Laboratory Procedure Manual

Analyte: Panel of Steroid Hormones

Matrix: Serum

Method: Steroid Panel Analysis in Serum by ID LC-MS/MS

Method No: 1036

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:

	VARIABLE NAME	SAS LABEL (and SI units)
TST_K_R	LBX17H	17α-hydroxyprogesterone (ng/dL)
	LBD17HSI	17α-hydroxyprogesterone (nmol/L)
	LBXAND	Androstenedione (ng/dL)
	LBDANDSI	Androstenedione (nmol/L)
	LBXDHE	Dehydroepiandrosterone (ug/dL)
	LBDDHESI	Dehydroepiandrosterone (µmol/L)
	LBXEST	Estradiol (pg/mL)
	LBDESTSI	Estradiol (pmol/L)
	LBXESO	Estrone (ng/dL)
	LBDESOSI	Estrone (pmol/L)
	LBXES1	Estrone Sulfate (pg/mL)
	LBDES1SI	Estrone Sulfate (pmol/L)
	LBXPG4	Progesterone (ng/dL)
	LBDPG4SI	Progesterone (nmol/L)
	LBDTST	Testosterone, total (ng/dL)
	LBDTSTSI	Testosterone, total (nmol/L)

Summary of Test Principle and Clinical Relevance

1.1 Intended Use

The measurement procedure described in this document is intended to measure concentrations of the circulating steroid hormones listed in Table 1, *in vitro*, in human serum.

This standard operation procedure addresses all aspects related to the measurement process (specimen collection, storage, processing, analysis and reporting). It was evaluated for measurements in human serum and may not be suitable for other sample matrices such as saliva, plasma, and urine, or specimens from animals. Results obtained with this method may be used to assess levels of these hormones in humans and to define population-based clinical reference ranges. The results obtained with this method are not intended for diagnosis and treatment of patients. This document is not intended to provide information on interpretation of hormone concentrations.

Specific details related to equipment maintenance and operation is provided in the manufacturers' manuals and maintained by the Protein Biomarker Laboratory personel.

Table 1. Analytes measured with this method and their Identification (ID) Codes

Analyte	ID Code
17α-Hydroxyprogesterone	17-OHP
Androstenedione	AD
Progesterone	P4
Testosterone	TT
Estrone	E1
17β-Estradiol	E2
Estrone sulfate	E1S
Dehydroepiandrosterone sulfate	DHEAS

1.2 Clinical and Public Health Relevance

Steroid hormones are polar and non-polar cholesterol-derived compounds that regulate a wide range of metabolic processes, and are essential to physical development, sexual differentiation and maturation of the human body [1]. They are secreted by three steroid glands: the adrenal cortex, testes and ovaries; and during pregnancy by the placenta. Steroid hormones have lipophilic structures, and therefore are not soluble in serum or other body fluids. As a results, the majority of steroids are bound to binding proteins. In blood, the protein bound steroids are in equilibrium with free steroids, which are considered to be biologically "active".

Information about steroid hormones and their metabolites help to improve understanding of the steroidogenic pathway and factors that potentially affect this pathway, and may lead to identifying risks associated with diseases and disorders [2-4].

To correctly and consistently identify concerning levels of these steroid hormones in patients, normal or reference ranges for these analytes are needed. Such normal or reference ranges can be derived from the general population or specific study cohorts. This method is used to measure steroid hormones in these populations.

1.3 Test Principle

This measurement procedure describes the measurement of eight circulating steroid hormones, including some of their conjugates, in human serum.

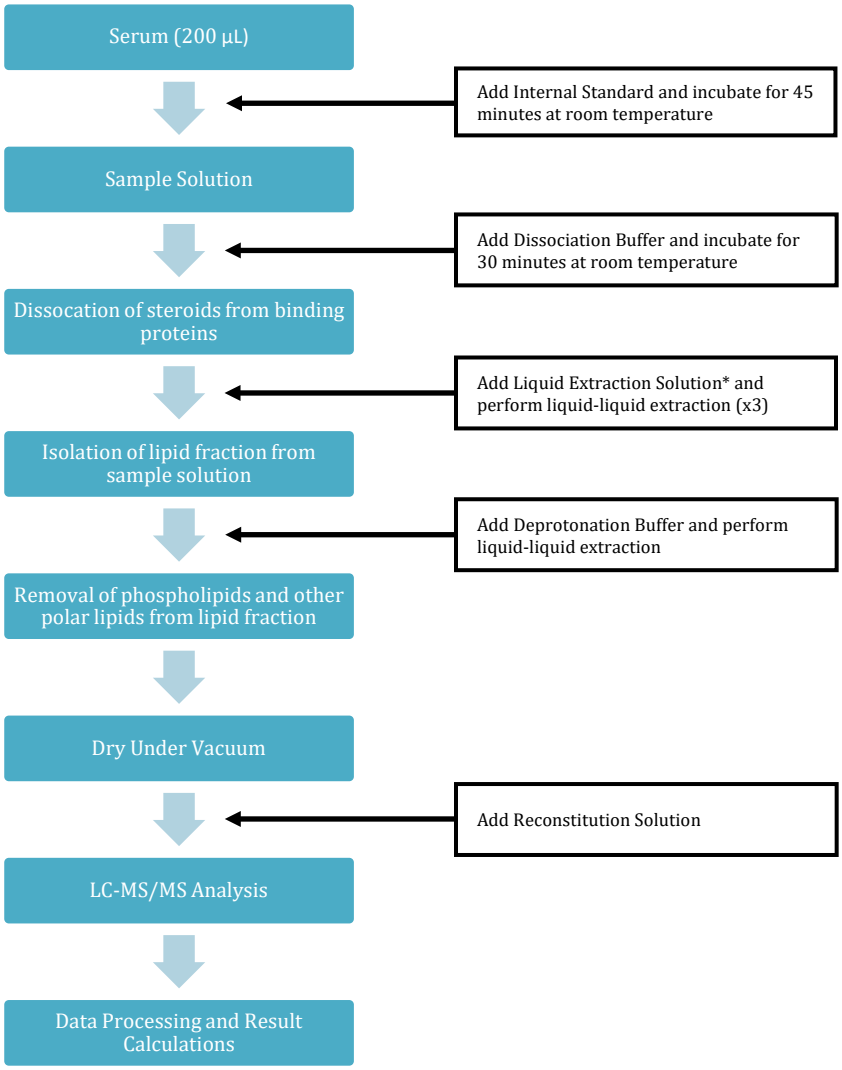
The measurement procedure consists of the following main steps:

1. Dissociation of steroids from binding proteins
2. Isolation of progestogens, androgens, estrogens, conjugated steroids and other lipids by sequential liquid-liquid extraction (LLE)
3. Removal of phospholipids and other interfering lipophilic compounds from the extract by LLE
4. Analysis and quantitation of the analytes by isotope dilution liquid chromatography tandem mass spectrometry (ID LC-MS/MS)

Isolation of the analytes is achieved by sequential LLE using three unique solvents to maximize analyte extraction. ID LC-MS/MS is performed on a triple quadrupole mass spectrometer using electrospray ionization in both positive and negative modes. The analytes are identified based on chromatographic retention time and on specific mass to charge ratio transitions using selected reaction monitoring (SRM). Isotopically labeled internal standards are used for each analyte.

Scheme 1 summarizes the measurement procedure for this serum panel method:

Scheme 1: Measurement Procedure for Steroid Panel in Serum



*_Note_: This method uses a three-step LLE process with different extraction solvents to maximize extraction of all analytes based on polarity. Therefore, the LLE step is performed three times in total.

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.

Ammonium Acetate: Hazardous in case of skin contact (irritant, permeator), of eye contact (irritant), of ingestion, of inhalation. Hygroscopic. Keep lid closed tightly in place when not in use. Store in desiccator to prevent adsorption of moisture.

Ammonium Bicarbonate: Hazardous in case of skin contact (irritant, permeator), of eye contact (irritant), of ingestion, of inhalation. Hygroscopic. Keep lid closed tightly in place when not in use. Store in desiccator to prevent adsorption of moisture

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 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.

Butanol: Flammable liquid. Hazardous in case of skin contact (irritant), very hazardous in case of eye contact (corrosive). Acute toxicity (oral, dermal, inhalation).

Ethanol: Highly flammable liquid and vapor. Causes serious eye irritation.

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.

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.

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.

Methanol: Flammable liquid and vapor. Toxic if swallowed, in contact with skin, or inhaled. May cause respiratory irritation, drowsiness or dizziness. Causes damage to organs through prolonged or repeated exposure. Causes eye irritation. Poison, may be fatal or cause blindness if swallowed.

Safety data sheets (SDS) for these chemicals are readily accessible as hard copies in the laboratory. SDS can be viewed at http://www.ilpi.com/msds/index.html or at http://intranet.cdc.gov/ohs. Laboratory personnel are advised to review the SDS before using chemicals

CAUTION! Glacial Acetic Acid, Hexane, Ethyl Acetate, and Methanol 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 and autoclaved.

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)
- CDC–OHS Hazardous Chemical Waste Management Course
- Security Awareness Training
- Records Management Training

Further, the analyst must have received training on the specific instrumentation and procedures used with this measurement procedure from designated staff or the instrument manufacturer found in the New Analyst Checklist document located on the laboraotry network. At a minimum, the analysts performing this measurement procedure must be familiar with the following:

- Exposure Control Plan
- Chemical Hygiene Plan
- Relevant SDS
- 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 SCIEX Triple Quad Mass Spectrometer (using Analyst 1.6 Software version or higher) and Hamilton Microlab STARlet (using Hamilton VENUS Software version 4.3 or higher). Specific training to operate this software is required to ensure appropriate and safe instrument function, as indicated by the New Analyst Checklist document.

Further, calculations of results obtained with the LC-MS/MS instrument 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 specially 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.

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 steroid hormones from sweat, gloves need 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 0.02 g, (Mettler Toledo, Columbus, OH)
2. Hanna HI5221-01 Laboratory Research Grade Benchtop pH/mV Meter with 0.001 pH Resolution with Hanna HI1131B Refillable Combination pH Electrode with BNC Connector, pH range 0-13, Operating Temperature 0-100^{o}C (Hanna Instruments, Woonsocket, RI)
3. SATO CL612e Thermal Printer and NiceLabel Software (SATO America, Charlotte, NC)
4. 500 mL, 1000 mL, and 2000 mL Glass Beakers (Corning Incorporated, Lowell, MA)
5. 50 mL class A Graduated Cylinder (Pyrex, Cat. No: 3062)
6. 500 mL class A Graduated Cylinder (Kimax, Cat. No. 20036, Kimble, USA)
7. Fisherbrand Octagonal stirring bars, 1 inch length, 0.312 inch diameter (Fisher Scientific, Cat No: 14-513-59, Suwanee, GA)
8. Scholar™ 5 x 5 Inch PC-171 Magnetic Stirrer (Corning Incorporated, Lowell, MA)
9. 1 L Glass Bottles with Screw Tops (Wheaton Industries Inc., Cat. No: 219440, Millville, NJ)
10. 500 mL, 1 L, and 2 L Pyrex Storage Media Bottles (Corning Inc., Corning, NY)
11. Fisherbrand Disposable Borosilicate Glass Pasteur Pipets, 5’ 3/4” (Fisher Scientific, Cat. No: 13-678-20A, Suwanee, GA)
12. Milli-Q Water, Resistivity, 18.1 MΩ·cm at 25°C, 18.2 (Aqua Solutions, Jasper, GA)
13. Ammonium Acetate (Crystalline/Certified ACS), CAS No: 631-61-8 (Fisher Scientific, Cat. No: A637, Suwanee GA)
14. Ammonium Bicarbonate, 99%, for analysis, CAS No: 1066-33-7 (Fisher Scientific, Cat. No: AC393212500, Suwanee, GA)
15. Ammonium Fluoride, CAS No: 12125-01-8 (Fisher Scientific, Cat. No: A665, Suwanee, GA)
16. Butanol, CAS No: 71-36-3 (Fisher Scientific, Cat. No: A399-4, Suwanee, GA)
17. Ethyl Acetate, HPLC grade, CAS No: 141-78-6 (Fisher scientific, Cat. No: E195SK, Suwanee, GA)

18. Hexane, HPLC/ACS grade, CAS No: 110-54-3 (Fisher Scientific, Cat. No: H302, Suwanee, GA)
19. Ammonium Hydroxide 30%, Aqueous, CAS No: 1336-21-6, (Sigma-Aldrich, Cat. No: 320145, St. Louis, MO)
20. Glacial Acetic Acid, Certified ACS grade, CAS No: 64-19-7 (Fisher Scientific, Cat. No: BP2401, Suwanee, GA)
21. Water, Optima grade, CAS No: 7732-18-5 (Fisher Scientific, Cat. No: W5, Suwanee, GA)
22. Methanol, HPLC Grade, CAS: 67-56-1 (Fisher Scientific, Cat. No: A452, Suwannee, GA)

4.1.2 Equipment, Chemicals and Consumables Used for Preparation of Calibration Materials

Formula weights and structures for analytes and internal standards used can be found in Appendix 1.

1. Mettler Toledo AX205 Analytical Balance, Electronic “0.000 g”, Max 220.0 g, d 0.01 mg (Mettler Toledo, Columbus, OH)
2. Water Bath–IsoTemp 3016 Regulator Apparatus (Fisher Scientific, Suwanee, GA)
3. 50 mL, 100 mL, 200 mL, 500 mL, and 1000 mL Pyrex Volumetric Flasks (Kimble Chase Life Science and Research Products LLC, Vineland, NJ)
4. 1 mL, 2 mL, 5 mL, 10 mL, and 20 mL Glass Volumetric Class A Pipette (Fisher Scientific, Suwanee, GA)
5. 10-100 µL Positive Displacement Pipette (Gilson, Inc., Cat. No: F148504, Middleton, WI)
6. 50-250 µL Positive Displacement Pipette (Gilson, Inc., Cat. No: F148505, Middleton, WI)
7. 100-1000 µL Positive Displacement Pipette (Gilson, Inc., Cat. No: F148506, Middleton, WI)
8. 100 µL Microman Tips, (Gilson, Inc., Cat. No: F148414, Middleton, WI)
9. 250 µL Microman Tips, (Gilson, Inc., Cat. No: F148014, Middleton, WI)
10. 1000 µL Microman Tips, (Gilson, Inc., Cat. No: F148560, Middleton, WI)
11. 2 mL Cryogenic Tubes with Screw Cap (Sigma-Aldrich, Cat. No: BR114832, St. Louis, MO)
12. Ethanol, 200 proof, CAS NO: 64-17-5 (Sigma-Aldrich, Cat. No: E7023, St. Louis, MO)
13. 17α-Hydroxyprogesterone (CAS Number: 68-96-2, Cerilliant, Cat. No: H-085, 1.0 mg/mL in methanol)*
14. Androstenedione (CAS Number: 63-05-8, Cerilliant, Cat. No: A-075, 1.0 mg/mL in acetonitrile)*
15. Progesterone (powder) (CAS Number: 57-83-0, National Metrology Institute of Japan, Cat. No: 6003-a)*

16. Testosterone (CAS Number: 58-22-0, Cerilliant, Cat. No: T-037, 1.0 mg/mL in acetonitrile)*
17. Estrone (CAS Number: 53-16-7, Cerilliant, Cat. No: E-075, 1.0 mg/mL in methanol)*
18. 17β-Estradiol (CAS Number: 50-28-2, Cerilliant, Cat. No: E-060, 1.0 mg/mL in acetonitrile)*
19. Estrone 3-sulfate sodium salt unlabeled (powder) (CAS Number: 438-67-5, Cambridge Isotope Laboratories, Cat. No: ULM-8132-0.1MG)*
20. Dehydroepiandrosterone 3-sulfate sodium salt (CAS Number: 1099-87-2, Cerilliant, Cat. No: D-065, 1.0 mg/mL free sulfate in methanol)*
21. 17α-Hydoxyprogesterone-2,3,4-$^{13}C_3$ (CAS Number: 1356154-92-1, Cerilliant, Cat. No: H-100, 100 µg/mL in methanol)*
22. Androstene-3,17-dione-2,3,4-$^{13}C_3$ (CAS Number: 327048-86-2, Cerilliant, Cat. No: A-084, 100 µg/mL in acetonitrile)*
23. Progesterone-2,3,4-$^{13}C_3$ (CAS Number: 327048-87-3, Cambridge Isotope Laboratories, Cat. No: CLM-9162-C, 100 µg/mL in acetonitrile)*
24. Testosterone-2,3,4-$^{13}C_3$ (CAS Number: 327048-83-9, Cerilliant, Cat. No: T-070, 100 µg/mL in acetonitrile)*
25. Estrone-2,3,4-$^{13}C_3$ (CAS Number: 1241684-29-6, Cerilliant, Cat. No: E-108, 100 µg/mL in methanol)*
26. 17β-Estradiol-2,3,4-$^{13}C_3$ (CAS Number: 1261254-48-1, Cerilliant, Cat. No: E-073, 100 µg/mL in acetonitrile)*
27. Estrone-13,14,15,16,17,18-$^{13}C_6$ 3-sulfate sodium (CAS Number: N/A, Cambridge Isotope Laboratories, Cat. No: CLM-8018-0.1MG)*
28. Dehydroepiandrosterone-2,2,3,4,4-d_5 3-sulfate sodium salt (CAS Number: N/A, Cerilliant, Cat. No: D-066, 100 µg/mL free sulfate in methanol)*

*All materials are tested for identity and purity, and findings are compared to certificates of analysis provided by the manufacturer

4.1.3 Equipment, Chemicals and Consumables Used for Sample Processing

1. Eppendorf Centrifuge 5810R with Eppendorf Swing-bucket Rotor (Eppendorf, Ramsey, MN)
2. Hamilton Microlab STARlet Liquid Handler with 8-chanel and 96-chanel pipettors (using Hamilton VENUS software version 4.3.5 (Hamilton Company, Reno, NV))
3. Glas-Col MultiPulse Vortexer (Glas-Col, Terre Haute, IN)
4. Fisher Digital Multi-tube Vortex Mixer (Fisher Scientific, Cat. No: 02-215-452, Suwanee, GA).
5. Eppendorf Repeater Plus Pipetter (Eppendorf, Cat. No: 022260201, Ramsey, MN)
6. Eppendorf Combitips plus Pipet tips, 5 mL (Eppendorf, Cat. No: 022266403, Ramsey, MN)
7. SATO CL612e Thermal Printer and NiceLabel Software (Sato America, Charlotte, NC)

8. 100-250 µL Positive Displacement Pipette (Gilson, Inc., Cat. No: F148505, Middleton, WI)
9. 250 µL Microman Tips, (Gilson, Inc., Cat. No: F148014, Middleton, WI)
10. Co-RE Tips, Standard Volume Tips (300 µL) with Filters (Hamilton Company, Cat. No: 235903, Reno, NV)
11. 96-well, 2 mL Square Well Plates (Seahorse Labware, Cat. No: S30009, Chicopee, MA)
12. 96-well, 2 mL Round-bottom Well Plates (Microliter Analytical Supplies INC, Product No: 07-8000, Suwanee, GA)
13. Robotic Reservoirs, Convoluted Bottom (Thermo Scientific, Cat. No: 1200-2300, Waltham, MA)
14. ArctiSeal 96-Well Square Silicone w/ PTFE Spray Coating (Arctic White LLC, Cat. No: AWSM-1003SX, Bethlehem, PA)
15. GeneVac EZ-2.3 Elite Evaporation System with Side Bridge Holders and Universal Rotor (GeneVac Inc., Valley Cottage, NY)
16. Orbitron Rotator II, Model 26250, (Boekel Scientific, Feasterville, PA)

4.1.4 Equipment, Chemicals and Consumables Used for Sample Measurement

1. SCIEX API 6500 Triple Quad Mass Spectrometer with ESI source (Foster City, CA)
2. Shimadzu Prominence CTO-20AC Column Oven (Columbia, MD)
3. 4 Shimadzu Nexera LC-30AD LC Pumps (Columbia, MD)
4. Shimadzu Nexera SIL-30AC MP Autosampler (Columbia, MD)
5. Shimadzu DGU-20A5R Degasser (Columbia, MD)
6. Shimadzu CBM-20A Communication Bus Module (Columbia, MD)
7. Shimadzu Prominence LC-20AD LC Pump (Columbia, MD)
8. 2 Thermo Fisher Scientific Accucore Phenyl-Hexyl Column, 150 **x** 3.0 mm, 2.6 µm particle size (Cat. No: 17926-153030, Waltham, MA)
9. 2 Phenomenex SecuriGuard Guard Cartridge Kit (Cat. No: KJ0-4282, Torrance, CA)
10. 2 Phenomenex SecuriGuard Standard C18 Cartridges, 4 x 2.0 mm, (Cat. No: AJ0-4286, Torrance, CA)
11. BioChromato RAPID Slit Seal for 96 Well Plates (Cat. No: RSS-S96-80122, Kanagawa-ken, Japan)

4.2 Preparation of Reagents Used For Sample Preparation

4.2.1 Dissociation Buffer

This is a solution of 0.5 M ammonium acetate at pH 5.5 used to dissociate steroid hormones from binding globulins as described in Section 6.3.
Preparation of 1 L of Dissociation Buffer which is sufficient for a maximum of 1200 serum samples. If more samples are to be processed volumes can be adjusted accordingly.

1. Add 800 mL of DI water to a 1 L beaker using a 500 mL graduated cylinder
2. Weigh 38.56 g ammonium acetate and transfer into the same 1 L beaker

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 Glacial Acetic Acid using a glass disposable pipette
6. Add DI water to adjust the total volume to 1 L
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 as needed and verify its pH prior to use each day.

4.2.2 Liquid Extraction Solution

The Liquid Extraction Solution is used to separate non-polar serum components ("lipid fraction") from polar serum components as described in Section 6.4. This method utilizes a three-step liquid extraction process using three different extraction solvents to maximize extraction efficiency based on analyte affinity.

Liquid Extraction Solution (A) is 100% ethyl acetate.
Liquid Extraction Solution (B) is a 70:30 (v/v) ethyl acetate:hexane mixture, described below.
Liquid Extraction Soultion (C) is 100% butanol.

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

1. Using a graduated cylinder, transfer 700 mL of ethyl acetate in a 1 L bottle with screw top
2. Using a graduated cylinder, transfer 300 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, step 7.

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 M ammonium bicarbonate at pH 8.0 used to deprotonate phospholipids and other similar compounds prior to extraction as described in Section 6.4.

Preparation of 1 L of Deprotonation Buffer is sufficient for 1200 samples. If more samples are to be processed, volumes need be adjusted accordingly.

1. Add 800 mL of DI water to a 1 L beaker using a 500 mL graduated cylinder
2. Weigh out 15.4 g ammonium carbonate and transfer into the same 1 L beaker
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 30% ammonium hydroxide using a glass disposable pipette
6. Add DI water to adjust the total volume to 1 L

7. Transfer to a glass bottle with plastic screw cap and appropriately label the bottle as described in 4.2.1, step 7

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

4.2.4 Sample Reconstitution Solution

This solution is a mixture of water, methanol, and ethanol (75/20/5 v/v/v) used to reconstitute samples prior to injection on LC-MS/MS as described in Section 6.5.

1. Using a graduated cylinder, measure out 750 mL of water and transfer to a 1 L glass bottle with screw top
2. Using a graduated cylinder, measure out 200 mL of methanol and transfer to the same 1 L bottle
3. Using a graduated cylinder, measure out 50 mL of ethanol and transfer to the same 1 L bottle
4. Close glass bottle and mix thoroughly
5. Appropriately label the bottle as described in 4.2.1, step 7

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 4.3.1.

3 L 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, step 7

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, methanol, and ethanol (75/20/5 v/v/v) used as LC Mobile Phase A. LC Mobile Phase B used in this procedure is methanol.

1. Using a graduated cylinder, measure transfer 750 mL of water to a 1 L bottle with screw top
2. Using a graduated cylinder, transfer 200 mL of methanol to the same 1 L bottle
3. Using a graduated cylinder, transfer 50 mL of ethanol to the same 1 L bottle
4. Weigh 7.4 mg Ammonium Fluoride and transfer to the 1 L bottle

5. Close the glass bottle and mix thoroughly
6. Appropriately label the bottle as described in 4.2.1, step 7

Prepare a fresh batch of this solution as needed. Discard solution after 1 month.

4.3 Calibration Materials

Note: Some of the steroids in powder form are controlled substances and handling of such materials must comply with DEA regulations and CDC policies for use of controlled substances. Use of such materials require approval and oversight by the designated custodian.

4.3.1 Preparation of Calibrator Stock Solutions and Working Standard Solutions

The Calibrator Working Solutions are prepared from Calibrator Stock Solutions which are prepared from certified or commercial solutions with an assigned concentration (see Section 4.1.2). If standards with different concentrations are used, the preparation procedures need to be adjusted accordingly. This procedure yields 200 mL of each calibrator level, equating to approximately 330 vials, and is sufficient for the analyses of approximate 23,000 samples, assuming use of 1 vial per sample batch.

I. The following Calibrator Stock A solutions (Table 2) were prepared:

Table 2. Desired Steroid Hormone Calibrator Stock Solution A Concentrations (in *µg/mL* and *nmol/L*)

Analyte	Concentration*		Dilution Scheme
	µg/mL	*nmol/L*	
17-OHP	0.786	2,377	160 µL (certified solution) → 200 mL
AD	0.786	2,776	160 µL (certified solution) → 200 mL
P4	3.06	9,726	3.0 mg (certified standard) → 1000 mL
TT	0.968	3,358	1.0 mL (certified solution) → 100 mL
E1	0.993	3,673	200 µL (certified solution) → 200 ml
E2	0.656	2,409	5.4 mL (certified solution) → 50 mL
E1S	1.98	5,650	0.1 mg (certified standard) → 50 mL
DHEAS	189	512,400	10.0 mL (certified solution) → 50 mL

**Note*: Concentration calculations include adjustments based on purity of the certified standards used to prepare the calibrators. Detailed information on each certified standard/solution can be found in their respective Certificates of Analysis.

1. Preparation of 17-Hydroxyprogesterone (17-OHP) Calibrator Stock Solution A
   a. Add 40 mL of ethanol to a 200 mL volumetric flask

b. Transfer 160 µL of a 1 mg/mL certified 17-OHP solution into the volumetric flask using a calibrated positive displacement pipette
c. Add ethanol to just below the fill line of the volumetric flask
d. Place flask in a water bath for 15 minutes to reach 20°C and add ethanol (at 20°C) to the fill line
e. Label the volumetric flask appropriately with content, concentration, preparation date, analyst initials, safety precautions and hazard information, and store in the refrigerator

2. Preparation of Androstenedione (AD) Calibrator Stock Solution A
   a. Add 40 mL of ethanol to a 200 mL volumetric flask
   b. Transfer 160 µL of a 1 mg/mL certified AD solution into the volumetric flask using a calibrated positive displacement pipette
   c. Add ethanol to just below the fill line of the volumetric flask
   d. Place flask in a water bath for 15 minutes to reach 20°C and add ethanol (at 20°C) to the fill line
   e. Label the volumetric flask appropriately with content, concentration, preparation date, analyst initials, safety precautions and hazard information, and store in the refrigerator

3. Preparation of Progesterone (P4) Calibrator Stock Solution A
   a. Add 200 mL of ethanol to a 1000 mL volumetric flask
   b. Measure and transfer 3.0 mg of certified P4 standard into the volumetric flask using a clean and dried weighing boat
   c. Add ethanol to just below the fill line of the volumetric flask
   d. Place flask in a water bath for 15 minutes to reach 20°C and add ethanol (at 20°C) to the fill line
   e. Label the volumetric flask appropriately with content, concentration, preparation date, analyst initials, safety precautions and hazard information, and store in the refrigerator

4. Preparation of Testosterone (TT) Calibrator Stock Solution A
   a. Add 20 mL of ethanol to a 100 mL volumetric flask
   b. Transfer 1.0 mL of a certified TT solution into the volumetric flask using a calibrated positive displacement pipette
   c. Add ethanol to just below the fill line of the volumetric flask
   d. Place flask in a water bath for 15 minutes to reach 20°C and add ethanol (at 20°C) to the fill line
   e. Label the volumetric flask appropriately with content, concentration, preparation date, analyst initials, safety precautions and hazard information, and store in the refrigerator

5. Preparation of Estrone (E1) Calibrator Stock Solution A
   a. Add 40 mL of ethanol to a 200 mL volumetric flask
   b. Transfer 200 µL of a 1 mg/mL certified E1 solution into the volumetric flask using a calibrated positive displacement pipette
   c. Add ethanol to just below the fill line of the volumetric flask
   d. Place flask in a water bath for 15 minutes to reach 20°C and add ethanol (at 20°C) to the fill line

e. Label the volumetric flask appropriately with content, concentration, preparation date, analyst initials, safety precautions and hazard information, and store in the refrigerator

6. Preparation of Estradiol (E2) Calibrator Stock Solution A
   a. Add 10 mL of ethanol to a 50 mL volumetric flask
   b. Transfer 5.4 mL of a certified E2 solution into the volumetric flask using an appropriate volumetric glass pipettes and calibrated positive displacement pipette
   c. Add ethanol to just below the fill line of the volumetric flask
   d. Place flask in a water bath for 15 minutes to reach 20°C and add ethanol (at 20°C) to the fill line
   e. Label the volumetric flask appropriately with content, concentration, preparation date, analyst initials, safety precautions and hazard information, and store in the refrigerator

7. Preparation of Estrone sulfate (E1S) Calibrator Stock Solution A
   a. Add 10 mL of ethanol to a 50 mL volumetric flask
   b. Measure and transfer 0.1 mg of certified E1S standard into the volumetric flask using a clean and dried weighing boat
   c. Add ethanol to just below the fill line of the volumetric flask
   d. Place flask in a water bath for 15 minutes to reach 20°C and add ethanol (at 20°C) to the fill line
   e. Label the volumetric flask appropriately with content, concentration, preparation date, analyst initials, safety precautions and hazard information, and store in the refrigerator

8. Preparation of Dehydroepiandrosterone sulfate (DHEAS) Calibrator Stock Solution A
   a. Add 10 mL of ethanol to a 50 mL volumetric flask
   b. Transfer 10 mL of a 1 mg/mL certified DHEAS solution into the volumetric flask using a volumetric glass pipette
   c. Add ethanol to just below the fill line of the volumetric flask
   d. Place flask in a water bath for 15 minutes to reach 20°C and add ethanol (at 20°C) to the fill line
   e. Label the volumetric flask appropriately with content, concentration, preparation date, analyst initials, safety precautions and hazard information, and store in the refrigerator

Note: To minimize evaporation loss of ethanol and changes to concentrations, seal volumetric flasks tightly with Parafilm, where possible, and avoid reusing stock solutions long-term. This solution is stable for 6 months.

II. Using the Calibrator Stock A solutions, the following Calibrator Stock Solution B (Table 3) and Calibrator Stock Solution B' (Table 4) are prepared:

Table 3. Calibrator Stock Solution B Concentrations (in *µg/mL* and *nmol/L*)

[redacted]	**Analyte**	**Concentration**		**Dilution Scheme**
		µg/mL	*nmol/L*	

Stock Solution B	17-OHP	0.00393	11.89	5 mL (17-OHP Calibrator Stock Solution A)	→ 1000 mL
	AD	0.00477	16.66	6 mL (AD Calibrator Stock Solution A)	
	P4	0.0245	77.81	8 mL (P4 Calibrator Stock Solution A)	
	TT	0.0116	40.30	12 mL (TT Calibrator Stock Solution A)	
	E1	0.000497	1.837	0.5 mL (E1 Calibrator Stock Solution A)	
	E2	0.00131	4.818	2 mL (E2 Calibrator Stock Solution A)	
	E1S	0.00198	5.650	1 mL (E1S Calibrator Stock Solution A)	
	DHEAS	3.78	10,250	20 mL (DHEAS Calibrator Stock Solution A)	

1. Preparation of Steroid Hormone Calibrator Stock Solution B
    a. Add 200 mL of ethanol to a 1000 mL volumetric flask
    b. Transfer 5 mL of 17-OHP Calibrator Stock Solution A into the volumetric flask using a volumetric glass pipette
    c. Transfer 6 mL of AD Calibrator Stock Solution A into the volumetric flask using the appropriate volumetric glass pipettes and/or calibrated positive displacement pipettes
    d. Transfer 8 mL of P4 Calibrator Stock Solution A into the volumetric flask using the appropriate volumetric glass pipettes and/or calibrated positive displacement pipettes
    e. Transfer 12 mL of TT Calibrator Stock Solution A into the volumetric flask using the appropriate volumetric glass pipettes and/or calibrated positive displacement pipettes
    f. Transfer 0.5 mL of E1 Calibrator Stock Solution A into the volumetric flask using a calibrated positive displacement pipette
    g. Transfer 2 mL of E2 Calibrator Stock Solution A into the volumetric flask using a volumetric glass pipette
    h. Transfer 1 mL of E1S Calibrator Stock Solution A into the volumetric flask using a volumetric glass pipette
    i. Transfer 20 mL of DHEAS Calibrator Stock Solution A into the volumetric flask using a volumetric glass pipette
    j. Add ethanol to just below the fill line of the volumetric flask
    k. Place flask in a water bath for 15 minutes to reach 20°C and add ethanol (at 20°C) to the fill line
    l. Label the volumetric flask appropriately with content, concentration, preparation date, analyst initials, safety precautions and hazard information, and store in the refrigerator

Table 4. Calibrator Stock Solution B' Concentrations (in *µg/mL* and *nmol/L*)

[redacted]	**Analyte**	**Concentration**		**Dilution Scheme**	
		µg/mL	*nmol/L*		
	17-OHP	0.00786	23.77	5 mL (17-OHP Calibrator Stock Solution A)	→ 500 mL

Stock Solution B	AD	0.00954	33.32	6 mL (AD Calibrator Stock Solution A)
	P4	0.0490	155.6	8 mL (P4 Calibrator Stock Solution A)
	TT	0.0232	80.60	12 mL (TT Calibrator Stock Solution A)
	E1	0.000993	3.674	0.5 mL (E1 Calibrator Stock Solution A)
	E2	0.00263	9.636	2 mL (E2 Calibrator Stock Solution A)
	E1S	0.00396	11.30	1 mL (E1S Calibrator Stock Solution A)
	DHEAS	7.55	20,500	20 mL (DHEAS Calibrator Stock Solution A)

2. Preparation of Steroid Hormone Calibrator Stock Solution B'
    a. Add 100 mL of ethanol to a 500 mL volumetric flask
    b. Repeat steps *b* to *l* of 1. Preparation of Steroid Hormone Calibrator Stock Solution B, but using the new 500 mL volumetric flask instead

Note: To minimize evaporation loss of ethanol and changes to concentrations, seal volumetric flasks tightly with Parafilm, where possible, and avoid reusing stock solutions long-term. This solution is stable for 6 months.

III. Calibrator Working Solutions (Table 5) are prepared using Calibrator Stock Solution B and Calibrator Stock Solution B' (for higher concentration samples). The following levels of Calibrator Working Solutions are used:

Table 5. Calibrator Working Solutions and Their Concentrations (in *ng/dL*, *pg/mL*, or *µg/dL*)

Calibrator Working Solution Code	Concentrations								Dilution Scheme
	17-OHP (ng/dL)	*AD (ng/dL)*	*P4 (ng/dL)*	*TT (ng/dL)*	*E1 (ng/dL)*	*E2 (pg/mL)*	*E1S (pg/mL)*	*DHEAS (µg/dL)*	
CC01	0.197	0.239	1.22	0.581	0.0248	0.656	0.990	0.189	100 µL (Stock Solution B) → 200 mL
CC02	0.786	0.954	4.90	2.32	0.0993	2.63	3.96	0.755	400 µL (Stock Solution B) → 200 mL
CC03	2.95	3.58	18.4	8.72	0.372	9.84	14.9	2.83	1.5 mL (Stock Solution B) → 200 mL
CC04	9.83	11.9	61.2	29.1	1.24	32.8	49.5	9.44	5 mL (Stock Solution B) → 200 mL
CC05	19.7	23.9	122	58.1	2.48	65.6	99.0	18.9	10 mL (Stock Solution B) → 200 mL
CC06	49.1	59.6	306	145	6.21	164	248	47.2	25 mL (Stock Solution B) → 200 mL
CC07	147	179	918	436	18.6	492	743	142	75 mL (Stock Solution B) → 200 mL
CC08	197	239	1220	581	24.8	656	990	189	100 mL (Stock Solution B) → 200 mL
CC09	255	310	1590	755	32.3	853	1290	245	65 mL (Stock Solution B') → 200 mL
CC10	314	382	1960	930	39.7	1050	1580	302	80 mL (Stock Solution B') → 200 mL
CC11	393	477	2450	1160	49.7	1310	1980	378	100 mL (Stock Solution B') → 200 mL

Table 6. Calibrator Working Solutions and Their Concentrations (in *nmol/L*)

Calibrator Working Solution Code	Concentrations (*nmol/L*)								Dilution Scheme
	17-OHP	*AD*	*P4*	*TT*	*E1*	*E2*	*E1S*	*DHEAS*	
CC01	0.00595	0.00833	0.0389	0.0201	0.000918	0.00241	0.00283	5.12	100 µL (Stock Solution B) → 200 mL
CC02	0.0238	0.0333	0.156	0.0806	0.00367	0.00964	0.0113	20.5	400 µL (Stock Solution B) → 200 mL
CC03	0.0892	0.125	0.584	0.302	0.0138	0.0361	0.0424	76.9	1.5 mL (Stock Solution B) → 200 mL
CC04	0.297	0.416	1.95	1.01	0.0459	0.120	0.141	256	5 mL (Stock Solution B) → 200 mL
CC05	0.595	0.833	3.89	2.01	0.0918	0.241	0.283	512	10 mL (Stock Solution B) → 200 mL
CC06	1.49	2.08	9.73	5.04	0.230	0.602	0.706	1280	25 mL (Stock Solution B) → 200 mL
CC07	4.46	6.25	29.2	15.1	0.689	1.81	2.12	3840	75 mL (Stock Solution B) → 200 mL
CC08	5.95	8.33	38.9	20.1	0.918	2.41	2.83	5120	100 mL (Stock Solution B) → 200 mL
CC09	7.73	10.8	50.6	26.2	1.19	3.13	3.67	6660	65 mL (Stock Solution B') → 200 mL
CC10	9.51	13.3	62.3	32.2	1.47	3.85	4.52	8200	80 mL (Stock Solution B') → 200 mL
CC11	11.9	16.7	77.8	40.3	1.84	4.82	5.65	10200	100 mL (Stock Solution B') → 200 mL

1. Preparation of Steroid Hormone Calibrator Working Solutions
    a. Place Calibrator Stock Solution B and B' in a water bath and allow temperature to reach 20°C
    b. To eleven 200 mL volumetric flasks, add 40 mL of Calibrator Storage Solution
    c. Transfer the volumes of Calibrator Stock Solution B or B' to separate volumetric flasks using the appropriate volumetric glass pipettes and/or calibrated positive displacement pipettes
    d. Add Calibrator Storage Solution to just below the fill line of the volumetric flasks
    e. Place each flask in a water bath for 15 minutes to reach 20°C and add Calibrator Storage Solution (at 20°C) to the fill line
    f. Aliquot each Calibrator Working Solution flask into 0.6 mL aliquots in appropriately labeled 2 mL cryovials, and store at -70°C

Note: These aliquot vials are for single use only. Do not reuse these vials as contents may evaporate and result in changes in 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 B, which is prepared from pure, certified or commercial solutions (see Section 4.1.2). If standards with different concentrations are used, the preparation procedures need to be adjusted accordingly. For this procedure, the following Internal Standard Stock Solution B is prepared (Table 7):

Table 7. Internal Standard Stock Solution B Concentrations (in *µg/mL* and *nmol/L*)

	Analyte	Concentration		Dilution Scheme	
		µg/mL	*nmol/L*		
IS Stock Solution B	17-OHP	0.150	450	300 µL (17-OHP-$^{13}C_3$ certified solution)	→ 200 mL
	AD	0.200	691	400 µL (AD-$^{13}C_3$ certified solution)	
	P4	1.00	3,150	1.00 mL (P4-$^{13}C_3$ IS Stock Solution A)	
	TT	0.800	2,930	1.60 mL (TT-$^{13}C_3$ certified solution)	
	E1	0.100	366	200 µL (E1-$^{13}C_3$ certified solution)	
	E2	0.250	908	500 µL (E2-$^{13}C_3$ certified solution)	
	E1S	0.800	2,260	2.00 mL (E1S-$^{13}C_6$ IS Stock Solution A)	
	DHEAS	7.50	20,100	15 mL (DHEAS-d_5 certified solution)	

1. Preparation of Steroid Hormone Internal Standard Stock Solution B
    a. Add 40 mL of ethanol to a 200 mL volumetric flask
    b. Transfer 300 µL of a 100 µg/mL certified 17-OHP-$^{13}C_3$ solution into the volumetric flask using a calibrated positive displacement pipette
    c. Transfer 400 µL of a 100 µg/mL certified AD-$^{13}C_3$ solution into the volumetric flask using a calibrated positive displacement pipette
    d. Transfer 1.00 mL of a 200 µg/mL P4 IS Stock Solution A into the volumetric flask using a calibrated positive displacement pipette
        i. P4 IS Stock Stock Solution A (concentration: 2 µg/mL) is prepared by dissolving 2 mg of certified P4-$^{13}C_3$ standard into a 10 mL volumetric flask using ethanol
        ii. Label the volumetric flask appropriately with content, concentration, preparation date, analyst initials, safety precautions and hazard information, and store in the refrigerator
    e. Transfer 1.60 mL of a 100 µg/mL certified TT-$^{13}C_3$ solution into the volumetric flask using a calibrated positive displacement pipette
    f. Transfer 200 µL of a 100 µg/mL certified E1-$^{13}C_3$ solution into the volumetric flask using a calibrated positive displacement pipette
    g. Transfer 500 µL of a 100 µg/mL certified E2-$^{13}C_3$ solution into the volumetric flask using a calibrated positive displacement pipette
    h. Transfer 2.00 mL of E1S IS Stock Solution A into the volumetric flask using the appropriate volumetric glass pipettes and/or calibrated positive displacement pipettes

i. E1S IS Stock Solution A (concentration: 80.0 µg/mL) is prepared by dissolving 100 µg of certified E1S-$^{13}C_6$ standard (80% purity) into 1 mL using ethanol

i. Transfer 15.0 mL of a 100 µg/mL certified DHEAS-d_5 solution into the volumetric flask using the appropriate volumetric glass pipettes and/or calibrated positive displacement pipettes
j. Add ethanol to just below the fill line of the volumetric flask
k. Place flask in a water bath for 15 minutes to reach 20°C and add ethanol (at 20°C) to the fill line
l. Label the volumetric flask appropriately with content, concentration, preparation date, analyst initials, safety precautions and hazard information, and store in the refrigerator

The Internal Standard Working Solution (Table 8) is prepared by diluting 500 µL of Internal Standard Stock Solution B up to 100 mL of water. This Internal Standard Working Solution should be prepared fresh each sample preparation day, and 100 µL of this solution is added to each sample during the sample prep procedure.

Table 8. Concentration of Labeled Compounds in Internal Standard Working Solution (in ng/dL)

Labeled Compound	Concentrations	Concentrations (nmol/L)
17α-Hydroxyprogesterone-$^{13}C_3$	75.0 (ng/dL)	2.25
Androstenedione-$^{13}C_3$	100 (ng/dL)	3.46
Progesterone-$^{13}C_3$	500 (ng/dL)	15.8
Testosterone-$^{13}C_3$	400 (ng/dL)	13.7
Estrone-$^{13}C_3$	50.0 (ng/dL)	1.83
17β-Estradiol-$^{13}C_3$	1,250 (pg/mL)	4.54
Estrone-6$^{13}C_3$sulfate	4,000 (pg/mL)	11.3
Dehydroepiandrosterone-d_5 sulfate	3.75 (µg/dL)	100

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

5.1 General Specimen Requirements

For analysis of steroid hormones in serum using this measurement procedure, a minimum of 300 µL of fresh or frozen serum is needed. A sample volume of at least 600 µL is preferred to allow for repeat analyses.

Serum should be processed within 6 hours of collection, if blood is kept at room temperature, or within 24 hours if blood is stored at 4°C. Morning fasting samples (i.e., samples collected in the morning after an overnight fast) are recommended to minimize biological variability. Serum collected with regular serum tubes ("Red Top Tubes") and serum serparator tubes are acceptable. Red Top Tubes are preferred.

The specimen should be transported in a cryogenic vial with external screw caps. These cryovials should be labeled in accordance with CDC and DLS policies and regulations. Barcodes are scanned upon receipt of samples, during the process of sample preparation, and during sample transfer in order to ensure that individual samples can be tracked throughout the process. Other specimen handling conditions are outlined in the latest version of the DLS Policies and Procedures Manual.

5.2 Specimen Storage

Specimens collected in the field can be shipped frozen on dry ice by overnight mail. The status of samples should be documented upon arrival. Specimens should be stored frozen at -70^{o}C upon acceptance, unless they are to be analyzed immediately. Samples should be analyzed immediately after thawing and reaching room temperature, with any residual samples returning to storage at -70^{o}C.

5.3 Unacceptable Specimens

Specimens that do not meet the above mentioned criteria (e.g., sample volume), were transported at room temperature, have evidence of leakage, or are in a condition that may compromise sample integrity are not acceptable.

6 PROCEDURE OPERATION INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

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

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

6.1 Specimen Storage and Handling during Testing

All vials are labeled according to the 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 prior to the sample preparation procedure. Any unused portion of the patient specimen is returned to the freezer and stored at -70^{o}C. Samples ready for analysis by LC-MS/MS are either stored in the refrigerator at 5^{o}C or in the LC sample tray at 5^{o}C.

6.2 Sample Processing using Hamilton Liquid Handler

Samples are processed in one batch along with 3 bench QC samples in duplicate (2 low, 2 medium, and 2 high level samples), 4 reagent blanks (1 double blank, and 3 saline), and 1 set of calibrators (11 levels: CC01-11 as defined in Section 4.3.1). Up to 75 patient samples can be processed in one batch (total number of samples per batch: 96 samples, including 6 QCs, 4 reagent blanks, 11 calibrators, and 75 samples).

1. Assess all samples for acceptability using the criteria described in Section 5
2. Thaw all samples at room temperature. Allow frozen serum samples, QC samples, Calibrator Working Solutions, and Internal Standard Working Solutions to reach room temperature. Homogenize all samples by placing them on the rotator at medium speed for 1.5 hours
3. Place pipette tips, all patient samples, reagent blanks, QC samples, and calibrators in designated locations on the Hamilton Microlab STARlet Liquid Handler instrument. See Hamilton Microlab STARlet Liquid Handler deck layout in Appendix 2
4. Scan the barcodes for all coded sample vials. 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 a particular sample on the instrument, and the current date and time is automatically created on the Hamilton computer. This file is transferred to a defined location on the CDC network and is used to create a run sequence for the LC-MS/MS instrument, and to verify run log sheets. An Example of Analytical Sequence for the LC-MS/MS can be found in Appendix 3
5. Using the Hamilton Microlab STARlet Liquid Handler, individually transfer 200 µL of reagent blanks, QCs, calibrators (CC01-11), and serum samples from individual vials into

appropriate wells of a 96-Well, 2 mL square bottom well plate (Plate A)

6. Transfer 100 µL of Internal Standard Working Solution to all reagent blanks (excluding double blank), QCs, calibrators, and serum samples
7. Cover Plate A with ArctiSeal cover, and allow the samples and internal standard to equilibrate on the MultiPulse Vortexer at a speed of 1800 (with pulse) for 45 minutes at room temperature
8. Centrifuge Plate A at 3700 RPM for 15 minutes at 5°C to remove any residual droplets from the ArctiSeal cover
9. Recap all sample vials, and store any remaining samples at -70°C

6.3 Dissociation of Steroid Hormones from Binding Proteins

1. Place Plate A, Dissociation Buffer, and pipette tips in designated locations on the Hamilton Microlab STARlet Liquid Handler
2. Transfer 100 µL of Dissociation Buffer* to all sample wells

   **Note*: If the Dissociation Buffer was not prepared in the same day, test its pH prior to use. If the pH is not within desired range (5.5 ± 0.1), or the integrity of the solution is in doubt, discard the entire buffer solution, and prepare a fresh batch for usage
3. Cover Plate A with ArctiSeal cover, and allow the sample solution to equilibrate on the MultiPulse Vortexer at a speed of 1800 (with pulse) for 30 minutes at room temperature
4. Centrifuge Plate A at 3700 RPM for 15 minutes at 5°C to remove any residual droplets from the ArctiSeal cover

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

1. Place Plate A, and Liquid Extraction Solution (A) (100% ethyl acetate) in designated locations on the Hamilton Microlab STARlet Liquid Handler instrument
2. Transfer 500 µL of Liquid Extraction Solution (A) to all sample wells
3. Cover Plate A with ArctiSeal cover, and place it on the MultiPulse Vortexer at a speed of 1000 (no pulse) for 15 minutes at room temperature
4. Centrifuge Plate A at 3700 RPM for 15 minutes at 5°C
5. While centrifuging Plate A, place a new 96-Well, 2 mL square bottom well plate (Plate B), and pipette tips in designated locations on the Hamilton Microlab STARlet Liquid Handler instrument
6. Transfer 200 µL of Deprotonation Buffer* to all wells in Plate B

   **Note*: If the Deprotonation Buffer was not prepared in the same day, test its pH prior to use. If the pH is not within desired range (8.0 ± 0.1), or the integrity of the solution is in doubt, discard the entire buffer solution and prepare a fresh batch for usage
7. Once centrifugation is complete, place Plate A in the designated location on the Hamilton Microlab STARlet Liquid Handler instrument
8. Extract the organic (top) layer from Plate A to Plate B using the Hamilton Microlab STARlet Liquid Handler instrument. Do not discard Plate A
9. Cover Plate B with ArctiSeal cover, and allow the sample solution to equilibrate on the MultiPulse Vortexer at a speed of 1000 (no pulse) for 15 minutes at room temperature

10. Centrifuge Plate B at 3700 RPM for 15 minutes at 5°C
11. While centrifuging Plate B, place a new 96-Well, 2 mL round-bottom well plate (Plate C), and pipette tips in designated locations on the Hamilton Microlab STARlet Liquid Handler instrument
12. Transfer 200 µL of ethanol to all wells in Plate C
13. Once centrifugation is complete, place Plate B in the designated position on the Hamilton Microlab STARLet Liquid Handler instrument
14. Extract the organic (top) layer from Plate B to Plate C using the Hamilton Microlab STARlet Liquid Handler instrument. Temporarily store Plate C in a safe, uncontaminated location
15. Place Plate A, and Liquid Extraction Solution (B) (70:30 ethyl acetate:hexane) in designated locations on the Hamilton Microlab STARlet Liquid Handler instrument
16. Transfer 500 µL of Liquid Extraction Solution (B) to all sample wells
17. Repeat steps 3 and 4, and continue with steps 7 through 10, followed by steps 13 to 14
18. Place Plate A, and Liquid Extraction Solution (C) (100% butanol) in designated locations on the Hamilton Microlab STARlet Liquid Handler instrument
19. Transfer 500 µL of Liquid Extraction Solution (C) to all sample wells
20. Repeat steps 3 and 4, and continue with steps 7 through 10, followed by steps 13 to 14
21. Cover Plate C with ArctiSeal cover, and allow the sample solution to equilibrate on the MultiPulse Vortexer at a speed of 1000 (no pulse) for 10 minutes at room temperature
22. Centrifuge Plate C at 3700 RPM for 10 minutes at 5°C to remove any residual droplets from the ArctiSeal cover

6.5 Preparation of Samples for Analysis by LC-MS/MS

1. Dry down the combined organic layers in Plate C using the Genevac Evaporator using the "High and Low BP" setting for 4.0 hours
2. Place Plate C, and Sample Reconstitution Solution in designated locations on the Hamilton Microlab STARlet Liquid Handler instrument
3. Transfer 150 µL of Sample Reconstitution Solution to all sample wells
4. Cover Plate C with ArctiSeal cover, and place it on the MultiPulse Vortexer at a speed of 1800 (with pulse) for 60 minutes at room temperature
5. Centrifuge Plate C at 3700 RPM for 15 minutes at 5°C

6.6 Analysis of Steroid Panel by LC-MS/MS

All samples prepared in one batch are analyzed together on the same instrument. Two matrix-free System Suitability Samples (SSS) containing the analytes and internal standards are added to each batch (one in the beginning of the run and one at the end of the run) to verify appropriate functioning of the instrument and chromatographic conditions. Additionally, a sample containing the Sample Reconstitution Solution ("Blank" or "BL") is added after every 8 samples to assess carryover. The SSS and Blanks are kept in a separate well plate 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 Microlab STARlet instrument (Section 6.2) to the appropriate Excel Worksheet. This worksheet combines the sample ID information from the instrument with additional information required by DLS policy and CLIA regulation, such as sample location on the well plate, and acquisition method name. It also inserts the SSS and Blank samples where appropriate. The Excel Worksheet creates the appropriate data file names for each individual sample
2. Save the Excel worksheet as a text file (.txt), and import it into the LC-MS/MS instrument software (Analyst). Save the final run sequence file as an Analyst sequence file. For every batch, the first sample in the sequence is a Blank followed by the SSS. See Appendix 3 for an Example of Analytical Sequence
3. Remove the ArctiSeal cover from Plate C and apply a RAPID Slit Seal
4. Load Plate C onto the autosampler on the LC-MS/MS instrument. Verify that the information in the sequence file agrees with the positions of plates in the autosampler
5. Check the basic instrument functions and settings according to the LC-MS/MS manufacturer's instructions. Ensure that the correct instrument method is selected and all method parameters are stable
6. Start the instrument run sequence using Analyst
7. Using the SSS, assess the performance of the LC-MS/MS system by inspecting retention times, peak intensities, and peak shapes. Retention times and peak intensities should fall between 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
8. Upon completion of the LC-MS/MS analysis, apply a new ArctiSeal on Plate C and store the plate in a designated space in the freezer at -70^{o}C.

LC-MS/MS Parameters:

LC: Shimadzu HPLC system as described in Section 4.1.4. The HPLC gradient is shown in Table 9. Post-column addition of 10 mM ammonium bicarbonate at 60 µL/min is used to stabilize and improve ionization in the negative ion mode.

HPLC Column:	Accucore Phenyl-Hexyl
Guard Column:	C18
Column Oven:	40 (±2) ^{o}C
Injection Volume:	50 µL
Mobile Phase A:	0.2 mM Ammonium Fluoride in Water:Methanol:Ethanol (75:20:5 v/v)
Mobile Phase B:	Methanol
Flow Rate:	250 µL/min

Table 9: LC Gradient Conditions

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0.00	84	16
1.00	84	16
7.00	44	56
17.0	16	74

A representative sample chromatogram is shown in Appendix 4.

MS: SCIEX API 6500 Triple Quad Mass Spectrometer as described in Section 4.1.4. Instrument settings are described in Table 10 and Table 11. Two transitions are monitored for each analyte and internal standard (Quantitation Ion – QI and Confirmation Ion – CI).

Acquisition Mode: Selected Reaction Monitoring (SRM)
Ionization Mode: Electrospray Ionization (ESI) in Positive and Negative Ion Modes

Table 10: Representative MS Ion Source Conditions

Parameters	Positive Ion Mode	Negative Ion Mode
Curtain Gas (CUR)	45.0	45.0
Collision Gas (CAD)	9	9
IonSpray Voltage (IS)	4500.0	-4500.0
Temperature (TEM)	650.0	650.0
Ion Source Gas 1 (GS1)	55.0	55.0
Ion Source Gas 2 (GS2)	70.0	70.0
Entrance Potential (EP)	10	-7.0

Table 11: SRM Conditions for each Analyte

Analyte	ESI Mode	Analyte CE (V) (QI / CI)	Analyte DP (V)	Analyte	IS
				m/z (QI, CI)	*m/z (QI, CI)*
17-OHP	+	31 / 33	11	331.2 > 97, 109	334.2 > 100, 112
AD	+	27 / 29	31	287.2 > 97, 109	290.3 > 100, 112
P4	+	29 / 31	16	315.2 > 97, 109	318.2 > 100, 112
TT	+	25 / 25	160	289.3 > 97, 109	292.3 > 100, 112
E1	-	-62/-40	-140/-120	269.0 > 143,145	272.0 > 146, 148
E2	-	-50 / -52	-100	271.0 > 145, 183	274.0 > 148, 186
E1S	-	-40/-60	-55	349.0 > 269, 145	355.1 > 275, 145
DHEAS	-	-40/-15	-45	367.0 > 80, 97	372.1 > 80, 98

6.7 Data Processing

1. Transfer data files generated by the LC-MS/MS system to the dedicated location on the CDC network sharedrive.
2. Using a dedicated data processing method ("Quantitation Method") via Analyst or MultiQuant software, identify relevant chromatographic peaks based on their retention times. The Quantitation Method should automatically integrate the area under the curve. Manual integration maybe required if automatic processing fails to integrate the peaks correctly.
3. Document integrated peaks as electronic files and generate integration report as electronic file ("pdf" file).
4. Export the integration results as a text file (.txt) and import into the appropriate Excel Template where final results are calculated.
5. Review of integrations and results is conducted by the analyst and, independently, by the project lead or a dedicated, specially trained staff member. Any coorections to the integration or data are documented and approved by laboratory chief or designated individual.

6.8 Data Calculations

1. As part of quality assurance, the ratio between the analyte quantitation ion (QI) area counts and the analyte confirmation ion (CI) area counts is used ("QI/CI Ratio") to identify potential interferences. Only analytes with a QI/CI Ratio within 20% of the target value are considered for processing and reporting. The target value is computed by averaging the ratios of the calibrators used in the run being assessed.
2. To calculate the analyte concentration, determine area ratio between the analyte quantitation ion (QI) area count and internal standard quantitation ion (QI) area count ("Area Ratio")
3. Generate calibration curves by plotting the Area Ratios of the calibrators against their assigned concentration values, using an appropriate weighted linear regression

   [*]*Note*: Do not further process 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 samples and the regression parameters of the corresponding weighted calibration curve

   [*]*Note*: Do not use area ratios for analytes outside of 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 their calibration is verified 6 months after calibration. Mass spectrometers 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, are verified regularly according to the manufacturer's recommendation.

7.1.2 Calibration of Measurement

When available, calibrators used in this measurement procedure are from commercial standard solutions that are tracable to SI according to ISO 17511. For further information, see certificates of analysis provided by the vendors. Calibrator mateirals are assessed in-house for idnetly and purity. Calibration solutions are prepared starting with volumetric measurements. Calibrators are analyzed together with each set of samples. The accuracy of calibration of the measurement is assessed using certified reference materials where available as described in section 8.4.

7.2 Calibration Verification

With each batch of samples, 11 levels of calibrators, and 3 levels (low, mid, high) of quality control materials covering the clinical range of reported steroid hormones 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 Section 9.1: Quality Control Procedures).

Calibration is further verified every 6 months by analyzing serum material with assigned reference values, where available, and by spike recovery experiment according to CLSI C62 guidelines for analytes where serum material with assigned reference values are unavailable [5].

8 METHOD PERFORMANCE CHARACTERISTICS

The method performance documentation for this method including accuracy, precision, sensitivity, specificity, and stability is provided in Appendix A of this method documentation. The signatures of the branch chief and director of the Division of Laboratory Sciences on the first page of this procedure denote that the method performance is fit for the intended use of the method.

8.1 Reportable Range of Results and Linearity Limits

The analytical measurement range (AMR) is the range of the calibration curve in which linearity was verified. The linearity for the analytes measured in this measurement procedure was determined following CLSI guideline EP6 [6]. The calibration curve is linear for all analytes over the calibration range listed in Table 12 below. Unknown serum samples with concentrations exceeding the upper calibration limit are reanalyzed after dilution. Dilutions of samples with saline up to 10 times for 17-OHP, AD, P4, TT, E1 and E2, and up to 7 times for DHEAS were found to provide accurate measurements. The extended reportable range using dilution is also indicated in Table 12. E1S appears sensitive to dilution and should not be diluted using saline as it results in decreased accuracy.

8.2 Limit of Detection (LOD)

The limits of detection (LOD) for this method was evaluated following the Taylor method [7], using the 5 lowest calibrator levels. Upon acquiring more data, the LOD will be updated as described in the DLS Policies and Procedures Manual. The limits of quantitation (LOQ), LOD, and calibration ranges of the measurement method are shown in Table 12. Further information about the LOD of this method is provided in Appendix A.

Table 12. LOD, LOQ, and Calibration Range of the Method

Analyte	LOD	LOQ	AMR	Reportable Range
17-OHP (ng/dL) / (nmol/L)	0.41 / 0.012	1.35 / 0.041	0.79 – 432 / 0.024 – 13.1	0.41 – 4320 / 0.012 – 131
AD (ng/dL) / (nmol/L)	0.82 / 0.029	2.75 / 0.096	0.95 – 525 / 0.033 – 18.3	0.82 – 5250 / 0.029 – 183
P4 (ng/dL) / (nmol/L)	0.86 / 0.027	2.85 / 0.091	1.22 – 1960 / 0.039 – 62.3	0.86 – 19600 / 0.027 – 623.3
TT (ng/dL) / (nmol/L)	0.57 / 0.020	1.91 / 0.066	0.58 – 1280 / 0.020 – 44.4	0.57 – 12800 / 0.020 – 444
E1 (ng/dL) / (nmol/L)	0.13 / 0.005	0.44 / 0.016	0.37 – 54.6 / 0.014 – 2.02	0.13 – 546 / 0.005 – 20.2
E2 (pg/mL) / (nmol/L)	1.72 / 0.006	5.74 / 0.021	2.63 – 1710 / 0.010 – 6.28	1.72 – 17100 / 0.006 – 62.8
E1S (pg/mL) / (nmol/L)	2.04 / 0.006	6.81 / 0.019	3.92 – 2548 / 0.011 – 6.84	2.04 – 25480 / 0.006 –68.42
DHEAS (µg/dL) / (nmol/L)	0.22 / 5.970	0.75 / 20.35	0.75 – 415 / 20.35 – 11300	0.22 – 2905 / 5.970 – 78800

8.3 Analytical Specificity

Analytical specificity of this method 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 from other compounds and allows for compound identification based on chromatographic retention time using reference compounds and stable isotope labeled internal standards
- Mass selective detection mode that allows for detection of the mass-to-charge (M/Z) ratios specific to a precursor and two fragment ions per compound (Quantitation Ion – QI and Confirmation Ion – CI). The most intense product ion is typically chosen to be the QI and the second most intense to be the CI. However, other traces may be used, if the most intense ion does not allow for realiable quantitation.

Analytical specificity was tested by:

> Assessing chromatographic coelution and MS detection using other steroid hormones that may potentially interfer. Refer to Section 8.5: Interfering Substances for a list of steroid analytes tested.

8.4 Accuracy (Trueness) and Precision

To ensure accuracy of this method, isotope dilution with stable, isotopically labeled standards for each analyte is used, and gravimetrically prepared certified, pure reference standards are used to prepare calibrators wherever available.

Accuracy is confirmed with higher-order serum reference materials, such as materials from the U.S. National Institute of Standards and Technology (NIST) for testosterone and progesterone, and material from the European Union Institute for Reference Materials and Methods for estradiol. Where reference materials were unavailable, accuracy was confirmed by spike recovery experiment of two serum based material with three varying concentration levels covering the measurement range. Each sample was measured over two days with triplicate measurements on each day. This method showed excellent agreement with the certified materials, and yielded excellent recovery under the spiked recovery experiments. The results of the experiments are summarized in Table 13 and 14. Further information on method accuracy is provided in Appendix A.

Table 13. Method Accuracy, based on comparisons to Certified Reference Standards

Analyte	Reference Material	Nominal Value	Measured Value (n=10)	Difference from Nomial Value (%)
P4 (ng/dL)	971F	194.96	198.70	1.9
TT (ng/dL)	971F	27.69	29.03	4.8
	971M	642.94	629.6	-2.1
E2 (pg/mL)	BCR576	31.05	30.99	-0.2
	BCR577	187.96	186.8	-0.6
	BCR578	365.02	364.5	-0.1

Table 14. Method Accuracy, based on Spiked Recovery Experiments

	Sample 1		Sample 2			
Analyte	**Spike Concentration**	**Recovery (%) (n=6)**	**Spike Concentration**	**Recovery (%) (n=6)**	**Mean Recovery (%)**	**SD (%)**
17-OHP (ng/dL)	0	-	0	-	**99.3**	**3.5**
	40	106.0	60	99.3		
	80	97.2	140	99.4		
	180	97.3	220	96.4		
AD (ng/dL)	0	-	0	-	**104.9**	**4.5**
	80	96.75	120	107.16		
	160	108.38	280	103.11		
	350	108.57	430	105.58		
P4 (ng/dL)	0	-	0	-	**97.7**	**5.4**
	245	91.38	370	91.0		
	490	100.77	860	103.2		
	1100	102.14	1350	97.8		
TT (ng/dL)	0	-	0	-	**100.0**	**1.4**
	190	99.8	290	99.4		
	390	101.4	680	97.7		
	870	101.4	1060	100.4		
E1 (ng/dL)	0	-	0	-	**102.9**	**5.3**
	15	97.2	20	111.0		
	30	100.1	50	104.7		
	70	98.3	80	105.9		
E2 (pg/mL)	0	-	0	-	**100.5**	**1.7**
	260	102.0	390	100.0		
	525	101.9	920	100.5		
	1180	100.9	1440	97.4		
E1S (pg/mL)	0	-	0	-	**101.3**	**5.8**
	200	104.9	300	110.8		
	400	95.7	700	100.1		
	900	95.8	1100	100.5		
DHEAS (µg/dL)	0	-	0	-	**96.0**	**4.9**
	40	102.0	60	99.2		
	80	98.9	140	93.1		
	180	93.9	220	88.8		

To assess the within-day imprecision for this method, 10 measurements of quality material of low, medium, and high concentration for each analyte was evaluated made in duplicate. Similarly, among-day variability was determined by evaluating these quality materials in duplicate over 20 distinct days. The percent coefficients of variation (%CV) are listed in Table 15 below. The results fell within acceptability criteria, based on established biological variability data from Ricos [8].

Where biological variability data was not available, criteria set by CLSI and the FDA was used [5, 9]. Further infomraiton on method precision is provided in Appenix A.

Table 15. Within-day and Among-day Precision

Analyte	Mean Concentration Results	Within-day Precision (%CV) (n=10)	Among-day Imprecision (%CV) (n=20)	Total Imprecision (%CV)
17-OHP (ng/dL)	8.12	8.96	9.70	**13.2**
	40.0	8.87	0.00	**8.87**
	120	3.07	4.19	**5.20**
AD (ng/dL)	15.5	3.60	5.36	**6.45**
	84.2	4.01	2.42	**4.68**
	272	2.07	5.87	**6.22**
P4 (ng/dL)	45.9	10.4	4.29	**11.3**
	231	6.20	1.84	**6.47**
	784	6.21	5.43	**8.25**
TT (ng/dL)	39.9	5.96	4.61	**7.54**
	203	5.10	3.71	**6.31**
	601	3.64	5.46	**6.57**
E1 (ng/dL)	3.17	9.50	0.00	**9.50**
	16.3	4.45	2.96	**5.34**
	45.5	2.70	3.70	**4.58**
E2 (pg/mL)	51.1	7.27	3.27	**7.97**
	253	8.86	4.44	**9.91**
	785	3.98	8.25	**9.16**
E1S (pg/mL)	43.5	7.75	0.00	**7.75**
	199	9.13	6.94	**11.5**
	591	6.17	4.90	**7.88**
DHEAS (µg/dL)	8.35	5.31	6.59	**8.46**
	40.4	2.19	3.90	**4.48**
	117	7.73	0.00	**7.73**

8.5 Stability of the Analytes

The stability of the analytes was determined for three scenarios:

1. Freeze and Thaw Stability (Three Freeze-Thaw Cycles)

    - Samples were removed from a -70^{o}C freezer and thawed at room temperature for two hours, during which they were placed on a bench rotator and allowed to homogenize. The samples were then placed back in the freezer and allowed to refreeze. This cycle was completed three times prior to the samples undergoing the sample preparation procedure and LC-MS/MS analysis

2. Bench-Top Stability

- Samples were removed from a -70ºC freezer and stored on a lab bench for 12 hours at room temperature. After the 12 hours, the samples underwent sample preparation procedure and analyzed

3. Processed Sample Stability

- Processed samples were stored at 20ºC for 24 hours, and then were reinjected into the LC-MS/MS system

Note: Due to unavailability of samples that have been previously analyzed, a fourth stability condition, Long-Term Stability, could not be assessed at this time. The samples that were tested for stability in these experiments will be stored at -70ºC and reassessed at a future date

For each of these stability conditions, three replicates of four quality materials of varying concentrations were evaluated. All stability testing results, as shown in Table 16, fell within ±15% of initial measurements, indicating that all analytes tested are sufficiently stable under the conditions tested. Further information on analyte stability is provided in Appendix A.

Table 16. Stability of Analytes Under Various Conditions

Analyte	Sample ID	Initial Result (n=3)	Three Freeze-Thaw Cycles		Bench-Top Stability		Processed Sample Stability	
			Mean Result (n=3)	Difference from Initial (%)	Mean Result (n=3)	Difference from Initial (%)	Mean Result (n=3)	Difference from Initial (%)
17-OHP (ng/dL)	Sample 1	16.6	16.3	**-1.60**	16.5	**-0.45**	16.5	**-0.40**
	Sample 2	120	120	**-0.24**	120	**-0.01**	119	**-0.94**
	Sample 3	65.2	65.1	**-0.25**	64.3	**-1.39**	62.8	**-3.76**
	Sample 4	64.4	64.6	**0.29**	63.5	**-1.40**	62.3	**-3.29**
AD (ng/dL)	Sample 1	35.1	35.3	**0.70**	34.2	**-2.54**	34.8	**-0.75**
	Sample 2	199	198	**-0.50**	196	**-1.23**	198	**-0.23**
	Sample 3	34.4	34.6	**0.65**	33.9	**-1.45**	34.0	**-1.04**
	Sample 4	182	181	**-0.68**	173	**-5.11**	178	**-2.36**
P4 (ng/dL)	Sample 1	105	106	**1.03**	110	**5.27**	105	**-0.12**
	Sample 2	746	749	**0.42**	719	**-3.65**	743	**-0.40**
	Sample 3	5.15	5.23	**1.51**	5.28	**2.57**	4.95	**-3.88**
	Sample 4	5.60	5.60	**-0.03**	5.46	**-2.64**	5.40	**-3.56**
TT (ng/dL)	Sample 1	51.6	52.5	**1.78**	51.0	**0.34**	52.4	**1.57**
	Sample 2	368	374	**1.65**	369	**0.26**	361	**-1.93**
	Sample 3	509	513	**0.85**	505	**-0.82**	499	**-2.01**
	Sample 4	55.9	56.2	**0.59**	55.7	**0.33**	55.9	**0.12**
E1 (ng/dL)	Sample 1	4.66	4.74	**1.73**	4.73	**1.66**	4.68	**0.55**
	Sample 2	15.7	15.4	**-2.23**	15.5	**-1.76**	15.1	**-3.86**
	Sample 3	1.65	1.64	**-0.56**	1.64	**-0.56**	1.72	**4.57**
	Sample 4	10.0	9.99	**-0.07**	9.97	**-0.29**	9.85	**-1.45**
E2 (pg/mL)	Sample 1	24.0	24.6	**2.22**	22.8	**-5.11**	21.0	**-12.7**
	Sample 2	411	392	**-4.62**	404	**-1.56**	399	**-2.78**

	Sample 3	74.8	74.1	**-0.94**	71.2	**-4.76**	65.7	**-12.1**
	Sample 4	86.1	85.3	**-0.97**	78.5	**-8.88**	80.4	**-6.71**
E1S (pg/mL)	Sample 1	32.6	32.7	**0.05**	31.6	**-3.09**	32.2	**-1.22**
	Sample 2	351	356	**1.19**	351	**-0.06**	348	**-0.88**
	Sample 3	52.8	52.0	**-1.59**	52.6	**-0.50**	52.8	**-0.07**
	Sample 4	422	415	**-1.75**	415	**-1.54**	428	**1.42**
DHEAS (µg/dL)	Sample 1	17.4	17.0	**-2.21**	17.2	**-0.82**	17.8	**2.30**
	Sample 2	144	148	**3.09**	142	**-1.12**	143	**-0.17**
	Sample 3	81.1	82.6	**1.86**	83.1	**2.42**	82.9	**2.19**
	Sample 4	77.8	78.6	**1.09**	76.9	**-1.13**	75.7	**-2.68**

8.6 Limitations of Method, Interfering Substances, and Ruggedness

Limitations of the Method
This method was tested for steroid hormone analysis in human serum and may not be suitable for other specimens such as plasma, whole blood, serum, and/or saliva. The analytical performance needs to be reassessed when using other specimen matrices.

Interfering Substances
The following potentially interfering compounds were evaluated: dehydroepiandrosterone (DHEA), dihydroxytestosterone (DHT), pregnenolone (P5), estriol (E3), etiocholanolone (EC), testosterone glucuronide (TG), and compounds (TS). None of these compounds interfered with the analytes measured by this method. The QI/CI ratio are monitored in samples and compared to that of neat standard solutions to detect unknown interferences. No interferences were detected when analyzing 105 serum samples from individual donors.

Ruggeness
Ruggedness testing is performed to determine whether variations in factors such as pH, incubation time, and oven temperature may affect the measurement result. Five critical elements within the method measurement procedure are evaluated and tested for ruggedness. The following parameters were assessed:

1. pH of the Dissociation Buffer

    - The pH of the dissociation buffer as described in Section 6.3 should be within the pH range of 5.5 ± 0.1. Decrease in method performance was observed at pH 6.

2. pH of the Deprotonation Buffer

    - The pH of the deprotonation buffer as described in Section 6.4 should be within the pH range of 8.0 ± 0.1. Decrease in method performance was observed at pH 9.

3. Incubation Time

    - The IS-Analyte incubation time for this method as described in Section 6.2 is 60 minutes. Decrease in method performance was observed at 30 or 90 min incubation times

4. Dissociation Time

    - The dissociation time for this method as describebed in Section 6.3 is 30 minutes. No change in method performance was observed at 15 or 45 min dissociation times

5. Oven Temperature

    - The oven temperature for LC/MS/MS analysis as described in Section 6.6 should be within the range of 40 ± 2^{o}C. Decrease in method performance was observed at temperatures below 40^{o}C

Analytes may be subject to oxidation from oxygen in the air under elevated temperatures. Thus, samples sould 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 used in this measurement procedure consist of three spiked serum materials with levels of concentration spanning the low to high ranges for the analytes measured.

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 [10].

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 are not reported. Specimen processing and analysis are stopped and will only resume when corrective action have been performed and measurements are within the control and quality contro limits.

9.2 Proficiency Testing

For analytes where external Proficiency Testing (PT) Programs (i.e., those offered by the College of American Pathologists [CAP]) exist, these programs are used. If external PT Programs are unavailable, the PT for this method is administered by an in-house PT coordinator following CLSI GP29-A guidelines [11]. Five serum samples within the linear range of the method are analyzed. The coordinator then establishes the mean and confidence limits for each analyte concentration.

For the assessment, the PT coordinator randomly selects five PT materials for analysis. These samples are treated as unknown patient samples, and the analytical results are forwarded directly to the PT coordinator for interpretation. A passing score is obtained if at least four of the five samples fall within the prescribed limits, previously established. The PT coordinator will notify the laboratory of its PT status (i.e. pass/fail). All PT results are appropriately documented. Proficiency Testing for this procedure is performed a minimum of once every 6 months.

10 REFERENCE RANGES (NORMAL VALUES)

Population-based reference ranges have not yet been established for the analytes included in this panel method. Normal ranges for serum concentrations for adults as suggested in literature are as follows [12, 13] (Table 17).

Table 17. Normal Ranges of Steroid Hormones in Serum

Analyte – Sex	Group	Reference Interval	
		ng/dL	nmol/L
17-OHP – Male	Adult	41 – 183	1.2 – 5.5
17-OHP – Female	Follicular	45 – 1185	1.3 – 35.7
	Luteal	42 – 450	1.3 – 13.5
	Postmenopausal	18 – 48	0.5 – 1.4

Analyte – Sex	Group	Reference Interval	
		ng/dL	nmol/L
AD – Male	Adult	75 – 205	2.6 – 7.2
AD – Female	Adult	85 – 275	3.0 – 9.6

Analyte – Sex	Group		Reference Interval	
			ng/dL	nmol/L
P4 – Male	Adult		13 – 97	0.4 – 3.1
P4 – Female	Follicular		15 – 70	0.5 – 2.2
	Luteal		200 – 2500	6.4 – 79.5
	Pregnancy	First trimester	1025 – 4400	32.6 – 139.9
		Second trimester	1950 – 8250	62.0 – 262.4
		Third trimester	6500 – 22,900	206.7 – 728.2

Analyte – Sex	Group	Reference Interval	
		ng/dL	nmol/L
TT – Male	Adult	280 – 1100	9.71 – 38.17
TT – Female	Adult	15 – 70	0.52 – 2.43
	Postmenopausal	8 – 35	0.28 – 1.22

Analyte – Sex	Group	Reference Interval	
		ng/dL	pmol/L
E1 – Male	Adult	1.5 – 6.5	55 – 240
E1 – Female	Early follicular phase	1.5 – 15	55 – 555
	Late follicular phase	10 – 25	370 – 925
	Luteal phase	1.5 – 20	55 – 740
	Postmenopausal	1.5 – 5.5	55 – 204

Analyte – Sex	Group	Reference Interval	
		pg/mL	pmol/L
E2 – Male	Adult	10 – 50	37 – 184
E2 – 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

Analyte – Sex	Group	Reference Interval	
		pg/mL	pmol/L
E1S – Male	Adult	230 – 2200	656 – 6278
E1S – Female	Follicular	300 – 2600	856 – 7419
	Luteal	100 – 3200	285 – 9132
	Postmenopausal	100 – 1300	285 – 3710

Analyte – Sex	Group		Reference Interval	
			µg/dL	nmol/L
DHEAS – Male	Adult	19 – 30 yr	125 – 619	3.4 – 16.7
		31 – 50 yr	59 – 452	1.6 – 12.2
		51 – 60 yr	20 – 413	0.5 – 11.1
		61 – 83 yr	10 – 285	0.3 – 7.7
DHEAS – Female	Adult	19 – 30 yr	29 – 781	0.8 – 21.1
		31 – 50 yr	12 – 379	0.8 – 10.2
		Postmenopausal	30 – 260	0.8 – 7.0

Note: For more complete reference ranges, including infant and tanner stage intervals, refer to Tietz Clinical Guide to Laboratory Tests [12].

11 TEST RESULT REPORTING SYSTEM

Results are typically reported in ng/dL for 17-OHP, AD, P4, TT, E1, in pg/mL for E2, E1S, and in µg/dL for DHEAS. Reports may be issued in other units, if requested by the prinicla investigator.

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^{o}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 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

Insufficient data exists to correlate serum concentrations of these analytes with serious health effects in humans. Therefore, critical values have not yet been stablished.

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

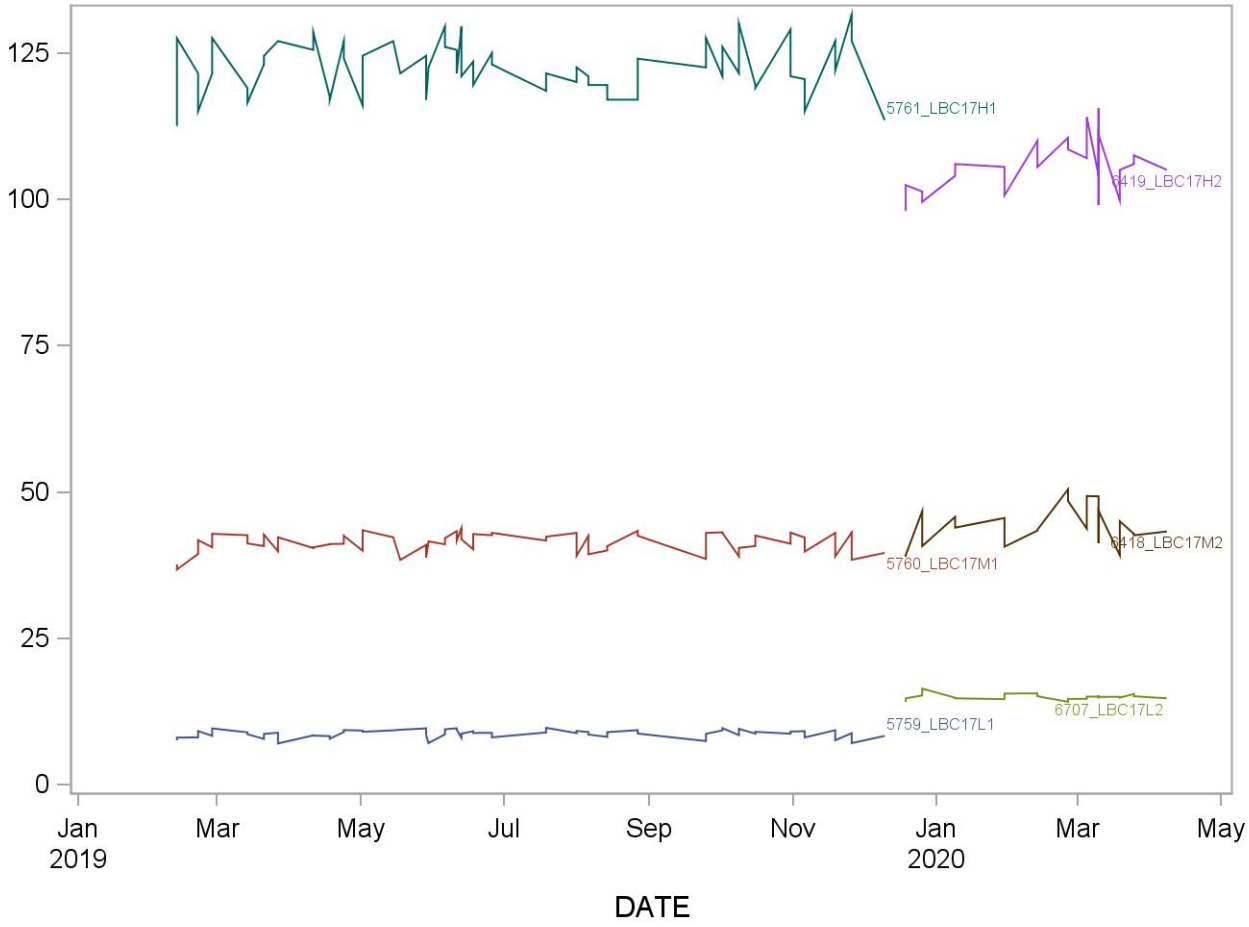
Not applicable for this procedure.

17 SUMMARY STATISTICS AND QC GRAPHS

See following pages.

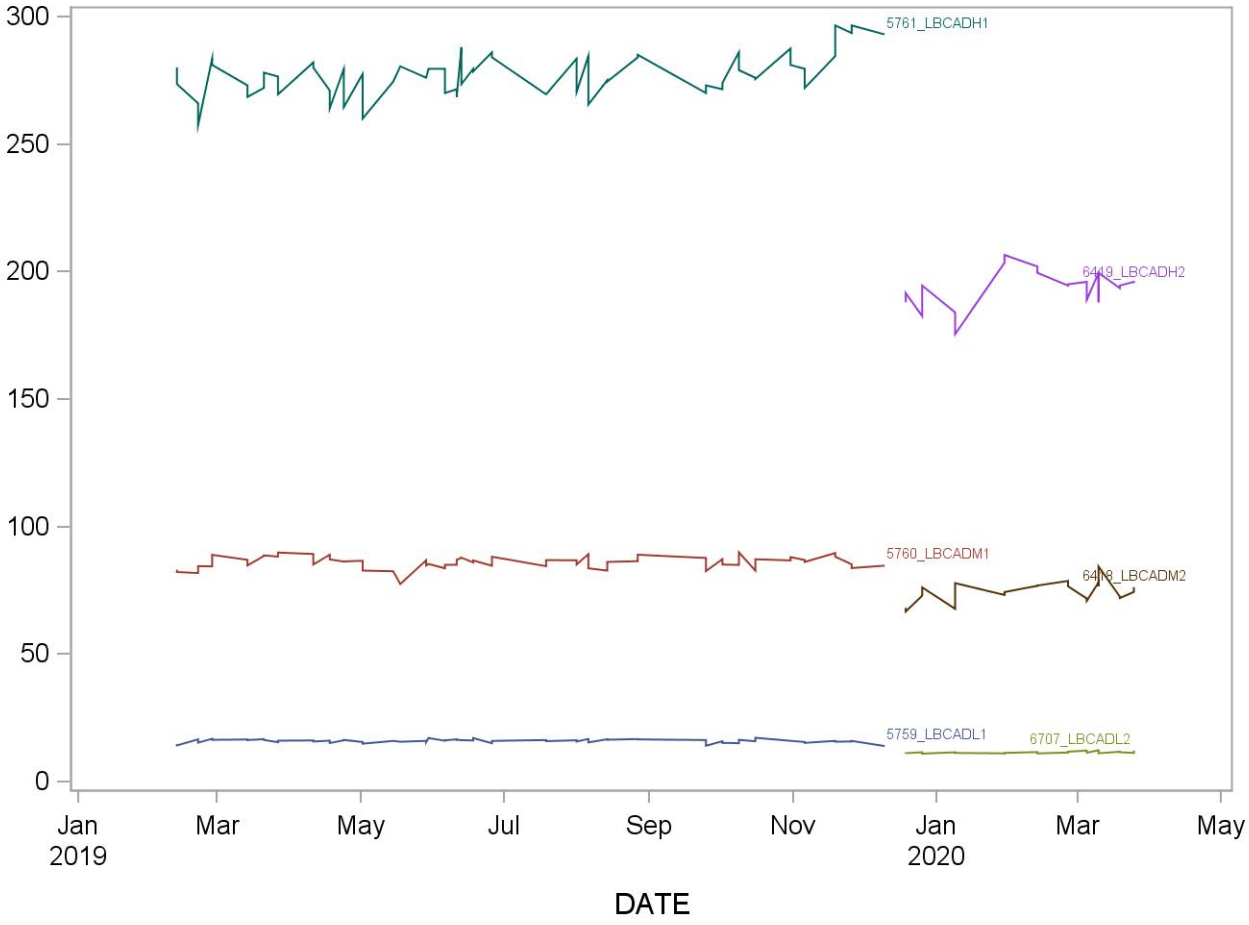
2019-2020 Summary Statistics and QC Chart
LBX17H (Hydroxyprogesterone (ng/dL))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
5761_LBC17H1	62	12FEB19	10DEC19	122.4677	4.42874	3.6
5759_LBC17L1	62	12FEB19	10DEC19	8.69411	0.66481	7.6
5760_LBC17M1	62	12FEB19	10DEC19	41.24355	1.65645	4.0
6419_LBC17H2	23	19DEC19	08APR20	105.4630	4.70196	4.5
6707_LBC17L2	23	19DEC19	08APR20	14.98696	0.48341	3.2
6418_LBC17M2	23	19DEC19	08APR20	44.26304	3.40538	7.7



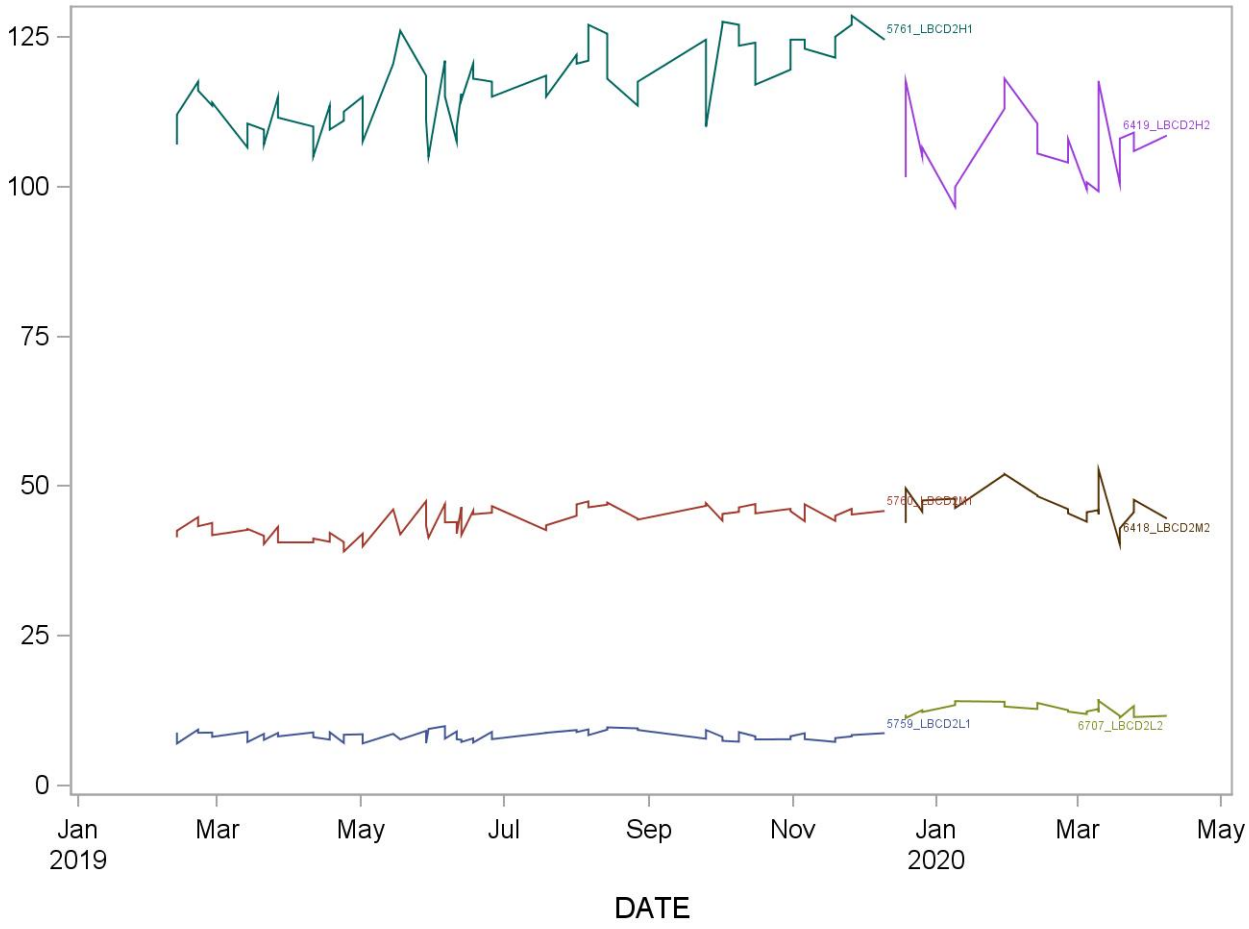
2019-2020 Summary Statistics and QC Chart
LBXAND (Androstenedione (ng/dL))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
5761_LBCADH1	62	12FEB19	10DEC19	276.9919	8.20061	3.0
5759_LBCADL1	62	12FEB19	10DEC19	15.94919	0.69249	4.3
5760_LBCADM1	62	12FEB19	10DEC19	85.98065	2.38768	2.8
6419_LBCADH2	22	19DEC19	25MAR20	193.9546	7.27505	3.8
6707_LBCADL2	22	19DEC19	25MAR20	11.49318	0.40979	3.6
6418_LBCADM2	22	19DEC19	25MAR20	74.58409	4.10552	5.5



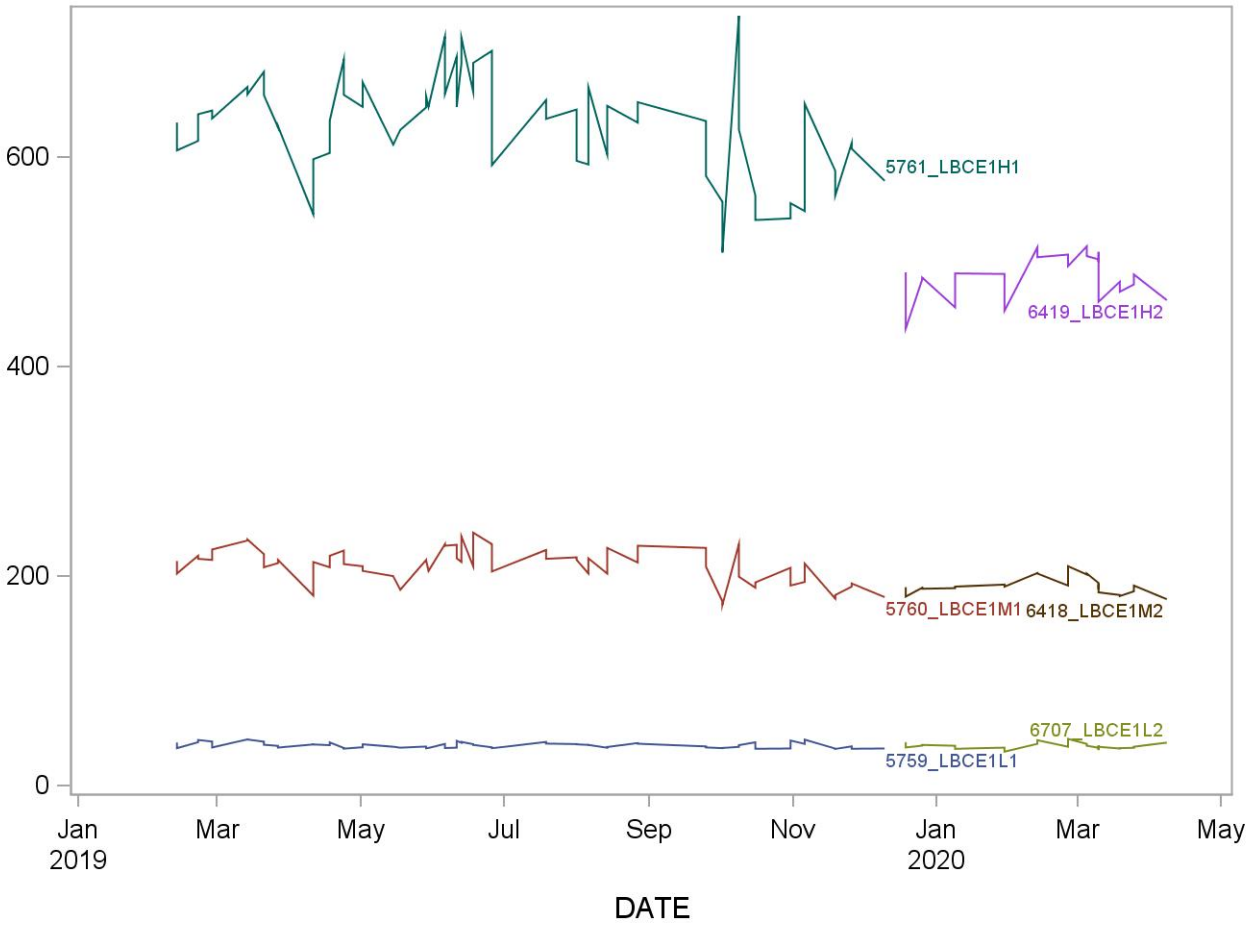
2019-2020 Summary Statistics and QC Chart
LBXDHE (Dehydroepiandrosterone (ug/dL))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
5761_LBCD2H1	62	12FEB19	10DEC19	117.0403	6.581954	5.6
5759_LBCD2L1	62	12FEB19	10DEC19	8.367500	0.765091	9.1
5760_LBCD2M1	62	12FEB19	10DEC19	44.15403	2.283338	5.2
6419_LBCD2H2	23	19DEC19	08APR20	106.7543	6.487994	6.1
6707_LBCD2L2	23	19DEC19	08APR20	12.68478	0.968649	7.6
6418_LBCD2M2	23	19DEC19	08APR20	46.95870	3.134400	6.7



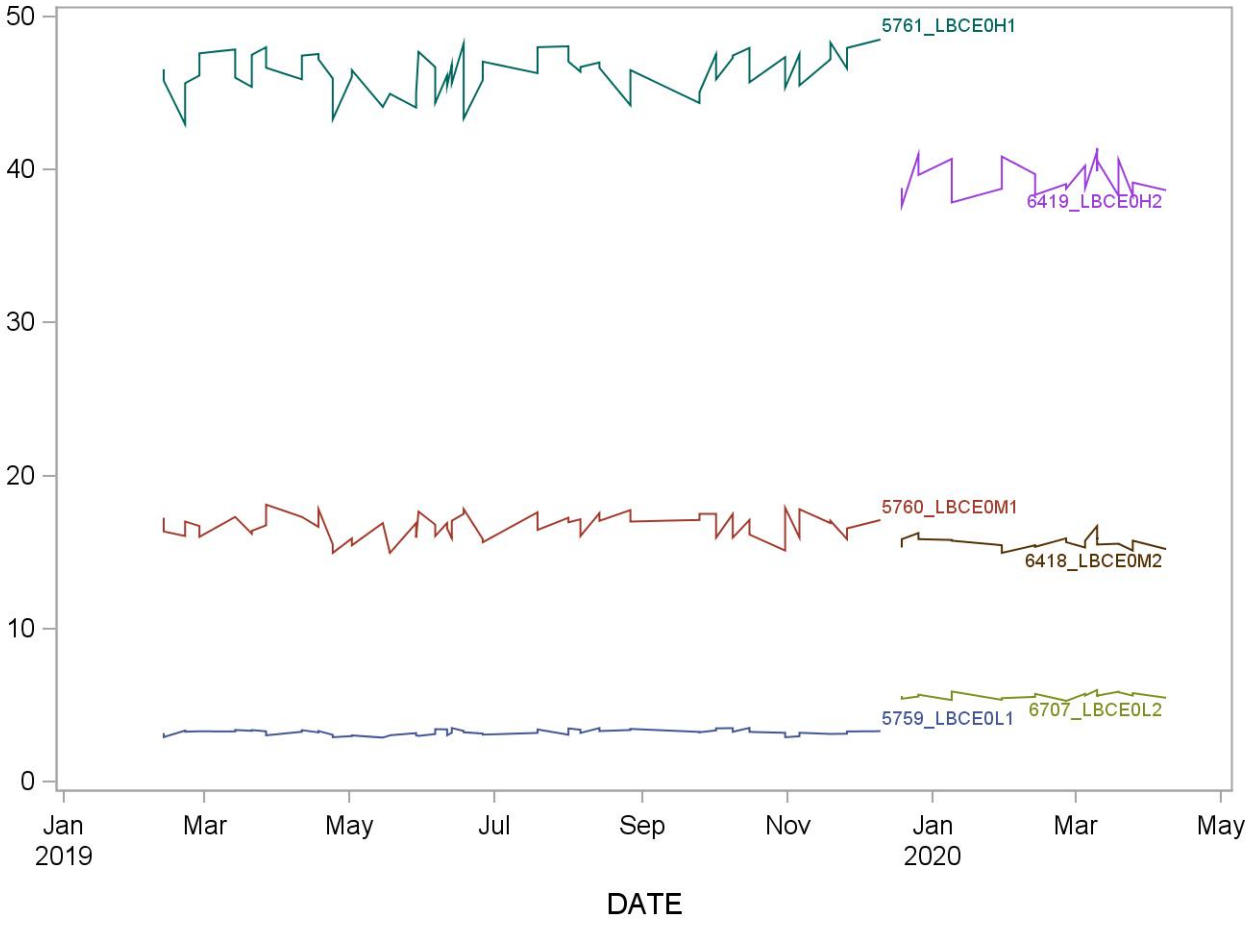
2019-2020 Summary Statistics and QC Chart
LBXES1 (Estrone Sulfate (pg/mL))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
5761_LBCE1H1	62	12FEB19	10DEC19	629.7258	48.14278	7.6
5759_LBCE1L1	62	12FEB19	10DEC19	38.60242	2.66397	6.9
5760_LBCE1M1	62	12FEB19	10DEC19	210.5323	16.39294	7.8
6419_LBCE1H2	23	19DEC19	08APR20	486.1304	20.99958	4.3
6707_LBCE1L2	23	19DEC19	08APR20	37.89565	2.88519	7.6
6418_LBCE1M2	23	19DEC19	08APR20	191.0000	8.08759	4.2



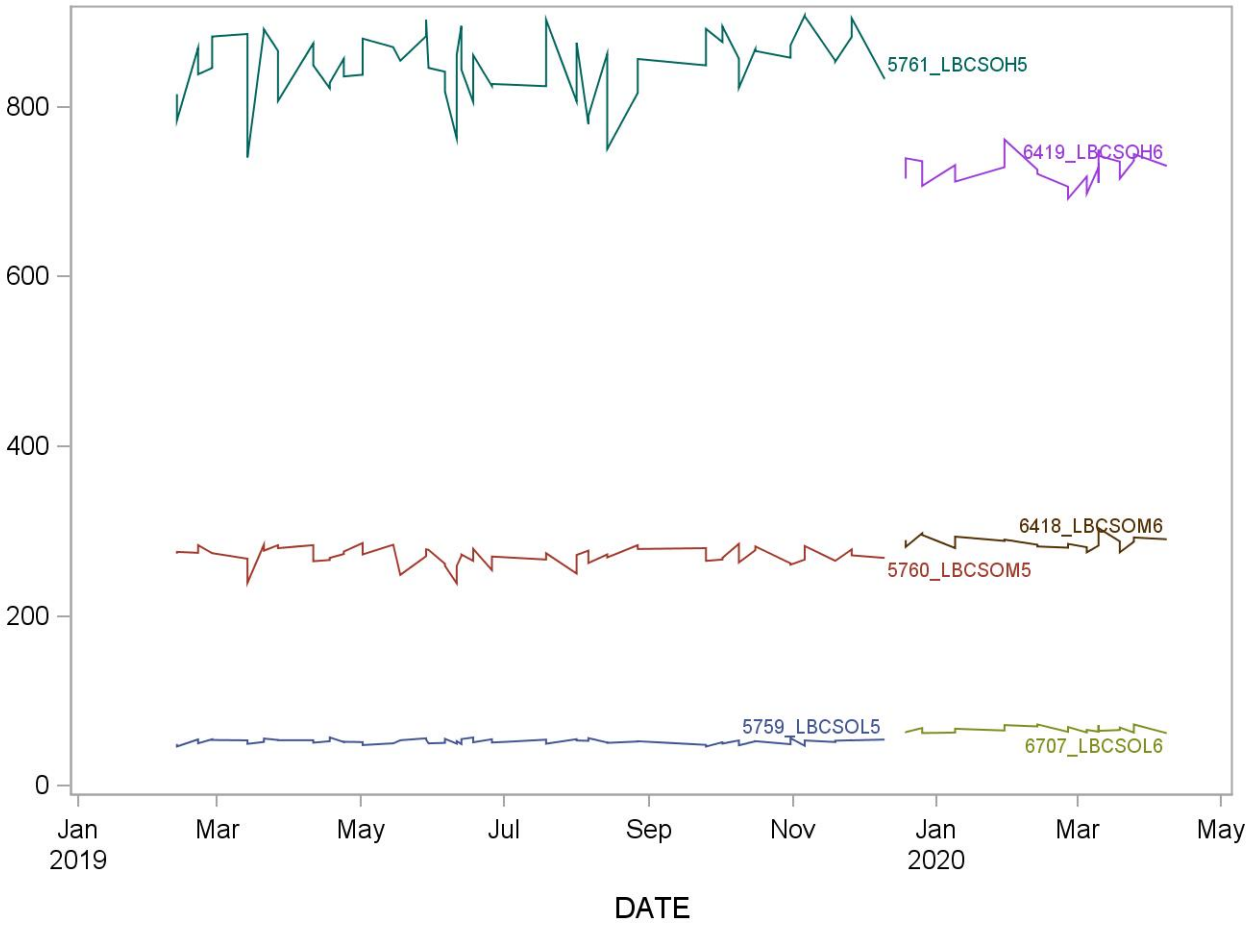
2019-2020 Summary Statistics and QC Chart
LBXESO (Estrone (ng/dL))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
5761_LBCE0H1	62	12FEB19	10DEC19	46.35242	1.33872	2.9
5759_LBCE0L1	62	12FEB19	10DEC19	3.22653	0.16919	5.2
5760_LBCE0M1	62	12FEB19	10DEC19	16.71613	0.77902	4.7
6419_LBCE0H2	23	19DEC19	08APR20	39.49130	1.14472	2.9
6707_LBCE0L2	23	19DEC19	08APR20	5.60326	0.19889	3.5
6418_LBCE0M2	23	19DEC19	08APR20	15.64130	0.38366	2.5



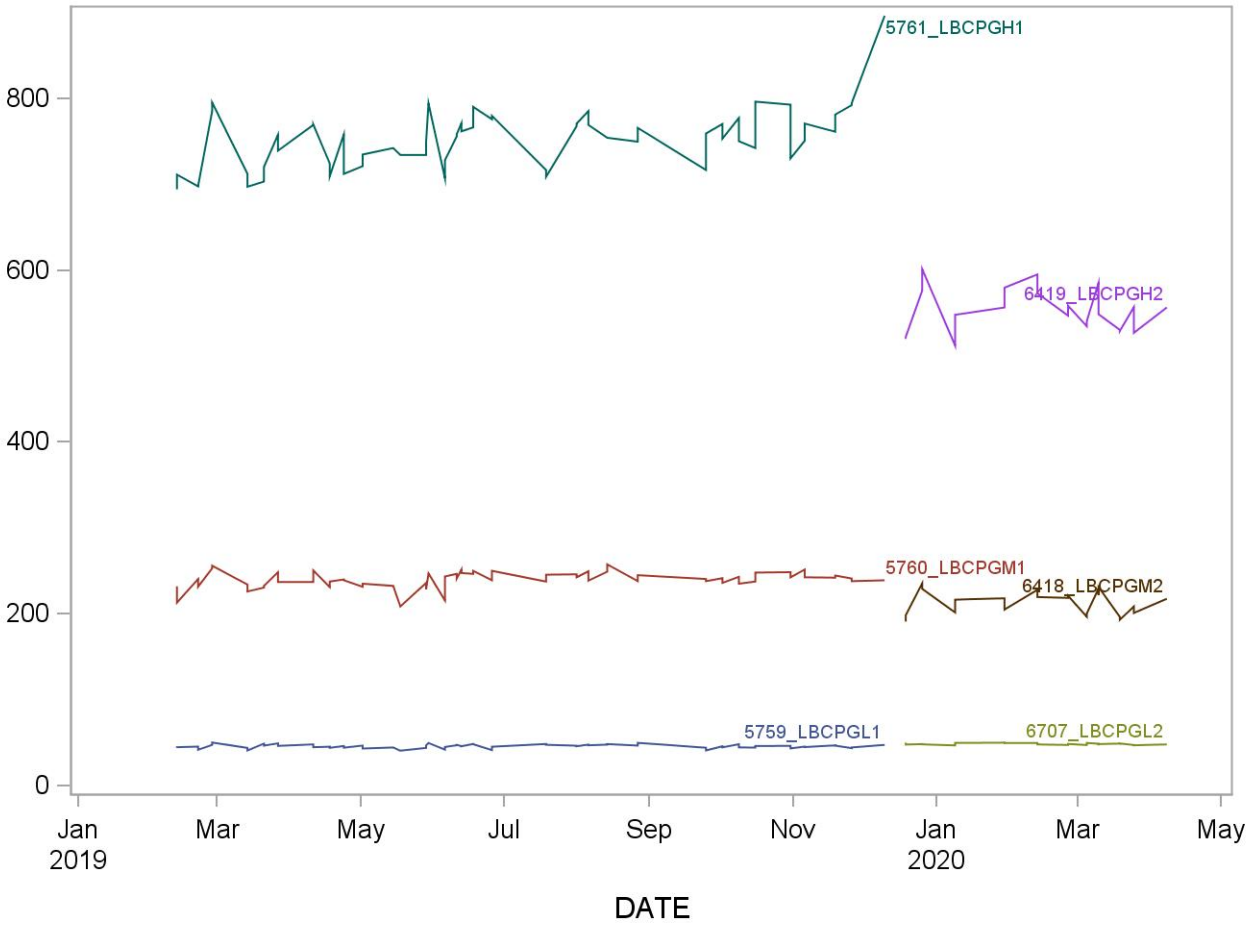
2019-2020 Summary Statistics and QC Chart
LBXEST (Estradiol (pg/mL))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
5761_LBCSOH5	62	12FEB19	10DEC19	849.4436	38.54677	4.5
5759_LBCSOL5	62	12FEB19	10DEC19	52.05242	2.61558	5.0
5760_LBCSOM5	62	12FEB19	10DEC19	270.6210	10.42329	3.9
6419_LBCSOH6	23	19DEC19	08APR20	725.5217	17.08234	2.4
6707_LBCSOL6	23	19DEC19	08APR20	66.10652	3.48697	5.3
6418_LBCSOM6	23	19DEC19	08APR20	287.4348	7.26027	2.5



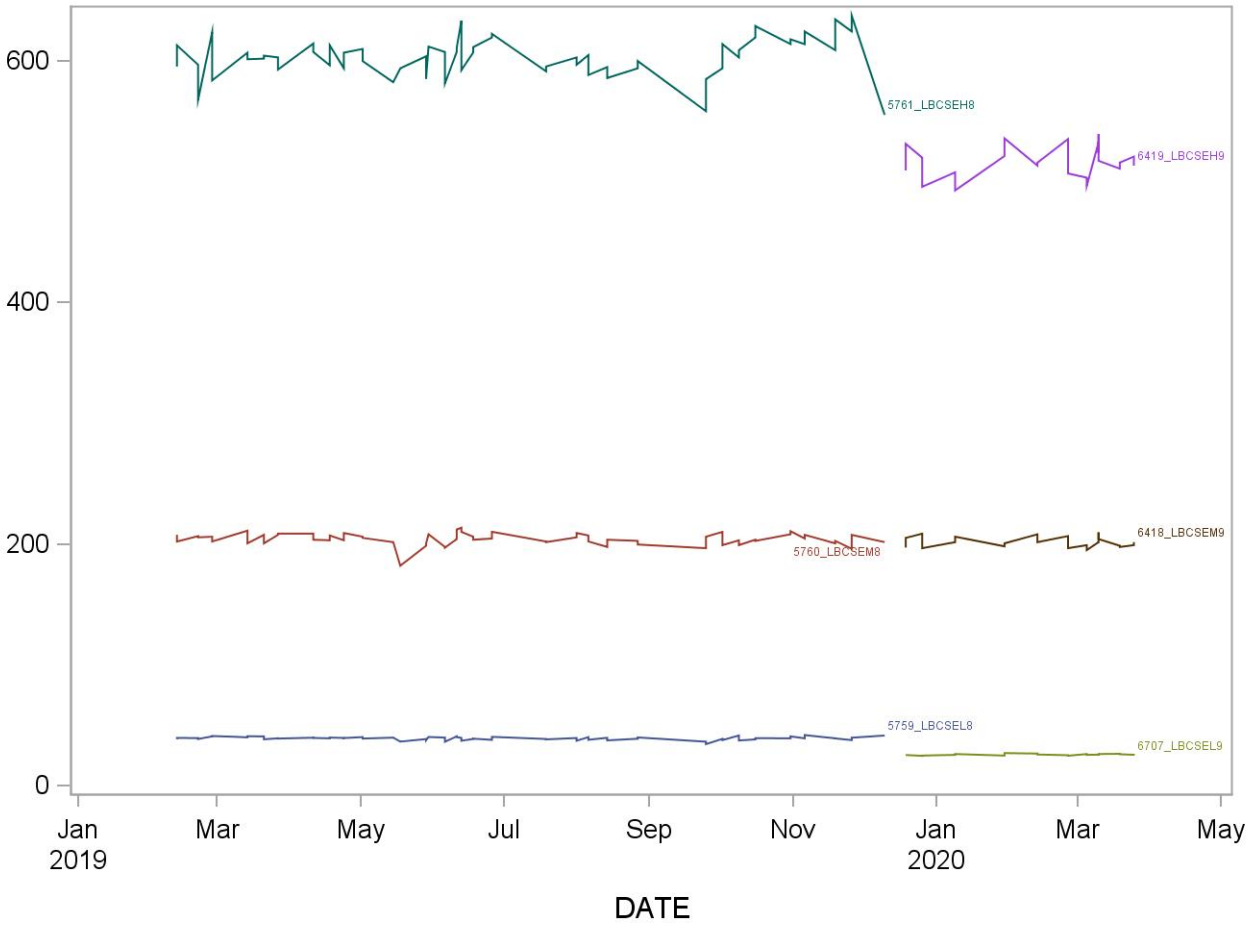
2019-2020 Summary Statistics and QC Chart
LBXPG4 (Progesterone (ng/dL))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
5761_LBCPGH1	62	12FEB19	10DEC19	751.9839	34.82014	4.6
5759_LBCPGL1	62	12FEB19	10DEC19	45.78629	2.26645	5.0
5760_LBCPGM1	62	12FEB19	10DEC19	239.9597	9.36478	3.9
6419_LBCPGH2	23	19DEC19	08APR20	553.8044	24.76606	4.5
6707_LBCPGL2	23	19DEC19	08APR20	48.45435	1.03495	2.1
6418_LBCPGM2	23	19DEC19	08APR20	213.2609	13.83085	6.5



2019-2020 Summary Statistics and QC Chart
LBXTST (Testosterone, total (ng/dL))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
5761_LBCSEH8	62	12FEB19	10DEC19	602.8226	16.32661	2.7
5759_LBCSEL8	62	12FEB19	10DEC19	39.08468	1.39365	3.6
5760_LBCSEM8	62	12FEB19	10DEC19	203.9758	4.98558	2.4
6419_LBCSEH9	22	19DEC19	25MAR20	516.0227	13.20937	2.6
6707_LBCSEL9	22	19DEC19	25MAR20	25.65227	0.64022	2.5
6418_LBCSEM9	22	19DEC19	25MAR20	201.4773	4.26636	2.1



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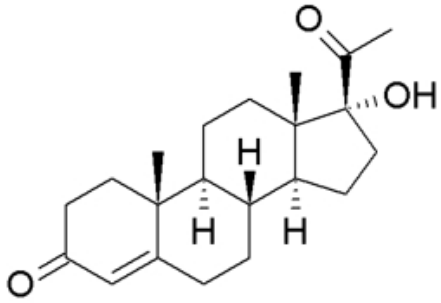
19 APPENDICES

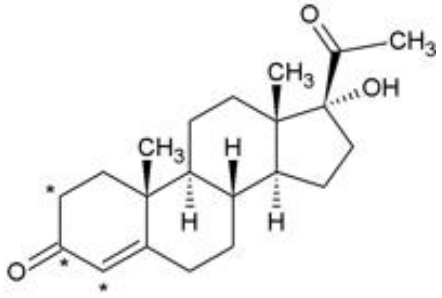
Appendix 1. Analyte Structures
Appendix 2. Hamilton Microlab STARlet Liquid Handler Deck Layout
Appendix 3. Example of Analytical Sequence
Appendix 4. Representative Sample Chromatogram
Appendix 5. Related Documents
Appendix 6. Symbols, Abbreviations, Terminology
Appendix 7. Document Compliance Tables
Appendix A. Method Performance Documentation

Appendix 1. Analyte Structures

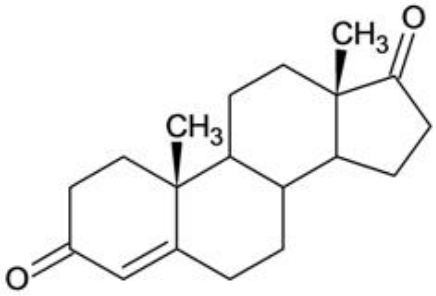
17α-Hydroxyprogesterone (17-OHP)
Chemical Formula: $C_{21}H_{30}O_3$
Molecular Weight: 330.46 g/mol



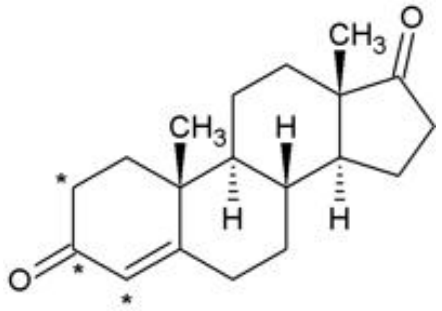
17α-Hydroxyprogesterone-2,3,4-$^{13}C_3$
Chemical Formula: $^{13}C_3C_{18}H_{30}O_3$
Molecular Weight: 333.44 g/mol



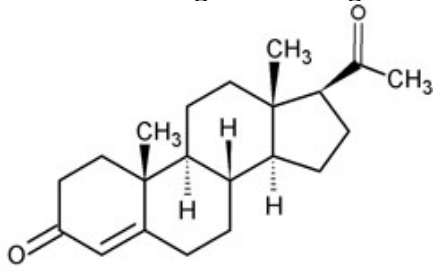
Androstenedione (AD)
Chemical Formula: $C_{19}H_{26}O_2$
Molecular Weight: 286.41 g/mol



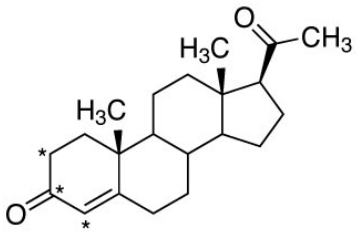
Androstene-3,17-dione-2,3,4-$^{13}C_3$
Chemical Formula: $^{13}C_3C_{16}H_{26}O_2$
Molecular Weight: 289.39 g/mol



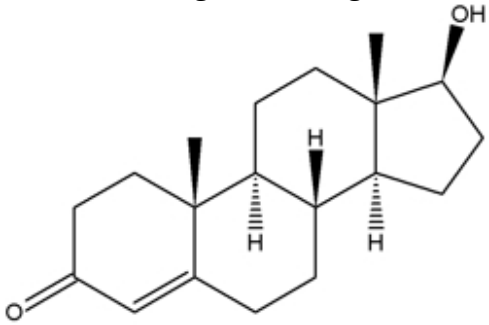
Progesterone (P4)
Chemical Formula: $C_{21}H_{30}O_2$
Molecular Weight: 314.46 g/mol



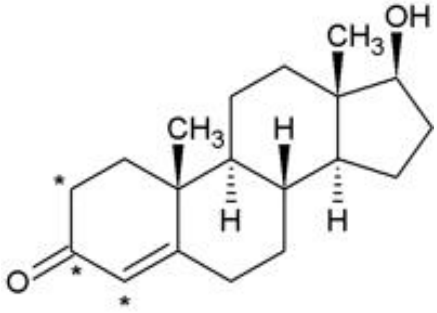
Progesterone-2,3,4-$^{13}C_3$
Chemical Formula: $^{13}C_3C_{18}H_{30}O_2$
Molecular Weight: 317.44 g/mol



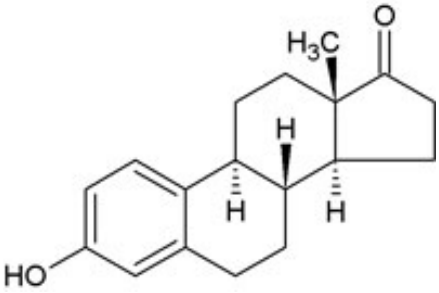
Testosterone (TT)
Chemical Formula: $C_{19}H_{28}O_2$
Molecular Weight: 288.42 g/mol



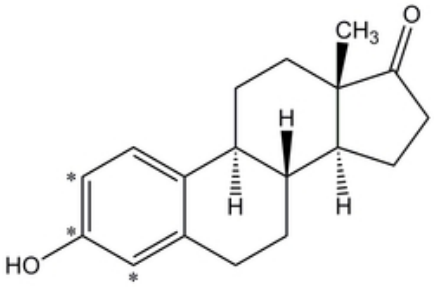
Testosterone-2,3,4-$^{13}C_3$
Chemical Formula: $^{13}C_3C_{16}H_{28}O_2$
Molecular Weight: 291.40 g/mol



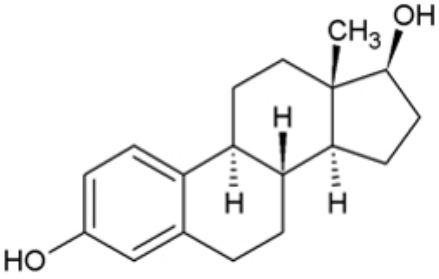
Estrone (E1)
Chemical Formula: $C_{18}H_{22}O_2$
Molecular Weight: 270.37 g/mol



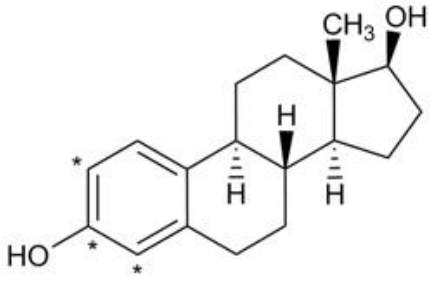
Estrone-2,3,4-$^{13}C_3$
Chemical Formula: $^{13}C_3C_{15}H_{22}O_2$
Molecular Weight: 273.34 g/mol



17β-Estradiol (E2)
Chemical Formula: $C_{18}H_{24}O_2$
Molecular Weight: 272.38 g/mol

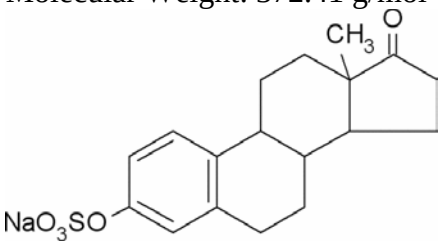


17β-Estradiol-2,3,4-$^{13}C_3$
Chemical Formula: $^{13}C_3C_{15}H_{24}O_2$
Molecular Weight: 275.36 g/mol



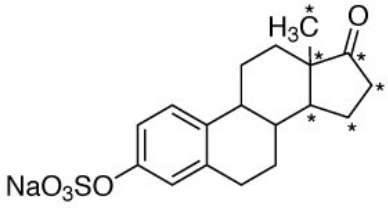
Estrone 3-sulfate sodium salt (E1S)

Chemical Formula: $C_{18}H_{21}O_5SNa$
Molecular Weight: 372.41 g/mol



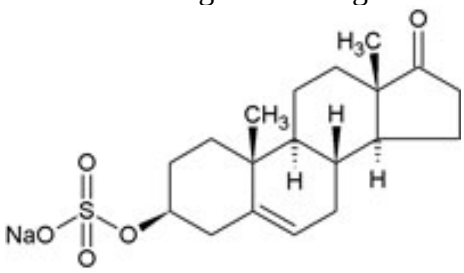
Estrone-13,14,15,16,17,18-$^{13}C_6$-3-sulfate sodium salt

Chemical Formula: $C_{18}D_4H_{17}O_5SNa$
Molecular Weight: 376.44 g/mol



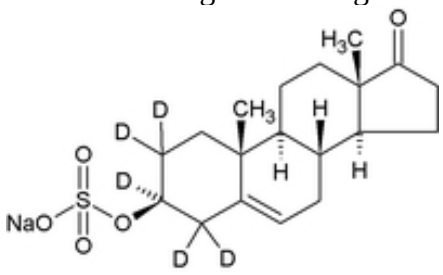
Dehydroepiandrosterone 3-sulfate sodium salt (DHEAS)

Chemical Formula: $C_{19}H_{27}O_5SNa$
Molecular Weight: 390.47 g/mol

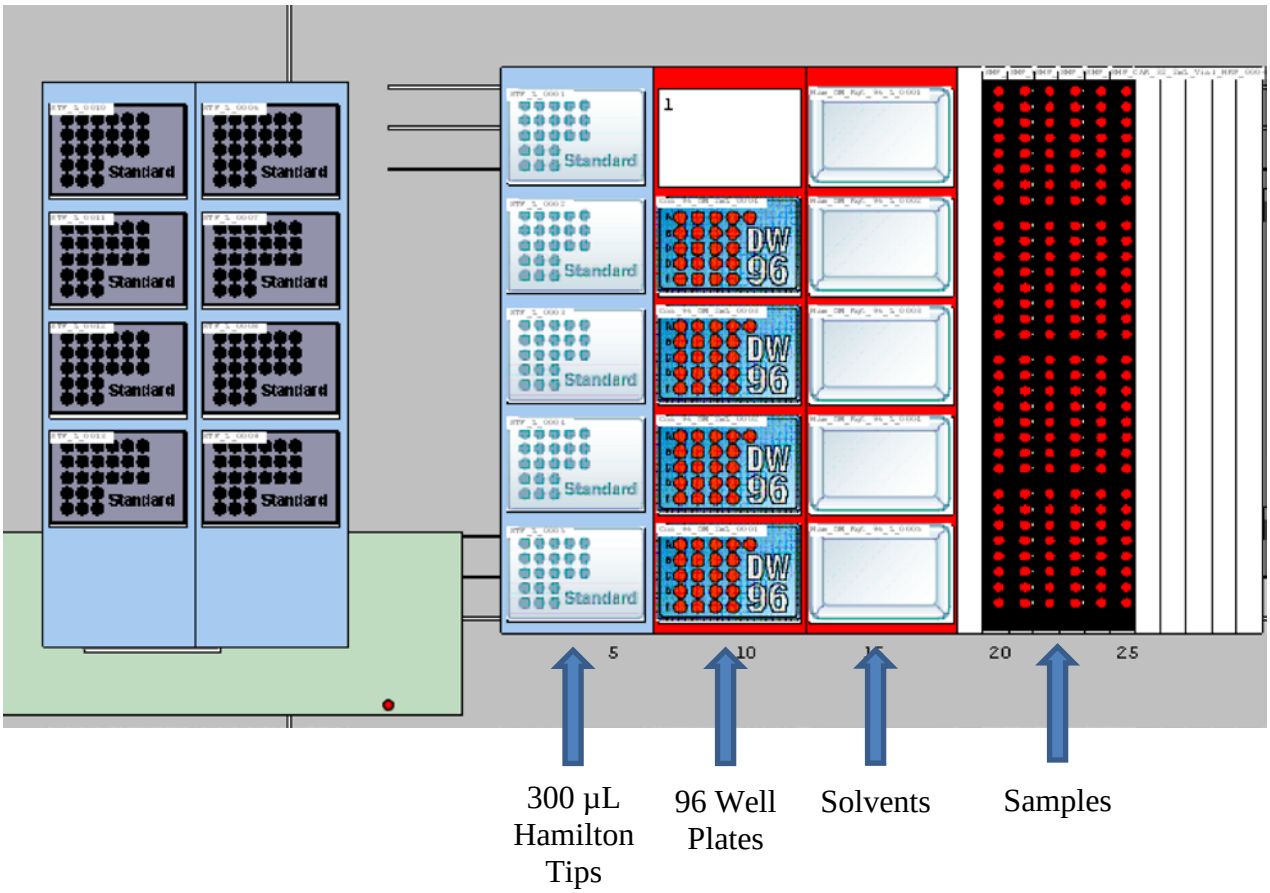


Dehydroepiandrosterone-2,2,3,4,4-d_5-3-sulfate sodium salt

Chemical Formula: $C_{19}D_5H_{22}O_5SNa$
Molecular Weight: 395.50 g/mol



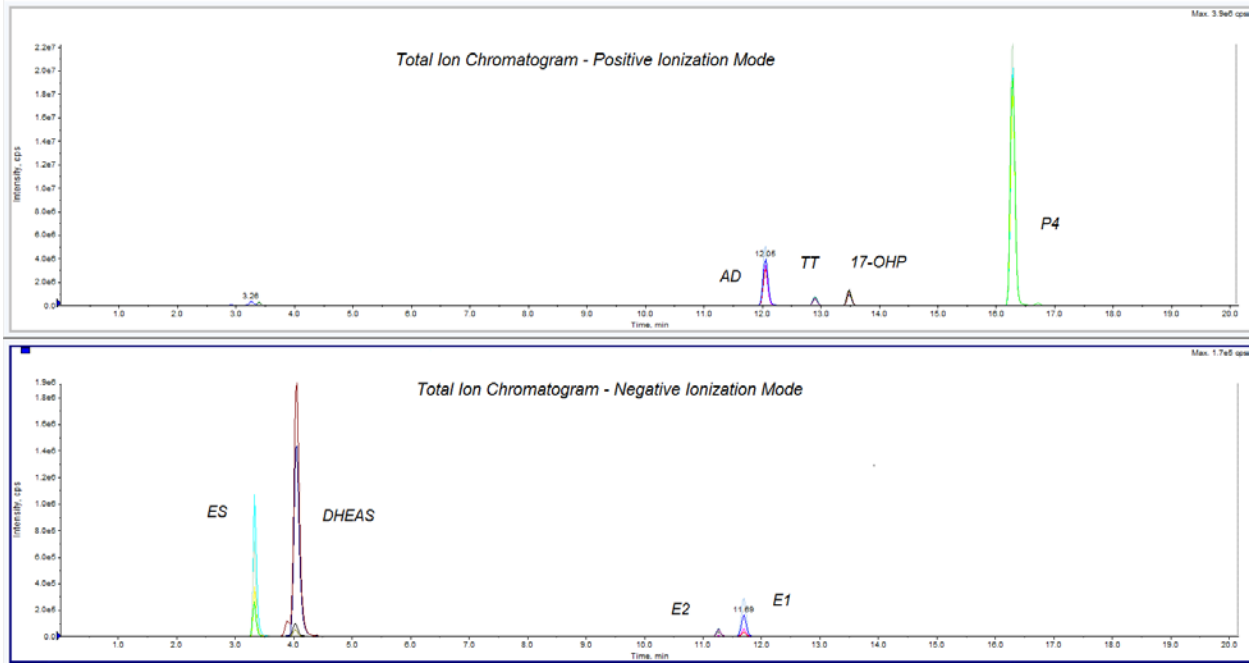
Appendix 2. Hamilton Microlab STARlet Liquid Handler Deck Layout



Appendix 3. Example of Analytical Sequence

Sample Name	AcqMethod	PlateCode	VialPos	SmplInjVol	RackPos	PlatePos	OutputFile
BL1	1036_112117_C2A_C1W	Deep Well MTP 96	1	50	1	3	11152017_HSP_065_001_301
BL2	1036_112117_C2A_C1W	Deep Well MTP 96	2	50	1	3	11152017_HSP_065_002_302
SSS_01	1036_112117_C2A_C1W	Deep Well MTP 96	85	50	1	3	11152017_HSP_065_003_385
BL3	1036_112117_C2A_C1W	Deep Well MTP 96	3	50	1	3	11152017_HSP_065_004_303
Double Blank	1036_112117_C2A_C1W	Deep Well MTP 96	1	50	1	1	11152017_HSP_065_005_101
SP_Saline	1036_112117_C2A_C1W	Deep Well MTP 96	13	50	1	1	11152017_HSP_065_006_113
A36C01L01	1036_112117_C2A_C1W	Deep Well MTP 96	25	50	1	1	11152017_HSP_065_007_125
A36C02L01	1036_112117_C2A_C1W	Deep Well MTP 96	37	50	1	1	11152017_HSP_065_008_137
A36C03L01	1036_112117_C2A_C1W	Deep Well MTP 96	49	50	1	1	11152017_HSP_065_009_149
A36C04L01	1036_112117_C2A_C1W	Deep Well MTP 96	61	50	1	1	11152017_HSP_065_010_161
A36C05L01	1036_112117_C2A_C1W	Deep Well MTP 96	73	50	1	1	11152017_HSP_065_011_173
A36C06L01	1036_112117_C2A_C1W	Deep Well MTP 96	85	50	1	1	11152017_HSP_065_012_185
BL4	1036_112117_C2A_C1W	Deep Well MTP 96	4	50	1	3	11152017_HSP_065_013_304
A36C07L01	1036_112117_C2A_C1W	Deep Well MTP 96	2	50	1	1	11152017_HSP_065_014_102
A36C08L01	1036_112117_C2A_C1W	Deep Well MTP 96	14	50	1	1	11152017_HSP_065_015_114
A36C09L01	1036_112117_C2A_C1W	Deep Well MTP 96	26	50	1	1	11152017_HSP_065_016_126
A36C10L01	1036_112117_C2A_C1W	Deep Well MTP 96	38	50	1	1	11152017_HSP_065_017_138
A36C11L01	1036_112117_C2A_C1W	Deep Well MTP 96	50	50	1	1	11152017_HSP_065_018_150
SP_Saline	1036_112117_C2A_C1W	Deep Well MTP 96	62	50	1	1	11152017_HSP_065_019_162
A36QC01L01	1036_112117_C2A_C1W	Deep Well MTP 96	74	50	1	1	11152017_HSP_065_020_174
A36QC02L01	1036_112117_C2A_C1W	Deep Well MTP 96	86	50	1	1	11152017_HSP_065_021_186
BL5	1036_112117_C2A_C1W	Deep Well MTP 96	5	50	1	3	11152017_HSP_065_022_305
A36QC03L01	1036_112117_C2A_C1W	Deep Well MTP 96	3	50	1	1	11152017_HSP_065_023_103
Double Blank	1036_112117_C2A_C1W	Deep Well MTP 96	95	50	1	1	11152017_HSP_065_024_195
BL14	1036_112117_C2A_C1W	Deep Well MTP 96	14	50	1	3	11152017_HSP_065_025_314
SP_Saline	1036_112117_C2A_C1W	Deep Well MTP 96	12	50	1	1	11152017_HSP_065_026_112
BL15	1036_112117_C2A_C1W	Deep Well MTP 96	15	50	1	3	11152017_HSP_065_027_315
SSS_02	1036_112117_C2A_C1W	Deep Well MTP 96	86	50	1	3	11152017_HSP_065_028_386
BL16	1036_112117_C2A_C1W	Deep Well MTP 96	16	50	1	3	11152017_HSP_065_029_316

Appendix 4. Representative Sample Chromatogram



Example total ion chromatogram for a female serum sample

Appendix 5. Related Documents

Normative References

DLS Policies and Procedures Manual. http://intranet.nceh.cdc.gov/dls/qaqc.aspx.
CDC Safety Policies and Practices Manual. http://isp-v-ehip-asp/dlsintranet/safety_manual/
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CLSI. Evaluation of the linearity of quantitative measurement procedures: A statistical approach. NCCLS document EP6. NCCLS, Wayne, PA, USA, 2003.
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.
International Organization for Standardization (ISO). General requirements for the competence of testing and calibration laboratories. ISO 17025:2003(E), ISO Geneva, Switzerland. 2003.
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.
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.
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

Symbols

Not applicable

Abbreviations

ACS	American Chemical Society
ANMI	Australian National Measurement Institute
ASTM	American Society for Testing and Material
CDC	Centers for Disease Control and Prevention
CC	Calibrators
CCB	Clinical Chemistry Branch
CLIA	Clinical Laboratory Improvement Act/Amendment
CV	Coefficient of Variation
DLS	Division of Laboratory Sciences
EMV	Electron Multiplier Voltage
ESI	Electrospray Ionization
FDA	Food and Drug Administration
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
ID	Identification
IS	Internal Standards
ISO	International Organization for Standardization
ITSO	Information Technology Services Office
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LIMS	Laboratory Information Management Systems
MRM	Multiple Reaction Monitoring
M/Z	Mass-to-Charge
N/A	Not Applicable
NCEH	National Center for Environmental Health
$(NH_4)CH_3COO$	Ammonium Acetate
$(NH_4)HCO_3$	Ammonium Bicarbonate
$(NH_4)OH$	Ammonium Hydroxide
NIST	National Institute of Standards and Technology
OHS	Occupational Health and Safety
PT	Proficiency Testing
QA	Quality Assurance
QC	Quality Control
SAS	Statistical Analysis Software
SD	Standard Deviation
SDS	Safety Data Sheets
SRM	Selected Reaction Monitoring

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	9
Remedial action to be taken when calibration or control results are outside acceptable limits	9.1.3
Limitation in methods, including interfering substances	8.6
Reference range (normal values)	10
Life-threatening or "panic values"	15
Pertinent literature references	17
Specimen storage criteria	5.2
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	9
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	8.6
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	11, 13, 15
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	4.1
Reference standards and reference materials required	4.3, 7.1.2
Environmental conditions required and any stabilization period needed	N/A
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	9
Data to be recorded and method of analysis and presentation	3, 6
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	Contents
Foreword	N/A
Warning and safety precautions	2
Introduction	1
Title	Title Page
Scope	1
Normative references	Appendix 5
Definitions	N/A
Symbols and abbreviations	Appendix 6
Terminology	Appendix 6
Principle and method of measurement	1
Check list	N/A
Reagents	4.2
Apparatus	4.1
Sampling and sample	6.1
Preparation of measuring system and analytical portion	6
Operation of measuring system	6
Data processing	6.5
Analytical reliability	8
Special cases	N/A
Validation by inter-laboratory studies	N/A
Reporting	11, 13, 15
Quality assurance	9
Bibliography (Annex)	17
Dates of authorization and revision	Page 1, Page 2

Appendix A. Method Performance Documentation

Table A1. LOD, Specificity and Fit for Intended Use

Analytes	Limit of Detection (LOD)	Interferences successfully checked in at least 50 human samples	Accuracy, precision, LOD, specificity and stability meet performance specifications for intended use
17-OHP	0.41 (ng/dL)	Yes	Yes
AD	0.82 (ng/dL)	Yes	Yes
P4	0.86 (ng/dL)	Yes	Yes
TT	0.57 (ng/dL)	Yes	Yes
E1	0.13 (ng/dL)	Yes	Yes
E2	1.72 (pg/mL)	Yes	Yes
E1S	2.04 (pg/mL)	Yes	Yes
DHEAS	0.22 (µg/dL)	Yes	Yes

Table A2. Accuracy Compared to Reference Material

Progesterone (P4)			Measured concentration (ng/dL)								
Reference material	Replicate	Nominal value	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	CV (%)	Difference from nominal value (%)
971F	1	194.96	200.5	200.1	200.4	201.8	196.1	198.70	2.58	1.30	1.9
	2		194.1	195.8	198.4	199.0	201.0				

Testosterone (TT)			Measured concentration (ng/dL)								
Reference material	Replicate	Nominal value	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	CV (%)	Difference from nominal value (%)
971F	1	27.69	27.9	27.7	29.9	29.1	28.1	29.03	1.22	4.20	4.8
	2		28.2	28.4	29.4	31.6	30.0				
971M	1	642.94	630.7	644.3	634.2	641.4	642.7	629.6	16.84	2.67	-2.1
	2		631.6	641.8	589.5	614.9	625.1				

Estradiol (E2)			Measured concentration (pg/mL)								
Reference material	Replicate	Nominal value	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	CV (%)	Difference from nominal value (%)
BCR576	1	31.05	29.7	29.6	30.3	32.4	31.6	30.99	1.17	3.79	-0.2
	2		30.3	30.3	31.1	31.4	33.2				
BCR577	1	187.96	178.9	187.3	181.6	190.8	198.3	186.8	7.08	3.79	-0.6
	2		189.0	194.3	190.6	177.9	179.1				
BCR578	1	365.02	365.3	358.1	372.4	352.1	352.9	364.5	11.52	3.16	-0.1
	2		381.9	357.1	360.1	360.4	384.7				

Table A3. Accuracy Using Spike Recovery

17-OHP		Sample 1					Sample 2				
	Replicate		Measured concentration (ng/dL)					Measured concentration (ng/dL)			
		Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)
Sample	1	0	20.03	21.29	21.2		0	21.75	20.04	20.4	
	2		23.03	21.60				20.54	23.26		
	3		21.80	19.51				18.49	18.13		
Sample + Spike 1	1	40	59.24	62.79	63.6	106.0	60	77.18	81.08	80.0	99.3
	2		65.60	73.00				84.14	86.31		
	3		57.54	63.36				76.19	74.86		
Sample + Spike 2	1	80	100.42	98.26	98.9	97.2	140	161.89	160.09	159.6	99.4
	2		98.16	103.00				164.18	171.25		
	3		94.77	99.08				154.53	145.43		
Sample + Spike 3	1	180	198.11	204.94	196	97.3	220	242.52	240.19	233	96.4
	2		214.83	193.28				248.46	226.65		
	3		180.98	185.83				214.71	222.59		
								Mean Recovery (%)			99.3
								SD (%)			3.5

AD		Sample 1					Sample 2				
	Replicate		Measured concentration (ng/dL)					Measured concentration (ng/dL)			
		Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)
Sample	1	0	43.89	44.52	43.52		0	41.04	45.69	43.46	
	2		43.48	41.97				44.31	44.05		
	3		43.77	43.47				43.25	42.46		
Sample + Spike 1	1	80	123.08	121.19	120.91	96.75	120	169.63	173.16	172.06	107.16
	2		125.82	115.37				181.14	174.59		
	3		120.27	119.76				163.93	169.90		
Sample + Spike 2	1	160	205.98	213.59	216.93	108.38	280	326.53	346.25	332.18	103.11
	2		217.80	218.29				327.80	341.98		
	3		210.57	235.34				323.68	326.83		
Sample + Spike 3	1	350	418.15	457.00	423.52	108.57	430	488.43	507.21	497.46	105.58
	2		437.57	410.45				507.79	492.93		
	3		414.80	403.14				464.56	523.83		
								Mean Recovery (%)			104.9
								SD (%)			4.5

P4		Sample 1					Sample 2				
	Replicate		Measured concentration (ng/dL)					Measured concentration (ng/dL)			
		Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)
Sample	1	0	120.06	121.07	123.58		0	134.74	136.28	134.6	
	2		124.93	122.34				137.07	133.63		
	3		128.10	124.98				130.96	135.14		
Sample + Spike 1	1	245	327.80	354.97	347.45	91.38	370	460.49	466.37	471.3	91.0
	2		349.99	347.18				464.01	480.46		
	3		354.53	350.24				483.06	473.53		
Sample + Spike 2	1	490	603.24	621.18	617.34	100.77	860	998.50	997.98	1021.8	103.2
	2		576.79	609.20				1,056.12	1,047.27		
	3		664.18	629.42				1,028.05	1,003.11		
Sample + Spike 3	1	1100	1,241.11	1,226.71	1,247.13	102.14	1350	1,429.56	1,434.91	1455	97.8
	2		1,314.08	1,172.66				1,542.83	1,439.18		
	3		1,259.00	1,269.21				1,434.59	1,448.42		
								Mean Recovery (%)			97.7
								SD (%)			5.4

TT		Sample 1					Sample 2				
	Replicate		Measured concentration (ng/dL)					Measured concentration (ng/dL)			
		Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)
Sample	1	0	94.14	96.58	97.5		0	98.23	100.71	100.5	
	2		105.02	97.73				99.03	104.03		
	3		96.40	94.90				98.91	101.94		
Sample + Spike 1	1	190	280.27	292.46	287.1	99.8	290	396.49	403.80	388.7	99.4
	2		288.83	285.29				405.65	370.13		
	3		286.85	288.85				376.66	379.36		
Sample + Spike 2	1	390	470.39	507.77	493.0	101.4	680	781.35	753.50	764.7	97.7
	2		492.28	506.70				762.44	804.55		
	3		497.63	483.45				743.46	742.99		
Sample + Spike 3	1	870	995.52	1,017.33	980	101.4	1060	1,171.25	1,184.08	1165	100.4
	2		1,014.52	970.65				1,216.69	1,124.82		
	3		921.22	960.16				1,147.46	1,143.14		
								Mean Recovery (%)			100.0
								SD (%)			1.4

E1		Sample 1					Sample 2				
	Replicate		Measured concentration (ng/dL)					Measured concentration (ng/dL)			
		Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)
Sample	1	0	8.55	8.66	8.3		0	7.77	8.24	8.1	
	2		8.78	7.86				7.71	8.32		
	3		8.00	8.03				7.99	8.57		
Sample + Spike 1	1	15	22.39	22.39	22.9	97.2	20	31.02	29.26	30.3	111.0
	2		23.64	23.07				31.10	30.95		
	3		23.73	22.12				29.98	29.43		
Sample + Spike 2	1	30	39.41	38.10	38.4	100.1	50	60.15	60.47	60.4	104.7
	2		38.45	39.36				61.62	60.91		
	3		38.86	35.97				58.76	60.78		
Sample + Spike 3	1	70	77.31	77.76	77	98.3	80	89.25	92.59	93	105.9
	2		76.85	77.99				98.23	94.87		
	3		76.45	76.44				91.40	90.62		
								Mean Recovery (%)			102.9
								SD (%)			5.3

E2		Sample 1					Sample 2				
	Replicate		Measured concentration (pg/mL)					Measured concentration (pg/mL)			
		Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)
Sample	1	0	129.17	132.40	128.6		0	127.88	135.18	131.6	
	2		125.72	113.44				142.73	130.74		
	3		134.19	136.45				133.79	119.49		
Sample + Spike 1	1	260	384.01	400.96	393.7	102.0	390	544.49	540.53	521.7	100.0
	2		385.53	380.20				511.49	522.71		
	3		410.62	400.65				506.61	504.64		
Sample + Spike 2	1	525	684.90	700.26	663.7	101.9	920	1,054.71	1,066.52	1056.3	100.5
	2		632.25	647.95				1,025.07	1,075.29		
	3		640.02	676.72				1,048.85	1,067.28		
Sample + Spike 3	1	1180	1,338.11	1,298.95	1319	100.9	1440	1,529.68	1,555.50	1534	97.4
	2		1,304.33	1,344.46				1,485.58	1,535.60		
	3		1,350.96	1,280.17				1,530.02	1,567.08		
								Mean Recovery (%)			100.5
								SD (%)			1.7

E1S		Sample 1					Sample 2				
	Replicate		Measured concentration (pg/mL)					Measured concentration (pg/mL)			
		Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)
Sample	1	0	125.40	98.27	106.5		0	110.01	92.82	97.7	
	2		90.73	111.79				88.55	107.70		
	3		99.29	113.40				91.40	95.84		
Sample + Spike 1	1	200	294.03	318.65	316.3	104.9	300	449.76	468.76	430.2	110.8
	2		304.57	282.19				458.70	424.95		
	3		371.11	327.28				403.57	375.72		
Sample + Spike 2	1	400	560.41	504.29	489.2	95.7	700	812.67	892.69	798.3	100.1
	2		456.78	460.76				750.46	801.21		
	3		451.17	501.50				740.61	791.90		
Sample + Spike 3	1	900	974.48	1,016.28	968	95.8	1100	1,209.45	1,175.45	1203	100.5
	2		970.31	978.46				1,183.03	1,205.98		
	3		932.14	938.54				1,267.80	1,177.18		
								Mean Recovery (%)			101.3
								SD (%)			5.8

DHEAS		Sample 1					Sample 2				
	Replicate		Measured concentration (µg/dL)					Measured concentration (µg/dL)			
		Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)
Sample	1	0	21.48	22.89	22.6		0	21.14	24.13	21.8	
	2		20.83	22.70				20.49	21.46		
	3		24.38	23.36				21.65	22.08		
Sample + Spike 1	1	40	64.32	61.28	63.4	102.0	60	83.09	83.41	81.3	99.2
	2		61.00	64.47				83.09	85.47		
	3		59.99	69.38				76.59	76.39		
Sample + Spike 2	1	80	103.60	106.72	101.8	98.9	140	142.44	155.77	152.2	93.1
	2		96.68	105.75				151.99	157.82		
	3		95.54	102.22				148.11	157.21		
Sample + Spike 3	1	180	190.76	188.73	192	93.9	220	205.95	210.08	217	88.8
	2		194.43	186.78				208.07	206.57		
	3		194.30	194.56				239.41	233.51		
								Mean Recovery (%)			96.0
								SD (%)			4.9

Table A4. Precision

Quality material low – 17-OHP						
Run	**Result 1 (ng/dL)**	**Result 2 (ng/dL)**	**Mean (ng/dL)**	**SS 1**	**SS 2**	**2*mean^2**
1	6.31	7.00	6.66	0.12	0.12	88.67
2	9.23	7.39	8.31	0.85	0.85	138.14
3	6.38	7.08	6.73	0.12	0.12	90.59
4	9.99	8.41	9.20	0.62	0.62	169.36
5	8.70	8.44	8.57	0.02	0.02	146.84
6	8.66	6.84	7.75	0.83	0.83	120.24
7	7.78	7.68	7.73	0.00	0.00	119.53
8	9.21	9.19	9.20	0.00	0.00	169.23
9	8.83	9.43	9.13	0.09	0.09	166.68
10	7.93	7.96	7.95	0.00	0.00	126.27
Grand sum	162.46	**Grand mean**	8.12			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	5.30	0.53	0.73	8.96
		Between Run	15.95	1.77	0.79	9.70
		Total	21.25		1.07	**13.21**
Quality material medium – 17-OHP						
Run	**Result 1 (ng/dL)**	**Result 2 (ng/dL)**	**Mean (ng/dL)**	**SS 1**	**SS 2**	**2*mean^2**
1	43.95	37.62	40.79	10.00	10.00	3,327.14
2	38.79	40.35	39.57	0.61	0.61	3,131.33
3	41.23	35.72	38.47	7.59	7.59	2,960.24
4	43.51	39.36	41.44	4.31	4.31	3,434.10
5	40.46	36.72	38.59	3.50	3.50	2,978.29
6	41.00	46.85	43.92	8.55	8.55	3,858.40
7	42.62	35.90	39.26	11.30	11.30	3,082.94
8	41.41	38.42	39.91	2.24	2.24	3,186.00
9	43.15	37.20	40.18	8.86	8.86	3,228.31
10	40.68	35.76	38.22	6.05	6.05	2,921.47
Grand sum	800.69	**Grand mean**	40.03			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	126.01	12.60	3.55	8.87
		Between Run	52.65	5.85	0.00	0.00
		Total	178.65		3.55	**8.87**
Quality material high – 17-OHP						
Run	**Result 1 (ng/dL)**	**Result 2 (ng/dL)**	**Mean (ng/dL)**	**SS 1**	**SS 2**	**2*mean^2**
1	113.35	114.74	114.05	0.48	0.48	26,013.22
2	120.57	121.32	120.95	0.14	0.14	29,256.33
3	120.81	129.31	125.06	18.06	18.06	31,279.75
4	111.28	113.46	112.37	1.19	1.19	25,253.28
5	111.98	117.18	114.58	6.74	6.74	26,256.66
6	119.51	122.24	120.88	1.86	1.86	29,222.92
7	116.79	118.91	117.85	1.12	1.12	27,778.43
8	122.07	130.31	126.19	16.98	16.98	31,848.16
9	133.24	125.12	129.18	16.48	16.48	33,375.87
10	118.82	114.63	116.72	4.39	4.39	27,247.74
Grand sum	2,395.65	**Grand mean**	119.78			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	134.88	13.49	3.67	3.07
		Between Run	575.72	63.97	5.02	4.19
		Total	710.59		6.22	**5.20**

Quality material low – AD						
Run	**Result 1 (ng/dL)**	**Result 2 (ng/dL)**	**Mean (ng/dL)**	**SS 1**	**SS 2**	**2*mean^2**
1	15.71	16.03	15.87	0.03	0.03	503.88
2	15.68	16.69	16.18	0.26	0.26	523.70
3	15.57	14.50	15.03	0.28	0.28	452.02
4	15.61	15.25	15.43	0.03	0.03	476.24
5	15.04	15.80	15.42	0.14	0.14	475.34
6	15.88	17.01	16.44	0.32	0.32	540.83
7	12.96	13.58	13.27	0.10	0.10	352.30
8	15.93	16.84	16.39	0.21	0.21	537.02
9	15.47	14.98	15.22	0.06	0.06	463.59
10	16.08	15.35	15.72	0.13	0.13	494.10
Grand sum	309.96	**Grand mean**	15.50			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	3.11	0.31	0.56	3.60
		Between Run	15.19	1.69	0.83	5.36
		Total	18.30		1.00	**6.45**

Quality material medium – AD						
Run	**Result 1 (ng/dL)**	**Result 2 (ng/dL)**	**Mean (ng/dL)**	**SS 1**	**SS 2**	**2*mean^2**
1	78.59	85.05	81.82	10.44	10.44	13,388.01
2	84.23	76.27	80.25	15.86	15.86	12,880.10
3	75.14	81.89	78.51	11.39	11.39	12,327.75
4	83.41	85.21	84.31	0.80	0.80	14,216.75
5	85.52	82.98	84.25	1.61	1.61	14,195.82
6	85.70	85.10	85.40	0.09	0.09	14,586.84
7	85.59	87.70	86.64	1.11	1.11	15,013.43
8	90.03	87.44	88.73	1.68	1.68	15,747.73
9	90.24	82.80	86.52	13.82	13.82	14,972.16
10	85.79	85.09	85.44	0.12	0.12	14,601.15
Grand sum	1,683.76	**Grand mean**	84.19			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	113.84	11.38	3.37	4.01
		Between Run	177.15	19.68	2.04	2.42
		Total	290.99		3.94	**4.68**

Quality material high – AD						
Run	**Result 1 (ng/dL)**	**Result 2 (ng/dL)**	**Mean (ng/dL)**	**SS 1**	**SS 2**	**2*mean^2**
1	240.24	249.94	245.09	23.50	23.50	120,139.12
2	254.29	260.00	257.14	8.17	8.17	132,246.51
3	253.52	259.31	256.41	8.40	8.40	131,496.54
4	262.45	274.75	268.60	37.82	37.82	144,291.41
5	289.48	279.90	284.69	22.95	22.95	162,095.71
6	291.36	292.68	292.02	0.43	0.43	170,549.88
7	260.34	270.44	265.39	25.53	25.53	140,864.72
8	286.32	279.48	282.90	11.69	11.69	160,068.36
9	297.44	290.96	294.20	10.51	10.51	173,106.58
10	280.45	274.11	277.28	10.05	10.05	153,770.27
Grand sum	5,447.46	**Grand mean**	272.37			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	318.11	31.81	5.64	2.07
		Between Run	4,885.80	542.87	15.99	5.87
		Total	5,203.91		16.95	**6.22**

Quality material low – P4						
Run	**Result 1 (ng/dL)**	**Result 2 (ng/dL)**	**Mean (ng/dL)**	**SS 1**	**SS 2**	**2*mean^2**

1	50.11	40.98	45.54	20.84	20.84	4,148.62
2	44.32	43.61	43.96	0.12	0.12	3,865.59
3	52.48	36.14	44.31	66.73	66.73	3,926.31
4	46.11	48.07	47.09	0.97	0.97	4,434.96
5	43.17	46.03	44.60	2.05	2.05	3,977.54
6	48.11	50.52	49.32	1.46	1.46	4,864.25
7	39.55	34.36	36.96	6.71	6.71	2,731.34
8	53.38	49.29	51.33	4.19	4.19	5,270.54
9	48.04	47.63	47.84	0.04	0.04	4,576.93
10	51.10	44.35	47.72	11.38	11.38	4,554.80
Grand sum	917.34	**Grand mean**	45.87			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	228.97	22.90	4.79	10.43
		Between Run	275.64	30.63	1.97	4.29
		Total	504.61		5.17	**11.28**

Quality material medium – P4

Run	**Result 1 (ng/dL)**	**Result 2 (ng/dL)**	**Mean (ng/dL)**	**SS 1**	**SS 2**	**2*mean^2**
1	268.52	228.09	248.30	408.67	408.67	123,309.15
2	248.46	249.32	248.89	0.18	0.18	123,891.81
3	231.43	207.79	219.61	139.73	139.73	96,459.14
4	208.48	243.13	225.81	300.13	300.13	101,977.06
5	239.38	229.13	234.26	26.25	26.25	109,752.40
6	217.76	219.90	218.83	1.15	1.15	95,774.22
7	231.52	213.08	222.30	84.99	84.99	98,834.99
8	234.35	239.48	236.92	6.59	6.59	112,257.75
9	218.15	231.57	224.86	45.03	45.03	101,127.86
10	229.44	237.30	233.37	15.43	15.43	108,920.75
Grand sum	4,626.29	**Grand mean**	231.31			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	2,056.31	205.63	14.34	6.20
		Between Run	2,175.42	241.71	4.25	1.84
		Total	4,231.72		14.96	**6.47**

Quality material high – P4

Run	**Result 1 (ng/dL)**	**Result 2 (ng/dL)**	**Mean (ng/dL)**	**SS 1**	**SS 2**	**2*mean^2**
1	753.07	740.61	746.84	38.78	38.78	1,115,533.56
2	746.82	737.95	742.39	19.70	19.70	1,102,274.32
3	765.22	722.33	743.77	459.80	459.80	1,106,401.89
4	725.26	807.57	766.42	1,693.74	1,693.74	1,174,791.80
5	766.65	785.19	775.92	86.01	86.01	1,204,103.52
6	716.65	849.85	783.25	4,435.32	4,435.32	1,226,957.13
7	694.98	744.25	719.61	606.92	606.92	1,035,681.24
8	898.77	788.12	843.44	3,061.35	3,061.35	1,422,798.90
9	880.28	917.56	898.92	347.58	347.58	1,616,113.74
10	781.56	848.05	814.81	1,105.08	1,105.08	1,327,822.23
Grand sum	15,670.74	**Grand mean**	783.54			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	23,708.57	2,370.86	48.69	6.21
		Between Run	53,877.83	5,986.43	42.52	5.43
		Total	77,586.40		64.64	**8.25**

Quality material low – TT						
Run	**Result 1 (ng/dL)**	**Result 2 (ng/dL)**	**Mean (ng/dL)**	**SS 1**	**SS 2**	**2*mean^2**
1	44.52	36.22	40.37	17.22	17.22	3,259.00
2	39.01	41.47	40.24	1.51	1.51	3,238.50
3	35.24	34.66	34.95	0.08	0.08	2,442.91
4	36.23	40.89	38.56	5.44	5.44	2,973.34
5	38.48	39.85	39.17	0.47	0.47	3,068.10
6	45.32	42.71	44.01	1.71	1.71	3,874.53
7	36.96	37.98	37.47	0.26	0.26	2,808.28
8	40.75	42.04	41.40	0.42	0.42	3,427.51
9	42.10	40.34	41.22	0.77	0.77	3,398.01
10	40.70	41.82	41.26	0.31	0.31	3,405.26
Grand sum	797.29	**Grand mean**	39.86			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	56.40	5.64	2.37	5.96
		Between Run	111.67	12.41	1.84	4.61
		Total	168.07		3.00	**7.54**

Quality material medium – TT						
Run	**Result 1 (ng/dL)**	**Result 2 (ng/dL)**	**Mean (ng/dL)**	**SS 1**	**SS 2**	**2*mean^2**
1	178.22	179.10	178.66	0.19	0.19	63,837.59
2	210.89	209.31	210.10	0.63	0.63	88,283.01
3	214.61	188.71	201.66	167.63	167.63	81,334.86
4	196.61	207.82	202.21	31.41	31.41	81,780.34
5	208.94	194.26	201.60	53.89	53.89	81,285.97
6	211.86	199.72	205.79	36.81	36.81	84,698.14
7	188.72	202.50	195.61	47.48	47.48	76,523.89
8	217.57	216.00	216.79	0.61	0.61	93,992.84
9	214.38	186.45	200.41	195.10	195.10	80,331.17
10	211.67	212.80	212.24	0.32	0.32	90,087.86
Grand sum	4,050.13	**Grand mean**	202.51			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	1,068.14	106.81	10.34	5.10
		Between Run	1,978.67	219.85	7.52	3.71
		Total	3,046.82		12.78	**6.31**

Quality material high – TT						
Run	**Result 1 (ng/dL)**	**Result 2 (ng/dL)**	**Mean (ng/dL)**	**SS 1**	**SS 2**	**2*mean^2**
1	539.92	501.76	520.84	364.02	364.02	542,546.40
2	623.22	567.02	595.12	789.54	789.54	708,341.97
3	583.11	602.29	592.70	91.96	91.96	702,579.32
4	575.26	567.23	571.25	16.12	16.12	652,646.05
5	601.27	582.96	592.12	83.80	83.80	701,208.66
6	605.50	611.74	608.62	9.76	9.76	740,837.37
7	629.15	629.61	629.38	0.05	0.05	792,233.57
8	620.85	619.57	620.21	0.41	0.41	769,315.07
9	655.06	641.47	648.26	46.19	46.19	840,490.15
10	662.58	599.54	631.06	993.60	993.60	796,472.06
Grand sum	12,019.11	**Grand mean**	600.96			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	4,790.89	479.09	21.89	3.64
		Between Run	23,726.16	2,636.24	32.84	5.46
		Total	28,517.05		39.47	**6.57**

Quality material low – E1						
Run	**Result 1 (ng/dL)**	**Result 2 (ng/dL)**	**Mean (ng/dL)**	**SS 1**	**SS 2**	**2*mean^2**
1	3.10	2.54	2.82	0.08	0.08	15.93
2	3.44	2.66	3.05	0.15	0.15	18.61
3	3.28	3.06	3.17	0.01	0.01	20.13
4	3.09	3.22	3.16	0.00	0.00	19.93
5	3.11	3.44	3.28	0.03	0.03	21.50
6	3.03	3.03	3.03	0.00	0.00	18.36
7	3.14	3.56	3.35	0.04	0.04	22.43
8	3.61	3.31	3.46	0.02	0.02	23.98
9	3.32	2.86	3.09	0.05	0.05	19.05
10	3.49	3.00	3.25	0.06	0.06	21.06
Grand sum	63.31	**Grand mean**	3.17			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	0.90	0.09	0.30	9.50
		Between Run	0.59	0.07	0.00	0.00
		Total	1.50		0.30	**9.50**

Quality material medium – E1						
Run	**Result 1 (ng/dL)**	**Result 2 (ng/dL)**	**Mean (ng/dL)**	**SS 1**	**SS 2**	**2*mean^2**
1	14.93	15.97	15.45	0.27	0.27	477.29
2	15.74	15.46	15.60	0.02	0.02	486.57
3	16.44	16.23	16.33	0.01	0.01	533.53
4	16.86	15.80	16.33	0.28	0.28	533.25
5	15.29	16.73	16.01	0.51	0.51	512.70
6	16.86	15.22	16.04	0.68	0.68	514.65
7	17.98	17.71	17.85	0.02	0.02	637.06
8	17.71	16.44	17.07	0.41	0.41	583.09
9	16.41	16.77	16.59	0.03	0.03	550.36
10	16.72	15.44	16.08	0.41	0.41	517.38
Grand sum	326.71	**Grand mean**	16.34			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	5.28	0.53	0.73	4.45
		Between Run	8.97	1.00	0.48	2.96
		Total	14.25		0.87	**5.34**

Quality material high – E1						
Run	**Result 1 (ng/dL)**	**Result 2 (ng/dL)**	**Mean (ng/dL)**	**SS 1**	**SS 2**	**2*mean^2**
1	43.93	44.41	44.17	0.06	0.06	3,902.10
2	44.45	43.82	44.13	0.10	0.10	3,895.52
3	40.74	44.07	42.41	2.76	2.76	3,596.50
4	46.86	44.87	45.86	0.98	0.98	4,206.94
5	45.15	47.11	46.13	0.97	0.97	4,255.83
6	45.10	45.23	45.16	0.00	0.00	4,079.26
7	48.41	48.18	48.30	0.01	0.01	4,665.28
8	49.52	47.65	48.59	0.87	0.87	4,721.36
9	45.70	44.90	45.30	0.16	0.16	4,104.61
10	45.78	43.26	44.52	1.60	1.60	3,963.76
Grand sum	909.14	**Grand mean**	45.46			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	15.03	1.50	1.23	2.70
		Between Run	64.38	7.15	1.68	3.70
		Total	79.41		2.08	**4.58**

Quality material low – E2						
Run	**Result 1 (pg/mL)**	**Result 2 (pg/mL)**	**Mean (pg/mL)**	**SS 1**	**SS 2**	**2*mean^2**
1	47.06	59.77	53.42	40.40	40.40	5,706.62
2	53.07	50.56	51.82	1.58	1.58	5,369.88
3	47.24	46.86	47.05	0.04	0.04	4,427.50
4	49.83	55.93	52.88	9.32	9.32	5,592.24
5	48.93	51.33	50.13	1.43	1.43	5,025.76
6	47.13	44.40	45.77	1.85	1.85	4,189.15
7	51.66	58.28	54.97	10.95	10.95	6,042.85
8	51.58	53.84	52.71	1.28	1.28	5,556.59
9	52.54	55.43	53.99	2.08	2.08	5,829.22
10	48.29	48.77	48.53	0.06	0.06	4,710.48
Grand sum	1,022.50	**Grand mean**	51.13			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	137.97	13.80	3.71	7.27
		Between Run	174.61	19.40	1.67	3.27
		Total	312.59		4.07	**7.97**

Quality material medium – E2						
Run	**Result 1 (pg/mL)**	**Result 2 (pg/mL)**	**Mean (pg/mL)**	**SS 1**	**SS 2**	**2*mean^2**
1	234.09	217.86	225.97	65.83	65.83	102,126.77
2	276.92	253.84	265.38	133.21	133.21	140,853.23
3	276.09	233.82	254.96	446.50	446.50	130,004.11
4	233.79	231.12	232.46	1.78	1.78	108,073.20
5	203.74	262.15	232.95	853.00	853.00	108,527.02
6	254.57	238.10	246.34	67.77	67.77	121,362.05
7	284.66	276.78	280.72	15.53	15.53	157,606.72
8	283.73	227.86	255.79	780.49	780.49	130,861.17
9	285.32	278.74	282.03	10.82	10.82	159,079.19
10	238.07	260.69	249.38	127.89	127.89	124,382.04
Grand sum	5,051.94	**Grand mean**	252.60			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	5,005.65	500.57	22.37	8.86
		Between Run	6,772.87	752.54	11.22	4.44
		Total	11,778.53		25.03	**9.91**

Quality material high – E2						
Run	**Result 1 (pg/mL)**	**Result 2 (pg/mL)**	**Mean (pg/mL)**	**SS 1**	**SS 2**	**2*mean^2**
1	700.66	621.77	661.21	1,555.79	1,555.79	874,405.52
2	760.36	768.36	764.36	15.97	15.97	1,168,494.99
3	804.30	760.51	782.40	479.47	479.47	1,224,309.24
4	794.64	805.00	799.82	26.81	26.81	1,279,433.40
5	751.63	744.20	747.91	13.79	13.79	1,118,749.08
6	797.40	717.74	757.57	1,586.62	1,586.62	1,147,823.80
7	911.92	938.39	925.15	175.13	175.13	1,711,815.78
8	782.33	736.92	759.62	515.54	515.54	1,154,048.77
9	860.21	815.80	838.01	493.05	493.05	1,404,514.05
10	820.46	811.36	815.91	20.70	20.70	1,331,414.25
Grand sum	15,703.95	**Grand mean**	785.20			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	9,765.77	976.58	31.25	3.98
		Between Run	84,310.34	9,367.82	64.77	8.25
		Total	94,076.11		71.92	**9.16**

Quality material low – E1S						
Run	**Result 1 (pg/mL)**	**Result 2 (pg/mL)**	**Mean (pg/mL)**	**SS 1**	**SS 2**	**2*mean^2**
1	41.97	49.95	45.96	15.95	15.95	4,224.44
2	42.73	42.42	42.57	0.02	0.02	3,625.05
3	39.72	41.17	40.45	0.53	0.53	3,271.93
4	43.93	38.70	41.32	6.83	6.83	3,414.52
5	46.27	49.36	47.82	2.40	2.40	4,572.60
6	44.19	42.23	43.21	0.96	0.96	3,734.53
7	45.63	42.15	43.89	3.02	3.02	3,852.67
8	44.00	40.30	42.15	3.43	3.43	3,553.60
9	47.62	43.00	45.31	5.33	5.33	4,105.88
10	38.34	46.94	42.64	18.50	18.50	3,636.80
Grand sum	870.64	**Grand mean**	43.53			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	113.93	11.39	3.38	7.75
		Between Run	91.31	10.15	0.00	0.00
		Total	205.23		3.38	**7.75**

Quality material medium – E1S						
Run	**Result 1 (pg/mL)**	**Result 2 (pg/mL)**	**Mean (pg/mL)**	**SS 1**	**SS 2**	**2*mean^2**
1	153.39	211.44	182.41	842.27	842.27	66,549.80
2	216.65	210.28	213.47	10.16	10.16	91,136.34
3	203.66	194.38	199.02	21.55	21.55	79,215.90
4	225.81	222.60	224.20	2.58	2.58	100,533.25
5	212.70	201.72	207.21	30.13	30.13	85,869.44
6	217.67	217.64	217.65	0.00	0.00	94,744.20
7	168.98	175.22	172.10	9.76	9.76	59,237.20
8	205.51	178.24	191.87	185.91	185.91	73,629.83
9	194.07	230.30	212.19	328.15	328.15	90,046.30
10	187.02	157.10	172.06	223.86	223.86	59,209.55
Grand sum	3,984.36	**Grand mean**	199.22			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	3,308.74	330.87	18.19	9.13
		Between Run	6,416.61	712.96	13.82	6.94
		Total	9,725.35		22.85	**11.47**

Quality material high – E1S						
Run	**Result 1 (pg/mL)**	**Result 2 (pg/mL)**	**Mean (pg/mL)**	**SS 1**	**SS 2**	**2*mean^2**
1	622.02	609.12	615.57	41.60	41.60	757,844.39
2	699.17	638.52	668.84	919.55	919.55	894,705.30
3	622.01	593.02	607.52	210.15	210.15	738,155.57
4	529.06	609.33	569.19	1,610.63	1,610.63	647,962.42
5	631.45	579.47	605.46	675.58	675.58	733,162.36
6	540.12	551.56	545.84	32.73	32.73	595,885.89
7	588.49	596.90	592.69	17.66	17.66	702,570.51
8	583.72	599.10	591.41	59.19	59.19	699,530.27
9	527.44	635.65	581.54	2,927.50	2,927.50	676,383.41
10	541.70	517.40	529.55	147.66	147.66	560,852.38
Grand sum	11,815.24	**Grand mean**	590.76			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	13,284.51	1,328.45	36.45	6.17
		Between Run	27,055.28	3,006.14	28.96	4.90
		Total	40,339.79		46.55	**7.88**

Quality material low – DHEAS						
Run	**Result 1 (µg/dL)**	**Result 2 (µg/dL)**	**Mean (µg/dL)**	**SS 1**	**SS 2**	**2*mean^2**
1	9.23	10.00	9.61	0.15	0.15	184.87
2	7.68	8.26	7.97	0.08	0.08	127.04
3	7.56	7.55	7.56	0.00	0.00	114.17
4	8.08	8.21	8.15	0.00	0.00	132.69
5	8.18	8.19	8.19	0.00	0.00	133.99
6	8.31	8.61	8.46	0.02	0.02	143.08
7	8.14	8.83	8.49	0.12	0.12	144.02
8	8.49	9.94	9.22	0.52	0.52	169.88
9	7.82	7.70	7.76	0.00	0.00	120.33
10	8.44	7.88	8.16	0.08	0.08	133.10
Grand sum	167.09	**Grand mean**	8.35			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	1.97	0.20	0.44	5.31
		Between Run	7.23	0.80	0.55	6.59
		Total	9.19		0.71	**8.46**

Quality material medium – DHEAS						
Run	**Result 1 (µg/dL)**	**Result 2 (µg/dL)**	**Mean (µg/dL)**	**SS 1**	**SS 2**	**2*mean^2**
1	45.05	44.94	44.99	0.00	0.00	4,049.06
2	39.84	40.66	40.25	0.17	0.17	3,239.73
3	41.75	39.18	40.46	1.65	1.65	3,274.29
4	40.12	40.32	40.22	0.01	0.01	3,235.65
5	38.91	39.10	39.01	0.01	0.01	3,043.32
6	40.75	39.86	40.31	0.20	0.20	3,249.30
7	40.75	39.79	40.27	0.23	0.23	3,243.77
8	40.63	38.28	39.45	1.38	1.38	3,113.23
9	39.10	39.68	39.39	0.08	0.08	3,103.19
10	39.04	39.92	39.48	0.19	0.19	3,117.88
Grand sum	807.68	**Grand mean**	40.38			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	7.85	0.78	0.89	2.19
		Between Run	51.77	5.75	1.58	3.90
		Total	59.62		1.81	**4.48**

Quality material high – DHEAS						
Run	**Result 1 (µg/dL)**	**Result 2 (µg/dL)**	**Mean (µg/dL)**	**SS 1**	**SS 2**	**2*mean^2**
1	92.83	131.07	111.95	365.58	365.58	25,063.77
2	115.05	117.10	116.07	1.05	1.05	26,946.07
3	130.32	120.07	125.19	26.26	26.26	31,345.31
4	112.84	113.95	113.40	0.31	0.31	25,716.88
5	124.14	125.55	124.85	0.50	0.50	31,173.89
6	117.11	113.42	115.26	3.40	3.40	26,570.95
7	125.43	125.64	125.53	0.01	0.01	31,517.98
8	116.94	114.23	115.58	1.84	1.84	26,717.93
9	113.98	108.24	111.11	8.25	8.25	24,691.54
10	111.80	109.40	110.60	1.44	1.44	24,463.75
Grand sum	2,339.08	**Grand mean**	116.95			
			Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
		Within Run	817.27	81.73	9.04	7.73
		Between Run	643.17	71.46	0.00	0.00
		Total	1,460.44		9.04	**7.73**

Table A5. Stability

17-OHP (ng/dL)					
Quality Material 1	**Sample 1 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	19.08	16.40	16.06	17.54	-
Replicate 2	14.04	16.64	17.74	16.29	-
Replicate 3	16.66	15.93	15.75	15.75	-
Mean	16.59	16.32	16.52	16.52	-
% difference from QC		**-1.60**	**-0.45**	**-0.40**	**-**
Quality Material 2	**Sample 2 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	121.15	113.66	112.37	122.32	-
Replicate 2	117.91	125.81	128.30	120.40	-
Replicate 3	121.68	120.37	120.01	114.63	-
Mean	120.24	119.95	120.23	119.12	-
% difference from QC		**-0.24**	**-0.01**	**-0.94**	**-**
Quality Material 3	**Sample 3 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	64.14	64.71	64.39	57.58	-
Replicate 2	64.86	65.92	64.77	71.36	-
Replicate 3	66.69	64.57	63.81	59.39	-
Mean	65.23	65.07	64.32	62.78	-
% difference from QC		**-0.25**	**-1.39**	**-3.76**	**-**
Quality Material 4	**Sample 4 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	62.30	64.57	63.29	59.57	-
Replicate 2	66.29	68.54	63.63	61.21	-
Replicate 3	64.59	60.62	63.55	66.05	-
Mean	62.30	64.57	63.49	62.28	-
% difference from QC		**0.29**	**-1.40**	**-3.29**	**-**

AD (ng/dL)					
Quality Material 1	**Sample 1 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	34.99	35.82	34.55	35.36	-
Replicate 2	35.68	35.51	34.17	36.60	-
Replicate 3	34.59	34.67	33.86	32.51	-
Mean	35.09	35.33	34.20	34.82	-
% difference from QC		**0.70**	**-2.54**	**-0.75**	**-**
Quality Material 2	**Sample 2 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	194.40	203.18	193.63	201.57	-
Replicate 2	195.18	197.35	192.42	193.48	-
Replicate 3	206.53	192.59	202.71	199.67	-
Mean	198.70	197.71	196.25	198.24	-
% difference from QC		**-0.50**	**-1.23**	**-0.23**	**-**
Quality Material 3	**Sample 3 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	35.00	34.64	33.48	34.15	-
Replicate 2	32.25	33.58	34.88	34.36	-
Replicate 3	35.91	35.59	33.29	33.56	-
Mean	34.38	34.61	33.88	34.03	-
% difference from QC		**0.65**	**-1.45**	**-1.04**	**-**
Quality Material 4	**Sample 4 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	173.94	176.52	175.19	169.24	-
Replicate 2	184.86	185.00	171.44	184.30	-
Replicate 3	186.78	180.37	171.09	179.16	-
Mean	181.86	180.63	172.57	177.57	-
% difference from QC		**-0.68**	**-5.11**	**-2.36**	**-**
P4 (ng/dL)					

Quality Material 1	Sample 1 (Initial)	Three freeze-thaw cycles	Bench-top stability	Processed sample stability	Long-Term Stability
Replicate 1	113.46	107.78	106.09	108.59	-
Replicate 2	100.46	111.13	120.16	98.89	-
Replicate 3	100.50	98.75	104.73	106.56	-
Mean	104.81	105.89	110.33	104.68	-
% difference from QC		**1.03**	**5.27**	**-0.12**	**-**
Quality Material 2	**Sample 2 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	750.70	750.14	746.46	706.33	-
Replicate 2	763.95	762.37	679.51	765.11	-
Replicate 3	722.46	734.00	729.54	756.70	-
Mean	745.70	748.84	718.51	742.71	-
% difference from QC		**0.42**	**-3.65**	**-0.40**	**-**
Quality Material 3	**Sample 3 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	5.42	5.04	5.53	5.22	-
Replicate 2	4.71	5.19	5.19	4.51	-
Replicate 3	5.31	5.44	5.13	5.11	-
Mean	5.15	5.23	5.28	4.95	-
% difference from QC		**1.51**	**2.57**	**-3.88**	**-**
Quality Material 4	**Sample 4 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	5.42	5.40	5.28	5.22	-
Replicate 2	5.73	5.58	5.90	5.53	-
Replicate 3	5.66	5.83	5.19	5.46	-
Mean	5.60	5.60	5.46	5.40	-
% difference from QC		**-0.03**	**-2.64**	**-3.56**	**-**

TT (ng/dL)					
Quality Material 1	**Sample 1 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	50.54	51.35	50.84	53.62	-
Replicate 2	55.27	50.92	50.84	52.18	-
Replicate 3	49.06	55.35	51.40	51.51	-
Mean	51.62	52.54	51.03	52.44	-
% difference from QC		**1.78**	**0.34**	**1.57**	**-**
Quality Material 2	**Sample 2 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	361.58	386.65	356.49	342.84	-
Replicate 2	373.60	361.87	356.71	372.57	-
Replicate 3	368.98	373.89	394.41	367.42	-
Mean	368.05	374.14	369.21	360.94	-
% difference from QC		**1.65**	**0.26**	**-1.93**	**-**
Quality Material 3	**Sample 3 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	513.82	511.40	496.94	503.33	-
Replicate 2	502.79	517.02	493.55	496.06	-
Replicate 3	510.73	511.90	524.29	497.26	-
Mean	509.11	513.44	504.93	498.89	-
% difference from QC		**0.85**	**-0.82**	**-2.01**	**-**
Quality Material 4	**Sample 4 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	55.31	55.57	55.95	54.35	-
Replicate 2	56.57	55.99	54.04	57.27	-
Replicate 3	55.75	57.05	56.96	56.22	-
Mean	55.88	56.21	55.65	55.94	-
% difference from QC		**0.59**	**0.33**	**0.12**	**-**

E1 (ng/dL)

Quality Material 1	Sample 1 (Initial)	Three freeze-thaw cycles	Bench-top stability	Processed sample stability	Long-Term Stability
Replicate 1	4.84	4.84	4.84	4.53	-
Replicate 2	4.59	4.95	4.94	4.58	-
Replicate 3	4.53	4.42	4.42	4.93	-
Mean	4.66	4.74	4.73	4.68	-
% difference from QC		**1.73**	**1.66**	**0.55**	**-**
Quality Material 2	**Sample 2 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	15.09	15.59	15.66	14.97	-
Replicate 2	16.29	15.14	15.26	15.10	-
Replicate 3	15.82	15.41	15.45	15.31	-
Mean	15.73	15.38	15.46	15.12	-
% difference from QC		**-2.23**	**-1.76**	**-3.86**	**-**
Quality Material 3	**Sample 3 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	1.63	1.63	1.63	1.65	-
Replicate 2	1.65	1.65	1.65	1.81	-
Replicate 3	1.66	1.63	1.63	1.71	-
Mean	1.65	1.64	1.64	1.72	-
% difference from QC		**-0.56**	**-0.56**	**4.57**	**-**
Quality Material 4	**Sample 4 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	9.78	10.03	9.76	9.68	-
Replicate 2	10.22	9.71	9.67	10.20	-
Replicate 3	9.99	10.23	10.48	9.68	-
Mean	10.00	9.99	9.97	9.85	-
% difference from QC		**-0.07**	**-0.29**	**-1.45**	**-**

E2 (pg/mL)					
Quality Material 1	**Sample 1 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	23.94	22.23	22.48	20.71	-
Replicate 2	24.51	26.77	23.77	20.95	-
Replicate 3	23.59	24.63	22.11	21.23	-
Mean	24.01	24.55	22.79	20.96	-
% difference from QC		**2.22**	**-5.11**	**-12.70**	**-**
Quality Material 2	**Sample 2 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	382.24	385.45	361.81	383.63	-
Replicate 2	416.47	395.62	437.41	396.38	-
Replicate 3	432.81	393.60	413.04	417.24	-
Mean	410.51	391.56	404.09	399.08	-
% difference from QC		**-4.62**	**-1.56**	**-2.78**	**-**
Quality Material 3	**Sample 3 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	72.10	67.70	67.08	69.54	-
Replicate 2	74.35	69.68	61.96	63.97	-
Replicate 3	77.95	84.92	84.69	63.69	-
Mean	74.80	74.10	71.24	65.74	-
% difference from QC		**-0.94**	**-4.76**	**-12.12**	**-**
Quality Material 4	**Sample 4 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	97.90	88.98	92.89	83.13	-
Replicate 2	81.05	89.02	74.57	77.86	-
Replicate 3	79.49	77.92	68.03	80.09	-
Mean	86.14	85.30	78.50	80.36	-
% difference from QC		**-0.97**	**-8.88**	**-6.71**	**-**

E1S (pg/mL)

Quality Material 1	Sample 1 (Initial)	Three freeze-thaw cycles	Bench-top stability	Processed sample stability	Long-Term Stability
Replicate 1	33.45	31.40	31.70	28.89	-
Replicate 2	32.62	33.22	31.81	37.26	-
Replicate 3	31.84	33.34	31.37	30.57	-
Mean	32.64	32.65	31.63	32.24	-
% difference from QC		**0.05**	**-3.09**	**-1.22**	**-**
Quality Material 2	**Sample 2 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	348.48	345.24	361.81	339.82	-
Replicate 2	336.23	377.00	339.70	345.10	-
Replicate 3	369.74	344.80	352.33	360.23	-
Mean	351.48	355.68	351.28	348.38	-
% difference from QC		**1.19**	**-0.06**	**-0.88**	**-**
Quality Material 3	**Sample 3 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	53.22	51.93	52.73	52.61	-
Replicate 2	52.95	52.84	53.35	53.23	-
Replicate 3	52.29	51.17	51.61	52.51	-
Mean	52.82	51.98	52.56	52.79	-
% difference from QC		**-1.59**	**-0.50**	**-0.07**	**-**
Quality Material 4	**Sample 4 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	422.33	415.23	414.59	425.93	-
Replicate 2	412.50	414.47	412.74	425.16	-
Replicate 3	430.98	413.92	419.01	432.69	-
Mean	421.94	414.54	415.45	427.93	-
% difference from QC		**-1.75**	**-1.54**	**1.42**	**-**

DHEAS (µg/dL)					
Quality Material 1	**Sample 1 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	17.44	16.87	17.07	18.03	-
Replicate 2	17.02	17.17	17.39	17.95	-
Replicate 3	17.69	16.97	17.27	17.37	-
Mean	17.38	17.00	17.24	17.78	-
% difference from QC		**-2.21**	**-0.82**	**2.30**	**-**
Quality Material 2	**Sample 2 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	141.33	147.46	141.91	141.81	-
Replicate 2	147.18	146.04	143.20	144.87	-
Replicate 3	142.68	151.03	141.23	143.77	-
Mean	143.73	148.18	142.12	143.48	-
% difference from QC		**3.09**	**-1.12**	**-0.17**	**-**
Quality Material 3	**Sample 3 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	81.14	85.40	79.39	80.32	-
Replicate 2	80.46	81.90	81.24	79.93	-
Replicate 3	81.77	80.60	88.62	88.46	-
Mean	81.12	82.63	83.08	82.90	-
% difference from QC		**1.86**	**2.42**	**2.19**	**-**
Quality Material 4	**Sample 4 (Initial)**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-Term Stability**
Replicate 1	77.05	78.56	75.81	76.15	-
Replicate 2	77.67	77.30	77.27	73.02	-
Replicate 3	78.55	79.95	77.55	77.85	-
Mean	77.76	78.60	76.88	75.67	-
% difference from QC		**1.09**	**-1.13**	**-2.68**	**-**