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Enhanced throughput of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) real-time RT-PCR panel by assay multiplexing and specimen pooling

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ABSTRACT

A multiplex real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) assay for detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was developed based on the same primer and probe sequences of an existing U.S. CDC Emergency Use authorized test panel, targeting SARS-CoV-2 N1, N2 and human RNase P genes in singleplex. Both singleplex and multiplex assays demonstrated linear dynamic ranges of 8 orders of magnitude and analytical limits of detection of 5 RNA transcript copies/reaction. Both assays showed 100 % agreement with 364 previously characterized clinical specimens (146 positive and 218 negative) for detection of SARS-CoV-2 RNA. To further increase testing throughput, 40 positive and 20 negative four-specimen pools were tested by the multiplex assay and showed 97.75 % and 100 % congruence with individual specimen tests, respectively. rRT-PCR assay multiplexing and sample pooling, individually or in combination, can substantially increase throughput of SARS-CoV-2 testing.

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) poses unprecedented challenges to medical and public health systems throughout the world (Sharfstein et al., 2020). The U.S. Centers for Disease Control and Prevention (CDC) 2019-Novel Coronavirus (2019-nCoV) Real-Time Reverse Transcriptase (RT)-PCR Diagnostic Panel (2019-nCoV rRT-PCR panel) for detection of SARS-CoV-2, which was granted Emergency Use Authorization (EUA) by the Food and Drug Administration (FDA), comprises three singleplex assays: two that target the virus nucleoprotein gene (N1 and N2) and a third that targets the human RNase P gene (RP) (CDC, 2020a; Lu et al., 2020). The test panel requiring three separate rRT-PCR reactions, limits the throughput of SARS-CoV-2 detection. The CDC singleplex assays have been multiplexed successfully by some commercial and clinical laboratories (Kudo et al., 2020; LabCorp, 2020; Perchetti et al., 2020a; Quest, 2020; Waggoner et al., 2020).

Sample pooling, that allows laboratories to test more samples using fewer resources, is especially useful when the disease prevalence is 20 % or lower (CDC, 2020a) and has been used previously for other pathogens for large scale testing (Hsiang et al., 2010; Keys et al., 2014; Sullivan et al., 2011). Recent studies demonstrated that a pooled screening strategy was feasible to increase testing throughput for SARS-CoV-2 (Abdalhamid et al., 2020; Griesemer et al., 2020; Hogan et al., 2020; Perchetti et al., 2020b). FDA granted the first EUA for sample pooling in COVID-19 diagnostic testing on July 18, 2020 (Quest, 2020). "CDC Diagnostic or Screening Testing Using a Pooling Strategy General Guidance" (CDC, 2020b) also gives instruction that laboratories certified under the Clinical Laboratory Improvement Amendments (CLIA) can use a specimen pooling strategy to expand SARS-CoV-2 nucleic acid diagnostic or screening test capacity when using an FDA-authorized test.

In this study, we compared our routine test procedure using three singleplex rRT-PCR assays to test individual samples with a multiplex assay, also testing four-sample pools to detect SARS-CoV-2. We demonstrate that a combination of assay multiplexing with sample pooling substantially increases test throughput with negligible loss of sensitivity.

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2. Material and methods

2.1. Viruses and specimens

Inactivated SARS-CoV-2 stock USA-WA1/2020 (2.01×10^6 50 % tissue culture infectious dose [$TCID_{50}$]) (Lu et al., 2020) was used as reference material for assay evaluation. RNA transcript of the SARS-CoV-2 nucleocapsid (N) gene was used for assessing assay analytical sensitivity as previously described (Lu et al., 2020). A convenience collection of archived nucleic acid extracts from 364 nasopharyngeal swab (NPS) specimens in viral transport medium (VTM) submitted to the CDC from January to May 2020 for testing with the 2019-nCoV rRT-PCR panel was used for assay clinical performance evaluation. All residual samples and nucleic acid extracts were stored at −70 °C. Of the 364 archived nucleic acid extracts analyzed, 146 were positive and 218 were negative for SARS-CoV-2 RNA upon arrival at CDC prior to being frozen for storage. A pool of 94 negative NPS samples was used as clinical matrix for the reproducibility study. Forty SARS-CoV-2 positive NPS specimens with viral loads that span the clinical range for natural infection (CDC, 2020a), with N1 cycle threshold (Ct) values ranging from 23.48 to 38.01 [median, 29.40; interquartile range (IQR), 7.55] and N2 Ct values ranging from 23.71 to 38.52 (median, 29.76; IQR, 8.13) of which 9 (22.5 %, 9/40) were very weakly positive specimens (36 < Ct<40), and 80 previously characterized negative NPS samples were selected for sample pooling assessment with the multiplex rRT-PCR assay.

2.2. Primers and probes

The primer and probe sequences used in the singleplex and multiplex assays were identical. Probes used in the multiplex assays were labeled with different fluorescence reporter and quenchers dyes (Table 1).

2.3. Multiplex rRT-PCR assay

The multiplex rRT-PCR assay was performed using the TaqPath™ 1-Step Multiplex Master Mix (No ROX) (Thermo Fisher Scientific). Concentrations of primers and probes were optimized to achieve best performance of the multiplex assay (Table 1). Each 20-μL reaction mixture contained 5 μL of 4X Master Mix, 0.5 μL of each probe, 0.5 μL of each forward and reverse primer, with final concentrations listed in Table 1, 5.5 μL of nuclease-free water and 5 μL of nucleic acid extract. Amplification was carried out in 96-well plates on an Applied Biosystems™ 7500 Fast Dx Real-Time PCR (Thermo Fisher Scientific). Thermocycling conditions consisted of 2 min at 25 °C for UNG incubation, 10 min at 53 °C for reverse transcription, 2 min at 95 °C for activation of the *Taq* enzyme and 45 cycles of 3 s at 95 °C and 30 s at 55 °C. The positive and no-template controls were included in all runs. A positive test result was defined as an exponential fluorescence curve that crossed the threshold within 40 cycles. Test result interpretation was consistent with the 2019-nCoV rRT-PCR panel (CDC, 2020a; Lu et al., 2020). 2019-nCoV rRT-PCR panel was performed as previously described (Lu et al., 2020).

2.4. Sample pooling and nucleic acid extraction

Eighty known negative specimens were used to make 20 negative 4-specimen pools. Forty 4-specimen pools each containing one positive specimen and three negative specimens were constructed. For un-pooled individual specimens, 120 μL of respiratory specimen was extracted into 120 μL of eluate using the EZ1 DSP Virus Kit (QIAGEN) as previously described (CDC, 2020a; Lu et al., 2020). To reduce specimen dilution effect during pooling, the extraction input and elution volumes were modified for the pooled specimens. Fifty microliters of positive specimen together with 50 μL from each of three negative samples, for a final volume of 200 μL, were extracted into 60 μL of eluate using the EZ1 DSP Virus Kit. Either positive or inconclusive pooled specimen results required each pool member to be tested individually (CDC, 2020a). Extracts were either tested by the multiplex rRT-PCR assay immediately or stored at −70 °C until use.

2.5. Assay efficiency, sensitivity, and reproducibility evaluation

Serial 10-fold dilutions of quantified N gene RNA transcript were prepared in 10 mM Tris−HCl, pH 8.0 buffer containing 50 ng/μL of yeast tRNA (Thermo Fisher Scientific) and tested in 5 replicates by the multiplex assay to assess assay efficiency. Serial two-fold dilutions of the same material from 2 to 0.25 copies/μL were prepared in buffer as above and tested by the multiplex assay in 24 replicates per dilution to assess assay sensitivity. The highest dilution of transcript at which all replicates were positive was defined as the limit of detection (LoD). Assay reproducibility was evaluated with three contrived respiratory specimens constructed from pooled negative nasopharyngeal swabs and spiked with high (1.0×10^3 TCID50/mL), moderate (1.0×10^1 TCID50/mL), and low (1.0×10^{-1} TCID50/mL) concentrations of cultured inactivated SARS-CoV-2. The contrived specimens were extracted in triplicate and the extracts were tested by the multiplex rRT-PCR assay on three different days.

Table 1
SARS-CoV-2 multiplex real-time RT-PCR assay primers and probes.

Assay	Primers & probes	Sequence (5' > 3')[d]	Working Concentration (μM)	Final Concentration (nM)
N1	Forward primer	GACCCCAAAATCAGCGAAAT	5	125
	Reverse primer	TCTGGTTACTGCCAGTTGAATCTG	5	125
	Probe[a]	5'FAM-ACCCCGCAT/ZEN/TACGTTTGGTGGACC-3'IBFQ	2.5	62.5
N2	Forward primer	TTACAAACATTGGCCGCAAA	20	500
	Reverse primer	GCGCGACATTCCGAAGAA	20	500
	Probe[b]	5'Yak-ACAATTTGC/ZEN/CCCCAGCGCTTCAG-3'IBFQ	5	125
RP	Forward primer	AGATTTGGACCTGCGAGCG	5	125
	Reverse primer	GAGCGGCTGTCTCCACAAGT	5	125
	Probe[c]	5'Cy5-TTCTGACCT/TAO/GAAGGCTCTGCGCG-3'IBRQ	2.5	62.5

[a] Probe labeled at the 5′-end with the reporter molecule 6-carboxyfluorescein (FAM), with a ZEN™ quencher between the 9th and 10th nucleotide, and with an Iowa Black™ FQ quencher (IBFQ) at the 3′-end (Integrated DNA Technologies, Coralville, IA).

[b] Probe labeled at the 5′-end with the Yakima Yellow® (Yak), with a ZEN™ quencher between the 9th and 10th nucleotide, and with an IBFQ at the 3′-end (Integrated DNA Technologies).

[c] Probe labeled at the 5′-end with the Cy® 5 reporter, with a TAO™ quencher between the 9th and 10th nucleotide, and with an Iowa Black™ RQ quencher (IBRQ) at the 3′-end (Integrated DNA Technologies).

[d] Primer/probe sequences from Lu et al., 2020.

2.6. Statistical analysis

Positive percent agreement (PPA), negative percent agreement (NPA), and the overall percent agreement of the multiplex assay were calculated using the 2019-nCoV rRT-PCR panel as the reference standard and were reported with exact Clopper-Pearson confidence intervals (95 % CI) (Clopper and Pearson, 1934). The percent overall agreement between two assays was measured using the Cohen's Kappa statistic (Cohen, 1960) where 0 indicates no agreement and 1 indicates perfect agreement. The coefficient of variation (CV) was used to measure assay reproducibility. For Ct value pairwise comparison between the multiplex rRT-PCR assay and the 2019-nCoV rRT-PCR panel, pooled specimens and un-pooled specimens, the Bland-Altman analysis (Bland and Altman, 1986) was used to depict the magnitude of agreement between assays. Paired *t*-test and Wilcoxon matched pairs test were used to test for statistical differences in Ct values between the two assays. A p-value <0.05 was considered statistically significant.

3. Results

3.1. Assay efficiency, sensitivity, and reproducibility

Linear amplification was achieved over an 8-log dynamic range from 5 to 5×10^7 copies per reaction for N1 and N2 targets with calculated efficiency of 100.7 % and 100.8 %, respectively (Fig. 1).The LoD for the N1 and N2 targets in the multiplex assay were 5 RNA transcript copies/reaction (Table 2), which was identical with the 2019-nCoV rRT-PCR panel (Lu et al., 2020). Inter-assay variation of Ct values of the three contrived respiratory specimens with high, moderate, and low concentrations of SARS-CoV-2 was low for all the targets in the multiplex rRT-PCR assay (CV range for N1, 0.39–2.23 %; N2, 1.20–1.37 %; RP, 0.44-0.70 %) (Table 3).

3.2. Performance with discrete clinical specimens

Archived nucleic acid extracts from 364 clinical specimens (146 positive and 218 negative) were tested by the multiplex assay and the 2019-nCoV rRT-PCR panel concurrently to compare their diagnostic performance. For the 146 specimens previously tested positive by the panel, the Ct values for N1 target ranged from 16.11 to 38.82 (median, 28.80; IQR, 7.57) and the Ct values for N2 target ranged from 16.05 to 39.18 (median, 29.18; IQR, 7.34) in the initial testing. Twenty-one positive specimens (14.4 %, 21/146) were weakly positive (Ct values $\geq$36) and 45 specimens (30.8 %, 45/146) were moderately positive (Ct values 30–35.99). Among the 146 previously positive nucleic acid extracts, all tested positive by the multiplex assay. In contrast, the panel detected fewer positive samples compared to the result from the initial test on 6 (4 inconclusive, 2 negative) archived extracts that had been stored at −70 °C for months. All 6 specimens were confirmed positive by the 2019-nCoV rRT-PCR panel after re-extraction and retesting from the original specimens and all were very weakly positive with Ct >36 for both N1 and N2 targets in the initial testing upon arrival at CDC. Overall, the Kappa coefficient of the multiplex assay with the 2019-nCoV rRT-PCR panel was 1 (95 % CI, 0.987–1) and a PPA and NPA of 100 % (95 % CI, 97.4–100 %) and 100 % (95 % CI, 98.3–100 %), respectively (Table 4). Although Ct values obtained from the 146 positive specimens by the multiplex assay and the panel correlated well for both N1 and N2 targets ($R^2 = 0.97$ and 0.92 for N1 and N2, respectively) (Fig. 2A), Ct values of the multiplex assay were significantly lower ($p < 0.0001$) than those of the singleplex assays in the panel, on average by 0.34 Ct [95 % limits of agreement (lower and upper limits of agreement), -2.28, 1.59] and 0.93 Ct (95 % limits of agreement, -3.99, 2.12) for N1 and N2 targets, respectively (Fig. 2B). The ΔCt (difference in Ct value between the multiplex assay and the 2019-nCoV rRT-PCR panel) for the majority of specimens tested was within the 95 % limit of agreement for both N1 and N2 targets. A few outlier results with greater ΔCt values were observed when the Ct values were high (>34). The RP rRT-PCR assay results were unambiguously positive with all samples tested (data not shown).

Table 2
SARS-CoV-2 multiplex real-time RT-PCR assay limits of detection with RNA transcripts.

Predicted RNA copies/reaction	No. of positive tests/no. of transcript replicates (%)	
	N1	N2
10	24/24 (100)	24/24 (100)
5	24/24 (100)	24/24 (100)
2.5	23/24 (95.8)	21/24 (87.5)
1.25	21/24 (87.5)	20/24 (83.3)

Lowest RNA copies at which 100 % of multiplex rRT-PCR replicates were positive are underlined.

3.3. Performance with pooled clinical specimens

One positive pool gave inconclusive results (N1 not detected, N2 Ct: 39.08) by the multiplex assay, while the individual specimen tested weakly positive (N1 Ct: 36.15 and N2 Ct: 36.19); the remaining 39 positive pools were positive, giving a 97.5 % positive pool agreement with the expected results (95 % CI, 87.1–99.6%) (Table 5). Following the CDC pooled specimen testing algorithm from the 2019-nCoV rRT-PCR panel EUA that either positive or inconclusive pooled specimen results require testing of all pool members individually (CDC, 2020a), the positive pool agreement with expected results (positive and inconclusive) would be 100 % (95 % CI, 91.2–100%). The negative pool agreement with expected results was 100 % (95 % CI, 83.9–100%). For the 39 pools that had positive testing results, the pools' Ct values were an average of 1.51 cycles (95 % limits of agreement, -0.27, 3.29) and 1.51 cycles (95 % limits of agreement, -0.83, 3.84) higher than the

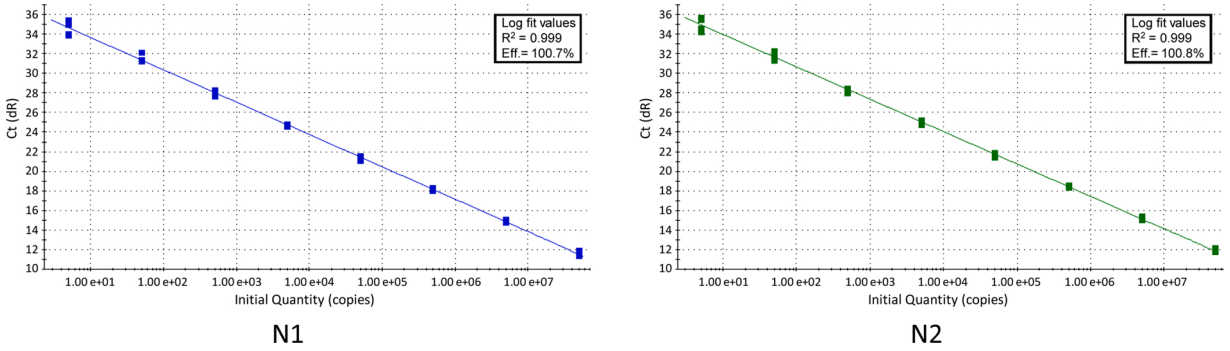

Fig. 1. Standard curves of serial 10-fold dilutions ranging from 5 to 5×10^7 copies/reaction of the nucleocapsid synthetic RNA transcripts tested in five replicates by the multiplex SARS-CoV-2 rRT-PCR. Plot inserts show calculated linear correlation coefficients (R^2) and amplification efficiencies (Eff.) for N1 and N2 targets.

Table 3
SARS-CoV-2 Multiplex real-time RT-PCR assay reproducibility with virus-spiked respiratory specimen matrix[a].

Virus titer, $TCID_{50}$/mL	N1 Ct[b]			N2 Ct			RP Ct		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Day 1									
1.0×10^3	21.41	21.18	21.19	20.38	20.37	20.35	27.89	27.97	27.83
1.0×10^1	28.14	28.11	28.01	27.29	27.31	27.00	29.28	29.46	29.18
1.0×10^{-1}	35.34	34.61	34.46	34.35	33.72	33.92	28.67	28.32	28.58
Day 2									
1.0×10^3	21.10	21.20	21.23	20.72	20.92	21.07	28.19	28.00	28.15
1.0×10^1	28.05	28.18	27.99	27.95	28.01	27.60	29.35	29.61	29.36
1.0×10^{-1}	35.34	33.93	34.07	34.17	33.28	34.08	28.41	28.80	28.54
Day 3									
1.0×10^3	21.19	21.24	21.18	20.70	20.59	20.78	27.92	27.87	27.96
1.0×10^1	28.20	28.45	28.14	27.62	27.80	27.46	29.07	29.37	29.73
1.0×10^{-1}	35.24	33.00	34.48	34.46	33.92	34.92	28.63	28.24	28.80
Summary results	Mean	SD[c]	CV[d]	Mean	SD	CV	Mean	SD	CV
1.0×10^3	21.21	0.08	0.39 %	20.65	0.25	1.23 %	27.97	0.12	0.44 %
1.0×10^1	28.14	0.14	0.49 %	27.56	0.33	1.20 %	29.38	0.20	0.69 %
1.0×10^{-1}	34.50	0.77	2.23 %	34.09	0.47	1.37 %	28.56	0.20	0.70 %

[a] Specimen matrix constructed from combined nasopharyngeal swabs obtained from 94 persons.
[b] Ct: cycle threshold.
[c] SD: standard deviation.
[d] CV: coefficient of variation.

Table 4
Comparison of the SARS-CoV-2 multiplex real-time RT-PCR assay with the CDC 2019-nCoV real-time RT-PCR Diagnostic Panel (2019-nCoV rRT-PCR panel).

Multiplex Assay	2019-nCoV rRT-PCR panel [a]				
	Positive	Negative	PPA[b] (95% CI)[c]	NPA[d] (95% CI)	Kappa (95% CI)[e]
Positive	146	0	100 (97.4–100)	100 (98.3–100)	1 (0.987–1)
Negative	0	218			

[a] 2019-nCoV rRT-PCR panel was used as the reference standard.
[b] PPA: positive percent agreement.
[c] 95 % CI, 95 % confidence interval.
[d] NPA: negative percent agreement.
[e] Kappa value representing level of agreement: <0-0.2 = poor; 0.21-0.4 = fair; 0.41-0.6 = moderate; 0.61-0.8 = good; and 0.81–1 = very good.

individual positive samples for N1 and N2 targets, respectively, by Bland-Altman analysis (Fig. 3B). Strong Ct value correlations were obtained between pooled and individual specimens for both N1 and N2 targets ($R^2 = 0.96$ and 0.94 for N1 and N2, respectively) (Fig. 3A).

4. Discussion

One of the main challenges of multiplex rRT-PCR assay is potential oligonucleotide interaction and competition for reagents within the same reaction, which could reduce amplification efficiency and sensitivity (Sint et al., 2012). In this study, we found that the efficiency and LoD of the multiplex SARS-CoV-2 rRT-PCR assay was comparable to the 2019-nCoV rRT-PCR panel for detection of SARS-CoV-2 serially diluted RNA transcripts. In fact, the multiplex assay had slightly better clinical performance than the panel for detection of archived SARS-CoV-2 RNA with high Ct values. Side-by-side comparisons of the multiplex rRT-PCR assay and the 2019-nCoV rRT-PCR panel showed that the multiplex assay was able to detect 6 archived extracts with very low concentrations of SARS-CoV-2 RNA that were missed by the panel after being frozen. This discrepancy was resolved by re-extraction and retesting of the original specimens by the panel and was therefore likely due to RNA degradation occurring during long-term storage or the freeze-thaw process, even though the RNA was frozen at −70 °C. Overall, the retrospective comparison of 364 NPS specimens showed excellent agreement using the Cohen's Kappa statistic between the multiplex and singleplex assays.

The decreased Ct values in the multiplex assay was likely caused by the different real-time RT-PCR master mixes used and different quencher/reporter dye for the N2 assay in the multiplex and singleplex assays. Other factors that could impact the multiplex rRT-PCR performance include primer/probe concentrations. Limited examination of other commercial multiplex rRT-PCR reagents kits showed that the multiplex assay performed poorly with UltraPlex 1-Step ToughMix (Quantabio) (data not shown) although the reagent worked well with the 2019-nCoV rRT-PCR panel (CDC, 2020a). Similar multiplex assays using different reagents and running conditions may not perform comparably with the one specified here. For example, a SARS-CoV-2 multiplex assay based on the same primer/probe sequences of 2019-nCoV rRT-PCR panel in a recent study that used different rRT-PCR reagents, fluorescence reporter dyes and primer/probe concentrations gave a LoD of 500 copies of RNA transcripts/reaction, two logs less sensitive than that obtained in our study (Kudo et al., 2020). The multiplex rRT-PCR assay developed and validated in this study is for research use only and has not been authorized by the FDA for patient care purposes. Laboratories choosing to use the assay must validate the assay independently as a laboratory developed test for in vitro diagnostic test in accordance with FDA and CLIA regulations.

Specimen pooling can increase SARS-CoV-2 testing capability and preserving testing resources (Abdalhamid et al., 2020; Griesemer et al., 2020; Hogan et al., 2020; Perchetti et al., 2020b). The major concern of sample pooling is loss of test sensitivity due to specimen dilution. Modified extraction input and elution volume to increase the concentration of the final eluant helps compensate for the dilution effect of pooling. Our data showed that pooling of 4 specimens with modified extraction protocol is suitable for use with the multiplex assay. A web-based application for pooling (A Shiny App, 2020), with an assay having 100 % sensitivity and specificity and a two-stage pooling algorithm, predicted that a sample pool size of 4 would reduce the expected number of tests by 56 % with a COVID-19 prevalence of 5 %, when compared to individual sample testing. However, if COVID-19 prevalence increased to 20 %, the expected number of tests would only be reduced by 16 %. Reduction in test volume with pooling is inversely related to COVID-19 prevalence. Therefore, sample pooling to increase throughput should only be considered with populations where the prevalence of infection is expected to be low (CDC, 2020a; 2020b). Since

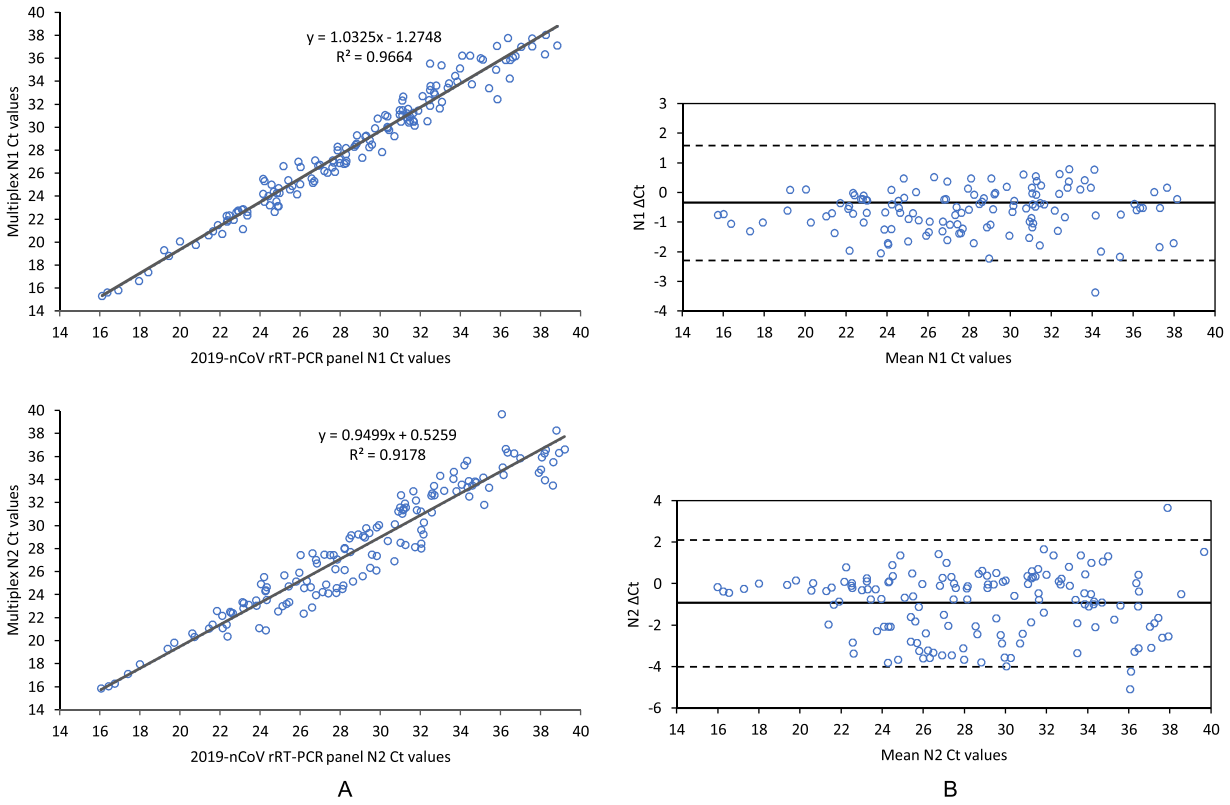

Fig. 2. A) Comparison of the SARS-CoV-2 multiplex Real-Time RT-PCR (rRT-PCR) assay with the singleplex assays in the CDC 2019-nCoV rRT-PCR panel with 146 SARS-CoV-2 positive clinical specimens. Linear regression lines fitted to cycle threshold (Ct) data with regression equations and coefficients of determination (R^2) insets. B) Bland-Altman plot analysis: ΔCt (Ct value difference between the SARS-CoV-2 rRT-PCR multiplex assay and the singleplex assays in the 2019-nCoV rRT-PCR panel with 146 positive specimens) vs average Ct values obtained from the SARS-CoV-2 multiplex rRT-PCR and the 2019-nCoV rRT-PCR panel. Solid lines represent the mean ΔCt and dashed lines represent the upper and lower limits of agreement (mean ΔCT ± 1.96 standard deviation of ΔCt).

Table 5
Comparison of 4-pooled specimens with individual specimens with the SARS-CoV-2 multiplex real-time RT-PCR assay.

Pooled specimens	Individual specimens[a]		Positive pool agreement with expected results (95 % CI)[b]	Negative pool agreement with expected Results (95 % CI)	Kappa[c] (95 % CI)
	Positive	Negative			
Positive	39	0	97.5 (87.1–99.6)[d]	100 (83.9–100)	0.983 (0.911-0.997)[e]
Negative	0	20			
Inconclusive	1	0	100 (91.2–100)[e]		1 (0.940–1)[e]

[a] Individual specimen testing was used as the reference standard.
[b] 95 % CI, 95 % confidence interval.
[c] Kappa value representing level of agreement: <0-0.2 = poor; 0.21-0.4 = fair; 0.41-0.6 = moderate; 0.61-0.8 = good; and 0.81–1 = very good.
[d] Only positive was considered as expected result.
[e] Both positive and inconclusive were considered as expected result.

SARS-CoV-2 positivity rates vary in different regions of the U.S. and fluctuate with time (CDC, 2020c), laboratories should monitor specimen positivity rates over time to determine if pooling of 4 specimens continues to provide a test throughput advantage (CDC, 2020a). It must also be noted that specimen pooling would not reduce the time spent on the specimen accessioning and reporting processes and may complicate electronic data management systems (Griesemer et al., 2020).

Both multiplexing and specimen pooling can substantially increase testing throughput and reduce reagents and consumable costs. With extraction and rRT-PCR controls being included in the test properly, at least three-times as many samples with multiplexing can be tested in a single run per plate per real-time PCR instrument. Moreover, multiplexing and specimen pooling will reduce pipetting steps and staffing costs associated with setup time.

In conclusion, the multiplex rRT-PCR detected SARS-CoV-2 with the same sensitivity and specificity as the 2019-nCoV rRT-PCR Panel, and when used to test 4-specimens pools, showed nearly perfect agreement with individually tested specimens. While specimen pooling has the potential to slightly reduce the sensitivity of SARS-CoV-2 RNA detection, it could substantially increase testing throughput and preserving testing reagents and resources. Although the multiplex assay is currently for research use only, it could be validated by users for clinical testing and could be further expanded through sample pooling among populations with low COVID-19 prevalence.

Disclaimer

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Author statement

Xiaoyan Lu: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing - Original Draft, Writing - Review & Editing.

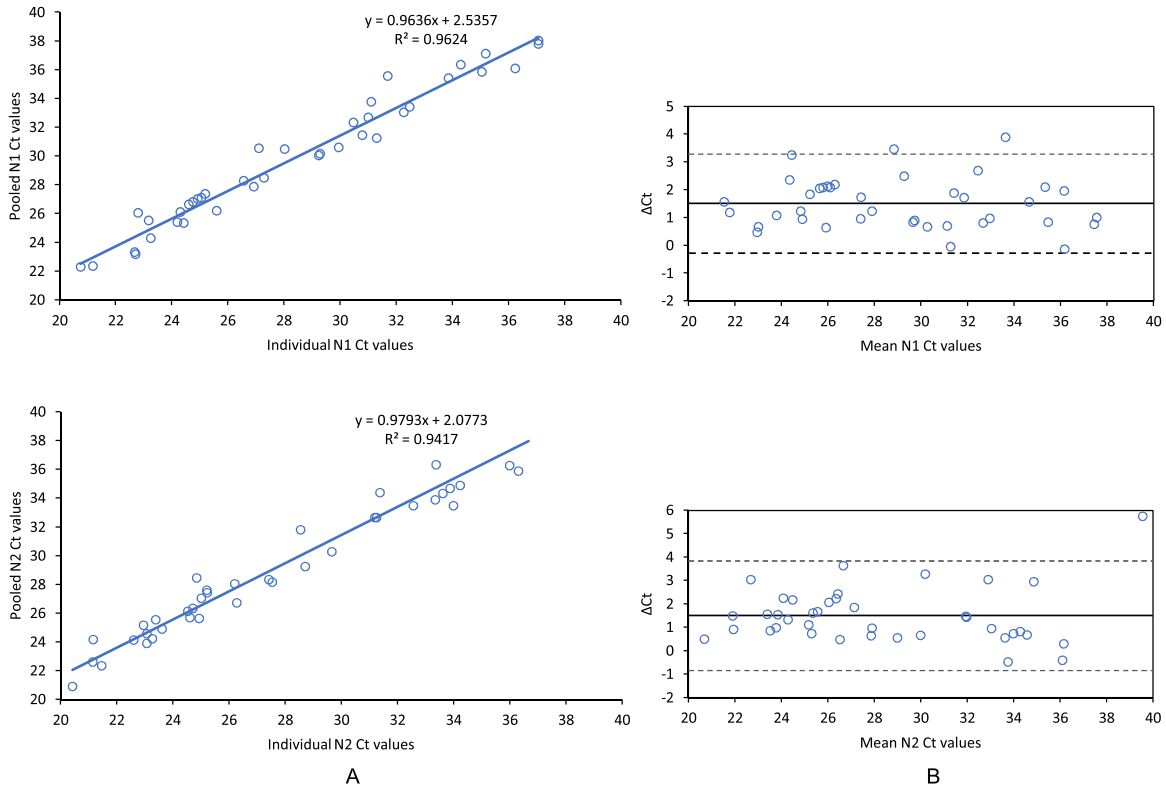

Fig. 3. A) Comparison of cycle threshold (Ct) values of 39 positive 4-specimen pools containing one SARS-CoV-2 positive sample with three negative specimens vs individual positive specimens tested with the SARS-CoV-2 multiplex real-time RT-PCR (rRT-PCR) assay. Linear regression lines fitted to Ct values with regression equations and coefficients of determination (R^2) insets. B) Bland-Altman plot analysis: ΔCt (Ct value difference between 39 positive 4-specimen pools each containing one SARS-CoV-2 positive sample and 3 negative specimens and individual positive specimens) vs average Ct values of individual and pooled specimens obtained from the SARS-CoV-2 multiplex rRT-PCR assay. Solid lines represent the mean ΔCt and dashed lines represent the upper and lower limits of agreement (mean ΔCT ± 1.96 standard deviation of ΔCt).

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Declaration of Competing Interest

The authors report no declarations of interest.

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