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Standard Operation Procedure

HBM4EU-SOP-QA-002

Preparation of control materials for Interlaboratory Comparison Investigations (ICI) and External Quality Assurance Schemes (EQUAS)

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1 Aim and application area

This SOP is one out of four SOPs describing how Interlaboratory Comparison Investigations (ICIs) and External Quality Assurance Schemes (EQUAS) are done in HBM4EU:

HBM4EU-SOP-QA-001 "Organisation of Interlaboratory Comparison Investigations (ICI) and External Quality Assurance Schemes (EQUAS) of interlaboratory studies"

HBM4EU-SOP-QA-002 "Preparation of control materials for Interlaboratory Comparison Investigations (ICI) and External Quality Assurance Schemes (EQUAS)"

HBM4EU-SOP-QA-003 "Evaluation of results from Interlaboratory Comparison Investigations (ICI) and External Quality Assurance Schemes (EQUAS)"

HBM4EU-SOP-QA-004 "Reporting of results of Interlaboratory Comparison Investigations (ICI) and External Quality Assurance Schemes (EQUAS)"

This SOP describes general procedures for preparation of the control material and test samples, and procedures for homogeneity and stability assessment of the control material. Test samples are aliquots of control material that are distributed to participating laboratories to assess their proficiency in the determination of a specified biomarker/matrix combination.

For drafting this SOP, requirements as outlined in ISO/IEC 17043:2010, "Conformity assessment – General requirements for proficiency testing" have been taken into account.

2 Definitions

All definitions have been compiled in HBM4EU-SOP-QA-001, and the reader is referred to that SOP.

3 Chemicals and Reagents

3.1 Chemicals

3.1.1 Reference substances of the biomarkers or certified solutions of the reference substance (only in case of preparation of spiked control materials)

3.2 Reagents

3.2.1 Stock solution.

Dissolve at least 10 mg of reference substance in an appropriate type and volume of solvent, considering solubility/stability of reference substance and compatibility with matrix in mixing ratio according to 3.2.2. Prepare freshly unless data on storage stability are available and the expiration date is not exceeded. Some reference substances are available as solutions only, in that case the purchased solution is the stock solution.

3.2.2 Spiking solutions.

Dilute the stock solution to a concentration such that addition of 0.5% (v/m) solution to the control material results in the desired concentration in control material. Prepare freshly unless data on storage stability are available and the expiration date is not exceeded.

4 Equipment

4.1 Analytical balance, accuracy 0.1 mg.

4.2 Laboratory balance, accuracy 0.01 g.

4.3 Pipettes, suited for organic solvents if applicable.

4.4 Vortex mixer, stirrer, ultrasonic homogenizer for homogenisation of liquid materials.

4.5 Blender, e.g. Moulinette, or Ultra-turrax for homogenisation of semi-solid materials.

4.6 Ball mill, for homogenisation of keratinous matrices (hair or nails).

4.7 Large container/flask with screw cap for sample material, free of the biomarker of interest.

4.8 Small containers/tubes/vials with screw cap for test samples, free of the biomarker of interest.

Note: make sure that all equipment and containers used are free of the biomarker(s) of interest. Pre-test to confirm if not yet established before.

5 Procedures

5.1 Control material to be used

For ICI / EQUAS purposes, the use of burdened human material is preferable. If this is not available, then blank material can be spiked or surrogate materials (e.g. of animal origin) may be used.

Collect an amount of control material, burdened and/or blank, that is sufficient to cover the items below, plus an additional 20-50%.

- homogeneity testing (10 samples, amount sufficient for duplicate analysis)

- stability testing (a minimum of 6 samples is required)

- samples to be sent to the laboratories participating in the ICI / EQUAS

- in case of EQUAS, sample set (at least 6 replicates) to be analysed by the expert laboratories for determination of the assigned value.

For use of human materials for ICI/EQUAS purposes, the ethics requirements from WP1 need to be taken into account. Register all control materials collected with a unique code and document origin and storage location/conditions.

5.2 Preparation of control material

5.2.1 Use of burdened control material

Determine the concentration of the biomarker(s) of interest in burdened control material(s) collected. Calculate the amounts of burdened control material(s) and blank material to be blended to in order to obtain the anticipated concentrations for the ICI / EQUAS.

5.2.2 Use of blank control material

When no burdened material is available, calculate the amount and concentration of spiking solution (3.2.2) to be prepared for spiking the blank material. The volume of solution added for spiking should not exceed 0.5% (v/m).

5.2.3 Liquid materials (urine, blood/serum/plasma, milk)

- Make sure the sample is clear and free of precipitates as much as possible (filter if necessary)

- Homogenize the control material (burdened material(s) or spiked material) by ultrasonication (typically 10 min, avoiding heat generation) and vortex or stir mixing using a suitable device (4.4).

- Transfer the control material into containers or tubes with screw cap (4.8). The amount should be sufficient for at least one analysis. In case the material is turbid or new precipitates have been formed, ensure that no settling in the bulk control material occurs. Store the containers freezer (≤-18°C).

Note: urine may contain sediment/precipitates which may result in inhomogeneity. These can be removed before preparation and homogenisation of the control material, e.g. through filtration and/or freeze-out. However, after preparation and aliquotation in test sample tubes, new precipitates may be formed upon storage. Therefore, re-homogenisation of the content of the test tube is typically needed before taking out an aliquot for analysis.

5.2.4 Semi-solid materials (meconium, faeces)

- Homogenize the control material (burdened material(s) or spiked material) with a blender (4.5) during 10-60 min, avoiding overheating. If necessary add water to obtain a slurry to improve homogeneity. In case the amount of sample exceeds the blender capacity, divide in portions which after homogenisation are combined and manually mixed.

- Transfer appropriate amounts of the control material into containers (4.8). Store the containers in the freezer (≤-18°C).

5.2.5 Keratinous materials

- If appropriate: wash/decontaminate the intact hair/nail material

- Cut the material into < 1 cm pieces/sections

- Pulverize the keratinous matrix using a ball mill (25 Hz, 4 min) to obtain a powder. Repeat if necessary avoiding overheating of the material.

- Distribute appropriate amounts of the control material into tubes (4.8). Store them under dry and ambient conditions in the dark.

5.2.6 Records of control materials and test samples

Label each individual container/tube containing test samples with a unique (random) code. For each ICI / EQUAS study, register all codes of test samples with description and link to original control material in a database.

5.3 Homogeneity testing

- Randomly take 10 containers/tubes with the test samples from the storage.

- Thaw frozen samples (note: once thawed, do not freeze again)

- Re-homogenise the thawed sample by ultrasonication and/or vortex shaking

- Analyse the 10 samples in duplicate according to the applicable SOP for sample analysis

Note: the result for homogeneity of the control material is only valid for aliquots/portions equal or larger than taken during homogeneity testing.

- Calculate the concentrations for the individual 20 aliquots/portions analysed and the overall mean

Note: the overall mean is not the assigned values as used in EQUAS, for this, analysis of the test samples by multiple expert laboratories is required as described in HBM4EU-QA-001.

5.4 Stability testing

For evaluation of the stability of the biomarker in the test samples, the samples are stored at conditions representative for transport to and storage at the participant's laboratory until analysis (typically frozen, <-18°C). The stability will be determined by analysis of at least six test samples at one or more time intervals. The time interval should always cover the time between shipment and the (extended) deadline of submission of the results.

There are three approaches for determination of the stability:

A) A set of test samples (n≥6) are stored after preparation (and homogeneity testing) at conditions representative for transport and storage at the participant's laboratory (typically frozen, <-18°C) for 28 days and are analysed. Assessment of the stability is done by comparing the mean of the stored samples and the mean of the homogeneity testing.

B) six test samples are taken from the storage and analysed at t=0 (date of shipment) and t=end (=deadline submission of results). Optionally, additional sets can be taken and analysed at a time in between the start and end of the analysis period allowed for the participants. Assessment of the stability is done by comparing the means of the replicates.

C) At t=0 (date of shipment), six test samples are taken from the storage location and placed in an ultra-freezer (≤-80°C). At or after t=end, the six samples are taken from the ultra-freezer and analysed together with 6 samples stored at ICI/EQUAS conditions (typically -18°C) in one batch. The assumption here is that at ≤-80°C the samples are stable.

6 Results

6.1 Homogeneity

Assessment whether or not the control material is sufficiently homogenous, is based on ISO 13528:2015, Fearn et al [2001]. The 20 concentrations obtained by duplicate analysis of the 10 different test samples are copied into an Excel sheet that calculates the relevant statistical parameters described in detail below. Alternatively, dedicated software programs may be used when providing equivalent results.

6.1.1 Outliers

The duplicate analysis results will be tested for outliers using the Cochran's test [Fearn 2001]. For each set of duplicates, the difference (w_t) is calculated:

$$w_t = duplo_1 - duplo_2 \quad (1)$$

With: w_t = difference in duplicate concentrations;
$duplo_1$ = analysis result of first portion of a test sample;
$duplo_2$ = analysis result of second portion of a test sample.

Calculate for each duplicate analysis the square of the duplicate difference (w_t^2).
Calculate the sum of squares of the duplicate-difference ($\sum w_t^2$) and determine the highest square of all duplicate-differences (w_{max}^2).

Calculate the Cochrans's value (C):

$$C = \frac{w_{max}^2}{\sum w_t^2} \quad (2)$$

With: C = Cochran's value;
w_{max}^2 = the highest square of all duplicate-differences;
$\sum w_t^2$ = sum of squares of the duplicate-difference.

Look up the critical value (C_{crit}) for the applicable number of duplicates in the table 'Critical values for Cochran's test'. There are no outliers when $C < C_{crit}$. The duplicate-difference with the highest square of all duplicate-differences (w_{max}^2) is considered an outlier when $C > C_{crit}$.

When an outlier has been identified, the duplicate result will be discarded from the data set and further calculations of homogeneity. Repeat the outlier test with the remaining data. If another outlier is detected, then the data set is considered unfit, e.g. an issue during analysis occurred which need to be resolved and the analysis for homogeneity assessment needs to be repeated.

6.1.2 Calculations for testing homogeneity

The calculations described below can be done using an Excel sheet. Alternatively, a dedicated software tools can be used when providing equivalent results.

First, a target standard deviation (σ_T) as used in the ICI/EQUAS needs to be set that is considered fit-for-purpose for the biomarker/technique used.

This value is set based on expert opinion, taking into account what is technically feasible and realistic in current routine practises. By default the value is set at 0.25*concentration of the control material:

$$\sigma_T = 0.25 * \bar{x} \quad (5)$$

Calculate the standard deviation of the mean of the test samples by:

$$s_x = \sqrt{\frac{\sum (x_t - \bar{x})^2}{g-1}} \quad (6)$$

With s_x = standard deviation of mean of test samples;
x_t = mean of duplicates of one test sample;
$\bar{x}$ = overall mean of analysis results;
g = number of test samples (10 in case there are no outliers).

Calculate the within-sample standard deviation by:

$$s_w = \sqrt{\frac{\sum w_t^2}{2g}} \quad (7)$$

With s_w = within-sample standard deviation;
w_t = duplicate-difference of test samples (formula 1);

g = number of test samples (10 in case there are no outliers).

Calculate the between-sample standard deviation by:

$$s_s = \sqrt{s_x^2 - \frac{s_w^2}{2}} \qquad (8)$$

With s_s = between-sample standard deviation;
s_x = standard deviation of the sample means (formule 6);
s_w = within-sample standard deviation (formula 7).

Calculate the critical value (*c*) by:

$$c = 0{,}3\hat{\sigma} \qquad (9)$$

With c = critical value;
$\hat{\sigma}$ = expected standard deviation in ICI (= σ_T , formula 3-5).

Compare the analytical deviation (s_w) with 0.5*σ_T to assess whether or not the analysis method used is suited to determine inhomogeneity. The method is considered suited when $s_w < 0.5*\sigma_T$.

Compare the between-sample standards deviation (s_s) with the calculated critical value. The control material is insufficiently homogeneous (with 95% confidence) when $s_s > c$. In other cases the sample is considered sufficiently homogeneous for ICI / EQUAS.

6.2 Stability

The stability analyses for ICI are done in line with ISO 13528:2015 and the International harmonised protocol for the proficiency testing of analytical laboratories [Thompson 2006]. The criterion for stability of the control material for an ICI is related to the influence of potential instability on the evaluation of the results provided by the participants to the ICI.

Calculate the mean for the replicates analysed at t_0 (date of shipment of samples) and t_e (deadline of submission of results). The biomarkers in the control material are considered sufficiently stable when the difference between the means is ≤ 0,3σ_T [ISO 13528:2015].

The calculations are done using an Excel spreadsheet or dedicated software.

In case the above criterion is not met, the statistical significance of the differences between the mean values at the different storage times will be determined. For this, determine whether the variances of the analyses at t_0 en t_a are equal using an F-test. Calculate the F-values using equation (10) or (11), whichever results in F>1.

$$F = \frac{s_{t_0}^2}{s_{t_e}^2} \qquad (10)$$

$$F = \frac{s_{t_e}^2}{s_{t_0}^2} \quad (11)$$

With: F = F-value;
s_{t_0} = standard deviation of the analyses at t_0;
s_{t_e} = standard deviation of the analyses at t_e.

Determine the critical values (F_{crit}) with n_0-1 and n_e-1 degrees of freedom using the table 'F-distribution, critical values for α=0,05' [Thompson 2006]. Compare the calculated F-values (F) with the critical value (F_{crit}) from the table.

The variances of the two analyses are considered significantly different when $F > F_{crit}$. If they do not differ, then a t-test can be used to test whether or not the analysis results obtained for t_0 en t_a are equal. For this the following hypothesis is used:

$$x_0 - x_a = 0 \quad (12)$$

With: x_0 = calculated mean at t_0;
x_e = calculated mean at t_e.

Calculate the standard deviation for x_0-x_e ($s_{x_0 - x_e}$) with:

$$s_{x_0 - x_a} = \sqrt{\frac{(n_0 - 1)s_{t_0}^2 + (n_e - 1)s_{t_a}^2}{(n_0 - 1) + (n_e - 1)}} \times \sqrt{\frac{1}{n_0} + \frac{1}{n_e}} \quad (13)$$

With: $s_{x_0 - x_a}$ = standard deviation of x_0-x_e ;
n_0 = number of analyses at t_0;
n_a = number of analyses at t_e;
s_{t_0} = standard deviation of analyses at t_0;
s_{t_e} = standard deviation of analyses at t_e.

Calculate t:

$$t = \frac{x_0 - x_e}{s_{x_0 - x_e}} \quad (14)$$

With: t = Student t-value;
x_0 = calculated mean at t_0;
x_e = calculated mean at t_e.
$s_{x_0 - x_e}$ = standard deviation of x_0-x_e .

Determine the critical t-value (t_{crit}) for α=0.05 with n_0+n_e-2 degrees of freedom using the 'Student t-distribution table'. Compare the calculated t-value (t) with the critical t-value (t_{crit}) from the table.

The means at t_0 en t_e are considered not significantly different when $t < t_{crit}$.

Note: in case the sample sets from t_0 and t_e are not analysed on the same day: when the means are significantly different but still in the range of what is to be expected from intermediate precision data, the control material may still be considered sufficiently stable.

7 Registration

Archive all relevant records regarding the analyses and the Excel sheets with calculations either electronically (with at least one backup) or as paper files for a period of at least 5 years. In case the control material is not prepared by the organiser, a copy of the relevant records should be send to the organiser.

8 References

HBM4EU-SOP-QA-001 "Organisation of Interlaboratory Comparison Investigations (ICI) and External Quality Assurance Schemes (EQUAS) of interlaboratory studies"

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HBM4EU-SOP-QA-004 "Reporting of results of Interlaboratory Comparison Investigations (ICI) and External Quality Assurance Schemes (EQUAS)"

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