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Report on the In-house Validation of a DNA Extraction Method from the meat analogue Impossible™ burger

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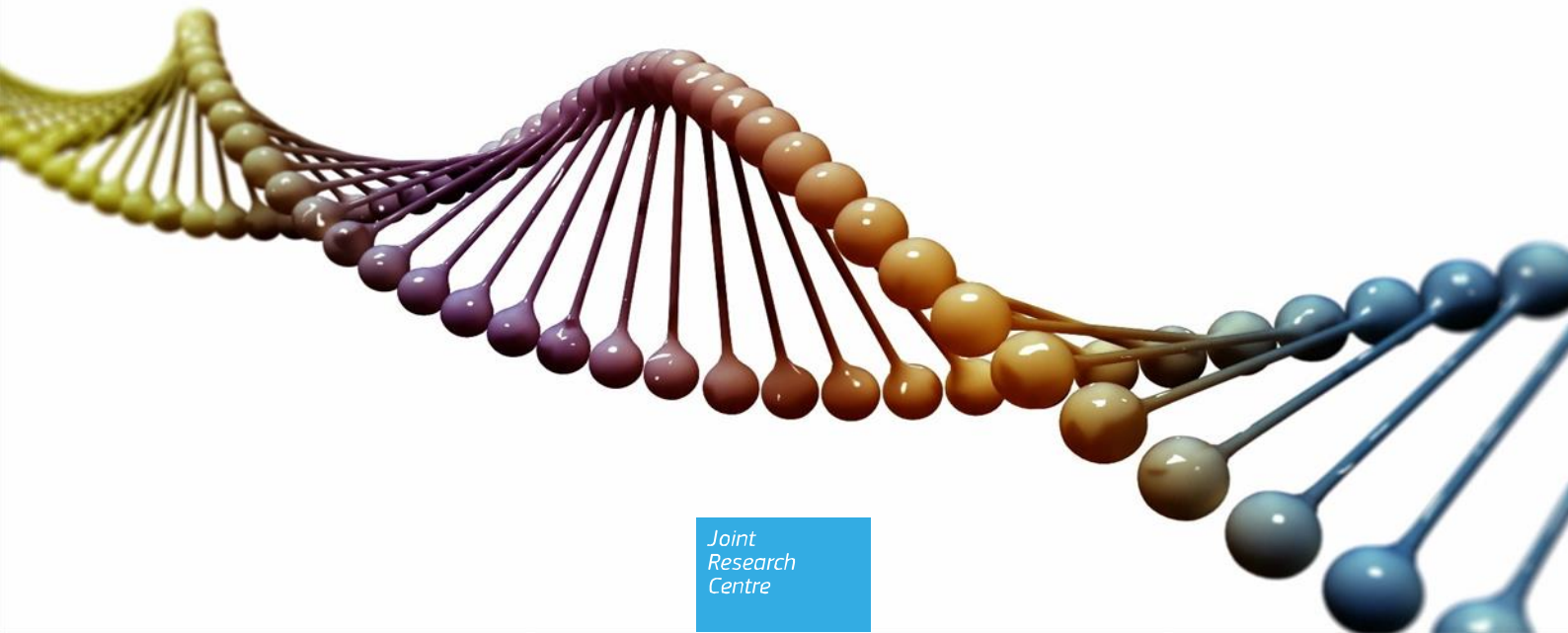

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Contents

Abstract

In accordance with relevant EU legislation, Impossible Foods Inc, represented by Intertek Scientific & Regulatory Consultancy, submitted to the European Union Reference Laboratory for GM Food and Feed (EURL GMFF) a DNA extraction method from Impossible™ burger meat analogue, developed by Biosafe – Biological Safety Solutions Ltd and the relevant samples.

In line with its mandate, the EURL GMFF conducted an in-house validation of this DNA extraction method. 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 ENGL, and that it satisfies the provisions of Annex III-3.C.2 to Regulation (EU) No 641/2004.

The method is therefore fit for the purpose of producing oilseed rape DNA of suitable quantity and quality for subsequent PCR-based analysis.

This report is published at https://gmo-crl.jrc.ec.europa.eu/method-validations.

Quality assurance

The EURL GMFF is ISO/IEC 17025:2017 accredited [certificate number: BELAC 268 TEST (Flexible Scope for determination of Genetically Modified content in % (m/m) and % (cp/cp) in food and feed by DNA extraction, DNA identification and Real-time PCR and for determination of Genetically Modified content in % (cp/cp) in food and feed by DNA extraction and digital PCR)].

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

Report on the In-house Validation of a DNA Extraction Method from the **meat analogue Impossible™ burger**

11 October 2024

European Union Reference Laboratory for GM Food and Feed

1 Introduction

The yeast *Komagataella phaffii* strain MxY0541 is genetically modified to produce soybean Leghemoglobin protein. Soy leghemoglobin is used in Impossible Foods' plant-based meat analogue products such as Impossible™ Burger. This report describes the validation of DNA extraction method based on a Wizard® Magnetic DNA Purification System for Food (Promega) of DNA followed by an anion exchange chromatography with commercially available columns (Promega Wizard® DNA Clean-Up System). This protocol can be used for the extraction of DNA from Impossible™ burger.

The purpose of the DNA extraction method described is to provide DNA with purity and quantity suitable for real-time PCR based detection methods.

It is advised to take particular notice of products safety recommendations and guidelines.

2 Materials (Equipment/Chemicals/Plastic ware)

2.1 Equipment

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

- Pipettes (Rainin-Mettler Toledo)
- Balances (Mettler Toledo XS2002S)
- Pestles and mortars
- Disposable rubber glovesCentrifuges (Eppendorf 5810R and 5415D)
- Vortex (MS1 Minishaker IKA)
- Incubator (for 50 mL tubes, 55 °C and 65 °C, with agitation) (Hybridization Incubator Combi-SV12-FINEPCR) or water bath with temperature options of 55 °C and 65 °C (any model appropriate)
- Freezer -20oC and Fridge 4oC (any model appropriate)
- Fume hood (any model appropriate)

2.2 Chemicals

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

- Wizard Magnetic DNA Purification System for Food kit (Promega FF3750)
- PolyATtract® System 1000 Magnetic Separation Stands (Promega Z5410)
- Glass beads acid washed 425-600 m (e.g., Sigma, G8772)
- Proteinase K (Sigma P2308)
- RNase A Solution, 4mg/ml (A7973 part of Promega kit)
- Isopropanol (Sigma I9516)
- Ethanol (Fluka 02860) diluted to 70% in nuclease free water (AMBION AM9937)
- Nuclease free water (AMBION AM9937)
- TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8)

2.3 Plasticware

- 50 mL conical tubes (BD 352098)
- 2.0 mL microcentrifuge DNA LoBind tubes (Eppendorf 0030108078)
- 1.5 mL microcentrifuge DNA LoBind tubes (Eppendorf 0030108051)
- Rainin filter tips for pipettes, aerosol-free

Note: all plastic ware should be sterile and free of DNases, RNases and nucleic acids.

2.4 Precautions

Consideration of notice of products and safety recommendations and guidelines is strongly recommended.

Chloroform, and Isopropanol and ethanol are hazardous chemicals; therefore, all manipulations have to be performed according to safety guidelines, under a fume hood.

Strictly separate working areas for DNA extraction, PCR set-up and amplifications are advised.

All equipment and lab benches should be free of DNA residues.

All tubes and pipette tips have to be discarded as biological hazardous material.

2.5 Abbreviations

PCR polymerase chain reaction

TE Tris EDTA

3 Description of the method

3.1 Scope and applicability

The method for DNA extraction described below is suitable for the isolation DNA from Impossible™ burger.

The sample matrices used were provided by Impossible Foods and are uncooked versions of their meat analogue products. The sample matrix is a processed food product, with most of the DNA being degraded.

3.2 Practicability

The primary downstream use of DNA from the Impossible™ burger meat analogue is for the real-time PCR-based qualitative detection method. Therefore, the preparation of DNA from the matrix sample should take place in an area dedicated for this purpose. All equipment (e.g. pipettes, centrifuges), lab ware and reagents used in this process should be stored and used only in the dedicated area. The entire procedure, after melting the burger patties, takes about 3-4 hours.

3.3 Principle

According to information reported by the applicant, the Wizard® Magnetic DNA Purification System for Food uses paramagnetic particles for DNA isolation. Binding of nucleic acids to magnetic particles can occur in solution, resulting in increased binding and efficiency.

Initially, a one-gram sample is homogenized and then 500 µL of glass beads and 2.5 mL of Lysis Buffer A are added. The mixture is vortexed for two minutes to ensure thorough mixing. Next, 25 µL of RNase A is introduced, and the sample is incubated for five minutes at room temperature to enhance the lysis process. Afterward, proteinase K is added to achieve a final concentration of 200 µg/mL, and the sample is incubated for an additional hour at 55 ºC. Finally, 1.25 mL of Lysis Buffer B is added, and the DNA extraction is completed according to the manufacturer's instructions.

The application states that the proposed DNA extraction method is based on some criteria: the method should provide adequate results from processed food samples and yeast; it should also fit to other sample matrices from the same category (plant-based meat replacement products) potentially subject for testing; the sample treatments and processing times are easily completed during in one work day; and the cost per sample should not be considerably higher than with other methods.

According to the applicant, the Wizard® Magnetic DNA Purification System for Food (Promega) purifies DNA from a variety of food samples including corn seed, cornmeal, soybean, soya flour and soya milk and it is also widely applicable for highly processed food. The variety of sample matrices covers the sample matrix.

3.4 Grinding

Grinding of the meat analogue not only facilitates the lysis by mechanically disrupting cellular structures and increasing the surface area, but is also indispensable for the generation of representative test portions by reducing the particle size.

3.5 DNA extraction protocol

1. After melting, a burger sample is grinded and homogenized with a mortar and pestle for around three minutes.

2. From each homogenized sample, collect two subsamples of 1 gram (1000 ±10 mg) in 50 mL tubes.

3. Add ~ 500 µL of glass beads (Sigma, G8772) and 2.5 mL of Lysis Buffer A in each tube and vortex for two minutes.

4. Add 25 µL of RNase A to each sample. Cap the tube and mix by vortexing. Incubate for 5 minutes at room temperature.

5. Note the volume in the tubes and add proteinase K to a final concentration of 200 µg/mL. Incubate the tubes at 55 ºC for 1 hour.

6. Add 1.25 mL of Lysis Buffer B and vortex for 10–15 seconds to mix.

7. Incubate for 10 minutes at room temperature.

8. Add 3.75 mL of Precipitation Solution, then vortex vigorously.

9. Spin the tubes for a suitable time and speed to achieve separation, such as 1780 x g for 30 minutes.

10. Carefully transfer the supernatant to a fresh 50-mL tube (preferably using 1000 µL pipette). Around 5 mL of supernatant can be separated from each sample.

11. Vigorously mix the bottle of MagneSil PMPs for 15–30 seconds. Add 100 µL of resuspended MagneSil PMPs to the supernatant. Vortex the tube vigorously. Note the volume of liquid in the tube.

12. Add 0.8 volume of isopropanol and invert the tube 10–15 times to mix. Incubate for 5 minutes at room temperature (22–25°C) with occasional mixing. Insert tubes into a PolyATtract System 1000 Magnetic Separation Stand until beads separate (usually takes 3 minutes). Discard the liquid phase by pipetting.

13. Remove the tube from the stand and add 1.25 mL of Lysis Solution B to the particles. Invert the tube 2–3 times to mix, washing the inner surfaces of the tube as well as the particles. Replace the tube on the Magnetic Separation Stand, and allow the particles to separate, then discard the liquid phase by pipetting.

14. Resuspend the particles in 5.0 mL of 70% ethanol wash solution. Place the tube back on the Magnetic Separation Stand and allow the particles to separate. Discard the liquid phase by pipetting.

15. Repeat Step 14 twice for a total of 3 washes. After the final wash, remove and discard as much of the liquid phase as possible using a pipette.

16. Dry the particles at room temperature for 30 minutes.

17. Add 100 µL of TE buffer or Nuclease-Free water, vortex to mix and incubate at 65°C for 5 minutes. Insert the tube in the Magnetic Separation Stand for 1 minute. Collect the DNA by leaving the tube in the stand and carefully transferring the liquid phase to a fresh tube.

4 Testing of the DNA extraction method by the EURL GMFF

The EURL GMFF tested the method submitted by the applicant and described above on samples consisting of meat analogue, raw Impossible Burger patty, provided by the applicant. DNA extraction procedures should result in repeatable recovery, fragmentation profile, concentration and DNA extracts suitable for PCR analysis.

The extracted DNA should be of suitable quantity and quality for the intended purpose[1].

4.1 Preparation of samples

Samples of raw Impossible™ Burger meat analogue with leghemoglobin and samples of raw Impossible Burger without leghemoglobin were received from the applicant and used for DNA extraction. The extraction method was validated in-house on the matrix with leghemoglobin and further verified on the meat analogue matrix without leghemoglobin.

4.2 DNA extraction

The matrix for the DNA extraction was the raw Impossible Burger patty. The in-house validation of the DNA extraction method was carried out on six test portions (replicates) of the meat analogue following the method described in paragraph 3.5 "DNA extraction protocol". The procedure was repeated in three days, for a total of 18 DNA extractions. The performance of the procedure was also verified on 6 test portions of the meat analogue without leghemoglobin.

4.3 DNA concentration, yield and repeatability

The concentration of the extracted DNA solutions was determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Invitrogen) and a Biorad VersaFluor fluorometer. DNA concentrations were established on the basis of a five point standard curve ranging from 1 to 500 ng/µL. Each DNA extract was measured three times and the three values were averaged. The recovered volume was 60 µL per each DNA extract.

Table 1 reports the DNA concentration, using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen by Thermo Fisher Scientific #P7589), for the 18 extracted samples from the raw meat analogue with leghemoglobin and the 6 extracted samples from the raw meat analogue without leghemoglobin. The yield per each extraction was estimated based on the recovered volume of 60 µL per extract.

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

Table 1. DNA concentration (ng/µL) and yield (µg DNA / gram of sample intake) of extracted samples

	DNA extract	Concentration **(ng/µL)**	Yield **(µg)**
Raw Impossible™ burger supplemented with Leghemoglobin	1	2.58	0.15
	2	2.73	0.16
	3	2.81	0.17
	4	2.35	0.14
	5	3.12	0.19
	6	2.35	0.14
	7	4.13	0.25
	8	4.59	0.28
	9	2.96	0.18
	10	3.97	0.24
	11	3.12	0.19
	12	4.43	0.27
	13	2.42	0.15
	14	2.65	0.16
	15	2.65	0.16
	16	2.96	0.18
	17	2.50	0.15
	18	2.35	0.14
Impossible™ burger without leghemoglobin	19	1.03	0.06
	20	1.03	0.06
	21	1.03	0.06
	22	1.03	0.06
	23	0.95	0.06
	24	1.03	0.06

Samples from the raw meat analogue supplemented with leghemoglobin: yellow boxes, DNA samples extracted on day 1; green boxes, DNA samples extracted on day 2; blue boxes, DNA samples extracted on day 3.

Samples from the raw meat analogue without leghemoglobin: pink boxes

The average DNA concentration and yield for all extracted samples are reported in Table 2A and Table 2B below.

Table 2A. Concentration and yield of the DNA extraction procedure on the raw meat analogue supplemented with leghemoglobin

DNA concentration, mean (ng/µL)	3.0
Coefficient of variation (%)	24
Yield (µg DNA / g sample intake)	0.18

In-house validation based on eighteen extracted samples

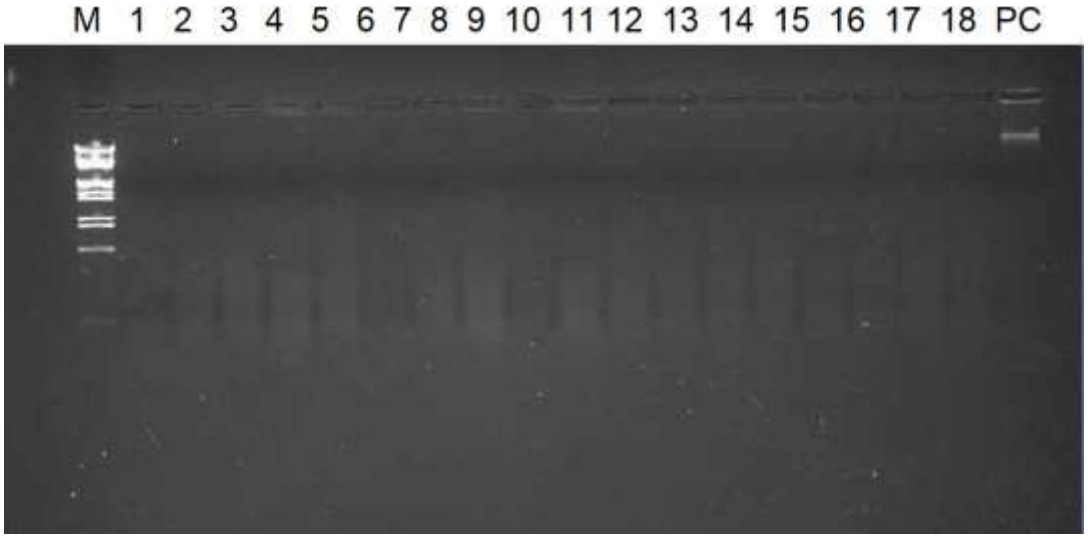
Table 2B. Concentration and yield of the DNA extraction procedure on the raw meat analogue without leghemoglobin

DNA concentration, mean (ng/µL)	1.0
Coefficient of variation (%)	3.3
Yield (µg DNA / g sample intake)	0.061

Verification based on six extracted samples

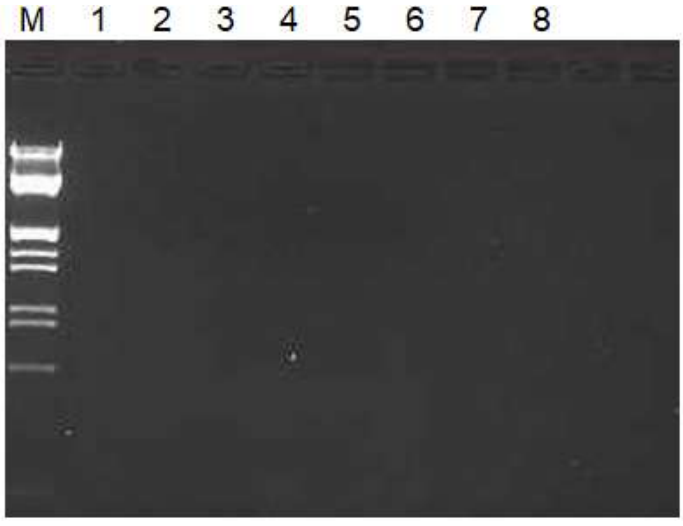
The size of the DNA extracted was evaluated by analysis on electrophoresis. For each sample 5 µL of DNA extract were loaded in a 1.0% agarose gel. (Figure 1A and 1B).

Figure 1A. Agarose gel electrophoresis of 18 extracted samples from the raw meat analogue with leghemoglobin



Lanes 1-6: samples extracted on day 1, lanes 7-12: samples extracted on day 2, lanes 13-18: samples extracted on day 3; M: Lambda DNA/EcoRI+HindIII marker 3 (Thermofisher, SM0193), PC= positive control, plant genomic DNA.

Figure 1B. Agarose gel electrophoresis of six genomic DNA samples extracted from raw meat analogue without leghemoglobin.



Lanes 1-6: samples extracted from raw meat analogue without leghemoglobin. Lanes 7: blank DNA extraction controls; Lane 8: environmental control; M: Lambda DNA/EcoRI+HindIII marker 3 (Thermofisher, SM0193).

The extracted DNA from the Impossible™ burger is degraded as expected from a processed material, thus confirming the findings of the applicant.

4.5 Purity / Absence of PCR inhibitors

In order to assess the purity and to conduct a test for the presence of PCR inhibitors, the extracted DNA solutions were used as "undiluted" samples). The six samples extracted from raw meat analogue without leghemoglobin were spiked with 500 pg of purified DNA from K. phaffi event MxY0541.

Subsequently, four-fold serial dilutions (1:4, 1:16, 1:64, 1:256) of each extract were prepared with TE low buffer (1 mM Tris, 10 µM EDTA, pH 8.0). Each sample was amplified in duplicate using a real-time PCR method detecting the target sequence of the MxY0541 event.

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 of MxY0541.

	Cq values					
	DNA extract	Undiluted	Diluted extracts			
			1:4	1:16	1:64	1:256
Raw Impossible™ burger supplemented with Leghemoglobin	1	26.73	28.77	30.89	32.61	34.86
	2	26.87	28.96	30.79	32.84	34.90
	3	26.87	28.83	30.99	32.58	34.55
	4	26.81	28.73	30.68	32.67	34.49
	5	26.70	28.69	30.86	32.46	34.75
	6	27.30	29.33	30.96	33.18	34.87
	7	28.38	30.46	32.46	34.20	36.17
	8	27.65	29.75	31.55	33.54	35.76
	9	27.43	29.28	31.24	33.37	35.07
	10	27.37	29.27	31.33	33.10	35.05
	11	27.36	29.27	31.32	33.15	35.50
	12	26.92	28.73	30.73	32.61	34.45
	13	26.92	28.89	30.77	32.80	35.34
	14	26.64	28.75	30.69	32.79	34.94
	15	26.81	28.79	30.78	32.57	34.76
	16	26.82	28.78	30.73	32.38	35.04
	17	27.19	29.21	30.96	32.86	34.98
	18	26.70	28.67	30.74	33.12	34.71
Impossible™ burger without leghemoglobin	1	24.51	26.47	28.44	30.59	32.63
	2	24.50	26.33	28.30	30.23	32.18
	3	24.47	26.34	28.33	30.20	32.24
	4	24.51	26.48	28.40	30.26	32.42
	5	24.46	26.27	28.21	30.14	32.31
	6	24.48	26.28	28.24	30.14	32.13

- Samples from the raw meat analogue supplemented with leghemoglobin: yellow boxes, DNA samples extracted on day 1; green boxes, DNA samples extracted on day 2; blue boxes, DNA samples extracted on day 3.
- Samples from the raw meat analogue without leghemoglobin: pink boxes.

To check for inhibition the Cq values of the four diluted samples were plotted against the logarithm of the dilution and the Cq values for the "undiluted" samples were extrapolated from the equation calculated by linear regression.

Subsequently, the extrapolated Cq values for the "undiluted" samples were compared with the measured Cq data. It is assumed that PCR inhibitors are present if the measured Cq value for the "undiluted" sample is > 0.5 cycles from the calculated Cq value. Table 4 below reports the comparison of extrapolated Cq values versus measured Cq values for all samples and the values of R^2 and slope of all measurements.

Table 4. R^2 and slope values of the dilution series used to estimate the presence of inhibitors (inhibition runs) for each DNA extract. Comparison of extrapolated Cq values versus measured Cq values.

	DNA extraction	R2	Slope	Cq extrapolated	Mean Cq measured	*ΔCq
Raw Impossible™ burger supplemented with Leghemoglobin	1	0.99	-3.29	26.82	26.73	0.09
	2	0.99	-3.30	26.91	26.87	0.04
	3	0.99	-3.11	27.06	26.87	0.19
	4	1.00	-3.20	26.83	26.81	0.02
	5	1.00	-3.28	26.75	26.70	0.05
	6	0.99	-3.14	27.36	27.30	0.06
	7	0.98	-3.14	28.60	28.38	0.22
	8	1.00	-3.28	27.69	27.65	0.03
	9	0.99	-3.27	27.33	27.43	0.10
	10	1.00	-3.17	27.41	27.37	0.04
	11	0.99	-3.37	27.22	27.36	0.14
	12	0.99	-3.16	26.87	26.92	0.06
	13	0.99	-3.55	26.60	26.92	0.31
	14	1.00	-3.42	26.64	26.64	0.00
	15	0.99	-3.27	26.80	26.81	0.01
	16	0.99	-3.39	26.62	26.82	0.20
	17	1.00	-3.19	27.20	27.19	0.01
	18	0.99	-3.40	26.69	26.70	0.01
Impossible™ burger without leghemoglobin	1	1.00	-3.43	24.37	24.51	0.14
	2	1.00	-3.24	24.39	24.50	0.11
	3	1.00	-3.25	24.39	24.47	0.08
	4	1.00	-3.27	24.47	24.51	0.04
	5	1.00	-3.33	24.22	24.46	0.23
	6	1.00	-3.23	24.33	24.48	0.15

- Samples from the raw meat analogue supplemented with leghemoglobin: yellow boxes, DNA samples extracted on day 1; green boxes, DNA samples extracted on day 2; blue boxes, DNA samples extracted on day 3.
- Samples from the raw meat analogue without leghemoglobin: pink boxes.
- *ΔCq = abs (Cq extrapolated - Cq measured)

The table indicates that all ΔCq values of extrapolated versus measured Cq are < 0.5. The R^2 coefficient of linear regression is ≥ 0.98 for all DNA samples and the slopes of the curves are between -3.1 and -3.6 for all samples. Therefore, all the samples were amplified despite the matrix processing, the low molecular weight of the extracted DNA; the limited yield did not make it practical to monitor the average size of DNA fragments via migration in agarose gel electrophoresis. No inhibition was detected in the extracted DNA samples from the raw Impossible™ burger supplemented with leghemoglobin or without it.

5 Conclusions

The results confirm that the DNA extraction method from samples of the raw meat analogue supplemented with leghemoglobin produces DNA of suitable quantity and quality for subsequent PCR-based analyses.

List of abbreviations and definitions

EURL GMFF	European Union Reference Laboratory for GM Food and Feed
PCR	Polymerase chain reaction
ENGL	European Network of GMO Laboratories

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