WORLD ANTI-DOPING AGENCY

World Anti-Doping Program

GUIDELINES

Human GROWTH HORMONE (hGH) BIOMARKERS TEST

for *Doping Control* Analyses

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1. Objective

This guideline has 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 guideline provides direction on the *Sample* pre-analytical preparation procedure, the performance of the test and the interpretation of the test results.

2. Scope

This guideline follows the rules established in the *World Anti-Doping Agency's (WADA) International Standards* for Laboratories (ISL) [1] and relevant Technical Documents regarding the testing of blood *Sample*s. These requirements are still fully applicable and shall be respected. This guideline contains additional recommendations to facilitate the implementation of the *Testing* procedures particular to hGH detection by the Biomarkers Test.

3. Introduction to the 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 guidelines lists the main publications produced during the development and validation of the method. These measurements are combined in sex-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, sex [2], ethnicity [12], exercise [8, 9], diurnal and day-to-day variation, intra-individual variation [13], bony and soft tissue injury [14], sporting discipline, and body habitus (physique) [15-17].

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

The GH-2000 discriminant function formulae are sex-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 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 [18].

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, whereas two different IGF-I/P-III-NP assay pairings shall be used for the Confirmation Procedures (see Table 2 below). One IGF-I/P-III-NP assay pairing may be the same as that used in the Initial Testing Procedure. It is recommended that the LC-MS/MS assay for IGF-I be applied as part of the Confirmation Procedure where 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 assay (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-I 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 assay (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 [19].

   The iSYS IGF-I assay is calibrated using the new WHO recombinant IGF-I IRP standard 02/254.

3) Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) IGF-I assay [18].

   This is a bottom-up approach based on the quantification of IGF-I-specific tryptic peptides. Serum samples are incubated with an acidic solution in the presence of excess IGF-II and ^{15}N-labeled IGF-I as internal standard. Following reduction and alkylation, 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 liquid chromatography and measured by tandem mass spectrometry.

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 assay [(Siemens Healthcare Laboratory Diagnostics, Camberley, UK)] [20]

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. Assay 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, for example, 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 the Table 1 below:

Table 1: Acceptance Criteria for some parameters of assay performance.

Validation parameter	Immunoassays	LC-MS/MS [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 the two peptides T1 and T2.

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

- In addition, the Laboratory shall determine 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 section 7 below.
- Demonstrate readiness for assay implementation through test 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 from a relevant accreditation body for the inclusion of the hGH Biomarkers Test in the Laboratory scope of accreditation.

4.1 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) in accordance with the *WADA* Guidelines for Blood *Sample* Collection [21]. Such blood *Samples* should have been kept in a refrigerated state (not frozen) following collection and during transportation to the Laboratory[1].
- 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. In line with this, the *Sample* Collection Authorities are provided with Guidelines for collection of blood *Samples* for hGH analysis, which specify that the matrix of analysis is serum [21]. The Laboratory shall notify and seek advice from the *Testing* Authority regarding rejection and Analytical Testing of *Samples* for which irregularities are noted (as per ISL 6.2.2.4). In cases of *Sample* collection in the incorrect matrix (to be identified at the results management level), the results of such analysis of the *Sample* shall be disregarded.
- Check the status of the *Sample(s)* (for example, evidence of hemolysis) and the integrity of the collection tubes (for example, 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.

[1] Previous studies have demonstrated that IGF-I and P-III-NP concentrations remain stable if the sample remains refrigerated for up to 5 days [22].

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

  "A" *Sample*

  - Centrifuge the "A" *Sample* for 10-15 min at 1300-1500 g as soon as possible after reception.

  - The whole separated serum fraction from the "A" *Sample* should be transferred into another tube or aliquoted into new vials, which shall be properly labelled to ensure Laboratory Internal Chain of Custody documentation. One Aliquot should be used for the Initial Testing Procedure. The remaining "A'' *Sample* Aliquot(s) not used for the Initial Testing Procedure must be stored frozen[2] until the "A" Confirmation Procedure, if needed.

  - For the Initial Testing Procedure, ''A'' *Sample* Aliquots may be analyzed immediately after aliquoting or stored at approximately 4 °C for a maximum of 24 h before analysis (within a maximum of 5 days from *Sample* collection). Alternatively, the ''A'' *Sample* Aliquots must be frozen[2] until analysis.

  "B" *Sample*

  - Centrifuge the "B" *Sample* 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 S*ample* collection tube and step-frozen according to the tube manufacturer's instructions[3] until analysis, if needed.

  - Once the "B" *Sample* is thawed and opened (according to ISL 6.2.4.2.2), an Aliquot of the "B" *Sample* shall be used for the "B" Confirmation Procedure. The remaining "B" *Sample* serum should be transferred into a new tube/vial and shall be sealed in front of the *Athlete* or the *Athlete's* representative or a Laboratory-appointed independent witness using a tamper-proof evident method and frozen[2] until further analysis, if needed.

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[2] For storage of Aliquots frozen, 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.

Thawing of the *Sample(s)* for analysis shall not be done 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.

[3] Place the tube into a dedicated isolating box before transferring into a –20 °C freezer. In order to maintain the integrity of the separation gel, allow the freezing to proceed for at least 2 hours 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.

- For *Samples* received as separated serum *Samples*:

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

  - These *Samples* should remain frozen[2] until analysis.

  - Once thawed, an Aliquot of *Sample* "A" shall be taken to be used for the Initial Testing Procedure. This Aliquot of *Sample* "A" may be stored at approximately 4 °C if the Initial Testing Procedure is scheduled to take place within 24 hours of thawing. The remaining "A" *Sample* serum fraction 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[2] until the "A" Confirmation Procedure, if needed.

  - Once the "B" *Sample* is thawed and opened (according to ISL 6.2.4.2.2), an Aliquot of the "B" *Sample* shall be used for the "B" Confirmation Procedure. The remaining "B" *Sample* serum shall be kept in the *Sample* collection tube and shall be sealed in front of the *Athlete* or the *Athlete's* representative or a Laboratory-appointed independent witness using a tamper-proof evident method and frozen[2] until further analysis, if needed.

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

  - Take an Aliquot of the "A" *Sample* as soon as possible upon reception. For the Initial Testing Procedure, "A" *Sample* Aliquots may be analyzed immediately after aliquoting or stored at approximately 4 °C for a maximum of 24 h before analysis (within a maximum of 5 days from *Sample* collection). Alternatively, ''A'' *Sample* Aliquots must be frozen[2] until analysis.

  - The remainder of the "A'' *Sample* not used for the Initial Testing Procedure 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[2] until the "A" Confirmation Procedure, if needed.

  - For "B" *Samples*, freeze[2] the *Samples* as soon as possible upon reception and thaw before analysis. Once the "B" *Sample* is thawed and opened (according to ISL 6.2.4.2.2), an Aliquot of the "B" *Sample* shall be used for the "B" Confirmation Procedure. 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-proof evident method and stored frozen[2] until further analysis, if needed.

4.2 Assay Analytical Procedure

For the performance of the assay(s) analytical procedure, refer to the test procedure described in the Instructional Insert provided with the test assays and the Laboratory SOP.

In cases of contradiction between the Instructional Insert provided with the assays and the Laboratory SOP, or between the Instructional Insert and these Guidelines, the latter document shall prevail in each case.

4.3 Analytical Testing Strategy

- One assay pairing (*e.g.* Immunotech IGF-I + Orion P-III-NP) should be used for the Initial Testing Procedure (Table 2).
- In the case of an initial Presumptive *Adverse Analytical Finding*, two different assay pairings shall be used for the Confirmation Procedure of the "A" *Sample* (Table 2) using three new Aliquots of the original "A" *Sample* [4]. One of the assay pairings may be the same as the one used for the Initial Testing Procedure.
- For the "B" Confirmation Procedure, both assay pairings used during the confirmation of the "A" *Sample* shall be applied on three Aliquots taken from the original "B" *Sample* [5]. The Laboratory shall follow the requirements of the *ISL* 6.2.4.2.2.1 for the performance of the "B" *Sample* confirmation analysis.
- For both "A" and "B" Confirmation Procedures, three *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* provisions 6.2.4.2.1.4 and 6.2.4.2.2.8, the Laboratory shall have a policy to define those circumstances where the Confirmation Procedure 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[5] should be:

  - **QC_{low}**: Serum obtained from healthy individual(s), which is shown to have a value of ≤ 200 ng/mL IGF-I and < 5 ng/mL P-III-NP.

[4] Laboratories that do not have the analytical capacity to perform the Confirmation Procedure 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.

[5] Four QC samples may also be used, as long as they contain IGF-I and P-III-NP at the necessary concentration levels (*e.g.* $QC_{IGF\text{-}I\_low}$, $QC_{IGF\text{-}I\_high}$, QC_{PIIINP_low} and QC_{PIIINP_high}).

- **QC_{high}**: Serum obtained from hGH administration studies or another appropriate source that have been shown to contain concentrations of ≥ 500 ng/mL IGF-I and ≥ 10 ng/mL P-III-NP.

- Assay Repeatability (s_r) and Intermediate Precision (s_w) will be assessed by analyzing each QC sample in triplicates on 5-6 separate occasions.
- With every new batch of reagents (new lot number)[6], the following evaluation steps should be implemented before accepting the new batch:
    - Each of the internal QC samples shall be determined at least three 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, a cumulative sum (CUSUM) chart should be constructed for each QC, based on the difference between the mean for the new batch and the initial value. The result should be assessed using customary CUSUM procedures as detailed at

      http://itl.nist.gov/div898/handbook/pmc/section3/pmc323.htm;
    - The age-corrected mean concentrations for at least 20 normal samples obtained with the new batch should be calculated[7] and compared with those from the previous batch. All samples should be from subjects of the same sex. If the mean difference (calculated on a log scale) between the batches is statistically significant at the 5% level, investigation of the new batch will be required (see worked-out example in **Appendix I**).

[6] Information about batch-to-batch variability may also be available from the manufacturers. In addition, it is proposed that a central Laboratory could co-ordinate this work.

[7] Age adjustments to be used are:
For IGF-I Men: - exp[23.7*(1/25-1/age)]
For IGF-I Women: - exp[20.9*(1/25-1/age)]
For P-III-NP Men: - exp[15.9*(1/25-1/age)]
For P-III-NP Women: - exp[11.6*(1/25-1/age)]

5. Reporting and Interpretation 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 decimal places) with the corresponding gender-specific DLs established for the assay pairings [23]. The DL values are given in Table 2 below [8].

Table 2. Possible assay pairings for the Initial Testing Procedure and Confirmation Procedure(s) and applicable sex-specific Decision Limits.

Initial Testing Procedure: Assay Pair 1 OR Assay Pair 2				
Confirmation Procedure: Assay Pair 1 AND Assay Pair 2				
Sex	**Assay Pair 1** (IGF-I + P-III-NP)	**DL1**	**Assay Pair 2** (IGF-I + P-III-NP)	**DL2**
Males	LC-MS/MS + Orion	9.35	IDS-Sys + Advia Centaur	10.29
	LC-MS/MS + Orion	9.35	Immunotech + Advia Centaur	11.18
	IDS-Sys + Orion	8.63	LC-MS/MS + Advia Centaur	10.97
	IDS-Sys + Orion	8.71	Immunotech + Advia Centaur	11.22
	ImmunoTech + Orion	9.52	IDS-Sys + Advia Centaur	10.29
	ImmunoTech + Orion	9.61	LC-MS/MS + Advia Centaur	10.93
Females	LC-MS/MS + Orion	8.18	IDS-Sys + Advia Centaur	8.98
	LC-MS/MS + Orion	8.21	Immunotech + Advia Centaur	9.73
	IDS-Sys + Orion	7.40	LC-MS/MS + Advia Centaur	9.76
	IDS-Sys + Orion	7.48	Immunotech + Advia Centaur	9.77
	ImmunoTech + Orion	8.18	IDS-Sys + Advia Centaur	9.00
	ImmunoTech + Orion	8.22	LC-MS/MS + Advia Centaur	9.74

[8] The DL values specified above have been derived from the analysis of *Samples* from *Athletes* treated under *Doping Control* conditions of *Sample* collection, transportation, storage and analysis [23]. The established DL values define a combined test specificity (between the two assay parings used for the Confirmation Procedure) 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:

-6.586 + 2.905*ln(*P-III-NP*) + 2.100*ln(*IGF-I*) - 101.737/ age

GH-2000 score for females:

-8.459 + 2.454*ln(*P-III-NP*) + 2.195*ln(*IGF-I*) – 73.666/ age

where ln(*P-III-NP*) and ln(*IGF-I*) are the natural logarithms (ln) of the mean concentration values obtained from the measured replicates of the *Sample* Aliquot and age is rounded down to the nearest year[9].

5.1.1 Presumptive *Adverse Analytical Finding*

- The Initial Testing Procedure shall produce a Presumptive *Adverse Analytical Finding* for *Sample* "A" if the corresponding GH-2000 score (rounded to two decimal places) exceeds the pre-established sex-specific DL (Table 2) for the assay pairing used for the screening procedure.

5.1.2 *Adverse Analytical Finding*

- The Confirmation Procedure shall produce an *Adverse Analytical Finding* if the *Sample's* GH-2000 scores (rounded to two decimal places) exceed the sex-specific DLs (Table 2) established for the two assay pairings used for the Confirmation Procedure.

5.1.3 *Atypical Finding*

- The Confirmation Procedure shall produce an *Atypical Finding* if the GH-2000 scores (rounded to two decimal places) exceed the DL (Table 2) for only one of the two assay pairings employed for the Confirmation Procedure.

The MU of the assays has already been considered and incorporated in the reference population-based statistical estimation of the DL[10] [24, 25]. Therefore, for declaration of an *Adverse Analytical Finding* or an *Atypical Finding* the assay MU shall not be added.

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[9] For calculation of the GH-2000 scores, the natural logarithms (ln) of the mean concentrations of IGF-I and P-III-NP shall be expressed to 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 decimal places.

[10] According to *WADA*'s Technical Document on Decision Limits for the Confirmatory Quantification of Threshold Substances (TDDL) [24], the decision rule applicable to assays 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) or not (*Adverse Analytical Finding*) with this decision rule would be defined by the Threshold value itself, which constitutes the DL.

5.2 Reporting of Test Results

- When reporting an *Adverse Analytical Finding* or an *Atypical Finding*, the Laboratory Test Report shall include the mean GH-2000 scores from triplicate determinations (obtained during the Confirmatory Procedure) expressed to two 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.

  In addition, the Laboratory Documentation Package shall include the mean concentration values of IGF-I and P-III-NP from triplicate determinations (obtained during the Confirmatory Procedure, expressed to the nearest integer for IGF-I and two decimal places for P-III-NP) and the expanded MU equivalent to the 95% coverage interval ($U_{95\%}$, k = 2) for the value of the GH-2000 score for the *Sample*.

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

The analysis of the *Sample* identified above by using 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 10.29 and 9.35, 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.

6. Assay Measurement Uncertainty

6.1 Combined Standard Uncertainty (u_c)

- Laboratories shall generally refer to the TDDL [24] 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 expertise in the 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) [26].
- 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 formulae (1)[11]:

(1) 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 formula (2)[12].

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

- 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 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[12]. These QCs should be aliquoted and stored frozen (preferably at -80°C for long term storage) until use.
- QC sample(s) and four different ½ dilutions should be measured in triplicates over 5-6 days by at least 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 values of 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* educational EQAS round or inter-Laboratory collaborative study. The bias is expressed as % deviation from the expected value (RMS_{bias}).

6.2 Maximum levels of u_c

- In accordance with the TDDL [24], 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 $u_{c\,Max}$.

[11] In formula (1) and (2), the u_c (score) and the contributing u_c associated with the values of the natural logarithms of the measured concentrations should be expressed as standard deviations (*SD*).

[12] 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.

6.3 Expanded Uncertainty ($U_{95\%}$)

For determination of the expanded uncertainty $U_{95\%}$ a coverage factor k=2 can be applied if u_c has a 95 % confidence level.

(3) $U_{95\%} = k * u_c$, where k=2

6.4 Verification of Measurement Uncertainty

Laboratories shall refer to the TDDL [24] for ongoing verification of the assay MU estimates.

7. Definitions

7.1 *Code* Defined Terms

Adverse Analytical Finding: A report from a *WADA*–accredited laboratory or other *WADA* -approved laboratory that, consistent with the International Standard for Laboratories and related Technical Documents, identifies in a *Sample* the presence of a *Prohibited Substance* or its *Metabolites* or *Markers* (including elevated quantities of endogenous substances) or evidence of the *Use* of a *Prohibited Method*.

Athlete: Any *Person* who competes in sport at the international level (as defined by each International Federation) or the national level (as defined by each *National Anti-Doping Organization).* An *Anti-Doping Organization* has discretion to apply anti-doping rules to an *Athlete* who is neither an *International-Level Athlete* nor a *National-Level Athlete*, and thus to bring them within the definition of "Athlete." In relation to *Athletes* who are neither *International-Level* nor *National-Level Athletes*, an *Anti-Doping Organization* may elect to: conduct limited *Testing* or no *Testing* at all; analyze *Samples* for less than the full menu of *Prohibited Substances*; require limited or no whereabouts information; or not require advance *TUEs*. However, if an Article 2.1, 2.3 or 2.5 anti-doping rule violation is committed by any *Athlete* over whom an *Anti-Doping Organization* has authority who competes below the international or national level, then the *Consequences* set forth in the *Code* (except Article 14.3.2) must be applied. For purposes of Article 2.8 and Article 2.9 and for purposes of anti-doping information and education, any *Person* who participates in sport under the authority of any *Signatory*, government, or other sports organization accepting the *Code* is an *Athlete*.

Atypical Finding: A report from a *WADA*-accredited laboratory or other *WADA* -approved laboratory which requires further investigation as provided by the International Standard for Laboratories or related Technical Documents prior to the determination of an *Adverse Analytical Finding*.

Code: The World Anti-Doping Code.

Doping Control: All steps and processes from test distribution planning through to ultimate disposition of any appeal including all steps and processes in between such as provision of whereabouts information, *Sample* collection and handling, laboratory analysis, *TUEs*, results management and hearings.

International Standard: A standard adopted by *WADA* in support of the *Code*. Compliance with an *International Standard* (as opposed to another alternative standard, practice or procedure) shall be sufficient to conclude that the procedures addressed by the *International Standard* were performed properly. *International Standards* shall include any Technical Documents issued pursuant to the *International Standard.*

Marker: A compound, group of compounds or biological variable(s) that indicates the *Use* of a *Prohibited Substance* or *Prohibited Method.*

Sample or *Specimen:* Any biological material collected for the purposes of *Doping Control*.

Testing: The parts of the *Doping Control* process involving test distribution planning, *Sample* collection, *Sample* handling, and *Sample* transport to the laboratory.

WADA: The World Anti-Doping Agency.

7.2 *ISL* Defined Terms

Aliquot: A portion of the *Sample* of biological fluid or tissue (e.g. urine, blood) obtained from the *Athlete* used in the analytical process.

Analytical Testing: The parts of the *Doping Control* process involving *Sample* handling, analysis and reporting following receipt in the Laboratory.

Confirmation Procedure: An analytical test procedure whose purpose is to identify the presence or to measure the concentration/ratio of one or more specific *Prohibited Substances*, *Metabolite(s)* of a *Prohibited Substance*, or *Marker(s)* of the *Use* of a *Prohibited Substance* or *Method* in a *Sample*.

[Comment: A Confirmation Procedure for a Threshold Substance shall also indicate a concentration/ratio of the Prohibited Substance greater than the applicable Decision Limit (as noted in the TD DL).]

Decision Limit: a concentration, accounting for the maximum permitted combined uncertainty, above which an *Adverse Analytical Finding* shall be reported.

Initial Testing Procedure: An analytical test procedure whose purpose is to identify those *Samples* which may contain a *Prohibited Substance*, *Metabolite(s)* of a *Prohibited Substance*, or *Marker(s)* of the *Use* of a *Prohibited Substance* or *Prohibited Method* or the quantity of a *Prohibited Substance*, *Metabolite(s)* of a *Prohibited Substance*, or *Marker(s)* of the *Use* of a *Prohibited Substance* or *Prohibited Method*.

Intermediate Precision: Variation in results observed when one or more factors, such as time, equipment, or operator are varied within a Laboratory.

International Standard for Laboratories (ISL): The *International Standard* applicable to Laboratories as set forth herein.

Laboratory Internal Chain of Custody: Documentation of the sequence of *Persons* in custody of the *Sample* and any Aliquot of the *Sample* taken for Analytical Testing.

[Comment: Laboratory Internal Chain of Custody is generally documented by a written record of the date, location, action taken, and the individual performing an action with a Sample or Aliquot.]

Laboratory(ies): (A) *WADA*-accredited laboratory(ies) applying test methods and processes to provide evidentiary data for the detection of *Prohibited Substances, Methods* or *Markers* on the *Prohibited List* and, if applicable, quantification of a Threshold Substance in *Samples* of urine and other biological matrices in the context of anti-doping activities.

Laboratory Documentation Packages: The material produced by the Laboratory to support an analytical result such as an *Adverse Analytical Finding* as set forth in the *WADA* Technical Document for Laboratory Documentation Packages.

Measurement Uncertainty (MU): Parameter associated with a measurement result that characterizes the dispersion of quantity values attributed to a measurand.

[Comment: Knowledge of the MU increases the confidence in the validity of a measurement result].

Presumptive *Adverse Analytical Finding*: The status of a *Sample* test result for which there is a suspicious result in the Initial Testing Procedure, but for which a confirmation test has not yet been performed.

Repeatability, sr*:* Variability observed within a Laboratory, over a short time, using a single operator, item of equipment, etc.

Threshold Substance: An exogenous or endogenous *Prohibited Substance, Metabolite* or *Marker* of a *Prohibited Substance* which is analyzed quantitatively and for which an analytical result (concentration, ratio or score) in excess of a pre-determined Decision Limit constitutes an *Adverse Analytical Finding*. Threshold Substances are identified as such in the Technical Document on Decision Limits (TD DL).

7.3 *International Standard* for *Testing* and Investigations (ISTI) Defined Terms

Sample Collection Authority: The organization that is responsible for the collection of *Samples* in compliance with the requirements of the *International Standard* for *Testing* and Investigations, whether (1) the *Testing* Authority itself; or (2) another organization (for example, a third party contractor) to whom the *Testing* Authority has delegated or sub-contracted such responsibility (provided that the *Testing* Authority always remains ultimately responsible under the *Code* for compliance with the requirements of the *International Standard* for *Testing* and Investigations relating to collection of *Samples*).

Testing Authority: The organization that has authorized a particular *Sample* collection, whether (1) an *Anti-Doping Organization* (for example, the International Olympic Committee or other *Major Event Organization*, *WADA*, an International Federation, or a *National Anti-Doping Organization*); or (2) another organization conducting *Testing* pursuant to the authority of and in accordance with the rules of the *Anti-Doping Organization* (for example, a National Federation that is a member of an International Federation).

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Appendix 1: Worked example for testing between batches of reagents.

The first block of data below represents a "previous batch". The second block represents results from a "new batch" being evaluated. The factor is as given at the foot of page 11 of the Guidelines. Column 4 gives the age-adjusted IGF-I values (product of columns 2 and 3), and the final column is the natural logarithm of column 4.

Original batch

age	IGF-I value	factor	Adjusted IGF-I	ln
34	128	1.28523	164.510	5.103
27	265	1.07275	284.278	5.650
27	205	1.07275	219.913	5.393
31	217	1.20140	260.703	5.563
23	276	0.92087	254.161	5.538
30	296	1.17117	346.665	5.848
31	286	1.20140	343.599	5.839
23	321	0.92087	295.600	5.689
27	402	1.07275	431.244	6.067
29	159	1.13969	181.211	5.200
34	168	1.28523	215.919	5.375
30	236	1.17117	276.395	5.622
34	128	1.28523	164.510	5.103
28	265	1.10691	293.331	5.681
27	205	1.07275	219.913	5.393
31	217	1.20140	260.703	5.563
24	276	0.96127	265.311	5.581
30	296	1.17117	346.665	5.848
33	286	1.25837	359.894	5.886
23	321	0.92087	295.600	5.689
				====
			mean	5.582

New batch

age	IGF-I value	factor	Adjusted IGF-I	ln
32	114	1.23044	140.271	4.944
25	260	1.00000	260.000	5.561
25	186	1.00000	186.000	5.226
29	198	1.13969	225.659	5.419
21	253	0.83479	211.203	5.353
27	286	1.07275	306.806	5.726
30	290	1.17117	339.638	5.828
18	308	0.69166	213.030	5.361
23	384	0.92087	353.615	5.868
26	157	1.03713	162.830	5.093
32	150	1.23044	184.567	5.218
25	216	1.00000	216.000	5.375
32	117	1.23044	143.962	4.970
23	245	0.92087	225.614	5.419
23	183	0.92087	168.519	5.127
29	202	1.13969	230.218	5.439
23	274	0.92087	252.319	5.531
28	275	1.10691	304.400	5.718
27	278	1.07275	298.224	5.698
19	313	0.74129	232.023	5.447
				====
			mean	5.416

The distributions of age-adjusted IGF-I values are to be compared. Often, as here, the most appropriate test will be Student's t test based on the logged values. Other tests, including non-parametric ones, are available; these may be more appropriate, especially if outliers are present.

For the data in this example, summary statistics are:

	N	Mean	St Dev
Previous batch	20	5.582	0.261
New batch	20	5.416	0.269

Estimate for mean difference: Mean 1 – Mean 2 = 0.166
Test of "mean difference = 0": t = 1.98, p = 0.056

In this case, the result is not significant at the 5% level, so no further investigation is required.