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GM Food and Feed

Event-specific Method for the Detection of Dried-killed Bacterial Biomass PT73 (TM) Derived from *E. coli* GM Strain AG3139 Using Real-time PCR

Validation Report

24 June 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, in collaboration with the European Network of GMO Laboratories (ENGL), has carried out a collaborative study to assess the performance of a qualitative event-specific method to detect the AG3139 transformation event in bacterial biomass derived from *E. coli* K-12 DNA. The collaborative trial was conducted according to internationally accepted guidelines [1, 2].

In accordance with 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", Ajinomoto Eurolysine S.A.S. provided the detection method and the samples (genomic DNA from *E. coli* K-12 harbouring the AG3139 event, bacterial biomass derived thereof, genomic DNA from the parental organism *E. coli* K-12 MG1655, and plasmids as positive and negative control samples). The JRC prepared the validation samples. The collaborative trial involved twelve laboratories from eight European countries.

The results of the international collaborative trial met the ENGL performance requirements (see Annex 1). The method is, therefore, considered applicable to the control and feed 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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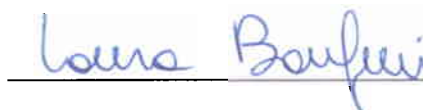

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Report on Steps 1-3 of the Validation Process

Ajinomoto Eurolysine S.A.S. submitted the detection method and control samples for dried killed bacterial biomass PT73 (TM) containing event AG3139 under Article 8 and 20 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 applicable 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). During step 2, a scientific assessment was performed for bacterial biomass PT73 (TM) and positively concluded in July 2008.

In July-September 2008, the CRL-GMFF experimentally verified the method characteristics (step 3, experimental testing of samples and methods) by assessing the method specificity, limit of detection (LOD) and dynamic range on feed sample on a weight basis and on the positive control sample on a genome copy number basis. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, LOD and specificity were within the limits established by the ENGL. The DNA extraction module of the method was tested on samples of feed and a report is published on the CRL-GMFF website (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

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

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1. Introduction

Ajinomoto Eurolysine S.A.S. submitted the detection method, control samples for *E. coli* K-12 GM event AG3139 (hereinafter referred to as 'AG3139') and samples of feed PT73 (TM) dried-killed bacterial biomass under Articles 8 and 20 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 for Health and Consumer Protection) as Community Reference Laboratory for GM Food and Feed (see Commission Regulation (EC) No 1829/2003) organised the international collaborative study for the event-specific method for the detection of event AG3139 in both the feed and the control samples. The study involved twelve laboratories 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 JRC carried out the assessment of the documentation (step 2) and the in-house evaluation of the method (step 3) according to the requirements of Commission Regulation (EC) No 641/2004 and following its operational procedures.

The internal experimental evaluation of the method was carried out between July and August 2008.

Following the evaluation of the data and the results of the internal laboratory tests, the international collaborative study was organised (step 4) and took place in October 2008.

A method for DNA extraction from PT73 (TM) biomass, 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/.

The operational procedure of the collaborative study included the following modules:

- DNA extraction procedure to extract DNA from dried killed bacterial biomass of *E. coli* K-12 event AG3139. A report on the method testing is available at http://gmo-crl.jrc.ec.europa.eu/.

- Qualitative real-time PCR (Polymerase Chain Reaction). The method is an event-specific real-time qualitative TaqMan® PCR procedure for the detection of event AG3139 DNA.

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. List of participating laboratories

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

In September 2008 the CRL-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 traceability real-time PCR method for the detection and identification of event AG3139 in *E. coli* K-12 AG3139 and PT73 (TM) dried killed bacterial biomass derived thereof.

Eighteen laboratories expressed in writing their willingness to participate, two declined the invitation, while fifty-two did not answer. The CRL-GMFF performed 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 the standard operational procedures to follow for the execution of the protocol. The participating laboratories are listed alphabetically in Table 1.

Table 1. Laboratories participating in the validation of the detection method for *E. coli* K-12 event AG3139.

Laboratory	Country
E.N.S.E. - Seed Testing Station	IT
Genetically Modified Organism Controlling Laboratory	PL
Institute for Agricultural and Fisheries Research (ILVO)	BE
Institute for Hygiene and Environment	DE
Institute for Consumer Protection, Department 3 - Food Safety	DE
Laboratory of DNA analysis, Department of Gene Technology (GT), Tallinn University of Technology (TUT)	EE
Lower Saxony Federal State Office for Consumer Protection and Food Safety, State Food Laboratory Braunschweig	DE
National Centre for Food, Spanish Food Safety Agency	ES
National Diagnostic Centre of Food and Veterinary Service	LV
National Institute of Biology	SI
Scientific Institute of Public Health (IPH)	BE
Walloon Agricultural Research Centre (CRA-W) - Department Quality of Agricultural Products	BE

3. Materials

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

i) dried killed bacterial biomass PT73 (TM) made of cells of bacterial strain AG3139,
and
ii) genomic DNA extracted from *E. coli* K-12 event AG3139

were provided by the applicant in accordance to the provisions of Commission 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)].

The control sample consisting of genomic DNA from bacterial strain AG3139 at the starting concentration of 26214 copies/μL was prepared by the CRL-GMFF. Samples of the dilution series were prepared by the participant laboratories using DNA stock solutions extracted from *E. coli* K-12 event AG3139 and the PT73 (TM) biomass.

Participants received the following materials:

- ✓ PT73 (TM) biomass (2 grams of finely ground powder).
- ✓ Internal Positive Control (200 µL of DNA solution at 10 ng/µL) extracted from the PT73 (TM) biomass.
- ✓ Genomic DNA of strain AG3139 (310 µL of DNA solution at 26214 copies/µL).
- ✓ Reaction reagents as follows:
  - universal PCR Master Mix (2x), two bottles: 5 mL each
  - distilled sterile water, one tube: 15 mL

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

  TMD system
  - *TMD*-F (10 µM): 715 µL
  - *TMD*-R (10 µM): 715 µL
  - *TMD*-P (5 µM): 420 µL

4. Experimental design

The method module focusing on the testing of the qualitative real-time PCR was divided in two phases to assess the method performance in the detection of event AG3139 respectively in the feed sample PT73 (TM) and in the positive control sample *E. coli* K-12 event AG3139.

4.1. Accuracy rate and detection range of the TMD system for detection of event AG3139 in bacterial biomass PT73 (TM)

In the first phase of the collaborative study, four independent DNA extractions have been performed by each laboratory on the feed sample, the bacterial biomass PT73 (TM).

Laboratories were requested to estimate the DNA concentration by fluorometric means with the Picogreen kit provided (details in the report of DNA extraction at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). Four independent dilution series were built by the laboratories starting from each of the DNA extracts adjusted to a starting concentration of 10 ng/μL (100 ng of total DNA per reaction).

Twelve samples (labelled from E1 to E12) were prepared for each dilution series (Table 2) for a total of four dilution series. On each PCR plate, the samples were analysed for detection of the AG3139 event by the *TMD* specific system; two dilution series were loaded per PCR plate, with each dilution point prepared in triplicate. Two plates were run per participating laboratory; in total, four replicates per dilution point were analysed. On each plate, the no template control, the DNA extraction control and an internal positive control provided by the CRL-GMFF (at 10 ng/μL of DNA extracted from the bacterial biomass) were analysed in triplicate. Participating laboratories were requested to set the same baseline and thresholds in both plates. Raw data were sent to the CRL-GMFF according to the instructions provided in the protocol and using the electronic tool provided (Excel spreadsheet).

Table 2. DNA content of the samples analysed for each dilutions series

Sample Name	DNA content	Dilution
E1	100 ng	-
E2	25 ng	1:4
E3	6.25 ng	1:4
E4	1.56 ng	1:4
E5	390 pg	1:4
E6	100 pg	1:4
E7	24 pg	1:4
E8	6.10 pg	1:4
E9	1.52 pg	1:4
E10	380 fg	1:4
E11	95 fg	1:4
E12	24 fg	1:4

4.2. Accuracy rate and dynamic range of the TMD system for detection of event AG3139 in *E. coli* K-12 event AG3139

In the second phase of the collaborative study, laboratories tested the method for detecting event AG3139 in DNA purified from bacterial strain *E. coli* K-12 event AG3139. The DNA stock solution was delivered to the participating laboratories at a DNA content of 26214.4 DNA copies/μL.

Twelve samples of a dilution series (labelled from S1 to S12) were built for each series (Table 3) for a total of four dilution series. On each PCR plate, the samples were analysed for event AG3139 with the TMD specific system; two dilution series were loaded per PCR plate, with each dilution point prepared in triplicate. Two plates were run per participating laboratory; in total, four replicates per dilution point were analysed. On each plate, the no template control was analysed in triplicate. Participating laboratories were requested to set the same baseline and thresholds in both plates. Raw data were provided to the CRL-GMFF according to the instructions provided in the protocol and using the electronic tool provided (Excel spreadsheet).

Table 3. DNA contents of the samples analysed for each dilution series

Sample Name	DNA copies	Dilution
S1	262144	-
S2	65536	1:4
S3	16384	1:4
S4	4096	1:4
S5	1024	1:4
S6	256	1:4
S7	64	1:4
S8	16	1:4
S9	8	1:2
S10	4	1:2
S11	1	1:4
S12	0.1	1:10

5. Method

Description of operational steps followed

For the specific detection of event AG3139 genomic DNA, a 90 bp fragment of the region that spans the 5' bacterial-to-insert junction in AG3139 event is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM as reporter dye at its 5' end and TAMRA as quencher dye at its 3' end (*TMD* system).

Standard curves are generated for the *TMD* specific system by plotting the Ct values measured for the calibration points against the logarithm of the DNA amount expressed in weight [feed sample, PT73 (TM)] or in copy numbers (positive control sample AG3139) and by fitting a regression line into these data. Thereafter, the standard curves are used to estimate the extension of the dynamic range.

The *TMD* assay utilises a qualitative approach for detecting of the target sequence over the dynamic range. This is determined through a standard curve made of serially diluted samples. In each real-time PCR run, the Ct values are determined for all the samples in triplicate for the target GM specific- sequence.

The absolute copy numbers in the positive control sample S1 (pure DNA from *E coli* K-12 event AG3139) was determined by considering that an amount of 2.2 ng corresponds to 417,360 DNA copies of *E. coli* K-12 event AG3139 (source: applicant's dossier). The determination of DNA concentration of the AG3139 DNA stock was made on the basis of 10 readings by fluorometric method with the Picogreen kit.

6. Deviations reported

Eight laboratories reported no deviations from the protocol.

One laboratory did not follow the indicated loading order for the "E" dilution series but introduced the correct Ct values for the corresponding samples in the excel spreadsheet for data analysis.

One laboratory had to re-run one series of dilution "E" and one series of dilution "S".

One laboratory re-run plates A and B due to an error in the cycling program.

One laboratory re-calculated reagents concentration to allocate a 20 µL volume in the wells of a 384 well plate with no consequences on the E dilution series but with a total DNA copy number of 209,712 instead of 262,140 in the S1 samples. The values for the slope of the regression line, efficiency and linearity were recalculated by the CRL-GMFF taking into account this deviation.

One laboratory measured the DNA concentration of the DNA extracts from the feed sample PT73 (TM) via spectrophotometric instead of fluorometric means. This was a major protocol violation. The laboratory overestimated consistently the DNA content in the extracts compared to the remaining eleven laboratories (Report on DNA extraction method at http://gmo-crl.jrc.ec.europa.eu/); consequently, data from the dilution series E_A to E_D were not computed in data analysis for this laboratory.

7. Summary of results

7.1. Performance of the detection method on bacterial biomass PT73 (TM) containing *E. coli* K-12 event AG3139

Detection accuracy rate

Table 4 illustrates the average Ct value corresponding to each of the 12 levels (samples E1 to E12) across the four dilution series (A to D); DNA content in reaction per dilution level is indicated.

Within each dilution series, the detection and Ct value of any given sample resulted from the average of three replicates. The sample was scored as 'positive' if at least two out of three replicate reactions showed amplification. Similarly, the sample was scored as "not detected" if at least two out of the three replicate reactions did not result in detectable amplification.

Table 4. Samples of the dilution series E, average Ct* values across series per laboratory and accuracy rate

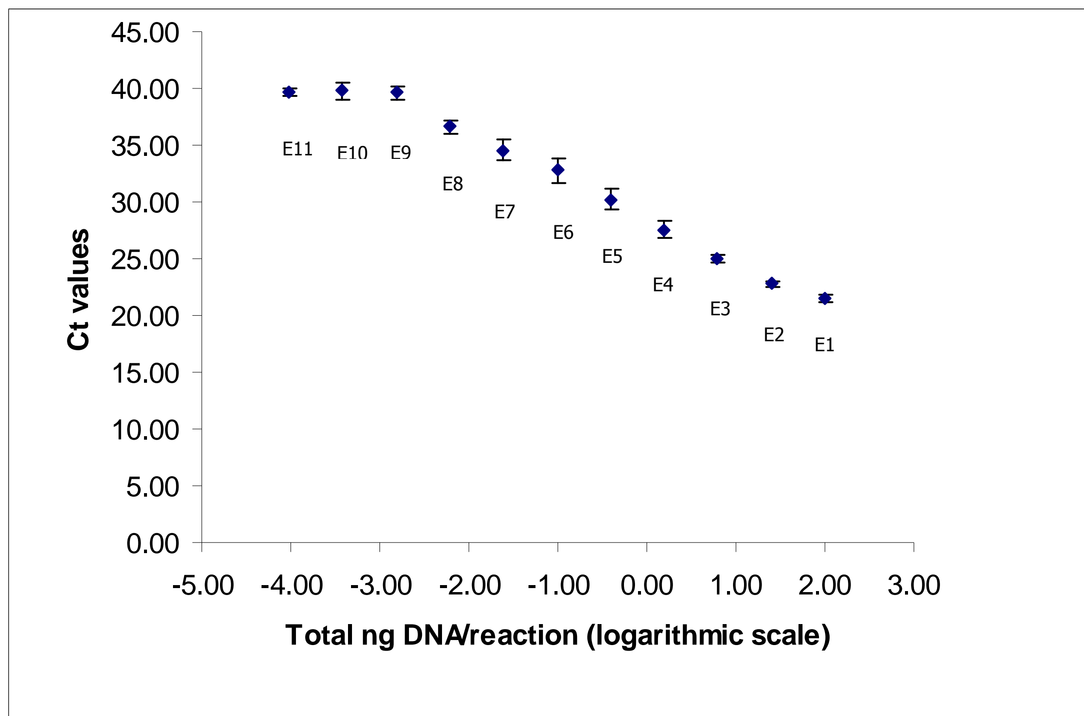
Sample	Total DNA (weight) / reaction	Lab 01	Lab 02	Lab 03	Lab 04	Lab 05	Lab 06	Lab 07	Lab 08	Lab 09	Lab 10	Lab 11	Accuracy rate
E1	100 (ng)	20.99	21.28	23.78	21.12	21.50	21.88	21.71	19.83	20.28	22.51	21.06	100
E2	25 (ng)	23.01	23.10	22.85	22.80	22.18	23.94	23.47	21.55	21.57	23.36	22.71	100
E3	6.25 (ng)	25.93	24.99	25.10	24.74	24.04	27.99	25.38	23.61	23.56	25.11	24.67	100
E4	1.56 (ng)	29.40	27.04	27.44	26.77	26.08	34.01	27.46	25.55	25.65	27.11	26.76	100
E5	390 (pg)	33.30	29.36	32.12	28.70	28.26	37.72	29.58	27.47	27.92	29.50	28.67	97.73
E6	100 (pg)	38.43	31.63	34.93	30.86	30.43	40.21	31.81	29.83	30.06	31.97	30.62	95.45
E7	24.4 (pg)	40.27	35.30	37.95	32.85	33.30	n.d.	34.45	31.88	32.17	34.68	32.90	88.64
E8	6.10 (pg)	n.d.	38.96	39.08	35.04	37.12	n.d.	37.53	34.60	34.62	37.36	35.31	77.27
E9	1.52 (pg)	n.d.	39.97	42.01	37.95	39.99	n.d.	41.72	38.51	36.78	41.43	38.09	59.09
E10	380 (fg)	n.d.	n.d.	n.d.	41.52	n.d.	n.d.	n.d.	38.30	40.44	n.d.	38.95	18.18
E11	95 (fg)	n.d.	n.d.	n.d.	38.84	n.d.	n.d.	n.d.	39.21	40.38	n.d.	40.19	11.36
E12	24 (fg)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

* For each laboratory values are the average Ct of the replicates of the same dilution level across the four dilution series

The detection accuracy rate, calculated as percent of detection across the four replicates of each level of the dilution series (44 data per dilution level) is shown in Table 4. At 100 pg level of total DNA in reaction more than 95% of the replicates resulted in detectable amplification (sample E6). This value dropped steadily as the total PT73 (TM) DNA in reaction decreased with further dilution.

The average Ct value per DNA level was influenced by the starting DNA amount in sample E1, this being affected by the accuracy of DNA quantification of the DNA extracts from PT73 (TM) bacterial biomass and by the threshold settings performed by each laboratory; therefore the average Ct values reported should be taken as 'indicative'. Figure 1 shows the distribution of Ct figures associated to the levels of the dilution series.

Figure 1. Distribution of the means of Ct values per laboratory over the dilution series.



Black diamonds represent the average of the laboratories Ct means. Bars indicate standard deviation. Number of available data set per each dilution point can be retrieved from Table 4. The last point (E12) of the dilution series has been omitted for clarity of representation.

The average of the laboratories Ct means at the lower end of the dilution series (E9 to E11) are very similar to each other and close to 40 and the associated variability around the mean is very narrow due to the small number of data sets available.

Efficiency and linearity of the method

The efficiency and linearity of the *TMD* system on the PT73 (TM) bacterial biomass were therefore assessed for all laboratories over the first six DNA levels (sample E1 to E6); the slopes and linearity of the PCR reactions was calculated thereof.

Table 5 illustrates the values of the slopes, from which the PCR efficiency is calculated using the formula ((10^(-1/slope))-1) x 100), of the reference curve and of the R^2 (expressing the linearity of the regression) reported by participating laboratories for the *TMD* system.

Table 5. Values of slope, PCR efficiency and linearity (R^2)

Laboratory	Dilution series	Slope	PCR Efficiency (%)	R^2
Lab 01	EA	-5.8	49	0.965
Lab 01	EB	-5.6	51	0.984
Lab 01	EC	-5.6	51	0.940
Lab 01	ED	-6.0	46	0.974
Lab 02	EA	-3.4	96	0.997
Lab 02	EB	-3.4	97	0.996
Lab 02	EC	-3.4	95	0.995
Lab 02	ED	-3.5	92	0.989
Lab 03	EA	-4.1	74	0.923
Lab 03	EB	-3.9	79	0.878
Lab 03	EC	-4.1	74	0.872
Lab 03	ED	-4.2	72	0.883

Laboratory	Dilution series	Slope	PCR Efficiency (%)	R^2
Lab 04	EA	-3.3	103	0.997
Lab 04	EB	-3.3	102	0.997
Lab 04	EC	-3.2	103	0.997
Lab 04	ED	-3.2	104	0.998
Lab 05	EA	-3.1	110	0.978
Lab 05	EB	-3.2	106	0.984
Lab 05	EC	-2.9	120	0.977
Lab 05	ED	-3.1	110	0.981
Lab 06	EA	-6.5	43	0.960
Lab 06	EB	-5.9	47	0.970
Lab 06	EC	-8.6	31	0.960
Lab 06	ED	-7.3	37	0.904
Lab 07	EA	-3.4	97	0.993
Lab 07	EB	-3.4	96	0.996
Lab 07	EC	-3.3	100	0.996
Lab 07	ED	-3.3	99	0.998
Lab 08	EA	-3.3	99	0.985
Lab 08	EB	-3.3	99	0.992
Lab 08	EC	-3.2	103	0.990
Lab 08	ED	-3.3	101	0.992
Lab 09	EA	-3.3	102	0.991
Lab 09	EB	-3.4	95	0.994
Lab 09	EC	-3.3	100	0.993
Lab 09	ED	-3.3	103	0.994
Lab 10	EA	-3.4	95	0.979
Lab 10	EB	-3.0	114	0.966
Lab 10	EC	-3.4	98	0.979
Lab 10	ED	-3.0	115	0.982
Lab 11	EA	-3.0	114	0.996
Lab 11	EB	-3.1	109	0.995
Lab 11	EC	-3.2	104	0.996
Lab 11	ED	-3.5	95	0.998

With the exception of two laboratories the efficiencies calculated from the corresponding slopes ranged from more than 70% to around 120%. In most cases, the slopes of the regression lines for detection of AG3139 event in PT73 (TM) biomass extended from -3.0 to -4.2.

7.2. Assessment of method performance requirements for detection of event AG3139 in bacterial biomass PT73 (TM)

Further to the identification and removal of outlier laboratories according to ISO 5725-2, through Cochran and Grubbs tests based on the slope dataset, the mean value of the slope of the regression lines of the method of detection of event AG3139 in PT73 (TM) bacterial biomass (in the range from 100 ng to 100 pg of total DNA) was -3.37 with a relative repeatability standard deviation (RSDr) of 3.5% and a relative reproducibility standard deviation (RSDR) of 9.4% (Table 6).

Table 6. Detection of AG3139 in PT73 (TM) bacterial biomass: summary of validation results for the slope

Laboratories having returned valid results	11
Samples per laboratory	4
Number of outliers	2
Reason for exclusion	1 C, 1G
Mean value of the slope	-3.37
Relative repeatability standard deviation, RSD_r (%)	3.5
Repeatability standard deviation	0.117
Relative reproducibility standard deviation, RSD_R (%)	9.4
Reproducibility standard deviation	0.317

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

The PCR efficiency and the coefficient of determination, giving method linearity (R^2), were subsequently analysed (Table 7) and evaluated according to the limits set by the ENGL document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

The reaction efficiency was 99% with a limited variability (RSDr % of 5.25 and RSD_R of 11%), thus within the ENGL requirements; the method linearity was 0.97, just below the lower limit of 0.98 indicated by the ENGL minimum acceptance criteria.

Table 7. Mean values, RSDr and RSDR for PCR efficiency and coefficient of determination

	Efficiency	**R^2**
Number of data	36	36
Mean value	99	0.97
Relative repeatability standard deviation, RSD_r (%)	5.2	1.5
Repeatability standard deviation	5.149	0.014
Relative reproducibility standard deviation, RSD_R (%)	11	6.3
Reproducibility standard deviation	11.205	0.061

When the two outlying laboratories were excluded from the computation of the accuracy rate, this showed a far better performance (compare to Table 4), with the remaining laboratories detecting the target at a total DNA amount per reaction down to 6.1 pg (Table 8), close to 95% of the times (94.4%).

Table 8. Samples of the dilution series E, average Ct* across series per laboratory and accuracy after identification and removal of outlying laboratories through Cochran and Grubbs tests, according to ISO 5725-2

Sample	Total DNA (weight) / reaction	Lab 02	Lab 03	Lab 04	Lab 05	Lab 07	Lab 08	Lab 09	Lab 10	Lab 11	Accuracy rate
E1	100 (ng)	21.28	23.78	21.12	21.50	21.71	19.83	20.28	22.51	21.06	100
E2	25 (ng)	23.10	22.85	22.80	22.18	23.47	21.55	21.57	23.36	22.71	100
E3	6.25 (ng)	24.99	25.10	24.74	24.04	25.38	23.61	23.56	25.11	24.67	100
E4	1.56 (ng)	27.04	27.44	26.77	26.08	27.46	25.55	25.65	27.11	26.76	100
E5	390 (pg)	29.36	32.12	28.70	28.26	29.58	27.47	27.92	29.50	28.67	100
E6	100 (pg)	31.63	34.93	30.86	30.43	31.81	29.83	30.06	31.97	30.62	100
E7	24.4 (pg)	35.30	37.95	32.85	33.30	34.45	31.88	32.17	34.68	32.90	100
E8	6.10 (pg)	38.96	39.08	35.04	37.12	37.53	34.60	34.62	37.36	35.31	94.44
E9	1.52 (pg)	39.97	42.01	37.95	39.99	41.72	38.51	36.78	41.43	38.09	72.22
E10	380 (fg)	n.d.	n.d.	41.52	n.d.	n.d.	38.30	40.44	n.d.	38.95	22.22
E11	95 (fg)	n.d.	n.d.	38.84	n.d.	n.d.	39.21	40.38	n.d.	40.19	13.89
E12	24 (fg)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0

* For each laboratory values are the average Ct of the replicates of the same dilution level across the four dilution series

Overall, the data reported confirm the appropriate performance characteristics of the method proposed to detect *E coli* K-12 event AG3139 in feed material the dried killed bacterial biomass PT73 (TM).

7.3. Performance of the detection method on DNA from control sample *E. coli* K-12 event AG3139

Detection accuracy rate

Table 9 illustrates the average Ct value corresponding to each of the 12 levels (samples S1 to S12) across the four dilution series (A to D); the DNA content in copy number per dilution level is indicated.

Within each dilution series, the Ct value of any given sample resulted from the average of three replicates. At least two out of three replicate reactions needed to show amplification for scoring the sample as 'positive'. Similarly, whenever at least two out of the three replicate reactions did not result in detectable amplification, the result was considered negative, i.e. sample not detected (n.d.).

Table 9. Samples of the dilution series S, average Ct* across series per laboratory and accuracy rate

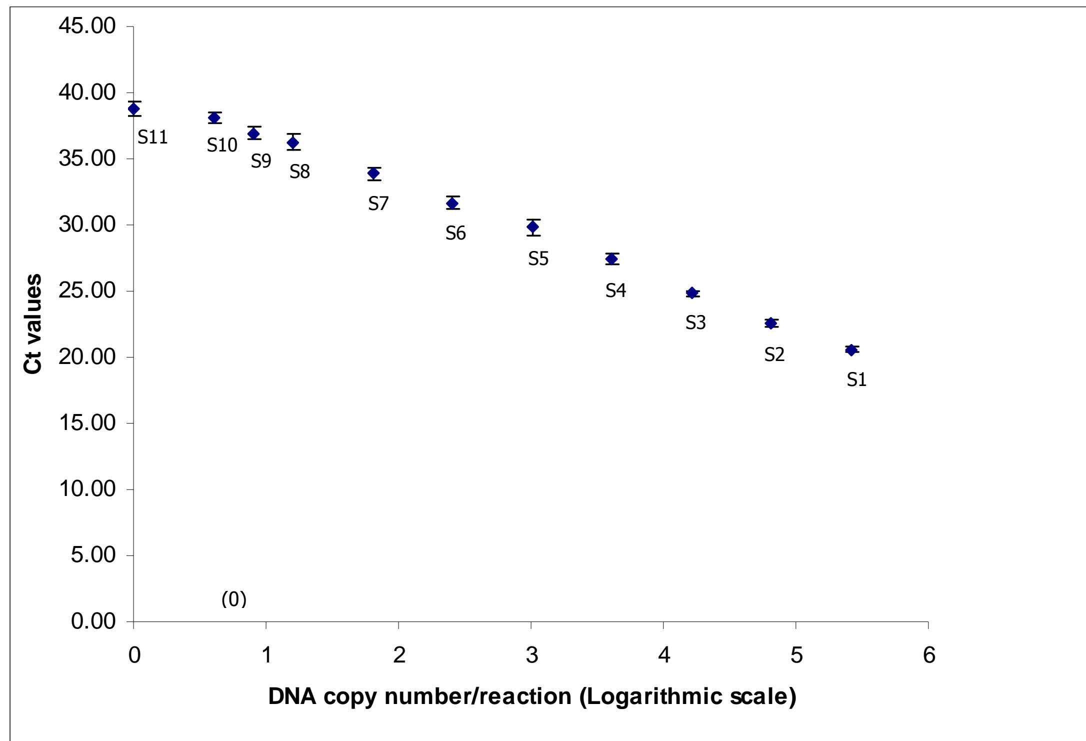
Sample	DNA copies/ reaction	Lab 01	Lab 02	Lab 03	Lab 04	Lab 05	Lab 06	Lab 07	Lab 08	Lab 09	Lab 10	Lab 11	Lab 12	Accuracy rate
S1	262144	21.09	20.81	20.99	20.12	20.36	19.19	20.71	19.72	19.60	21.83	20.77	21.66	100.00
S2	65536	23.04	22.84	23.88	22.09	22.00	21.26	22.66	21.55	21.60	23.98	22.75	23.65	100.00
S3	16384	25.47	24.87	25.81	24.06	24.05	24.76	24.64	23.53	23.74	26.14	24.76	25.79	100.00
S4	4096	28.37	27.11	28.47	26.13	26.12	31.00	26.80	25.66	25.98	28.27	26.92	28.01	100.00
S5	1024	30.72	29.37	30.90	28.10	28.27	35.01	28.93	27.68	27.89	30.48	29.00	31.29	100.00
S6	256	33.07	31.69	34.17	30.09	30.98	31.60	30.76	29.99	29.94	32.76	30.89	34.11	93.75
S7	64	35.02	34.06	36.40	32.06	32.62	34.36	32.80	32.25	32.24	35.04	33.04	36.86	93.75
S8	16	37.10	36.66	39.36	34.02	34.86	n.d.	35.04	34.34	34.66	37.11	35.31	40.18	89.58
S9	8	38.08	37.80	35.42	35.30	36.15	n.d.	37.45	35.30	35.90	38.09	36.65	40.01	81.25
S10	4	38.79	38.60	39.54	36.17	37.28	n.d.	n.d.	36.94	36.64	39.54	37.54	39.88	68.75
S11	1	40.39	n.d.	n.d.	37.79	n.d.	n.d.	n.d.	37.31	38.03	40.84	38.38	n.d.	25.00
S12	0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

* For each laboratory values are the average of the replicates of the same level across the four dilution series

The accuracy rate, calculated as percent detection across the four samples of each level of the dilution series (48 data per dilution level) is shown in Table 9. The accuracy rate was 100% down to 1024 DNA copies per reaction; it slightly lowered to almost 94% at 64 DNA copies (sample S7). This value dropped steadily as the total amount of AG3139 DNA in reaction decreased with further dilution.

Figure 2 shows the distribution of Ct figures associated to the levels of the dilution series. The variability expressed in this study was overall modest.

Figure 2. Distribution of the means of Ct values over the dilution series.



Blue diamonds represent the average of the laboratories Ct means. Bars indicate standard deviation. The number of available data set for each dilution point can be retrieved from Table 9. The last point (S12) of the dilution series was omitted for clarity of representation.

Efficiency and linearity of the method

The dynamic range of the *TMD* system on the *E. coli* K-12 event AG3139 positive control sample was assessed for all laboratories over the first five DNA levels (sample S1 to S5); the slopes and linearity of the PCR reactions were calculated thereof.

Table 10 illustrates the values of the slopes of the reference 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 regression) reported by participating laboratories for the *TMD* system.

Table 10. Values of linear dilution slope, PCR efficiency and linearity (R^2)

Laboratory	Dilution series	Slope	PCR Efficiency (%)	R^2
Lab 01	SA	-3.8	82	0.984
Lab 01	SB	-4.0	78	0.992
Lab 01	SC	-4.3	70	0.957
Lab 01	SD	-4.2	73	0.985
Lab 02	SA	-3.7	88	0.998
Lab 02	SB	-3.5	92	0.998
Lab 02	SC	-3.5	92	0.998
Lab 02	SD	-3.5	93	0.999
Lab 03	SA	-3.9	82	0.969
Lab 03	SB	-4.3	72	0.970
Lab 03	SC	-3.9	80	0.975
Lab 03	SD	-4.2	74	0.972
Lab 04	SA	-3.3	102	0.998
Lab 04	SB	-3.3	99	0.999
Lab 04	SC	-3.3	99	0.998
Lab 04	SD	-3.3	101	0.999
Lab 05	SA	-3.4	96	0.998
Lab 05	SB	-3.4	96	0.998
Lab 05	SC	-3.4	95	0.998
Lab 05	SD	-3.4	95	0.994
Lab 06	SA	-8.4	32	0.975
Lab 06	SB	-4.7	63	0.984
Lab 06	SC	-6.6	42	0.946
Lab 06	SD	-7.6	35	0.936
Lab 07	SA	-3.4	98	0.999
Lab 07	SB	-3.4	97	0.995
Lab 07	SC	-3.5	94	0.999
Lab 07	SD	-3.4	95	0.994
Lab 08	SA	-3.4	98	0.997
Lab 08	SB	-3.3	100	0.997
Lab 08	SC	-3.4	98	0.997
Lab 08	SD	-3.2	103	0.993
Lab 09	SA	-3.5	92	0.999
Lab 09	SB	-3.5	94	0.999
Lab 09	SC	-3.5	92	0.998
Lab 09	SD	-3.4	98	0.997
Lab 10	SA	-3.6	88	0.998
Lab 10	SB	-3.5	94	0.999
Lab 10	SC	-3.7	85	0.998
Lab 10	SD	-3.5	93	0.999
Lab 11	SA	-3.4	96	0.996
Lab 11	SB	-3.4	97	0.999
Lab 11	SC	-3.4	97	0.998
Lab 11	SD	-3.5	93	0.999
Lab 12	SA	-3.9	81	0.990
Lab 12	SB	-3.9	80	0.982
Lab 12	SC	-4.1	75	0.978
Lab 12	SD	-3.8	84	0.982

With the exception of one laboratory, the efficiencies calculated from the corresponding slopes ranged between 70% to around 105%. Overall, more than 90% of the data showed that the slopes of the regression lines, built to estimate the dynamic range of the *TMD* system for the detection of AG3139 event in *E. coli* K-12, extended from -3.2 to -4.3.

7.4. Assessment of method performance requirements for detection of event AG3139 in *E. coli* K-12 event AG3139

Further to the identification and removal of outlier laboratories according to ISO 5725-2, through Cochran and Grubbs tests based on the slope dataset, the mean value of the slope of the regression lines of the method for detection of event AG3139 on DNA from *E. coli* K-12 event AG3139 (in the range from 262,144 to 1024 DNA copies) was -3.6 with a relative repeatability standard deviation (RSDr) of -3.2% and a relative reproducibility standard deviation (RSD_R) of 8.4% (Table 11).

Table 11. Detection of AG3139 event in AG3139 positive control sample: summary of validation results for the slope

Laboratories having returned valid results	12
Samples per laboratory	4
Number of outliers	1
Reason for exclusion	1 C
Mean value	-3.6
Relative repeatability standard deviation, RSD_r (%)	3.2
Repeatability standard deviation	0.115
Relative reproducibility standard deviation, RSD_R (%)	8.4
Reproducibility standard deviation	0.301

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

The PCR efficiency and coefficient of determination (R^2) were subsequently analysed (Table 12) and evaluated according to the limits set by the ENGL document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

The reaction efficiency was 91% with a limited variability (RSDr % of 3.6 and RSD_R of 10%), while the method linearity was 0.99, thus both within the ENGL requirements.

Table 12. Mean values, RSDr and RSD_R for PCR efficiency and coefficient of determination R^2

	Efficiency	**R^2**
Number of data	44	44
Mean value	91	0.99
Relative repeatability standard deviation, RSD_r (%)	3.6	0.5
Repeatability standard deviation	3.250	0.005
Relative reproducibility standard deviation, RSD_R (%)	10	1.0
Reproducibility standard deviation	9.327	0.011

When the outlying laboratory was excluded from the computation of the accuracy rate, this was higher than 95% at total DNA amount per reaction of 16 copies, with a 64-fold improvement compared to the accuracy of 95% at 1024 copies per reaction when the outlying laboratory was retained (Table 9).

Table 13. Samples of the dilution series S, average Ct* across series per laboratory and accuracy rate following identification and removal of outlying laboratories through Cochran and Grubbs tests, according to ISO 5725-2

Sample	DNA copies/ reaction	Lab 01	Lab 02	Lab 03	Lab 04	Lab 05	Lab 07	Lab 08	Lab 09	Lab 10	Lab 11	Lab 12	Accuracy rate
S1	262144	21.09	20.81	20.99	20.12	20.36	20.71	19.72	19.60	21.83	20.77	21.66	100
S2	65536	23.04	22.84	23.88	22.09	22.00	22.66	21.55	21.60	23.98	22.75	23.65	100
S3	16384	25.47	24.87	25.81	24.06	24.05	24.64	23.53	23.74	26.14	24.76	25.79	100
S4	4096	28.37	27.11	28.47	26.13	26.12	26.80	25.66	25.98	28.27	26.92	28.01	100
S5	1024	30.72	29.37	30.90	28.10	28.27	28.93	27.68	27.89	30.48	29.00	31.29	100
S6	256	33.07	31.69	34.17	30.09	30.98	30.76	29.99	29.94	32.76	30.89	34.11	100
S7	64	35.02	34.06	36.40	32.06	32.62	32.80	32.25	32.24	35.04	33.04	36.86	100
S8	16	37.10	36.66	39.36	34.02	34.86	35.04	34.34	34.66	37.11	35.31	40.18	97.73
S9	8	38.08	37.80	35.42	35.30	36.15	37.45	35.30	35.90	38.09	36.65	40.01	88.64
S10	4	38.79	38.60	39.54	36.17	37.28	n.d.	36.94	36.64	39.54	37.54	39.88	75.00
S11	1	40.39	n.d.	n.d.	37.79	n.d.	n.d.	37.31	38.03	40.84	38.38	n.d.	27.27
S12	0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

* For each laboratory values are the average of the replicates of the same level across the four dilution series

Though the experimental design for the determination of the accuracy rate of the *TMD* system in *E. coli K-12* event AG3139 DNA was not intended to confirm the limit of detection (LOD) of the method within the context of a collaborative trial, these findings are in very good agreement with the results for the absolute LOD study performed at CRL-GMFF on the same sample and reported to be around 16-32 DNA copies (protocol of validated method at http://gmo-crl.jrc.ec.europa.eu/).

Overall, the data reported confirm the appropriate performance characteristics of the method proposed to detect *E. coli* event AG3139 both in the bacterial biomass PT73 (TM) and in the control sample *E. coli* K-12 event AG3139.

9. Conclusions

The overall method performance has been evaluated with respect to the applicable method acceptance criteria and method performance requirements recommended by the ENGL (as detailed at http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). Data supporting the meeting of 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 method submitted by the applicant complies with the applicable ENGL performance criteria.

Therefore, the method is considered applicable to the control and the feed 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 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, Genève, Switzerland.

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 ($-3.1 \geq$ 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 ≤ 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 ± 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 ± 25% of the accepted reference value over the whole dynamic range.