A Comparative Study of Real-Time RT-PCR–Based SARS-CoV-2 Detection Methods and Its Application to Human-Derived and Surface Swabbed Material

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Abstract: Real-time RT-PCR remains a gold standard in the detection of various viral diseases. In the coronavirus 2019 pandemic, multiple RT-PCR-based tests were developed to screen for viral infection. As an emergency response to increasing testing demand, we established a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) PCR diagnostics platform for which we compared different commercial and in-house RT-PCR protocols. Four commercial, one customized, and one in-house RT-PCR protocols were evaluated with 92 SARS-CoV-2-positive and 92 SARS-CoV-2-negative samples. Furthermore, economical and practical characteristics of these protocols were compared. In addition, a highly sensitive digital droplet PCR (ddPCR) method was developed, and application of RT-PCR and ddPCR methods on SARS-CoV-2 environmental samples was examined. Very low limits of detection (1 or 2 viral copies/L), high sensitivities (93.6% to 97.8%), and high specificities (98.7% to 100%) for the tested RT-PCR protocols were found. Furthermore, the feasibility of downsizing two of the commercial protocols, which could optimize testing capacity, was demonstrated. Tested commercial and customized RT-PCR detection kits show very good and comparable sensitivity and specificity, and the kits could be further optimized for use on SARS-CoV-2 viral samples derived from human and surface swabbed samples.

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A Comparative Study of Real-Time RT-PCR–Based SARS-CoV-2 Detection Methods and Its Application to Human-Derived and Surface Swabbed Material

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Real-time RT-PCR remains a gold standard in the detection of various viral diseases. In the coronavirus 2019 pandemic, multiple RT-PCR–based tests were developed to screen for viral infection. As an emergency response to increasing testing demand, we established a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) PCR diagnostics platform for which we compared different commercial and in-house RT-PCR protocols. Four commercial, one customized, and one in-house RT-PCR protocols were evaluated with 92 SARS-CoV-2–positive and 92 SARS-CoV-2–negative samples. Furthermore, economical and practical characteristics of these protocols were compared. In addition, a highly sensitive digital droplet PCR (ddPCR) method was developed, and application of RT-PCR and ddPCR methods on SARS-CoV-2 environmental samples was examined. Very low limits of detection (1 or 2 viral copies/μL), high sensitivities (93.6% to 97.8%), and high specificities (98.7% to 100%) for the tested RT-PCR protocols were found. Furthermore, the feasibility of downscaling two of the commercial protocols, which could optimize testing capacity, was demonstrated. Tested commercial and customized RT-PCR detection kits show very good and comparable sensitivity and specificity, and the kits could be further optimized for use on SARS-CoV-2 viral samples derived from human and surface swabbed samples. (J Mol Diagn 2021, 1–9; https://doi.org/10.1016/j.jmoldx.2021.04.009)

On March 11, 2020, the World Health Organization (WHO) (Geneva, Switzerland) declared a pandemic because of the quick spread of a respiratory disease caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). With cases increasing in multiple countries and high transmissibility of SARS-CoV-2, eradication is rather unrealistic in the short term. In Switzerland, the second wave of SARS-CoV-2 is predicted to be slower than the first one but with a higher case fatality rate. The same situation was reported by the WHO for Spanish influenza for which the second and third waves of the infection claimed more lives and the pandemic lasted for almost 2 years and resulted in at least 50 million deaths worldwide [Centers for Disease Control and Prevention (CDC), https://www.cdc.gov/flu/pandemic-resources/1918-commemoration/three-waves.htm, last accessed September 7, 2020]. Another important factor contributing to the rapid spread of the coronavirus disease 2019 (COVID-19) pandemic is an unusually high number

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of asymptomatic spreaders. Therefore, continuous testing and reliable detection of the virus are essential parts of controlling the spread of SARS-CoV-2 (WHO, https://www.who.int/emergencies/diseases/novel-coronavirus-2019/strategies-and-plans, last accessed September 7, 2020).

In March 2020, an in-house platform for SARS-CoV-2 diagnostics was initiated as part of an emergency response to the increasing demand for test capacity in a routine microbiology laboratory at University Hospital in Zurich, Switzerland. Currently, the gold standard for the detection and diagnosis of SARS-CoV-2 infection is based on the real-time RT-PCR. The overall goal was to provide in-house SARS-CoV-2 diagnosis to all patients and personnel to ensure the safe and efficient continuation of the health care work within the hospital and the protection of high-risk patients. The aims of this study were i) to evaluate four commercially available, one customized, and one in-house RT-PCR test by comparing the limit of detection (LoD), sensitivity using a panel of SARS-CoV-2 confirmed cases, and specificity using a group of non-COVID-19 respiratory samples; ii) to examine the feasibility of down-scaling two commercial protocols to optimize the testing capacity; iii) to develop a droplet digital PCR (ddPCR) assay to increase test sensitivity and provide more accurate quantitation of viral RNA; and iv) to examine applicability of two validated RT-PCR protocols as well as of a ddPCR protocol on SARS-CoV-2 environmental samples.

### Table 1 Description of Real-Time RT-PCR Assays Compared in the Study

| RT-PCR protocol | Abbreviated name | RT-PCR kit/primer and probes | Mastermix used in this study | Positive control |
|-----------------|------------------|------------------------------|-----------------------------|-----------------|
| CDC 2019-Novel Coronavirus Real-Time RT-PCR Diagnostic Panel (for in vitro diagnostic) | CDC | 2019-nCoVEUA-01 Diagnostic Panel Box, catalog number 10006606, IDT, Newark, NJ | TaMan, Fast Virus 1-step Master Mix, 4444436, 10 mL, Applied Biosystems/Thermo Fisher Scientific, Waltham, MA | 2019-nCoV N_Positive Control, catalog number 10006625, IDT |
| Applied Biosystems TaqMan 2019-nCoV Assay Kit version 1 | TF-SinglePlex | TaqMan 2019-nCoV Assay Kit v1, catalog number A47532, Applied Biosystems/Thermo Fisher Scientific | TaMan, Fast Virus 1-step Master Mix, catalog number 4444436, 10 mL, Applied Biosystems/Thermo Fisher Scientific | 2019-nCoV Control version 1, catalog number A47533, Applied Biosystems/Thermo Fisher Scientific |
| Applied Biosystems Multiplex TaqMan 2019-nCoV Assay Kit version 2 (research use only) kit | TF-MultiPlex | TaqPath COVID-19 Combo Kit, catalog number A47813/A47814, Applied Biosystems/Thermo Fisher Scientific | TaqPath1-Step Multiplex Master Mix (No ROX) (4×), catalog number A28523, Applied Biosystems/Thermo Fisher Scientific | TaqPath COVID-19 Control Kit, catalog number A47816, Applied Biosystems/Thermo Fisher Scientific |
| EURORealTime SARS-CoV-2 (for research use only) Real-time RT-PCR assays for the detection of SARS-CoV-2, Pasteur Institute, Paris, France | Euroimmun | Catalog number MP 2606-0425 | Provided with the kit | In Vitro Interferon Suppressive III Platinum One-Step Quantitative RT-PCR system, catalog number 11732-088 |
| In-house customized RT-PCR protocol | Oncobit | https://www.who.int/docs/default-source/coronaviruse/real-time-rtpcr-assays-for-the-detection-of-sars-cov-2-institutepasteur-paris.pdf, last accessed November 12, 2020; ordered from Microsynth (Baligach, Switzerland) | https://www.cdc.gov/coronavirus/2019-ncov/lab/rtpcr-panel-primer-probes.html, last accessed September 7, 2020; ordered from Microsynth | TaqPath 1-Step Multiplex Master Mix (no ROX), catalog number A28521, Thermo Fisher Scientific |

CDC, Centers for Disease Control and Prevention; nCoV, novel coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; WHO, World Health Organization.
Materials and Methods

Clinical Samples

Patient samples were collected by nasopharyngeal and/or oropharyngeal swabs (CM-FS913, iClean, San Ramon, CA) at the University Hospital Zurich and at ADMed Laboratory in La Chaux-de-Fonds, Switzerland (Copan Diagnostics, Brescia, Italy). The non-COVID-19 samples (other respiratory disease samples) were provided by ADMed Laboratory and were selected after having been tested on the Respiratory disease samples) were provided by ADMed Laboratory and were selected after having been tested on the Respiratory Panel FilmArray on Biofire (bioMérieux, Marcy-l’Étoile, France). Households samples were collected by swabbing of the different surfaces in a quarantined household of a SARS-CoV-2—positive patient. All swabs were stored in a viral transport medium (CDC, https://www.cdc.gov/coronavirus/2019-ncov/downloads/Viral-Transport-Medium.pdf, Accessed March 20, 2020) or Eswab (Copan Diagnostics, Murrieta, CA) at 4°C for a maximum of 48 hours or stored at −80°C until further analyses. All household swabbing participants provided informed consent for the study, and both the assay establishment and household studies were approved by the Cantonal Ethics Committee (BASEC-Nr-2020-00660 and BASEC-Nr-2020-00659, respectively).

Table 2 Oligonucleotide Sequences of Primers and Probes of Oncobit Real-Time RT-PCR and Digital Droplet PCR Protocols

| Primer/probe name | Sequence |
|-------------------|----------|
| N2 forward primer | 5’-TACAACAGTGGCGGCGAAA-3’ |
| N2 reverse primer | 5’-GATAGTCAGGTGTAACACTCTA-3’ |
| N2 probe (FAM) | 5’-AGTCTGCGGTATGTTGAAAGGTTATGG-3’ |
| ORF1ab forward primer | 5’-AGTTGAGCTCGCTCGAGG-3’ |
| ORF1ab reverse primer | 5’-GAGCGGCTGCTTCCCAAGT-3’ |
| ORF1ab probe (Cy5) | 5’-TTCTGACCTGAGGCTTCGGCG-3’ |
| RnaseP forward primer | 5’-AGTCGATCTACACTCTTACCT-3’ |
| RnaseP reverse primer | 5’-GCAAGTGGAGAGCAGG-3’ |
| RnaseP probe (HEX) | 5’-TGAGCTGCTTCCCAAGT-3’ |

RNA Extraction

Viral RNA was extracted as previously described using a magnetic bead-based (SpeedBeads, GE Healthcare, Darmstadt, Germany) extraction kit for the KingFisher instrument (MagMax, Thermo Fisher Scientific, Waltham, MA).

Detection of SARS-CoV-2 by RT-PCR Protocols

Four commercially available, one customized (Pasteur Institute, Paris, France), and in-house optimized RT-PCR protocols (Table 1) were compared. Primer probes design, reaction mix, and thermal cycling conditions are given in Tables 2–4 respectively. All RT-PCR protocols were run according to manufacturer instructions on a QuantStudio 5 DX real-time PCR system (catalog number A36324, Thermo Fisher Scientific), and data were analyzed with the Design and Analysis Software DA version 2.4 (Thermo Fisher Scientific) except for the Euroimmun protocol, which was run on LightCycler 480 II (RocheDiagnostics, Basel, Switzerland). Fast cycling mode was used, and a comparative Ct analysis method was performed.

For the CDC protocol, an RT-PCR result was defined as inconclusive if only the N1 gene (±N3 gene) was positive or if only the N2 gene (±N3 gene) was positive. For the TF-MultiPlex (Thermo Fisher Scientific), TF-SinglePlex (Thermo Fisher Scientific), and Oncobit protocols, an RT-PCR result was considered inconclusive if only one of two or three of the viral genes was positive. Inconclusive results were not repeated. The Euroimmun protocol (Luebeck, Germany) does not have the inconclusive category.

Detection of SARS-CoV-2 by ddPCR

The ddPCR protocol for SARS-CoV-2 detection targets two viral genomic regions of the SARS-CoV-2 gene (ORF1ab and N2) and uses the human RnaseP gene as an in-process control. The following probes for the three genes were used: ORF1ab (FAM and HEX), N2 (FAM), and RnaseP (HEX) (Table 2). Briefly, 20 μL of reaction mix (containing 1-Step RT-ddPCR Advanced Kit for Probes Mastermix; BioRad, Luxembourg, Luxembourg) was combined with 10 μL
of RNA sample for a final reaction volume of 30 μL. The final concentrations were 90 nmol/L for primers (ORF1ab, N2, RNSaP), 19.5 nmol/L for RdRP probes, 30 nmol/L for the N2 probe, and 40 nmol/L for the RNase P probe. The SARS-CoV-2 Positive Run Control (catalog number COV019CE, Bio-Rad) was used as positive control. ddPCR was run according to the program listed in Table 5 using QX200 Droplet Digital PCR System (Bio-Rad). The swabbing household samples from a laptop, newspaper, or door handle as well as the nontemplate control were tested in two independent runs.

LoD, Sensitivity, and Specificity Calculation

The LoD of four published SARS-CoV-2 detection protocols (CDC, TF-MultiPlex, TF-SinglePlex, and Euroimmun) was determined using a dilution of an external quality assessment quantitative test sample (Instand, https://www.instand-ev.de/en/news/detail/news/neuartiges-coronavirus-sars-cov-2-2019-ncov-im-vorgeto nen-instand-ringsversuch-virusgenom-nachw/%3Ftx_news_pi l%5Bcontroller%5D=News&tx_news_pil%5Baction%5 D=detail&cHash=f91865b86a1f67390788c7f40b16e7e, last accessed November 12, 2020). Linear regression was used to determine the line of best fit for the relationship between Ct and viral copies. A Ct value of 40 was set as the minimum amount of viral copies detected by RT-PCR. LoD for Oncobit ddPCR protocol was determined using a dilution of the SARS-CoV-2 Positive Run Control (catalog number COV019CE, Bio-Rad).

For sensitivity and specificity value calculations of each assay, the results of RT-PCR obtained from the ADMed Laboratory were used as the gold standard reference. The sensitivity was defined with the formula TP/(TP + FN), whereas specificity was defined as TN/(TN + FP), where TP indicates true positive, FP indicates false positive, TN indicates true negative, and FN indicates false negative. If the result of tested assays matched the reference, it was labeled concordant. If the result from the tested assays did not match the gold reference, it was labeled discordant. Inconclusive results were excluded from sensitivity and specificity calculations.

SARS-CoV-2 Infectivity Assay

The viral infectivity assay was performed as previously described8–10 with slight modifications. Briefly, 5 x 10⁴ Vero E6 cells (catalog number CCL-81, ATCC, Manassas, VA) were seeded on 96-well flat bottom cell culture plates in 200 μL of high glucose Dulbecco’s modified Eagle’s medium supplemented with l-glutamate, sodium pyruvate, nonessential amino acids, HEPES, 5% fetal cow serum, and Normocin (catalog number ant-nr-1, InvivoGen, Toulouse, France). After 24 hours of incubation (37°C, 5% CO₂), the medium was removed, and 100 μL of a virus test solution or the positive SARS-CoV-2 control (provided by Prof. Volker Thiel, Inst. Virology & Immunology, University of Berne, Switzerland) was added in twofold serial dilutions to the cells. The plates were incubated for 48 hours at 37°C. The cells were then fixed with 10% formaldehyde solution for 15 minutes at room temperature, rinsed with phosphate-buffered saline, and stained with 1% crystal violet stain solution (catalog number 252532.1211, Pan Reac AppliChem, Darmstadt, Germany) for 15 minutes at room temperature. The staining solution was removed, the cells were rinsed twice with phosphate-buffered saline, and the plates dried at room temperature before assessment for viral plaques.

Results

Description and Comparison of SARS-CoV-2 RT-PCR Detection Protocols

The six RT-PCR protocols compared in this study use the same principle of isolating viral RNA from the nasopharyngeal and/or oropharyngeal swabs or bronchial fluid and running a 1-step RT reaction followed by real-time amplification of two or three SARS-CoV-2 target genes (Figure 1). Summary and comparison of all tested RT-PCR protocols is given in Table 6. All protocols have internal controls, non-template controls and positive controls. In TF-MultiPlex, the phage MS2 is added as the internal control that serves as both RNA isolation and reaction control. All other protocols except for Euroimmun (where the type of internal control is not indicated) use a widely accepted reaction control RNaseP to ensure that RNA isolation worked and RT-PCR reaction
Figure 1 Summary of different severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) real-time RT-PCR detection protocols. SARS-CoV-2 genome structure and coverage by different protocols are shown. Continuous line indicates relative gene coverage by the detection protocol. The Euroimmun and TF-MultiPlex, protocols were for research use only. CDC, Centers for Disease Control and Prevention; WHO, World Health Organization.

was not inhibited. The protocol design is single plex, double plex, or multiplex. Euroimmun protocol stands out with its design, with two target probes coupled to the same reporter color FAM. The viral RNA input is 5 to 10 μL. Because of unspecified E-gene amplification (Supplemental Table S1), the protocol developed by Pasteur Institute was not used further in this comparative study.

LoD of Real-Time RT-PCR and ddPCR SARS-CoV-2 Detection Protocols

With a Ct value cut-off of 40, the five RT-PCR SARS-CoV-2 detection protocols (CDC, TF-MultiPlex, TF-SinglePlex, Euroimmun, and Oncobit) as well as the Oncobit ddPCR protocol had an LoD between 1 and 2 viral copies/μL (Figure 2, A and B). Values <1 copy/μL indicate high sensitivity of the tested protocol (Figure 2, A and B).

Specificity and Sensitivity of Real-Time RT-PCR SARS-CoV-2 Detection Protocols

For the sensitivity and specificity of the SARS-CoV-2 detection protocols (CDC, TF-SinglePlex, TF-MultiPlex, Euroimmun, and Oncobit), a cohort of 92 SARS-CoV-2-positive samples and 92 SARS-CoV-2-negative samples was used that were provided by ADMed Laboratory. A comparison to SARS-CoV-2-positive results showed similar sensitivity of all tested protocols, with a 93.6% sensitivity for TF-SinglePlex and 96.7% to 97.8% sensitivity for the other protocols (Figure 3A). In the specificity cohort, 22 samples had a confirmed diagnosis of other respiratory diseases (Supplemental Table S2), and 70 samples tested negative for all listed respiratory diseases, including SARS-CoV-2. All protocols, except TF-SinglePlex, had no cross-reactivity (Figure 3A), including samples that tested positive for four other...
types of coronaviruses (Supplemental Table S2). The specificity was thus 100% for all protocols except for TF-SinglePlex, which had a specificity of 98.7% (Figure 3A).

Inconclusive results were found in 0.5% to 3.2% of these 184 samples, with TF-MultiPlex and Oncobit providing the most accuracy (Figure 3A). Comparing RT-PCR results (positive, negative, or inconclusive) of all 184 samples, the
Table 1: Performance of real-time RT-PCR detection protocols. Performance calculation (sensitivity/speciﬁcity) as well as calculation of percentage of inconclusive results of ﬁve real-time RT-PCR detection protocols [Centers for Disease Control and Prevention (CDC), TF-SinglePlex, TF-MultiPlex, Euroimmun, and Oncobit]. The Euroimmun RT-PCR detection protocol does not have the inconclusive category; inconclusive for Euroimmun equals an invalid result.

| Protocol       | Sensitivity (%) | Specificity (%) | Inconclusive (%) |
|----------------|-----------------|-----------------|------------------|
| CDC            | 96.7            | 1               | 2.7              |
| TF-SinglePlex  | 96.7            | 98.7            | 3.2              |
| TF-MultiPlex   | 93.6            | 1               | 0.5              |
| Euroimmun      | 97.8            | 1               | -                |
| Oncobit        | 97.8            | 1               | 1.6              |

Figure 3: Specificity and sensitivity of real-time RT-PCR severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) detection protocols. A: Performance calculation (sensitivity/speciﬁcity) as well as calculation of percentage of inconclusive results of ﬁve real-time RT-PCR detection protocols [Centers for Disease Control and Prevention (CDC), TF-SinglePlex, TF-MultiPlex, Euroimmun, and Oncobit]. The Euroimmun RT-PCR detection protocol does not have the inconclusive category; inconclusive for Euroimmun equals an invalid result. B: Heatmap summarizing concordance of ﬁve real-time RT-PCR detection protocols (CDC, TF-SinglePlex, TF-MultiPlex, Euroimmun, and Oncobit) for both sensitivity (bottom) and speciﬁcity (top) sample cohorts.
Application of SARS-CoV-2 Detection Protocols on Swabbed Surfaces

Having compared and established the RT-PCR protocols for SARS-CoV-2 diagnostics, the possibility of application of the RT-PCR and ddPCR protocol for SARS-CoV-2 detection on environmental samples was examined. Swabs of different surfaces from a SARS-CoV-2 quarantined household were collected and analyzed by two validated RT-PCR protocols. In addition, an in-house ddPCR protocol was developed to accurately detect and quantify virus.

On the day of household surface swabbing (April 25, 2020) of the SARS-CoV-2—positive family, only patient 2 was swabbed again and tested positive but reported no symptoms (Supplemental Figure S1A). The pharyngeal swab as well as the swabbed surface samples were collected on the same day and tested with three different SARS-CoV-2 detection protocols (CDC, TF-MultiPlex, and ddPCR). The pharyngeal swab tested positive (cycle thresholds >30) on three different protocols. The laptop keyboard and two more swabbed surface (the door handle and newspaper) samples had positive and inconclusive results, respectively (Supplemental Table S3), whereas no infectivity for any of the samples was detected (Supplemental Figure S1B).

Discussion

Real-time RT-PCR remains the most sensitive method for early detection of SARS-CoV-2. We report a comparison of LoD, specificity, sensitivity, economic, and practical advantages of four commercial SARS-CoV-2 detection kits as well as one optimized in-house RT-PCR SARS-CoV-2 protocol. A study comparing RT-PCR with rapid fluorescence immunochromatographic assay—based SARS-CoV-2 nucleocapsid protein antigen detection method showed that sensitivity of the rapid method was only approximately 75.6%; therefore, RT-PCR remains a more sensitive detection method for SARS-CoV-2. Most of the reported multiprotocol comparison studies on real-time RT-PCR SARS-CoV-2 detection performed the benchmarking only on a limited number of samples and tested only commercial detection kits,10,12,13 and some studies limited the comparison only to sensitivity assessment.14

In this study, a low LoD and high sensitivity for four commercial SARS-CoV-2 RT-PCR detection protocols were observed by using standard quantitative test samples and a cohort of 92 SARS-CoV-2—positive samples, respectively. Furthermore, specificity of those protocols was tested and confirmed with 92 samples that had confirmed SARS-CoV-2—negative result or were collected in pre-pandemic times from patients presenting with respiratory symptoms (Supplemental Table S2).

In addition, downscaling of two commercial protocols that were chosen for the diagnostic routine (CDC and TF-
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Supplemental Data

Supplemental material for this article can be found at http://doi.org/10.1016/j.jmoldx.2021.04.009.

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Supplemental Figure S1  Patients Ct values and symptom progression as well as infectivity examination of swabbed patient and surface samples. A: The patient's Ct values and symptom progression. Mean of Ct values of the N1, N2, and N3 viral genes are shown (Centers for Disease Control and Prevention protocol). Patient 1 was a 42-year-old woman; patient 2, 42-year-old man; patient 3, 6-year-old boy; and patients 4, 4-year-old boy. The Ct values at the time of diagnosis for patient 1 are missing because the patient was tested in a different laboratory. On April 7, 2020, the patient experienced shortness of breath, which lasted for 3 days. On April 7, 2020, the 42-year-old man (father, patient 2) tested positive for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), with symptoms resolving after 10 days. As the symptoms gradually resolved, we observed increasing Ct values in both patients. On April 12, the two children (patients 3 and 4) also tested positive for SARS-CoV-2 but remained asymptomatic at all times. Family members in the household were swabbed on the April 25, with patients being asymptomatic for at least 2 days. B: An infectivity assay using Vero E6 cells found no plaque formation for any of the samples that tested positive by real-time RT-PCR (patient's throat, laptop keyboard, newspaper, and toilet rim).