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## Report on the In-house Validation of a DNA Extraction Method for PL73 (LM) Dried-killed Bacterial Biomass Derived from *Escherichia coli* K-12 GM Strain 19E

European Union Reference Laboratory for  
Genetically Modified Food and Feed

2015



**European Commission**

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# **Report on the In-house Validation of a DNA Extraction Method for PL73 (LM) Dried-killed Bacterial Biomass Derived from *Escherichia coli* K-12 GM Strain 19E**

**17 september 2015**

**European Union Reference Laboratory for GM Food and Feed**

## **Executive Summary**

In accordance with relevant EU legislation<sup>1</sup>, Ajinomoto Eurolysine S.A.S. previously provided to the European Union Reference Laboratory for GM Food and Feed (EURL GMFF) a DNA extraction method suitable for DNA extraction from PT73 (TM) dried-killed bacterial biomass, used as feed. This method was validated by the EURL GMFF in June 2009 in the context of the application for PT73 (TM) dried-killed bacterial biomass derived from *Escherichia coli* GM Strain AG3139 (<http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>).

In line with its mandate<sup>2</sup>, the EURL GMFF has conducted an in-house verification of this DNA extraction method on dried-killed bacterial biomass of PL73 (LM) derived from *Escherichia coli* GM K12 strain E19. To this end it tested the DNA extraction method on the samples provided and evaluated its performance in terms of DNA yield, integrity and quality.

The in-house validation study confirmed that the method meets the method performance requirements as established by the EURL GMFF and the ENGL<sup>3</sup>, and that it satisfies the provisions of Annex I-2.C.2 to Regulation (EC) No 641/2004.

The method is therefore fit for the purpose of producing DNA from PL73 (LM) dried-killed bacterial biomass of suitable quantity and quality for subsequent PCR-based analysis.

This report is published at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.htm>.

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<sup>1</sup>Regulation (EC) No 641/2004 of 6 April 2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003".

<sup>2</sup>Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed".

<sup>3</sup>Definition of minimum performance requirements for analytical methods of GMO testing. [http://gmo-crl.jrc.ec.europa.eu/doc/Min\\_Perf\\_Requirements\\_Analytical\\_methods.pdf](http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf)

## 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](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.

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## 1. Introduction

This report describes the in-house verification of the applicability of a method for extracting genomic DNA from dried-killed GM-bacterial biomass (DNA extraction report on GM-event PT73 *E. coli* TM dried killed bacterial biomass at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.htm>) from samples of feed provided by the applicant, Ajinomoto Eurolysine S.A.S. According to the applicant this method can be used for the extraction of DNA of appropriate quality for subsequent PCR-analysis from bacterial biomass, consisting of the bacterial cells separated from the fermentation broth after the latter has been subjected to an 'inactivation treatment'. This product consists of bacterial cells but does not contain viable cells and the size of the degraded recombinant DNA is reduced. According to the applicant, the method was successfully used to isolate and purify DNA from the biomass of product PT73 (TM), PT73 *E. coli* THR, PL73 (LM), PL73 *E. coli* Lys and from a series of feed materials.

The purpose of the DNA extraction method described is to provide DNA with purity suitable for real-time PCR based methods for detecting, identifying, and quantifying the GM-event PT73.

This protocol is recommended to be executed by skilled laboratory personnel since hazardous chemicals and materials are used at some steps. It is strongly advised to take particular notice of all product safety recommendations and guidelines.

## 2. Materials (Equipment/Chemicals/Plastic ware)

### 2.1. Equipment

The following equipment is used in the DNA extraction procedure described (equivalents may be used):

1. Blender
2. Balance
3. Vortex Mixer
4. Thermomixer
5. Rotator
6. Centrifuge (for 2 ml tube 12,000 rpm)
7. Fluorometer
8. Minicolumns (Promega)
9. Micropipettes
10. Rack for reaction tubes
11. Platform for real-time PCR and analysis software
12. Vacuum manifold

## 2.2. Chemicals

The following chemicals are used in the DNA extraction procedure described (equivalents may be used):

1. Tris-base (Sigma, T-1503)
2. Sodium chloride (Merck, 106404)
3. Na<sub>2</sub>EDTA\*2H<sub>2</sub>O (Merck, 108418)
4. Sodium dodecyl sulphate, 'SDS' (Merck, 113760)
5. Hydrochloric acid (Merck, 100317)
6. Guanidine hydrochloride (Sigma, G-7153)
7. Proteinase K (Boehringer, 745723)
8. Chloroform (Lab-scan A 3505 E)
9. Wizard™ resin and minicolumns (Promega, A 7280)
10. 2-Propanol (baker 8068)
11. DNase free distilled sterile water
12. Picogreen dsDNA Quantitation Kit (Molecular probes P7589)

## 2.3. Solutions

The following buffers and solutions are used in the DNA extraction procedure described:

### 1. DNA Extraction Buffer (1xTNE), pH=8.0, 1000 mL (store at 2 – 10 °C)

- 1.21 g Tris base (10 mmol/L Tris-HCl)
- 744 mg Na<sub>2</sub>EDTA\*2H<sub>2</sub>O (2 mmol/L EDTA)
- 8.76 g NaCl (150 mmol/L NaCl)

Add 6 mmol/L HCl to pH=8.0

10 g SDS (1% w/V SDS)

DNase free distilled sterile water to 1000 mL

### 2. Guanidine hydrochloride (5 mol/L) (store at room temperature)

### 3. Proteinase K (20 mg/mL) (store at -20 °C)

### 4. 2-Propanol (80% v/v) (store at room temperature)

## 2.4. Plasticware

- MicroAmp Optical 96-Well Reaction Plates
- Optical adhesive covers
- 1.5 mL DNase free reaction tubes
- 2.0 mL DNase free reaction tubes
- 5.0 (or 15.0) mL DNase free reaction tubes

Note: plasticware should be sterile and free of DNases, RNases and nucleic acids.

## 2.5. Precautions

- Chloroform, isopropanol, hydrochloric acid (HCl), guanidine hydrochloride and sodiumdodecylsulphate are hazardous chemicals; therefore, all manipulations have to be performed according to safety guidelines, under fume hood.
- All tubes and pipette tips have to be discarded as biological hazardous material.

## 2.6. Abbreviations

EDTA	ethylenediaminetetraacetic acid
HCl	Chloridric acid
NaCl	Sodium chloride
PCR	polymerase chain reaction
SDS	sodiumdodecylsulphate
TNE	Tris, NaCl, EDTA
Tris	Tris(hydroxymethyl)aminomethane

## 3. Description of the method

### 3.1. Scope and applicability

The method can be used to isolate and purify DNA from the bacterial dried-killed biomass. Application of the method to other matrices may require adaptation and possibly a further specific validation.

### 3.2. Practicability

The DNA extraction method described below requires only standard molecular biology equipment, e.g. a centrifuge, an incubator and pipettes. The procedure takes about 4 hours of hands-on time.

### 3.3. Principle

The basic principle of the DNA extraction procedure consists in first releasing into aqueous solution the DNA present in the matrix and then in further purifying it from PCR inhibitors. The first step of the extraction procedure involves the use of guanidine hydrochloride, a chaotropic agent, and proteinase K, followed by extraction with chloroform to remove contaminants such as lipophilic molecules and proteins. The extracted DNA is further purified using a Wizard resin and finally dissolved in distilled water.

### 3.4. Sampling and samples grinding procedure

The animal feed PL73 (LM), consisting of dried-killed bacterial biomass derived from *E. coli* K-12 event E19, is 100% GM. To the applicant's knowledge no bacterial biomass from non-GM *E. coli*

is used as feed material. Therefore any detection of event E19 identifies 100% GM sample. As such, no sampling scheme is applicable.

Samples should be milled prior to extraction procedure. Possible methods of processing include a commercial blender.

### 3.5 Extraction of genomic DNA from *E. coli* dried-killed bacterial biomass

Note: Two samples of 100 mg each are processed separately from step 1 to step 9 and pooled at step 10.

1. Weigh out 2 times 100 mg of the sample into two 2 mL tubes.
2. To each tube, add 860  $\mu$ L of TNE extraction buffer, 100  $\mu$ L of 5M Guanidine-HCl and 40  $\mu$ L of Proteinase K.
3. Mix well by vortexing and incubate for 1 hour between 65  $^{\circ}$ C and 75  $^{\circ}$ C in thermomixer.
4. Centrifuge the two 2 mL tubes at 12,000 rpm for 5 min and transfer 750  $\mu$ L of the supernatant to new 2 mL tubes containing 750  $\mu$ L of chloroform.
5. Mix 10 min with the rotator.
6. Heat the Wizard<sup>TM</sup> resin 10 min at 37  $^{\circ}$ C and mix before use.
7. Centrifuge the sample extract with chloroform for 2 min at 12,000 rpm.
8. Transfer 500  $\mu$ L of the upper layer into new 2 mL tubes and add 500  $\mu$ L of the Wizard<sup>TM</sup> resin (within 5 min following heating) in each tube. Mix 10 min by inverting.
9. Attach 1 syringe barrel to the extension of 1 minicolumn. Insert the tip of the minicolumn/syringe barrel assembly into the vacuum manifold.
10. Pour the two extracted samples (containing each 500  $\mu$ L of Wizard resin and 500  $\mu$ L of supernatant) into the syringe barrel. Apply a vacuum to draw the solution through the minicolumn. Break the vacuum to the minicolumn.
11. To wash the minicolumn, add 2 mL of 80% isopropanol to the syringe barrel, and re-apply the vacuum.
12. Dry the resin by applying a vacuum. After the solution has been completely aspirated continue to apply the vacuum for additional 30 seconds. Do not dry the resin any longer. To remove residual isopropanol, put the minicolumn into a 1.5 mL tube and centrifuge at 10,000 rpm for 2 min.
13. Transfer the minicolumn into a new 1.5 mL tube and apply 100  $\mu$ L of sterile water pre-warmed at 65-70  $^{\circ}$ C. Wait 1 min and elute the DNA solution by centrifugation at 12,000 rpm. Repeat the elution step with another 100  $\mu$ L of pre-warmed water.

Store the DNA extract at -20  $^{\circ}$ C.

DNA concentration is determined by fluorometric measurement.

## 4. Testing of the DNA extraction method by the EURL GMFF

The aim of the experimental testing was to verify that the DNA extraction method provides DNA of quantity and quality appropriate for the detection and identification of the target analyte from the dried-killed bacterial biomass of PL73 (LM).

The EURL GMFF tested the method proposed by the applicant on samples of feed consisting of 100% dried-killed bacterial biomass, made available by the applicant.

To assess the suitability of the extracted DNA for real-time PCR analysis, the extracted DNA was tested using a qualitative PCR run on real-time PCR equipment.

### 4.1. DNA extraction

The samples were ground and DNA was extracted following the method described above (see section 3. "Description of the method"); the DNA extraction was carried out on eighteen test portions over three days (six replicates/day).

### 4.2. DNA concentration, yield and repeatability

The concentration of the DNA extracted was determined by fluorescence detection using the PicoGreen® dsDNA Quantitation Kit (Molecular Probes).

Each DNA extract was measured twice, and the two values were averaged. DNA concentration was determined on the basis of a five point standard curve ranging from 1 to 500 ng/μL using a BioRad VersaFluor fluorometer. The DNA concentration for the replicates is reported in the Table 1 below.

Table 1. Average DNA concentration and yield

	<b>Concentration (ng/μL)</b>	<b>Yield (μg)</b>
Overall average	137.5	27.5
Standard deviation of all samples	38.3	7.7
Coefficient of variation (%)	27.8	27.8

Table 2 reports the data of DNA concentration and yield for the 18 extracted samples.

Table 2. DNA concentration (ng/ $\mu$ L) of DNA samples extracted from PL73 (LM) bacterial biomass

Test portion	Concentration (ng/ $\mu$ L)	Yield* ( $\mu$ g)
1	117.8	23.56
2	207.4	41.48
3	141.6	28.32
4	83.9	16.78
5	77.9	15.58
6	137.5	27.5
7	150.8	30.16
8	86.6	17.32
9	167.5	33.5
10	154.9	30.98
11	108.5	21.7
12	165.6	33.12
13	142.6	28.52
14	139.8	27.96
15	168.6	33.72
16	75.5	15.1
17	181.1	36.22
18	166.6	33.32

Note: In yellow boxes samples extracted on day 1; in green boxes samples extracted on day 2; in blue boxes samples extracted on day 3.

\* total volume of the extracted DNA solution: 200  $\mu$ L

#### 4.3. Purity/Absence of PCR inhibitors

In order to assess the purity and to confirm the absence of PCR inhibitors, the extracted DNA solutions were adjusted to a concentration of 25 ng/ $\mu$ L (hereafter referred to as "undiluted" samples).

Subsequently fourfold serial dilutions of each extract were prepared with 0.2x TE buffer (1:4, 1:16, 1:64, 1:256) and analysed using a real-time PCR system detecting the LMA system (specific for a border region DNA sequence that spans the insert-to-*E. coli* junction of strain K-12 event 19E). The Cq values obtained for "undiluted" and diluted DNA samples are reported in Table 3.

Table 3. Cq values of undiluted and fourfold serially diluted DNA extracts after amplification with the LMA system.

<b>Cq values</b>					
<b>DNA extract</b>	<b>Undiluted (30 ng/μL)</b>	<b>Diluted extracts</b>			
		<b>1:4</b>	<b>1:16</b>	<b>1:64</b>	<b>1:256</b>
<b>1</b>	26.38	28.07	30.73	32.72	34.31
<b>2</b>	27.66	28.51	30.53	32.52	34.28
<b>3</b>	26.74	28.35	30.24	32.12	n.d.
<b>4</b>	26.42	27.90	29.97	31.75	33.78
<b>5</b>	26.22	27.77	29.72	31.71	33.83
<b>6</b>	26.58	28.30	30.54	32.48	34.31
<b>7</b>	27.32	29.46	31.57	33.53	n.d.
<b>8</b>	26.40	28.06	30.31	32.38	34.25
<b>9</b>	27.20	28.96	30.89	32.66	34.65
<b>10</b>	27.24	28.94	30.79	32.45	34.43
<b>11</b>	27.09	28.85	30.72	32.51	34.56
<b>12</b>	26.94	28.63	30.72	32.67	34.41
<b>13</b>	26.94	29.00	30.87	32.86	35.15
<b>14</b>	26.63	28.43	30.45	32.48	34.08
<b>15</b>	26.86	28.73	30.68	32.69	34.69
<b>16</b>	26.32	28.00	30.02	31.97	34.13
<b>17</b>	26.84	28.75	30.78	32.93	34.43
<b>18</b>	26.86	28.90	30.95	32.71	n.d.

n.d. the target was not detected

Table 4 below reports the comparison of extrapolated Cq values versus measured Cq values for all samples and the values of linearity ( $R^2$ ) and slope for all measurements.

To measure inhibition, the Cq values of the four diluted samples were plotted against the logarithm of the dilution and the Cq value for the "undiluted" sample (25 ng/μL) was extrapolated from the equation calculated by linear regression.

Subsequently the extrapolated Cq for the "undiluted" sample was compared with the measured Cq. The evaluation is carried out considering that PCR inhibitors are present if the measured Cq value for the "undiluted" sample is  $> 0.5$  cycles compared the calculated Cq value ( $\Delta Cq > 0.5$ ). In addition, the slope of the curve should be between -3.6 and -3.1.

Table 4. Comparison of extrapolated Cq values versus measured Cq values (amplification of PL73 (LM) dried-killed bacterial biomass LMA system).

DNA extraction	R <sup>2</sup>	Slope	Cq extrapolated	mean Cq measured	ΔCq*
<b>1</b>	0.98	-3.44	26.28	26.38	0.10
<b>2</b>	1.00	-3.21	26.63	27.66	1.02
<b>3</b>	1.00	-3.13	26.47	26.74	0.27
<b>4</b>	0.99	-3.22	25.99	26.42	0.43
<b>5</b>	1.00	-3.34	25.73	26.22	0.49
<b>6</b>	0.99	-3.31	26.42	26.58	0.16
<b>7</b>	1.00	-3.38	27.46	27.32	0.14
<b>8</b>	1.00	-3.43	26.09	26.40	0.31
<b>9</b>	1.00	-3.13	27.08	27.20	0.12
<b>10</b>	0.99	-3.01	27.13	27.24	0.11
<b>11</b>	0.99	-3.14	26.93	27.09	0.16
<b>12</b>	1.00	-3.21	26.78	26.94	0.16
<b>13</b>	1.00	-3.40	26.86	26.94	0.09
<b>14</b>	0.99	-3.15	26.61	26.63	0.02
<b>15</b>	1.00	-3.30	26.73	26.86	0.14
<b>16</b>	1.00	-3.38	25.95	26.32	0.38
<b>17</b>	0.99	-3.19	26.92	26.84	0.08
<b>18</b>	1.00	-3.16	27.05	26.86	0.19

Note: In yellow boxes samples extracted on day 1; in green boxes samples extracted on day 2; in blue boxes samples extracted on day 3.

\*delta Cq = abs (Cq extrapolated - Cq measured)

According to ENGL definition of minimum performance requirements for analytical methods of GMO testing<sup>4</sup> the expected slope for a PCR with 100% efficiency is -3.3; the accepted average value should be in the range of -3.6 and -3.1. In addition the average value of R<sup>2</sup> shall be ≥0.98<sup>5</sup>.

The table indicates that all ΔCq values of extrapolated versus measured Cq are < 0.5, except for the second test portion. R<sup>2</sup> of linear regression is > 0.98 for all DNA samples. The slopes of the curve are all between -3.1 and -3.6, except for the tenth sample.

<sup>4</sup> EURL/ENGL guidance document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>)

<sup>5</sup> R<sup>2</sup> is the coefficient of determination of a standard curve obtained by linear regression analysis.

## 5. Conclusion

The data reported confirm that the extraction method, applied to samples of feed consisting to 100% of dried-killed GM-bacterial biomass provided by the applicant, produced DNA of suitable quantity and quality for subsequent PCR based detection applications.

The method is consequently applicable to samples of 100% bacterial biomass provided as samples of feed in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

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In accordance with relevant EU legislation , Ajinomoto Eurolysine S.A.S. previously provided to the European Union Reference Laboratory for GM Food and Feed (EURL GMFF) a DNA extraction method suitable for DNA extraction from PT73 (TM) dried-killed bacterial biomass, used as feed. This method was validated by the EURL GMFF in June 2009 in the context of the application for PT73 (TM) dried-killed bacterial biomass derived from Escherichia coli GM Strain AG3139 (<http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>).

In line with its mandate , the EURL GMFF has conducted an in-house verification of this DNA extraction method on dried-killed bacterial biomass of PL73 (LM) derived from Escherichia coli GM K12 strain E19. To this end it tested the DNA extraction method on the samples provided and evaluated its performance in terms of DNA yield, integrity and quality.

The in-house validation study confirmed that the method meets the method performance requirements as established by the EURL GMFF and the ENGL , and that it satisfies the provisions of Annex I-2.C.2 to Regulation (EC) No 641/2004.

The method is therefore fit for the purpose of producing DNA from PL73 (LM) dried-killed bacterial biomass of suitable quantity and quality for subsequent PCR-based analysis.

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