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

PROTOTYPE

Diagnostic System for Detection of Salmonella Typhimurium by Real-Time PCR Using a STM4200 - Specific Primer Set and SYTO 9 Fluorescent Dye

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Introduction

Salmonella enterica subsp. enterica serovar Typhimurium is a Gram-negative, facultatively anaerobic bacterium belonging to the family Enterobacteriaceae and represents one of the major non-typhoidal Salmonella serovars associated with human gastroenteritis. S. Typhimurium has a broad host range and occurs in domestic and wild animals, including poultry, pigs, cattle, companion animals and reptiles. Human infection is predominantly associated with the consumption of contaminated foods of animal origin, particularly meat, poultry, eggs and milk, but contamination of vegetables and other plant-derived foods may also occur. Transmission by direct contact with infected animals and by the faecal-oral route is also possible. Salmonellosis is typically characterized by diarrhoea, abdominal pain, fever, nausea and occasionally vomiting; invasive infection can occur particularly in young children, elderly persons and immunocompromised patients. Salmonellosis remains one of the most frequently reported foodborne zoonoses in the European Union.[1–3]

Microbiological control of Salmonella in foods within the European Union is governed primarily by Commission Regulation (EC) No 2073/2005. The regulation specifies food safety criteria requiring absence of Salmonella spp. in defined quantities of sample for a range of food categories. A serovar-specific criterion is defined for fresh poultry meat from specified Gallus gallus and turkey populations, for which Salmonella Typhimurium and Salmonella Enteritidis must be absent in 25 g using a sampling plan of n = 5 and c = 0; for monophasic Salmonella Typhimurium, the antigenic formula :- is included.[4] In the Czech Republic, Decree No. 121/2023 Coll. requires Salmonella spp. to be absent in 25 g or ml of meals outside food categories for which criteria are established by Regulation (EC) No 2073/2005, with testing according to ČSN EN ISO 6579-1.[5]

The reference procedure for detection of Salmonella spp. is EN ISO 6579-1. The method is based on non-selective pre-enrichment, selective enrichment, isolation on selective solid media and confirmation of presumptive colonies. ISO 6579-1:2017, together with Amendment 1:2020, remains the current horizontal method for detection of Salmonella spp. in foods, feed, food-production environments and primary-production samples.[6,7] Serotyping of pure Salmonella cultures is addressed by ISO/TR 6579-3:2014.[8] ISO 6579-4:2025 specifies PCR-based identification and differentiation of monophasic S. Typhimurium :- starting from isolates.[9] Alternative microbiological methods may be assessed and validated against the corresponding reference method according to ISO 16140-2:2016 and Amendment 1:2024.[10]

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1. Field of Technology

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

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

2.1. Overview

Detection of Salmonella in food microbiology is based primarily on culture-dependent detection, biochemical and serological confirmation, immunological methods and molecular detection of specific DNA sequences. EN ISO 6579-1 remains the principal horizontal reference method for detection of Salmonella spp. in the food chain. Subsequent serotyping can be performed by determination of somatic and flagellar antigens. PCR, quantitative real-time PCR (qPCR), digital PCR (dPCR), loop-mediated isothermal amplification (LAMP), immunomagnetic separation and combinations of these methods are used for rapid detection or characterization of Salmonella and individual serovars

The analytical outputs differ. Culture-based procedures determine the presence of viable organisms capable of recovery and growth under the prescribed conditions. Immunological methods detect bacterial antigens or use antibodies for selective capture of bacterial cells. Nucleic-acid methods detect defined genomic targets. Genus-level detection of Salmonella and serovar-specific identification of S. Typhimurium are separate analytical objectives and require different target-selection strategies.

2.2. Diagnostic Methods

Culture-based methods. EN ISO 6579-1:2017 specifies detection of Salmonella spp. in products intended for human consumption and animal feeding, environmental samples from food production and handling, and samples from primary production. The procedure includes non-selective pre-enrichment, selective enrichment, isolation on selective media and confirmation of presumptive colonies. The method is intended to detect most Salmonella serovars, although additional culture steps may be required for specific serovars. The result represents viable Salmonella capable of recovery under the prescribed conditions. Identification of an isolate as S. Typhimurium requires subsequent serological or molecular characterization. [1,2]

Serological identification is traditionally based on determination of somatic O antigens and flagellar H antigens according to the White-Kauffmann-Le Minor scheme. ISO/TR 6579-3 provides guidance for serotyping pure Salmonella cultures. For classical S. Typhimurium, antigenic characteristics include O:4 and the flagellar antigens H:i and H:1,2, while monophasic variants lack expression of the second flagellar phase. ISO 6579-4:2025 provides a PCR-based procedure for differentiation of monophasic S. Typhimurium from isolates by detection of sequence regions associated with the first and second flagellar phases. [3,4]

Immunological methods are used mainly for rapid screening, concentration or confirmation. Antibody-based immunomagnetic separation can selectively concentrate Salmonella cells before cultivation or PCR and can reduce the effect of competing microbiota and selected matrix components. Immunochromatographic and lateral-flow assays detect Salmonella antigens after enrichment and are generally qualitative. These methods do not inherently provide serovar-level identification unless antibodies with appropriate specificity are used. [5,6]

Endpoint and multiplex PCR are used for genus-level detection and for identification of epidemiologically important serovars. The invA gene is one of the most frequently used Salmonella-associated PCR targets and is suitable for broad detection of Salmonella spp., but an invA-positive result alone does not identify S. Typhimurium. Serovar-specific PCR assays have therefore used additional genomic regions including fliC, STM4492 and STM4497. Comparative-genomic approaches identified STM4497 as a useful S. Typhimurium-associated target, while multiplex systems combine a genus-level target with one or more serovar-associated targets. [7–10]

qPCR measures fluorescence during amplification and supports qualitative detection and calibration-based quantification. Detection chemistries include intercalating dyes and sequence-specific probes. Intercalating dyes produce fluorescence from amplified double-stranded DNA and therefore require specificity to be provided by primer design, reaction conditions and, where applicable, melting-curve analysis. Probe-based assays add an independent sequence-specific hybridization step. Hadjinicolaou et al. developed a real-time PCR system combining invA for Salmonella spp. detection with fliC for S. Typhimurium and prot6E for S. Enteritidis, whereas Park et al. used the STM4497 region for S. Typhimurium-specific real-time PCR in artificially contaminated chicken meat. [10,11]

dPCR partitions the reaction into multiple individual reactions and calculates target-copy concentration from the number of positive and negative partitions using Poisson statistics. The method allows absolute target-copy estimation without a conventional calibration curve and can show increased tolerance to selected PCR inhibitors. dPCR has been evaluated for detection and quantification of S. Typhimurium in bacterial cultures and food matrices. [12]

LAMP performs amplification under isothermal conditions using multiple primers recognizing several regions of the target sequence. S. Typhimurium-specific LAMP assays have been developed using serovar-associated genomic regions including STM4497. Detection may be based on fluorescence, turbidity or visual readout. Compared with conventional PCR, LAMP reduces the need for thermal cycling but remains dependent on target specificity, sample preparation and matrix-related inhibition.

2.3. Reported Analytical Performance

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

1. Molecular-beacon real-time PCR; Hadjinicolaou et al., 2009 [10]

Targets: invA for Salmonella spp., fliC for S. Typhimurium and prot6E for S. Enteritidis

Reported performance: analytical detection limit of approximately 10 target copies per 25 µl reaction; the tested strain collection was correctly classified by the multiplex assay.

2. Real-time PCR; Park et al., 2009 [11]

Target: STM4497

Reported performance: specific amplification of S. Typhimurium; experimentally contaminated chicken samples containing approximately 4.5×10^5 to 4.5 CFU/ml were analysed, and calculated bacterial concentrations correlated with plate-count results.

3. Immunomagnetic separation–PMA–multiplex PCR; 2013 [13]

Targets: S. Typhimurium and two additional foodborne pathogens

Reported performance: detection limit for S. Typhimurium of approximately 1.2×10^2 CFU/ml in pure culture and approximately 5.1×10^3 CFU/g in experimentally contaminated food samples. PMA treatment was included to reduce amplification from membrane-compromised cells.

4. Real-time PCR combined with immunomagnetic separation; Zheng et al., 2014 [14]

Target: S. Typhimurium

Reported performance: the method was evaluated for detection of both healthy and heat-injured S. Typhimurium on raw duck wings after antibody-based magnetic capture. Analytical performance depended on capture conditions and recovery of injured cells.

5. qPCR and dPCR; Wang et al., 2018 [12]

Target: S. Typhimurium DNA

Reported performance: dPCR detection limit of 10^{-4} ng/µl DNA or 10^2 CFU/ml; under the tested conditions, dPCR was reported to be approximately one order of magnitude more sensitive than qPCR and showed greater resistance to selected inhibitors.

6. Immunomagnetic separation and PCR; Jenikova et al., 2000 [5]

Target: Salmonella spp.

Reported performance: Salmonella detection from non-fatty food matrices after pre-enrichment and immunomagnetic separation within approximately 24 h; the method combined antibody-based bacterial capture with PCR identification and reduced selected matrix effects.

2.4. Examples of commercial Assays

1. SureFast® Salmonella ONE, R-Biopharm [15]

Target: Salmonella spp.; exact proprietary locus not disclosed

Reported performance: real-time PCR analytical LOD ≤5 DNA copies. The manufacturer states that the assay is applied after cultural enrichment and that the whole-procedure detection limit depends on sample matrix, processing, DNA preparation and DNA content

2. foodproof® Salmonella Detection LyoKit, Hygiena [16]

Target: Salmonella spp.; proprietary sequence

Reported performance: real-time PCR assay intended for detection of Salmonella spp. after enrichment; manufacturer documentation states a whole-procedure detection limit of approximately 1–10 CFU per 25 g for validated applications. The assay is genus-level and does not independently identify S. Typhimurium.

3. foodproof® Salmonella Genus plus Enteritidis & Typhimurium Detection LyoKit, Hygiena [17]

Target: proprietary sequences for Salmonella spp., S. Enteritidis and S. Typhimurium

Reported performance: multiplex real-time PCR for simultaneous Salmonella genus detection and identification

of S. Enteritidis and S. Typhimurium. The manufacturer reports a matrix-level sensitivity of approximately 0.6 CFU per 25 g for specified validated applications

4. SureTect™ Salmonella species, Typhimurium and Enteritidis Multiplex PCR Assay, Thermo Fisher Scientific [18]

Target: proprietary Salmonella spp., S. Typhimurium and S. Enteritidis sequences

Reported performance: multiplex real-time PCR intended for simultaneous detection of Salmonella species and presumptive identification of S. Typhimurium and S. Enteritidis in food and environmental samples. Validated workflows include subsequent confirmation according to the applicable reference procedure.

These products differ in analytical scope. Genus-level Salmonella assays cannot by themselves establish S. Typhimurium identity, whereas multiplex serovar assays include additional discriminating targets. Manufacturer-reported analytical LOD values and matrix-level detection limits are not directly interchangeable and depend on enrichment, extraction and sample type.

2.5. Representative Primer Sequences

Published primer sequences are retained below as reported.

Rahn et al. — invA, Salmonella spp. [7]

F: 5′-GTGAAATTATCGCCACGTTCGGGCAA-3′
R: 5′-TCATCGCACCGTCAAAGGAACC-3′

Reported amplicon: 284 bp.

This primer pair is widely used for genus-level Salmonella detection and is not specific for S. Typhimurium.

Kim et al., 2006 — STM4497, S. Typhimurium [8]

F: 5′-GGAATCAATGCCCGCCAATG-3′
R: 5′-CGTGCTTGAATACCGCCTGTC-3′

Reported amplicon: 523 bp.

The primer pair was derived from comparative genomic analysis and was developed for S. Typhimurium identification. [PubMed Central

fliC-based S. Typhimurium PCR system [9]

F: 5′-CGGTGTTGCCCAGGTTGGTAAT-3′
R: 5′-ACTCTTGCTGGCGGTGCGACTT-3′

Reported amplicon: approximately 559 bp.

The target is associated with the phase-1 flagellin of S. Typhimurium, but fliC-based identification requires evaluation against other Salmonella serovars because flagellar antigen-associated sequences are not necessarily unique under all assay designs.

2.6. Analytical Limitations

Detection of Salmonella spp. and identification of S. Typhimurium are distinct analytical objectives. Broadly conserved Salmonella targets, including invA, provide genus-level information but do not alone establish the Typhimurium serovar. Conversely, serovar-associated targets such as flagellar genes may show sequence similarity or antigenic overlap with other Salmonella strains. Specificity therefore depends on target selection, primer and probe design, amplification conditions and the composition of the inclusivity and exclusivity panel.

PCR detects target DNA and does not inherently distinguish viable cells from dead or severely damaged cells or extracellular DNA. Molecular copy number is consequently not equivalent to CFU. PMA or PMAxx treatment can reduce selected contributions from membrane-compromised cells but does not universally establish viability. Immunomagnetic separation can concentrate target organisms and reduce some matrix effects but does not completely remove intracellular or co-isolated PCR inhibitors. [5,13]

Salmonella contamination in foods can occur at low and heterogeneous levels. For this reason, enrichment is commonly incorporated before PCR, immunological testing or rapid molecular confirmation. Enrichment increases the probability of detecting low initial contamination and permits recovery of stressed cells, but the resulting signal reflects organisms or DNA present after growth and therefore does not directly quantify the original concentration in the food sample.

Food composition, DNA extraction efficiency, bacterial recovery, inhibitor removal and reaction input volume affect analytical sensitivity. An amplification-stage LOD obtained using purified DNA or a bacterial suspension cannot be assigned to the complete food-testing procedure without matrix-specific evaluation. Values reported in DNA copies, DNA mass, CFU/reaction, CFU/ml and CFU/g are therefore not directly comparable.

Culture according to EN ISO 6579-1 establishes the presence of viable Salmonella recoverable under the prescribed conditions, while molecular methods establish the presence of selected nucleic-acid sequences. Identification as S. Typhimurium additionally requires validated serovar discrimination. Application of a molecular method as an alternative to the reference method requires validation of the complete analytical procedure for the intended food categories according to the applicable ISO 16140 framework. [1,19,20]

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[17] Hygiena. foodproof® Salmonella Genus plus Enteritidis & Typhimurium Detection LyoKit. Manufacturer documentation.

[18] Thermo Fisher Scientific. SureTect™ Salmonella species, Typhimurium and Enteritidis Multiplex PCR Assay. Manufacturer documentation.

[19] 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.

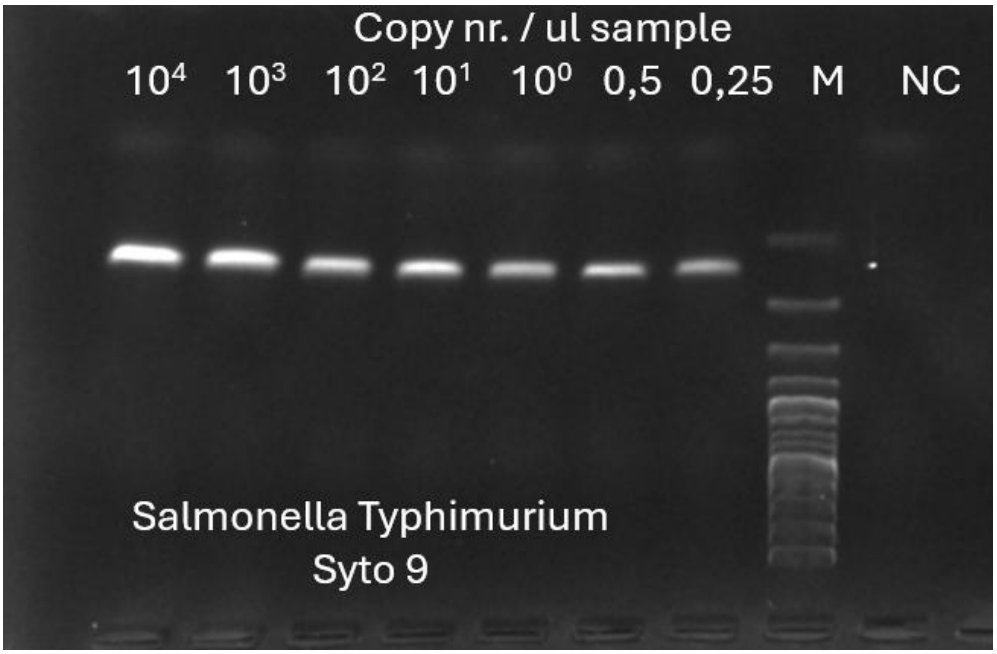
[20] International Organization for Standardization. ISO 16140-2:2016/Amd 1:2024. Amendment 1. Geneva: ISO; 2024.

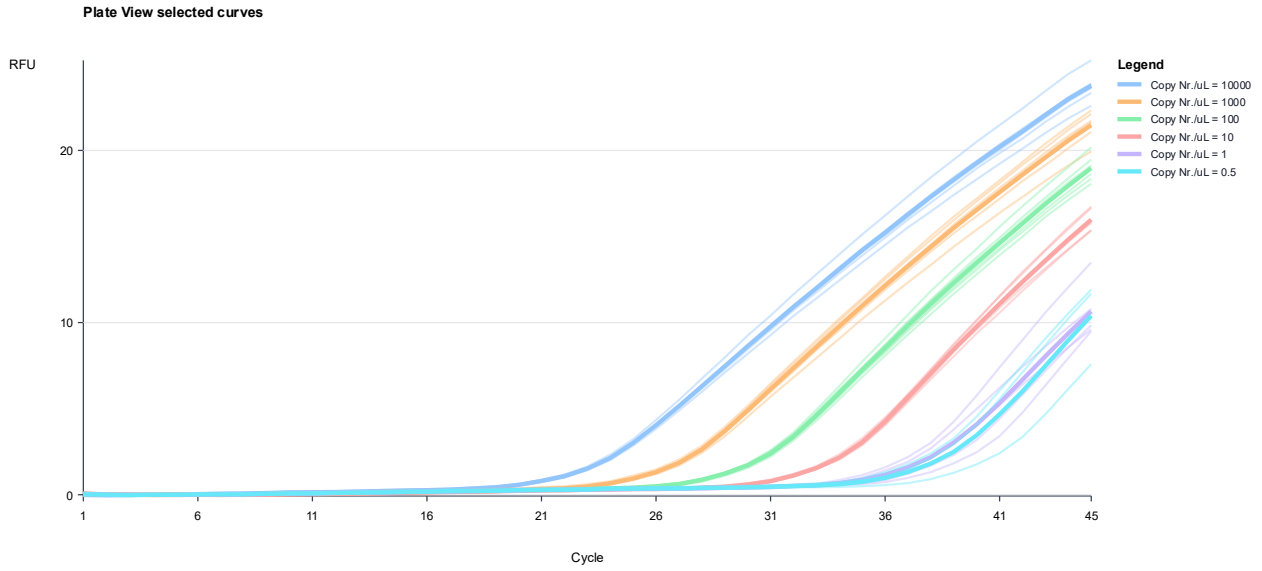
3. Description of the Technical Solution

The technical solution is based on real-time PCR detection of Salmonella enterica subsp. enterica serovar Typhimurium DNA by amplification of a 138-bp fragment of the STM4200 genomic region. Amplification is performed using the forward primer STM4200_FW (5′-CAGAATTCCCAGCCTGTCCTACT-3′) and the reverse primer STM4200_Rv (5′-TTGCTATTGTCCCCGTTATTGCG -3′) each at a final concentration of 0.5 µM. Detection is based on SYTO 9, an intercalating fluorescent dye used at a final concentration of 2 µM. Fluorescence is recorded at the end of the extension step in each amplification cycle.

A ready-to-use master mix is prepared as a 1.33× concentrate. For 750 µl of master mix, 238 µl of PCR-grade water, 500 µl of 2× PCR premix, 2 µl of 1 mM SYTO 9, 5 µl of 100 µM forward primer and 5 µl of 100 µM reverse primer are combined. Addition of 250 µl of sample gives a final volume of 1,000 µl and final concentrations of 1× PCR premix, 2 µM SYTO 9 and 0.5 µM of each primer. The sample therefore represents 25% of the final reaction volume and the master mix-to-sample ratio is 3:1. The reaction format was tested at final volumes of 10, 20 and 50 µl, corresponding to master mix/sample volumes of 7.5/2.5 µl, 15/5 µl and 37.5/12.5 µl, respectively. Three commercially available 2× PCR premixes were evaluated: Maxima Probe qPCR, QuantiTect PCR and Platinum SuperMix. The method was tested on Bio-Rad CFX and Roche LightCycler real-time PCR platforms.

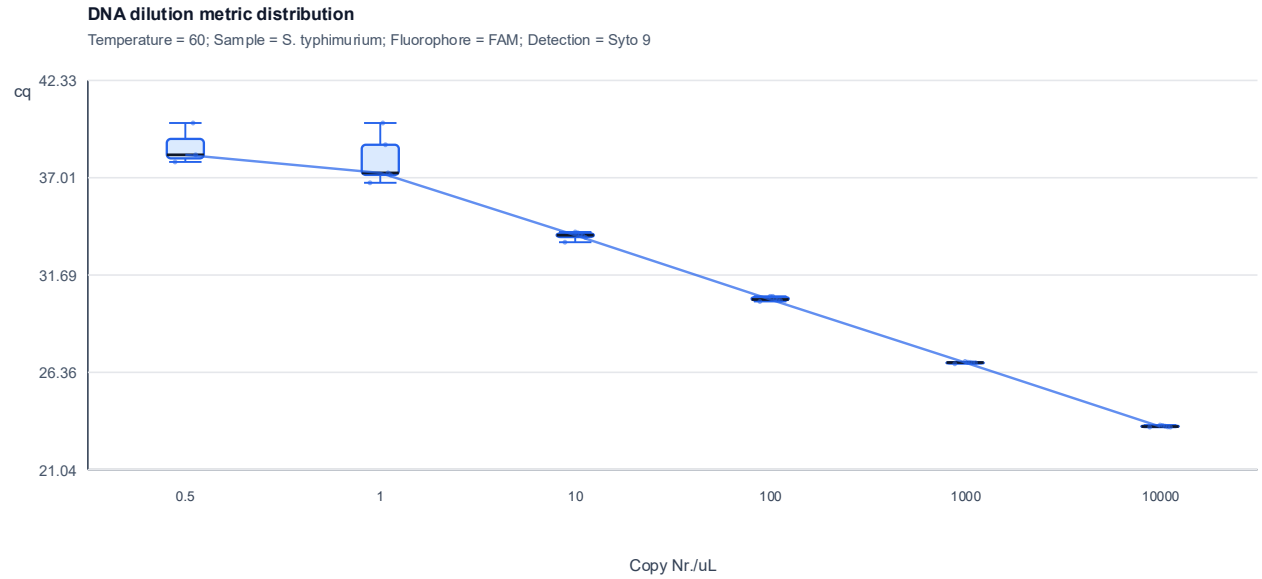
PCR product 138 bp
CAGAATTCCCAGCCTGTCCTACTTGCACCACGTTTCCAGTAGGGTTGCTATTAGGTAATACACCACCAGCCCCACTAGCGCCCC
CAGCCCCCACGGTTACTGGATAATTATTTCCCGCAATAACGGGGACAATAGCAA





Amplification consists of initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 3 min. SYTO 9 fluorescence is measured at the end of each extension step at 72 °C.

Analytical performance was evaluated using target DNA concentrations from 0.25 to 10,000 copies/µl. No positive amplification was observed in target-free controls or at 0.25 copies/µl. Detection frequencies were 50% at 0.5 copies/µl and 83% at 1 copy/µl, whereas 100% detection was obtained at concentrations from 10 to 10,000 copies/µl.

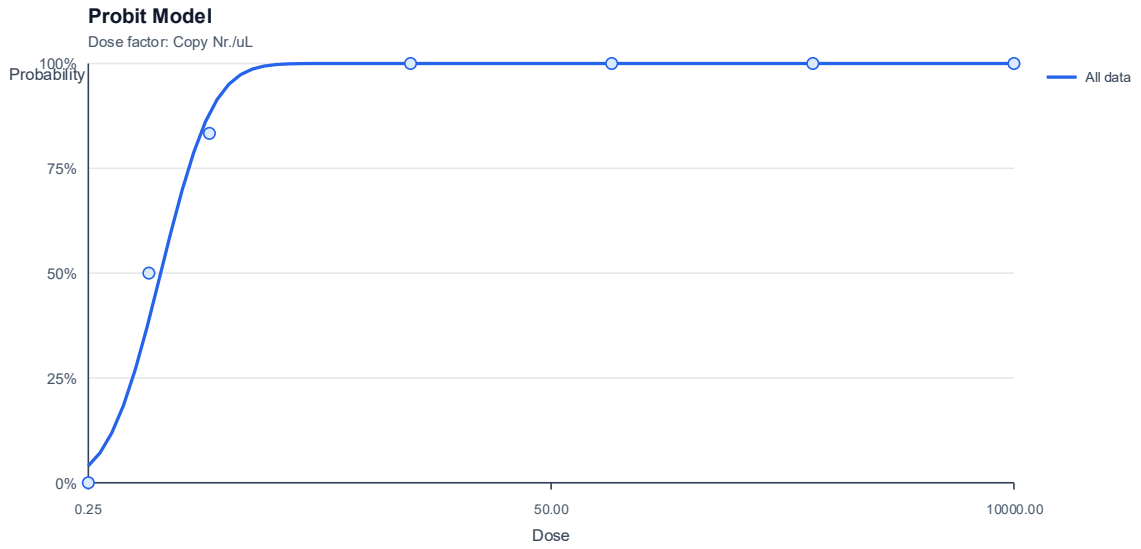


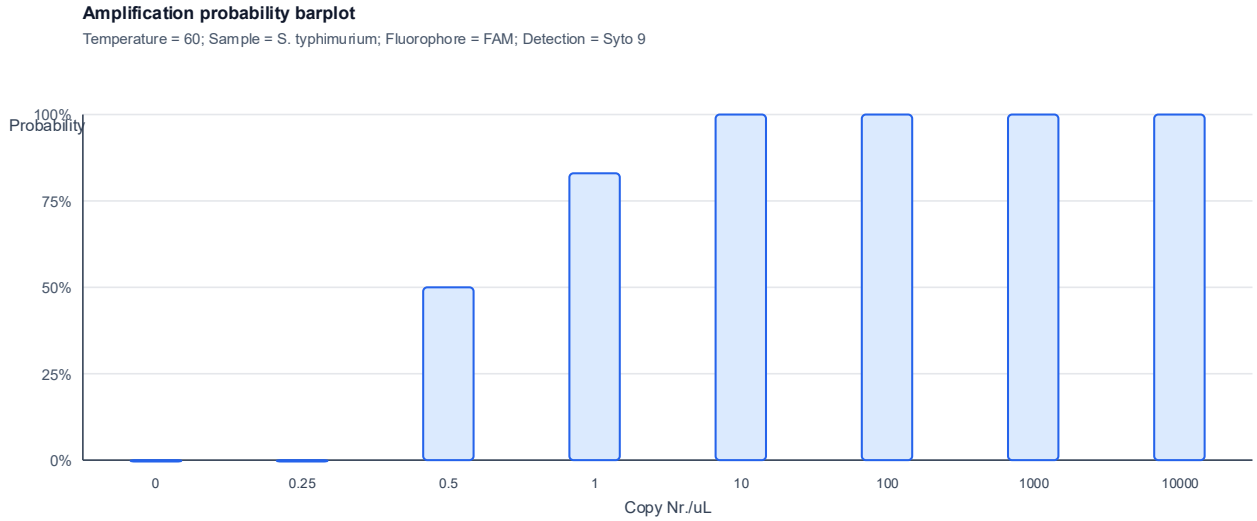
dna_factor	metric	dna_high	dna_low	n_high	n_low	mean_high	mean_low	median_high	sd_low	abs_delta_median	test	statistic	p_value	distance_per_log10_dilution
Copy Nr./uL	cq	1	0.5	5	3	37.994	38.71	37.27	1.134063	0.99	Welch t-test	-0.798486	0.460232	2.378501
Copy Nr./uL	cq	10	1	6	5	33.83	37.994	33.865	1.369974	3.405	Welch t-test	-6.735378	0.002224	4.164
Copy Nr./uL	cq	100	10	6	6	30.378333	33.83	30.35	0.202583	3.515	Welch t-test	-36.006775	0	3.451667
Copy Nr./uL	cq	1000	100	6	6	26.9	30.378333	26.9	0.118729	3.45	Welch t-test	-67.491344	0	3.478333
Copy Nr./uL	cq	10000	1000	6	6	23.413333	26.9	23.4	0.042895	3.5	Welch t-test	-132.628388	0	3.486667

Separation of adjacent concentrations was evaluated using the absolute difference in Cq values. The |ΔCq| between 0.5 and 1 copy/µl was 0.72. Successive tenfold concentration steps from 1 to 10,000 copies/µl produced |ΔCq| values of 4.16, 3.45, 3.48 and 3.49 cycles, respectively. Because detection was incomplete at 0.5 and 1

copy/µl and the difference between these two concentrations was substantially lower than the separation observed for the higher concentration levels, reliable quantitative interpretation is not supported below 10 copies/µl under the tested conditions. A formal limit of quantification was not independently determined; 10 copies/µl represents the lowest tested concentration showing 100% detection together with clear separation from the subsequent higher concentration levels.

Probit regression of the complete detection dataset yielded an intercept of 1.18 and a slope of 4.86. The estimated LOD50 was 0.57 copies/µl and the estimated LOD95 was 1.25 copies/µl. These values represent model-based estimates of detection probability and are distinct from the lowest experimentally tested concentration showing complete observed detection.





4. Claims for Protection

1. A primer pair for detection of Salmonella enterica subsp. enterica serovar Typhimurium DNA by amplification of a 138-bp fragment of the STM4200 genomic region, comprising the forward primer STM4200_FW having the nucleotide sequence 5′- CAGAATTCCCAGCCTGTCCTACT -3′ and the reverse primer STM4200_Rv having the nucleotide sequence 5′- TTGCTATTGTCCCCGTTATTGCG -3′.

2. Use of a PCR reaction mixture comprising a 2× PCR premix, SYTO 9 fluorescent dye, the forward primer STM4200_FW and the reverse primer STM4200_Rv as a ready-to-use master mix for real-time PCR detection of Salmonella enterica subsp. enterica serovar Typhimurium 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, 2 µM SYTO 9 and 0.5 µM of each primer.

VÚVeL

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