Validation of TaqMan screening qPCR method specific to the GMM protease2 in targeting the junction between the pUB110 shuttle vector and a Bacillus gene coding for a protease (GMM protease2 marker)

This dossier describes the validation of a Taq®Man qPCR method for the detection of an unauthorized GMM producing protease (GMM protease2). This GMM protease2 marker targets the junction between the pUB110 shuttle vector and a *Bacillus* (probably *amyloliquefaciens*) gene coding for a protease. This method is intended for use in the GMOlab of the Transversal activities in Applied Genomics (TAG) for the detection of the above mentioned GMM producing protease2 in food and feed microbial fermentation products.

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1 qPCR method

The method described here uses the TaqMan® qPCR chemistry for amplification of a 120 bp fragment of the junction of a GMM producing protease (Fraiture et al., 2021 in preparation).

1.1. Oligonucleotides

The primer and probe sequences are shown in table 1.

Table 1. Primer and probe sequences. The sequence of the forward primer from the GMM protease2 marker (Fraiture et al., 2021 in preparation) is identical to the one of the forward primer from the GMM protease marker (Fraiture et al., 2020) and the GMM alpha-amylase marker (Fraiture et al., 2021 submitted).

Oligonucleotide type	Oligonucleotide name	Sequence (5'-3')
Forward primer	GMM_protease2-F	GAAAAACGAGGAAAGATGCTG
Reverse primer	GMM_protease2-R	TGACAAATGAAGACGGGAAA
Probe	GMM_protease2-P	FAM- TTGAGCAACTGGATCTTAACATTTTTCCCCT-TAMRA

1.2. qPCR mix

The qPCR mix is given in table 2 (Fraiture et al., 2021 in preparation).

Table 2. qPCR mix for the GMM protease2 marker.

Component	Stock concentration	Final concentration	µl per reaction
TaqMan mastermix	2X	1 X	12.50 µl
Primer GMM_protease2-F	20 µM	400 nM	0.50 µl
Primer GMM_protease2-R	20 µM	400 nM	0.50 µl
Probe GMM_protease2-P	10 µM	200 nM	0.50 µl
Nuclease-free water			6.00 µl
		Total volume	20 µl
		DNA 5 ng/µl	5 µl

1.3. qPCR programme

The qPCR programme is given in table 3. See also Fraiture et al., 2021 in preparation.

Table 3. qPCR programme

Step	Stage	T (°C)	Time (s)	Acquisition	Cycles
1	Activation UNG	50	120	No	1x
2	Taq Activation	95	600	No	1x
3	Amplification Denaturing Annealing and Extension	 95 60	 15 60	 No Mesure	45x

1.4. Control materials

No Certified Reference Materials (CRM) or other Reference Materials (RM) are commercially available for this method. As positive control material, gDNA extracted from a food enzyme product, labelled as containing protease, contaminated by a GMM producing protease is used (sample 1, see Table 4). In addition, a plasmid, artificially synthetized by genecust, carrying one copy of the target sequence (Figure 1) is used (PC plasmid carrying one copy of the target sequence).

2 Scope of the validation

The method can be used in the GMO screening procedure for the detection of an unauthorized GMM producing protease (GMM protease2) in food and feed microbial fermentation products. The proposed Taq®Man qPCR method is intended for use as a qualitative detection method. Therefore, the validation parameters tested are the specificity and the limit of detection ($LOD_{95\%}$).

3 Method development

The GMM protease2 marker was developed by TAG in the frame of the SPECENZYM project and is described in Fraiture et al. 2021 (in preparation). This TaqMan® qPCR method is targeting the junction of an unauthorized GMM producing protease. More precisely, the target sequence corresponds to the junction between the pUB110 shuttle vector and a gene coding for a protease belonging to *Bacillus* species (probably *amyloliquefaciens*).

3.1. Experimental set-up for testing in situ specificity

3.1.1 Bacterial strains and isolates

The specificity tests were done using positive and negative materials:

A food enzyme product, labelled as containing protease, contaminated by a GMM producing protease was used as positive material (sample 1, see Table 4). In addition, a plasmid, artificially synthetized by genecust, carrying one copy of the targeted sequence (Figure 1) was used as positive material (PC plasmid carrying one copy of the target sequence, see Table 4).

Different types of negative materials were used: 1/ WT bacterial species reported as used for the production of food and feed fermentation products; 2/ WT fungal species reported as used for the production of food and feed fermentation products; 3/ GM *Bacillus subtilis* RASFF2014.1249 strain producing vitamin B2; 4/ GM *Bacillus velezensis* RASFF2019.3332 strain producing protease; 5/ a food enzyme product (labelled as containing alpha-amylase; Dextzyme HT from The Alchemist's Pantry) contaminated by a GMM overproducing alpha-amylase (RASFF2019.3332).

In addition, a no template control (NTC = water) was used.

The list of all tested materials is given in table 4.

Table 4: Materials used to test the specificity of the GMM protease2 qPCR method. All materials were used at 10 ng , excepted the PC plasmid, carrying one copy of the target sequence, that was used at 100 copies of the target (see Table 6).

Species	Origin	Reference
Positive samples		
gDNA extracted from a food enzyme product, labelled as containing protease, contaminated by the GMM producing protease2	/	sample 1
Positive control plasmid carrying one copy of the target sequence	/	PC plasmid carrying one copy of the target sequence
Negative samples		
<u>Fungal strains</u>		
Aspergillus acidus	BCCM	IHEM 26285
Aspergillus aculeatus	BCCM	IHEM 05796
Aspergillus fijiensis	BCCM	IHEM 22812
Aspergillus melleus	BCCM	IHEM 25956
Aspergillus niger	BCCM	IHEM 25485
Aspergillus oryzae	BCCM	IHEM 25836
Candida cylindracea	BCCM	MUCL 041387
Candida rugose	BCCM	IHEM 01894
Chaetomium gracile	BCCM	MUCL 053569
Cryphonectria parasitica	BCCM	MUCL 007956
Disporotrichum dimorphosporum	BCCM	MUCL 019341
Fusarium venenatum	BCCM	MUCL 055417
Hansenula polymorpha	BCCM	MUCL 027761
Humicola insolens	BCCM	MUCL 015010
Kluyveromyces lactis	BCCM	IHEM 02051
Leptographium procerum	BCCM	MUCL 008094
Mucor javanicus	BCCM	IHEM 05212
Penicillium camemberti	BCCM	IHEM 06648
Penicillium chrysogenum	BCCM	IHEM 03414
Penicillium citrinium	BCCM	IHEM 26159
Penicillium decumbens	BCCM	IHEM 05935
Penicillium funiculosum	BCCM	MUCL 014091
Penicillium multicolor	CBS	CBS 501.73
Penicillium roqueforti	BCCM	IHEM 20176
Pichia pastori	BCCM	MUCL 027793
Rhizomucor miehei	BCCM	IHEM 26897
Rhizopus niveus	ATCC	ATCC 200757
Rhizopus oryzae	BCCM	IHEM 26078

Species	Origin	Reference
Saccharomyces cerevisiae	BCCM	IHEM 25104
Sporobolomyces singularis	BCCM	MUCL 027849
Talaromyces cellulolyticus/pinophilus	BCCM	IHEM 16004
Talaromyces emersonii	DSMZ	DSMZ 2432
Trametes hirsuta	BCCM	MUCL 030869
Trichoderma citrinoviride	BCCM	IHEM 25858
Trichoderma longibrachiatum	BCCM	IHEM 00935
Trichoderma reesei	BCCM	IHEM 05651
Trichoderma viride	BCCM	IHEM 04146
Bacterial strains		
Arthrobacter ramosus	BCCM	LMG 17309
Bacillus amyloliquefaciens	BCCM	LMG 98140
Bacillus brevis	BCCM	LMG 7123
Bacillus cereus	ATCC	ATCC 14579
Bacillus circulans	BCCM	LMG 6926T
Bacillus coagulans	BCCM	LMG 6326
Bacillus firmus	BCCM	LMG 7125
Bacillus flexus	BCCM	LMG 11155
Bacillus lentus	Sciensano	TIAC 101
Bacillus licheniformis	BCCM	LMG 6933T
Bacillus megaterium	BCCM	LMG 7127
Bacillus pumilus	DSMZ	DSMZ 1794
Bacillus smithii	BCCM	LMG 6327
Bacillus subtilis	BCCM	LMG 7135 T
Bacillus subtilis	Sciensano	W04-510
Bacillus subtilis	Sciensano	E07-505
Bacillus subtilis	Sciensano	S10005
Bacillus subtilis	Sciensano	SUB033
Bacillus subtilis	Sciensano	BNB54
Bacillus velezensis	BCCM	LMG 12384
Bacillus velezensis	BCCM	LMG 17599
Bacillus velezensis	BCCM	LMG 22478
Bacillus velezensis	BCCM	LMG 23203
Bacillus velezensis	BCCM	LMG 26770
Bacillus velezensis	BCCM	LMG 27586
Cellulosimicrobium cellulans	BCCM	LMG 16121
Corynebacterium glutamicum	BCCM	LMG 3652
Enterococcus faecium	BCCM	LMG 9430
Escherichia coli	BCCM	LMG2092T
Geobacillus caldoproteolyticus	DSMZ	DSMZ 15730
Geobacillus pallidus	BCCM	LMG 11159T

Species	Origin	Reference
Geobacillus stearothermophilus	BCCM	LMG 6939T
Klebsiella pneumonia	BCCM	LMG 3113T
Lactobacillus casei	BCCM	LMG 6904
Lactobacillus fermentum	BCCM	LMG 6902
Lactobacillus plantarum	BCCM	LMG 9208
Lactobacillus rhamnosus	BCCM	LMG 18030
Lactococcus lactis	BCCM	LMG 6890T
Leuconostoc citreum	BCCM	LMG 9824
Microbacterium imperiale	BCCM	LMG 20190
Paenibacillus alginolyticus	BCCM	LMG 18723
Paenibacillus macerans	BCCM	LMG 6324
Protaminobacter pubrum	CBS	CBS 574.77
Pseudomonas amyloderamosa	ATCC	ATCC-21262
Pseudomonas fluorescens	BCCM	LMG1794T
Pullulanibacillus naganoensis	BCCM	LMG 12887
Streptomyces aureofaciens	BCCM	LMG 5968
Streptomyces mobaraensis	DSMZ	DSMZ 40847
Streptomyces murinus	BCCM	LMG 10475
Streptomyces netropsis	BCCM	LMG 5977
Streptomyces rubiginosus	BCCM	LMG20268
Streptomyces violaceoruber	BCCM	LMG 7183
Streptoverticillium mobaraense	CBS	CBS 199.75
GMM samples		
gDNA extracted from a food enzyme product (labelled as containing alpha-amylase; Dextzyme HT from The Alchemist's Pantry) contaminated by a GMM producing alpha-amylase (RASFF2019.3332)	Dextzyme HT, The Alchemist's Pantry	Dextzyme HT
GM Bacillus subtilis producing vitamin B2 (RASFF2014.1249)	SCL	GMM producing vitamin B2
GM Bacillus velezensis producing protease (RASFF2019.3332)	Sciensano	GMM producing protease
PCR controls		
No template control (NTC)		

BCCM: Belgian co-ordinated collections of micro-organisms (http://bccm.belspo.be/).
CBS: Convention of Biological Diversity (http://www.wi.knaw.nl/Collections/).
DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; (https://www.dsmz.de/).
ATCC: American Type Culture Collection (http://www.lgcstandards-atcc.org/) ;
Sciensano: Service "Foodborne pathogenes, J. Wytsmanstraat 14, 1050 Brussels.
SCL : Service Commun des Laboratoires, France

3.1.2 Equipment:

qPCR: CFX96 Touch Real-Time PCR Detection System (BioRad).

3.1.3 Reagents:

qPCR: Taq®Man 2x Master Mix (Diagenode BE, ref: GMO-MM2X-A300), Nuclease free water (Acros organics, BE), primers and probe (Eurogentec, BE).

3.1.4 Bacterial DNA isolation and measurement

Bacterial gDNA from GM *B. subtilis* and *velezensis* isolates was extracted as described in Fraiture et al. 2020. The wild-type microbial strains were provided as gDNA extracted by using Quick-DNA™ Fungal/Bacterial Miniprep Kit" (Zymo, cat. No. D6005).

The DNA concentration was measured spectrophotometrically using Nanodrop® 2000 (ThermoFisher) and DNA purity was evaluated using the A260/A280 and A260/A230 ratios.

3.1.5 Taq®Man qPCR:

The qPCR conditions are described in part 1.1. – 1.3. The reactions were carried out in two replicates for each sample.

3.1.6 Analysis of the amplicon sequence

The PCR product from the GMM protease2 marker was purified using USB ExoSAP-IT PCR Product Cleanup (Affymetrix) to be sequenced on a Genetic Sequencer 3500 (ThermoFisher) using the Big Dye Terminator Kit v3.1 (Applied Biosystems). The amplicon sequence is shown in Figure 1.

GAAAAACGAGGAAAGATGCTGTTCTTGTAAATGAGTTGCTAGTAACATCTGACCGAGATTTTT**TT**
GAGCAACTGGATCTTAACATTTTTCCCCTATCATT**TTTCCCGTCTTCATTTGTCA**

Figure. 1 Amplicon sequence of GMM protease 2 qPCR method. The primers and probe are indicated in bold.

As detailed in Fraiture et al. 2021 (in preparation), the amplicon sequence from the GMM protease2 marker was also compared to the ones from the GMM protease markers (Figure 2). On this basis, although the GMM producing protease and the GMM producing protease2 present both unnatural associations of sequences between the pUB110 shuttle vector and a *Bacillus* gene coding a protease, these two GMM were demonstrated as differentiable (Fraiture et al. 2021 in preparation).

```
(A)
GMM protease2       GAAAAACGAGG--AAAGATGCTGTTCTTGTAAATGAGTTGCTAGTAACATCTGACCGAGA
GMM protease-L      CGAGAATGCAGCTGAAACAGCACTTGGCGGAAAAAAGGAAGGACGGACA---GATCAAGA
                      * ** *  *   **   **  **   * ***  **     *   ***   ** * ***

GMM protease2       TTTTTTTGAGCAACTGGATCTTAACATTTTTCCCCTATCATTTTTCCCGTCTTCATTTGT
GMM protease-L      ACTGTTATGGCTACAAGAT----AAA---TTCCCCGAGCATATG----------------
                      * **   ** **  ***    * *   ****** * *** *

GMM protease2       CA
GMM protease-L      --

(B)
GMM protease2          GAAAAACGAGGAAAGATGCTGTTCTTGTAAATGAGTTGCTAGTAACATCTGACCGAGATT
GMM alpha-amylase      GAAAAACGAGGAAAGATGCTGTTCTTGTAAATGAGTTGCTAGT-ACATCTGACCGAGATT
                       ******************************************* ****************

GMM protease2          TTTTTGAGCAACTGGATCTTAACATTTTTCCCCTATCATTTTTCCCG--------TCTTC
GMM alpha-amylase      TTTTTGAGCAACTGGATCCCACGTTGTGATTAAAAGCAGCGATCCCGATGAACAATCCAT
                       ******************  *   * *       * **    *****        **

GMM protease2          ATTTG------------TCA
GMM alpha-amylase      ATTGGAAGCAGCTACGATCG
                       *** *            **
```

Figure. 2 Alignments of the amplicon sequences from the GMM protease2 marker against the ones from the GMM protease Left (A) and Right (B) markers .

3.1.7 Data processing

The following analyses of the data obtained by the qPCR specificity testing were done based on:

- Calculation of the false negatives rate. The false negatives rate was calculated according to the formula:

$$False\ neg\ rate = \frac{100x(No.misclassified\ known\ pos\ samples)}{total\ No.known\ pos\ samples}$$

- Calculation of the false positives rate. The false positives rate was calculated according to the formula:

$$False\ pos\ rate = \frac{100x(No.misclassified\ known\ neg\ samples)}{total\ No.known\ neg\ samples}$$

The GMM protease2 qPCR method is considered specific when the following conditions are obtained:

- Positive amplification signal in materials containing the targeted sequence from the junction between the pUB110 shuttle vector and a gene coding for a protease belonging to *Bacillus* species with a false negative rate of 0%.

- No positive amplification signal in materials that do not contain the targeted sequence from the junction between the pUB110 shuttle vector and a gene coding for a protease belonging to *Bacillus* species with a false positives rate of 0%.

3.2. Results

3.2.1 Specificity of the qPCR method

The positive materials (2 samples) gave a positive amplification signal. No misclassified positive samples were observed. The false negatives rate was 0%.

All negative materials (93 samples) and the negative PCR control (1 sample) gave no amplification signal. No misclassified negative samples were observed. The false positives rate was 0%.

The C_t values obtained for the positive material is given in table 5. As no C_t was recorded for the negative materials, it is not included in the table 5. The full C_t dataset and amplification curves are shown in Annex 1.

Table 5 Results from the specificity test of the qPCR method for the positive material. Information about the estimated target copy number is not available for the sample 1, as it is DNA extracted from a food enzyme product contaminated with an unknown amount of GMM protease2. For the PC plasmid, the test was performed on 100 estimated target copies.

Bacterial species	C_t value
sample 1	*23.5*
PC plasmid carrying one copy of the target sequence	*33.8*

3.3. Conclusions on specificity

The experimental results on specificity showed that:

- The false positives rate is 0%.

- The false negatives rate is 0%.

Based on this, it can be concluded that the GMM protease2 qPCR method is specific for the detection of an unauthorized GMM producing protease2 (GMM protease2).

4 Limit of detection ($LOD_{95\%}$) of the GMM protease2 qPCR method

4.1. Experimental set-up

4.1.1 Material:

Plasmid DNA synthetized by genecust is carrying the target sequenced (Figure 1) (Fraiture et al., 2021, in preparation). The plasmid was used under the native/supercoiled form.

4.1.2 Preparation of the serial dilutions

The plasmid DNA was first diluted in nuclease free water (Acros organics, BE) in order to obtain 1,600 copies template in the final reaction.
The copy number calculation was done according to the formula hereunder taking into account that the plasmid size is 2,929 bp (Fraiture et al., 2020):

$$N = \frac{m \times N_A}{\langle Mw \rangle \times L}$$

Formula symbols:
N = number of plasmid molecules
m = amount of plasmid (grams)
N_A = Avogadro constante = $6{,}0221415 \times 10^{23}$ mol^{-1}
<MW> = average base pair weight = 649Da

On this basis, serial dilutions, going from 100 copies to 0.1 copy as indicated in Table 6, were prepared in nuclease free water (Acros organics, BE). To determine the $LOD_{95\%}$, 12 replicates of each dilution indicated in Table 6 were tested.

Table 6: Preparation of dilution series from 1,600 copies.

DNA concentration (ng)	Copy number	Dilution factor	Volume from the previous dilution (µl)	Water (µl)	Total volume (µl)
0.000005	1,600				
0.0000003125	100	16	100	1500	1600
0.0000000625	20	5	200	800	1000
0.00000003125	10	2	300	300	600
0.000000015625	5	2	200	200	400
0.000000003125	1	5	100	400	500
0.0000000003125	0.1	10	100	900	1000

4.1.3 Equipment

qPCR: CFX96 Touch Real-Time PCR Detection System (BioRad).

4.1.4 Reagents

qPCR: Taq®Man 2x Master Mix (Diagenode BE, ref: GMO-MM2X-A300), Nuclease free water (Acros organics, BE), primers and probe (Eurogentec, BE).

4.1.5 Taq®Man qPCR

The qPCR conditions are described part 1.1 – 1.3.

4.1.6 Data analysis

The $LOD_{95\%}$, defined as the number of copies of the target required to ensure a 95% probability of detection (POD), was determined by using Quodata web tool (Uhlig et al. 2015; Grohmann 2016; https://quodata.de/content/validation-qualitative-pcr-methods-single-laboratory) accessed in December

2020.

4.2. Results

The $LOD_{95\%}$ of the method was determined as described in part 4.1.6. The summarized data are shown in table 7 and in Fraiture et al., 2021 (in preparation). The full C_t data set and the amplification plots are shown in Annex 2. As indicated in Figure 2, the $LOD_{95\%}$ was set at **13** copies using DNA from a plasmid carrying one copy of the target sequence, complying with the acceptance criteria in the “Minimum Performance Requirements for Analytical Methods of GMO Testing” of the European Network of GMO Laboratories (ENGL, 2015). The plausibility check of the curve did not give any irregularities.

Table 7: Number of positive replicates and average Ct obtained for each dilution tested.

Copy number	Positive replicate number	Average C_t
100	12/12	33.8
20	12/12	36.3
10	10/12	37.3
5	10/12	37.9
1	2/12	40.4
0.1	0/12	/
0	0/12	/

Please enter your data

1st column: Number of copies of the target DNA sequence (= nominal copies)

2nd column: Number of positive test results

3rd column: Number of PCR replicates

Click here to insert example data

```
0.1   0    12
1     2    12
5     10   12
10    10   12
20    12   12
100   12   12
```

Clear data

Start the calculation

Results

Plausibility Check

The plausibility check indicates no irregularities.

POD curve and $LOD_{95\%}$

The $LOD_{95\%}$ is 12.529 with a 95 % confidence interval of [8.015, 19.557].

The figure below summarises the results. The blue diamonds characterise the laboratory-specific RODs. The blue curve denotes the mean POD curve along with the corresponding 95 % confidence range highlighted as the grey band. The POD curve under ideal conditions is displayed as the black dashed curve.

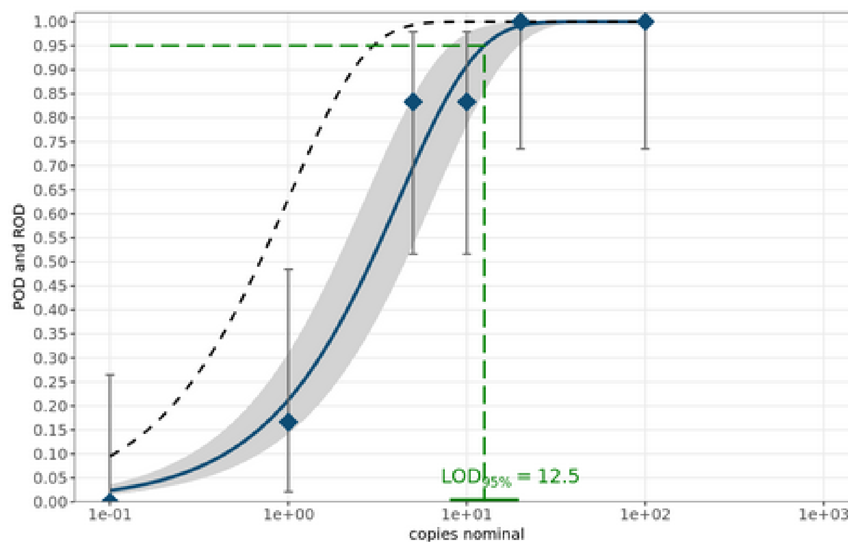


***Figure. 2** Calculation of $LOD_{95\%}$ based on the POD curve.*

5 Final conclusions for in-house validation

The results from the specificity and sensitivity testing showed that the tested parameters comply with the acceptance criteria described in the “Minimum Performance Requirements for Analytical Methods of GMO Testing” of the European Network of GMO Laboratories (ENGL, 2015).

As results of this, it can be concluded that the method can be included in the flexible scope of accreditation and can be used in routine analysis in food and feed microbial fermentation products under ISO17025.

As control material, gDNA extracted from food enzyme product, labelled as containing protease, contaminated by a GMM producing protease2 (sample 1) as well as plasmid DNA harboring the targeted sequence (PC plasmid) are recommended to be used.

6 Method transferability

In order to evaluate whether the in-house validated GMM protease2 marker performs in the same manner when performed in another laboratory with different operators using different equipment and reagents, a transferability study was carried out. The experiments were performed in Unità Operativa Semplice a valenza Direzionale - Ricerca e controllo degli organismi geneticamente modificati at the Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "M.Aleandri" (Roma, Italy).

6.1. Experimental set-up

The experimental setup was the same as described in section 4, for determination of the $LOD_{95\%}$. Serial dilutions of the control plasmid DNA, from 100 to 0.1 estimated target copy number, used in-house was tested with the same real-time PCR protocol, were sent to the second laboratory. All reagents were ordered by the external laboratory, including oligonucleotides (Metabion International AG) and TaqMan™ Universal PCR Master Mix (Applied Biosystems®). All runs were performed on a QuantStudio™ 7 Flex Real-Time PCR System (Life Technologies). The $LOD_{95\%}$ was determined based on the results of the second laboratory as described in the section 4.1.6.

6.2. Results

The summarized data are shown in table 7 and in Fraiture et al., 2021 (in preparation). The full C_t data set and the amplification plots are shown in Annex 3. As indicated in Figure 3, the $LOD_{95\%}$ was set at **9** copies using DNA from a plasmid carrying one copy of the target sequence, complying with the acceptance criteria in the "Minimum Performance Requirements for Analytical Methods of GMO Testing" of the European Network of GMO Laboratories (ENGL, 2015). The plausibility check of the curve did not give any irregularities.

Table 7: Number of positive replicates and average Ct obtained for each dilution tested.

Copy number	Positive replicate number	Average C_t
100	12/12	34.3
20	12/12	36.7
10	11/12	37.6
5	12/12	39.8
1	2/12	39.7
0.1	0/12	/
0	0/12	/

Please enter your data

1st column: Number of copies of the target DNA sequence (= nominal copies)

2nd column: Number of positive test results

3rd column: Number of PCR replicates

Click here to insert example data

0.1	0	12
1	2	12
5	12	12
10	11	12
20	12	12
100	12	12

Clear data

Start the calculation

Results

Plausibility Check

The plausibility check indicates no irregularities.

POD curve and $LOD_{95\%}$

The $LOD_{95\%}$ is 8.590 with a 95 % confidence interval of [5.330, 13.840].

The figure below summarises the results. The blue diamonds characterise the laboratory-specific RODs. The blue curve denotes the mean POD curve along with the corresponding 95 % confidence range highlighted as the grey band. The POD curve under ideal conditions is displayed as the black dashed curve.

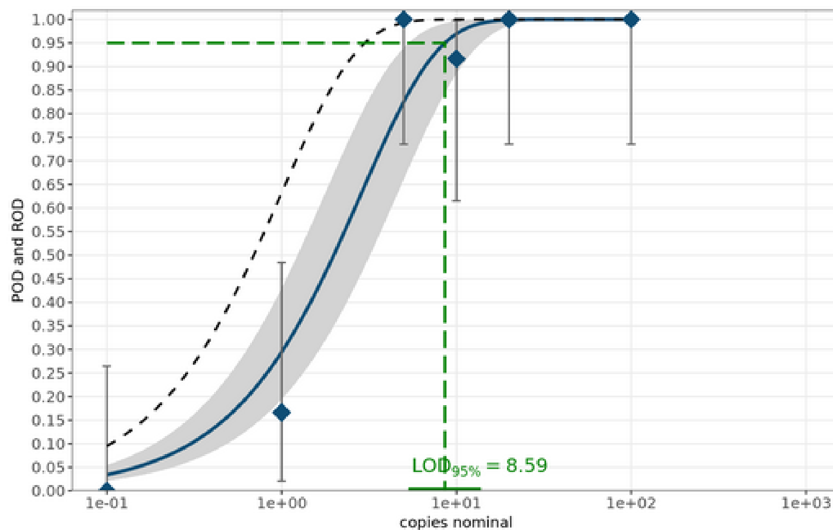


Figure. 3 *Calculation of $LOD_{95\%}$ based on the POD curve.*

6.3. Conclusion on the method transferability

The results obtained by the external laboratory were comparable with the ones observed during the in-house validation. These results demonstrate that the method can be successfully performed in another laboratory using different operators, infrastructure, equipment and reagents.

7 References

ENGL (2015) Definition of Minimum Performance Requirements for Analytical Methods for GMO Testing. http://gmo-crl.jrc.ec.europa.eu/doc/MPR%20Report%20Application%2020_10_2015.pdf.

Fraiture et al. (in preparation) 2021. Construct/Event-specific real-time PCR method targeting a second unauthorized GMM protease.

Fraiture et al. (submitted) 2021. Development of a real-time PCR method targeting an unauthorized genetically modified microorganism producing alpha-amylase.

Fraiture et al. 2020. Next-generation sequencing: a key tool to identify unauthorized genetically modified microorganisms in food enzyme preparations. Sci. Rep. 10:7094.

Grohmann 2016; Guidelines for the validation of qualitative real-time PCR methods by means of a collaborative study.

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

International Standard: ISO5725-2 (1994). Accuracy (trueness and precision) of measurement methods and results - Part 2. International Organisation for Standardization, Genéve, Switzerland.

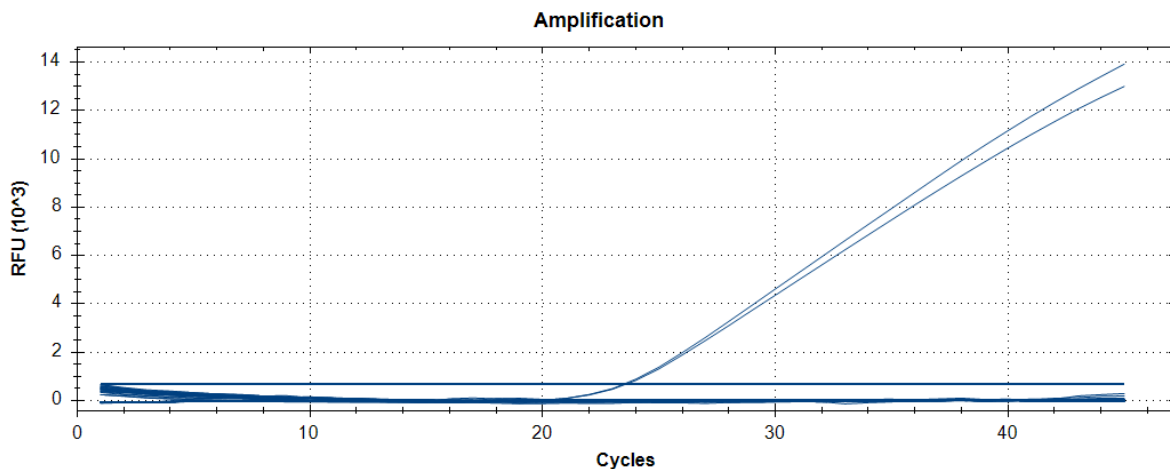
Uhlig, S., Frost, K., Colson, B. et al. Validation of qualitative PCR methods on the basis of mathematical–statistical modelling of the probability of detection. Accred Qual Assur 20, 75–83 (2015). https://doi.org/10.1007/s00769-015-1112-9

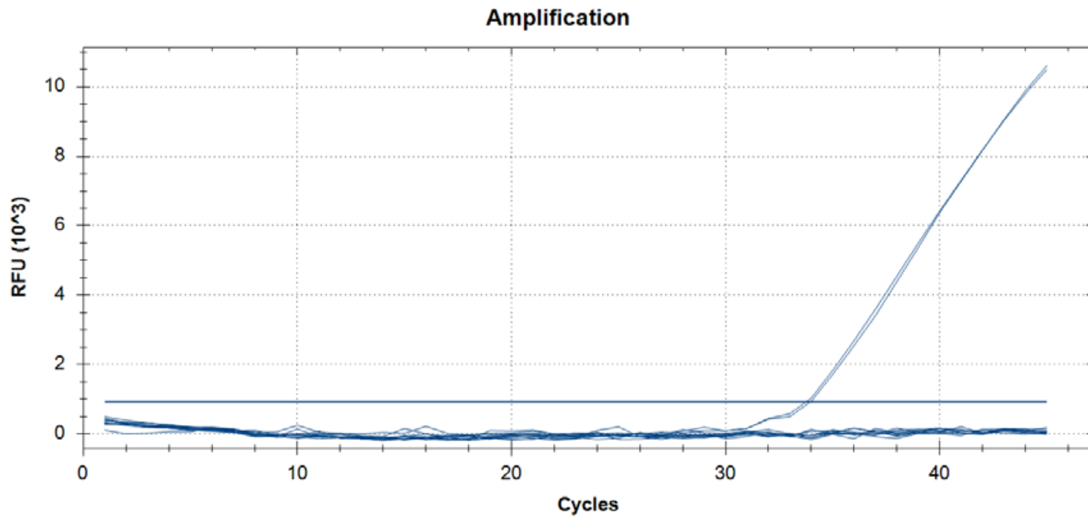
8 Annexes

Annex 1: Specificity: full C_t dataset and amplification plots

Samples		Ct values	
Positive samples			
Sample 1		23.5	23.5
PC plasmid carrying one copy of the target sequence		33,7	33.9
Negative samples			
Fungal strains			
Aspergillus acidus	IHEM 26285	No signal	No signal
Aspergillus aculeatus	IHEM 05796	No signal	No signal
Aspergillus fijiensis	IHEM 22812	No signal	No signal
Aspergillus melleus	IHEM 25956	No signal	No signal
Aspergillus niger	IHEM 25485	No signal	No signal
Aspergillus oryzae	IHEM 25836	No signal	No signal
Candida cylindracea	MUCL 041387	No signal	No signal
Candida rugose	IHEM 01894	No signal	No signal
Chaetomium gracile	MUCL 053569	No signal	No signal
Cryphonectria parasitica	MUCL 007956	No signal	No signal
Disporotrichum dimorphosporum	MUCL 019341	No signal	No signal
Fusarium venenatum	MUCL 055417	No signal	No signal
Hansenula polymorpha	MUCL 027761	No signal	No signal
Humicola insolens	MUCL 015010	No signal	No signal
Kluyveromyces lactis	IHEM 02051	No signal	No signal
Leptographium procerum	MUCL 008094	No signal	No signal
Mucor javanicus	IHEM 05212	No signal	No signal
Penicillium camemberti	IHEM 06648	No signal	No signal
Penicillium chrysogenum	IHEM 03414	No signal	No signal
Penicillium citrinium	IHEM 26159	No signal	No signal
Penicillium decumbens	IHEM 05935	No signal	No signal
Penicillium funiculosum	MUCL 014091	No signal	No signal
Penicillium multicolor	CBS 501.73	No signal	No signal
Penicillium roqueforti	IHEM 20176	No signal	No signal
Pichia pastori	MUCL 027793	No signal	No signal
Rhizomucor miehei	IHEM 26897	No signal	No signal
Rhizopus niveus	ATCC 200757	No signal	No signal
Rhizopus oryzae	IHEM 26078	No signal	No signal
Saccharomyces cerevisiae	IHEM 25104	No signal	No signal
Sporobolomyces singularis	MUCL 027849	No signal	No signal
Talaromyces cellulolyticus/pinophilus	IHEM 16004	No signal	No signal
Talaromyces emersonii	DSMZ 2432	No signal	No signal
Trametes hirsuta	MUCL 030869	No signal	No signal
Trichoderma citrinoviride	IHEM 25858	No signal	No signal
Trichoderma longibrachiatum	IHEM 00935	No signal	No signal
Trichoderma reesei	IHEM 05651	No signal	No signal
Trichoderma viride	IHEM 04146	No signal	No signal
Bacterial strains			
Arthrobacter ramosus	LMG 17309	No signal	No signal
Bacillus amyloliquefaciens	LMG 98140	No signal	No signal
Bacillus brevis	LMG 7123	No signal	No signal
Bacillus cereus	ATCC 14579	No signal	No signal
Bacillus circulans	LMG 6926T	No signal	No signal
Bacillus coagulans	LMG 6326	No signal	No signal
Bacillus firmus	LMG 7125	No signal	No signal
Bacillus flexus	LMG 11155	No signal	No signal
Bacillus lentus	TIAC 101	No signal	No signal
Bacillus licheniformis	LMG 6933T	No signal	No signal
Bacillus megaterium	LMG 7127	No signal	No signal
Bacillus pumilus	DSMZ 1794	No signal	No signal
Bacillus smithii	LMG 6327	No signal	No signal
Bacillus subtilis	W04-510	No signal	No signal
Bacillus subtilis	E07-505	No signal	No signal
Bacillus subtilis	LMG 7135 T	No signal	No signal
Bacillus subtilis	S10005	No signal	No signal
Bacillus subtilis	SUB033	No signal	No signal
Bacillus subtilis	BNB54	No signal	No signal
Bacillus velezensis	LMG 12384	No signal	No signal

Bacillus velezensis	LMG 17599	No signal	No signal
Bacillus velezensis	LMG 22478	No signal	No signal
Bacillus velezensis	LMG 23203	No signal	No signal
Bacillus velezensis	LMG 26770	No signal	No signal
Bacillus velezensis	LMG 27586	No signal	No signal
Cellulosimicrobium cellulans	LMG 16121	No signal	No signal
Corynebacterium glutamicum	LMG 3652	No signal	No signal
Enterococcus faecium	LMG 9430	No signal	No signal
Escherichia coli	LMG2092T	No signal	No signal
Geobacillus caldoproteolyticus	DSMZ 15730	No signal	No signal
Geobacillus pallidus	LMG 11159T	No signal	No signal
Geobacillus stearothermophilus	LMG 6939T	No signal	No signal
Klebsiella pneumonia	LMG 3113T	No signal	No signal
Lactobacillus casei	LMG 6904	No signal	No signal
Lactobacillus fermentum	LMG 6902	No signal	No signal
Lactobacillus plantarum	LMG 9208	No signal	No signal
Lactobacillus rhamnosus	LMG 18030	No signal	No signal
Lactococcus lactis	LMG 6890T	No signal	No signal
Leuconostoc citreum	LMG 9824	No signal	No signal
Microbacterium imperiale	LMG 20190	No signal	No signal
Paenibacillus alginolyticus	LMG 18723	No signal	No signal
Paenibacillus macerans	LMG 6324	No signal	No signal
Protaminobacter pubrum	CBS 574.77	No signal	No signal
Pseudomonas amyloderamosa	ATCC-21262	No signal	No signal
Pseudomonas fluorescens	LMG1794T	No signal	No signal
Pullulanibacillus naganoensis	LMG 12887	No signal	No signal
Streptomyces aureofaciens	LMG 5968	No signal	No signal
Streptomyces mobaraensis	DSMZ 40847	No signal	No signal
Streptomyces murinus	LMG 10475	No signal	No signal
Streptomyces netropsis	LMG 5977	No signal	No signal
Streptomyces rubiginosus	LMG20268	No signal	No signal
Streptomyces violaceoruber	LMG 7183	No signal	No signal
Streptoverticillium mobaraense	CBS 199.75	No signal	No signal
GMM samples			
Dextzym HT	/	No signal	No signal
GM Bacillus subtilis producing vitamin B2 (RASFF2014.1249)	/	No signal	No signal
GM Bacillus velezensis producing protease (RASFF2019.3332)	/	No signal	No signal
PCR controls			
NTC	/	No signal	No signal

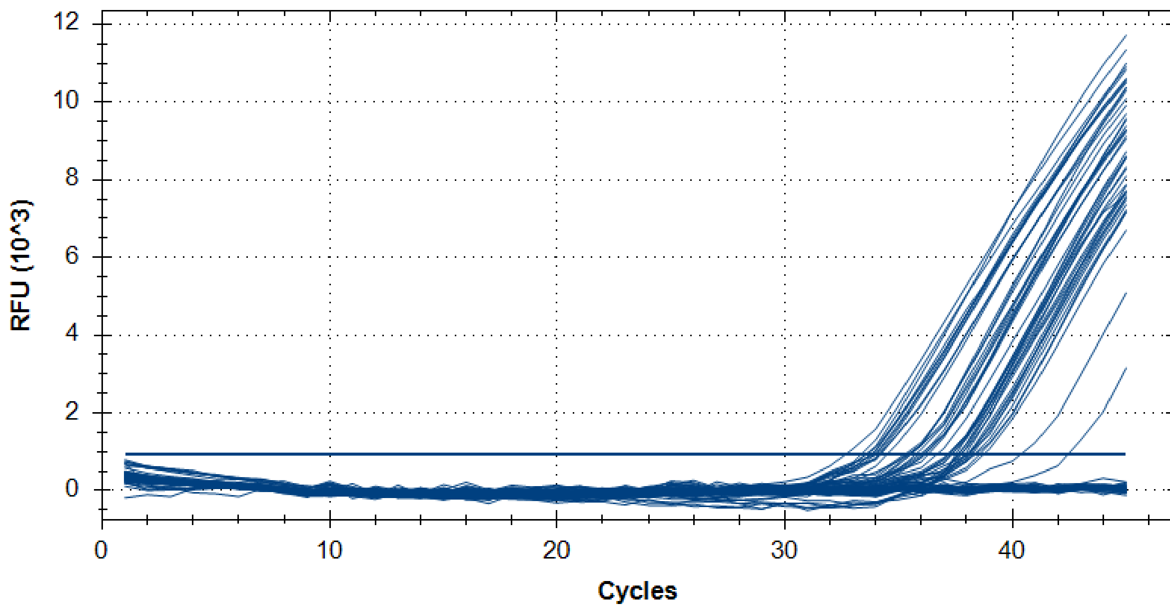




Annex 2: Sensitivity: full C_t dataset

Estimated full genome copy number	C_t values
100	32,63
	33,52
	33,76
	33,74
	33,94
	34,18
	34
	34,1
	34,15
	34,47
	33,32
	33,54
20	35,35
	36,12
	35,89
	36,65
	38,12
	37,3
	37,7
	35,38
	35,71
	35,44
	36,11
	35,5
10	No signal
	No signal
	37,31
	37,19
	35,23
	36,27
	37,17
	38,53
	40,16
	36,02
	37,12
	38,19
5	No signal
	No signal
	37,51
	37,6
	37,61
	38,06

	37,74 38,02 38,29 37,89 38,02 38,71
1	38,33 42,42 No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal
0.1	No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal
0	No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal



Annex 3: Transferability: full C_t dataset

Estimated full genome copy number	**C_t values**
100	33,8 34,2 34,6 34,3 34,5 34,6 34,0 34,1 34,1 34,4 34,5 34,2
20	37,4 36,0 37,0 39,2 36,4 36,6 35,5 36,9 36,6 36,4 36,0

	36,5
10	No signal 36,9 37,0 39,0 39,4 37,1 36,5 36,8 36,8 36,7 36,9 38,0
5	38,8 39,5 40,3 38,9 41,5 38,1 40,0 40,0 38,8 38,3 40,9 39,0
1	39,7 42,2 No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal
0.1	No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal
0	No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal; No signal