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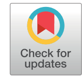
Short communication

Multiplexing primer/probe sets for detection of SARS-CoV-2 by qRT-PCR



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ABSTRACT

Background: The novel respiratory virus SARS-CoV-2, responsible for over 380,000 COVID-19 related deaths, has caused significant strain on healthcare infrastructure and clinical laboratories globally. The pandemic's initial challenges include broad diagnostic testing, consistent reagent supply lines, and access to laboratory instruments and equipment. In early 2020, primer/probe sets distributed by the CDC utilized the same fluorophore for molecular detection - requiring multiple assays to be run in parallel - consuming valuable and limited resources.
Methods: Nasopharyngeal swabs submitted to UW Virology for SARS-CoV-2 clinical testing were extracted, amplified by our laboratory developed test (LDT) - a CDC-based quantitative reverse transcriptase PCR reaction - and analyzed for agreement between the multiplexed assay. Laboratory- confirmed respiratory infection samples were included to evaluate assay cross-reaction specificity.
Results: Triplexing correctly identified SARS-CoV-2 in 98.4% of confirmed positive or inconclusive patient samples by single-plex LDT (n = 183/186). All 170 SARS-CoV-2 negative samples tested by single-plex LDT were negative by triplexing. Other laboratory-confirmed respiratory infections did not amplify for SARS-CoV-2 in the triplex reaction.
Conclusions: Multiplexing two virus-specific gene targets and an extraction control was found to be comparable to running parallel assays independently, while significantly improving assay throughput.

1. Introduction

The novel virus responsible for causing coronavirus disease 2019 (COVID-19), Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), has infected more than six million individuals in 188 countries as of writing [1]. Emerging from Wuhan, China in late 2019, the ongoing pandemic has been intensified by lack of adequate diagnostic testing in the US and internationally [2]. SARS-CoV-2 is highly communicable with significant morbidity and mortality [3–5]. Early detection of SARS-CoV-2 can identify patients who are more likely to experience significant disease and so curb pathogen transmission and scope of global contagion.

Many labs use the Centers for Disease and Control and Prevention (CDC) primer and probe sets targeting N1 and N2 for SARS-CoV-2 and RPP30 as a human control [6]. As the CDC kits utilize the same fluorescent reporter for each of the primer/probe sets, reactions are required to be run separately, leading to fewer than 30 samples per 96-well plate. To increase throughput of SARS-CoV-2 testing in clinical laboratories, we designed a multiplexed real-time quantitative reverse transcription PCR (qRT-PCR) assay utilizing primers and probe sets from the CDC combined with an internal extraction control.

Multiplexed qRT-PCR is a powerful tool in laboratory medicine, able to detect infectious disease pathogens effectively and efficiently. Multiple target assays are critical for accurate SARS-CoV-2 detection, as it is possible to miss low viral load infections if only a single gene amplicon is used. After running a duplex reaction with N1 and N2 in separate wells with internal control, we developed a three-target single-reaction triplex assay with the same viral nucleocapsid gene targets. Multiplexing offers increased throughput of SARS-CoV-2 detection by reducing the quantity of qRT-PCR reactions run in parallel [7]. Here, we describe a single-reaction, triplex assay for SARS-CoV-2 that demonstrates comparable sensitivity to individual parallel assays.

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2. Methods

2.1. Clinical specimens

The SARS-CoV-2 positive control consisted of a wild-type clinical nasopharyngeal (NP) swab tested at UW Virology in late February, 2020. HeLa cells for extraction and no template controls of water for amplification were included as negative standards. NP swabs in viral transport media were submitted to UW Virology for COVID-19 clinical testing by LDT beginning in March 2020. Specimens were subsequently compared to triplex assay performance by C_Ts and percent of positive samples detected.

2.2. Extraction

Nucleic acid (NA) extraction was performed on Roche's MagNA Pure 96 instrument enabling high-throughput total NA extraction using the pathogen universal kit [8]. In brief, 200 µL of sample was extracted and eluted into 50 µL elution buffer and 5 µL of eluted template was utilized for each subsequent 25 µL LDT assay, whereas 11 µL of eluted RNA was used for triplexing.

2.3. qRT-PCR

Distinct amplicons within the N gene, the region encoding a nucleocapsid protein of SARS-CoV-2, were targeted for detection: N1 and N2. Each target was combined with EXO (a 130-base RNA transcript derived from jellyfish DNA) to serve as an internal extraction control [9,10]. If all targets amplified, the result was determined positive. If only one of the N gene targets amplified with EXO, then the result is inconclusive and subsequently re-tested. Not detected (NDET) test results required the amplification of EXO, without N1 or N2 amplification.

AgPath-ID One-Step RT-PCR Kit (Life Technologies, Carlsbad, CA) constituting ArrayScript reverse transcriptase, AmpliTaq Gold DNA polymerase, and buffer, was utilized for qRT-PCR. Each 25 µL reaction consisted of 0.09 µL H_20, 12.5 µL of 2X reaction mix buffer, 1 µL 25X enzyme, and 11 u L of extracted RNA template. Final primer concentrations were [200 nmol/L] for N1, N2, and EXO reverse: 5'-GGAACCTAAGACAAGTGTGTTTATGG-3', and [100 nmol/L] for EXO forward: 5'-GGCGGAAGAACAGCTATTGC-3'. FAM (G-carboxyfluorescein) and Cy5: 5'-Cy5-ACAATTTGCCCCCAGCGCTTCAG-BHQ-3' (Sulfo-Cyanin5) probes had a final concentration of 100 nmol/L each. EXO's reporter VIC: 5'-VIC-AACGCCATCGCACAAT-MGB-3' (proprietary fluorescent dye to ABI) was included at [62.5 nmol/L]. Complete probe and primer sequences are outlined in the CDC SARS-CoV-2 protocol [11,12].

Thermocycling conditions were 48 °C (10 min), 95 °C (10 min), followed by 40 cycles of 95 °C (15 s) and 60 °C (45 s). Viral amplification utilized ABI 7500 Real-Time PCR Systems (Applied Biosystems, Foster City, CA) and PCR analysis was performed on Life Technologies Applied Biosystems' 7500 software v2.3. Reactions were evaluated with water, extracted HeLa cells, and extracted SARS-CoV-2 as standards. A baseline from 6 to 15 and threshold of 0.1 was used across clinical and LoD validation runs for LDT PCR. For triplexing three targets in a single reaction, the threshold was set at 0.025 for N1 and N2, while EXO's threshold was set at 0.02 [13].

3. Results

3.1. Optimization of multiplexing

We compared the performance of a novel triplex three-target assay run in parallel to the Washington state emergency use authorization LDT performed at UW Virology. Positive samples of unique patients (n = 186) from clinical runs were compared (N1 C_T range: 14–36.3, N2: 13.8–39.5). Our triplex assay detected 183/186 (98.4%) positives or inconclusive results for SARS-CoV-2. The mean and median C_Ts were comparable between UW Virology's LDT and novel triplex assays, varying by less than two C_Ts (Table 1). Triplexing was also performed on samples testing negative for SARS-CoV-2 by LDT (n = 170) and corroborated all 170 samples as negative for SARS-CoV-2, demonstrating 100% assay specificity (Table 2).

Table 1
Clinical LDT CT comparison to triplex assay.

Target	Mean C_T	Median C_T	Range C_T
N1 LDT	24.0	23.0	14.0-36.3
N1 Triplex	23.1	22.2	13.7-36.5
N2 LDT	24.0	23.0	13.8-39.5
N2 Triplex	25.4	24.9	14.0-39.6

Abbreviations: C_T, cycle threshold, LDT, laboratory developed test.
Positive or inconclusive SARS-CoV-2 samples (n = 183) tested by triplex have comparable mean and median C_T values to LDT.

Table 2
Results of clinical LDT assay and triplex comparison.

	Triplex Assay			
LDT	N1/N2 Positive	Inconclusive	NDET	Total
N1/N2 Positive	158	15	1	174
Inconclusive	5	5	2	12
NDET	0	0	170	170
Total	163	20	173	356

Abbreviations: LDT, laboratory developed test, NDET, not detected.
Total SARS-CoV-2 positives, inconclusives, and NDETS in clinical LDT and triplex assays (n = 356). Including additional laboratory-confirmed respiratory infections (n = 20), testing by triplex demonstrated 99.2% agreement (n = 373/376).

Laboratory-confirmed non-SARS-CoV-2 infections from unique patient NP swabs (n = 20) by respiratory panel were extracted, amplified, and compared for assay specificity [14–16]. NP swabs included were rhinovirus (n = 2), parainfluenza (n = 6), respiratory syncytial virus (n = 2), adenovirus (n = 2), bocavirus (n = 1), non-SARS-CoV-2 coronavirus (n = 3), influenza (n = 2), and human metapneumovirus (n = 2). C_T ranges for laboratory-confirmed respiratory infections spanned from 19.9 to 29.9. All respiratory infection samples were negative for SARS-CoV-2 by the triplex assay. Of all 356 samples assayed by LDT, 353 (99.2%) showed agreement by triplexing.

4. Discussion

Here, we demonstrate that a sensitive, specific triplex assay has the ability to accurately and quickly detect SARS-CoV-2 for the vast majority of infected individuals. Since the CDC kits utilize the same reporter, parallel PCRs are required to confirm multiple targets from the same sample. Separate, individual assays targeting N1, N2, and RNaseP were initially implemented as our LDT. However, as SARS-CoV-2 testing demands increased exponentially, significant strain on thermocycler capacity can be a major throttle in diagnostic COVID-19 testing. Multiple PCR reactions require more reagents, controls, thermocyclers, and labor. In a resource-limited setting, instruments and reagents can be in short supply, especially during a pandemic where PCR thermocyclers can be a rate limiting step [17].

Waggoner et al. recently described a triplex RT-PCR using the N2 target from the CDC and the E gene target published by Corman et al [18,19]. They similarly report negligible loss in sensitivity when all targets are included together in a single-reaction and also demonstrate assay specificity against other respiratory viral infections. However, our triplex assay increased the unique patients suspected of SARS-CoV-2

infection tested from n = 27 to n = 186. Furthermore, although both triplex assays utilize automated extraction platforms, ours has been optimized for improved high-throughput, allowing simultaneous extraction of 96 specimens on Roche's MP96 compared to Waggoner and colleagues' EMAG (Biomérieux, Marcy-l'Étoile, France) platform, which limits extraction to 48 samples at a time.

Multiplexing offers a two-reaction or even single-reaction assay that significantly reduces reagent consumption, labor, inconsistencies in reporting, and frees up valuable lab equipment when it is critically needed. We demonstrated that the CDC individual assays can be triplexed into a single-reaction without substantially compromising sensitivity, detecting 98.4% of samples determined positive or inconclusive by SARS-CoV-2 LDT.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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