WORLD ANTI-DOPING AGENCY
play true

Laboratory Guidelines

Human Growth Hormone (hGH) Biomarkers Test

Version 3.0
January 2021

WORLD ANTI-DOPING AGENCY
play true

TABLE OF CONTENTS

[For the purpose of these Laboratory Guidelines, *Code* definitions are in *Italics*. *International Standard* definitions are Underlined.]

1.0 Objective

These Laboratory Guidelines have been developed to ensure a harmonized approach in the application of the GH-2000 Biomarkers Test for the detection of doping with human Growth Hormone (hGH) in sport. The document provides direction on the pre-analytical *Sample* preparation procedure, the performance of the assay and the interpretation of the test results.

2.0 Scope

These Laboratory Guidelines follow the rules established in the *WADA International Standard* for Laboratories (ISL) [1] and relevant *Technical Documents* (*TD*s) regarding the Analytical *Testing* of blood *Sample*s. These requirements are still fully applicable and shall be respected. These Laboratory Guidelines contain additional recommendations to facilitate the implementation of the Analytical *Testing* procedures particular to hGH detection by the Biomarkers Test.

3.0 Introduction to the Test Method

The hGH Biomarkers Test involves the measurement of two hGH-sensitive *Markers*, namely insulin-like growth factor-I (IGF-I) and N-terminal pro-peptide of type III collagen (P-III-NP), which are present in serum. The bibliography at the end of these Laboratory Guidelines lists the main publications produced during the development and validation of the method. These measurements are combined in gender-specific discriminant function formulae which improve the sensitivity and specificity of the test based on a score (the GH-2000 score) [2] to detect hGH misuse compared with single-*Marker* analysis. The hGH Biomarkers Test may also have utility in detecting GH secretagogues and IGF-I abuse in sport [3, 4].

A series of placebo-controlled recombinant (r)hGH administration studies performed in Europe (lead centers in the UK and Germany) and Australia has shown that both IGF-I and P-III-NP rise substantially following rhGH administration in a dose-dependent manner [2, 5-11]. These *Markers* have been evaluated for several confounding factors that might influence the scores of the discriminant functions, including age [12-14], gender [2], ethnicity [15], exercise [8, 9], diurnal and day-to-day variation, intra-individual variation [16], bone and soft tissue injury [17], sporting discipline, and body habitus (physique) [12, 18, 19].

Except gender and age, no other factor has been shown to affect the hGH discriminant function scores substantially.

The GH-2000 discriminant function formulae are gender-specific, based on the natural logarithm of IGF-I and P-III-NP serum concentrations (required to normalize the data distribution) and include an adjustment for age to reflect the age-related decline in hGH and *Marker* concentrations [2].

3.1. Principle of the Test Method

The hGH Biomarkers Test is based on the measurement of IGF-I and P-III-NP by immunoassays or mass spectrometry (MS)-based approaches [20].

In order to perform the test, an assay pairing formed by an IGF-I and a P-III-NP assay is utilized for the Initial *Testing* Procedure (ITP), whereas two (2) different IGF-I/P-III-NP assay pairings shall be

used for the Confirmation Procedures (CP) (see Table 2 below). One (1) IGF-I/P-III-NP assay pairing may be the same as that used in the ITP. It is recommended that the Liquid Chromatography (LC)-tandem MS (LC-MS/MS) or LC-High Resolution MS (LC-HRMS) assay for IGF-I be applied as part of the CP whenever possible. The results of each assay pairing are then used to calculate the GH-2000 score.

The assays currently used are:

IGF-I Assays

1) **Immunotech A15729 IGF-I IRMA** (Immunotech SAS, Marseille, France)

The Immunotech assay is a two-site, solid-phase, immunoradiometric assay (IRMA) using two monoclonal antibodies prepared against two different antigenic sites of the IGF-1 molecule. The first is coated on a solid phase and the second is radiolabelled with ^{125}I. IGF-I is separated from IGFBPs by acidification and excess IGF-II is added to prevent further interference with the assay from IGFBPs. The Immunotech assay is calibrated using the WHO IGF-I IRP standard 87/518.

2) **IDS-iSYS IGF-I** (Immunodiagnostics Systems Limited, Boldon, UK).

The iSYS IGF-I assay is an automated sandwich, chemiluminescent immunoassay (CLIA). Samples are incubated with an acidic solution to dissociate IGF-I from the IGFBPs. A portion of this, along with a neutralization buffer containing excess IGF-II to prevent re-aggregation with IGFBPs, a biotinylated anti-IGF-I monoclonal antibody directed against the N-terminal, and an acridinium labeled anti-IGF-I monoclonal antibody are incubated. Streptavidin labeled magnetic particles are then added and, following an additional incubation step, the magnetic particles are captured using a magnet. After a washing step and addition of trigger reagents, the light emitted by the acridinium label is directly proportional to the concentration of IGF-I in the original sample [21]. The iSYS IGF-I assay is calibrated using the new WHO recombinant IGF-I IRP standard 02/254.

3) **LC-MS/MS or LC-HRMS IGF-I** [20].

This is a bottom-up approach based on the quantification of peptides derived from trypsin digestion of IGF-I. Serum samples are incubated with an acidic solution in the presence of excess IGF-II and ^{15}N-labeled IGF-I as internal standard. Proteins are precipitated with acetonitrile. Following reduction and alkylation of the dried supernatant, the solution is enzymatically hydrolyzed with trypsin. Two peptides corresponding to amino acids 1–21 (T1) and 22–36 (T2) of IGF-I are separated by LC and measured by MS/MS or HRMS.

P-III-NP assays

1) **Orion UniQ™ P-III-NP RIA** (Orion Diagnostica, Espoo, Finland)

The Orion UniQ™ P-III-NP RIA is a competitive radioimmunoassay based on the formation of a complex between solid-phase anti-P-III-NP polyclonal rabbit antibodies and P-III-NP in the serum samples in competition with ^{125}I-labelled P-III-NP. A sample volume of 100 µL is used.

2) **Siemens ADVIA Centaur P-III-NP** [(Siemens Healthcare Laboratory Diagnostics, Camberley, UK)] [22]

The Siemens ADVIA Centaur P-III-NP assay is an automated, two-site sandwich, chemiluminescent immunoassay. The assay uses two 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.

4.0 Assay Requirements

4.1. Test Method Validation Requirements

Prior to the implementation of the Biomarkers Test in routine *Doping Control* analysis, the Laboratory shall fulfill the following requisites:

- Validate the assays' performance on-site, including the determination of the assays' Limit of Quantification (LOQ), Repeatability (s_r), Intermediate Precision (s_w) and Bias;
- The acceptance values for parameters of assay performance, applicable to the separate determinations of IGF-I and P-III-NP concentrations, are specified in Table 1 below;
- In addition, the Laboratory shall estimate the assay Measurement Uncertainty (MU) from Laboratory validation data. The combined standard uncertainty (u_c) shall be not higher than a maximum value of u_{c_Max} = 0.50 for either assay pairing, expressed as Standard Deviations (SD) and applied to the GH-2000 scores at values close to the corresponding *Decision Limits* (*DLs*), as described in Article 6.0 below.

4.2. Test Method Accreditation Requirements

- Demonstrate readiness for assay implementation through method validation data and/or 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 hGH Biomarkers Test 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).

Table 1: Acceptance Criteria for Parameters of Assay Performance.

Validation Parameter	Immunoassays	**LC-MS/MS** or **LC-HRMS** [a]
s_r (within-assay Relative Standard Deviation, *RSD* %)	≤ 10%	≤ 10%
s_w (between-assay *RSD* %)	≤ 20%	≤ 15%
LOQ [b] IGF-I P-III-NP	 ≤ 50 ng/mL ≤ 1 ng/mL	 ≤ 50 ng/mL N/A

[a] when applied to the mean of the measured concentrations of T1 and T2.

[b] LOQ is defined as the lowest concentration meeting the criteria for s_r and s_w.

4.3. Assay Pre-analytical Procedure

Upon reception of the "A" and "B" *Samples* in the Laboratory, the following steps should be followed:

- Check that the blood *Samples* have been collected in tubes containing an inert polymeric serum separator gel and a clotting activation factor (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 *WADA* Blood *Sample* Collection Guidelines [23]. Such blood *Samples* should have been kept in a refrigerated state (not frozen) following collection and during transportation to the Laboratory;

  [Comment: Previous studies have demonstrated that IGF-I and P-III-NP concentrations remain stable if the sample remains refrigerated for up to 5 days [24].]

- Alternatively, *Samples* may be received in the Laboratory as frozen or refrigerated serum *Samples*, following the clotting and centrifugation of the blood and separation of the serum fraction at the site of *Sample* collection;

- Any *Samples* delivered to the Laboratory as plasma shall not be accepted for the purposes of hGH analysis with the current assays. The Laboratory shall notify and seek advice from the *Testing* Authority regarding rejection and Analytical *Testing* of *Samples* for which irregularities are noted (see ISL [1]). In cases of *Sample* collection in the incorrect matrix, the results of such analysis of the *Sample* shall be disregarded;

- 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*, record such condition(s) and include it in the Test Report to the *Testing* Authority in *ADAMS*.

4.3.1. *Samples* received as whole blood in SST™-II tubes or SST™-II Plus Advance tubes or SST™ tubes:

a) "A" *Sample*

- Centrifuge the "A" *Sample* for 10-15 min at 1300-1500 g as soon as possible after reception;
- A serum Aliquot of the "A" *Sample* shall be taken to be used for the ITP. The remaining of the "A" Sample serum fraction not used for the ITP may be kept in the *Sample* collection tube or aliquoted into new vials, which shall be properly labelled to ensure Laboratory Internal Chain of Custody documentation, and stored frozen until the "A" CP, if needed;.

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 needed.

[Comment: For storage of Aliquots frozen in new vials, well-closing vials should be used (for optimal storage cryovials with an "O-ring" are recommended) and the following conditions are recommended:

- *For short-term storage (up to three months) at approximately –20 °C;*
- *For long-term periods (more than three months) freeze at approximately –20 °C and transfer to approximately –70 to –80 °C.*

For the step-freezing of ("A" or "B") Sample collection tubes, place the tube into a dedicated isolating box (e.g. foam box) before transferring it into a –20°C freezer. In order to maintain the integrity of the separation gel, allow the freezing to proceed for at least 2 h before moving or transferring the frozen tubes. Moving the tubes before the separating gel is frozen and stable may lead to contamination of serum by cellular material.

It is recommended that thawing of the Sample(s) for analysis is also done stepwise. Samples shall not be thawed under hot water or any other similar process that would raise the temperature of the Sample above room temperature. Thawing overnight at 4°C is recommended.]

- For the ITP, ''A'' *Sample* Aliquots may be analyzed immediately after aliquoting or stored at approximately 4°C for a maximum of 24h before analysis (within a maximum of 5 days from *Sample* collection). Alternatively, the ''A'' *Sample* Aliquots must be frozen until analysis.

b) "B" Sample

- The "B" *Sample* shall be centrifuged for 10-15 minutes at 1300-1500g as soon as possible after reception. The whole of the "B" *Sample* separated serum fraction should be kept in the SST™-II or SST™-II Plus Advance or SST™ S*ample* collection tube and step-frozen according to the tube manufacturer's instructions until analysis, if needed;
- Once the "B" *Sample* is thawed and opened, an Aliquot of the "B" *Sample* shall be used for the "B" CP. The remaining "B" *Sample* serum fraction should be kept in the *Sample* collection tube or transferred into a new tube/vial and shall be (re)sealed in front of the *Athlete* or the *Athlete's* representative or an Independent Witness using a tamper-evident system and frozen until further analysis, if needed.

4.3.2. *Samples* received as separated serum *Samples*:

a*) Samples* received as frozen separated serum fractions:

- These *Samples* should remain frozen until analysis;
- Once thawed, an Aliquot of *Sample* "A" shall be taken to be used for the ITP. This Aliquot of *Sample* "A" may be stored at approximately 4°C if the ITP is scheduled to take place within 24 h of thawing. The remaining "A" *Sample* serum fraction not used for the ITP may be kept in the *Sample* collection tube or aliquoted into new vials, which shall be properly labelled to ensure Laboratory Internal Chain of Custody documentation, and stored frozen until the "A" CP, if needed;
- Once the "B" *Sample* is thawed and opened, an Aliquot of the "B" *Sample* shall be used for the "B" CP. The remaining "B" *Sample* serum shall be kept in the *Sample* collection tube and shall be re-sealed in front of the *Athlete* or the *Athlete's* representative or a Laboratory-appointed Independent Witness using a tamper-evident system and frozen until further analysis, if needed*.*

b) *Samples received* as refrigerated separated serum fractions:

- An Aliquot of the "A" *Sample* shall be taken as soon as possible upon reception. For the ITP, "A" *Sample* Aliquots may be analyzed immediately after aliquoting or stored at approximately 4°C for a maximum of 24h before analysis (within a maximum of 5 days from *Sample* collection). Alternatively, ''A'' *Sample* Aliquots must be frozen until analysis;
- The remainder of the "A'' *Sample* not used for the ITP may be kept in the *Sample* collection tube or aliquoted into new vials, which shall be properly labelled to ensure Laboratory Internal Chain of Custody documentation, and stored frozen until the "A" CP, if needed;
- "B" *Samples* shall be frozen as soon as possible upon reception and thaw before analysis. Once the "B" *Sample* is thawed and opened, an Aliquot of the "B" *Sample* shall be used for the "B" CP. The remaining "B" *Sample* serum shall be kept in the *Sample* collection tube and shall be re-sealed in front of the *Athlete* or the *Athlete's* representative or an Independent Witness using a tamper-evident system and stored frozen until further analysis, if needed.

4.4. Analytical *Testing* Procedure

- For the performance of the assay(s) Analytical *Testing* Procedure, refer to the test procedure described in the Instructional Insert provided with the test assays and the Laboratory Standard Operating Procedure (SOP);
- In cases of contradiction between the Instructional Insert provided with the assays and the Laboratory SOP, or between the Instructional Insert and these Laboratory Guidelines, the latter document shall prevail in each case.

4.4.1. Analytical *Testing* Strategy

- One (1) assay pairing (*e.g.* Immunotech IGF-I + Orion P-III-NP) should be used for the ITP (Table 2);
- In the case of an initial Presumptive *Adverse Analytical Finding* (PAAF), two (2) different assay pairings shall be used for the CP of the "A" *Sample* (Table 2) using three (3) new Aliquots of the original "A" *Sample*. One of the assay pairings may be the same as the one used for the ITP;

  [Comment: Laboratories that do not have the analytical capacity to perform the CP with an additional assay pairing shall have, upon consultation with the responsible Testing Authority, the Sample shipped to and analyzed by another Laboratory that has such analytical capacity.]
- For the "B" CP, both assay pairings used during the confirmation of the "A" *Sample* shall be applied on three (3) Aliquots taken from the original "B" *Sample.* The Laboratory shall follow the requirements of the ISL [1] for the performance of the "B" *Sample* confirmation analysis;
- Either the LC-MS/MS or LC-HRMS IGF-I assay may be applied as the unique test for IGF-I quantification (*i.e.* either assay may be used for the ITP and also combined with two (2) different P-III-NP assays for the CP(s);
- For both "A" and "B" CP, three (3) *Sample* Aliquots shall be measured, except in cases of limited *Sample* volume, in which case a lower maximum number of replicates may be used;
- In accordance with the ISL [1], the Laboratory shall have a policy to define those circumstances where the CP of an "A" or "B" *Sample* should be repeated (for example, values of within-assay s_r > 10%);
- It is recommended that the Laboratories implement well-characterized and stable internal quality control (QC) sample(s), which are not subject to assay lot variations, for the performance of the tests under different assay conditions (different lots of assay, 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 be:
  - **QC_{low}**: Serum obtained from healthy individual(s), which is shown to have a value of IGF-I ≤ 200 ng/mL and P-III-NP < 5 ng/mL;
  - **QC_{high}**: Serum obtained from hGH administration studies or another appropriate source that has been shown to contain concentrations of IGF-I ≥ 500 ng/mL and P-III-NP ≥ 10 ng/mL.
    [Comment: Four (4) 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}).]
- Assay Repeatability (s_r) and Intermediate Precision (s_w) will be assessed by analyzing each QC sample in triplicates (3x) on 5-6 separate occasions;
- With every new batch of reagents (new lot number), the following evaluation steps should be implemented before accepting the new batch:

- 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 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%, investigation of the new batch will be required;
- In order to detect small but systematic changes with time, it is recommended that the performance of a new batch of reagents be controlled, for example, through a cumulative sum (CUSUM) chart/table, which is built for each QC based on the difference between the mean(s) for 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

5.0 Interpretation and Reporting of Results

5.1. Interpretation of Test Results

For determination of compliance of the analytical result, the Laboratory shall compare the *Sample's* GH-2000 score (rounded to two (2) decimal places) with the corresponding gender-specific *DLs* established for the assay pairings [25].

- The DL values are given in Table 2 below;

  [Comment: The DL values specified in Table 2 have been derived from the analysis of Samples from Athletes treated under Doping Control conditions of Sample collection, transportation, storage and analysis [25, 26]. The established DL values define a combined test specificity (between the two assay parings used for the CP) of at least 99.99%. These DL values are conservative values and will be periodically refined as more data are accumulated from normative studies and Doping Control tests performed by WADA-accredited laboratories.]

- The GH-2000 score for the *Sample* is calculated applying the following discriminant function formulae:

GH-2000 score for males [14]:

$$\mathbf{GH_{2000\_M} = -6.586 + 2.905 \cdot \ln(P\text{-}III\text{-}NP) + 2.100 \cdot \ln(IGF\text{-}I) - 101.737 / age - 0.02 \cdot (age - 25.09)}$$

GH-2000 score for females:

$$\mathbf{GH_{2000\_F} = -8.459 + 2.454 \cdot \ln(P\text{-}III\text{-}NP) + 2.195 \cdot \ln(IGF\text{-}I) - 73.666 / age}$$

where ln(*P-III-NP*) and ln(*IGF-I*) are the natural logarithms (ln) of the mean concentration values (expressed in ng/mL) obtained from the measured replicates of the *Sample* Aliquot and age is rounded down to the nearest year.

[Comment: For calculation of the GH-2000 scores, the natural logarithms (ln) of the mean concentrations (ng/mL) of IGF-I and P-III-NP shall be expressed to three (3) decimal places. However, for compliance decisions (comparison to the assay pairing- and gender-specific DLs), the resulting GH-2000 score shall be rounded to two (2) decimal places.

Table 2. Possible assay pairings for the ITP and CP(s) and applicable gender-specific *DLs* [25]

Gender	Assay Pair (IGF-I + P-III-NP)	*DL1*
Males	LC-MS/MS or LC-HRMS + Orion	9.70
	LC-MS/MS or LC-HRMS + Siemens Advia Centaur	11.34
	IDS-Sys + Orion	9.00
	IDS-Sys + Siemens Advia Centaur	10.61
	ImmunoTech + Orion	9.98
	ImmunoTech + Siemens Advia Centaur	11.53
Females	LC-MS/MS or LC-HRMS + Orion	8.56
	LC-MS/MS or LC-HRMS + Siemens Advia Centaur	10.13
	IDS-Sys + Orion	7.79
	IDS-Sys + Siemens Advia Centaur	9.35
	ImmunoTech + Orion	8.62
	ImmunoTech + Siemens Advia Centaur	10.10

5.1.1. Presumptive *Adverse Analytical Finding* (PAAF)

- The ITP shall produce a PAAF for *Sample* "A" if the corresponding GH-2000 score (rounded to two decimal places) exceeds the gender-specific *DL* (Table 2) applicable for the assay pairing used for the screening procedure;
- When the LC-MS/MS or LC-HRMS method is used for IGF-I quantification during the ITP, the test result shall be considered a PAAF if the GH-2000 score, calculated on the basis of the IGF-I concentration determined from the quantification of the T1 or the T2 diagnostic peptide (Table 3), exceeds the gender-specific *DL* applicable for the assay pairing used (Table 2).

5.1.2. *Adverse Analytical Finding* (*AAF*)

- The CP shall produce an *AAF* if the *Sample's* GH-2000 scores (rounded to two (2) decimal places) exceed the sex-specific *DLs* (Table 2) established for the two (2) assay pairings applied for the CP;

- When the LC-MS/MS or LC-HRMS method is used for IGF-I quantification during the CP, the test result shall be considered an *AAF* if:
  - the GH-2000 scores calculated on the basis of the **average** IGF-I concentration determined from the quantification of T1 and T2 exceed the gender-specific *DLs* established in Table 2 for the two assay pairings applied, and the T1- and T2-derived IGF-I concentrations do not differ by more than 20% (Table 3).

5.1.3. *Atypical Finding (ATF)*

- The CP shall produce an *ATF* if the GH-2000 scores (rounded to two (2) decimal places) exceed the *DL* (Table 2) for only one (1) of the two (2) assay pairings employed for the CP;
- When the LC-MS/MS or LC-HRMS method is used for IGF-I quantification during the CP, the test result shall also be considered an *ATF* if:
  - the GH-2000 scores calculated on the basis of the **average** IGF-I concentration determined from the quantification of T1 and T2 exceed the gender-specific *DLs* established in Table 2, BUT
  - the IGF-I concentrations determined from the quantification of T1 and T2 differ by more than (>) 20% (Table 3);
  - In such cases, the Laboratory shall repeat the LC-MS/MS or LC-HRMS analysis to verify the IGF-I T1, T2 concentration difference before reporting the finding.

[Comment: The decision rule applicable to CPs used for the analysis of endogenous Threshold Substances, for which the Threshold value(s) have been established based on reference population statistics, already incorporates a guard band that reflects the uncertainty of the measurements provided by the assay(s). Therefore, the zone of analytical values considered compliant (Negative Finding) or not (AAF) with this decision rule would be defined by the Threshold value itself, which constitutes the DL. The assay MU shall not be added to the test result for reporting an AAF or an ATF.]

5.2. Reporting of Test Results

- When reporting an *AAF* or an *ATF*, the Laboratory Test Report shall include the mean GH-2000 scores from triplicate determinations (obtained during the CP) expressed to two (2) decimal places, the values of the applicable *DL* as well as the combined standard uncertainty of the assay (u_c, expressed as SD) at values close to the *DL* as determined by the Laboratory during Test Method validation;
- In addition, the Laboratory Documentation Package shall include the mean concentration values of IGF-I and P-III-NP from triplicate (3x) determinations (obtained during the CP, expressed to the nearest integer for IGF-I and two (2) decimal places for P-III-NP).

Test Report Example (*e.g.* for a *Sample* from a male *Athlete*):

The analysis of the *Sample* with the hGH Biomarkers Test has produced the following GH-2000 scores: 10.90 for assay pair '1' [IDS IGF-I + Centaur P-III-NP] and 9.90 for assay pair '2' [LC-MS/MS IGF-I + Orion P-III-NP], which are greater than the corresponding male-specific *DLs* of 10.61 and

9.70, respectively. The combined standard uncertainty (u_c) estimated by the Laboratory at levels close to the *DL* is 0.40 for assay pair '1' and 0.35 for assay pair '2'. This constitutes an *Adverse Analytical Finding* for hGH.

Table 3. Examples of interpretation of tests findings when applying LC-MS/MS or LC-HRMS for IGF-I quantification.

Procedure	$\frac{\lvert T_1 - T_2 \rvert}{\text{MEAN}(T_1 ; T_2)}$	GH-2000 score			Interpretation/ Reporting
		IGF-I (T1)	IGF-I (T2)	Mean IGF-I (T1, T2)	
ITP	N/A	N/A	***> DL***	N/A	**PAAF**
		> DL	N/A	N/A	**PAAF**
CP	**≤ 0.2**	> *DL*	> *DL*	***> DL***	***AAF***
		> *DL*	< *DL*	***> DL*** < *DL*	***AAF*** Negative Finding
		< *DL*	> *DL*	***> DL*** < *DL*	***AAF*** Negative Finding
		< *DL*	< *DL*	< *DL*	Negative Finding
	> 0.2	> *DL*	> *DL*	***> DL***	***ATF***
		> *DL*	< *DL*	***> DL*** < *DL*	***ATF*** Negative Finding
		< *DL*	> *DL*	***> DL*** < *DL*	***ATF*** Negative Finding
		< *DL*	< *DL*	< *DL*	Negative Finding

6.0 Assay Measurement Uncertainty

6.1. Combined Standard Uncertainty (u_c)

- Laboratories shall generally refer to the TD DL [27] for estimation of assay MU;
- The Laboratories shall determine each assay's u_c based on their assay validation data.

The u_c is a dynamic parameter that can be reduced with increasing improvement in performance of the assays. The establishment of a confident value of u_c would be based on multiple measurements done throughout a long period of time, when certain sources of uncertainty (such as environmental changes, instrument performance, different analysts, etc.) would be accounted for.

ISO/IEC 17025 recommends that u_c be estimated using an approach consistent with the principles described in the ISO/IEC Guide to the Expression of Uncertainty in Measurement (GUM) [28];

For application to the hGH marker method, the following approach for calculation of the u_c budget is recommended:

The value of u_c, applicable to the GH-2000 scores close to the *DLs*, will result from the contributing u_c of the component assays (applicable to the natural logarithms (ln) of the values of the measured concentrations) using the law of propagation of uncertainty, according to the following equations:

For males:

$$u_c(\text{score}) = \sqrt{8.44^* \, u_c{}^2 [\ln(\text{P-III-P})] + 4.41^* \, u_c{}^2 [\ln(\text{IGF-I})]}$$

For females:

$$u_c(\text{score}) = \sqrt{6.02^* \, u_c{}^2 [\ln(\text{P-III-P})] + 4.82^* \, u_c{}^2 [\ln(\text{IGF-I})]}$$

- The u_c associated with the values of the natural logarithms (ln) of the concentrations determined with the IGF-I and P-III-NP assays, shall be estimated from the Intermediate Precision (s_w) and the bias of the ln determinations according to the following equation:

$$u_c = \sqrt{s_w{}^2 + u_{bias}{}^2}$$

[Comment: The u_c (score) and the contributing u_c associated with the values of the natural logarithms of the measured concentrations, s_w and bias should be expressed as standard deviations (SD).

- For calculation of u_c, either a single QC sample, containing IGF-I and P-III-NP in appropriate concentrations (*e.g.* QC_{high}) or two (2) separate QC samples containing IGF-I at ~ 500 - 800 ng/mL (*e.g.* $QC_{IGFI\text{-}high}$) and P-III-NP at ~ 10 - 20 ng/mL (*e.g.* $QC_{PIIINP\text{-}high}$), should be used. These QCs should be aliquoted and stored frozen (preferably at - 80°C for long term storage) until use;

[Comment: Since the GH-2000 scores depend on the age of the donor, in order to produce relevant values of the GH-2000 scores (close to the DLs), the age of the donors should ideally be between 20 – 40 years old.]

- QC sample(s) and four (4) different ½ dilutions should be measured in triplicates (3x) over 5-6 days by at least two (2) different analysts. This would ensure that the s_w is calculated over the physiological range of concentrations of hGH *Markers* that may be found in *Samples* producing GH-2000 scores close to the *DLs*;
- The bias will be established by comparison of the Laboratory's long-term means of the ln of concentration values obtained *e.g.* for the QC_{low} and QC_{high} samples with the expected values determined through a *WADA* EQAS round or inter-laboratory collaborative study. Where information is available from separate bias determinations, the bias contribution to u_c is expressed as RMS_{bias} [27].

6.2. Maximum Levels of u_c

Laboratories shall have values of u_c, applicable to values close to the *DL* for each assay pairing, not higher than (≤) the maximum values of $u_{c\,Max}$.

6.3. Verification of MU

Laboratories shall refer to the TD DL [27] for ongoing verification of the assay u_c estimates.

7.0 Bibliography

1. World Anti-Doping Code *International Standard* for Laboratories.

   https://www.wada-ama.org/en/what-we-do/science-medical/laboratories

2. Powrie JK, Bassett EE, Rosen T *et al.* Detection of growth hormone abuse in sport. *Growth Horm IGF Res* **17**(3):220-6 (2007).

3. Guha N1, Erotokritou-Mulligan I, Bartlett C *et al*.. Biochemical markers of insulin-like growth factor-I misuse in athletes: the response of serum IGF-I, procollagen type III amino-terminal propeptide, and the GH-2000 score to the administration of rhIGF-I/rhIGF binding protein-3 complex.. *J Clin Endocrinol Metab.* **99**(6):2259-68 (2014). doi: 10.1210/jc.2013-3897. Epub 2014 Feb 25.

4. Holt RI, Sonksen PH. Growth hormone, IGF-I and insulin and their abuse in sport. *Br J Pharmacol* **154**(3):542-56 (2008).

5. Holt RI, Erotokritou-Mulligan I, McHugh C *et al*. The GH-2004 project: the response of IGF1 and type III pro-collagen to the administration of exogenous GH in non-Caucasian amateur athletes. *Eur J Endocrinol* **163**(1):45-54 (2010).

6. Erotokritou-Mulligan I, Bassett EE, Kniess A, Sonksen PH, Holt RI. Validation of the growth hormone (GH)-dependent marker method of detecting GH abuse in sport through the use of independent data sets. *Growth Horm IGF Res* **17**(5):416-23 (2007).

7. Longobardi S, Keay N, Ehrnborg C *et al*. Growth hormone (GH) effects on bone and collagen turnover in healthy adults and its potential as a marker of GH abuse in sports: a double blind, placebo-controlled study. The GH-2000 Study Group. *J Clin Endocrinol Metab* **85**(4):1505-12 (2000).

8. Wallace JD, Cuneo RC, Lundberg PA *et al*. Responses of markers of bone and collagen turnover to exercise, growth hormone (GH) administration, and GH withdrawal in trained adult males. *J Clin Endocrinol Metab* **85**(1):124-33 (2000).

9. Wallace JD, Cuneo RC, Baxter R *et al*. Responses of the growth hormone (GH) and insulin-like growth factor axis to exercise, GH administration, and GH withdrawal in trained adult males: a potential test for GH abuse in sport. *J Clin Endocrinol Metab* **84**(10):3591-601 (1999).

10. Dall R, Longobardi S, Ehrnborg C *et al.* The effect of four weeks of supraphysiological growth hormone administration on the insulin-like growth factor axis in women and men. GH-2000 Study Group. *J Clin Endocrinol Metab* **85**(11):4193-200 (2000).

11. Nelson AE, Meinhardt U, Hansen JL *et al*. Pharmacodynamics of growth hormone abuse biomarkers and the influence of gender and testosterone: a randomized double-blind placebo-controlled study in young recreational athletes. *J Clin Endocrinol Metab* **93**(6):2213-22 (2008).

12. Healy ML, Dall R, Gibney J *et al.* Toward the development of a test for growth hormone (GH) abuse: a study of extreme physiological ranges of GH-dependent markers in 813 elite athletes in the postcompetition setting. *J Clin Endocrinol Metab* **90**(2):641-9 (2005).

13. Böning D, Böning W, Guha N *et al*. Statistical methodology for age-adjustment of the GH-2000 score detecting growth hormone misuse. *BMC Medical Research Methodology* **16**:147-155 (2016).

14. Böning D, Böning W, Guha N *et al*. A correction to the age-adjustment of the GH-2000 score used in the detection of growth hormone misuse. *BMC Res Notes* **11**:650-654 (2018).

15. Erotokritou-Mulligan I, Bassett EE *et al*. Influence of ethnicity on IGF-I and procollagen III peptide (P-III-P) in elite athletes and its effect on the ability to detect GH abuse. *Clin Endocrinol (Oxf)* **70**(1):161-8 (2009).

16. Erotokritou-Mulligan I, Bassett E.E., Cowan DA *et al*. The use of growth hormone (GH)-dependent markers in the detection of GH abuse in sport: Physiological intra-individual variation of IGF-I, type 3 pro-collagen (P-III-P) and the GH-2000 detection score. *Clin Endocrinol (Oxf)* **72**(4):520-6 (2010).

17. Erotokritou-Mulligan I, Bassett EE, Bartlett C, Cowan D *et al.* The effect of sports injury on insulin-like growth factor-I and type 3 procollagen: implications for detection of growth hormone abuse in athletes. *J Clin Endocrinol Metab* **93**(7):2760-3 (2008).

18. Nelson AE, Howe CJ, Nguyen TV *et al*. Influence of demographic factors and sport type on growth hormone-responsive markers in elite athletes. *J Clin Endocrinol Metab* **91**(11):4424-32 (2006).

19. Healy ML, Gibney J, Pentecost C, *et al.* Endocrine profiles in 693 elite athletes in the postcompetition setting. *Clin Endocrinol (Oxf)*. **81**(2):294-305 (2014). doi: 10.1111/cen.12445. Epub 2014 Apr 2.

20. Cox HD, Lopes F, Woldemariam GA *et al*. Interlaboratory Agreement of Insulin-like Growth Factor 1 Concentrations Measured by Mass Spectrometry. *Clinical Chemistry* **60**:541–548 (2014).

21. Bidlingmaier M, Friedrich N, Emeny RT *et al*. Reference intervals for insulin-like growth factor-1 (igf-i) from birth to senescence: results from a multicenter study using a new automated chemiluminescence IGF-I immunoassay conforming to recent international recommendations. *Clin Endocrinol Metab*. **99**: 1712-21 (2014).

22. Knudsen CS, Heickendorff L, Nexo E. Measurement of amino terminal propeptide of type III procollagen (PIIINP) employing the ADVIA Centaur platform. Validation, reference interval and comparison to UniQ RIA. *Clin Chem Lab Med* **52**(2): 237-241 (2014).

23. World Anti-Doping Program. Guidelines for Blood Sample Collection.

    https://www.wada-ama.org/en/resources/search?f[0]=field_resource_collections%3A190

24. Holt RI, Erotokritou-Mulligan I, Ridley SA *et al*. A determination of the pre-analytical storage conditions for insulin like growth factor-I and type III procollagen peptide. *Growth Horm IGF Res* **19**(1):43-50 (2009).

25. Holt RI, Böhning W, Guha N *et al*. The development of decision limits for the GH-2000 detection methodology using additional insulin-like growth factor-I and amino-terminal pro-peptide of type III collagen assays. *Drug Test Anal* **7**: 745-755 (2015).

26. Bassett EE, Erotokritou-Mulligan I. Statistical issues in implementing the marker method. *Growth Horm IGF Res* **19**(4):361-5 (2009).

27. *WADA Technical Document* TD DL: *Decision Limits* for the Confirmatory Quantification of Exogenous Threshold Substances by Chromatography-based Analytical Methods.

    https://www.wada-ama.org/en/what-we-do/science-medical/laboratories

28. ISO/IEC Guide 98-3: 2008. Evaluation of Measurement Data – Guide to the Expression of Uncertainty in Measurement (GUM) (2008).

    http://www.bipm.org/en/publications/guides/gum.html

WORLD ANTI-DOPING AGENCY
play true

Extended Bibliography describing the hGH Biomarkers Test

- Abellan R, Ventura R, Palmi I *et al*. Immunoassays for the measurement of IGF-II, IGFBP-2 and -3, and ICTP as indirect biomarkers of recombinant human growth hormone misuse in sport. Values in selected population of athletes. *J Pharm Biomed Anal*. **48**(3):844-52 (2008).
- Abellan R, Ventura R, Pichini S *et al*. Effect of physical fitness and endurance exercise on indirect biomarkers of recombinant growth hormone misuse: insulin-like growth factor I and procollagen type III peptide. *Int J Sports Med.* **27**(12):976-83 (2006).
- Abellan R, Ventura R, Pichini S *et al*. Evaluation of immunoassays for the measurement of insulin-like growth factor-I and procollagen type III peptide, indirect biomarkers of recombinant human growth hormone misuse in sport. *Clin Chem Lab Med*.43(1):75-85 (2005).
- Armanini D, Faggian D, Scaroni C, Plebani M. Growth hormone and insulin-like growth factor I in a Sydney Olympic gold medalist. *Br J Sports Med.* **36**(2):148-9 (2002). Bassett EE, Erotokritou-Mulligan I. Statistical issues in implementing the marker method. *Growth Horm IGF Res*. **19**(4):361-5 (2009).
- Chung L, Baxter RC. Detection of growth hormone responsive proteins using SELDI-TOF mass spectrometry. *Growth Horm IGF Res*. **19**(4):383-7 (2009).
- Di Luigi L, Rigamonti AE, Agosti F *et al*. Combined evaluation of resting IGFI, N-terminal propeptide of type III procollagen and C-terminal cross-linked telopeptide of type I collagen levels might be useful for detecting inappropriate GH administration in female athletes. *Eur J Endocrinol.* **160**(5):753-8 (2009).
- Di Luigi L, Guidetti L. IGF-I, IGFBP-2, and -3: do they have a role in detecting rhGH abuse in trained men? *Med Sci Sports Exerc*. **34**(8):1270-8 (2002).
- Ding J, List EO, Okada S, Kopchick JJ. Perspective: proteomic approach to detect biomarkers of human *growth hormone. Growth Horm IGF Res.* **19**(4):399-407 (2009).
- Ehrnborg C, Lange KH, Dall R *et al*; GH-2000 Study Group. The growth hormone /insulin-like growth factor-I axis hormones and bone markers in elite athletes in response to a maximum exercise test. *J Clin Endocrinol Metab.* **88**(1):394-401 (2003).
- Erotokritou-Mulligan I, Eryl Bassett E, Cowan DA *et al.* The use of growth hormone (GH)-dependent markers in the detection of GH abuse in sport: Physiological intra-individual variation of lGF-I, type 3 pro-collagen (P-III-P) and the GH-2000 detection score*. Clin Endocrinol (Oxf).* **72**(4):520-6 (2010).
- Erotokritou-Mulligan I, Bassett EE, Cowan DA *et al;* GH-2004 group. Influence of ethnicity on IGF-I and procollagen III peptide (P-III-P) in elite athletes and its effect on the ability to detect GH abuse. *Clin Endocrinol (Oxf).* **70**(1):161-8 (2009).
- Erotokritou-Mulligan I, Bassett EE, Bartlett C *et al*; GH-2004 Group. The effect of sports injury on insulin-like growth factor-I and type 3 procollagen: implications for detection of growth hormone abuse in athletes. *J Clin Endocrinol Metab*. **93**(7):2760-3 (2008).
- Erotokritou-Mulligan I, Bassett EE, Kniess A, Sonksen PH, Holt RI. Validation of the growth hormone (GH)dependent marker method of detecting GH abuse in sport through the use of independent data sets. *Growth Horm IGF Res.* **17**(5):416-23 (2007).
- Guha N, Erotokritou-Mulligan I, Burford C *et al*. Serum insulin-like growth factor-I and pro-collagen type III N-terminal peptide in adolescent elite athletes: implications for the detection of growth hormone abuse in sport. *J Clin Endocrinol Metab.* **95**(6):2969-76 (2010).

- Holt RI, Erotokritou-Mulligan I, McHugh C *et al*. The GH-2004 project: the response of IGF1 and type III pro-collagen to the administration of exogenous GH in non-Caucasian amateur athletes. *Eur J Endocrinol.* **163**(1):45-54 (2010).
- Holt RI, Bassett EE, Erotokritou-Mulligan I, McHugh C *et al*; GH-2004 group. Moving one step closer to catching the GH cheats: The GH-2004 experience. *Growth Horm IGF Res.* **19**(4):346-51 (2009).
- Holt RI, Erotokritou-Mulligan I, Sonksen PH. The history of doping and growth hormone abuse in sport. *Growth Horm IGF Res.* **19**(4):320-6 (2009).
- Kicman AT, Miell JP, Teale JD *et al.* Serum IGF-I and IGF binding proteins 2 and 3 as potential markers of doping with human GH. *Clin Endocrinol (Oxf)*. **47**(1):43-50 (1997).
- Kniess A, Ziegler E, Kratzsch J, Thieme D, Muller RK. Potential parameters for the detection of hGH doping. *Anal Bioanal C*hern. **376**(5):696-700 (2003).
- Longobardi S, Keay N, Ehrnborg C *et al.* Growth hormone (GH) effects on bone and collagen turnover in healthy adults and its potential as a marker of GH abuse in sports: a double blind, placebo-controlled study. The GH-2000 Study Group. *J Clin Endocrinol Metab*. **85**(4):1505-12 (2000).
- McHugh CM, Park RT, Sonksen PH, Holt RI. Challenges in detecting the abuse of growth hormone in sport. *Clin Chem.* **51**(9):1587-93 (2005).
- Minuto F, Barreca A, Melioli G. Indirect evidence of hormone abuse. Proof of doping? *J Endocrinol Invest.* **26**(9):919-23 (2003).
- Nelson AE, Ho KK. Demographic factors influencing the GH system: Implications for the detection of GH doping in sport. *Growth Horm IGF Res.* **19**(4):327-32 (2009).
- Nelson AE, Howe CJ, Nguyen TV *et al*. Influence *of* demographic factors and sport type on growth hormone-responsive markers in elite athletes. *J Clin Endocrinol Metab*. **91**(11):4424-32 (2006).
- Nguyen lV, Nelson AE, Howe CJ *et al.* Within subject variability and analytic imprecision of insulin like growth factor axis and collagen markers: implications for clinical diagnosis and doping tests. *Clin Chem*. **54**(8):1268-76 (2008).
- Pichini S, Ventura R, Palmi R *et al*. Effect of physical fitness and endurance exercise on indirect biomarkers of growth hormone and insulin misuse: Immunoassay-based measurement in urine samples. *J Pharm Biomed Anal.* **53**(4):1003-10 (2010).
- Powrie JK, Bassett EE, Rosen T *et al*; GH-2000 Project Study Group. Detection of growth hormone abuse in sport. *Growth Horm IGF Res.* **17**(3):220-6 (2007).
- Sartorio A, Jubeau M, Agosti F *et al*. A follow-up of GH dependent biomarkers during a 6-month period of the sporting season of male and female athletes. *J Endocrinol Invest.* **29**(3):237-43 (2006).
- Sartorio A, Agosti F, Marazzi N *et al*. Combined evaluation of resting IGF-I, N-terminal propeptide of type III procollagen (P-III-NP) and Cterminal cross-linked telopeptide of type I collagen (ICTP) levels might be useful for detecting inappropriate GH administration in athletes: a preliminary report. *Clin Endocrinol (Oxf).* **61**(4):487-93 (2004).
- Thevis M, Bredehöft M, Kohler M, Schänzer W. Mass spectrometry-based analysis of IGF-I and hGH. *Handb Exp Pharmacol.* **195**:201-7 (2010).
- Thevis M, Thomas A, Schänzer W. Doping control analysis of selected peptide hormones using LCMS(/MS). *Forensic Sci. Int.* **213**:35-41 (2011)

- Wallace JD, Cuneo RC, Baxter R *et al*. Responses of the growth hormone (GH) and insulin-like growth factor axis to exercise, GH administration, and GH withdrawal in trained adult males: a potential test for GH abuse in sport. *J Clin Endocrinol Metab.* **84**(10):3591-601 (1999).