Laboratory Guidelines

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Analytical Requirements for the Endocrine Module of the *Athlete Biological Passport*

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1.0 Objective

These Laboratory Guidelines have been developed to ensure a harmonized application of Analytical *Testing* Procedures for the measurement of *Markers* of human Growth Hormone (hGH) as part of the Endocrine 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* Procedures for the quantification of hGH *Markers* as part of the Endocrine Module of the *ABP*, which allows the detection of hGH doping and may also have utility in detecting GH secretagogues and IGF-I abuse in sport [1,2]. These Laboratory Guidelines follow the rules established in the *WADA International Standard for* Laboratories (ISL) [3] and relevant *Technical Documents* (*TDs*) regarding the Analytical *Testing* of blood *Samples*.

3.0 Introduction to the Analytical *Testing* Procedures

The Analytical *Testing* Procedures for the Endocrine Module involve the measurement of two (2) *Markers* of hGH biological activity, namely Insulin-like Growth Factor-I (IGF-I) and N-terminal Pro-peptide of Type III Collagen (P-III-NP), which are naturally present in blood and whose concentrations are increased following hGH administration [4–11]. The measured concentrations of these two (2) *Markers* are then combined in a discriminant function formulae to calculate a GH-2000 score, which is gender-specific and includes an adjustment for age to reflect the age-related decline in hGH and *Marker* concentrations [4].

In order to generate individual *Athlete* longitudinal data that are comparable between Laboratories, a specific IGF-I / P-III-NP assay pairing is applied for the measurement of concentrations of IGF-I and P-III-NP in blood (serum) for the purposes of the *ABP*. The assays used for the Endocrine Module of the *ABP* are limited to:

- Intact IGF-I quantification by top-down Liquid Chromatography-(tandem) Mass Spectrometry ($LC\text{-}MS^n$; $n \geq 1$) [12], as detailed in Table 2 below.
- P-III-NP quantification using Siemens ADVIA Centaur P-III-NP chemiluminescence immunoassay (Siemens Healthcare Laboratory Diagnostics, Camberley, UK). The Siemens ADVIA Centaur P-III-NP assay is an automated, two-site sandwich, chemiluminescent immunoassay [13]. The assay uses two (2) monoclonal mouse antibodies: the first antibody is an acridinium ester-labeled anti-P-III-NP antibody. The second antibody is a biotin-labeled anti-P-III-NP antibody. The solid phase contains streptavidin-coated paramagnetic particles and during the reaction, the light emitted by the acridinium label is directly proportional to the concentration of P-III-NP in the sample. The Siemens P-III-NP assay is calibrated by the manufacturer using a standard derived from bovine P-III-NP.

For the purposes of the *ABP*, an initial quantification of the "A" *Sample* is performed. When requested, a confirmatory quantification of the "A" *Sample* may additionally be performed using the same assay pairing (see, Article 6.2) to confirm the concentrations and to perform identification of IGF-I (as per TD IDCR[14]).

The concentrations of IGF-I and P-III-NP reported by the Laboratories, as well as the GH-2000 score automatically calculated in *ADAMS*, are integrated in the Endocrine Module of *ADAMS* using a similar Bayesian approach to that applied in the Steroidal and Hematological Modules of the *ABP* [15].

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) [16];

  [Comment: Previous studies have demonstrated that IGF-I and P-III-NP concentrations remain stable if the Sample is maintained at a refrigerated temperature for up to 5 days [17].]

- 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;

- 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 [3]).

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

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	Two (2) Aliquots 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 Aliquots may be analyzed immediately after aliquoting; or • the Aliquots shall be stored at approximately 4 °C if analyzed within 24h (within a maximum of five (5) days from *Sample* collection); or • the Aliquots shall be frozen (-20°C) if the analysis will be conducted more than 24h after aliquoting. For the confirmatory quantification, two (2) new Aliquots 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[3].]*
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, two (2) Aliquots shall be taken for initial quantification. These Aliquots 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, two (2) new Aliquots 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* Procedures for the quantification of IGF-I and P-III-NP in routine *Doping Control* analysis, the Laboratory shall fulfil the following requisites:

- Validate the Analytical *Testing* Procedures, 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* Procedures shall meet the acceptance values for the parameters of IGF-I and P-III-NP assay performance, as specified in Table 1 and Table 2 (as applicable).

Table 1: Acceptance Criteria for Parameters of Assay Performance for the Endocrine Module

Validation Parameters	IGF-I	P-III-NP
Maximum LOQ	≤ 50 ng/mL	≤ 1 ng/mL
Maximum Relative Combined Standard Measurement Uncertainty (u_{c_Max}, %)	≤ 20%	≤ 15%

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* Procedures for the quantification of hGH *Markers* in blood as part of the Endocrine Module 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).

5.3. Quality Controls (QCs) and Reagents

- QC samples: Laboratories shall implement well-characterized and stable internal QC sample(s), which are not subject to assay lot variations, for the performance of the tests under different assay conditions (different assay lots, different analysts, *etc*.). Following preparation/reception by the Laboratory, all QC material should be aliquoted and stored frozen (preferably at – 80°C for long-term storage) until use. These QC samples should include:
  - QC_{low}: Serum obtained from healthy individual(s), which is demonstrated to contain concentrations of IGF-I not greater than (≤) 200 ng/mL and P-III-NP not greater than (≤) 5 ng/mL;
  - QC_{high}: Serum obtained from hGH administration studies or another appropriate source that has been demonstrated to contain concentrations of IGF-I greater than (≥) 500 ng/mL and P-III-NP greater than (≥) 10 ng/mL.

  [Comment: Four (4) separate QC samples may also be used, as long as they contain IGF-I and P-III-NP at the necessary concentrations (e.g., $QC_{IGF\text{-}I\_low}$, $QC_{IGF\text{-}I\_high}$, QC_{PIIINP_low} and QC_{PIIINP_high}).]
- Reagents: With every new batch of reagents (new lot number), the following evaluation steps should be implemented before including the new batch into routine operations for P-III-NP quantification:
  - Each of the QC samples shall be determined at least three (3) times whenever a new batch of reagents is obtained. The number of replicates per determination shall be conducted as stipulated by the assay manufacturers. The QCs may be measured in a single assay or over a range of assays. If, for any QC, the difference between the mean concentration for the new batch and that for the preceding batch is more than 20%, the new batch shall not be implemented into routine operations and an investigation of the new batch shall be conducted.
  - In order to detect small but systematic changes over time, it is recommended that the performance of a new batch of reagents is controlled, for example, through a cumulative sum (CUSUM) chart/table, which is established for each QC based on the difference between the mean(s) of the new batch and the initial value(s). When using the CUSUM, results should be assessed using customary procedures as detailed at *http://itl.nist.gov/div898/handbook/pmc/section3/pmc323.htm*

6.0 Analytical *Testing* Procedure and Reporting of Test Results

6.1. Initial Quantification of the *Markers*

- Two (2) Aliquots taken from the original "A" *Sample* shall be analyzed once (x1) to quantify intact IGF-I and P-III-NP;
- QC Sample(s), at low- and high-levels of the *Marker*s (see Article 5.3), shall be included in each initial quantification analytical batch;
- The coefficient of variation (CV%) between the duplicate determinations of the IGF-I and P-III-NP concentrations shall not be higher (≤) than the associated u_{c_Max} (see Table 1). If the CV%

between duplicate determinations of only one *Marker* (IGF-I or P-III-NP) exceeds the respective u_{c_Max}, the analysis of only that *Marker* shall be repeated;

- The mean *Marker* concentration from the duplicate measurement of IGF-I and P-III-NP shall be reported in *ADAMS* in nanograms per milliliter (ng/mL);

  [Comment: for the purposes of the Endocrine Module of the ABP, the GH-2000 score does not need to be calculated or reported by the Laboratory since it will be automatically calculated in ADAMS [15]*].*

- 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 Endocrine 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 quantifications.]

When a confirmatory quantification analysis is requested:

- Two (2) new Aliquots taken from the original "A" *Sample* shall be analyzed once (x1) to:
  - quantify intact IGF-I and P-III-NP; and
  - identify IGF-I (as per the TD IDCR [14]);
- At least one QC Sample (see Article 5.3), depending on initial quantification results, shall be included in each confirmatory quantification analytical batch;
- The CV (%) between the duplicate determinations of the IGF-I or P-III-NP concentrations shall not be higher (≤) than the associated u_{c_Max} (see Table 1). If the CV% between duplicate determinations of only one *Marker* (IGF-I or P-III-NP) exceeds the respective u_{c_Max}, the analysis of only that *Marker* shall be repeated;
- The mean *Marker* concentration from the duplicate measurement of IGF-I and P-III-NP shall be reported in *ADAMS* in nanograms per milliliters (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 2. Analytical *Testing* Procedure Validation and Performance Requirements for the initial and confirmatory quantification of IGF-I in blood (serum) *Samples* by top-down LC-MS^n for the Endocrine Module of the *ABP*.

Method and Instrumentation	Top-down (intact IGF-I) Liquid Chromatography combined with (Tandem) Mass Spectrometry based on triple quadrupole or HRMS (LC-MS^n; n ≥ 1).
Range of the Method	Shall cover the ranges of IGF-I concentrations normally found in males and females and demonstrate linearity between **50–1000 ng/mL**, at least.
Limit of Quantification (LOQ)	The LOQ shall not be greater than (≤) **50 ng/mL**.
Maximum Relative Combined Standard Measurement Uncertainty u_c (%)	The estimated u_c (%) shall not be greater than (≤) **20%**.
Sample	IGF-I quantification shall be conducted in duplicate (using two Aliquots of the "A" *Sample*) using a volume not greater than (≤) **50 µL** of serum per replicate.
Internal Standard	Stable isotope-labeled IGF-I (*e.g.,* NIST[ii] or ProSpec[iii] ^{15}N-IGF-I).
Calibration	A freshly prepared single point calibrator (SPC) shall be included in each analytical batch. The Recombinant Human IGF-I calibrator from NIST (SRM 2926[iv]) should be used to prepare the SPC. Any other calibration material shall be validated against the NIST SRM 2926 calibrator.

Applicable links
[ii] https://shop.nist.gov/ccrz__ProductDetails?sku=2927&cclcl=en_US
[iii] https://www.prospecbio.com/igf1_n15_human
[iv] https://shop.nist.gov/ccrz__ProductDetails?sku=2926&cclcl=en_US

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