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World Anti-Doping Program

GUIDELINES

hGH ISOFORM DIFFERENTIAL IMMUNOASSAYS

for anti-doping analyses

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TABLE OF CONTENTS

1. Objective 3

2. Scope 3

3. Definitions 3

4. Introduction to the Method 3

4.1 Principle of the method 3

5. Assay Requirements 4

5.1 Laboratory Requirements prior to the application of the hGH isoform differential immunoassay method to Samples for Anti-doping analyses 4

5.2 Assay Pre-analytical Procedure 5

5.3 Assay Analytical Procedure 7

5.4 Testing Strategy 8

6. Reporting and Interpretation of Results 8

7. Assay Measurement Uncertainty 10

7.1 Laboratory standard Uncertainty (u) 10

7.2 Maximum levels of u 12

7.3 Expanded Uncertainty $U_{95\%}$ 12

7.4 Verification of Measurement Uncertainty 12

8. Bibliography 13

1. Objective

This guideline has been developed 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. The guideline provides direction on the *Sample* pre-analytical preparation procedure, the performance of the test(s) and the interpretation of the test results.

2. Scope

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

3. Definitions

All relevant *Code* and ISL definitions apply to this Guideline.

4. 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[2, 3].

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 only comprised of the 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[4].

4.1 Principle of the method

This hGH isoform differential immunoassay method is based on a dual-antibody, sandwich-type immunoassay system. The hGH-specific capture monoclonal antibody is pre-coated on the surface of the assay tubes and the detection antibody is labelled with acridinium ester, a chemical that gives a luminescent signal when excited at a specific energy in the detection instrument (luminometer).

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[5]. Either kit may be utilized for the Initial Testing Procedures, whereas both kit1 and kit2 shall be used for the Confirmation Procedures. Each kit contains one 'recombinant' and one 'pituitary' assay. In the 'recombinant' (recGH)

assay, the coated capture antibody preferentially binds to the 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 concentration values recGH / pitGH for each particular kit.

5. Assay Requirements

5.1 Laboratory Requirements prior to the application of the hGH isoform differential immunoassay method to Samples for anti-doping analyses

Prior to the implementation of this assay in routine anti-doping analysis, the Laboratory shall fulfill the following requisites:

- Validate the assay performance on-site, including, for example, the determination of the assay limit of detection (LOD), limit of quantification (LOQ), intra- and inter-assay variability, and uncertainty of measurement.

  The acceptance values for these parameters of assay performance are:

  - LOQ of 0.05 ng/mL or less[ℵ];
  - Intra-assay CV not higher than 10%;
  - Inter-assay CV not higher than 20%;
  - Assay measurement uncertainty (MU), determined from Laboratory validation data, not higher than the maximum levels of MU set from inter-laboratory External Quality Assessment Scheme (EQAS) data (relative combined standard uncertainty u = 14% for both kits, applied to the assay ratios at the value of the corresponding Decision Limits and expressed in units).
- Participate successfully in at least one WADA-organized educational EQAS in order to demonstrate readiness for assay implementation. In cases of identified deficiencies, proper implementation of the necessary corrective action(s);
- Obtain ISO/IEC 17025 certification from a relevant accreditation body for the inclusion of the hGH differential immunoassays in the Laboratory scope of accreditation.

[ℵ] The LOQ of the assays is defined as the lowest concentration with intra-assay CV < 10% and inter-assay CV < 20%.

- If the concentration at the LOD, where LOD = mean (blank) + 3SD (blank), meets these assay requirements, then LOQ = LOD.
- If these analytical requirements are not met at the LOD, higher hGH concentrations must be tested to determine the LOQ. The lowest calibration sample may be further diluted in sheep serum with the objective to determine the LOQ of the assays. At least, 10-fold measurements must be performed to validate the LOQ.

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5.2 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 tubes, EU ref 367955) in accordance with the WADA Guidelines for Blood *Sample* Collection. Such blood *Samples* should have been kept in a refrigerated state (not 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. The Laboratory shall notify and seek advice from the Testing Authority regarding rejection and 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 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:

  "A" *Sample*

  - Centrifuge the "A" *Sample* for 10-15 min at 1300-1500g as soon as possible after reception.
  - Take one Aliquot of the separated serum fraction into a new vial for the Initial Testing Procedure;

    The remaining of the "A" *Sample* separated serum fraction should be kept in the S*ample* collection tube and step-frozen according to the tube manufacturer's instructions[⊗, §]. This fraction shall be kept frozen and thawed before analysis, if needed[*].
  - Alternatively, and if allowed by the criteria defined in the assays' ISO/IEC 17025 accreditation, the whole separated serum fraction from the "A" *Sample* may be aliquoted into new vials, which shall be properly labelled to ensure 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 must be stored frozen and thawed before analysis, 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 24h before analysis (within a maximum of 96h from *Sample* collection). Alternatively, the ''A'' *Sample* Aliquots must be frozen§ and thawed before 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 S*ample* collection tube and step-frozen according to the tube manufacturer's instructions⊗, §. This fraction shall be thawed before analysis, if needed*;
- Once the "B" *Sample* is thawed and opened, it shall be aliquoted following the same procedure as for the "A" *Sample*. An Aliquot of the "B" *Sample* shall be used for the "B" Confirmation Procedure. The remaining serum shall be sealed by the *Athlete* or the *Athlete's* representative using a tamper-proof evident method and stored frozen§ until further analysis, if needed*;*

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

- *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 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 of the "A" *Sample* serum fraction should be kept in the S*ample* collection tube and frozen§ until the "A" Confirmation Procedure, if needed;
  Once the "B" *Sample* is thawed* and opened, it shall follow the same procedure as for the "A" *Sample*. An Aliquot of the "B" *Sample* shall be used for the "B" Confirmation Procedure. The remaining serum shall be re-sealed by the *Athlete* or the *Athlete's* representative using a tamper-proof evident method and stored frozen§ until further analysis, if needed;

- *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 aliquotting or stored at approximately 4°C for a maximum of 24h before analysis (within a maximum of 96h from *Sample* collection). Alternatively, ''A'' *Sample* Aliquots must be frozen§ and thawed before analysis*;

The remaining of the "A'' *Sample* not used for the Initial Testing Procedure should be kept in the *Sample* collection tube and stored frozen and thawed before analysis[§, *];
For "B" *Samples*, freeze the *Samples*[§] 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" Confirmation Procedure. The remaining serum shall be re-sealed by the *Athlete* or the *Athlete's* representative using a tamper-proof evident method and stored frozen[§] until further analysis, if needed.

[⊗] 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 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. This might be the case, for example, for "B" *Samples* not needing confirmation due to a negative "A" *Sample* result, but which may be stored for up to 8 years for any future re-testing.

***** Thawing of the *Sample(s)* 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.

5.3 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 SOP.

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

Note 1: *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 must be set at 1 second.*

5.4 Testing Strategy

- Either kit '1' or kit '2' may be used for the Initial Testing Procedure using at least duplicates of an Aliquot taken from the original "A" *Sample*;
- In the case of an initial Presumptive Analytical Finding, both kit '1' and kit '2' shall be used for the Confirmation Procedure of the "A" *Sample* using an additional Aliquot of the original "A" *Sample*;
- For the "B" Confirmation Procedure, both kit '1' and kit '2' shall be used on an Aliquot 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;
- For both "A" and "B" Confirmation Procedures, triplicates of *Sample* Aliquots should be measured, except in cases of limited *Sample* volume, in which case a lower maximum number of replicates may be used (as per ISL 5.4.4.1.2);
- 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* may be repeated (for example, values of intra-assay CV > 10%);
- It is recommended that the Laboratories implement well-characterized and stable internal quality control sample(s), 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. These quality control samples might be, for example, recGH-spiked human serum sample(s) to serve as positive control(s) (the addition of such a sample to a positive doping-control *Sample* would render it more positive) or a low-hGH, negative control sample (the addition of which to a positive doping-control *Sample* would render it less positive or negative).

6. Reporting and Interpretation of Results

- The final determination of an *Adverse Analytical Finding* (*AAF*) is dependent on the kit ratio for the *Sample*, which is calculated by dividing the mean value of the results of the 'recombinant' assay (concentration of recGH in ng/mL) by the mean value of the results of the 'pituitary' assay (pitGH in ng/mL), obtained from the measured replicates of the *Sample* Aliquot (ratio1 = rec1 / pit1; ratio2 = rec2 / pit2);
- For *Samples* where the measured values of pitGH concentrations are below the assay LOQ, as determined by the Laboratory, the LOQ value of the pitGH concentration shall be utilized for the purposes of calculating the kit ratio;
- For declaration of an *AAF*, the Laboratory shall compare the *Sample* analytical result for the kit ratio (expressed to two decimal places) with

the corresponding gender-specific Decision Limits (DL) established for the test kit used. The DL values are the following ♣:

Kit ‘1’: Males (**1.81**); Females (**1.46**)
Kit ‘2’: Males (**1.68**); Females (**1.55**)

- The Initial Testing Procedure shall produce a Presumptive Analytical Finding on *Sample* “A” if the corresponding ratio of recGH to pitGH exceeds the pre-established gender-specific DL for the kit used (kit ‘1’ or kit ‘2’);
- A *Sample* shall be reported as an *AAF* if the analytical results (ratios) from the “A” *Sample* Confirmation Procedure exceed the DL values for both kit ‘1’ and kit ‘2’.

  The MU of the assay has already been considered and incorporated in the reference population-based statistical estimation of the DL (see Note 2 below). Therefore, for declaration of an *AAF* the assay MU shall not be applied;
- All *Samples* with confirmed mean values of **recGH below 0.1 ng/mL** shall be reported as **negative**, irrespective of the corresponding values of pitGH and the resulting kit ratio;

Note 2*: According to WADA’s TD2010DL (Technical Document on Decision Limits for the Confirmatory Quantification of Threshold Substances)[6], the decision rule applicable to assays for which the threshold value(s) have been established based on reference population statistics does not consider the inclusion of a guard band for the determination of a DL. In such cases, such a guard band that reflects the uncertainty of the measurements provided by the assay(s) would have already been incorporated in the definition of the threshold(s). Therefore, the zone of analytical values considered compliant (negative) or not (AAF) with this decision rule would be defined by the threshold value itself, which constitutes the DL.*

♣ *The DL values specified above 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 99.99%. These DL values are conservative values and will be periodically refined as more data is accumulated from Doping Control tests performed by WADA-accredited Laboratories.*

- When reporting an *AAF*, the Laboratory Test Report shall include the **ratio** of mean concentration values 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 ($\boldsymbol{u_c}$) at the value of the DL as determined by the Laboratory (expressed in units).

In addition, the Documentation Package shall include the mean concentration values of recGH and pitGH from triplicate determinations (obtained during the Confirmatory Procedure) and the expanded measurement uncertainty ($U_{95\%}$) equivalent to the 95% coverage interval (k = 2) for the analytical value of the ratio recGH /pitGH for the *Sample* (expressed in units).

Reporting Example (Test Report):

The analysis of the *Sample* identified above by using the CMZ hGH differential immunoassays has produced the following analytical values of assay ratios: X for kit '1' (e.g. 2.50) and Y for kit '2' (e.g. 2.40), which are greater than the corresponding DL of W (e.g. 1.81) and Z (e.g. 1.68), respectively. The combined standard uncertainty (u_c) estimated by the Laboratory at the DL is '*a*' (e.g. 0.22 if 12% relative u_c at the DL) for kit '1' and '*b*' (e.g. 0.17 if 10% relative u_c at the DL) for kit '2'. This constitutes an *Adverse Analytical Finding* for hGH.

Note 3*: As stated in Note 2, the assay MU is not applied for compliance decisions (declaration or not of an AAF) since the estimate of the MU has already been incorporated into the values of the DL. The inclusion of the u_c in the test report is in compliance with the TD2010DL[6] and serves to demonstrate that the Laboratory has performed the assay(s) in accordance with the technical specifications established in these Guidelines. According to TD2010DL[6], the value of u_c reported by the Laboratory must be not greater than the maximum acceptable values of $u_{c\ Max}$ established from inter-Laboratory EQAS studies (see section 7 below).*

7. Assay Measurement Uncertainty

7.1 Combined Standard Uncertainty (u_c)

- Laboratories shall generally refer to TD2010DL[6] 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)[7];
- 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;

  For application to the hGH assays, two top-down approaches for calculation of the u_c budget have been recommended:

A) The u_c budget will include elements of within- and between-day variabilities (expressed as coefficient of variations – CV %) as well as bias.

(1) $$u_c = \sqrt{(u_{bias})^2 + (u_{within-day})^2 + (u_{between-day})^2}$$

- For calculation of u_c, a standard control sample, prepared by spiking pitGH and recGH in human zero serum to yield an approximate ratio of recGH / pitGH = 1.50 – 2.00, should be used. Five different dilutions, containing values of recGH ~ 50, 10, 5, 1 and 0.2 ng/mL, should be measured in triplicates over different days (5-6) by different analysts (at least 2). This would ensure that the u_c is calculated over a large range of concentrations;
- The within-day and between-day variabilities should be calculated for each assay (CV);
- The element of bias will be calculated for each assay from the results reported by the Laboratory in the EQAS (% deviation from the inter-laboratory consensus value);
- The value of u_c, applicable to the ratios, will result from the u_c of the component assays, according to formula (2).

(2) $$u_{ratio} = \sqrt{(u_{rec})^2 + (u_{pit})^2}$$

B) Alternatively, the Laboratories may calculate the u_c based on the long-term multiple measurements of the kit control samples QC1 and QC2 (since these are lyophilized samples, which are reconstituted just before the analysis, thus ensuring their long-term stability);

- In this case, the u budget includes elements of long-term precision and bias[8]

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

- The long-term precision would be determined based on a minimum of 30 measurements over a period of 6-months. The long-term imprecision, expressed as CV, of the ratio can be calculated by combining the long term imprecision of recGH and pitGH using equation 2.

Note 4*: All measurements of QC samples shall be considered unless the intra-assay acceptance criteria are not met (CV<10%), in which case the assay shall be repeated (as for Doping Control Samples).*

- The bias will be established by comparison of the long-term mean of all values obtained for both QC1 and QC2 for a particular assay with the true assay value mentioned by the kits' manufacturer (batch-specific). The bias is expressed as % deviation from the manufacturer's value;

- The kit u_c will be calculated as the mean of u_c for QC1 and QC2, applied to the ratio:

(4) $$u_C = \frac{u_{QC1} + u_{QC2}}{2}$$

7.2 Maximum levels of u_c

- In accordance with TD2010DL[6], Laboratories shall have values of u_c, applicable to the DL for each test kit, not higher than the maximum values of $u_{c\,Max}$ obtained from relevant rounds of inter-laboratory 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.

7.3 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.

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

7.4 Verification of Measurement Uncertainty

Laboratories shall refer to TD2010DL[6] for ongoing verification of the assay MU estimates.

8. Bibliography

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