Event-specific Method for the Quantification of Maize MIR162 by Real-time PCR

Validation Report

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Joint Research Centre
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Executive Summary

The European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF), established by Regulation (EC) No 1829/2003, in collaboration with the European Network of GMO Laboratories (ENGL), has carried out a collaborative study to assess the performance of a quantitative event-specific method to detect and quantify the MIR162 transformation event (unique identifier SYN-IR162-4) in maize DNA. The collaborative 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 with Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Syngenta Seeds S.A.S. provided the detection method and the control samples (genomic DNA extracted from homogenised seeds containing the transformation event and from conventional homogenised seeds). The EURL-GMFF prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The collaborative study involved twelve laboratories from nine European countries.

The results of the international collaborative study met the ENGL performance requirements. The method is therefore considered applicable to the control samples provided, in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

The results of the collaborative study are made publicly available at http://gmo-crl.jrc.ec.europa.eu/.

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Report on Steps 1-3 of the Validation Process

Syngenta Seeds S.A.S. provided the detection method and the control samples for maize event MIR162 (unique identifier SYN-IR162-4) according to 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 Genetically Modified Food and Feed (EURL-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 internal procedures ("Description of the EURL-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 and listed in the "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). The scientific evaluation of the detection method for event MIR162 was positively concluded in January 2009.

In February-April 2009, the EURL-GMFF verified experimentally the method characteristics (step 3, experimental testing of samples and methods) by quantifying five GM levels within the range 0.1%-5.0% on a DNA/DNA ratio. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, trueness and precision were within the limits established by the ENGL. The DNA extraction method was previously tested on samples of food and feed and a report was published on the EURL-GMFF web site (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

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

Content

1. Introduction

Syngenta Seeds S.A.S. submitted the detection method and control samples for maize event MIR162 (unique identifier SYN-IR162-4) according to 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, established by Regulation (EC) No 1829/2003, organised the international collaborative study for the validation of the method of detection and quantification of maize MIR162. The study involved twelve laboratories from nine European countries among those listed in Annex II ("National reference laboratories assisting the CRL for testing and validation of methods for detection") of Commission Regulation (EC) No 1981/2006 of 22 December 2006.

Upon reception of method, samples and related data (step 1), the EURL-GMFF carried out the assessment of the documentation (step 2) and the in-house evaluation of the method (step 3) according to the requirements of Regulation (EC) No 641/2004.

The internal experimental evaluation of the method was carried out in February-April 2009.

Following the evaluation of the data and the results of the internal tests, the international collaborative study was organised (step 4) and took place in May 2009.

The collaborative study aimed at validating a quantitative real-time PCR (Polymerase Chain Reaction) method. The methodology is an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of MIR162 DNA to total maize DNA. The procedure is a simplex system, in which a maize *adh1* (alcohol dehydrogenase1) endogenous assay (targeting the taxon-specific *adh1* gene) and the target assay (MIR162) are performed in separate wells.

The international collaborative study was carried out in accordance with the following internationally accepted guidelines:

- ✓ ISO 5725 (1994).
- ✓ The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995).

2. Selection of participating laboratories

As part of the international collaborative study the method was tested in twelve laboratories to determine its performance.

On 16th April 2009 the EURL-GMFF invited all National Reference Laboratories nominated under Commission Regulation (EC) No 1981/2006 of 22 December 2006 and listed in Annex II ("National reference laboratories assisting the CRL for testing and validation of methods for

detection") of that Regulation to express the availability to participate in the validation study of the quantitative real-time PCR method for the detection and quantification of maize event MIR162.

Thirty-six laboratories expressed in writing their willingness to participate, while thirty-six did not express their interest. The EURL-GMFF carried out a random selection of twelve laboratories out of those that responded positively to the invitation, making use of a validated software application.

Clear guidance was given to the selected laboratories with regards to standard operational procedures to follow for the execution of the protocol. The participating laboratories are listed in Table 1.

Table 1. Laboratories participating in the validation of the detection method for maize MIR162.

Laboratory	Country
Crop Research Institute - Reference Laboratory for GMO Detection and DNA fingerprinting	CZ
E.N.S.E. - Seed Testing Station	IT
Genetically Modified Organism Controlling Laboratory	PL
Institute for Agricultural and Fisheries Research (ILVO)	BE
Institute for Consumer Protection, Department 3 - Food Safety	DE
National Diagnostic Centre of Food and Veterinary Service	LV
National Food Administration	SE
National Food and Veterinary Risk Assessment Institute	LT
National Health Laboratory, Food Control Department	LU
Office for Social Affairs, Health and Consumer Protection of the German Federal State Saarland	DE
Scientific Institute of Public Health (IPH)	BE
Veterinary Public Health Institute for Lazio and Toscana Regions; National Reference Centre for GMO Analysis	IT

3. Materials

For the validation of the quantitative event-specific method, samples consisted of:

i) genomic DNA extracted from maize seeds harbouring the event MIR162
ii) genomic DNA extracted from conventional maize seeds.

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 maize MIR162 and non-GM maize genomic DNA at different GM percentages were prepared by the EURL-GMFF, using the control samples provided, in a constant amount of total maize DNA.

Participants received the following materials:

- ✓ Five calibration samples (175 µL of DNA solution each) for the preparation of the standard curve, labelled from S1 to S5.
- ✓ Twenty unknown DNA samples (87.5 µL of DNA solution each), labelled from U1 to U20.
- ✓ Reaction reagents:
  - ❑ Sigma JumpStart Taq Ready mix 2x, 1 tube: 8.0 mL
  - ❑ Sulphorhodamine 1.5 mM 500 µL
  - ❑ Sterile distilled water: 5 mL

- ✓ Primers and probes (1 tube each) as follows:

adh1 taxon-specific assay

❑ Zm Adh1 primer F, one tube	(10 µM):	240 µL
❑ Zm Adh1 primer R, one tube	(10 µM):	240 µL
❑ Zm Adh1 probe, one tube	(10 µM):	160 µL

MIR162 specific assay

❑ MIR162–f1, one tube	(10 µM):	240 µL
❑ MIR162–r1, one tube	(10 µM):	240 µL
❑ MIR162–p1, one tube	(10 µM):	160 µL

4. Experimental design

Twenty unknown samples (labelled from U1 to U20), representing five GM levels, were used in the validation study (Table 2). On each PCR plate, the samples were analysed for the MIR162 specific system and the *adh1* taxon-specific system. In total, two plates were run per laboratory and four replicates for each GM level were analysed. PCR was performed in triplicate for all samples. Participating laboratories carried out the estimation of the GM% according to the instructions provided in the protocol and using the application provided.

Table 2. MIR162 GM contents

MIR162 GM% (GM copy number/maize genome copy number x 100)
0.10
0.40
0.90
2.00
5.00

4. Method

For the detection of event MIR162, a 92 bp fragment of the region spanning the 3' insert-to-plant junction is amplified. PCR products are measured at each cycle (real-time) by means of a specific oligonucleotide probe labelled with FAM dye (6-carboxyfluorescein) and TAMRA (carboxytetramethylrhodamine) as quencher dye.

For the relative quantification of GM event MIR162, a maize specific reference system amplifies a 135-bp fragment of the maize endogenous gene *adh1* (alcohol dehydrogenase 1, GenBank Accession No. AY691949), using two *adh1* gene-specific primers and an *adh1* probe labelled with VIC and TAMRA.

For relative quantification of event MIR162 DNA in an unknown sample, the normalised ΔCt values of the calibration samples are used to calculate, by linear regression, the parameters of a standard curve (plotting ΔCt values against the logarithm of the relative amount of event MIR162). The normalised ΔCt values of the unknown samples are measured and, by means of the regression formula, the relative amount of event MIR162 is estimated.

Calibration samples (S1-S5) were prepared by mixing the appropriate amount of MIR162 DNA from the stock solution with non-GM maize DNA to obtain the following five relative contents of MIR162: 8.0%, 4.0%, 1.0%, 0.5% and 0.08%. The total DNA amount per reaction was 250 ng, with 5 µL of a DNA solution at the concentration of 50 ng/µL for each reaction.

The MIR162 contents of the calibration samples and the total DNA quantity used in the PCR are provided in Table 3.

Table 3. % GMO copy number values of the standard curve samples.

Sample	S1	S2	S3	S4	S5
Total amount (ng) of DNA in reaction	250	250	250	250	250
GM% (GM copy number/maize genome copy number x 100)	8.00	4.00	1.00	0.50	0.08

6. Deviations reported

Eight laboratories reported no deviations from the protocol.

One laboratory did not take into account one of the triplicates of standard S3 (for the taxon reference system) in the analysis.

One laboratory did not take into account two Ct values of the taxon reference system in both plates (for standard S1 and S2 respectively).

One laboratory used the Stratagene Mx-3005P thermo cycler with an adaptive baseline for the Mx-3005 software.

One laboratory did not calibrate the ABI 7000 detection system with the sulphorhodamine but used ROX as passive reference dye in combination with VIC and FAM as reporter dyes. In this laboratory the first step of the PCR cycle (i.e. 2 minutes at 50.0°C) was not followed.

7. Results

PCR efficiency and linearity

The values of the slopes of the standard curve, from which the PCR efficiency is calculated using the formula [((10^(-1/slope))-1) x 100] and of the R^2 (expressing the linearity of the method) reported by participating laboratories are summarised in Table 4.

The mean PCR efficiency was 104%, and the R^2 of the method was 0.99. Data reported in Table 4 confirm the appropriate performance characteristics of the method in terms of efficiency and linearity.

Table 4. Values of slope, PCR efficiency and R^2

LAB	PLATE	Slope	PCR Efficiency (%)	Linearity (R^2)
1	**A**	-3.34	99	0.99
	B	-2.87	123	0.98
2	**A**	-3.07	112	1.00
	B	-3.45	95	1.00
3	**A**	-3.19	106	1.00
	B	-3.33	100	1.00
4	**A**	-3.37	98	1.00
	B	-3.39	97	1.00
5	**A**	-3.40	97	1.00
	B	-3.37	98	1.00
6	**A**	-3.35	99	1.00
	B	-3.20	105	1.00
7	**A**	-3.31	100	1.00
	B	-3.37	98	1.00
8	**A**	-3.30	101	1.00
	B	-3.11	110	1.00
9	**A**	-3.21	105	0.99
	B	-3.24	104	0.94
10	**A**	-3.09	110	1.00
	B	-3.19	106	1.00
11	**A**	-3.32	100	1.00
	B	-3.23	104	1.00
12	**A**	-3.16	107	0.99
	B	-3.01	115	1.00
	Mean	**-3.25**	**104**	**0.99**

GMO quantification

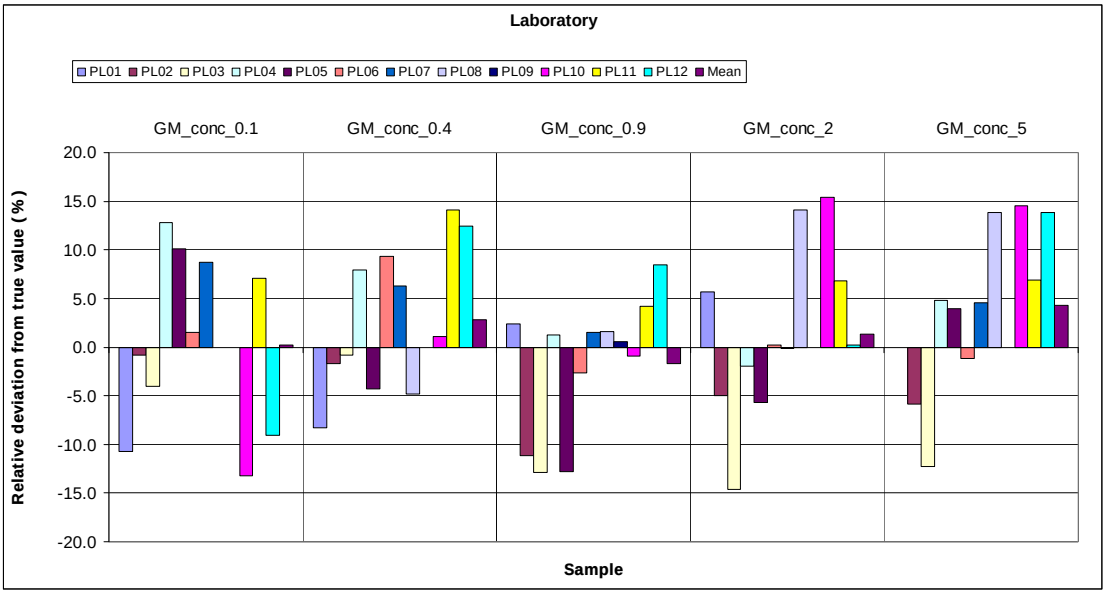
Table 5 reports the mean values of the four replicates for each GM level as estimated by all laboratories. Each mean value is the average of three PCR repetitions.

Table 5. GM% determined by laboratories in the unknown samples.

	GMO content GMO % = (GMO copy number / maize genome copy number) x 100																			
LAB	0.10				0.40				0.90				2.00				5.00			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.09	0.07	0.11	0.08	0.43	0.33	0.37	0.33	1.02	0.79	0.90	0.97	2.42	2.36	1.61	2.06	7.77	7.51	5.46	5.84
2	0.13	0.11	0.07	0.09	0.44	0.41	0.41	0.32	0.81	0.81	0.81	0.77	2.03	1.93	1.87	1.77	4.45	5.08	4.34	4.95
3	0.12	0.10	0.08	0.09	0.39	0.43	0.39	0.38	0.75	0.87	0.76	0.75	1.73	1.63	1.73	1.74	4.49	4.44	4.63	3.98
4	0.10	0.11	0.12	0.12	0.42	0.43	0.44	0.45	0.96	0.88	0.89	0.91	1.95	1.90	2.04	1.96	5.04	5.04	5.23	5.65
5	0.12	0.10	0.09	0.12	0.37	0.43	0.41	0.32	0.74	0.87	0.78	0.74	1.72	2.02	1.86	1.94	5.66	5.26	4.51	5.36
6	0.10	0.10	0.11	0.10	0.43	0.42	0.44	0.46	0.91	0.87	0.89	0.83	2.02	2.16	1.94	1.90	5.34	4.73	4.34	5.36
7	0.10	0.10	0.11	0.12	0.40	0.39	0.43	0.48	0.93	0.82	1.01	0.89	2.06	2.09	1.88	1.97	5.14	5.04	5.13	5.60
8	0.08	0.08	0.14	0.20	0.40	0.34	0.38	0.41	0.80	1.00	0.94	0.93	2.31	2.36	2.31	2.15	5.38	6.34	5.43	5.63
9	0.16	0.17	0.07	0.05	0.33	1.04	0.51	0.33	0.66	1.12	0.97	0.87	1.43	0.54	2.33	2.69	6.64	2.22	13.72	5.71
10	0.08	0.10	0.08	0.08	0.42	0.28	0.49	0.43	0.91	0.74	0.96	0.95	2.77	2.29	1.91	2.27	5.40	5.23	6.33	5.95
11	0.11	0.10	0.11	0.11	0.43	0.43	0.52	0.45	0.92	0.97	1.11	0.74	2.08	2.31	2.09	2.07	5.75	5.82	4.90	4.91
12	0.09	0.09	0.10	0.08	0.47	0.36	0.43	0.54	1.07	0.88	1.13	0.82	1.94	2.37	1.75	1.96	5.57	6.09	5.19	5.92

In Figure 1 the relative deviation from the true value for each GM level tested is shown for each laboratory, following removal of statistical outliers. The coloured bars represent the relative deviations obtained by the participating laboratories; the purple bar on the right hand side represents the overall relative deviation for each GM level.

Figure 1. Relative deviation (%) from the true value of MIR162 for all laboratories



The mean relative deviations from the true values are slightly positive for GM levels 0.4%, 2% and 5% and slightly negative for the 0.9% GM level, being however well within the acceptance

criterion of maximum 25%. The average bias generated by all laboratories is modest, being below 5% at all GM levels tested, indicating a satisfactory trueness of the method.

8. Method performance requirements

Among the performance criteria established by the ENGL and adopted by the EURL-GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm, see also Annex 1), repeatability and reproducibility are assessed through an international collaborative trial, carried out with the support of twelve European laboratories (see Table 1). Table 6 illustrates the estimation of repeatability and reproducibility at various GM levels, according to the range of GM percentages tested during the collaborative trial.

The relative reproducibility standard deviation (RSD_R), describing the inter-laboratory variation, should be below 33% over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range.

As it can be observed in Table 6, the method satisfies this requirement at all GM levels tested. In fact, the highest value of RSD_R (%) is 15% at the 0.1% GM level.

Table 6. MIR162: summary of validation results.

Sample	Target value (GMO%)				
	0.10	**0.4**	**0.9**	**2.0**	**5.0**
Laboratories having returned results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	2	1	0	1	2
Reason for exclusion	C	C	-	C	C
Mean value	0.10	0.41	0.88	2.03	5.22
Relative repeatability standard deviation, RSD_r (%)	13	12	12	10	8
Repeatability standard deviation	0.013	0.049	0.102	0.193	0.414
Relative reproducibility standard deviation, RSD_R (%)	15	13	12	12	11
Reproducibility standard deviation	0.015	0.052	0.108	0.242	0.569
Bias (absolute value)	0.000	0.011	-0.015	0.027	0.216
Bias (%)	0.2	2.9	-1.7	1.4	4.3

C = Cochran's test; identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2.

Bias is estimated according to ISO 5725.

Table 6 further documents the relative repeatability standard deviation (RSD_r), as estimated for each GM level. In order to accept methods for collaborative study, the EURL-GMFF requires that RSD_r value is below 25%, as indicated by ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

As it can be observed from the values reported in Table 6, the method shows a relative repeatability standard deviation below 25% over the dynamic range with a maximum of 13% at 0.1%.

The trueness of the method is estimated using the measures of the method bias for each GM level. According to ENGL method performance requirements, trueness should be ± 25% across the entire dynamic range. The method fully satisfies this requirement; in fact, the highest value of bias (%) is 4.3% at the 5% level, thus within the acceptance criterion.

9. Conclusions

The method performance has been evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (as detailed at http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). The method acceptance criteria were reported by the applicant and were used to evaluate the method prior to the international collaborative study (see Annex 1 for a summary of method acceptance criteria and method performance requirements).

The results obtained during the collaborative study indicate that the analytical module of the method submitted by the applicant complies with ENGL performance criteria.

The method is 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 EURL-GMFF operates according to ISO 9001:2000 (certificate number: 32231) and ISO 17025:2005 (certificate number: DAC-PL-0459-06-00) [DNA extraction, qualitative and quantitative PCR in the area of Biology (DNA extraction and PCR method validation for the detection and identification of GMOs in food and feed materials)].

11. References

1. Horwitz, W. (1995) Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem*, 67, 331-343.
2. International Standard (ISO) 5725. 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Swizerland.

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 a 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. Euro/sample) of the method.

Acceptance Criterion: The practicability statement should provide indication on the required equipment for the application of the method with regards to the analysis *per se* and the sample preparation. An indication of costs, timing, practical difficulties and any other factor that could be of importance for the operators should be indicated.

Specificity

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

Acceptance Criterion: The method should be event-specific and be functional only with the GMO or GM based product for which it was developed. This should be demonstrated by empirical results from testing the method with non-target transgenic events and non-transgenic material. This testing should include closely related events and cases where the limit of the detection is tested.

Dynamic Range

Definition: The range of concentrations over which the method performs in a linear manner with an acceptable level of accuracy 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 acceptable level of accuracy and precision are described below. The range of the standard curve(s) should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) end.

Accuracy

Definition: The closeness of agreement between a test result and the accepted reference value.

Acceptance Criterion: The accuracy should 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 = $[10^{(-1/slope)}] - 1$.

Acceptance Criterion: The average value of the slope of the standard curve should 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 should be ≥ 0.98.

Repeatability Standard Deviation (RSD_r)

Definition: The 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 below 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 (1994).

Limit of Quantitation (LOQ)

Definition: The limit of quantitation 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 $RSD_r \leq 25\%$. Target concentration should be intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below.

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 $\leq$ 5% false negative results. Target concentration should be intended as the threshold relevant for legislative requirements.

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.

Acceptance Criterion: The response of an assay with respect to these small variations should not deviate more than $\pm$ 30%. Examples of factors that a robustness test could address are: use of different instrument type, operator, brand of reagents, concentration of reagents, and temperature of reaction.

Method Performance Requirements

Dynamic Range

Definition: In the collaborative trial the dynamic range is the range of concentrations over which the reproducibility and the trueness of the method are evaluated with respect to the requirements specified below.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least five times the target concentration. Target concentration should be intended as the threshold relevant for legislative requirements.

Reproducibility Standard Deviation (RSD_R)

Definition: The 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 should be below 35% at the target concentration and over the entire dynamic range. An RSD_R < 50 % is acceptable for concentrations below 0.2%.

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 $\pm$ 25% of the accepted reference value over the whole dynamic range.