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Event-specific Method for the Detection of Dried-killed Bacterial Biomass PL73 (LM) Derived from Escherichia coli K-12 GM Strain 19E Using Real-time PCR

European Union Reference Laboratory for Genetically Modified Food and Feed

2015

Joint Research Centre

EURL European Union Reference Laboratory for GM Food & Feed

European Commission
Joint Research Centre
Institute for Health and Consumer Protection

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Validation Report

17 september 2015

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In line with its mandate[a] the European Union Reference Laboratory for GM Food and Feed (EURL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), has validated an event-specific polymerase chain reaction (PCR) method for detecting the 19E transformation event in *E. coli* K-12 DNA. The validation study was conducted according to the EURL GMFF validation procedure (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) and internationally accepted guidelines [1, 2, 3, 4, 5].

In accordance with current EU legislation[b], Ajinomoto Eurolysine S.A.S. provided the detection method and the positive and negative control samples (genomic DNA extracted from *E. coli* K-12 harbouring the 19E event as positive control DNA, plasmid pUC-LMA as positive control DNA, genomic DNA extracted from *E. coli* K-12 strain VKPMB7 and plasmid pUC19 as negative control DNA). The EURL GMFF verified the performance data provided by the applicant, where necessary experimentally, prepared the validation samples, organised an international collaborative study, and analysed the results.

The EURL GMFF in-house verification and the collaborative study confirmed that the method meets the method performance requirements as established by the EURL GMFF and the ENGL and according to Annex I-2.C.2 to Regulation (EC) No 641/2004.

[a] Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed".

[b] Regulation (EC) No 641/2004 of 6 April 2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003".

Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative/quantitative PCR)] Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7.

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EURL GMFF quality system.

The EURL GMFF is also ISO 17043:2010 accredited (proficiency test provider) and applies the corresponding procedures and processes for the management of ring trials during the method validation.

The EURL GMFF conducts its activities under the certification ISO 9001:2008 of the Institute for Health and Consumer Protection IHCP provided by SGS.

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Content

1. Timeline

In line with Regulation (EC) No 1829/2003, Ajinomoto Eurolysine S.A.S. provided the EURL GMFF with a copy of the official application for authorisation of an event-specific method for the detection of DNA from *E. coli* K-12 strain 19E together with genomic and plasmid DNA as negative and positive control samples (September 2008).

In response to this submission of the method, the EURL GMFF started its step-wise procedure with step 1: dossier reception, followed by step 2: dossier evaluation in October 2008, i.e. at the same time EFSA declared the official dossier as complete and valid (October 2008).

The scientific dossier evaluation (step 2) focused on the reported method performance characteristics assessed against the ENGL method acceptance criteria[c] (see http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf for a summary of method acceptance criteria and method performance requirements). The assessment was positively concluded in October 2008.

In step 3 of the procedure (experimental testing), the EURL GMFF verified the purity of the control samples and conducted an in-house verification of the method provided.

The positive and negative control DNA samples, submitted in accordance with Article 17(3)(j) of Regulation (EC) No 1829/2003, were found to be of good quality.

The method characteristics were verified in-house. The limit of detection (LOD) and dynamic range were tested on the positive control samples 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. A validated DNA extraction protocol was tested in the context of the present application on dried-killed bacterial biomass from *E. coli* K-12 GM strain 19E and the report is available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx. Step 3 was completed in August 2009 with the conclusion that the method could be submitted to collaborative study (step 4).

The collaborative study (step 4) took place from December 2008 to January 2009. It demonstrated that the method is well suited for analysing suitable DNA extracted from dried-killed bacterial biomass of *E. coli K-12 harbouring the 19E event* and for identifying the event.

The preparation of the report (step 5) was aligned with the timelines communicated by EFSA for its risk assessment, which is going to be finalised early in 2015.

[c] EURL/ENGL guidance doc "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm)

2. Step 1 (dossier reception and acceptance) and step 2 (scientific dossier assessment)

The documentation and data provided by the applicant were evaluated by the EURL GMFF for completeness (step 1) and compliance with the ENGL method acceptance criteria (step 2).

The specificity of the event-specific assay, named LMA system, was assessed by the applicant. The method exploits a unique DNA sequence in the region of recombination between the insert and the bacterial genome; the sequence is specific to 19E transformation event in *E. coli* K-12 DNA and thus imparts event-specificity to the method. The specificity of the event-specific assay was experimentally tested by the applicant in real-time PCR against samples containing 10 ng of pUC19 (plasmid negative control sample), 262,144 copies of 19E *E. coli* K-12 DNA (genomic DNA positive control sample) and 1,000 copies of pUC-LMA plasmid positive control sample (at a different laboratory). Additionally, the method was tested on 10 ng of genomic DNA extracted from cotton, soybean, maize, wheat, pea, oat, barley, linseed, oilseed rape, buckwheat, lentil, lupin, rice, potato, sunflower, milk, egg. Only the positive controls 19E transformation event and pUC-LMA gave consistent amplifications.

The specificity was also verified and confirmed by the EURL GMFF by means of bioinformatics analysis, on the basis of the sequence data provided by the applicant. Bioinformatics analysis indicated the bacterial origin of the bases 1-49 of the amplicon and the vector origin for the remaining part of it, thus confirming the event specificity of the LMA system.

The parameters of the calibration curve (slope, R^2 coefficient) were appropriately determined by the applicant by testing the LMA system in different real-time PCR experiments using:

a) Dried bacterial biomass 'PL73 (LM)': the mean slope calculated over the range from 500 ng to 0.98 ng (per reaction) in two-fold dilution steps over 15 dilution series was -3.299 and -3.185 for ABI Prism 7700 and Roche LC 480 real-time PCR instruments, respectively. The mean R^2 coefficient was 0.99;
b) DNA of the GM strain 19E *E. coli* K-12: the slopes calculated over three independent dilution series in the range from 262,144 to 4 copies in two-fold dilution steps ranged from -3.403 to -3.533 for ABI Prism 7700 and from -3.399 to -3.544 for Roche LC 480, respectively. The mean R^2 coefficients were 0.99;
c) plasmid pUC-LMA containing the entire amplicon of the LMA system cloned in pUC vector: the slope calculated over three independent dilution series in the range from 262,144 to 4 copies in two-fold dilution steps ranged from -3.331 to -3.471 for ABI Prism 7700 and from -3.312 to -3.409 for Roche LC 480. The mean R^2 coefficient was 0.99 and 0.98 for ABI Prism 7700 and Roche LC 480, respectively.

The applicant also tested the dynamic range of the method in an alternative laboratory on 19E *E. coli* K-12 and on pUC-LMA and it was confirmed in the range from 262,144 to 16 copies. According to the ENGL method acceptance criteria, the average value of the slope of the curve shall be within the range of – 3.1 to – 3.6, and the R^2 shall be ≥ 0.98; therefore all values are within ENGL acceptance criteria.

According to the applicant, the absolute LOD of the method is 4 copies of 19E *E. coli* K-12 genomic DNA and 4 copies of the synthetic pUC-LMA plasmid containing the full amplicon cloned in pUC vector and suitable as qualitative positive control sample.
The applicant also tested the LOD of the method in a third laboratory and the results were confirmed.

3. Step 3 (EURL experimental testing of samples and methods)

3.1 DNA extraction

Genomic DNA was isolated by the applicant from *E. coli* K-12 GM strain 19E and non-GM strain *E. coli* K-12 using the DNA extraction method validated for the dried killed bacterial biomass of *E. coli* K-12 event AG3139. That DNA extraction method has been in-house verified by the EURL GMFF in the context of this application and was found to function suitably for the assessed matrix. The assessment report is published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

3.2 Method protocol for the PCR analysis

The PCR method provided by the applicant (see the corresponding Validated Method at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and in Annex 1 to this report) and subsequently validated by the EURL GMFF is an event-specific, qualitative, real-time TaqMan® PCR procedure for the detection of *E. coli* K-12 event 19E.

For the specific detection of *E. coli* K-12 event 19E, an 85-bp fragment of the region that spans the 5' bacterial genome-to-insert junction 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 (6-carboxyfluorescein) as reporter dye at its 5' end and TAMRA (6-carboxytetramethylrhodamine) as quencher dye at its 3' end (LMA system).

The LMA system makes use of a qualitative approach for detecting the target sequence over the dynamic range. The dynamic range of the assay is determined by testing serially diluted samples.

The dynamic range of the method for the detection of *E. coli* K-12 event 19E is estimated by plotting the Cq values measured for the diluted samples against the logarithm of the DNA amount expressed in copy numbers (positive control sample 19E *E. coli* K-12 DNA or plasmid pUC-LMA) and by fitting a regression line into these data. The absolute copy numbers in the positive control sample S1 (pure DNA from *E coli* K-12 event 19E) was determined by considering that an amount of 1 ng of DNA corresponds to 186,300 DNA copies of *E. coli* K-12 event 19E (source: applicant's dossier). The determination of the DNA concentration of the 19E *E. coli* K-12 DNA stock was made on the basis of 10 readings by fluorometric method with Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen ™).

3.3 EURL GMFF experimental testing of the method performance

3.3.1 Dynamic range

The EURL GMFF verified the dynamic range of the method for the detection of event 19E on genomic DNA extracted from *E. coli* K-12 GM strain 19E and on the plasmid pUC-LMA. Both control samples were diluted to a concentration of 26,214 copies/μL. Ten microliters were assayed in reaction. From this first dilution point (D1) a dilution series was built as per Table 1 for both the 19E *E. coli* K-12 genomic DNA and the pUC-LMA materials. Each dilution sample was tested in six replicates. The R^2 of the method over the dilution series and the slope of the regression line were evaluated against the ENGL acceptance criteria.

Table 1. DNA contents of the samples analysed

Sample Name	DNA copies	Dilution
D1	262144	-
D2	65536	1:4
D3	16384	1:4
D4	4096	1:4
D5	1024	1:4
D6	512	1:2
D7	256	1:2
D8	128	1:2
D9	64	1:2
D10	32	1:2
D11	16	1:2
D12	8	1:2

3.3.2 Estimation of the sample size in the determination of the Limit of Detection

The optimal sample size to determine the absolute or relative LOD was defined by estimating the number (n) of replicates per GM level that would generate a 95% confidence interval around the proportion of GM-negative samples with an upper boundary not exceeding 5%.

For an accurate estimate of the 95% confidence interval (depending on the degrees of freedom used to compute p), the F-distribution was used based on the relationship between such distribution and the binomial distribution. This method, derived from Bliss and recently re-proposed by Zar (see Annex 2), leads to an estimate of n = 100. Additionally, the standard approach based on the normal approximation was also considered, as suggested by Cochran. This alternative method returns an estimate of n = 60. Computational details are given in Annex 2.

Given the experimental design for an LOD study where it is required to test a large number of replicates for each sample, characterised by a defined analyte content (DNA copy number content) over a linearly decreasing series of concentrations, the Cochran approach was accepted as the most feasible. Hence, every sample of the dilution series was tested in 60 replicates.

3.3.3 Limit of Detection (LOD)

The determination of the LOD has been carried out during the step 3 (experimental testing) of the EURL GMFF validation process on DNA from the positive control sample 19E *E. coli* K-12 and plasmid control sample pUC-LMA provided by Ajinomoto Eurolysine S.A.S., containing the 85-bp amplicon corresponding to the whole PCR fragment amplified from event 19E.

The concentration of the positive control sample was estimated with the Picogreen kit by means of a Bio-Rad fluorometer Versafluor as an average of ten readings. The starting point for the study of the LOD of the LMA system on 19E *E. coli* K-12 sample was set at 128 copies, based on the assumption that 1 ng of 19E *E. coli* K-12 genomic DNA corresponds to 186,300 copies (from the applicant's dossier). Eight GM levels were built thereof by serial dilution, from 128 copies to 0.1 copies (see 4.1.2). Similarly, a mirroring dilution series was built with the pUC-LMA plasmid control sample knowing that the plasmid is 3,175 bp long and that the average molecular weight for a nucleotide pair is assumed to correspond to 660 Daltons.

3.4 International collaborative study (step 4)

The international collaborative study (step 4) involved twelve laboratories, all being National Reference Laboratories, assisting the EURL GMFF for testing and validation of methods for detection, as listed in annex to Regulation (EC) No 1981/2006. The study was carried out in accordance with the following internationally accepted guidelines:

- The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995) [1]
- ISO 5725 "Accuracy (trueness and precision) of measurement methods and results", Part 1 and Part 2 (ISO, 1994); ISO 5725-1:1994/Cor 1 (ISO 1998) and ISO 5725-2:1994/Cor 1 (ISO, 2002) [2, 3, 4, 5]

The objective of the international collaborative study was to assess in twelve laboratories the performance of the PCR analytical method that was provided by the applicant and which is described under 3.2 above and in the attached "Validated Method" (Annex 1).

3.4.1 List of participating laboratories

The 12 participants to the PL73 (LM) validation study where randomly selected from the 22 national reference laboratories (NRL) that offered to participate.

Clear guidance was given to the selected laboratories to strictly follow the standard operational procedures that were provided for the execution of the protocol. The participating laboratories are listed in Table 2.

Table 2. Laboratories participating in the international collaborative validation study of the detection method for *E. coli* K-12 event 19E.

Laboratory	Country
Agricultural Institute of Slovenia	SI
Central Science Laboratory	UK
Federal Environment Agency Austria	AT
Genetically Modified Organism Controlling Laboratory	PL
Institute for Agricultural and Fisheries Research (ILVO)	BE
Lower Saxony Federal State Office for Consumer Protection and Food Safety, State Food Laboratory Braunschweig	DE
National Diagnostic Centre of Food and Veterinary Service	LV
National Food Administration	SE
National Food and Veterinary Risk Assessment Institute, Laboratory Department, Molecular Biology and GMO Section	LT
Office for Social Affairs, Health and Consumer Protection of the German Federal State Saarland	DE
Scientific Institute of Public Health (IPH)	BE
State Institute of Chemical and Veterinarian Analysis	DE

3.4.2 Real-time PCR equipment used in the study

The laboratories involved in the collaborative study used a range of real-time PCR equipment: five laboratories used the ABI 7500, four used the ABI 7900, one used the Biorad IQ5 cycler, one used ABI 7000, and one used Bio-Rad CFX96.

The variability of equipment, with its known potential influence on PCR results, reflects the real situation of the control laboratories and the fact that in this case it did not significantly influence the performance of the method provides additional assurance that the method is useable under real conditions.

3.4.3 Materials used in the international collaborative study

For the validation of the qualitative event-specific method, control samples were provided by the EURL GMFF to the participating laboratories. They were derived from:

i) genomic DNA extracted from *E. coli* K-12 event 19E provided by the applicant

The control samples were prepared by the EURL GMFF from the genomic DNA provided by the applicant in accordance to Regulation (EC) No 1829/2003, Art 2.11[d].

The control sample, consisting of genomic DNA from bacterial strain *E. coli* K-12 event 19E at the starting concentration of 26,214 copies/µL (stock solution), was prepared by the EURL GMFF and samples of the dilution series were prepared by the participant laboratories using the stock solution provided (see 3.4.4).

The twelve NRLs participating to the validation study received the following materials:

- ✓ Genomic DNA of *E. coli* K-12 event 19E, 310 µL of DNA solution at 26,214 copies/µL).
- ✓ Reaction reagents:
  - TaqMan® Universal PCR Master Mix (2x), one tube: 5 mL
  - sterile distilled water, one tube: 5 mL
- ✓ Primers and probes (1 tube each) as follows:

  LMA system
  - LMA for (10 µM): 400 µL
  - LMA rev (10 µM): 400 µL
  - LMA probe (5 µM): 300 µL

[d] 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). Regulation (EC) No 1829/2003, Art. 2 (11).

3.4.4 Design of the collaborative study

Participating laboratories received a detailed validation protocol that included the exact design of the PCR plates, ensuring that on each PCR plate all samples were analysed for *E. coli* K-12 event 19E with the LMA system. In total, four dilution series were prepared by each participating laboratory starting from the stock solution at 26,214 copies/µL, 10 µL of template were loaded in each reaction. Two dilution series were loaded per PCR plate, with each dilution point prepared in triplicate according to the dilution shown in Table 3. In total, two plates (A and B) were run by each participating laboratory. On each plate, the no-template control was analysed in triplicate. Participating laboratories were requested to set the same baseline and threshold in both plates. The laboratories prepared the PCR master-mixes for *E. coli* K-12 event 19E in accordance with the description provided in the validation protocol.

Table 3. DNA content 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

The amplification reactions followed the cycling program specified in the protocol.

Laboratories reported raw data to the EURL GMFF on an Excel sheet that was designed, validated and distributed by the EURL GMFF. All data are stored by the EURL GMFF on a dedicated and protected server. The EURL GMFF analysed the data against the parameters and the limits set by the ENGL, concerning amplification efficiency and R^2.

3.4.5 Deviations reported from the protocol

Ten laboratories reported no deviations from the protocol.

One laboratory reported an unspecified error in one well of the first plate, therefore data from that well were excluded from the analysis.

One laboratory repeated plate B twice. Since this repetition was not justified by major technical problems in the protocol execution only the first plate B was processed in data analysis.

4. Results

4.1 EURL GMFF experimental testing

4.1.1 Dynamic range

The dynamic range of the method for detection of 19E was verified on genomic DNA extracted from E coli K12 No 19E. A 12-point serial dilution was built and each sample tested six times. Table 3 shows the linearity of the method over the dilution series, the slope of the regression line was evaluated against the ENGL acceptance criteria ($R^2 \geq 0.98$; $-3.1 \leq \text{slope} \leq -3.6$).

Table 3. DNA contents of the 19E samples analysed and respective average of Cq values

Sample Name	DNA copies	Cq values
D1	262144	19.81
D2	65536	21.79
D3	16384	23.88
D4	4096	25.92
D5	1024	28.09
D6	512	29.08
D7	256	30.04
D8	128	31.09
D9	64	32.23
D10	32	33.42
D11	16	34.39
D12	8	34.93
Slope	-3.43	
R^2	1.00	

Overall it can be observed that the method performs linearly over the dilution series from 262,144 copies to 8 copies of E19 *E. coli* K-12 genomic DNA, with a slope of -3.43 and an R^2 of 1.00, well within the ENGL acceptance criteria.

Similarly the method was applied to the control plasmid pUC-LMA (Table 5) and the results showed that the method also performed in a linear way: the average R^2 was equal to 1.00 with a slope of -3.33. Also in this case the parameters meet the ENGL acceptance requirements.

Table 5. DNA contents of the plasmid pUC-LMA samples analysed and respective average of Cq values, slope and R^2

Sample Name	DNA copies	Cq values
D1	262144	20.52
D2	65536	22.55
D3	16384	24.48
D4	4096	26.54
D5	1024	28.59
D6	512	29.50
D7	256	30.58
D8	128	31.42
D9	64	32.46
D10	32	33.72
D11	16	34.51
D12	8	35.59
Slope	-3.33	
R^2	1.00	

4.1.2 LOD

The EURL GMFF determined the LOD of the LMA system on *E. coli* K-12 event 19E control sample (Table 6) and on plasmid control sample pUC-LMA (Table 7) according to the validated method and under the statistical considerations described above.

Table 6. GM-levels, Cq average and ratio of positive replicates for *LMA* system on *E. coli* K-12 event 19E control sample

19E copy numbers	Average Cq/ Standard deviation	Positive/Total amplifications
128	31.06	60/60
64	32.12	60/60
32	32.92	60/60
16	34.24	60/60
8	35.4	60/60
4	36.76*	59/60
1	38.36*	35/60
0.1	38.76*	6/60

* available Cqs were computed; LOD level in grey

Table 7. GM-levels, Cq average and ratio of positive replicates for *LMA* system on pUC-LMA control sample

pUC-LMA copy number	Average Cq/ Standard deviation	Positive/Total amplifications
128	31.11	60/60
64	32.02	60/60
32	33.03	60/60
16	33.92	60/60
8	34.90	60/60
4	35.83	60/60
1	37.72*	54/60
0.1	38.60*	13/60

* available Cqs were computed; LOD level in grey

In conclusion, the LOD of the LMA system is estimated at 4 copies on *E. coli* K-12 event 19E and between 4 and 1 copies on the plasmid pUC-LMA.

4.2 Results of the international collaborative study

4.2.1 Performance of the detection method on DNA from control sample *E. coli* K-12 event 19E

Comparison of measured versus expected ΔCq values over the dilution series

A first insight of the performance of the method was obtained by inspecting how closely the dilution series compared with the expectation (Table 8).

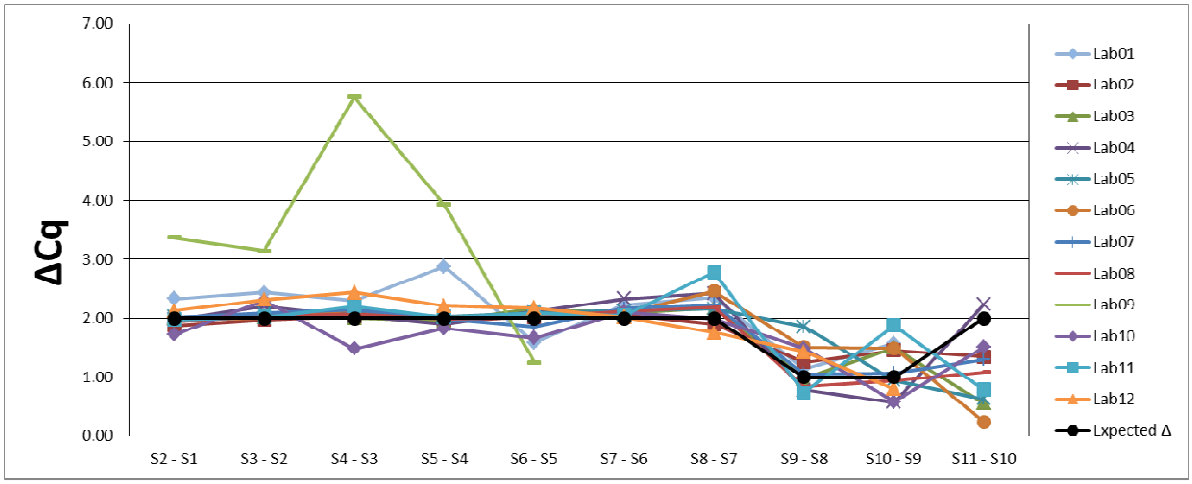
Table 8. Expected ΔCq values for the dilution intervals (assumed method efficiency = 100%)

Dilution interval	Expected ΔCq
S2 - S1	2
S3 - S2	2
S4 - S3	2
S5 - S4	2
S6 - S5	2
S7 - S6	2
S8 - S7	2
S9 - S8	1
S10 - S9	1
S11 - S10	2

To this aim, the mean ΔCq values of the four replicates of each dilution point for each laboratory were analysed. In particular, the ΔCq values of the consecutive dilution points were compared with the values expected from the designed linear dilution (S1 to S11), assuming 100% method efficiency.

Figure 1 shows the distribution of the measured ΔCq values per laboratory over the dilution series. The thick black line represents the expected ΔCq for each point of the series. Overall, the results are in line with the theoretical values; in particular eleven out of twelve laboratories shows ΔCq values consistent with the expectations at least down to S9 (corresponding to 8 target copies in reaction). Notably, this is the level down to which the dynamic range of the method was found linear during the EURL GMFF in-house testing (see 4.1.1). However, Lab09 clearly showed a pattern deviating from the expectations and truncated at S6 (no further detection of target from S7 onwards). Therefore, since this deviation did not appear to be related to the method and may have a relevant impact on the method assessment, data from Lab09 were excluded from subsequent analyses.

Figure 1. ΔCq values over the dilution series per laboratory. S12-S11 ΔCq was omitted due to insufficient data for S12



Detection accuracy rate

Table 9 illustrates the average Cq value corresponding to each of the 12 levels (samples S1 to S12), the DNA content in copy number per dilution level is indicated. Four dilution series (A to D) were tested in each laboratory, thus providing four replicate Cq values for each level. The Cq value of each replicate resulted from the average of three adjacent wells in a reaction plate: the replicate was scored 'positive' if at least two out of three adjacent wells showed amplification. If not the replicate was considered 'negative', i.e. not detected (N.D.).

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

SAMPLE	DNA COPIES/ REACTION	LAB01	LAB02	LAB03	LAB04	LAB05	LAB06	LAB07	LAB08	LAB10	LAB11	LAB12	ACCURACY RATE
S1	262,140	17.71	20.07	19.33	20.86	20.14	19.57	20.71	20.96	20.20	18.89	20.07	100
S2	65,535	20.04	21.95	21.34	22.84	22.17	21.54	22.73	22.90	21.93	20.87	22.20	100
S3	16,384	22.48	23.92	23.36	25.04	24.24	23.55	24.82	24.93	24.22	22.89	24.52	100
S4	4,096	24.79	25.95	25.37	27.08	26.35	25.69	26.96	27.01	25.70	25.09	26.96	100
S5	1,024	27.66	27.91	27.33	28.98	28.38	27.70	28.95	29.00	27.53	27.11	29.16	100
S6	256	29.25	29.93	29.49	31.09	30.46	29.78	30.82	31.01	29.20	29.21	31.34	100
S7	64	31.48	31.98	31.58	33.43	32.59	31.87	32.98	33.13	31.30	31.26	33.37	100
S8	16	33.83	33.89	33.75	35.85	34.74	34.32	35.21	35.31	33.30	34.03	35.12	98
S9	8	34.97	35.14	34.74	36.63	36.60	35.84	36.26	36.15	34.79	34.77	36.55	95
S10	4	36.54	36.59	36.23	37.21	37.54	37.33	37.33	37.10	35.37	36.65	37.35	89
S11	1	N.D.	37.94	36.79	39.44	38.17	37.57	38.63	38.18	36.88	37.43	N.D.	52
S12	0.1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	39.59	N.D.	N.D.	N.D.	2

* For each laboratory values are the mean of the replicates of the same level across the four dilution series. N.D. = Not Detected

The accuracy rate, calculated as percent detection across the four dilution series for each level of the dilution series (48 data per dilution level, four replicates by 12 laboratories) is shown in Table 9. The accuracy rate was ≥95% down to 8 DNA copies (S9) per reaction; it lowered to 89% at 4 DNA copies (S10) and yet all retained laboratories could detect the *E. coli* K-12 event 19E at this dilution level. The accuracy rate value importantly decreased as the total amount of 19E *E. coli* K-12 DNA in reaction decreased with further dilution.

Given the above considerations, the dynamic range of the LMA system on the *E. coli* K-12 event 19E DNA was assessed for all retained laboratories over the first nine DNA levels (from 262,144 to 8 copies); the slopes and the R^2 of the PCR reactions were calculated thereof. The PCR efficiency (%) and R^2 values for the serial dilutions reported by the participating laboratories (excluding Lab09) are reported in Table 10. The PCR efficiency (%) was calculated using the formula:

$$\text{Efficiency} = (10^{(-1/\text{slope})}) - 1) \times 100)$$

Table 10. Values of slope, PCR efficiency and R^2 obtained during the validation study

Laboratory	Dilution Series	Slope	PCR Efficiency (%)	R^2
Lab01	SA	-3.57	91	1.00
Lab01	SB	-3.67	87	1.00
Lab01	SC	-3.93	80	0.92
Lab01	SD	-4.06	76	1.00
Lab02	SA	-3.33	100	1.00
Lab02	SB	-3.28	102	1.00
Lab02	SC	-3.34	99	1.00
Lab02	SD	-3.35	99	1.00
Lab03	SA	-3.38	98	1.00
Lab03	SB	-3.43	96	1.00
Lab03	SC	-3.51	93	1.00
Lab03	SD	-3.36	99	1.00
Lab04	SA	-3.49	94	0.99
Lab04	SB	-3.47	94	1.00
Lab04	SC	-3.52	93	1.00
Lab04	SD	-3.61	89	0.99
Lab05	SA	-3.48	94	1.00
Lab05	SB	-3.53	92	1.00
Lab05	SC	-3.62	89	0.98
Lab05	SD	-3.51	93	1.00
Lab06	SA	-3.69	87	0.99
Lab06	SB	-3.53	92	0.99
Lab06	SC	-3.54	92	1.00
Lab06	SD	-3.44	95	1.00
Lab07	SA	-3.36	99	1.00
Lab07	SB	-3.41	96	0.99
Lab07	SC	-3.59	90	0.99
Lab07	SD	-3.37	98	1.00
Lab08	SA	-3.40	97	1.00
Lab08	SB	-3.40	97	1.00
Lab08	SC	-3.36	99	1.00
Lab08	SD	-3.42	96	1.00
Lab10	SA	-3.14	108	1.00
Lab10	SB	-3.31	100	0.99
Lab10	SC	-2.98	117	0.97
Lab10	SD	-3.16	107	0.99
Lab11	SA	-3.57	90	1.00
Lab11	SB	-3.52	92	0.99
Lab11	SC	-3.59	90	0.99
Lab11	SD	-3.50	93	1.00
Lab12	SA	-3.77	84	1.00
Lab12	SB	-3.37	98	0.99
Lab12	SC	-3.88	81	1.00
Lab12	SD	-3.51	93	1.00
Mean		**-3.48**	**94**	**0.99**

The efficiencies calculated from the corresponding slopes ranged between 76% and 117%. Mean efficiency was 94%. Overall, approximately 80% of the slopes of the regression lines, used to estimate the dynamic range, were within the limits set by the ENGL acceptance criteria ($-3.1 \leq$ slope ≤ -3.6); the mean R^2 was 0.99, again within the ENGL acceptance criteria ($R^2 \geq 0.98$).

Although the experimental design for the determination of the accuracy rate of the LMA system in *E. coli K-12* event 19E DNA was not intended to confirm the LOD of the method within the context of a collaborative trial, these findings are in good agreement with the absolute LOD study performed at EURL GMFF on the same sample and reported to be around 4 DNA copies.

Overall, the data reported confirm the appropriate performance characteristics of the method proposed to detect event 19E in the control sample *E. coli* K-12 event 19E.

5. Conclusions

A qualitative method for detection and identification of GM event 19E in *E. coli* K-12 event 19E DNA was provided by the applicant. It is described in detail under 3.2 (and available as the "Validated Method" in Annex 1 and at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx). The method has been fully validated in accordance to the EURL GMFF validation scheme, respecting all requirements of the relevant EU legislation and international standards for method validation.

The validation study confirmed that the method is applicable to suitable DNA extracted from the the control samples provided by the applicant (see paragraph 3.4.3), in accordance with the requirements of Annex I-2.C.2 to Regulation (EC) No 641/2004 and meets the ENGL method performance requirements applicable to detection methods. The method is therefore valid to be used for regulatory purposes. It can be assumed that it is applicable to any appropriately extracted PL73 (LM) DNA.

6. References

1. Horwitz W., 1995. Protocol for the design, conduct and interpretation of method - performance studies, *Pure & Appl. Chem.* 67, 331-343.
2. International Standard (ISO) 5725-1, 1994. Accuracy (trueness and precision) of measurement methods and results. Part 1: General principles and definitions. International Organization for Standardization, Genève, Switzerland.
3. ISO 5725-1:1994/Cor 1:1998.
4. International Standard (ISO) 5725-2, 1994. Accuracy (trueness and precision) of measurement methods and results. Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method. International Organization for Standardization, Genève, Switzerland.
5. ISO 5725-2:1994/Cor 1:2002.

Annex 1: Event-specific Method for the Detection of Dried-killed Bacterial Biomass PL 73 (LM) Derived from *E. coli* GM Strain 19E Using Real-time PCR

Validated Method

Method development:

Ajinomoto Eurolysine S.A.S.

Method validation:

European Union Reference Laboratory for GM Food and Feed (EURL GMFF)

1. General information and summary of the methodology

This protocol describes an event-specific real-time qualitative TaqMan® PCR procedure for the traceability of the bacterial biomass 'PL73 (LM)' and for the detection of the *Escherichia coli* K-12 GM strain 19E.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the specific detection of *E. coli* K-12 strain harbouring the event 19E, an 85-bp fragment of the integration site of event 19E corresponding to the 5' flanking region of bacterial origin and the integration sequence inserted into the bacterial genome (insert to *E. coli* junction) is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye FAM (6-carboxyfluorescein) as a reporter at its 5' end and TAMRA (6-carboxytetramethylrhodamine) as quencher dye at its 3' end. The primers/probe set targeting *E. coli* K-12 event 19E is referred to as 'LMA'.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Cq" value. For detection of *E. coli* K-12 event 19E DNA in a test sample, Cq values for the LMA system are determined.

The product PL73 (LM) consists of the dried killed cells of a genetically modified strain of *Escherichia coli* K-12 (*E. coli* K-12), named 19E. The *E. coli* K-12 strain 19E was constructed from a specific strain of *E. coli* K-12 – strain VKPMB7 –- using techniques of genetic modification. The purpose of the genetic modifications was to obtain a high production rate of L-Lysine.

Ajinomoto Eurolysine S.A.S. stated that to their knowledge no conventional counterpart to PL73 (LM) existed on the EU market at the time of method submission, i.e. no bacterial biomass by-product of lysine production consisting of the conventional strain of *E. coli* K-12 used as feed material. Consequently, if the bacterial biomass PL73 (LM) is present in animal feed, it should always be 100% GM and not a potential mixture of GM *E. coli* K-12 biomass plus wild-type *E. coli* K-12 biomass; thus no relative quantification is needed for such product. As a consequence, a real-time PCR based qualitative method was proposed by the applicant and validated by the EURL GMFF for the detection of PL73 (LM) product within the investigated dynamic range.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction vessels. Glass capillaries are not recommended for the buffer composition described in this method.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from the bacterial biomass PL73 (LM).

The repeatability and reproducibility of the method were tested through an international collaborative ring trial using linearly diluted genomic DNA samples produced from the control sample 19E *E. coli* K-12, received from the applicant.

2.2 Collaborative trial

The method was validated in a collaborative study by the European Union Reference Laboratory for GM Food and Feed (EURL GMFF) in collaboration with the European Network of GMO Laboratories.

A detailed validation report can be found at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

2.3 Limit of detection (LOD)

According to the applicant, the absolute LOD of the method is 4 copies of 19E *E. coli* K-12 genomic DNA and 4 copies of a synthetic pUC-LMA plasmid containing the full amplicon cloned in pUC vector and suitable as qualitative positive control sample.

The EURL GMFF verified the absolute LOD of the method on 19E *E. coli* K-12 and confirmed that it is 4 copies of 19E *E. coli* K-12 genomic DNA. The EURL GMFF also verified the LOD of the synthetic plasmid control sample pUC-LMA and found it to be between 4 and 1 copies.

2.4 Molecular specificity

The method targets a unique DNA sequence in the region of recombination between the insert and the bacterial genome; the sequence is specific to E*. coli* K-12 event 19E and thus imparts event-specificity to the method.

The specificity of the event-specific assay was experimentally tested by the applicant in real-time PCR against samples containing 10 ng of pUC19, 10 ng per reactions over ten replicates of two strains of *E coli* (AG3139 and VKPM B7), 262,144 copies of *E. coli* K-12 event 19E (as positive control) and 1,000 copies of pUC-LMA (in a different laboratory). Moreover, the method was tested on 10 ng of genomic DNA extracted from cotton, soybean, maize, wheat, pea, oat, barley,

linseed, oilseed rape, buckwheat, lentil, lupin, rice, potato, sunflower, milk, egg. Only the positive controls 19E *E. coli* K-12 and pUC-LMA gave consistent amplifications.

The specificity was also verified and confirmed by the EURL GMFF by means of bioinformatics analysis, on the basis of the sequence data provided by the applicant and by testing, against the pUC vector and *E. coli* GM strain AG3139. Bioinformatics analysis indicated the bacterial origin of the bases 1-49 of the amplicon and the vector origin for the remaining part of it, thus confirming the event specificity of the method.

3. Procedure

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Laboratory organisation, e.g. "forward flow direction" during PCR-setup, should follow the international guidelines, e.g. ISO 24276.
- PCR-reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
- All the equipment used should be sterilised prior to use and any residue of DNA has to be removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.
- Filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed frequently.
- Laboratory benches and equipment should be cleaned periodically with 10% sodium hypochlorite solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps - unless specified otherwise - should be carried out at 0 - 4°C.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for detection of event 19E in PL73 (LM) bacterial biomass

3.2.1 General

The method was optimised for suitable DNA extracted from dried killed bacterial biomass PL73 (LM). The detection range and LOD were tested through an international collaborative trial using serially diluted DNA samples.

The method was developed and validated for a total volume of 25 µL per reaction.

3.2.2 Preparation of the dilution series

A 12-point dilution series is prepared starting from the first sample of the dilution series (S1)

The DNA concentration of each sample of the four dilution series is reported in Table 1

Table 1. DNA concentration of the samples of the dilution series

Sample Name	Concentration (copies/µL)	Dilution factor
S1	26,214	-
S2	6,553	1:4
S3	1,638	1:4
S4	410	1:4
S5	102	1:4
S6	26	1:4
S7	6.4	1:4
S8	1.6	1:4
S9	0.8	1:2
S10	0.4	1:2
S11	0.1	1:4
S12	0.01	1:10

3.2.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the components needed for the run. Keep thawed reagents on ice.
2. To prepare the reaction mixture add the following components (Table 2) in one reaction tube on ice in the order mentioned below (except DNA).

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	0.97x	12.07
Primer Forward (10 µM)	439 nM	1.097
Primer Reverse (10 µM)	439 nM	1.097
Probe (5 µM)	146 nM	0.732
Template DNA (max 100 ng)	#	10.0
Total reaction volume:		25 µL

3. Mix gently and centrifuge briefly.
4. Prepare one reaction tube (with the master mix) for each DNA sample to be tested (samples of the dilution series). Add to each reaction tube the correct amount of master mix (e.g. 15 x 3.5 = 52.5 µL master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 10 x 3.5 = 35 µL DNA for three PCR repetitions). The additional 0.5 repetition included will ensure adequate volume when loading the samples. Vortex each tube for approx. 10 sec. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.
5. Spin down the tubes in a micro-centrifuge. Aliquot 25 µL in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250 x *g* for 1 minute at 4 °C) to spin down the reaction mixture.
6. Place the plate into the instrument.
7. Run the PCR following the cycling conditions described in Table 3.

Table 3. Cycling program

Step	Stage		T (°C)	Time (s)	Acquisition	Cycles
1	UNG		50	120	No	1X
2	Initial denaturation		95	600	No	1X
3	Amplification	Denaturation	95	15	No	45X
		Annealing & Extension	60	60	Yes	

3.3 Data analysis

Subsequent to the real-time PCR, analyse the run following the procedure below:

a) Set the threshold: display the amplification curves in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no "fork effect" between repetitions of the same sample. Press the "update" button to ensure changes affect Cq values (only needed for some analysis software). Switch to the linear view mode by clicking on the Y axis of the amplification plot and check that the threshold previously set falls within the exponential phase of the curves.

b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Cq = 25, set the baseline crossing at Cq = 25 – 3 = 22).

e) Save the settings and export for further calculations.

3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument's software calculates the Cq values for each reaction.

The dynamic range of the method is assessed by plotting the Cq values measured over the dilution series against the logarithm of the respective DNA copy numbers and by fitting a linear regression line into these data.

The accuracy rate of detection was calculated as percent detection across the four replicates for each level of the dilution series (48 data per dilution level, four replicates by 12 laboratories).

4. Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)

- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Centrifuge for PCR plates and reaction tubes
- Micropipettes
- Vortex
- Rack for reaction tubes
- 0.2/1.5/2.0 mL reaction tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix (2X). Applied Biosystems Part No 4304437

4.3 Primers and Probes

Oligonucleotides	Name	DNA Sequence (5' to 3')	Length (nt)
Primer Forward	LMA for	5'-ggT TAT CCA gTA ATA gCC ATC TTC ATC-3'	27
Primer Reverse	LMA rev	5'-CCT CCC ggT TTT TTT CgT ACT T-3'	22
Probe	LMA probe	5'-6FAM-CCg TCg CCg CTg TAT TgA TTC ACT Tg-TAMRA-3'	26

FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine

Annex 2: Estimation of the sample size in the determination of the Limit of Detection

According to the method from Bliss[i] and Zar[ii], in a sample of (n) data, (X) of which showing the character of interest, confidence limits (L_1: lower limit, L_2: upper limit) of a proportion (p) are computed as follows:

$$L_1 = \frac{X}{X + (n - X + 1) \cdot F_{\alpha/2,\nu1,\nu2}}$$

$$L_2 = \frac{(X + 1) \cdot F_{\alpha/2,'\nu1,'\nu2}}{n - X + (X + 1) \cdot F_{\alpha/2,'\nu1,'\nu2}}$$

where the degrees of freedom v1 and v2 are:

$$\nu1 = 2 \cdot (n - X + 1)$$

$$\nu2 = 2 \cdot X$$

and the degrees of freedom ‘v1 and ‘v2 are:

$$'\nu1 = \nu2 + 2$$

$$'\nu2 = \nu1 - 2$$

Based on this method, with X = 1, α= 0.05, and L_2 = 0.05, (n) is equal to 100.

According to Cochran[iii] the simplest approach to estimate the confidence interval of a sample proportion (p), is the use of the normal distribution (z) and its standard deviation p (1 - p):

$$L_1 = p - z_{\alpha/2} \cdot \sqrt{\frac{p \cdot (1 - p)}{n - 1}}$$

$$L_2 = p + z_{\alpha/2} \cdot \sqrt{\frac{p \cdot (1 - p)}{n - 1}}$$

Based on this simplified approach, with X = 1 and α= 0.05, L2 = 0.05 (n) would be equal to 60, thus resulting for determining the absolute LOD in an experimental set at 59 positive tests (n - X) over 60 replicates (see: "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing"[iv]).

i Bliss C.I. (1967) Statistical biology, Vol. 1. McGraw-Hill, New York, USA, 558 pp.
ii Zar J.H. (1999) Biostatistical analysis, 4th edition. Prentice Hall, New Jersey, 663 pp.
iii Cochran W.G. (1977) Sampling techniques, 3rd edition. John Wiley, New York, 428 pp.
iv ENGL. Definition of minimum performance requirements for analytical methods of GMO testing. http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm

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European Commission
Joint Research Centre – Institute for Health and Consumer Protection

Title: **Event-specific Method for the Detection of Dried-killed Bacterial Biomass PL73 (LM) Derived from Escherichia coli K-12 GM Strain 19E Using Real-time PCR**

Author(s): European Union Reference Laboratory for GM Food and Feed

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Abstract

In line with its mandate the European Union Reference Laboratory for GM Food and Feed (EURL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), has validated an event-specific polymerase chain reaction (PCR) method for detecting the 19E transformation event in E. coli K-12 DNA. The validation study was conducted according to the EURL GMFF validation procedure (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) and internationally accepted guidelines (1, 2, 3, 4, 5).

In accordance with current EU legislation , Ajinomoto Eurolysine S.A.S. provided the detection method and the positive and negative control samples (genomic DNA extracted from E. coli K-12 harbouring the 19E event as positive control DNA, plasmid pUC-LMA as positive control DNA, genomic DNA extracted from E. coli K-12 strain VKPMB7 and plasmid pUC19 as negative control DNA). The EURL GMFF verified the performance data provided by the applicant, where necessary experimentally, prepared the validation samples, organised an international collaborative study, and analysed the results.

The EURL GMFF in-house verification and the collaborative study confirmed that the method meets the method performance requirements as established by the EURL GMFF and the ENGL and according to Annex I-2.C.2 to Regulation (EC) No 641/2004.

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