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Evaluation of direct-to-PCR (D2P) method for molecular diagnosis of infectious diseases

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ABSTRACT

This study evaluates the performance of the Direct-to-PCR (D2P) method as a streamlined, extraction-independent alternative to conventional nucleic acid extraction techniques for diagnosing urinary tract infections, sexually transmitted infections, and respiratory tract infections. The D2P approach employs proprietary antimicrobial peptide-based lysis buffers tailored for bacterial, fungal, and viral targets, enabling direct amplification from clinical and contrived specimens without column- or bead-based purification. Comparative analyses were conducted against silica column-based (QIAGEN) and magnetic bead-based (KingFisher) extraction methods using both microbial reference isolates and 116 residual clinical samples. Results demonstrate that the D2P method yields comparable sensitivity and specificity to conventional extraction workflows across a diverse panel of pathogens—including Gram-negative and Gram-positive bacteria, *Candida* species, ssRNA viruses (e.g., CoV-229E, Parainfluenza Virus 1 and 2), and dsDNA viruses (e.g., HSV, HAdV). Notably, D2P outperformed both QIAGEN and KingFisher in extracting nucleic acids from *Candida auris*, a multidrug-resistant fungal pathogen. Limit of detection and amplification efficiency remained within acceptable ranges across all platforms, with R^2 values between 0.92 and 0.99, and slopes consistent with MIQE standards. The D2P protocol reduced total sample processing time from ~120 min to ~45 min, minimized hands-on steps, and demonstrated effective performance in turbid or hemolyzed samples—making it suitable for high-throughput and resource-limited settings. However, limitations were observed in samples with high PCR-inhibitor content or low target yield, and broader validation across additional matrices is recommended. These findings support D2P as a reliable, efficient, and scalable molecular diagnostic alternative with broad clinical utility. Integration of D2P into diagnostic workflows could enhance access to rapid, cost-effective pathogen detection in both centralized laboratories and decentralized or point-of-care environments.

1. Introduction

Molecular diagnostic tools, like polymerase chain reaction (PCR), have improved infectious disease detection by enabling rapid and precise identification of pathogens through genetic level analysis (Shahrajabian and Sun, 2023; Templeton et al., 2005; Yang and Rothman, 2004). A crucial component of these technologies is nucleic acid extraction, which isolates and purifies DNA or RNA, serving as the

Abbreviations: PCR, Polymerase Chain Reaction; DNA, Deoxyribonucleic Acid; RNA, Ribonucleic Acid; COVID-19, Coronavirus Disease 2019; UTI, Urinary Tract Infection; STI, Sexually Transmitted Infection; RTI, Respiratory Tract Infections; qPCR, Quantitative Polymerase Chain Reaction; EEC, Exogenous Extraction Control; ATCC, American Type Culture Collection; VTM, Viral Transport Medium; KF, KingFisher; QI, QIAGEN; PO, PCROpsis; D2P, Direct to PCR; PPV, Positive Predictive Value; NPV, Negative Predictive Value; RT, Reverse Transcription; Ct, Cycle Threshold; RVD, Reagent Viral Detection; RVD-Support, RVD Support Solution; MS2-Phage, RNA bacteriophage MS2; HSV, Herpes Simplex Virus; HAdV, Human Adenovirus; ssRNA, Single-Stranded RNA; dsDNA, Double-Stranded DNA; AL, Buffer AL; AW1, Washing Buffer 1; AW2, Washing Buffer 2; AE, Elution Buffer; MgB, Magnetic Bead; RPP, Respiratory Pathogen Panel.

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foundation for advanced applications like PCR, quantitative PCR (qPCR) and next-generation sequencing in the research and clinical laboratory (Almas et al., 2023; Carpenter et al., 2023).

Established extraction methods, such as enzymatic lysis with alcohol-based precipitation, are reliable for specific (research) contexts but are labor-intensive, requiring extensive manual handling and centrifugation (Thatcher, 2015). Column-based methods, which rely on silica matrices, are widely recognized for their robustness and reliability. However, their implementation often involves a mix of manual and semi-automated steps, which can present scalability challenges, particularly in high-throughput settings (Oberacker et al., 2019; Wallace and Rochfort, 2023). Magnetic bead-based techniques have emerged as a preferred method for nucleic acid extraction due to their efficiency and ability to deliver high-purity outputs. These methods are particularly advantageous in high-throughput clinical settings, as they offer excellent compatibility with automated workflows and scalability (Haile et al., 2022; Na et al., 2024). But their dependence on specialized staff, equipment and consumables can restrict their accessibility in certain settings. Altogether, conventional extraction methods often involve intricate workflows with multiple processing steps, contributing to procedural complexity that increases operational costs and limits the scalability of molecular diagnostics, particularly in resource-limited settings or point-of-care applications (Liu et al., 2021; Carpenter et al., 2022; Peeling and McNerney, 2014).

More recently, crude lysate extraction methods have been highlighted for their potential in point-of-care applications and low-resource settings, as they bypass extensive purification steps and allow direct analysis of nucleic acids from cell lysates, making them ideal for rapid diagnostics (Shatzkes et al., 2014). During the COVID-19 pandemic, various crude lysate PCR methods were introduced as alternatives to conventional column-based and organic solvent-dependent protocols, simplifying workflows and providing accessible testing options in diverse environments (Morecchiato et al., 2021). For example, the crude lysate SalivaDirect method, developed by Yale University, gained widespread global adoption as a lysis-to-amplification protocol for COVID-19 and received FDA Emergency Use Authorization in 2020 (Vogels et al., 2021). This method demonstrated lower sensitivity in detecting viruses—identifying only 40 copies/mL in 200 mL of gargle or saliva sample (Liu et al., 2022) compared to the 5 copies/mL achieved by bead-based extraction (Fei et al., 2022).—clinical sensitivity remained above 95 % with SalivaDirect. Similarly, although some targets displayed higher sensitivity (lower Ct values) with traditional extraction methods compared to direct lysis, clinical sensitivity consistently exceeded 95 % across targets.

However, nucleic acid extraction for on-site detection in resource-limited settings presents several challenges. Specialized equipment, lengthy procedures and the need for trained personnel render many extraction methods impractical in low-resource environments. Furthermore, minimal amounts of target DNA/RNA in samples necessitate amplification, a process that is both time-intensive and reliant on sophisticated equipment (Adams et al., 2015). Pathogen diversity further complicates extraction; for example, RNA viruses are particularly susceptible to degradation by RNases, making RNA integrity crucial for accurate respiratory virus detection (Barr and Fearns, 2010). Despite these complexities, cell lysate-based PCR approaches show promise for reducing testing time, costs and the risk of contamination or sample loss, while simplifying labor-intensive processes (Paul et al., 2020). Still, the clinical adoption of lysis buffers remains limited, in large part due to insufficient validation across diverse sample types and pathogens. This lack of comprehensive validation raises concerns about their reliability, reproducibility and applicability, posing significant scientific and operational challenges for integration into standardized diagnostic workflows.

While various extraction-free or crude lysate methods have been proposed to simplify molecular workflows, particularly for SARS-CoV-2 and select veterinary pathogens, their clinical adoption remains limited. Prior work has demonstrated successful pathogen detection in unpurified matrices such as saliva, serum, swabs, and tissue homogenates (Vogels et al., 2021; Miyachi et al., 2024; Nishi et al., 2022). However, these studies typically focused on single pathogen categories or highly specific use cases. The present study builds upon this foundation by evaluating the analytical and clinical performance of a Direct-to-PCR (D2P) method across a wide range of infectious agents and sample types. By comparing D2P to traditional extraction techniques in a multiplex qPCR setting, we aim to provide a broader framework for its integration into routine diagnostic workflows.

The objective of this study is twofold. First, we aimed to evaluate the analytical and diagnostic performance of the D2P sample processing method (Scienetix, Tyler, Texas, USA) in comparison to two conventional nucleic acid extraction techniques—silica column-based (QIAGEN) and magnetic bead-based (KingFisher). This assessment was conducted using both contrived microbial isolates and residual clinical specimens, encompassing a broad range of pathogen types—including Gram-negative and Gram-positive bacteria, fungi, and RNA and DNA viruses—across three major infection categories: urinary tract infections (UTIs), sexually transmitted infections (STIs), and respiratory tract infections (RTIs). The experimental design is depicted in Fig. 1, and representative assay results are presented in Fig. 2. Second, we sought to assess the operational potential of the D2P method as a streamlined, extraction-independent alternative for molecular diagnostics in clinical laboratory settings. Unlike traditional workflows, the D2P approach eliminates the need for extensive nucleic acid purification steps and specialized instruments by utilizing antimicrobial peptide-based lysis buffers—D2P-RP for respiratory samples and D2P-UN for bacterial and fungal pathogens. This simplified protocol reduces hands-on time and resource requirements while maintaining high diagnostic accuracy, thereby supporting its scalability and utility in high-throughput or resource-constrained environments.

This research contributes to the expanding evidence base advocating for simplified and efficient diagnostic workflows that uphold high standards of accuracy and reliability (Graf and Pancholi, 2020). Furthermore, the findings hold substantial implications for advancing molecular diagnostics in both the research and clinical laboratory settings, particularly in contexts requiring rapid, cost-effective and scalable PCR testing solutions.

2. Materials and methods

2.1. Methodological framework

This study employed a comparative framework to evaluate nucleic acid extraction methodologies using both reference microbial isolates (Phase 1) and residual clinical specimens (Phase 2). In Phase 1, the workflow was systematically designed to benchmark reference microbial isolates. The D2P method (Scienetix, Tyler, Texas, USA) was compared with conventional column-based extraction using the QIAamp and DNAEasy Kits (QIAGEN, Hilden, Germany) and magnetic bead-based extraction with the KingFisher™ Flex Purification System (Thermo Fisher Scientific, Waltham, USA). In Phase 2, residual clinical samples were assessed using both the D2P and KingFisher methods to evaluate their ability to isolate nucleic acids from a diverse array of pathogens associated with clinically significant infection categories: UTI, STI and RTI.

2.1.1. Phase 1: reference microbial isolates

Phase 1 focused on microbial isolates sourced from the American Type Culture Collection (ATCC) and BEI Resources (Manassas, VA, USA). The isolates were thoroughly characterized to confirm their identity and pathogenic relevance and suitability for experimental applications and downstream analyses (Sibley et al., 2012). Isolates encompassing key STI- and UTI-associated pathogens ($n = 9$) were strategically spiked into 20 mL of target-negative urine under tightly

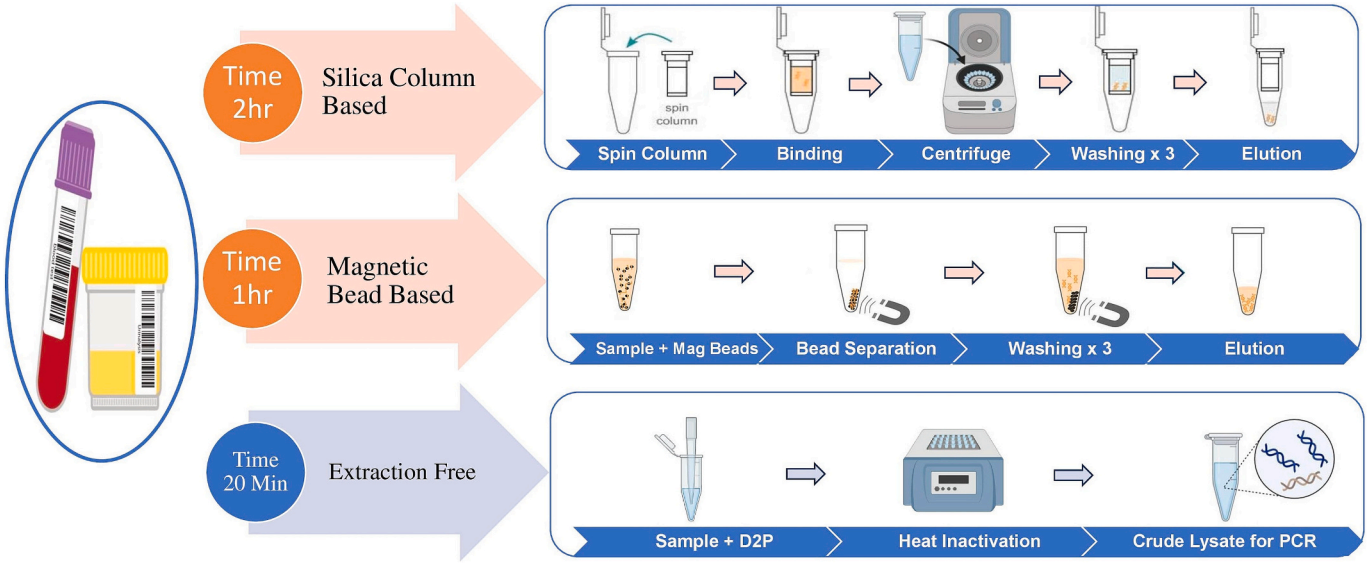

Fig. 1. Comparison of nucleic acid extraction methods and workflows.

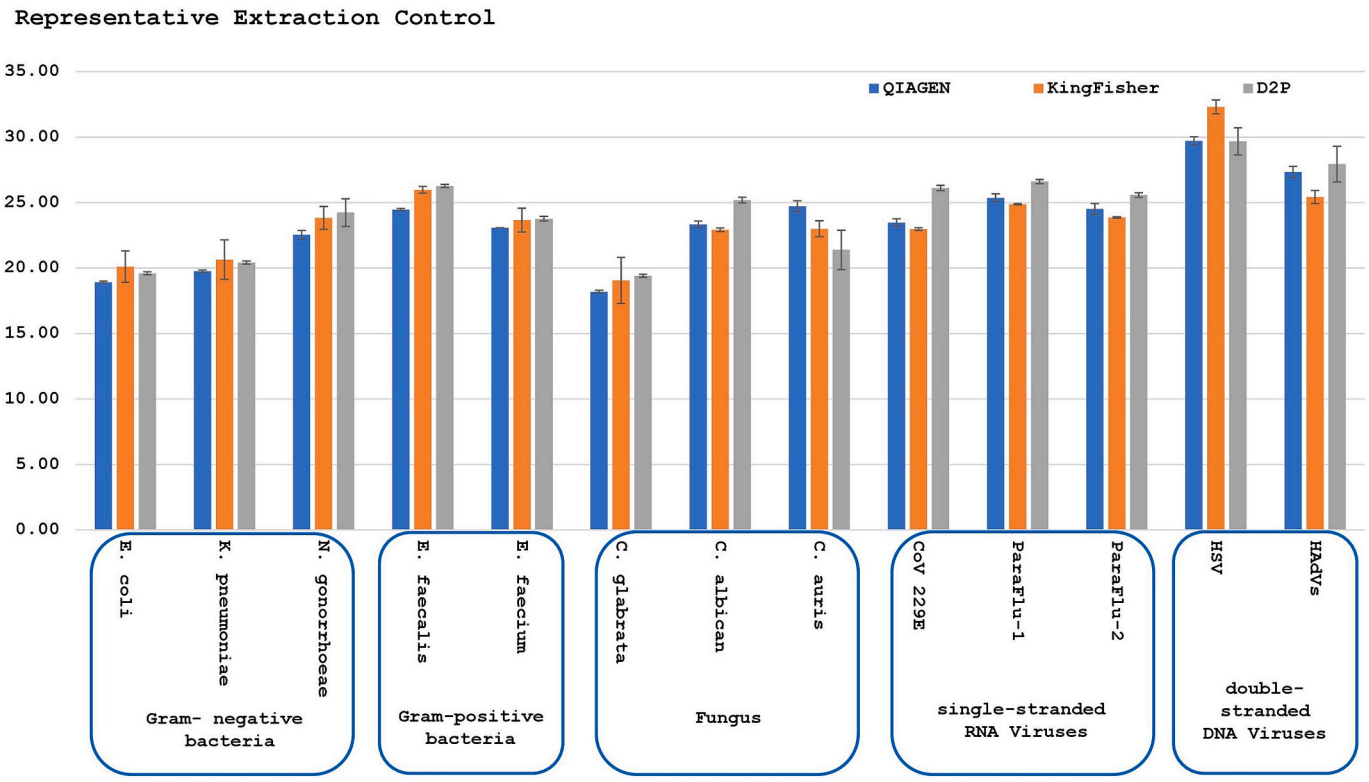

Fig. 2. Performance of extraction methods across various pathogens.

controlled conditions to ensure homogeneity and reproducibility. This created a microbial panel designed to encompass a diverse array of clinically significant taxa, including three Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*), two Gram-positive bacteria (*Enterococcus faecalis*, *Enterococcus faecium*), three fungal species (*Candida glabrata*, *Candida albicans*, *Candida auris*) and one viral pathogen (Herpes Simplex Virus-1). Then, each prepared sample was divided into 1 mL aliquots for processing. Likewise, for respiratory isolates, four representative viral pathogens (*Coronavirus 229E, Parainfluenza Virus 1, Parainfluenza Virus 2* and *Human Adenovirus*) were spiked into 3 mL of BioSci® viral transport medium (VTM) (Rhino diagnostics Pleasanton, CA). The contrived VTM was then divided into four equal aliquots for processing.

2.1.2. Analytical limit of detection (LOD) experiment design using spiked isolates

A known concentration "(~1.0 × 10^6 CFU/mL) of *Escherichia coli* (Gram-negative bacteria), *Enterococcus faecium* (Gram-positive bacteria), and *Candida glabrata* (fungi) were spiked into a human urine sample confirmed to be negative for these targets. Additionally, an exogenous extraction control (*Bacillus atrophaeus*, ~1.0 × 10^6 CFU/mL) was introduced into the sample. The mixture was then serially diluted in

natural urine that tested negative for the target organisms.

Similarly, MS2 bacteriophage (1.00 million PFU/mL) was added to a VTM and subjected to 10-fold serial dilutions. Each dilution was processed using three different extraction methods: D2P, KingFisher, and QIAGEN. For downstream analysis, 2.5 μL of the D2P-processed sample or purified DNA from each dilution was used in target-specific qPCR assays. CFU/mL were plotted against the corresponding cycle threshold (Ct) values, and key PCR amplification efficiency parameters—including the coefficient of determination (R (Almas et al., 2023)), slope, and intercept—were calculated in accordance with MIQE guidelines (Bustin et al., 2009). The lowest genomic copy number detected in the processed samples was defined as the limit of detection (LOD) for each pathogen.

2.1.3. Phase 2: clinical samples

Clinical samples for this study were sourced from Advanta Genetics, LLC (Tyler, Texas, USA), a laboratory accredited by the College of American Pathologists (CAP). All samples were handled and tested in compliance with institutional guidelines to ensure ethical standards in sample collection, processing and analysis. A total of 116 deidentified residual clinical specimen aliquots were collected, representing the three distinct infection categories: UTI ($n = 40$), STI, ($n = 24$) and RTI, ($n = 52$) including those visibly turbid, and haemolyzed samples.

To ensure consistency and assess inhibitor tolerance, sample condition and pre-analytical processing steps were standardized and explicitly documented. For urine samples, each 1 mL aliquot was centrifuged at 10,000 ×*g* for 10 min at 4 °C. Approximately 970 μL of the supernatant was removed, and the remaining concentrated pellet (~30 μL) was retained for downstream processing using D2P, KingFisher, and QIAGEN methods. Turbid and visibly hemolyzed urine specimens were intentionally included to assess performance in complex matrices.

For respiratory tract samples, nasopharyngeal/oropharyngeal swabs were eluted in VTM, and a 40 μL aliquot was used directly without centrifugation or filtration. Sample condition, including mucus content, discoloration, or particulate matter, was recorded.

For STI-associated specimens, vaginal or urogenital swabs were similarly processed without pretreatment. Samples exhibiting reddish discoloration or turbidity—potentially due to hemoglobin or debris—were included. Successful amplification of endogenous (RNaseP) and exogenous controls (MS2 and *Bacillus atrophaeus*) across all sample types confirmed compatibility with both conventional and D2P workflows, even under potentially inhibitory conditions.

2.2. DNA/RNA extraction methods

2.2.1. Column-based extraction

Urine samples spiked with uropathogens and sexually transmitted infection (STI)-associated microorganisms and residual clinical specimens, were subjected to nucleic acid extraction using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany). Briefly, a 1 mL urine sample (for UTI and STI testing) was centrifuged, and 800 μL of the supernatant was discarded. The remaining 200 μL sediment was processed according to the manufacturer's instructions, starting with cell lysis, followed by nucleic acid binding to a silica membrane, and concluding with sequential wash steps to remove contaminants and inhibitors (Shin, 2013). DNA was eluted in 100 μL of elution buffer. Similarly, reference viral pathogens spiked into VTM and clinical nasopharyngeal/oropharyngeal swab samples were processed using the QIAamp Viral RNA Kit (QIAGEN, Hilden, Germany). Briefly, 200 μL VTM sample was directly processed according to the manufacturer's instructions. Both extraction protocols leverage silica column technology to facilitate the efficient purification of high-quality nucleic acids from diverse biological matrices.

2.2.2. Magnetic bead-based extraction

Both the microbial isolate and residual clinical sample aliquots were processed using the KingFisher™ Flex system in conjunction with the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit, following the manufacturer's protocol. A 1 mL urine sample for STI and UTI testing was centrifuged and 200 μL of the resulting sediment was used for DNA purification according to the manufacturer's instructions. Clinical or contrived respiratory sample were processed directly without any concentration step. The workflow began with sample matrices, including urine and VTM, undergoing lysis in a chaotropic buffer enriched with Proteinase K. This step disrupted membranes while denaturing proteins, facilitating the efficient release of nucleic acids into the solution (Miller et al., 2022). The released nucleic acids were captured on paramagnetic beads engineered for high surface area and binding efficiency, enhancing the recovery of both RNA and DNA targets. The KingFisher™ Flex system automated the extraction process, incorporating precise robotic handling to transfer the beads through multiple wash steps. These wash steps utilized ethanol-based and proprietary wash buffers to remove proteins, lipids and other impurities while maintaining the integrity of the nucleic acids. Finally, the purified nucleic acids were eluted into a low-salt buffer formulated to preserve nucleic acid stability and maximize compatibility with downstream molecular analysis. This automated, high-throughput process not only standardized nucleic acid recovery but also minimized human error, ensuring high-purity yields suitable for accepted scientific analyses (Chaves et al., 2024).

2.2.3. Direct to PCR (D2P)

All D2P sample processing began with appropriate handling tailored to the sample type. Where applicable, physical sedimentation steps were used to concentrate microbial and host cellular components. For urine samples, this included centrifugation at 10,000 ×*g* for 10 min at 4 °C, followed by removal of the supernatant. In contrast, respiratory and STI (Uro-genital swab) samples were processed directly using fixed volumes without pretreatment. To evaluate the robustness of the D2P method under real-world conditions, the study included samples that were visibly hemolyzed, turbid, or discolored. Across all matrices, consistent amplification of endogenous (RNaseP) and exogenous controls (MS2 and *Bacillus atrophaeus*) confirmed compatibility of the D2P method even in the presence of potential PCR inhibitors.

Both contrived and residual clinical samples were processed using the D2P method. Briefly, 1 mL urine sample was processed to sediment the cellular components by centrifugation at 10,000 ×*g* for 10 min at 4 °C, ensuring optimal separation of cellular material. Post-centrifugation, the supernatant (~970 μL) was carefully aspirated with a micropipette, avoiding disturbance of the cellular pellet. This step is critical to minimize contamination from extracellular components. The resulting pellet was resuspended in ~30 μL of residual fluid, maintaining a concentrated cellular fraction to optimize nucleic acid yield.

To initiate nucleic acid extraction, 30 μL of D2P-Universal Extraction Buffer (Cat. D2P-UN) was added to the resuspended pellet. The sample was vortexed vigorously to ensure complete mixing and homogenization of cellular material with the extraction buffer. Subsequently, the tube was subjected to heat treatment at 95 °C for 10 min in a heat block, facilitating the disruption of cellular membranes and efficient release of nucleic acids. Heat treatment at this temperature is essential not only for cell lysis but also for the inactivation of potential nucleases and other inhibitory substances. Following heat treatment, the sample was cooled for 15–20 s at room temperature to reduce thermal denaturation of released nucleic acids, which can adversely affect downstream applications. An additional 30 μL of D2P-Sample Dilution Buffer (SDB) was introduced to the sample post-cooling to ensure optimal reagent concentrations for subsequent steps. The tube was gently mixed by pipetting to achieve uniform distribution, avoiding vigorous agitation that could shear nucleic acids. A brief centrifugation step (10 s) at 8000 ×g was performed to collect all liquid at the tube's base, ensuring maximal recovery for downstream processing.

For respiratory samples in VTM, a standardized approach was employed in accordance with the manufacturer's protocols. 40 μL of the

respiratory sample was aliquoted and combined with 10 μL of D2P Respiratory Extraction Buffer (Cat. D2P-RP-192). The sealed tube was incubated at 95 °C for 10 min to achieve efficient cell lysis and inactivation of potential PCR inhibitors, including mucinous or proteinaceous debris. Post-incubation, the sample was cooled at room temperature for 15–20 s to stabilize the nucleic acids. This step minimizes thermal degradation, particularly for RNA, which is inherently more prone to hydrolysis under prolonged exposure to high temperatures. After cooling, the tube contents were gently mixed by pipetting, followed by a brief centrifugation step (10 s) at 8000 ×g to ensure recovery of all sample liquid. This standardized protocol ensured the consistent preparation of samples, minimizing variability and maximizing the efficiency of downstream molecular analyses.

2.3. Amplification and detection

2.3.1. Exogenous extraction and reverse transcription controls

The human RNAseP gene was used as endogenous control, while *Bacillus atrophaeus* and MS2-Phage served as exogenous extraction control (EEC) and reverse transcription (RT) control, respectively. A suspension containing 0.2×10^5 copies/μL of either MS2-Phage (Dreier et al., 2005) and/or *Bacillus atrophaeus* (Picard et al., 2009) was spiked into samples at a concentration of 5 μL/mL prior to conventional nucleic acid extraction or D2P processing. Primers for the detection of MS2-Phage and *Bacillus atrophaeus* were included in the PCR reagents used for this study. The consistent amplification of these exogenous control targets confirmed the availability of nucleic acid and un-inhibited qPCR reaction using D2P method (Lim et al., 2016).

2.3.2. Quantitative PCR

Quantitative PCR was performed using 7.5 μL pre-formulated multiplex qPCR reaction mix containing the 1× one-step RT PCR Masrer Mix (Azura Genomics, Raynham, MA) 125 nM TaqMan probe and 250 nM primer for each target (Scienetix Tyler, Texas, USA), and 2.5 μL of extracted nucleic acid (DNA, RNA), or D2P processed lysate was added.

The thermal cycling protocol of 10 μL reaction was conducted using the Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, California, USA). The protocol began with an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of a denaturation step at 95 °C for 5 s and an annealing and extension step at 60 °C for 30 s. For RNA virus detection, the protocol incorporated a preliminary reverse transcription step at 50 °C for 15 min before the initial denaturation phase.

Amplification results were analyzed using Ct values accompanied with sigmoid amplification curve and Ct values <35 for the target gene (s) were classified as positive, a threshold established through validation studies to optimize sensitivity and specificity. Negative samples were those in which only the endogenous control (RNaseP) and the EEC were amplified, confirming the absence of target nucleic acids. Invalid samples, identified by the failure of either endogenous (RNaseP) or exogenous control amplification, indicated issues such as sample degradation, incomplete nucleic acid extraction, reagent failure, or instrumental error. Such samples were flagged for re-extraction and re-analysis, provided sufficient material was available.

2.3.3. Data analysis

Three distinct extraction protocols were systematically analyzed using contrived microbial isolates to assess their relative performance metrics. Column-based extraction (QIAGEN kits), widely recognized as the gold standard for nucleic acid extraction due to its high purity and efficiency (Alabi et al., 2020; Thatcher, 2018), served as the benchmark. Results were directly compared with those obtained using a magnetic bead-based approach (KingFisher) and the D2P (Scienetix) crude lysate-based method, offering valuable insights into their advantages and limitations for downstream applications. Residual clinical samples were also tested using both the KingFisher and D2P protocols to further validate their performance.

Amplification curves were manually reviewed to ensure result validity, with only curves demonstrating a clear sigmoidal shape and a Ct value <35 classified as positive for qualitative detection, following established criteria (Shain and Clemens, 2008). For quantitative analysis, valid Ct values were exported in CSV format and subjected to statistical analysis using Microsoft Excel. Key performance metrics—including sensitivity, specificity, accuracy positive predictive value (PPV) and negative predictive value (NPV)—were calculated using standard formulas and validated across multiple independent replicates to ensure robustness and reproducibility. This systematic approach enabled a comprehensive evaluation and comparison of extraction methods across datasets.

3. Results

3.1. Comparative analysis

3.1.1. Microbial isolates (Phase 1)

A comparative analysis was conducted to evaluate the performance of QIAGEN, KingFisher and D2P nucleic acid extraction methods. This analysis utilized a panel of well-characterized microbial reference isolates, representing a diverse range of bacteria, fungi and viruses (Table 1).

Ct value for each organism after extraction with different methods were compared. Relative to QIAGEN, KingFisher and D2P demonstrated ΔCt values ranging from 0.23 to 1.69, indicating minor difference in extraction efficiency for Gram-negative bacterial isolates, including *Escherichia coli*, *Klebsiella pneumoniae* and *Neisseria gonorrhoeae*. The Ct difference between KingFisher and D2P ($\Delta Ct^{(KF\text{-}D2P)} = 0.51$ to -0.40) supports their comparable performance, making both methods viable alternatives for these bacterial targets.

For Gram-positive bacterial isolates, including *Enterococcus faecalis* and *Enterococcus faecium*, QIAGEN exhibited slightly better performance (ΔCt < 2) with KingFisher and D2P demonstrating ΔCt values of 0.58 to 1.79 relative to QIAGEN. The minimal Ct differences between KingFisher and D2P ($Ct^{(KF\text{-}D2P)} = -0.29$ to -0.10) highlight their reliability as alternatives. These findings suggest that both methods are suitable for diagnostic applications involving Gram-positive bacteria.

Among fungal reference isolates, including *Candida glabrata*, *Candida albicans* and *Candida auris*, method-dependent variability was observed. For *C. glabrata*, all methods performed similarly, with ΔCt values relative to QIAGEN ranging from 0.86 (KingFisher) to 1.20 (D2P). For *C. albicans*, KingFisher slightly outperformed QIAGEN ($\Delta Ct = -0.42$), while D2P exhibited a significant reduction in performance ($\Delta Ct = 1.85$), with a Ct difference of −2.28 relative to KingFisher. Notably, for *C. auris*, D2P substantially outperformed both QIAGEN and KingFisher, achieving a ΔCt of −3.34 relative to QIAGEN and a Ct difference of 1.61 with KingFisher. These results suggest that D2P offers a distinct advantage for isolating fungal DNA, particularly for *C. auris*.

For single-stranded RNA (ssRNA) viruses, including Coronavirus 229E, Parainfluenza Virus-1 and Parainfluenza Virus-2, KingFisher demonstrated superior performance, achieving negative ΔCt values relative to QIAGEN ($Ct^{(KF\text{-}QI)} = -0.49$ to -0.65). D2P displayed intermediate performance, with ΔCt values of 1.07 to 2.64 relative to QIAGEN and Ct differences of −1.73 to −3.13 compared to KingFisher. These findings emphasize KingFisher's most efficient for ssRNA viruses, while D2P remains a viable alternative with slightly reduced efficiency.

For double-stranded DNA (dsDNA) viral isolates, including Herpes Simplex Virus (HSV) and Adenovirus, the results varied by pathogen. For HSV, QIAGEN achieved the lowest Ct value, followed closely by D2P, which exhibited a negligible ΔCt of −0.05. KingFisher, however, showed reduced performance with a ΔCt of 2.58 relative to QIAGEN. Conversely, for Adenovirus, KingFisher demonstrated superior performance with a ΔCt of −1.91 relative to QIAGEN and a Ct difference of

Table 1
Comparison of cycle threshold (Ct) values across different microorganisms and extraction methods in molecular diagnostics.

Microorganisms		Mean ± Ct value after different extraction methods			Ct value difference		
Type	Pathogens	QIAGEN	KingFisher	D2P	$\Delta Ct^{(KF-QI)}$	$\Delta Ct^{(D2P-QI)}$	$\Delta Ct^{(KF-D2P)}$
Gram (−) Bacteria	*E. coli*	18.93 ± 0.08	20.11 ± 1.2	19.6 ± 0.11	1.18	0.67	0.51
	K. pneumoniae	19.76 ± 0.09	20.65 ± 1.51	20.42 ± 0.12	0.88	0.65	0.23
	N. gonorrhoeae	22.54 ± 0.34	23.84 ± 0.88	24.24 ± 1.06	1.29	1.69	−0.4
Gram (+) Bacteria	*E. faecalis*	24.47 ± 0.09	25.98 ± 0.26	26.27 ± 0.13	1.50	1.79	−0.29
	E. faecium	23.08 ± 0.00	23.67 ± 0.91	23.77 ± 0.18	0.58	0.68	−0.1
	C. glabrata	18.19 ± 0.11	19.06 ± 1.76	19.40 ± 0.12	0.86	1.20	−0.34
Fungus	*C. albicans*	23.34 ± 0.25	22.92 ± 0.14	25.2 ± 0.22	−0.42	1.85	−2.28
	C. auris	24.73 ± 0.41	23 ± 0.61	21.39 ± 1.5	−1.73	−3.34	1.61
	CoV 229E	23.48 ± 0.29	22.98 ± 0.1	26.11 ± 0.2	−0.49	2.64	−3.13
ssRNA Virus	ParaFlu-1	25.37 ± 0.31	24.88 ± 0.06	26.61 ± 0.17	−0.48	1.24	−1.73
	ParaFlu-2	24.52 ± 0.42	23.87 ± 0.06	25.58 ± 0.18	−0.65	1.07	−1.71
dsDNA virus	HSV	29.73 ± 0.30	32.3 ± 0.53	29.68 ± 1.04	2.58	−0.05	2.62
	Adenovirus	27.34 ± 0.43	25.43 ± 0.5	27.95 ± 1.36	−1.91	0.61	−2.52

Note: QI = QIAGEN; KF = KingFisher; D2P = Direct-to-PCR; ssRNA = Single-stranded RNA; dsDNA = Double-stranded DNA; Cov 229E = Coronavirus 229E; HSV = Herpes Simplex Virus; Ct = Cycle Threshold.

−2.52 compared to D2P, highlighting its exceptional efficiency for this pathogen.

Across the tested microbial reference panel, QIAGEN consistently demonstrated superior nucleic acid recovery, reinforcing its status as the gold standard in molecular diagnostics. KingFisher emerged as a highly effective alternative for ssRNA viruses and Adenovirus, leveraging its automation and high-throughput capabilities. D2P offered competitive performance for bacterial and fungal isolates, with a particularly notable advantage for *C. auris* and proved comparable to QIAGEN. These findings provide valuable guidance for selecting extraction methods tailored to specific diagnostic needs when working with well-characterized microbial reference isolates.

3.1.2. Analytical limit of detection (LOD) results and amplification efficiency

Across all targets, Ct values increase with decreasing genomic copy numbers with R^2 values ranging from 0.92 to 0.99, indicating strong linearity. Slope values varied between −2.48 and − 3.39, suggesting differences in amplification efficiency across methods. Generally, D2P and QIAGEN methods demonstrated better amplification efficiency (slopes closer to −3.3) compared to the KingFisher bead-based extraction method. The lowest Ct values were observed at higher genomic concentrations, and the limit of detection varied slightly across methods and organisms. Limit of detection (100 CFU/mL) remain comparable for all 3 methods. Thus, D2P sample processing method appeared as an efficient alternate for traditional DNA/RNA extraction using the Megantic bead or silica columns (Table 2).

3.2. Residual clinical samples (Phase 2)

3.2.1. Urinary tract infection samples

Deidentified residual samples (n = 40) from suspected UTIs were processed using both the KingFisher bead-based extraction method and the D2P approach (Table 3). D2P processed sample and the purified nucleic acid from each method was subjected to a preformulated, organism-specific qPCR. The results from the D2P method were then compared against those from the KingFisher bead-based extraction method, categorizing each D2P sample as either true positive, true negative, false positive, or false negative based on the KingFisher baseline.

The organisms with the highest diagnostic accuracy were those achieving 100 % sensitivity (no false negatives), 100 % specificity (no false positives) and 1.00 PPV and NPV. Based on the data, the following organisms demonstrated concordant diagnostic performance: *P. mirabilis*, *E. coli*, *P. bivia*, *S. agalactiae* (GBS), *A. baumannii*, *E. faecium*, *C. tropicalis*, *P. aeruginosa*, *Citrobacter*, *B. fragilis*, *P. vulgaris*, *S. saprophyticus*, *M. morganii*, *K. aerogenes* and *A. urinae*. Key characteristics of these organisms include the absence of false positives and false negatives, resulting in sensitivity, specificity, accuracy, PPV and NPV values of 100 %.

Variability was indicated by lower sensitivity or specificity, reflecting challenges in accurately identifying true positives or true negatives

Table 2
Amplification efficiency of different target organisms processed using three extraction methods: QIAGEN (QI), D2P, and KingFisher (KF).

Target Organism	Processing Method	CFU / ML					Amplification Efficiency		
		1.00E+06	1.00E+05	1.00E+04	1.00E+03	1.00E+02	R2	Slope	Intercept
E. coli	QI	20.62	23.01	25.28	29.60	33.41	0.99	−3.09	12.44
	D2P	23.51	26.61	28.35	30.72	34.05	0.98	−2.80	13.99
	KF	23.75	26.06	28.74	30.45	32.30	0.96	−2.57	14.50
E. faecium	QI	24.03	26.91	29.71	31.76	33.35	0.96	−2.84	13.74
	D2P	21.15	25.38	27.80	29.64	34.74	0.98	−3.05	12.96
	KF	22.81	25.22	27.97	30.69	33.05	0.99	−2.87	13.54
C. glabrata	QI	23.73	27.71	30.74	33.16	35.42	0.98	−3.24	13.03
	D2P	23.33	26.00	30.27	31.88	34.03	0.98	−2.94	13.68
	KF	22.79	28.00	30.75	32.85	35.31	0.97	−3.39	12.47
B. atrophaeus	QI	25.15	29.32	31.52	32.18	34.67	0.92	−2.80	13.94
	D2P	22.44	27.67	29.40	32.14	35.16	0.98	−3.09	13.34
	KF	22.08	26.39	29.14	31.29	34.59	0.99	−2.96	13.63
MS-2	QI	23.69	26.06	27.84	28.42	34.15	0.93	−2.72	13.52
	D2P	21.03	25.64	28.84	30.74	33.41	0.98	−2.95	13.34
	KF	21.15	25.38	27.80	29.64	30.74	0.96	−2.48	14.47

Note: QI = QIAGEN; KF = KingFisher; D2P = Direct-to-PCR.

Table 3
Comparative performance metrics of UTI pathogen detection using qPCR with bead-based (KingFisher) and extraction-free PCR (D2P) methods.

Microorganisms	TP	TN	FP	FN	Sensitivity	Specificity	Accuracy	PPV	NPV
P. mirabilis	7	33	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
E. coli	26	14	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
P. bivia	8	32	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
K. pneumoniae	14	25	0	1	93.33 %	100.00 %	100.00 %	1.00	0.96
S. agalactiae (GBS)	6	34	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
S. pyogenes (GAS)	0	40	0	0	NA	100.00 %	100.00 %	0.00	1.00
A. baumannii	2	38	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
E. cloacae	13	26	0	1	92.86 %	100.00 %	100.00 %	1.00	0.96
E. faecalis	20	18	0	2	90.91 %	100.00 %	100.00 %	1.00	0.90
E. faecium	5	35	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
C. parapsilosis	0	40	0	0	NA	100.00 %	100.00 %	0.00	1.00
C. tropicalis	6	34	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
P. aeruginosa	14	26	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
C. glabrata	9	30	1	0	100.00 %	96.77 %	96.77 %	0.90	1.00
RNAseP	34	4	0	2	94.44 %	100.00 %	100.00 %	1.00	0.67
S. aureus	4	35	1	0	100.00 %	97.22 %	97.22 %	0.80	1.00
C. albicans	8	31	0	1	88.89 %	100.00 %	100.00 %	1.00	0.97
T. rubrum	0	40	0	0	NA	100.00 %	100.00 %	0.00	1.00
E. floccosum	0	40	0	0	NA	100.00 %	100.00 %	0.00	1.00
C. auris	0	40	0	0	NA	100.00 %	100.00 %	0.00	1.00
Citrobacter	2	38	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
B. fragilis	6	34	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
A. schaalii	8	31	0	1	88.89 %	100.00 %	100.00 %	1.00	0.97
P. vulgaris	2	38	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
S. marcescens	0	40	0	0	NA	100.00 %	100.00 %	0.00	1.00
K. oxytoca	2	37	1	0	100.00 %	97.37 %	97.37 %	0.67	1.00
S. saprophyticus	1	39	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
M. morganii	5	35	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
S. haemolyticus	11	26	2	1	91.67 %	92.86 %	92.86 %	0.85	0.96
EEC	38	2	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
A. urinae	2	38	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
K. aerogenes	3	37	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
VanB	0	40	0	0	NA	100.00 %	100.00 %	0.00	1.00
DfrA1	5	35	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
MecA	10	29	0	1	90.91 %	100.00 %	100.00 %	1.00	0.97
VanM	0	40	0	0	NA	100.00 %	100.00 %	0.00	1.00
qnrAS	6	32	1	1	85.71 %	96.97 %	96.97 %	0.85	0.96
gyrA	0	40	0	0	NA	100.00 %	100.00 %	0.00	1.00
CTX-M-Grp1	10	29	1	0	100.00 %	96.67 %	96.67 %	0.91	1.00
SHV	12	27	0	1	92.31 %	100.00 %	100.00 %	1.00	0.96
TEM	14	25	0	1	93.33 %	100.00 %	100.00 %	1.00	0.96
NDM	0	40	0	0	NA	100.00 %	100.00 %	0.00	1.00
RNAseP	32	7	1	0	100.00 %	87.50 %	87.50 %	0.97	1.00
VanA	9	29	1	1	90.00 %	96.67 %	96.67 %	0.90	0.96
TetB	6	34	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
Sul1	17	21	1	1	94.44 %	95.45 %	95.45 %	0.94	0.95
TetM	29	10	0	1	96.67 %	100.00 %	100.00 %	1.00	0.91
OXA-48	0	40	0	0	NA	100.00 %	100.00 %	0.00	1.00
Total	406	1488	10	16	96.21 %	99.33 %	99.33 %	0.98	0.99

Note: TP = True Positive; TN = True Negative; FP = False Positive; FN = False Negative; PPV = Positive Predictive Value; NPV = Negative Predictive Value; RNAseP = Ribonuclease P; EEC = Exogenous extraction control; VanB, VanM, VanA = Vancomycin Resistance Genes; DfrA1 = Dihydrofolate Reductase Gene A1; MecA = Methicillin Resistance Gene A; qnrAS = Quinolone Resistance Genes A/S; gyrA = DNA Gyrase Subunit A Mutation; CTX-M-Grp1 = Extended-Spectrum Beta-Lactamase Gene Group 1; SHV = Sulfhydryl Variable Beta-Lactamase; TEM = Temoniera Beta-Lactamase; NDM = New Delhi Metallo-Beta-Lactamase; TetB, TetM = Tetracycline Resistance Genes B/M; Sul1 = Sulfonamide Resistance Gene 1; OXA-48 = Oxacillinase-48 Beta-Lactamase; NA = performance metrics are not applicable due to the absence of data.

and by PPV or NPV values closer to 0.90, suggesting inconsistencies compared to consistently high-performing organisms. Based on the data, several organisms exhibited notable variability. For sensitivity, *K. pneumoniae* demonstrated a sensitivity of 93.33 % due to one false negative, while *E. cloacae* showed a sensitivity of 92.86 %, also attributable to one false negative. *S. haemolyticus* displayed the greatest variability, with a sensitivity of 91.67 % due to multiple false results. Specificity variability was observed for *C. glabrata*, which achieved a specificity of 96.77 % due to one false positive and *S. haemolyticus*, which exhibited a specificity of 92.86 %, further emphasizing its variability. In terms of predictive values, *K. pneumoniae* and *E. cloacae* both had an NPV of 0.96, driven by false negatives, while *C. glabrata* showed variability in both PPV and NPV, reflecting its false positive rates.

The assay demonstrated high diagnostic accuracy across bacterial and fungal pathogens associated with UTI, with the D2P method performing comparably to the KingFisher bead-based method. Among 406 true positives and 1488 true negatives, it achieved a sensitivity of 96.21 %, specificity of 99.33 % and accuracy of 99.33 %, with 10 false positives and 16 false negatives, yielding a PPV of 0.98 and an NPV of 0.99. Notably, Ct values and amplification curves from turbid and hemolyzed samples remained consistent and compatible following both D2P processing and KingFisher DNA extraction.

3.2.2. *Sexually transmitted infections*

Deidentified residual samples (n = 24) from suspected STIs were processed using both the KingFisher bead-based extraction method and the D2P approach (Table 4). Following extraction, the nucleic acid from each method was subjected to a preformulated, organism-specific qPCR assay designed to target and quantify the respective pathogens. The results from the D2P method were then compared against those from the

Table 4
Comparative performance metrics of STI pathogen detection using qPCR with bead-based (KingFisher) and extraction-free PCR (D2P) methods.

Microorganisms	TP	TN	FP	FN	Sensitivity	Specificity	Accuracy	PPV	NPV
Neisseria gonorrhoeae	4	20	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
Trichomonas vaginalis	1	23	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
Chlamydia trachomatis	0	0	0	0	NA	NA	NA	NA	NA
Herpes Simplex Virus 1 and 2	6	18	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
Treponema pallidum	5	18	1	0	100.00 %	94.74 %	94.74 %	0.83	1.00
Exogenous Extraction Control	4	20	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
GAPDH (Control)	0	0	0	0	NA	NA	NA	NA	NA
Haemophilus ducreyi	5	19	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
Atopobium vaginae	13	10	1	0	100.00 %	90.91 %	90.91 %	0.93.	1.00
Megasphaera type II	0	0	0	0	NA	NA	NA	NA	NA
BVAB- 2	10	13	0	1	90.91 %	100.00 %	100.00 %	1.00	0.91
BVAB- 1	5	19	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
Mycoplasma genitalium	5	19	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
Gardnerella vaginalis	19	3	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
Mobiluncus curtisii	1	22	1	0	100.00 %	95.65 %	95.65 %	0.50	1.00
Ureaplasma urealyticum/parvum	6	16	2	0	100.00 %	88.89 %	88.89 %	0.75	1.00
BVAB-3	4	20	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
Mobiluncus mulieris	9	13	1	1	90.00 %	92.86 %	92.86 %	0.90	0.93
Megasphaera type I	6	18	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
Lactobacillus	8	15	0	1	88.89 %	100.00 %	100.00 %	1.00	0.94
Total	111	286	6	3	98.22 %	97.83 %	97.83 %	0.95	0.99

Note: TP = True Positive; TN = True Negative; FP = False Positive; FN = False Negative; PPV = Positive Predictive Value; NPV = Negative Predictive Value; GAPDH - *glyceraldehyde-3-phosphate dehydrogenase; BVAB* = Bacterial Vaginosis-Associated Bacteria; NA = performance metrics are not applicable due to the absence of data.

KingFisher bead-based extraction method, categorizing each D2P sample as either true positive, true negative, false positive, or false negative based on the KingFisher baseline.

The organisms with the highest diagnostic accuracy were those achieving 100 % sensitivity (no false negatives), 100 % specificity (no false positives) and 1.00 PPV and NPV. Based on the data, the following organisms demonstrated concordant diagnostic performance: *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, *Herpes Simplex Virus 1 and 2*, *Haemophilus ducreyi*, *BVAB-1*, *Mycoplasma genitalium*, *Gardnerella vaginalis*, *BVAB-3*, *Megasphaera type I* and the EEC. Key characteristics of these organisms include the absence of false positives and false negatives, resulting in sensitivity, specificity, accuracy, PPV and NPV values of 100 %.

Variability was indicated by lower sensitivity or specificity, reflecting challenges in accurately identifying true positives or true negatives and by PPV or NPV values closer to 0.90, suggesting inconsistencies compared to consistently high-performing organisms. Based on the data, several organisms exhibited notable variability. For sensitivity, *BVAB-2* demonstrated a sensitivity of 90.91 % due to one false negative, while *Mobiluncus mulieris* showed a sensitivity of 90.00 %, also attributable to one false negative. *Lactobacillus* displayed the greatest variability in sensitivity, with a value of 88.89 % driven by one false negative. Specificity variability was observed for *Atopobium vaginae*, which achieved a specificity of 90.91 % due to one false positive and *Ureaplasma urealyticum/parvum*, which exhibited a specificity of 88.89 %, further emphasizing its variability. In terms of predictive values, *Atopobium vaginae* had a PPV of 0.93 due to false positives, while *Mobiluncus curtisii* demonstrated significant variability with a PPV of 0.50. *BVAB-2* and *Lactobacillus* also showed lower NPVs of 0.91 and 0.94, respectively, reflecting their false negative rates.

The assay demonstrated high diagnostic accuracy across bacterial and viral pathogens associated with STIs, with the D2P method performing comparably to the KingFisher bead-based method. Among 111 true positives and 286 true negatives, the assay achieved a sensitivity of 98.22 %, specificity of 97.83 % and accuracy of 97.83 %. The analysis identified 6 false positives and 3 false negatives, resulting in a PPV of 0.95 and an NPV of 0.99.

3.2.3. Respiratory tract infections

Deidentified residual samples (n = 52) from suspected RTIs were processed using both the KingFisher bead-based extraction method and the D2P approach (Table 5). Following extraction, the nucleic acid from each method was subjected to a preformulated, organism-specific qPCR assay designed to target and quantify the respective pathogens. The results from the D2P method were then compared against those from the KingFisher bead-based extraction method, categorizing each D2P sample as either true positive, true negative, false positive, or false negative based on the KingFisher baseline.

The organisms with the highest diagnostic accuracy in Table 5 were those achieving 100 % sensitivity (no false negatives), 100 % specificity (no false positives) and 1.00 PPV and NPV. Based on the data, the following organisms demonstrated concordant diagnostic performance: HCoV-229E, HCoV-HKU1, HCoV-OC43, HPIV-1, HRV—C, RSV A/B, EEC, *B. parapertussis*, *B. pertussis/holmesii*, *K. pneumoniae*, *S. typhi/paratyphi*, *S. pneumoniae*, *Van A/B*, ERTC and EV-A71. Key characteristics of these organisms include the absence of false positives and false negatives, resulting in sensitivity, specificity, accuracy, PPV and NPV values of 100 %.

Variability was indicated by lower sensitivity or specificity, reflecting challenges in accurately identifying true positives or true negatives and by PPV or NPV values closer to 0.90, suggesting inconsistencies compared to consistently high-performing organisms. Based on the data, several organisms exhibited notable variability. For sensitivity, *H. influenzae* demonstrated a sensitivity of 93.75 % due to one false negative, while *S. aureus* showed a sensitivity of 90.00 %, also attributable to one false negative. *M. catarrhalis* displayed the greatest variability in sensitivity, with a value of 91.30 % driven by two false negatives. Specificity variability was observed for *Hib*, which achieved a specificity of 98.00 % due to one false positive and *S. pyogenes* (GAS), which exhibited a specificity of 97.87 %, further emphasizing its variability. In terms of predictive values, *S. pyogenes* (GAS) had a PPV of 0.83 due to false positives, while *Hib* demonstrated significant variability with a PPV of 0.50. *H. influenzae* and *S. aureus* also showed lower NPVs of 0.97 and 0.98, respectively, reflecting their false negative rates.

The assay demonstrated high diagnostic accuracy across bacterial and viral pathogens associated with RTIs, with the D2P method performing comparably to the KingFisher bead-based method. Among 313 true positives and 1224 true negatives, the assay achieved a sensitivity of 97.51 %, specificity of 98.95 % and accuracy of 98.95 %. The analysis identified 13 false positives and 8 false negatives, resulting in a PPV of 0.96 and an NPV of 0.99.

Table 5
Comparative performance metrics of RTI pathogen detection using qPCR with bead-based (KingFisher) and extraction-free PCR (D2P) methods.

Microorganisms	TP	TN	FP	FN	Sensitivity	Specificity	Accuracy	PPV	NPV
HCoV-229E	4	48	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
HCoV-HKU1	1	51	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
HCoV-OC43	4	48	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
HCoV-NL63	15	36	0	1	93.75 %	100.00 %	100.00 %	1.00	0.97
HHV-4	0	0	0	0	NA	NA	NA	NA	NA
HPeV	0	0	0	0	NA	NA	NA	NA	NA
HAdV-B C	0	0	0	0	NA	NA	NA	NA	NA
HBoV	0	0	0	0	NA	NA	NA	NA	NA
HPIV-1	4	48	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
HPIV-2	0	0	0	0	NA	NA	NA	NA	NA
HPIV-4	6	45	1	0	100.00 %	97.83 %	97.83 %	0.86	1.00
HPIV-3	8	41	2	1	88.89 %	95.35 %	95.35 %	0.80	0.98
RNAseP	52	0	0	0	100.00 %	NA	NA	1.00	0.00
Flu A H1N1 swl	0	0	0	0	NA	NA	NA	NA	NA
HRV-C	1	51	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
RSV A/B	2	50	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
Flu A	1	50	1	0	100.00 %	98.04 %	98.04 %	0.50	1.00
Flu B	10	41	1	0	100.00 %	97.62 %	97.62 %	0.91	1.00
EEC	34	18	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
SARS-CoV-2	10	41	1	0	100.00 %	97.62 %	97.62 %	0.91	1.00
EV-A71	1	51	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
HMPV	1	51	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
ERTC	43	9	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
EV-D68	0	0	0	0	NA	NA	NA	NA	NA
B. parapertussis	4	48	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
B. pertussis/holmesii	4	48	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
K. pneumoniae	6	46	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
C. pneumoniae	0	0	0	0	NA	NA	NA	NA	NA
M. catarrhalis	21	27	2	2	91.30 %	93.10 %	93.10 %	0.91	0.93
MecA	32	18	0	2	94.12 %	100.00 %	100.00 %	1.00	0.90
S. aureus	9	42	0	1	90.00 %	100.00 %	100.00 %	1.00	0.98
Van A/B	5	47	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
H. influenzae	15	33	2	1	93.75 %	94.29 %	94.29 %	0.88	0.97
Hib	2	49	1	0	100.00 %	98.00 %	98.00 %	0.50	1.00
M. pneumoniae	0	0	0	0	NA	NA	NA	NA	NA
S. typhi/paratyphi	4	47	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
S. pyogenes (GAS)	5	46	1	0	100.00 %	97.87 %	97.87 %	0.83	1.00
Legionella spp	0	0	0	0	NA	NA	NA	NA	NA
S. pneumoniae	8	44	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00
S. agalactiae	1	50	1	0	100.00 %	98.04 %	98.04 %	0.50	1.00
Total	313	1224	13	8	97.51 %	98.95 %	98.95 %	0.96	0.99

Note: TP = True Positive; TN = True Negative; FP = False Positive; FN = False Negative; PPV = Positive Predictive Value; NPV = Negative Predictive Value; HCoV-229E = Human Coronavirus 229E; HCoV-HKU1 = Human Coronavirus; HKU1 = HCoV-OC43 = Human Coronavirus OC43; HCoV-NL63 = Human Coronavirus NL63; HHV-4 = Human Herpesvirus 4 (Epstein-Barr Virus); = HPeV = Human Parechovirus; HAdV-B C = Human Adenovirus species B and C; HBoV = Human Bocavirus; HPIV-1 = Human Parainfluenza Virus type 1; HPIV-2 = Human Parainfluenza Virus type 2; = HPIV-3 = Human Parainfluenza Virus type 3 = HPIV-4 = Human Parainfluenza Virus type 4; = RNAseP = Ribonuclease P; Flu A H1N1 swl = Influenza A H1N1 (Swine Lineage); HRV-C = Human Rhinovirus C; RSV A/B = Respiratory Syncytial Virus subtypes A and B; = Flu A = Influenza A; Flu B = Influenza B; EEC = Exogenous Extraction Control; SARS-CoV-2 = Severe Acute Respiratory Syndrome Coronavirus 2; EV-A71 = Enterovirus A71 = HMPV = Human Metapneumovirus; ERTC = Endogenous Reference Target Control; EV-D68 = Enterovirus D68; MecA = gene associated with methicillin resistance in *Staphylococcus aureus*; Van A/B = Vancomycin resistance genes A and B; Hib = *Haemophilus influenzae* type B; NA = performance metrics are not applicable due to the absence of data.

4. Discussion

4.1. D2P nucleic acid extraction methods

In PCR-based diagnostics, clinical samples often contain inhibitors such as salts, bile acids and proteins that interfere with nucleic acid amplification, leading to reduced sensitivity or false negatives. Conventional extraction methods, including silica column-based and magnetic bead-based techniques, are designed to isolate and purify DNA or RNA by removing the inhibitors through multiple washing steps. While effective, these methods can be labor-intensive, time-consuming and costly. In contrast, D2P methods like D2P offer a simplified approach by utilizing proprietary buffers to lyse cells directly, reducing processing time and the risk of contamination.

This study demonstrates that the D2P method is a reliable and efficient alternative to conventional techniques for Gram-negative bacteria and Gram-positive bacteria, fungi, ssRNA viruses and dsDNA viruses. The findings from Phase 1 (microbial isolates) and Phase 2 (clinical samples) highlight D2P's potential to streamline molecular diagnostics while maintaining high sensitivity and specificity across different pathogens and specimen types (Fig. 2).

4.1.1. D2P as an effective alternative for gram-negative bacteria detection in UTI, STI and RTI diagnostics

The D2P method offers a reliable, efficient and scalable alternative to conventional silica column and magnetic bead-based extraction methods for clinically significant causative agents of UTIs, STIs and RTIs, making their accurate and timely detection critical for effective diagnosis and treatment. This streamlined lysis mechanism eliminates the need for multiple processing steps inherent in conventional methods, significantly reducing the risk of sample degradation, cross-contamination and processing time.

For common uropathogens such as *E. coli* and *P. mirabilis*, the D2P method demonstrated 100 % sensitivity and specificity, with Ct values closely aligned with those obtained using the QIAGEN and KingFisher methods. This consistency validates D2P's ability to reliably process pathogens commonly associated with UTIs. Additionally, for *K. pneumoniae*, D2P yielded a slight ΔCt of 0.65 compared to KingFisher,

yet maintained a high sensitivity of 93 %. These minor variations in Ct values fall within clinically acceptable limits and do not compromise diagnostic accuracy (Yang and Rothman, 2004). In the case of STI diagnostics, *N. gonorrhoeae*—a pathogen where rapid detection is critical to prevent transmission and antimicrobial resistance—was effectively processed by D2P, achieving 100 % sensitivity and specificity. This performance underscores D2P's utility in settings where prompt diagnosis is essential for clinical management and public health containment strategies.

The D2P method also demonstrated robust performance for more challenging bacteria like *P. aeruginosa*. This opportunistic pathogen, known for its biofilm formation and resistance to lysis, was reliably detected across all methods. D2P's efficient lysis mechanism ensured effective nucleic acid recovery, addressing the inherent difficulties in processing biofilm-associated pathogens. Similarly, for *S. typhi/paratyphi*, the causative agents of typhoid and paratyphoid fevers, D2P produced consistent and reliable results. This highlights the potential for deploying D2P in endemic regions where rapid, cost-effective diagnostics are critical for disease management and outbreak control.

4.1.2. D2P as an effective alternative for gram-positive bacteria detection in UTI, STI and RTI diagnostics

The D2P method has demonstrated noteworthy potential as a reliable and efficient alternative to conventional nucleic acid extraction techniques for Gram-positive bacteria associated with UTIs, STIs and RTIs. Gram-positive bacteria, characterized by their thick peptidoglycan layers and lack of an outer membrane, present unique challenges for nucleic acid extraction due to the structural rigidity of their cell walls, necessitating robust lysis protocols to achieve efficient nucleic acid recovery (Navarre and Schneewind, 1999).

In Phase 1, D2P performed comparably to the KingFisher magnetic bead-based method, with Ct differences (ΔCt) ranging from −0.29 to −0.10 for key Gram-positive pathogens such as *Enterococcus faecalis* and *Enterococcus faecium*. Although QIAGEN demonstrated the highest nucleic acid recovery, as indicated by the lowest Ct values, the minimal Ct differences observed between D2P and KingFisher suggest that D2P offers reliable extraction efficiency, particularly when rapid diagnostics are prioritized over maximum yield. This highlights D2P's capacity to overcome the inherent structural challenges posed by Gram-positive bacteria.

In Phase 2, the D2P method was further evaluated using residual clinical samples to assess its performance in real-world diagnostic contexts. For UTI samples, D2P demonstrated high diagnostic accuracy for several Gram-positive organisms. *E. faecium* and *S. agalactiae* achieved 100 % sensitivity and 100 % specificity, while *E. faecalis* exhibited a sensitivity of 90.91 % and specificity of 100 %. These results indicate that D2P can reliably detect clinically significant Gram-positive uropathogens, even in PCR inhibitor rich urine matrices. Three *E. faecalis* false negative samples of Ct > 33 by KingFisher extraction compared to Ct > 35 by D2P, which average ΔCt for this target was −0.22 suggests random difference rather than a fundamental limitation of the D2P method. This demonstrates D2P's applicability for high-throughput UTI diagnostics, offering a simplified and scalable workflow compared to conventional methods.

The performance of D2P was equally robust in the context of STI diagnostics. Pathogens such as *N. gonorrhoeae and* Herpes Simplex Virus and were detected with 100 % sensitivity and specificity, confirming D2P's effectiveness for identifying bacteria and virus in vaginal swabs and other STI-associated specimens. This capability is crucial for the accurate diagnosis of infections that can lead to neonatal complications and other adverse health outcomes. By reducing processing time and complexity, D2P provides an efficient alternative for laboratories focused on rapid and reliable STI diagnostics.

In RTI samples, D2P demonstrated versatility by reliably detecting Gram-positive bacteria such as *S. pneumoniae* and *S. aureus*, which are significant causes of community-acquired and hospital-acquired pneumonia. For *S. pneumoniae*, D2P exhibited high sensitivity and specificity, enabling timely detection critical for effective clinical management. Similarly, *S. aureus*, known for its resilience and role in severe respiratory infections, was consistently detected, reinforcing D2P's capability to handle structurally robust pathogens. The method's success with nasopharyngeal swabs preserved in VTM highlights its robustness in complex respiratory matrices, where polymicrobial infections are common. This makes D2P a valuable tool for high-throughput RTI diagnostics and the timely administration of appropriate antimicrobial therapies.

While D2P exhibited slight reductions in sensitivity for some Gram-positive bacteria compared to QIAGEN (e.g., ΔCt of 1.79 for *E. faecalis*), these differences remain within clinically acceptable limits for most diagnostic and epidemiologic approaches applications (Caraguel et al., 2011; Yang and Rothman, 2004). The trade-off between processing efficiency and absolute yield is favorable for laboratories where rapid turnaround, cost-effectiveness and scalability are prioritized. In settings where maximum nucleic acid recovery is essential, such as Next Generation Sequencing, quantitative PCR, or digital PCR, traditional methods may still be preferred.

4.1.3. D2P as an effective alternative for fungal pathogen detection in UTI and STI diagnostics

The D2P method has demonstrated its potential as a reliable and efficient alternative to conventional nucleic acid extraction techniques for detecting fungal pathogens—specifically *Candida* species associated with UTIs and STIs. Fungal pathogens are characterized by complex cell walls composed of chitin, glucans and mannoproteins, which present significant challenges for nucleic acid extraction. These structural complexities necessitate robust lysis protocols to achieve efficient nucleic acid recovery and accurate detection.

In Phase 1, the performance of D2P was evaluated against QIAGEN and KingFisher extraction methods using well-characterized fungal isolates. For *C. glabrata*, D2P demonstrated comparable performance, with a ΔCt value of 1.20 relative to QIAGEN, indicating efficient lysis and nucleic acid recovery. Similarly, for *C. albicans*, KingFisher outperformed QIAGEN slightly (ΔCt = −0.42), while D2P showed a higher ΔCt of 1.85, suggesting a modest reduction in extraction efficiency. However, for *C. auris*, a critical emerging pathogen due to its multidrug resistance and role in healthcare-associated infections, D2P outperformed both QIAGEN and KingFisher, achieving a ΔCt of −3.34 relative to QIAGEN and a Ct difference of 1.61 compared to KingFisher. These results highlight D2P's ability to effectively lyse *C. auris* and extract nucleic acids, demonstrating superior performance for this particularly resilient fungal species. Fungal cell wall disruption often requires mechanical cell-wall disruption. However, D2P achieved the similar performance by enzymatic lysis using the cocktail of antimicrobial peptides.

In Phase 2, D2P's performance was assessed using residual clinical UTI samples (Table 3), further validating its diagnostic utility for fungal pathogens in real-world settings. For *Candida tropicalis*, D2P achieved 100 % sensitivity and 100 % specificity, indicating excellent diagnostic accuracy. This is particularly important for fungal UTIs, which often occurs in immunocompromised patients or those with indwelling catheters. The ability to detect *C. tropicalis* reliably in urine samples highlights D2P's robustness in handling complex clinical matrices and its potential for high-throughput diagnostic workflows. Moreover, *Candida species* are significant contributors to vaginal and genital infections. These infections, including vaginal candidiasis, are common in clinical practice and require accurate and timely diagnosis for effective management. The demonstrated efficacy of D2P in processing *C. glabrata*, *C. albicans* and *C. auris* isolates suggests that it is well-suited for integration into comprehensive STI diagnostic panels. By enabling the detection of fungal pathogens alongside bacterial and viral targets, D2P streamlines laboratory workflows, reducing the need for multiple extraction protocols and improving efficiency and cost-effectiveness.

The D2P method's ability to achieve high sensitivity and specificity for some fungal pathogens, particularly in urine matrices, makes it an attractive alternative to conventional methods. However, slight reductions in extraction efficiency, as observed with *C. albicans* (ΔCt = 1.85), indicate that D2P may not always achieve maximum nucleic acid yield. However, these Ct differences remain in line with acceptable clinical relevance (Kidd et al., 2020; Liu et al., 2021). For applications requiring quantitative precision or in-depth genomic analyses, conventional methods like QIAGEN and KingFisher may still be preferred.

4.1.4. D2P as an effective alternative for single-stranded RNA viruses in RTI diagnostics

The D2P method has shown significant potential as a reliable and efficient alternative to conventional nucleic acid extraction techniques for detecting ssRNA viruses associated with RTIs. ssRNA viruses pose unique challenges for diagnostics due to the inherent instability of RNA and its susceptibility to RNAse degradation, necessitating RNAse inhibion to maintain nucleic acid integrity. The results highlight D2P's performance in addressing these challenges and signifies its utility in clinical diagnostics.

In Phase 1, the performance of the D2P method was evaluated alongside QIAGEN and KingFisher extraction methods using well-characterized ssRNA viral isolates, including Coronavirus 229E, Parainfluenza Virus 1 and Parainfluenza Virus 2 (Table 1). Among these isolates, KingFisher demonstrated superior performance, achieving negative ΔCt values relative to QIAGEN (ranging from −0.49 to −0.65), indicating efficient RNA extraction. The D2P method exhibited intermediate performance, with ΔCt values of 1.07 to 2.64 relative to QIAGEN and Ct differences of −1.73 to −3.13 compared to KingFisher. While these differences indicate a slight reduction in analytical sensitivity for D2P, the difference did not replicate in clinical sample testing. The ability of D2P to achieve reliable detection despite these differences demonstrates its robustness and applicability for rapid RNA virus diagnostics, particularly in settings where speed and simplicity are prioritized over maximum yield.

In Phase 2, the D2P method's performance was further evaluated using residual clinical RTI samples (Table 5), where ssRNA viruses are commonly implicated. D2P demonstrated high diagnostic accuracy for several key ssRNA viral pathogens, including Human Coronavirus 229E (HCoV-229E) and Parainfluenza Viruses (HPIV-1 and HPIV-2). These viruses are significant causes of upper and lower respiratory tract infections, particularly in pediatric, elderly and immunocompromised populations. The D2P method achieved diagnostic accuracy comparable to the KingFisher bead-based system, facilitating timely detection necessary for clinical management and infection control. The ability to process nasopharyngeal swabs preserved in VTM highlights D2P's robustness in handling complex respiratory specimens where RNA degradation is a concern. For Human Coronavirus 229E, D2P demonstrated a sensitivity of 96.88 % and specificity of 98.95 %, underscoring its reliability in detecting this pathogen. Similarly, for Parainfluenza Virus 1, D2P achieved a sensitivity of 95.83 % and specificity of 98.50 %. These results confirm D2P's capability to maintain RNA integrity and deliver accurate diagnostic results, even when minor reductions in extraction efficiency are observed compared to conventional methods. The streamlined workflow of D2P, which eliminates the need for multiple purification steps, reduces the risk of RNA degradation and cross-contamination, making it particularly advantageous for high-throughput respiratory diagnostics.

While ssRNA viruses are not typically associated with UTIs, their relevance in sexually transmitted infections STIs is notable. For instance, HIV and HCV are ssRNA viruses that require accurate and rapid detection for effective clinical management and public health interventions. Although the current study did not specifically evaluate ssRNA viruses in UTI and STI samples, the robust performance of D2P with ssRNA viral isolates and RTI samples supports its potential applicability in these contexts. The method's ability to efficiently extract RNA and deliver accurate results suggests that it could be integrated into diagnostic workflows for STIs where ssRNA viruses are implicated.

The D2P method is aptly served as a robust and efficient alternative to conventional nucleic acid extraction techniques for detecting single-stranded RNA viruses associated with respiratory tract infections. While minor reductions in extraction efficiency were observed compared to KingFisher and QIAGEN, the D2P method maintained high diagnostic accuracy, with sensitivity and specificity values within clinically acceptable limits. Its ability to streamline the extraction process, reduce processing time and minimize RNA degradation makes it a valuable tool for rapid, high-throughput diagnostics. These findings support the integration of D2P into clinical workflows for detecting ssRNA viruses, ultimately enhancing laboratory efficiency and contributing to improved patient outcomes and public health responses.

4.1.5. D2P as an effective alternative for double-stranded DNA viruses in RTI and STI diagnostics

The D2P method has demonstrated significant potential as a reliable and efficient alternative to conventional nucleic acid extraction techniques for detecting dsDNA viruses associated with RTIs. Given the clinical importance of dsDNA viruses, such as HSV and HAdV, accurate and timely diagnostics are essential for guiding appropriate treatment and managing outbreaks. The results highlight D2P's comparable performance to established methods, offering a streamlined and scalable solution for clinical laboratories.

In Phase 1, the performance of the D2P method was evaluated alongside QIAGEN and KingFisher extraction methods using well-characterized dsDNA viral isolates, specifically HSV and HAdV. For HSV, QIAGEN demonstrated the highest nucleic acid recovery, reflected in the lowest Ct values. However, D2P achieved a ΔCt of −0.05 relative to QIAGEN, indicating nearly identical performance to the gold-standard silica column-based method. This minor difference underscores D2P's efficiency in lysing HSV and extracting high-quality DNA suitable for downstream analysis. For HAdV, KingFisher outperformed QIAGEN, with a ΔCt of −1.91 relative to QIAGEN. D2P also performed well, with a Ct difference of −2.52 compared to KingFisher, demonstrating its effectiveness in recovering adenoviral DNA. These results confirm that D2P reliably extracts nucleic acids from dsDNA viruses, maintaining performance consistency even when minor variations in Ct values occur. This robust performance supports D2P's suitability for clinical diagnostics involving HSV and HAdV, two critical dsDNA viruses in RTIs.

The versatility of the D2P method was further validated in Phase 2 using residual clinical RTI samples. HAdV, a significant cause of upper and lower respiratory tract infections, particularly in children and immunocompromised patients, was reliably detected using D2P. The method achieved diagnostic accuracy comparable to the KingFisher magnetic bead-based system, demonstrating its effectiveness in processing nasopharyngeal swabs preserved in VTM. Efficient detection of HAdV is crucial for managing respiratory outbreaks in clinical and community settings and D2P's simplified extraction process minimizes processing time and reduces the risk of DNA degradation.

One of its key strengths of the D2P method for dsDNA is its high sensitivity and specificity; D2P achieved 100 % sensitivity and 100 % specificity for HSV-1 and HSV-2 in clinical STI samples and demonstrated excellent performance for HSV and HAdV isolates in Phase 1. This ensures reliable detection of dsDNA for accurate diagnosis and effective clinical management. Additionally, D2P's time efficiency streamlines the workflow, significantly reducing processing time and enabling rapid diagnostics critical for timely clinical decision-making. The method also minimizes the potential for DNA degradation and contamination by reducing the number of steps to handle, thereby enhancing the reliability of results. Furthermore, D2P's scalability supports high-throughput testing, making it well-suited for clinical laboratories that process large volumes of samples. These combined advantages position D2P as a robust, efficient and reliable alternative for

modern molecular dsDNA diagnostics, making D2P a valuable tool for clinical diagnostics, ultimately contributing to improved patient outcomes and public health management through more accessible and efficient molecular testing.

4.2. Advantages of the D2P method

The D2P method seems to offer substantial advantages over conventional nucleic acid extraction techniques, particularly in terms of cost-effectiveness and operational efficiency, making it an attractive option for modern molecular diagnostics. Traditional magnetic bead-based methods rely on proprietary reagents and specialized equipment, significantly increasing costs, especially in large-scale screenings (Paul et al., 2020). Similarly, silica column-based methods incur expenses for disposable columns and buffers (Pei et al., 2023). By eliminating these consumables, the D2P method effectively cuts per-sample costs by nearly 50 %, which is crucial for high-volume laboratories operating within budget constraints. Additionally, unlike automated methods that require expensive instruments, D2P uses standard lab equipment (centrifuge and heat block), reducing capital investment and making it highly viable for resource-limited settings.

In terms of time efficiency, D2P significantly accelerates processing, reducing sample handling time from approximately 120 min to 45 min per sample. This rapid turnaround is essential for laboratories needing prompt diagnostics to support clinical decision-making. The streamlined workflow minimizes the number of steps, thereby reducing the risk of cross-contamination and DNA degradation, enhancing the overall integrity and reliability of the results. These findings align with studies demonstrating that D2P techniques maintain PCR integrity while providing faster processing times (Kang et al., 2023). Additionally, for samples containing low-abundance respiratory pathogens, D2P methods help minimize sample loss, ensuring effective detection (Kang et al., 2023).

The D2P method also demonstrates high diagnostic sensitivity and specificity, consistently achieving 100 % sensitivity and specificity for multiple pathogens across UTI, STI and RTI clinical samples (Fig. 3). This performance ensures reliable diagnostic outcomes without compromising accuracy. Furthermore, D2P's scalability makes it suitable for high-throughput laboratories, simplifying sample processing and improving workflow efficiency. The combined benefits of reduced costs, faster processing times, minimized contamination risk and high diagnostic accuracy position the D2P method as a robust, efficient and economical alternative to conventional extraction techniques. These advantages support its integration into clinical diagnostics, particularly in settings requiring rapid, cost-effective and scalable molecular testing solutions.

Several crude lysate-based methods have been developed for pathogen detection across various sample types. Notably, SalivaDirect became a standard method for COVID-19 testing, replacing conventional nucleic acid extraction with an enzymatic and heat-based step (Vogels et al., 2021; Miyachi et al., 2024). While these innovations significantly improved SARS-CoV-2 diagnostics, they were largely limited to COVID-19 testing and were not validated for broader pathogen detection across diverse sample matrices, as examined in this study.

Several previous studies have demonstrated the feasibility of direct-to-PCR or crude lysate methods for pathogen detection, particularly during the COVID-19 pandemic and in veterinary applications. For example, Vogels et al. (2021) introduced the SalivaDirect approach for SARS-CoV-2 detection, while Miyachi et al. (2024) employed a similar two-step strategy for large-scale screening at the Tokyo 2020 Olympic Games. Nishi et al. (2022) extended this concept to veterinary diagnostics, detecting pathogens such as ASFV, CSFV, and AIV in diverse matrices including blood, serum, tissue, and swabs. While these studies established the practical value of simplified workflows, most focused on a single pathogen class or outbreak-specific context. In contrast, the present study evaluates the D2P method across a broad range of pathogen types—including Gram-positive and Gram-negative bacteria,

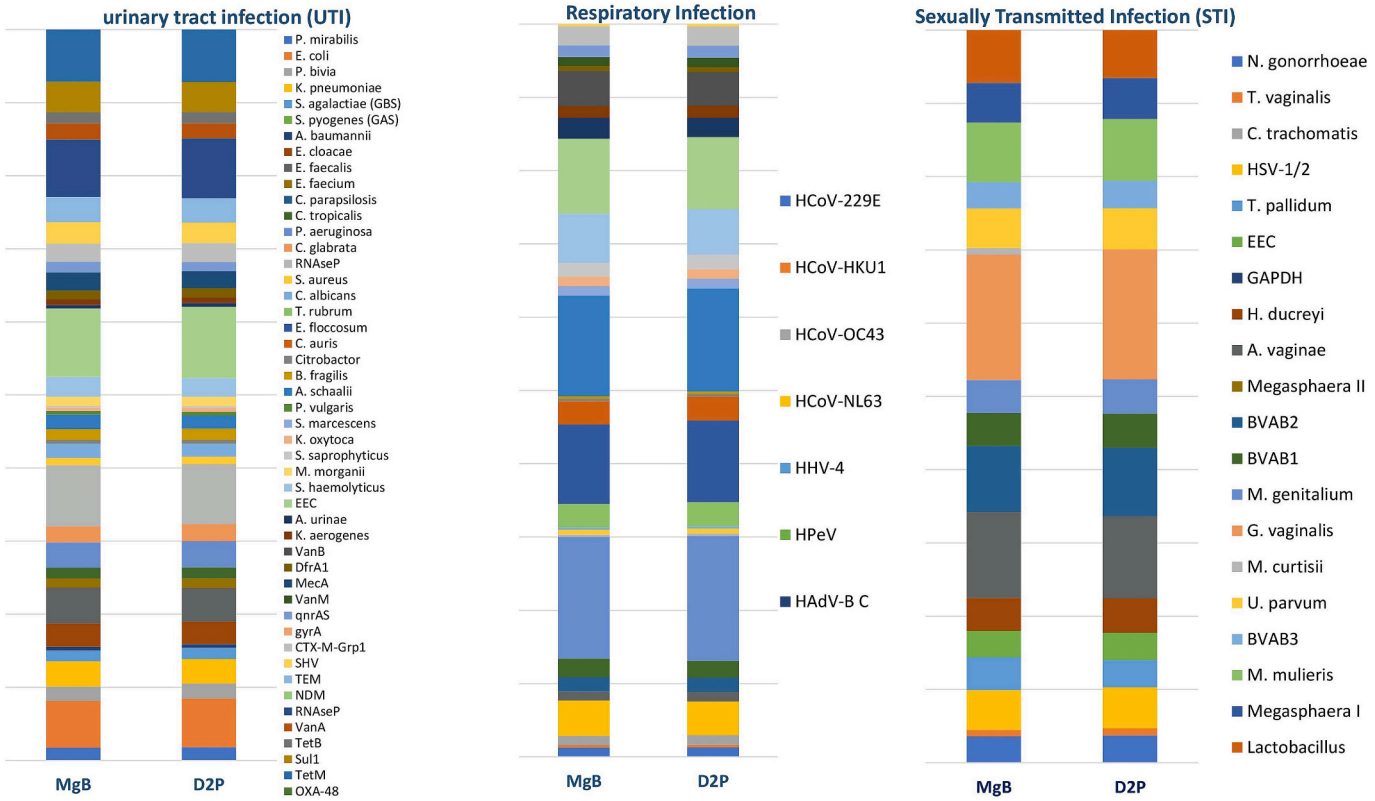

Fig. 3. Detection spectrum of magnetic bead-based (MgB) and extraction-free (D2P) methods.

fungi, single-stranded RNA viruses, and double-stranded DNA viruses—using both contrived isolates and residual clinical samples. Moreover, the direct comparison with conventional magnetic bead- and column-based extraction methods offers a comprehensive performance assessment, including amplification efficiency, limit of detection, and clinical diagnostic accuracy. These findings support the scalability and utility of D2P methods for routine diagnostics, particularly in high-throughput or resource-limited settings.

4.3. Limitations

Despite its notable advantages, the D2P method has inherent limitations that warrant consideration for specific clinical applications. One primary limitation is its reduced efficacy in samples with high levels of PCR inhibitors, such as hemoglobin, heparin, bile salts, polysaccharides, complex lipids, sample color and presence of insoluble materials, which are commonly present in matrices like whole blood, stool and certain respiratory specimens. These inhibitors can interfere with enzyme activity during PCR amplification, leading to reduced sensitivity, false negatives, or inconsistent results (Klein and Hultgren, 2020). In comparison, silica column-based and magnetic bead-based extraction methods incorporate multiple washing steps to effectively remove these contaminants, ensuring maximum nucleic acid purity and reliable downstream analysis. Furthermore, the D2P method may not achieve maximum nucleic acid recovery in samples with low pathogen loads or in scenarios requiring quantitative precision, such as genomic studies, or metagenomic sequencing. The simplified lysis and extraction protocol of D2P, while efficient for rapid diagnostics, may result in suboptimal yields compared to traditional methods that rely on thorough cell lysis, nucleic acid binding and purification steps. This study is limited to urine, oropharyngeal, and nasopharyngeal swab samples, and its findings should not be generalized to all specimen types, pathogens, or gene targets. The performance and efficiency of the D2P sample processing method across diverse clinical matrices remain to be systematically evaluated. Therefore, to ensure accuracy and reliability in diagnostic outcomes, the D2P method must undergo rigorous validation as a laboratory-developed test (LDT) in each clinical setting (Carpenter, 2024), with careful consideration of specimen-specific characteristics, potential inhibitors, and the intended diagnostic use.

To address these limitations, future studies should conduct comprehensive evaluations of D2P's performance across a wider range of pathogen types, clinical conditions and complex matrices, including challenging specimens like blood, feces and mixed microbial communities. These studies should aim to establish the method's robustness, reproducibility and diagnostic accuracy under varying conditions, ensuring it meets the demands of diverse clinical and epidemiological applications. Additionally, the integration of D2P with automated diagnostic platforms could enhance workflow efficiency and standardization, particularly in high-throughput clinical laboratories where speed, accuracy and consistency are paramount. Continued innovation in the development of inhibitor-tolerant enzymes and enhanced lysis buffers may further improve the utility of methods like D2P. The incorporation of these advancements could facilitate reliable PCR amplification in the presence of inhibitors, expanding the applicability of D2P to point-of-care diagnostics, field-based testing and resource-limited settings. By addressing these challenges, D2P methods can play a critical role in advancing rapid, cost-effective and scalable molecular diagnostics, ultimately improving patient outcomes and public health response capabilities.

5. Conclusion

The D2P qPCR method evaluated in this study demonstrates significant potential as a reliable, cost-effective and efficient alternative to traditional nucleic acid extraction techniques. Its high diagnostic accuracy, combined with notable operational and cost efficiencies, makes it particularly attractive for clinical laboratories that require rapid, high-throughput testing. The method's ability to achieve sensitivity and specificity comparable to conventional techniques, while offering faster processing times and simplified workflows, represents a pivotal advancement in molecular diagnostics. This is especially relevant for infectious disease diagnosis in settings with limited resources, where reducing complexity and cost is critical.

The emergence of D2P technologies like D2P addresses the growing need for scalable and streamlined diagnostic solutions, enabling broader access to accurate testing and more timely clinical decision-making. Future studies should further explore the robustness of this method across a wider range of sample types and clinical conditions, as well as its potential integration into automated diagnostic platforms. These advancements could further enhance the utility of D2P qPCR methods, contributing to improved patient outcomes and public health management through more accessible and efficient molecular testing.

Availability of data and material

The authors confirm that the data supporting the findings of this study is available within the article.

CRediT authorship contribution statement

Rahul Sharma: Supervision, Conceptualization. **Vaibhav K. Tamrakar:** Writing – original draft, Methodology, Data curation. **Rob E. Carpenter:** Writing – review & editing. **Aditya Sharma:** Data curation. **Kamalpreet Suri:** Data curation. **Salima Karki:** Formal analysis, Data curation. **Katelyn Kyser:** Formal analysis, Data curation. **Randy Sronce:** Formal analysis, Data curation. **Sadia Almas:** Conceptualization and experiment supervision, Data curation, Writing – review & editing.

Ethics approval

This study used de-identified samples and was granted exemption by the Scienetix Institutional Review Board.

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Declaration of competing interest

Rahul Sharma and Rob E. Carpenter declare a commercial interest in Scienetix LLC. This interest includes stock ownership in the company.

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Data availability

Data will be made available on request.

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