Report on the Verification of the Performance of MON87701 and MON89788 Event-specific Methods on the Soybean Event MON87701 x MON89788 Using Real-time PCR

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Executive Summary

The European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF), established by Regulation (EC) No 1829/2003, has carried out an in-house verification study to assess the performance of two quantitative event-specific methods on the soybean event MON87701 x MON89788 (unique identifier MON-877Ø1-2 x MON-89788-1) which combines the MON87701 and the MON89788 transformation events. The two methods have been validated individually on single-trait events, to detect and quantify each event in soybean samples. This study was conducted according to internationally accepted guidelines [1, 2].

In accordance to Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and to Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Monsanto Company provided the detection methods and the control samples: genomic DNA from homogenised seeds of MON87701 x MON89788 soybean and from homogenised seeds of conventional soybean. The EU-RL GMFF prepared the verification samples (calibration samples and blind samples at different unknown GM percentages [DNA/DNA]).

The results of the verification study were evaluated with reference to ENGL method performance requirements (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) and to the validation results on the individual parental events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

The results of this EU-RL GMFF in-house verification studies are made publicly available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm

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Report on Steps 1-3 of the Validation Process

Monsanto submitted the detection methods and control samples of the soybean event MON87701 x MON89788 (unique identifier MON-877Ø1-2 x MON-89788-1) under Articles 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF), following reception of the documentation and material including control samples (step 1 of the validation process), carried out the scientific assessment of documentation and data (step 2) in accordance with Commission Regulation (EC) No 641/2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation" and according to its operational procedures ("Description of the EU-RL GMFF Validation Process", http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

The scientific assessment focused on the method performance characteristics assessed against the method acceptance criteria set out by the European Network of GMO Laboratories (ENGL) and listed in the "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/doc/Method%20requirements.pdf) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, a scientific assessment of the detection method for soybean MON87701 x MON89788 was positively concluded in September 2009.

The event-specific detection methods for the two soybean lines hosting the single events MON87701 and MON89788 were validated by the EU-RL GMFF following the conclusion of the respective international collaborative studies and the publication of the validation reports (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). Hence, the detection methods applied on the soybean event MON87701 x MON89788 did not undergo a full validation process. The EU-RL GMFF performed an in-house verification of the detection methods to assess whether they exhibit a comparable performance on samples of event MON87701 x MON89788 combining the two traits (as provided in accordance to Annex I.2.C.2 of Commission Regulation (EC) No 641/2004).

In March 2010, the EU-RL GMFF concluded the verification of the method characteristics (step 3, experimental testing of the samples and methods) by quantifying, with each specific method, five blind GM levels within the range 0.085%-8.10% on a genome copy number basis. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, trueness and repeatability of the quantification were within the limits established by the ENGL.

A Technical Report summarising the results of tests carried out by the EU-RL GMFF (step 3) is available on request.

Content

1. Introduction

Monsanto Company submitted the detection methods and control samples of the soybean event MON87701 x MON89788 (unique identifier MON-877Ø1-2 x MON-89788-1) under Articles 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF), established by Regulation (EC) 1829/2003, carried out an in-house verification of the two event-specific methods for the detection and quantification of MON87701 and MON89788 in the MON87701 x MON89788 soybean event combining the two traits. The single methods had been previously validated by international collaborative studies on the single-trait soybean events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

Upon reception of methods, samples and related data (step 1), the EU-RL GMFF carried out the assessment of the documentation (step 2) and the in-house evaluation of the methods (step 3) according to the requirements of Regulation (EC) 641/2004 and following EU-RL GMFF procedures. The EU-RL GMFF method verification was concluded in March 2010.

A method for DNA extraction from soybean seeds, submitted by the applicant, was evaluated by the EU-RL GMFF in order to confirm its performance characteristics. The protocol for DNA extraction is available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

The operational procedure of the in-house verification included the following module:

- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The methodology consists of two event-specific real-time quantitative TaqMan® PCR procedures for the determination of the relative content of events MON87701 and MON89788 DNA to total soybean DNA in the MON87701 x MON89788 soybean event. The procedures are simplex systems, in which the events MON87701 and MON89788 were quantified in reference to a soybean *Le1 (lectin*) endogenous gene.

The study was carried out in accordance to the following internationally accepted guidelines:

- ISO 5725: 1994. (1)
- The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies". (2)

2. Materials

For the verification of the quantitative event-specific methods, control samples consisted of:

- genomic DNA extracted from homogenised seeds of MON87701 x MON89788 soybean, and
- genomic DNA extracted from homogenised seeds of conventional soybean.

Samples were provided by the applicant, in accordance to the provisions of Regulation (EC) No 1829/2003, Art 2.11 [control sample defined as "the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample)"].

Samples containing mixtures of 100% MON87701 x MON89788 and non-GM soybean genomic DNA at different GMO concentrations were prepared by the EU-RL GMFF in a constant amount of total soybean DNA, using the control samples provided.

The protocols (reagents, concentrations, primer/probe sequences) followed in the in-house verification are those already published as validated methods for the individual MON87701 and MON89788 events and are available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

Table 1 shows the five GM levels of unknown samples used in the verification of the MON87701 and MON89788 methods.

Table 1. MON87701 and MON89788 GM contents in soybean event MON87701 x MON89788.

MON87701 GM% (GM DNA / Non-GM DNA x 100)	**MON89788 GM%** (GM DNA / Non-GM DNA x 100)
0.085	0.1
0.26	0.4
0.90	0.9
2.70	4.0
8.10	8.0

3. Experimental design

Eight runs for each event-specific method were carried out. In each run, samples were analysed in parallel with both the GM-specific system and the reference system (*Le1*). Five GM levels per run were examined and two replicates for each GM level were analysed. PCR analysis was performed in triplicate for all samples. In total, for each method (MON87701 and MON89788), the quantification of the five GM levels was performed as an average of sixteen replicates per GM level. An Excel spreadsheet was used for determination of GM%.

4. Method

Description of the operational steps

For specific detection of events MON87701 and MON89788 in soybean event MON 87701 x MON 89788, two specific fragments, of 89-bp and 139-bp respectively, of the integration regions of the constructs inserted into the plant genome (5′ insert-to-plant junction) are amplified using specific primers. PCR products are measured during each cycle (real-time) by means of target-specific oligonucleotide probes labelled with two fluorescent dyes: FAM (6-carboxyfluorescein) is used as reporter dye at its 5′-end and TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at its 3′-end.

For relative quantification of events MON87701 and MON89788, a soybean-specific reference system amplifies a 74 bp fragment of the soybean endogenous gene *Le1* (*lectin*), using two *Le1* gene-specific primers and a *Le1* gene-specific probe labelled with FAM and TAMRA.

Standard curves are generated for each GM specific system (MON87701 and MON89788), by plotting Ct values of the calibration standards against the logarithm of the DNA copy numbers of MON87701 or MON89788, and by fitting a linear regression into these data. Thereafter, the normalised Ct values of the unknown samples are measured and the relative amount of MON87701 or MON89788 DNA, respectively, is estimated using the regression formula.

For detailed information on the preparation of the standard curve calibration samples please refer to the protocols of the validated methods at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

5. Deviations reported

No deviations have been reported.

6. Summary of results

PCR efficiency and linearity

The values of the slopes of the standard curves, from which the PCR efficiency is calculated using the formula $[10^{(-1/slope)} - 1] \times 100$, and of the R^2 (expressing the linearity of the regression) reported for all PCR systems in the eight runs, are presented in Tables 2 and 3 for MON87701 and MON89788 methods, respectively.

Table 2. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON87701 method on event MON87701 x MON89788.

Run	MON87701			*lec*		
	Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	-3.48	94	1.00	-3.33	100	1.00
2	-3.40	97	1.00	-3.41	96	1.00
3	-3.44	95	1.00	-3.34	99	1.00
4	-3.40	97	1.00	-3.33	100	1.00
5	-3.51	93	1.00	-3.35	99	1.00
6	-3.38	98	1.00	-3.32	100	1.00
7	-3.43	96	1.00	-3.37	98	1.00
8	-3.48	94	1.00	-3.33	99	1.00
Mean	-3.44	95	1.00	-3.35	99	1.00

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON89788 method on event MON87701 x MON89788.

Run	MON89788			*lec*		
	Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	-3.38	98	1.00	-3.36	99	1.00
2	-3.44	95	1.00	-3.32	100	1.00
3	-3.43	96	1.00	-3.33	100	1.00
4	-3.37	98	1.00	-3.32	100	1.00
5	-3.42	96	1.00	-3.38	98	1.00
6	-3.43	96	1.00	-3.36	98	1.00
7	-3.46	95	1.00	-3.33	100	1.00
8	-3.44	95	1.00	-3.33	100	1.00
Mean	-3.42	96	1.00	-3.34	99	1.00

The mean PCR efficiencies of the GM and species-specific systems are above 90% (95% and 99% for MON87701 and 96% and 99% for MON89788, respectively). The linearity of the methods (R^2) is 1.00 for both systems in all cases. Overall, data reported in Tables 2 and 3 confirm the appropriate performance characteristics of the two methods tested on MON87701 x MON89788 soybean samples in terms of PCR efficiency and linearity.

7. Method performance requirements

The results of the in-house verification study for the MON87701 and MON89788 detection methods applied to soybean event MON87701 x MON89788 DNA are reported in Tables 4 and 5, respectively. Results were evaluated with respect to the method acceptance criteria, as established by ENGL and adopted by the EU-RL GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm, see also Annex 1). In addition, Tables 4 and 5 report the trueness and precision for each GM level and for all methods.

Table 4. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON87701 method on event MON87701 x MON89788 soybean DNA.

MON87701					
Unknown sample GM%	**Expected value (GMO%)**				
	0.085	**0.26**	**0.90**	**2.70**	**8.10**
Mean	0.088	0.25	0.97	2.68	8.25
SD	0.01	0.01	0.05	0.20	0.59
RSD_r (%)	13	5.1	4.7	7.5	7.2
Bias (%)	3.0	-2.8	7.9	-0.9	1.9

Table 5. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation of the MON89788 method on event MON87701 x MON89788 soybean DNA.

MON89788					
Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.4**	**0.9**	**4.0**	**8.0**
Mean	0.10	0.40	0.93	3.89	8.06
SD	0.01	0.01	0.05	0.23	0.51
RSD_r (%)	7.0	3.5	4.9	5.9	6.3
Bias (%)	3.9	1.0	3.4	-2.7	0.8

The trueness of the method is estimated using the measurements of the method bias for each GM level. According to the ENGL acceptance criteria and method performance requirements, the trueness of the method, measured as bias from the accepted value, should be ± 25% across the entire dynamic range. As shown in Tables 4 and 5, the values range from -2.8% to 7.9% for MON87701 and from -2.7% to 3.9% for MON89788. Therefore, the two methods satisfy the above mentioned requirement throughout their respective dynamic ranges.

Tables 4 and 5 further document the relative repeatability standard deviation (RSD_r) as estimated for each GM level. As indicated by ENGL ("Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing", http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), the EU-RL GMFF requires the RSD_r values to be below 25%. As it can be observed from Tables 4 and 5, the values range between 4.7% and 13% for MON87701 and between 3.5% and 7.0% for MON89788. Therefore, the two methods satisfy this requirement throughout their respective dynamic ranges.

8. Comparison of method performance between event MON87701 x MON89788 and the single trait events

A comparison of the method performances assessed by the EU-RL on the soybean event MON87701 x MON89788 and the single trait events is shown in Tables 6 and 7. The performance of the methods on the single lines was previously assessed through international collaborative trials.

Table 6. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the MON87701 detection method on event MON87701 x MON89788 and on event MON87701.

Trueness and repeatability of MON87701 quantification on MON87701 x MON89788			**Trueness and repeatability of MON87701 quantification on single event MON87701***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.085	3.0	13	**0.085**	8.6	18
0.26	-2.8	5.1	**0.26**	6.4	21
0.90	7.9	4.7	**0.90**	5.2	15
2.7	-0.9	7.5	**2.7**	5.6	14
8.1	1.9	7.2	**8.1**	0.1	10

*method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Table 7. Trueness (bias %) and relative repeatability standard deviation (RSDr %) of the MON89788 detection method on event MON87701 x MON89788 and on event MON89788.

Trueness and repeatability of MON89788 quantification on MON87701 x MON89788			**Trueness and repeatability of MON89788 quantification on single event MON89788***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.1	3.9	7.0	**0.1**	-14	16
0.4	1.0	3.5	**0.4**	-5.0	22
0.9	3.4	4.9	**0.9**	-0.9	15
4.0	-2.7	5.9	**4.0**	11	13
8.0	0.8	6.3	**8.0**	2.8	12

*method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

For trueness, the MON87701 even-specific method (Table 6) shows generally lower bias when applied to event MON87701 x MON89788 compared to the single trait event, except for the 0.9% and 8.1% GM levels where similar and higher values are observed, respectively. The MON89788 event-specific method (Table 7) shows in general significantly lower bias when is applied to the stack event than the single line event, with an only slightly higher value at the 0.9% GM level. In all cases, the trueness of the two event-specific methods when applied to the

stacked event is within the acceptance range set by ENGL (± 25%) for the whole dynamic ranges studied.

For relative repeatability standard deviation (RSD_r %), the two event-specific methods (Tables 6 and 7) show significantly higher precision when applied to the stacked event than to the single trait events. In all cases, the relative repeatability standard deviations (RSD_r %) of the two event-specific methods when applied to the stacked event are below the ENGL maximum acceptance level established (25%).

Therefore, the in-house method verification has demonstrated that the MON87701 and MON89788 detection methods developed to detect and quantify the single events can be equally applied for the quantification of the respective events combined in event MON87701 x MON89788.

9. Conclusions

The overall method performance of the two event-specific methods for the quantitative detection of events MON87701 and MON89788 combined in soybean event MON87701 x MON89788 has been evaluated with respect to the method acceptance criteria and the method performance requirements recommended by the ENGL (as detailed under http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), and to the validation results obtained for the single trait events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

The results obtained during the present verification study indicate that the analytical modules of the methods submitted by the applicant comply with ENGL performance criteria. The methods are therefore applicable to the control samples provided (see paragraph 3 "Materials"), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

10. Quality assurance

The EU-RL GMFF operates according to ISO 9001:2008 (certificate number: CH-32232) and technical activities under ISO 17025:2005 [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative / quantitative PCR) – Accredited tests available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7]

11. References

1. International Standard (ISO) 5725:1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization.

2. Horwitz W., 1995. Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem*, 67: 331-343.

12. Annex 1: method acceptance criteria and method performance requirements as set by the European Network of GMO Laboratories (ENGL)

Method Acceptance Criteria should be fulfilled at the moment of submission of a method (Phase 1: acceptance for the collaborative study).

Method Performance Requirements should be fulfilled in a collaborative study in order to consider the method as fit for its purpose (Phase 2: evaluation of the collaborative study results).

Method Acceptance Criteria

Applicability

Definition: The description of analytes, matrices and concentrations to which the method can be applied.

Acceptance Criterion: The applicability statement should provide information on the scope of the method and include data for the indices listed below for the product/s for which the application is submitted. The description should also include warnings to known interferences by other analytes, or inapplicability to certain matrices and situations.

Practicability

Definition: The ease of operations, the feasibility and efficiency of implementation, the associated unitary costs (e.g. cost/sample) of the method.

Acceptance criteria: The method should generally be practicable in line with other methods for a similar purpose. More specifically the method is deemed unacceptable, unless suitable justification is supplied, if:

- it requires a new type of apparatus (not generally available) or expensive equipment; or
- the resources required to perform the method (time, workload, reagents, costs) are considerably higher than the resources required to perform other methods for similar purpose.

Other practicability considerations may also deem the method impracticable.

DNA Extraction and Purification

The aim of a DNA extraction procedure is to provide DNA of suitable quality for subsequent analysis. DNA quality depends on the average length, structural integrity and chemical purity of the extracted DNA.
It is recognised that highly fragmented DNA and co-extracted impurities of a DNA preparation may hinder the correct process of detecting and quantifying genetically modified DNA. Food and feed made of various ingredients may exert a matrix effect, depending on the DNA extraction method employed, and impair the sensitivity of the following analytical approach. For this purpose, critical steps of DNA extraction and purification should be clearly highlighted in the technical documentation accompanying a method and acceptance criteria are established to allow objective determination of PCR quality of DNA

extracts which can be considered suitable for subsequent detection experiments (e.g. qualitative and/or quantitative PCR).
DNA extraction procedures should result in repeatable recovery, fragmentation profile, concentration and PCR quality of DNA extracts. As such, it is recommended to process the given DNA extraction protocol on different days (e.g. 3 days) with an adequate number of test portions (e.g. 6 per day).
In agreement with international guidelines (e.g. EN ISO 21571, EN ISO 24276) the following criteria are used to assess method performance.

a) DNA concentration

Definition: amount of an analyte per unit volume of solution

Acceptance criterion: The DNA extraction method employed shall be appropriate to obtain the quantity of nucleic acid required for the subsequent analysis. The DNA concentration measured as weight of the analyte/volume of solution should be higher than the working concentration described in the protocol of the detection method.
Example: if the RT-PCR protocol indicates 40 ng/µL as the DNA concentration of the DNA solution to be added to the master-mix, the concentration of the DNA extract should be > 40 ng/µL.

b) DNA fragmentation state

Definition: Breakage of genomic (high molecular weight) DNA into smaller DNA fragments

Acceptance criterion: For quantitative (real time-based) analysis, the molecular weight of the extracted DNA sample should be at least higher than the amplicon size produced by the event specific and the taxon specific reference systems as established by comparison with a reference nucleic acid marker.
For qualitative analysis, in case of DNA suspensions to be used in qualitative analysis, the presence of a certain proportion of DNA molecules of molecular weight lower than the amplicon size produced by the method may be considered acceptable.

c) Purity of DNA extracts

Definition: the absence of co-extracted compounds in a DNA sample impairing the efficiency of the PCR reactions and leading to a delay in the onset of the exponential phase of the amplification profile

Acceptance criterion: The difference (ΔCt) average between the measured Ct value and the extrapolated Ct value of the first diluted sample of the inhibition test should be <0.5. [(measured Ct – extrapolated Ct)] <0.5 and the slope of the inhibition curve should be within -3.6 and -3.1.
The preferred PCR assay for the inhibition test is the internal control assay (e.g. the taxon specific reference system). The total DNA amount in the first sample of the dilution series should be not less than the total DNA amount used in the submitted method (e.g. the DNA amount indicated in the PCR protocol of the taxon specific reference system).

Specificity

Definition: Property of a method to respond exclusively to the characteristic or analyte of interest.

Acceptance Criterion: The method should not produce amplification signals with target sequences different for the target sequence for which the method was developed. This should be demonstrated by similarity searches against databases (e.g. EMBL, GenBank, Patent, etc.) and with empirical results from testing the method with non-target transgenic events and non-transgenic material.
For detection of specific GM events, the target sequence shall be event specific.
For taxon specific target sequences (target sequence), the absence of allelic and copy-number variation across a globally representative and diverse sample of the species variety shall be demonstrated. Allelic and/or copy-number variation in other lines shall be reported if such variation is known by the applicant.
The specificity of the target sequence shall be verified by *in silico* studies against publicly available sequence databases (e.g. EMBL, GenBank, etc.) and experimentally by demonstrating the absence of amplification products when the target sequence specific assay is applied to individual PCRs of pure genomic DNA of a representative sample of the closest relatives to the target taxa as well as of the most important food crops.

Dynamic Range

Definition: The range of concentrations over which the method performs in a linear manner with an acceptable level of trueness and precision.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least 5 times the target concentration. Target concentration is intended as the threshold relevant for legislative requirements. The range of the standard curve(s) for real-time PCR should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) ends.
Example: 0.09% and 4.5% for a 0.9% GMO concentration or 50 and 2500 genome copies if the target is 500 copies.

Trueness

Definition: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Acceptance Criterion: The trueness shall be within ±25% of the accepted reference value over the whole dynamic range.

Amplification Efficiency

Definition: The rate of amplification that leads to a theoretical slope of –3.32 with an efficiency of 100% in each cycle. The efficiency of the reaction can be calculated by the following equation:

$$Efficiency = 10^{\left(\frac{-1}{slope}\right)} - 1$$

Acceptance Criterion: The average value of the slope of the standard curve shall be in the range of (-3.1 ≥ slope ≥ -3.6)

R^2 Coefficient

Definition: The R^2 coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: The average value of R^2 shall be ≥0.98.

Precision - Relative Repeatability Standard Deviation (RSDr)

Definition: The relative standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time.

Acceptance Criterion: The relative repeatability standard deviation should be ≤25% over the whole dynamic range of the method.
Note: Estimates of repeatability submitted by the applicant should be obtained on a sufficient number of test results, at least 15, as indicated in ISO 5725-3**Error! Reference source not found.**.

Limit of Quantification (LOQ)

Definition: The limit of quantification is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy.

Acceptance Criterion: LOQ should be less than $1/10^{th}$ of the value of the target concentration with an RSD_r ≤25%. Target concentration should be intended as the threshold relevant for legislative requirements.
Example: For a 0.9% nominal value LOQ <0.09%.

Limit of Detection (LOD)

Definition: The limit of detection is the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified, as demonstrated by single-laboratory validation.

Acceptance Criterion: LOD should be less than $1/20^{th}$ of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring ≤5% false negative results. Target concentration should be intended as the threshold relevant for legislative requirements.
Example: For a 0.9% nominal value LOD <0.045%.

Robustness

Definition: The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.
The adequacy of the robustness testing needs to be demonstrated on a method-by-method basis. For instance, for a real-time PCR method, the following factors and their origin/source shall be taken into account: different thermal cycler models, DNA polymerase, uracyl-n-glycosylase, magnesium chloride

concentration, primer forward and reverse concentration, probe concentration, temperature profile, time profile, dNTP including dUTP concentrations.

Acceptance Criterion: The response of an assay with respect to these small changes shall not deviate more than ±30%.
Alternatively, robustness can be demonstrated through the application of formal robustness tests using factorial designs such as those published by Plackett Burman [1] or Yuden[2]

Method Performance Requirements

Precision - Relative Reproducibility Standard Deviation (RSDR)

Definition: The relative standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where test results are obtained with the same method, on identical test items, in different laboratories, with different operators, using different equipment. Reproducibility standard deviation describes the inter-laboratory variation.

Acceptance Criterion: The relative reproducibility standard deviation RSD_R should be <35% over the whole dynamic range. However, at concentrations <0.2% then RSD_R values <50% are deemed acceptable.

Trueness

Definition: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Acceptance Criterion: The trueness should be within ±25% of the accepted reference value over the whole dynamic range.

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1) R.L. Plackett and J.P. Burman, "The Design of Optimum Multifactorial Experiments", Biometrika 33 (4), pp. 305-25, June 1946.

2) Statistical Manual of the AOAC, W.J. Youdens and E.H. Steiner, 1987.