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Endogenous Anabolic Androgenic Steroids
Measurement and Reporting

1.0 Introduction

The purpose of this Technical Document (TD) is to harmonize the approaches to the measurement and reporting of Endogenous Anabolic Androgenic Steroids (EAAS) in urine *Samples*, including data in support of the steroidal module of the *Athlete Biological Passport* (*ABP*) (the steroidal Passport).

EAAS concentrations and their ratios form the urinary "steroid profile", which may be altered following the administration of synthetic forms of EAAS, in particular testosterone (T), its precursors [for example androstenediol, androstenedione and prasterone (dehydroepiandrosterone or DHEA)], or its active metabolite [dihydrotestosterone (DHT)], as well as epitestosterone (E).

The steroidal module of the *ABP* utilizes the Adaptive Model to identify an *Atypical Passport Finding* (*ATPF*), which triggers the performance of Confirmation Procedures. It is also useful for intelligent longitudinal *Target Testing* of an *Athlete*. Furthermore, an abnormal "steroid profile" (obtained from a single urine *Sample*) or an atypical steroidal Passport (including "steroid profiles" obtained from a series of *Samples* collected over a period of time), may be used as a means to pursue an anti-doping rule violation (ADRV).

EAAS Analytical Testing and reporting follows a two-step procedure. An Initial Testing Procedure is conducted to estimate the "steroid profile" of the *Athlete's Sample*. A subsequent Confirmation Procedure is performed when the estimated "steroid profile" constitutes an *ATPF*, as determined by the Adaptive Model, or represents a "suspicious steroid profile" (SSP) finding, or upon request from the Athlete Passport Management Unit (APMU), the Testing Authority or *WADA*.

The Confirmation Procedure includes the quantification of the *Markers* of the "steroid profile" as described in this TD as well as Gas Chromatography – Combustion - Isotope Ratio Mass Spectrometry (GC/C/IRMS) analysis, which is considered in a separate TD (TD IRMS) [1].

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1.1 The "Steroid Profile"

Each urine *Sample* shall be analyzed to determine its "steroid profile".

For the purposes of this TD, the "steroid profile" is composed of the following *Markers* (as free steroid content obtained from the free steroid fraction plus those released from the conjugated fraction after hydrolysis with β-glucuronidase from *E. coli*):

- Androsterone (A)
- Etiocholanolone (Etio)
- 5α-Androstane-3α,17β-diol (5αAdiol)
- 5β-Androstane-3α,17β-diol (5βAdiol)
- Testosterone (T)
- Epitestosterone (E).

and the following ratios:

- T/E
- A/T
- A/Etio
- 5αAdiol/5βAdiol
- 5αAdiol/E.

The administration of EAAS can alter one or more of the *Markers* and/or ratios of the urinary "steroid profile", resulting in increase or decrease of concentrations and/or ratios of specific pairs of steroid *Metabolites* [2-4].

Additionally, alteration of the urinary "steroid profile" can occur for a number of reasons including, but not limited to, the following confounding factors:

- the administration of other anabolic steroids (*e.g.* stanozolol);
- the administration of human chorionic gonadotrophin (hCG) in males;
- the administration of aromatase inhibitors and anti-estrogens;
- the administration of inhibitors of 5α-reductase (*e.g.* finasteride);
- intake of alcohol (ethanol);
- the administration of ketoconazole or other similar compounds;
- the use of masking agents (*e.g.* probenecid) and diuretics; or
- microbial growth.

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2.0 Initial Testing Procedure

The Laboratory shall use a validated Initial Testing Procedure that is Fit-for-Purpose to estimate the *Markers* of the urinary "steroid profile" in the range of values determined in males and females.

The Initial Testing Procedure is conducted on a single Aliquot.

2.1 Method Characteristics

- Gas chromatography combined with mass spectrometry (GC-MS or GC-MS/MS) of TMS derivatives (keto- and hydroxyl- groups) is required;
- Calibration standard(s) or a calibration curve should be included in each sequence of analysis;
- At least two urine quality control (QC) samples containing varying and representative concentrations of the *Markers* of the "steroid profile" should be included in each sequence of analysis;
- The enzymatic hydrolysis shall be carried out with purified β-glucuronidase from *E. coli* (*H. pomatia* mixtures are not acceptable);
- The completeness of hydrolysis of the glucuroconjugated urinary steroids shall be controlled with isotopically labeled A-glucuronide (or an equivalent scientifically recognized alternative);
- The completeness of the derivatization shall be controlled through the monitoring of mono-O-TMS vs. di-O-TMS derivative of A;
- When needed, the volume[1] of the *Sample* Aliquot may be adjusted as a function of its specific gravity (SG) and of the sex of the *Athlete*;
- The T/E ratios shall be determined from the ratios of the corrected chromatographic peak areas or peak heights[2];

[1] Much smaller concentrations of T and E are generally present in *Samples* from females and in those *Samples* with low SG; therefore, larger Aliquot volumes may be required for a reliable measurement.

[2] Ratios of T and E peak heights or peak areas corrected against a calibrator or a calibration curve (same mass or same ion transition screened for both steroids).

- The linearity of the method, established during method validation, shall cover the ranges of *Marker* concentrations normally found in males and females - the limit of quantification (LOQ) for T and E shall not be greater than 2 ng/mL[3];
- The relative standard combined Measurement Uncertainty [u_c (%)] for the determination of A, Etio, 5αAdiol, 5βAdiol, T and E, as estimated during method validation of the Initial Testing Procedure, shall be:
  - Not greater than 30% at the respective LOQ;
  - Not greater than 20% (for A and Etio) or 25% (for the Adiols) at five (5) times the LOQ;
  - Not greater than 20% (for T and E) when the concentration is greater than 5 ng/mL.
- The u_c (%) for determinations of T/E ratios calculated from the corrected chromatographic peak areas or heights shall be:
  - Not greater than 15% when the concentrations of T and E are both greater (>) than 5 ng/mL;
  - Not greater than 30% when the concentrations of T and/or E are equal to or lower (≤) than 5 ng/mL.
- Evidence of microbial degradation [*e.g.* presence of indicators of 3α-hydroxysteroid dehydrogenase (HSD) activity] and the presence of 5α-reductase inhibitors (*e.g.* finasteride), ethanol *Metabolite(s)* and ketoconazole (and similar substances) shall be monitored by the Laboratory[4].

[3] The LOQ for the "steroid profile" *Markers* shall be determined as the lowest concentration that can be measured within a u_c (%) of 30%.

The LOQ determined from the method validation of T, E, A, Etio, 5αAdiol and 5βAdiol shall be recorded in *ADAMS* by the Laboratory. The LOQ values shall be updated in *ADAMS* whenever a significant change is made to the analytical method.

[4] The direct enzymatic hydrolysis of urine *Samples* may increase the effects of microbial contamination.

2.2. Reporting the "steroid profile" from the Initial Testing Procedure

Following the performance of the Initial Testing Procedure, the Laboratory shall report in *ADAMS* the "steroid profile" for each *Sample* analyzed[5, 6], including:

- the SG[7] of the *Sample*;
- the concentrations of T, E, A, Etio, 5αAdiol and 5βAdiol[8, 9, 10];

[5] This also applies when more than one (1) *Sample* from the same *Athlete*, which are linked to a single Sample Collection Session, are analyzed.

[6] The Laboratory shall report in *ADAMS* the *Sample*'s "steroid profile", as determined during the Initial Testing Procedure, in cases when no *Prohibited Substance* or *Prohibited Method* is detected in the *Sample* [while reporting the test result as a Negative Finding], as well as in cases when the Laboratory confirms the presence of a *Prohibited Substance* or *Prohibited Method* [while reporting the result as an *Adverse Analytical Finding (AAF*) or *Atypical Finding (ATF)*, as applicable, for the *Prohibited Substance* or *Prohibited Method* detected].

[7] As determined by the Laboratory using, for example, a refractometer.

[8] When reporting the "steroid profile" in *ADAMS*, the Laboratory shall report the values of concentrations for T, E, A, Etio, 5αAdiol and 5βAdiol, and the T/E ratio (**without adjustment for the urine SG or correction to a specific number of significant figures**). An automatic correction of reported values to 2 significant figures will be made in *ADAMS* upon application of the Adaptive Model of the *ABP*.

[9] When the Initial Testing Procedure measurement of a "steroid profile" *Marker* is not possible due to, for example, dilution, unusual matrix interferences, inhibition of the enzymatic hydrolysis or incomplete derivatization, the Laboratory should repeat the analysis with an alternative, validated *Sample* preparation procedure (*e.g.* concentrating the *Sample* or taking larger Aliquot volumes, application of solid phase extraction, extraction with a different solvent or other equivalent procedure). If, however, the *Marker* of the "steroid profile" cannot be quantified, the concentration of the *Marker* shall be reported as "-1". When the chromatographic peak signal for a *Marker* cannot be detected (*i.e.* is below the detection capability of the assay), the concentration of the *Marker* shall be reported as "-2" (see Table 1).

[10] The Laboratory may also provide information on other steroidal parameters such as dehydroepiandrosterone (DHEA) and 6α-hydroxy-androstenedione at the request of the Testing Authority, Results Management Authority or the APMU.

- the T/E ratio[2, 11];
- signs of microbial activity in the *Sample*, *e.g.* ratios of 5α-androstanedione (5αAND) to A and 5β-androstanedione (5βAND) to Etio[12];
- the presence or absence in the *Sample* of substance(s) that may alter the "steroid profile" [12].

In cases when a *Sample* is not consistent with human urine (*e.g.* SG ≤ 1.001, creatinine ≤ 5 mg/dL [5], non-physiological salt concentration, abnormal pH values, absence or abnormally low levels of endogenous steroids, corticosteroids, proteins), the Laboratory shall:

- report the finding as an *AAF* for *Tampering* or *Attempted Tampering* (class M2.1 of the *Prohibited List*) if the Laboratory can unequivocally identify the nature of the liquid (*e.g.* water, liquor, synthetic urine) provided as the adulterated *Sample;* or
- report the finding as an *AAF* for *Tampering* or *Attempted Tampering* if the Laboratory has reason to believe that the *Sample* could have been altered in any manner, improperly interfered with, or potentially been the subject of any fraudulent conduct that could alter the results of Analytical Testing; or
- inform the Testing Authority about the suspicious finding and request further information which may support the reporting of this finding as an *AAF* for *Tampering* or *Attempted Tampering* (e.g. longitudinal "steroid profile" data for the *Athlete*); or
- report the finding as an *ATF* for *Tampering* or *Attempted Tampering* and include a comment in *ADAMS* advising the Testing Authority to perform further investigations (*e.g.* additional analyses on the *Sample*, *Target Testing* the *Athlete*) in order to establish whether *Tampering* of the

[11] The values of A/T, A/Etio, 5αAdiol/5βAdiol and 5αAdiol/E ratios are automatically computed in *ADAMS* after the reporting of the "steroid profile" by the Laboratory.

[12] A *Sample* showing signs of microbial degradation or containing any of the substances that may cause an alteration of the "steroid profile" (see section 1.1) may not be suitable for inclusion in the "longitudinal steroid profile". These findings are to be considered by the APMU during the results management process when evaluating the analytical data for the *Sample* and assessing the possible pathological or confounding conditions that may have impacted the *Sample's* "steroid profile".

Sample has occurred and the finding be treated as an Anti-Doping Rule Violation.

2.2.1 Validity of (the "steroid profile" of) the *Sample*

The validity of the *Sample* will be determined automatically upon reporting the "steroid profile" in *ADAMS* in accordance to:

a) **"Invalid"**: **only when the *Sample* shows signs of extensive degradation**[13], as determined by:

- 5αAND/A ≥ 0.1, and/or
- 5βAND/Etio ≥ 0.1

b) "**Valid"**: **in all other situations**, including:

- LOD ≤ [T and/or E] < LOQ

  When the concentration of either T and/or E in the *Sample* Aliquot analyzed cannot be quantified, but its chromatographic peak signal is still detectable (e.*g.* S/N > 3) and the T/E ratio can be determined from the corrected chromatographic peak areas or peak heights[2], the calculated value of the T/E ratio shall be reported in *ADAMS*, whereas the concentration of T and/or E, as applicable, shall be reported as "-1" (Table 1)[9].

- [T] < LOD

  If the chromatographic peak signal for T cannot be detected, the concentration of T shall be reported as "-2" and the T/E value shall be reported as "-1" (Table 1)[9] and:

  i. for [E] ≥ LOQ, a comment shall be included in *ADAMS* stating that the T/E ratio could not be measured because the concentration of T was below the detection capability of the assay; or

  ii. for LOD ≤ [E] < LOQ, the concentration of E shall be reported as "-1" [9] and a comment shall be included in *ADAMS* stating that the

[13] In addition, following the reporting of the "steroid profile" in *ADAMS* by the Laboratory, the *Sample* may be evaluated as "invalid" by the APMU upon review of the "steroid profile" data, for example, by considering the presence of substances that may alter the "steroid profile" in the *Sample*.

T/E ratio could not be measured because the concentrations of T and E could not be measured.

- [E] < LOD

  If the chromatographic peak signal for E cannot be detected, the concentration of E shall be reported as "-2" [9] (Table 1) and:

  i. for [T] ≥ LOQ, the T/E ratio shall be calculated on the basis of the Laboratory's LOD value for E (*e.g.* if T concentration is 3 ng/mL and E cannot be detected, and the Laboratory's LOD for E is 0.5 ng/mL, the T/E shall be reported as 6.0) (Table 1). A comment shall be included in *ADAMS* stating that the T/E ratio could not be measured accurately because the concentration of E was below the detection capability of the assay; or

  ii. for LOD ≤ [T] < LOQ, the T/E ratio and the concentration of T shall be reported as "-1" [9] and a comment shall be included in *ADAMS* stating that the T/E ratio could not be measured accurately because the concentrations of T and E could not be measured (Table 1).

- Both [T and E] < LOD:

  If the chromatographic peak signals for both T and E cannot be detected, the concentrations of T and E shall be reported as "-2" and the T/E value shall be reported as "-2" (Table 1)[9]. A comment shall be included in *ADAMS* stating that the T/E ratio could not be measured because the concentrations of both T and E were below the detection capability of the assay.

- When other *Marker*(s) of the "steroid profile" cannot be measured accurately:

  o LOD ≤ [*Marker*] < LOQ

  If the concentration of the *Marker* in the Aliquot is below the LOQ of the assay, but its chromatographic peak signal is still detectable (*i.e.* above the LOD of the assay), the concentration of the *Marker* shall be reported as "-1" [9].

  o [*Marker*] < LOD

  If the chromatographic peak signal for the *Marker* cannot be detected (*i.e.* below the LOD of the assay), the concentration shall be reported as "-2" [9].

- When less extensive microbial contamination is detected which shall be reported in *ADAMS*[12] as:

  5αAND/A ratio and/or 5βAND/Etio ratio between 0.05 and 0.1.

- When the Laboratory reports an *AAF* or an *ATF* for a *Prohibited Substance* that may alter the "steroid profile" (*e.g.* an anabolic steroid, hCG in males, a diuretic or masking agent)[12];
- When the Laboratory detects and reports the presence in the *Sample* of other substances that may cause an alteration of the "steroid profile" (see section 1.1)[12, 14].

[14] It is mandatory that the Laboratory tests at least for the presence of conjugated *Metabolite(s)* of ethanol [*e.g.* ethanol glucuronide (EtG)], inhibitors of 5α-reductase and ketoconazole during the Initial Testing Procedure and report the estimated concentration of EtG if above 5 μg/mL (without the need to report the Measurement Uncertainty).

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Table 1. Summary of conditions for reporting T and E concentrations and T/E ratio.

Concentration of T	Concentration of E	T/E ratio
Chromatographic peak signal of T measured at or above the LOQ. $[T] \geq LOQ_{(T)}$ **Report T as measured**	Chromatographic peak signal of E measured at or above LOQ. $[E] \geq LOQ_{(E)}$ **Report E as measured**.	**Report T/E as determined** from corrected peak heights/areas
	Chromatographic peak signal of E detected, but below LOQ. $LOD_{(E)} \leq [E] < LOQ_{(E)}$ **Report E as "-1"** [9]	
	Chromatographic peak signal of E not detected. $[E] < LOD_{(E)}$ **Report E as "-2"** [9]	**Report T/E as T/LOD$_{(E)}$** *Comment in ADAMS*: T/E ratio could not be measured accurately because the concentration of E was below the detection capability of the assay
Chromatographic peak signal of T detected, but below the LOQ. $LOD_{(T)} \leq [T] < LOQ_{(T)}$ **Report T as "-1"** [9]	Chromatographic peak signal of E measured at or above LOQ. $[E] \geq LOQ_{(E)}$ **Report E as measured**	**Report T/E as measured** from corrected peak heights/areas
	Chromatographic peak signal of E detected, but below LOQ. $LOD_{(E)} \leq [E] < LOQ_{(E)}$ **Report E as "-1"** [9]	
	Chromatographic peak signal of E not detected. $[E] < LOD_{(E)}$ **Report E as "-2"** [9]	**Report T/E as "-1"** *Comment in ADAMS*: T/E ratio could not be measured accurately because the concentrations of T and E could not be measured
Chromatographic peak signal of T not detected. $[T] < LOD_{(T)}$ **Report T as "-2"** [9]	Chromatographic peak signal of E measured at or above LOQ. $[E] \geq LOQ_{(E)}$ **Report E as measured**	**Report T/E as "-1"** *Comment in ADAMS*: T/E ratio could not be measured because the concentration of T was below the detection capability of the assay
	Chromatographic peak signal of E detected but below LOQ. $LOD_{(E)} \leq [E] < LOQ_{(E)}$ **Report E as "-1"** [9]	**Report T/E as "-1"** *Comment in ADAMS*: T/E ratio could not be measured because the concentrations of T and E could not be measured
	Chromatographic peak signal of E not detected. $[E] < LOD_{(E)}$ **Report E as "-2"** [9]	**Report T/E as "-2"** *Comment in ADAMS*: T/E ratio could not be measured because the concentrations of both T and E were below the detection capability of the assay

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3.0 Confirmation Procedures

Confirmation Procedures for the exogenous administration of EAAS include the GC-MS or GC-MS/MS quantification[15] and GC/C/IRMS analysis of the *Marker(s)* of the "steroid profile".

In addition, the Laboratory shall confirm the presence or absence, as applicable, of the confounding factors of the "steroid profile" as described in section 1.1, *i.e.* conjugated *Metabolite(s)* of ethanol (*e.g.* EtG), inhibitors of 5α-reductase (*e.g.* finasteride), ketoconazole as well as the signs of microbial degradation including, for example, the presence of the free forms of T, 5αAND or 5βAND.

3.1 "*Atypical Passport Finding* Confirmation Procedure Request (*ATPF*-CPR)"

Following the Laboratory's reporting of a *Sample*'s "steroid profile" in *ADAMS*, the *Sample* record is matched with a Doping Control Form (DCF), which allows the inclusion of the *Sample*'s "steroid profile" in the *Athlete's* steroidal Passport in *ADAMS*.

The Adaptive Model will generate an "*ATPF*-CPR" notification when the *Sample's* T/E ratio is abnormally high, as determined by the Adaptive Model, when compared with the previous longitudinal T/E values of the *Athlete*.

The Laboratory shall proceed with the Confirmation Procedures when receiving an "*ATPF*-CPR" notification for the *Sample*, except in the following cases:

- If the APMU advises the Laboratory, in writing, not to confirm the "steroid profile" of the *Sample* based on justifiable reason(s). Justification for not proceeding with a Confirmation Procedure for an *ATPF* may include:
    - the presence of EtG in a *Sample* from an *Athlete* with previous similar findings in his/her Passport with negative GC/C/IRMS results (indicating a pattern of alcohol abuse); or
    - if other *AAFs* have been reported for the *Sample*, which would likely lead to a maximum sanction.

[15] For T/E values, only T needs to be confirmed if the concentration levels of E or the volume of the *Sample* is not sufficient.

In such cases, the Laboratory shall update the *ADAMS* report for the *Sample* with a comment stating that the APMU requested not to perform the Confirmation Procedure(s). The APMU shall also update the APMU Report in *ADAMS* with an explanation of why the Confirmation Procedure(s) were not necessary.

- In addition, the GC/C/IRMS Confirmation Procedure for an *ATPF* is not mandatory if the GC-MS or GC-MS/MS quantitative analysis does not confirm the abnormally high T/E ratio of the *Sample* (see section 3.5 below). In such cases, the Laboratory shall report the confirmed values of the *Markers* of the "steroid profile" in *ADAMS* (see section 3.6 below) with a comment stating that the GC/C/IRMS analysis was not performed because the abnormally high T/E ratio was not confirmed.

The Adaptive Model will also determine abnormal values of the other ratios of the "steroid profile" (A/T, A/Etio, 5αAdiol/5βAdiol, 5αAdiol/E). However, in such cases the Laboratory will not receive an automatic "*ATPF*-CPR" notification through *ADAMS*. Instead, the Athlete Passport Management Unit (APMU) will advise the Testing Authority on whether the *Sample* shall be subjected to Confirmation Procedures. Therefore, in these cases the Laboratory shall receive a request from the Testing Authority before proceeding with the Confirmation Procedure(s)[16].

3.2 "Suspicious Steroid Profile Confirmation Procedure Request (SSP-CPR)"

The Laboratory will receive a "SSP-CPR" notification through *ADAMS* if:

1) The *Sample* is matched with a DCF in *ADAMS*, but there is no existing steroidal Passport of the *Athlete* in *ADAMS* (*i.e.* this is the first *Sample* in the *Athlete's* steroidal Passport), or

   The *Sample* cannot be matched with a DCF in *ADAMS* within fourteen (14) calendar days after the reception date of the *Sample* by the Laboratory, and therefore the "steroid profile" of the *Sample* cannot be processed by the Adaptive Model in *ADAMS*,

   and

[16] Unless covered by an agreement between the Laboratory and the Testing Authority.

2) The *Sample's* "steroid profile" meets **any** of the following criteria:

- o T/E ratio (calculated from the corrected chromatographic peak areas or heights) greater than 4.0;
- o A/T ratio less than 20;
- o 5αAdiol/5βAdiol ratio greater than 2.4;
- o concentration of T or E (adjusted for the SG[7, 17]) greater than 200 ng/mL in males or greater than 50 ng/mL in females;
- o concentration of A or Etio (adjusted for the SG[7, 17]) greater than 10,000 ng/mL;
- o concentration of 5αAdiol (adjusted for the SG[7, 17]) greater than 250 ng/mL in males or greater than 150 ng/mL in females, combined with a 5αAdiol/E ratio greater than 10 in either sex.

- Upon receipt of the "SSP-CPR" notification, the Laboratory shall proceed with the Confirmation Procedure(s) unless, after contacting the Testing Authority, the Testing Authority can justify in writing within seven (7) calendar days that the Confirmation Procedure(s) is not necessary. Justification for not proceeding with the Confirmation Procedure may include, for example, a naturally elevated T/E ratio confirmed by previous Analytical Testing, or a T/E ratio between 4.0 and 6.0 for the first test on the *Athlete*, or if other *AAF*s have been reported for the *Sample*, which would likely lead to a maximum sanction;
- If the Testing Authority justifies that confirmation is not necessary, the Laboratory shall update the *ADAMS* report for the *Sample* with a comment stating that the Testing Authority considered that the Confirmation Procedure(s) was not necessary and detail the explanation provided by the Testing Authority. If the Testing Authority does not justify that confirmation is not necessary, the Laboratory shall proceed with the confirmation analyses.

[17] The concentrations are adjusted to a urine SG[7] of 1.020 based on the following equation (free and hydrolyzed glucuroconjugated steroids).

$$\mathbf{Conc_{corr} = Conc_{measured} * (1.020 - 1)/(SG - 1)}$$

In cases when the Laboratory receives "*ATPF*-CPR" or "SSP-CPR" for two (2) or more *Samples*, which are linked to a single *Sample* collection session from the same *Athlete*, the Laboratory, in consultation with the Testing Authority, shall prioritize the confirmation of the *Sample* with the highest concentration levels of the *Markers* of the "steroid profile".

When the Laboratory receives an "*ATPF*-CPR" or a "SSP-CPR" for a *Sample* for which *AAF(s)* have been reported for other *Prohibited Substance(s)* or *Method(s)*, the Laboratory should consult the Testing Authority about the need to conduct the Confirmation Procedures for the *Markers* of the "steroid profile".

3.3 Confirmation Procedure Requests from the APMU, the Testing Authority or *WADA*.

Confirmation Procedures for the "steroid profile" may be also performed on *Samples* at the request of the APMU, the Testing Authority or *WADA*.

In addition, a Laboratory may have a contractual agreement in place with the Testing Authority to conduct the Confirmation Procedures when a *Sample* meets any of the analytical criteria of a "suspicious steroid profile" or at the Laboratory's discretion based on its expertise. In such circumstances, the Laboratory may proceed to the confirmation of the "suspicious steroid profile" immediately without waiting for an "*ATPF*-CPR" or a "SSP-CPR" through *ADAMS.*

3.4 GC-MS or GC-MS/MS quantification Confirmation Procedure

The Laboratory shall identify (in compliance with the TD IDCR [6]) and quantify all the *Markers* of the "steroid profile" in one additional *Sample* Aliquot by a validated Fit-for-Purpose GC-MS or GC-MS/MS quantification method.

The Laboratory shall confirm quantitatively all the *Markers* of the "steroid profile" before proceeding with the GC/C/IRMS analysis.

3.4.1 Method Characteristics for the GC-MS or GC-MS/MS quantification Confirmation Procedure

The same analytical requirements presented in section 2.1 shall apply, with the following modifications:

- A Solid Phase Extraction (SPE) shall be performed prior to the enzymatic hydrolysis of the *Sample*;

- Calibration standards and urine QC samples containing representative levels of the *Markers* of the "steroid profile" shall be included;
- The u_c (%) shall be not greater than 15% for determinations of A, Etio, 5αAdiol and 5βAdiol at concentrations representing five times the respective LOQ;
- For determinations of T, E and T/E ratios, the u_c (%) shall be not greater than 15% when the concentrations of T and E are greater than 5 ng/mL.

3.5 GC/C/IRMS Confirmation Procedure

Technical and reporting requirements for the GC/C/IRMS Confirmation Procedure are specified in the TD IRMS [1].

- In the case of an *ATPF*-CPR, GC/C/IRMS analysis is not mandatory when the confirmed T/E value is below the confirmation T/E threshold calculated by the Adaptive Model and provided within the *ATPF*-CPR notification received from *ADAMS;*
- For other Confirmation Procedure requests (*i.e.* SSP-CPR or upon APMU/Testing Authority/*WADA* request), when the quantitative GC-MS or GC-MS/MS Confirmation Procedure does not confirm the values reported from the Initial Testing Procedure (taking into consideration the expanded uncertainty of the measurement), the Laboratory shall consult the Testing Authority to determine if the GC/C/IRMS analysis is necessary. In such cases, the Testing Authority shall consult with the APMU of the Passport Custodian in order to assess whether the GC/C/IRMS analysis is still necessary. In the event that GC/C/IRMS analysis is deemed unnecessary, the Laboratory shall update the *ADAMS* report for the *Sample* with the newly confirmed values of the "steroid profile" and include a comment that GC/C/IRMS analysis was not necessary. The APMU shall also update the APMU Report in *ADAMS* with an explanation of why the GC/C/IRMS Confirmation Procedure was not necessary.

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3.6 Reporting Results from the Confirmation Procedures

Following the performance of the Confirmation Procedure(s) on the "A" or the "B" *Sample*[18], the Laboratory shall report in *ADAMS*:

- the SG[7] of the *Sample* (determined from a new Aliquot of the "A" or "B" *Sample*, as applicable)*;*
- the confirmed values (*e.g.* concentrations, T/E ratio) of the *Markers* of the "steroid profile", without adjustment for the SG of the *Sample* [8, 9, 11];
- the associated u_c expressed in units;
- the GC/C/IRMS confirmation results, if determined (see section 3.5 and TD IRMS [1]);
- the confirmed results for signs of microbial contamination (*e.g.* 5αAND/A, 5βAND/Etio, T_{free} / T_{total} [19]);
- the confirmed presence or absence of conjugated *Metabolite(s)* of ethanol, inhibitors of 5α-reductase (*e.g.* finasteride), ketoconazole or any other substances that might have altered the "steroid profile", if applicable. The Laboratory shall report the confirmed estimated levels of EtG if above 5 μg/mL (without the need to report the Measurement Uncertainty for this determination).

Following the confirmation of the "steroid profile", the Laboratory shall update the *ADAMS* test result record for the *Sample* (as *AAF*, *ATF*, or "Negative") based on the results of the GC/C/IRMS Confirmation Procedure, if performed, in accordance with the TD IRMS [1]).

[18] When an *AAF* is reported for the *Marker(s)* of the "steroid profile" based on the results of a GC/C/IRMS analysis performed on the "A" *Sample*, only the GC/C/IRMS analysis shall be repeated during the "B" *Sample* Confirmation Procedure, if applicable. Refer to the TD IRMS [1].

[19] In addition to the determination of the 5αAND/A and 5βAND/Etio ratios as signs of microbial contamination, as described in section 2.2.1 for the Initial Testing Procedure, the determination during the Confirmation Procedure of an elevated ratio of free Testosterone to total Testosterone (T_{free} / T_{total} > 0.05) will also invalidate (the "steroid profile" of) the *Sample*.

3.7 Additional Analyses: Steroid Ester(s) and DNA

When matched blood *Samples* have been collected during the same Sample Collection Session as urine *Samples* identified with an atypical or suspicious "steroid profile", Laboratories, in consultation with the Testing Authority, should consider conducting analysis to detect the presence of steroid ester(s) in the associated serum/plasma.

It is recommended that confirmation analyses for steroid ester(s) in serum/plasma be conducted prior to the performance of the GC/C/IRMS analysis in urine. The detection of steroid ester(s) in serum/plasma also constitutes an unequivocal demonstration of the exogenous origin of the steroid(s). On the other hand, the absence of detectable steroid ester(s) in serum/plasma shall not invalidate an *AAF* based on the GC/C/IRMS analysis in urine.

The performance of a DNA analysis may also be considered to establish, in conjunction with the *Athlete's* "longitudinal steroid profile", the origin of the *Sample*(s).

4.0 References

1. *WADA* Technical Document TD IRMS (current version): Detection of synthetic forms of Endogenous Anabolic Androgenic Steroids by GC/C/IRMS.

https://www.wada-ama.org/en/resources/search?f[0]=field_resource_collections%3A30

2. Mareck U, Geyer H, Opfermann G, Thevis M, Schänzer W. Factors influencing the steroid profile in doping control analysis. *J Mass Spectrom*. **43**(7):877-91, 2008.

3. Ayotte C. Detecting the administration of endogenous anabolic androgenic steroids. *Handb Exp Pharmacol.* **195**:77-98, 2010.

4. Kuuranne T, Saugy M, Baume N. Confounding factors and genetic polymorphism in the evaluation of individual steroid profiling. *Br J Sports Med*. **48**(10):848-55, 2014.

5. J D Cook, Caplan YH, LoDico CP and Bush DM. The Characterization of Human Urine for Specimen Validity Determination in Workplace Drug Testing: A Review. *J Anal Toxicol* **24**: 579-588, 2000

6. *WADA* Technical Document TDIDCR (current version): Minimum Criteria for Chromatographic-Mass Spectrometric Confirmation of the Identity of Analytes for Doping Control Purposes.

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