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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

Validated Method

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Method development:

Ajinomoto Eurolysine S.A.S.

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 CERMET.

Address of contact laboratory:

European Commission, Joint Research Centre (JRC)
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit (MBG)
European Union Reference Laboratory for GM Food and Feed
Via E. Fermi 2749, 21027 Ispra (VA) – Italy
Functional mailbox: eurl-gmff@jrc.ec.europa.eu

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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 μ L 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/ μ L)	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	$\mu\text{L}/\text{reaction}$
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

- Mix gently and centrifuge briefly.
- 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. $15 \times 3.5 = 52.5 \mu\text{L}$ master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. $10 \times 3.5 = 35 \mu\text{L}$ 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.
- 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 $250 \times g$ for 1 minute at $4 \text{ }^\circ\text{C}$) to spin down the reaction mixture.
- Place the plate into the instrument.
- Run the PCR following the cycling conditions described in Table 3.

Table 3. Cycling program

Step	Stage	T ($^\circ\text{C}$)	Time (s)	Acquisition	Cycles
1	UNG	50	120	No	1X
2	Initial denaturation	95	600	No	1X
3	Denaturation	95	15	No	45X
	Amplification Annealing & Extension	60	60	Yes	

3.3 Data analysis

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

- 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