

VALIDATION REPORT

PATHq *Klebsiella* spp.
Real-Time PCR Kit
PATHq-KLB-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 *Klebsiella* spp. Real-Time PCR Kit, a duplex qualitative Real-Time PCR assay 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 *Klebsiella* spp. Real-Time PCR Kit is intended for the qualitative detection of DNA from organisms belonging to the genus *Klebsiella* within the validated assay scope.

The kit is classified as Research Use Only (RUO) and is intended for laboratory research, assay development, molecular monitoring and internal analytical evaluation. It is not intended for 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 PCR-level analytical performance of the PATHq *Klebsiella* spp. Real-Time PCR Kit as a qualitative Real-Time PCR assay.

The validation includes Analytical Specificity, Analytical Sensitivity, Repeatability, PCR Efficiency, Intermediate Precision, Robustness and Instrument Equivalency for the detection of target *Klebsiella* DNA under controlled analytical 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 *Klebsiella* spp. DNA detection workflows.

2. Scientific&Technical background

2.1. THE ORGANISM

Klebsiella spp. are Gram-negative, non-motile, facultatively anaerobic bacteria belonging to the Enterobacteriaceae family. Members of the genus are widely distributed in the environment and may be found in soil, water, plants and the gastrointestinal and respiratory tracts of animals and humans.

The genus comprises several species of veterinary, environmental and public health relevance, including *Klebsiella pneumoniae*, *Klebsiella oxytoca* and other closely related species. These bacteria may behave as opportunistic pathogens and are capable of persisting in humid environments and forming biofilms on surfaces and equipment, facilitating their survival and dissemination in laboratory and animal production settings.

2.2. RELEVANCE IN MOLECULAR TESTING

Members of the genus *Klebsiella* are increasingly recognized as relevant in veterinary and animal production settings because of their ability to contaminate biological materials, persist in humid environments and affect reproductive performance. Although historically regarded primarily as environmental contaminants, their presence in critical biological 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, reduce fertilizing capacity and negatively affect reproductive outcomes.

Artificial insemination centers 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, *Klebsiella* spp. may also be detected in tissues, biological fluids, environmental samples and surfaces within animal facilities. Their 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 *Klebsiella* spp. 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 optimized 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 *Klebsiella* spp. 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 that prioritizes 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 hybridize 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 *Klebsiella* spp. 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 *Klebsiella* spp. DNA using primers and a hydrolysis probe designed to target a conserved genomic region within the genus. This target region was selected based on sequence analysis to ensure broad strain coverage while maintaining analytical exclusivity with respect to non-target organisms, including closely related members of 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 optimized 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 *Klebsiella* spp. 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 *Klebsiella* spp. The primer–probe systems assigned to the FAM (target) and VIC (IPC) channels were optimized to ensure efficient co-amplification under shared reaction conditions while minimizing 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 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 *Klebsiella* spp. 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 *Klebsiella* spp. 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 characterize qualitative Real-Time PCR, including Specificity, analytical Sensitivity, Repeatability, Reproducibility and Robustness.

Although the PATHq *Klebsiella* spp. 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 recognized analytical validation standards 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 Organization 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 *Klebsiella* spp. isolates) 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 *Klebsiella* spp. 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 target *Klebsiella* isolates relevant to the intended application setting.

Strain	Origin	Matrix / Nature	Identification Method	PATHq <i>Klebsiella</i> spp. Real-Time PCR Kit
<i>Klebsiella pneumoniae</i> subsp. <i>pneumoniae</i> CECT 143	Spain	Reference strain	Real-Time PCR	Detected
<i>Klebsiella oxytoca</i>	USA	Semen	MALDI-TOF	Detected
<i>Klebsiella oxytoca</i>	Spain	Semen	MALDI-TOF	Detected
<i>Klebsiella oxytoca</i>	Spain	Semen	MALDI-TOF	Detected
<i>Klebsiella oxytoca</i>	Spain	Semen	MALDI-TOF	Detected
<i>Klebsiella oxytoca</i>	Spain	Semen	MALDI-TOF	Detected
<i>Klebsiella pneumoniae</i>	Spain	Porcine semen	MALDI-TOF / Real-Time PCR	Detected
<i>Klebsiella oxytoca</i>	Spain	Porcine semen	MALDI-TOF	Detected
<i>Klebsiella variicola</i>	Spain	Porcine semen / environmental sample	MALDI-TOF / sequencing	Detected
<i>Klebsiella quasipneumoniae</i>	Spain	Porcine semen / reference material	Sequencing	Detected
<i>Klebsiella aerogenes</i>	Spain	Environmental / AI facility sample	MALDI-TOF	Detected
<i>Klebsiella pneumoniae</i>	Spain	Porcine semen	MALDI-TOF	Detected
<i>Klebsiella pneumoniae</i>	Spain	Porcine semen	MALDI-TOF	Detected
<i>Klebsiella oxytoca</i>	Spain	Porcine semen	MALDI-TOF	Detected
<i>Klebsiella variicola</i>	Spain	Environmental swab	MALDI-TOF / sequencing	Detected
<i>Klebsiella quasipneumoniae</i>	Spain	Reference strain / culture collection	Sequencing / culture collection	Detected

Strain	Origin	Matrix / Nature	Identification Method	PATHq <i>Klebsiella</i> spp. Real-Time PCR Kit
<i>Klebsiella michiganensis</i>	Spain	Water / AI facility environment	MALDI-TOF / sequencing	Detected
<i>Klebsiella grimontii</i>	Spain	Environmental sample	Sequencing	Detected
<i>Klebsiella aerogenes</i>	Spain	Semen processing equipment	MALDI-TOF	Detected
<i>Klebsiella ornithinolytica</i>	Spain	Environmental / water system	MALDI-TOF	Detected
<i>Klebsiella planticola</i>	Spain	Environmental sample	MALDI-TOF / sequencing	Detected
<i>Klebsiella terrigena</i>	Spain	Water / environmental sample	MALDI-TOF / sequencing	Detected
<i>Klebsiella mobilis</i>	Spain	Environmental sample	MALDI-TOF / sequencing	Detected

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

The PATHq *Klebsiella* spp. Real-Time PCR Kit detected 100% of the *Klebsiella* spp. isolates included in the tested panel. The assay reliably detected genetic and epidemiologically diverse *Klebsiella* spp. isolates representative of field conditions in artificial insemination centres. These results support inclusivity of the assay within the evaluated target panel, with no false-negative results observed under the defined analytical conditions.

EXCLUSIVITY: Exclusivity evaluates the ability of the assay to avoid amplification of non-target microorganisms, including phylogenetically related taxa, organisms associated with porcine semen and artificial insemination environments, and other microorganisms relevant to laboratory workflows.

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>Klebsiella</i> spp. Real-Time PCR Kit
Escherichia coli	Spain	Semen	PCR	Not detected
Staphylococcus aureus	Spain	Skin swab	MALDI-TOF	Not detected
Staphylococcus epidermidis	Spain	Skin swab	MALDI-TOF	Not detected
Streptococcus suis	Spain	Porcine genital tract	MALDI-TOF	Not detected
Corynebacterium spp.	Spain	Skin swab	MALDI-TOF	Not detected
Mycoplasma hyopneumoniae	Spain	Semen	Real-Time PCR	Not detected
Actinobacillus pleuropneumoniae	Spain	Semen	Real-Time PCR	Not detected
Lactobacillus spp.	Spain	Semen	MALDI-TOF	Not detected
Citrobacter freundii	Spain	Semen	MALDI-TOF	Not detected
Proteus mirabilis	Spain	Semen	MALDI-TOF	Not detected
Serratia marcescens	Spain	Semen	MALDI-TOF	Not detected
Salmonella enterica	Spain	Semen	Real-Time PCR	Not detected
Morganella morganii	Spain	Semen	MALDI-TOF	Not detected
Providencia stuartii	Spain	Semen	MALDI-TOF	Not detected
Pseudomonas aeruginosa	Spain	Semen	MALDI-TOF	Not detected
Bacillus cereus	Spain	Environmental swab	MALDI-TOF	Not detected
Staphylococcus spp.	Spain	Semen	MALDI-TOF	Not detected
Enterobacter cloacae	Spain	Semen	MALDI-TOF	Not detected
Glaesserella parasuis	Spain	Semen	PCR	Not detected
Proteus vulgaris	Spain	Semen	MALDI-TOF	Not detected
Pasteurella multocida	Spain	Semen	Real-Time PCR	Not detected
PCV2	Spain	Semen	Real-Time PCR	Not detected
PRRSV	Spain	Semen	Real-Time PCR	Not detected

Strain ID	Origin	Matrix / Nature	Identification Method	PATHq <i>Klebsiella</i> spp. Real-Time PCR Kit
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

The PATHq *Klebsiella* spp. Real-Time PCR Kit showed no amplification of the non-target organisms included in the evaluated panel. These results support analytical exclusivity under the defined test conditions.

Under the experimental conditions described above, the PATHq *Klebsiella* spp. Real-Time PCR Kit demonstrated appropriate analytical specificity, detecting the target *Klebsiella* spp. isolates included in the inclusivity panel and showing no cross-reactivity with the evaluated non-target organisms.

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

The analytical Sensitivity of the PATHq *Klebsiella* spp. 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 *Klebsiella pneumoniae* subsp. *pneumoniae* CECT 143 was used as the reference material. The material contained 5 µg of genomic DNA. Based on an estimated genome size of approximately 5.5 Mbp, the total number of genome copies in the lyophilized material was calculated to be approximately 8.3×10^8 copies.

The DNA was reconstituted in 664 µL of MagMAX CORE Elution Buffer, resulting in an estimated concentration of 1.25×10^6 genome copies/µL. Since 8 µL of DNA were added per PCR reaction, this concentration corresponded to approximately 1×10^7 genome copies per reaction. As the material was supplied with a specified DNA mass, no additional extraction or external quantification was performed prior to dilution preparation.

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 %
100	24/24	100%	24/24	100%
50	24/24	100%	24/24	100%
25	19/24	79.2%	22/24	91.7%
12.5	15/24	62.5%	12/24	62.5%

Under the experimental conditions described above, the LD_{PCR} of the PATHq *Klebsiella* spp. Real-Time PCR Kit was determined to be 50 genome copies per reaction in both Fast and Standard cycling modes on the QuantStudio™ 5 (0.1mL format).

5.3. PCR REPEATABILITY

Repeatability evaluates the precision of the PATHq *Klebsiella* spp. Real-Time PCR Kit under controlled analytical conditions. The study was performed using target DNA concentrations covering high, medium and low levels of the validated working range.

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.

	PATHq <i>Klebsiella</i> spp. Real-Time PCR Kit	
	Mean Ct ± SD	CV%
High (10 ⁶ copies/PCR)	20.50±0.5	2.4%
Medium (10 ⁴ copies/PCR)	27.60±0.57	2.1%
Low (10 ² copies/PCR)	34.59±1.13	3.3%

Across all concentrations tested, the assay demonstrated low dispersion of Ct values, with coefficients of variation around 3%. 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 performance under repeatability conditions across high, medium and low target concentrations.

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

5.4. PCR EFFICIENCY

PCR amplification efficiency of the PATHq *Klebsiella* spp. Real-Time PCR Kit was evaluated using the same commercially quantified genomic DNA standard of *Klebsiella pneumoniae* subsp. *pneumoniae* CECT 143 described in Section 5.2.

The working concentration was estimated from the specified DNA mass and the estimated genome size, as described in Section 5.2, and the material was used without additional extraction or external quantification.

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

- 1.50 × 10⁵ copies/PCR
- 1.50 × 10⁴ copies/PCR
- 1.50 × 10³ copies/PCR
- 1.50 × 10² 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, 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:

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

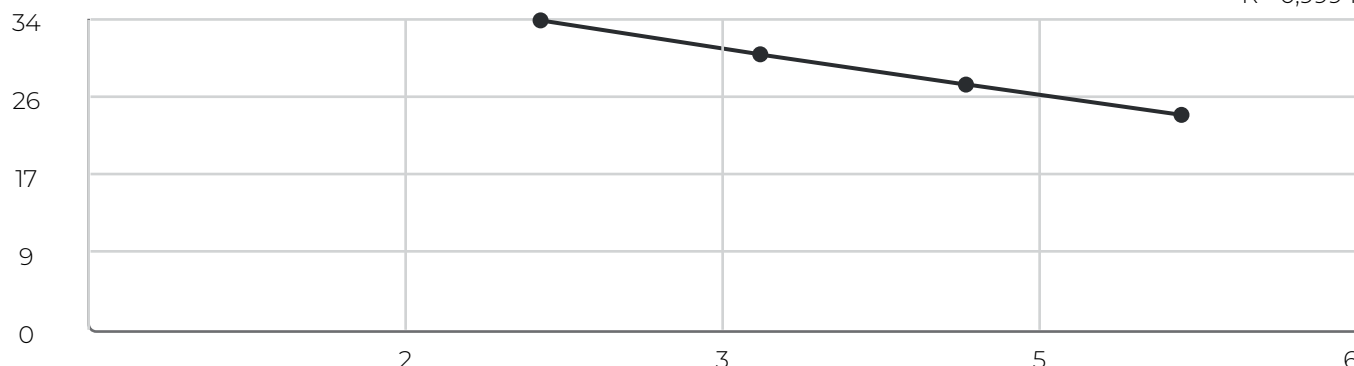
The amplification curves demonstrated consistent exponential behaviour across all dilution levels, and linearity was maintained throughout the tested range.%

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.

PATHq *Klebsiella* spp. Real-Time PCR Kit

$$y = -3,4397x + 41,158$$

$$R^2 = 0,9994$$



The calculated PCR efficiency was 95.3%. 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 *Klebsiella* spp. Real-Time PCR Kit demonstrated a PCR efficiency of 95.3%, confirming robust and reliable amplification performance across the evaluated dynamic range.

5.5. PCR INTERMEDIATE PRECISION (REPRODUCIBILITY)

Intermediate Precision (Reproducibility) of the PATHq *Klebsiella* spp. 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
7500 Fast	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.

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.

	PATHq <i>Klebsiella</i> spp. Real-Time PCR Kit	
	Mean Ct $\pm$ SD	CV%
High (10^6 copies/PCR)	20.80 $\pm$ 0.48	2.29%
Medium (10^4 copies/PCR)	27.71 $\pm$ 0.39	1.91%
Low (10^2 copies/PCR)	34.61 $\pm$ 0.63	3.05%

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

Under the experimental conditions described above, the PATHq *Klebsiella* spp. Real-Time PCR Kit demonstrated strong intermediate precision across operators, instruments and days of analysis. Coefficients of variation ranged from 1.91% to 3.05%, remaining well within the predefined acceptance criterion (CV < 5%), thereby confirming robust reproducibility of the amplification step.

5.6. PCR ROBUSTNESS

Robustness of the PATHq *Klebsiella* spp. 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. 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 genomic DNA reference material of *Klebsiella pneumoniae* subsp. *pneumoniae* was used for this evaluation. Serial dilutions were prepared from the stock solution using MagMAX CORE Elution Buffer 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 PATHq <i>Klebsiella</i> spp. Real-Time PCR Kit					
		Replicate 1	Replicate 2	Replicate 3	Replicate 4	Mean Ct ± SD	CV%
Volume of PCR mix	13.2 µL	33.83	34.94	34.37	33.99	34.28 ± 0.49	1.44%
	10.8 µL	33.50	33.84	33.17	33.87	33.59 ± 0.33	0.98%
Volume of DNA	8.8 µL	33.31	33.97	34.23	33.98	33.87 ± 0.39	1.16%
	7.2 µL	33.46	33.87	35.20	34.25	34.20 ± 0.74	2.17%
T °C hybridization	61°C	35.10	34.85	33.92	33.97	34.46 ± 0.60	1.75%
	59°C	34.19	33.92	33.40	32.95	33.62 ± 0.55	1.64%
Time hybridization	33 sec	31.31	31.20	32.95	32.46	31.98 ± 0.86	2.70%
	27 sec	32.38	34.25	32.90	32.28	32.95 ± 0.91	2.75%

Under the experimental conditions described above, the PATHq *Klebsiella* spp. Real-Time PCR Kit demonstrated stable performance despite controlled variations in PCR mix volume, DNA template volume, hybridization temperature and hybridization time. All evaluated conditions yielded coefficients of variation below the predefined acceptance criterion (CV < 5%), confirming the robustness of the amplification step.

5.7. PCR EQUIVALENCY STUDY

An equivalency study was conducted to compare the performance of the PATHq *Klebsiella* spp. Real-Time PCR Kit on two Real-Time PCR platforms: the QuantStudio™ 5 and 7500 Fast instruments, using Fast mode.

In accordance with AFNOR NF U47-600-2 validation principles, the evaluation was performed at a concentration corresponding to $2 \times LD_{PCR}$ (100 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).

	PATHq <i>Klebsiella</i> spp. Real-Time PCR Kit	
	QUANTSTUDIO™ 5-01	7500 FAST
Frequency of detection $2 \times LD_{PCR}$	4/4 - 100%	4/4 - 100%

Under the experimental conditions described above, the PATHq *Klebsiella* spp. Real-Time PCR Kit demonstrated equivalent qualitative performance on the QuantStudio™ 5 and 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 *Klebsiella* spp. Real-Time PCR Kit was developed with particular consideration for porcine semen testing workflows routinely used in artificial insemination laboratories. A central objective during assay development was to ensure that the kit could be applied directly to purified nucleic-acid extracts already generated within established semen-processing workflows, avoiding the need for a dedicated PATHq-specific extraction procedure.

6.1. INTEGRATION WITH ESTABLISHED PORCINE SEMEN EXTRACTION WORKFLOWS

The assay was evaluated using porcine semen extracts obtained following the MagMAX™ CORE-based semen purification workflow used with the VetMAX™ PRRSV EU & NA 3.0 Kit.

This workflow includes specific pre-treatment steps adapted to the characteristics of semen, followed by magnetic-bead nucleic-acid purification. It is applicable to individual semen samples and to pooled preparations of up to five samples.

PATHq was developed to be compatible with the resulting purified extracts, allowing laboratories already using this workflow to incorporate *Klebsiella* spp. testing into their existing molecular analysis scheme without introducing an additional extraction step.

This compatibility is particularly relevant in laboratories where the same purified extract is used for several molecular determinations performed from a common semen preparation.

The PATHq assay also uses the same Xeno Internal Positive Control (IPC) system associated with this extraction workflow. The IPC is carried through the sample-processing procedure and subsequently detected in the VIC channel during amplification, providing an internal control linked to the processed sample and supporting identification of potential PCR inhibition.

6.2. EVALUATION OF PORCINE SEMEN-DERIVED EXTRACTS

During development and analytical characterisation, the PATHq *Klebsiella* spp. Real-Time PCR Kit was tested using multiple nucleic-acid extracts derived from porcine semen.

The objective of this evaluation was to assess assay performance in extracts representative of the matrix and sample-preparation conditions expected in routine use.

Klebsiella-positive semen-derived extracts produced target amplification in the FAM channel under the evaluated conditions. Xeno IPC amplification was also observed in the VIC channel, indicating that the purified extracts were suitable for downstream amplification and that no relevant PCR inhibition was detected in the evaluated reactions.

These data support the compatibility of the PATHq *Klebsiella* spp. Real-Time PCR Kit with porcine semen nucleic-acid extracts obtained using the referenced MagMAX™ CORE-based workflow.

6.3. USE WITH POOLS OF THREE SEMEN SAMPLES

Pooling is frequently incorporated into semen-monitoring programmes to increase sample throughput and reduce the number of extraction and amplification reactions required.

Because the referenced semen purification workflow is designed to accommodate pools containing up to five individual semen samples, the PATHq assay was also evaluated under this sample configuration.

Detection studies performed with extracts obtained from pools of three demonstrated successful amplification of *Klebsiella* spp. DNA under the evaluated experimental conditions.

When one positive sample is combined with two negative samples in equal proportions, the target contributed by the positive sample is theoretically diluted approximately three-fold before extraction. This effect becomes increasingly relevant as target concentration approaches the analytical detection limit.

The results generated during assay development nevertheless support the use of pool-of-three semen extracts with the PATHq *Klebsiella* spp. Real-Time PCR Kit under the evaluated workflow conditions.

6.4. PERFORMANCE WITH ADDITIONAL SAMPLE MATRICES

Although porcine semen was the principal matrix considered during development of the assay, the evaluation of PATHq *Klebsiella* spp. was not restricted to semen-derived material.

The inclusivity panel comprised different *Klebsiella* species obtained from a range of sources relevant to artificial insemination facilities and veterinary laboratory environments. These included porcine semen, water and water-system samples, environmental samples and swabs, semen-processing equipment and reference culture material.

All target isolates included in the evaluated panel were detected by the PATHq assay under the defined analytical conditions.

The diversity of both species and sample origin represented in the panel supports the analytical applicability of the assay to purified nucleic-acid preparations beyond porcine semen, provided that the upstream sample preparation and extraction procedure is suitable for the corresponding matrix.

6.5. SCOPE OF THE SEMEN WORKFLOW EVALUATION

The studies described in this section were intended to characterise the behaviour of the PATHq *Klebsiella* spp. Real-Time PCR Kit when used with purified nucleic-acid extracts representative of its main application context.

The MagMAX™ CORE-based semen extraction workflow itself was not independently validated within the PATHq PCR characterisation study. Instead, this established extraction procedure was used to generate representative semen-derived nucleic-acid extracts for subsequent PATHq evaluation.

The results therefore demonstrate compatibility and detectability of the PATHq assay with extracts generated using this workflow, including pool-of-five semen preparations, without extending the scope of the study to complete validation of the upstream extraction method.

Together with the analytical performance data described in Section 5, these results support the use of the PATHq *Klebsiella* spp. Real-Time PCR Kit with purified nucleic-acid extracts from porcine semen and with other evaluated sample types under the defined experimental conditions.

Under the experimental conditions evaluated, the PATHq *Klebsiella* spp. Real-Time PCR Kit demonstrated compatibility with purified nucleic-acid extracts obtained from porcine semen using the referenced MagMAX™ CORE-based workflow, including pool-of-three preparations. Successful detection was also demonstrated across additional evaluated sample types, supporting the applicability of the assay beyond porcine semen while maintaining the defined PCR-characterisation scope.

7. Conclusions

The analytical validation of the PATHq *Klebsiella* spp. 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>Klebsiella</i> spp. 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 *Klebsiella* spp. DNA under controlled analytical conditions.

8. Version history

VERSION	DATE	DESCRIPTION
V01	08-2026	Initial Release

Validation report PATHq *Klebsiella* spp. Real-Time PCR Kit

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