Application Note

Rapid processing of human saliva for SARS-CoV-2 detection by digital PCR

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Introduction

The current SARS-CoV-2 screening tests, such as the RNA-based RT-qPCR and antigen-based tests, rely on the nasopharyngeal swab for sampling. However, such swabs are uncomfortable and invasive, which is problematic for those who have to get tested frequently, including individuals who need to travel, older people in retirement homes, healthcare workers and more recently, school students. Therefore, saliva samples have been proposed as a suitable alternative to swabs, since screening (i) requires less material and is, therefore, less expensive, (ii) does not require qualified personnel to perform the sampling and (iii) can be processed faster and scaled up to meet industrial throughput requirements (Tan et al. 2021). The third generation of PCR techniques, digital PCR (dPCR), has proven to be more sensitive and precise than qPCR, especially when detecting low levels of the virus in saliva. Here we report the use of saliva samples in combination with dPCR as a suitable alternative to screen for individuals infected with SARS-CoV-2.

Materials

Viral lysis

The QIAprep&amp Buffer AB (Cat. No. 221515) was used to prepare the saliva sample. Buffer AB was prepared by thoroughly mixing 3.75 µl of Saliva Prep Buffer A with 2.25 µl of Saliva Prep Buffer B. 6 µl of the mix was then pre-dispensed into a PCR tube or the well of a PCR plate. 18 µl of saliva sample to be analyzed was added to a tube or well containing Buffer AB. The mixture was homogenized by pipetting up and down at least twice.

Note: Saliva samples have high viscosity. Heating the primary sample at 80°C for 10 min can lower the viscosity and facilitate easy pipetting of such samples. This heating step does not replace the manufacturer's recommended heating step at 95°C for 5 min. Tubes or plates were sealed and incubated for 5 min at 95°C (Eppendorf ThermoMixer C, ref. 5382000015) before being briefly centrifuged (Eppendorf Centrifuge 5804R, ref. 5805000.010) at 11,000 g for 2 min with gradual deceleration to pellet unwanted cell residue. 8 µl of heat pre-treated sample was then transferred into a new PCR tube or well before proceeding with the dPCR reaction. ▷

One-step viral RT-dPCR

The RT-dPCR reaction was performed following the manufacturer's instructions (QIAGEN, Germany) using the QIAcuity Eight Platform System, 5-plex (Cat. No. 911052), the QIAcuity One-Step Viral RT-PCR Kit (Cat. No. 1123145), QIAcuity Nanoplate 8.5K 96-well (Cat. No. 250021) and QIAcuity Nanoplate 26K 24-well (Cat. No. 250001).

Common SARS-CoV-2 strain detection

Ten donors negative for SARS-CoV-2, as determined by RT-PCR, and seven donors positive for SARS-CoV-2, as determined by RT-PCR and NGS, were assayed with RT-dPCR for the presence of wild-type SARS-CoV-2 (Table 1). RT-dPCR reaction mixtures were prepared in a pre-plate as follows. For Nanoplate 26K reactions, 10 µl of 4x One-Step Viral RT-PCR Master Mix, 0.4 µl of 100x Multiplex Reverse Transcription Mix, 2 µl of each of the three 20x set of primers and probes 0131, 0170, 0171 (ref. IAGE; total 6 µl), 2 to 8 µl of saliva lysis sample and RNase-free water were combined to reach a final reaction volume of 40 µl. For Nanoplate 8.5K reactions, 3 µl of 4x One-Step Viral RT-PCR Master Mix, 0.12 µl of 100x Multiplex Reverse Transcription Mix, 0.4 µl of each of the three 30x set of primers and probes 0131, 0170, 0171 (ref IAGE) (1.2 µl total), 1 to 7.68 µl of saliva lysis sample, and RNase free water were combined to reach a final reaction volume of 12 µl.

Probe 0171 targets 2019-nCoV_N1 region NC_045512v2 with fluorophore HEX on a 72 bp amplicon length. Probe 0131 targets E_Sarbecco region NC_004718 with fluorophore FAM on a 125 bp amplicon length. Probe 0170 targets two regions in the human RPP30 gene ID ENST00000487998.5 with fluorophore TAMRA on a 62 bp amplicon length. The CDC has published all sequences of target genes.

Following assembly in the pre-plate, reaction mixtures were transferred into an appropriate QIAcuity Nanoplate and loaded onto the fully automated QIAcuity Eight instrument to perform RT-dPCR. The RT-dPCR workflow includes i) a priming and rolling step to fill and then seal the reaction chamber partitions, ii) an amplification step following this cycling protocol: 10 min at 50°C for reverse transcription, 2 min at 95°C for enzyme activation, 5 s at 95°C for denaturation and 30 s at 58°C for annealing/extension in 40 cycles and iii) an imaging step completed by reading in the following channels: GREEN, YELLOW and ORANGE. The entire workflow, comprising the three steps, takes about 2 hours. Data were analyzed using the QIAcuity Software Suite V1.2 and expressed as copies/µl.

Variant SARS-CoV-2 strain detection

Variants of SARS-CoV-2 have emerged which are distinguished by particular molecular signatures. To screen for important SARS-CoV-2 variants, IAGE has developed a 5-plex assay that takes full advantage of the five channels of detection available on the QIAcuity One 5plex, QIAcuity Four and QIAcuity Eight instruments. The 5-plex assay uses a HEX probe (0171) to detect SARS-CoV-2 wild-type N1 region NC_045512v2, a second FAM probe (0149) to detect the Del H69-V70 mutations associated with the so-called English variant: 20I/501Y.V1 (B.1.1.7), a third Cy5 probe (0150) to detect the N501Y mutation associated mainly with global variants: 20I/501Y.V1 (B.1.1.7); 20H/501Y.V2 (B.1.351); 20J/501Y.V3 (P.1), a fourth ROX probe (0155) to detect the E484K mutation mainly associated with the South African and Brazilian variants: 20H/501Y.V2 (B.1.351); 20J/501Y.V3 (P.1) and a fifth TAMRA probe (0170) targeting RPP30 (ENST000004879 98.5) serves as an internal control of human RNA extraction.

To demonstrate the utility of these assays, the 5-plex IAGE assay was used to analyze two saliva samples positive for the so-called English SARS-CoV-2 variant and ten saliva samples negative for SARS-CoV-2 with RT-dPCR (Table 2). The English SARS-CoV-2 variant is distinguished by the presence of an N501Y mutation, the presence of a Del69-70 deletion and the absence of an E484K mutation. Synthetic control templates for the E484K mutation were run in parallel.

The RT-dPCR reaction mixture for variant strain detection was prepared in a pre-plate as follows, depending on Nanoplate type. For Nanoplate 26K reactions, 10 µl of 4x One-Step Viral RT-PCR Master Mix, 0.4 µl of 100x Multiplex Reverse Transcription Mix, 5 µl of the primers/probes mix from the PENTA-CoV Saliva Kit (ref IAGE), 2 to 8 µl of saliva lysis sample and RNase-free water were combined to reach a final reaction volume of 40 µl.

For Nanoplate 8.5K reactions, 3 µl of 4x One-Step Viral RT-PCR Master Mix, 0.12 µl of 100x Multiplex Reverse Transcription Mix, 2 µl of the primers/probes mix from the PENTA-CoV Saliva Kit (ref IAGE), 1 to 7.68 µl of saliva lysis sample and RNase free water were combined to reach a final reaction volume of 12 µl.

dPCR reactions were run as described above, with the exception that all available channels (GREEN, YELLOW, ORANGE, RED and CRIMSON) were used during the imaging step.

Results and Discussion

In this proof-of-concept study, we observed equivalent dPCR results when the same saliva samples were assayed in both 8.5K and 26K Nanoplates. Thus, in this application, the 8.5K Nanoplates allow users to increase throughput while decreasing the cost per sample.

Table 1. Detection with a liquid prep method and 3plex dPCR assay. Triplex reaction with 8.5K and 26K events per well: N1, E and Rpp30 genes. Positive donor (n=7), detection=7; Negative donor (n=10), detection=0.

			dPCR assay					
	Donor	Reference method (RT-qPCR targeting E and N1 genes)	N1		E		Rpp30	
Positive donor	Saliva 1	+	+	+	+	+	+	+
	Saliva 2	+	+	+	+	+	+	+
	Saliva 3	+	+	+	+	+	+	+
	Saliva 4	+	+	+	+	+	+	+
	Saliva 5	+	+	+	+	+	+	+
	Saliva 6	+	+	+	+	+	+	+
	Saliva 7	+	+	+	+	+	+	+
Negative donor	Saliva 8	–	–	–	–	–	+	+
	Saliva 9	–	–	–	–	–	+	+
	Saliva 10	–	–	–	–	–	+	+
	Saliva 11	-	–	–	–	–	+	+
	Saliva 12	–	–	–	–	–	+	+
	Saliva 13	–	–	–	–	–	+	+
	Saliva 14	–	–	–	–	–	+	+
	Saliva 15	–	–	–	–	–	+	+
	Saliva 16	–	–	–	–	–	+	+
	Saliva 17	–	–	–	–	–	+	+
NTC	H_2O	–	–	–	–	–	–	–
Type of plate (n event per well)			8500	26,000	8500	26,000	8500	26,000

Quantitative analyses of fluorescence intensity shows clear separation with an approximately two-fold increase in fluorescence of positive partitions compared to negative partitions (i.e. signal-to-noise ratio), with no false positives in the no-template control (Figures 1–3).

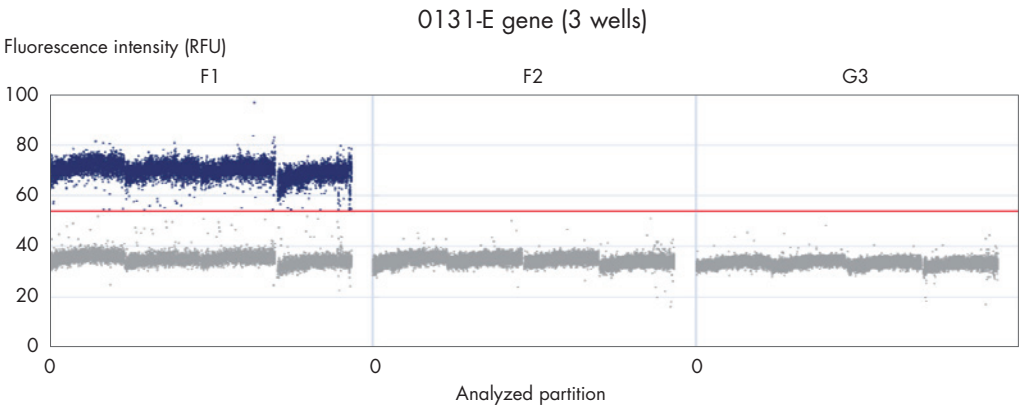

Figure 1. Fluorescence intensities as measured by the QIAcuity instrument. 0131-E gene: F1 = Positive donor; F2 = Negative donor; G3 = NTC.

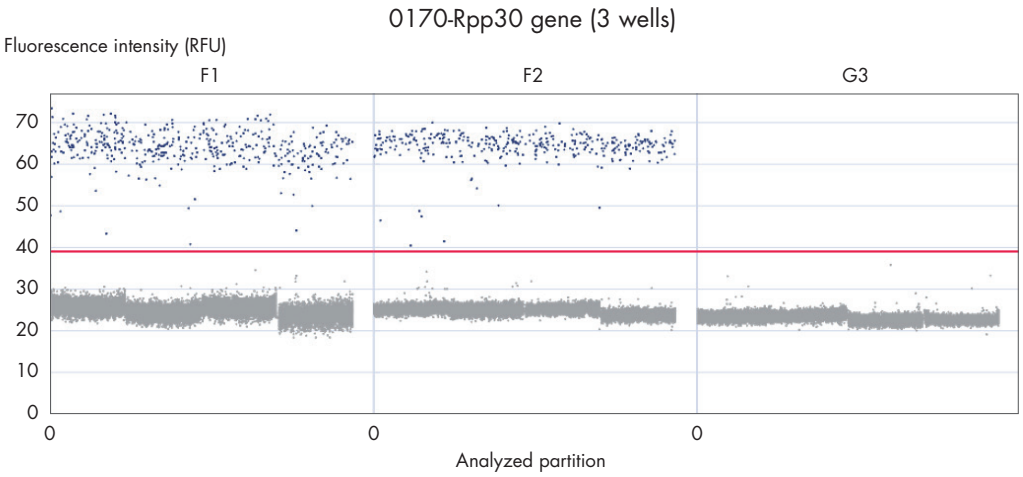

Figure 2. Fluorescence intensities as measured by the QIAcuity instrument. 0170-Rpp30 gene: F1 = Positive donor; F2 = Negative donor; G3 = NTC.

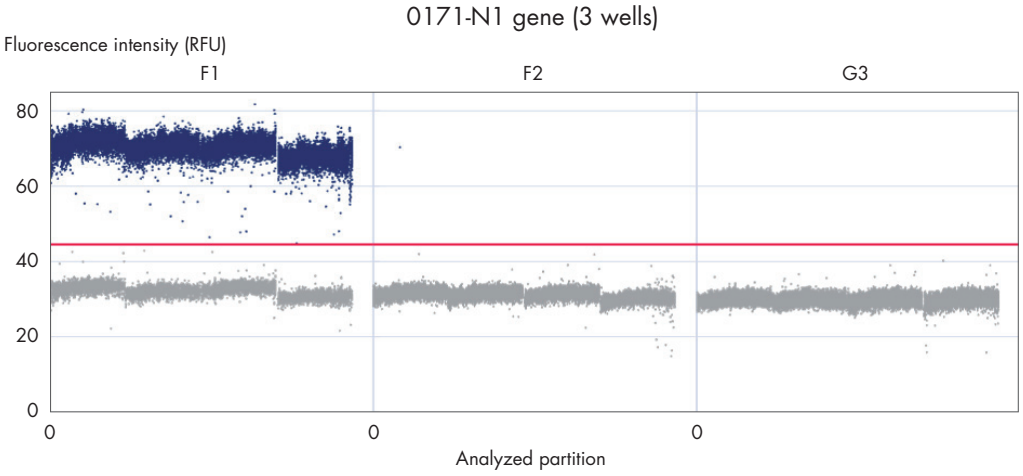

Figure 3. Fluorescence intensities as measured by the QIAcuity instrument. 0171-N1 gene: F1 = Positive donor; F2 = Negative donor; G3 = NTC.

Table 2. Detection with a liquid prep method and 5plex dPCR assay. Fiveplex reaction with 8.5K and 26K events per well: N1, E and Rpp30 genes. Positive donor (n=2), detection=2; Negative donor (n=10), detection=0.

			dPCR assay									
	Donor	Reference method (RT-qPCR targeting E and N1 genes)	N1		N501Y		Del69-70		E484K		Rpp30	
Positive donor UK variant	Saliva 1	+	+	+	+	+	+	+	–	–	+	+
	Saliva 2	+	+	+	+	+	+	+	–	–	+	+
Negative donor	Saliva 8	–	–	–	–	–	–	–	–	–	+	+
	Saliva 9	–	–	–	–	–	–	–	–	–	+	+
	Saliva 10	–	–	–	–	–	–	–	–	–	+	+
	Saliva 11	–	–	–	–	–	–	–	–	–	+	+
	Saliva 12	–	–	–	–	–	–	–	–	–	+	+
	Saliva 13	–	–	–	–	–	–	–	–	–	+	+
	Saliva 14	–	–	–	–	–	–	–	–	–	+	+
	Saliva 15	–	–	–	–	–	–	–	–	–	+	+
	Saliva 16	–	–	–	–	–	–	–	–	–	+	+
	Saliva 17	–	–	–	–	–	–	–	–	–	+	+
WT E484*	WT	–	–	–	–	–	–	–	–	–	–	–
Positive E484K*	S2	+	–	–	–	–	–	–	+	+	–	–
NTC	H_2O	–	–	–	–	–	–	–	–	–	–	–
Type of plate (n event per well)			8500	26,000	8500	26,000	8500	26,000	8500	26,000	8500	26,000

*Synthetic control

The 5plex assay developed to simultaneously detect SARS-CoV-2 and its variants is effective. The small number of samples tested in this study does not allow us to make any statistical analysis. Nonetheless, this assay allowed us to identify two donors positive for the UK variant (Figures 4–8), which was subsequently validated by sequencing.

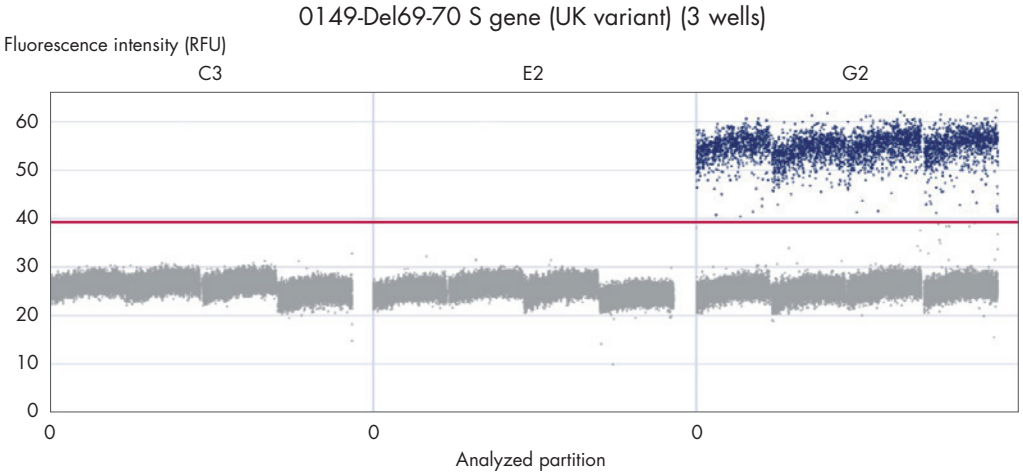

Figure 4. Fluorescence intensities as measured by the QIAcuity instrument in the GREEN (FAM) channel.
C3 = NTC;
E2 = Negative donor;
G2 = Positive donor for the UK variant.

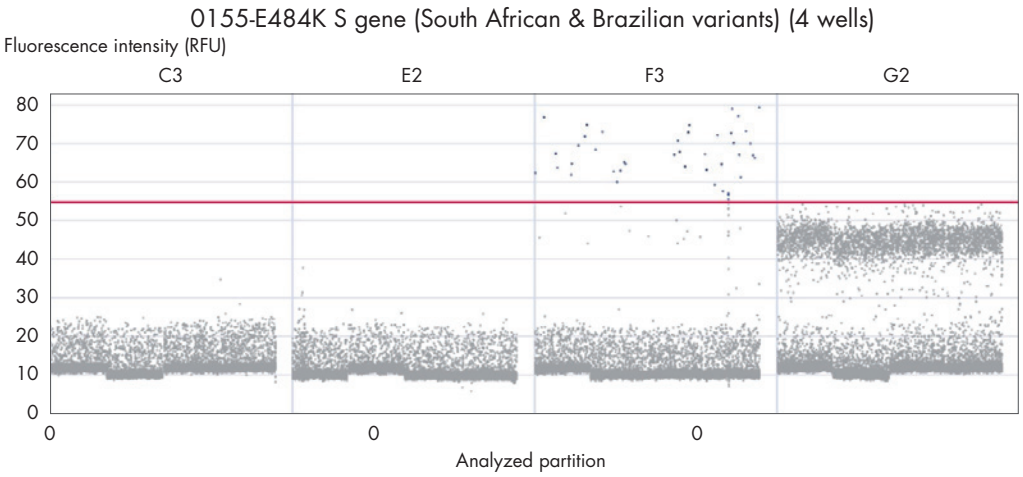

Figure 5. Fluorescence intensities as measured by the QIAcuity instrument in the RED (ROX) channel.
C3 = NTC;
E2 = Negative donor;
F3 = Mix synthetic control E484wt and E484K;
G2 = Positive donor for the UK variant.

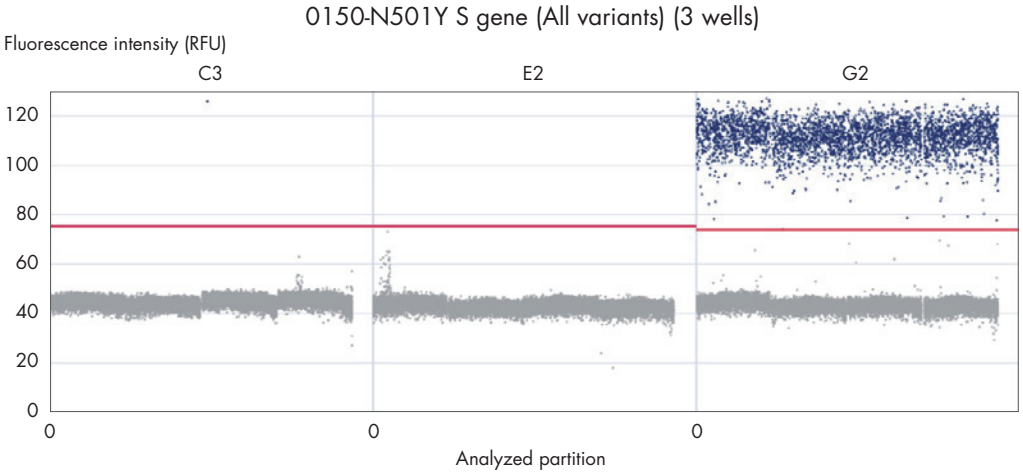

Figure 6. Fluorescence intensities as measured by the QIAcuity instrument in the CRIMSON (Cy5) channel.
C3 = NTC;
E2 = Negative donor;
G2 = Positive donor for the UK variant.

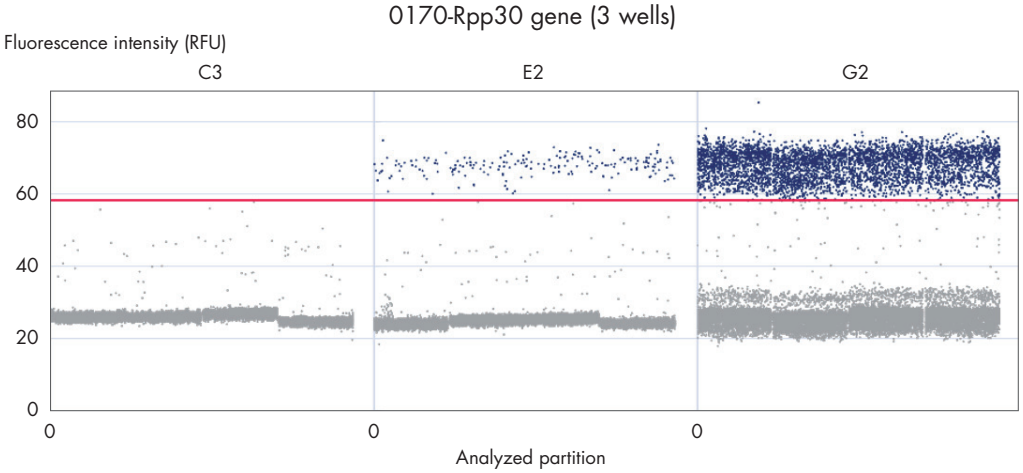

Figure 7. Fluorescence intensities as measured by the QIAcuity instrument in the ORANGE (TAMRA) channel.
C3 = NTC;
E2 = Negative donor;
G2 = Positive donor for the UK variant.

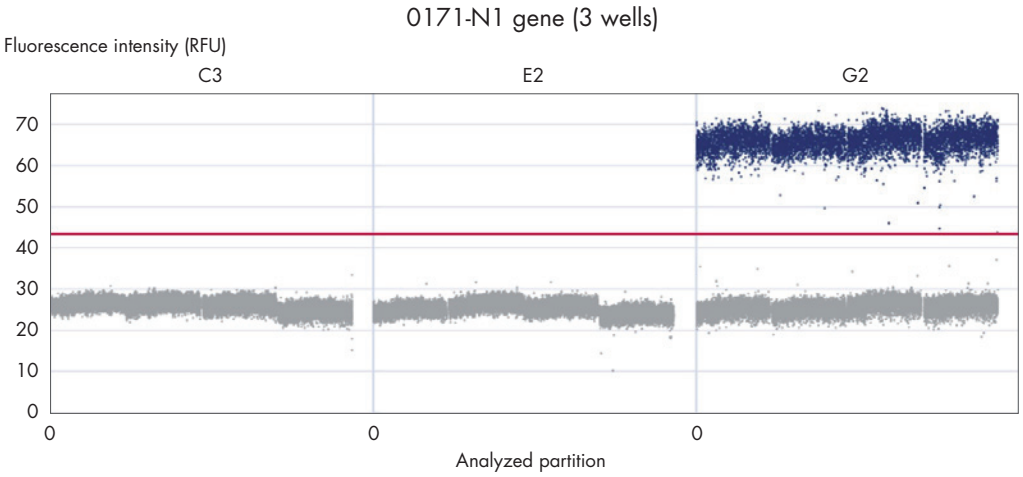

Figure 8. Fluorescence intensities as measured by the QIAcuity instrument in the YELLOW (HEX) channel.
C3 = NTC;
E2 = Negative donor;
G2 = Positive donor for the UK variant.

Conclusion

The QIAprep&amp Buffer AB coupled with a QIAcuity Eight dPCR instrument and a QIAcuity One-Step Viral RT-PCR Kit enables precise detection of SARS-CoV-2 in saliva. The workflow described here dramatically reduces the time required for analysis to under two hours. Thanks to this system, laboratories can perform more than 1300 saliva tests per day while achieving superior detection sensitivity. Multiplexing allows detection and genotyping of SARS-CoV-2 in a single reaction with improved time and resource efficiency. This is transformative in resource-limited settings and supports the role of dPCR as an alternative to qPCR when applied to saliva for high-frequency sampling and testing. We are currently developing a new version of this analytical method to individually characterize the mutations associated with the Brazilian, South African and Indian variants.

References

Tan, S.H., Allicock, O., Armstrong-Hough, M. and Wyllie, A.L. (2021) Saliva as a gold-standard sample for SARS-CoV-2 detection. Lancet Respir Med. S2213-2600(21)00178-8.

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Conflict of interest

None declared.

Research funding

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Ordering Information

Product	Contents	Cat. no.
QIAprep&amp Buffer AB (4.1 ml)	For the preparation of 600 samples requiring pre-treatment, including but not limited to saliva, gargle and sputum	221515
QIAprep&amp Viral RNA UM Kit (600)	For 600 x 20 µl reactions: 1.2 ml Viral RNA UM Prep Buffer, 2x 1.5 ml Viral RNA Master Mix, 4x, 1.2 ml RNA IC Template + Assay, 0.6 ml Human Sampling IC Assay, 1ml QN ROX, 2x 1.9 ml RNase-Free Water	221415
QIAcuity One-Step Viral RT-PCR Kit	4x One-Step Master Mix, 100x Multiplex Reverse Transcription Mix, RNase-free water, includes special reference dye needed for dPCR analysis and counting analyzable partitions	1123145
QIAcuity Nanoplate 26K 24-well (10)	10 QIAcuity Nanoplate 26k 24-well, 11 Nanoplate Seals	250001
QIAcuity Nanoplate 8.5K 96-well (10)	10 QIAcuity Nanoplate 8.5k 96-well, 11 Nanoplate Seals	250021
QIAcuity Eight Platform System	Eight-plate digital PCR instrument for detecting up to 5 fluorescent dyes, notebook computer, barcode scanner, nanoplate roller, USB flash memory and QIAcuity Software Suite: includes installation, training, and 1 preventive maintenance visit. 1 year warranty on labor, travel, and parts.	911052

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