VÚVeL VÝZKUMNÝ ÚSTAV VETERINÁRNÍHO LÉKAŘSTVÍ, v. v. i.

PROTOTYPE

Diagnostic System for Detection of Bacillus cereus by Real-Time PCR Using a gyrB-Specific Primer and Probe Set

Mgr. Adam Norek, Ph.D.
Mgr. Radek Machát, Ph.D.

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Introduction

Bacillus cereus is a Gram-positive, spore-forming bacterium belonging to the Bacillus cereus group and is widely distributed in the environment. Due to its ability to form resistant endospores, the organism can persist under adverse environmental conditions and can enter the food chain at different stages of primary production, processing, storage, and distribution. B. cereus is an important foodborne pathogen associated mainly with two types of gastrointestinal disease: an emetic syndrome caused by the heat-stable toxin cereulide and a diarrhoeal syndrome associated primarily with the enterotoxins haemolysin BL (Hbl), non-haemolytic enterotoxin (Nhe), and cytotoxin K (CytK). In addition to its role in foodborne disease, B. cereus may also cause opportunistic local or systemic infections. Its widespread occurrence, persistence of spores, and ability of individual strains to produce different virulence factors make reliable detection of B. cereus relevant for food safety and microbiological diagnostics.[1–3]

Microbiological control of Bacillus cereus in foods within the European Union is governed by Commission Regulation (EC) No 2073/2005 and, in the Czech Republic, is further specified by Decree No. 121/2023 Coll. Where B. cereus is included among the monitored microorganisms, its level is determined by culture-based enumeration according to EN ISO 7932. Under Commission Regulation (EC) No 2073/2005, a process hygiene criterion is defined for dried infant formulae and dried foods for special medical purposes intended for infants below six months of age, with limits of m = 50 CFU/g and M = 500 CFU/g using a sampling plan of n = 5 and c = 1.[4] In the Czech Republic, a limit of $<10^3$ CFU/g or ml is specified for B. cereus in meals, and B. cereus enterotoxin is required to be undetectable in the amount of sample examined.[5] The reference procedure specified by EN ISO 7932 is based on cultivation of the sample and its decimal dilutions, incubation at 30 °C, enumeration of characteristic colonies and their subsequent phenotypic confirmation; the final result is expressed as the number of colony-forming units per gram or millilitre of sample.[6] The method determines viable presumptive B. cereus, since the applied confirmation procedure does not provide unambiguous differentiation of B. cereus from all closely related members of the B. cereus group. Alternative microbiological methods for detection or quantification may be assessed and validated against the corresponding reference method according to ISO 16140-2.[7]

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7. International Organization for Standardization. ISO 16140-2:2016. Microbiology of the food chain — Method

validation — Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method. Geneva: ISO; 2016. Amendment 1:2024.
[ISO](https://www.iso.org/standard/83200.html?utm_source=chatgpt.com)

1. Field of Technology

The product is intended for diagnostic applications in the fields of food safety.

2. Current State of the Art: Molecular Diagnostics of Bacillus cereus

2.1. Overview

Detection of Bacillus cereus in food microbiology is based on culture-dependent enumeration, molecular detection of organism- or virulence-associated DNA, and immunological detection of toxins. EN ISO 7932 remains the reference method specified by Commission Regulation (EC) No 2073/2005 for the applicable process hygiene criterion. PCR, quantitative real-time PCR (qPCR), digital PCR (dPCR), and loop-mediated isothermal amplification (LAMP) are used for detection or characterization of B. cereus and related organisms.

The analytical outputs differ: cultivation determines viable presumptive B. cereus in CFU/g or CFU/ml, nucleic-acid methods determine specific DNA targets, and immunoassays detect toxin antigens. These measurements are not directly interchangeable.

2.2. Diagnostic Methods

Culture-based methods. EN ISO 7932:2004 specifies enumeration of presumptive B. cereus at 30 °C in food, feed, and samples from food-production environments. Characteristic colonies on selective media are enumerated and phenotypically confirmed using properties including haemolysis. The result represents organisms capable of colony formation under the prescribed conditions. The designation presumptive B. cereus reflects limited differentiation from genetically related members of the B. cereus group, including B. thuringiensis, B. anthracis, and B. mycoides. Supplementary biochemical identification does not necessarily resolve these species. ISO 7932:2004 remains published, with Amendment 1:2020 introducing optional tests; a revision is under development. [2]

Endpoint and multiplex PCR are used for organism identification and virulence-gene profiling. Carter et al. analysed toxin-associated genes in isolates from dried foods, infant formula, feed, and dietary supplements. Arunrut et al. developed multiplex PCR for simultaneous detection of Salmonella spp., Staphylococcus aureus, and B. cereus in pork products. [8,9]. qPCR measures fluorescence during amplification and supports qualitative detection or calibration-based quantification. Detection chemistries include intercalating dyes such as SYBR Green and sequence-specific hydrolysis probes. Organism-associated targets include gyrB, groEL, 16S rRNA-associated sequences, PLC-associated sequences, and essC. Virulence-associated targets include cesA, cesB, nheA, nheB, hblD, cytK, bceT, and entFM. Detection of a toxin gene does not establish expression or production of the encoded toxin. [7,10–13] dPCR partitions the reaction into individual microreactions and calculates target-copy concentration from positive and negative partitions using Poisson statistics. Absolute copy-number estimation does not require a conventional external calibration curve. The method has been applied to multiplex detection of foodborne pathogens, including B. cereus. [15]

2.3. Reported Analytical Performance

Published detection limits differ according to analytical target, amplification chemistry, extraction procedure, matrix, and enrichment. Values expressed as copies/reaction, DNA mass, CFU/ml, or CFU/g describe different experimental measurements and are retained below in their reported units.

1. Multiplex PCR; Arunrut et al., 2018 [8]
Target(s): B. cereus and two other pathogens

Reported performance: LOD 10^3 CFU/ml in mixed cultures; initial 10 CFU/ml detected after enrichment in artificially contaminated pork products

2. PMA-multiplex PCR; Forghani et al., 2015 [6]
Target(s): nheA, entFM, hblD, cytK, ces
Reported performance: 2 pg DNA/reaction; 3.8–4.0 × 10^2 CFU/ml in suspensions; 4.8 × 10^3 and 3.6 × 10^3 CFU/g for emetic and enterotoxic strains in contaminated food

3. qPCR; Wei et al., 2018 [7]
Target(s): groEL, gyrB
Reported performance: Specificity 98% and 96%, respectively, against a 120-strain panel; food LOD 3.5 and 3.7 log CFU/g in lettuce, spinach, and kimbap

4. PMA-qPCR; Cattani et al., 2016 [5]
Target(s): B. cereus group
Reported performance: Quantification limit 7.5 × 10^2 CFU/ml in UHT milk

5. PMA-qPCR; Zhou et al., 2019 [10]
Target(s): cesB
Reported performance: LOD 10^2 CFU/ml in pure culture and spiked milk; PMA concentration 3 µg/ml; no significant Ct interference from tested E. coli O157:H7

6. SYBR Green qPCR; Liang et al., 2022 [11]
Target(s): gyrB
Reported performance: Milk LOD 200 CFU/ml; range 2 × 10^2–2 × 10^8 CFU/ml; efficiency 96.6%; purified-target theoretical LOD 2.57 × 10^4 copies/ml, efficiency 94.0%

2.4. Examples of commercial Assays

1. SureFast® Bacillus cereus group PLUS, R-Biopharm [17]
Target: Group-associated DNA; exact locus not disclosed
Reported performance: Analytical LOD ≤5 DNA copies

2. SureFast® Emetic Bacillus cereus PLUS, R-Biopharm [18]
Target: Cereulide-synthetase-associated DNA
Reported performance: Analytical LOD ≤5 DNA copies

3. foodproof® SL Bacillus cereus Detection Kit, Hygiena [19]
Target: Specific groEL sequence
Reported performance: LOD 10–100 genetic equivalents; manufacturer reports 100% exclusivity for tested non-target bacteria

These products use real-time PCR for qualitative DNA detection. Public specifications do not consistently provide complete matrix-level LODs in CFU/g or full diagnostic sensitivity and specificity data. R-Biopharm states that whole-procedure detection limits depend on sample matrix, preparation, extraction, and DNA content. Manufacturer specifications do not independently demonstrate regulatory equivalence to EN ISO 7932.

2.5. Representative Primer Sequences

Published primer sequences are retained below as reported.

Liang et al., 2022 — gyrB qPCR [11]

F: 5′-AAGGGCTTCACCATCTTGTAT-3′
R: 5′-TTACATGGTGTTGGGGCATCG-3′

Busch et al., 2022 — LAMP [12]

Target gene: groEL
F3: 5′-GAAGAAGCACGTCGTTCG-3′
B3: 5′-TTTTGCTAGCAACTTCTGC-3′
FIP: 5′-AGTGGTGAACCAAATTTTTTCTCAA-ACTCTTGCAAACGCAGTA-3′
BIP: 5′-AATGATGGTGTAACAATCGCAAAAG-CTAATTTCGCACCCATGTT-3′
LF: 5′-TGGTCCAAGCGTAACTTT-3′
LB: 5′-TCGAATTAGAAGATGCATTCG-3′

Target gene: nheB
F3: 5′-CTATTATGATACTTTAGTTGCTGC-3′
B3: 5′-CGTTGTAATTTGATTTTGCAGAAG-3′
FIP: 5′-CTGATCCACTTGCGCTTTATTTTCA-TAAAGCGACTCTTACGAAAGG-3′
BIP: 5′-CCGAAATAAAATGACTTCGGATACG-TCCTGCATCTTGACTAGC-3′
LF: 5′-CTACTTGATAATCTTGTTAAG-3′
LB: 5′-CAAAACTTCAAGGGTGAT-3′

Haque et al., 2026 — LAMP [16]

Target gene: cytK
F3: 5′-CTAGCGTATCTTATCAACTTGG-3′
B3: 5′-CCGTTAAAGAATACGTTCCAT-3′
FIP: 5′-GACCAGTTGCACCAGCTTCATGGCTCTGTTAAAGCTTCTG-3′
BIP: 5′-AAGTCACTTGGTCTGACTCTGTTTTACGTTTTTGTTCGTTTGG-3′
LF: 5′-GACCAGTTGCACCAGCTTCAT-3′
LB: 5′-CGCTAGGGCCATTAGGCGT-3′

Target gene: entFM
F3: 5′-GGTTATGTAAGTGCAGACTTC-3′
B3: 5′-TCAAAACCAGCAGGTGTT-3′
FIP: 5′-CGTCTTTACCTGGTTGTTGAACGGTTAAGTTTGTAAAAGGCGGA-3′
BIP: 5′-CCAACAACAGGTGGAGATACATCGCAGTTCTGTATGGTGAAC-3′
LF: 5′-TCGCTGGATTCGCTAGATCTTT-3′
LB: 5′-GGTATTGCTGATGGAAACACAG-3′

2.6. Analytical Limitations

Discrimination between B. cereus sensu stricto and related group members depends on the selected target and amplification conditions. In the Liang et al. assay, reduced annealing-extension temperature produced a false-positive B. thuringiensis signal; 62 °C was selected for the reported differentiation. [11]

DNA amplification can detect material from viable, non-culturable, damaged, or dead cells, spores, and extracellular DNA. PMA/PMAxx reduces selected contributions from membrane-compromised cells but does not universally establish viability. Molecular copy number is consequently not equivalent to CFU.

Food composition, cell lysis, spore disruption, extraction yield, inhibitor removal, and reaction input volume

affect analytical sensitivity. An amplification-stage LOD cannot be assigned to the whole testing procedure without evaluating these factors.

Enrichment may increase detection probability, as demonstrated by PCR and LAMP studies, but the subsequent result identifies DNA after microbial growth rather than directly quantifying the original contamination.

Organism-associated genes, virulence genes, and toxin proteins are distinct analytical targets. A toxin-gene-positive result does not directly demonstrate toxin production; toxin immunoassays do not establish organism abundance or complete genomic identity.

Analytical specificity, diagnostic specificity, LOD, and quantification limit depend on their experimental definitions. Reported values in DNA copies, DNA mass, CFU/reaction, CFU/ml, and CFU/g cannot be compared without accounting for matrix and sample processing. Application as an alternative to EN ISO 7932 requires validation under the applicable regulatory framework. [1,3,4]

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[14] Yossa N, et al. qPCR detection of viable B. cereus group cells in cosmetic products. Sci Rep. 2023;13:4477. doi:10.1038/s41598-023-31128-3.
[15] Sun X, et al. Quadruplex ddPCR detection and quantification of foodborne pathogens, including B. cereus. Sci Rep. 2025;15:32092. doi:10.1038/s41598-025-17272-y.
[16] Haque MA, et al. LAMP detection of enterotoxigenic B. cereus in food and feed. Front Cell Infect Microbiol. 2026;16. doi:10.3389/fcimb.2026.1815090.

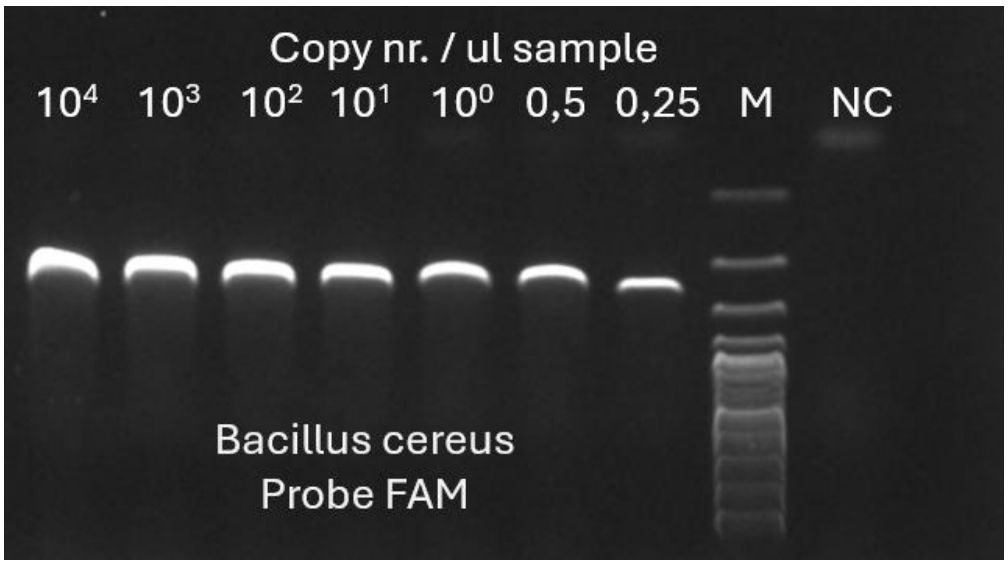
3. Description of the Technical Solution

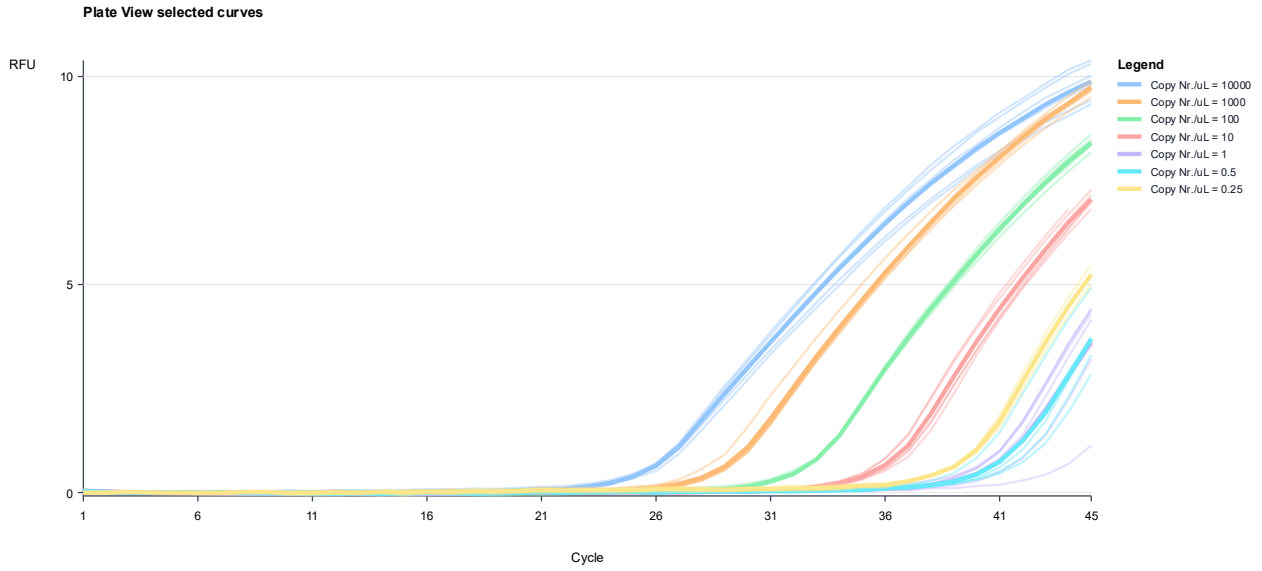
The technical solution is based on real-time PCR detection of Bacillus cereus DNA using amplification of a 256-bp fragment of the gyrB gene and sequence-specific fluorescence detection. Amplification is performed using the forward primer gyrB_Fw (5′-AAGGGCTTCACCATCTTGTTTGG-3′), the reverse primer gyrB_Rv (5′-CCAACACCATGTAAACCACCAGA-3′), and the probe gyrB_Pr (5′-CCACGCCGGTGGTAAATTTGGCGGTGGCGG-3′). The probe is labelled with HEX at the 5′ end and BHQ1 at the 3′ end. The final concentrations in the PCR reaction are 0.5 µM for each primer and 0.1 µM for the probe.

PCR Product 256 bp
AAGGGCTTCACCATCTTGTTTGGGAAATCGTAGATAATAGTATTGATGAAGCACTTGCAGGTTATTGTGATGAAAT
TAATGTTAGTATCGAAGAAGATAATAGTATTCGTGTAACGGATAATGGGCGTGGTATTCCGGTTGGTATACAAGA
AAAAATGGGACGTCCAGCTGTAGAAGTTATTATGACTGTTCTCCACGCCGGTGGTAAATTTGGCGGTGGCGGTTA
TAAAGTTTCTGGTGGTTTACATGGTGTTGG

The method employs a ready-to-use master mix prepared as a 1.33× concentrate relative to the final PCR reaction. A 750-µl volume of the master mix contains 239 µl of PCR-grade water, 500 µl of 2× PCR premix, 1 µl of 100 µM gyrB_Pr probe, 5 µl of 100 µM gyrB_Fw primer and 5 µl of 100 µM gyrB_Rv primer. Addition of 250 µl of sample results in a final volume of 1,000 µl containing 1× PCR premix, 0.1 µM probe and 0.5 µM of each primer. The sample therefore constitutes 25% of the final reaction volume and the master mix-to-sample ratio is 3:1. The reaction format was evaluated at final volumes of 10, 20 and 50 µl. These correspond to 7.5 µl of master mix and 2.5 µl of sample, 15 µl of master mix and 5 µl of sample, and 37.5 µl of master mix and 12.5 µl of sample, respectively. Three commercially available 2× PCR premixes were included in the evaluation: Maxima Probe qPCR, QuantiTect PCR and Platinum SuperMix. The method was tested on Bio-Rad CFX and Roche LightCycler real-time PCR platforms.

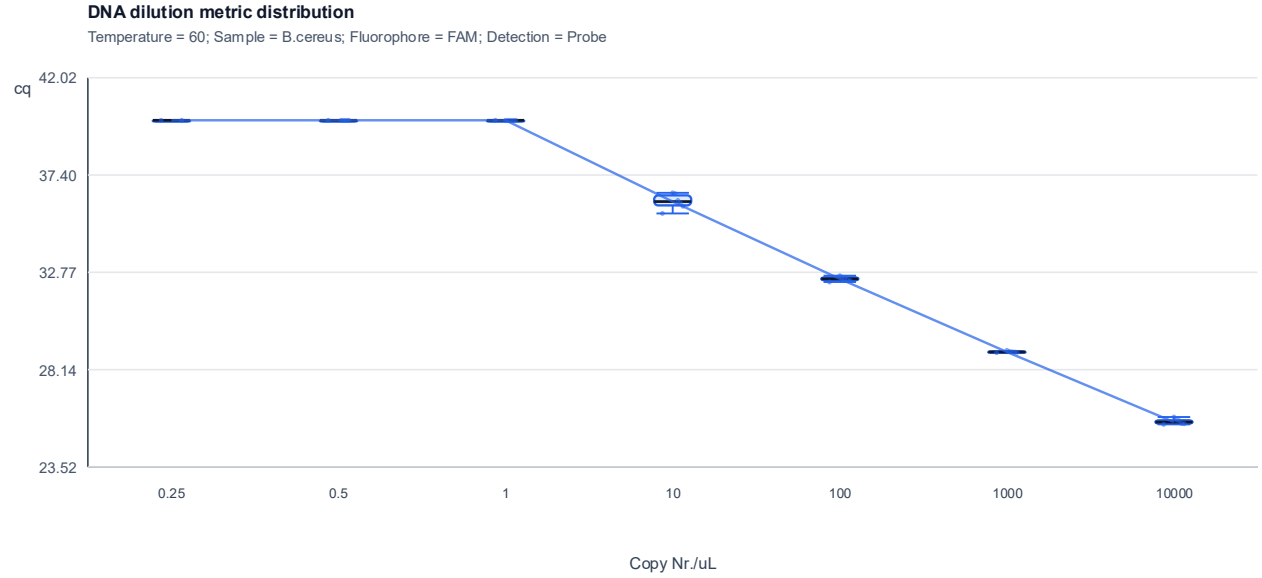
Amplification is performed by initial denaturation at 95 °C for 10 min, followed by 45 cycles consisting of denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s and extension at 72 °C for 30 s. A final extension at 72 °C for 3 min is performed after completion of the amplification cycles. Fluorescence is recorded in the HEX channel at the end of the extension step at 72 °C during each amplification cycle.





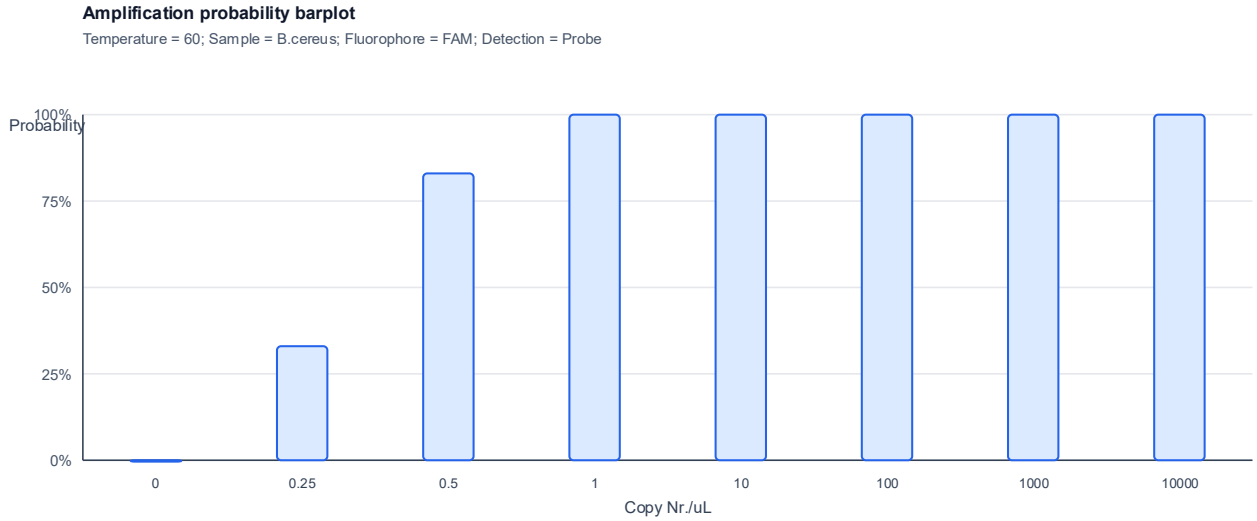
Analytical performance was evaluated using target DNA concentrations from 0 to 10,000 copies/µl. No positive amplification was observed in target-free controls. Detection frequencies were 33% at 0.25 copies/µl and 83% at 0.5 copies/µl. All tested reactions were positive at concentrations of 1, 10, 100, 1,000 and 10,000 copies/µl.

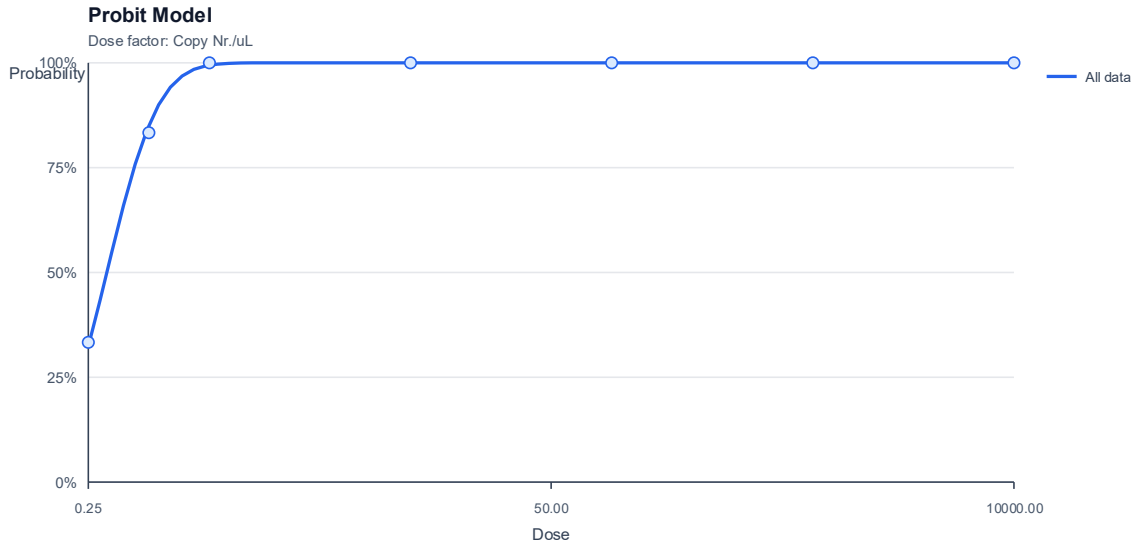
Separation of adjacent DNA concentrations was evaluated using the absolute difference in Cq values. The |ΔCq| values were 0.00 for 0.25–0.5 copies/µl, 0.00 for 0.5–1 copy/µl, 3.87 for 1–10 copies/µl, 3.67 for 10–100 copies/µl, 3.47 for 100–1,000 copies/µl and 3.32 for 1,000–10,000 copies/µl. The concentrations below 1 copy/µl were therefore not quantitatively distinguishable by Cq under the tested conditions. Clear separation between successive tenfold concentrations was observed from 1 copy/µl upward. A formal limit of quantification was not independently established; 1 copy/µl represents the lowest tested concentration from which quantitative separation of the subsequent concentration levels was observed.



dna_factor	metric	dna_high	dna_low	n_high	n_low	mean_high	mean_low	sd_high	sd_low	delta_low_minus_high	delta_median_low_minus_high	abs_delta_median	test	statistic	p_value	delta_cycles_low_minus_high
Copy Nr./uL	cq	0.5	0.25	5	2	40	40	0	0	0	0	0				0
Copy Nr./uL	cq	1	0.5	6	5	40	40	0	0	0	0	0				0
Copy Nr./uL	cq	10	1	6	6	36.135	40	0.371147	0	3.865	3.865	3.865	Welch t-test	-25.508171	0.000002	3.865
Copy Nr./uL	cq	100	10	6	6	32.46	36.135	0.112071	0.371147	3.675	3.675	3.675	Welch t-test	-23.21876	0	3.675
Copy Nr./uL	cq	1000	100	6	6	28.993333	32.46	0.038297	0.112071	3.466667	3.475	3.475	Welch t-test	-71.698566	0	3.466667
Copy Nr./uL	cq	10000	1000	6	6	25.673333	28.993333	0.13471	0.038297	3.32	3.335	3.335	Welch t-test	-58.068152	0	3.32

The relationship between target DNA concentration and probability of detection was further evaluated by probit regression. The fitted model yielded an intercept of 2.54 and a slope of 4.98. The estimated LOD50 was 0.31 copies/µl and the estimated LOD95 was 0.66 copies/µl. These values represent model-based estimates of detection probability and are distinct from the concentration range in which quantitative discrimination between DNA levels was demonstrated.





4. Claims for Protection

1. A primer and probe set for PCR detection of Bacillus cereus DNA by amplification of a 256-bp fragment of the gyrB gene, comprising the forward primer gyrB_Fw having the nucleotide sequence 5′-AAGGGCTTCACCATCTTGTTTGG-3′, the reverse primer gyrB_Rv having the nucleotide sequence 5′-CCAACACCATGTAAACCACCAGA-3′, and the probe gyrB_Pr having the nucleotide sequence 5′-CCACGCCGGTGGTAAATTTGGCGGTGGCGG-3′, wherein the probe is labelled with HEX at the 5′ end and BHQ1

at the 3′ end.

2. Use of a PCR reaction mixture comprising a 2× PCR premix, the primer pair gyrB_Fw and gyrB_Rv, and the HEX/BHQ1-labelled gyrB_Pr probe as a ready-to-use master mix for real-time PCR detection of Bacillus cereus DNA, wherein the master mix is prepared as a 1.33× concentrate and the analysed sample is added in an amount corresponding to 25% of the final reaction volume, thereby providing final concentrations of 1× PCR premix, 0.5 µM of each primer and 0.1 µM of the probe.

VÚVeL

Výzkumný ústav veterinárního lékařství, v.v.i.
Hudcova 296/70
621 00 Brno
Czech Republic
Tel.: +420 5 3333 1111; www.vri.cz; e-mail: vri@vri.cz