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EVALUATION OF MEASUREMENT UNCERTAINTY

ANNEX 4

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1. INTRODUCTION

The measurement uncertainty of biological assays can be estimated using the top-down approach or bottom-up approach described in Evaluation of Measurement Uncertainty – Core Document PA/PH/OMCL (18) 145 and its Annexes for physico-chemical methods. However, the top-down approach is mainly used for biological methods. While the general principles of these approaches apply, there are some specific features that require particular attention:

1) Some biological assays may lack reference preparations of known potency. Therefore, it may not be possible to estimate the bias component. It is recommended, in such cases, to clearly indicate that the reported standard uncertainty represents the precision component only and it may underestimate the actual measurement uncertainty of the assay.

2) Results of biological assays may not follow a normal distribution. The asymmetry of data distributions typically increases with the assay variability. For right-skewed data distributions, log-normal distributions are usually preferred, as shown in Figure 1. When such distributions are used, geometric means (GM) and geometric coefficients of variation (GCV) are reported. In addition, assay validity criteria and acceptance limits are asymmetrical to mimic the shape of the log-normal distribution, e.g. 80%-125%, 50%-200%.

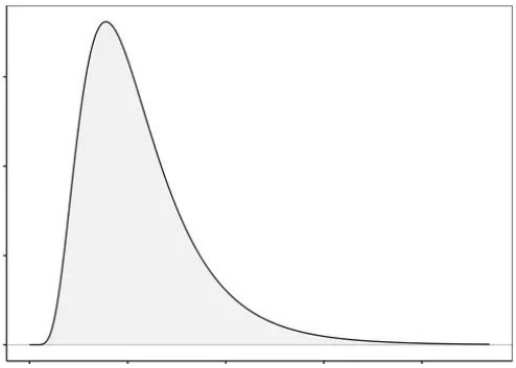


Figure 1. Example of a log-normal distribution

3) A log transformation of the assay results is carried out prior to the calculation of the precision and bias components. However, this data transformation may not be required for GCVs lower than 15% to 20%. In such cases, the skewness of the data distribution is still not very pronounced, and the normal approximation may remain acceptable.

4) The standard uncertainties (u) calculated on log-transformed results are expressed on a log scale, e.g. ln(IU/mL), $\log_{10}(CCID_{50}/mL)$. It is up to the analyst to back-transform these estimates depending on their routine use. For example, the assay results of viral titration are often reported on a $\log_{10}$ scale (along with the associated specification limits).

Table 1 shows some examples of anti-log calculations. Back-transformed results are expressed on a multiplicative scale. In particular, relative expanded uncertainty (U_{rel}) should be used as follows: assuming a reported value GM = 25.4 IU/mL and U_{rel} = 57% (k = 2), a 95% uncertainty interval is equal to 25.4 / (1 + 57/100) = 16.2 IU/mL and 25.4 × (1 + 57/100) = 39.9 IU/mL, where the factor 1.57 is the so-called fold ratio.

Alternatively, the uncertainty interval could be calculated using log-transformed values and the limits back-transformed: assuming the arithmetic mean (AM) = 3.236 ln(IU/mL) and U = 0.451 ln(IU/mL), a 95% uncertainty interval is equal to 3.236 ln(IU/mL) ± 0.451 ln(IU/mL) = [2.785, 3.687] ln(IU/mL). The corresponding back-transformed interval is [exp (2.785), exp (3.687)] = [16.2, 39.9] IU/mL.

Whatever the logarithm transformation applied, the final results (e.g. GM, GCV) are the same, as long as the proper anti-log transformation is used.

Table 1. Examples of anti-log calculations

Scale	Log-transformed results (additive scale)	Formula	Back-transformed results (multiplicative scale)
ln	AM = 3.236 ln(IU/mL)	exp (AM)	GM = 25.4 IU/mL
	SD = 0.148 ln(IU/mL)	100·(exp (SD)-1)	GCV = 16%
	U = 0.451 ln(IU/mL)	100·(exp(U)-1)	U_{rel} = 57%
log_{10}	AM = 4.805 log_{10}(CCID$_{50}$/mL)	10^{AM}	GM = 63826 CCID$_{50}$/mL
	SD = 0.086 log_{10}(CCID$_{50}$/mL)	100·(10^{SD}-1)	GCV = 22%
	U = 0.204 log_{10}(CCID$_{50}$/mL)	100·(10^{U}-1)	U_{rel} = 60%

AM = arithmetic mean, SD = standard deviation, GM = geometric mean, GCV = geometric coefficient of variation, U = expanded uncertainty, U_{rel} = relative expanded uncertainty.

Notes:

Another formula exists for the back-transformation of SD and U. This formula results in values lower than those obtained by the formulae given in Table 1.

ln scale: GCV = 100 x sqrt(exp(SD^2)-1), e.g. for SD = 0.148 ln(IU/mL), GCV = 15%.

log_{10} scale: GCV = 100 x sqrt(exp(SD^2 x $ln(10)^2$)-1), e.g. for SD = 0.086 log_{10}(CCID$_{50}$/mL), GCV = 20%.

There is no relationship between the SD and U values given in Table 1. In particular, U is not equal to 2 × SD in these examples. Recall that SD values represent random errors, while U values represent random errors and systematic errors.

This Annex contains 5 Appendices with different examples:

- Appendix 1: the precision and bias components are estimated from results (PFU/mL) of a biological reference preparation used in a cells-based assay. A logarithmic transformation of the data is carried out and the combined uncertainty expressed in log_{10} PFU/mL as well as in PFU/mL for two assay formats (single values and means of three replicates in a run).
- Appendix 2: the precision component is estimated from results (IU/mL) of an internal control used in a vaccine potency assay by ELISA. As in Appendix 1, a logarithmic transformation is applied. The bias component cannot be estimated in the absence of appropriate material/preparation. As a result, the method uncertainty may be underestimated unless the bias component is deemed negligible.
- Appendix 3: the precision and bias components are estimated from results (EU/mL) of samples spiked with a known concentration of endotoxin using the recombinant factor C

method for quantification of bacterial endotoxins. The combined uncertainty is calculated for endotoxin concentrations about 0.2 EU/mL.

- Appendix 4: the precision and bias components are estimated from results (EU/mL) of the chromogenic kinetic method for quantification of bacterial endotoxins. The combined uncertainty is calculated using spikes of 0.1 EU/mL added at the mid-point concentration of standard preparation.
- Appendix 5: the precision and bias components are estimated from results (IU/mL) of a series of PT studies carried out using the assay of human coagulation factor VIII by chromogenic method. The combined uncertainty is reported as a percentage, as PT results cover a range of about 10 IU/mL to 1000 IU/mL.

Appendix 1. Measurement uncertainty for a routine cell-based assay

1. Introduction

This example is intended to cover two potential situations that may arise when bioassay results clearly do not follow a normal distribution but rather a log-normal one (e.g. PFU/mL, $CCID_{50}$/dose, ED_{50}, relative potency). For some of these assays, specification limits can be given in log_{10} (or ln), for some other they are given in original units.

The virus concentration of a vaccine is determined by a cell-based assay and is indicative of the batch potency (measured in PFU/mL).

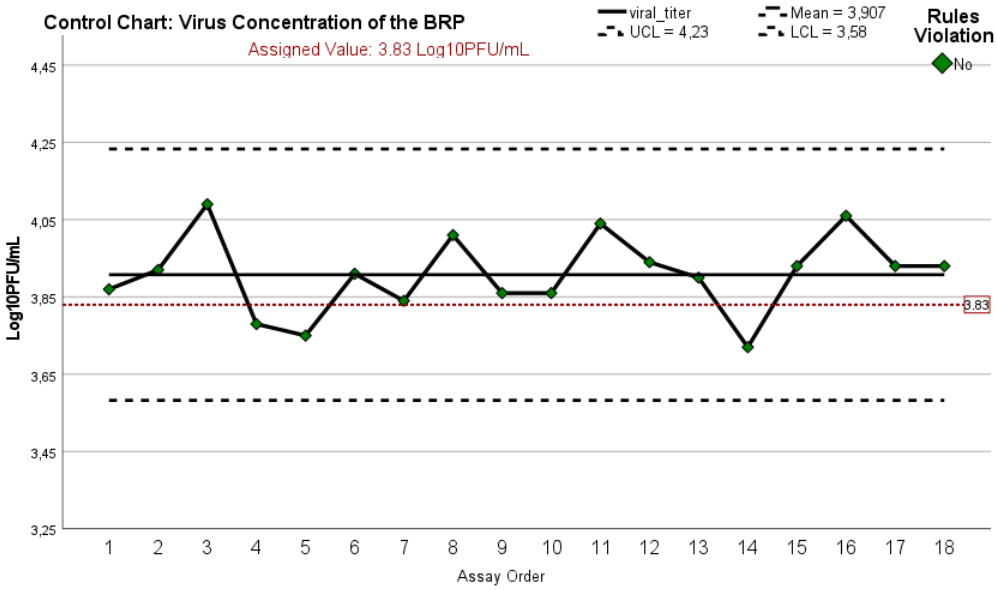
For this method (Ph. Eur. 01/2019:0648 corrected 10.0, *Varicella vaccine, live*), a Biological Reference Preparation (BRP) is tested in the same way as for product batches and its calculated virus concentration is compared to an acceptance criterion to check the validity of each assay (i.e. it is not directly involved in the titre calculation of product batches). This BRP has an assigned value of 3.83 log_{10}PFU/mL reported in the Certificate of Analysis; this is already an indication that PFU/mL data follows a log-normal distribution and therefore data analysis should be performed after log_{10} transformation. The log_{10} results obtained routinely for this BRP are monitored using a control chart.

The laboratory performed n = 18 runs (assays). Raw data are reported in Table 1 and plotted in Figure 1. As no control limit or alert rule were violated, all data were considered for the evaluation of the measurement uncertainty. All the routine assays were performed by the same operator.

Table 1. Raw data

BRP control chart data (log_{10} PFU/mL)					
Run	Date	Rep.1	Rep.2	Rep.3	Mean
1	25-May-18	3.91	3.88	3.81	3.87
2	27-Jun-18	3.89	3.94	3.93	3.92
3	02-Jul-18	4.10	4.12	4.05	4.09
4	24-Aug-18	3.80	3.76	3.78	3.78
5	10-Sep-18	3.72	3.83	3.71	3.75
6	13-Oct-18	3.86	3.96	3.90	3.91
7	24-Oct-18	3.92	3.90	3.71	3.84
8	08-Nov-18	3.98	4.05	4.00	4.01
9	29-Nov-18	3.90	3.89	3.80	3.86
10	18-Dec-18	3.91	3.85	3.82	3.86
11	11-Jan-19	4.02	4.06	4.04	4.04
12	21-Feb-19	3.92	3.91	3.98	3.94
13	30-Mar-19	3.91	3.91	3.88	3.90
14	18-Apr-19	3.75	3.61	3.79	3.72
15	12-Jun-19	3.99	3.84	3.97	3.93
16	15-Jul-19	4.05	4.10	4.02	4.06
17	27-Jul-19	4.00	3.89	3.90	3.93
18	10-Sep-19	3.94	3.93	3.91	3.93

Figure 1. – Individual Control Chart (performed on the mean values of each Assay)



Assuming that all critical sources of variation are taken into account, the data available in the control chart can be used to estimate the uncertainty of measurement of the method; the (absolute) combined standard uncertainty (u_c) is calculated as:

$$u_c = \sqrt{u_p^2 + u_b^2}$$

where:

- u_p is the precision component which depends on the random variation between results.
- u_b is the bias component which depends on the difference between the mean of results and the assigned value of the BRP (3.83 $\log_{10}$PFU/mL).

Warning: as the underlying distribution of PFU/mL is log-normal, the calculation for the combined uncertainty should not be based on relative terms (i.e. u_c/x). The absolute combined uncertainty (i.e. u_c) is the appropriate approach.

This example provides two different ways to proceed (A and B), depending on how many replicates are used in routine assays to estimate the potency of the unknown batch to be released onto the market.

2. Specification of measurand

The **measurand** is the viral concentration of a vaccine batch, expressed as PFU/mL.

3. Data and calculation

Note: calculated results, including intermediate results, are shown with a maximum of 3 significant digits for the sake of clarity. However, non-rounded values are used in intermediate calculation steps. Therefore, some discrepancy may be found between written formulae and reported results. In other words, to obtain the final results, the calculation steps described below should be reproduced without intermediate rounding.

3.1. Precision component

In section A, routine assays are performed with one replicate only while in triplicates (as for the BRP above described) in section B. This will affect the contribution of the precision component to the overall uncertainty.

A) Routine assays are performed with one replicate only

The precision component (for $\log_{10}$ transformed data) is estimated by a one-way random analysis of variance (ANOVA) to calculate the *within* and the *between* assay variances. Table 2 shows the calculated results.

Table 2. Variance estimates

Components	Variance estimates	% of total.	*Std-deviation estimates*
Between-assays_g^2	s_g^2 = 0.00914	75	0.0956
Within-assay (repeatability) s_r^2	s_r^2 = 0.00306	25	0.0553
Intermediate precision (total)	0.0122		**0.110**

(calculated on log-transformed data).

$$u_p = \sqrt{{s_r}^2 + {s_g}^2} = \sqrt{0.00306 + 0.00914} = 0.110 \log_{10} \text{PFU/mL}.$$

B) Routine assays are performed in triplicates (as for the BRP)

The precision component is equal to the standard deviation of the 18 run-means (of 3 replicates each) but can also be calculated using the variance estimates shown in Table 2 by dividing the within-assay variance by 3:

$$u_{p(3)} = \sqrt{\frac{{s_r}^2}{3} + {s_g}^2} = \sqrt{\frac{0.00306}{3} + 0.00914} = 0.101 \log_{10} \text{PFU/mL}.$$

Warning: this formula is applicable only if there are 3 valid replicates for all assays. Otherwise, different calculation procedures are required.

3.2. Bias component

With the overall mean of the BRP being equal to 3.91 $\log_{10}$PFU/mL and its assigned (target) value being equal to 3.83 $\log_{10}$PFU/mL, the bias is calculated as:

$$B = 3.91 - 3.83 = 0.08 \log_{10}\text{PFU/mL}.$$

The standard-error (uncertainty) of the bias (i.e. BE: Bias Error) is calculated as the standard deviation of the run-means (i.e. $u_{p(3)}$ for 3 replicates per run) divided by the square-root of the number of runs:

$$BE = \frac{u_{p(3)}}{\sqrt{k}}$$

$$BE = \frac{0.101}{\sqrt{18}}$$

$$BE = 0.0238 \log_{10} \text{PFU/mL}$$

The bias is more than 3 times higher than its standard-error and thus likely to be statistically significant. Indeed, the Student t-test leads a t-value equal to 3.26 which is higher than the t-critical value (2.11 calculated for a 5% significance threshold and k = 17 degrees of freedom). Therefore, the bias cannot be neglected and will be included in the uncertainty budget.

t_{obs} = (B/BE) = (0.08/0.0238) = 3.26

t_{crit} = 2.11 (alpha = 0.05, df = 17)

The bias component is equal to:

$$u_{\mathrm{b}} = \sqrt{B^2 + BE^2}$$

$$u_{\mathrm{b}} = \sqrt{0.08^2 + 0.0238^2}$$

$$u_{\mathrm{b}} = 0.081 \log_{10} \text{PFU/mL}$$

3.3. Combined standard uncertainty

Considering the intermediate precision calculated for routine assays of batches tested with 1 replicate:

$$u_{\mathrm{c}} = \sqrt{u_p^2 + u_b^2}$$

$$u_{\mathrm{c}} = \sqrt{0.110^2 + 0.081^2}$$

u_c = 0.137 $\log_{10}$PFU/mL

The expanded (absolute) uncertainty, at an approximated 95% confidence level, is:

$$U = 2 \times u_{\mathrm{c}} = 2 \times 0.137 = 0.274 \log_{10}\text{PFU/mL}.$$

Expression of the measurement uncertainty for a batch potency result equal to 4.06 $\log_{10}$PFU/mL:

Reported results are rounded to the second decimal place, consistent with the specification limits when given in $\log_{10}$PFU/mL [3].

4.06 ± 0.27 $\log_{10}$PFU/mL (k = 2)

The corresponding uncertainty range is 3.79 to 4.33 $\log_{10}$PFU/mL,

However, as in this case the specification limits are given in PFU/mL, the corresponding uncertainty range should be obtained by back-calculating the upper and lower uncertainty limits:

- Upper Limit: 4.06 + 0.27 = 4.33 $\log_{10}$PFU/mL → $10^{4.33}$ = 21573 PFU/mL
- Lower Limit: 4.06 - 0.27 = 3.79 $\log_{10}$PFU/mL → $10^{3.79}$ = 6111 PFU/mL

In this case, the uncertainty range is asymmetrical around the reported result which is equal to $10^{4.06}$ = 11482 PFU/mL.

This uncertainty of measurement can be alternatively expressed as a “fold ratio” and associated with results in PFU/mL as follows:

➢ calculate the antilog$_{10}$ of the expanded uncertainty: $10^{0.274}$ = 1.88, and express the result as:
11482 PFU/mL with U (fold ratio) = 1.88 (k = 2)

The uncertainty range is equal to:

- Upper limit: 11482 × 1.88 = 21573 PFU/mL.
- Lower limit: $\frac{11482}{1.88}$ = 6111 PFU/mL.

Intermediate calculations are performed without rounding; reported results are rounded at the integer, the consistent with the specification limits [3].

Note: the standard deviations calculated above using log-transformed data are not informative of the precision of non-transformed data. As explained in the introduction, back transformations should be carried out to:

1. For the central location in PFU/mL, the geometric mean should be reported:
$$GM = 10^{AMlog} = 10^{3.91} = 8080 \text{ PFU/mL}$$
2. For the variability of results in PFU/mL, the geometric coefficient of variation should be reported:
$$GCV = 100 \times \sqrt{\exp\left(SD_{log}^2 \times \ln(10)^2\right) - 1}$$

where AM_{log} and SD_{log} are the arithmetic mean and standard deviation, respectively, calculated on log_{10}-transformed data.

For example, the mean bias is equal to 0.08 log_{10}, which corresponds to a geometric ratio of 1.20 ($10^{0.08}$). This means that the geometric mean calculated by the laboratory for the BRP is 1.2 times higher than the assigned value. Finally, with respect to method precision, GCVs (Table 3) can be calculated using variance estimates shown in Table 2:

Table 3. GCV estimates

Components	Variance estimates	GCV estimates	
Between-assay	0.00914	$100 \cdot \sqrt{exp(0.00914 \cdot ln(10)^2) - 1}$	= 22%
Within-assay (repeatability)	0.00306	$100 \cdot \sqrt{exp(0.00306 \cdot ln(10)^2) - 1}$	= 13%
Intermediate precision (total)	0.0122	$100 \cdot \sqrt{exp(0.0122 \cdot ln(10)^2) - 1}$	= 26%

The GCV of repeatability is equal to 13% and the GCV of intermediate precision is equal to 26%

The formulae reported above is not applicable if a different log transformation is used (including the common case of a ln; natural log transformation for which the factor ln(10)² is not required in the GCV formula). For further details, please see Ref. [2].

Next, considering the intermediate precision calculated for routine assays of batches tested with 3 replicates:

$$u_c = \sqrt{u_{p(3)}^2 + u_b^2}$$

$$u_c = \sqrt{0.101^2 + 0.081^2}$$

u_c = 0.129 log_{10}PFU/mL

The expanded (combined) uncertainty, at an approximately 95% confidence level, is:

$$U = 2 \times u_c = 2 \times 0.129 = 0.259 \log_{10}\text{PFU/mL}.$$

Expression of the measurement uncertainty for a batch potency of 4.06 log_{10}PFU/mL:

For specification limits given in log_{10}PFU/mL: 4.06 ± 0.26 log_{10}PFU/mL (k = 2),
or for specification limits given in PFU/mL: 11482 PFU/mL with U (fold-ratio) = 1.81 (k = 2).

The corresponding expanded uncertainty limits are equal to:

- Lower limit: 11482 / 1.81 = 6341 PFU/mL;
- Upper limit: 11482 × 1.81 = 20823 PFU/mL.

Comments:

There is no big difference between the results obtained from the two options above, due to the small within-assay variability (25% contribution to the intermediate precision) and the important contribution of the bias component.

Therefore, considering the high contribution of between-assay variation, i.e. 75% of the precision component (to be calculated on $\log_{10}$ data), the measurement uncertainty could be reduced by increasing the number of assays, e.g. 2 assays performed with 3 replicates each ($u_{p(3,2)}$), with the final titre of a batch calculated as the average of the results of the 2 assays:

$$u_{p(3,2)} = \sqrt{\frac{{s_r}^2}{3 \times 2} + \frac{{s_g}^2}{2}}$$

$$u_{p(3,2)} = \sqrt{\frac{0.0553^2}{6} + \frac{0.0956^2}{2}} = 0.0713 \log_{10} \text{PFU/mL}$$

$$u_c = \sqrt{u_{p(3,2)} + u_b^2}$$

$$u_c = \sqrt{0.0713^2 + 0.081^2} = 0.108 \log_{10} \text{PFU/mL}$$

The corresponding expanded uncertainty is U = 2 × 0.108= 0.216 $\log_{10}$PFU/mL (for an approximately 95% level of confidence). That is, for a batch potency of 4.06 $\log_{10}$PFU/mL calculated as the mean of 2 independent assays (of 3 replicates each), the reported results are:

For specification limits given in $\log_{10}$PFU/mL: 4.06 ± 0.22 $\log_{10}$PFU/mL (k = 2),
Or for specification limits given in PFU/mL: 11482 PFU/mL with U (fold-ratio) = 1.64 (k = 2).

The corresponding expanded uncertainty limits are equal to:

- Lower limit: 11482 / 1.64 = 6987 PFU/mL;
- Upper limit: 11482 × 1.64 = 18868 PFU/mL.

Table 4 shows the expanded uncertainty for further assay formats (number of assays and replicates).

Table 4. Expanded uncertainty magnitude in ± $\log_{10}$ and in fold ratio according to various potential formats of the analytical procedure

N. assays	N. replicates	U in $\log_{10}$	U as fold-ratio
1	1	0.274*	1.88*
	2	0.262	1.83
	3	0.259*	1.81*
2	1	0.225	1.68
	2	0.218	1.65
	3	0.216*	1.64*
3	1	0.206	1.61
	2	0.201	1.59
	3	0.199	1.58

* values calculated above.

Therefore, by increasing the number of assays to 2 (with 3 replicates), U is reduced from a 1.81-fold ratio to a 1.64-fold ratio. It is still not a huge reduction, due to the important remaining contribution of

the uncertainty components not related to the precision (i.e. the bias and its standard error). In fact, the bias component (B + BE) contributes to 35% of the total uncertainty (see results in section A):

$$\text{Bias contribution} = \frac{u_b^2}{u_c^2} = \frac{0.0810^2}{0.137^2} = 0.35.$$

4. References

1. PA/PH/OMCL (18) 150 - EVALUATION OF MEASUREMENT UNCERTAINTY - ANNEX 2.2
2. PA/PH/OMCL (22) 54 – INTRODUCTION TO STATISTICAL PROCESS CONTROL
3. PA/PH/OMCL (21) 1 – ROUNDING

Appendix 2. Vaccine potency assay by routine ELISA method

1. Introduction

This example is based on an ELISA method used as potency assay for inactivated polio virus (IPV) vaccine within the framework of batch release. The method was first transferred from the manufacturer and validated by the OMCL. Modifications were made later on (mainly dilutions in plates instead of tubes) and a new validation exercise performed to compare the performance of the OMCL and manufacturer methods for each vaccine serotype (serotypes 1, 2 and 3).

As the validation run by the manufacturer had been performed using a log transformation of the potency results, the log transformation was also applied by the OMCL for a direct comparison of the method performance. In addition, as BRP batch 3 is used as standard in this ELISA, there is no other international reference available to determine the bias. Therefore, the measurement uncertainty is based on the precision component only.

2. Validation plan

A validation plan was established to evaluate the repeatability, the intermediate precision and the range of linearity of the ELISA. The evaluation was based on an internal control (IC) used to monitor the method and a multivalent vaccine (MV) batch. The range of linearity consisted of 4 concentration levels (100%, 80%, 70%, and 60% of the IC concentration) covering the lower part of the specification window (no spiking experiments were possible in the absence of monovalent or concentrated bulks).

Four sessions were carried out by different operators on different days using different equipment (3 plates per serotype in each assay).

3. Data and calculation

3.1. Raw data

Table 1 show the potency results of the IC obtained for serotype 1 taken as an example.

Table 1. IC potency results (IU/dose) for serotype 1

Internal control	Session 1	Session 2	Session 3	Session 4
Rep. 1	30.8	27.3	30.4	33.4
Rep. 2	28.5	30.4	32.1	28.8
Rep. 3	28.8	28.1	30.7	28.2
Mean	29.4	28.6	31.1	30.1

3.2. Repeatability and intermediate precision

The repeatability is defined as the variability between potency results within a session. In this example, it represents the variability between the results of different plates.

The repeatability standard deviation, in $\log_{10}$ IU/dose, is equal to:

Session 1: $s_1 = 0.0183$ Session 2: $s_2 = 0.0242$

Session 3: $s_3 = 0.0126$ Session 4: $s_4 = 0.0401$

There is no significant difference between the corresponding variances (Levene's test p-value = 0.11), and the pooled standard deviation can be calculated:

$$s_r = \sqrt{\frac{\sum s_i^2}{k}},$$

where i is the session number, and k is the total number of sessions.

The repeatability standard deviation is:

$$s_r = \sqrt{(0.0183^2 + 0.0242^2 + 0.0126^2 + 0.0401^2)/4}$$

s_r = 0.0259 $\log_{10}$ IU/dose.

As a next step, the standard deviation (S_m) between session means is calculated and reported in $\log_{10}$ IU/dose:

Session 1: m_1 = 1.468 Session 2: m_2 = 1.456

Session 3: m_3 = 1.492 Session 4: m_4 = 1.478

S_m = 0.0154 $\log_{10}$ IU/dose.

Noting that S_m can be expressed in terms of repeatability (s_r) and inter-session variation (s_g):

$$S_m = \sqrt{s_g^2 + s_r^2/n},$$

where n is the number of replicates per session, the inter-session variation is equal to:

$$s_g = \sqrt{S_m^2 - s_r^2/n},$$

$$s_g = \sqrt{0.0154^2 - 0.0259^2/3}\,,$$

s_g = 0.0037 $\log_{10}$ IU/dose.

Note: this calculation approach requires the same number of results per session. If this is not the case, a one-way random ANOVA is recommended to estimate the repeatability and inter-session variation.

4. Summary

In this example of a vaccine potency assay where the bias component cannot be determined, the measurement uncertainty depends on the precision component only. This means that the reported measurement uncertainty may be underestimated, unless we can assume that the bias component is not significant.

Table 2 shows the standard measurement uncertainty of mean potency results calculated for different numbers of sessions (k) and replicates (n) according to the formula:

$$u(p) = \sqrt{\frac{s_g^2}{k} + \frac{s_r^2}{k \cdot n}}$$

Table 2. Standard uncertainty (log_{10} IU/dose) of mean results according to different assay formats

Sessions (s_g = 0.0037)	Replicates (s_r = 0.0259)			
	1	2	3	4
1	0.026	0.019	0.015	0.013
2	0.018	0.013	0.011	0.010
3	0.015	0.011	0.009	0.008
4	0.013	0.009	0.008	0.007

The expanded measurement uncertainty (U) is equal to 2 times the standard measurement uncertainty (for a 95% confidence level). As examples:

- For one replicate in one session, U = 0.052 log_{10} IU/dose;
- For two replicates in two sessions, U = 0.026 log_{10} IU/dose.

As the repeatability is the major source of variation (s_r is 7 times higher than s_g), there is the opportunity to reach the same expanded uncertainty as in the last example by performing 4 replicates in a single session (U = 0.026 log_{10} IU/dose), which may be more convenient.

Application: mean potency value = 1.477 log_{10} IU/dose ± 0.026 log_{10} IU/dose. The corresponding interval ranges from 1.451 log_{10} IU/dose to 1.503 log_{10} IU/dose.

In cases where the results are reported in IU/dose, the mean potency value is equal to $10^{1.477}$ = 30.0 IU/dose and the expanded measurement uncertainty interval ranges from $10^{1.451}$ = 28.3 IU/dose to $10^{1.503}$ = 31.8 IU/dose. Alternatively, these values can be calculated considering the fold-uncertainty U_{fold} = $10^{0.026}$ = 1.06. The uncertainty interval ranges from 30.0 / 1.06 = 28.3 IU/dose to 30.0 x 1.06= 31.8 IU/dose.

5. References

PA/PH/OMCL (18) 150 - EVALUATION OF MEASUREMENT UNCERTAINTY - ANNEX 2.2

PA/PH/OMCL (21) 1 - ROUNDING

Appendix 3. Quantification of bacterial endotoxins by recombinant factor C method

1. Introduction

The recombinant factor C (rFC) method (Ph. Eur. 2.6.32 01/2021:20632) is used for the quantification of bacterial endotoxins using a fluorometric method. This compendial method was validated internally to demonstrate its efficiency and the absence of interference for each vaccine complex matrix tested routinely. Validation data were used to determine the measurement uncertainty following a top-down approach.

The recoveries of samples spiked with endotoxin are calculated to validate the assay. The spiking solution has a concentration corresponding to the endotoxin concentration of the mid-point of the calibration curve used routinely. In addition, spiked samples are required to have an endotoxin concentration higher than the LOQ of the assay.

The results of the repeatability and intermediate precision exercises as well as the results of the spiking experiments are used to estimate the precision component (u_p) and bias component (u_b) of the combined standard uncertainty (u_c) (Formula 1). The recoveries of the vaccine matrices tested fall well within the 50% to 200% valid range stated in the monograph, with a minimum value of 86% and maximum of 116%, which allows the assumption of normal distribution, as the obtained results are precise enough and approximately symmetrical around 100%.

$$u_c = \sqrt{u_p^2 + u_b^2} \qquad (1)$$

2. Data and calculation

2.1. Precision component

Table 1 shows the results of a vaccine batch tested on 3 different occasions (different days and operators) using 3 rFC kit lots.

Table 1. Endotoxin results (EU/mL) obtained in intermediate precision conditions.

Run	Rep. 1	Rep. 2	Rep. 3	m_i	s_i
1	0.16	0.14	0.14	0.147	0.0115
2	0.22	0.26	0.20	0.227	0.0306
3	0.26	0.24	0.28	0.260	0.0200
	s_g = **0.0568**				**s_r = 0.0221**

The repeatability and inter-run variation were calculated as follows:

$$s_r = \sqrt{\frac{\sum s_i^2}{k}},$$

where i is the run number, and k is the total number of runs.

The repeatability standard deviation is equal to:

$$s_r = \sqrt{(0.0115^2 + 0.0306^2 + 0.0200^2)/3}$$

s_r = 0.0221 EU/mL.

As a next step, the standard deviation (S_m) between run means is calculated:

$$m_1 = 0.147 \; ; \; m_2 = 0.227 \; ; \; m_3 = 0.260$$

$$S_m = 0.0582 \text{ EU/mL}.$$

Noting that S_m can be expressed in terms of repeatability (s_r) and inter-session variation (s_g):

$$S_m = \sqrt{s_g^2 + s_r^2/n},$$

where n is the number of replicates per run, the inter-run variation is equal to:

$$s_g = \sqrt{S_m^2 - s_r^2/n},$$

$$s_g = \sqrt{0.0582^2 - 0.0221^2/3}\,,$$

$$s_g = 0.0568 \text{ EU/mL}.$$

The repeatability is equal to s_r = 0.0221 EU/mL and the between-run variation is equal to s_g = 0.0568 EU/mL; s_g is 2.6 times higher than s_r, showing the significant effect of the kit lot on the expected (mean) results.

In routine testing, assuming that the mean of n = 2 independent replicates obtained in k = 1 run (i.e. by 1 kit) is reported for each vaccine batch, the precision about the reported mean value is equal to 0.0589 EU/mL (Formula 2). For k = 2 test kits times 2 replicates, the precision becomes 0.0417 EU/mL.

$$u_p = \sqrt{\frac{s_g^2}{k} + \frac{s_r^2}{k \cdot n}} \qquad (2)$$

2.2. Bias component

Table 2 shows the results of the spiking experiments where each sample (a vaccine batch diluted 1:10) was spiked with a known concentration of endotoxin (0.5 EU/mL) corresponding to the mid-point of the calibration curve used routinely.

Table 2. Endotoxin results (EU/mL) of spiking experiments

Sample	1	2	3	4	5	6	7	8	9	10	11	12
S^-	0.136	0.149	0.136	0.165	0.004	0.002	0.001	0.153	0.140	0.148	0.189	0.253
S^+	0.639	0.660	0.628	0.702	0.468	0.529	0.485	0.701	0.610	0.674	0.767	0.683
Diff	0.503	0.511	0.492	0.537	0.464	0.527	0.484	0.548	0.470	0.526	0.578	0.430
b	0.003	0.011	-0.008	0.037	-0.036	0.027	-0.016	0.048	-0.030	0.026	0.078	-0.070

S^-: measurements before spiking. S^+: measurements after spiking. Diff: ($S^+ - S^-$) b = Diff - 0.5.

The uncertainty associated with the bias component, calculated according to Formula 3, is equal to u_b = 0.0395 EU/mL (q = 12 samples). Note that the uncertainty related to the preparation of the spiked solutions is considered as negligible.

$$u_b = \sqrt{\frac{\sum_{i=1}^{q} b_i^2}{q}} \qquad (3)$$

Note: the bias component is not statistically significant (t-test, p-value = 0.68) and could be neglected.

3. Combined uncertainty

The precision and bias components are equal to u_p = 0.0589 EU/mL (for the mean of 2 replicates by one kit) and u_b = 0.0395 EU/mL, respectively. Their contributions to the combined standard uncertainty (u_c) are equal to 69% and 31%, respectively, and u_c = 0.0709 EU/mL (Formula 1).

The corresponding expanded uncertainty (U), with k = 2 (for a 95% confidence level) is equal

$$U = 2 \times 0.0709 = 0.1418 \approx 0.14 \text{ EU/mL}.$$

This measurement uncertainty estimate was obtained for endotoxin concentrations about 0.2 EU/mL to 0.6 EU/mL. Further analysis at higher concentration levels would help to identify whether the measurement uncertainty remains similar or if it increases. Should this be the case, the calculation approach may be reconsidered, including a logarithm transformation of the results to take into account the dependence between the variability and the mean of the results.

4. References

1. PA/PH/OMCL (18) 149 - EVALUATION OF MEASUREMENT UNCERTAINTY - ANNEX 2.1: Use of Data from Validation Studies for the Estimation of Measurement Uncertainty
2. PA/PH/OMCL (18) 145 CORR EVALUATION OF MEASUREMENT UNCERTAINTY CORE DOCUMENT

Appendix 4. Quantification of bacterial endotoxins by chromogenic kinetic method (method D)

1. Introduction

There are several different methods to test for bacterial endotoxins according to the compendial method (Ph. Eur. 2.6.14. Bacterial endotoxins). This example focuses on the chromogenic kinetic method (method D), which measures the time needed for the reaction mixture to reach a predetermined absorbance.

In the chromogenic kinetic method, a standard curve with five endotoxin concentrations is prepared. Different samples are added with the mid-point concentration of the standard to test for interfering factors. This spiking is performed in duplicate, and each preparation is tested twice giving four results for each sample. These results are used to determine the uncertainty associated with the precision (u_p) and the bias (u_b).

2. Specification of measurand

The measurand is the concentration of bacterial endotoxin expressed as EU/mL.

3. Data and calculation

Data shown is an example for the estimation of measurement of uncertainty, assuming a normal distribution after a log transformation. The combined standard uncertainty of measurement u_c is given by the following general formula:

$$u_c = \sqrt{u_p^2 + u_b^2}$$

Where:

- u_p - uncertainty associated with the precision;
- u_b - uncertainty associated with the bias.

3.1. Use of spiked samples

For the interfering factors test, a total of four results were obtained with samples spiked with a known concentration of a standard (0.1 EU/mL). Table 1 shows the corresponding results that can be used to determine the precision and the bias components because they were carried out by all the qualified operators and cover all critical analytical process steps (and all test conditions (sample matrix, preparation of the sample, different days, different equipment).

The repeatability and inter-run variation were calculated as follows:

$$s_r = \sqrt{\frac{\sum s_i^2}{k}},$$

where i is the run number, and k is the total number of runs.

The repeatability standard deviation is equal to:

$$s_r = \sqrt{(0.033^2 + 0.046^2 + \cdots + 0.020^2)/27}$$

s_r = 0.045 log10 EU/mL.

Table 1. Results in EU/mL for samples spiked with endotoxin 0.1 EU/mL (different operators and days)

	EU/mL				$\log_{10}$ EU/mL	
Run	**Rep.1**	**Rep.2**	**Rep.3**	**Rep.4**	**Mean**	**s_i**
1	0.120	0.118	0.105	0.104	-0.953	0.033
2	0.079	0.088	0.092	0.102	-1.046	0.046
3	0.118	0.129	0.149	0.171	-0.853	0.071
4	0.115	0.121	0.099	0.107	-0.958	0.038
5	0.098	0.110	0.106	0.113	-0.972	0.027
6	0.083	0.088	0.077	0.079	-1.088	0.026
7	0.076	0.083	0.076	0.074	-1.113	0.022
8	0.085	0.085	0.083	0.090	-1.067	0.015
9	0.085	0.091	0.114	0.124	-0.990	0.078
10	0.098	0.118	0.111	0.118	-0.955	0.038
11	0.166	0.168	0.177	0.187	-0.759	0.024
12	0.161	0.165	0.166	0.168	-0.783	0.008
13	0.150	0.152	0.147	0.143	-0.830	0.012
14	0.148	0.162	0.121	0.136	-0.851	0.054
15	0.132	0.149	0.121	0.143	-0.867	0.040
16	0.126	0.148	0.143	0.156	-0.845	0.039
17	0.179	0.186	0.155	0.160	-0.771	0.038
18	0.117	0.127	0.111	0.118	-0.928	0.024
19	0.083	0.090	0.131	0.136	-0.969	0.110
20	0.130	0.148	0.159	0.179	-0.815	0.058
21	0.160	0.154	0.146	0.112	-0.849	0.070
22	0.089	0.097	0.105	0.114	-0.996	0.046
23	0.103	0.106	0.091	0.093	-1.009	0.033
24	0.093	0.098	0.092	0.093	-1.027	0.012
25	0.093	0.092	0.085	0.082	-1.056	0.027
26	0.142	0.128	0.148	0.132	-0.862	0.029
27	0.175	0.185	0.193	0.192	-0.730	0.020

s_i is the repeatability between 4 replicates of a given run.

$$s_r = \sqrt{0.033^2 + 0.046^2 + \cdots + 0.020^2/27}$$

Run means are equal to:

m_1 = -0.953 ; m_2 = -1.046 ; ... ; m_{26} = -0.862 ; m_{27} = -0.730

with a corresponding standard deviation (S_m) between run means equal to:

S_m = 0.109 log10 EU/mL.

Noting that S_m can be expressed in terms of repeatability (s_r) and inter-session variation (s_g):

$$S_m = \sqrt{s_g^2 + s_r^2/n},$$

where n is the number of replicates per run, the inter-run variation is equal to:

$$s_g = \sqrt{S_m^2 - s_r^2/n},$$

$$s_g = \sqrt{0.109^2 - 0.045^2/4}\,,$$

s_g = 0.107 log10 EU/mL.

3.2. Precision component (u_p)

The uncertainty associated with the precision can be estimated by:

$$\boldsymbol{u}(p) = \sqrt{\frac{s_g^2}{k} + \frac{s_r^2}{k \times n}}$$

where:

k and n are the numbers of runs and replicates (per run) foreseen in routine testing,

- s_r = 0.045 is the standard deviation between replicates (within a run) or repeatability,
- s_g = 0.107 is the standard deviation between runs.

Therefore, for the mean of n = 4 replicates in k = 1 run:

$$\boldsymbol{u_p} = \sqrt{\frac{0.107^2}{1} + \frac{0.045^2}{1\times4}} = 0.109 \text{ log10 EU/mL.}$$

As s_g is more than 2 times higher than s_r, a better precision can be achieved by increasing the number of runs. For the mean of n = 2 replicates in k = 2 runs, as an example:

$$\boldsymbol{u_p} = \sqrt{\frac{0.107^2}{2} + \frac{0.045^2}{2\times2}} = 0.079 \text{ log10 EU/mL.}$$

3.3. Bias component (u_b)

The uncertainty associated with the bias can be estimated by:

$$u_b = \sqrt{\frac{\sum_{i=1}^{q} b_i^2}{q} + {u_{add}}^2}$$

where:

b_i is the difference between a run-mean and the target value: $mean_{log10} - log_{10}(0.1)$. For q = 27 runs in Table 1, $\frac{\sum_{i=1}^{q} b_i^2}{q}$ = 0.0172.

u_{add} is the uncertainty contribution due to the concentration of the analyte added. It can be estimated taking into account the relative error and the repeatability from the equipment used.

The analyte added was prepared with two micropipettes, one with a nominal value of 200 µL and another with a nominal value of 1000 µL, both used twice.

Micropipette	200 µL	1000 µL
Relative Error (RE) (%)	0.57	0.14
Repeatability (r_p) (%)	0.25	0.15

Assuming a rectangular distribution and a negligible uncertainty of the assigned value of the standard, the uncertainty contribution due to the concentration of the analyte added (u_{add}) is estimated by:

$$u_{add} = \sqrt{\left(\frac{RE_{200}}{\sqrt{3}}\right)^2 \times 2 + \left(r_{p200}\right)^2 \times 2 + \left(\frac{RE_{1000}}{\sqrt{3}}\right)^2 \times 2 + \left(r_{p1000}\right)^2 \times 2}$$

$$u_{add} = \sqrt{\left(\frac{0.0057}{\sqrt{3}}\right)^2 \times 2 + (0.0025)^2 \times 2 + \left(\frac{0.0014}{\sqrt{3}}\right)^2 \times 2 + (0.0015)^2 \times 2} = 0.00632$$

That is, u_{add} is equal to 0.6% only and can be neglected in subsequent calculations.

The uncertainty associated with the bias is:

$$u_b = \sqrt{0.0172}$$

u_b = 0.131 log10 EU/mL

3.4. Combined uncertainty

The combined standard uncertainty is equal to:

$$u_c = \sqrt{u_p^2 + u_b^2}$$

For the mean of four replicates in one run,

$u_c = \sqrt{0.109^2 + 0.131^2}$ = 0.171 log10 EU/mL

The expanded uncertainty calculated for a 95% confidence level (k=2) is equal to:

$U = 2 \times u_c$ = 0.341 log10 EU/mL.

This value can be back-transformed in case where results should be reported in EU/mL rather than log10 EU/mL:

$U_{fold} = 10^{0.341}$ = 2.19 (119%)

3.5. Application

For a value of 0.25 EU/mL the uncertainty is:

- Lower limit = 0.25 / 2.19 = 0.11 EU/mL;
- Upper limit = 0.25 x 2.19 = 0.55 EU/mL.

4. References

1. PA/PH/OMCL (18) 145 EVALUATION OF MEASUREMENT UNCERTAINTY CORE DOCUMENT
2. PA/PH/OMCL (18) 149 - EVALUATION OF MEASUREMENT UNCERTAINTY - ANNEX 2.1: Use of Data from validation studies for the Estimation of Measurement Uncertainty

Appendix 5. Assay of human coagulation factor VIII by chromogenic method

1. Introduction

The assay of human coagulation factor VIII by chromogenic method follows European Pharmacopoeia method *2.7.4*.

The chromogenic assay method consists of 2 consecutive steps: the factor VIII-dependent activation of factor X in a coagulation-factor reagent composed of purified components, and the enzymatic cleavage of a chromogenic factor Xa substrate to yield a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between the rate of factor Xa formation and the factor VIII concentration (Ph. Eur. 2.7.4). In this method the samples are tested in duplicate.

2. Specification of measurand

The measurand is the concentration of factor VIII expressed as IU/mL.

3. Data and calculation

The combined standard uncertainty of measurement u_c is given by the following general formula:

$$u_c = \sqrt{u_p^2 + u_b^2}$$

where:

- u_p - uncertainty associated with the precision;
- u_b - uncertainty associated with the bias.

The estimation of measurement uncertainty can be performed using PTS results from different rounds, as long as they are representative of all steps of the method. PTS samples have the same matrix as real samples and all qualified operators took part to the PTS studies.

3.1. Precision component (u_p)

The uncertainty associated with the precision can be estimated using independent results obtained by different operators for the same sample. The results obtained are described in Table 1.

The uncertainty associated with the precision can be estimated by S_{pool}:

$$u_p = S_{pool} = \sqrt{\frac{\sum\left(DF_j \times S_{round(j)}^2\right)}{\sum DF_j}}$$

where:

- DF_j are the degrees of freedom of sample j. DF_j = n. rep. -1 = 2 – 1 = 1 for every sample in this example.
- S_{round} is standard deviation for the results obtained by each sample. As the magnitude of results in IU/mL are different, the relative results were considered, i.e. the obtained result divided by their mean value.

$$u_p = S_{pool} = \sqrt{\frac{281.5}{12}} = 4.84\%$$

Table 1. Results obtained by different operators in different PTS studies

		Laboratory results (IU/mL)				PT statistics			Rel. (%)			
Study	Sample	Oper.1	Oper.2	Mean	S_{Round}	N labs	Assigned	$U_{Assigned}$	Mean	Bias	S_{Round}	$U_{Assigned}$
PTS244	1	38.9653	37.905	38.4352	0.750	24	42	0.5	91.51	-8.49	1.95	1.19
PTS244	2	135.386	137.234	136.310	1.307	24	140	1.79	97.36	-2.64	0.96	1.28
PTS244	3	38.1399	39.9670	39.0535	1.292	24	40	0.59	97.63	-2.37	3.31	1.48
PTS244	4	74.656	69.362	72.009	3.743	24	72	1.06	100.01	0.01	5.20	1.47
PTS182	5	757	845	801	62.23	24	755	11.58	106.09	6.09	7.77	1.53
PTS182	6	196	185	191	7.78	24	180	3.32	105.83	5.83	4.08	1.84
PTS182	7	851	745	798	74.95	24	720	8.73	110.83	10.83	9.39	1.21
PTS182	8	791	770	781	14.85	24	720	8.11	108.40	8.40	1.90	1.13
PTS146	9	276.314	300.459	288.387	17.07	29	265	3.78	108.83	8.83	5.92	1.43
PTS146	10	10.6102	10.2080	10.4091	0.284	29	10	0.07	104.09	4.09	2.73	0.70
PTS146	11	291.413	273.458	282.436	12.70	29	265	3.23	106.58	6.58	4.50	1.22
PTS146	12	1054.69	1015.35	1035.02	27.82	29	930	11.47	111.29	11.29	2.69	1.23
								Σ^2 =		609.1	281.5	
								k =		12	12	
								SQRT(Σ^2/k) =		7.12	4.84	

3.2. Bias component (u_b)

The uncertainty associated with the bias can be estimated using the following equation:

$$u_b = \sqrt{RMS_b^2 + u_{assigned}^2}$$

Where:

- $RMS_b = \sqrt{\sum bias^2/k}$ is the root mean square of individual relative biases;
-

$$RMS_b = \sqrt{609.1/12} = 7.1$$

- $u_{assigned}$ is taken as the median of the relative standard uncertainties of the assigned values.

$$u_{assigned} = 1.26$$

And the uncertainty associated with the bias is:

$$u_b = \sqrt{7.12^2 + 1.26^2} = 7.23$$

Note: the above calculated values are percentages, so are the values calculated in the next section.

3.3. Combined uncertainty

The combined relative standard uncertainty is equal to

$$u_{rel} = \sqrt{u_p^2 + u_b^2}$$

$$u_{rel} = \sqrt{4.84^2 + 7.23^2} = 8.61$$

The expanded relative uncertainty calculated for a 95% confidence level (k=2) is equal to:

$$U_{rel} = 2 \times 8.61\% = 17.22 \approx 17.$$

Application

For a factor VIII result equal to 106 IU/mL, the uncertainty would be 106 × 0.17 = 18 IU/mL and the results reported as 106 ± 18 IU/mL.

4. References

1. PA/PH/OMCL (18) 145 EVALUATION OF MEASUREMENT UNCERTAINTY CORE DOCUMENT
2. PA/PH/OMCL (18) 153 EVALUATION OF MEASUREMENT UNCERTAINTY – Annex 2.5: use of data from proficiency testing studies for the estimation of measurement uncertainty
3. ISO 11352:2012(en) Water quality — Estimation of measurement uncertainty based on validation and quality control data