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Event-specific Method for the Detection and Identification of K. phaffii event MxY0541 Using Real-time PCR

Validation Report

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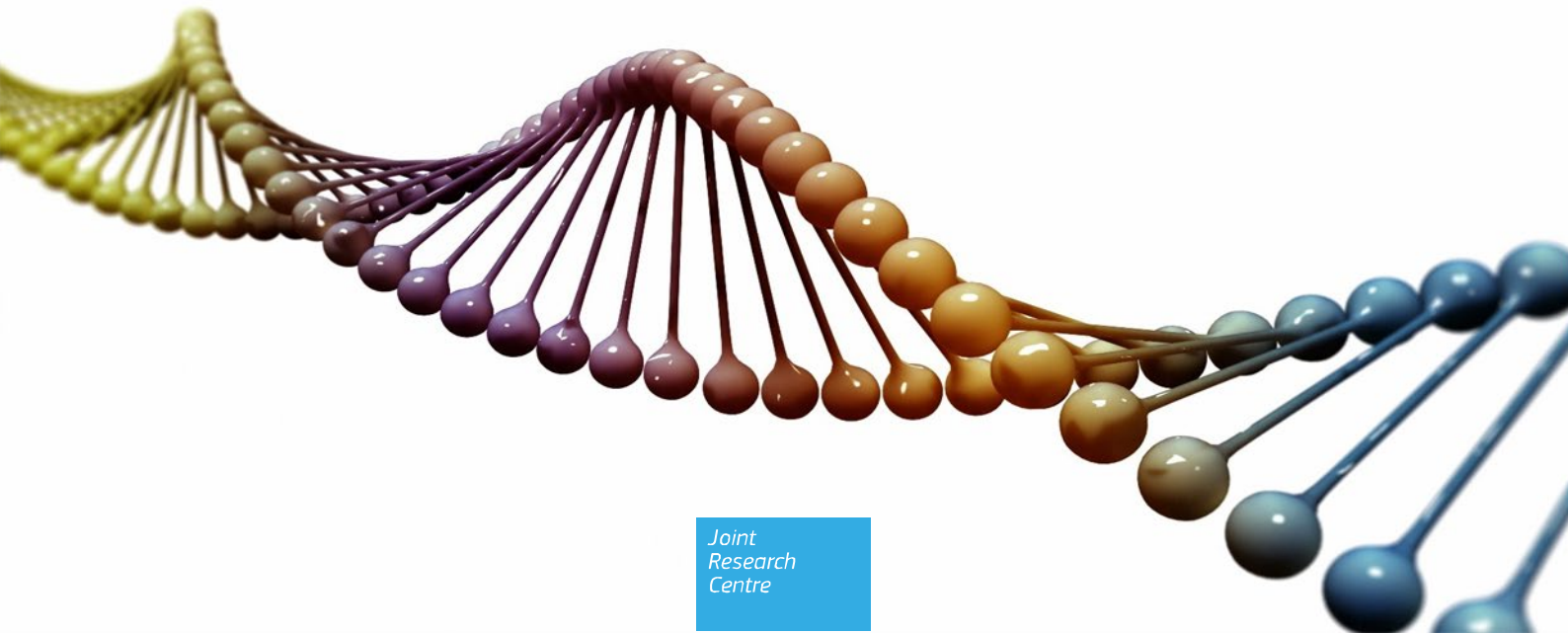
2024



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Contents

Abstract

In line with its mandate (1[1]) the European Union Reference Laboratory for GM Food and Feed (EURL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), validated an event-specific real-time polymerase chain reaction (qPCR) method for detection and identification of genetically modified yeast *K. phaffii* strain MxY0541 (unique identifier IF-KPØ541-7). The validation study was conducted according to the EURL GMFF validation procedure https://gmo-crl.jrc.ec.europa.eu/guidance-documents and the relevant internationally accepted guidelines (2-6).

An application for authorisation under Regulation (EC) No 1829/2003 was submitted by Impossible Foods Inc. for the use of soy leghemoglobin produced from genetically modified *Pichia pastoris*, also referred to as *Komagataella phaffii*, as a flavouring ("meaty taste") in meat analogue products.

In accordance with current EU legislation (1), Impossible Foods Inc., represented by Intertek Assuris, provided a DNA extraction method from meat-analogue burger and the real-time PCR detection method with the positive and negative control samples (genomic DNA from *Komagataella phaffii* strain MxY0541 as positive control DNA, genomic DNA from conventional yeast *K. phaffii* CBS 7435 as negative control DNA and genomic DNA from *K. pastoris*). Based on the information provided by the applicant, a conventional counterpart of the food product is not available on the market. A traceability method was submitted for detection and identification of *K. phaffii* event MxY0541. The EURL GMFF verified the data on method performance provided by the applicant, where necessary experimentally, prepared the validation samples for the determination of the limit of detection, of the false negative and of the false positive rate, organised an international collaborative study and analysed the results.

The EURL GMFF in-house verification and the collaborative study confirmed that the method meets the method performance requirements for qualitative methods as established by the EURL GMFF and the ENGL (6), therefore the method is applicable to the control samples provided by the applicant (see paragraph 5.1), in accordance with the requirements of Annex 1.2.C.2 to Commission Regulation (EC) No 641/2004. This validation 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.

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11/10/2024

European Union Reference Laboratory for GM Food and Feed

1 Introduction

An application for authorisation under Regulation (EC) No 1829/2003 was submitted by Impossible Foods Inc. for the use of soy leghemoglobin produced from genetically modified *Pichia pastoris*, also referred to as *Komagataella. phaffii* (hereinafter *K. phaffii*), as a flavouring ("meaty taste") in meat analogue products.

In line with Regulation (EC) No 1829/2003 (1), Impossible Foods Inc., represented by Intertek Assuris provided the EURL GMFF with an event-specific method for detection and identification of yeast *K. phaffii* GM strain MxY0541 (unique identifier IF-KPØ541-7) together with genomic DNA as positive and negative control samples.

The applicant stated that "the *K. phaffii* strain MxY0541 is genetically modified to produce soybean leghemoglobin protein. Soybean leghemoglobin is used in Impossible Foods' plant-based meat analogue product, e.g. Impossible™ Burger. Soy leghemoglobin is delivered in a preparation (LegH Prep) that consists of soy leghemoglobin protein, water, proteins (i.e. cellular proteins expressed by *K. phaffii*), residual DNA from *K. phaffii,* and added stabilizers. The soy leghemoglobin protein cannot be isolated from the *K. phaffii* cellular lysate without denaturing the protein and causing a loss of flavouring properties; thus, the final ingredient contains no viable cells but does contain residual *K. phaffii* DNA". For the reason above, the applicant submitted a method for detection of the transformation event under Regulation (EC) 1829/2003. The EURL GMFF, further to consultation with DG SANTE E.3, accepted a qualitative detection method based on the following statement by the applicant: "no relative quantification is possible for such a product because any DNA present from the GM-strain *K. phaffii* in foodstuff MxY0541 is always 100 % GM and cannot be a potential mixture of *K. phaffi* MxY0541 with wild-type *K. phaffi*; *K. phaffii* is not viable in the product for which authorisation is requested".

The EURL GMFF informed the applicant that any change in the present knowledge about this information should be promptly communicated.

In case a conventional counterpart would become available on the market, a quantitative detection method may become necessary.

The traceability method consists of a DNA extraction procedure from the food matrix Impossible™ Burger containing leghemoglobin and a real-time PCR method targeting the junction between the insert and the yeast genome . The method was found complete (step 1 of the EURL GMFF validation procedure). The scientific assessment (step 2) was conducted in line with the "Definition of minimum performance requirements for analytical methods of GMO testing - Part 1", (ENGL, 2015) for a qualitative real-time PCR based method (§ 3.1 and 3.4).

The assessment concluded that the reported method performance characteristics were in compliance with the ENGL method acceptance criteria (8) and allowed moving the method forward to step 3 of the procedure (experimental testing), where the EURL GMFF verified the purity of the control samples provided and conducted an in-house testing of samples and method.

The positive and negative control DNA, submitted in accordance with Art 5(3)(j) and Article 17(3)(j) of Regulation (EC) No 1829/2003, were found of good quality.

Step 3 was completed with the conclusion that the method could be submitted to the collaborative study (step 4). This study confirmed that the method had sufficient sensitivity for the detection of DNA of GM *K. phaffii event MxY0541*, and that it could be applied to DNA extracted from the food matrix.

The preparation of the report (step 5) was aligned with the timeline communicated by EFSA for its risk assessment.

2 Dossier reception and acceptance (step 1)

Impossible Foods represented by Intertek Assuris submitted the detection and identification method, with data to demonstrate its adequate performance when applied to genomic DNA from yeast *K. phaffii* MxY0541.

The dossier was found to be complete and was thus moved to step 2.

3 Scientific assessment and bioinformatics analysis (step 2)

Documentation and data supplied by the applicant were evaluated by the EURL GMFF for compliance with the ENGL method acceptance criteria.

The specificity of the event-specific method was verified by the applicant and confirmed by the EURL GMFF by means of bioinformatics analysis, on the basis of the sequence data provided by the applicant.

3.1 Specificity assessment conducted by the applicant

The specificity of the event-specific method was assessed by the applicant in duplicate real-time PCR reactions containing 2500 copies of each non-target DNA, according to the method described in Annex 1 (Tables 1, 2, 3, 4 and 5), using genomic DNA extracted from: *K. phaffii* MxY0541, the non-modified *K. phaffii* CBS 7435 (i.e., the recipient strain), the closest related taxa *Komagataella pastoris* (strain DSM 70382) and genomic DNA from plant for which GMOs exist on the market, that is conventional maize, soybean, canola, cotton, rice, potato and sugar beet, and from soybean events FG72 and A2704-12. No amplification was recorded from any of the non-target organisms.

The applicant declared that all the PCR reactions were then repeated in duplicate with 2500 copies of each non-target DNA and 100 copies of target DNA (corresponding to 1 pg DNA of *K. phaffii* MxY0541) in order to exclude possible inhibition. The spiking resulted in consistent amplification in all the spiked reactions with Cq value ranging between 33.45 and 34.42.

The specificity of the real-time PCR method was also assessed against DNA extracted from different food matrices Tofu, Beanit® vegetable protein product and minced meat (60% beef, 40% pork; 20% fat). The applicant informed that no amplification was reported, except when 100 copies of target DNA (1 pg DNA of *K. phaffii* MxY0541) was spiked in reaction.

The specificity of the primers and probe and the target DNA template was evaluated by *in silico* analysis. The DNA sequences and flanking regions were analysed by BLAST searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the absence of similar sequences in public databases. In addition, the JRC detection method matrix and GMO event finder were applied (https://gmo-crl.jrc.ec.europa.eu/jrcgmomatrix/) to exclude the possibility to similar GMO-constructs. The search of the 130 bp amplicon produced with primers T1318_F10 and T1318_R10 resulted in no hits from the GMO-matrix database.

The PCR primer specificity was evaluated using primer-BLAST searches (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) against the nr-database. The tests were conducted against bacteria, yeasts and the organisms currently having GMO approvals in the EU market area. The search included nr-database organisms limited to (Bacteria (taxid:2), yeasts (*Komagataella phaffii* GS115 (taxid:644223), *Komagataella pastoris* (taxid:4922), *Komagataella* (taxid:460517), *Pichia* (taxid:4919) and *Saccharomycetes* (taxid:4891)), GM plant genomes from maize (*Zea mays*; taxid:4577), cotton (*Gossypium hirsutum*; taxid:3635), soybean (*Glycine max*; taxid:3847), potato (*Solanum tuberosum*; taxid:4113), sugarbeet (*Beta vulgaris* subsp. *vulgaris*; taxid:3555), rice (*Oryza sativa*; taxid:4530), human (*Homo sapiens*; taxid:9606), beef (*Bos*; taxid:9903) and pork (*Suidae*; taxid:9821) and no target templates were found in the selected databases.

3.2 Specificity assessment conducted by the EURL GMFF

The detection method spans the 3' insert-to-yeast junction in *K. phaffii* MxY0541. The MxY0541-R10 binding site was found in the a region annotated by the applicant as the insert, the MxY0541-P10 probe anneals on the insert-to-yeast junction, the MxY0541-F10 primer lays in the 3'-flanking region of *K. phaffii*.

The amplicon size is expected to be 130 bp, consistent to what reported by the applicant. The sequence of the amplicon was analysed by BLAST (NCBI) against local copies of the "nt" and "patents" databases.

Only partial matches of the amplicon sequence were found to entries of both databases.

The amplicon was also blasted against the NCBI Refseq prokaryote and Univec database with no significant hits.

In addition, primers were tested against the sequences of the other GMO events present in the Central Core Sequence Information System (CCSIS) of the JRC, as well as the whole genomes of more than 500 plants (including *Brassica rapa, Glycine max, Oryza sativa, Solanum lycopersicum and Zea mays*) using the e-PCR prediction tool (NCBI). No full length alignment was found with GMO sequences stored in the CCSIS, except for event MxY0541. A limited number of hits were found against plant species genomes but with mismatches and gaps in the putative primers annealing sites. To investigate whether the probe could anneal to the potential amplicons that were predicted in several plant species, the amplicon sequences were extracted and analysed using EMBOSS matcher (version 6.6.0). In one case (*H. bulbosum*) the amplicon was predicted with the forward and reverse primers of the MxY0541 method (primer pair 1 x 2); in all other cases, the amplification was predicted considering twice the forward primer or twice the reverse primer (Table 1).

Table 1. Summary of the potential amplifications predicted in plant species checked for the binding of the probe

Assembly Accession	Organism name	Sequence Genbank ID	Start	End	Length (bp)	Strand	Primer pair
GCA_963506655.1	*Hordeum bulbosum*	OY737390.1	357317820	357318949	1129	+	1 x 2
GCA_001447015.2	*Pinus lambertiana*	LMTP010212639.1	114801	115271	470	+	1 x 1
GCA_001447015.2	*Pinus lambertiana*	LMTP010212639.1	114801	115271	470	-	1 x 1
GCA_001447015.2	*Pinus lambertiana*	LMTP010338216.1	621	710	89	+	1 x 1

GCA_001447015.2	*Pinus lambertiana*	LMTP010338216.1	621	710	89	-	1 x 1
GCA_002284615.2	*Dunaliella salina*	MU069835.1	103160	103970	810	+	1 x 1
GCA_002284615.2	*Dunaliella salina*	MU069835.1	103160	103970	810	-	1 x 1
GCA_014633365.1	*Diospyros lotus*	CM025703.1	21020096	21020742	646	+	1 x 1
GCA_014633365.1	*Diospyros lotus*	CM025703.1	21020096	21020742	646	-	1 x 1
GCA_001447015.2	*Pinus lambertiana*	LMTP010451228.1	27855	28478	623	+	2 x 2
GCA_001447015.2	*Pinus lambertiana*	LMTP010451228.1	27855	28478	623	-	2 x 2
GCA_001447015.2	*Pinus lambertiana*	LMTP010555939.1	1076	1723	647	+	2 x 2
GCA_001447015.2	*Pinus lambertiana*	LMTP010555939.1	674	1723	1049	+	2 x 2
GCA_001447015.2	*Pinus lambertiana*	LMTP010555939.1	1076	1723	647	-	2 x 2
GCA_001447015.2	*Pinus lambertiana*	LMTP010555939.1	674	1723	1049	-	2 x 2
GCA_001447015.2	*Pinus lambertiana*	LMTP010642365.1	966	1399	433	+	2 x 2
GCA_001447015.2	*Pinus lambertiana*	LMTP010642365.1	966	1399	433	-	2 x 2
GCA_001447015.2	*Pinus lambertiana*	LMTP010683820.1	1776	2541	765	+	2 x 2
GCA_001447015.2	*Pinus lambertiana*	LMTP010683820.1	1776	2541	765	-	2 x 2
GCA_009746045.1	*Mesostigma viride*	RPF001000384.1	652095	652478	383	+	2 x 2
GCA_009746045.1	*Mesostigma viride*	RPF001000384.1	652095	652478	383	-	2 x 2

According to this *in silico* analysis, none of the amplicons was predicted to anneal to the method probe considering a threshold of 80% identity.

An analysis was then conducted to understand whether the proposed primers could find multiple annealing sites in *K. phaffii* event MxY0541. It was found that four amplicons are potentially generated. The first, of 130 nt, is the event-specific amplicon. The second potential amplicon shows perfect primers and perfect probe match to the target and thus it could be possibly detected if amplified; however, the expected length (1315 bp) is significantly large for a reliable concurring reaction. Further to a request for information, the applicant explained that agarose gel electrophoresis and SYBR Green PCR were both carried out in the context of the method development project to confirm the amplification of the correct-sized amplicon and the absence of other products resulting in no visible amplification products other than the 130 bp product (primer pair F10 and R10). A similar result was obtained with a melting curve analysis. The third and fourth potential amplicons are larger than the second predicted amplicon and with no probe annealing site.

An *in silico* analysis was performed to verify whether the PCR method targeting the MxY0541 event could produce amplification in the wild-type strain CBS 7435 that was used to generate the GM-yeast. A simulation with e-PCR was performed (e-PCR version: 2.3.12; e-PCR parameters: -n 2 -g 2 -f 3 -t 4 -m 1000 -d 20-1000). These parameters allow for a maximum of 2 mismatches and 2 gaps per primer, and a size range of 20-1000 bp for the amplicon. No amplification was predicted. The analysis was repeated allowing for 3 mismatches and 3 gaps per primer, and no amplification was predicted. Therefore, the genome of the *K. phaffii* strain CBS 7435 does not include any sequence that could potentially be amplified with the primers of this method. Moreover, this genome does not contain any sequence similar to the full amplicon sequence of the method targeting this event.

The analysis was further extended to the Genbank genomes of *Komagataella* and *Pichia* species with quality "chromosome" or "complete" available on NCBI. (Table 2).

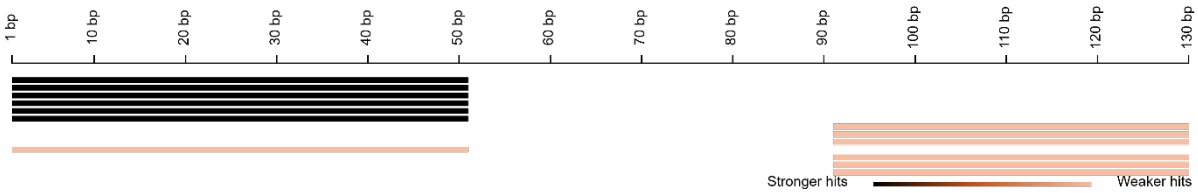
Table 2. Genbank genomes of *Komagataella* and *Pichia* species with quality "chromosome" or "complete" (01/07/2024)

Assembly Accession	**Assembly Name**	**Organism Name**
GCA_003054445.1	ASM305444v1	*Pichia kudriavzevii*
GCA_030168465.1	ASM3016846v1	*Pichia terricola*
GCA_000027005.1	ASM2700v1	*Komagataella phaffii GS115*
GCA_030062975.1	ASM3006297v1	*Pichia kluyveri*
GCA_001708105.1	ASM170810v1	*Komagataella pastoris*
GCA_003054405.1	ASM305440v1	*Pichia kudriavzevii*
GCA_014873065.1	ASM1487306v1	*Pichia kudriavzevii*
GCA_003033855.1	ASM303385v1	*Pichia kudriavzevii*

GCA_900235035.2	KP_7435-4	*Komagataella phaffii CBS 7435*
GCA_000223565.1	PicPas_Mar2011	*Komagataella phaffii CBS 7435*
GCA_001708085.1	ASM170808v1	*Komagataella phaffii*
GCA_001746955.1	ASM174695v1	*Komagataella phaffii GS115*
GCA_029632965.1	ASM2963296v1	*Komagataella pastoris*
GCA_031179465.1	ASM3117946v1	*Pichia kudriavzevii*

The genomes were locally downloaded and analysed with blastn (Blast version: 2.12.0; e-value < 10^{-5}) looking for matches to the expected amplicon sequence of MxY0541. Although some partial alignments were found with the 5' end or with the 3' end of the amplicon, no chromosome sequence had matches to both ends or to the central part of the amplicon.

Figure 1. Results of the blastn analysis



Finally, the MxY0541 contains genetic modifications at different chromosomes. Further to a request for clarifications on the possible segregation, the applicant informed that the production strain (*K. phaffi*) is not viable in the final product (burger) and is therefore not able to escape to the environment.

3.3 Experimental Specificity conducted by the EURL GMFF

A DNA sample of *Komagataella pastoris* DSM 70382 (from the Leibniz Institute DSMZ) was indicated by the applicant as a closely related species and its DNA was supplied upon request for confirmatory specificity testing.

Therefore, an experiment was set out to verify the specificity of the MxY0541 detection method. For each of three different samples, ten replicates were analysed: the untargeted DNA of DSM 70382 and of the conventional yeast *K. phaffii* were tested at 2500 copies per reaction; the target DNA of strain MxY0541 was tested at 50 copies per reaction. Each reaction was stabilised with salmon sperm DNA (100 ng/reaction), All the replicates of the untargeted DNA samples (i.e. DSM 70382 and the conventional yeast *K. phaffii* DNA) resulted negative, while all the replicates of the positive control MxY0541 DNA resulted positive.

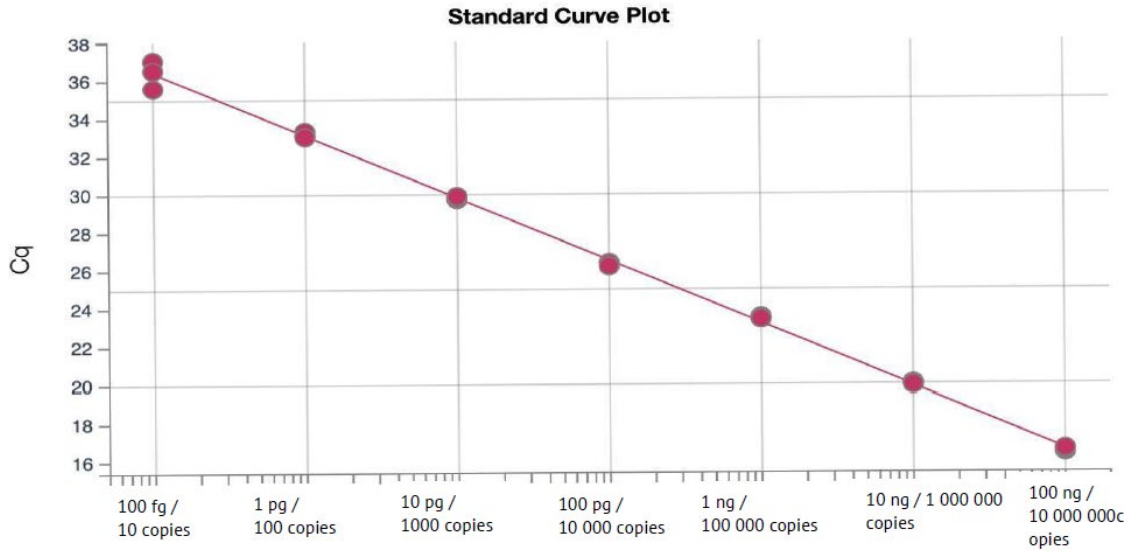
3.4 Verification of the ENGL acceptance parameters

Amplification efficiency. The amplification efficiency of the real-time PCR method was analysed by the applicant. For this purpose, DNA of *K. phaffii* MxY0541 was serially diluted 1:10 from 100 ng/µL to 1 fg/µL. Each DNA dilution (1 µL of template) was then run in real-time PCR in triplicate. One genome of *K. phaffi* MxY0541 weights approximately 10 femtograms (fg) ([1]). Hence the dilution series spanned from 10,000,000 copies to 0.1 copy (one tenth dilution of 1 copy).

[1] The weight of one genome of *K. phaffi* is estimated considering its genome length of 9,402,723 base pairs (bp) and assuming a weight of 650 Daltons per bp, or 650 g/mol per bp. Therefore, the molecular weight of *K. phaffi* MxY0541 genome is 9,402,723 x 650 = 6,111,769,950 g/mol. Given that a mole represents a substance containing the Avogadro's number (6.022 x 10^{23}) of molecules, one *K. phaffi* MxY0541 genome weighs approximately 10 femtograms (6,111,769,950 / 6.022 x 10^{23}, expressed in fg).

Figure 2 shows the standard curve plot for 100 ng to 100 fg DNA. All the dilutions down to 100 fg/µL gave consistent amplification in the three replicates, whereas the 10 and 1 fg/µL dilutions both gave amplification in two out of the three replicates. Therefore, the working range from 10,000,000 to 10 genome yeast copies per reaction showed an R^2 of 0.998, a slope of -3.301 and a PCR efficiency of 100.88%. These values are consistent with the ENGL acceptance criteria for the R^2, slope and efficiency of the regression line for a calibration curve.

Figure 2. Trendline of the serial dilution series used for the estimation of the method's amplification efficiency and R^2 from 100 ng to 100 fg DNA.



Sensitivity. The sensitivity was evaluated through the determination of the Limit of Detection ($LOD_{95\%}$). Sixty reactions were run for each dilution levels: 20, 10, 5, 1 copies and 1/10 of 1 copy per reaction. Each replicate was spiked with 5 µL of DNA extract from the burger without leghemoglobin as the background DNA. The 20, 10 and 5 target copies returned amplification for all the 60 replicates, but the 1 copy gave amplification for 40 out of the 60 replicates (33% negative results) and the 0.1 copy gave amplification for 7 out of the 60 replicates (Table 3).

Table 3. Summary of LOD testing performed by the applicant (numbers are not rounded)

K. phaffii event MxY0541		
Copy number	**Positive/Total reactions**	**Mean Cq value**
20	60/60	35.43
10	60/60	36.30
5	60/60	37.24
1	40/60	38.93
0.1	7/60	39.19

The number of the positive reactions reported in Table 3 were also compared to the expected positive reactions from the Poisson distribution, which are (a) 60 for the copy numbers of 5, 10, 20, (b) 38 for the copy number of 1 and (c) 6 for the copy number of 0.1. This comparison confirmed that the results match with the expected results.

Therefore, 5 target copies or 50 fg genomic DNA of *K. phaffii* MxY0541 was considered as the LOD of the PCR. Thus, the sensitivity of the method meets the minimum performance requirements set (< 25 copies with a level of confidence of 95%, ensuring ≤ 5% false negative results).

Robustness. The robustness of the method was assessed in eight combinations of the following variations to the method: exact/-10% master mix concentration, exact/-30% primer concentration, exact/-30% probe concentration, +/-1 µL master mix volume, +/-1 °C in annealing temperature. The combination scheme applied was the one reported in Annex 3 to the ENGL MPR, 2015. The combinations 1-4 were run on QuantStudio 3 Real-Time PCR System, and the combinations 5-8 on LightCycler 480 II System. The robustness experiments were run with 15 genome copies (150 fg DNA, representing 3x LOD) of *K. phaffii* MxY0541. PCR reactions were also spiked with 5 µL DNA from Impossible Burger with no leghemoglobin, and all the combinations were run in triplicates. According to the applicant, all combinations and replicates provided amplifications, thus indicating that the method tolerates small and deliberate deviations from the experimental conditions described in the procedure.

Interlaboratory transferability. A second operator performed the DNA extraction and the qualitative PCR test for the detection of *K. phaffi* event MxY0541 from samples of Impossible™ Food burger either containing leghemoglobin or produced without leghemoglobin (4 test samples per type). The PCR runs were carried out with LightCycler 480 II Real-Time PCR System at a second laboratory. The DNA concentrations reported are close to those obtained in the DNA extraction method. The average Cq values obtained using LightCycler 480 II are close to the Cq values for non-diluted samples analysed in the DNA extraction procedure and no false positive or false negative results were recorded**.**

4 DNA extraction

Genomic DNA was isolated from raw Impossible™ burger meat analogue, using a Wizard® Magnetic DNA Purification System for Food that has been validated in-house by the EURL GMFF in the context of the present submission. The protocol for DNA extraction and a report on testing are published at https://gmo-crl.jrc.ec.europa.eu/method-validations. According to the results provided by the applicant, the protocol for DNA extraction generated DNA of sufficient quantity and quality for PCR-based applications when applied to the raw meat analogue Impossible™ burger.

The applicant discussed the validity and limitations of the detection method stating that "Impossible Foods has developed plant-based meat analogue products to substitute for animal meat. Impossible Foods produces leghemoglobin, by genetically modifying the yeast *K. phaffii*, to provide the meaty flavour in their plant-based meat analogue products. The soy leghemoglobin protein is delivered in a preparation (LegH Prep) that contains *K phaffii* DNA. Thus, the qualitative method for the detection of GM DNA is designed to be applicable to samples of the food matrix (i.e., meat analogue products). The sample matrices used in the GMM testing 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. The LegH Prep used to deliver the soy leghemoglobin protein during the manufacture of the meat analogue product is in a liquid format. From the DNA extraction point of view, the LegH Prep, containing *K. phaffii* MxY0541 DNA, is distributed homogeneously throughout the product, and thus does not require extensive breakage of the sample matrix for DNA recovery. The selected DNA extraction method was based on the following criteria: 1) the method should provide adequate results from processed food samples and yeast; 2) it should also fit to other sample matrices from the same category (plant-based meat replacement products) potentially subject for testing; 3) the sample treatments and processing times should be convenient and the processing easily completed during office hours in one work day; and 4) the cost per sample should not be considerably higher than with other methods submitted for similar purpose (qualitative/quantitative GMO analytics).

The selected DNA extraction method, Wizard® Magnetic DNA Purification System for Food (Promega) purifies DNA from a variety of food samples including corn seed, corn meal, soybean, soya flour and soya milk. It is also widely applicable for highly processed food, such as corn chips, chocolate and chocolate-containing foods, lecithin and vegetable oils. The variety of sample matrices covers the sample matrix, the production strain and the reference materials required for the validation process.

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. The method does not require the use of harmful substances, such as phenol or β-mercaptoethanol and it is thus also a worker-safe option for DNA processing. The Wizard® Magnetic DNA Purification System for Food has been previously accepted and validated by EURL-AP for animal protein detection in feeding stuff (EURL-AP, 2014)."

Whenever DNA is extracted from more complex and difficult matrices, a thorough control of the quality of the DNA is recommended in order to ensure that it has the required quality for subsequent PCR analysis.

5 Materials and method

5.1 Samples

The following positive and negative control samples for the validation of the real-time PCR method were provided and described by the applicant to the EURL GMFF in accordance to Regulation (EC) No 1829/2003 Art 2.11 ([2]):

- genomic DNA extracted by the applicant from yeast *K. phaffii* GM-strain MxY0541, and
- genomic DNA extracted by the applicant from conventional yeast *K. phaffii* strain CBS 7435, the recipient strain used in the development of MxY0541, and
- genomic DNA provided by the applicant from the strain DSM 70382 *Komagataella* pastoris (closely related to *K. phaffii*)

5.2 Method for the PCR analysis

The PCR method provided by the applicant is an event-specific, qualitative, real-time TaqMan® PCR procedure for the detection and identification of GM event MxY0541. The procedure is a simplex system. The validated protocol is published by the EURL GMFF at https://gmo-crl.jrc.ec.europa.eu/method-validations and in Annex 1 to this report.

For the detection of GM event *K. phaffii* event *MxY0541*, a 130 bp fragment of the region spanning the 3' insert-to-yeast junction in *K. phaffii* event MxY0541 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and MBG (minor groove binder) Eclipse as non-fluorescent quencher dye at its 3' end.

5.3 EURL GMFF experimental testing (step 3)

5.3.1 In-house verification of the method performance against ENGL method acceptance criteria

Amplification efficiency and R^2 of the trendline of the MxY0541 dilution series

The amplification efficiency and the coefficient of determination (R^2) of the real-time PCR method for the detection and identification of *K. phaffii* strain MxY0541 was verified experimentally. The positive control sample (DNA) was tested over a 5-step dilution series starting from 1,000,000 copies down to 10 copies per reaction (dilution factor 10). Three replicates were tested in the range of 1,000,000 to 1000 copies per reaction while six replicates were used at the last two levels (100 and 10 copies). Additionally, the EURL GMFF verified the method's efficiency with the real-time PCR platforms ABI 7500 and QuantStudio 7 using a range from 2500 copies to 10 copies per reaction.

[2] Control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample).

Limit of Detection

The limit of detection is the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified. The LOD should be < 25 copies with a level of confidence of 95%, ensuring ≤ 5% false negative results (ENGL, 2015). The EURL GMFF followed two approaches to estimate the LOD. The first approach consisted in serially diluting the target DNA of *K. phaffi* event MxY0541 and testing 60 replicates per each dilution level, containing respectively 10, 5, 1 and 0.1 (1/10 dilution of the 1 copy-level) copies per reaction, in 100 ng of salmon DNA as background DNA.

Probability of Detection

As a second approach, the LOD for the MxY0541 method is estimated via the probability of detection (POD) curve, specifying the relationship between the POD and the DNA copy number. The corresponding statistical model applied in this study is based on the Poisson distribution and is used to assess an analytical method's ability to identify the presence or absence of a specific target DNA sequence in a sample. For a qualitative real-time PCR method, the model describes the probability of detecting the target DNA by PCR amplification, at a given concentration level of target DNA (3,11). In particular, the model is applied to estimate the $LOD_{95\%}$ of the GM target equal to POD = 0.95 (95% probability of detection).

The statistical model has been modified by introducing the average amplification probability (λ) and the slope parameter (*b)* to reflect possible deviations of the PCR reaction from the ideal POD function defined by the Poisson distribution (9). In detail, the theoretical $LOD_{95\%}$ derived from the Poisson distribution is 3 copies; however, when applying the PCR method, the actual $LOD_{95\%}$ could be above 3. In the statistical model, this deviation of the observed LOD from the ideal value of 3 is accounted for by the amplification probability λ. For instance, if the amplification probability λ is 0.75 (or 75%), the $LOD_{95\%}$ would be calculated as 3/0.75 =4.

To this aim, a stock solution containing 500 copies/µL (intended for testing at 2500 copies/reaction) of MxY0541 GM DNA was serially diluted. The $LOD_{95\%}$ was determined by testing 12 replicates at respectively 20, 10, 5, 2, 1 and 1/10 of 1 copy (i.e. 0.1) of the MxY0541 GM target per reaction, with the QuantStudio 7 and ABI 7500 real-time PCR instruments. Each reaction contained 100 ng of salmon DNA as background nucleic acid. The testing and modelling was in accordance to the CEN/TS 17329-1 "General guidelines for the validation of qualitative real-time PCR methods - Part 1: Single- laboratory validation". The results were analysed with the "Validation of qualitative PCR methods within a single laboratory", tool available online ([3]).

False Negative and False Positive rate

A false negative occurs when the test result is negative (i.e., the GM target is not detected) even though the true condition is positive (i.e., the GM target is present at a concentration ≥ LOD).

The acceptance criterion for the false negative rate (FNR) is that the percentage of misclassified known positive samples does not exceed 5% of the total number of positive samples.

In the FNR tests, 8 replicates of MxY0541 at 50 copies per reaction were stabilised in 100 ng of salmon DNA and spiked with 10 ng of maize DNA (as positive DNA target control) and amplified with the MxY0541 event-

[3] https://quodata.de/content/validation-qualitative-pcr-methods-single-laboratory

specific method. In parallel, the same number of replicates was run with an *hmg* maize method as a reaction control (§ Annex 1).

A false positive result occurs when the test result is classified positive (GM target is detected) when the true condition is negative (GM target is absent).

The acceptance criterion for the false positive rate (FPR) is that the percentage of misclassified known negative samples does not exceed 5% of the total number of negative samples.

In the FPR tests, 8 replicates containing 50 copies of the non-modified *K. phaffi* strain per reaction were analysed; samples were stabilised in 100 ng of salmon DNA and 10 ng of maize DNA (as positive DNA target control) and amplified with the MxY0541 event-specific method.

5.4 International collaborative study (step 4)

The international collaborative trial involved twelve randomly selected laboratories, all being "national reference laboratories, assisting the EURL GMFF for testing and validation of methods for detection", as listed in annex to Regulation (EC) No 120/2014 (9) who had expressed their interest in participation. The study was carried out in accordance with the following internationally accepted guidelines:

- The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies." (Horwitz, 1995) (2)

- CEN/TS 17329-1: 2021-06 "Foodstuffs - General guidelines for the validation of qualitative real-time PCR methods - Part 1: Single-laboratory validation".

- CEN/TS 17329-2:2019-06 "Foodstuffs - General guidelines for the validation of qualitative real-time PCR methods - Part 2: Collaborative study".

- ISO 24276:2006, Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - General requirements and definitions

- ISO 24276:2006/Amd 1:2013-Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions-Amendment 1

The objective of the international collaborative study was to verify the limit of detection, the false positive rate and the false negative rate of the analytical method provided by the applicant and previously verified in-house by the EURL GMFF.

5.4.1 List of participating laboratories

The twelve laboratories participating in event MxY0541international collaborative study were randomly selected from 24 national reference laboratories (NRL) that offered to participate.

Clear guidance was given to the selected laboratories for strictly following the validation protocol that was provided to them. The participating laboratories are listed in Table 4.

Table 4. Laboratories participating in the validation of the detection method for yeast *K. phaffii* MxY0541

Laboratory	**Country**
AGES -Austrian Agency for Health and Food Safety	AT
Crop Research Institute - Reference Laboratory for GMO Detection and DNA fingerprinting	CZ
INIAV	PT
Institute for Diagnosis and Animal Health, Molecular Biology and GMOs Unit	RO
Institute for Hygiene and Environment- Hamburg	DE
Laboratory for the Detection of GMO in Food - Bad Langensalza	DE
National Institute of Biology	SI
Service commun des laboratoires du ministère de l'économie et des finances	FR
State Institute of Chemical and Veterinarian Analysis - Freiburg	DE
State Veterinary and Food Institute Dolny Kubin	SK
Wageningen Food Safety Research (WFSR)	NL
Walloon Agricultural Research Centre	BE

5.4.2 Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: four laboratories used QuantStudio 5, two laboratories used QuantStudio 7, two of them used ABI 7500, one used Bio-Rad Opus 96, one had QuantStudio 6, one utilized qTower3G, and one used the BioRad CFX 96.

This variability of equipment, with its known potential influence on PCR results, reflects the real-life situation in the control laboratories and provides additional assurance that the method is robust and usable under real conditions.

5.4.3 Materials used in the international collaborative study

For the validation of the qualitative event-specific method, blind test samples (of undisclosed GM content = blind samples) were provided by the EURL GMFF to the participating laboratories

The twelve NRLs participating in the validation study received the following materials:

1. One DNA stock containing genomic DNA from *K. phaffii* event MxY0541 at a concentration of 500 copies/µL, in background Salmon Sperm DNA, (100 µL);

2. One positive control sample containing genomic DNA from *K. phaffii* event MxY0541, in background Salmon Sperm DNA, (50 µL);
3. Sixteen blind samples containing or not genomic DNA from *K. phaffii* event MxY0541 in background Salmon Sperm DNA and maize DNA (30 µL each);
4. One positive control sample containing genomic DNA from *K. phaffii* event MxY0541, in background Salmon Sperm DNA and maize DNA (100 µL).

✓ Reaction reagents:

- 2x TaqMan Fast Advanced Master Mix: one tube 4 mL
- Distilled sterile water: one tube 1 mL
- Dilution Buffer: Salmon Sperm DNA (20 ng/µL), one tube 4 mL

✓ Primers and probes (1 tube each) as follows:

MxY0541

- MxY0541-F10 (10 µM): 585 µL
- MxY0541-R10 (10 µM): 585 µL
- MxY0541-P10 (10 µM): 165 µL

hmg

- hmg primer 1 (10 µM): 50 µL
- hmg primer 2 (10 µM): 50 µL
- hmg probe (10 µM): 25 µL

5.4.4 Design of the collaborative study

Participating laboratories received a detailed validation protocol that included the exact design of the PCR plates, ensuring that on each PCR plate the samples were analysed for the MxY0541 event-specific method. In total, two plates were run by each participating laboratory: plate A for the determination of the LOD and POD; plate B for the determination of the false-negative, false-positive rate.

For the determination of the LOD (in copies of the GM-target sequence) and for the estimation of the 95% interval by means of a probability of detection curve, a serial dilution was prepared by each laboratory using the sample provided as stock DNA at 500 genomic copies of *K. phaffii* MxY0541/µL and the dilution buffer, according to Table 2 of the validated method (§ Annex 1). The serial dilution comprised the eleven levels indicated in Table 5.

Table 5. Dilution scheme for the preparation of the samples for the POD curve for the MxY0541 method

DNA Dilution	DNA copy number of the target sequence (in 5 µL)	Number of qPCR replicates
L1	2500	3
L2	500	3
L3	100	3
L4	50	3
L5	20	6
L6	10	6
L7	5	6
L8	2	6
L9	1	6
L10	0.5	6
L11	0.1	6

The statistical analysis was carried out by QuoData GmbH according to the principles of DIN CEN/TS 17329-2:2019-06 "Foodstuffs - General guidelines for the validation of qualitative real-time PCR methods - Part 2: Collaborative study", and reviewed by the EURL GMFF. Lab-specific POD curves were analysed via the web tool for the validation of qualitative PCR methods within a single laboratory (5). Subsequently, the model parameters of the POD curve (across all laboratories) and the performance characteristics of the method were determined. These include the average amplification probability λ_0, the standard deviation σ_L reflecting the variability of λ across laboratories on a logarithmic scale, the slope parameter *b* and the $LOD_{95\%}$ value in copies. In addition, the confidence interval for the $LOD_{95\%}$ of a median laboratory and the prediction interval of the various $LOD_{95\%}$ values across laboratories were calculated.

In order to determine the false negative and false positive rates, laboratories tested sixteen blind samples. Eight of these samples were spiked with 50 copies of the MxY0541 target DNA, while the remaining eight samples contained the DNA of the non-modified strain. All samples were mixed with salmon sperm DNA at a final amount of 100 nanograms per reaction and with maize DNA as the positive control at a final amount of 10 nanograms per reaction. Control samples provided by the EURL GMFF were loaded with no further dilution in the two respective plates. Each laboratory prepared the "no template" PCR controls.

The laboratories prepared the PCR master-mixes in accordance with the description provided in the validation protocol. Test samples were loaded on the PCR plates as per pre-determined plate layout.

The amplification reaction followed the cycling program specified in the protocol. Participants determined the Cq values of the test samples according to the instructions and also reported the raw data to the EURL GMFF on an Excel sheet that was designed, validated and distributed by the EURL GMFF. All data are stored by the EURL GMFF on a dedicated and protected server.

The EURL GMFF analysed the data against the parameters and the limits set by the ENGL, for the amplification efficiency, R^2 of the trendline over the dilutions series, as well as the LOD, false negative and false positive rates.

5.4.5 Deviations reported from the protocol

Eleven laboratories reported no deviations from the validation protocol. One laboratory reported a minimal deviation due to an extension time of 31 seconds instead of the 30 seconds indicated for ABI 7500.

6 Results

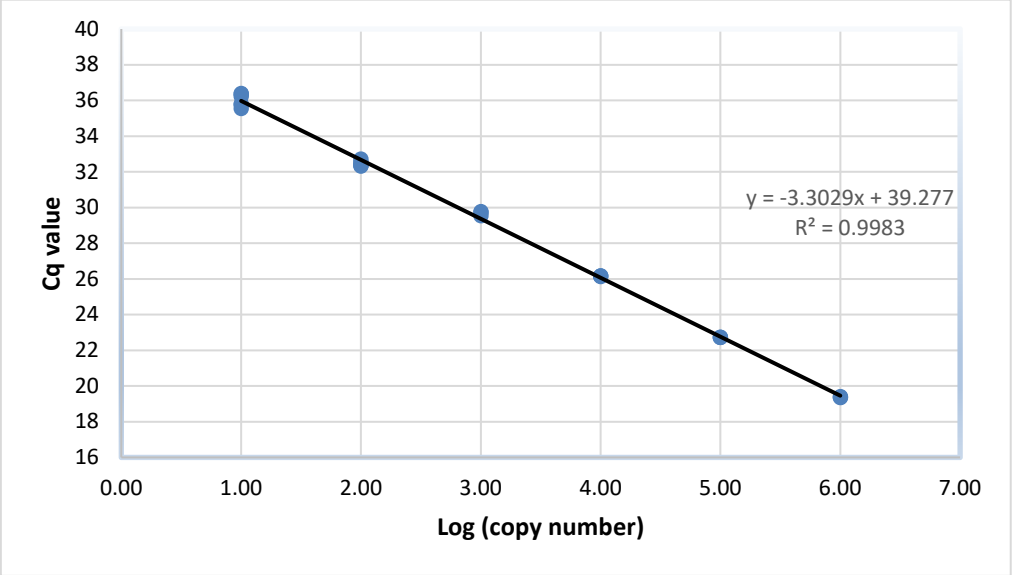
6.1 EURL GMFF experimental testing

6.1.1 In-house verification of method performance against ENGL method acceptance criteria

Amplification efficiency of the serial dilution. The samples of the dilutions series from 1,000,000 copies down to 10 copies were assessed with the MxY0541 event-specific method.

Figure 3 shows the trend of the dilution series after plotting the Cq values measured at each level (mean of three replicates per level) versus the logarithm of the target copy number in reaction.

Figure 3. Regression line of the serial dilution series used for the estimation of the method's amplification efficiency



The slope is -3.30 and an R^2 is close to 1.00. Those values are in line with the findings of the applicant and confirm the linear response of the method from 1,000,000 to 10 target copies per reaction. Both parameters aligns well with the ENGL acceptance criteria for the amplification efficiency (-3.1 ≤ slope ≤- 3.6) and R^2 (≥0.98).

Additionally, the EURL GMFF verified the method's efficiency with the real-time PCR platforms ABI 7500 and QuantStudio 7 over a narrower range, from 2500 copies to 10 copies per reaction: a slope of -3.47 and a R^2 of 1.00 were obtained (data not shown).

Limit of Detection

The EURL GMFF followed two approaches to estimate the LOD. The first approach consisted in serially diluting the target DNA of *K. phaffi* MxY0541 and testing 60 replicates per each dilution level, respectively 10, 5, 1 and 0.1 (1/10 dilution of the 1 copy) copies per reaction, in 100 ng of salmon DNA as background DNA.

The dilution series was tested in two real-time PCR platforms QuantStudio 7 and ABI 7500 for the determination of the LOD. Table 6 shows the results obtained for the determination for the LOD via serial dilutions.

Table 6. Summary of the LOD testing performed at the EURL GMFF

Copy number	QuantStudio 7	ABI 7500
	Positive/Total reactions	**Positive/Total reactions**
10	60/60	60/60
5	57/60	53/60
1	40/60	24/60
0.1	5/60	2/60

The LOD is estimated between 10 and 5 copies per reaction in both QuantStudio7 and ABI 7500 real-time PCR platforms. According to the ENGL, 2015 the $LOD_{95\%}$ is in fact set at 59/60 positive replicates. The acceptance criterion for the limit of detection (absolute) according to the MPR should be < 25 copies. Therefore, the method meets the LOD criterion when tested via serial dilution on different PCR apparatus, with an LOD comprised between 10 and 5 copies per reaction.

Probability of Detection (POD)

The EURL GMFF estimated the LOD of the MxY0541 method also following the POD approach, in relation to the parameter definition and acceptance criterion established in the ENGL, 2015 and following the provisions of the Technical Specification CEN/TS 17329-1: 2021 "General guidelines for the validation of qualitative real-time PCR methods - Part 1: Single-laboratory validation".

The $LOD_{95\%}$ was determined by testing 12 replicates at each dilution level of the MxY0541 target at respectively 20, 10, 5, 2, 1, 0.5 and 0.1 copies per reaction with the QuantStudio 7 and ABI 7500 real-time PCR instruments. Each reaction contained 100 ng of salmon DNA as background nucleic acid. Table 7 reports the outcome of the testing on QuantStudio 7 and ABI 7500.

Table 7. Summary of the results of the serial dilution prepared for the probability of detection further to testing with the MxY0541 method.

Copy number per reaction	Positive / Total replicates	
	QuantStudio 7	ABI 7500
20	9/9	9/9
10	12/12	12/12
5	12/12	11/12
2	8/12	11/12
1	6/12	8/12
0.5	7/12	4/12
0.1	2/12	1/12

The plausibility check indicates no irregularities. On QS7 real-time PCR platform, the $LOD_{95\%}$ is estimated at 3.5 copies (95%CI: 2.3-5.3). Similar results were obtained when the same dilution series was analysed on ABI 7500 real-time PCR platform, with a $LOD_{95\%}$ estimated as 3.5 (95%CI: 2.3-5.2).

Overall, the two approaches led to an $LOD_{95\%}$ estimate within the acceptance criteria of the ENGL, 2015.

False negative and false positive rate.

Results of the in-house determination of the method's false negative rate and false positive rate are reported in Table 8.

Table 8. Results of the tests for the false negative rate and false positive rate further to testing with the MxY0541 event-specific method and with *hmg*.

	MxY0541			*hmg*		
	Mean Cq	St Dev Cq	Positive/Total replicates	Mean Cq	St Dev Cq	Positive/Total replicates
FNR test	34.2	0.24	8/8	28.3	0.05	8/8
FPR test	n.d.	n.d.	0/8	28.4	0.07	8/8

n.d.: not detected

In the FNR test, the MxY0541 method could amplify all the eight samples containing the GMM target, thus showing a FNR of 0.00% (no misclassified known positive in eight total positive samples).

In the FPR test, none of the eight samples without the GMM target was amplified. However, they amplified with the *hmg* method indicating that no unexpected amplification failure occurred during the experiment. Therefore, the method FPR was 0.00% (no misclassified known negative sample in eight total negative samples)

The FNR and the FPR were found in agreement with the ENGL acceptance criteria (ENGL, 2021), being ≤ 5%.

In conclusion, the method for identification of the *K. phaffii* event MxY0541 shows acceptable false positive and false negative rates and performs linearly over the range of concentration from 1,000,000 copies down to 10 copies. Finally, the $LOD_{95\%}$ complies with the ENGL requirement, being less than 25 copies.

6.2 Results of the international collaborative study

6.2.1 PCR efficiency and linearity

The laboratories taking part in the collaborative study received one DNA stock containing genomic DNA from *K. phaffii* MxY0541 at a concentration of 500 copies/µL, in background Salmon Sperm DNA (100 µL). A serial dilution with the dilution buffer provided (§ 4.4.3) was prepared by each laboratory, ranging from 2500 copies to 0.1 copies per reaction, as shown in Table 5.

All laboratories successfully detected the replicates at each dilution level down to 10 copies per reaction. Hence, for each laboratory a linear regression line was generated by plotting the Cq values obtained by the participant laboratory versus the logarithm of the DNA copy number, from 2500 copies to 10 copies per reaction.

The PCR efficiency (%) and R^2 values (expressing the linearity of the regression), reported by participating laboratories are reported in Table 9. The PCR efficiency (%) was calculated from the standard curve slopes using the formula:

$$\text{Efficiency (\%)} = \left(10^{\frac{-1}{slope}} - 1\right) \times 100 \qquad \text{eq.1}$$

Table 9. Values of slope, PCR efficiency and R^2 obtained during the international collaborative trial. Slope and R^2 coefficient values were rounded to two digits.

		K. phaffii event MxY0541		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.45	95	1.00
2	A	-3.40	97	1.00
3	A	-3.47	94	1.00
4	A	-3.57	91	1.00
5	A	-3.48	94	1.00
6	A	-3.58	90	1.00
7	A	-3.47	94	1.00
8	A	-3.51	93	1.00
9	A	-3.38	98	1.00
10	A	-3.72	86	0.99
11	A	-3.32	100	1.00
12	A	-3.46	95	1.00
	Mean	**-3.48**	**94**	**1.00**

Table 9 indicates that the efficiency of amplification for the event MxY0541 method ranges from 90 % to 100 % and the linearity from 0.99 to 1.00. The mean PCR efficiency was 94% and the average R^2 was 1.00. Both PCR efficiency and linearity values were within the ENGL acceptance criteria.

These results confirm the appropriate performance characteristics of the regression line over the dilution series in terms of efficiency and linearity from 2500 to 10 target copies per reaction.

6.2.2 False negative and false positive rate

Table 10 reports the Cq values determined by each laboratory in relation to the eight samples containing the GMM target and used for the estimation of the false negative rate.

Table 10. False negative rate. Cq values recorded by laboratories for the samples containing the MxY0541 target

	Laboratory											
Sample	PL01	PL02	PL03	PL04	PL05	PL06	PL07	PL08	PL09	PL10	PL11	PL12
1	33.0	33.9	32.7	33.3	34.0	33.8	31.2	35.4	33.2	32.7	33.2	34.0
2	33.2	33.5	33.1	33.2	34.4	33.5	31.6	35.1	33.1	32.7	33.7	34.3
3	32.7	33.3	33.5	33.5	33.8	33.8	31.2	35.3	33.1	32.4	33.6	34.3
4	33.0	33.6	33.8	33.4	34.1	34.0	31.5	34.9	33.2	32.7	34.1	34.3
5	32.9	33.6	33.5	32.9	33.6	33.5	30.5	35.5	33.0	32.8	34.0	34.5
6	32.9	33.6	33.3	33.2	33.9	33.9	31.4	35.5	33.0	32.6	33.7	34.3
7	32.8	33.5	33.4	33.3	34.2	33.6	30.9	34.8	33.1	32.8	34.0	34.6
8	32.9	33.4	33.2	33.3	33.9	33.8	31.3	35.0	33.9	32.7	33.3	34.6

All samples were amplified with the MxY0541 method by all laboratories, thus leading to FNR equal to 0.00% (no misclassified known positive samples in 96 total positive samples).

Table 11 reports the Cq values determined by each laboratory in relation to the eight samples without the GMM target and used for the estimation of the false positive rate.

Table 11. False positive rate. Cq values recorded by laboratories for the samples without the MxY0541 target

	Laboratory											
Sample	PL01	PL02	PL03	PL04	PL05	PL06	PL07	PL08	PL09	PL10	PL11	PL12
1	n.d	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2	n.d	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3	n.d	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	37.9	n.d.
4	n.d	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5	n.d	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	n.d	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
7	n.d	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8	n.d	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d., not detected

None of the samples, except one for PL11 (Cq = 37.9), was amplified by the MxY0541 method, whilst the same samples were in all cases amplified by the *hmg* maize-specific method. Therefore, the FPR was of 1.04% (1 misclassified known negative in 96 total negative samples).

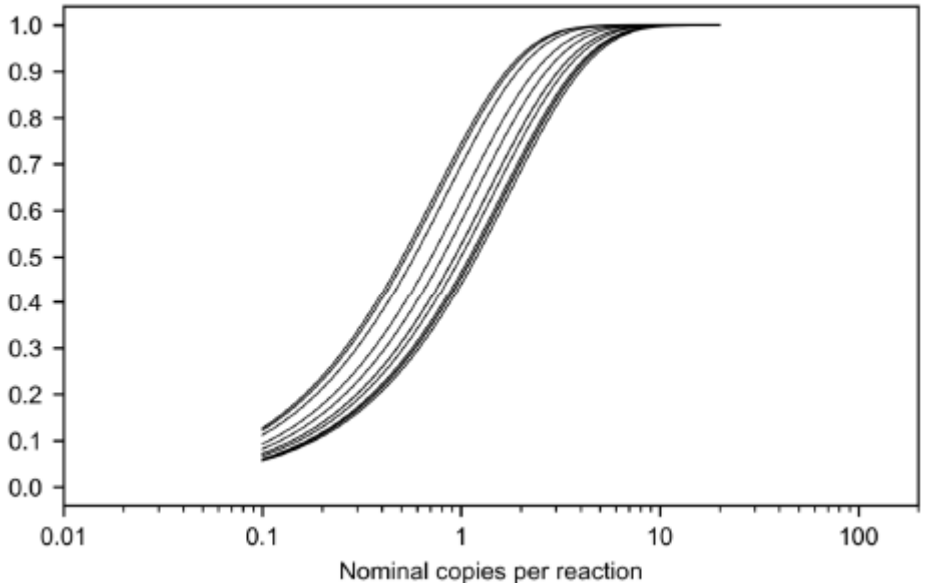
Therefore, the MxY0541 method meets the ENGL acceptance criteria for FNR and FPN since both parameters are ≤ 5%.

6.2.3 Lab-specific POD curves

The laboratory-specific results were analysed using the Quodata GmbH web tool for qualitative PCR methods within a single laboratory (§ 4.4.4), to determine whether they meet the criteria for single-lab validation.

No statistically significant deviating values were identified, and a value of 1 was assumed for the slope parameter *b* for all lab-specific POD curves shown in Figure 4.

Figure 4. Laboratory-specific POD curves (slope parameter *b* = 1)



The laboratory-specific POD curves are shifted in parallel along the x-axis, indicating that the λ values vary between the laboratories. Consequently, also the lab specific values for the $LOD_{95\%}$ vary as explained in chapter 5.3.1. In addition, the Grubbs test was applied to the laboratory specific λ values and no outliers were identified.

6.2.4 Performance parameters of the POD curve (across all laboratories)

Table 12 provides the number of positive test results per dilution level obtained by each laboratory participating in the collaborative study.

Table 12. Number of positive test results (out of a total of 6 PCR replicates) per laboratory and dilution level

Laboratory	Target GMM copy number per reaction						
	20	**10**	**5**	**2**	**1**	**0.5**	**0.1**
PL01	6	6	6	5	2	3	1
PL02	6	6	6	5	5	3	0
PL03	6	6	5	3	5	3	0
PL04	6	6	6	5	2	1	0
PL05	6	6	6	4	3	1	0
PL06	6	6	6	4	3	3	0
PL07	6	6	6	5	6	2	1
PL08	6	6	5	6	3	3	0
PL09	6	6	6	4	1	2	1
PL10	6	6	6	3	3	3	1
PL11	6	6	6	5	5	3	1
PL12	6	6	6	6	3	2	0

The performance parameters of the POD curve, i.e. the average amplification probability λ_0, the slope parameter *b*, the laboratory standard deviation σ_L and the $LOD_{95\%}$ value are provided in Table 13.

Table 13. Performance parameters of the POD curve

Parameter		**Value**
Number of participating laboratories		12
Number of PCR replicates per dilution level		6
POD curve	Average amplification probability λ_0	0.80
	95% confidence interval for λ_0	0.67 to 0.96
	Slope *b* (across laboratories)	1
	95% confidence interval for σ_L	0.00 to 0.36
	Specified estimated value for σ_L	0.18
	95% variation range of lab-specific $LOD_{95\%}$ values based on $\sigma_L = 0.18$ (expressed as relative values in relation to the $LOD_{95\%}$ for a theoretical median laboratory)	70% to 142%
$LOD_{95\%}$ in copies	Theoretical median laboratory (average sensitivity)	3.7
	95% confidence interval for a laboratory with average sensitivity	3.1 to 4.4
	95% prediction interval for a randomly selected laboratory	2.5 to 5.5

The average amplification probability λ_0 is about 0.80, i.e. 80%. The 95% confidence interval for λ_0 is [0.67, 0.96] and does not include the optimal value of 1. This means that the achievable LOD of the method is somewhat above the ideal LOD calculated from the Poisson distribution.

The slope parameter *b* can be assumed to be 1. This means that there is no noticeable dependence of the amplification probability across laboratories on the number of copies of the target sequence for the PCR method.

Due to the strongly asymmetric 95% confidence interval (0.00 to 0.36), the maximum likelihood estimate for σ_L (0.02) is replaced by the centre of this interval, i.e. by 0.18. The latter value can be interpreted as follows: by taking into account this value, the lab-specific $LOD_{95\%}$ values will range between 70% and 142% (95% variation range) of the $LOD_{95\%}$ for a (theoretical) median laboratory.

The $LOD_{95\%}$ value expected for a theoretical median laboratory is about 3.7 with the 95% confidence interval [3.1, 4.4]. This means that, for a laboratory with average sensitivity, the best $LOD_{95\%}$ value can be expected to lie around 3.1, and the worst $LOD_{95\%}$ value around 4.4.

Moreover, for a randomly selected laboratory, the best $LOD_{95\%}$ value can be expected to lie around 2.5 ([4]), and the worst $LOD_{95\%}$ value around 5.5. This range corresponds to the 95% prediction interval, which reflects not only the laboratory standard deviation σ_L, i.e. 0.18, but also the standard error of the natural logarithm of the average amplification probability λ_0, since the latter is not negligible.

Overall, the upper limit of the 95% CI of the LOD is estimated at 5.5 copies that is well below 25 copies set by the ENGL as the acceptance criteria for the LOD of PCR-based analytical methods.

7 Conclusion

The method provided by the applicant has been validated in accordance to the EURL GMFF validation process, respecting all the requirements applicable of the relevant EU legislation and international standards and guidelines for method validation for a qualitative method for detection and identification of a GMO.

This validation study confirmed that the method is applicable to the control samples provided by the applicant (see paragraph 4.1), in accordance with the requirements of Annex 1.2.C.2 to Commission Regulation (EC) No 641/2004 (7) and meets all method performance requirements established by the ENGL and the EURL GMFF for qualitative methods. The method is therefore valid to be used for regulatory purposes, for the traceability of the GM event in the food matrix and applicable to any appropriately extracted yeast *K. phaffii* genomic DNA.

In any case the user of the method is advised to verify the quality of the extracted DNA in order to ensure that it is suitable for the subsequent PCR analysis. This is particularly relevant for more complex matrices of samples from food and feed products.

The validated method is described in detail as “Validated Method” at https://gmo-crl.jrc.ec.europa.eu/method-validations and in Annex 1.

[4] According to the Poisson distribution the best theoretically achievable $LOD_{95\%}$ value is about 3 copies per reaction, i.e. lower values indicate a sensitivity that is better than that achievable according to the theoretical POD curve.

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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
FNR	False negative rate
FPR	False positive rate
LOD	Limit of Detection
LOQ	Limit of Quantification
POD	Probability of Detection

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Annex 1 – Event-specific Method for the detection and identification of DNA from genetically modified *Komagataella phaffii* strain MxY0541 using Real-time PCR

Validated Method

Method development:

Impossible Foods Inc., represented by Intertek Assuris

1 General information and summary of the methodology

This protocol describes an event-specific real-time TaqMan® PCR (polymerase chain reaction) procedure for the detection and identification of *K. phaffii* event MxY0541 DNA in a sample.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR assays. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are also recommended to ensure suitability of the extracted DNA.

For the detection of GM event MxY0541, a 130 bp fragment of the region spanning the 3' insert-to-yeast junction in *K. phaffii* MxY0541 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and MBG (minor groove binder) as non-fluorescent quencher dye at its 3' end. The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Cq" value. DNA target is detected when the amplification curve crosses the threshold (the measured Ct is above the PCR threshold), indicating exponential amplification of the target sequence

2 Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from Impossible Foods burger and genetically modified yeast *K. phaffii*. The method sensitivity, the false positive and false negative rates were tested through an international collaborative ring trial using DNA samples at different levels of genetically modified organisms (GMM). Examples for assessing the LOD, the false positive and the false negative rates of the method are provided in this protocol (see 3.2.3 and 3.2.4).

2.2 Collaborative trial

The method was validated in an international collaborative study by the European Union Reference Laboratory for GM Food and Feed (EURL GMFF). The study was undertaken with twelve participating laboratories in March 2024.

A detailed validation report can be found at https://gmo-crl.jrc.ec.europa.eu/method-validations.

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 5 copies of genomic DNA from *K.phaffi* event MxY0541. The absolute LOD was determined in the collaborative study.

2.4 Limit of quantification (LOQ)

The method for detection of *K. phaffii* event MxY0541 is not quantitative as further detailed in the validation report. Consequently, the LOQ was not determined.

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 3' insert-to-yeast junction in *K. phaffii* event MxY0541 and is event-specific for the event MxY0541 (§ 3.1, 3.2 and 3.3 in the Validation Report).

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 international guidelines, e.g. ISO 24276:2006.
- 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 the equipment should be sterilised prior to use and any residue of DNA should have been removed. All material used 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 regularly
- 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 room temperature.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for qualitative analysis of K. phaffii event MxY0541

3.2.1 General

The real-time PCR set-up the GMM (event MxY0541) target sequence is a simplex method.

The method is developed for a total volume of 25 µL per reaction mixture for the GMM (event MxY0541) with the reagents as listed in Table 2.

3.2.2 Amplification reaction mixture for the K. phaffii event MxY0541

Thaw, mix and centrifuge the components needed for the run. Keep thawed reagents on ice.

In a 1.5 mL tubes on ice, add the components in the order mentioned below (except DNA) to prepare the reaction mix for the MxY0541 method (Table 1).

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the *K. phaffii* event MxY0541 method.

Component	Final concentration	µL/reaction
TaqMan® Fast Advanced Master Mix (2x)	1x	12.5
MxY0541-F10 forward primer (10 µM)	900 nM	2.25
MxY0541-R10 reverse primer (10 µM)	900 nM	2.25
MxY0541-P10 probe (*) (10 µM)	250 nM	0.625
Nuclease free water	-	2.375
DNA	-	5
Total reaction volume:		25 µL

*TaqMan® probe is labelled with FAM at its 5'-end and MGB at its 3'-end

3.2.3 Real-time PCR set-up for the determination of the Limit of Detection

For the determination of the LOD (in copies of the GM-target sequence) and for the estimation the 95% interval via POD, a serial dilution is prepared using a stock DNA sample at 500 genomic copies of MxY0541/ µL and the dilution buffer. The dilution scheme is shown in Table 2:

Table 2. Dilution scheme for the preparation of the samples for the estimation of LOD and POD.

DNA Dilution Level	DNA copy number of the target sequence (in 5µL)	Volume from previous DNA Dilution Level	Volume dilution buffer	Number of qPCR replicates
L1	2500	Stock solution	None	3
L2	500	10 µL	40 µL	3
L3	100	20 µL	80 µL	3
L4	50	50 µL	50 µL	3
L5	20	40 µL	60 µL	6
L6	10	50 µL	50 µL	6
L7	5	50 µL	50 µL	6
L8	2	40 µL	60 µL	6
L9	1	50 µL	50 µL	6
L10	0.5	50 µL	50 µL	6
L11	0.1	20 µL	80 µL	6

1. For dilution levels L1 to L4, add into each reaction tube the amount of reaction mix needed for 3.5 repetitions (70 µL). Add into each tube the proper amount of DNA for 3.5 repetitions (17.5 µL DNA). For dilution levels 5 to 11, add into each reaction tube the amount of reaction mix needed for 7 repetitions (140 µL). Add into each tube the proper amount of DNA for 7 repetitions (35 µL DNA). The excess amount will ensure adequate volume when loading the samples.
2. Vortex each tube for approx. 10 seconds. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
3. Spin down the tubes. Aliquot 25 µL for MxY0541 system in each well.

4. In the collaborative trial a positive control in triplicate was used, containing 5 copies per reaction of DNA from *K. phaffi* event MxY0541.
5. Place an optical cover on the reaction plate and briefly centrifuge the plate.
6. Place the reaction plate in the real-time PCR apparatus according to the manufacturer's instructions and your Standard Operating Procedures and start the run.
7. Select FAM as reporter dye for the event MxY0541. Define MGB or non-fluorescent as quencher dye for. Select ROX as the passive reference dye if needed. Enter the correct reaction volume (25 µL).
8. Run the PCR with the cycling program described in Table 4.

3.2.4 Real-time PCR set-up for the determination of the false negative and false positive rate

For the testing of the false negative rate, samples containing 50 copies per reaction of DNA from *K. phaffi* event MxY0541 should be used; for the testing of the false positive rate, samples did not contain DNA from *K. phaffi* event MxY0541.

The samples for the false negative and false positive rates are tested in single replicate with the MxY0541 method, with amplification reaction mixture as described in §3.2.2 Table 1.

As a monitoring run, as in the collaborative trial, samples for the false negative and false positive rates where spiked with maize genomic DNA (10 ng/reaction) and analysed also with the *hmg* maize-specific reference system (Table 3).

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

Component	Final concentration	µL/reaction
TaqMan® Fast Advanced Master Mix (2x)	1x	12.5
hmg primer 1(10 µM)	300 nM	0.75
hmg primer 2 (10 µM)	300 nM	0.75
hmg probe (*) (10 µM)	160 nM	0.40
Nuclease free water	-	5.6
DNA	-	5
Total reaction volume:		25 µL

*TaqMan® probe is labelled with FAM at its 5'-end and TAMRA at its 3'-end

1. Add into each well 20 µL reaction mix for MxY0541 system and for *hmg*.
2. Add into each well 5 µL of the DNA samples (masked samples in the collaborative trial).
3. In the collaborative trial a positive control in triplicate was used, containing 50 copies per reaction of DNA from *K. phaffi* event MxY0541.
4. The no template controls (NTC) are prepared by the participating laboratory by adding into each well 5 µL of H_2O and are loaded before any other sample.

5. Place an optical cover on the reaction plate and briefly centrifuge the plate.
6. Place the reaction plate in the real-time PCR apparatus (possibly apply a compression pad, depending on the model), according to the manufacturer's instructions and your Standard Operating Procedures and start the run.
7. Select FAM as reporter dye and MGB or non fluorescent as a quencher for MxY0541. Select FAM as reporter dye and TAMRA as a quencher for *hmg*. Select ROX as the passive reference dye if needed. Enter the correct reaction volume (25 µL).
8. Run the PCR with the cycling program described in Table 4

3.2.5 Cycling program

Table 4 reports the cycling program for MxY0541 and *hmg*.

Table 4. Cycling program for MxY0541/*hmg*.

Step	Stage		T (°C)	Time (s)	Acquisition	Cycles
1	UNG (*)		50	120	No	1
2	Initial denaturation		95	120	No	1
3	Amplification	Denaturation	95	1[i] or 3[ii]	No	45
		Annealing & Extension	60	20[i] or 30[ii]	Yes	

(*) UNG: Uracil-N-glycosylase

The EURL GMFF tested this protocol on QuantStudio 7[i] and ABI 7500[ii].

According to the method developer, time for denaturation step can be reduced to 1 second and time for annealing/extension step to 20 seconds when using QuantStudio 3 and 5 Real-Time PCR Instruments, QuantStudio 6 and 7 Flex Real-Time PCR System, QuantStudio 12K Flex Real-Time PCR System, 7900HT Real-Time PCR Instrument, 7900HT Fast Real-Time PCR Instrument, ViiA 7 Real-Time PCR System, or StepOnePlus Real-Time PCR System

3.3 Data analysis

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

a) Set the threshold following the automatic or the manual mode. In the manual mode display the amplification curves of the event specific assay 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 following the automatic or the manual mode. In the manual mode: 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).

c) Save the settings.

d) Repeat the procedure described in a), b) and c) on the amplification plots of the taxon specific system.

e) Save the settings and export all the data 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 latter are transferred into an excel spreadsheet to determine the method performance requirements for qualitative methods: the efficiency of the amplification, the false negative rate, the false positive rate and the Limit of Detection as POD = 0.95 (probability of detection).

4 Equipment and Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition) and appropriate analysis software
- 96-well reaction plates
- Optical caps/adhesion covers
- Microcentrifuge
- Micropipettes
- Standard bench top centrifuge with rotor or standard microfuge fit for 0.5 mL reaction tubes, centrifuge for 96-Well reaction plates
- Vortex
- Racks for reaction tubes, also cooled
- 0.5, 1.5 mL and 5 or 15 mL DNAse free reaction tubes

4.2 Reagents

- TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific #4444557)

4.3 Primers and Probes

Table 5. Primers and probes for the MxY0541 and *hmg* methods

		DNA Sequence (5' to 3')		**Length (nt)**
	MxY0541			
Forward primer	MxY0541-F10	CTG CAT CTC TCA GGC AAA TGG C		22
Reverse primer	MxY0541-R10	AAC TCA CTT CCG TAC GCA ACC A		22
Probe	MxY0541-P10	FAM-TCC TCT TGA GCT AGC TAC CGT-MGB		21
	hmg			
Forward primer	hmg primer 1	TTG GAC TAG AAA TCT CGT GCT GA		23
Reverse primer	hmg primer 2	GCT ACA TAG GGA GCC TTG TCC T		22
Probe	hmg probe	FAM-CAA TCC ACA CAA ACG CAC GCG TA-TAMRA		23

FAM: 6-carboxyfluorescein; MGB: minor groove binder.

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
LOD	Limit of Detection
LOQ	Limit of Quantification
POD	Probability of Detection

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