Laboratory Procedure Manual

Analyte: Total Estradiol and Total Testosterone

Matrix: Serum

Method: Simultaneous Measurement of Estradiol and Testosterone in Human Serum by ID LC-MS/MS

Method No: 1033

Revised:

as performed by:

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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 for SAS file **TST_H**:

VARIABLE NAME	SAS LABEL (and SI units)
LBXTST	Testosterone, total (nmol/L)
LBXEST	Estradiol (pg/mL)

Contents

1 SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

1.1 Intended Use

INTENDED USE OF ASSAY: The CDC DLS total estradiol/total testosterone assay is intended to quantitatively measure total estradiol and testosterone concentrations in vitro in human serum.

The measurement procedure described in this document is intended for quantitatively measuring all unconjugated (free and protein-bound) estradiol (EST) and testosterone (TST) in human serum. Measurement of conjugated estradiol or testosterone requires different methodologies. This method addresses all aspects related to the measurement process (specimen collection, storage, processing, analysis and reporting). This method was evaluated for measurements in serum and may not be suitable for other sample matrices such as plasma and urine. Results obtained from this method may be used to define population-based clinical reference ranges. The results obtained with this method are not used for direct diagnosis and treatment of patients. As outlined in section 15 of this document, results obtained with this method may be used to alert study participants of possible medical issues that may require further medical attention and evaluation by a professional.

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

1.2 Clinical and Public Health Relevance

Clinical guidelines recommend testing for testosterone to aid in the diagnosis of certain diseases and disorders such as hypogonadism and polycystic ovary syndrome, and to monitor patients on certain treatments (1-7). Research found that testosterone levels are associated with certain chronic diseases and conditions, such as metabolic syndrome (8), diabetes (9), cardiovascular disease (10, 11), fractures (12, 13), neurodegenerative disorder (14, 15), and higher mortality in men with lower testosterone levels (16, 17).

Estradiol levels in serum can be used to detect hormone-secreting tumors found in the breast and ovaries (18). Patients undergoing assisted reproduction procedures are monitored for estradiol to detect ovarian hyperstimulation syndrome (19). Elevated estradiol levels in postmenopausal women have been suggested as biomarker for breast cancer risk (18). Estradiol treatment has been recommended for certain conditions to reduce menopausal symptoms and to prevent osteoporosis (20, 21).

To correctly and consistently identify concerning levels of testosterone and estradiol in patients, information about TST and EST levels in the population and specific study cohorts are needed to allow the determination of normal or reference ranges (22, 23). This method is used to address this need.

1.3 Test Principle

This measurement procedure describes the measurement of total estradiol and total testosterone (free and protein bound testosterone) in human serum.

The ISO/IUPAC definition of the quantity measured with this method is 'total testosterone' and 'total estradiol, the measurands are 'serum total testosterone; amount of substance concentration equal to x nmol/L' and serum total estradiol; amount of substance concentration equal to x pmol/L'. To facilitate the clinical use of these measurements, results are converted into ng/dL for testosterone and pg/ml for estradiol.

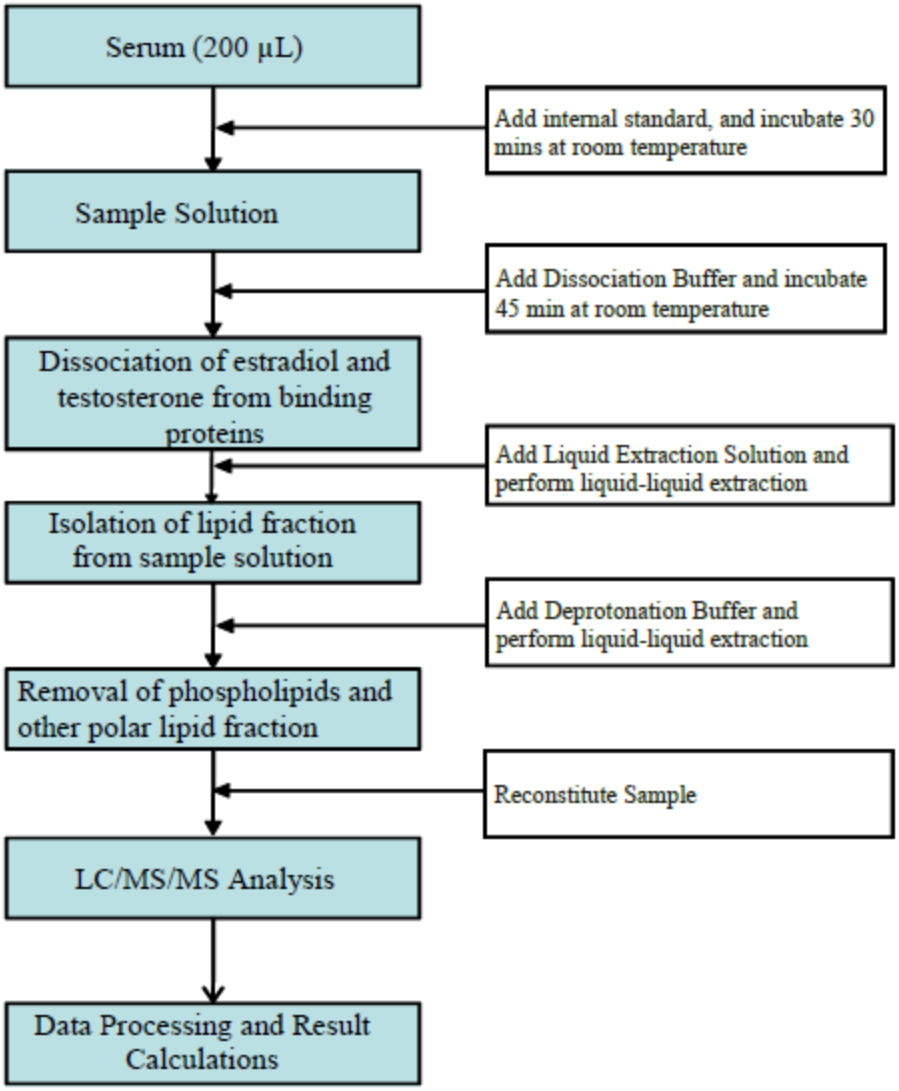
The four principle steps in this measurement procedure are: Dissociation of the analytes from binding proteins, extraction of the analytes from the sample matrix, removal of potentially interfering compounds, and quantitation of the analytes by isotope dilution high performance liquid chromatography tandem mass spectrometry (ID-LC-MS/MS) using stable isotope labeled internal standards and external calibrators.

Isolation of the analytes is achieved using liquid-liquid extraction. ID-LC-MS/MS is performed with a triple quadrupole mass spectrometer using electrospray ionization in positive ion mode for testosterone, and negative ion mode for estradiol. Estradiol and testosterone are identified based on chromatographic retention time and on specific mass to charge ratio transitions using selected reaction monitoring (SRM). A ^{13}C isotope-labeled testosterone and a ^{13}C isotope-labeled estradiol are used as internal standards.

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

1. Preparation of samples solution
2. Dissociation of estradiol and 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 estradiol and testosterone by ID-LC-MS/MS
6. Data processing and result calculations

Scheme 1: Measurement Procedure for Total Estradiol and Total Testosterone in Serum



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, in 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.

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.

Methanol: 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.

Ammonium Hydroxide: Very hazardous in case of skin contact (corrosive, irritant, permeator), of eye contact (irritant), of ingestion. Liquid or spray mist may produce tissue damage particularly on mucous membranes of eyes, mouth and respiratory tract.

Ammonium Fluoride: Very hazardous in case of skin contact (irritant), of eye contact (irritant), of ingestion, of inhalation. Hazardous in case of eye contact, skin contact(corrosive). Slightly hazardous in case of skin contact (permeator). Severe over-exposure can result in death. Inflammation of the eye is characterized by redness, watering, and itching. Skin inflammation is characterized by itching, scaling, reddening, or, occasionally, blistering.

Ammonium bicarbonate: Hazardous in case of skin contact (irritant, permeator), of eye contact (irritant), of ingestion, of inhalation.

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 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 4.11or higher). Specific training to operate this software is required to ensure appropriate and safe instrument function.

Further, calculations of results obtained with the LC-MS/MS instrument are performed using calculation templates created with Microsoft Excel and Indigo. 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 be able to identify so called “panic values” information about the gender and age group of the patient 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. 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 PREPARATION FOR REAGENTS, CALIBRATION MATERIALS, CONTROL MATERIALS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION.

To avoid contamination of equipment and consumables with testosterone and estradiol from sweat, gloves needs to be worn at all times when preparing reagents, handling samples, and operating equipment.

4.1 Equipment, Chemicals and Consumables

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

4.1.1 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)
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. Fisherbrand Octagonal stirring bars, 1 inch length; 0.312 inch diameter (Fisher Scientific, Cat No: 14-513-59, Suwanee, GA)
6. Scholar™ 5 x 5 Inch PC-171 Magnetic Stirrer (Corning Incorporated, Lowell, MA)
7. 1L glass bottles with screw tops (Wheaton Industries Inc., Cat. No: 219440, Millville, NJ)
8. Fisherbrand Disposable Borosilicate Glass Pasteur Pipets, 5’3/4” (Fisher Scientific, Cat. No: 13-678-20A, Suwannee, GA)
9. Milli-Q Water, Resistivity, 18.1 MΩ·cm at 25 ° C, 18.2 (Aqua Solutions, Jasper, GA)
10. Ammonium Bicarbonate, Bioultra ≥99.5%, CAS No: 1066-33-7 (Fluka, St. Louis, MO).
11. Ethyl Acetate, HPLC grade, CAS No: 141-78-6 (Fisher scientific, Cat. No: E195SK, Suwanee, GA)
12. Hexane, HPLC/ACS grade, CAS No: 110-54-3 (Fisher Scientific, Cat. No: H302, Suwanee, GA)
13. Ammonium Hydroxide 30%, Aqueous, CAS No: 1336-21-6, (Sigma-Aldrich, Cat. No: 320145, St. Louis, MO)
14. Methanol, HPLC Grade, CAS: 67-56-1 (Fisher Scientific, Cat. No: A452, Suwannee, GA)
15. Glacial Acetic Acid, Certified ACS grade, CAS No: 64-19-7 (Fisher Scientific, Cat. No: BP2401, Suwanee, GA)
16. Water, Optima grade, CAS No: 7732-18-5 (Fisher Scientific, Cat. No: W5, Suwanee, GA)
17. Ammonium Fluoride, CAS No: 12125-01-8 (Fisher Scientific, Cat. No: A665, Suwanee, GA)

4.1.2 Equipment, Chemicals and Consumables Used for Preparation of 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. 10-mL glass volumetric pipette (Fisher Scientific, Cat. No: 13-650-2L, Suwanee, GA)
5. 7-mL Pyrex brand glass tubes (Corning Inc., Cat. No: 9826-16, Lowell, MA)
6. 1-mL aqueous pipette (Gilson, Inc., Cat. No: F148505, Middleton, WI)
7. 1-mL positive displacement pipette (Gilson, Inc., Cat. No: F148506, Middleton, WI)
8. Gilson Pipetman, Serial: W62622K (Gilson, Inc., 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 Reference Material), testosterone CAS No: 58-22-0, Acetonitrile CAS NO: 75-08-8 (Cerillant, Cat. No: Round Rock, TX)
11. 17β-Estradiol in Acetonitrile, 1 mg/ml (Certified Reference Material), Estradiol CAS No: 50-28-2, Acetonitrile CAS NO: 75-08-8 ((Cerillant, Cat. No: E-060, Round Rock, TX)
12. [2,3,4-$^{13}C_3$]-Testosterone, purity ≥98%, CAS No: 327048-83-9 (IsoSciences, Cat. No: 6066, King of Prussia, PA)
13. 17β-Estradiol-[2,3,4-$^{13}C_3$], purity ≥98%, CAS No: 1261254-48-1, (IsoSciences, Cat. No: 9124, King of Prussia, PA)

4.1.3 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. Fisher Digital Multi-tube Vortex Mixer (Fisher Scientific, Cat. No: 02-215-452, Suwanee, GA).
6. Eppendorf Repeater Plus Pipetter (Eppendorf, Cat. No: 022260201, Ramsey, MN)
7. Sato Label Maker CL612e and Label Making Software (Sato America, Charlotte, NC)
8. 100-µL Positive displacement pipette (Gilson, Inc., Cat. No: F148504, Middleton, WI)
9. 96-Well, 2-ml square well plates (Seahorse Labware, Cat. No: S30009, Chicopee, MA)
10. 96-Well, 2-mL square well Round (Microliter Analytical Supplies INC, Product No: 07-8000, Suwanee, GA)
11. Robotic Reservoirs, Convoluted bottom (Thermo Scientific, Cat. No: 1200-2300, Waltham, MA)
12. ArctiSeal 96-Well Square Silicone w/ PTFE Spray Coating (Arctic White LLC, Cat No: AWSM-1003SX, Bethlehem, PA)
13. Eppendorf Combitips plus Pipet tips, 5 ml (Eppendorf, Cat. No: 022266403, Ramsey, MN)
14. GeneVac EZ-2.3 Elite Evaporation System with side bridge holders and universal rotor (GeneVac Inc., Valley Cottage, NY)

15. Co-RE Tips, 480 standard volume tips (300 µL) with Filters (Hamilton Company, Reno, NV)Orbitron Rotator II, Model 26250, (Boekel Scientific, Feasterville, PA)Eppendorf Swing-bucket Rotor (Eppendorf, Cat. No: A-2-DWP, Ramsey, MN)
16. Gilson Pipetman, Serial: W62622K (Gilson, Inc., Middleton, WI)
17. D1000 100-1000 µL volume Diamond for pipetman, (Cat. No: F171500, Gilson, Middleton, WI)

4.1.4 Equipment, Chemicals and Consumables Used for Sample Measurement

1. AB/Sciex API 5500 Triple Quad Mass Spectrometer with ESI source (AB/Sciex, Foster City, CA)
2. Shimadzu Nexera Column Oven CTO-30A (Columbia, Maryland)
3. 4 Shimadzu Nexera LC-30AD LC Pumps (Columbia, Maryland)
4. Shimadzu SIL-30 ACMP Autosampler (Columbia, Maryland)
5. Shimadzu Prominence DGU-20A5 Degasser (Columbia, Maryland)
6. Shimadzu Prominence DGU-20A5R Degasser (Columbia, Maryland)
7. Shimadzu Prominence Communication Bus CBM-20A (Columbia, Maryland)
8. 2 Accucore Phenyl/Hexyl Column, 150 **x** 3.0 mm, 2.6 um particle size (Thermo Scientific, Cat. No: 17926-153030, Waltham, MA)
9. Clear Reconstruction Micro Plate Seal (BioChromato, Inc., Kanagawa-Ken, Japan)

4.2 Preparation of Reagents Used For Sample Preparation

4.2.1 Dissociation Buffer

This is a solution of 0.5 mol/L ammonium acetate at pH 5.5 used to dissociate total testosterone from binding globulins as described in section 6.3.

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 9.64 g ammonium acetate and transfer into a 500-mL beaker
2. Add 200 mL of DI water using a 250-mL graduated cylinder
3. Add stir bar to the beaker and mix until completely dissolved
4. Measure pH of solvent with a 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 glass disposable pipette
6. Add DI water to adjust the total volume to 250 mL
7. Transfer to a glass bottle with plastic screw cap and label the bottle with content, concentration, preparation date, expiration date, analyst initials, safety precautions and hazard information.

Prepare a fresh batch of this solution each day and verify its pH prior to use.

4.2.2 Liquid Extraction Solution

This solution is a mixture of ethyl acetate and hexane (400 ml + 600 ml) used to separate non-polar serum components ("lipid fraction") from polar serum components as described in section 6.4.

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

1. Using a graduated cylinder transfer 400 mL of ethyl acetate in a 1-L bottle with screw top
2. Using a graduated cylinder transfer 600 mL of hexane and transfer to same 1-L container
3. Close glass bottle and mix thoroughly
4. Appropriately label the bottle as described in 4.2.1

Store this solution in a flammable cabinet. This Solution is stable for at least 1-2 months.

4.2.3 Deprotonation Buffer

This is a solution of 0.2 mol/L ammonium bicarbonate at pH 8.0 used to deprotonate phospholipids and similar compounds prior to extraction as described in section 6.4.

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 3.85 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 calibrated pH meter
5. Adjust pH to 8.0 (±0.1) with 6 mol/L NaOH using a glass disposable pipette.
6. Add DI water to adjust the total volume to 250 mL
7. Transfer to a glass bottle with plastic screw cap and appropriately label the bottle, including safety precautions and hazard information

Prepare a fresh batch of this solution each day and verify its pH prior to use.

4.2.4 Sample Reconstitution Solution

This solution is a mixture of water and methanol (800 mL + 200 mL) used to reconstitute samples prior to injection on LC-MS/MS as described in section 6.5.

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

1. Using a graduated cylinder measure out 800 mL of water and transfer to a 1-L bottle with screw top
2. Using a graduated cylinder measure out 200 mL of methanol and transfer to the same 1 L bottle
3. Close glass bottle and mix thoroughly

4. Appropriately label the bottle as described in 4.2.1.

Store this solution in a flammable cabinet. This solution is stable for at least 6 months.

4.2.5 Calibrator Storage Solution

This solution is a mixture of water and ethanol (800 mL + 200 mL) used to prepare calibrators as described in section 4.3.

Three liter of Calibrator Storage Solution is sufficient for preparation of 1 set of calibrators. If more calibrators are to be processed, volumes need to be adjusted.

1. Using a graduated cylinder transfer 600 mL of ethanol to a 4-L bottle with screw top
2. Using a graduated cylinder transfer 2400 mL of water and transfer to the same 4-L bottle.
3. Close glass bottle and mix thoroughly
4. Appropriately label the bottle as described in 4.2.1

Store this solution in a flammable cabinet. This solution is stable for at least 6 months.

4.2.6 LC Mobile Phase A

This solution is a mixture of 0.2 mM ammonium fluoride in a mixture of water and methanol (800 mL + 200 mL) used as LC Mobile Phase A. Mobile phase B used in this procedure is methanol.

Preparation of 1 L of LC Mobile Phase A is sufficient for 300 samples. If more samples are to be processed, volumes need to be adjusted.

1. Using a graduated cylinder measure transfer 800 mL of water to a 1-L bottle with screw top
2. Using a graduated cylinder transfer 200 mL of methanol and transfer to the same 1-L bottle with screw top
3. Weigh 7.4 mg Ammonium Fluoride and transfer to the 1-L bottle
4. Close the glass bottle and mix thoroughly
5. Appropriately label the bottle as described in 4.2.1

Prepare a fresh batch of this solution prior to use.

4.3 Calibration Materials

4.3.1 Preparation of Calibrator Working Solutions

The Calibrator Working Solutions are prepared from Calibrator Stock Solutions which are prepared from certified, commercial solutions 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 300 vials per calibrator level, which is sufficient for 20,000 samples assuming use of 1 vial per sample batch.

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

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

Stock Solution	Analyte	Concentration µg/mL	Concentration nmol/L	Dilution
Stock Solution A	TST	1	3467	500 µL (certified solution) → 500 mL
Stock Solution A	EST	1	3671	500 uL (certified solution) → 500 mL
TST/EST Stock Solution B	TST	0.02	69.3	10 mL (TST Calibrator Stock solution A) → 500 mL
	EST	0.002	7.34	1 mL (EST Calibrator Stock solution A) → 500 mL

1. Preparation of Testosterone Calibrator Stock Solution A
    a. Transfer 500 µL of a 1-mg/mL certified testosterone solution into a 500-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. Aliquot 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 Estradiol Calibrator Stock Solution A
    a. Transfer 500 µL of a 1-mg/mL certified Estradiol solution into a 500 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. Aliquot 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
3. Preparation of Testosterone/Estradiol Calibrator Stock Solution B
    a. Transfer 10 mL of Testosterone Calibrator Stock Solution A into a 100-mL volumetric flask using a volumetric glass pipette
    b. Transfer 1 mL of Estradiol Calibrator Stock Solution A into a 100 mL volumetric flask using a volumetric glass pipette
    c. Add 20% Ethanol to just below the fill line of the volumetric flask
    d. Place flask in the water bath for 15 minutes to reach 20°C and add 20% ethanol (at 20°C) to the fill line
    e. Aliquot solution in 7-mL aliquots in 14, 7-mL Pyrex glass tubes
    f. Label the tubes appropriately and store them in the refrigerator. Tubes are for single use only

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 are used for measuring testosterone levels in humans:

Table 2: Desired Estradiol and Testosterone Calibrator Working Solution Concentration

Calibrator Working Solution Code	Testosterone Target Concentration		Estradiol Target Concentration		Dilution
	ng/dL	nmol/L	pg/mL	pmol/L	
CC01	1000	34.7	1000	3671	100 mL (TST/EST Calibrator Stock Solution B) → 200 mL
CC02	750	26.0	750	2754	75 mL (TST/EST Calibrator Stock Solution B) → 200 mL
CC03	500	17.3	500	1836	50 mL (TST/EST Calibrator Stock Solution B) → 200 mL
CC04	250	8.67	250	918	25 mL (TST/EST Calibrator Stock Solution B) → 200 mL
CC05	100	3.47	100	367	10 mL (TST/EST Calibrator Stock Solution B) → 200 mL
CC06	50	1.73	50	184	5 mL (TST/EST Calibrator Stock Solution B) → 200 mL
CC07	25	0.867	25	91.8	2.5 mL (TST/EST Calibrator Stock Solution B) → 200 mL
CC08	10	0.347	10	36.7	1 mL (TST/EST Calibrator Stock Solution B) → 200 mL
CC09	4	0.139	4	14.7	400 µL (TST/EST Calibrator Stock Solution B) → 200 mL
CC10	1	0.0347	1	3.67	100 µL (TST/EST Calibrator Stock Solution B) → 200 mL
CC11	0.1	0.00347	0.1	0.367	25 µL (TST/EST Calibrator Stock Solution B) → 500 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 (200 mL or 500 mL) using positive displacement pipettes
3. Add Calibrator Storage Solution 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 20% ethanol (at 20°C) to the fill line
5. Aliquot solution 0.6 ml each in 1.5 mL aliquots in appropriately labeled cryovials and store them in the freezer -70 °C. 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.

4.3.2 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 are 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 following internal standard solutions (Table 3) are prepared:

Table 3: Desired Internal Standard Estradiol and testosterone Solution Concentration

IS Stock Solution	Analyte	Concentration ng/dL	Concentration nmol/L	Dilution
IS Stock Solution A	TST	1,000,000	34,315	1 mg → 100 mL
IS Stock Solution A	EST	1,000,000	36,314	1 mg → 100 mL
IS Stock Solution B	TST	40,000	1373	8 mL (TST IS Stock Solution A) → 200 mL
	EST	2,000	72.6	0.4 mL (EST IS Stock Solution A) → 200 mL
IS Work Solution	TST	100	3.43	0.25 mL (IS Stock Solution B) → 100 mL
	EST	5	0.182	

1. Preparation of Testosterone 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 staying at 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 ºC and add ethanol (at 20 ºC) to the fill line
    g. Aliquot 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 Estradiol Internal Standard Stock Solution A
    a. Clean one glass weighing funnel with Ethanol and allow to dry
    b. Remove $^{13}C_3$-labeled Estradiol from refrigerator and allow to stay at 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 Estradiol 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 ºC and add ethanol (at 20 ºC) to the fill line
    g. Aliquot solution in 15 mL aliquots in 6 15-mL pyrex glass tubes, label tubes appropriately and store them in the -70 °C freezer

3. Preparation of Estradiol/Testosterone Internal Standard Stock Solution B
    a. Transfer 8 mL of Testosterone Internal Standard Stock Solution A (at 20ºC) into a 200 mL volumetric flask using a 1-mL positive displacement pipette
    b. Transfer 0.4 mL of Estradiol Internal Standard Stock Solution A (at 20ºC) into the same 200 mL volumetric flask using a 1 mL positive displacement pipette
    c. Add Ethanol to the flask just below the fill line of the volumetric flask
    d. Place flask in the water bath for 15 minutes to reach 20ºC and add ethanol (at 20ºC) to the fill line
    e. Aliquot solution in 1.5-mL aliquots in 66, 1.5-mL cryovials
    f. 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

4. Preparation of Internal Standard Working Solution
    a. Transfer 0.25 mL of Internal Standard Stock Solution B into a 100-mL volumetric flask using a 1-mL positive displacement pipette
    b. Add HPLC grade water 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.

5 PROCEDURE FOR COLLECTING, STORING, AND HANDLING SPECIMENS; CRITERIA FOR SPECIMEN REJECTION

5.1 General Specimen Requirements

For analysis of total testosterone and total estradiol using the measurement procedure, a minimum of 300 µL of fresh or frozen serum is needed. A sample volume of 200 µL is used for analysis. A sample volume of 0.6 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 (24). 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 4ºC. 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)(25).

5.2 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 no notable effect on total testosterone and estradiol concentrations (26) . Studies have shown that storage of serum at -25ºC resulted in negligible changes in total testosterone concentration over 40 years (27), and at -80 ºC total estradiol and total testosterone remained almost the same for a 3-year study period (28).

5.3 Unacceptable Specimens

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

6 PROCEDURE OPERATION INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

To avoid contamination of equipment and consumable with testosterone and estradiol from sweat, gloves needs to be worn at all times when preparing reagents, handling samples and operating equipment.

All instruments are checked before use for correct function using the manufacturer's acceptance criteria.

6.1 Specimen Storage and Handling during Testing

All vials are labeled according to DLS Policies and Procedures Manual. Samples are tracked by scanning the sample ID barcode and handling samples in well plates using defined pipetting schemes during sample preparation and analysis.

Specimens are homogenized and 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 analyses by LC-MS/MS are either stored at 5 ^{o}C in the refrigerator or at 5 °C in the LC-MS/MS instrument sample tray.

6.2 Preparation of Samples for Analysis

Samples processed in one batch are processed together with 4 bench QC samples, 2 reagent blank (saline), and 1 set of calibrators (11 levels). Approximately 75 patient samples are processed in one batch (total number of samples per batch: 96 including 2 reagent blank, 11 calibrators, 8 QCs, 75 samples).

1. Assess all samples for acceptability using the criteria described in section 5.2 and 5.3.
2. Thaw all samples at room temperature: Frozen serum samples, QC samples, Internal Standard Working Solutions and Calibrators are allowed to reach room temperature and are homogenized by placing them on the rotator at medium speed for about 1.5 hrs.
3. Place pipette tips, all patient samples, QC samples and Calibrators 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. When a barcode cannot be read, the instrument software prompts and allows 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 LC-MS/MS instrument and to verify run log sheets.

5. Transfer 200 µL of each Calibrator (CC01-CC11), patient samples, QCs, and blanks into appropriate wells of a 96 2.0-mL deep-well plate (“Sample Plate” or “Plate-A”).
6. Transfer 100 µL of Internal Standard Working Solution to all patient samples, QCs, blanks and calibrators.
7. Cover Sample Plate with ArctiSeal and allow serum and Internal Standard Working Solution to equilibrate using a multivortexer for approximately 45 minutes at room temperature at a setting of 1950 with pulse.
8. Centrifuge the Sample Plate for 3 minutes at room temperature and 2000 rpm.
9. Recap sample and QC vials and store remaining samples and QCs at dedicated location in -70 ˚C freezer.

6.3 Dissociation of Estradiol and Testosterone from Binding Proteins

1. Place Sample Plate (Plate-A), 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 (Plate-A) with ArctiSeal and equilibrate sample solutions using a multivortexer for approximately 30 min at room temperature at a setting of 1950 with pulse.
4. Centrifuge the Sample Plate for 3 minutes at room temperature and 2000 rpm.

6.4 Isolation of Lipids Fraction from Sample and Removal of Phospholipids and Similar Compounds from Lipid Fraction

1. Following step 4 at 6.3, place Sample Plate (Plate-A) in the Hamilton Microlab STARLet Liquid Handler instrument in the designated location.
2. Add 600 µL of Lipid Extraction Solution to all samples on the Sample Plate (Plate-A).
3. Cover Sample Plate (Plate-A) with ArctiSeal and place the well plate on a multivortexer for 5 minutes at a setting of 1950 (no pulse).
4. Centrifuge the Sample Plate (Plate-A) for 5 minutes at 5 ^{o}C and 3700 rpm.
5. While centrifuging the Sample Plate Plate A), place a new 96-2.0 mL deep-well plate ("Lipid Fraction Plate" or "Plate-B") and pipette tips on the Hamilton Microlab STARLet Liquid Handler instrument in the designated locations.
6. Add 200 µL of Deprotonation Buffer to the Lipid Fraction Plate (Plate-B).
7. Transfer Sample Plate (Plate-A) from centrifuge to designated position on the Hamilton Microlab STARLet Liquid Handler instrument.
8. Extract the organic layer (top layer) of the Sample Plate (Plate-A) into Lipid Fraction Plate (Plate-B) using the Hamilton Microlab STARLet Liquid Handler instrument. Keep Sample Plate (Plate-A) for second extraction.
9. Cover Lipid Fraction Plate (Plate-B) with the ArctiSeal and place the well plate on a multivortexer for 5 minutes at a setting of 1950 with pulse.
10. Centrifuge the Lipid Fraction Plate (Plate-B) for 5 minutes at 5 ^{o}C and 3700 rpm.
11. Place Lipid Fraction Plate (Plate-B) from centrifuge to designated position on the Hamilton Microlab STARLet Liquid Handler instrument and extract the organic layer (top layer) of the Lipid Fraction Plate (Plate-B) into a new 96-2.0 mL round-bottom deep-well plate ("Sample Analysis Plate" or "Plate-C") using the Hamilton Microlab STARLet.
12. Place Sample Plate (Plate-A) to designated position on the Hamilton Microlab STARLet Liquid Handler instrument and add 600 µL of Lipid Extraction Solution to all samples on the Sample Plate (Plate-A).
13. Repeat step 3 and 4, and then continue with step 7 through step 12 (second extraction).
14. Evaporate the combined organic layers in the Sample Analysis Plate (Plate-C) to dryness using the Genevac Evaporator at 'Low BP Mix' setting.
15. Place Sample Analysis Plate (Plate-C) to designated position on the Hamilton Microlab STARLet Liquid Handler instrument and add 135 µL of Sample Reconstitution Solution to the samples using the Hamilton Microlab STARLet, vortex thoroughly on a multivortexer for 60 minutes at a setting of 1950 with pulse.

6.5 Analysis of Total Estradiol and Total Testosterone by LC-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 well plate or vials in the autosampler of the LC-MS/MS instrument.

1. Create an analytical run sequence file by importing the file containing the sample barcode information from the Hamilton instrument (section 6.2) to an Excel Worksheet. This template combines the sample ID information with additional information required by DLS policy and CLIA regulation to analyze the samples on the LC-MS/MS system such as sample ID, Sample location on the 96-well plate, and instrument method name. The Excel Worksheet creates the appropriate data file names for the individual sample data.
2. Save the Excel worksheet as a text file, and import it into the LC-MS/MS Instrument software and save the final run sequence file 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. Load the Sample Analysis Plate (Plate C) onto the autosampler on the LC-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.
4. Check the basic instrument functions and settings according to the LC-MS/MS manufacturer's instructions. Ensure that the correct instrument method is loaded and all method parameters are stable.
5. Start the instrument run sequence using Analyst software.
6. Using the Instrument Control Sample, assess the performance of the LC-MS/MS system 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. If instrument malfunction is indicated, abort the sequence and store the samples in the refrigerator until the instrument error is resolved.
7. Upon completion of the LC-MS/MS analysis, apply a new seal on the Sample Analysis Plate, and store the plate in the designated space in the freezer at -70 ^{0}C.

LC-MS/MS parameters:

MS: AB/Sciex API 5500 Triple Quad Mass Spectrometer as described in section 4.1. Instrument settings described in Table 4 and Table 5.

Acquisition mode: SRM
Ionization: ESI in the Positive Ion and Negative Ion Mode

Table 4: Parameters of representative MS conditions

Parameters	Estradiol	Testosterone
Curtain Gas (CUR)	45	45
Collision Gas (CAD)	9	9
IonSpray Voltage (IS)	-4000	4750
Temperature (TEM)	650	650
Ion Source Gas 1 (GS1)	35	35
Ion Source Gas 2 (GS2)	65	65
Entrance Potential (EP)	-12	8
Collision Energy (CE)	-51	27
Collision Cell Exit Potential (CXP)	-17	12
Decluster Potential (DP)	-140	96

Table 5: SRM transitions

Analyte	SRM (m/z)	Transition use	Ion Mode
Estradiol	271>145	Quantitation	Negative
	271>183	Confirmation	Negative
$^{13}C_3$-Estradiol	274>148	Quantitation	Negative
	274>186	Confirmation	Negative
Testosterone	289>97	Quantitation	Positive
	289>109	Confirmation	Positive
$^{13}C_3$-Testosterone	292>100	Quantitation	Positive
	292>112	Confirmation	Positive

LC: Shimadzu LC system as described in section 4.1.

Column: Accucore Phenyl/Hexyl Column
Column Oven: 40 (±2) ºC
Injection volume: 50 µL
Mobile Phase A: 0.2 mM ammonium fluoride in Water:Methanol (80:20 v/v)
Mobile Phase B: Methanol
Flow Rate: 450 µL/min
HPLC Gradient is shown described in Table 6.

Table 6: A representative LC gradient

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	100	0
0.2	100	0
1.5	60	40
8.0	27.5	72.5
8.2	100	0

Representative samples chromatograms are shown in Appendix 3.

6.6 Data Processing

1. Transfer data files generated by the LC-MS/MS system to the dedicated place on the CDC network.
2. Use a dedicated data processing method within the Analyst or Indigo software to identify relevant chromatographic peaks based on their retention times. Integrate the area under the curve. Manual integration maybe required if automatic processing fails to integrate the peaks properly.
3. Document integrated peaks as electronic files (in ".pdf format") and save integration results.
4. Import the integration results text file into an MS Excel template where final results are calculated.
5. Review integrations and integration results by the project lead or a dedicated and specially trained analyst. Correct detected errors. Only consider data that passed this review process for further processing.

6.7 Data Calculations

1. For quality control, calculate area ratios from the quantitation ion and the confirmation ion ("Confirmation Ion Ratio"). Only consider analytes with a Confirmation Ion Ratio ±20% of the target value for further processing.
2. Calculate area ratios for calculating analyte concentration using the analyte and internal standard area counts.
3. Generate calibration curves with the area ratios from the calibrator samples and their assigned values using weighted linear regression.
   Do not process further for sample batches with calibration curves not meeting DLS and laboratory specific quality criteria.
4. Calculate the analyte concentration in serum using the area ratio calculated for the unknown sample and the regression parameters of the corresponding weighted calibration curve.
   Do not use area ratios for analytes outside the established linear range to calculate reportable results. Reanalyze these samples after appropriate dilution or concentration.

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 and calibration is verified 6 months after calibration. 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(29) 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 11 levels of calibration material and a low, mid, and high quality control material covering the clinical range of reported total estradiol and 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 estradiol and total testosterone every 6 months and comparing the results obtained against predefined acceptance limit, for total estradiol, which is ± 8.3 %(30) from target value, and for total testosterone, which is ± 5.3 % (31).

8 METHOD PERFORMANCE CHARACTERISTICS

8.1 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 (32). The reportable range of results for total Estradiol is 0.603-1400 pg/mL or 2.21-5140 pmol/L, and total Testosterone: 0.241-1400 ng/dL or 0.00836-48.5 nmol/L.

8.2 Limit of detection (LOD)

The limit of detection for this method was determined by following the method described in chapter 22.1 from DLS Policies and Procedures Manual. The method is the equivalent of CLSI for including Type II error in estimates of LOD, and requires 60 measurements that should be made in 60 different runs over at least 2 months. After examining the data and excluding the outliers, a mean fit was chosen to calculate the limits of detection. The LOD was calculated to be 2.994 pg/mL or 10.99 pmol/L for total estradiol, and 0.36 ng/dL and 0.241 ng/dL after the method change or 0.012492 nmol/L and 0.0083627 nmol/L respectively for total testosterone.

8.3 Analytical Specificity

Analytical specificity is achieved through:

- A sample preparation that isolates the analytes of interest from other components in the sample matrix.
- Ultra 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 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.
3. By participating the CDC HoSt program in which individual donor sera with reference values are compared against measurement results obtained with this method.

8.4 Accuracy (Trueness and Precision)

Within-day imprecision was determined from 11 replicates of low, medium and high QC samples (Table 7). 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 (34).

Table 7: Within-day and among-day precision

Analyte	Conc.	Among-day Variability (%CV) n=20	Within-day Variability (%CV) n=11
Total Estradiol (pg/mL)	27.8	4.6	4.2
	124	3.2	3.1
	684	3.4	3.1
Total Testosterone (ng/dL)	37.2	2.8	2.8
	178	3.5	2.3
	862	2.9	2.4

The accuracy was verified by analyzing 39 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 (Table 8) showed no or negligible bias between this method and the reference methods.

Table 8: Deming regression and different plot analysis of patient measured values against reference values.

	Total Estradiol		Total Testosterone	
	Intercept (95% CI)	Slope (95% CI)	Intercept (95% CI)	Slope (95% CI)
Deming Regression n=49	-0.16 (-2.19 to 1.87)	0.97 (0.92 to 1.01)	2.92 (-0.14 to 5.97)	0.99 (0.97 to 1.01)
	Bias in Percent (95% CI)		Bias in Percent (95% CI)	
Difference Plot n=49	-2.80% (-5.5% to 0.0%)		0.016 (0.7% to 2.6%)	

The predefined acceptance bias, for total estradiol, is ± 8.3 % (30) from target value, and for total testosterone, ± 5.3 % (31).

8.5 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.

Interfering Substances
No interfering substances were identified.

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

9 QUALITY ASSESSMENT AND PROFICIENCY TESTING

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

9.1 Quality Control Procedures

9.1.1 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.

9.1.2 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.(34)

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.

9.1.3 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.

9.2 Proficiency Testing

Participation in a Proficiency Testing Program such as the one offered by the College of American Pathologists is assured (35).

10 REFERENCE RANGES (NORMAL VALUES)

Population-based reference ranges have not been established yet for total estradiol and total testosterone. Normal ranges suggested in literature (36) (Table 9) for adults are:

Table 9: Literature normal ranges of total estradiol and total serum in serum

Analyte - Sex	Group	Value Range	
		pg/mL	pmol/L
Total Estradiol - Male	Adult	10 - 50	37-184
Total Estradiol - Female	Early follicular phase	20 - 150	73 - 551
	Late follicular phase	40 - 350	147 - 1285
	midcycle peak	150 - 750	551 - 2753
	luteal phase	30 - 450	110 - 1652
	postmenopausal	≤ 20	≤73

Total Testosterone	Group	Value Range	
		ng/dL	nmol/L
Male	Adult	280-1100	9.71-34.7
Female	Adult	15-70	0.52-2.43

11 TEST RESULT REPORTING SYSTEM

Results are reported to 3 significant digits based on assay sensitivity calculations. Data for Total Estradiol and total testosterone are reported in pg/ml and ng/dL, respectively.

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.

12 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.

13 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, and 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.

14 TRANSFER OR REFERRAL OF SPECIMENS

Transfer or referral of specimens will follow the procedures outlined in the most recent version of the DLS Policies and Procedures Manual.

15 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 for the total testosterone:

1. Men <150 ng/dL concern for pituitary and/or hypothalamic tumors(5)
2. Women >200 ng/dL concerns for tumors. (37, 38)

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

16 PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTING INADEQUATELY PREPARED SLIDES

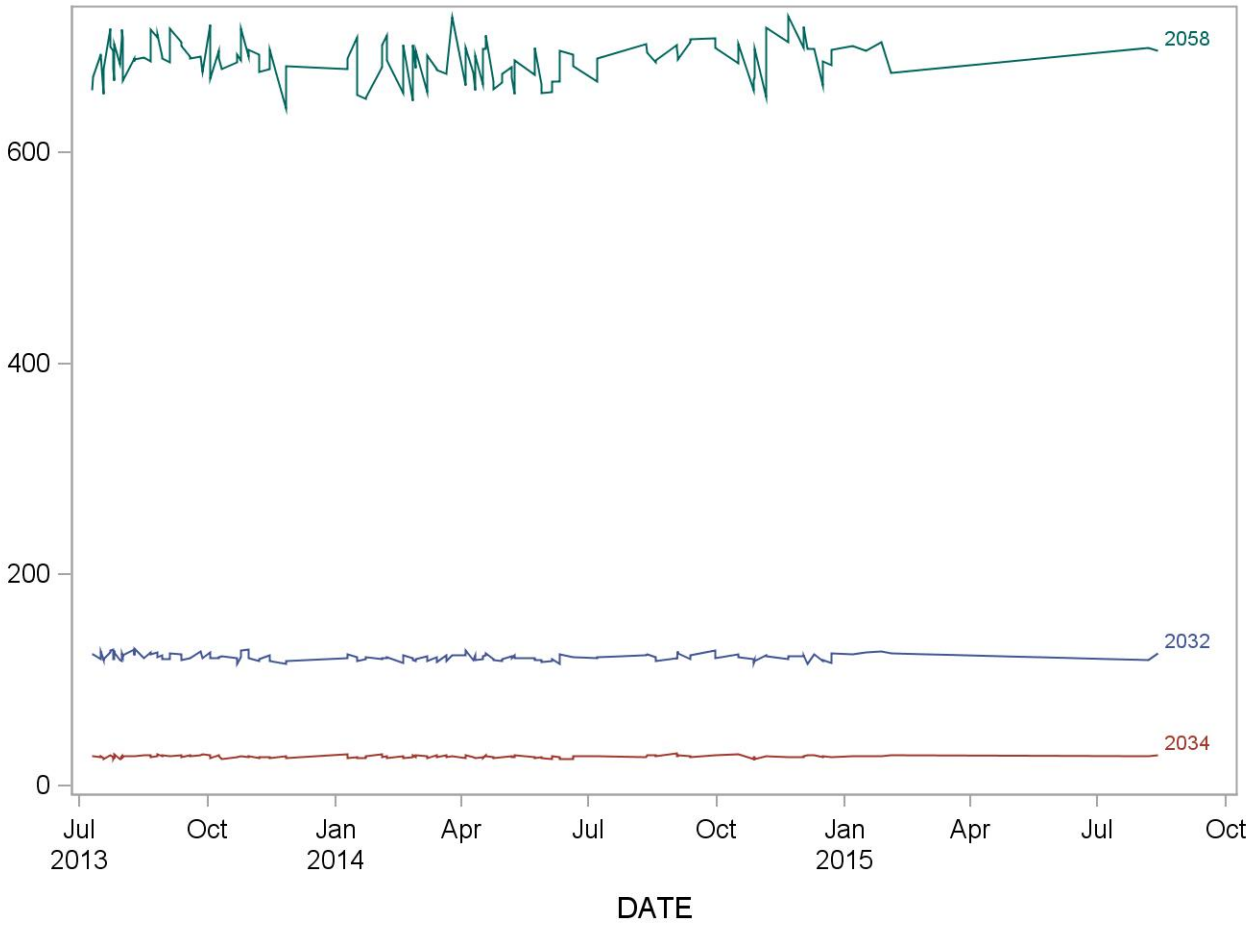
Not applicable for this procedure.

17 SUMMARY STATISTICS AND QC GRAPHS

See following pages.

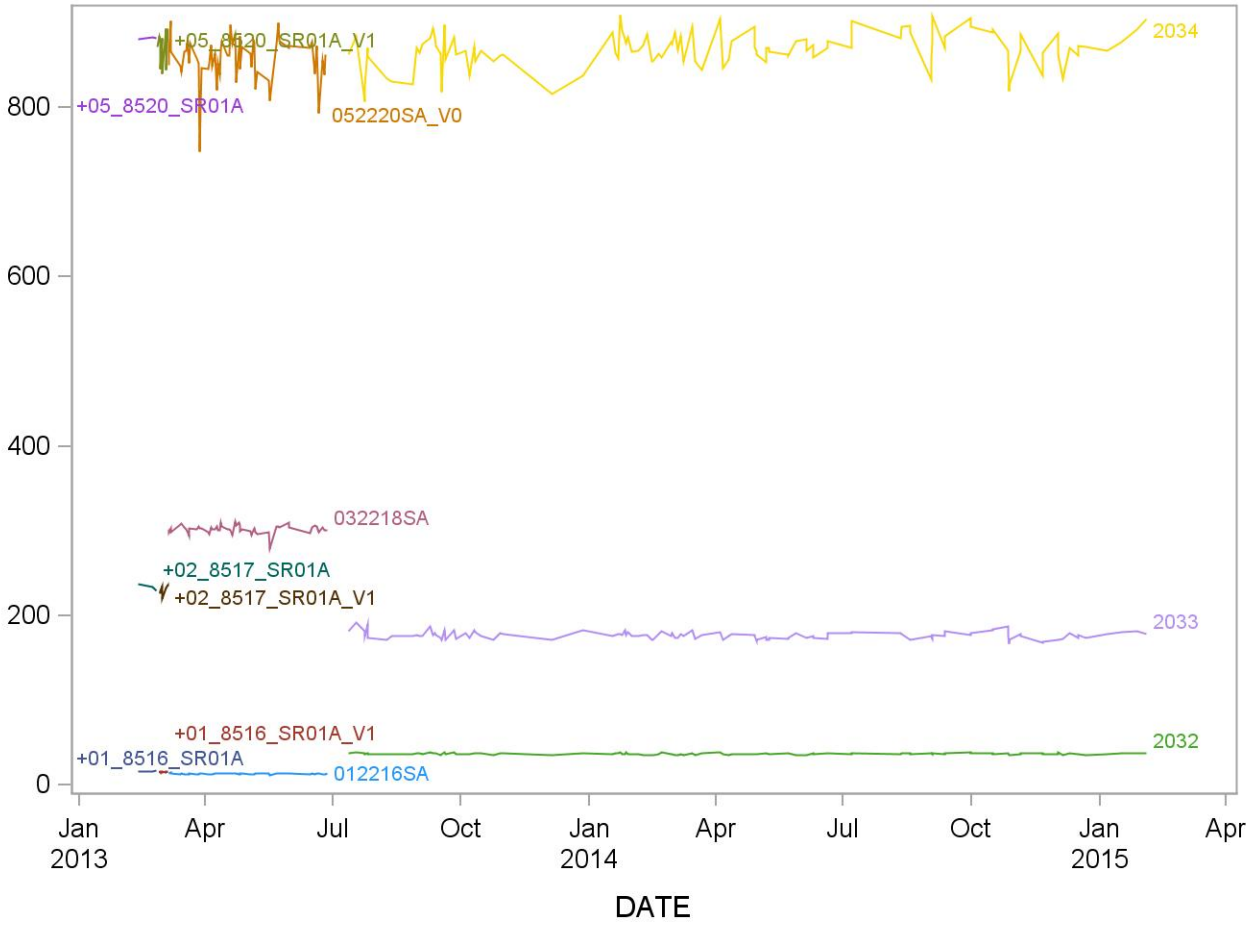
2013-2014 Summary Statistics and QC Chart for Estradiol (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2058	141	10JUL13	13AUG15	687.0106	18.31144	2.7
2034	141	10JUL13	13AUG15	27.94716	1.17455	4.2
2032	141	10JUL13	13AUG15	122.2021	3.29175	2.7



2013-2014 Summary Statistics and QC Chart for Testosterone, total (nmol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
+05_8520_SR01A	3	12FEB13	25FEB13	881.4817	1.20638	0.1
+01_8516_SR01A	3	12FEB13	25FEB13	16.23167	0.34990	2.2
+02_8517_SR01A	3	12FEB13	25FEB13	233.4867	3.83192	1.6
+05_8520_SR01A_V1	9	26FEB13	05MAR13	870.2651	21.26408	2.4
+01_8516_SR01A_V1	9	27FEB13	05MAR13	15.58445	0.33263	2.1
+02_8517_SR01A_V1	9	27FEB13	05MAR13	230.7325	5.15585	2.2
032218SA	57	06MAR13	27JUN13	302.0356	4.97456	1.6
012216SA	57	06MAR13	27JUN13	13.27894	0.46311	3.5
052220SA_V0	57	06MAR13	26JUN13	857.3447	25.86187	3.0
2034	114	12JUL13	03FEB15	868.1404	20.82571	2.4
2032	114	12JUL13	03FEB15	36.84737	0.91774	2.5
2033	114	12JUL13	03FEB15	176.7281	4.39435	2.5



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19 APPENDICES

Appendix 1. Metrological Traceability of Total Estradiol and Total Testosterone Measurements

Appendix 2. Example of Analytical Sequence

Appendix 3. Representative Samples Chromatograms

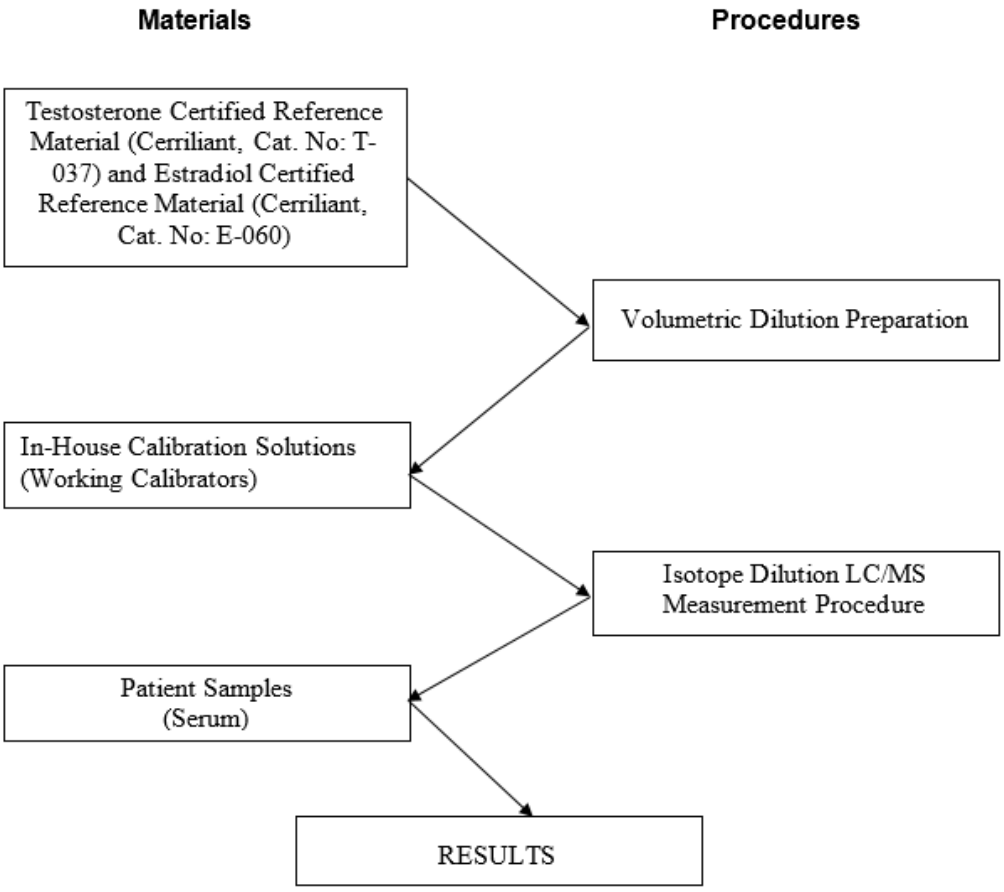
Appendix 4. List of Compounds Tested for Interference

Appendix 5. Related Documents

Appendix 6. Symbols, Abbreviations, Terminology

Appendix 7. Document Compliance Tables

Appendix 1. Metrological Traceability of Total Estradiol and Total Testosterone Measurements

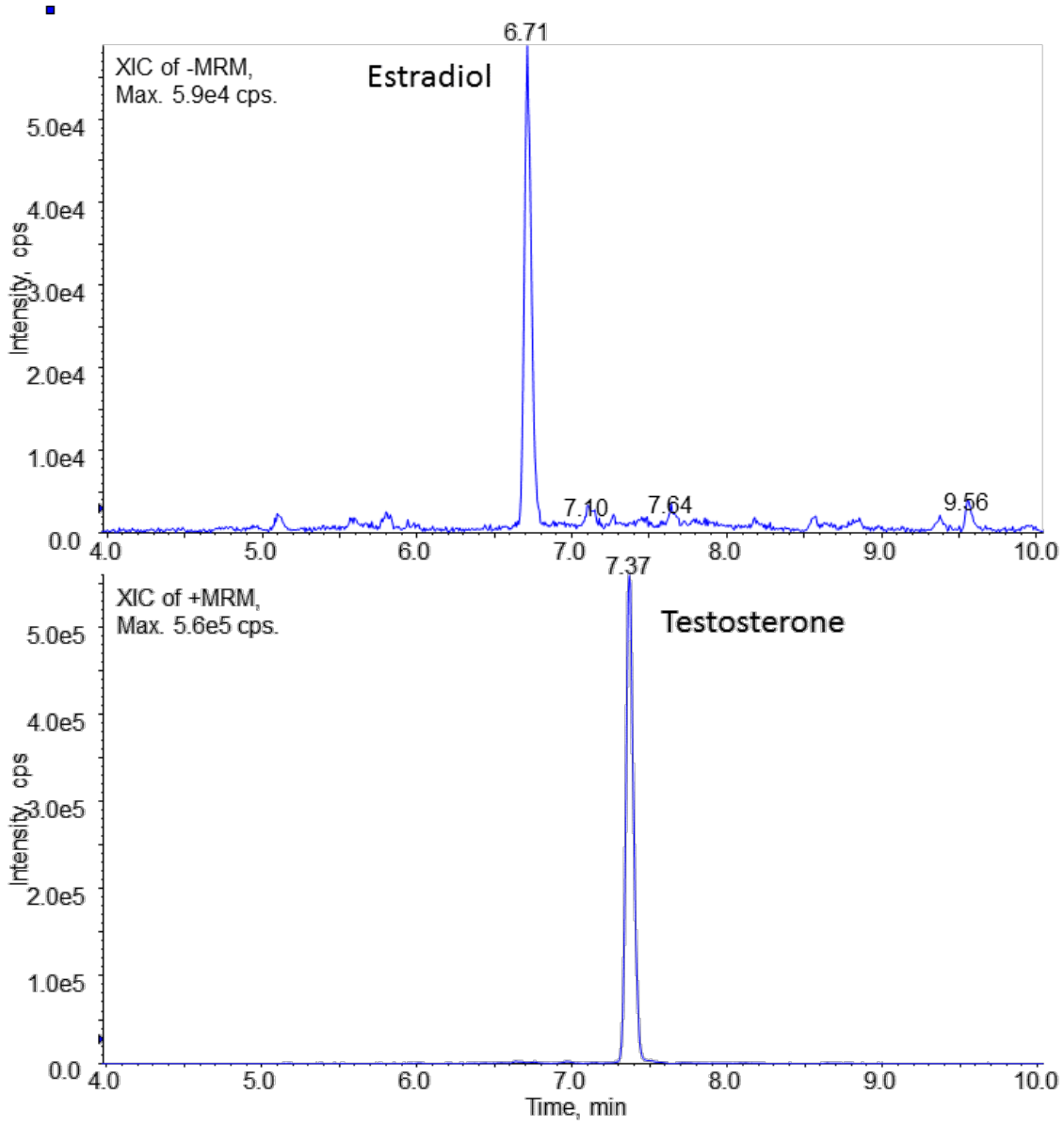


Appendix 2. Example of Analytical Sequence

SampleID	RackCode	PlateCode	VialPos	SmplInjVol	RackPos	OutputFile
BL1	Stk1-03	DW96	1	20	3	20130112_HN34001_001_301
BL2	Stk1-03	DW96	2	20	3	20130112_HN34001_002_302
INST_L1_01	Stk1-03	DW96	85	20	3	20130112_HN34001_003_385
BL3	Stk1-03	DW96	3	20	3	20130112_HN34001_004_303
H22C1102	Stk1-01	DW96	1	20	1	20130112_HN34001_005_101
H22C1002	Stk1-01	DW96	13	20	1	20130112_HN34001_006_113
H22C0902	Stk1-01	DW96	25	20	1	20130112_HN34001_007_125
H22C0802	Stk1-01	DW96	37	20	1	20130112_HN34001_008_137
H22C0702	Stk1-01	DW96	49	20	1	20130112_HN34001_009_149
H22C0602	Stk1-01	DW96	61	20	1	20130112_HN34001_010_161
H22C0502	Stk1-01	DW96	73	20	1	20130112_HN34001_011_173
H22C0402	Stk1-01	DW96	85	20	1	20130112_HN34001_012_185
H22C0302	Stk1-01	DW96	2	20	1	20130112_HN34001_013_102
H22C0202	Stk1-01	DW96	14	20	1	20130112_HN34001_014_114
H22C0102	Stk1-01	DW96	26	20	1	20130112_HN34001_015_126
SP_Water	Stk1-01	DW96	38	20	1	20130112_HN34001_016_138
SP_Saline	Stk1-01	DW96	50	20	1	20130112_HN34001_017_150
BL4	Stk1-03	DW96	4	20	3	20130112_HN34001_018_304
042220SA	Stk1-01	DW96	62	20	1	20130112_HN34001_019_162
062226SA	Stk1-01	DW96	74	20	1	20130112_HN34001_020_174
022222SA	Stk1-01	DW96	86	20	1	20130112_HN34001_021_186
042220SA	Stk1-01	DW96	3	20	1	20130112_HN34001_022_103
042220SA	Stk1-01	DW96	15	20	1	20130112_HN34001_023_115
022222SA	Stk1-01	DW96	27	20	1	20130112_HN34001_024_127
022222SA	Stk1-01	DW96	39	20	1	20130112_HN34001_025_139
062226SA	Stk1-01	DW96	51	20	1	20130112_HN34001_026_151
BL5	Stk1-03	DW96	5	20	3	20130112_HN34001_027_305
012216SA	Stk1-01	DW96	76	20	1	20130112_HN34001_028_176
052220SA	Stk1-01	DW96	88	20	1	20130112_HN34001_029_188
052220SA	Stk1-01	DW96	5	20	1	20130112_HN34001_030_105
032218SA	Stk1-01	DW96	17	20	1	20130112_HN34001_031_117
SRM 971 M	Stk1-01	DW96	29	20	1	20130112_HN34001_032_129
SRM 971 F	Stk1-01	DW96	41	20	1	20130112_HN34001_033_141
ERM DA345	Stk1-01	DW96	53	20	1	20130112_HN34001_034_153
ERM DA346	Stk1-01	DW96	65	20	1	20130112_HN34001_035_165
BL6	Stk1-03	DW96	15	20	3	20130112_HN34001_036_315
INST_L1_02	Stk1-03	DW96	86	20	3	20130112_HN34001_037_386
BL7	Stk1-03	DW96	16	20	3	20130112_HN34001_038_316

Appendix 3. Representative Sample Chromatograms

Representative Sample Chromatogram



Extracted Ion Chromatograms of one pooled serum sample with the estradiol concentration (80.1 pg/ml or 294 pmol/L) and testosterone concentration (50.9 ng/dL or 1.76 nmol/L).

Appendix 4. List of Compounds Tested for Interference

1,4-androstadien-17b-ol-3-one
11-Deoxycortisol
16, (5a)-Androsten-3-one
17a-Ethyinylestradiol
17-alpha methyl testosterone
17α-Hydroxypregnenolone
17α-hydroxyprogesterone
19-Norethindrone
2,(5a)-Androsten-17-one
21-Hydroxyprogesterone
4,16-Androstadien-3b-ol
4,6-androsten 17b-ol-3-one
5,16-Androstadien-3b-ol
5-androsten-3b 17-diol
5-Pregnen-3β-ol-20-one
Androstenediol
Androstenedione
Androsterone
Corticosterone
Cortisone
D(-)-Norgestrel
Dehydroandrosterone
Dehydroepiandrosterone
Dehydroepiandrosterone sulfate
Dihydrotesosterone
Epitestosterone
Estriol
Estrone
Etiocholan-3a-ol-17-one
Hydrocortisone
Pregnenolone
Progesterone

For interference testing, each compound was prepared in a solution of 200 ng/dL 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.
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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

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
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
NCEH	National Center of Environmental Health
$(NH_4)CH_2COO$	Ammonium Acetate
$(NH_4)HCO_3$	Ammonium Bicarbonate
$(NH_4)OH$	Ammonium Hydroxide
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
TST	Total Testosterone
EST	Total Estradiol

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	0

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, 7.1.2
environmental conditions required and any stabilization period needed	4, 6
description of the procedure, including affixing of identification marks, handling, transporting, storing and preparation of items, checks to be made before the work is started, checks that the equipment is working properly and, where required, calibration and adjustment of the equipment before each use, the method of recording the observations and results, 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

Provide section	Section# in this Document
Title page	Title Page
Contents list	List of Content
Foreword	N/A
Warning and safety precautions	0
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