WADA Technical Document – ~~TD2021NA~~ISL TD2027NA

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Written by: Reviewed by:	*WADA* Science ~~/~~ /NA Working Group *WADA* Laboratory Expert Group	Approved by:	*WADA* Executive Committee
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HARMONIZATION OF ANALYSIS AND REPORTING OF 19-NORSTEROIDS ~~RELATED TO NANDROLONE~~

1.0 Introduction

This *Technical Document* (*TD~~)~~*), which constitutes an integral part of the *International Standard* for Laboratories (ISL [1]), has been established to harmonize the ~~Confirmation Procedure (CP) for the~~ analysis of 19-norsteroids in urine and the reporting of 19-norsteroids findings ~~for 19-norsteroids related to nandrolone~~ by Laboratories.

The detection of the *Use* of ~~nandrolone (19-nortestosterone) and other~~ 19-norsteroids (*e.g.,* 19-nortestosterone (nandrolone), 19-norandrostenedione, 19-norandrostenediol) is based primarily upon the identification of the main urinary *Metabolite*, 19-norandrosterone (19-NA). More than one *Metabolite* of administered 19-norsteroids may be detected in urine *Samples* and reported [*e.g.,* 19-noretiocholanolone (19-NE)]; however, the identification of 19-NA, including the demonstration, ~~when~~where required, that the 19-NA is not of endogenous origin, is generally sufficient to report an *Adverse Analytical Finding (AAF)*. In some cases, the analysis of 19-NE may be needed to establish the *Use* of 19-norsteroids (see Article 3.2.1.2.1)

[Comment to Article 1.0: In the context of this ISL TD, "endogenous" origins of 19-NA~~,~~ at levels higher than 2.5 ng/mL~~,~~ include: i~~),~~) pregnancy [1-3~~,~~; 2-4], and ii) in- situ microbial degradation of androsterone (A) to 19-NA [4]~~; and iii) consumption of edible parts of non-castrated male pigs [3, 5-10].~~ [5]. This ISL TD ~~aims at providing~~provides elements to determine the source of 19-NA in each of these cases~~.~~]. In rare cases, the consumption of edible parts of non-castrated male pigs or cryptorchids can also result in 19-NA at levels higher than 2.5 ng/mL [4, 6-12].]

2.0 ~~Initial *Testing* Procedure (ITP)~~for Analysis of 19-Norsteroids

The ~~initial test must detect the presence of 19-NA in urine *Samples* at levels greater than or equal to (≥)~~ analysis of 19-norsteroids follows a two (2)-step procedure:

a) An Initial *Testing* Procedure (ITP) (see Article 3.1 ~~ng/mL~~) based on the Gas Chromatography-Mass Spectrometric ($GC\text{-}MS^n$) detection and estimation of the concentration of 19-NA (and 19-NE, where necessary – see also Article 3.2.1.2.1), and

b) A subsequent Confirmation Procedure (CP) (see Article 3.2), which consists of the $GC\text{-}MS^n$ identification (as per ISL *TD* IDCR [13]) and estimation of the concentration of 19-NA and/or 19-NE

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(where necessary), and the eventual performance of Gas Chromatography/Combustion/Isotope Ratio Mass Spectrometry (GC/C/IRMS) analysis (see Article 3.2.1.2).

For the estimation of 19-NA and 19-NE concentrations by GC-MS^n, the Laboratory shall target, both during the ITP and the CP, the total content of free steroid obtained from the non-conjugated steroid plus the free steroid fraction released after hydrolysis of the respective phase-II glucuronidated *Metabolite* with an enzyme with substrate specificity for β-D-glucuronide linkages (*e.g.*, purified β-glucuronidase from *E. coli* or other Fit-for-Purpose enzyme with the same substrate specificity, as determined by the Laboratory during Test Method validation).

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3.0 Analytical *Testing* Procedure Requirements

3.1 Initial *Testing* Procedure ~~also provide its~~

The ITP of 19-norsteroids includes the GC-MS^n detection and estimation of the concentration of 19-NA (and 19-NE, where necessary).

a) The ITP shall be validated according to the ITP validation requirements established in the ISL *TD* VAL [14].

- The Limit of Detection (LOD) of the ITP for 19-NA and 19-NE, as estimated ~~concentration when lower than~~ during Test Method validation, shall be less than or equal to (≤) ~~15~~1.25 ng/mL ~~in order to guide the CP~~.

b) ~~The~~ The ITP shall include the enzymatic hydrolysis of the 19-NA (and 19-NE, where necessary) glucuronide using an enzyme with substrate specificity for β-D-glucuronide linkages (*e.g.*, purified β-glucuronidase from *E. coli*). The efficiency of hydrolysis shall be monitored in each test Aliquot with isotopically labeled Androsterone (A)-glucuronide (or an equivalent scientifically recognized steroid glucuronide alternative).

c) The Aliquot shall be subject to derivatization to produce TMS derivatives of the target *Metabolite*(s) (TMS enol ethers and/or TMS ethers). The efficiency of the derivatization shall be controlled in each test Aliquot through the monitoring of mono-O-TMS vs. di-O-TMS derivative of A.

~~a)~~d) The ITP ~~Initial *Testing* Procedure (ITP)~~ shall include the following characteristics:

~~• A single calibration point at 15 ng/mL;~~

i. A Single Point Calibrator (SPC) prepared in urine at a concentration of 19-NA (or 19-NE, where necessary) between 2.5-15 ng/mL.

[Comment to Article 3.1 d): Alternatively, at the Laboratory's discretion and according to its Test Method validation results, a calibration curve in urine with a working range of at least 2.5-15 ng/mL may also be used.]

ii. A urine NQC with an estimated concentration of 19-NA (or 19-NE, where necessary), if detected in the sample, not higher than (≤) 1.25 ng/mL.

iii. A urine PQC containing 19-NA (or 19-NE, where necessary) at a concentration higher than (>) 2.5 ng/mL. It is recommended that the PQC be prepared at a different concentration than the SPC.

~~i.~~iv. An appropriate labeled (*e.g.,* ^{2}H- or ^{13}C~~)~~-) steroid internal standard~~;~~ (ISTD).

~~• The use of a negative (NQC) and a positive quality control (PQC) sample.~~

e) The 19-NA (or 19-NE) concentration estimated during the ITP shall not be corrected for the specific gravity (SG) to subject a *Sample* to the CP.

3.2 Confirmation ~~Procedures (CP)~~Procedure

3 ~~In addition to meeting the~~ "A" *Sample* Confirmation Procedure

The "A" CP of 19-norsteroids includes the GC-MS^n estimation of the concentration and identification ~~criteria described~~ (in compliance with the ISL *TD* IDCR [~~11~~], ~~the Laboratory shall confirm the estimated concentration of 19-NA (and 19-NE, if necessary) and/or perform~~[13]), as well as the eventual GC/C/IRMS analysis ~~[12]~~, of 19-NA (or 19-NE, where necessary) to establish the origin (endogenous or exogenous) of the ~~19-NA~~*Metabolite*(s) detected ~~(or 19-NE, if applicable).~~ .

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~~3.1 Identification and Estimation of Concentration~~

~~The CP to estimate the concentration of 19-NA in the *Sample* shall include the following characteristics:~~

~~• A single calibration point at 15 ng/mL, preferably with the 19-NA concentration based on/traceable to a Certified Reference Material (CRM);~~

3.2.1.1 "A" *Sample* GC-MS^n CP

The GC-MS^n CP for 19-norsteroids shall be validated according to the CP Qualitative Procedure validation requirements established in the ISL *TD* VAL [14].

a) The Limit of Identification (LOI) of the GC-MS^n CP for 19-NA and 19-NE, as estimated during Test Method validation, shall be less than (<) 2.5 ng/mL.

b) Like the ITP, the GC-MS^n CP shall include the enzymatic hydrolysis of the glucuronidated *Metabolite*(s) and the derivatization to produce TMS derivatives (TMS enol ethers and/or TMS ethers). The efficiency of both processes shall be monitored in each CP Aliquot (see Articles 3.1 b) and c).

c) The GC-MS^n CP for 19-NA shall follow the requirements for the confirmation of Non-Threshold Substances subject to a *Minimum Reporting Level* (*MRL*), as specified in the ISL *TD MRL* [15], including the use of:

i. A PQC urine sample at the *MRL* of 15 ng/mL.

ii. A Single Point Calibrator (SPC) [1] prepared in urine at 150% *MRL* (22.5 ng/mL).

iii. A urine NQC with an estimated concentration of 19-NA (or 19-NE, where necessary), if detected in the sample, not higher than (≤) 1.25 ng/mL.

~~i.~~iv. An appropriate labeled ~~internal standard~~ISTD (*e.g.*, ^{2}H-19-NA-~~$^2H_4$~~-glucuronide~~);~~).

[1] The SPC (or calibration curve) shall be prepared from a different batch or different stock solution of Reference Material than the PQC. For the SPC, it is preferably that the concentration is based on/traceable to a Certified Reference Material.

~~• The use of a NQC (at less than (<) 2.5 ng/mL) and a PQC (at greater than (>) 15 ng/mL).~~

[Comment 1 to Article 3.2.1.1: No quantification (and, therefore, no Measurement Uncertainty estimation) is required in the GC-MS^n CP ~~for 19-NA~~. The ~~application~~use of ~~a one-point calibrator~~the SPC at ~~15~~22.5 ng/mL and appropriate QC samples is sufficient to confirm the estimated ~~19-NA~~ concentration of 19-NA (or 19-NE, where necessary.]

[Comment 2 to Article 3.2.1.1: The SPC, NQC and PQC shall be subjected to the same sample preparation procedure as the Sample Aliquot.]

~~3.2~~

~~3.1.1.1~~3.2.1.2 "A" Sample GC/C/IRMS Analysis

~~3.1.1.1.1~~3.2.1.2.1 ~~3.2.1~~ Conducting GC/C/IRMS Analysis

a) The ~~decision to proceed to the~~conditions for performing GC/C/IRMS analysis ~~shall be guided by the following:~~for 19-NA are described in Table 1 below.

~~• GC/C/IRMS analysis is not necessary on *Samples* in which the (SG-adjusted, if needed) concentration of 19-NA is estimated above (>) 15 ng/mL (except in cases of pregnancy), or when the presence of 3,5-tetrahydronorethisterone (THNE) has been detected in the *Sample* of a female *Athlete* [13];~~

~~*[Comment: When the estimated concentration of 19-NA is greater than (>) 15 ng/mL, to decide whether the GC/C/IRMS analysis shall be performed or not, the 19-NA concentration in the Sample shall be adjusted for the urine specific gravity (SG), if $SG_{Sample} > 1.018$, according to:*~~

~~$$Conc_{adj} = \frac{(1.020 - 1)}{SG_{Sample\ Max} - 1} \cdot Conc_{measured}$$~~

~~*Refer to the effective TD DL [14] for instructions on calculating SG_{Sample_Max}].*~~

~~• GC/C/IRMS analysis is mandatory in cases of pregnancy, when the estimated 19-NA concentration is greater than (>) 15 ng/mL. However,~~ The Laboratory should consider conducting GC/C/IRMS analysis for 19-NE when the GC/C/IRMS analysis ~~may also be performed to ascertain the endogenous origin~~ of 19-NA ~~when the estimated concentration of 19-NA in a urine *Sample* of a pregnant female is between (≥) 2.5 and (≤) 15 ng/mL.~~

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~~*[Comment: For Samples from pregnant females, for which the GC/C/IRMS analysis is only mandatory for 19-NA concentrations greater than (>) 15 ng/mL, the adjustment of the 19-NA concentration for a high SG (> 1.018)*~~ is not ~~*strictly necessary].*~~

~~• GC/C/IRMS analysis is mandatory on *Samples* in which the concentration of 19-NA is estimated between (≥) 2.5 and (≤) 15 ng/mL, except in cases of pregnancy or in the~~conclusive (*e.g.*, presence of ~~THNE;~~

~~• GC/C/IRMS analysis may be performed on *Samples* containing 19-NA at estimated concentrations lower than (<) 2.5 ng/mL.~~

~~*[Comment: For Samples with 19-NA lower or equal to (≤) 15 ng/mL, the adjustment of the 19-NA concentration for a high SG (> 1.018) is not needed, since such adjustment may only lead to lower concentrations. For such Samples, GC/C/IRMS analysis is required to determine the origin of the 19-NA and may be performed at concentrations below 2.5 ng/mL, depending on the Laboratory's analytical capacity.]*~~

b) ~~When 5α-~~interferences or when 5α-reductase inhibitor activity is ~~suspected (*e.g.*, abnormally low A/Etio ratio) or~~ confirmed ~~(*e.g.*, detection of finasteride and/or dutasteride *Metabolites*) in a urine~~ in the *Sample*~~, and this~~).

~~•~~ If the Laboratory does not ~~allow a reliable analysis of 19-NA, Laboratories should target 19-NE (which is detected at a higher concentration than 19-NA) for GC/C/IRMS analysis to determine the administration of 19-norsteroids. The GC/C/IRMS method characteristics and data evaluation criteria shall follow the same requirements as for 19-NA determination (see Article 3.2.3).~~

~~b)~~c) ~~Laboratories that do not~~ have the analytical capacity to perform GC/C/IRMS analysis for 19-NA (or 19-NE, if applicable~~)~~), the Laboratory shall, in consultation with the *Testing* Authority (TA) (or the *Results Management* Authority~~,~~ (RMA), if different), have *Samples* transferred to ~~and analyzed by~~ another Laboratory that has such analytical capacity (Analytical Method included in the Laboratory's Scope of ISO/IEC 17025 Accreditation).

Table 1 GC/C/IRMS analysis of 19-NA

GC/C/IRMS analysis	**Estimated concentration**	***Samples***

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Not needed	• > 15 ng/mL [2, 3] **(*AAF*)**	• Males • Females – Non-pregnant, and – Absence of 3,5-tetrahydronorethisterone (THNE)
	• ≤ 15 ng/mL [2, 3]	• Females – Presence of THNE
Required	• 2.5 - 15 ng/mL [2, 4]	• Males • Females – Non-pregnant, and – Absence of THNE
	• > 15 ng/mL [2, 3]	• Females – Pregnant females
Optional	• 2.5 - 15 ng/mL [4]	• Females – Pregnant females
	• < 2.5 ng/mL	• Males • Females – Non-pregnant – Absence of THNE

[2] According to the ISL *TD MRL* [15], only when the estimated concentration of 19-NA (or 19-NE, where applicable) in the *Sample* exceeds that of the 150% *MRL* SPC, and the estimated concentration of 19-NA (or 19-NE, where applicable) in the 100% *MRL* PQC is lower than (<) the respective concentration in the SPC, the Laboratory can conclude with enough confidence that the concentration of 19-NA in the *Sample* is higher than (>) the *MRL* of 15 ng/mL.

For the avoidance of doubt, when the analysis does not fulfill these requirements, *e.g.*, when the estimated concentration of 19-NA is higher than (>) the 100% *MRL* PQC but lower than (<) the 150% *MRL* SPC, the Laboratory cannot conclude with sufficient confidence that the concentration of 19-NA in the *Sample* is higher than (>) the *MRL*. Therefore, in those cases, the Laboratory shall subject the *Sample* to GC/C/IRMS analysis (or subcontract the analysis to another Laboratory with the required analytical capacity) to establish the origin of 19-NA in the *Sample* and determine whether the result constitutes an *AAF* or not.

[3] When the SG of the *Sample* (SG_{Sample}), as measured by the Laboratory during the CP, is higher than (>) 1.018, and the GC-MS^n CP-estimated concentration of 19-NA in the *Sample* is confidently greater than (>) the *MRL* of 15 ng/mL (as determined in accordance with the ISL *TD MRL*) [15], the estimated 19-NA concentration shall be adjusted for the SG of 1.020 [15]. Only when the SG-adjusted concentration is > 15 ng/mL the result can be reported as an *AAF* without the need for GC/C/IRMS analysis.

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[4] In such cases, the adjustment of the 19-NA concentration for a high SG_{Sample} (> 1.018) is not necessary, since it would only lead to a lower adjusted concentration, whereas the actual concentration in the *Sample*, without the adjustment for the SG_{Sample}, should allow the performance of the GC/C/IRMS analysis

3.2.1.2.2 GC/C/IRMS CP Validation and Analysis Requirements

Refer to the ISL *TD* IRMS [16] for general GC/C/IRMS Test Method validation and analysis requirements.

3.2.1.2.3 Interpretation of GC/C/IRMS Results

a) To reject the hypothesis of endogenous or *in situ* 19-NA (or 19-NE, where applicable) formation based on the application of GC/C/IRMS analysis (*i.e.*, to report the finding as an *AAF*), the $|\Delta\delta^{13}C|$ values between two (2) ERCs and 19-NA (or 19-NE, where applicable), *i.e.*, $|\Delta\delta^{13}C| = |\delta^{13}C_{ERC} - \delta^{13}C_{19\text{-NA (or 19-NE)}}|$, shall be greater than (>) 3.0 ‰ (refer also to the ISL *TD* IRMS [16] for use of ERCs and interpretation of GC/C/IRMS test results) .

[Comment to Article 3.2.1.2.4 a): Androsterone (A) shall not be used as an ERC for 19-NA GC/C/IRMS analysis when there are indications of the administration of testosterone or its precursors (e.g., prasterone). In such cases, an alternative ERC as described in ISL TD IRMS [16] shall be used.]

~~a)~~b) Due to the occurrence of preparations of 19-norsteroids with a carbon isotopic signature ($^{13}C/^{12}C$) close to that of endogenous human urinary steroids (*e.g.*, $\delta^{13}C_{19\text{-NA}}$ ~~=~~between -16~~ ‰ to~~.000 ‰ and -24~~ ‰)~~ [9, 10, 15].000 ‰) [11, 17], the result of the GC/C/IRMS analysis of the excreted 19-NA (or 19-NE) may not always readily indicate its exogenous origin in *Samples*~~. Therefore, in *Samples* from males and non-pregnant females, when the estimated concentration of 19-NA is equal to or less than (≤) 15 ng/mL and the~~ (result of the GC/C/IRMS analysis ~~is~~ negative ~~(*i.e.* not consistent with an exogenous origin of 19-NA)~~ or inconclusive~~, the Laboratory shall consider the ratio of 19-NA to 19-NE (based on the respective estimated concentrations) as a possible indicator of the administration of 19-norsteroids when the *in situ* formation of 19-NA and 19-NE is excluded [3].~~).

~~*[Comment: The possible Use for doping purposes of 19-norsteroid preparations with a pseudo-endogenous carbon isotopic signature may be established on the basis of the pharmacokinetics of 19-NA excretion, as determined from the analysis of previously collected and/or follow-up Samples [3, 5, 16-18].*~~

b)c) ~~*Following consumption of the edible parts of non-castrated male pigs, concentrations of excreted 19-NA in urine are usually in the low ng/mL range (< 10 ng/mL), although higher concentrations have been exceptionally reported [3]. The origin of the urinary 19-NA may not be established by GC/C/IRMS analysis, since the varying diets of migrating wild boars lead to dissimilar $\delta^{13}C$ values which may range between -15 ‰ and -25 ‰ [9]. Therefore, if*~~ If the consumption of edible parts of intact pigs or cryptorchids is invoked by an *Athlete* as the unlikely origin of a 19-NA finding, ~~*this may be established based on the pharmacokinetics of 19-NA*~~ the TA (or RMA, if different) shall consider the compatibility of any previously collected and/or follow-up *Samples* (viz. the analytical results) with that explanation in terms of 19-NA excretion pattern in urine. The excretion ~~*[3, 5, 16-18]. Profiles*~~kinetics of 19-NA and 19-NE ~~*excretion*~~ following oral ingestion will have a different time course than ~~*following*~~after an injection of 19-norsteroids~~.~~ [6, 9, 12, 19-21].

~~3.2.2 GC/C/IRMS Test Method Validation Requirements~~

- ~~The Laboratory shall refer to the TD IRMS [12] for general GC/C/IRMS Test Method validation requirements;~~
- ~~The Laboratory shall validate the use of at least two (2) ERCs [*e.g.*, Androsterone (A) and pregnanediol (PD)];~~
- ~~The standard combined uncertainty (u_c) associated with the determination of $\delta^{13}C$ values, as estimated by the Laboratory during the GC/C/IRMS method validation, shall not be greater than (≤) the u_{c_Max} of 1.0 ‰ for 19-NA or 0.7 ‰ for the ERCs.~~

~~3.2.3 GC/C/IRMS Analysis Requirements~~

~~The GC/C/IRMS method to establish the origin of the 19-NA detected shall include the following characteristics (also refer to the TD IRMS [12] for general method characteristics):~~

- ~~Each sequence of analysis by GC/C/IRMS shall include:~~
  - ~~A NQC: $\delta^{13}C$ values of 19-NA and endogenous reference compound(s) (ERC) in a normal endogenous range (*i.e.* between -16 ‰ and -26 ‰), with an absolute difference in $\delta^{13}C$ values ($|\Delta\delta^{13}C|$) between ERC and 19-NA not greater than (≤) 3 ‰;~~

~~*[Comment: The normal endogenous range of $\delta^{13}C$ values between -16 ‰ and -26 ‰ reflects the range of $\delta^{13}C$ isotopic signatures of urinary steroid Metabolites in humans around the world; the QC samples will reflect the geographical location of the Laboratory and do not have to cover the entire possible range of $\delta^{13}C$ values.]*~~

~~- A PQC: $\delta^{13}C$ value of ERC in a normal endogenous range (*i.e.*, between -16 ‰ and -26 ‰), with a $|\Delta\delta^{13}C|$ between ERC and 19-NA greater than (>) 3 ‰.~~

~~*[Comment: The NQC and PQC shall be subjected to the same sample preparation procedure as the Sample Aliquot.]*~~

~~• The same *Sample* Aliquot(s) that were subjected to GC/C/IRMS analysis shall be analyzed by GC-MS under similar chromatographic conditions to ensure the identity of the peaks of 19-NA and ERC(s), and the absence of significant interference prior to reporting an *AAF* or an *ATF* based on GC/C/IRMS results.~~

~~*[Comment: Minor differences in retention times (RT) between the two techniques are expected. The provisions of the TD IDCR [11] shall be followed. In addition, a full scan spectrum shall be obtained over the complete width of the steroid chromatographic peak(s) of interest to document the lack of interference. GC-MS identification is not necessary when the GC/C/IRMS results are negative.]*~~

~~• The GC/C/IRMS confirmation analyses shall include the identification of the 19-NA peak identity by GC-MS according to the identification criteria described in the TD IDCR [11].~~

~~*[Comment: Minor differences in retention time between the two techniques are expected.]*~~

~~3.1.1.1.2~~3.1.1.1.1 ~~3.2.4 Interpretation of GC/C/IRMS Results~~

~~To reject the hypothesis of endogenous or *in-situ* 19-NA formation based on the application of GC/C/IRMS analysis (*i.e.*, to report the finding as an *AAF*), the following criterion shall be met:~~

~~• The $|\Delta\delta^{13}C|$ values between two (2) ERCs and 19-NA, *i.e.*, $|\Delta\delta^{13}C| = |\delta^{13}C_{ERC} - \delta^{13}C_{19\text{-}NA}|$, is greater than (>) 3 ‰ (refer to the TD IRMS [12]).~~

~~*[Comment: Androsterone (A) shall not be used as an ERC when there are indications of the administration of testosterone (T) or its precursors (e.g., prasterone). In such cases, an alternative ERC as described in TD IRMS [12] shall be used.]*~~

~~3.1.1.2~~3.2.1.3 ~~3.3~~ Additional Tests

~~3.1.1.2.1~~3.2.1.3.1 ~~3.3.1 Test~~Tests for Norethisterone and Pregnancy

19-NA is excreted during pregnancy [~~1 3~~2-4] and as a minor *Metabolite* of norethisterone [~~13~~18, 22, 23], a progestogen agent ~~of permitted use~~ that is present in some oral contraceptives of permitted use in some countries. Therefore, when the estimated concentration of 19-NA is equal to or exceeds (≥) 2.5 ng/mL in the urine *Sample* of a female *Athlete,* the Laboratory shall:

a) Test for pregnancy based on the measurement of urinary human Chorionic Gonadotrophin (hCG) in the urine *Sample*.

~~a)~~b) Establish the presence or absence of 3,5-tetrahydronorethisterone (THNE~~,~~), the main *Metabolite* of norethisterone, and ~~if not~~ whether it is compatible with the 19-NA ~~level:~~concentration.

~~• Test for pregnancy based on the measurement of urinary human Chorionic Gonadotrophin (hCG).~~

[Comment~~:~~ to Article 3.2.1.3.1 b): When testing for the presence of THNE, Laboratories should target the ~~detection of the 3α,5β~~3α,5β isomer, namely 5~~β~~-β-estran-17~~α~~-α-ethynyl-3~~α~~,α,17~~β~~-β-diol [~~13~~]~~.~~[23]. With 19-NA being a minor Metabolite of norethisterone, 19-NA concentrations at levels higher than (>)~~10~~15 ng/mL should be associated with ~~a very intense THNE peak signal~~an estimated concentration ratio of 19-NA / 5β-estran-17α-ethynyl-3α,17β-diol ≤ 2 in the Sample.]

~~3.1.1.2.2~~3.2.1.3.2 ~~3.3.2~~ Test for Demethylation

In addition, ~~but rarely,~~ 19-NA and 19-NE may be produced in urine *Samples*, in small concentrations, by *in~~-~~ situ* 19-demethylation of androsterone (A) [4]~~.~~and Etiocholanolone (Etio), respectively [5]. This potential *in~~-~~ situ* formation of 19-NA ~~shall be~~and 19-NE is verified by GC/C/IRMS analysis [~~3, 19~~24].

~~3.1.2~~3.2.2 ~~3.4~~ "B" *Sample* ~~CP~~Confirmation Procedure

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a) In cases when the *AAF* for the "A" *Sample* is based on the ~~results of a~~ GC/C/IRMS ~~analysis~~results, the "B" *Sample* CP ~~also~~ requires only the GC/C/IRMS analysis ~~and identification of 19-NA in accordance with the TD IDCR [11];~~.

b) In cases when the estimated concentration of 19-NA (SG-adjusted, if needed) is shown to be greater than (>) 15 ng/mL (in accordance with ISL *TD MRL* [15] requirements) in a *Sample* collected from a male or a non-pregnant female *Athlete*, the "B" *Sample* CP requires only the identification of 19-NA only, in accordance with the ISL *TD* IDCR [~~11~~13].

~~3.0~~4.0 Interpretation and Reporting of Results

a) The Laboratory shall report 19-NA ~~detected~~(and/or 19-NE, where applicable) findings in a *Sample* from a male or a female *Athlete* as defined in the Table below~~:~~ (see also flow diagram in Annex A).

b) Where GC/C/IRMS results are to be reported, the Laboratory shall refer to the ISL *TD* IRMS [16] for GC/C/IRMS results reporting requirements.

Table 2 Interpretation and Reporting of Results for 19-Norsteroids

***Sample* Origin** ~~/ Test Report~~	***Adverse Analytical Finding***	***Atypical Finding***	**Negative Finding**
4.1 Pregnant Female *Athletes*	**Results**		

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	~~o~~ Results of the GC/C/IRMS analysis are consistent with the exogenous origin of 19-NA ~~(see Art. 3.2.3).~~ .	– Estimated concentration of 19-NA > 15 ng/mL~~;~~. [2, 3] AND – Results of ~~the mandatory~~ GC/C/IRMS analysis are inconclusive or do not meet the criteria supporting an exogenous origin of 19-NA ~~(see Art. 3.2.3).~~.	~~No other *Prohibited Substance* or *Prohibited Method* has been confirmed in the *Sample*;~~ ~~AND~~ – Estimated concentration of 19-NA ~~≤~~≤ 15 ng/mL~~;~~. [2, 3] ~~AND~~ AND – GC/C/IRMS analysis ~~was~~is either not performed, or the results are ~~inconclusive or~~ consistent with an endogenous origin of 19-NA ~~(see Art. 3.2.3).~~.
	Test Report **(No reference to the pregnancy status of the *Athlete* shall be reported)**		
	– Estimated concentration of 19-NA, expressed as "≤15 ng/mL" or ">15 ng/mL~~", as applicable;~~" [2, 3]. ~~o~~ Results of GC/C/IRMS analysis, including a comment indicating that the GC/C/IRMS finding is consistent with an exogenous origin of 19-NA, the $\delta^{13}C$ values for 19-NA and the ~~two~~ ERCs, as well as the ~~associated~~respective u_c (‰).	– Estimated concentration of 19-NA >15 ng/mL [2, 3]. ~~o~~ Results of GC/C/IRMS analysis, including a comment indicating that the GC/C/IRMS finding is inconclusive or ~~do~~does not meet the criteria supporting an exogenous origin of 19-NA, the $\delta^{13}C$ values for 19-NA and the ~~two~~ ERCs as well as the ~~associated~~respective u_c (‰). – ~~No reference to the pregnancy status of the *Athlete* shall be reported.~~	~~No reference to the pregnancy status of the *Athlete* shall be reported~~If GC/C/IRMS analysis is performed: – Estimated concentration of 19-NA ≤ 15 ng/mL [2, 3]. – Include a comment indicating that the GC/C/IRMS finding is consistent with an endogenous origin of 19-NA, the $\delta^{13}C$ values for 19-

	– ~~No reference to the pregnancy status of the *Athlete* shall be reported.~~		NA and the ERC, as well as the respective u_c (‰).
***Sample* Origin**	***Adverse Analytical Finding***	***Atypical Finding***	**Negative Finding**
4.2 Female *Athletes* using Norethisterone	**Results**		
	– ~~N/A~~Estimated concentration of 19-NA > 15 ng/mL [2, 3]. – The estimated concentration ratio of 19-NA/THNE is > 2.	– Estimated concentration of 19-NA > ~~10~~15 ng/mL~~;~~ [2, 3]. ~~OR~~ – The ~~level of THNE appears incompatible with that~~estimated concentration ratio of 19-NA/THNE is ≤ 2.	~~○ No other *Prohibited Substance* or *Prohibited Method* has been confirmed in the *Sample*;~~ ~~AND~~ – Estimated concentration of 19-NA ~~≤ 10~~≤ 15 ng/mL~~*~~ ~~and is compatible with that of THNE.~~ [2, 3]
	Test Report		
	– ~~N/A~~Estimated concentration of 19-NA >15 ng/mL [2, 3]. – A comment describing the presence of THNE in the *Sample*. *Example:* *"19-NA was found in the Sample at an estimated concentration > 15 ng/mL.*	– Estimated concentration of 19-NA >~~10~~15 ng/mL~~;~~ [2, 3]. – A comment describing the ~~finding that demonstrates the use~~presence of ~~norethisterone or of any other substance that is converted to norethisterone and further metabolized to tetrahydronorethisterone (*e.g.*,~~ THNE in the *Sample*.	**No reference to the use of norethisterone shall be reported.**

	Tetrahydronorethisterone, a Metabolite of norethisterone, was also detected in the Sample. The estimated concentration ratio 19-NA/THNE is incompatible with norethisterone being the exclusive source of 19-NA".	*Example:* *"19-NA was found in the Sample at an estimated concentration > ~~10~~15 ng/mL. Tetrahydronorethisterone, a Metabolite of norethisterone, was also detected in the Sample~~ at a~~. The estimated concentration ~~that~~ratio 19-NA/THNE is compatible with ~~that~~norethisterone being the exclusive source of 19-NA~~".~~"*	
4.3 Male or Female *Athletes* (neither pregnant nor using norethisterone)	**Results**		

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4.3 Male or Female *Athletes* (neither pregnant nor using norethisterone) (cont.)	

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4.3 Male or Female *Athletes* (neither pregnant nor using norethisterone) (cont.)			
	– Estimated concentration of 19-NA > 15 ng/mL~~,~~. [2, 3] OR ~~o~~ Estimated concentration of 19-NA ≤ 15 ng/mL~~;~~ ~~AND~~ – [2,3] and GC/C/IRMS results are consistent with an exogenous origin of 19-NA ~~(see Art. 3.2.3).~~. OR – 19-NA cannot be reliably analyzed in the *Sample* (*e.g.*, due to the presence of 5α-reductase inhibitor activity) and the GC/C/IRMS results for 19-NE are consistent with an exogenous origin of 19-NE.	~~o~~ Estimated concentration of 19-NA ≤ 15 ng/mL~~;~~ ~~AND~~ – [2,3] and GC/C/IRMS results are inconclusive or do not meet the criteria supporting an exogenous origin of 19-NA ~~(see Art. 3.2.3);~~. ~~AND~~ ~~19-NA/19-NE > 3.~~OR – 19-NA cannot be reliably analyzed in the *Sample* (*e.g.*, due to the presence of 5α-reductase inhibitor activity) and the GC/C/IRMS results for 19-NE are inconclusive or do not meet the criteria supporting an exogenous origin of 19-NE.	~~o~~ ~~No other *Prohibited Substance* or *Prohibited Method* has been confirmed in the *Sample*; AND~~ – Estimated concentration of 19-NA < 2.5 ng/mL (and too low to perform GC/C/IRMS analysis). OR ~~o~~ Estimated concentration of 19-NA ≥between 2.5 ~~ng/mL but ≤~~ 15 ng/mL~~;~~ ~~AND~~ – ~~the~~ [2, 3] and GC/C/IRMS results ~~of the GC/C/IRMS analysis~~ are consistent with an endogenous origin ~~(*i.e.*, *in-situ* formation) of~~ of 19-NA~~;~~. ~~AND~~ ~~19-NA/19-NE ≤ 3~~ OR

			– 19-NA cannot be reliably analyzed in the *Sample* (*e.g.*, due to the presence of 5α-reductase inhibitor activity) and the GC/C/IRMS results for 19-NE are consistent with an endogenous origin of 19-NE.
	Interpretation of Results		
	– If 19-NA > 15 ng/mL with no GC/C/IRMS analysis: • ~~-~~Estimated concentration of 19-NA >15 ng/mL~~;~~ [2, 3]. • For *Samples* from female *Athletes*~~: A~~, include a comment explaining that pregnancy and the use of norethisterone were excluded as the source of 19-NA ~~(*e.g.*,~~. *Example:* *"The 19-NA finding is not consistent with pregnancy or the use of norethisterone~~").~~."* – If 19-NA ≤ 15 ng/mL with positive GC/C/IRMS results~~:~~ • Estimated concentration of 19-NA ≤ 15 ng/mL~~;~~. • Results of GC/C/IRMS analysis, including a comment indicating that the	– If 19-NA ≤ 15 ng/mL with inconclusive GC/C/IRMS results: • Estimated ~~19-NA~~ concentration of 19-NA ≤ 15 ng/mL~~;~~ [2, 3]. • Results of GC/C/IRMS analysis, including a comment indicating that the GC/C/IRMS results are inconclusive (*e.g.*, due to the presence of interfering compound(s) or any other factor preventing a reliable GC/C/IRMS analysis) or do not meet the criteria supporting an exogenous origin of 19-NA, the $\delta^{13}C$ values for 19-NA and the ~~two~~ ERCs as well as the ~~associated~~respective u_c ~~(‰);~~(‰). ~~○ Ratio of 19-NA to 19-NE;~~	– If GC/C/IRMS analysis is performed: • Estimated concentration of 19-NA (either < 2.5 ng/mL or between 2.5-15 ng/mL). • Results of GC/C/IRMS analysis, including a comment indicating that the GC/C/IRMS analysis is consistent with an endogenous origin of 19-NA, the $\delta^{13}C$ values for 19-NA and ERC as well as the respective u_c (‰). – If the finding is based on negative GC/C/IRMS results for 19-NE: • Results of GC/C/IRMS analysis, including a comment indicating that the GC/C/IRMS analysis

	GC/C/IRMS finding is consistent with an exogenous origin of 19-NA, the $\delta^{13}C$ values for 19-NA and the ~~two~~ ERCs as well as the ~~associated~~respective u_c ~~(‰);~~(‰). • For *Samples* from female *Athletes*, a comment explaining that the use of norethisterone was excluded as the source of 19-NA (*e.g.,* "the 19-NA finding is not consistent with the use of norethisterone"). – If the finding is based on positive GC/C/IRMS results for 19-NE: – Results of GC/C/IRMS analysis, including a comment indicating that the GC/C/IRMS finding is consistent with an exogenous origin of 19-NE, the $\delta^{13}C$ values for 19-NE and the ERCs as well as the respective u_c (‰).	• For *Samples* from female *Athletes*, clarify that pregnancy was excluded as the source of 19-NA ~~(*e.g.,* "the~~ *Example: "The 19-NA finding is not consistent with pregnancy"~~);~~".* • Recommend ~~to the *Testing* Authority/Results *Management* Authority~~the TA (or RMA, if different) to conduct follow-up no-notice tests on the *Athlete* as soon as possible and evaluate the ~~pharmacokinetics~~kinetics of 19-NA excretion in urine. – If the finding is based on inconclusive GC/C/IRMS results for 19-NE: – Results of GC/C/IRMS analysis, indicating that the GC/C/IRMS results are inconclusive (*e.g.,* due to the presence of interfering compound(s) or any other factor preventing a reliable GC/C/IRMS analysis) or do not meet the criteria supporting an exogenous origin of 19-NE, the $\delta^{13}C$ values for 19-NE and the ERCs as well as the respective u_c (‰).	is consistent with an endogenous origin of 19-NE, the $\delta^{13}C$ values for 19-NE and the ERC as well as the respective u_c (‰).

~~5.0~~

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~~Annex A – Flowchart for 19-NA Findings~~

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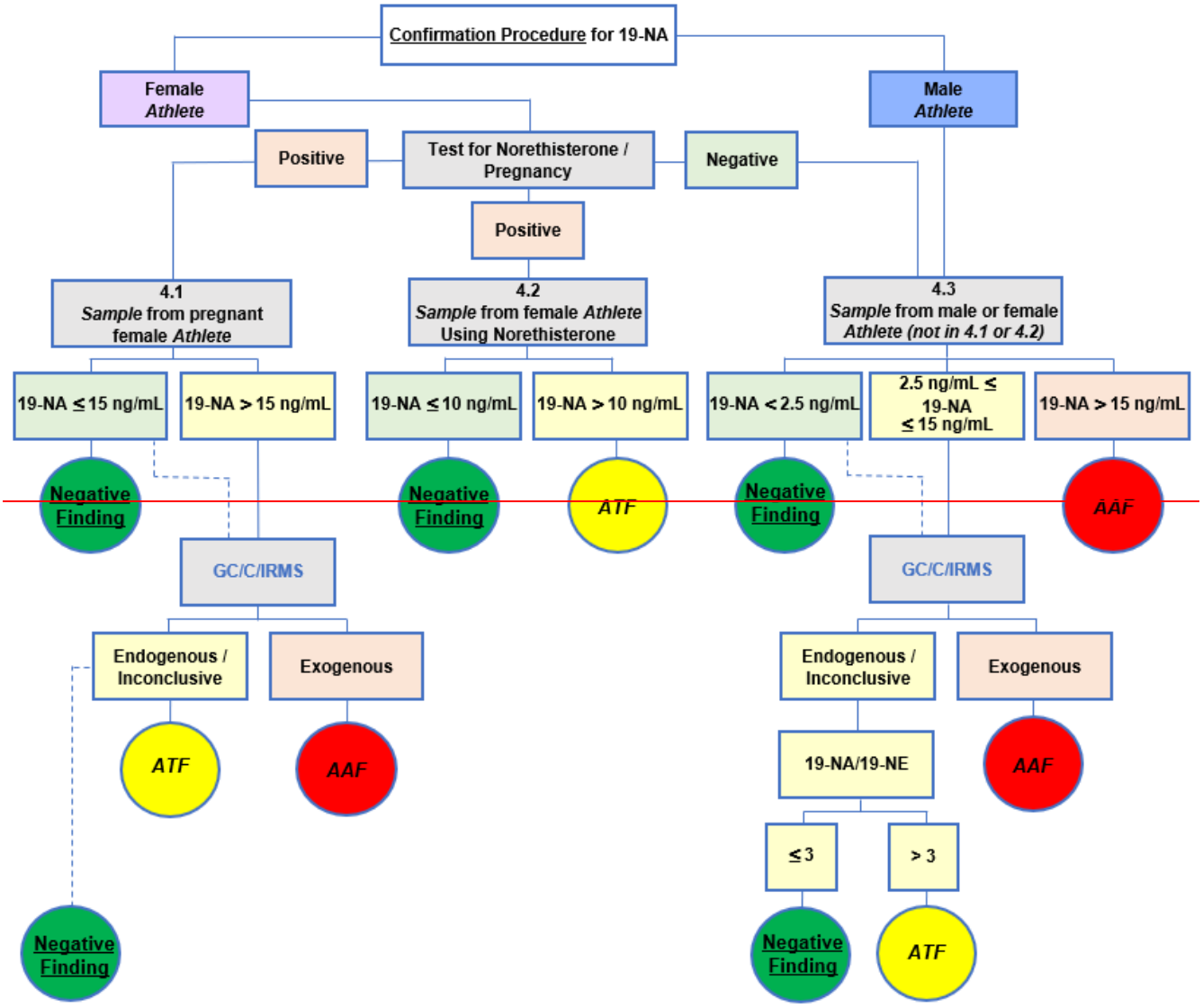

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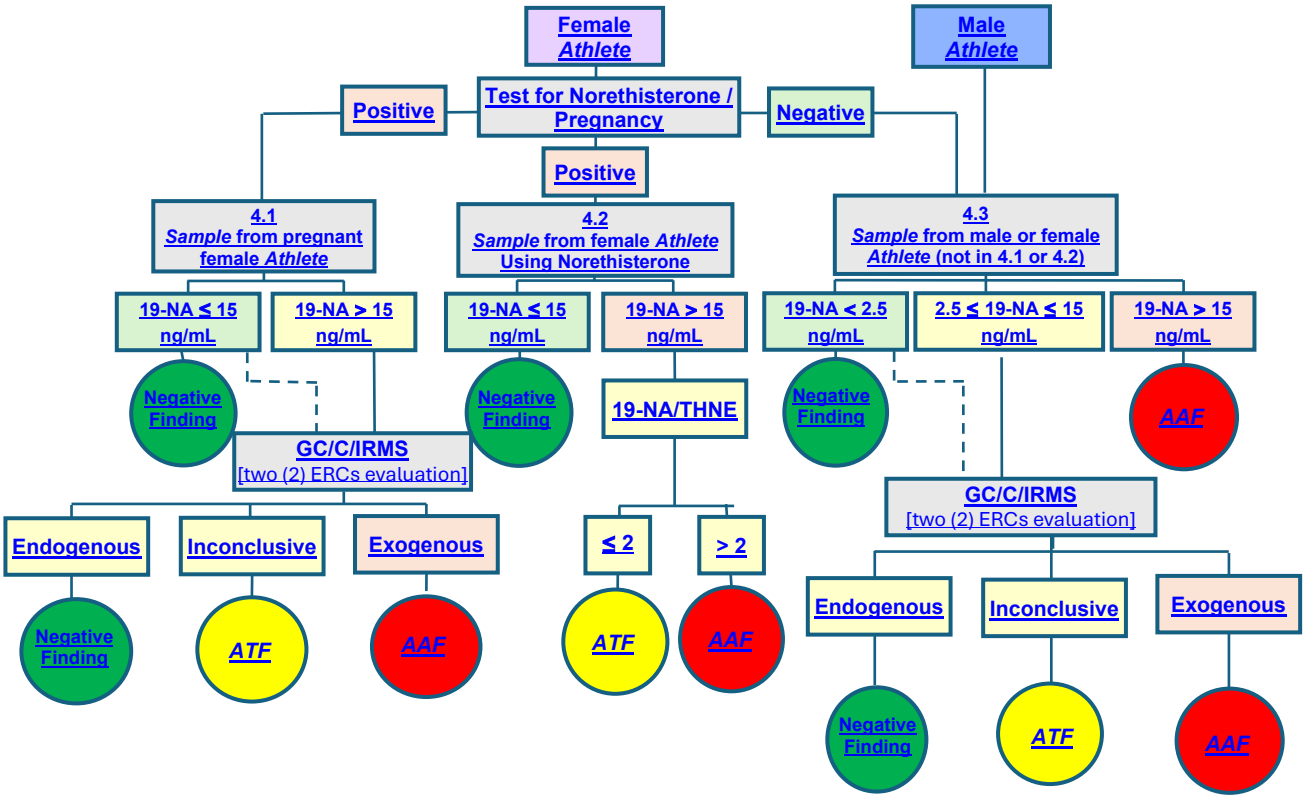
ANNEX A. Confirmation Procedure for 19-Norsteroids

A1. Confirmation Procedure for 19-NA



A.2 Confirmation Procedure for 19-NE