

# Report on the Verification of the Performance of MON 88017 and MON 810 Event-specific Methods on the Maize Event MON 88017 x MON 810 Using Real-Time PCR

15 July 2009

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

## Executive Summary

The JRC as Community Reference Laboratory for GM Food and Feed (CRL-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 maize event MON 88017 x MON 810 (unique identifier MON-88Ø17-3 x MON-ØØ810-6) which combines the MON 88017 and MON 810 transformation events. The two methods have been previously validated individually on single-trait event, to detect and quantify each event in maize samples; a validation report for each method is available at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>. This study was conducted according to internationally accepted guidelines <sup>(1, 2)</sup>.

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 Europe S.A. provided the detection methods and the control samples: whole seeds of MON 88017 x MON 810 maize (TPX151-DT, GLP-0409-15526-S) and whole conventional maize seeds of EXP258 (GLP-0409-15528-S). The JRC prepared the in-house verification samples (calibration samples and blind samples at different GM percentages).

The results of the in-house 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 CRL-GMFF in-house verification studies are made publicly available at <http://gmo-crl.jrc.ec.europa.eu/>.

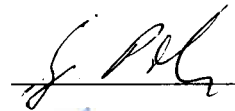
*Drafted by:*  
C. Delobel

  
\_\_\_\_\_

*Report Verification Team:*  
1) M. Ermolli

  
\_\_\_\_\_

2) G. Pinski

  
\_\_\_\_\_


*Scientific and technical approval:*  
M. Mazzara

  
\_\_\_\_\_

*Compliance with CRL Quality System:*  
S. Cordeil

  
\_\_\_\_\_

*Authorisation to publish:*  
G. Van den Eede

  
\_\_\_\_\_

**Address of contact laboratory:**

European Commission, Joint Research Centre (JRC)  
Institute for Health and Consumer Protection (IHCP)  
Molecular Biology and Genomics Unit – Community Reference Laboratory for Genetically  
Modified Food and Feed  
Via Fermi 2749, I-21027 Ispra (VA), Italy

## Report on Steps 1-3 of the Validation Process

Monsanto Europe S.A. submitted the detection methods and control samples of the maize event MON 88017 x MON 810 (unique identifier MON-88Ø17-3 x MON-ØØ810-6) under Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Community Reference Laboratory for GM Food and Feed (CRL-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 CRL-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/doc/Method%20requirements.pdf>) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, two scientific assessments were performed and requests of complementary information were addressed to the applicant. Upon reception of the complementary information, the scientific assessment of the detection method for the MON 88017 x MON 810 maize was concluded in March 2006.

The event-specific detection methods for the two maize lines hosting the single event MON 88017 and MON 810 were validated by the CRL-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 maize event MON 88017 x MON 810 did not undergo a full validation process. The CRL-GMFF performed an in-house verification of the detection methods to verify that they exhibit a comparable performance on samples of event MON 88017 x MON 810 combining the two traits (as provided in accordance to Annex 1.2.C.2 of Commission Regulation (EC) No 641/2004).

In February 2008, the CRL-GMFF concluded the experimental 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 0.09%-8%, on a DNA/DNA ratio. 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 European Network of GMO Laboratories (ENGL).

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

## Content

<b>1. INTRODUCTION.....</b>	<b>5</b>
<b>2. MATERIALS.....</b>	<b>6</b>
<b>3. EXPERIMENTAL DESIGN.....</b>	<b>6</b>
<b>4. METHOD.....</b>	<b>7</b>
DESCRIPTION OF THE OPERATIONAL STEPS.....	7
<b>5. DEVIATIONS REPORTED .....</b>	<b>7</b>
<b>6. SUMMARY OF RESULTS .....</b>	<b>7</b>
PCR EFFICIENCY AND LINEARITY .....	7
<b>7. METHOD PERFORMANCE REQUIREMENTS.....</b>	<b>8</b>
<b>8. COMPARISON OF METHOD PERFORMANCE BETWEEN EVENT MON 88017 X MON 810 AND THE SINGLE TRAIT EVENTS.....</b>	<b>10</b>
<b>9. CONCLUSIONS.....</b>	<b>11</b>
<b>10. QUALITY ASSURANCE .....</b>	<b>11</b>
<b>11. REFERENCES.....</b>	<b>11</b>
<b>12. ANNEX 1: METHOD ACCEPTANCE CRITERIA AND METHOD PERFORMANCE REQUIREMENTS AS SET BY THE EUROPEAN NETWORK OF GMO LABORATORIES (ENGL).....</b>	<b>12</b>

## 1. Introduction

The applicant Monsanto Europe S.A. submitted the detection methods for MON 88017 and MON 810 and the control samples of the maize event MON 88017 x MON 810 (unique identifier MON-88Ø17-3 x MON-ØØ810-6) under Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Joint Research Centre (JRC, Molecular Biology and Genomics Unit of the Institute of Health and Consumer Protection) as Community Reference Laboratory for GM Food and Feed (CRL-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 MON 88017 and MON 810 in the MON 88017 x MON 810 maize event combining the two traits. The single methods had been previously validated by international collaborative studies on the single-trait maize events (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>).

Upon reception of methods, samples and related data (step 1), the CRL-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 CRL-GMFF operational procedures. The CRL-GMFF method verification was concluded in February 2008.

A method for DNA extraction from maize seeds, submitted by the applicant, was evaluated by the CRL-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<sup>®</sup> PCR procedures for the determination of the relative content of events MON 88017 and MON 810 DNA to total maize DNA in the MON 88017 x MON 810 maize event. The procedures are simplex systems, in which the events MON 88017 and MON 810 are quantified in reference to the maize *hmg* (high mobility group) endogenous gene.

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

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

## 2. Materials

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

- seeds of MON 88017 x MON 810 maize (TPX151-DT, GLP-0409-15526-S),
- seeds of conventional maize EXP258 (GLP-0409-15528-S),

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% MON 88017 x MON 810 and conventional maize genomic DNA at different GMO concentrations were prepared by the CRL-GMFF in a constant amount of total maize 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 MON 88017 and MON 810 events and available at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>.

Table 1 shows the GM% used in the verification of the MON 88017 and MON 810 methods.

Table 1. MON 88017 and MON 810 GM contents in maize event MON 88017 x MON 810.

MON 88017 GM%	MON 810 GM%
(GM DNA / Non-GM DNA x 100)	(GM DNA / Non-GM DNA x 100)
0.09	0.10
0.50	0.50
0.90	1.00
5.00	2.00
8.00	5.00

## 3. Experimental design

Eight runs for each event-specific method were carried out. In each run, samples were analysed in parallel with the GM-specific system and the reference system (*hmg*). 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 (MON 88017 and MON 810), the quantification of the five GM levels was calculated as average of sixteen replicates per GM level.

## 4. Method

### *Description of the operational steps*

For specific detection of events MON 88017 and MON 810 in maize event MON 88017 x MON 810, two specific fragments of the integration regions of the constructs inserted into the plant genome, of 95 bp and 92 bp respectively, 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: 6-FAM is used for MON 88017 and MON 810 as reporter dye at its 5' end and TAMRA as a quencher dye at its 3' end.

For relative quantification of events MON 88017 and MON 810 DNA, a maize-specific reference system which amplifies a 79 bp fragment of the maize endogenous gene *hmg* (high mobility group), using two *hmg* gene-specific primers and an *hmg* gene-specific probe labelled with FAM and TAMRA, was used.

Standard curves are generated for each GM specific system (MON 88017 or MON 810), by plotting Ct values of the calibration standards against the logarithm of the DNA copy numbers MON 88017 or MON 810 DNA, and fitting a linear regression into these data. Thereafter, the Ct values of the blind samples are measured and, by means of the regression formula, the relative amount of event MON 88017 or MON 810 DNA is estimated respectively.

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 were introduced.

## 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  $[10^{(-1/\text{slope})}-1] \times 100$ , and of the  $R^2$  (expressing the linearity of the regression) reported for all PCR systems in the eight runs, are presented in Table 2 and 3 for MON 88017 and MON 810 methods, respectively.

Table 2. Values of standard curve slope, PCR efficiency and linearity ( $R^2$ ) for the MON 88017 method on event MON 88017 x MON 810.

Run	MON 88017			<i>hmg</i>		
	Slope	PCR Efficiency (%)	Linearity ( $R^2$ )	Slope	PCR Efficiency (%)	Linearity ( $R^2$ )
1	-3.48	94	0.99	-3.11	110	1.00
2	-3.41	97	1.00	-3.07	112	1.00
3	-3.66	87	0.99	-3.11	110	1.00
4	-3.44	95	0.99	-3.01	115	1.00
5	-3.39	97	1.00	-2.98	116	0.99
6	-3.63	89	0.99	-3.27	102	1.00
7	-3.63	89	1.00	-3.15	108	1.00
8	-3.70	86	0.99	-3.17	107	1.00
<b>Mean</b>	<b>-3.54</b>	<b>92</b>	<b>0.99</b>	<b>-3.11</b>	<b>110</b>	<b>1.00</b>

Table 3. Values of standard curve slope, PCR efficiency and linearity ( $R^2$ ) for the MON 810 method on event MON 88017 x MON 810.

Run	MON 810			<i>hmg</i>		
	Slope	PCR Efficiency (%)	Linearity ( $R^2$ )	Slope	PCR Efficiency (%)	Linearity ( $R^2$ )
1	-3.40	97	0.99	-3.44	95	1.00
2	-3.58	90	0.98	-3.54	92	1.00
3	-3.77	84	0.99	-3.58	90	1.00
4	-3.65	88	0.97	-3.53	92	1.00
5	-3.64	88	0.98	-3.43	96	0.99
6	-3.44	95	0.99	-3.48	94	0.99
7	-3.66	88	0.98	-3.48	94	1.00
8	-3.55	91	0.97	-3.57	91	1.00
<b>Mean</b>	<b>-3.58</b>	<b>90</b>	<b>0.98</b>	<b>-3.51</b>	<b>93</b>	<b>1.00</b>

The mean PCR efficiencies of the GM specific systems are 92% and 90% respectively for the MON 88017 and MON 810 specific systems. The linearity of the methods is equal to or above 0.98 for both systems. Overall, data reported in Table 2 and 3 confirm the appropriate performance characteristics of the two methods tested on MON 88017 x MON 810 maize samples in terms of PCR efficiency and linearity.

## 7. Method performance requirements

The results of the in-house verification study for the MON 88017 and MON 810 detection methods applied to event MON 88017 x MON 810 maize DNA are reported in Tables 4 and 5, respectively. Results were evaluated with respect to the method acceptance criteria and



performance requirements, as established by ENGL and adopted by the CRL-GMFF (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>, see also Annex 1). In addition, Tables 4 and 5 report estimates of the trueness and repeatability standard deviation for each GM level and for both methods.

Table 4. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD<sub>r</sub>, %) of the MON 88017 method on event MON 88017 x MON 810 maize DNA.

MON 88017					
Blind sample GM%	Expected value (GMO%)				
	0.09	0.5	0.9	5.0	8.0
Mean	0.09	0.44	0.99	4.60	7.97
SD	0.02	0.08	0.12	0.48	0.68
RSD <sub>r</sub> (%)	24.9	18.3	11.6	10.5	8.5
Bias (%)	2.6	-11.4	10.0	-8.0	-0.3

Table 5. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation of the MON 810 method on event MON 88017 x MON 810 maize DNA.

MON 810					
Blind sample GM%	Expected value (GMO%)				
	0.1	0.5	1.0	2.0	5.0
Mean	0.11	0.48	1.09	1.92	5.25
SD	0.02	0.07	0.07	0.23	0.60
RSD <sub>r</sub> (%)	18.8	15.5	6.3	12.2	11.5
Bias (%)	12.0	-3.9	9.4	-4.1	5.0

The *trueness* of the method is estimated using the measures 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 within  $\pm 25\%$  across the entire dynamic range. As shown in Tables 4 and 5, both methods satisfy the above requirement throughout their respective dynamic ranges.

Tables 4 and 5 further document the relative repeatability standard deviation (RSD<sub>r</sub>) as estimated for each GM level. In order to accept methods for collaborative trial, the CRL-GMFF requires that RSD<sub>r</sub> values are 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 Tables 4 and 5, the two methods satisfy this requirement throughout their respective dynamic ranges.

## 8. Comparison of method performance between event MON 88017 x MON 810 and the single trait events

An indicative comparison of the two method performances on the maize event MON 88017 x MON 810 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 MON 88017 detection method on event MON 88017 x MON 810 and on event MON 88017.

Trueness and repeatability of MON 88017 quantification on MON 88017 x MON 810			Trueness and repeatability of MON 88017 quantification on single event MON 88017*		
GM%	Bias (%)	$RSD_r$ (%)	GM%	Bias (%)	$RSD_r$ (%)
0.09	2.6	24.9	0.09	-2.6	28
0.5	-11.4	18.3	0.5	2.9	13
0.9	10.0	11.6	0.9	-9.6	19
5.0	-8.0	10.5	5.0	-4.8	19
8	-0.3	8.5	8	-7.6	18

\*method validated (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>)

Table 7. Trueness (bias %) and relative repeatability standard deviation ( $RSD_r$ , %) of the MON 810 detection method on event MON 88017 x MON 810 and on event MON 810.

Trueness and repeatability of MON 810 quantification on MON 88017 x MON 810			Trueness and repeatability of MON 810 quantification on single event MON 810*		
GM%	Bias (%)	$RSD_r$ (%)	GM%	Bias (%)	$RSD_r$ (%)
0.1	12.0	18.8	0.1	-14	19.1
0.5	-3.9	15.5	0.5	-9	22.2
1.0	9.4	6.3	1.0	-5	7.5
2.0	-4.1	12.2	2.0	-7	10.7
5.0	5.0	11.5	5.0	-8	11.1

\*method validated (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>)

For trueness, the MON 88017 even-specific method (Table 6), when applied to event MON 88017 x MON 810 and compared to the single line, shows a higher absolute bias (%) at 0.5% and 5% and a lower bias at 8%. The MON 810 event-specific method (Table 7), when applied to event MON 88017 x MON 810, shows a lower absolute bias (%) at all levels of GM, except for 1% level. The trueness is within the acceptance value set by ENGL ( $\pm 25\%$ ) over the whole dynamic range.

With the exception of the 0.5% GM level of the MON 88017 method and the 2.0% of the MON 810 method, both the MON 88017 and MON 810 (Table 6 and 7) event-specific methods show lower or equal values for relative repeatability standard deviation (RSD, %) when applied to the hybrid and compared to the single events. The results are in all cases within the ENGL acceptance level established at maximum 25%.

Therefore, the in-house method verification has demonstrated that the MON 88017 and MON 810 detection methods developed to detect and quantify the single events can be equally applied for the quantification of the respective events combined in event MON 88017 x MON 810.

## 9. Conclusions

The overall method performance of the two event-specific methods for the quantitative detection of events MON 88017 and MON 810 combined in maize event MON 88017 x MON 810 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 in this 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 section 2 "Materials"), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

## 10. Quality assurance

The CRL-GMFF carries out all operations according to ISO 9001:2000 (certificate number: CH-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.

## 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  $\pm 25\%$  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 =  $[10^{-(1/\text{slope})}] - 1$ .

Acceptance Criterion: The average value of the slope of the standard curve should be in the range of  $(- 3.1 \geq \text{slope} \geq - 3.6)$ .

#### ***R<sup>2</sup> Coefficient***

Definition: The R<sup>2</sup> coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: The average value of R<sup>2</sup> should be  $\geq 0.98$ .

#### ***Repeatability Standard Deviation (RSD<sub>r</sub>)***

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  $1/10^{\text{th}}$  of the value of the target concentration with an RSD<sub>r</sub>  $\leq 25\%$ . 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  $1/20^{\text{th}}$  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.