinologists position statement on metabolic and cardiovascular consequences of polycystic ovary syndrome, *Endocr. Pract.* **11** (2005), pp. 126–134.
3. N.F. Goodman, M.B. Bledsoe, R.H. Cobin, W. Futterweit, J.W. Goldzieher, S.M. Petak, K.D. Smith and E. Steinberger, American Association of Clinical Endocrinologists Hyperandrogenic Disorders Task Force, American Association of Clinical Endocrinologists Medical Guidelines for the clinical practice for the diagnosis and treatment of hyperandrogenic disorders, *Endocr. Pract.* **7** (2001), pp. 120–134.
4. S.M. Petak, H.R. Nankin, R.F. Spark, R.S. Swerdloff and L.J. Rodriguez-Rigau, American Association of Clinical Endocrinologists, American Association of Clinical Endocrinologists Medical Guidelines for clinical practice for the evaluation and treatment of hypogonadism in adult male patients—2002 update, *Endocr. Pract.* **8** (2002), pp. 440–456.
5. C. Wang, E. Neischlag, R. Swerfloff, H.M. Behre, W.J. Hellstrom, L.J. Gooren, J.M. Kaufman, J.-J. Legros, B. Lunenfeld, A. Morales, J.E. Morley, C. Shulman, I.M. Thompson, W. Weidner and F.C.W. Wu, Review: ISA, ISSAM, EAU, EAA, and ASA recommendations: investigation, treatment and monitoring of late-onset hypogonadism in males, *Int. J. Impot. Res.* **21** (2009), pp. 1–8.
6. National Institute of Health. www.clinicaltrials.gov, 2009 (accessed 09/15/2009).
7. M.E. Wierman, R. Basson, S.R. Davis, S. Khosla, K.K. Miller, W. Rosner and N. Santoro, Androgen therapy in women: an Endocrine Society Clinical Practice guideline, *J. Clin. Endocrinol. Metab.* **91** (2006), pp. 3697–3710
8. V. Nisenblat and R.J. Norman, Androgens and polycystic ovary syndrome, *Curr. Opin. Endocrinol. Diabetes Obes.* **16** (2009), pp. 224–231
9. F. Saad and L. Gooren, The role of testosterone in the metabolic syndrome: a review,*J. Steroid Biochem. Mol. Biol.* **114** (2009), pp. 40–43
10. E. Selvin, M. Feinleib, L. Zhang, S. Rohrmann, N. Rifai, W.G. Nelson, A. Dobs, S. Basaria, S.H. Golden and E.A. Platz, Androgens and diabetes in men: results from the Third National Health and Nutrition Examination Survey (NHANES III), *Diabetes Care* **30** (2007), pp. 234–238
11. P. Hakimian, M. Blute Jr., J. Kashanian, S. Chan, D. Silver and R. Shabsigh, Metabolic and cardiovascular effects of androgen deprivation therapy, *BJU Int.* **102** (2008), pp. 1509–1514.
12. G.M. Rosano, A. Cornoldi and M. Fini, Effects of androgens on the cardiovascular system, *J. Endocrinol. Invest.* **28** (2005), pp. 32–38.

13. S.P. Tuck and R.M. Francis, Testosterone, bone and osteoporosis, *Front. Horm. Res.* **37** (2009), pp. 123–132.

14. M.I. Hu, R.F. Gagel and C. Jimenez, Bone loss in patients with breast or prostate cancer, *Curr. Osteoporos. Rep.* **5** (2007), pp. 170–178.

15. C.J. Pike, J.C. Carroll, E.R. Rosario and A.M. Barron, Protective actions of sex steroid hormones in Alzheimer's disease, *Front. Neuroendocrinol.* **30** (2009), pp. 239–258.

16. M.M. Cherrier, Testosterone effects on cognition in health and disease, *Front. Horm. Res.* **37** (2009), pp. 150–162.

17. G.A. Laughlin, E. Barrett-Connor and J. Bergstrom, Low serum testosterone and mortality in older men, *J. Clin. Endocrinol. Metab.* **93** (2008), pp. 68–75.

18. M.M. Shores, A.M. Matsumoto, K.L. Sloan and D.R. Kivlahan, Low serum testosterone and mortality in male veterans, *Arch. Intern. Med.* **166** (2006), pp. 1660–1665.

19. M.J. Wheeler, Measurement of testosterone in the diagnosis of hypogonadism in the ageing male, Clin. Endocrinol. **69** (2008), PP. 515-525.

20. Policies and Procedures Manual, Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, May 16, 2008.

21. H. Raff, P. M. Sluss, Pre-analytical issues for testosterone and estradiol assays, *Steroids*. **73** (2008) 1297-1304.

22. L. R. Strouda,, C. Solomona, E. Shenassab, G. Papandonatosc, R. Niaurad, L.P. Lipsitte, K.L.Winnf, S.L. Bukab, Long-term stability of maternal prenatal steroid hormones from the National Collaborative Perinatal Project: Still valid after all these years, *Psychoneuroendocrinology*, **32 (**2007), PP. 140–150

23. International Organization for Standardization (ISO). In vitro diagnostic medical devices — Measurement of quantities in biological samples — Metrological traceability of values assigned to calibrators and control materials. ISO 17511:2003(E), ISO Geneva, Switzerland. 2003.

24. CLSI. Evaluation of the linearity of quantitative measurement procedures: A statistical approach. NCCLS document EP6. NCCLS, Wayne, PA, USA, 2003.

25. Taylor JK, Lewis Publishers, Chelsea, MI2525. 1987. pp. 78-84

26. Caudill SP, Schleicher RL, Pirkle JL. Multi-rule quality control for the age-related eye disease study. *Stat. Med.* **27** (2008), PP 4094-106

27. CLSI. Assessment of laboratory tests when proficiency testing is not available. NCCLS document GP29. NCCLS, Wayne PA, USA. 2002.

28. Guideline: Diagnosis and management of polycystic ovarian syndrome. University of TX, School of Nursing; 2006 May

# 20. APPENDICES

Appendix 1. Metrological Traceability of Total Testosterone Measurements

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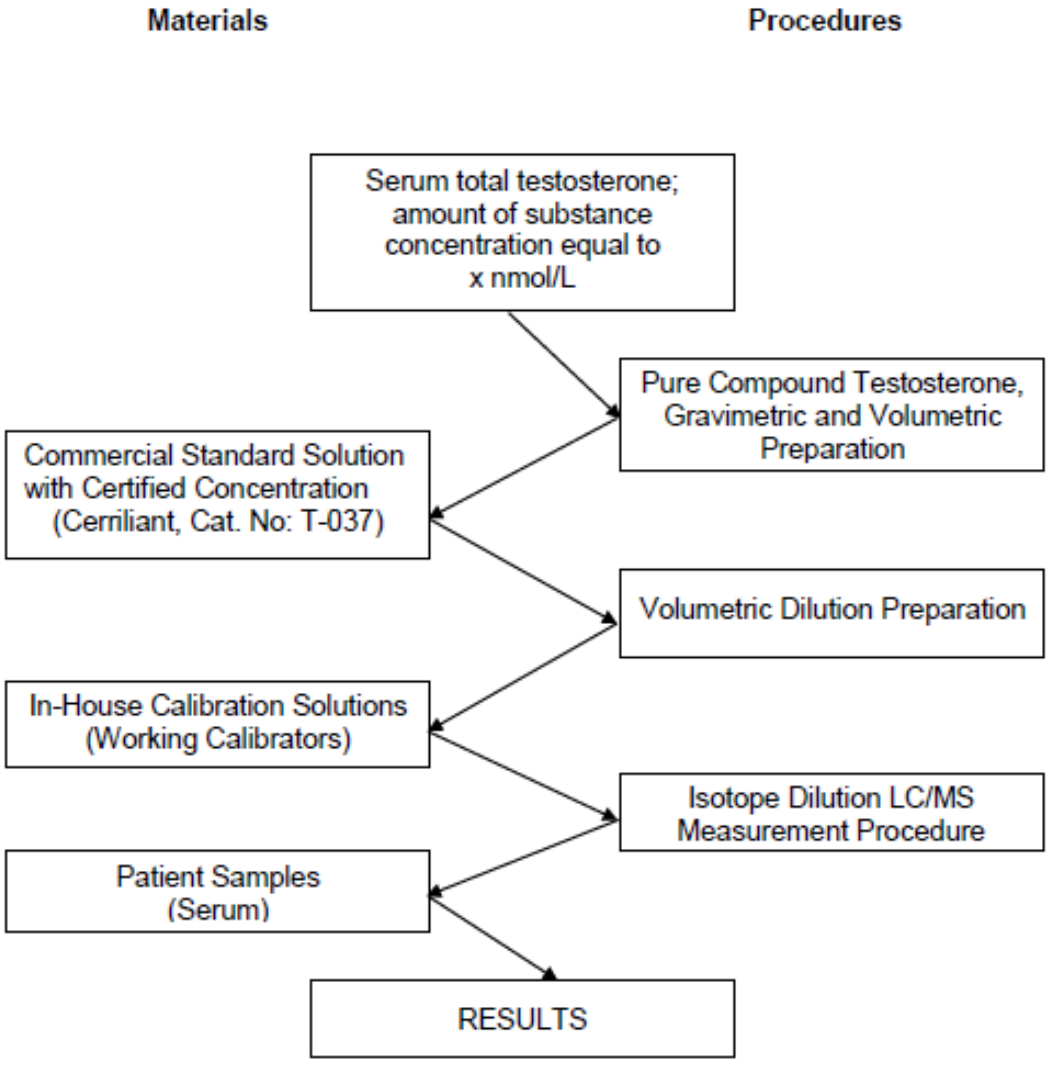
# Appendix 1. Metrological Traceability of Total Testosterone Measurements



# Appendix 2. Example of Analytical Sequence

| Sample ID | Sample Name | Tray | Vial | Volume | Type | Comment | Data File |
|---|---|---|---|---|---|---|---|
| 122210_Water | Water | CStk1-03 | 1 | 100 | Solvent | YW | 122210_TTP_0416_001_301 |
| 122210_INST_1 | INST_1 | CStk1-03 | 95 | 100 | Standard | YW | 122210_TTP_0416_002_395 |
| 122210_Water | Water | CStk1-03 | 2 | 100 | Solvent | YW | 122210_TTP_0416_003_302 |
| 122210_Blank | Blank | CStk1-01 | 13 | 100 | Solvent | YW | 122210_TTP_0416_004_113 |
| 122210_CC2.50_L01 | CC2.50_L01 | CStk1-01 | 25 | 100 | Standard | YW | 122210_TTP_0416_005_125 |
| 122210_CC5.00_L01 | CC5.00_L01 | CStk1-01 | 37 | 100 | Standard | YW | 122210_TTP_0416_006_137 |
| 122210_CC7.50_L01 | CC7.50_L01 | CStk1-01 | 49 | 100 | Standard | YW | 122210_TTP_0416_007_149 |
| 122210_CC10.0_L01 | CC10.0_L01 | CStk1-01 | 61 | 100 | Standard | YW | 122210_TTP_0416_008_161 |
| 122210_CC25.0_L01 | CC25.0_L01 | CStk1-01 | 73 | 100 | Standard | YW | 122210_TTP_0416_009_173 |
| 122210_CC50.0_L01 | CC50.0_L01 | CStk1-01 | 85 | 100 | Standard | YW | 122210_TTP_0416_010_185 |
| 122210_CC75.0_L01 | CC75.0_L01 | CStk1-01 | 2 | 100 | Standard | YW | 122210_TTP_0416_011_102 |
| 122210_CC100._L01 | CC100._L01 | CStk1-01 | 14 | 100 | Standard | YW | 122210_TTP_0416_012_114 |
| 122210_CC250._L01 | CC250._L01 | CStk1-01 | 26 | 100 | Standard | YW | 122210_TTP_0416_013_126 |
| 122210_CC500._L01 | CC500._L01 | CStk1-01 | 38 | 100 | Solvent | YW | 122210_TTP_0416_014_138 |
| 122210_CC750._L01 | CC750._L01 | CStk1-01 | 50 | 100 | Standard | YW | 122210_TTP_0416_015_150 |
| 122210_CC1000_L01 | CC1000_L01 | CStk1-01 | 62 | 100 | Standard | YW | 122210_TTP_0416_016_162 |
| 122210_Water | Water | CStk1-03 | 3 | 100 | Solvent | YW | 122210_TTP_0416_017_303 |
| 122210_147598535SA | 147598535SA | CStk1-01 | 3 | 100 | Sample | YW | 122210_TTP_0416_018_103 |
| 122210_141118745SA | 141118745SA | CStk1-01 | 15 | 100 | Sample | YW | 122210_TTP_0416_019_115 |
| 122210_142522254SA | 142522254SA | CStk1-01 | 27 | 100 | Sample | YW | 122210_TTP_0416_020_127 |
| 122210_142604346SA | 142604346SA | CStk1-01 | 39 | 100 | Sample | YW | 122210_TTP_0416_021_139 |
| 122210_142342531SA | 142342531SA | CStk1-01 | 51 | 100 | Sample | YW | 122210_TTP_0416_022_151 |
| 122210_142390482SA | 142390482SA | CStk1-01 | 63 | 100 | Sample | YW | 122210_TTP_0416_023_163 |
| 122210_142441161SA | 142441161SA | CStk1-01 | 75 | 100 | Sample | YW | 122210_TTP_0416_024_175 |
| 122210_142482442SA | 142482442SA | CStk1-01 | 87 | 100 | Sample | YW | 122210_TTP_0416_025_187 |
| 122210_Water | Water | CStk1-03 | 4 | 100 | Solvent | YW | 122210_TTP_0416_026_304 |
| 122210_147496174SA | 147496174SA | CStk1-01 | 4 | 100 | Sample | YW | 122210_TTP_0416_027_104 |
| 122210_147932483SA | 147932483SA | CStk1-01 | 16 | 100 | Sample | YW | 122210_TTP_0416_028_116 |
| 122210_147948093SA | 147948093SA | CStk1-01 | 28 | 100 | Sample | YW | 122210_TTP_0416_029_128 |
| 122210_147989501sa | 147989501sa | CStk1-01 | 40 | 100 | Sample | YW | 122210_TTP_0416_030_140 |
| 122210_148032122SA | 148032122SA | CStk1-01 | 52 | 100 | Sample | YW | 122210_TTP_0416_031_152 |
| 122210_0372788SA | 0372788SA | CStk1-01 | 64 | 100 | Sample | YW | 122210_TTP_0416_032_164 |
| 122210_147656886SA | 147656886SA | CStk1-01 | 76 | 100 | Sample | YW | 122210_TTP_0416_033_176 |
| 122210_147394404SA | 147394404SA | CStk1-01 | 88 | 100 | Sample | YW | 122210_TTP_0416_034_188 |
| 122210_Water | Water | CStk1-03 | 5 | 100 | Solvent | YW | 122210_TTP_0416_035_305 |
| 122210_+016207PA | +016207PA | CStk1-01 | 5 | 101 | Standard | YW | 122210_TTP_0416_036_105 |
| 122210_+026208PA | +026208PA | CStk1-01 | 17 | 100 | Standard | YW | 122210_TTP_0416_037_117 |
| 122210_+036209PA | +036209PA | CStk1-01 | 29 | 99 | Standard | YW | 122210_TTP_0416_038_129 |
| 122210_Water | Water | CStk1-03 | 6 | 98 | Solvent | YW | 122210_TTP_0416_039_306 |
| 122210_INST_2 | INST_2 | CStk1-03 | 96 | 97 | Standard | YW | 122210_TTP_0416_040_396 |
| 122210_Water | Water | CStk1-03 | 7 | 96 | Solvent | YW | 122210_TTP_0416_041_307 |

# Appendix 3. QC Samples Chromatograms

## Low QC Sample Chromatogram

**Plot removed as it could not be made 508 compliant.**

| Approximate Concentration (ng/dL) | |
|---|---|
| Analyte (Testosterone) | 14.8±0.7 |
| Internal Standard (13C Testosterone) | 100±4 |

## High QC Sample Chromatogram

**Plot removed as it could not be made 508 compliant.**

| Concentration (ng/dL) | |
|---|---|
| Analyte (Testosterone) | 883±40 |
| Internal Standard (13C Testosterone) | 100±4 |

## Appendix 4. List of Compounds Tested for Interference

1. 5b-androstan 3,17 dione, CAS No: 1229-12-5, (Steraloids Inc., Newport, RI )
2. 4-androsten 3a-ol-17-one, CAS No: 2791-99-3, (Steraloids Inc., Newport, RI )
3. 4-androsten 3b-ol-17-one, CAS No: 571-44-8, (Steraloids Inc., Newport, RI )
4. 4,6-androsten 17b-ol-3-one, CAS No: n/a, (Steraloids Inc., Newport, RI )
5. 5-androsten 3b,17b-diol, CAS No: 521-17-5, (Steraloids Inc., Newport, RI )
6. 1,(5a)-androsten-17b-ol-3-one, CAS No: n/a, (Steraloids Inc., Newport, RI )
7. 1,4-androstadien-17b-ol-3-one, CAS No:846-48-0, (Steraloids Inc., Newport, RI )
8. 5a-androstan-17b-ol-3-one, CAS No: 521-18-6, (Steraloids Inc., Newport, RI )
9. Dehydroepiandrasterone, CAS No: 53-43-0, Aust. Gov't. NMI
10. Androsterone 10.3mg, CAS No: 53-41-8, Aust. Gov't. NMI
11. 17-a methyl, 5, a-androstane-3 a 17 b diol, CAS No: n/a, (Cerilliant, Round Rock, Texas)
12. 6b-Hydroxytestosterone, CAS No: 62-99-7, (Cerilliant, Round Rock, Texas)
13. 17-alpha methyl testosterone, CAS No: 58-18-4, (Cerilliant, Round Rock, Texas)

For interference testing, each compound was prepared in a solution of 0.35 nmol/L using Sample Reconstitution Solution.

# Appendix 5. Related Documents

### Normative References

1. DLS Policies and Procedures Manual. http://intranet.nceh.cdc.gov/dls/qaqc.aspx.
2. CDC Safety Policies and Practices Manual. http://isp-v-ehip-asp/dlsintranet/safety_manual/
3. Clinical Laboratory Improvement Amendments of 1988 (CLIA). 42CFR493 from February 28, 1992.
4. CLSI. Evaluation of the linearity of quantitative measurement procedures: A statistical approach. NCCLS document EP6. NCCLS, Wayne, PA, USA, 2003.
5. International Organization for Standardization (ISO). In vitro diagnostic medical devices — Measurement of quantities in biological samples — Metrological traceability of values assigned to calibrators and control materials. ISO 17511:2003(E), ISO Geneva, Switzerland. 2003.
6. International Organization for Standardization (ISO). General requirements for the competence of testing and calibration laboratories. ISO 17025:2003(E), ISO Geneva, Switzerland. 2003.
7. International Organization for Standardization (ISO). *In vitro* diagnostic medical devices —Measurement of quantities in samples of biological origin — presentation of reference measurement procedures. ISO 15193:2002(E), ), ISO Geneva, Switzerland. 2002.
8. International Organization for Standardization (ISO).*In vitro* diagnostic medical devices —Measurement of quantities in samples of biological origin — Description of reference materials. ISO 15194:2002(E), ), ISO Geneva, Switzerland. 2002.
9. International Organization for Standardization (ISO). Laboratory medicine — Requirements for reference measurement laboratories. ISO 17195:2003(E), ISO Geneva, Switzerland. 2003.

# Appendix 6. Symbols, Abbreviations, Terminology

## Abbreviations

**ACN** Acetonitrile
**ACS.ASTM** American Chemical Society. American Society for Testing and Material
**BP** Boiling Point
**CDC** Centers for Disease Control and Prevention
**CC** Calibrators
**CCB** Clinical Chemistry Branch
**$C_2H_3O_2Na$** Sodium Acetate
**CLIA** Clinical Laboratory Improvement Act/Amendment
**CV** Coefficient of Variant
**DLS** Division of Laboratory Sciences
**EMV** Electron Multiplier Voltage
**ESI** Electrospray Ionization
**FDA** Food and Drug Administration
**HCl** Hydrochloric Acid
**HIV** Human Immunodeficiency Virus
**HPLC** High Performance Liquid Chromatography
**ID** Identification
**IS** Internal Standards
**ISO** International Organization for Standardization
**ITSO** Information Technology Service Office
**LC/MS/MS** Liquid Chromatography/Tandem Mass Spectrometry
**MSDS** Material Safety Data Sheets
**SRM** Selected Reaction Monitoring
**N/A** Not Applicable
**NaOH** Sodium Hydroxide
**NCEH** National Center of Environmental Health
**$(NH_4)_2CO_2$** Ammonium Carbonate
**NMI** Australian National Measurement Institute
**OHS** Occupational Health and Safety
**PT** Proficiency Testing
**QA** Quality Assurance
**QC** Quality Control
**SAS** Statistical Analysis Software
**SD** Standard Deviation
**SAS** Statistical Analysis System
**TT** Total Testosterone

## Symbols

**Not applicable**

**Terminology**

The terminology defined in CLIA ’88 (57 FR 7139 Subpart A Sec Sec. 493.2) is used in this document. Otherwise the terminology described in the Clinical and Laboratory Standards Institute’s terminology database was used. The database can be accessed at: (http://www.clsi.org/Content/NavigationMenu/Resources/HarmonizedTerminologyDatabase/Harmonized_Terminolo.htm)

# Appendix 7. Document Compliance Tables

Table 1: Location of information required by the DLS Policies and Procures Manual

| Required section | Section in this Document |
|---|---|
| requirements for specimen collection and processing, including criteria for specimen rejection | 5 |
| step-by-step performance of the procedure, including test calculations and interpretation of results | 6 |
| preparation of reagents, calibrators, controls, solutions and other materials used in testing | 4 |
| calibration and calibration verification procedures | 7 |
| the reportable range for patient test results | 8.1 |
| quality control procedures, including PT materials and programs/procedures used | 8 |
| remedial action to be taken when calibration or control results are outside acceptable limits | 9.1.3 |
| limitation in methods, including interfering substances | 8.5 |
| reference range (normal values) | 10 |
| life-threatening or "panic values" | 15 |
| pertinent literature references | 17 |
| specimen storage criteria | 5.2, 7.1 |
| protocol for reporting panic values | 15 |
| course of action if test system becomes inoperable | 9.1.3, 12 |
| criteria for referral of specimens (usually not needed) | 14 |
| safety considerations for performing the method | 2 |

Table 2: Location of information as required by CLIA

| Required section | Section in this Document |
|---|---|
| Requirements for patient preparation; specimen collection, labeling, storage, preservation, transportation, processing, and referral; and criteria for specimen acceptability and rejection | 3.2, 5, |
| Microscopic examination, including the detection of inadequately prepared slides | 16 |
| Step-by-step performance of the procedure, including test calculations and interpretation of results | 6 |
| Preparation of slides, solutions, calibrators, controls, reagents, stains, and other materials used in testing | 4 |
| Calibration and calibration verification procedures | 7 |
| The reportable range for test results for the test system as established or verified | 8.1 |
| Control procedures | 8 |
| Corrective action to take when calibration or control results fail to meet the laboratory's criteria for acceptability | 9.1.3 |
| Limitations in the test methodology, including interfering Substances | 8.5 |
| Reference intervals (normal values) | 10 |
| Imminently life-threatening test results or panic or alert Values | 15 |
| Pertinent literature references | 17 |
| The laboratory's system for entering results in the patient record and reporting patient results including, when appropriate, the protocol for reporting imminent life threatening results, or panic, or alert values | 3, 7.7, 13 |
| Description of the course of action to take if a test system becomes inoperable | 9.1.3, 12 |

Table 3: Location of information as required by ISO 17025

| Required section | Section in this Document |
|---|---|
| appropriate identification | Title Page |
| Scope | 1 |
| description of the type of item to be tested or calibrated | 1 |
| parameters or quantities and ranges to be determined | 1, 8.1 |
| apparatus and equipment, including technical performance requirements | 4 |
| reference standards and reference materials required | 4.3, 0 |
| environmental conditions required and any stabilization period needed | 4, 6 |
| description of the procedure, including<br>affixing of identification marks, handling, transporting, storing and preparation of items,<br>checks to be made before the work is started,<br>checks that the equipment is working properly and, where required, calibration and adjustment of the equipment before each use,<br>the method of recording the observations and results,<br>any safety measures to be observed | 6 |
| criteria and/or requirements for approval/rejection | 5, 8 |
| data to be recorded and method of analysis and presentation | 3, 7.8 |
| the uncertainty or the procedure for estimating uncertainty | 8.4 |

Table 4: Location of information as required by ISO 15193

| Required section | Section in this Document |
|---|---|
| Title page | Title Page |
| Contents list | List of Content |
| Foreword | N/A |
| Warning and safety precautions | 2 |
| Introduction | 1 |
| Title | Title Page |
| Scope | 1 |
| Normative references | 0 |
| Definitions | 0 |
| Symbols and abbreviations | 0 |
| Terminology | 0 |
| Principle and method of measurement | 1 |
| Check list | |
| Reagents | 4 |
| Apparatus | 4 |
| Sampling and sample | 5, 6.1 |
| Preparation of measuring system and analytical portion | 6 |
| Operation of measuring system | 6 |
| Data processing | 3, 7.8 |
| Analytical reliability | 8 |
| Special cases | N/A |
| Validation by inter-laboratory studies | N/A |
| Reporting | 7.8, 11 |
| Quality assurance | 8 |
| Bibliography (Annex) | 16 |
| Dates of authorization and revision | Second page of document |