



PATHOGEN ANALYSIS THROUGH
HIGH-QUALITY QPCR

VALIDATION REPORT

PATHq *Serratia marcescens*
Real-Time PCR Kit
PATHq-SMA-001



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1. Introduction

1.1. PURPOSE OF THE DOCUMENT

The purpose of this document is to present the analytical validation data supporting the PCR characterization of the PATHq *Serratia marcescens* Real-Time PCR Kit, a duplex qualitative Real-Time PCR classified as Research Use Only (RUO).

Although RUO products are not intended for regulated diagnostic use, the validation described herein follows the performance principles defined in the NF U47-600-2 framework for qualitative Real-Time PCR.

This document summarizes the analytical characterization of the assay, including Specificity, Sensitivity, Repeatability, Reproducibility and Robustness, to support scientific and technical evaluation by laboratories.

1.2. INTENDED USE

The PATHq *Serratia marcescens* Real-Time PCR Kit is a duplex qualitative Real-Time PCR intended for the detection of *Serratia marcescens* DNA.

The kit is classified as Research Use Only (RUO) and is intended for use in laboratory research, assay development, and internal analytical evaluations. It is not intended for use in regulated diagnostic procedures.

The assay is designed for use on compatible Real-Time PCR platforms employing hydrolysis probe chemistry and may be applied to the analysis of purified nucleic acids obtained from porcine semen and other laboratory-controlled sample types. The assay is particularly intended for molecular monitoring and evaluation of bacterial contamination in porcine semen and related laboratory workflows.

1.3. SCOPE OF VALIDATION

This validation report covers the analytical performance of the PATHq *Serratia marcescens* Real-Time PCR Kit, focusing on parameters relevant to the characterization of a qualitative Real-Time PCR.

The scope of the validation includes the evaluation of Analytical Specificity (Inclusivity and Exclusivity), Analytical Sensitivity, Repeatability, Reproducibility, Robustness and overall Analytical Performance, as applicable to PCR-based detection of *Serratia marcescens* DNA under controlled laboratory conditions.

The validation activities described herein were primarily designed to characterize the intrinsic PCR performance of the assay independently of routine laboratory variability associated with sample preparation procedures. The assay was evaluated using purified nucleic-acid extracts, including extracts derived from porcine semen samples representative of the intended application context.

The validation was conducted using Real-Time PCR instrumentation compatible with FAM and VIC detection channels, including the Applied Biosystems QuantStudio 5 (0.1 mL format) as the reference platform and the Applied Biosystems 7500Fast as a secondary instrument.

This report does not include operational procedures or end-user workflow instructions; rather, it provides analytical evidence supporting the technical characterization of the assay for molecular monitoring, research applications and laboratory evaluation of *Serratia marcescens* DNA detection workflows.

2. Scientific&Technical background

2.1. THE ORGANISM

Serratia marcescens is a Gram-negative, motile, facultatively anaerobic bacterium of the family Enterobacteriaceae. It is widely distributed in the environment and readily colonises humid surfaces, water systems, veterinary equipment and biofilms. Some strains produce the red pigment prodigiosin, although pigmentation is not universal. Its ecological adaptability supports persistence in diverse animal production environments.

Serratia marcescens is considered an opportunistic pathogen affecting ruminants, poultry, equines, companion animals and aquatic species, typically as a secondary agent or environmental contaminant. Its survival in moist environments, biofilm formation capacity and intrinsic or acquired antimicrobial resistance increase its relevance in veterinary facilities where strict hygiene is required.

Genetically, the species possesses a circular chromosome typical of enterobacteria, and some strains carry plasmids associated with antimicrobial resistance or environmental persistence. Genetic variability arising from mutation, recombination and horizontal gene transfer results in differences in pigmentation, virulence, resistance and environmental fitness. This diversity requires robust molecular detection methods capable of identifying *Serratia marcescens* across diverse strains and genomic backgrounds.

2.2. RELEVANCE IN MOLECULAR TESTING

Serratia marcescens is increasingly recognised as relevant in veterinary and animal production settings due to its ability to contaminate biological materials, persist in humid environments and affect reproductive performance. Although historically regarded as an environmental contaminant, its presence in critical materials may have sanitary, productive and economic consequences.

In swine production, it is particularly relevant in boar semen used for artificial insemination. Contamination may occur during collection, processing or storage, and the bacterium can proliferate in liquid-preserved semen, even under refrigeration. This growth may impair sperm motility, membrane integrity and viability, reducing fertilising capacity and negatively affecting reproductive outcomes.

Artificial insemination centres face additional risks due to the organism's persistence on moist surfaces, water systems and equipment, often through biofilm formation. The use of semen extenders with reduced antibiotic concentrations, particularly in the context of current antimicrobial stewardship strategies, further increases the risk of uncontrolled bacterial growth.

Beyond swine reproduction, *Serratia marcescens* may also be detected in tissues, biological fluids, environmental samples and surfaces within animal facilities. Its persistence and ecological versatility highlight the importance of effective monitoring strategies in veterinary laboratories and production environments.

Sensitive and specific molecular assays may therefore support reliable molecular monitoring across diverse strains. The PATHq assay provides a duplex Real-Time PCR approach with internal control monitoring, supporting detection in porcine semen and potential application to other matrices following user validation.

2.3. RATIONALE FOR DEVELOPING A PATHq ASSAY

The PATHq *Serratia marcescens* Real-Time PCR Kit was developed to provide a robust, sensitive and highly specific molecular tool capable of detecting genetically diverse strains in complex laboratory contexts. Given the species' variability and coexistence with closely related organisms, high Analytical Specificity is essential without compromising Sensitivity.

Real-Time PCR technology offers rapid detection, high Sensitivity and strong discriminatory capacity when combined with optimised primer and probe design. The PATHq assay was therefore designed as a qualitative Real-Time PCR test focused on reliable target detection, consistent with screening, monitoring and research applications.

A duplex format enables simultaneous detection of *Serratia marcescens* DNA and an IPC in a single reaction. The IPC verifies PCR performance and identifies potential amplification inhibition, enhancing result reliability.

Overall, the PATHq assay follows a platform-based design prioritising analytical Robustness and Reproducibility, allowing consistent Performance across different laboratory and research settings as demonstrated through analytical validation.

3. Principle of the PATHq assay

3.1. REAL-TIME PCR CONCEPT

Real-Time polymerase chain reaction (Real-Time PCR) is a molecular technique based on the exponential amplification of a specific nucleic acid target through repeated cycles of denaturation, annealing and extension. Amplification is monitored in real time through the detection of fluorescent signals generated during the reaction, allowing the presence of the target sequence to be identified as amplification progresses.

In probe-based Real-Time PCR, sequence-specific oligonucleotide probes labelled with a fluorescent reporter and a quencher hybridise to the target region between the primers. During amplification, probe hydrolysis by the polymerase separates the reporter from the quencher, resulting in a measurable increase in fluorescence proportional to the accumulation of amplicons. This approach provides high analytical Specificity by requiring simultaneous primer and probe binding to the target sequence.

Real-Time PCR is particularly suited to qualitative detection applications, where the objective is to determine the presence or absence of a specific target rather than to quantify its abundance. When appropriately designed and validated, qualitative Real-Time PCR offers high Analytical Sensitivity, rapid turnaround time and robust performance across a wide range of laboratory conditions.

Within the PATHq framework, Real-Time PCR serves as the core analytical technology supporting reliable pathogen detection, forming the basis for the assay design principles described in the following sections.

3.2. PATHq DUPLEX DESIGN

The PATHq *Serratia marcescens* Real-Time PCR Kit employs a duplex Real-Time PCR design that enables simultaneous detection of the target organism and Real-Time monitoring of assay performance within a single amplification well. The assay integrates two independent detection channels, each assigned to a specific primer–probe system optimised for selective amplification under shared reaction conditions.

The FAM channel is dedicated to the detection of *Serratia marcescens* DNA using primers and a hydrolysis probe designed to target a conserved genomic region of the species. This target region was selected based on sequence analysis to ensure broad strain coverage while maintaining analytical Exclusivity with respect to other members of the *Serratia* genus and the Enterobacteriaceae family.

The VIC channel is assigned to the detection of the Xeno Internal Positive Control (IPC), an exogenous nucleic acid sequence included to monitor the integrity of the amplification process. Amplification of the IPC provides assurance of PCR performance and supports the correct interpretation of negative results by enabling the identification of potential reaction inhibition.

The duplex configuration enhances analytical Robustness without compromising Specificity, as both primer–probe systems were optimised to perform efficiently under common thermal cycling conditions. This design supports reliable detection in analytically challenging laboratory contexts where internal control monitoring is essential for result confidence.

3.3. INTERNAL POSITIVE CONTROL (IPC)

The PATHq *Serratia marcescens* Real-Time PCR Kit incorporates an exogenous Internal Positive Control (IPC) designed to monitor the integrity of the amplification process and to detect potential PCR inhibition within each reaction. The IPC consists of a non-target DNA sequence (Xeno) that is co-amplified alongside the target using a dedicated fluorescent detection channel (VIC).

The primary function of the IPC is to support the reliable interpretation of negative results by providing confirmation that the PCR reaction has proceeded correctly. In the absence of target amplification, the presence of a valid IPC signal indicates that the negative result is not attributable to reaction failure or inhibition.

Within the duplex configuration of the PATHq assay, IPC amplification does not interfere with the detection of *Serratia marcescens*. The primer–probe systems assigned to the FAM (target) and VIC (IPC) channels were optimised to ensure efficient co-amplification under shared reaction conditions while minimising competition for reaction components.

A valid IPC signal supports the classification of samples as negative when no FAM signal is detected. Conversely, the absence of IPC amplification in a target-negative sample reaction indicates an invalid result, necessitating repeat analysis. This IPC strategy enhances the Robustness and interpretability of qualitative PCR results in analytically challenging laboratory contexts.

3.4. INSTRUMENT COMPATIBILITY

The PATHq *Serratia marcescens* Real-Time PCR Kit is compatible with Real-Time PCR instruments supporting hydrolysis probe chemistry and Real-Time fluorescence acquisition in the FAM and VIC channels. Analytical validation was performed using two Real-Time PCR platforms commonly employed in veterinary and research molecular laboratories.

The Applied Biosystems QuantStudio 5 (0.1 mL format) was used as the reference instrument, while the Applied Biosystems 7500 Fast was evaluated as a secondary platform.

Both instruments demonstrated consistent target detection and IPC within the duplex assay configuration.

The validation confirms that the assay performs reliably across different Real-Time PCR instrument architectures when equivalent optical configurations and thermal cycling performance are available. Use of the assay on platforms not included in this validation should be verified internally by end users under their specific laboratory conditions.

4. Validation framework

4.1. REFERENCE STANDARDS

The analytical validation of the PATHq *Serratia marcescens* Real-Time PCR Kit was conducted in accordance with the performance principles recommended for qualitative Real-Time PCR, using the AFNOR NF U47-600-2 framework as the primary methodological reference. This standard defines the key analytical parameters required to characterise qualitative Real-Time PCR, including Specificity, analytical Sensitivity, Repeatability, Reproducibility and Robustness.

Although the PATHq *Serratia marcescens* Real-Time PCR Kit is classified as Research Use Only (RUO) and is not subject to formal regulatory approval, the validation strategy was deliberately aligned with recognised analytical validation standards in order to generate transparent, reproducible and scientifically robust performance data.

Additional methodological concepts were informed by internationally accepted quality frameworks, including ISO/IEC 17025 principles related to method reliability and result interpretation, as well as recommendations from the World Organisation for Animal Health (WOAH/OIE) concerning molecular detection methods. These references support the analytical rigor of the validation without implying regulatory status.

4.2. VALIDATION PARAMETERS

The parameters evaluated include Analytical Specificity, addressing both Inclusivity (ability to detect *Serratia marcescens* strains) and Exclusivity (absence of cross-reactivity with non-target microorganisms relevant to the validated context); Analytical Sensitivity, assessing the assay's ability to detect low concentrations of target DNA; Repeatability, evaluating result consistency under identical analytical conditions; Reproducibility, assessing performance consistency across independent runs and analytical settings; and Robustness, determining the assay's tolerance to controlled minor variations.

These parameters define the analytical validation scope of the assay at the PCR characterization level, providing a comprehensive description of its analytical behaviour without extending into full method validation.

5. PCR Characterisation

5.1. ANALYTICAL SPECIFICITY

Analytical Specificity evaluates the ability of the PATHq *Serratia marcescens* Real-Time PCR Kit to correctly detect the target organism (Inclusivity) and to avoid amplification of non-target microorganisms (Exclusivity). The study was conducted in accordance with the analytical performance principles defined in AFNOR NF U47-600-2 for qualitative Real-Time PCR.

INCLUSIVITY: Inclusivity was evaluated using a representative panel of *Serratia marcescens* isolates covering the genetic and epidemiological diversity expected in artificial insemination (AI) centers.

The panel comprised 25 field isolates recovered mainly from porcine semen samples collected in Spain between 2021 and 2025, together 2 well characterized reference strains. All isolates were taxonomically confirmed prior to molecular testing using MALDI-TOF MS, sequencing and/or validated Real-Time PCR.

DNA extracted from each isolate was analyzed using the PATHq duplex Real-Time PCR under the defined analytical conditions.

Representative *Serratia marcescens* strains included in the Inclusivity study are summarized in the table below:

Strain ID	Origin	Matrix / Nature	Year	Identification Method	PATHq <i>Serratia marcescens</i> detection
<i>Serratia marcescens</i> CECT 846	Spain	Reference strain	–	Culture collection reference	Detected
<i>Serratia marcescens</i> ATCC 13880	USA	Reference strain	–	Culture collection reference	Detected
3303	Spain	Porcine semen	2021	MALDI-TOF	Detected
3304	Spain	Porcine semen	2021	MALDI-TOF	Detected
3305	Spain	Porcine semen	2021	MALDI-TOF	Detected

Strain ID	Origin	Matrix / Nature	Year	Identification Method	PATHq <i>Serratia marcescens</i> detection
3306	Spain	Porcine semen	2021	MALDI-TOF	Detected
3360	Spain	Porcine semen	2022	MALDI-TOF	Detected
3361	Spain	Porcine semen	2022	MALDI-TOF	Detected
3362	Spain	Porcine semen	2022	MALDI-TOF	Detected
3437	Spain	Porcine semen	2023	MALDI-TOF	Detected
3463	Spain	Porcine semen	2024	MALDI-TOF	Detected
3464	Spain	Porcine semen	2024	MALDI-TOF	Detected
3465	Spain	Porcine semen	2024	MALDI-TOF	Detected
3466	Spain	Porcine semen	2024	MALDI-TOF	Detected
3467	Spain	Porcine semen	2024	MALDI-TOF	Detected
3468	Spain	Porcine semen	2024	MALDI-TOF	Detected
3469	Spain	Porcine semen	2024	MALDI-TOF	Detected
3471	Spain	Porcine semen	2025	MALDI-TOF	Detected
3472	Spain	Porcine semen	2025	MALDI-TOF	Detected
3439	Spain	Water	2024	MALDI-TOF	Detected
3441	Spain	Water	2024	MALDI-TOF	Detected
3432	Spain	Environmental swab (AI facility surface)	2023	MALDI-TOF	Detected
3445	Spain	Semen processing equipment	2024	MALDI-TOF	Detected
3492	Spain	Porcine semen	2025	MALDI-TOF	Detected
3443	Spain	Porcine semen	2024	MALDI-TOF	Detected
3482	Spain	Porcine semen	2025	MALDI-TOF	Detected
3485	Spain	Porcine semen	2025	MALDI-TOF	Detected

All tested isolates produced clear amplification signals in the FAM channel. In all reactions, the IPC was correctly amplified in the VIC channel, confirming successful IPC recovery and absence of significant PCR inhibition.

The PATHq *Serratia marcescens* Real-Time PCR Kit demonstrated 100% Inclusivity for all tested strains. The assay reliably detected genetic and epidemiologically diverse *Serratia marcescens* isolates representative of field conditions in artificial insemination centers. These results confirm that the assay target region is conserved within the evaluated population and that no false-negative results were observed under the defined analytical conditions.

EXCLUSIVITY: Exclusivity evaluates the ability of the assay to avoid amplification of non-target microorganisms, including species that are phylogenetically related, ecologically associated, or commonly present in porcine semen or artificial insemination environments.

A total of 27 non-target microorganisms were included in the Exclusivity study. All isolates were previously identified using MALDI-TOF, Real-Time PCR or validated microbiological methods to ensure taxonomic accuracy prior to molecular testing. The Exclusivity panel was constructed according to predefined criteria including genetic proximity (Enterobacteriaceae members), ecological overlap (microorganisms commonly detected in porcine semen and artificial insemination facilities), and environmental or laboratory relevance (frequent contaminants of water systems, equipment or laboratory workflows). In addition, relevant porcine viruses (PRRSV, PCV2 and PPV) were included to assess potential cross-reactivity with unrelated pathogens frequently tested in the same laboratory environment.

All non-target organisms were processed using the same extraction workflow as field samples and analyzed with the PATHq duplex Real-Time PCR. None of the tested non-target microorganisms produced amplification in the FAM channel, while IPC amplification was consistently observed in the VIC channel across all reactions.

Representative non-target microorganisms included in the Exclusivity study are summarized in the next table:

Strain ID	Origin	Matrix / Nature	Identification Method	PATHq <i>Serratia marcescens</i> detection
<i>Escherichia coli</i>	Spain	Semen	PCR	Not detected
<i>Staphylococcus aureus</i>	Spain	Skin swab	MALDI-TOF	Not detected
<i>Staphylococcus epidermidis</i>	Spain	Skin swab	MALDI-TOF	Not detected
<i>Streptococcus suis</i>	Spain	Porcine genital tract	MALDI-TOF	Not detected
<i>Corynebacterium spp.</i>	Spain	Skin swab	MALDI-TOF	Not detected
<i>Mycoplasma hyopneumoniae</i>	Spain	Semen	Real-Time PCR	Not detected
<i>Actinobacillus pleuropneumoniae</i>	Spain	Semen	Real-Time PCR	Not detected
<i>Lactobacillus spp.</i>	Spain	Semen	MALDI-TOF	Not detected
<i>Citrobacter freundii</i>	Spain	Semen	MALDI-TOF	Not detected
<i>Proteus mirabilis</i>	Spain	Semen	MALDI-TOF	Not detected
<i>Klebsiella pneumoniae</i>	Spain	Semen	Real-Time PCR	Not detected

Strain ID	Origin	Matrix / Nature	Identification Method	PATHq <i>Serratia marcescens</i> detection
<i>Salmonella enterica</i>	Spain	Semen	Real-Time PCR	Not detected
<i>Morganella morganii</i>	Spain	Semen	MALDI-TOF	Not detected
<i>Providencia stuartii</i>	Spain	Semen	MALDI-TOF	Not detected
<i>Pseudomonas aeruginosa</i>	Spain	Semen	MALDI-TOF	Not detected
<i>Bacillus cereus</i>	Spain	Environmental swab	MALDI-TOF	Not detected
<i>Staphylococcus spp.</i>	Spain	Semen	MALDI-TOF	Not detected
<i>Enterobacter cloacae</i>	Spain	Semen	MALDI-TOF	Not detected
<i>Glaesserella parasuis</i>	Spain	Semen	PCR	Not detected
<i>Proteus peogeri</i>	Spain	Semen	MALDI-TOF	Not detected
<i>Pasteurella multocida</i>	Spain	Semen	Real-Time PCR	Not detected
PCV2	Spain	Semen	Real-Time PCR	Not detected
PRRSV	Spain	Semen	Real-Time PCR	Not detected
PPV	Spain	Semen	Real-Time PCR	Not detected
<i>Serratia liquefaciens</i>	Spain	Semen	MALDI-TOF	Not detected
<i>Serratia rubidaea</i>	Spain	Semen	MALDI-TOF	Not detected
<i>Serratia plymuthica</i>	Spain	Semen	MALDI-TOF	Not detected

5.2. ANALYTICAL SENSITIVITY (LD_{PCR} ACCORDING TO THE EXPERIMENTAL MODEL)

The analytical Sensitivity of the PATHq *Serratia marcescens* Real-Time PCR Kit was determined in accordance with AFNOR NF U47-600-2, using a commercially supplied quantified genomic DNA standard. The objective of this study was to establish the intrinsic limit of detection (LD_{PCR}) of the Real-Time PCR Kit under controlled analytical conditions, independently of extraction steps or biological matrix effects.

A lyophilized genomic DNA preparation of *Serratia marcescens* CECT 846 was used as the reference material. The material contained 5 µg of genomic DNA. Based on an estimated genome size of approximately 5.1 Mbp, the total number of genome copies in the lyophilized material was calculated to be approximately 8.9×10^8 copies.

The DNA was reconstituted in 712 µL of MagMAX CORE Elution Buffer to obtain a working concentration of 1.25×10^6 genome copies/µL. Since 8 µL of DNA are added per PCR reaction, this concentration corresponds to 1×10^7 genome copies per reaction.

Serial dilutions were prepared directly from the primary DNA standard using DNA storage solution supplemented with carrier DNA to ensure stability and minimize adsorption losses at low concentrations. All dilutions were expressed in genome copies per PCR reaction (copies/PCR).

The assay was evaluated under the two thermal cycling profiles available on the QuantStudio 5 (0.1 mL format): Fast mode and Standard mode.

Preliminary 10-fold dilution screening

An initial 10-fold dilution series ranging from 10^6 to 10^1 genome copies per reaction was tested in both cycling modes. Each concentration was evaluated in duplicate.

This screening phase served three purposes:

- To confirm expected amplification behavior of the quantified DNA standard.
- To identify the concentration interval in which stochastic detection begins.
- To compare duplex configuration (target + IPC) with target-only reactions.

Amplification was consistent from 10^6 down to 10^2 copies in both cycling modes. Reduced detection frequency was observed between 10^2 and 10^1 copies, defining the concentration interval for AFNOR-compliant LD_{PCR} refinement.

During this preliminary phase, duplex and target-only reactions showed comparable amplification performance, demonstrating that the IPC did not interfere with target detection within the evaluated concentration range.

Refined LD_{PCR} determination

Based on the screening results, refined concentrations of 80, 40 and 20 genome copies per reaction were selected for LD_{PCR} determination.

In accordance with AFNOR NF U47-600-2 requirements:

- Three independent PCR runs were performed.
- Eight replicates per concentration were tested per run.
- A total of 24 replicates per concentration were analyzed.
- Both Fast and Standard cycling profiles were evaluated.

For the refined LD determination, only the target channel (FAM) was analyzed. The IPC was not included in this phase, as the objective was to determine the intrinsic detection capability of the amplification system without introducing additional reaction components that could theoretically compete for reagents at very low template concentrations. The absence of IPC-related interference had already been demonstrated during the preliminary duplex evaluation.

For Standard mode, three independent PCR runs containing complete replicate sets were included in the final LD_{PCR} (PCR subindexado) analysis.

Copies / Reaction	Fast – Pos/24	Fast %	Standard – Pos/24	Standard %
80	24/24	100%	24/24	100%
40	21/24	87.5%	21/24	87.5%
20	8/24	33.3%	8/24	33.3%

Under the experimental conditions described above, the LD_{PCR} of the PATHq *Serratia marcescens* Real-Time PCR Kit was determined to be 80 genome copies per reaction in both Fast and Standard cycling modes on the QuantStudio 5 (0.1 mL format).

5.3. PCR REPEATABILITY

Repeatability was evaluated to assess the intra-run precision of the PATHq *Serratia marcescens* Real-Time PCR Kit under controlled analytical conditions. The study was performed using three target DNA concentrations covering the working dynamic range of the assay (1×10^6 , 1×10^4 and 1×10^2 genome copies per reaction).

Testing was conducted across four independent PCR runs. In each run, four replicate reactions per concentration were prepared and amplified under identical conditions. Ct values obtained from all replicates were pooled per concentration (n = 16 per level), and the mean Ct, standard deviation (SD) and coefficient of variation (CV%) were calculated.

	<i>Serratia marcescens</i>	
	Mean Ct $\pm$ SD	CV%
High (10^6 copies/PCR)	21.47 $\pm$ 0.18	0.85%
Medium (10^4 copies/PCR)	28.19 $\pm$ 0.18	0.65%
Low (10^2 copies/PCR)	35.19 $\pm$ 0.55	1.55%

Across all concentrations tested, the assay demonstrated low dispersion of Ct values, with CV% below 2%. Variability increased slightly at the lowest concentration, as expected near the lower analytical range, but remained well within predefined acceptance criteria (CV < 5%).

These results confirm consistent amplification performance within a single analytical setting and demonstrate stable intra-run performance across high, medium and low target concentrations.

Under the experimental conditions described above, the PATHq *Serratia marcescens* Real-Time PCR Kit demonstrated acceptable PCR Repeatability, with CV values below 5% at all tested concentrations. The results confirm strong intra-run precision across the evaluated dynamic range of the assay.

5.4. PCR EFFICIENCY

PCR amplification efficiency of the PATHq *Serratia marcescens* Real-Time PCR Kit was evaluated using the same *Serratia marcescens* CECT 846 genomic DNA preparation described in Section 5.2. The working concentration was calculated and prepared as described above.

A four-point serial dilution series was prepared to cover the dynamic analytical range of the assay. The tested concentrations corresponded to:

- 1.20×10^5 copies/PCR
- 1.20×10^4 copies/PCR
- 1.20×10^3 copies/PCR
- 1.20×10^2 copies/PCR

Each concentration was tested in single replicate across four independent PCR runs, performed on separate plates under identical analytical conditions. Amplification was carried out using the Fast thermal cycling profile on the QuantStudio 5 (0.1 mL format), following the validated PATHq protocol.

Ct values obtained from the four runs were used to generate a standard curve by plotting Ct against the logarithm of genome copy number. PCR efficiency (E) was calculated from the slope of the regression line using the formula:

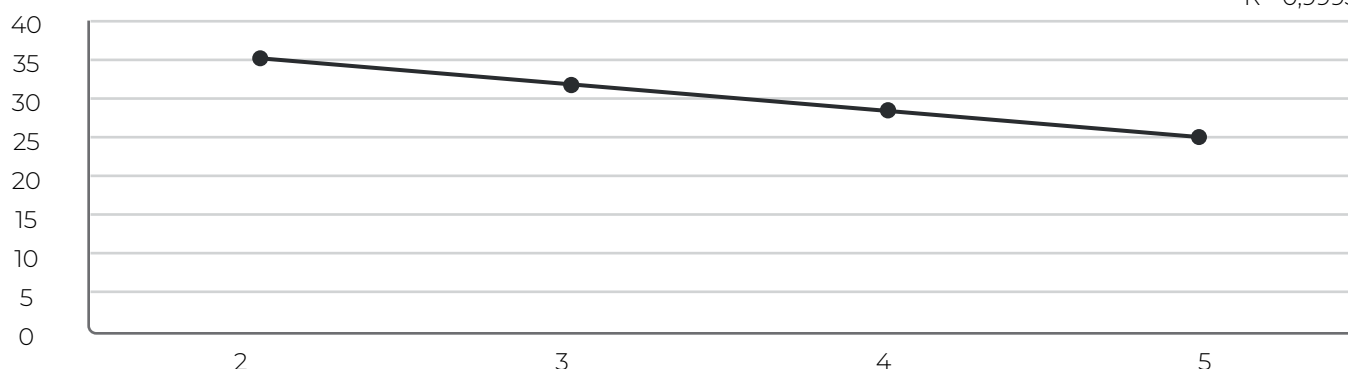
$$E = (10^{(-1/\text{slope})} - 1) \times 100$$

The amplification curves demonstrated consistent exponential behaviour across all dilution levels, and linearity was maintained throughout the tested range.

Serratia marcescens

$$y = -3,2348x + 37,992$$

$$R^2 = 0,9995$$



The calculated PCR efficiency was: 103.7%

This value falls within commonly accepted Real-Time PCR performance criteria (90–110%), indicating efficient and stable target amplification without evidence of significant inhibition or suboptimal primer–probe performance.

Under the experimental conditions described above, the PATHq *Serratia marcescens* Real-Time PCR Kit demonstrated a PCR efficiency of 103.7%, confirming robust and reliable amplification performance across the evaluated dynamic range.

5.5. PCR INTERMEDIATE PRECISION (REPRODUCIBILITY)

Intermediate Precision (Reproducibility) of the PATHq *Serratia marcescens* Real-Time PCR Kit was evaluated to assess inter-run variability under controlled but deliberately varied laboratory conditions. In accordance with the principles outlined in AFNOR NF U47-600-2, Reproducibility reflects the ability of the assay to maintain consistent performance when key experimental factors are modified within a single laboratory environment.

The following sources of variability were intentionally introduced:

- Operator
- Thermal cycler
- Day of analysis

Three target DNA concentrations were selected to represent the upper, mid and lower regions of the validated dynamic range:

- 1×10^6 copies/PCR

- 1×10^4 copies/PCR
- 1×10^2 copies/PCR

The experimental design comprised four independent PCR plates distributed as follows:

Thermal Cycler	Operator	Day
QuantStudio 5	1	1
7500Fast	1	1
QuantStudio 5	2	1
QuantStudio 5	1	2

For each plate, all three concentrations were tested in four replicate reactions per concentration. All reactions were performed in Fast cycling mode following the standard PATHq protocol.

Ct values obtained across all plates were pooled per concentration and used to calculate mean Ct, standard deviation (SD) and coefficient of variation (CV), thereby integrating instrument, operator and day effects into a single Reproducibility metric.

	<i>Serratia marcescens</i>	
	Mean Ct $\pm$ SD	CV%
High (10^6 copies/PCR)	21.40 $\pm$ 0.48	2.23%
Medium (10^4 copies/PCR)	28.28 $\pm$ 0.39	1.39%
Low (10^2 copies/PCR)	35.19 $\pm$ 0.63	1.78%

The results demonstrate low inter-run variability across all tested concentrations. Variation remained below 3% in all cases, including the lowest target level, where stochastic effects are typically more pronounced.

Under the experimental conditions described above, the PATHq *Serratia marcescens* Real-Time PCR Kit demonstrated strong intermediate precision across operators, instruments and days of analysis. Coefficients of variation ranged from 1.39% to 2.23%, remaining well within the pre-defined acceptance criterion (CV < 5%), thereby confirming robust Reproducibility of the amplification step.

5.6. PCR ROBUSTNESS

Robustness of the PATHq *Serratia marcescens* Real-Time PCR Kit was evaluated to determine the capacity of the method to remain unaffected by small, deliberate variations in critical analytical parameters. According to AFNOR NF U47-600-2 principles, Robustness assessment verifies that minor deviations within acceptable laboratory limits do not compromise assay performance.

The study was performed in Fast cycling mode on the QuantStudio 5 (0.1 mL format). Testing was conducted at a target concentration corresponding to $2 \times LD_{PCR}$ in order to ensure consistent detection under standard conditions while remaining close to the Sensitivity threshold.

Eight controlled variations were introduced to simulate realistic deviations that may occur during routine laboratory practice:

- +10% and -10% of PCR mix volume:
 - Test 1: +10% volume of PCR mix (13.2 µL)
 - Test 2: -10% volume of PCR mix (10.8 µL)
- +10% and -10% of DNA template volume
 - Test 3: +10% volume of DNA (8.8 µL)
 - Test 4: -10% volume of DNA (7.2 µL)
- ±1°C of annealing/hybridization temperature
 - Test 5: +1°C T of hybridization
 - Test 6: -1°C T of hybridization
- ±10% of hybridization time
 - Test 7: +10% time of hybridization (33 seconds)
 - Test 8: -10% time of hybridization (27 seconds)

A commercially supplied quantified genomic DNA standard of *Serratia marcescens* was used for this evaluation. Serial dilutions were prepared from the quantified stock solution using DNA storage buffer supplemented with carrier DNA to obtain the required working concentration.

Each Robustness condition was tested in quadruplet reactions. Ct values obtained under each variation were used to calculate mean Ct, standard deviation (SD) and coefficient of variation (CV).

Critical parameters		Robustness <i>Serratia marcescens</i>					
		Replicate 1	Replicate 2	Replicate 3	Replicate 4	Mean Ct ± SD	CV%
Volume of PCR mix	13.2 µL	35,77	37,83	37,06	35,05	36.43±1.25	3,43%
	10.8 µL	35,76	35,52	36,15	35,52	35.74±0.30	0,83%
Volume of DNA	8.8 µL	36,99	35,97	36,09	36,11	36.29±0.47	1,30%
	7.2 µL	38,00	35,16	37,21	35,85	36.56±1.29	3,52%
T °C hybridization	61°C	35,09	34,71	34,83	35,03	34.92±0.18	0,50%
	59°C	36,73	38,00	37,00	37,35	37.27±0.55	1,47%
Time hybridization	33 sec	35,78	35,41	36,41	35,99	35.90±0.42	1,16%
	27 sec	35,56	36,32	37,56	35,72	36.29±0.91	2,50%

Under the experimental conditions described above, the PATHq *Serratia marcescens* Real-Time PCR Kit demonstrated stable performance despite controlled variations in reaction volume, template volume, hybridization temperature and hybridization time. All evaluated conditions yielded coefficients of variation below the predefined acceptance criterion ($CV < 5\%$).

5.7. PCR EQUIVALENCY STUDY

An equivalency study was conducted to compare the performance of the PATHq *Serratia marcescens* Real-Time PCR Kit on two Real-Time PCR platforms: the QuantStudio 5 (QS5-01, 0.1 mL format) and the Applied Biosystems 7500 Fast instrument (Fast mode).

In accordance with AFNOR NF U47-600-2 validation principles, the evaluation was performed at a concentration corresponding to $2 \times LD_{PCR}$ (160 genome copies per reaction). This concentration was selected to ensure reliable detection while remaining sufficiently close to the analytical detection limit to allow meaningful comparison of instrument performance.

Each condition was tested in quadruplet reactions on both platforms under identical analytical parameters (Fast cycling mode).

	<i>Serratia marcescens</i>	
	QS5-01	7500 FAST
Frequency of detection $2 \times LD_{PCR}$	4/4 - 100%	4/4 - 100%

Under the experimental conditions described above, the PATHq *Serratia marcescens* Real-Time PCR Kit demonstrated equivalent qualitative performance on the QuantStudio 5 and Applied Biosystems 7500 Fast platforms at $2 \times LD_{PCR}$. No differences in detection frequency were observed between instruments, confirming analytical equivalency under Fast cycling conditions.

6. Application to porcine semen and other sample matrices

The PATHq *Serratia marcescens* Real-Time PCR Kit was specifically developed for use with purified nucleic-acid extracts obtained from porcine semen and for integration into established molecular testing workflows routinely used in veterinary and artificial insemination laboratories.

6.1. COMPATIBILITY WITH ESTABLISHED PORCINE SEMEN EXTRACTION WORKFLOWS

A major consideration during development of the PATHq assay was compatibility with the porcine semen extraction workflow commonly used with the VetMAX™ PRRSV EU & NA 3.0 Kit and the MagMAX™ CORE Nucleic Acid Purification Kit.

This workflow includes a dedicated sample preparation procedure for porcine semen followed by magnetic-bead nucleic-acid purification. It is applicable to individual semen samples and to pooled samples containing up to five individual samples.

The PATHq assay was developed so that nucleic-acid extracts generated using this established workflow can be used directly for *Serratia marcescens* detection without requiring an additional or PATHq-specific extraction procedure.

This approach allows laboratories already using the MagMAX™ CORE semen workflow to incorporate PATHq testing into their existing sample-processing scheme and, where appropriate, to perform different molecular analyses using the same purified nucleic-acid extract.

The PATHq assay also uses the same Xeno Internal Positive Control (IPC) system used in this extraction workflow. The IPC is introduced during sample processing and recovered together with the sample nucleic acids. Subsequent amplification of the Xeno IPC in the VIC channel therefore provides an internal control associated with the complete processed sample, supporting assessment of nucleic-acid recovery and identification of potential PCR inhibition.

6.2. EXPERIMENTAL EVALUATION USING PORCINE SEMEN-DERIVED EXTRACTS

Compatibility with porcine semen was evaluated experimentally during development of the PATHq *Serratia marcescens* Real-Time PCR Kit using nucleic-acid extracts obtained from semen samples processed according to the MagMAX™ CORE-based semen workflow described above.

The purpose of these studies was to confirm that nucleic-acid preparations obtained using a workflow representative of the intended routine application were suitable for direct analysis with the PATHq assay.

Multiple porcine semen-derived extracts were analysed during assay development and characterisation. Target-positive materials consistently produced amplification in the FAM channel under the evaluated conditions, while appropriate Xeno IPC amplification was observed in the VIC channel.

These studies demonstrated that components associated with the semen matrix and remaining after the evaluated purification procedure did not prevent reliable amplification of the PATHq target under the experimental conditions tested.

The results therefore support direct use of the PATHq *Serratia marcescens* Real-Time PCR Kit with nucleic-acid extracts generated from porcine semen using this established MagMAX™ CORE-based workflow.

6.3. APPLICATION TO POOLED SEMEN SAMPLES

Pooling of porcine semen samples is frequently used in molecular monitoring workflows to increase analytical efficiency while reducing the number of extractions and PCR reactions required.

The semen extraction workflow considered during development of the PATHq assay supports pooling of up to five individual semen samples. Consequently, the PATHq assay was also developed and experimentally evaluated taking this pool-of-five configuration into consideration.

Detection studies were performed using nucleic-acid extracts obtained from pooled porcine semen samples processed according to the same extraction workflow. The results demonstrated detection of *Serratia marcescens* DNA in extracts derived from pools of five under the evaluated experimental conditions.

Pooling necessarily reduces the concentration of a target initially present in only one of the individual samples. For an equal-volume pool of five samples, the target concentration contributed by one positive sample is theoretically reduced approximately five-fold before extraction.

This dilution effect is particularly relevant for samples containing very low target concentrations and should therefore be considered when interpreting results close to the analytical detection limit.

Within these limitations, the experimental data generated during PATHq development support the use of pool-of-five semen extracts with the assay under the evaluated workflow conditions.

6.4. EVALUATION IN ADDITIONAL SAMPLE MATRICES

Although porcine semen represents the principal matrix considered during development of the PATHq *Serratia marcescens* Real-Time PCR Kit, assay performance was not evaluated exclusively using semen-derived material.

During analytical development and specificity studies, *Serratia marcescens* DNA originating from different types of material relevant to artificial insemination centres and laboratory environments was successfully detected.

Evaluated materials included bacterial isolates and reference cultures as well as nucleic-acid preparations originating from water samples, environmental swabs collected from artificial insemination facilities and samples associated with semen-processing equipment.

These results demonstrate that target amplification is not intrinsically restricted to nucleic acids obtained from porcine semen and support the use of the PATHq assay for detection of *Serratia marcescens* DNA in purified nucleic-acid preparations from other sample types under appropriate laboratory conditions.

Sample preparation and nucleic-acid extraction procedures should nevertheless be appropriate for the characteristics of the matrix being analyzed.

6.5. SCOPE OF THE SEMEN WORKFLOW EVALUATION

The studies described in this section were designed to evaluate compatibility and detectability of the PATHq *Serratia marcescens* Real-Time PCR Kit using nucleic-acid extracts representative of its intended application, with particular emphasis on porcine semen and pool-of-five semen samples.

The extraction workflow itself was not independently validated as part of the PATHq PCR characterisation study. Rather, an established MagMAX™ CORE-based porcine semen extraction procedure was used to generate representative nucleic-acid extracts on which PATHq performance was experimentally evaluated.

Accordingly, the evidence generated during development demonstrates compatibility of the PATHq assay with nucleic-acid extracts obtained using this workflow and supports its integration into established porcine semen molecular testing processes.

Together with the analytical characterization presented in Section 5, these studies support the application of the PATHq *Serratia marcescens* Real-Time PCR Kit to purified nucleic-acid extracts from porcine semen, including pool-of-five samples, and to other evaluated sample matrices under the described experimental conditions.

7. Conclusions

The analytical validation of the PATHq *Serratia marcescens* Real-Time PCR Kit demonstrates that the assay fulfils the performance principles defined for qualitative Real-Time PCR methods under the AFNOR NF U47-600-2 framework for PCR Characterisation.

The assay showed:

Parameter	Acceptance Criteria	Result	Validation
Analytical Inclusivity	100% positive detection of target isolates	100% detection of tested <i>Serratia marcescens</i> isolates	Yes
Analytical Exclusivity	No amplification of non-target organisms	No cross-reactivity with non-target bacteria or relevant porcine viruses	Yes
Analytical Sensitivity (LD _{PCR})	Consistent detection at defined LD _{PCR}	80 genome copies/reaction under Fast and Standard profiles	Yes
Repeatability	CV ≤ 5% at tested concentration levels	CV < 5% across tested concentration levels	Yes
Intermediate precision	CV ≤ 5% at tested concentration levels	CV < 5% across tested concentration levels	Yes
Robustness	Results remain within predefined performance limits under parameter variation	No significant impact under controlled reaction parameter variations	Yes
Platform equivalency	Equivalent qualitative detection across platforms	Equivalent detection performance across validated Real-Time PCR platforms	Yes

Collectively, these results confirm that the PATHq assay provides reliable, sensitive and specific detection of *Serratia marcescens* DNA under controlled analytical conditions.

8. Version history

VERSION	DATE	DESCRIPTION
V01	08-2026	Initial Release

Validation report PATHq *Serratia marcescens* Real-Time PCR Kit

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