Methods 232 (2024) 72–80

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Development of novel digital PCR assays for the rapid quantification of Gram-negative bacteria biomarkers using RUCS algorithm

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ARTICLE INFO

Keywords:
Digital PCR (dPCR)
Gram-negative bacteria
Pathogen detection
Respiratory infections
Biomarkers
RUCS algorithm

ABSTRACT

Rapid and accurate identification of bacterial pathogens is crucial for effective treatment and infection control, particularly in hospital settings. Conventional methods like culture techniques and MALDI-TOF mass spectrometry are often time-consuming and less sensitive. This study addresses the need for faster and more precise diagnostic methods by developing novel digital PCR (dPCR) assays for the rapid quantification of biomarkers from three Gram-negative bacteria: *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*.

Utilizing publicly available genomes and the *rapid identification of PCR primers for unique core sequences* or RUCS algorithm, we designed highly specific dPCR assays. These assays were validated using synthetic DNA, bacterial genomic DNA, and DNA extracted from clinical samples. The developed dPCR methods demonstrated wide linearity, a low limit of detection (~30 copies per reaction), and robust analytical performance with measurement uncertainty below 25 %. The assays showed high repeatability and intermediate precision, with no cross-reactivity observed. Comparison with MALDI-TOF mass spectrometry revealed substantial concordance, highlighting the methods' suitability for clinical diagnostics.

This study underscores the potential of dPCR for rapid and precise quantification of Gram-negative bacterial biomarkers. The developed methods offer significant improvements over existing techniques, providing faster, more accurate, and SI-traceable measurements. These advancements could enhance clinical diagnostics and infection control practices.

1. Introduction

Infectious diseases, particularly respiratory infections, continue to impose a substantial burden on global healthcare, accounting for over 15 % of deaths worldwide [1]. Gram-negative bacteria, in particular from genera *Pseudomonas* and *Acinetobacter*, and *Enterobacteriaceae* family (*Klebsiella*, *Citrobacter*, *Escherichia*) cause the majority of hospital acquired respiratory tract infections, accounting for 45 % to 70 % of ventilator-associated pneumonia and 20 % to 30 % of catheter-related bloodstream infections [2–6]. These infections often lead to severe complications, including sepsis [7,8]. In such situations, timely administration of adequate antibiotics is vital for patient outcome [9]. Hence, rapid and accurate identification of Gram-negative bacteria is crucial not only for effective treatment but also for infection control and prevention, surveillance, and epidemiological purposes.

Pathogens in clinical samples are typically identified and quantified through various diagnostic tests, including phenotypic and molecular approaches [10]. Although standard microbiological techniques, such as bacterial cultures combined with matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF), are considered the gold standard for detection and identification, they are time-consuming and may delay effective infection-control measures.

To expedite pathogen identification, molecular approaches that do not require initial cultures have been introduced. Polymerase chain reaction (PCR), notably quantitative real-time PCR (qPCR), has been widely adopted in life sciences, agriculture, medicine, and molecular diagnostics due to its heightened sensitivity, specificity, and rapid turnaround times. More recently, digital PCR (dPCR) has emerged. Differing from qPCR in reaction partitioning and end-point fluorescence measurement, it enables absolute quantification of nucleic acids using

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https://doi.org/10.1016/j.ymeth.2024.10.011
Received 3 September 2024; Received in revised form 28 October 2024; Accepted 28 October 2024
Available online 30 October 2024

Poisson statistics without the need for a calibrator, while exhibiting enhanced sensitivity even in complex backgrounds and higher resilience to PCR inhibitors. Its high accuracy enables traceability to the International System of Units (SI) for copy number unit 1 through counting, thus contributing to method standardization [11–13].

Here we introduce three dPCR methods capable of detecting and quantifying biomarkers specific to *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* – prominent Gram-negative bacteria often responsible for hospital-acquired respiratory infections that may lead to sepsis. Assay development was a combination of an in-house design, and a literature survey aimed at creating highly specific assays targeting single-copy biomarkers on the selected organisms' genomes (Fig. 1). Leveraging publicly available genome sequence data of the selected bacteria and their close relatives, alongside the rapid identification of PCR primers for unique core sequences (RUCS) algorithm [14], we designed and developed dPCR methods in-house. RUCS identifies unique sequences within genomic data, prepares primers and probes based on these sequences, and validates them *in silico*. In our study, we used RUCS to find species-specific sequences, by providing a positive genome dataset with genomes of the target organism, and a negative genome dataset, with genomes of other species within the genus. Based on the identified unique sequences, we subsequently designed primer and probe sets using Applied Biosystems' Primer Express version 2.0. The significance of this approach lies in its ability to translate biomarker concentrations into pathogenic agent, as one copy of a biomarker/target DNA sequence should represent a single bacterial cell. Ensuring the analytical performance met ISO guidelines [15,16], we evaluated various factors to guarantee accurate quantification. Validating dPCR methods against the gold standard method – MALDI TOF, demonstrated the precision and reliability of developed methods for intended use (Fig. 1). Furthermore, owing to their accuracy, these methods have already been utilized to evaluate DNA biomarker extraction from complex matrices and have the potential to serve as candidate reference measurement procedures.

2. Materials and methods

All experiments were conducted at the National Institute of Biology and University Clinic of Respiratory and Allergic Diseases Golnik, following dMIQE guidelines [17,18]. See also Supplementary Data.

2.1. *In silico identification of novel biomarkers*

Novel biomarkers specific to *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa* were identified using RUCS [14] (Fig. 2). Ališ et al.'s approach [19] guided positive and negative dataset assembly for each species: the former contained full genomic sequences of the target species, while the latter encompassed sequences of other species within the same genus. Data relevance was ensured through average nucleotide identity analysis using the pyani algorithm [20]. Sequences with identity below 95 % to the reference genome were excluded from the positive dataset, and sequences above 95 % identity were removed from the

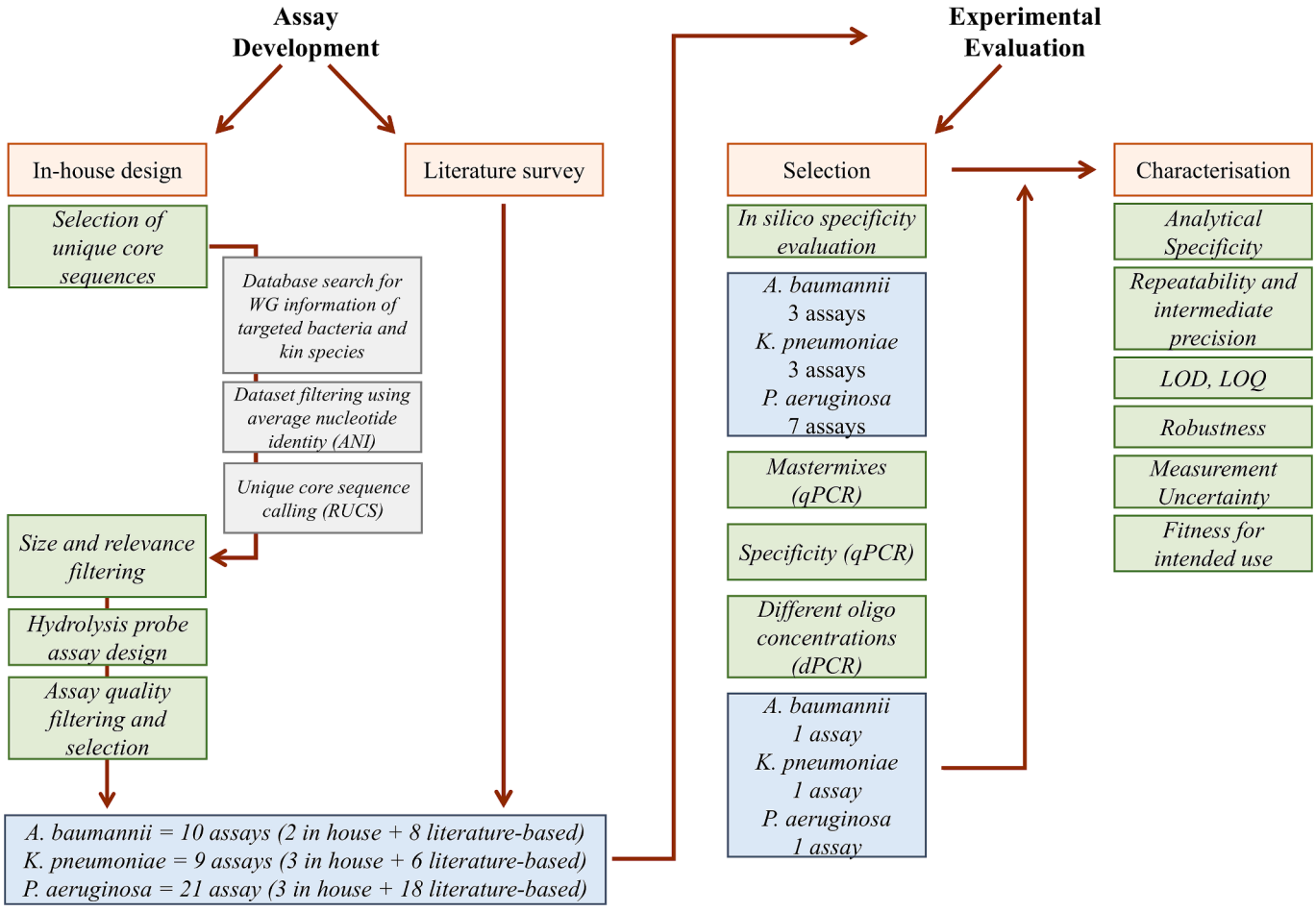

Fig. 1. Approach for the development and evaluation of dPCR methods. The schematics show the experimental series from the assay development through the characterisation in compliance with ISO 20395:2019. Orange boxes represent the main steps of the process, green boxes the selection and performance criteria and blue boxes the number of assays that pass the individual evaluation criteria. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

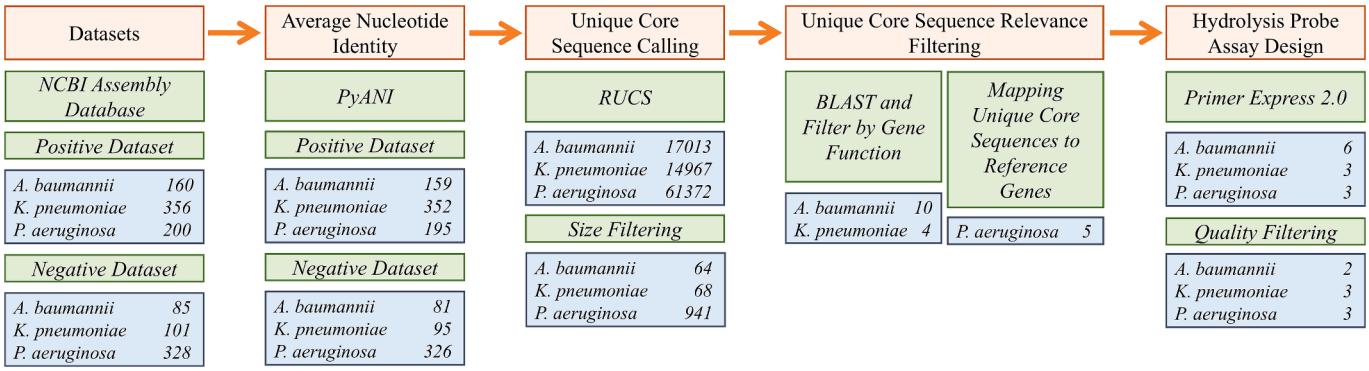

Fig. 2. A detailed description of in-house assay design. Orange boxes show the main steps in the workflow, from dataset compiling to identification of unique sequences and assay design. Green boxes show tools and operations used, and blue boxes show the number of sequences used in each step as well as the number of final assays. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

negative dataset. NCBI Assembly database provided all genomic sequences (Supplementary Data, section 1). RUCS identified 17,013, 14,967, and 61,372 unique sequences for *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa*, respectively. Size filtering (≥100 bp) and relevance filtering yielded target sequences for assay design (Fig. 2 and Supplementary Data, section 1).

2.2. Design of dPCR assays

Primer Express version 2.0 (Applied Biosystems), default parameters, facilitated primer and hydrolysis probe design, encompassing classical and minor groove binding (MGB) probes (Supplementary Data, section 2.2). Assays with the lowest penalty score were selected, eliminating those with scores above 100. *In silico* validation using Oligo Analyser (Integrated DNA Technologies) and BLASTn [21] was conducted to assess oligonucleotide quality and specificity. A literature survey identified additional qPCR assays (Supplementary Data, section 2.2), which were subjected to *in silico* specificity, keeping only those with no cross-reactivity and a single copy amplicon in the genome. The list of assays passing *in silico* evaluation and subject to further testing is provided in Supplementary Data, section 2.2.

2.3. Assay selection

Three assays were selected for *A. baumannii* and *K. pneumoniae*, and seven for *P. aeruginosa* for further evaluation (Supplementary Data section 2.2). The assays underwent high-throughput qPCR (BioMark, Standard BioTools, formerly Fluidigm) and dPCR (QX200, BioRad) testing (Supplementary Data sections 2.5 and 2.6) to determine the optimal assay for each of the target organisms. In the first step high-throughput qPCR was used to determine amplification under different conditions and assess assay specificity, while in the second step both qPCR and dPCR were employed to evaluate amplification under varied primer and probe concentrations.

2.4. Material preparation

Four material types were used; i) synthetic double stranded (ds)DNA gene fragments (Supplementary Data, section 2.3), ii) bacterial gDNA from type strains obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), including *A. baumannii* – DSM No. 30007, *K. pneumoniae* – DSM No. 30,104 and *P. aeruginosa* – DSM No. 50071, iii) DNA extracted from sputum containing an asymmetric mix of all three bacteria, and iv) DNA extracted from real-life and mock sputum samples. Material preparation methods for iii) and iv) are detailed in section 2.3.2 of the Supplementary Data. In short: both material iii) and iv) were prepared at University Clinic for Respiratory and Allergic Diseases in Golnik. For preparation of material iii) 16 sputum samples were collected, pooled and digested with Liquillizer (1:1; MetaSystems). The digested sputum was divided into three parts and spiked with suspensions of the three target bacteria at different concentrations (5.5×10^5, 5.5×10^4 and 5.5×10^3 cells/mL). Type iv) material was prepared by collecting sputa from routine sampling. These were inoculated on solid culture media, with subcultured colonies characterized using MALDI-TOF. Out of 20 samples, 10 contained pathogenic bacteria, 10 contained normal mixed respiratory flora. The later were spiked with different pathogenic bacteria and presented the mock samples. Approximately 1 mL of homogenized sputum was spiked with 100 μL of bacterial suspension (0.5 McF). DNA was extracted as described in Supplementary Data section 2.4.

Synthetic DNA facilitated determination of linearity, limits of detection (LOD) and quantification (LOQ), repeatability, and assay robustness. Sputum samples were used to assess the validation parameters in a complex background, while real-life and mock samples evaluated fitness for intended use and/or analytical specificity. A three-point gDNA dilution series (3-log intervals) was prepared for each model organism.

2.5. DNA extraction method

Bacterial gDNA was extracted using Chelex, and DNA from all sputum samples was extracted using the GXT NA extraction kit (Hain Lifescience) with the Arrow extraction system (NorDiag) (Supplementary Data section 2.4). Extracted DNA was stored in DNA low-binding tubes at −20 °C, except for real-life and mock samples which were initially stored at −80 °C until the first use, and subsequently maintained at temperatures below −20 °C.

2.6. Validated range

Two dilution series were analysed; i) synthetic DNA fragments from 2.4×10^5 to $\sim 10^{-1}$ copies per reaction (copy/rnx) and ii) gDNA in human DNA background (derived from spiked sputum samples) ranged from $\sim 5.5 \times 10^5$ to $\sim 5.5 \times 10^3$ cells/mL (Supplementary Data, section 2.3).

2.7. Data analysis

Fluidigm Real-Time PCR Analysis (Standard BioTools) and QuantaSoft 1.7.4.0917 (BioRad) softwares were used to analyzed high-throughput qPCR and dPCR, respectively. Data were further processed in Microsoft Excel 2016 (Supplementary Data sections 2.5, 2.6, and 3).

2.8. High-throughput qPCR

High-throughput qPCR was performed using a 48.48 Dynamic Array

(Fluidigm). Three different mastemixes were tested: Premix Ex Taq (Takara Bio), Maxima Probe/ROX qPCR Master Mix (Thermo Fisher Scientific) and TaqMan Universal PCR mastermix (Applied Biosystems). Final oligonucleotide concentrations were 900 nM for primers and 300 nM for probes, with additional concentrations tested for assay selection. After priming, 5 μL of each assay and sample were loaded onto the chip, which was then transferred to the BioMark instrument. PCR conditions were: 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 60 s at 60 °C. Each sample was tested in triplicate at three concentrations, with three non-template controls per assay. Data were analysed using Fluidigm Real-Time PCR Analysis software and Microsoft Excel.

2.9. Digital PCR

The dPCR reactions (20 μL) included ddPCR Supermix for Probe (no dUTP) (BioRad), primers and probe mix, and sample. Droplets were generated using manual droplet generator and transferred to 96-well plates. PCR conditions were: 10 min at 95 °C, 40 cycles of 30 s at 95 °C and 60 s at 60 °C, followed by 10 min at 98 °C and cooling to 4 °C. Final oligonucleotide concentrations were 900 nM for primers and 300 nM for probes, with additional concentrations tested for assay selection, and robustness. Positive controls and no-template controls were used in each run. Data were analysed using QuantaSoft software and Microsoft Excel. Reactions with fewer than 8,000 accepted droplets were excluded. For assay selection, each sample was tested in triplicate. For assay characterization, each sample was tested in 15 technical repeats over three days. For the robustness study five additional technical repeats were tested with ddPCR Supermix for Probes (BioRad) and five using automated droplet generator.

3. Results

3.1. Assay development and selection

Two assays for *K. pneumoniae* and three for *A. baumannii* and *P. aeruginosa* passed RUCS pipeline selection rules (Fig. 2), forming a set for experimental evaluation, combined with qPCR assays from the literature (Fig. 1, Supplementary Data, section 2.2). *In silico* specificity assessment led to the selection of specific assays for each organism, confirmed through high-throughput qPCR on the BioMark system. To evaluate amplification efficiency under different conditions a three-

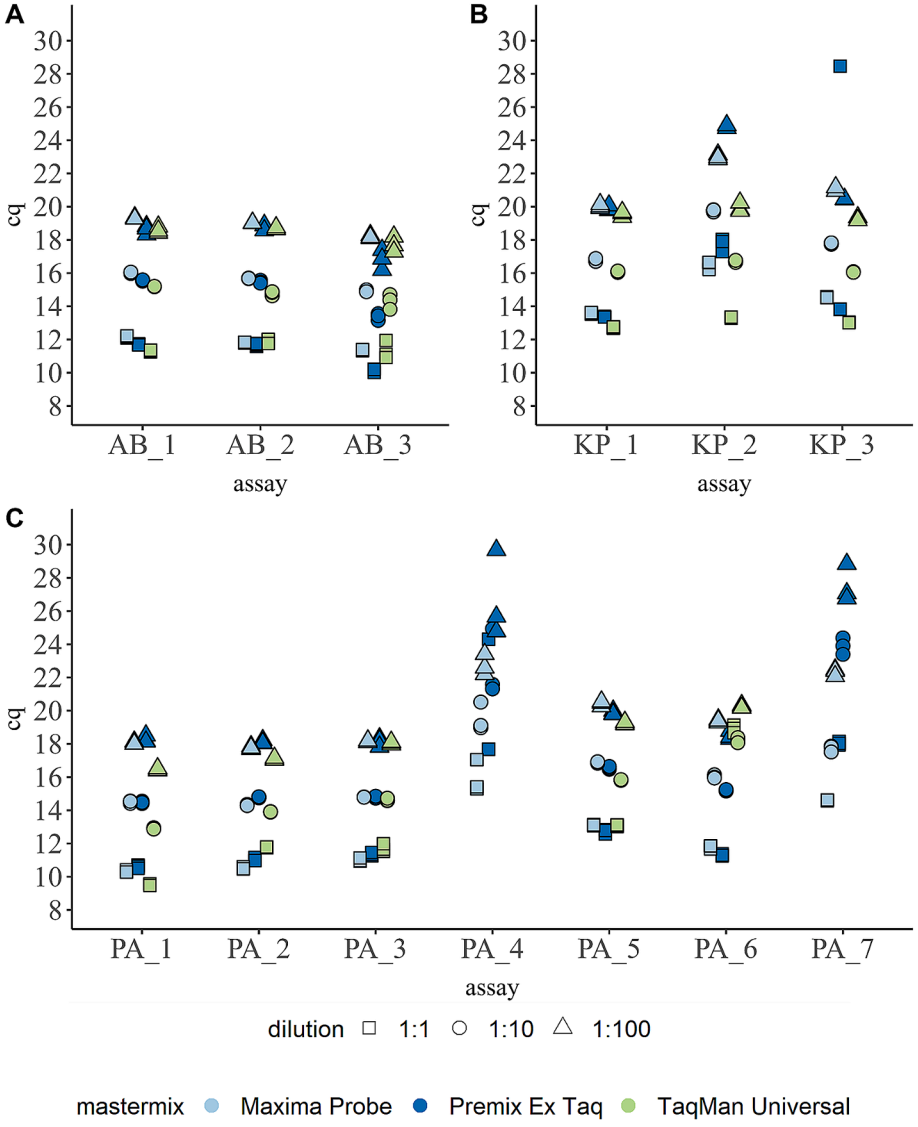

Fig. 3. Selection of the assays based on amplification efficiency using high-throughput qPCR. A three-point dilution series with 10 × increments was used to determine the efficiency of amplification for assays targeting A) *A. baumannii*, B) *K. pneumoniae* and C) *P. aeruginosa*. Each assay was tested with three mastermixes. Each data point presents a mean Cq of three technical repeats.

point dilution series of gDNA (3-logs apart), was measured for each model organisms by their intended assays, using three different mastermixes; Premix Ex Taq (Takara Bio), Maxima Probe/ROX qPCR Master Mix (Thermo Fisher Scientific) and TaqMan Universal PCR mastermix. Assays showing suitable amplification efficiency (Fig. 3), with slopes between −4.1 and −2.9 (Supplementary Table S1), sigmoid amplification curves for all three mastermixes (Supplementary Figures S1-S3) and did not exhibit any cross-reactivity with any of the mastermixes (Supplementary Figure S4) were shortlisted.

The remaining assays, AB_1, AB_2, KP_1, KP_2; PA_1 and PA_5, were then subjected to testing at three different primer and probe concentrations and two gDNA concentrations, using both qPCR and dPCR methods (Supplementary Figures S5-S7). Selection of the final assay for each organism was based on the combination of differences in qPCR Cq values (Supplementary Table S2), concentration bias (Supplementary Table S3), and resolution at the highest primer and probe concentration in dPCR (Supplementary Table S4). The assays chosen for the development of dPCR methods were: AB_1 targeting the multidrug efflux RND transporter outer membrane channel subunit AdeK (*adeK*) gene for *A. baumannii*; KP_1 targeting the RpoS response regulator (*rssB*) gene for *K. pneumoniae*; and PA_1 targeting the DNA primase gene (*dnaG*) for *P. aeruginosa*.

3.2. Evaluation of dPCR methods

3.2.1. Analytical specificity

Specificity was tested on gDNA of two strands of *Pseudomonas fluorescence*, methicillin resistant *Staphylococcus aureus*, methicillin resistant *Staphylococcus epidermidis* and two strains of *Escherichia coli*, as well as *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* where applicable. No cross-reactivity was observed, confirming adequate specificity of the assays (Supplementary Figures S8-S10).

3.2.2. Repeatability, intermediate precision, limits of detection and quantification

The performance of the methods was characterized near the lower limit, using synthetic linear dsDNA (Supplementary Data, section 2.3). Nominal concentration of the stock solutions was assigned using Qubit (Thermo Fisher Scientific), followed by the creation of a 12-point volumetric dilution series. Each dilution underwent testing in 15 technical replicates over three days. Repeatability, expressed as the percentage coefficient of variation (CV), remained below 25 % for concentrations ranging from ~ 4×10^4 to 40 copy/rnx for *A. baumannii*, and ~ 2×10^4 – 20 copy/rnx for *K. pneumoniae* and *P. aeruginosa* (Supplementary Tables S5-S7). Intermediate precision, reflecting the variation between mean concentrations across experimental days, stayed below 10 % for all data points above or at LOD (Table 1). The only exceptions were the KP_1 at around 80 copy/rnx and the PA_1 at approximately 16 copy/rnx, with CVs of 18.46 % and 21.67 %, respectively.

The definitions of LOD and LOQ for dPCR methods are outlined in ISO 20395:2019 [16]. LOD is defined as the lowest measured value that can be consistently detected with a defined level of certainty, typically set at 95 %. This means the LOD is the concentration at which at least 95 % of reactions are positive. In this study, one negative reaction would reduce the certainty below 95 %, so the LOD was set at the lowest concentration where all reactions yielded positive results. LOQ is defined as the lowest concentration or quantity of the nucleic acid target sequence per defined volume, that can be measured with reasonable statistic certainty [16], and was determined at a CV ≤ 25 % in this study [22]. For AB_1, the LOD and LOQ were 26 and 46 copy/rnx, for KP_1 17 and 30 copy/rnx, and for PA_1 16 and 22 copy/rnx (Supplementary Tables S5-S7). The methods demonstrated strong linearity, with R^2 values exceeding 0.999 across a ~ 3.5 log range (Fig. 4) and bias within ± 16 %, even encompassing the LOD (Supplementary Tables S5-S7).

3.2.3. Influence of a complex background on sensitivity

Following assessment of repeatability, intermediate precision, LOD and LOQ on a pure DNA solution, developed methods were tested on complex samples mimicking real-life scenarios. Sputum mixtures were spiked with bacterial suspensions. Each sample contained all three bacteria at ~ 5.5×10^3, ~5.5×10^4 or ~ 5.5×10^5 cells/mL, determined by turbidity (Supplementary Data, section 2.3). Over three days, DNA was extracted from nine vials of each sample (three vials daily), followed by triplicate dPCR assessments to determine biomarker concentrations. For each target, a total of 18 measurements were performed on each sample. Notably, all samples yielded positive results for each biomarker, with concentration differences of approximately 3-fold, 1.5-fold and 4-fold based on turbidity estimations (Table 2). Both within- and between-run repeatability were assessed, resulting in CV values below 20 % and less than 19 % for all methods and samples, except for sample A on day 1, where the CV exceeded 43 % (Supplementary Tables S8-S10).

3.2.4. Robustness

Method resilience was exhibited by varying primer and probe concentrations and through transitions to automated droplet generation and different mastermixes (Fig. 5, Supplementary Tables S2-S3). While the change in droplet generator did not affect concentration ($p > 0.05$; ANOVA), altering the mastermix significantly impacted AB_1 and PA_1 at higher concentrations (D1), with no effect at lower concentrations (D2). Conversely, for KP_1, the mastermix effect was observed at lower concentrations but not at higher ones (Fig. 5).

3.2.5. Expended measurement uncertainty

The expanded measurement uncertainty (MU), expressed as relative uncertainty, was assessed for synthetic DNA − biomarker measurements in pure solutions, and sputum samples − biomarker measurements in a complex matrix (calculation details in Supplementary Data section 3). For measurements in pure solutions MU remained below 21 % for all dilutions above LOD, and even at LOD, it did not exceed 30 %, except for PA_1 where it reached 32 % (Supplementary Tables S5-S7). In complex samples MU was ≤ 25 %, spanning ~ 150——1.4×10^4 copy/rnx, 70——7×10^3 copy/rnx, 180——1.5×10^4 copy/rnx for AB_1, KP_1 and

Table 1
Intermediate precision and measurement uncertainty (MU) for AB_1, KP_1 and PA_1 method. *at least one measurement negative, NA = not applicable.

AB_1			KP_1			PA_1		
Intermediate precision		**MU**	**Intermediate precision**		**MU**	**Intermediate precision**		**MU**
Mean copy/rnx	CV %	relative %	Mean copy/rnx	CV %	relative %	Mean copy/rnx	CV %	relative %
45,916	5.50	7.40	26,935	4.09	6.18	23,954	2.29	4.52
13,751	3.00	7.11	8889	3.59	7.13	7896	2.77	4.74
4584	3.09	9.33	883	1.11	14.35	639	6.02	9.32
1451	4.63	11.59	83	18.46	13.71	74	9.96	9.02
149	7.54	14.98	30	8.35	21.00	22	8.79	17.30
42	9.00	13.09	17	5.17	32.02	16	21.67	24.20
26	7.28	28.14	8	NA	NA	8	NA	NA

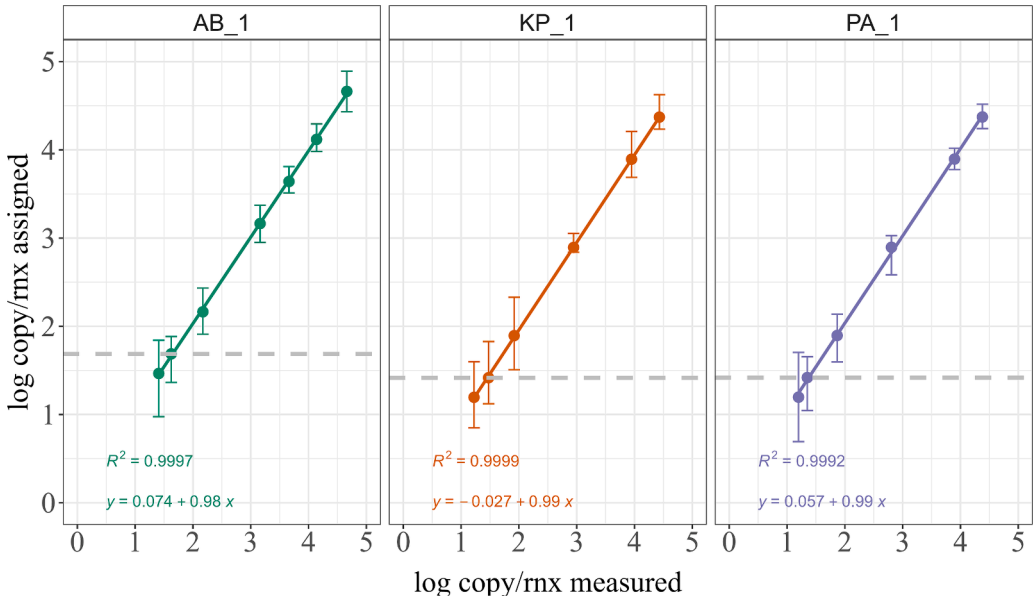

Fig. 4. Linear ranges of the developed assays. Solid lines denote correlations for each assay (AB_1 in green, KP_1 in orange, and PA_1 in purple), with associated CV for every data point. Linear equations are presented along with their respective R^2 values. The dashed horizontal line represents the observed LOQ, while the final point on the graph signifies the LOD. Each data point signifies the mean of 15 measurements obtained from three independent experiments, encompassing five measurements per experiment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Analytical sensitivity as assessed by turbidity (McFarland) and dPCR complete with relative expanded measurement uncertainty (MU %).

Organism	Measurement	Sample		
		A	B	C
Acinetobacter baumannii	McFarland copy/mL	5.5×10^4	5.5×10^5	5.5×10^3
	dPCR copy/mL	1.7×10^5	1.7×10^6	1.7×10^4
	MU %	12.77	11.89	19.30
Klebsiella pneumoniae	McFarland copy/mL	5.5×10^5	5.5×10^3	5.5×10^4
	dPCR copy/mL	8.5×10^5	8.7×10^3	8.7×10^4
	MU %	13.02	21.04	16.58
Pseudomonas aeruginosa	McFarland copy/mL	5.5×10^3	5.5×10^4	5.5×10^5
	dPCR copy/mL	2.3×10^4	1.9×10^5	1.9×10^6
	MU %	25.03	13.30	14.52

PA_1, respectively (Supplementary Tables S8-S10).

3.3. Comparison with MALDI-TOF

To assess the detection capabilities of the evaluated methods, dPCR measurements were compared to the gold standard method (MALDI-TOF). A set of 20 blind-evaluated samples was prepared, consisting of 10 real-life samples with bacterial species identified by MALDI-TOF and 10 spiked samples. The evaluations showed 93.75 % agreement, with no false negative results (Supplementary Table S11). Some false positive results were observed, with two real-life samples initially considered negative for *K. pneumoniae* testing positive using dPCR, and three samples (two real-life and one mock) testing positive for *P. aeruginosa*. In all five cases, the dPCR-determined concentrations were low, $\leq 2 \times 10^4$. No false positives were observed for *A. baumannii*.

4. Discussion

Traditional bacterial pathogen identification leans on culture-based techniques and MALDI-TOF, while DNA-based molecular methods offer both heightened specificity and speed. While there are still some limitations with the use of molecular methods for antibiotic resistance detection, specifically as molecular methods detect the presence of resistance genes, which are not always expressed, qPCR has made considerable progress in identification and detection of bacteria. However quantification with qPCR still relies on well-characterised control materials or standards. In contrast, dPCR directly quantifies DNA biomarkers, bypassing these limitations and ensuring precise measurements with SI-traceability, which has already been shown by entry of dPCR methods in JCTLM Database: Higher-order reference materials, methods and services [23]. While already prevalent in various fields, dPCR is progressively used in microbiology, including bacterial detection in clinical diagnostics [24].

Numerous qPCR methods have been reported for the detection of *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* [25–29]. While many target biomarkers present in multiple copies in the genome, this design, while aiding detection of low bacterial loads, complicates quantification in terms of one target per organism, which is more in line with the way concentration is typically expressed in microbiology, in colony forming units per amount of volume (e.g. CFU/mL). Our approach involves careful biomarker selection based on their intended purpose, whether it be accurate quantification for decision support in patient treatment or ultra-sensitive detection of biomarkers with low tolerance thresholds.

The abundance of whole genome sequences for all three organisms in publicly available databases, facilitated automated analysis for identifying species-specific genomic sequences. Challenges arose early in our in-house assay design process when RUCS failed to identify unique core sequences. Employing an average nucleotide identity algorithm [20], revealed misclassified sequences in both sets. As data in public domains often lack curation, taxonomy issues are not uncommon in metadata. After addressing this, RUCS generated numerous sequences of varying lengths. To align with optimal amplicon lengths for dPCR and qPCR (60–90 nucleotides) [30], we set a size filter of $\geq$ 100 bp. Our negative dataset contained genomic sequences only from organisms within the same genus as our studied bacteria, thus additional specificity filtering

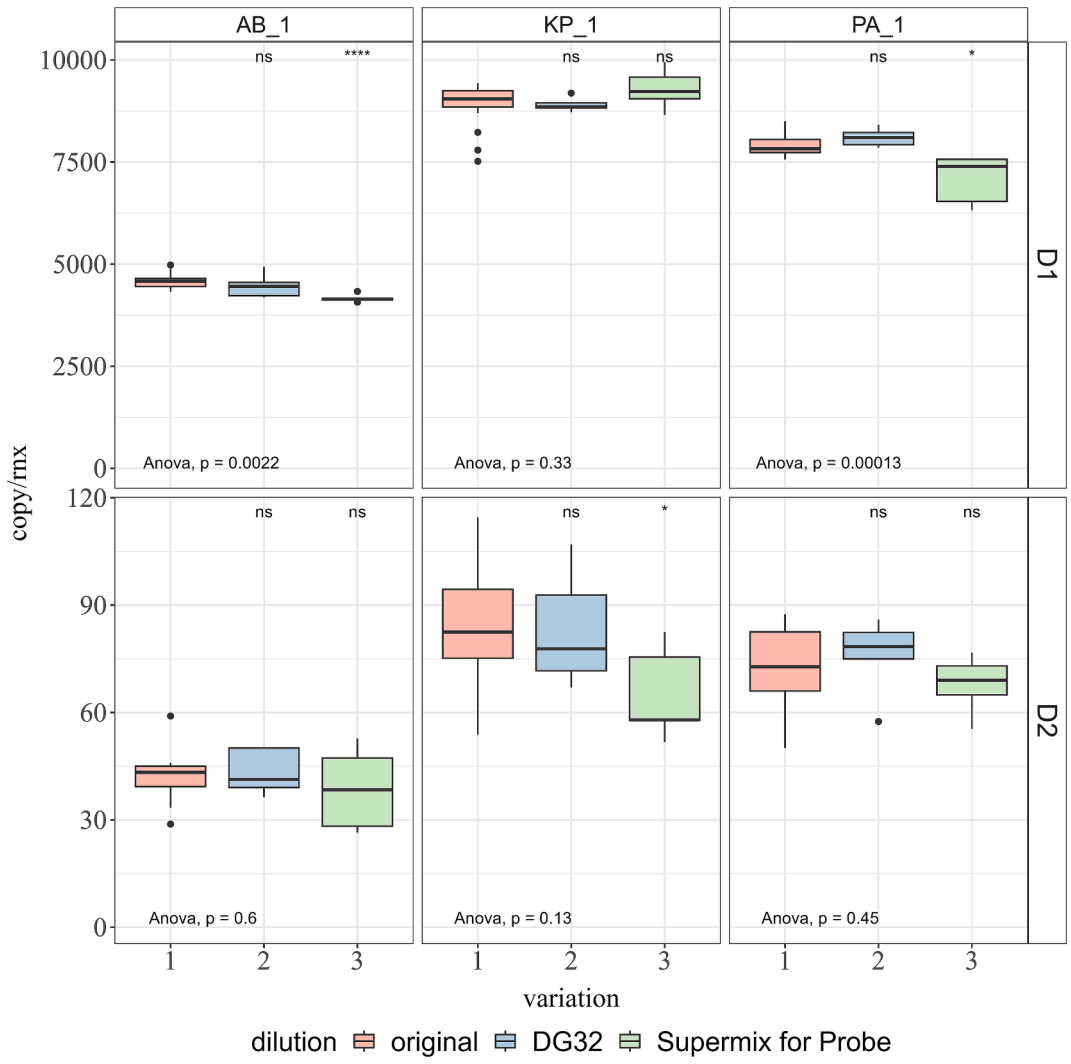

Fig. 5. Comparison of copy number concentration when introducing small changes in the method. Pink box plot represents the original method using DG8 (manual droplet generator) and Supermix for probe (no dUTP), blue using DG32 or automated droplet generator and Supermix for probe (no dUTP), and green box plot denotes the use of DG8 and Supermix for Probe. Each variability was tested on two different concentrations of DNA, D1 ~ 5×10^3 or 10^4 and D2 ~ 50 or 100 copy/rnx. The concentration is significantly different for AB_1 assay for both concentrations of input DNA and for PA_1 assay for the higher concentration when using Supermix for probe (ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$; ANOVA). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and BLAST-based filtering were employed. For *P. aeruginosa*, size filtering resulted in an overwhelming number of sequences. Flexibility in our pipeline allowed us to tailor approaches for each organism. By mapping unique core sequences to the reference genome and selecting those that mapped to reference or "housekeeping" genes, we managed to bring down the number of unique core sequences for *P. aeruginosa* to a manageable level (Fig. 2).

Coupled with assays from the literature, our in-house developed assays comprised a set for experimental evaluation. A significant reduction in assays occurred after *in silico* specificity assessment based on amplicon sequences (Fig. 1), largely due to cross-reactivity observed in assays from the literature. BLAST analysis revealed potential cross-reactivity with same-genus bacteria, a concern resolved early in the pipeline for in-house designed assays. This highlights the critical role of choosing the right negative dataset in RUCS-based analysis [14]. While dPCR's robustness was evident, qPCR helped identify potential assay limitations. Most assays remained unaffected by changes in the mastermix; however, PA_4 and PA_7 were significantly impacted, showing no signal at all for one of the mastermixes (Supplementary Figure S3 and Table S1). Although *in silico* analysis didn't predict cross-reactivity, strong cross-reactivity emerged for PA_4, and weak cross-reactivity was observed for KP_3 and PA_3 (Supplementary Figure S4). As a result, we eliminated these assays from further analysis. We were then left with two assays per organism, each meeting the desired criteria: single-copy presence in the genome and specificity, confirmed through *in silico* and *in vitro* analysis (both qPCR and dPCR, Supplementary Figures S8-S10). Finally, based on amplification profiles and the influence of primer and probe concentration changes on quantification, one assay per organism was chosen. Limits were set at bias < 20 % for dPCR and < Δ1 Cq for qPCR (where smaller differences can be attributed to inter-operator variability).

Method characterization adhered to ISO 20395:2019, which outlines criteria for evaluating quantification methods for nucleic acid testing, including qPCR and dPCR [16] and ISO 17511:2022, which establishes requirements for metrological traceability of values assigned to calibrators, trueness, control materials, and human samples in *in vitro* diagnostic medical devices [15]. The three dPCR methods demonstrated robust performance, showcasing both high sensitivity and specificity for the targeted biomarkers. With a substantial number of reactions conducted, a confident assessment of LOD and LOQ, was achieved. The

LOD, was estimated conservatively, potentially overestimating the LOD due to the limited number of dilution points tested. Notably, the AB_1 method had its LOD determined at approximately ~ 26 copy/rnx, with the subsequent dilution point (~13 copy/rnx) exhibiting 1 out of 15 negative reactions (Supplementary Table S5). While statistical modelling considering the limit of blank could provide a more precise LOD determination, such analysis, in this instance is unnecessary as the conservative approach yields satisfactory results. Regarding the upper LOQ, it's defined by the characteristics of dPCR platform (reaction volume, number, and size of partitions). The reference interval for the developed methods was based on the lower LOQ, with the more cautious estimation established at the dilution point where the coefficient of variation (CV) was $\leq$ 25 %.

Extracting a biomarker from a complex matrix significantly influences its availability [31]. Although not the primary focus of this study, we opted for a DNA extraction method typically employed in clinical settings, thereby maintaining alignment with real-world analysis procedures. Notably, all three methods showcased not just detection capabilities, but also the quantification of low target biomarker concentrations within a substantial background (Table 2 and Supplementary Tables S8-S10).

Most dPCR platforms, including the QX100 and QX200 utilized in this study, operate with proprietary mastermixes. Consequently, the robustness of the methods was evaluated using two mastermix options, ddPCR Supermix for Probe with dUTP and without dUTP Notably, a statistically significant concentration difference emerged when comparing the outcomes between the two mastermixes. Several studies have highlighted that droplet size can be influenced by the chosen mastermix [32–35]. The size of the partition plays a pivotal role in concentration calculations, and even subtle changes in mastermix can yield notable effects. It's important to emphasize that the partition volume used remained constant throughout this study, regardless of the mastermix used. Thus, the observed concentration variance likely stems from the inherent physics of the platform and partitions, rather than the methods themselves. This interpretation is reinforced by the fact that altering primer and probe concentrations, as well as utilizing different droplet generation platforms, did not produce an impact. For a more precise comparison of the results, an exhaustive droplet volume investigation specific to the mastermix would be required. However, such an undertaking might prove resource-intensive for most laboratories. This underscores the significance of acknowledging the mastermix as a critical variable influencing droplet volume, potentially affecting measurement outcomes, even with meticulously chosen biomarkers.

In the realm of quantitative outcomes, the provision of measurement uncertainty is integral. In order to enable reliable quantification an expanded measurement uncertainty (MU) below 30 %. Encouragingly, for all three methods, the expanded MU remains below 30 % across the entire reference range, encompassing pure DNA solutions as well as complex samples.

Proficiency across simple and complex samples was demonstrated by all methods. The intended use was assessed by comparing methodologies with the gold standard, MALDI-TOF. While a few false positive outcomes were recorded (Supplementary Table S11), these consistently featured exceedingly low biomarker concentrations determined by dPCR. Notably, results for both KP_1 and PA_1 were proximate to the LOD for sample 1 and above the LOQ for samples 7 and 16. Comparing molecular methods to culture-based ones is not straightforward. Molecular methods detect DNA indiscriminately, challenging the distinction between living and non-living entities, even with specialized chemistries. This inherent limitation is a major drawback. In contrast, culture-based techniques like MALDI-TOF exclusively identify cultivable organisms, potentially overlooking non-cultivable or slow-growing variants masked by faster-growing strains. Additionally, MALDI-TOF can struggle with closely related species, making them difficult or even impossible to differentiate. This complexity adds to the analysis process. However, given dPCR's greater sensitivity than MALDI-TOF, observed false positive outcomes could be true positives with concentrations below MALDI-TOF's LOD.

5. Conclusions

This investigation underscores the critical significance of pinpointing species-specific genomic biomarkers as the foundation for the creation of precise and reliable measurement procedures. The research has successfully designed and characterized dPCR methods tailored for detecting and quantifying three prevalent Gram-negative bacteria responsible for respiratory tract infections. The newly developed methods exhibit enhanced speed and sensitivity compared to conventional culture-based approaches. Notably, these methods yield outcomes characterized by accuracy and measurement uncertainty that meet or surpass current measurement standards, aligning seamlessly with the requisites outlined in the EU regulation 2017/746 [36] concerning *in vitro* diagnostic medical devices. The ability to provide SI-traceable values positions them as promising candidates for serving as dependable reference measurement procedures, enabling reliable quantification of reference and control materials, as well as materials used in external quality assessment schemes (EQAS). As this study concludes, the critical step of advancing into genuine clinical applications warrants further exploration, underlining the next crucial phase of translating these advancements to real-world medical contexts.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This study was financially supported by the Slovenian Research Agency (Contracts no. Z2-1860 and P4-0165) and SEPTIMET project (which has received funding from the EMPIR program co-financed by the Participating States and from the European Union's Horizon 2020 research and innovation program, project no. 18HLT03). Part of dPCR equipment used in this study was financed by the Metrology Institute of the Republic of Slovenia (MIRS), with financial support from the European Regional Development Fund. The authors would also like to thank Jim F. Huggett, the coordinator of the SEPTIMET project, for suggestions on improvement of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2024.10.011.

Data availability

Data will be made available on request.

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