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REVIEW

Digital PCR Applications in the SARS-CoV-2/COVID-19 Era: a Roadmap for Future Outbreaks

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SUMMARY The ongoing coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has led to a global public health disaster. The current gold standard for the diagnosis of infected patients is real-time reverse transcription-quantitative PCR (RT-qPCR). As effective as this method may be, it is subject to false-negative and -positive results, affecting its precision, especially for the detection of low viral loads in samples. In contrast, digital PCR (dPCR), the third generation of PCR, has been shown to be more effective than the gold standard, RT-qPCR, in detecting low viral loads in samples. In this review article, we selected publications to show the broad-spectrum applications of dPCR, including the development of assays and reference standards, environmental monitoring, mutation detection, and clinical diagnosis of SARS-CoV-2, while comparing it analytically to the gold standard, RT-qPCR. In summary, it is evident that the

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specificity, sensitivity, reproducibility, and detection limits of RT-dPCR are generally unaffected by common factors that may affect RT-qPCR. As this is the first time that dPCR is being tested in an outbreak of such a magnitude, knowledge of its applications will help chart a course for future diagnosis and monitoring of infectious disease outbreaks.

KEYWORDS RT-dPCR, RT-qPCR, SARS-CoV-2, COVID-19, ddPCR, diagnosis, quantification, viral load

INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic is still ongoing, affecting several countries around the world. Since the first report in December 2019 (1) from Wuhan, China, SARS-CoV-2 has spread worldwide and caused a public health crisis. Even though the emergency use of vaccines has been deployed worldwide, molecular diagnosis of the associated disease, coronavirus disease 2019 (COVID-19), remains important. This is mainly because not everyone in the world can or wants to be vaccinated, and vaccination strategies vary per country (2, 3). In addition, the emergence of new SARS-CoV-2 variants that can spread more easily or compromise vaccine protection has led to more COVID-19 cases (4, 5). Therefore, scientists have explored various nucleic acid amplification tests (NAATs) (6–9), including reverse transcription-quantitative PCR (RT-qPCR) (10–12) and isothermal amplification tests (such as clustered regularly interspaced short palindromic repeats [CRISPR] [13, 14] and loop-mediated isothermal amplification [LAMP] [15, 16], etc.) to rapidly detect and isolate infected patients. Despite the use of existing and the development of new NAATs, RT-qPCR remains the gold standard for the detection of SARS-CoV-2 and COVID-19 diagnosis.

Invented by Kary B. Mullis in the 1980s (17), PCR has undergone constant improvements, from the 1st-generation, gel-based/conventional PCR (17) to the 2nd-generation, quantitative/real-time PCR (qPCR) (18) and, finally, to the 3rd generation, digital PCR (dPCR) (19, 20). Among these, RT-qPCR is widely used for the detection and relative quantification of SARS-CoV-2 nucleic acid targets. However, this method suffers from false-positive results (FPRs) and false-negative results (FNRs) when quantifying less abundant targets (21–24). Moreover, RT-qPCR relies on a standard curve to relatively quantify nucleic acid targets (25). Therefore, a gap remains in the development of new techniques for the rapid and accurate diagnosis of SARS-CoV-2.

Digital PCR, the latest generation of PCR, has been developed to overcome the challenges of qPCR (20, 26–29). The working principle of dPCR is to divide a sample into thousands to millions of partitions so that each partition contains zero, one, or a few target copies. The partitions are then amplified by PCR. Unlike qPCR, where amplification occurs in a single bulk reaction, partitioning in dPCR ensures that thousands of reactions occur simultaneously, ultimately increasing the tolerance to inhibitors and practical sensitivity when detecting low copy numbers of nucleic acid targets in a complex background of other nucleic acids. After amplification, all positive partitions (with one or more targets at the beginning) are counted together with the negative partitions (without a target). The positive/negative ratio for all partitions is used to determine the original copy numbers of the nucleic acid target(s) in copies per microliter using Poisson statistics. This allows absolute quantification without the need for a standard curve. Several companies have developed commercial dPCR systems using this principle, as shown in Table 1 (26, 30–33). Since its advent, dPCR has been used for the detection of various pathogens (28, 32, 34), with new applications and methods being explored daily. Compared to qPCR, studies have shown that dPCR is associated with several advantages, including better sensitivity, precision, repeatability, and tolerance to inhibitors (27, 28, 30, 35). Despite these advantages, dPCR is not routinely used in clinical microbiology laboratories, likely due to high cost, low throughput, long sample turnaround time (TAT), technical complexity, and lesser availability of the

TABLE 1 Examples of commercially available dPCR systems and their properties

Company	System	No. of dyes	Vol/well (μL)	Type of partition	No. of partitions/well	Throughput(s) (no. of reactions/run)	TAT	Cost[a] (euros)
Bio-Rad	QX200	2	20	Droplet plate	20,000	96	8-h shift	~150,000
Bio-Rad	QX One	4	20	Droplet plate	20,00	Up to 480 (5 plates)	21 h for 5 plates	~400,000
Qiagen	QIAcuity One digital PCR	2 or 5	12 and 40	Nanoplate	8,500 or 26,000	96 or 24, depending on the nanoplate type	2 h	~60,000
Qiagen	QIAcuity Eight digital PCR	5	12 and 40	Nanoplate	8,500 or 26,000	Up to 1,248 (96-well plate), up to 312 (24-well plate)	8-h shift	~180,000
Stilla Technologies	Naica system	3 or 6	25	Microfluidic chips	20,000 or 30,000	Up to 12 or 48	<4 h	~120,000
Thermo Fisher Scientific	QuantStudio Absolute Q digital PCR	4	20	Microarray plate	20,000	16	~2 h 30 min/run	~30,000–40,000
Thermo Fisher Scientific	QuantStudio 3D digital real-time PCR system	3	14.5	Microfluidic chips	20,000	24	2–3 h/run	~30,000–40,000
RainDance Technologies	RainDrop Plus digital PCR System	2	25–50	Droplet chip	Up to 80 million	Up to 8 samples/run	8-h shift	~110,000
Targeting One	TD-1 digital PCR platform	2	30–50	Microfluidic chips	50,000	8 per 5 min to fill a 96-well plate	<3 h/8 samples	~120,000

[a]Approximated cost (in euros). The cost is approximated based on user experience and may vary between different regions. It does not account for consumables and extra instrumentation costs.

technology and reagents worldwide compared to qPCR. Applications that require a broad dynamic range, high throughput, and short sample TAT when screening numerous samples may benefit from qPCR. However, applications that require higher accuracy, good sensitivity in complex matrices, multiplexing, reproducibility, and superior precision may benefit from dPCR.

Taking advantage of these benefits, researchers have used dPCR to address and solve several problems caused by the SARS-CoV-2 pandemic. In this review, we focus on these applications and critically compare them to the recommended gold standard, RT-qPCR, where a reverse transcription step is added due to the RNA viral genome. The applications discussed will provide insight to future researchers in the event of another pandemic. Researchers can use this review article as a reference point to consider how dPCR can be used to diagnose emerging and/or reemerging infectious disease outbreaks.

SARS-CoV-2 GENOME AND DETECTION PROCESS

Understanding the viral genome of a novel coronavirus (nCoV) is critical for the development of effective molecular diagnostic tools. Among these tools, the design of new primer and/or probe (PP) sets that are highly specific and sensitive is critical to enable rapid screening, detection, isolation, and timely diagnosis of infected patients (7, 36, 37). Using SARS-CoV-2 as an example, we illustrate the process of the detection of an nCoV, as summarized in Fig. 1. In the event of an outbreak of an nCoV of unknown origin (such as SARS-CoV-2), a reference sequence should be obtained to help design specific primers to detect the emerging coronavirus. This reference sequence can be obtained directly from patient samples (e.g., Wu et al. [38] isolated SARS-CoV-2 from a patient sample and performed deep metatranscriptome analysis of the sample to generate the first full-length genome sequence of SARS-CoV-2 [WH-Human 1 coronavirus {GenBank accession no. MN908947}], which was later used to develop the first WHO-approved RT-qPCR assay by Corman et al. [39]) or through sequence alignments of publicly deposited sequences (Fig. 1A) in various domains such as the Global Initiative on Sharing All Influenza Data (GISAID) and the National Center for Biotechnology Information (NCBI). During sequence alignments, small base pair variations can be detected within different sequences. It is important to avoid such sites when designing primers and probes because they can lead to assay failure (36, 40, 41). After obtaining a consensus reference sequence, target genes can be identified (Fig. 1B) by comparing the nCoV genome with those of other closely related coronaviruses to identify conserved and nonconserved regions. For example, a comparison of the SARS-CoV-2 genome with other bat-associated SARS-related viral genomes reveals conserved regions such as open reading frame 1ab (ORF1ab), the envelope gene (E), and the nucleocapsid protein gene (N) that can be used for primer design (36). For confirmatory tests, the ORF1ab and N genes can be used, whereas for pan-sarbecovirus detection, the E gene is used, which is common to SARS-CoV-2 and other bat-associated SARS-related viral genes. Starting from these specific regions, primers are designed following the basic principles of specific primer/probe design software as reviewed elsewhere (36, 42). Using this workflow, several SARS-CoV-2 PP sets (Table 2 and Fig. 1B) have been designed by various public health laboratories around the world, including the China Center for Disease Control (CCDC), the U.S. CDC (43), Hong Kong University (HKU) (44), the Charité Institute of Virology, Universitätsmedizin Berlin (Charité) (Germany) (39), the National Institute of Infectious Disease Department of Virology III (NIID) (Japan) (45), the Institut Pasteur (IP) (France) (46), and the National Institute of Health (NIH) (Thailand) (47). Over time, the nCoV can mutate into new variants, e.g., the variants of concern (VoCs) observed in SARS-CoV-2 (Fig. 1D), that can be more lethal than the parental strain. Using the same reference sequence, common mutation sites on the new variants can be determined after sequence alignment. Since sequencing is costly, time-consuming, and impractical for analysis of all samples, new PP sets can be designed to screen for common/specific mutations of these variants in

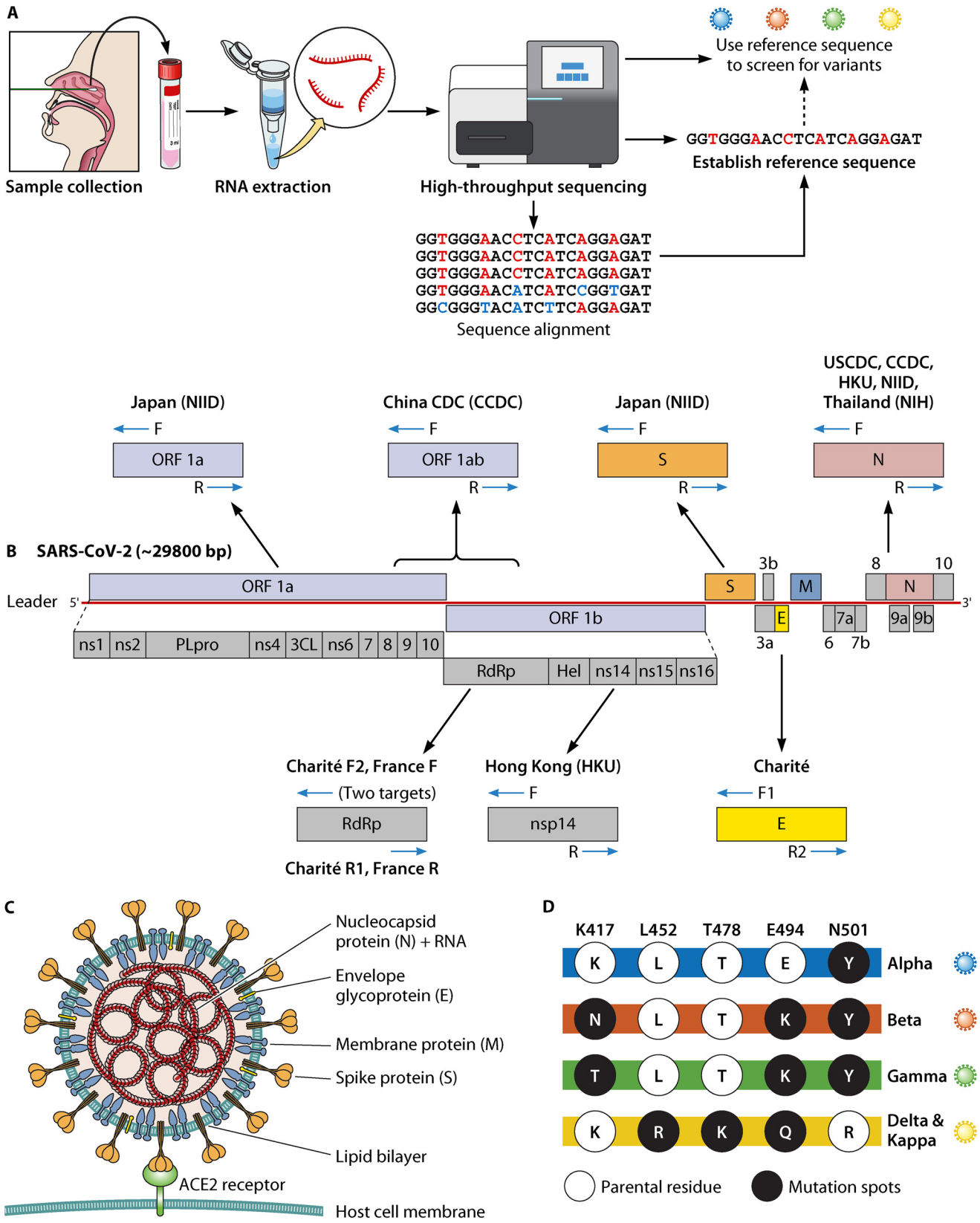

FIG 1 SARS-CoV-2 detection process. (A) Generation of a reference sequence from a COVID-19 patient's sample or by sequence alignment of publicly available sequences. The reference sequences can also be used to screen for emerging variants. (B) Target sites for developing RT-qPCR primers and probes, including targets commonly used by national public health institutions for RT-qPCR. (C) SARS-CoV-2 virion structure with locations of specific targets. (D) Common mutation spots associated with SARS-CoV-2 variants of concern.

TABLE 2 RT-dPCR-tested primers and probes from different institutes for SARS-CoV-2 diagnostics[a]

Institute	Target	Primer/probe name	Sequence (5′–3′)	GenBank accession no. of reference sequence	Nucleotide positions	Source or reference
CCDC (China)	ORF1ab	CCDC-ORF1-F	CCCTGTGGGTTTTACACTTAA	MN908947	13342–13362	CCDC (China)
		CCDC-ORF1-R	ACGATTGTGCATCAGCTGA	MN908947	13442–13460	CCDC (China)
		CCDC-ORF1-P	CCGTCTGCGGTATGTGGA AAGGTTATGG	MN908947	13377–13404	CCDC (China)
	N	CCDC-N-F	GGGGAACTTCTCCTGCTAGAAT	MN908947	28881–28902	CCDC (China)
		CCDC-N-R	CAGACATTTTGCTCTCAAGCTG	MN908947	28958–28979	CCDC (China)
		CCDC-N-P	TTGCTGCTGCTTGACAGATT	MN908947	28934–28953	CCDC (China)
HKU (Hong Kong)	ORF1b-nsp14	HKU-ORF1-F	TGGGGYTTTACRGGTAACCT	MN908947	18778–18797	44
		HKU-ORF1-R	AACRCGCTTAACAAAGCACTC	MN908947	18889–18909	44
		HKU-ORF1-P	TAGTTGTGATGCWATCATGACTAG	MN908947	18849–18872	44
	N	HKU-N-F	TAATCAGACAAGGAACTGATTA	MN908947	29145–29166	44
		HKU-N-R	CGAAGGTGTGACTTCCATG	MN908947	29236–29254	44
		HKU-N-P	GCAAATTGTGCAATTTGCGG	MN908947	29179–29198	44
Charité (Germany)	E	E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT	MN908947	26269–26294	39
		E_Sarbeco_R	ATATTGCAGCAGTACGCACACA	MN908947	26360–26381	39
		E_Sarbeco_P1	ACACTAGCCATCCTTACTGCGCTTCG	MN908947	26332–26357	39
CDC (USA)	N	2019-nCoV_N1-F	GACCCCAAAATCAGCGAAAT	MN908947	28287–28306	43
		2019-nCoV_N1-R	TCTGGTTACTGCCAGTTGAATCTG	MN908947	28335–28358	43
		2019-nCoV_N1-P	ACCCCGCATTACGTTTGGTGGACC	MN908947	28309–28332	43
	N	2019-nCoV_N2-F	TTACAAACATTGGCCGCAAA	MN908947	29164–29183	43
		2019-nCoV_N2-R	GCGCGACATTCCGAAGAA	MN908947	29213–29230	43
		2019-nCoV_N2-P	ACAATTTGCCCCCAGCGCTTCAG	MN908947	29188–29210	43
	N	2019-nCoV_N3-F	GGGAGCCTTGAATACACCAAAA	MN908947	28681–28702	43
		2019-nCoV_N3-R	TGTAGCACGATTGCAGCATTG	MN908947	28732–28752	43
		2019-nCoV_N3-P	AYCACATTGGCACCCGCAATCCTG	MN908947	28704–28727	43
NIID (Japan)	N	NIID_2019-nCOV_N_F2	AAATTTTGGGGACCAGGAAC	MN908947	29125–29144	45
		NIID_2019-nCOV_N_R2	TGGCAGCTGTGTAGGTCAAC	MN908947	29263–29282	45
		NIID_2019-nCOV_N_P2	ATGTCGCGCATTGGCATGGA	MN908947	29222–29241	45
NIH (Thailand)	N	WH-NIC N-F	CGTTTGGTGGACCCTCAGAT	MN908947	28320–28339	47
		WH-NIC N-R	CCCCACTGCGTTCTCCATT	MN908947	28358–28376	47
		WH-NIC N-P	CAACTGGCAGTAACCA	MN908947	28341–28356	47
Institut Pasteur (France)	ORF1a	IP2-F	ATGAGCTTAGTCCTGTTG	NC_045512.2	12690–12707	46
		IP2-R	CTCCCTTTGTTGTGTTGT	NC_045512.2	12780–12797	46
		IP2-P	AGATGTCTTGTGCTGCCGGTA	NC_045512.2	12717–12737	46
	ORF1b	IP4-F	GGTAACTGGTATGATTTCG	NC_045512.2	14080–14098	46
		IP4-R	CTGGTCAAGGTTAATATAGG	NC_045512.2	14105–14123	46
		IP4-P	TCATACAAACCACGCCAGG	NC_045512.2	14186–14167	46

(Continued on next page)

TABLE 2 (Continued)

Institute	Target	Primer/probe name	Sequence (5′–3′)	GenBank accession no. of reference sequence	Nucleotide positions	Source or reference
University of California (USA)	RdRp	RDRP_F	CCTCACTTGTTCTTGCTCGCAAAC	NC_045512.2	15341–15364	68
		RDRP_R	GAACCGCCACACATGACCA	NC_045512.2	15437–15456	68
		RDRP_P	ACGTGTTGTAGCTTGTCACACCGT	NC_045512.2	15370–15393	68
	N	N-ORF9_F	ATCACGTAGTCGCAACAG	NC_045512.2	28833–28851	68
		N-ORF9_R	AAGCAAGAGCAGCATCAC	NC_045512.2	28917–28934	68
		N-ORF9_P	AACTTCTCCTGCTAGAATGGCTG	NC_045512.2	28885–28907	68
	S/PBCS	S_PBCS_F	ACCCATTGGTGCAGGTATATGCG	NC_045512.2	23554–23576	68
		S_PBCS_R	GCACCAAGTGACATAGTGTAGGCA	NC_045512.2	23641–23664	68
		S_PBCS_P	CACTACGTGCCCGCCGAGG	NC_045512.2	23603–23622	68
	M	M-ORF5_F	CGCAATGGCTTGTCTTGTAGGC	NC_045512.2	26768–26789	68
		M-ORF5_R	GTACGCGCAAACAGTCTGA	NC_045512.2	26821–26840	68
		M-ORF5_P	TGTGGCTCAGCTACTTCATTGCT	NC_045512.2	26794–26816	68

[a]S/PBCS, polybasic cleavage site of the surface (S) glycoprotein.

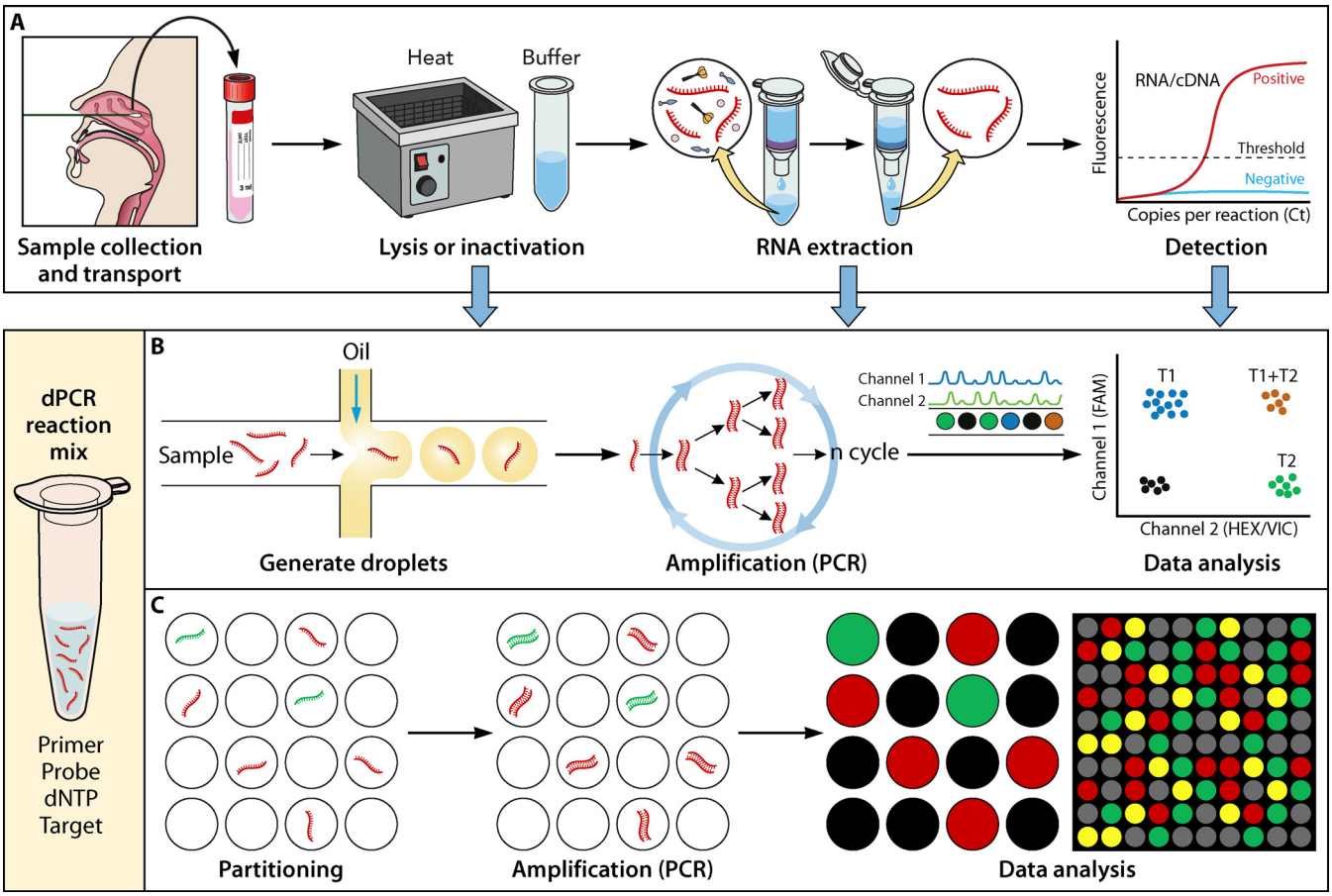

FIG 2 dPCR workflow and principles of ddPCR and cdPCR. (A) SARS-CoV-2 sample collection processing. Arrows point to specific points where samples can be used for detection. Samples can be detected as crude lysates after inactivation, as purified RNA after extraction, or after RT-qPCR for further analysis. Ct, threshold cycle. (B) Droplet digital PCR workflow. (C) Chip/chamber-based dPCR workflow. dNTP, deoxynucleoside triphosphate.

previously identified positive samples. Samples that show positive results for the specific variants can be further confirmed by sequencing.

BASIC PRINCIPLES AND WORKFLOW OF DIGITAL PCR

The history and basic principles of dPCR have been discussed in detail previously (20, 27, 28, 30, 48, 49). Briefly, dPCR was conceptualized in 1992 by Sykes et al., who referred to it as "limiting dilution PCR," which uses endpoint detection and Poisson statistics for the absolute quantification of nucleic acid targets (50). A few years later, in 1999, Vogelstein and Kinzler (19) introduced "digital PCR," a term still used today to describe a method in which a sample is diluted and distributed among individual reaction mixtures, called "partitions," prior to amplification and endpoint fluorescence detection. With the development of microfluidics for the effective division of the reaction mixture into very small partitions, dPCR as we know it today emerged and has undergone several transformations in the last 10 years (20, 31). For example, several chamber/chip-based dPCR (cdPCR) platforms and droplet-based dPCR (ddPCR) platforms have been developed that can generate thousands to millions of partitions from a single reaction mixture (30, 31, 51). Both forms, cdPCR and ddPCR, have been used to detect SARS-CoV-2 during the COVID-19 pandemic. The workflow from SARS-CoV-2 sample collection to detection by reverse transcription-digital PCR (RT-dPCR) is summarized in Fig. 2. Prior to detection by RT-dPCR, samples must be collected and processed in a manner similar to that for RT-qPCR, as summarized in Fig. 2A. The collected samples can be analyzed as crude lysates (52) or as purified nucleic acids directly by RT-dPCR. To save costs, samples can also be analyzed first by RT-qPCR, and discordant

or suspicious samples can then be further analyzed by RT-dPCR (21, 53). The decision to use RT-dPCR for detection and/or quantification depends solely on the nature of the sample (e.g., low-viral-load samples) and the overall goal of the detection process (e.g., absolute quantification, copy number variation, or gene expression, etc.).

After sample collection and processing, one of the two forms of dPCR can be used for the detection and quantification of SARS-CoV-2 in samples. In ddPCR (Fig. 2B), the reaction mixture is divided into thousands of nanoliter-sized droplets per well by a droplet generator using water-in-oil emulsion technology and microfluidics. The generated droplets are then transferred to a thermal cycler for endpoint PCR amplification. After amplification, the fluorescence of each droplet is measured using a droplet reader. Positive droplets show increased fluorescence, while negative droplets show only background fluorescence. Finally, a threshold is set to classify droplets as either negative or positive. In cdPCR (Fig. 2C), the reaction mixture is evenly distributed into separate, premade compartments on a chip (51). The chip is then PCR amplified to the endpoint using a thermal cycler. After amplification, a camera detects the fluorescence intensity in the partitions to distinguish between partitions with targets (positive partitions) and partitions without targets (negative partitions). In this way, a threshold can be set based on a normalized fluorescence intensity signal. Some cdPCR systems have the ability to also collect real-time data as amplification curves for each partition (54, 55).

After thresholds are set for both platforms, Poisson statistics is used to estimate the exact copy number of targets per partition (56). The results are automatically calculated by the platform-specific software and presented as copies per microliter of the reaction mixture. However, for most dPCR users, the number of copies in the starting material is of interest. For example, after running a 20-μL RT-dPCR reaction mixture using 4 μL of SARS-CoV-2 RNA as the template, the dPCR software may report a concentration of 10 copies/μL. Since there were 10 copies/μL in the whole PCR mixture and 20 μL of the PCR mixture was prepared, there were 200 copies ($10 \times 20 = 200$) of SARS-CoV-2 target RNA in the whole PCR mixture. This reaction mixture contained 4 μL of the original sample; hence, there were 50 copies/μL (200/4) of target RNA in the initial sample. For SARS-CoV-2, quantification results can be reported directly as copies per microliter or copies per reaction or can be converted to other forms such as copies per 1,000 cells (for respiratory samples) (57, 58), copies per milliliter (for plasma) (59, 60), and copies per gram (for stool samples) (61), as recently proposed (62, 63). Materials properly calibrated and quantified by RT-dPCR can be used for various applications, including the generation of RT-qPCR standard curves for relative quantification. Using this working principle, dPCR has been widely used to answer various nucleic acid research questions (27, 28, 48, 64, 65).

Currently, several companies have been able to develop dPCR systems that function differently, as shown in Table 1. Compared to qPCR, which has a constant sample throughput of 96, 384, or 1,356 samples/run, most dPCR systems, especially those based on chips, have a low sample throughput of $\leq$96 samples/run. Nevertheless, some companies like Bio-Rad and Qiagen have developed dPCR systems with higher throughput, comparable to that of qPCR. The sample turnaround time (TAT) is usually higher in dPCR than in qPCR, likely due to the additional steps for partitioning and detection at the endpoint. This TAT may be even higher if partitioning and amplification are done in separate instruments. Similar to qPCR, the reaction volume for dPCR often ranges from 20 to 50 μL. Unlike qPCR, where reactions occur in bulk, in dPCR, reaction mixtures are partitioned into thousands to millions of partitions, which theoretically enables dPCR to detect down to 1 copy of the nucleic acid target in a complex background of other targets. Although consumables for qPCR and dPCR are made by injection molding or hot embossing techniques, the dPCR chips and plates are usually costly, instrument specific, and not as readily available as those of qPCR. In addition, the reagents for qPCR are easily interchangeable between different systems, unlike dPCR, where the reagents and consumables are often instrument specific. These factors make dPCR more expensive than qPCR. Despite these facts, dPCR has its own

advantages that may make it beneficial for the detection of microorganisms, as summarized in this review.

SYNOPTIC OVERVIEW OF SARS-CoV-2 RT-dPCR APPLICATIONS

In this section, we analyze some of the applications of RT-dPCR that have emerged from the current SARS-CoV-2 pandemic. These applications can be used for future outbreaks or adapted for the diagnosis of current or emerging pathogens.

Validation of Primer-Probe Sets

Primers and probes are key components of any PCR assay, including dPCR. Since the beginning of the outbreak, several PP sets (Table 2 and Fig. 1B) for SARS-CoV-2 RT-qPCR detection have been developed by various public health institutions around the world. Due to the numerous available sequences of PP sets, it is difficult to decide which set to use (66). Therefore, an analytical comparison of the sensitivities, specificities, efficiencies, and detection limits of these PP sets for the detection of low-abundance targets is critical for any assay developed.

Studies have investigated the RT-dPCR performances of different primer-probe sets originally designed for RT-qPCR (21, 57, 67, 68), and their results are summarized in Table 3. In these studies, direct comparison with the gold standard, RT-qPCR, showed that the quantification cycle (C_q) value observed by RT-qPCR is significantly dependent on the sequences of the PP sets, whereas copy numbers determined by RT-dPCR are generally not affected by the sequences of the primers and probes (21, 67); RT-dPCR is capable of detecting low viral loads in samples even with suboptimal PP sets (21); and by developing a log-linear relationship between RT-qPCR C_q values and RT-dPCR copy numbers, a formula can be created for each assay to directly compare RT-qPCR C_q values to RT-dPCR copy numbers without having to retest samples (57, 69). Moreover, RT-dPCR can be used to validate novel PP sets and compare their efficiencies with those of existing PP sets (68). In these studies, both one-step and two-step RT-dPCR protocols were used. It was found that the efficiency of PP sets increased when a two-step RT-dPCR assay was used, as opposed to a one-step assay (57). At the same time, the PP sets with better amplification efficiencies may generate higher fluorescence amplitudes in the positive droplets under the same concentrations of primers and probes. It is also clear from the above-mentioned studies that both the reference standards and the clinical samples play an important role in evaluating the performance of the different PP sets. Currently, several companies have developed commercial reference materials that can be used to validate these assays. Additionally, in their studies, Zhou et al. (70) and Niu et al. (71) have shown that a reference candidate can be developed for head-to-head comparisons of different dPCR instruments and methods for SARS-CoV-2 detection. With the introduction of new variants, the analysis of novel PP sets that can readily detect the new SARS-CoV-2 variants will be of great importance.

Assay Development

Proper assay development is key to the success of detection/quantification using dPCR. dPCR assay development is highly dependent on the sample type (DNA or RNA), PP set (some primers are more efficient than others), and dPCR system (two-color or multicolor system). Developing a singleplex/duplex (one/two targets) assay is straightforward, whereas developing a multiplex assay for three or more targets is not, especially if a two-color detection system is used (26, 72, 73). Therefore, one strategy would be to purchase an already developed commercial assay if one is not familiar with multiplex dPCR assay development. However, the use of commercial assays can be challenging for users who wish to detect targets outside those included in the commercial assay. For this reason, some researchers have explored steps to develop RT-dPCR assays including multiplex assays for the detection and quantification of SARS-CoV-2 (53, 68, 74–76). Since most dPCR systems can detect at least two targets in separate channels (e.g., channel 1 [6-carboxyfluorescein {FAM}] and channel 2 [6-carboxy-2,4,4,5,7,7-hexachlorofluorescein {HEX}]), it is easy to develop singleplex (Fig. 3C) or

TABLE 3 Summary of results from analyses of multiple primer-probe sets for SARS-CoV-2 using RT-dPCR[a]

Sample type(s)	Primer-probe sets tested (target[s])	RT-dPCR type	Results	Reference
Serially diluted clinical sample	U.S. CDC (N1, N2, N3), Charité (E), HKU (N, ORF), CCDC (N, ORF)	2 step	All 8 PP sets could not significantly distinguish between FNRs and FPRs at low viral loads using RT-qPCR compared to RT-dPCR; the different characteristics of PP sets used in RT-dPCR can help in better optimization to avoid FPRs and/or FNRs; when handling samples with low viral loads, RT-dPCR is more sensitive than RT-qPCR regardless of the primer-probe sequence	21
N and E gene RNA standards	U.S. CDC (N1, N2), NIID (N), NIH (N), HKU (N), E-Sarbeco (E)	2 step	Unlike RT-qPCR, where the C_q value significantly varies depending on the PP sequences, RT-dPCR copy no. are unaffected by changes in PP sequences; the N gene PP set had a higher copy no. than those targeting the E gene	67
Synthetic RNA standards, clinical samples	U.S. CDC (N1), CCDC (ORF, N), IP2 (ORF1a), IP4 (ORF1b), HKU (ORF, N), E-Sarbeco (E)	1 step	Of the 8, Charité (E), IP2, and IP4 were the most efficient, precise, and sensitive PP sets for RT-dPCR; duplexing reduced the analytical efficiency and precision of IP2 and IP4; the LLODs of Charité (E), IP2, and IP4 were determined to be 4.4, 7.8, and 12.6 copies/reaction, respectively; this indicated that the Charité (E) PP set was the best of all 3, also with the highest analytical efficiency; using a reference standard, a formula can be generated to directly convert RT-qPCR C_q values to SARS-CoV-2 copy no. without the need for retesting samples by RT-dPCR	57
IVT RNA reference material, plasmid DNA, virion standard	N-ORF9 (N), U.S. CDC (N1, N2), RdRp (ORF1b), IP2 (ORF1a), E-Sarbeco (E), S-PBCS (ORF2), M (ORF5)	1 step	Developed 7 novel RT-dPCR assays that can be used to determine the transcriptional profile of SARS-CoV-2; the efficiency of the novel nucleocapsid PP set N-ORF9 was similar to those of U.S. CDC (N1, N2) PP sets for plasmid DNA, between those of U.S. CDC (N1, N2) PP sets for IVT RNA, and similar to that of U.S. CDC N1 for the virion standard; the efficiency of the novel PP set targeting RdRp/NSP12 was 1.20–1.28, compared to 1.11 for IP2; the efficiency of the E-Sarbeco (E) PP set (1.08) was similar to that of the IP2 set but may have been lower than those of the novel developed PP sets targeting neighboring genes (S-PBCS [ORF2], 1.32; M-ORF5, 1.51)	68

[a]IVT, *in vitro* transcribed; PP, primer-probe; LLODs, lower limits of detection.

duplex assays by using different probe labels (53, 72). However, if the dPCR system is a two-color system, one would need to use the higher-order multiplex technique to develop assays that can detect more than two targets, something that cannot be done by RT-qPCR. There are two main strategies for developing higher-order multiplex assays: probe mix and amplitude-based multiplexing (53, 72). In probe mix, two probes for different targets are mixed at different ratios (e.g., FAM/HEX ratios of 1:0 for target 1, 0:1 for target 2, and 1:1 for target 3), resulting in up to 8 droplet clusters (Fig. 3C, 3-plex) in the two-dimensional (2D) amplitude channel (26, 53, 72, 74). In amplitude-based multiplexing, the probes for different targets are labeled with the same dye but at different concentrations (e.g., 0.5× FAM for target 1, 1× FAM for target 2, 0.5× HEX for target 3, and 1× HEX for target 4) (26, 72–74). This also leads to differences in the target positions in both the one-dimensional (1D) and 2D graphs, resulting in up to 16 droplet clusters in the 2D channel (Fig. 3C, 4-plex). Importantly, amplitude multiplexing also allows the primer concentrations of the targets to be changed for optimal separation. Using these and other options (26, 72), multiplex assays can be developed to detect up to 6 or more SARS-CoV-2 targets (53, 68, 74–76). After assay development, it is often important to validate and analyze the performance of the developed assay. Common dPCR analysis parameters include optimization of PCR conditions (in the

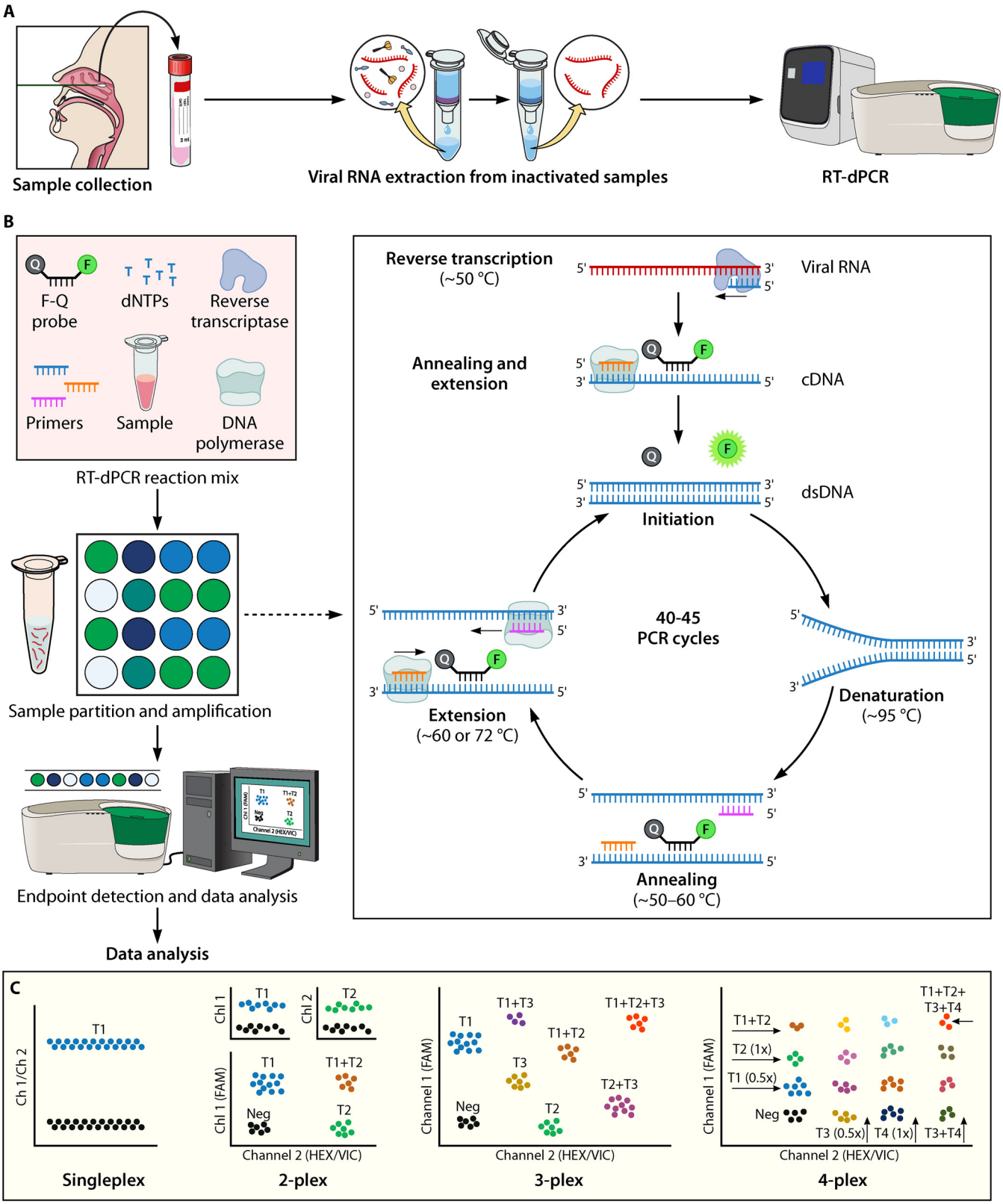

FIG 3 Multiplex assay development using a two-color dPCR system. (A) General workflow for the detection of SARS-CoV-2 using dPCR. (B) Assay mix composition and dPCR workflow, including reaction mix preparation, partitioning, PCR amplification to the endpoint, and data analysis. cDNA, complementary DNA; dsDNA, double-stranded DNA. (C) Schematic representation of expected results per well from a two-color dPCR system when one (singleplex), two (2-plex), three (3-plex), or four (4-plex) targets are detected. T1 to -4, positive targets 1 to 4; Chl, channel; Neg, negative droplets.

reverse transcription step and/or the annealing-and-extension step), determination of linear dynamic range, analytical efficiency, sensitivity, specificity, precision, and reproducibility tests of the developed assays (53, 57, 74, 75). When developing multiplex assays, researchers should strive to follow Minimum Information for Publication of Digital PCR Experiments (dMIQE) guidelines (77). It is also important to note that in addition to probe-based assays, intercalating dyes (e.g., SYBR green) can also be used for detection (78). However, the capabilities of these dyes for multiplex dPCR assay development are limited. Figure 3 provides an overview of the development of various dPCR assays, including higher-order multiplex assays, cycling conditions, and expected results, when one uses a two-color dPCR system. Generally, dPCR provides better capabilities to develop multiplex assays than qPCR.

Generation of Reference Materials

Reference materials have played an important role in the molecular diagnosis of SARS-CoV-2 since the beginning of the pandemic. The establishment of stable, well-characterized reference materials helps not only in the validation of test results but also in the analysis of assays, workflows, devices, and SARS-CoV-2 diagnostic kits (70, 71, 79–81). Quantification by qPCR requires a sample of a known quantity to establish a standard curve, which in turn is used for relative quantification (27, 30, 72). In contrast, dPCR can accurately quantify pathogens in absolute terms without the need for a standard curve. Throughout the pandemic, dPCR's advantage for absolute quantification has been used to develop commercial reference materials for SARS-CoV-2. For example, during the certification of the reference material "EURM-019 single-stranded RNA (ssRNA) fragments of SARS-CoV-2," dPCR was used to determine the RNA copy number. Another example is the "EDX SARS-CoV-2 standard" containing the E, N, ORF1ab, RNA-dependent RNA polymerase (RdRp), and S genes, each of which was quantitated at 200,000 copies/mL using dPCR. In the absence of a commercial assay, dPCR can still be used to support qPCR by assigning a copy number to a control sample, which in turn can be used to generate a standard curve for relative quantification (57, 82–84). This is particularly helpful in supporting standardization during the early stages of a large-scale pandemic where reference standards have not yet been developed or are taking a long time to be developed. For example, during the Wuhan outbreak, RT-dPCR was used to quantify reference RNA material that was used to generate an RT-qPCR standard curve for the relative quantification of wastewater samples collected in Wuhan postlockdown (85).

dPCR-quantified reference materials have also been found to give reproducible results across different laboratories, even when different dPCR instruments are used (70, 71). For example, an interlaboratory assessment of *in vitro*-transcribed (IVT) RNA reference materials was conducted by the National Institute of Metrology, China (NIMC), using 6 different platforms and 10 participating laboratories. The IVT RNA reference material contained three targets, the N, E, and ORF1ab genes of SARS-CoV-2, with known copy numbers. This IVT RNA reference material was analyzed by the 10 participating laboratories, and the results were found to be highly reproducible; i.e., the percent coefficient of variation (%CV) values between the laboratories were 17% for both the ORF1ab and E genes and 23% for the N gene. These results were also found to be acceptable as all three targets had a Z score of ≤ 2. The slight variations between different laboratories and instruments were also noted to arise from differences in PCR assays, partition volumes, and reverse transcription conditions (71). Similarly, Zhou et al. developed a reference standard from whole SARS-CoV-2 cultured in a cell and quantified by RT-dPCR (70). Unlike IVT RNA, the use of whole virus was thought to be beneficial, as it would include all targets and, hence, can be used to validate the analytical performances and claimed limits of detection (LODs) of different assays. Nine participating laboratories and six dPCR platforms were involved in this study to quantify the copies of N and ORF1ab genes from the developed reference standard. The results were found to be quite consistent across different laboratories and platforms, with mean %CVs of 43% for ORF1ab and 44% for the N gene (70). The higher %CVs than

those in the above-mentioned study might arise from the fact that in this study, different laboratories used their own assays, cycling conditions, input templates, and PP sets, etc., which may have increased the bias of the results. Nevertheless, the variance was found not to be significant, and the authors derived a consensus value to test the claimed LODs of 6 different NAATs. The test showed that most of the assays could meet or exceed their claimed sensitivity (LOD) and, hence, were found to be fit for use for the detection of SARS-CoV-2 (70). These two examples highlight the reproducibility of dPCR for the accurate quantification of reference materials and the development of reference materials that can be used to validate the analytical performances of other NAATs.

Environmental Monitoring

Recently, testing strategies have evolved from a purely patient-based approach to environmental monitoring of SARS-CoV-2. Environmental monitoring is a noninvasive method that plays a critical role in disease surveillance and can complement clinical tests in assessing population health (86, 87). However, due to many factors in the environment, including the presence of inhibitors, a highly sensitive and inhibition-tolerant method should be used for detection. Although RT-qPCR is widely used in environmental testing for SARS-CoV-2, comparative literature studies have shown that RT-dPCR is more sensitive in environmental monitoring, as summarized in Table 4. In addition, due to the partitioning of a sample that leads to increased tolerance to inhibitors, the CDC has also recommended the use of one-step RT-dPCR as the most sensitive technique for environmental sampling, as opposed to RT-qPCR (88). Using RT-dPCR, aerosol (86, 87, 89), surface (65, 90, 91), and wastewater (92–96) samples have been studied, and the amounts of SARS-CoV-2 in them were successfully quantified. In these studies, RT-dPCR was found to be sensitive, accurate, and highly tolerant to inhibitors. Generalized steps for the collection, processing, and detection of environmental samples using RT-dPCR are described in Fig. 4. To ensure successful detection, several factors must be considered when performing environmental testing. These factors include (i) sample transport after collection, including medium (e.g., viral transport medium) and/or cold chains that ensure viral integrity; (ii) sample processing, as due to the presence of inhibitors, stable controls (e.g., pepper mild mottle virus controls [92, 93]) should be extracted alongside test samples during environmental detection to avoid FPRs and FNRs; (iii) cost, as samples from large-population monitoring (e.g., wastewater treatment plants) are more cost-effective than monitoring small populations (e.g., in hospitals); and (iv) infectivity, as collected samples have the potential to harbor other infectious agents, so appropriate biosecurity should be considered. Despite the positive signal of SARS-CoV-2 from environmental samples by RT-dPCR, all these studies failed to establish the infectivity of the samples. This was possibly due to the lack of a biosafety level 3 (BSL-3) facility, as highlighted by Lv et al. (90). However, the infectivity and survivability of SARS-CoV-2 in complex environments can be demonstrated by various means, including cell culture (97). Since dPCR cannot directly determine virus infectivity, further studies, especially those devoid of cell culture, such as monoazide dyes (e.g., propidium monoazide [PMA]) and platinum compounds coupled with dPCR (Fig. 4D) (98–101), to determine the viability of positive SARS-CoV-2 samples will be critical in the future.

Viability PCR (v-PCR), compared to the gold-standard culture technique, is a rapid, sensitive, and specific technique for determining infectious microorganisms (98, 99, 102–104). This technique can even detect viable but nonculturable microorganisms. When using specific dyes such as PMA (Fig. 4D) during v-PCR, the dye is added to a sample (e.g., containing a mixture of dead and live cells). PMA then penetrates inactivated/dead cells (with damaged membranes) but not live cells (with intact membranes) and forms covalent bonds with the microorganism's DNA/RNA when exposed to intense light. Subsequent PCR amplification of the PMA-modified DNA/RNA template is inhibited by a combination of the removal of PMA-modified DNA/RNA during purification and nonaccessible PMA-modified templates by DNA polymerase. In

TABLE 4 Environmental monitoring of SARS-CoV-2 using dPCR[a]

Type(s) of surveillance	Sampling site(s)	Sample type(s)	Target gene(s)	Major findings and recommendations	Reference
Wastewater	WWTPs	Influent, primary settled solids	N1, N2	Detection of SARS-CoV-2 RNA from settled solids is more efficient by 1-step RT-dPCR than by 2-step RT-qPCR or RT-dPCR; settled solids offer a more sensitive approach in measuring SARS-CoV-2 RNA concns than influents	92
	Water resource recovery facilities	PGS, PCS	N1, N2	Despite lower inhibition of PCS in RT-dPCR than in RT-qPCR, PGS samples had similar quantifiable concns using both methods; PCS sampling offers an effective approach for SARS-CoV-2 quantification	93
	Commercial passenger aircraft and cruise ship	Cruise ship influent and effluent, aircraft wastewater tank	N1	SARS-CoV-2 could be detected in samples from both sites but with concns nearing the LOD; Samples should be tested in replicates to avoid false-negative results; RT-dPCR had a slightly higher detection frequency than RT-qPCR	94
	Hospitals, quarantine spots, WWTPs	Influent, effluent	ORF1ab, N, E	Sample concn helps detection; hospital sample RNA levels are higher, possibly due to fewer treatments than with WWTP RNA; RNA was detected from all sites; wastewater samples had some traces of chlorine	95
	WWTPs	Influent, grab, flow-weighted composite	N1, N2, N3	The N2 primer set was the most sensitive and, hence, was used for subsequent RT-dPCR; N2 sensitivity was different from those in other studies, possibly due to matrix recovery and specific workflows; levels of detected SARS-CoV-2 RNA ranged from 10^1–10^4 copies 100 mL^{-1}	173
	Campus where mass vaccination has taken place	Wastewater solids	N1	The SARS-CoV-2 detection rate in wastewater solids correlated with the no. of COVID-19 cases; vaccination led to a decrease in the rate of detection of SARS-CoV-2 shed in wastewater; the detection rate increased with an influx of visitors to the campus, meaning that RT-ddPCR wastewater surveillance can be a useful matrix for COVID-19 community surveillance at the subsewershed level	174
	WWTPs	Composite	N501Y, WT	RT-dPCR can detect, quantify, and discriminate between WT SARS-CoV-2 RNA and variants containing the N501Y mutation in wastewater samples; droplet partitioning helps detect SARS-CoV-2 RNA mutations even when present at low abundances; mutation detection in patients was related to detection in wastewater samples	96
Aerosol	Hospitals (patient, staff, and public areas)	TSPs, deposition, size segregated, field blanks	ORF1ab, N	SARS-CoV-2 aerosols were found mainly in two size ranges (submicrometer range and supermicrometer range); aerosolized SARS-CoV-2 RNA concns are low in isolation wards and ventilated patient rooms but high in mobile patient toilets; staff areas had high viral RNA levels, which were reduced later to undetectable limits after rigorous disinfection; airborne SARS-CoV-2 is undetectable in most public areas but detectable in crowded areas; use of PPE is recommended when visiting crowded areas, especially in hot spot regions	86
	2 regions of Italy	Air samples, virus particles	N1, N2	All air samples tested negative for SARS-CoV-2; RT-dPCR improved the LOD from 10 copies/μL by RT-qPCR to 0.625 copies/μL; in both northern and southern Italy, outdoor atmospheric concns of SARS-CoV-2 were low ($<$0.8 copies m^{-3}); virus-laden aerosol concns were generally $<$0.4 copies m^{-3}; measured concns are too low to transmit SARS-CoV-2 in the air	87

(Continued on next page)

TABLE 4 (Continued)

Type(s) of surveillance	Sampling site(s)	Sample type(s)	Target gene(s)	Major findings and recommendations	Reference
	13 locations within 10 cities in western Turkey	Particulate matter, TSPs, field blanks	N1	RT-dPCR was used as the definitive positivity test after samples were first screened by RT-qPCR and 2% agarose gel electrophoresis; of the 39 suspect samples (4 RT-qPCR-positive samples and 35 samples with a specific band) tested by RT-dPCR, 20 were found to be positive with an amplified copy no. above 10 copies/μL; max viral RNA concns were detected at 23 copies m^{-3} air; SARS-CoV-2 can be transported by ambient temps, especially in hot spot areas such as hospitals; use of PPE in outdoor hot spot regions is vital; viability tests are needed to determine the infectivity of aerosol samples	89
Surfaces	Homes, positive patient isolation rooms	Surface swab, passive sampler, bulk floor dust	N1	RT-ddPCR was more sensitive for detection than RT-qPCR and cdPCR; using RT-qPCR, cdPCR, and ddPCR, the avg detection rates were 88% for bulk dust samples, 55% for surface swabs, and lower for the passive sampler (19% for carpet, 29% for polystyrene); bulk floor dust has useful potential for virus outbreak surveillance	91
	Hospital	Medical waste, operator gloves	N, ORF1ab	Before nucleic acid testing, no RNA could be detected on wastes or operator gloves by RT-dPCR; in contrast, after nucleic acid testing, SARS-CoV-2 RNA was detected on operator gloves (avg of 19.54 copies/cm^2) and medical waste surfaces (avg of 22.84 copies/cm^2) before autoclaving; residual RNA (avg of 0.85 copies/cm^2) was still detectable on medical waste surfaces after autoclaving but found not to be infectious by cell culture; the concn increased from 0.85 to 3.36 copies/cm^2 when sterilized wastes were transferred with contaminated operator gloves; after sterilizing waste, operators should change their gloves or sterilize them before moving wastes to avoid contaminating them and possibly leaking pathogens from the lab; labs should routinely monitor their waste disposal techniques	22
	Hospital BSL-2 facility	Swabs from PPE, equipment, reception and transport facilities, other facilities	N, ORF1ab	No sample was positive by RT-qPCR; in contrast, 13 of 61 samples were positive for SARS-CoV-2 by RT-dPCR; outer operators' gloves had the highest contamination and are the potential source of most contaminated surfaces; RT-dPCR is advantageous in tracking environmental contamination compared to RT-qPCR	90
Multiple	Hospital	Air samples, surface swabs	Kit	The positivity rate of the air samples was higher than that of the surface samples; 5 environmental samples that previously tested negative by RT-qPCR were positive by RT-dPCR	65

[a]WWTPs, wastewater treatment plants; TSPs, total suspended particles; WT, wild type (WIV04/2019); LOD, limit of detection; PGS, postgrit solids; PCS, primary clarified sludge; PPE, personal protective equipment.

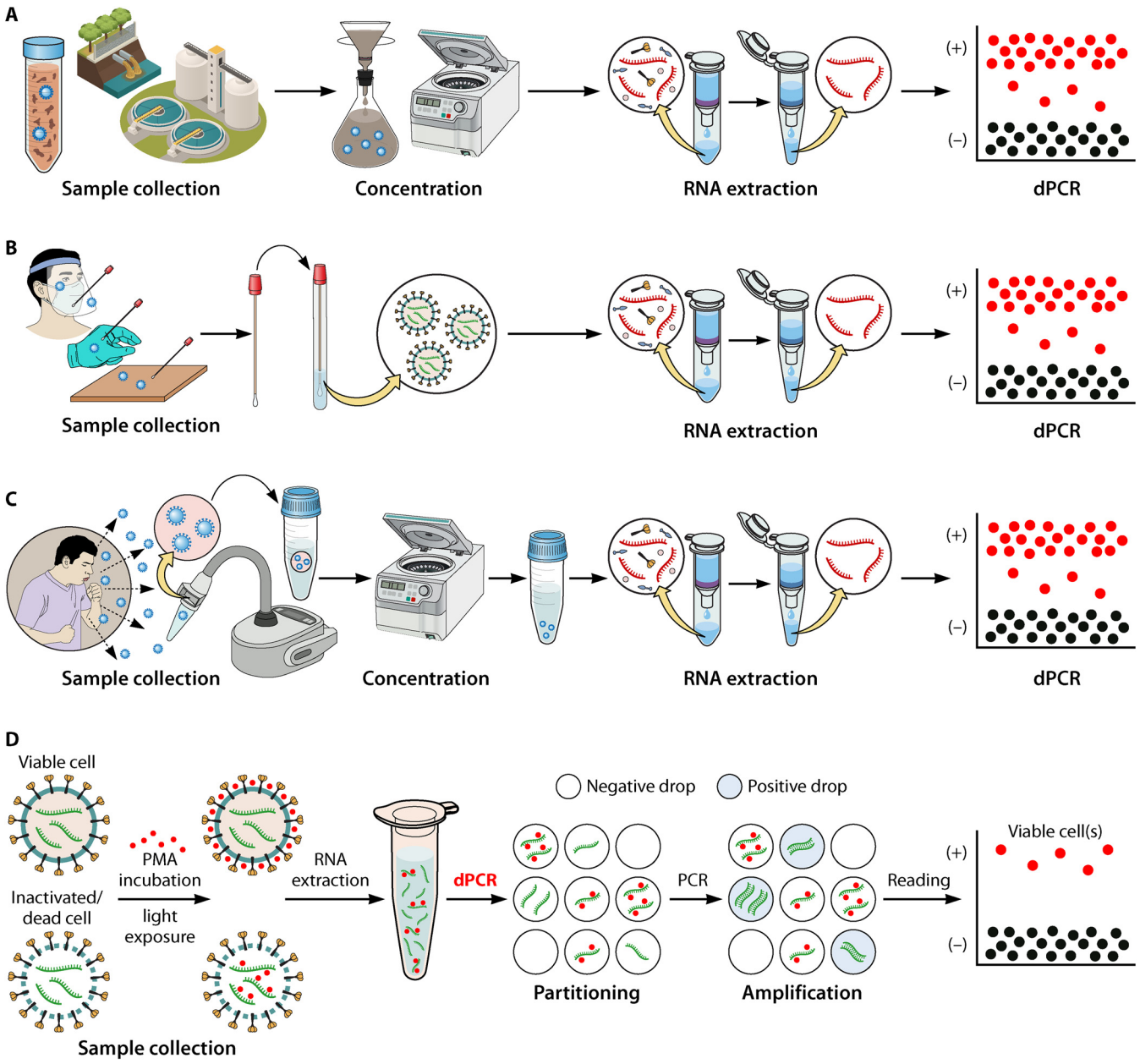

FIG 4 SARS-CoV-2 environmental sample detection using RT-dPCR and determination of viable cells using propidium monoazide (PMA)-coupled RT-dPCR. (A to C) Wastewater (92–95) (A), surface (65, 90) (B), and aerosol (86, 87) (C) sample collection, processing, and detection. (D) PMA-coupled RT-dPCR for the determination of viable cells (98, 99). The PMA dye enters inactivated/dead cells with compromised membranes, and after light treatment, PMA covalently modifies the RNA. Subsequent amplification (PCR) of PMA-modified RNA templates is inhibited, while PMA-free RNA is amplified, enabling the selective quantification of RNA from viable cells. (The figure was constructed based on the information from the above-mentioned references.)

contrast, PMA-free templates are not inhibited and amplify normally during PCR. In v-qPCR, this results in high or delayed C_q values if a mixture of dead and live cells is treated compared to a sample of only live cells (102). There will be no amplification if only dead cells are present. In v-dPCR, copy numbers are higher in samples with more live cells, as dead cells produce no amplification signal. Based on the results, cutoffs can be set to classify infectious or noninfectious agents. However, it is also important to note that v-PCR alone sometimes does not work because some microorganisms (such as coronaviruses) have intact membranes that are difficult to penetrate (102). In such cases, coupling v-PCR assays with surfactants such as Triton X-100 or sodium dodecyl sulfate (SDS) may prove beneficial in making the membrane more permeable to monoazide dyes (100, 102, 103, 105, 106). Following this principle, various SARS-CoV-2

viability RT-qPCR assays (107–109) have been tested on environmental, animal, and clinical samples to determine their infectivity. For example, an SDS-PMA-assisted viability RT-qPCR assay was recently established, which is similarly as effective as the gold-standard cell culture technique to detect infectious SARS-CoV-2 from environmental samples (102). In addition to monoazide dyes, platinum compounds can also be used to detect infectious SARS-CoV-2, as shown by Cuevas-Ferrando et al. (108). In their study, a platinum chloride ($PtCl_4$)-based viability assay demonstrated that infectious SARS-CoV-2 can be successfully isolated from complex samples, including environmental and clinical samples. The application of similar assays to dPCR will broaden its spectrum for the detection of SARS-CoV-2 and other microorganisms.

SARS-CoV-2 Sample Analysis

Since SARS-CoV-2 samples come from multiple sources and collection techniques are vast, it is important to analyze various parameters used to collect these samples and how they can be effectively detected using RT-dPCR. In this section, we discuss some of the conditions that were used to analyze SARS-CoV-2 samples using RT-dPCR, as summarized in Table 5.

Inactivation and direct/indirect quantification methods. Due to the infectious nature of SARS-CoV-2 (11, 110), inactivation of samples is used not only to protect medical laboratory personnel from infection but also to allow SARS-CoV-2 to be handled at lower containment levels (111–113). Although these methods are beneficial, they can affect the RNA levels of SARS-CoV-2 (114), which in turn can lead to FNRs (111). Therefore, a method that can directly quantify RNA levels, such as RT-dPCR, may play a critical role in evaluating these sample preparation methods. Currently, several inactivation methods are used to treat SARS-CoV-2 (114, 115), including the use of heat and lysis buffers (112). Furthermore, other methods have shown that SARS-CoV-2 RNA can be detected and quantified directly in crude lysates without upfront RNA purification after inactivation, highlighting the need for such methods, especially given the ongoing scarcity of extraction kits (52, 116–119). When using the direct approach, there is a possibility that unpurified cell lysates may inhibit the reaction efficiency and thus affect the quantification accuracy, leading to FNRs (52). In this regard, it may not be advantageous to perform detection in bulk as is the case with RT-qPCR. However, this can be circumvented with RT-dPCR by sample partitioning and sequestering inhibitors into individual partitions so that reactions can proceed normally, allowing accurate quantitation. High tolerance to inhibitors is one of the advantages of dPCR compared to qPCR (27, 28, 32, 120, 121). The impact of these methods on SARS-CoV-2 RNA, as determined by RT-dPCR, is summarized in Table 5. For example, while determining SARS-CoV-2 viral loads from crude lysates, Vasudevan et al. (52) showed that dPCR, but not qPCR, had high agreement with viral loads from purified RNA, possibly due to their hypothesized tolerance to inhibitors. This has also been observed in other studies (116, 119). Although the direct-quantification approach resulted in RNA copies similar to those after extraction, it has also been noted that copies can vary between different sampling media, possibly due to different volumes of the swab samples (116). It has therefore been suggested that additional treatment with proteinase K may be helpful. Marzinotto et al. (119) proved that this was possible by first pretreating the samples with proteinase K and then subjecting them to heating-cooling cycles before detection. This resulted in the detection of higher copy numbers of SARS-CoV-2 RNA using the direct method than with upfront RNA extraction (119). Furthermore, RT-dPCR can be used to show how different inactivation methods can affect SARS-CoV-2 copies, as shown by Chen et al. (111). In this study, three inactivation methods, (i) incubation with TRIzol LS reagent for 10 min at room temperature, (ii) heating in a water bath at 56°C for 30 min, and (iii) high-temperature treatment (autoclaving at 121°C, boiling at 100°C, and heating at 80°C for 20 min each), were tested by dPCR. Compared with the original group, the copy numbers of the N and ORF1ab genes decreased by 47.54% and 39.85% for TRIzol, 48.55% and 56.40% for 56°C, and 49.96% and 65.96% for 80°C, respectively, and almost no viral RNA was detected after treatment at 100°C and 121°C

TABLE 5 SARS-CoV-2 sample analysis using RT-dPCR

Type of sample analysis	Method(s)/sample type(s)[a]	Major finding(s)[b]	Reference(s)
Inactivation methods	TRIzol, heating, boiling	Heat inactivation resulted in reduced SARS-CoV-2 RNA copies compared to the original sample, which may cause FNRs in weakly positive cases; compared to heat treatment, TRIzol is recommended as it had the smallest effect on RNA copy no.	111
Crude vs purified RNA	Extracted RNA, pretreated RNA	Compared to automated RNA extraction, a direct method (pretreatment with proteinase K and a heating-cooling cycle) for amplification yielded high viral RNA levels	119
	Heat-inactivated RNA, extracted RNA	Generally, direct detection after heat inactivation yielded equal RNA copies compared to the extracted RNA	116
	Purified RNA, crude lysates	Without upfront RNA extraction, RT-dPCR can robustly detect low viral loads in nasopharyngeal swabs in UTM; compared to RT-qPCR, RT-dPCR crude lysate results show high concordance with purified RNA viral load measurements; the crude lysate has higher sensitivity and robustness when detected by RT-dPCR than with purified RNA as the input	52
Viral load	Sputum, throat swab, nasal swab	Viral load was highest in sputum samples, followed by throat swabs and nasal swabs; viral loads are higher in early and progressive stages of COVID-19 than in recovery stages	69
	Pharyngeal swab, stool, blood	RT-dPCR can be used to monitor patient treatment and accurately measure low viral loads compared to RT-qPCR; viral loads were highest in pharyngeal samples, followed by stool samples, and lowest in blood samples	129
	Plasma	The presence of SARS-CoV-2 RNA in plasma can be associated with critical illness in COVID-19 patients; an increase in the viral load concn in plasma increases the association strength; an increased viral load in plasma was correlated with key signatures of uncontrolled viral replication in COVID-19 patients; detection of viral load and RNAemia can help identify COVID-19 patients at risk of clinical deterioration, which can help assess treatment responses and predict disease outcomes	59, 131
	Nasopharyngeal swabs, anal swabs, saliva, blood, urine	Mean viral loads and positivity rates were highest in nasopharyngeal swabs, followed by anal swabs, saliva, blood, and urine specimens	130
Collection methods	Nasopharyngeal swab	Rotation of swabs does not increase RNA concns compared to in-out sampling quantified by RT-dPCR; comparison by ethnicity reveals that discomfort and nucleic acid recovery are highest in samples from Asian individuals compared to samples from white individuals, consistent with differences in nasal anatomy	134
	Nasopharyngeal swab	Quantification of human DNA levels demonstrated that suboptimal biological sampling can cause false-negative COVID-19 results; there were low DNA levels in suspected false-negative cases (median, 3,409 human cells/μL) that were later found to be positive compared to the control group (median, 5,539 human cells/μL); measurement of DNA levels presents a stable biomarker for sampling quality	58
	Plasma	Quantification of SARS-CoV-2 RNAemia by RT-dPCR presents a promising prognosis biomarker in COVID-19 patients; the presence of SARS-CoV-2 in plasma signifies severe disease; disease severity increases with increasing viral loads	60
	Saliva	Saliva presents significant advantages over other SARS-CoV-2 samples; a saliva-based testing pipeline was developed for both RT-qPCR and RT-dPCR; saliva has sensitivity similar to that of nasal swabs when testing community and hospital samples; combined with dPCR, saliva has potentially higher sensitivity in detecting low viral loads that are missed by traditional testing methods	133
Preservation, extraction, and quantification	Stool	Three preservative approaches in combination with three extraction methods were compared by both RT-qPCR and RT-dPCR; RT-dPCR and RT-qPCR assays targeting the N1 gene are reliable for estimating SARS-CoV-2 RNA; the use of preservatives is important compared to the commonly used stool storage without preservatives; the Zymo DNA/RNA preservative combined with the QIAamp viral extraction kit yields more detectable RNA in both RT-dPCR and RT-qPCR	61

[a]Sample type refers to the sample type used for sampling or viral load analysis, while method refers to the method used for sample inactivation or collection.
[b]UTM, universal transport medium.

for 20 min. This showed that heat treatment affects the amount of detectable RNA and that TRIzol may be better than heating for the inactivation of samples. This study also showed that RT-qPCR was not effective in detecting false-negative samples (111). A more comprehensive study, including direct lysis buffers such as Buffer AVL, multiple clinical samples, and reference standards, would be important to evaluate the effects of sample pretreatment on RNA yield and quantification.

Viral load quantification. The SARS-CoV-2 viral loads in COVID-19 patients may vary due to various factors such as collection methods, disease progression, severity, and sample type (7, 60, 110, 122). Recently, it was found that RT-qPCR C_q values cannot be directly interpreted as SARS-CoV-2 viral loads without relative quantification (62, 123). In addition, it has been shown that SARS-CoV-2 C_q values are not reproducible across laboratories and platforms even when using the same proficiency testing specimens (123–127). The C_q values may vary by $>$10 (124) and by $>$1,000-fold in copies per milliliter for a given C_q value when using relative quantification (125). This presents a challenge as there is no international, commutable, SARS-CoV-2 RT-qPCR quantitative reference standard material that can be used to harmonize assays and instruments across multiple testing laboratories (128). In contrast, RT-dPCR can overcome this limitation due to absolute quantification without relative quantification. Furthermore, SARS-CoV-2 copy numbers have been found to be quite reproducible across different platforms and laboratories when the same reference material was used (70, 71). Therefore, accurate and sensitive direct viral load quantification by RT-dPCR can be used to determine factors that may affect SARS-CoV-2 viral loads from different samples. In addition, monitoring viral loads in patients can help clinicians and researchers understand various factors such as COVID-19 disease progression and SARS-CoV-2 RNA concentrations in different sample types (69, 129). RT-dPCR has been used to measure SARS-CoV-2 viral loads in various types of clinical specimens, including sputum, throat swabs, pharyngeal swabs, stool, blood/plasma, urine, and nasal swabs, from COVID-19 patients during hospitalization (60, 69, 129, 130). These studies reported that the viral load is higher in sputum than in throat and nasal swabs (69) and in pharyngeal swabs than in stool, anal swabs, saliva, blood, and urine samples (129, 130). After viral load analysis, an appropriate biomarker can be selected to directly monitor the patient's viral load. For example, since sputum was found to have a high viral load, it was used to monitor the variation in viral loads in different patients during hospitalization. This indicated that the viral load of SARS-CoV-2 is generally higher in the early and progressive stages of COVID-19 and lower in the recovery stages (69). In addition, when plasma was used, SARS-CoV-2 viral loads were found to be significantly higher in COVID-19 patients in intensive care units, wards, and outpatient samples (59, 131). The concentration was also higher in patients who died from COVID-19 than in those who survived (59). Further analysis correlated increased viral loads in plasma with key signatures of dysregulated host responses (such as increased levels of chemokines and the activation of NK cells, etc.), all of which play an important role in the uncontrolled replication of SARS-CoV-2 in the pathogenesis of COVID-19 (59). This suggests that the viral RNA in plasma is more dependent on disease severity than on the time from disease onset (59, 60, 131).

Improper sample collection, especially by nasopharyngeal swabbing, can lead to false-negative results (130). SARS-CoV-2 viral loads can be determined by RT-dPCR to evaluate sample collection and handling techniques. For example, recent studies have begun to examine fecal shedding of SARS-CoV-2 RNA as a new indicator of COVID-19. However, few studies exist that explain the techniques for the collection, preservation, and extraction of these samples for the robust detection and quantification of SARS-CoV-2. Using both RT-qPCR (due to accessibility) and RT-dPCR (due to absolute quantification), Natarajan et al. (61) investigated three preservation approaches (using the OMNIgene-Gut kit, the Zymo DNA/RNA shield kit, and the commonly used storage without a preservative) in combination with three extraction kits (MagMAX viral/pathogen kit, QIAamp viral RNA minikit, and Zymo Quick-RNA viral kit) for the detection of SARS-CoV-2 RNA in stool samples. Using a series of reference standards and matrix

recovery controls, the study results revealed that the use of preservatives improves the yield of SARS-CoV-2 RNA in both standardized and nonstandardized samples, the Zymo DNA/RNA preservative in combination with QIAamp extraction provides the optimal RNA yield for detection by RT-qPCR and quantification by RT-dPCR, and bovine coronavirus (BCoV) was validated as a reliable matrix recovery control that does not interfere with the detection of SARS-CoV-2 RNA in stool samples. The authors (61) also concluded that the study could provide a "roadmap" for future fecal coronavirus testing. Another study also found that human DNA levels may serve as a stable biomarker of sampling quality as these levels were generally lower in false-negative RT-qPCR samples, possibly due to suboptimal sampling rather than RT-qPCR itself (58). In their study involving multiple samples, Gniazdowski et al. (132) agreed with Kinloch et al.'s (58) assessment of the inclusion of stable human reference controls during testing. This study reemphasizes the need to add a stable human reference control, e.g., RNase P or RPP30, to all tests, as is the case with most SARS-CoV-2 RT-qPCR tests. A comprehensive study following up on patients who have low viral loads of these stable human biomarkers in different sample types and who test negative for SARS-CoV-2 by RT-qPCR/RT-dPCR would be beneficial to support the study by Kinloch et al. (58). Other studies have also analyzed saliva (133), plasma (60), and nasopharyngeal swabs (58, 134) using RT-dPCR, as summarized in Table 5. Collectively, these studies demonstrate that when stable biomarkers and reference standards are used, dPCR's advantage of absolute quantification can be used to determine which method (collection, preservation, or extraction, etc.) maximizes RNA recovery and whether the quality of the collected samples could be the plausible cause of false-negative results in suspect patients.

Mutation Detection

RNA viruses tend to mutate frequently. These mutations can be the cause of new disease variants that can be more infectious or even lethal. Mutations in the spike protein of SARS-CoV-2 have resulted in new variants, including the variants of concern (Fig. 1D) Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2), Gamma (P.1.), and Omicron (B.1.1.529), which have the ability to spread rapidly, cause severe to mild disease, evade detection by diagnostic tests, reduce sensitivity to drugs, and evade natural or vaccine-induced immunity (135–139). Also, viral mutations pose a potential challenge to nucleic acid testing methods, leading to the occurrence of FNRs (140). For example, some assays have reported S gene dropout or S gene target failure (SGTF) in RT-qPCR and RT-dPCR assays targeting SARS-CoV-2's S gene due to mutations caused by the Alpha and Omicron variants (139, 141–144). Although specific mutations might compromise the sensitivity of the assays, it has been suggested that such assays may be used as a surrogate marker for the variants pending confirmation by sequencing (139, 143, 145). Sequencing remains the gold standard for the detection of new variants and mutations for SARS-CoV-2. Since sequencing is costly, not readily available, and impractical for the screening of all samples, assays can be developed for rapid screening by PCR. Once novel mutation sites are identified, specific primers for these sites can be developed for routine screening by sensitive techniques such as dPCR and qPCR. Of note, by partitioning of the sample, dPCR has the ability to detect mutations present at low frequencies in a background of wild-type (WT) RNA, which provides an advantage over qPCR (96, 144).

Using RT-dPCR, Wong et al. (146) developed a duplex assay to determine the transmission of bat-like SARS-CoV-2 proline-arginine-arginine-alanine (PRRA) variants (SARS-CoV-$2_{\Delta PRRA}$) in COVID-19 patients. They found that SARS-CoV-$2_{\Delta PRRA}$ occurs naturally in humans and remains transmissible in COVID-19 patients. Similarly, Heijnen et al. used a multiplex RT-dPCR assay to quantify the mutant N501Y and the wild-type sequence (WIV04/2019) in wastewater samples (96). The results showed that the N501Y mutation was detected in samples collected in Amsterdam and Utrecht, Netherlands. They also correlated the first weeks of detection of the Alpha variant B.1.1.7 in patient samples in the United Kingdom with the first detection of N501Y in wastewater from Amsterdam

and Utrecht (96). To detect the same variant, Perchetti et al. developed a novel RT-ddPCR assay to detect four mutations associated with the Alpha variant, including the important N501Y mutation (144). N501Y is also found in the Beta variant. Using this assay, 1,035 patient samples were examined, and all four mutations (a deletion at amino acids [aa] 69 and 70 [ACATGT], a deletion at aa 145 and 52 [TTA], an S982A mutation [GCA], and an N501Y mutation [TAT]) were found in two samples (144). It was also found that by RT-qPCR, inefficient binding to the wild-type strand cannot be detected, whereas by RT-dPCR, it can be detected because the template strands are amplified in separate droplets (144).

Building on the same assay (144), Mills et al. sought to further develop the assay to be capable of identifying key mutations in the spike region of SARS-CoV-2 and applied it to rapidly and accurately detect the recently emerged Omicron variant in clinical specimens (142). In this study, 390 clinical specimens were first screened using the assay, and the results matched 99% with whole-genome sequencing (WGS) results. Furthermore, 2,657 positive clinical specimens were screened by the TaqPath assay, and 16 specimens that showed SGTF along with 5 non-SGTF specimens were tested by the developed RT-dPCR assay to screen for the Omicron variant. The results showed that all 5 non-SGTF specimens and 1 SGTF specimen were Delta positive when screened by RT-dPCR and confirmed by sequencing. However, the remaining 15 SGTF specimens were all confirmed to be Omicron positive, with an RT-dPCR response indicating the presence of mutations at SARS-CoV-2 positions 484 and 501 not observed in other variants. The results of the 15 Omicron samples were also confirmed by sequencing results. This study also recorded the first Omicron case in Washington State (142). Finally, during the development of CoV2-ID, a 5-plex RT-qPCR assay capable of detecting the D614G mutation, RT-dPCR was used to delineate the quantification and detection limits of the assay (140). Taken together, these reports highlight the potential of RT-dPCR for validating RT-qPCR assays to detect mutations and also for developing novel RT-dPCR assays to detect and continuously monitor SARS-CoV-2 variants. Although sequencing is required to specifically identify a novel variant, existing dPCR assays could provide evidence that the mutation occurred in the region of the assay as observed with S gene target failure/dropout (142, 144). In the case of a mutation in the assay region, the PCR efficiency is lower, resulting in a lower fluorescence amplitude of positive partitions that may be clearly visible in the partition/droplet readout. This may indirectly indicate the presence of new variants. However, confirmation and identification of the mutation would still need to be done by sequencing.

Diagnosis

Digital PCR has been used to diagnose COVID-19 patients, with several benefits compared to the gold standard, RT-qPCR. In this section, we summarize some of these applications (as shown in Fig. 5) while comparing them to the commonly used RT-qPCR.

Sensitivity of detection in RT-qPCR-negative COVID-19 samples. RT-qPCR is the current gold standard for the diagnosis of SARS-CoV-2; however, this method has been associated with FPRs and FNRs, especially in low-viral-load samples (21, 24, 147, 148). This implies that a technique with better reproducibility at lower target concentrations can be used as a confirmatory test or replacement option for RT-qPCR, especially in analyzing low-viral-load samples. Due to its advantages, RT-dPCR has been used by several researchers to test COVID-19 samples that were RT-qPCR negative, as summarized in Table 6. For example, Suo et al. (147) compared the sensitivity of RT-dPCR with that of RT-qPCR for detecting SARS-CoV-2 in 63 suspected and 14 convalescent COVID-19 patients. Of the suspected cases, 21 were positive and 42 were negative by RT-qPCR. In contrast, for RT-dPCR, 49 positive, 4 suspected (results between 0 copies/reaction and the LOD of ddPCR for each PP set), and 10 negative results were recorded. Of the 49 RT-dPCR-positive cases, 47 were diagnosed as SARS-CoV-2 positive by ground-glass opacity (GGO) images of chest computed tomography (CT) scans, which were also confirmed by RT-qPCR within 2 to 10 days of hospitalization. The other two were

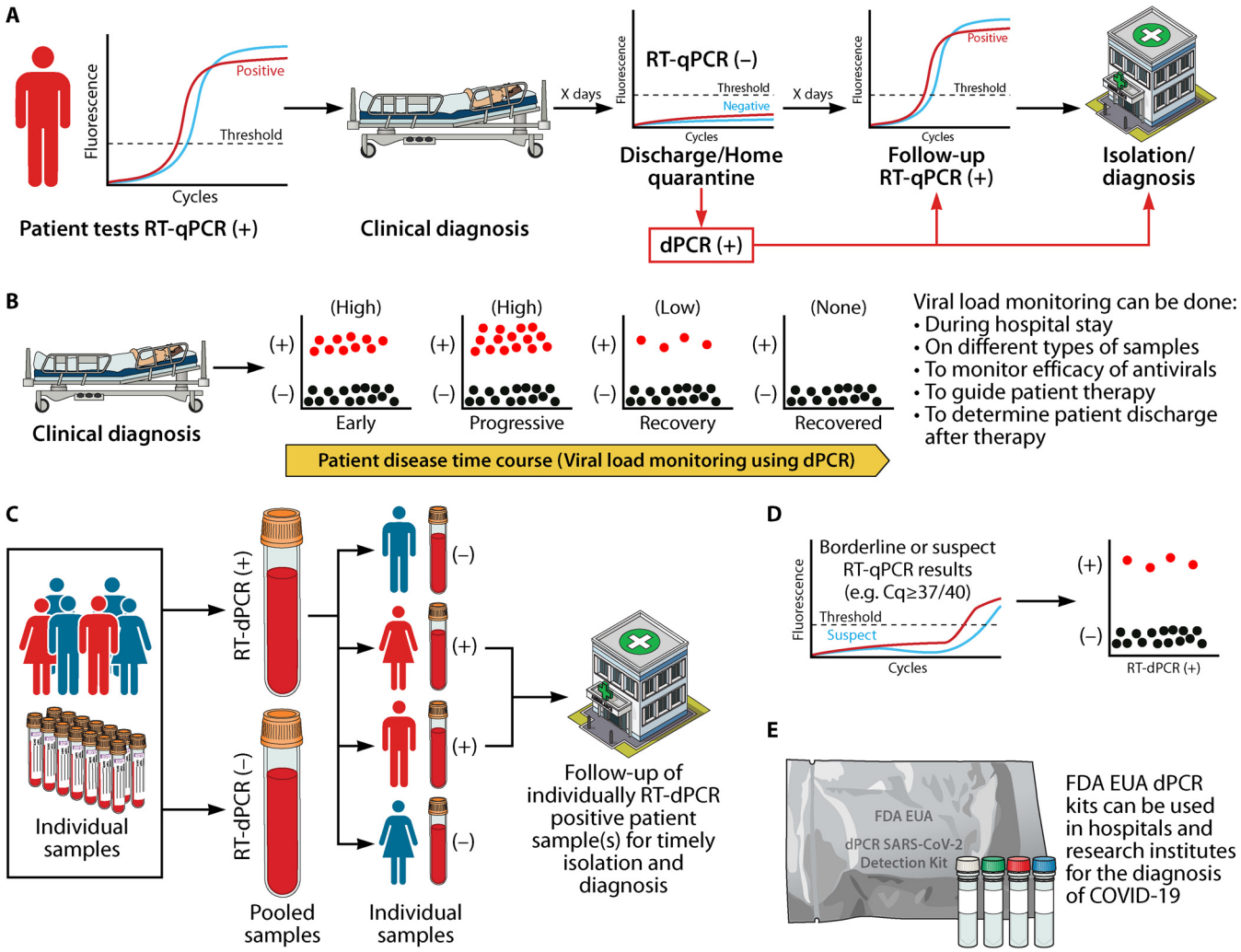

FIG 5 Applications of RT-dPCR in the diagnosis of COVID-19. (A) Diagnosis of RT-qPCR-negative patient samples, including patient discharge and follow-up. (B) COVID-19 patient viral load monitoring. (C) Pooled sample testing strategy to identify COVID-19 patients. (D) Resolving borderline RT-qPCR cases. (E) Development of commercial FDA EUA-authorized RT-dPCR diagnosis test kits.

reported as lost contacts. Two of the four suspected cases were also found to be positive upon follow-up, including one negative case and one lost contact. There were also 3 FNRs as indicated by the follow-up survey of the RT-dPCR-negative patients. From the supposed convalescent group, all 14 samples were RT-qPCR negative. However, using RT-dPCR, 7 positive, 5 negative, and 2 suspect cases were recorded. Upon follow-up, 5 of 9 (including 2 suspect cases) were diagnosed as SARS-CoV-2 positive by RT-qPCR within 5 to 12 days after discharge. The other 4 remained negative, and 1 result that was negative by RT-dPCR was found to be positive 7 days later upon follow-up. In another related study, Alteri et al. (24) found 19 SARS-CoV-2 RT-dPCR-positive samples that initially tested negative by RT-qPCR. At follow-up, 15 of the 19 positive patients developed pneumonia, while the other 4 showed signs of severe infection. Of note, of the 19 patients, subsequent tests on 12 patients showed persistent negative results by RT-qPCR, while the other 7 patients were later determined to be positive by RT-qPCR. Additionally, in their comprehensive study, Gniazdowski et al. tested several assays, including RT-qPCR for screening samples, cell culture to establish the infectivity of positive samples, WGS to establish the virus genotype in patients presenting prolonged viral RNA detection, and RT-dPCR to assess false-negative COVID-19 results (132). In the 2-month cohort study, a total of 29,686 specimens were tested, with 2,194 patients undergoing repeat testing. Notably, SARS-CoV-2 was isolated via cell culture in

TABLE 6 Sensitivity comparison of RT-qPCR and RT-dPCR in detecting SARS-CoV in clinical samples[a]

Sample type(s)	Total no. of samples tested	No. of samples with result						No. of RT-qPCR$^+$, RT-dPCR$^-$ samples	No. of RT-qPCR$^-$, RT-dPCR$^+$ samples	Sensitivity (%)		Confirmatory test(s)	Reference
		RT-qPCR			RT-dPCR								
		Positive	Negative	FNR	Positive	Negative	FNR			RT-qPCR	RT-dPCR		
Throat swabs	77	21	56	37	56	15[b]	4	0	35	40	94	Chest CT, follow-up survey	147
OPS, NPS	64	46	18	11	0	7[c]	7	0	11	72	89	Chest CT	148
NPS	55	0	55	19	19	36	1	0	19	NS	NS	Chest CT, serology	24
NPS	198[c]	0	198	11	NS	187	0	0	11	NS	>5.6	Cell culture, sequencing	132
Saliva	13[c]	8	5	5	11	2	2	0	3	62	85	Confirmed RT-PCR-positive NPS, clinical symptoms	76
PS, sputum	91	28	63	2	30	61	0	0	2	93	100	Clinical diagnosis	175
OPS, NPS, serum	10	7	3	3	10	0	0	0	3	NS	NS	Chest CT	65
NPS	208	13	187[b]	16	29	178[b]	0	0	16	NS	>8.6	Clinical symptoms	176
AS, sputum, throat	18	2	16	16	7	11	11	2	7	NS	NS	Recurrent or convalescent COVID-19 patients	166
PS	103	29	25[b]	25	90	13	13	0	19	28.2	87.4	Clinical symptoms, follow-up RT-qPCR	177
NPS, sputum, blood	366	173	193	0	236	130	4	4	63	NS	NS	Radiology, medical records	149

[a]RT-qPCR$^+$, RT-qPCR positive; RT-dPCR$^-$, RT-dPCR negative; TS, throat swab; NPS, nasopharyngeal swab; OPS, oropharyngeal swab; PS, pharyngeal swab; AS, anal swab; FNR, false-negative result; CT, computed tomography; NS, not specified.

[b]The remaining samples that were not positive or negative were judged to be suspect, i.e., values between negative and the LOD of the PCR assay.

[c]The study included multiple samples, and only a subset of them was used for RT-dPCR comparison to establish FNRs/FPRs.

specimens with a mean C_q value of 18.8 (standard deviation of 3.4) by RT-qPCR, with a high efficiency of virus recovery being observed in specimens with a C_q value of between 10 and 20. Furthermore, WGS results showed that a single genotype of SARS-CoV-2 in a patient was carried over time. Specimens that initially tested negative by RT-qPCR but later tested positive upon follow-up had C_q values of $>$29.5 and were not associated with cell culture. Finally, RT-dPCR could positively identify 5.6% of negative specimens obtained from patients with clinically suspected or confirmed COVID-19 (132).

Taken together, these and other studies (summarized in Table 6) showed that RT-dPCR could detect the presence of SARS-CoV-2 in RT-qPCR-negative samples. Therefore, RT-dPCR could be used as an alternative or complementary method to reduce the FNRs commonly observed with RT-qPCR in the quantification/detection of low viral loads in samples. However, despite the better practical sensitivity in these studies, RT-dPCR also had its faults as a few samples were also reported as FNRs and FPRs compared to other methods such as chest CT and clinical symptoms upon follow-up (Table 6). This was hypothesized to arise from RNA degradation (due to low copy numbers of the internal control gene), suboptimal sampling, or the absence of SARS-CoV-2 from collection sites (e.g., airways), necessitating the re-collection and/or retesting of different specimen types (148, 149). It is also important to note that although some of the patients presented with symptoms of COVID-19, including a positive SARS-CoV-2 RT-dPCR test and a negative RT-qPCR test, the patients may not have the ability to spread the disease due to low viral loads, as confirmed upon follow-up (132, 149). Some studies also reported suspect RT-dPCR results (positive copies observed below the RT-dPCR-set LOD). For such samples, it was proposed that the samples may need further detection by retesting the sample to confirm true positivity (147, 149). Finally, despite the improved sensitivity of dPCR in detecting SARS-CoV-2-positive samples, it is important to state that this may not always be the case for all samples or universal for all pathogens. Therefore, parameters such as sensitivity need to be validated using a well-established reference standard prior to adapting the technique.

Patient discharge and monitoring. In their updated guidelines (150), the WHO recognizes that some patients with severe disease may be symptomatic for a prolonged period of time. Therefore, a laboratory-based approach is needed to determine whether these patients may also require prolonged isolation. Their recommendations also include monitoring patients' viral loads (150), as studies have associated lower infectivity with a decrease in the SARS-CoV-2 viral load (151–153). Since dPCR has the capability of absolute quantification, it has been used to monitor viral loads of patients and to monitor disease progression in COVID-19 patients during hospitalization and after discharge (23, 69, 76, 129, 147, 154). For example, after analyzing the viral loads in various clinical samples, Yu et al. (69) discovered that the viral loads in the sputum samples of patients could reflect COVID-19 disease progression *in vivo*. Subsequently, they quantified the viral loads in sputum samples from 44 confirmed COVID-19-positive patients during the disease time course using RT-dPCR. Their results showed that the SARS-CoV-2 viral loads in the early and progressive stages were higher than those in the recovery stages of COVID-19. The same result was also highlighted by Lu et al. (129). However, in this study, RT-qPCR failed to detect SARS-CoV-2 during the time course of the disease compared with RT-dPCR. Taken together, these studies showed that RT-dPCR was better than RT-qPCR in monitoring patients' disease progression. Moreover, after discharge of RT-qPCR-negative patients, RT-dPCR detected positive cases among some of the patients who were later confirmed to be COVID-19 positive (24, 76, 147, 154). Although this is not recommended by the WHO, it also means that RT-dPCR could be used to monitor discharged patients in cases of relapse, which could lead to the timely identification and isolation of infected patients. Nevertheless, more is needed to validate RT-dPCR as an efficient technique to quantify viral loads in patients with persistent symptoms and to monitor discharged RT-qPCR-negative but

RT-dPCR-positive patients in cases of relapse. These findings may prove useful for the current SARS-CoV-2 pandemic and future disease outbreaks.

Pooled sample testing. Since its introduction in 1943 by Robert Dorfman (155), "pooling of samples" has become a commonly used method for the detection and diagnosis of infectious diseases (156–159). Specifically, pooling of samples means that different samples are mixed to form a pool before tests are performed on that pool as a single sample (160–162). If the result is negative after testing, no further action is required. However, if a single pool tests positive, each sample in that pool is tested individually according to the testing scheme to obtain accurate information about the positive sample(s) (160–162). Due to the mixing of samples, pooling can greatly expand the testing capacity of a single laboratory while saving the costs and time of batch sample processing. In the current SARS-CoV-2 case, this approach was used to test different SARS-CoV-2 samples using RT-qPCR (162, 163). In particular, this approach was used to test all Wuhan residents in different facilities, including ours, with great success. However, this method has not been widely used due to the limited sensitivity of RT-qPCR in testing samples with low viral loads. It has also been suggested that RT-dPCR may be more effective than RT-qPCR in pooled samples due to its higher sensitivity, specificity, and precision; same-day turnaround time; and relatively low cost (23, 164, 165). To test the suitability of RT-dPCR for pooling, Martin et al. divided 448 COVID-19 hospital samples into three groups (14 pools of 32 samples/pool, 28 pools of 16 samples/pool, and 56 pools of 8 samples/pool) for RT-dPCR testing and directly compared the results to those of individual testing by RT-qPCR (165). From these results, it was shown that the pooling of 16 samples and individually retesting the positive pools retained sensitivity to that of individual RT-qPCR testing. In addition, pooling was found to reduce reagent consumption by 80% and increase test capacity by 10-fold. However, we believe that this study would have benefited more if the pooled samples had also been tested by RT-qPCR. By doing so, a direct comparison between pooled sample testing by RT-qPCR and that by RT-dPCR could have been achieved. Also, only two samples were discordant from the whole group and, hence, not statistically significant to conclude that RT-dPCR was superior to individual RT-qPCR testing. This leaves room for further comparative studies in the future to establish the true sensitivity of group testing by RT-dPCR over individual testing by both RT-qPCR and RT-dPCR. In another, unrelated publication, Armendáriz et al. developed a pooling strategy for nested testing and showed, through a series of calculations, that RT-dPCR is the method of choice when samples are pooled to test individuals infected with SARS-CoV-2 (164).

Development of diagnostic test kits. Currently, only three commercially available RT-dPCR test kits have received emergency use authorization (EUA) from the Food and Drug Administration (FDA) for use in the *in vitro* diagnosis of SARS-CoV-2, as shown in Table 7. These numbers are staggering compared to the number of FDA EUA-approved RT-qPCR kits for SARS-CoV-2 and other infectious diseases, probably due to the scarcity of the method (8). Unlike RT-qPCR, where some of the kits can be used in laboratories that can perform moderately complex laboratory tests (M), all three approved RT-dPCR test kits should be used in laboratories that meet the requirements for performing highly complex tests (H) (8). Moreover, unlike most RT-qPCR kits that can be used on different platforms, the RT-dPCR kits are platform specific, which may be the reason why there are not many commercially available RT-dPCR kits. Despite this, RT-dPCR kits have been used in the clinical diagnosis of SARS-CoV-2 (60, 69, 166). These kits target already established SARS-CoV-2 genes such as the N and ORF1ab genes. In addition, a quick search of the websites of the companies producing these kits revealed that they also produce RT-dPCR test kits (albeit not FDA approved) for screening SARS-CoV-2 variants, which could play a critical role in detecting mutations caused by these variants. Furthermore, since the dPCR technology is not readily available in most parts of the world and these kits need to be used in complex laboratories (H), some companies like Biodesix (167) have offered a service to perform tests on behalf of interested parties.

TABLE 7 Commercially available SARS-CoV-2 RT-dPCR detection kits and assays[a]

Kit	Company	Approval(s)	Setting	Application	Target(s)	Platform(s)	Sample type(s)	LOD	Reference[b]
Bio-Rad SARS-CoV-2 ddPCR kit	Bio-Rad	U.S. FDA EUA, CE-IVD	H	IVD Rx only	N1 (FAM), N2 (FAM + HEX), RNase P (HEX)	QX200, QXDx	NPS, ANS, MNS, NPW/A	625 copies/mL	23
FastPlex triplex SARS-CoV-2 detection kit (RT-digital PCR)	PreciGenome LLC	U.S. FDA EUA	H	IVD Rx only	ORF1ab (FAM), N (HEX), RNase P (Cy5)	DropX-200	OPS	571.4 copies/mL	-
Gnomegen COVID-19 RT-digital PCR detection kit	Gnomegen LLC	U.S. FDA EUA	H	IVD Rx only	N1/N2 (FAM), RNase P (VIC)	Gnomegen, QuantStudio	NPS, NS, OPS	8 copies/reaction (both platforms)	166
Dr. PCR DiS20K SARS-CoV-2 detection kit	Optolane Technologies	KMFDS, CE-IVD	-	IVD Rx only	E (FAM), RdRp (FAM), ProC (FAM-Cy5)	LOAA analyzer system	NPS, OPS	2,138.0 (range, 1,513.6–3,020.0) copies/mL	178
Novel coronavirus (2019-nCoV) nucleic acid detection kit	Targeting One	Pending cFDA, CE-IVD	-	IVD Rx only	ORF1ab (FAM), N (FAM + VIC), RNase P (VIC)	TD-1 digital PCR	Upper and lower respiratory tract, blood, serum, tissues	10 copies/reaction	69
SARS-CoV-2 multiplex crystal digital PCR kit	ApexBio	Pending FDA	-	Research only	ORF1ab (FAM), N (HEX), human gene (Cy5)	Naica	-	N, 0.6 copies/μL ORF1ab, 0.9 copies/μL	60

[a]NS, nasal swab; NPS, nasopharyngeal swab; NPW/A, nasopharyngeal wash/aspirate; ANS, anterior nasal swab; MNS, midturbinate nasal swab; OPS, oropharyngeal swab; FDA, Food and Drug Administration; cFDA, Chinese Food and Drug Administration; EUA, emergency use authorization; IVD, *in vitro* diagnostic; Rx, prescription use; LOD, limit of detection; H, CLIA-certified high-complexity laboratory; KMFDSA Korean Ministry of Food and Drug Safety; -, not specified. CE-IVD indicates that the kit is approved for sale and *in vitro* diagnostic use in Europe.
[b]Example of citing the diagnostic application of the test kit.

TABLE 8 Performance comparison of RT-qPCR and RT-dPCR in SARS-CoV-2 diagnostics

	Description	
Factor	**RT-qPCR**	**RT-dPCR**
Inhibitors	Platform less tolerant to inhibitors	Platform generally resistant to inhibitors[a]
Cost	Lower than that of RT-dPCR[a]	Higher than that of RT-qPCR
Turnaround time	~2 to 3 h[a]	>4 h
Precision	Adequate in most cases	Generally better than that of RT-qPCR[a]
Expert personnel	Vast[a]	Limited
Availability	Global[a]	Limited
Sensitivity	High	Higher than that of RT-qPCR[a]
Reproducibility	Lower than that of RT-dPCR	Higher than that of RT-qPCR[a]
Viral load quantification	Dependent on reference standards	Direct, without reference standards[a]
Reagents	Can be used on different platforms[a]	Platform specific

[a]Better-performing platform.

This presents an avenue for the development of more dPCR testing centers in regions that cannot readily access the technology, like in developing countries.

Antiviral research. Antiviral drugs are essential for disease management of patients as they help to reduce the severity of the disease in infected patients (168). When a new pathogen such as SARS-CoV-2 emerges, researchers test the efficacy of new and/or existing drugs against the new pathogen. Quantification systems such as RT-qPCR and RT-dPCR are often used after cell culture to determine the percent inhibitory effect of these drugs (169). Using RT-dPCR as the method of choice, after cell experiments, Rojas et al. (170) tested the antiviral efficacy of the antimalarial drug quinacrine (Qx) against SARS-CoV-2. Their results showed that Qx can effectively inhibit the replication of SARS-CoV-2 *in vitro*. In addition, Nyaruaba et al. showed that multiplex RT-dPCR assays can be used to test the efficacy of drugs using the drug remdesivir as an example (53). Once the drugs are tested, their efficacy *in vivo* can be tested by monitoring viral loads in patients. When using RT-qPCR, reference standards must be used to establish standard curves for relative viral load quantification. Since this material is not readily available, a direct quantification method such as RT-dPCR may be the method of choice in these situations, as suggested previously by Yu et al. (69). Lu et al. demonstrated this potential by using RT-dPCR to measure viral loads in COVID-19 patients during therapy, which eventually led to the administration of the drugs moxifloxacin, umifenovir (Arbidol), and Pudilan (129).

Advantages and Disadvantages of RT-dPCR Compared to RT-qPCR for SARS-CoV-2 Diagnosis

Despite the advantages of RT-dPCR, fewer data and publications (especially from developing countries) are available online for SARS-CoV-2 RT-dPCR research than for RT-qPCR. This can be attributed to some of the factors highlighted in Table 8. More studies need to be conducted to fully explore the advantages of dPCR. For example, dPCR can be combined with other fast amplification systems to reduce detection times. Wu et al. combined dPCR and CRISPR to form a rapid digital CRISPR approach (RADICA) for the absolute quantification of nucleic acids in 40 to 60 min (171). Additionally, Yin et al. combined RT-dPCR with rapid PCR to achieve ultrafast detection of SARS-CoV-2 within 7 min with a detection accuracy similar to or better than that of RT-qPCR (172). These two examples show that dPCR can be improved to overcome some of its limitations.

FUTURE PROSPECTS

The current SARS-CoV-2 pandemic is the first to test the working potential of RT-dPCR compared to the commonly used RT-qPCR for SARS-CoV-2 detection and viral load quantification. Of all applications, RT-dPCR is praised for its sensitivity, absolute quantitation without standard curves, better flexibility for multiplex detection, tolerance to inhibitors, and ability to detect small percentages of mutations compared to

the gold standard, RT-qPCR. Because of these advantages, it makes sense to pay attention to and monitor these applications so that scientists can quickly respond to and control an outbreak of this magnitude in the future. Based on our literature review, we can summarize some of these applications. First, we believe that in the event of a new disease outbreak, the first step should be to develop assays that can rapidly detect and monitor the spread of the disease. The multiplex capability of dPCR could be used for the rapid evaluation of PP sets to find optimal PPs for detection. Second, the advantages of RT-dPCR in absolute quantification should be used to quantitate viral genome copies and generate stable reference standards that will be used to validate assays, including RT-qPCR assays, to detect the pathogen. Once sensitive, specific, and reproducible assays are developed, they can be used in conjunction with the stable reference standards for the detection of suspect samples and other related applications. Third, for samples with low viral loads and unknown inhibitors, such as environmental samples and pooled specimens, dPCR may be preferred over qPCR since dPCR generally shows better sensitivity because of its better tolerance to inhibitors. However, when analyzing low viral loads in patient samples, care should be taken as the significance of low viral loads with regard to patient management and diagnosis is not yet fully understood.

Our review also identified several gaps that may need to be addressed. These include the development of new assays that can detect viable SARS-CoV-2 cells, especially from environmental samples; the development and validation of new assays to monitor mutations caused by the new SARS-CoV-2 variants; increased research on pooling and other strategies for mass testing; analysis of various sampling techniques and follow-up on RT-qPCR-negative samples to reduce false-negative results, especially in samples that present low viral loads, with stable human biomarkers like RNase P and RPP30; improvement of dPCR sample TATs so that the test reports can be given as soon as possible; and the development of additional FDA EUA-approved RT-dPCR diagnostic test kits.

Finally, we also agree with critics from various reports (21, 27, 28) that more needs to be done to reduce the high costs associated with RT-dPCR, including instrument and reagent costs. One strategy could be to harmonize reagents that can be used across different platforms, as in RT-qPCR. This would mean that the technology can be easily accessible to many testing laboratories, and more competition may reduce the costs eventually, which could lead to the method being used as the gold standard for future diagnostic applications.

CONCLUSION

The new data summarized in this review suggest that RT-dPCR is superior to the gold standard, RT-qPCR, in certain aspects for the detection and diagnosis of SARS-CoV-2, especially in low-viral-load samples, but RT-qPCR still has some advantages due to its ubiquity. dPCR has been used in a wide range of applications for SARS-CoV-2, from assay development to clinical diagnosis. Its specificity, sensitivity, reproducibility, and detection limits are generally not affected by the common factors that can affect RT-qPCR. These applications will pave the way for the future diagnosis and monitoring of infectious disease outbreaks. Once suitable infrastructure is installed and the technique is adapted by many laboratories around the world, we believe that future pandemic monitoring would be performed using dPCR due to its numerous advantages and that this technique could eventually replace the currently used RT-qPCR as the gold standard for diagnosis.

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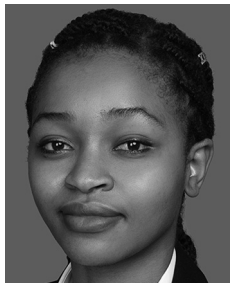

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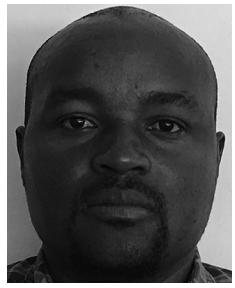

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