World Anti-Doping Program

GUIDELINES

hGH ISOFORM DIFFERENTIAL IMMUNOASSAYS

for anti-doping analyses

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Version 2.1

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1. Objective

These guidelines have 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 guidelines provide direction on the *Sample* pre-analytical preparation procedure, the performance of the test(s) and the interpretation of the test results.

2. Scope

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

3. Responsibility

These guidelines are intended for use by *WADA*-accredited laboratories.

4. Definitions

4.1 *Code* Defined Terms

Atypical Finding: A report from a Laboratory or other *WADA*-approved entity 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*.

Adverse Analytical Finding: A report from a Laboratory or other *WADA*-approved entity 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 participates in sport at the international level (as defined by each International Federation), the national level (as defined by each *National Anti-Doping Organization*, including but not limited to those *Persons* in its *Registered Testing Pool*), and any other competitor in sport who is otherwise subject to the jurisdiction of any *Signatory* or other sports organization accepting the *Code*. All provisions of the *Code*, including, for example, *Testing* and therapeutic use exemptions, must be applied to international- and national-level competitors. Some *National Anti-Doping Organizations* may elect to test and apply anti-doping rules to recreational-level or masters competitors who are not current or potential national caliber competitors. *National Anti-Doping Organizations* are not required, however, to apply all aspects of the *Code* to such *Persons*. Specific national rules may be established for *Doping Control* for non-international-level or

non-national-level competitors without being in conflict with the *Code*. Thus, a country could elect to test recreational-level competitors but not require therapeutic use exemptions or whereabouts information. In the same manner, a *Major Event Organization* holding an *Event* only for masters-level competitors could elect to test the competitors but not require advance therapeutic use exemptions or whereabouts information. For purposes of Article 2.8 (Administration or *Attempted* Administration) 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*.

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, therapeutic use exemptions, 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*.

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

4.2 *ISL* Defined Terms

Aliquot: A portion of the *Sample* or biological fluid or tissue (*e.g.* urine, blood, etc.) 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 of one or more specific *Prohibited Substance*, *Metabolite*(s) of a *Prohibited Substance*, or *Marker(s)* of the *Use* of a *Prohibited Substance* or *Method* in a *Sample*.

Initial Testing Procedure (Screen Testing Procedure): An analytical test procedure whose purpose is to identify those *Sample*s 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* in excess of a defined threshold.

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

International Standard for Laboratories (ISL): The *International Standard* applicable to Laboratories.

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.

Laboratory(ies): (An) accredited laboratory(ies) applying test methods and processes to provide evidentiary data for the detection of *Prohibited substance(s), Methods* and *Markers* on the *Prohibited List* and, if applicable, quantification of a Threshold Substance, in urine and other biological *Sample*s 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.

Presumptive 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 (s_r): Variability observed within a laboratory, over a short time, using a single operator, item of equipment, etc.

Testing Authority(ies): The *Anti-Doping Organization* that has authorized a particular test. For example, the International Olympic Committee, *World Anti-Doping* Agency, International Federation, National Sport Organization, *National Anti-Doping Organization*, *National Olympic Committee*, Major *Event Organization*, or other authority defined by the *Code* responsible for authorizing *Sample* Testing either *In-Competition* or *Out-of-Competition*.

Threshold Substance: A substance listed on the TD DL for which the detection and quantification of an amount in excess of a stated threshold is considered an *Adverse Analytical Finding*.

5. 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 comprised almost exclusively of 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 [4].

5.1 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 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 concentration values recGH / pitGH for each particular kit.

6. Assay Requirements

6.1 Laboratory Requirements prior to the Application of the hGH Isoform Differential Immunoassay Method to *Doping Control Samples*

Prior to the implementation of this method 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 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

[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.

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).

6.2 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 [6]. 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 [6]. 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; [1]). 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[2]. This fraction must be kept frozen[3] until analysis, if needed (*e.g.* until the "A" Confirmation Procedure).

- Alternatively, and if allowed by the criteria defined in the method's 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 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[3] 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 24h before analysis (within a maximum of 96h from *Sample* collection). Alternatively, the ''A'' *Sample* Aliquots must be frozen[3] 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 S*ample* collection tube and step-frozen according to the tube manufacturer's instructions[2, 3] until 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* should be used for the "B" Confirmation Procedure. The remaining serum shall be sealed in front of the *Athlete* or the *Athlete's* representative using a tamper-proof evident method and stored frozen[2, 3] until further analysis, if needed.

[2] Place the *Sample* collection 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.

[3] 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.

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

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

  - These *Samples* must remain frozen[3] until analysis.

  - Once thawed, an Aliquot of *Sample* "A" should 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 not used for the Initial Testing Procedure should be kept in the S*ample* collection tube and frozen[3] 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 in front of the *Athlete* or the *Athlete's* representative using a tamper-proof evident method and stored frozen[3] 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 24h before analysis (within a maximum of 96h from *Sample* collection). Alternatively, ''A'' *Sample* Aliquots must be frozen[3] until 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[3] until the "A" Confirmation Procedure, if needed.

  - For "B" *Samples*, freeze[3] the *Samples* as soon as possible upon reception until 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 in front of the *Athlete* or the *Athlete's* representative using a tamper-proof evident method and stored frozen[3] until further analysis, if needed.

6.3 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 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 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.

6.4 Analytical 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 a new 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 [1].
- 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 6.2.4.2.1.6 and 6.2.4.2.2.5) [1].
- In accordance with the ISL provisions 6.2.4.2.1.4 and 6.2.4.2.2.8 [1], 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 the 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. These iQC 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).

7. Compliance Decision Rules - Interpretation and Reporting of Results

7.1 Interpretation of 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 gender-specific Decision Limit (DL) established for the test kit used [7]. The DL values are the following[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.
- 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.

7.1.1 Presumptive Analytical Finding

- The Initial Testing Procedure shall produce a Presumptive Analytical Finding on *Sample* "A" if the ratio of recGH to pitGH exceeds the pre-established gender-specific DL for the kit used (kit '1' or kit '2').

7.1.2 *Adverse Analytical Finding*

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

[4] 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 at least 99.99% [7].

7.1.3 *Atypical Finding*

- The Confirmation Procedure shall produce an *Atypical Finding* if the analytical results (recGH / pitGH ratios) exceed the DL values 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 (see Note 2 below). Therefore, for declaration of an *Adverse Analytical Finding* or an *Atypical Finding* the assay MU shall not be added.

Note 2*:* According to WADA's Technical Document on Decision Limits for the Confirmatory Quantification of Threshold Substances (TD DL) [8], the decision rule applicable to assays for which the threshold value(s) have been established based on reference population statistics does not consider the addition 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 calculation of the threshold(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.

7.2 Reporting of 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 mean recGH and pitGH concentration values from replicate determinations (obtained during the Confirmatory Procedure), the values of the applicable **DL** as well as the $\boldsymbol{u_c}$ at values close to the DL as determined by the Laboratory (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 male *Sample*):

The analysis of the *Sample* identified above by 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 the DL is. 0.22 for kit '1' and 0.19 for kit '2'. This constitutes an *Adverse Analytical Finding* for hGH.

8. Assay Measurement Uncertainty

8.1 Combined Standard Uncertainty (u_c)

- Laboratories shall generally refer to the TD DL [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 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) [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, standard control samples, 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. Four different dilutions, containing values of recGH ~ 12.5, 2.5, 0.5 and 0.1 ng/mL, should be measured in triplicates 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[5] 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).

- 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 would be determined based on a minimum of 30 measurements over a period of at least 6-months.
- The bias will 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 true assay value mentioned 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:

$$(3) \qquad u_c = \frac{u_{c\,QC1} + u_{c\,QC2}}{2}$$

8.2 Maximum levels of u_c

- In accordance with the TD DL [8], Laboratories shall have values of u_c, applicable to 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.

8.3 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

[5] All measurements of QC samples shall be considered unless the intra-assay acceptance criteria are not met ($s_r \leq 15\%$), in which case the assay shall be repeated (as for *Doping Control Samples*).

8.4 Verification of Measurement Uncertainty

Laboratories shall refer to the TD DL [8] for ongoing verification of the assay u_c estimates.

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