Report on the Validation of a DNA Extraction Method for Dried-killed Bacterial Biomass

24 June 2009

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1. Introduction

This report describes the validation of a DNA extraction method to extract genomic DNA from dried-killed bacterial biomass and its applicability on the samples of food and feed provided by the applicant Ajinomoto Eurolysine S.A.S. This method can be used for the extraction of DNA from bacterial biomass consisting of the bacterial cells separated from the fermentation broth after the latter has been subject to an 'inactivation treatment', so that the final product does not contain viable cells and so that the size of the degraded recombinant DNA is reduced. According to the applicant, the method has been used to isolate and purify DNA from the biomass of product PT73 (TM), PT73 *E. coli* THR, PL73 (LM), PL 73 *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 detection methods.

This protocol is recommended to be executed by skilled laboratory personnel since hazardous chemicals and materials are exploited 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. $Na_2EDTA*2H_20$ (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 $Na_2EDTA*2H_20$ (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)**

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: All plasticware has to 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. Sampling

The feeding stuff PT73 (TM), consisting of dried-killed bacterial biomass derived from *E. coli* K-12 event AG3139, is 100% GM. To the applicant's knowledge no bacterial biomass from *E. coli* is used as feed material. Therefore any detection of event AG3139 identifies 100% GM sample. As such, no sampling scheme can be enforced.

3.2. 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.

3.3. Principle

The basic principle of the DNA extraction procedure consists in first releasing into aqueous solution the DNA present into 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. Samples grinding procedure

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

3.5 Extraction of genomic DNA from *E. coli* bacterial biomass

Note: the protocol describes the DNA extraction procedure for one sample only. 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 µL of TNE extraction buffer, 100 µL of 5M Guanidine-HCl and 40 µL of Proteinase K.
3. Mix well by vortexing and incubate for 1 hour at 65 ºC in thermomixer.
4. Centrifuge the two 2 mL tubes at 12,000 rpm for 5 min and transfer 750 µL of the supernatant to new 2 mL tubes containing 750 µL of chloroform.
5. Mix 10 min with the rotator.
6. Heat the Wizard™ resin 10 min at 37 ºC and mix before use.
7. Centrifuge the sample extract with chloroform for 2 min at 12,000 rpm.
8. Transfer 500 µL of the upper layer into new 2 mL tubes and add 500 µL of the Wizard™ resin (within 5 min following heating) in each tube. Mix 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 µL of Wizard resin and 500 µL 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 column, 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 of isopropanol, put the minicolum 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 µL of sterile water pre-warmed at 65-70 ºC. Wait 1 min and elute the DNA solution by centrifugation at 12,000 rpm. Repeat the elution step with another 100 µL of pre-warmed water.

Store the DNA extract at -20 ºC.

DNA concentration is determined by fluorometric measurement.

4. Testing of the DNA extraction method by the Community Reference Laboratory for GM Food and Feed

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 event-specific analyte.

The CRL-GMFF tested the method proposed by the applicant on samples of food and feed consisting of dried killed bacterial biomass.

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

4.1. Preparation of samples

About 200 g of feed material were ground using a GRINDOMIX GM 200 (Retsch GmbH) mixer; sample was ground once at 8000 rpm for 10 sec and twice at 10000 rpm for 15 sec.

4.2. DNA extraction

DNA was extracted following the method described above (see section 3. “Description of the method”); the DNA extraction was carried out on 6 test portions (replicates).

4.3. DNA concentration, yield and repeatability

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 six replicates is reported in the Table 1 below.

Table 1. DNA concentration (ng/µL) of six DNA samples extracted from PT73 (TM)

Sample	Concentration (ng/µL)
1	328
2	294
3	313
4	298
5	327
6	308

- ✓ DNA concentration (ng/μL)

Overall average	311.3 ng/μL
Standard deviation	14.3 ng/μL
Coefficient of variation	4.6%

- ✓ Yield (total volume of DNA solution: 200 µL)

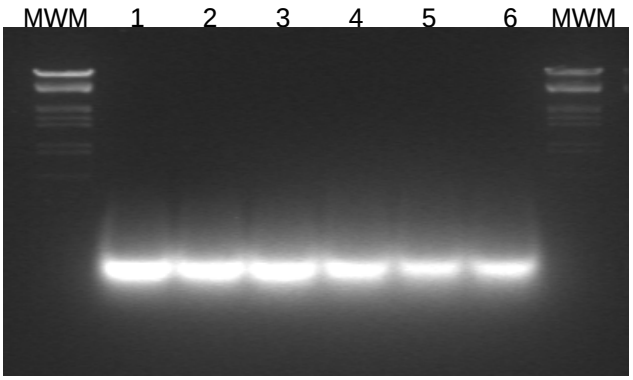
Overall average	62.27 µg
Standard deviation	2.85 µg
Coefficient of variation	4.6%

4.4. Fragmentation of DNA

The size of the extracted DNA was evaluated by agarose gel electrophoresis; 5 µL of the DNA solution were analysed on a 1.0% agarose gel (Figure 1).

The six genomic DNA samples extracted as described above appeared as fluorescent low molecular weight DNA bands (approx 100 bp), thus suggesting that extensive degradation of the genomic DNA may occur during the industrial processing required to produce the dried killed bacterial biomass for animal feeding from the inactivated *E. coli* K-12 event AG3139 strain in use.

Figure 1. Agarose gel electrophoresis of six genomic (lanes 1-6) DNA samples extracted from dried killed bacterial biomass; Molecular weight marker (MWM): Lambda EcoRI/Hind III.



4.5. 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/µL (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 TMD system (specific for a border region DNA sequence that spans the insert-to-*E. coli* junction of strain AG3139). The Ct values obtained for "undiluted" and diluted DNA samples are reported in Table 2.

Table 2. Ct values of undiluted and fourfold serially diluted DNA extracts after amplification with the TMD system.

	Undiluted (25 ng/µL)	Diluted			
DNA extract		1:4	1:16	1:64	1:256
1	19.97	21.62	23.56	25.76	27.81
2	19.79	21.67	23.52	25.38	27.43
3	20.33	22.21	24.00	26.24	28.25
4	19.82	21.46	23.34	25.32	27.41
5	20.33	22.19	24.13	26.10	28.17
6	20.36	22.02	23.84	26.14	28.05

Table 3 below reports the comparison of extrapolated Ct values versus measured Ct values for all samples and the values of linearity (R^2) and slope for all measurements.

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

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

Table 3. Comparison of extrapolated Ct values versus measured Ct values (amplification of dried bacterial biomass TMD system).

DNA extraction	R^2	Slope*	Ct extrapolated	mean Ct measured	ΔCt**
1	0.997	-3.451	19.49	19.97	0.47
2	0.996	-3.179	19.72	19.79	0.07
3	0.997	-3.383	20.08	20.33	0.25
4	0.998	-3.294	19.42	19.82	0.39
5	0.995	-3.306	20.17	20.33	0.15
6	0.996	-3.390	19.91	20.36	0.45

***The expected slope for a PCR with 100% efficiency is -3.32**

****delta Ct = abs (Ct extrapolated - Ct measured)**

All ΔCt values of extrapolated versus measured Ct are < 0.5.

R^2 of linear regression is > 0.99 for all DNA samples. The slopes of the curve are all between -3.1 and -3.6.

In conclusion, the DNA extracted according to the method tested fulfilled the acceptance criteria.

5. Testing of the DNA extraction method in the inter-laboratory collaborative study

5.1. List of participating laboratories

As part of the international collaborative study the method was tested in twelve laboratories to determine its performance. In September 2008 the CRL-GMFF invited all National Reference Laboratories nominated under Commission Regulation (EC) No 1981/2006 of 22 December 2006 and listed in Annex II ("National reference laboratories assisting the CRL for testing and validation of methods for detection") of that Regulation to express the availability to participate in the validation of the DNA extraction and of the real-time PCR method for the detection and identification of event AG3139 in *E. coli* K-12 AG3139 and PT73 (TM) dried killed bacterial biomass derived thereof.

Eighteen laboratories expressed in writing their willingness to participate, two declined the invitation, while fifty-two did not answer. The CRL-GMFF performed a random selection of twelve laboratories out of those that responded positively to the invitation, making use of a validated software application.

Clear guidance was given to the selected laboratories with regards to the standard operational procedures to follow for the execution of the protocol. The participating laboratories are listed alphabetically in Table 4.

Table 4. Laboratories participating in the validation of the DNA extraction method for dried-killed bacterial biomass.

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

The aim of the inter-laboratory collaborative study was to verify that the DNA extraction method provides DNA of suitable quantity and quality for the intended purpose.

The participating laboratories listed in Table 4 tested the method proposed by the applicant on samples of feed consisting of dried killed bacterial biomass.

To assess the suitability of the DNA extraction method for real-time PCR analysis, the extracted DNA was tested using the RT-PCR based event-specific method for the detection of *E. coli* K-12 event AG3139 (validated method available at http://gmo-crl.jrc.ec.europa.eu/).

5.2. Samples and reagents provided

A) Control and feed samples

- ✓ PT73 (TM) biomass (2 grams of fine ground powder), labelled '0408PT73 TM'
- ✓ Internal Positive Control IPC (200 µL of DNA solution) extracted from PT73 (TM) at 10 ng/µL

B) Material for DNA extraction procedure

✓ TNE extraction buffer, one tube	17.2 mL
✓ Guanidine hydrochloride 5 mol/L, one tube	2 mL
✓ Proteinase K, one tube	800 µL
✓ Wizard® resin, one tube	10 mL
✓ Minicolumns and syringe barrels, 10 units	
✓ Distilled sterile water, one tube	15 mL
✓ Picogreen, one tube	240 µL
✓ Lambda Phage DNA, one tube	60 µL
✓ TE 20X, two tubes	2 mL

C) Reaction reagents

- ✓ Universal PCR Master Mix (2x), two bottles: 5 mL each
- ✓ Primers and probes (1 tube each) as follows:
  - *TMD* system
    - *TMD*-F (10 µM): 715 µL
    - *TMD*-R (10 µM): 715 µL
    - *TMD*-P (5 µM): 420 µL

5.3. Reagents, Materials and equipments provided by the participating laboratory

- Chloroform
- Isopropanol
- Fluorometer
- MicroAmp Optical 96-Well Reaction Plates
- Optical adhesive covers

- Micropipettes
- Rack for reaction tubes
- 1.5 mL DNAse free reaction tubes
- 2.0 mL DNAse free reaction tubes
- 5.0 (or 15.0) mL DNAse free reaction tubes
- Platform for real-time PCR and analysis software
- Standard bench top centrifuge with 1.5 mL reaction tubes rotor or standard microfuge
- Vortexer
- Rotator
- Thermomixer
- Vacuum manifold

5.4. DNA extraction

DNA was extracted following the method described above (see section 3. “Description of the method”); the DNA extraction was carried out on 4 test portions (replicates).

5.5. Deviations reported

Seven laboratories reported no deviations from the protocol.

One laboratory used the centrifuge instead of vacuum manifold.

One laboratory loaded each sample extract of step 10 of the extraction procedure into separate columns instead of first combining and then loading them into one column.

Two laboratories found clumps of bacterial biomass powder in the respective tube and used only its finest part.

One laboratory heated briefly at 65˚C the TNE buffer to dissolve some precipitate,

One laboratory measured the DNA concentration of the DNA extracts from the feed sample via spectrophotometric instead of fluorometric means. DNA concentration resulted in evident overestimation.

Minute contaminations of the extraction control sample with traces of DNA extracts from the bacterial biomass were noted in several laboratories and were likely due to high powder volatility.

5.6. DNA concentration, yield and repeatability

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

Each DNA extract was measured in triplicate according to the laboratory procedure, lambda phage DNA at 100 ng/μL was used as a control.

Raw data were sent to the CRL-GMFF according to the instructions provided in the protocol and using the electronic tool provided (Excel spreadsheet).

The DNA concentration for all samples is reported in Table 5 below.

Table 5. DNA concentration (ng/μL) of the four replicate extracts obtained by all laboratories.

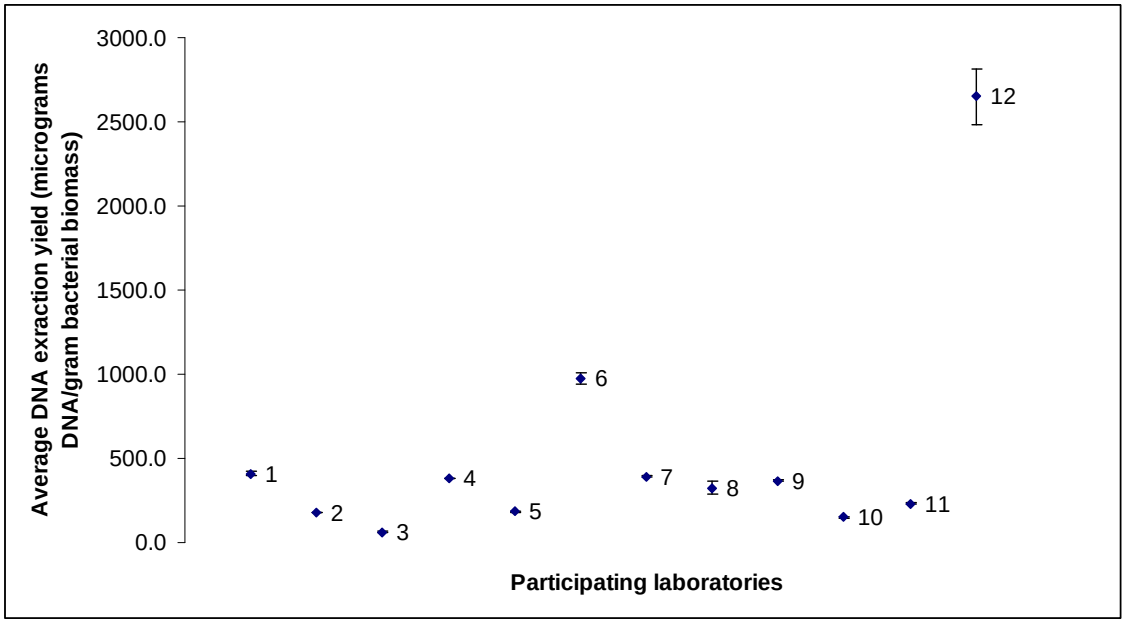
	PL1	PL2	PL3	PL4	PL5	PL6	PL7	PL8	PL9	PL10	PL11	PL12
EA	441	181	65	379	188	988	385	286	362	165	225	2446
EB	401	181	65	386	197	884	403	410	353	142	234	2712
EC	407	176	62	380	166	1014	391	231	373	144	244	3096
ED	385	171	63	375	178	1011	384	372	375	146	224	2347

Table 5 indicates that all laboratories were successful in extracting DNA from the four replicates of the PT73 (TM) feed sample.

The DNA extraction yield was calculated for each DNA extraction performed by the participating laboratories considering a total volume of 200 μL per extract and a sample amount of 200 mg as referred to in paragraph 3.5.

Figure 2 shows the data dispersion around the average for each laboratory.

Figure 2. Average yield of DNA extraction per participant



Blue circles represent the average of the laboratories mean yield. SD bars are indicated.

Two laboratories resulted in rather large DNA yield compared to the remaining participants. This could be either due to high nucleic acid extraction yield or to overestimation of DNA measurement in solution.

Following the determination of DNA concentration, the extracted DNA was diluted to the working concentration of 10 ng/μL as described in the protocol of the validated Real-time method (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). To test the accuracy of the detection method and its detection range (Validation Report, http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm), these samples were used to prepare four parallel dilution series.

Table 6 shows the Ct values for the first sample of the dilution series obtained for each DNA extract for all laboratories. Two dilution series were loaded on each single plate together with an internal positive control (IPC, provided by the CRL-GMFF) diluted to the same concentration of 10 ng/μL.

Table 6. Ct figures for DNA extracts, IPC and delta Ct for all laboratories

Lab	E1* mean	IPC*	Delta Ct	Lab	E1* mean	IPC mean	Delta Ct
1	21.23	20.48	0.76	7	21.93	21.27	0.66
	20.66		0.18		21.57		0.30
	21.07	20.89	0.18		21.92	21.59	0.33
	21.01		0.11		21.42		-0.17
2	21.12	21.41	-0.30	8	19.31	20.09	-0.78
	21.24		-0.18		20.25		0.16
	21.31	21.30	0.01		20.11	20.15	-0.04
	21.47		0.17		19.65		-0.50
3	24.44	23.05	1.39	9	20.32	20.32	0.00
	24.20		1.15		20.45		0.06
	23.18	22.37	0.81		20.38	20.37	0.01
	23.28		0.91		19.97		-0.39
4	21.15	20.75	0.41	10	22.50	23.89	-1.39
	21.11		0.36		23.20		-0.69
	21.09	20.79	0.30		21.70	23.33	-1.63
	21.14		0.35		22.64		-0.69
5	21.61	21.49	0.13	11	21.12	21.31	-0.19
	21.24		-0.25		20.82		-0.48
	21.72	21.56	0.16		21.15	21.34	-0.19
	21.43		-0.12		21.14		-0.20
6	21.47	19.89	1.58	12	24.60	23.00	1.60
	21.62		1.72		25.40		2.40
	22.20	20.48	1.72		25.44	22.82	2.62
	22.25		1.77		25.31		2.49

*Each Ct value is the mean of three replicates; Delta Ct according to the formula: $Ct_{extracts} - Ct_{IPC}$

These values clearly indicate that each DNA preparation ensued 100% of amplifiable DNA.

It was noted that in certain laboratories the absolute difference between the average Cts from the extracted DNA samples and the IPC titrated at the same total DNA amount per reaction (100 ng) was higher than 1, with DNA extracts showing greater Ct figures. These data suggest an overestimation of the DNA concentrations in the extracted samples and, as a consequence, an over-dilution of the DNA content into the final PCR reaction.

5.7. Assessment of method performance

In accordance with the performance criteria established by ENGL and adopted by the CRL-GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), the DNA extraction method has to be appropriate to obtain *i*) amplifiable DNA and *ii*) the amount of nucleic acid required for downstream analysis; in particular the DNA concentration should be higher than the working concentration described in the protocol of the validated method. In this case, the maximum working concentration tested is equivalent to 10 ng/μL (see protocol of validated method at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). This goal was achieved by all participating laboratories with no exception at a 100% success rate as shown in Tables 5 and 6.

Further elaboration of DNA extraction performance is summarised in Table 7.

Table 7. Mean values, RSDr and RSDR for DNA concentration

Number of laboratories having returned valid results	12
Samples per laboratory	4
Number of outliers	1
Reasons for exclusion	1C
Number of laboratories retained after eliminating outliers	11
Mean DNA concentration value	331.6
Relative repeatability standard deviation, RSD_r (%)	9.7
Repeatability standard deviation	32.2
Relative reproducibility standard deviation, RSD_R (%)	74
Reproducibility standard deviation	243.9

Following identification and removal of outlier laboratories, through Cochran and Grubbs tests based on the DNA concentration dataset, the mean value of DNA concentration was 330 ng/μL therefore in good agreement with the CRL-GMFF findings shown above. The intra-laboratory variability was shown to be quite modest indicating good repeatability among replicates (RSDr, ~ 10%); the inter-laboratory variability was however definitely large, accounting for a relative reproducibility standard deviation of 74%.

6. Conclusion

The data reported confirm that the extraction method, applied to samples of feed provided by the applicant, produces DNA of suitable quantity and quality for subsequent PCR based detection applications.

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

7. Quality assurance

The CRL-GMFF carries out all operations according to ISO 9001:2000 (certificate number: CH-32232) and ISO 17025:2005 (certificate number: DAC-PL-0459-06-00) [DNA extraction, qualitative and quantitative PCR in the area of Biology (DNA extraction and PCR method validation for the detection and identification of GMOs in food and feed materials)]

8. References

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