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Written by:	WADA Science / hGH Working Group	Approved by:	WADA Executive Committee
Reviewed by:	WADA <u>Laboratory Expert Group</u>		
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HUMAN GROWTH HORMONE (hGH) ISOFORM DIFFERENTIAL IMMUNOASSAYS FOR DOPING CONTROL ANALYSES

The purpose of this *Technical Document (TD)* is to ensure a harmonized approach in the application of the Isoform Differential Immunoassays for the detection of doping with human Growth Hormone (hGH) in sport. This *TD* provides direction on the *Sample* pre-analytical preparation procedure, the performance of the Test Method(s) and the interpretation of the test results.

1.0 Introduction to the Test Method

The Isoform Differential Immunoassays for the detection of doping with hGH were developed to distinguish between the proportions of hGH isoforms found under normal physiological conditions and those found after recombinant (rec) hGH injection [1, 2].

The Test Method is essentially based on the established principle that the normal composition of hGH in blood is a mixture of different isoforms, present at constant relative proportions. In contrast, recGH is comprised almost exclusively of the monomeric 22-kDa molecular form. The administration of exogenous recGH not only leads to an increase in the concentration of the 22-kDa isoform but also causes a reduction of the non-22-kDa concentrations, thus altering the natural ratios established between these hGH isoforms [3].

1.1 Principle of the Test Method

In order to perform the Test Method(s), two separate kits ('1' and '2', supplied by CMZ-Assay GmbH, Germany), are used for the measurement of the hGH isoforms for each *Sample* analysis [4]. Either kit may be utilized for the Initial Testing Procedure (ITP), whereas both kit '1' and kit '2' shall be used for the Confirmation Procedure(s) (CP).

Each kit contains one 'recombinant' and one 'pituitary' assay. In the 'recombinant' (recGH) assay, the coated capture antibody preferentially binds to the monomeric 22-kDa hGH present in the *Samples*, whereas the 'pituitary' (pitGH) assay employs a capture antibody that recognizes a variety of pituitary-derived hGH isoforms. The respective assays are referred to as "rec1", "pit1", "rec2" and "pit2". The result of the Test Method is expressed as the ratio of the concentrations recGH / pitGH for each particular kit.

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2.0 Assay Requirements

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

2.1 Test Method Validation Requirements

- Validate the assay performance on-site, including the determination of the assay Limit of Quantification (LOQ), within-Laboratory Repeatability (s_r) and Intermediate Precision (s_w).

The acceptance values for these parameters of assay performance, applicable to the separate determinations of recGH and pitGH concentrations (“rec1”, “pit1”, “rec2” and “pit2”), are:

- s_r (expressed as intra-assay Relative Standard Deviation, RSD) $\leq 15\%$;
- s_w (expressed as inter-assay RSD) $\leq 20\%$;
- LOQ ≤ 0.050 ng/mL, defined as the lowest concentration with $s_r \leq 15\%$ and $s_w \leq 20\%$.

[Comment: The Laboratory LOQs, established at ≤ 0.050 ng/mL on the basis of Test Method performance criteria ($s_r \leq 15\%$ and $s_w \leq 20\%$), should not be lower than the respective LOQ values established by the kits’ manufacturer.]

- In addition, the Laboratory shall estimate the assay Measurement Uncertainty (MU) from Laboratory validation data. The combined relative standard uncertainty (u_c , %), applied to the assay recGH / pitGH ratios, shall be not higher than the maximum allowed u_{c_Max} of 20% for both kits, at values close to the corresponding *Decision Limits (DLs)*].

2.2 Test Method Accreditation Requirements

- Participate successfully in at least one WADA-organized EQAS in order to demonstrate readiness for assay implementation. In cases of identified deficiencies, proper corrective action(s) shall be implemented;
- Obtain ISO/IEC 17025 accreditation for the hGH Isoform Differential Immunoassay method 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).

2.3 Assay Pre-Analytical Procedure

Upon reception of the “A” and “B” *Samples* in the Laboratory, the following steps shall be followed:

- Check that the blood *Samples* have been collected in tubes containing an inert polymeric serum separator gel and a clotting activation factor (e.g. BD Vacutainer® SST™-II 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 Sample Collection Guidelines* [5]. Such blood *Samples* should

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have been kept in a refrigerated state (shall not be frozen) following collection and during transportation to the Laboratory;

- 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 kits. The Laboratory shall notify and seek advice from the Testing Authority regarding rejection or Analytical Testing of *Samples* for which irregularities are noted (see *International Standard for Laboratories, ISL* ^[6]). In cases of *Sample* collection in the incorrect matrix, the results of such analysis shall be disregarded;
- Check the status of the *Sample(s)* (for example, evidence of haemolysis) 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 in ADAMS;
- For *Samples* received as whole blood in SST™-II tubes or SST™-II Plus Advance tubes or SST™ tubes:

“A” *Sample*

- The “A” *Sample* shall be centrifuged for 10-15 min at 1300-1500g 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 into 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 (≤ 3) months) at approximately – 20 °C;
- For long-term periods (more than three (> 3) 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.

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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 24 h before analysis (within a maximum of 4 days from Sample collection). Alternatively, the “A” Sample Aliquots shall be frozen until analysis.

“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 shall be kept in the SST™-II or SST™-II Plus Advance or SST™ Sample 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, as applicable, using a tamper-evident system and frozen until Further Analysis, if needed.

- For Samples received as separated serum Samples:

a) Samples received as frozen separated serum fractions:

- These Samples shall 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 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;

- 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 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, as applicable, 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 24 h before analysis (within a maximum of 4 days from Sample collection). Alternatively, “A” Sample Aliquots shall be frozen until analysis;

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- The remainder of the “A” *Sample* not used for the ITP shall 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 thawed before analysis. Once the “B” *Sample* is thawed and opened, an Aliquot 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, as applicable, using a tamper-evident system and stored frozen until Further Analysis, if needed.

2.4 Analytical Testing Procedure

For the performance of the Analytical Testing Procedure, refer to the test procedure described in the Instructional Insert provided with the test kits and the Laboratory Standard Operating Procedure (SOP).

In cases of contradiction between the Instructional Insert provided with the kits and the Laboratory SOP, or between the Instructional Insert and this *TD*, the latter document shall prevail in each case.

[Comment: In order to ensure the quality of the assay performance, attention must be paid to the time of Sample signal acquisition on the luminometer, which shall be set at 1 s.]

2.4.1 Analytical Testing Strategy

- Either kit ‘1’ or kit ‘2’ may be used for the ITP using at least two (2) Aliquots taken from the original “A” *Sample*;
- In the case of an initial Presumptive Adverse Analytical Finding (PAAF), both kit ‘1’ and kit ‘2’ shall be used for the CP of the “A” *Sample* using three (3) new Aliquots of the original “A” *Sample*;
- For the “B” CP, both kit ‘1’ and kit ‘2’ shall be used on three (3) Aliquots taken from the original “B” *Sample*. The Laboratory shall follow the requirements of the ISL ^[6] for the performance of the “B” *Sample* confirmation analysis;
- For both “A” and “B” CP, three (3) Sample Aliquots shall be measured, except in cases of limited *Sample* volume, in which case the maximum number of Aliquots that can be prepared should be analyzed ^[6];
- In accordance with the ISL ^[6], the Laboratory shall have a policy to define those circumstances where the CP of an “A” or “B” *Sample* may be repeated (for example, values of intra-assay RSD > 15%);
- It is recommended that Laboratories implement well-characterized and stable internal quality control sample(s) (iQCs), which are under direct control of the Laboratory and not subject to kit lot

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variations, for the performance of the Test Method(s) under different assay conditions (different lots of kits, different analysts, etc.) and/or to demonstrate the specificity of the assays.

3.0 Interpretation and Reporting of Test Results

3.1 Interpretation of Test Results

For determination of compliance of the analytical result, the Laboratory shall compare the recGH / pitGH ratio (expressed to two (2) decimal places), obtained from the measured replicates of the Sample Aliquots and calculated by dividing the mean value of the results of the ‘recombinant’ assay (concentration of recGH in ng/mL, expressed to three (3) decimal places) by the mean value of the results of the ‘pituitary’ assay (pitGH in ng/mL, expressed to three (3) decimal places), with the corresponding *DL* for males and females established for the test kit used ^[7]. The *DL* values to be used are:

Kit ‘1’: Males (**1.84**); Females (**1.63**)
 Kit ‘2’: Males (**1.91**); Females (**1.59**)

- For *Samples* with measured values of pitGH concentrations below the assay LOQ, as determined by the Laboratory, the LOQ value of the corresponding pitGH assay (expressed to three (3) decimal places) shall be utilized for the purposes of calculating the recGH / pitGH ratio;
- In such cases, the recGH / pitGH ratio for the *Sample* shall be reported as “greater than” (>) (e.g. if recGH is 0.200 ng/mL while the pitGH is below the assay’s LOQ, and the Laboratory’s LOQ for pitGH is 0.050 ng/mL, the ratio shall be reported as “> 4.00”);
- All *Samples* with values of **recGH lower than (<) 0.150 ng/mL** shall be reported as a Negative Finding, irrespective of the corresponding values of the recGH / pitGH ratio.

3.1.1 Presumptive Adverse Analytical Finding (PAAF)

The ITP shall produce a PAAF for *Sample* “A” if the ratio of recGH to pitGH exceeds the appropriate *DL* for the kit used (kit ‘1’ or kit ‘2’).

3.1.2 Adverse Analytical Finding (AAF)

The CP shall produce an AAF if the analytical results (recGH / pitGH ratios) exceed the appropriate *DL* for both kit ‘1’ and kit ‘2’.

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3.1.3 Atypical Finding (ATF)

The CP shall produce an *ATF* if the analytical results (recGH / pitGH ratios) exceed the appropriate *DL* for only one (kit '1' or kit '2') of the two kits employed.

*[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*.]*

3.2 Reporting of Test Results

When reporting an *AAF* or an *ATF*, the Laboratory Test Report shall include the recGH / pitGH ratio, expressed to two (2) decimal places, of the mean recGH and pitGH concentration values from replicate determinations (obtained during the CP), the values of the applicable *DL* as well as the u_c (%) at values close to the *DL* as determined by the Laboratory during Test Method validation (expressed in units to two (2) decimal places).

In addition, the Laboratory Documentation Package shall include the recGH and pitGH concentrations, expressed to three (3) decimal places, for the three (3) Sample Aliquots analyzed using kit-1 and kit-2, as well as the mean concentration values of recGH and pitGH from the triplicate determinations, expressed to three (3) decimal places.

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

The analysis of the *Sample* using the hGH differential immunoassays has produced the following analytical values of rec/pit ratios: 2.52 for kit '1' and 2.40 for kit '2', which are greater than the corresponding *DLs* of 1.84 and 1.91, respectively. The combined relative standard uncertainty (u_c , %) estimated by the Laboratory at levels close to the *DL* is 11.9% for kit '1' and 10% for kit '2'. This constitutes an *AAF* for hGH.

4.0 Assay Measurement Uncertainty

4.1 Relative Combined Standard Uncertainty (u_c , %)

- Laboratories shall generally refer to the TD DL ^[8] for estimation of assay MU;
- 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 stable value of u_c (%) would be based on multiple measurements done throughout a long period of time, when certain sources of uncertainty (such as kit

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variability, 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) [9].

For the hGH assays, whose results are expressed as the ratio of the concentration values recGH / pitGH, it is necessary to take into account the values of u_c (%) obtained for both assays of a particular test kit.

Two (2) top-down approaches for calculation of the u_c (%) budget are recommended:

- A) The relative u_c (%) budget will include elements of Intermediate Precision (s_w , expressed as *RSD*, %) as well as relative Bias (% deviation from expected or consensus values), applicable to the determinations of the recGH and pitGH concentrations with each particular kit:

$$(Eq. 1) \quad u_c(\%) = \sqrt{s_w^2(\%) + u_{bias}^2(\%)}$$

- For calculation of u_c (%), it is recommended that standard control samples, prepared by spiking pitGH and recGH in human zero (undetectable levels of hGH) serum to yield an approximate ratio of recGH / pitGH = 1.50 – 2.00, be used. Four (4) different dilutions, containing values of recGH ~ 12.5, 2.5, 0.5 and 0.1 ng/mL, should be measured in triplicate over 5-6 days by at least two (2) different analysts. This would ensure that the u_c (%) is calculated over the physiological range of hGH concentrations found in samples from healthy individuals;
- The value of u_c (%), applicable to the ratios, will result from the u_c (%) of the component assays, according to Eq. (2).

$$(Eq. 2) \quad u_{c_{ratio}}(\%) = \sqrt{u_{c_{rec}}^2(\%) + u_{c_{pit}}^2(\%)}$$

- B) Alternatively, the Laboratories may calculate the u_c (%) based on the long-term multiple measurements of the kit control samples QC1 and QC2.

[Comment: All measurements of QC samples shall be considered unless the intra-assay acceptance criterion ($s_r \leq 15\%$) is not met, in which case the assay shall be repeated (as for Samples).

- The u_c (%) budget will include elements of Intermediate Precision (s_w , expressed as *RSD*, %) as well as Bias (% deviation from the manufacturer's value), applicable to determinations of the recGH and pitGH concentrations for QC1 and QC2 with each particular kit [Eq.(1)];
- The s_w (%) should be determined based on a minimum of thirty (30) measurements over a period of at least six (6) months;

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- The Bias should be established by comparison of the long-term mean of recGH and pitGH concentration values obtained for both QC1 and QC2 with a particular kit with the accepted assay value determined by the kits' manufacturer (batch-specific).
- The u_c (%) of the recGH/pitGH ratio for each QC can be calculated by combining the u_c (%) of recGH and pitGH using Eq. (2).
- The kit u_c (%) will be calculated as the mean of u_c (%) (QC1) and u_c (%) (QC2), applied to the ratio.

4.2 Maximum levels of u_c (%)

Laboratories shall have values of u_c (%), applicable to the ratios at values close to the *DL* for each test kit, not higher than the maximum allowed values of u_{c_Max} (%) = 20%.

4.3 Verification of MU

Laboratories shall refer to the TD DL ^[8] for on-going verification of the assay u_c (%) estimates.

5.0 References

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- [4] Bidlingmaier M *et al.* High-Sensitivity Chemiluminescence Immunoassays for Detection of Growth Hormone Doping in Sports. *Clin Chem* **55**: 3 (2009).
- [5] World Anti-Doping Program *Sample Collection Guidelines*.
- [6] World Anti-Doping *Code International Standard* for Laboratories (ISL).
- [7] Hanley JA, Saarela O, Stephens DA, Thalabard JC. hGH isoforms differential immunoassays applied to blood samples from athletes: decision limits for anti-doping testing. *Growth Horm IGF Res* **24**: 205, 2014.
- [8] *WADA Technical Document TD DL: Decision Limits for the Confirmatory Quantification of Exogenous Threshold Substances* by Chromatography-based Analytical Methods.
- [9] ISO/IEC Guide 98-3: 2008. Evaluation of Measurement Data – Guide to the Expression of Uncertainty in Measurement (GUM) (2008).

[Current versions of WADA ISL and Technical Documents may be found at <https://www.wada-ama.org/en/what-we-do/science-medical/laboratories>]