Event-specific Method for the Detection of Dried-killed Bacterial Biomass PT 73 (TM) Derived from *E. coli* GM Strain AG3139 Using Real-time PCR

Protocol

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Content

1. General information and summary of the methodology

This protocol describes an event-specific real-time qualitative TaqMan® PCR procedure for the:

a. traceability of the bacterial biomass 'PT73 (TM)' and for the
b. detection of the *E. coli* GM strain 'AG3139'.

The product PT73 (TM) consists of the dried killed cells of a genetically modified strain of *Escherichia coli* K-12 (*E. coli* K-12), named AG3139. The strain AG3139 has been constructed from a specific strain of *E. coli* K-12 – strain MG1655 - using conventional and modern techniques of genetic modifications. The purpose of the genetic modifications is to obtain a high production rate of L-threonine.

Ajinomoto Eurolysine S.A.S. stated that to their knowledge no conventional counterpart to PT73 (TM) exists on the EU market, i.e. no bacterial biomass by-product of threonine production, consisting of conventional strain of *E. coli* K-12 used as feed material at the time of the method submission. Consequently, if the bacterial biomass PT73 (TM) is present in feeding stuffs it will always be 100% GM material and not a potential mixture of GM *E. coli* K12 biomass plus wild-type *E. coli* K-12 biomass: thus no relative quantification is possible for such product. As a consequence, a Real-time PCR based qualitative method has been proposed by the applicant and validated by the CRL-GMFF for the detection of PT73 (TM) 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.

Template DNA extracted by means of a suitable method 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 strain AG3139, a 90 bp fragment of the integration site of AG3139 corresponding to the border covering the 5' flanking region of bacterial origin and the integration sequence inserted into the bacterial genome (insert to *E. coli* junction) is amplified using two specific primers. PCR products are measured at each cycle (real-time) by means of a target-specific oligonucleotide probe labeled with FAM dye and TAMRA as quencher dye. The primers/probe set targeting the event AG3139 is referred to as '*TMD*'.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For detection of the event AG3139 DNA in a test sample, Ct values for the *TMD* system are determined for the sample.

2. Validation status and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from feeding stuffs which may use the bacterial biomass PT73 (TM) as a component.

The repeatability and reproducibility of the method was tested through an international collaborative ring trial using linearly diluted DNA samples from PT73 (TM) feed and from the control sample AG3139.

2.2 Collaborative trial

The method was validated in a collaborative study by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with twelve participating laboratories in October 2008.

Each participant received 2 grams of PT73 (TM) biomass ground to a fine powder.
Four independent DNA extractions were performed by each laboratory.
Extracted DNA was quantified by fluorimetric means and diluted to a working concentration of 10 ng/μL. Four 12-point dilution series (named E_A to E_D) were built thereof and each point of the dilution series was tested in triplicate with the *TMD* system.
Similarly, four independent 12-point dilution series (named S_A to S_D) were built from the positive control sample 'AG3139' and each point of the dilution series was analysed in triplicate with the *TMD* system.

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

2.3 Limit of detection (LOD)

According to the applicant, the absolute LOD of the method is 16 copies of AG3139 genomic DNA and 8 copies of a synthetic pUC-TMD plasmid containing the full amplicon cloned in pUC vector and suitable as qualitative positive control sample.
The CRL-GMFF experimentally verified the absolute LOD of the method on AG3139 and found it to be between 32 and 16 copies of AG3139 genomic DNA. The CRL-GMFF also verified the LOD of the synthetic plasmid control sample pUC-TMD and found it to be between 8 and 4 copies.

2.4 Molecular specificity

According to 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 AG3139 *E. coli* 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, 1000 copies of AG3139 and 1000 copies of pUC-TMD.
Only the positive control AG3139 and pUC-TMD gave consistent amplifications with Ct figures less than 45.

The CRL-GMFF further tested the PT73 (TM) specificity in real-time PCR against pUC19 and *E. coli* MG1655, parental organism for AG3139. No detectable amplifications were observed.

Bioinformatics analysis indicated the bacterial origin of the oligonucleotide bases 1-52 of the amplicon and the vector origin for the remaining part of it, thus confirming the event specificity of the PT73 (TM) system. In addition, though *in silico* analysis found several partial matches particularly for the forward primer against GMO sequences maintained at JRC. No alert was raised for possible cross-reactivity of the PT73 (TM) system with other GMOs whose methods were submitted to the CRL-GMFF.

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 guidelines given by relevant authorities, 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 hypochloride 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 qualitative detection of event AG3139 in PT73 (TM) bacterial biomass

3.2.1 General

The use of maximum 100 ng of template DNA per reaction well is recommended.
The method is developed for a total volume of 25 µL per reaction.

3.2.2 Feed sample preparation

The detection range of the *TMD* system has been investigated in collaborative trial on DNA extracted by each laboratory from the bacterial biomass. DNA concentration was determined by fluorometer and adjusted to a concentration of 10 ng/µL. Four independent dilution series (from EA to ED) were built from each DNA extract according to the intended concentrations indicated in Table 1, thus providing a total DNA amount per reaction (10 µL of DNA sample per reaction, see 3.2.4) from 100 ng down to 24 fg at the two ends of the dilution series.

Table 1. DNA concentration of the samples of the dilutions series EA (as an example of the four dilution series)

Sample Name*	DNA Concentration	Dilution rate
EA1	10 ng/µL	-
EA2	2.5 ng/µL	1:4
EA3	625 pg/µL	1:4
EA4	156 pg/µL	1:4
EA5	39 pg/µL	1:4
EA6	10 pg/µL	1:4
EA7	2.4 pg/µL	1:4
EA8	610 fg/µL	1:4
EA9	152 fg/µL	1:4
EA10	38 fg/µL	1:4
EA11	9.5 fg/µL	1:4
EA12	2.4 fg/µL	1:4

Samples were carefully mixed (vortex for 30 seconds) and spun down before taking an aliquot to constitute the following sample of the dilution series.

3.2.3 Control sample preparation

The detection range of the *TMD* system has been also investigated in collaborative trial on DNA from control sample S1 of AG3139 provided by the CRL-GMFF.

Four 12-point dilution series were prepared starting from sample S1 (series SA, SB, SC, SD):

Series A: from S1 to SA12;

Series B: from S1 to SB12;
Series C: from S1 to SC12;
Series D: from S1 to SD12.

The DNA concentration of each sample of the four dilution series is reported in Table 2 for series SA as an example.

Table 2. DNA concentrations of the samples of the dilutions series SA (as an example of the four dilution series)

Sample Name*	Concentration (copies/μL)	Dilution rate
S1	26214	-
SA2	6553	1:4
SA3	1638	1:4
SA4	410	1:4
SA5	102	1:4
SA6	26	1:4
SA7	6.4	1:4
SA8	1.6	1:4
SA9	0.8	1:2
SA10	0.4	1:2
SA11	0.1	1:4
SA12	0.01	1:10

3.2.4 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run. Keep thawed reagents at 1-4°C on ice.
2. In one reaction tube (for the *TMD* system) on ice, add the following components (Table 3) in the order mentioned below (except DNA) to prepare the reaction mixture.

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the *TMD* specific system.

Component	Final concentration	µL / reaction
TaqMan® Universal PCR Master Mix (2x)	0.96x	12.07
Primer F (10 µM)	439 nM	1.097
Primer R (10 µM)	439 nM	1.097
Probe (5 µM)	146 nM	0.732
Template DNA (max 100 ng)	#	10.0
Total reaction volume:		25

3. Mix gently and centrifuge briefly.

4. Prepare one reaction tube (with the *TMD* master mix) for each DNA sample to be tested (samples of the dilution series and control samples).
5. Add to each reaction tube the correct amount of master mix (e.g. 15 x 3 = 45 µL master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 10 x 3 = 30 µL DNA for three PCR repetitions). Vortex each tubes for approx. 10 sec. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.
6. Spin down the tubes in a microcentrifuge. 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.
7. Place the plate into the instrument.
8. Run the PCR following the cycling conditions described in Table 4:

Table 4. Cycling program for TMD system

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	UNG		50 °C	120	No	1
2	Initial denaturation		95 °C	600	No	1
3	Amplification	Denaturation	95 °C	15	No	45
		Annealing & Extension	60 °C	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 of the *TMD* system 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 Ct values. 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 geometric 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 Ct = 25, set the baseline crossing at Ct = 25 – 3 = 22).

e) Save the settings and export all the data to a text file 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 Ct-values for each reaction.

The 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 (mass/volume for the dilution series of the feed sample and copy numbers for the dilution series of the control sample) and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the repeatability, reproducibility of the detection range and for the control sample the accuracy rate corresponding to the dilution points.

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 plates and reaction tubes
- Micropipettes
- Vortex
- Rack for reaction tubes
- 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

Name	Oligonucleotide DNA Sequence (5' to 3')
TMD target sequence	
Primer F	5' – AAT ACC GTT AAA CGT AAA TTC TTT TTC TTT – 3'
Primer R	5' – TCC TCC CGG TTT TTT TCG TA – 3'
probe	6-FAM 5' – AGA TCG AGT ATT CAT TCG GTG TAT TGA TTC ACT TGA – 3' TAMRA

ANNEX 1. Determination of limit detection (LOD) at the CRL-GMFF

A1 Experimental design

The determination of the LOD has been carried out during the step 3 (experimental testing) of the CRL-GMFF validation process on DNA from the positive control sample event AG3139 and plasmid control sample pUC-TMD provided by Ajinomoto Eurolysine S.A.S., containing the 90 bp amplicon corresponding to the whole PCR fragment amplified from AG3139.
The concentration of the positive control samples 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 TMD system on AG3139 sample was set at 128 copies, based on the assumption that 1 ng of AG3139 genomic DNA corresponds to 189709 copies (from applicant's dossier). Seven GM levels were built thereof by serial dilution. Similarly, a mirroring dilution series was built with the pUC-TMD plasmid control sample knowing that the plasmid is 3231 bp long and that the average molecular weight for a nucleotide pair is assumed to correspond to 660 Daltons.

A.2 Optimum sample size for LOD determination

The optimal sample size (number of replicates n per assayed GM level) was estimated to determine the limit of detection (LOD), defined as the GM level (p) detected at least 95% of the time, thus ensuring ≤5% false negative results. The number n was estimated to generate a 0.95 confidence interval whose upper bound does not exceed 5%.
For an accurate estimate of the 0.95 (1-α) 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 (Fisher and Yates, 1963). The method is derived from Bliss (1967) and recently re-proposed by Zar (1999). According to this method, in a sample of n data, X of which showing the character of interest, confidence limits (L1: lower limit, L2: 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 $\nu1$ and $\nu2$ are:

$$\nu1 = 2 \cdot (n - X + 1)$$
$$\nu2 = 2 \cdot X$$

and the degrees of freedom $'\nu1$ and $'\nu2$ are:

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

Based on this method, with X = 1 and α = 0.05, L2 = 0.05 results from n = 100.

As suggested by various statisticians (e.g. Cochran, 1977), 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 results from n = 60, thus resulting in an experimental absolute LOD set at 59 positive tests (n - X) over 60 replicates

Given the experimental design for a LOD study where it is required to test a large number of replicates in each sample characterised by defined analyte content (GM level) over a linearly decreasing series of concentrations, the Cochran approach was accepted as the most feasible. Hence, 60 replicates were tested with the GM-specific system in each of eight GM levels (Table A.1 and A.2) set up as serial dilutions. This resulted in running six PCR experiments for a total of 480 test items excluding controls per each control sample.

A.3 Results and conclusions

The CRL-GMFF determined the LOD of the *TMD* system on event AG3139 (Table A.1.) and on plasmid control sample pUC-TMD (Table A.2.) according to the validated method and under the statistical considerations identified above.

Table A.1. GM-levels, Ct average and ratio of positive replicates for *TMD* system on AG3139 control sample

AG3139 copy number	Average Ct* / Standard deviation	Positive / Total amplifications
128	31.72	60/60
64	32.66	60/60
32	33.94	60/60
16	35.29	59/60
8	36.33	59/60
4	37.07	58/60
1	38.90	37/60
0.1	39.36	7/60

* available Cts were computed

Table A.2. GM-levels, Ct average and ratio of positive replicates for *TMD* system on pUC-TMD control sample

AG3139 copy number	Average Ct* / Standard deviation	Positive / Total amplifications
128	30.93	60/60
64	32.06	60/60
32	33.04	60/60
16	34.07	60/60
8	35.09	60/60
4	35.94	60/60
1	38.46	52/60
0.1	39.46	10/60

* available Cts were computed

In conclusion, the LOD is estimated between 8 and 32 copies for the TMD system on event AG3139 and between 1 and 4 copies of plasmid pUC-TMD

A.4 References

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