

WADA Technical Document – TD2014EAAS

Document Number:	TD2014EAAS	Version Number:	2.0
Written by:	WADA Laboratory Expert Group	Approved by:	WADA Executive Committee
Date:	17 May 2014	Effective Date:	1 September 2014

Endogenous Anabolic Androgenic Steroids Measurement and Reporting

1.0 Introduction

The purpose of this Technical Document is to harmonize the approaches to the measurement and reporting of endogenous anabolic androgenic steroids (EAAS) in urine, including data in support of the steroidal module of the Athlete Biological Passport (ABP) or “steroid profile”.

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 steroid module of the ABP uses the Adaptive Model to identify an Atypical Passport Finding (ATPF), which triggers the performance of Confirmation Procedures. It is also used to apply intelligent target *Testing* of the *Athlete* on a longitudinal basis. Furthermore, an abnormal “steroid profile” (obtained from a single urine *Sample*) or an atypical “longitudinal steroid profile” (including values obtained from a series of “steroid profiles” collected over a period of time), may be a means to pursue an anti-doping rule violation (ADRV).

EAAS *Testing* and reporting follows a two-step procedure: an Initial Testing Procedure aims to estimate the “steroid profile” in 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” finding. The Confirmation Procedure includes the quantification of the *Markers* of the “steroid profile” as described in this Technical Document as well as Gas Chromatography – Combustion - Isotope Ratio Mass Spectrometry (GC-C-IRMS) analysis which is considered in a separate Technical Document, the TDIRMS [1].

WADA Technical Document – TD2014EAAS

Document Number:	TD2014EAAS	Version Number:	2.0
Written by:	WADA Laboratory Expert Group	Approved by:	WADA Executive Committee
Date:	17 May 2014	Effective Date:	1 September 2014

1.1 The "Steroid Profile"

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

For the purposes of this Technical Document, 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 on hydrolysis by glucuronidase):

- Testosterone (T),
- Epitestosterone (E),
- Androsterone (A),
- Etiocholanolone (Etio),
- 5 α -androstane-3 α ,17 β -diol (5 α Adiol),
- 5 β -androstane-3 α ,17 β -diol (5 β Adiol), and
- The ratio of Testosterone to Epitestosterone (T/E).

Other urinary steroids or ratios of steroid metabolites could be useful in evaluating a "steroid profile" (e.g. A/T, A/Etio, 5 α Adiol/5 β Adiol, 5 α Adiol/E¹).

The administration of EAAS can alter one or more of the *Markers* and/or ratios of the urinary "steroid profile", resulting in increased or decreased concentrations and/or ratios of specific pairs of steroid metabolites. Additionally, alteration of the urinary "steroid profile" can occur for a number of reasons including, but not limited to:

- A large intake of alcohol (ethanol).
- The administration of ketoconazole, human chorionic gonadotrophin (hCG) in males or of other anabolic steroids (e.g. stanozolol).
- The administration of inhibitors of 5 α -reductase (e.g. finasteride).
- The use of masking agents (e.g. probenecid) and diuretics.
- Microbial growth.

¹ In *ADAMS*, the values of these four ratios are computed after the reporting of the "steroid profile" by the Laboratory.

WADA Technical Document – TD2014EAAS

Document Number:	TD2014EAAS	Version Number:	2.0
Written by:	WADA Laboratory Expert Group	Approved by:	WADA Executive Committee
Date:	17 May 2014	Effective Date:	1 September 2014

2.0 **Initial Testing Procedure**

In the Initial Testing Procedure, the Laboratory shall use a method validated in urine that is appropriate for estimating the *Markers* of the “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 standards should be analyzed periodically, and whenever a significant change is made to the analytical setup.
- A urine quality control (QC) sample containing representative levels of the analytes 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 verified with isotopically labeled A-glucuronide (or an equivalent scientifically recognized alternative).
- The completeness of the derivatization shall be verified through the monitoring of mono-O-TMS vs. di-O-TMS derivative of A.
- When needed, the volume² of the *Sample Aliquot* may be adjusted as a function of its specific gravity (SG) and of the gender of the *Athlete*.
- The T/E ratios shall be determined from the ratios of the corrected chromatographic peak areas or peak heights³.
- The linearity of the method, established during method validation, shall cover the ranges of values normally found in males and females -

² Much lower levels of T and E are generally present in female *Samples* and in those *Samples* with low SG; therefore, larger Aliquot volumes may be required for a reliable measurement.

³ 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).

WADA Technical Document – TD2014EAAS

Document Number:	TD2014EAAS	Version Number:	2.0
Written by:	WADA Laboratory Expert Group	Approved by:	WADA Executive Committee
Date:	17 May 2014	Effective Date:	1 September 2014

the limit of quantification (LOQ) for T and E shall not be higher than 2 ng/mL⁴.

- The relative standard combined uncertainty [$u_c(\%)$] for the determination of A, Etio, 5 α Adiol, 5 β Adiol, T and E, as estimated during method validation of the Initial Testing Procedure, shall be not higher than 30% at the respective LOQ;

For concentration values at five times the LOQ, the $u_c(\%)$ shall be not higher than 20% for A and Etio or 25% for the Adiols;

The $u_c(\%)$ for determinations of T and E shall not exceed 20% when the steroid concentrations are higher than 5 ng/mL;

The $u_c(\%)$ for determinations of T/E ratios calculated from the corrected chromatographic peak areas or heights shall not exceed 15% when the concentrations of T and E are higher than 5 ng/mL; for lower concentrations of T or E, the $u_c(\%)$ for the T/E determinations shall not exceed 30%.

- Evidence of microbial degradation (*e.g.* presence of 5 α - and 5 β -androstenedione or 4-androstenedione) and the presence of 5 α -reductase inhibitors (*e.g.* finasteride) shall be monitored.

⁴ The LOQ shall be determined as the lowest concentration that can be measured with the uncertainty criteria established for the given *Marker* of the “steroid profile” when applying the Initial Testing Procedure.

The LOQ for T, E, A, Etio, 5 α Adiol and 5 β Adiol shall be reported once in *ADAMS* by the Laboratory. The LOQ values shall be updated in *ADAMS* whenever a significant change is made to the analytical method.

WADA Technical Document – TD2014EAAS

Document Number:	TD2014EAAS	Version Number:	2.0
Written by:	WADA Laboratory Expert Group	Approved by:	WADA Executive Committee
Date:	17 May 2014	Effective Date:	1 September 2014

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

The Laboratory shall report in *ADAMS* the T/E ratio, the concentrations of T, E, A, Etio, 5 α Adiol and 5 β Adiol (without adjustment for the SG of the *Sample*), the SG and the validity of the *Sample*, as determined in the Initial Testing Procedure^{5, 6}.

The validity of the *Sample* shall be reported in *ADAMS* as "yes" or "no".

- A *Sample* showing signs of microbial degradation or containing any of the substances⁷ that may cause an alteration of the "steroid profile", as described in Section 1.0 above, may not be suitable for inclusion in the "longitudinal steroid profile". In such cases the validity of the "steroid profile" shall be reported in *ADAMS* as "no" and an explanation shall be included in the Test Report in *ADAMS*.
- When the measurement of a *Marker* of the "steroid profile" 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 a modified, validated *Sample* preparation and analysis (e.g. solid phase extraction, extraction with a different solvent or other equivalent procedure).
 - When (a) *Marker(s)* of the "steroid profile" cannot be measured accurately (i.e. below the LOQ of the assay), the concentration of the negatively impacted *Marker(s)* shall be reported as "-1"⁶. However, if the T/E ratio of the *Sample* can be determined from the ratios of the corrected chromatographic peak areas or peak

⁵ When reporting the "steroid profile" in *ADAMS*, the Laboratory shall report the values of concentrations for T, E, A, Etio, 5 α Adiol and 5 β Adiol, and the T/E ratio as measured (without correction for a specific number of significant figures).

However, an automatic correction of reported values to 2 significant figures will be made in *ADAMS* upon application of the Adaptive Model of the ABP to the "longitudinal steroid profile" of the *Athlete*.

⁶ Any concentration measured below the LOQ shall be reported as "-1" by the Laboratory.

⁷ It is not mandatory that the Laboratory tests for the presence of ethanol metabolite(s) or ketoconazole during the Initial Testing Procedure.

WADA Technical Document – TD2014EAAS

Document Number:	TD2014EAAS	Version Number:	2.0
Written by:	WADA Laboratory Expert Group	Approved by:	WADA Executive Committee
Date:	17 May 2014	Effective Date:	1 September 2014

heights, the “steroid profile” of the *Sample* shall be considered as valid and reported in *ADAMS* as “yes”.

- When the T/E ratio cannot be determined from the ratios of the corrected chromatographic peak areas or peak heights, the T/E value shall be reported as “-1” and the validity of the *Sample* shall be reported as “no”. A comment shall be included in the Test Report in *ADAMS* stating that the T/E ratio could not be measured reliably.

The Laboratory may recommend in the Test Report in *ADAMS* that a *Sample* be submitted to confirmation analyses by GC-C-IRMS.

3.0 Confirmation Procedure

Confirmation Procedures for the exogenous administration of EAAS include the GC-MS or GC-MS/MS quantification and GC-C-IRMS analyses of the relevant *Marker(s)* of the “steroid profile”. GC-C-IRMS analyses are considered in a separate Technical Document, the TDIRMS [1].

“ATPF Confirmation Procedure Request” Notification

The Laboratory shall confirm the relevant “steroid profile” *Marker(s)* or ratio (e.g. the T/E ratio) measured in the Initial Testing Procedure when, upon reporting the results in *ADAMS* and following the application of the Adaptive Model of the ABP to the “longitudinal steroid profile” of the *Athlete*, the Laboratory receives an automatic “ATPF Confirmation Procedure Request” notification through *ADAMS*.

The Adaptive Model will generate an ATPF notification when the following criteria are met:

- i). The *Sample* is matched with a Doping Control Form (DCF) in *ADAMS*, allowing the automatic inclusion of the *Sample’s* “steroid profile” in the *Athlete’s* steroidal passport,
- ii). There is an already existing “longitudinal steroid profile” of the *Athlete* in *ADAMS*,
- iii). The *Sample’s* T/E ratio is abnormal, as determined by the Adaptive Model, when compared with the previous longitudinal T/E values of the *Athlete*, and/or

WADA Technical Document – TD2014EAAS

Document Number:	TD2014EAAS	Version Number:	2.0
Written by:	WADA Laboratory Expert Group	Approved by:	WADA Executive Committee
Date:	17 May 2014	Effective Date:	1 September 2014

- iv). The *Sample's* "steroid profile" meets any of the following two criteria:
- o Concentration of T or E (adjusted for the SG⁸) greater than 200 ng/mL in males or greater than 50 ng/mL in females.
 - o Concentration of A or Etio (adjusted for the SG⁸) greater than 10,000 ng/mL combined with ratio of A/Etio lower than 0.4 in males or greater than 4 in either sex.
- Following the performance of the confirmation analyses, the Laboratory shall update the *ADAMS* record for the *Sample* based on the results of the Confirmation Procedure(s) (refer to the TDIRMS [1]).

"Suspicious Steroid Profile Confirmation Procedure Request" Notification

The Laboratory will receive an automatic "Suspicious Steroid Profile Confirmation Procedure Request" notification through *ADAMS* if:

- i). The *Sample* is matched with a DCF in *ADAMS*, but there is no existing "longitudinal steroid profile" of the *Athlete* in *ADAMS*, or
- ii). The *Sample* cannot be matched with a DCF in *ADAMS* within 14 calendar days after the reception of the *Sample* by the Laboratory, and therefore the "steroid profile" of the *Sample* cannot be processed by the Adaptive Model in *ADAMS*, and
- iii). The *Sample's* "steroid profile" meets any of the following three criteria:
 - o T/E ratio (calculated from the corrected chromatographic peak areas or heights) greater than 4.0.
 - o Concentration of T or E (adjusted for the SG⁸) greater than 200 ng/mL in males or greater than 50 ng/mL in females.
 - o Concentration of A or Etio (adjusted for the SG⁸) greater than 10,000 ng/mL combined with ratio of A/Etio lower than 0.4 in males (in the absence of inhibitors of 5 α -reductase) or greater than 4 in either sex.

⁸ The concentrations are adjusted to a urine SG of 1.020 based on the following equation (free and hydrolyzed glucuroconjugated steroids).

$$\text{Conc}_{\text{corr}} = \text{Conc}_{\text{measured}} * (1.020 - 1)/(SG - 1)$$

WADA Technical Document – TD2014EAAS

Document Number:	TD2014EAAS	Version Number:	2.0
Written by:	WADA Laboratory Expert Group	Approved by:	WADA Executive Committee
Date:	17 May 2014	Effective Date:	1 September 2014

- Upon reception of the “Suspicious Steroid Profile Confirmation Procedure Request” notification, the Laboratory shall proceed with the Confirmation Procedure(s) unless, after contacting the Testing Authority, the Testing Authority can justify within 7 calendar days that the Confirmation Procedure(s) is not necessary.
- 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) were not necessary, and the explanation provided by the Testing Authority.
- If the Testing Authority cannot justify that confirmation is not necessary, the Laboratory shall proceed with the confirmation analyses and subsequently update the *ADAMS* record for the *Sample* based on the results of the Confirmation Procedure(s) (refer to the TDIRMS [1]).

3.1 *GC-MS or GC-MS/MS quantification Confirmation Procedure*

The Laboratory shall identify (in compliance with the TDIDCR [2])⁹ and quantify the relevant *Markers* of an ATPF or a suspicious steroid profile finding in one additional *Sample Aliquot* by a validated fit-for-purpose GC-MS or GC-MS/MS quantification method.

- If GC-C-IRMS analysis has been performed with negative or inconclusive results, the Laboratory shall confirm the T/E ratio only.
- In cases when the GC-C-IRMS analysis demonstrates the exogenous administration of EAAS, the Laboratory shall confirm the relevant variable(s) of the “steroid profile”. When the exogenous administration involves T, only the T/E ratio shall be confirmed.

During the Confirmation Procedure, the presence of conjugated metabolite(s) of ethanol or ketoconazole shall be determined as well as the signs of microbial degradation including, for example, the presence of the free forms of T, 5 α - and 5 β -androstenedione, 4-androstenedione, or DHEA.

⁹ For T/E values, only T needs to be identified if the concentration level and volume of the *Sample* are sufficient.

WADA Technical Document – TD2014EAAS

Document Number:	TD2014EAAS	Version Number:	2.0
Written by:	WADA Laboratory Expert Group	Approved by:	WADA Executive Committee
Date:	17 May 2014	Effective Date:	1 September 2014

3.1.1 Method Characteristics for GC-MS or GC-MS/MS quantification Confirmation Procedure

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

- Calibration standards and urine QC samples shall be included;
- The $u_c(\%)$ shall be not higher than 15% for determinations of A, Etio, 5 α Adiol and 5 β Adiol at concentrations representing five times the respective LOQ;
- For determinations of T, E and T/E ratios, the $u_c(\%)$ shall be not higher than 15% when the concentrations of T and E are higher than 5 ng/mL.

3.1.2 Reporting Results from the GC-MS or GC-MS/MS Confirmation Procedures

The Laboratory shall report in *ADAMS* the confirmed values of the “steroid profile” (without adjustment for the SG of the *Sample*)^{5, 6}, the associated u_c expressed in units and the SG of the *Sample*.

The presence of signs of microbial degradation, of conjugated metabolite(s) of ethanol, of inhibitors of 5 α -reductase, or of any other substances that might have altered the “steroid profile” shall be reported.

4.0 References

<http://www.wada-ama.org/en/Science-Medicine/Anti-Doping-Laboratories/Technical-Documents/>

1. WADA Technical Document TDIRMS (current version): Detection of synthetic forms of Endogenous Anabolic Androgenic Steroids by GC-C-IRMS.
2. WADA Technical Document TDIDCR (current version): Identification Criteria for Qualitative Assays incorporating Column Chromatography and Mass Spectrometry.