Evaluating analysis settings and assay verification for the TaqCheck SARS-CoV-2 Fast PCR Assay

Optimizing pathogen detection using RT-PCR

Purpose

This paper provides an overview of considerations for defining analysis settings and evaluating assay verification for detecting pathogens using RT-PCR. An example is provided below utilizing data generated with the Applied Biosystems™ TaqCheck™ SARS-CoV-2 Fast PCR Assay. The following information is for reference only; the end user is responsible for validation of their own experimental design and analysis parameters through comprehensive studies before implementing them.

Introduction

Pathogen detection analysis via molecular methods such as RT-PCR allows laboratories to take advantage of increased analytical sensitivity and shorter processing times, compared to traditional cell culture methods. Pathogen detection assays require specific considerations to evaluate analysis settings and characterize the analytical performance of the assay. These settings and characteristics are different from those of other RT-PCR applications, such as relative gene expression analysis.

The TaqCheck SARS-CoV-2 Fast PCR Assay is a fast and robust solution that utilizes a streamlined sample collection and preparation process to increase throughput of RT-PCR testing in order to support population-level surveillance testing for SARS-CoV-2 infection.

The TaqCheck assay workflow is designed to utilize saliva samples, reducing the need for difficult and invasive sample collection. The simplified protocol to collect saliva samples and a direct PCR approach eliminate the need for additional RNA extraction equipment, reagents, and consumables. The multiplex Applied Biosystems™ TaqMan® assay targets the endogenous human RNase P gene as an indicator of sample adequacy, and detects SARS-CoV-2 by targeting both the nucleocapsid (N) gene and the spike (S) gene.

The assay configuration is shown in Table 1.

Table 1. TaqCheck SARS-CoV-2 Fast PCR Assay configuration.

Target	Dye	Quencher
SARS-CoV-2 N gene	VIC dye	QSY quencher
SARS-CoV-2 S gene		
Human RNase P RPP30 gene*	FAM dye	QSY quencher

* Serves as an internal positive control to monitor sample quality.

Considerations for analysis settings in pathogen detection

When using RT-PCR technology for pathogen detection, a specific and methodical process should be applied to define the optimal analysis parameters for the assay in question. In this paper, we outline the process undertaken to identify the appropriate analysis parameters for the TaqCheck SARS-CoV-2 Fast PCR Assay, but a similar approach may be applied to other pathogen detection workflows.

For pathogen detection applications on Applied Biosystems™ qPCR instruments, the use of the baseline threshold algorithm with manual threshold setting is recommended to determine C_t values. With this algorithm, there are two primary analysis settings that impact C_t value—baseline and threshold.

The baseline is set individually for each amplification curve and defines the region of the baseline before significant fluorescent signal is detected. This helps to normalize for well-to-well variance in background noise in the early cycles, as shown in Figure 1. For the TaqCheck SARS-CoV-2 Fast PCR Assay, Design and Analysis Software v2.5 (or higher) is recommended, with automatic baselining utilizing a start cycle of 5.

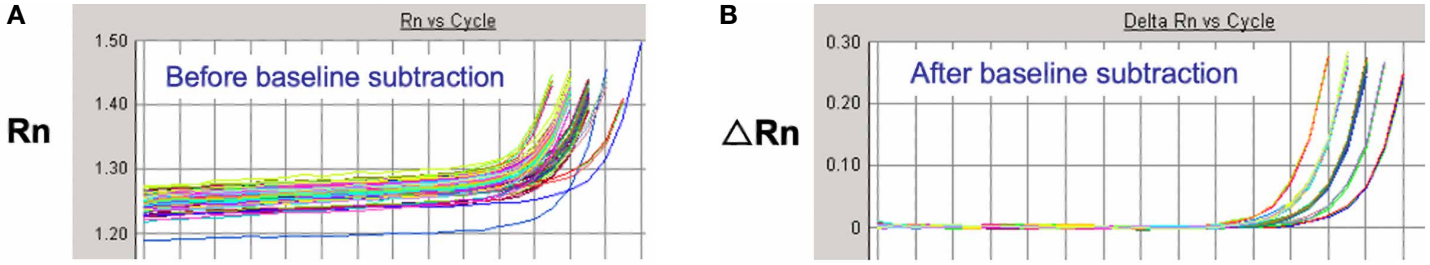

Figure 1. Normalization of baseline variation. Amplification plot in linear view showing **(A)** the variability of background fluorescence in ROX™ reference dye–normalized reporter signal (Rn), and **(B)** the impact of automatic baseline subtraction on ROX dye–normalized reporter signal (ΔRn).

The manual threshold is set independently for each target and defines the threshold fluorescence (ΔRn) level at which the C_t value will be collected for each sample. Even with multiplex assays such as the TaqCheck SARS-CoV-2 Fast PCR Assay, different thresholds may be required for each target—for example, in the TaqCheck experiment outlined in this paper, the ΔRn threshold is set at 0.2 for the RNase P target and 0.1 for the SARS-CoV-2 target.

The ΔRn at which the threshold is set impacts the C_t values collected. In pathogen detection analysis, manual ΔRn thresholds should be used rather than automatic thresholds because automatic thresholds are set based only on the positive targets present on an individual sample plate.

Manual threshold settings should be evaluated for each assay target as part of the experimental design phase, and locked in as part of the lab's standard operating procedures (SOPs) prior to routine processing. Changes in the threshold settings directly impact C_t values, as shown in Figure 2—raising the threshold shifts C_t values later (Figure 2A) and lowering the threshold shifts C_t values earlier (Figure 2B). Any changes in threshold settings require re-evaluation.

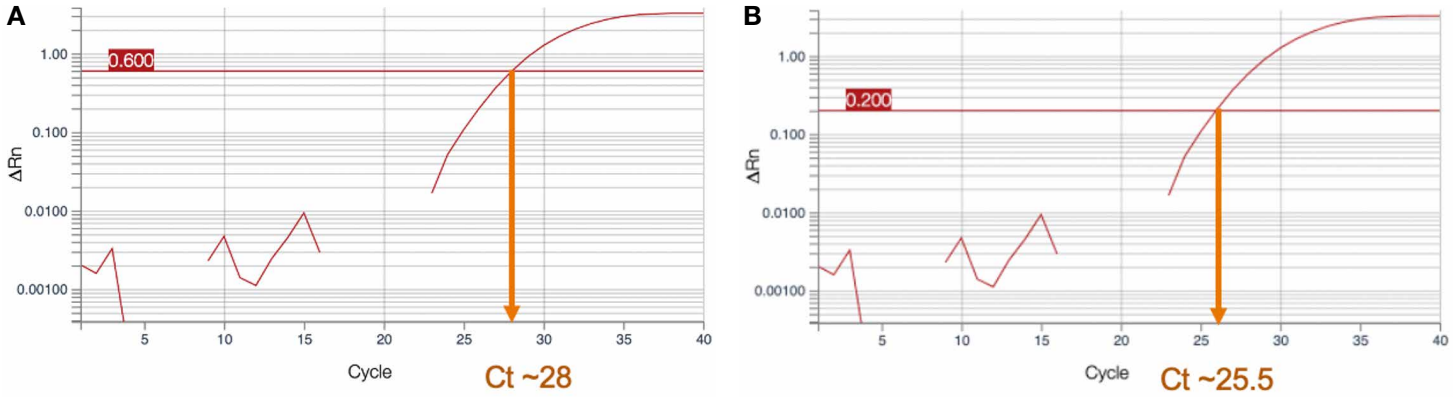

Figure 2. Effect of threshold settings on C_t values. Amplification plot in logarithmic view showing **(A)** a manual ΔRn set at 0.600 generates a C_t of ~28, and **(B)** for the same amplification data when the threshold is lowered to 0.200 the C_t shifts lower to ~25.5.

Once the primary analysis settings are established, C_t cutoffs should be defined for each target for both samples and controls. C_t cutoffs can be evaluated using data generated with no-template controls (NTCs) and other negative controls (if relevant) used to exclude spurious amplification such as from contamination introduced from the lab environment. C_t cutoffs should also be evaluated in the context of data to assess the dynamic range of the assay. For example, in the data set in Figure 3, a C_t cutoff of 37 excludes background contamination in the NTC while capturing true amplification within the verified dynamic range.

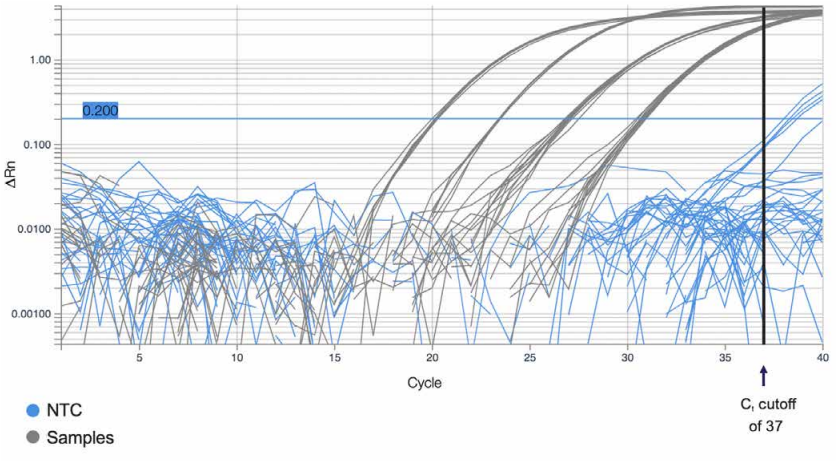

Figure 3. Assessment of C_t cutoff values.

For the TaqCheck SARS-CoV-2 Fast PCR Assay, the main considerations involve potential measurement variability from the following sources: 1) instrument-to-instrument variability, 2) well-to-well variability, 3) background RNase P amplification (due to human DNA sequences being ubiquitous in laboratory settings).

Experimental design

A set of example experiments evaluating ΔRn thresholds and C_t cutoff values for the TaqCheck SARS-CoV-2 Fast PCR Assay is presented in this section. A total of three main experiments were run to cover variability and other data conditions, such as high background and modest inhibition.

The first experiment was to determine the maximum level of background fluorescence signal and therefore the lowest ΔRn thresholds that could be set for both SARS-CoV-2 and RNase P targets. For a multiplex assay like the TaqCheck assay, it is important to assess what level of background signal is introduced when one target is present at a high concentration. As shown in Figure 4, we assessed whether a high concentration of VIC-labeled SARS-CoV-2 product contributed substantial background signal in the FAM channel in which RNase P is detected. For SARS-CoV-2, single-amplification reactions with Applied Biosystems™ SARS-CoV-2 IVT RNA were run in 4 corner wells and 4 wells in the center of a 384-well plate, on 8 different Applied Biosystems™ QuantStudio™ 5 instruments (Figure 4).

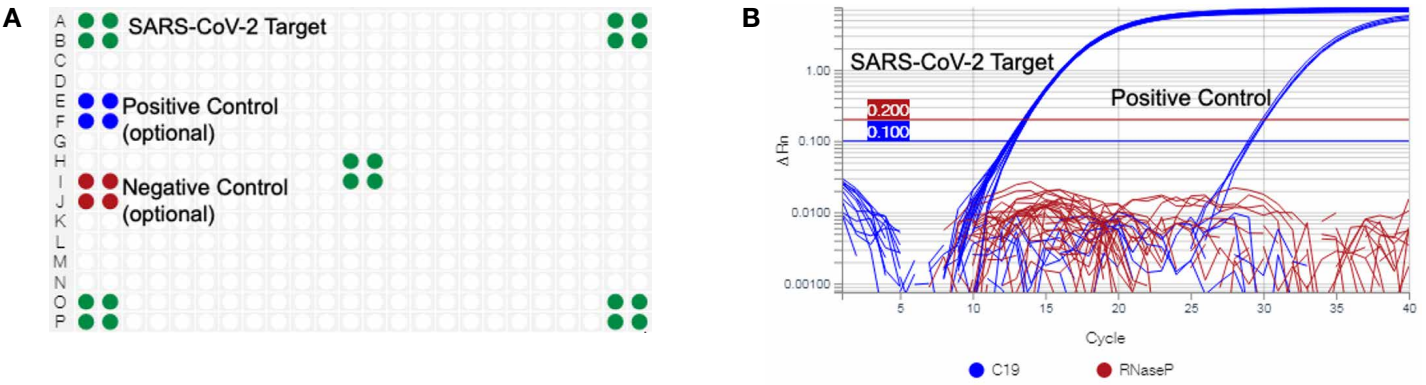

Figure 4. Identifying SARS-CoV-2 ΔRn thresholds for different instruments. Amplification plots can be used to determine the maximum background fluorescence on each instrument used by a lab. **(A)** SARS-CoV-2 IVT RNA was added at 1 x 10^7 copies/well in wells shown in green on the plate map. The resulting amplification produced the maximum ΔRn level. **(B)** The 0.2 threshold for RNase P (red line) is well above any background fluorescence introduced by the SARS-CoV-2 IVT RNA.

We also assessed whether a high concentration of FAM-labeled RNase P product contributed substantial background signal in the VIC channel in which SARS-CoV-2 is detected. To evaluate the lowest ΔRn threshold that could be set for RNase P, single reactions with Invitrogen™ Universal Human Reference (UHR) RNA were run in 4 corner wells and 4 wells in the center of a 384-well plate, on 8 different QuantStudio 5 instruments (Figure 5).

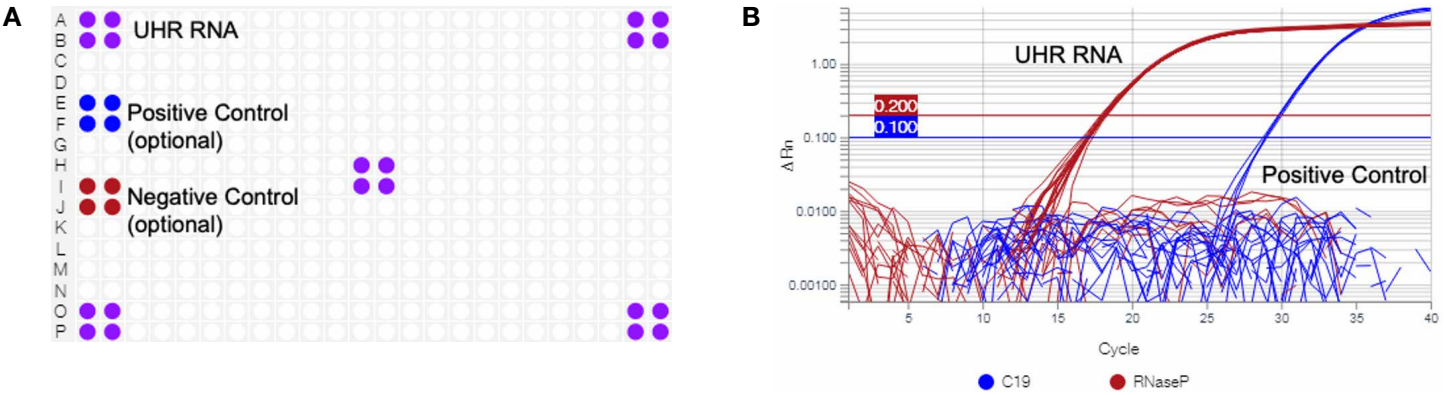

Figure 5. Identifying RNase P ΔRn thresholds for different instruments. Amplification plots can be used to determine the maximum background fluorescence for each target on different qPCR instruments used by a lab. **(A)** 1 µg of UHR RNA was added in wells shown in purple on the plate map. The resulting amplification produced the maximum ΔRn level. **(B)** The 0.1 threshold for SARS-CoV-2 (blue line) is well above any background fluorescence introduced by the RNase P target in the UHR RNA.

The second experiment assessed variability in C_t values for the RNase P target and the real-world effects of inhibition on the overall strength of qPCR amplification, and therefore how high the ΔRn thresholds could be set (Figure 6). This experiment was carried out using frozen SARS-CoV-2–negative saliva samples and spiking in inactivated virus.

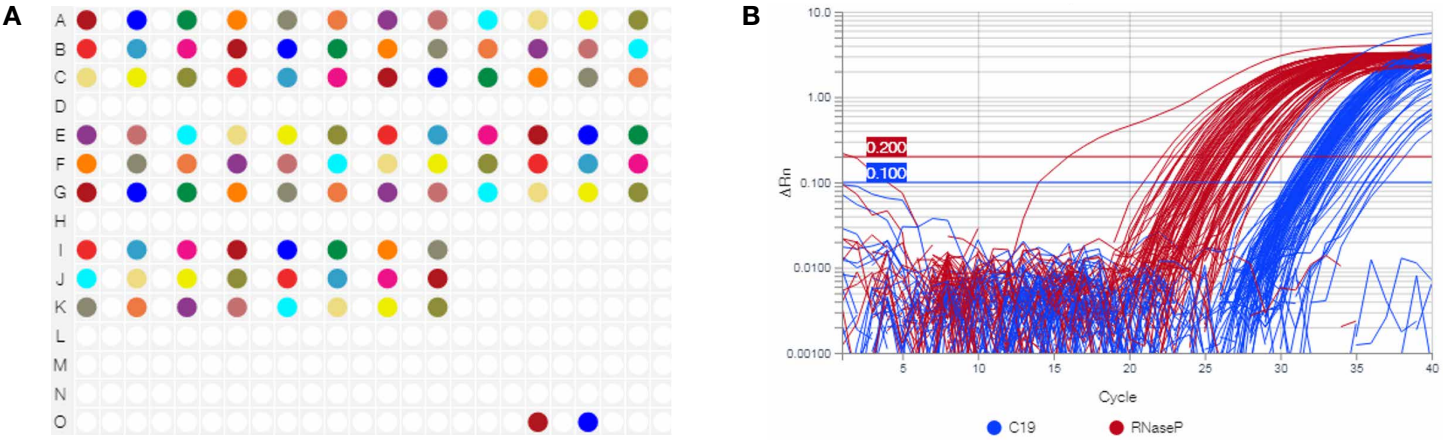

Figure 6. Determining ΔRn and C_t cutoff values for RNase P and SARS-CoV-2 assays. (A) Plate layout showing multiple saliva samples tested in triplicate, as well as a positive control well and negative control well. **(B)** Amplification plot from the same plate showing variation in C_t values in different saliva samples for RNase P (red). Inactivated SARS-CoV-2 was added to each saliva sample to a concentration of 10,000 genome copy equivalents (GCE) per mL to produce the amplification plots (blue).

The final experiment was to establish ΔRn and C_t cutoff values for both assay targets. This was accomplished by running a total of eight 384-well NTC plates at 3 different lab sites to determine the variability of RNase P background signal between different labs and establish the C_t cutoff settings for the assay in our labs. There is inherent variability between labs since human cells, though ubiquitous, exist at varying levels in different laboratories.

Results

Once the data were collected, the ΔRn values of representative present and absent samples for the RNase P gene were plotted at various cycles to determine a preliminary C_t cutoff and threshold value that separates the RNase P–present from the RNase P–absent samples (Figure 7).

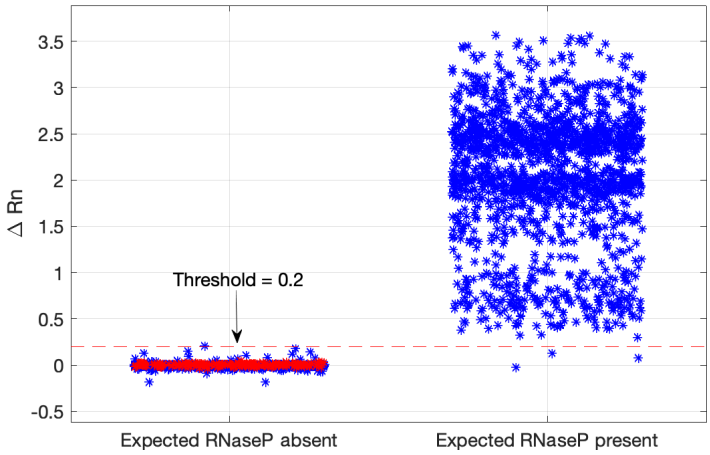

Figure 7. Determining C_t cutoff value for RNase P–present and RNase P–absent samples. ΔRn of NTC and RNase P–containing saliva samples plotted with proposed ΔRn threshold of 0.2 for samples.

Preliminary experiments indicated that a C_t cutoff of 32 for the RNase P target in samples would provide optimal analytical sensitivity and guard against false calls due to sample inadequacy. Based on these experiments, a more stringent C_t cutoff of 35 was selected for the RNase P target in NTCs and positive controls (PCs) to check for RNase P contamination, as these controls should not contain human genomic material. However, it is not uncommon to observe low levels of human genomic contamination in the lab environment, causing detection of RNase P with C_t values in the high 30s. Individual labs should assess RNase P cutoffs accordingly based on data generated during evaluation and verification studies.

Another important caveat regarding internal controls such as RNase P in a multiplex assay is that they can be impacted by competitive inhibition from the viral targets they are multiplexed with. It is possible for the viral titer in a sample to be high enough that the amplification of the RNase P internal control is inhibited or even extinguished as a result of this competition. For this reason, labs may choose to disregard the C_t cutoff for RNase P in reactions exhibiting a robust C_t for SARS-CoV-2. The C_t values of known negative samples were plotted using the ΔRn threshold of 0.2 and applying the cycle 32 cutoff to check for false calls (Figure 8).

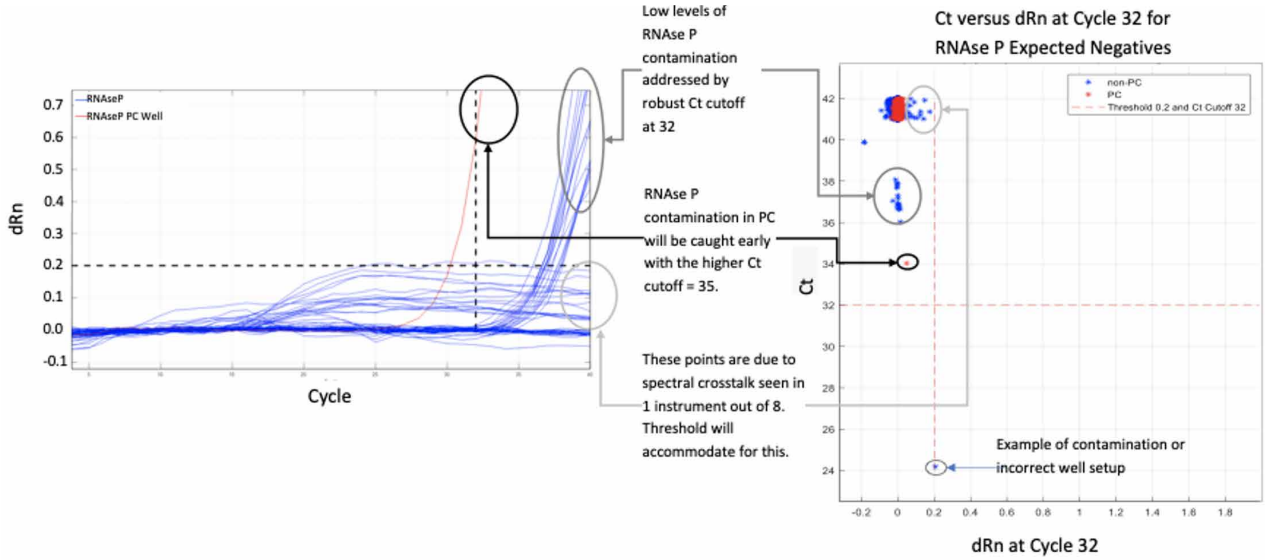

Figure 8. Example C_t value distribution for expected RNase P–negative samples, using a ΔRn threshold of 0.2 and a C_t cutoff at cycle 32 for RNase P. Cutoff and threshold shown with dotted lines.

Preliminary experiments indicated that a C_t cutoff value of 37 for SARS-CoV-2 provides optimal analytical sensitivity while addressing low levels of SARS-CoV-2 contamination, as shown in Figure 9. It is important to note that high levels of contamination (such as from a cross-contamination event) cannot be addressed by threshold and C_t cutoff values—best practices must be implemented in the lab SOP to prevent contamination.

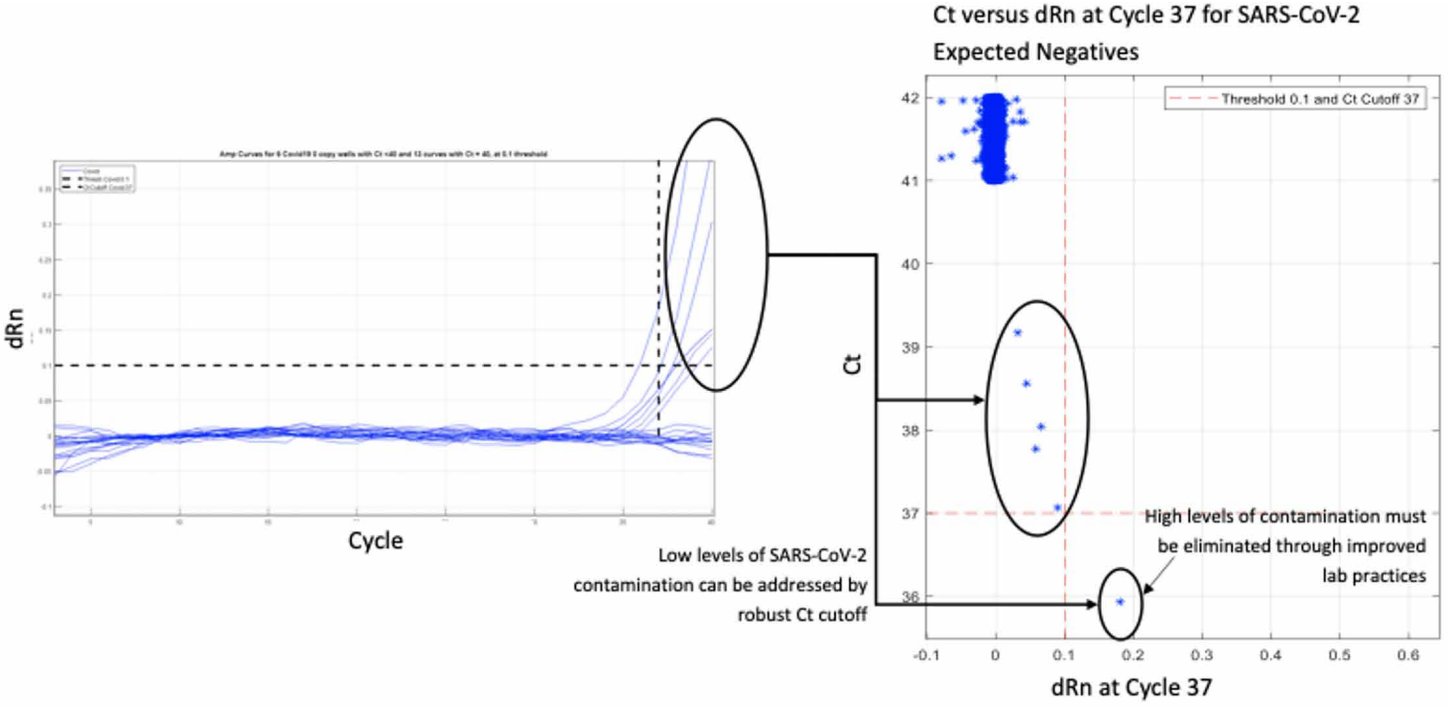

Figure 9. Example C_t value distribution for expected SARS-CoV-2–negative samples, using a ΔRn threshold of 0.1 and a C_t cutoff at cycle 37 for SARS-CoV-2. Cutoff and threshold shown with dotted lines.

Based on the experiments above, thresholds and C_t cutoff values were identified, as shown in Table 2.

Table 2. Proposed thresholds and C_t cutoff values for RNase P and SARS-CoV-2 targets.

Target	Sample type	ΔRn threshold	C_t cutoff value
RNase P	Sample	0.2	32
SARS-CoV-2	Sample, NTC	0.1	37
RNase P	Positive control, NTC	0.2	35
SARS-CoV-2	Positive control	0.1	37

Proposed C_t and ΔRn based on the experiments described in this paper.

Based on these experiments, C_t cutoff values for secondary analysis were applied for samples as described in Table 3, and for NTCs and positive controls as described in Table 4.

Table 3. C_t cutoff values for secondary analysis of SARS-CoV-2 assay samples.

C_t cutoff value		
SARS-CoV-2 N and S genes (VIC dye)	RNase P (FAM dye)	Result
≤37	≤32	Present
≤37	>32	Present
>37	≤32	Absent
>37	>32	Re-test*

* For re-testing it is recommended to reprocess sample from start to finish. It may be necessary to collect a new sample.

Table 4. C_t cutoff values for secondary analysis of SARS-CoV-2 assay controls.

Control	C_t cutoff value	
	SARS-CoV-2 N and S genes (VIC dye)	RNase P (FAM dye)
NTC	>37	>35
Positive control	≤37	>35

During assay validation, one should ensure that the chosen analysis parameters, such as thresholds and C_t cutoffs, are appropriate on all instruments to be used with the assay.

Analytical sensitivity

To determine the analytical sensitivity, an experiment was performed to determine the genome copy equivalents (GCE) per mL, where greater than 95% of the expected "present" samples were detected. Gamma-irradiated virus was spiked into SARS-CoV-2–negative saliva samples. Samples were then prepared as described in the TaqCheck SARS-CoV-2 Fast PCR Assay Quick Reference Guide and analyzed by RT-PCR. Data from a representative experiment, based on the determined thresholds and C_t cutoff values contained herein, are presented in Table 5. Based on the results of the experiment, analytical sensitivity was established at 6,000 GCE/mL.

Table 5. Results from an analytical sensitivity verification experiment.

Copies (GCE/mL)	Number of replicates	Number of positive samples detected	% positive
1,000	92	46	50%
3,320	96	87	91%
4,000	300	192	64%
6,000	80	79	99%
6,680	96	93	97%
9,000	60	60	100%
10,000	280	274	98%
12,000	60	60	100%
20,000	161	161	100%

applied
biosystems

Figure 10 illustrates the performance of expected SARS-CoV-2–absent and SARS-CoV-2–present samples at the proposed threshold for ΔRn.

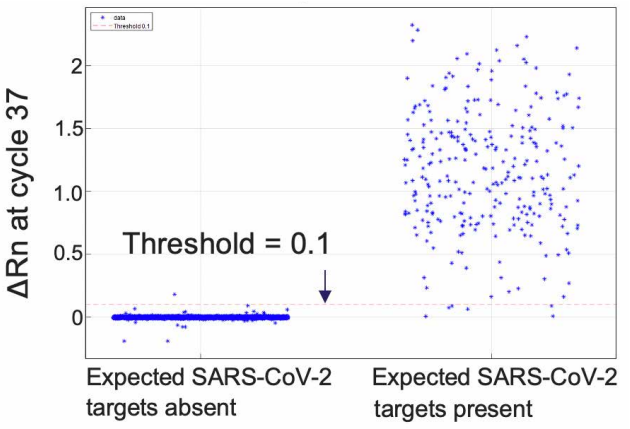

Figure 10. ΔRn values for SARS-CoV-2 samples at 10,000 GCE/mL and NTC.

Conclusion

The method outlined in this study produced analysis parameters for the TaqCheck SARS-CoV-2 Fast PCR Assay, which, when implemented, minimize incorrect calls and spurious retests due to failing RNase P results. The experiments reported here accounted for common sources of variability experienced with pathogen detection assays such as the TaqCheck SARS-CoV-2 Fast PCR Assay. The analysis of these experiments provided optimal parameters in terms of ΔRn thresholds and C_t cutoff values for use in routine analysis in these labs.

This study may only be used as a reference for any lab determining threshold values and performing analytical sensitivity verification.

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