WADA Technical Document – TD2015GH

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HUMAN GROWTH HORMONE (hGH) ISOFORM DIFFERENTIAL IMMUNOASSAYS FOR *DOPING CONTROL* ANALYSES

The purpose of this Technical Document 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 Technical Document provides direction on the *Sample* pre-analytical preparation procedure, the performance of the test(s) and the interpretation of the test results.

1.0 Introduction to the 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 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 method

In order to perform the test(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, whereas both kit '1' and kit '2' shall be used for the Confirmation Procedure(s).

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 is expressed as the ratio of the concentrations recGH / pitGH for each particular kit.

2.0 Assay Requirements

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

- Validate the assay performance on-site, including, for example, 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) ≤ 15%.
  - s_w (expressed as inter-assay RSD) ≤ 20%.
  - LOQ ≤ 0.050 ng/mL[1], defined as the lowest concentration with s_r ≤ 15% and s_w ≤ 20%.

- In addition, the Laboratory shall determine the assay Measurement Uncertainty (MU) from Laboratory validation data. The combined standard uncertainty (u_c), applied to the assay recGH / pitGH ratios, shall be not higher than the maximum levels of u_{c_Max} set from inter-Laboratory External Quality Assessment Scheme (EQAS) data [relative u_{c_Max} = 20% for both kits, at values close to the corresponding Decision Limits (DLs)].

- 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).

[1] The Laboratory LOQs, established at ≤ 0.050 ng/mL on the basis of method performance criteria (s_r ≤ 15% and s_w ≤ 20%), should not be lower than the respective LOQ values established by the kits' manufacturer.

2.1 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 (BD Vacutainer® SST™-II tubes, EU ref 367955; BD Vacutainer® SST™-II Plus Advance tubes, EU ref 367954) in accordance with the *WADA* Guidelines for Blood *Sample* Collection [5]. Such blood *Samples* should 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. 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 [5]. The Laboratory shall notify and seek advice from the *Testing* Authority regarding rejection or Analytical Testing of *Samples* for which irregularities are noted (as per ISL 6.2.2.4; [6]). In cases of *Sample* collection in the incorrect matrix (to be determined by the Results Management Authority), 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.

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

  "A" *Sample*

  - The "A" *Sample* shall be centrifuged for 10-15 min at 1300-1500g 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 shall be used for the Initial Testing Procedure. The remaining "A'' *Sample* Aliquot(s) not used for the Initial Testing

Procedure shall 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 4 days from *Sample* collection). Alternatively, the ''A'' *Sample* Aliquots shall be frozen[2] 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 *Sample* 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 fraction 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-evident system and frozen[2] until further analysis, if needed.

[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 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.

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

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

  - These *Samples* shall 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 h of thawing. The remaining of the "A" *Sample* serum fraction 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.

  - 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 fraction 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-evident system and frozen[2] 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 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 4 days from *Sample* collection). Alternatively, ''A'' *Sample* Aliquots shall be frozen[2] until analysis.

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

  - "B" *Samples* shall be frozen[2] as soon as possible upon reception and thawed before analysis. Once the "B" *Sample* is thawed and opened (according to ISL 6.2.4.2.2), an Aliquot 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-evident system and stored frozen[2] until further analysis, if needed.

2.2 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 kits and the Laboratory Standard Operation 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 Technical Document, the latter document shall prevail in each case.

Note: 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.

2.2.1 Analytical Testing Strategy

- Either kit '1' or kit '2' may be used for the Initial Testing Procedure using at least two Aliquots taken from the original "A" *Sample*.
- In the case of an initial Presumptive *Adverse Analytical Finding*, both kit '1' and kit '2' shall be used for the Confirmation Procedure of the "A" *Sample* using three new Aliquots of the original "A" *Sample*.
- For the "B" Confirmation Procedure, both kit '1' and kit '2' shall be used on three Aliquots taken from the original "B" *Sample*. The Laboratory shall follow the requirements of the ISL 6.2.4.2.2.1 for the performance of the "B" *Sample* confirmation analysis [6].
- For both "A" and "B" Confirmation Procedures, three *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 (as per ISL 6.2.4.2.1.6 and 6.2.4.2.2.5) [6].
- In accordance with the ISL provisions 6.2.4.2.1.4 and 6.2.4.2.2.8 [6], the Laboratory shall have a policy to define those circumstances where the Confirmation Procedure 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 variations, for the performance of the tests under different assay conditions (different lots of kits, different analysts, etc.) and/or to demonstrate the specificity of the assays.

3.0 Reporting and Interpretation of 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 decimal places), obtained from the measured replicates of the *Sample* Aliquot and calculated by dividing the mean value of the results of the 'recombinant' assay (concentration of recGH in ng/mL, expressed to 3 decimal places) by the mean value of the results of the 'pituitary' assay (pitGH in ng/mL, expressed to 3 decimal places), with the corresponding Decision Limit (DL) for males and females established for the test kit used [7]. The DL values to be used are[4]:

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[1] value of the corresponding pitGH assay (expressed to 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 "greater than 4.00").

- All *Samples* with values of **recGH below 0.150 ng/mL** shall be considered as **Negative**, irrespective of the corresponding values of the recGH / pitGH ratio.

3.1.1 Presumptive *Adverse Analytical Finding*

The Initial Testing Procedure shall produce a Presumptive *Adverse Analytical Finding* 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*

The Confirmation Procedure shall produce an *Adverse Analytical Finding* if the analytical results (recGH / pitGH ratios) exceed the appropriate DL values for both kit '1' and kit '2'.

[4] The DL values have been derived from the analysis of *Samples* from *Athletes* treated under real *Doping Control* conditions of *Sample* collection, transportation, storage and analysis (using the current commercial ISO-certified hGH kits and standardized analytical protocols and instrumentation). The established DL values define a combined test specificity (between the two kits) of at least 99.99% [7].

3.1.3 *Atypical Finding*

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

The MU of the assay has already been considered and incorporated in the reference population-based statistical estimation of the DL. Therefore, for declaration of an *Adverse Analytical Finding* or an *Atypical Finding* the assay MU shall not be added [5].

3.2 Reporting of Test Results

When reporting an *Adverse Analytical Finding* or an *Atypical Finding*, the Laboratory Test Report shall include the recGH / pitGH ratio, expressed to 2 decimal places, of the mean recGH and pitGH concentration values from replicate determinations (obtained during the Confirmatory Procedure), the values of the applicable DL as well as the u_c at values close to the DL as determined by the Laboratory during method validation (expressed in units to 2 decimal places).

In addition, the Laboratory Documentation Package shall include the mean concentration values of recGH and pitGH from replicate determinations (obtained during the Confirmatory Procedure, expressed to 3 decimal places) and the expanded MU ($U_{95\%}$) equivalent to the 95% coverage interval (k = 2) for the analytical value of the recGH /pitGH ratio for the *Sample* (expressed in units to 2 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 assay 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 standard uncertainty (u_c) estimated by the Laboratory at levels close to the DL is. 0.22 for kit '1' and 0.19 for kit '2'. This constitutes an *Adverse Analytical Finding* for hGH.

[5] According to *WADA*'s Technical Document on Decision Limits for the Confirmatory Quantification of Threshold Substances (TDDL) [8], 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.

4.0 Assay Measurement Uncertainty

4.1 Combined Standard Uncertainty (u_c)

- Laboratories shall generally refer to the TDDL [8] 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 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 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 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 bias (% deviation from expected or consensus values), applicable to the determinations of the recGH and pitGH concentrations with each particular kit:

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

- 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 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 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 formula (2).

$$(2) \quad u_{c_{ratio}} = \sqrt{\left(u_{c_{rec}}\right)^2 + \left(u_{c_{pit}}\right)^2}$$

B) Alternatively, the Laboratories may calculate the u_c based on the long-term multiple measurements [6] of the kit control samples QC1 and QC2.

- The relative u_c budget (%) will include elements of Intermediate Precision (s_w, expressed as *RSD*, %) as well as bias, applicable to determinations of the recGH and pitGH concentrations for QC1 and QC2 with each particular kit [formula (1)].
- The s_w should be determined based on a minimum of 30 measurements over a period of at least 6-months.
- 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 bias is expressed as % deviation from the manufacturer's value (RMS_{bias}).
- The u_c (%) of the recGH/pitGH ratio for each QC can be calculated by combining the u_c of recGH and pitGH using equation (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

In accordance with the TDDL [8], 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 values of u_{c_Max} obtained from relevant rounds of EQAS.

The target u_{c_Max} represents the minimum requirement to be achieved by a Laboratory for the uncertainty of measurement when reporting a result for the determination of a Threshold Substance.

4.3 Expanded Uncertainty $U_{95\%}$

For determination of the expanded uncertainty $U_{95\%}$, a coverage factor k=2 shall be applied for u_c at a 95% confidence level.

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

4.4 Verification of Measurement Uncertainty

Laboratories shall refer to the TDDL [8] for on-going verification of the assay u_c estimates.

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[6] 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 *Doping Control Samples*).

5.0 References

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