Laboratory Guidelines

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Quantification of Endogenous Steroids in Blood for the *Athlete Biological Passport*

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1.0 Objective

These Laboratory Guidelines have been developed to ensure a harmonized application of the Analytical *Testing* Procedure for the quantification of endogenous steroid *Markers* measured in blood (serum) as part of the Steroidal Module of the *Athlete Biological Passport* (*ABP*). The document provides guidance on the pre-analytical details, *Sample* preparation procedure, the performance of the analyses and the reporting of the test results.

2.0 Scope

These Laboratory Guidelines contain requirements for the implementation of the Analytical *Testing* Procedure for the quantification of endogenous *steroid Markers* in blood (serum) as part of the Steroidal Module of the *ABP* to uncover use of endogenous anabolic androgenic steroids (EAAS) administered exogenously. These Laboratory Guidelines follow the rules established in the *WADA International Standard for* Laboratories (ISL) [1] and relevant *Technical Documents* (*TDs*) regarding the Analytical *Testing* of blood *Samples*.

3.0 Introduction to the Analytical *Testing* Procedure

The Analytical *Testing* Procedure involves the measurement of two (2) *Markers*, namely Testosterone (T) and Androstenedione (Androst-4-ene-3,17-dione, A4), which are naturally present in blood, and the calculation of the T/A4 ratio. While the endogenous levels of these *Markers* are gender-specific, they have been identified as relevant target Analytes to detect T abuse with an increased sensitivity in female *Athletes* [2,3], as well as the transdermal application of T-related drugs in both genders [4–6].

The quantification of T and A4 concentrations is based on Liquid Chromatography (LC) combined with tandem Mass Spectrometry ($LC\text{-}MS^n$; $n \geq 1$). For the purposes of the *ABP*, an initial quantification from the "A" *Sample* is performed. When requested, a confirmatory quantification of the "A" *Sample* may additionally be performed (see Article 6.2) to confirm the concentrations and to perform identification of the *Markers* (as per TD IDCR[7]).

The concentrations of T and A4 in blood reported by the Laboratories are integrated in the Steroidal Module of *ADAMS*, using a similar Bayesian approach to that applied in the other Steroidal (urine), Hematological and Endocrine Modules of the *ABP*.

4.0 Assay Pre-analytical Procedure

- The Laboratory should (usually) receive refrigerated (not frozen[i]) "A" and "B" blood *Samples*, which have been collected in blood tubes containing an inert polymeric serum separator gel and a clotting activation factor (for example: BD Vacutainer® SST™-II Plus tubes, EU ref 367955; BD Vacutainer® SST™-II Plus Advance tubes, EU ref 367954; BD Vacutainer® SST™ tubes, US ref 367986) in accordance with the *International Standard* for *Testing* and Investigation (ISTI) [7];
- Alternatively, if the clotting and centrifugation of the *Sample* is performed prior to reception at the Laboratory (for example, at the site of *Sample* collection), *Samples* may be received at the Laboratory as frozen/refrigerated blood *Samples* either in the same *Sample* collection tubes or as separated serum in new tubes;

[i] unless the blood matrix components have been separated before shipment to the Laboratory.

- The Laboratory shall check the status of the *Sample(s)* (*e.g.,* evidence of hemolysis) and the integrity of the collection tubes (*e.g.,* evidence of breakage of the separating gel). The Laboratory shall note any unusual condition of the *Sample* and record such condition(s) in the Test Report in *ADAMS*;
- Any *Samples* delivered to the Laboratory in tubes containing an anti-coagulant (for example, *ABP* blood *Samples* collected in EDTA tubes), or as separated plasma, shall not be analyzed for *Markers* of the Endocrine Module;
- The Laboratory shall notify and seek advice from the *Testing* Authority regarding rejection or Analytical *Testing* of *Samples* for which irregularities are noted (see ISL [1]).

4.1. *Samples* received as non-separated blood in tubes containing an inert polymeric serum separator gel and a clotting activation factor:

Reception	Both *Samples* "A" and "B" shall be centrifuged for 10-15 min at 1300-1500 g as soon as possible after reception at the Laboratory. The "A" *Sample* shall be used for the initial and confirmatory (if needed) quantifications (see below). The "B" *Sample* shall be step-frozen and stored until use, if needed (see below).
Aliquoting and analysis	An Aliquot of the "A" *Sample* serum shall be taken for initial quantification. The remaining "A" serum fraction may be kept in the *Sample* collection tube or aliquoted into new vials with label(s) ensuring that Laboratory Internal Chain of Custody is maintained. For initial quantification: • the Aliquot may be analyzed immediately after aliquoting; or • the Aliquot shall be stored at approximately 4 °C°C if analyzed within 24h (within a maximum of five (5) days from *Sample* collection); or • the Aliquot shall be frozen (-20°C) if the analysis will be conducted more than 24h after aliquoting. For the confirmatory quantification, a new Aliquot of the "A" *Sample* shall be analyzed immediately after aliquoting. *[Comment: When analyses specific to the ABP are requested for blood (serum) Samples (i.e., Markers of the Endocrine Module or blood steroid Markers as part of the Steroidal Module), only the "A" Sample should be considered for the initial and the confirmatory quantifications of the Markers. In cases where the "A" Sample is not suitable for the performance of ABP Markers quantification (e.g., there is insufficient Sample volume; the Sample container has not been properly sealed or has been broken; the Sample's integrity has been compromised in any way; the "A" Sample is missing), a splitting procedure of the "B" Sample could be performed, as detailed in the ISL[1].]*
Storage [The same storage conditions apply for *Samples* received in conditions described in section 4.2]	Storage for up to three (3) months → at approximately -20 °C. Storage for more than three (3) months → freeze at approximately -20 °C and transfer to approximately -70 to -80 °C. *[Comment: If the separated serum fraction is kept in the Sample collection tube, it shall be step-frozen for storage according to the tube manufacturer's instructions until analysis.* *If the Laboratory transfers the Aliquot into new vials for frozen storage, the vials should ensure proper sealing for optimal storage (cryovials with an "O-ring").* *Thawing of Sample(s) for analysis should also be done stepwise. Samples shall not be thawed under hot water or any other similar process that risks raising the temperature of the Sample above room temperature. Thawing overnight at 4°C is recommended.]*

4.2. *Samples* received as frozen/refrigerated centrifuged blood/serum *Samples*:

Reception	If *Samples* are received frozen, they should remain frozen until analysis as described in this Article 4.2. If *Samples* are received refrigerated, they should be processed as soon as possible as per Article 4.1.
Aliquoting and analysis	Once the *Sample* "A" is thawed, an Aliquot shall be taken for initial quantification. This Aliquot may be stored at approximately 4 °C for a maximum of 24h before analysis. The remaining "A" serum fraction may be kept in the *Sample* collection tube or aliquoted into new vial(s) with label(s) ensuring Laboratory Internal Chain of Custody is maintained. For the confirmatory quantification, a new Aliquot of the "A" *Sample* shall be analyzed immediately after aliquoting.

5.0 Analytical *Testing* Procedure Requirements

5.1. Analytical *Testing* Procedure Validation Requirements

Prior to the implementation of the Analytical *Testing* Procedure for the quantification of blood endogenous steroids in routine *Doping Control* analysis, the Laboratory shall fulfil the following requisites:

- Validate the Analytical *Testing* Procedure, including the determination of the assays' Limit of Quantification (LOQ), Repeatability (s_r), Intermediate Precision (s_w), Bias and Measurement Uncertainty (u_c);
- The Analytical *Testing* Procedure shall meet the acceptance values for the parameters of assay performance applicable to the separate determination of T and A4 concentrations as specified in Table 1 below.

5.2. Analytical *Testing* Procedure Accreditation Requirements

- Demonstrate readiness for assay implementation through method validation data and successful participation in at least one *WADA*-approved educational External Quality Assessment Scheme (EQAS) round or inter-Laboratory collaborative study. In cases of identified deficiencies, proper corrective action(s) shall be documented and implemented;
- Obtain ISO/IEC 17025 accreditation for the Analytical *Testing* Procedure for quantification of endogenous steroids in blood from an Accreditation Body that is a full member of the International Laboratory Accreditation Cooperation (ILAC) and a signatory to the ILAC Mutual Recognition Agreement (ILAC MRA).

6.0 Analytical *Testing* Procedure and Reporting of Test Results

6.1. Initial Quantification of the *Markers*

- One (1) Aliquot taken from the original "A" *Sample* shall be analyzed once (x1) to quantify T and A4;
- QC Sample(s), at low- and high-levels of the *Marker*s (see Table 1), shall be included in each initial quantification analytical batch;

- The T and A4 *Marker* concentrations shall be reported in *ADAMS* in nanograms per milliliter (ng/mL);

  [Comment: for the purposes of the Steroidal Module of the ABP, the T/A4 ratio does not need to be calculated or reported by the Laboratory; it will be automatically calculated in ADAMS].

- If the measured *Marker* concentration is below the LOQ of the assay, the Laboratory shall report a value of "-1" for its concentration in *ADAMS* and the Laboratory shall make a comment in the Test Report on why the *Marker* could not be quantified (e.g., the measurement of the *Marker* is not possible due to unusual matrix interferences);
- An observation of hemolysis of the *Sample* should be recorded in the comments section of the Laboratory Test Report in *ADAMS*.

6.2. Confirmatory Quantification of the *Markers*

If requested by the *Testing* Authority (TA), *Results Management* Authority (RMA) or *WADA*, the Laboratory shall proceed with the confirmatory quantification of the *Markers* of the blood Steroidal Module.

[Comment: An APMU or Passport Custodian (PC), where the PC is not the TA, may request a confirmatory quantification on behalf of the TA or RMA. In such cases, the APMU or PC shall copy the relevant TA or RMA, as applicable, on all written requests to the Laboratory for confirmatory quantification.]

When a confirmatory quantification analysis is requested:

- One (1) new Aliquot taken from the original "A" *Sample* shall be analyzed once (x1) to identify (as per the TD IDCR [8]) and to quantify T and A4.
- At least one QC Sample (see Table 1), depending on initial quantification results, shall be included in each confirmatory quantification analytical batch;
- The T and A4 *Marker* concentrations shall be reported in *ADAMS* in nanograms per milliliter (ng/mL).
- If the measured *Marker* concentration is below the LOQ of the assay, the Laboratory shall report a value of "-1" for its concentration in *ADAMS* and the Laboratory shall make a comment in the Test Report on why the *Marker* could not be quantified (e.g., the measurement of the *Marker* is not possible due to unusual matrix interferences);
- An observation of hemolysis of the *Sample* should be recorded in the comments section of the Laboratory Test Report in *ADAMS*.

Table 1: Analytical *Testing* Procedure Validation and Performance Requirements for the initial and confirmatory quantification of blood (serum) endogenous steroid *Markers*.

Markers	**Testosterone (T)**, total unconjugated fraction **Androstenedione** (Androst-4-ene-3,17-dione, **A4**), total unconjugated fraction
Method and Instrumentation	Liquid Chromatography combined with tandem Mass Spectrometry based on triple quadrupole or HRMS analyzer (LC-MS^n; n ≥ 1).
Range of the Method	Shall cover the ranges of *Marker* concentrations normally found in males and females and demonstrate linearity between **0.1 – 10 ng/mL (~ 0.35 – 35 nmol/L)**, at least.
Limits of Quantification (LOQ)	The LOQ shall be determined during method validation and is defined as the lowest concentration with an associated u_c (%) not greater than (≤) 30% and shall be not greater than (≤) **0.1 ng/mL (~ 0.35 nmol/L)**.
Relative Standard Combined Measurement Uncertainty, u_c (%)	The estimated u_c (%) shall be no greater than (≤) **30%** at the LOQ; and not greater than (≤) **20%** when the *Marker* concentration is greater than (>) 0.3 ng/mL.
Sample	*Marker* quantification shall be conducted on one serum Aliquot of no greater than (≤) **100 µL**.
Internal Standards	Adequate isotopic-labelled internal standards shall be used for both *Markers* (*e.g.,* Testosterone-d3 (16,16,17-d3)[ii] and Androstenedione-d3 (19-d3)[iii]).
Calibration	Calibration standard(s) shall be included in each sequence of analysis. The "*Multilevel Serum Calibrator Set*" from Chromsystem[iv] is recommended. Other calibrators may be used as long as the method performance criteria are met.
Quality Control	At least two (2) quality control (QC) samples in serum containing representative low (*e.g*., 0.5 ng/mL) and high (*e.g.,* 5 ng/mL) concentrations of the *Markers* shall be included in each analytical batch. The QCs should be prepared from authentic samples, or by spiking with a standard solution independent from that used for the calibrator(s).

Applicable links:
[ii] https://www.lipomed-usa.com/en/testosterone-d3, for example.
[iii] https://www.lgcstandards.com/US/en/Androstenedione-d3/p/TRC-A637552-1MG, for example.
[iv] https://chromsystems.com/en/6plus1r-multilevel-serum-calibrator-set-masschromr-steroid-panel-2-72039.html

7.0 Bibliography

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