Validation of TaqMan screening qPCR method specific to the GMM protease1 in targeting the left transgene flanking region between the pUB110 shuttle vector and a Bacillus velezensis gene coding for a protease (GMM protease1 left border)

This dossier describes the validation of a Taq®Man qPCR construct-specific method for the detection of an unauthorized GMM producing protease (GMM protease1). This method targets the left transgene flanking region between the pUB110 shuttle vector and a *Bacillus velezensis* gene coding for a protease.
The method is intended for use to detect the above mentioned GMM producing protease 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 94 bp fragment of the left transgene flanking region of the GMM producing protease (Fraiture et al., 2020).

1.1. Oligonucleotides

The primer and probe sequences are shown in table 1.

Table 1. Primer and probe sequences.

Oligonucleotide type	Oligonucleotide name	Sequence (5'-3')
Forward primer	GMM_protease1_left_border-F	CGAGAATGCAGCTGAAACAG
Reverse primer	GMM_protease1_left_border-R	CATATGCTCGGGGAATTTATCT
Probe	GMM_protease1_left_border-P	FAM- GGACGGACAGATCAAGAACTGTTATGG-TAMRA

1.2. qPCR mix

The qPCR mix is given in table 2.

Table 2. qPCR mix for the GMM protease1 left border marker.

Component	Stock concentration	Final concentration	µl per reaction
TaqMan mastermix	2X	1 X	12.50 µl
Primer GMM_protease1_left_border-F	20 µM	400 nM	0.50 µl
Primer GMM_protease1_left_border-R	20 µM	400 nM	0.50 µl
Probe GMM_protease1_left_border-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.

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 an isolated strain of GM *B. velezensis* producing protease, related to RASFF2019.3332 (isolate indicated as 2019.3332) is used. In addition, a plasmid, artificially synthetized by genecust, carrying one copy of the target sequence (Figure 1) can be used.

2 Scope of the validation

The 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 ($LOD_{95\%}$), as described in Fraiture et al., 2020.

3 Method development

The GMM protease left border marker was developed by Service TAG in Sciensano within the SPECENZYM project and is described in Fraiture et al. 2020. This TaqMan® qPCR method is targeting the left transgene flanking region 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 velezensis* species.

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:

As positive materials, three samples were used: 1/ gDNA from isolate 2019-3332 related to RASFF2019.3332 (GM *B. velezensis* RASFF2019.3332 strain); 2/ DNA extract from a food enzyme product (labelled as containing protease; Sample 1) contaminated by the GM *B. velezensis* RASFF2019.3332 strain; 3/ a plasmid, artificially synthetized by genecust (https://www.genecust.com/en/) carrying one copy of the targeted sequence (Figure 1) (PC plasmid).

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.

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 protease1 left border 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		
Bacillus velezensis GM (2019-3332)	/	2019-3332
gDNA extracted from a food enzyme product, labelled as containing protease. This "real-life sample" was reported as being contaminated by the GMM producing protease1 (RASFF2019.3332).	/	sample 1
Positive control plasmid carrying one copy of the target sequence	/	PC plasmid carrying one copy of the target sequence
Negative samples		
Fungal strains		
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
Chaetomiumgracile	BCCM	MUCL 053569
Cryphonectria parasitica	BCCM	MUCL 007956
Disporotrichumdimorphosporum	BCCM	MUCL 019341
Fusariumvenenatum	BCCM	MUCL 055417
Hansenula polymorpha	BCCM	MUCL 027761
Humicola insolens	BCCM	MUCL 015010
Kluyveromyces lactis	BCCM	IHEM 02051
Leptographiumprocerum	BCCM	MUCL 008094

Species	Origin	Reference
Mucor javanicus	BCCM	IHEM 05212
Penicilliumcamemberti	BCCM	IHEM 06648
Penicilliumchrysogenum	BCCM	IHEM 03414
Penicilliumcitrinium	BCCM	IHEM 26159
Penicilliumdecumbens	BCCM	IHEM 05935
Penicilliumfuniculosum	BCCM	MUCL 014091
Penicilliummulticolor	CBS	CBS 501.73
Penicilliumroqueforti	BCCM	IHEM 20176
Pichia pastori	BCCM	MUCL 027793
Rhizomucor miehei	BCCM	IHEM 26897
Rhizopus niveus	ATCC	ATCC 200757
Rhizopus oryzae	BCCM	IHEM 26078
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

Species	Origin	Reference
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
Cellulosimicrobiumcellulans	BCCM	LMG 16121
Corynebacteriumglutamicum	BCCM	LMG 3652
Enterococcus faecium	BCCM	LMG 9430
Escherichia coli	BCCM	LMG2092T
Geobacillus caldoproteolyticus	DSMZ	DSMZ 15730
Geobacillus pallidus	BCCM	LMG 11159T
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
Microbacteriumimperiale	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
Streptoverticilliummobaraense	CBS	CBS 199.75
GMM samples		
GM Bacillus subtilis producing vitamin B2 (RASFF2014.1249)	SCL	GMM producing vitamin B2
gDNA extracted from a food enzyme product, labelled as containing protease. This "real-life sample" was reported as being contaminated by the GMM producing protease2 (RASFF2021.1641).	Sciensano	GMM producing protease2
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, BE).

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 protease1 left border 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 on Figure 1.

CGAGAATGCAGCTGAAACAGCACTTGGCGGAAAAAAGGAA**GGACGGACAGATCAAGAACTGT TATGG**CTACA**AGATAAATTCCCCGAGCATATG**

Figure. 1 Amplicon sequence of GMM protease1 left border qPCR method. The primers and probe are indicated in bold.

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 protease1 left border qPCR method is considered specific when the following conditions are obtained:

- Positive amplification signal in materials containing the targeted sequence from the left transgene flanking region of GM *B. velezensis* RASFF2019.3332 strain with a false negative rate of 0%.

- No positive amplification signal in materials that do not contain the targeted sequence from the left transgene flanking region of GM *B. velezensis* RASFF2019.3332 strain with a false positives rate of 0%.

3.2. Results

3.2.1 Specificity of the qPCR method

The positive materials (3 samples) gave a positive amplification signal. No misclassified positive samples were observed. The false negatives rate was 0%.

All negative materials (91 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 materials 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 materials. 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 protease1. For the PC plasmid, the test was performed on 100 estimated target copies. For the 2019.3332 isolate, the test was performed on 2,200,000 estimated genome copies.

Bacterial species	C_t value
Bacillus velezensis GM (2019-3332)	*15.5*
sample 1	*12.7*
PC plasmid carrying one copy of the target sequence	*33.2*

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 protease1 left border qPCR method is specific for the detection of the unauthorized GM *Bacillus velezensis* producing protease (GMM protease1).

4 Limit of detection ($LOD_{95\%}$) of the GMM protease1 left border qPCR method

4.1. Experimental set-up

4.1.1 Material:

Plasmid DNA synthetized by genecust is carrying the target sequenced (Figure 1). 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 1,595 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 2,903 bp:

$$N = \frac{m\ x\ N_A}{MW\ x\ L},$$

N is the number of plasmid molecules, m is the plasmid amount in g, N_A is Avogadro constante = 6,0221415 x 10^{23} $mol^{-1,}$ MW is average base pair weight = 649Da and L is the plasmid length in base pairs.
On this basis, dilution series starting 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.

DNA concentration (ng)	Copy number	Dilution factor	Volume from the previous dilution (µl)	Water (µl)	Total volume (µl)
0.000005	1595				
3.125E-07	100	16	100	1500	1600
6,25E-08	20	5	200	800	1000
3.125E-08	10	2	300	300	600
1.5625E-08	5	2	200	200	400
3.125E-09	1	5	100	400	500
3.125E-10	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 November 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. 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 **3** 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	33.2

20	12/12	35.7
10	12/12	36.8
5	12/12	38.2
1	8/12	40.2
0.1	0/12	/
0	0/12	/

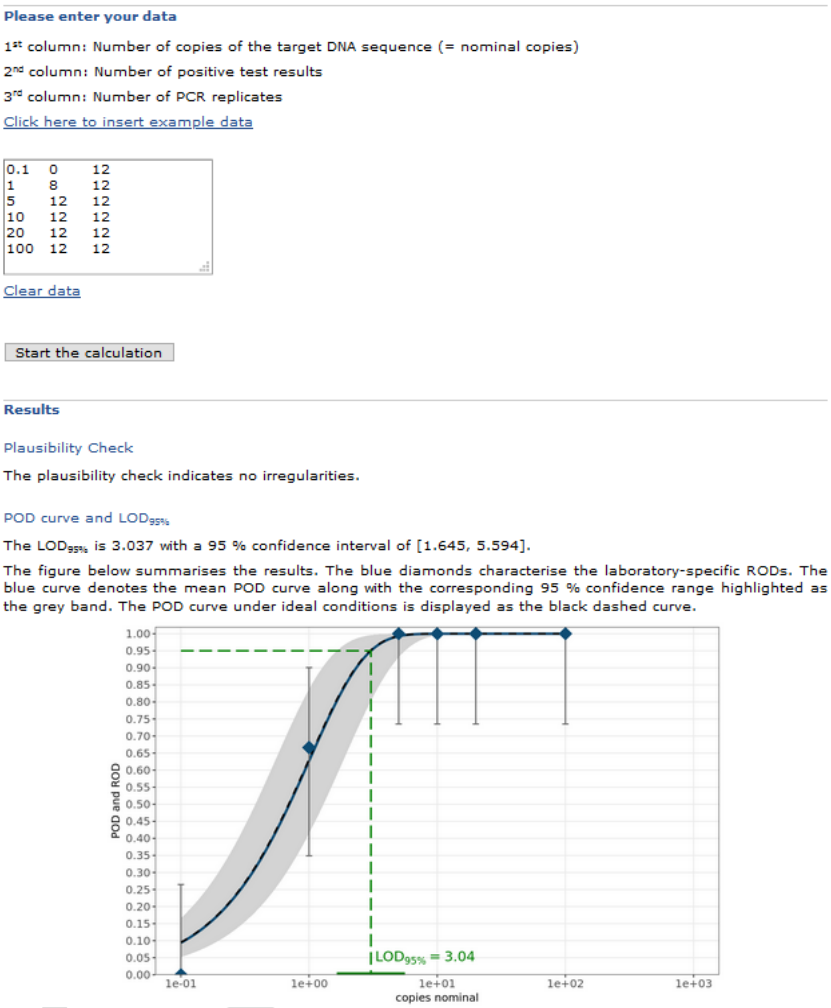

***Figure. 2** Calculation of $LOD_{95\%}$ based on the POD curve.*

5 Final conclusions on the 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 control material, gDNA extracted from the *B. velezensis* strain 2019-3332 as well as plasmid DNA harboring the targeted sequence (PC plasmid for GM protease1 left border marker) are recommended to be used. gDNA from the *B. velezensis* strain 2019-3332 is available in Sciensano. The plasmid was artificially synthetized by Genecust (https://www.genecust.com/en/). This plasmid consists in pUC57 where the sequence of interest (Figure 1) was introduced.

6 Method transferability

In order to evaluate whether the in-house validated GMM protease1 left border 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®, 4304437). 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 8. The full C_t data set and the amplification plots are shown in Annex 3. The number of the positive replicates per dilution was comparable to the results presented in Table 7.

As indicated in Figure 3, the $LOD_{95\%}$ was set at **2** 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).

Table 8: 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	36.6
10	12/12	37.5
5	12/12	39.1
1	10/12	40.2
0.1	2/12	41.0
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   2    12
1     10   12
5     12   12
10    12   12
20    12   12
100   12   12
```

Clear data

Start the calculation

Results

Plausibility Check

The calculated POD curve indicates sensitivity better than achievable according to the theoretical POD curve.

POD curve and $LOD_{95\%}$

The $LOD_{95\%}$ is 1.664 with a 95 % confidence interval of [0.889, 3.108].

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.

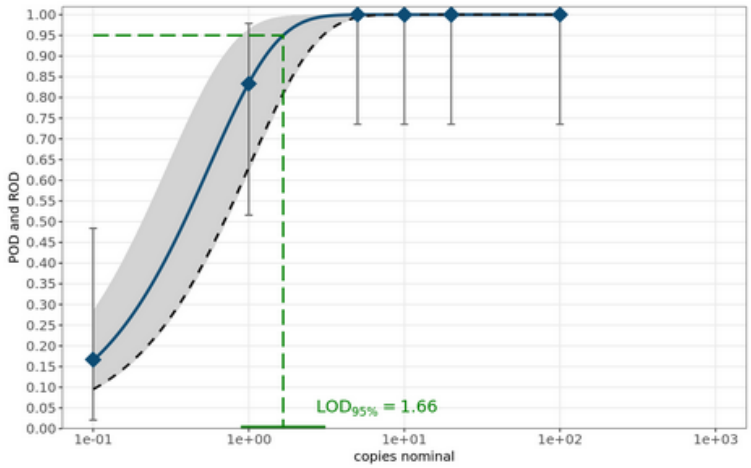

***Figure. 3** Calculation of $LOD_{95\%}$ based on the POD curve.*

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

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8 Annexes

Annex 1: Specificity: full C_t dataset and amplification plots. DNA extracts were previously assessed as amplifiable (Deckers et al., 2020a,b; Fraiture et al., 2020).

Samples		Ct values	
Positive samples			
Bacillus velezensis GM (2019-3332)		15.5	15.5
Sample 1		12.7	12.7
PC plasmid carrying one copy of the target sequence		33.2	33.4
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
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Hansenula polymorpha	MUCL 027761	No signal	No signal
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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
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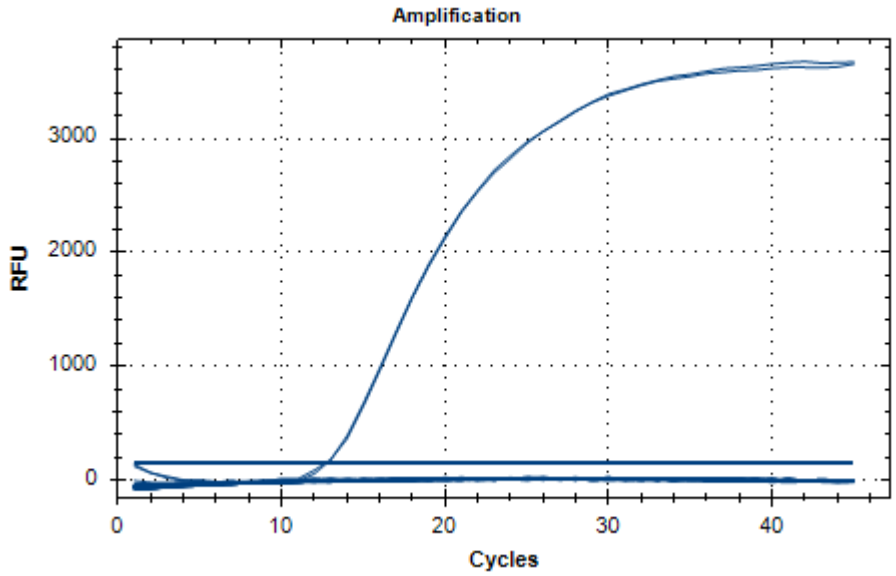
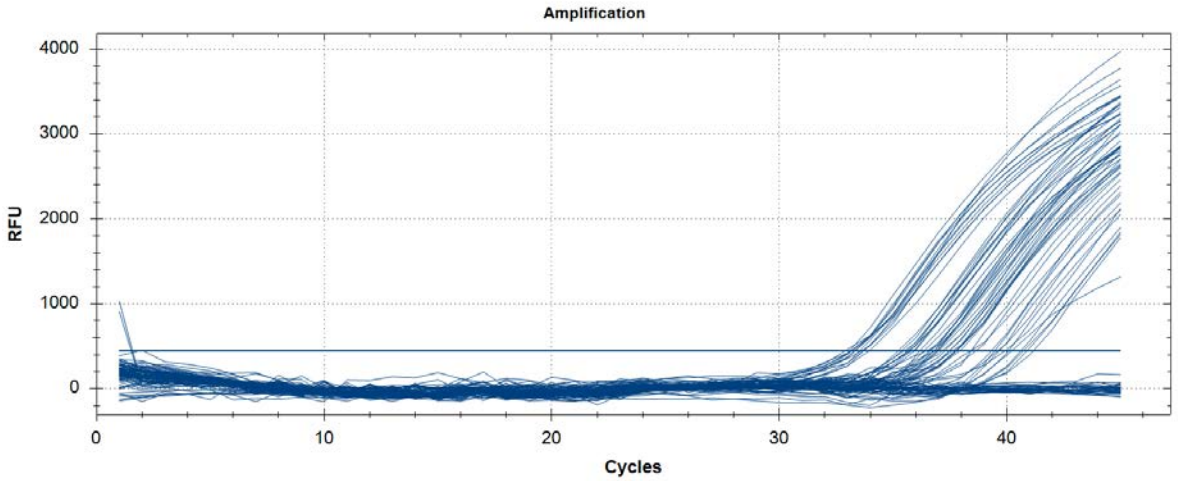
Bacillus brevis	LMG 7123	No signal	No signal
Bacillus cereus	ATCC 14579	No signal	No signal
Bacillus circulans	LMG 6926T	No signal	No signal
Bacillus coagulans	LMG 6326	No signal	No signal
Bacillus firmus	LMG 7125	No signal	No signal
Bacillus flexus	LMG 11155	No signal	No signal
Bacillus lentus	TIAC 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			
GM Bacillus subtilis producing vitamin B2 (RASFF2014.1249)	/	No signal	No signal
GMM producing protease2*	/	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	33,41 32,94 33,01 32,7 33,38 33,15 33,02 33,2 32,99 33,57 33,53 33,77
20	35,23 35,51 35,1 35,5 35,79 35,61 36,19 36,06 36,53 35,62 36,24 35,3
10	35,51 37,15 36,66 37,03 36,88 36,61 37,34 36,45 36,67 36,8 37,37 37,61
5	36,79 39,21 37,45 38,22 38,47 39,45 37,76 38,35 37,66 37,62 37,6 39,74
1	41,05 No signal 39,36 No signal No signal 40,08 No signal 40,61 39,55 39,99 40,77 40,51
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	34,322 34,233 34,519 34,125 34,254 34,498 34,123 34,817 34,119 34,296 34,132 34,276
20	36,647 36,035 36,468 36,857 36,275 36,189 36,718 37,013 36,692 37,030 36,073 37,370
10	37,590 38,025 37,342 37,356 37,416 37,763 36,882 37,596 37,567 37,701 37,772 37,315
5	38,506 39,933 37,717 40,245 39,827 39,063 39,178 38,911 38,706

	39,558 39,501 37,518
1	41,215 40,263 40,919 38,596 39,202 41,056 40,683 No signal No signal 40,045 39,976 39,927
0.1	40,945 41,120 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