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SHORT COMMUNICATION

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Implementation and clinical evaluation of an Mpox virus laboratory-developed test on a fully automated random-access platform

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Abstract

While Mpox virus (MPXV) diagnostics were performed in specialized laboratories only, the global emergence of Mpox cases in 2022 revealed the need for a more readily available diagnostic. Automated random-access platforms with fast nucleic acid extraction and PCR have become established in many laboratories, providing faster and more accessible testing. In this study, we adapted a previously published generic MPXV-PCR as a lab-developed test (LDT) on a NeuMoDx Molecular System and isolated MPXV clones from patient materials. To reduce the handling of infectious material, we evaluated a viral lysis buffer (VLB) for sample pretreatment. We further compared the MPXV-LDT-PCR to conventional real-time PCR, determined its sensitivity and specificity using positive swabs, and assessed its performance using external quality assessment samples. Pretreatment of samples with 50% VLB reduced MPXV infectivity by approximately 200-fold while maintaining PCR sensitivity. The assay demonstrated a sensitivity and specificity of 100% with no cross-reactivity in the samples tested and performed with a limit of detection of 262 GE/mL. In summary, the assay had a turnaround time of fewer than 2 h and can easily be transferred to other automated PCR platforms, providing a basis for developing rapid assays for upcoming pandemics.

KEYWORDS

automated random-access platforms, lab development test (LDT), monkeypox (MPOX), NeuMoDx, PCR, rapid testing

Jochen M. Wettengel and Till Bunse contributed equally to the work.

1 | INTRODUCTION

Over the past years, we have been facing an increase in emerging infectious diseases (EID). As the majority of EIDs are zoonotic, outbreaks are usually unprecedented and initial cases are rarely diagnosed.[1] Due to globalization, EIDs spread rapidly to other regions.[2] The recent SARS-CoV-2 pandemic has showcased the importance of rapid and reliable diagnostic assays that can be performed without specialized personnel, allowing quick and sensitive identification of cases for contact tracing and therapy decisions.[3–7] PCR offers high specificity and sensitivity in pathogen detection, but routine workflows are time-consuming and demand significant hands-on time for nucleic acid extraction and sample preparation.

Automated random-access platforms like the QIAGEN NeuMoDx Molecular System or the Abbott Alinity m System have been developed to combine the advantages of PCR analysis with on-demand sample processing, minimal hands-on time, and overall reduced turnaround time. Notably, most of these platforms offer the possibility to implement self-designed PCRs, so-called lab-developed tests (LDT), to extend the existing portfolio to cover pathogens that are not usually tested during routine work.

In 2022, a Mpox outbreak has affected so far 111 countries, with a total number of more than 85 000 cases to date.[8] Mpox virus (MPXV) is an enveloped DNA virus belonging to the genus of Orthopoxviruses and is genetically diverted into a Clade I present in the Congo Basin, a Clade IIa present in West Africa, and a Clade IIb from the recent outbreak.[9] Usually, only laboratories specializing in emerging and tropical diseases offer established and validated PCR diagnostics for MPXV. However, the recent outbreak has created a much broader need for rapid diagnostics.

2 | MATERIALS AND METHODS

2.1 | PCR set-up

Primers and probe, as previously published by Li et al.[10] (primer G2R_G-fw: 5′-GGAAAATGTAAAGACAACGAATACAG-3′; primer G2R_G-rv: 5′-GCTATCACATAATCTGGAAGCGTA-3′; probe G2R_G-probe: 5′-FAM—AAGCCGTAATCTATGTTGTCTATCGTGTCC—BHQ1-3′) (Eurofins Genomics) were added to NeuMoDx LDT Primer/Probe Stripes (QIAGEN) as a 6X primer/probe master mix to a single LDT well in the following order, volume, and concentration: 1 µL primer G2R_G-fw (10 µM to final 2.5 µM), 1 µL primer G2R_G-rv (10 µM to final 2.5 µM), 0.2 µL probe G2R_G-probe (10 µM to final 0.5 µM), and 1.8 µL nuclease-free water.

2.2 | Patient samples and positive controls

Diagnostic patient samples were collected at the University Hospital rechts der Isar of the Technical University of Munich between May 25, 2022 and June 10, 2023 using universal swabs (REST collection swabs; Nobel Bioscience) containing 2 mL clinical virus transport medium. Samples include swabs from skin lesions as well as the oropharynx.

Patient samples for bacterial cross-reactivity testing (eSwab 480CFA; Copan), collected between May 25, 2022 and August 12, 2022, were received from the Institute of Microbiology of the Technical University of Munich.

All samples were stored at 4°C and, if not otherwise indicated, pretreated with 50% viral lysis buffer (VLB) before MPXV-LDT-PCR.

2.3 | Generation of a positive control plasmid

A positive control plasmid was cloned using a synthesized fragment with overhangs (Thermo Fisher Scientific) (Supporting Information: Figure 1) of the Monkeypox virus isolate (Boende_DRC_2008, complete genome, sequence ID: KP849469.1, length: 197 422 bp) into a pcDNA3.1_Hygro vector via BglII and NotI. Plasmid concentration was analyzed using NanoDrop One (Thermo Fisher Scientific) and QX200 droplet digital PCR system (Bio-Rad) according to the manufacturer's instructions.

2.4 | Virus culture

Five hundred microliters collected patient samples medium was mixed with 2.5 mL of Dulbecco's modified eagle medium containing 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2 mM glutamine. Vero E6 cells were seeded and cultured for inoculation until cytopathic effect (CPE) was visible. Once cells detached, cells and supernatant were collected, vortexed, and subjected to three freeze-thaw cycles (−80°C/37°C). After removal of cell debris by centrifugation (10 min, 1000*xg*), the supernatant containing MPXV was aliquoted and stored at −80°C.

2.5 | Determination of infectious viral titers

Infectious viral titers were determined using a $TCID_{50}$ assay. In short, 1×10^3 Vero E6 cells were seeded into a flat-bottom 96-well plate. Twenty-four hours later, a 10-fold serial dilution of a cell culture-derived virus stock was added. To assess possible inactivation of VLB, the virus was preincubated for 10 min with indicated ratios of VLB before dilution. To ensure that the concentration of VLB did not cause any toxic effects, a dilution series was performed in parallel to assess cell viability using the CellTiter-Blue Cell Viability Assay (Promega). Only nontoxic dilution ratios were used for subsequent analysis. Cells were infected in four replicates per condition. After 3 days, cells were analyzed for CPE via microscopy, and the virus titer was determined by the Spearman−Karber method as previously described.[11]

2.6 | NeuMoDx MPXV-LDT-PCR

MPXV-LDT-PCR was performed on a NeuMoDx 96 and 288 Molecular System and with the LDT master mix, DNA (QIAGEN). Due to the sample pretreatment with VLB, we chose Lysis Buffer 2 (QIAGEN) with a reduced guanidinium chloride concentration for DNA extraction. NeuMoDx-LDT Assay definition file (ADF) was modified specifically from the preinstalled template TM DNA QUAL-0.0.1 according to the following parameters: C_t calling algorithm: second derivative; result type: qualitative; specimen type: transport medium; specimen aspirate volume (µL): 400; specimen mix volume (µL): 600; lysis conditions: 600 s, lysis buffer 2, 50°C (medium); target: SPC1 (sample process control); reporter: yellow (530/555); peak minimum cycle: 28; peak maximum cycle: 34; minimum end point fluorescence: 1000; minimum peak height: 100; target: MPOX; reporter: FAM (BHQ1), green (470/510); peak minimum cycle: 10; peak maximum cycle: 40; minimum end point fluorescence: 1000; minimum peak height: 100; PCR stage: activation (hold, 600 s, 95°C); PCR stage: cycle (cycle, 45 cycles); step denature: 5 s, at 95°C, no detect; and step anneal: 20 s, at 60°C, detect.

2.7 | Light-cycler 2.0 MPOX-PCR

For external quality assessment, DNA extraction was performed using QIAGEN QIAmp DNA Mini Kit (Qiagen), followed by a LightCycler 2.0 MPOX-PCR in a laboratory accredited for Mpox (DIN EN ISO 15189) at the Bundeswehr Institute of Microbiology in Munich according to the protocol Li et al.[10]

2.8 | Assessment of linearity performance

Performance of linearity was determined using a cell culture derived MPXV sample serial diluted in clinical virus transport medium in a 10-fold dilution.

2.9 | Analysis of the limit of detection (LoD)

LoD (95%-confidence) was determined by testing negative swab samples spiked with the positive control plasmid (Supporting Information: Figure 1) at decreasing concentrations and pretreated with 50% VLB. LoD was calculated by probit-analysis (MedCalc Software Ltd).

3 | RESULTS

3.1 | Optimization of probe concentration

A critical step in implementing a TaqMan PCR on new platforms is a sensitive fluorescence detection. To optimize probe concentration, we analyzed the fluorescence kinetics with increasing probe, and constant primer concentrations. A positive control plasmid (Supporting Information: Table 1 and Figure 1) was used to spike samples at a concentration of 5 × 10^6 copies/mL quantified by ddPCR. We determined 0.5–1 µM as optimal probe concentration range (Supporting Information: Figure 2) and thus prepared all following LDT primer/probe stripes with a probe concentration of 0.5 µM.

3.2 | Sample pretreatment for virus inactivation

Positive MPVX swab samples usually contain high viral titers and are very contagious. Sample pretreatment might reduce infectious viral titers but may influence subsequent PCR performance. To analyze this effect, we diluted MPXV samples with different concentrations of VLB and consecutively tested them by MPXV-LDT-PCR while determining the infectivity of VLB-pretreated samples by performing a cell-culture based $TCID_{50}$. VLB concentrations of ≤50% showed stable PCR performance and led to a >200-fold reduction of infectivity (Figure 1A). Therefore, all test samples were pretreated by adding 50% VLB before analysis.

3.3 | Primer/probe stripes open shelf-life stability

Preparation of primer/probe mix involves punctuating the sealing film of the LDT primer/probe stripes and the mix is exposed to air and subject to evaporation. Thus, we analyzed the open shelf-life at room temperature on the NeuMoDx. We prepared LDT primer/probe stripes and measured an aliquoted sample at different time points. All samples tested positive over a period of 14 days without significant variation in C_t values (Figure 1B) and 4/4 (100%) negative samples tested negative at Day 14.

3.4 | Assessment of performance

After the implementation of the assay on the NeuMoDx, we validated the PCR in accordance to the guidelines of the German Society of Virology and the In Vitro Diagnostic Regulation Annex 1 for in-house assays (Tables 1 and 2). In an external quality assessment using inactivated MPXV provided by the Robert-Koch-Institute, all samples (8/8) tested positive. Next, we compared the clinical performance of the MPXV-LDT-PCR to the results of a laboratory accredited for Mpox PCR by coanalyzing swabs suspected for Mpox with 100% agreement for positive (10/10) and negative (2/2) patient samples. To have a direct comparison between the two PCRs we analyzed cell-culture derived high-titer and low-titer MPXV samples on both systems (Supporting Information: Figure 3). Both PCRs determined the samples positive however with higher C_t values in the LightCycler 2.0 MPOX PCR, indicating a higher sensitivity of the MPXV-LDT-PCR. Further we validated the precision, linearity, intra- and interassay performance of the MPXV-LDT-PCR, demonstrating

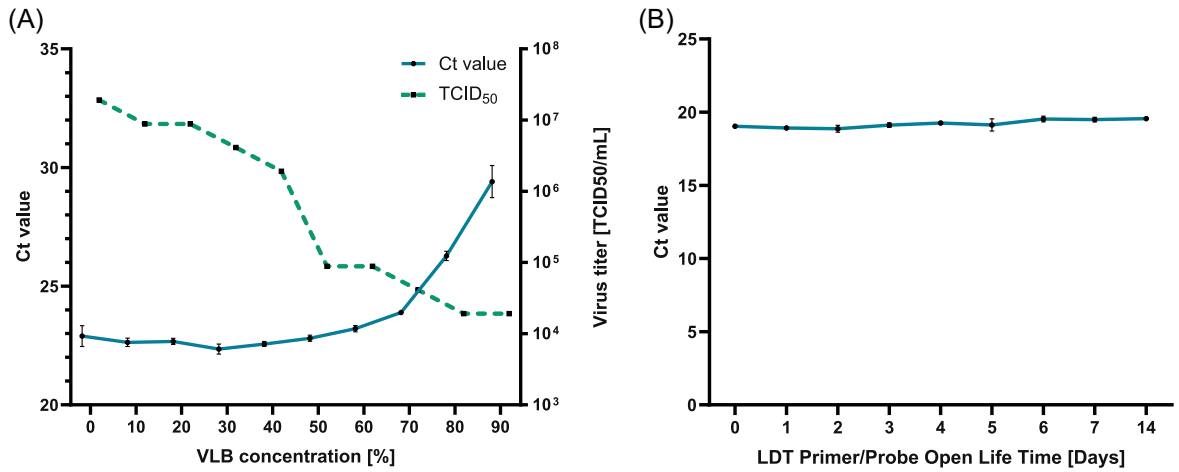

FIGURE 1 Sample pretreatment to reduce of infectious viral titers and assessment of reagent stability. (A) Swabs containing MPXV were diluted in ascending concentrations of VLB and subsequently analyzed in quadruplicates ($N = 4$) using MPXV-LDT-PCR (solid blue line) or by $TCID_{50}$ (dotted green line). (B) To analyze open shelf-life of LDT primer/probe stripes at room temperature, a positive sample was aliquoted, and the MPXV-LDT-PCR was performed in quadruplicates ($N = 4$) at indicated time points. Mean ± SEM of C_t values are shown. LDT, lab-developed test; MPXV, Mpox virus; VLB, viral lysis buffer.

high precision, a broad linear range and low C_t value variance within the days tested (Figure 2).

Finally, we analyzed the MPXV-LDT-PCR specificity and excluded cross-reactivity by testing swaps positive for pathogens that are frequently present in patients suffering from a MXPV infection or show a similar clinical presentation. Here, all 36 swab samples tested negative, indicating no cross-reactivity to these pathogens (Table 1).

Altogether, we performed 246 PCR reactions in our validation experiments (164 positive and 82 negative samples). Here, 164/164 results were positive (100%) and 82/82 negative (100%), indicating a sensitivity and specificity of 100%.

In a last step, we determined the LoD of the assay using negative swab samples spiked with decreasing concentrations of our control plasmid quantified by a QX200 droplet digital PCR system. All samples ($N = 12$) with a plasmid concentration of 500 GE/mL were tested positive (Table 2) and we calculated the LoD to be 262 GE/mL (95% CI: [118–405 GE/mL]).

3.5 | Clinical performance

Including the 10 positive samples from the external quality assessment, we identified 29 Mpox cases with a median C_t value of 17.67 (95% CI: [17.40–20.88]) (Figure 3).

4 | DISCUSSION

Here we describe the implementation and optimization of an MPXV-LDT-PCR on the random-access platform NeuMoDx Molecular System. Pandemic preparedness, as initiated by the WHO, includes the improvement of diagnostic workflows to enable faster responses to outbreaks of emerging diseases. This highlights the need for fast and flexible tests for pathogens not commonly included in routine diagnostics. While cartridge-based devices such as the QIAstat-Dx (QIAGEN) or the GeneXpert (Cepheid) allow for the analysis of single samples with minimal hands-on and turnaround times, automated plate-based solutions like the Roche Cobas, Altona AltoStar, or the Seegene Novaplex allow for high-throughput screenings but with turnaround times of 3–4 h. The NeuMoDx Molecular System combines a high-throughput sample processing with a short turnaround time and allow for the implementation of LDT assays.

After optimizing the ADF settings and probe concentrations, we demonstrated that pretreating patient samples with VLB (1:1) reduces the infectious viral titers and mitigates the risk of infection during sample processing while maintaining consistent MPXV-LDT-PCR performance. This is in line with a recommended pretreatment workflow for SARS-CoV-2 on the same platform.[3] A high stability of prepared LDT primer/probe stripes in terms of PCR performance over the course of 14 days allows for preparation of assay strips in advance and is especially important for on-call diagnostics and for infrequently used specialized assays.

The newly developed NeuMoDx MPXV-LDT-PCR showed a convincing clinical performance with a 100% agreement in all samples of our quality assessment. High specificity of this assay could be demonstrated by testing samples with pathogens that cause similar symptoms or are likely to be present in patients suffering from a MPXV infection. Moreover, we excluded cross reactivity for the Mpox pre- and post-exposition prophylaxis treatment, the modified vaccinia virus Ankara-based attenuated vaccine JYNNEOS/Imvanex.

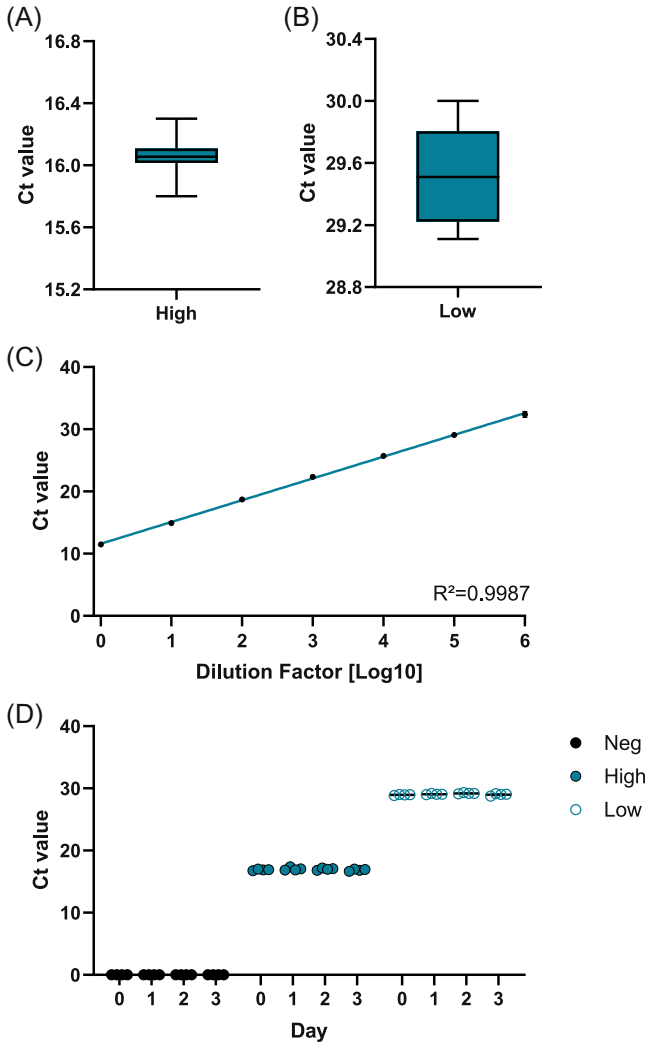

FIGURE 2 Precision, linearity, intra- and interassay performance of the MPXV-LDT-PCR. Different cell-culture-derived MPXV samples with variable MPXV concentrations were analyzed with the MPXV-LDT-PCR. Precision was analyzed using samples with a (A) high or (B) low virus concentration ($N = 12$). Box blots show median, interquartile ratio (box), and minimum to maximum (whiskers). (C) Linearity was analyzed using a serially diluted sample with a high MPXV concentration, tested in quadruplicates ($N = 4$). (D) Intra- and interassay performance was analyzed using an MPXV negative sample and positive samples with high and low MPXV concentrations. Negative results are indicated with a C_t value of 0. LDT, lab-developed test; MPXV, Mpox virus.

Finally, an MPXV-LDT-PCR LoD of 262 copies/mL demonstrated a high sensitivity compared to other commercial and LDT Mpox-PCR-assays which perform at a LoD-range within 57 and 14 495 GE/mL.[12,13] Notably, MPXV isolates of the current outbreak exhibit mismatches in the primer binding sites of both the forward and reverse primer.[14] A recent report indicates a fourfold increase of the LoD of the PCR described by Li et al.[10] on the current MPXV subvariants.[15] Since other mutations in the MPXV genome might further increase the LoD of this PCR,

TABLE 1 Quality and performance assessment.

External quality assessment (quality control samples)		Mpox reference samples	
		Positive	
NeuMoDx (MPXV-LDT-PCR)	Positive	8	
	Negative	0	
Samples total		8	
External quality assessment (method comparison)		LightCycler 2.0 MPOX-PCR	
		Positive	Negative
NeuMoDx (MPXV-LDT-PCR)	Positive	10	0
	Negative	0	2
Samples total		12	
Cross-reactivity for other pathogens		NeuMoDx (MPXV-LDT-PCR)	
		N	Negative
Mycoplasma genitalium		4	4
Chlamydia trachomatis		4	4
Neisseria gonorrhoeae		4	4
Herpes simplex virus 1		4	4
Herpes simplex virus 2		4	4
Human immunodeficiency virus		4	4
Modified vaccinia virus Ankara		4	4
Varicella zoster virus		4	4
SARS-CoV-2		4	4
Samples total		36	
Internal quality assessment			
		Positive	Negative
NeuMoDx (MPXV-LDT-PCR)	Positive	146	0
	Negative	0	44
Samples total		190	

Abbreviations: LDT, lab-developed test; MPXV, Mpox virus.

TABLE 2 Determination of the MPXV-LDT-PCR LoD.

Copies/mL	*N*	Positive	Negative
500	12 (100%)	12 (100%)	0 (0%)
250	15 (100%)	14 (93%)	1 (7%)
125	14 (100%)	12 (86%)	2 (14%)
62.5	11 (100%)	3 (27%)	8 (73%)
31.25	12 (100%)	3 (25%)	9 (75%)
15.625	8 (100%)	1 (13%)	7 (87%)

Note: LoD at 95% probability of detection: 262 copies/mL (95% CI: [118–405 copies/mL]).

Abbreviations: LDT, lab-developed test; LoD, limit of detection; MPXV, Mpox virus.

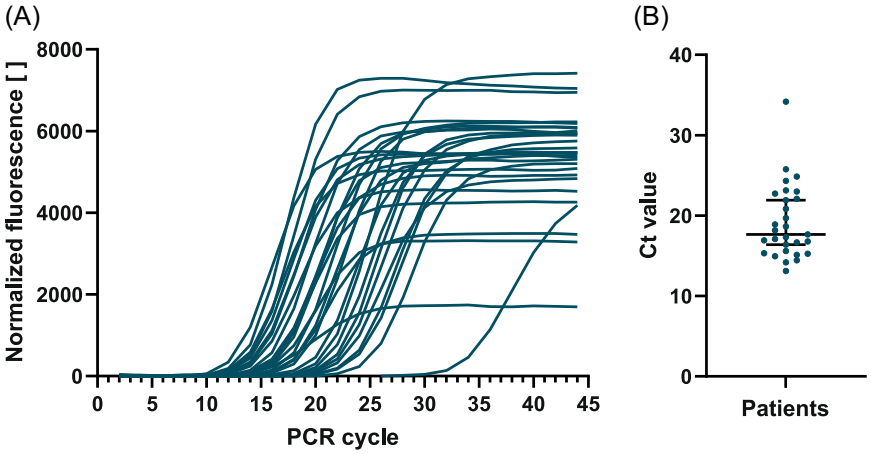

FIGURE 3 Clinical performance of the MPXV-LDT-PCR on MPXV-positive swab samples. (A) Normalized PCR curves of MPXV-positive swabs collected during this study. (B) C_t values of positive swabs, each dot represents one sample. $N = 29$, median $C_t = 17.67$ (95% CI: [17.40–20.88]). LDT, lab-developed test; MPXV, Mpox virus.

we recommend following current guidelines of local health authorities.

In summary, performing an MPXV-LDT-PCR on a fully automated random-access platform allows for highly specific and sensitive identification of MPXV-positive samples and is suitable for a broad range of laboratories as it does not require highly trained personnel. On-demand availability, minimal hands-on time and the short turnaround time of less than 2 h highlight the benefits of using LDT assays on random access platforms in the diagnostics of emerging pathogens such as MPXV.

AUTHOR CONTRIBUTIONS

Jochen Wettengel, Till Bunse, and Samuel Jeske performed the experiments. Julia Taeubner and Uta Goelnitz helped with analyzing the data and provided scientific advice. Roman Wölfel and Sabine Zange performed the external quality assessment via the LightCycler 2.0 MPOX-PCR. Figures, tables, and manuscript were prepared by Jochen Wettengel, Till Bunse, and Ulrike Protzer. Revising the manuscript critically was done by Samuel Jeske. All authors approved the final version of the manuscript.

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CONFLICTS OF INTEREST STATEMENT

Jochen Wettengel received speaker's fees from QIAGEN. Julia Taeubner and Uta Goelnitz work for QIAGEN, Strategic Lab Consultancy. The remaining authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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