# An Ultrafast One-Step qRT-PCR Assay for Detection of SARS-CoV-2

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**Method Article**

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# An Ultrafast One-Step qRT-PCR Assay for Detection of SARS-CoV-2

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**Keywords**

Ultrafast; one-step RT-qPCR assay; SARS-CoV-2 detection

**Abstract**

We developed an ultrafast one-step RT-qPCR assay for SARS-CoV-2 detection, which can be completed in only 30 mins on benchtop *Bio-Rad* CFX96. The assay significantly reduces the running time of conventional RT-qPCR: reduced RT step from 10 to 1 min, and reduced PCR cycle of denaturation from 10 to 1 s and extension from 30 to 1 s. A cohort of 60 nasopharyngeal swab samples testing showed that the assay had a clinical sensitivity of 100% and a clinical specificity of 100%.

## Introduction

The current highly transmissible outbreak of severe acute respiratory syndrome coronavirus (SARS-CoV-2) is the leading cause of morbidity and mortality across the globe (1-3). Real-time quantitative reverse transcription–polymerase chain reaction (qRT-PCR) of nasopharyngeal swabs is the current gold standard in clinical setting to confirm the clinical diagnosis of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (4-7). Conventional qRT-PCR for SARS-CoV-2 detection usually takes approximately 2 hours on benchtop qPCR instrument, with 10 minutes of reverse transcription, followed with initial denaturation for 1 minutes, and 45 PCR cycles of 10 seconds denaturation and 30 seconds extension (Fig. 1) (8). However, the ongoing COVID-19 pandemic pose substantial challenges for health-care systems and their infrastructure. Therefore, to meet to pandemic challenges, it is important to significantly shorten the turnaround time in the race for increasing number of diagnostic tests.

## Result and Discussion

Here, we described an ultrafast one-step qRT-PCR assay for the qualitative detection of SARS-CoV-2 that is fully compatible with conventional benchtop qPCR instruments. SARS-CoV-2 RNA was reverse transcribed for 1 minute into cDNA and amplified with 40 PCR cycles of 1 second denaturing and 1 second extension step (Fig. 1). This one-step qRT-qPCR assay can detect down to 25 copies of SARS-CoV-2 RNA in 10 µl reaction volume. The assay employs primers and probes developed by the United States Centers for Disease Control and Prevention (CDC) targeting N1 and N2 regions of Nucleocapsid gene of SARS-CoV-2 with the internal control human RNase P gene (RP). The total ultrafast one-step qRT-PCR can be completed in 30 minutes on benchtop *Bio-Rad* CFX96 platform.

In developing the ultrafast one-step qRT-PCR assay, we reasoned that the enzymes in the qRT-PCR are key to significantly shorten the qRT-PCR and to keep comparable sensitivity as conventional qRT-PCR for SARS-CoV-2 detection. We found that SpeedSTAR HS DNA Polymerase is optimized for PCR with extension time as fast as 10 second/kb. The amplicons of N1, N2, and RP are within length of 100 bp. Therefore, we investigated whether the N1/N2 SARS-CoV-2 RNA could be detected with the fast PCR cycle setting of 2 second/cycle (it includes 1 second denaturing and 1 second extension step) on conventional qPCR instrument by using SpeedSTAR HS DNA Polymerase (9). Using synthetic SARS-CoV-2 RNA from ATCC as the model, we found that 4 U of SpeedSTAR HS DNA Polymerase in the one-step qRT-qPCR assay can detect down to 25 copies of N1 and N2 of SARS-CoV-2 RNA (Fig. 2). Furthermore, in RT

step, we chosen SuperScript IV Reverse Transcriptase because of its fast speed in cDNA synthesis (10). We demonstrated that ultrafast one-step qRT-PCR can still detect down to 25 copies of N1 and N2 of SARS-CoV-2 RNA genome (Fig. 2) by reducing RT step from 10 minutes to 1 minute of with 160 U of SuperScript IV Reverse Transcriptase. The limit of detection of the developed ultrafast one-step qRT-PCR is comparable to the other CDC qRT-PCR tests (11). The recipe of ultrafast one-step qPCR-PCR master mix (Table 2) and running protocol of ultrafast one-step qPCR-PCR were detailed the Methods.

To validate the performance of the ultrafast one-step qRT-PCR in clinical setting, we performed a blinded and randomized study with 30 SARS-CoV-2–positive and 30 SARS-CoV-2–negative nasopharyngeal swab samples obtained from patients. Ultrafast one-step qRT-PCR testing showed that SARS-CoV-2 positive samples exhibited N1, N2 and RNase P gene, and the cycle threshold (Ct) values of N1, N2, and RP are very close to those obtained with FDA approved diagnostics (Fig 3, Table 3). In SARS-CoV-2 negative samples, N1 was not detected in all negative samples, Ct values of N2 in three negative samples of were above 35, which still qualifies as SARS-CoV-2 negative samples according to CDC guideline (Table 4). Overall, the testing results showed that the ultrafast one-step qRT-PCR had a clinical sensitivity of 100% and a clinical specificity of 100% (Table 5). The simplified format of ultrafast one-step qRT-PCR for detection of SARS-CoV-2 in nasopharyngeal swabs is suitable for use in clinical diagnostic laboratories. The limitation of this study includes that we have not explored other sample types. We will further validate ultrafast one-step qRT-PCR for SARS-CoV-2 detection in saliva samples without RNA extraction.

**Methods and Materials**

**Clinical Samples:** A cohort of 60 clinical nasopharyngeal swab samples including 30 SARS-COV-2 negative and 30 SARS-CoV-2 positive sample were pre-collected and deidentified, which meet the requirement of Institutional Review Board (IRB) Exemption 4. Those clinical nasopharyngeal swab samples were stored in virial transport media at -80 °C until future use. The nasopharyngeal swab samples have been tested by a Clinical Laboratory Improvement Amendments (CLIA)-certified diagnostic laboratory with an FDA approved diagnostic kit at Penn State Health Milton S. Hershey Medical Center.

**Ultrafast one-step qRT-PCR for SARS-CoV-2 detection:** The ultrafast one-step qRT-PCR was developed using primers and probes set targeting the N1 and N2 regions in nucleocapsid (N) gene of SARS-CoV-2 and the human RNase P gene as previously published by “United States Center for Disease Control and Prevention” (CDC) (Table 1). The primers and probes for N1,

nucleocapsid N2 and RNase P (RP) were purchased from Integrated DNA Technologies (IDT) and diluted as recommended. Synthetic SARS-CoV-2 RNA (ATCC, VT-3276T) was used as SARS-Cov-2 RNA standards in all condition optimization of ultrafast one-step qRT-PCR assay for detection of SARS-Cov-2. The ultrafast one-step qRT-PCR was performed as follows: the one-step qRT-PCR master mix (100 µL) was prepared according to the components in the Table 2. Then, in each sample, 2 µL of SARS-CoV-2 RNA standard or extracted RNA samples were added into to 8 µL of ultrafast one-step qRT-PCR master mix. Then, 10 µL of reaction solution with RNA sample and qRT-PCR master mix was loaded into 96 hard-shell PCR plates (Bio-Rad Laboratories), and the PCR plate was loaded in CFX96 Real-Time PCR detection system (Bio-Rad Laboratories). Thermal cycling conditions included 1 minute reverse transcription at 50°C, 1 minute at 95°C for reverse transcription deactivation and initial activation of SpeedStar HS DNA polymerase, followed by 40 cycles of 1 second denaturing at 95°C and 1 second extension at 55°C. All samples with cycle threshold (Ct) value of both N1, N2 and RP ≤40 was considered as positive according to CDC guideline.

**RNA extraction from nasopharyngeal swab samples:** Total RNA was isolated from the heat inactivated nasopharyngeal swabs samples using Direct-zol™ RNA Microprep (R2060, Zymo Research) by following the manufacturer's instruction. In brief, 300 µL of nasopharyngeal swabs samples were lysed in 400 µL of Trizol. Then 700 µL of 100% ethanol was added, followed by column purification using Zymo-Spin™ Column. Direct-zol™ RNA PreWash and RNA Wash Buffer were added sequentially to wash the column. Finally, RNA was eluted in 12 µL of nuclease free water and stored in -80 °C until future use.

**Statistical Analysis**: Continuous and categorical variables are expressed as means (SD) and number (%), respectively, analyzed with Prism 8.0.1 (GraphPad Software, La Jolla, CA). Clinical agreements were analyzed according to Clinical and Laboratory Standards Institute (CLSI) EP12-A2 as recommended in FDA Guidelines, performed with MedCalc® Statistical Software version 19.7.4 (MedCalc Software Ltd, Ostend, Belgium)

**Author contributions**

J.M., H.Z.H. and S.Y.Z. designed research; J.M and W. G. performed research; J.M and M.L. analyzed data; and J.M., H.Z.H. and S.Y.Z. wrote the paper with input from all authors.

**Data Availability**

All study data are included in the article and/or supporting information.

**Competing interests**

There is patent pending on the ultrafast one-step qRT-PCR assay for pathogen detection (US63/178797) method used in this work. S.Y.Z declares a competing interest in the form of consulting for and equity ownership in Captis Diagnostics.

**Reference**


1. Woolf SH, Chapman DA, & Lee JH (2021) COVID-19 as the Leading Cause of Death in the United States. *JAMA* 325(2):123-124.
2. Andrasfay T & Goldman N (2021) Reductions in 2020 US life expectancy due to COVID-19 and the disproportionate impact on the Black and Latino populations. *Proceedings of the National Academy of Sciences* 118(5):e2014746118.
3. Cohen J (2021) Is India's coronavirus death ‘paradox’ vanishing? *Science* 372(6542):552.
4. Carter LJ*, et al.* (2020) Assay Techniques and Test Development for COVID-19 Diagnosis. *ACS Central Science* 6(5):591-605.
5. Kevadiya BD*, et al.* (2021) Diagnostics for SARS-CoV-2 infections. *Nature Materials* 20(5):593-605.
6. Ji T*, et al.* (2020) Detection of COVID-19: A review of the current literature and future perspectives. *Biosensors and Bioelectronics* 166:112455.
7. Tang Y-W, Schmitz JE, Persing DH, & Stratton CW (2020) Laboratory Diagnosis of COVID-19: Current Issues and Challenges. *Journal of Clinical Microbiology* 58(6):e00512-00520.
8. Vogels CBF*, et al.* (2020) Analytical sensitivity and efficiency comparisons of SARS-CoV-2 RT–qPCR primer–probe sets. *Nat. Microbiol.* 5(10):1299-1305.
9. Giese H, Lam R, Selden R, & Tan E (2009) Fast Multiplexed Polymerase Chain Reaction for Conventional and Microfluidic Short Tandem Repeat Analysis. *Journal of Forensic Sciences* 54(6):1287-1296.
10. Martín-Alonso S, Frutos-Beltrán E, & Menéndez-Arias L (2021) Reverse Transcriptase: From Transcriptomics to Genome Editing. *Trends in Biotechnology* 39(2):194-210.
11. Arnaout R*, et al.* (2020) SARS-CoV2 Testing: The Limit of Detection Matters. *bioRxiv*:2020.2006.2002.131144.

Fig. 1. Workflow of ultrafast one-step qRT-PCR with comparison with conventional qRT-PCR for SARS-CoV-2 detection.

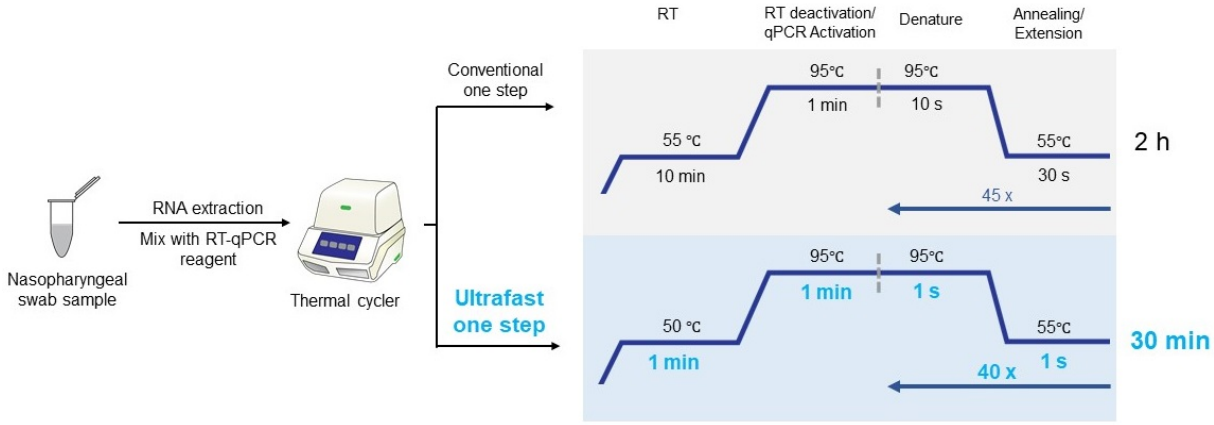


Fig. 2. Limit of detection of ultrafast one-step qRT-PCR assay. SARS-Cov-2 synthetic RNA genome was used as model sample. RNA templates were series diluted in the range of 25- $1\times10^5$ copies. Ultrafast one-step qRT-PCR assay detects both N1 (a) and N2 (b) regions of Nucleocapsid gene of SARS-CoV-2.

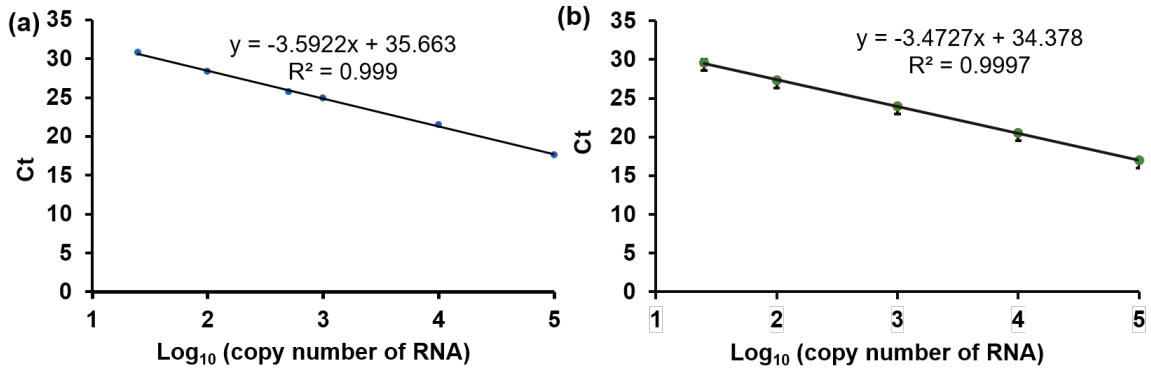

Fig. 3. Cycle threshold (Ct values) of N1 and N2 correlation of ultrafast one-step qRT-PCR with FDA-approved assay for SARS-CoV-2 positive samples. (a) Ct value of N1 and (b) Ct value of N2 from the 30 SARS-CoV-2 positive samples.

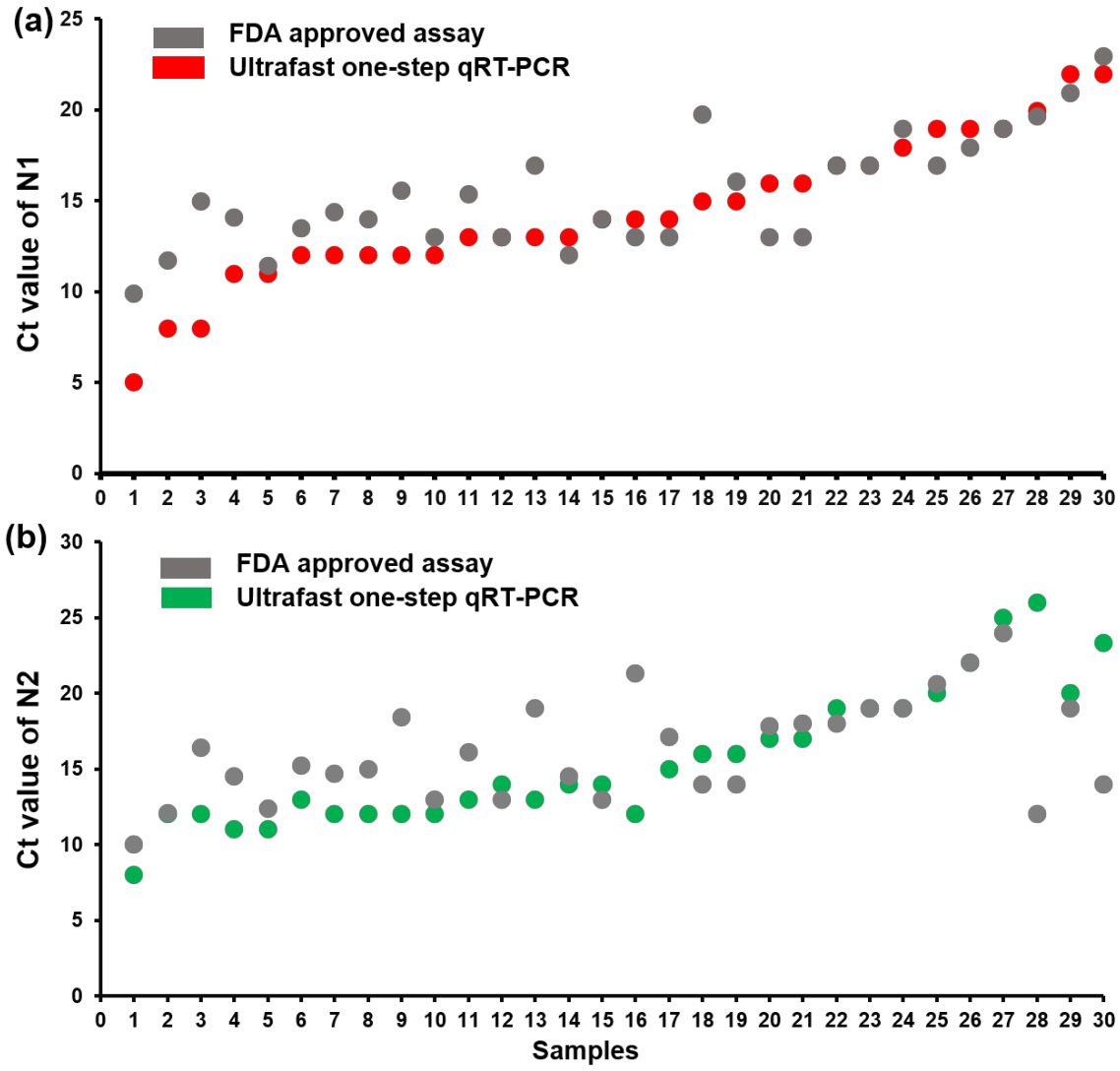

**Table 1.** Primers and probes for N1, N2 and RNase P (RP)

| Name | Oligonucleotide Sequence (5'-3') |
|---|---|
| 2019-nCoV_N1-Forward primer | GAC CCC AAA ATC AGC GAA AT |
| 2019-nCoV_N1-Revere primer | TCT GGT TAC TGC CAG TTG AAT CTG |
| 2019-nCoV_N1-Probe | FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1 |
| 2019-nCoV_N2- Forward primer | TTA CAA ACA TTG GCC GCA AA |
| 2019-nCoV_N2- Revere primer | GCG CGA CAT TCC GAA GAA |
| 2019-nCoV_N2-Probe | FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1 |
| RP- Forward primer | AGA TTT GGA CCT GCG AGC G |
| RP- Revere primer | GAG CGG CTG TCT CCA CAA GT |
| RP- Probe | FAM – TTC TGA CCT GAA GGC TCT GCG CG – BHQ-1 |

**Table 2.** Components of the ultrafast one-step qRT-PCR master mix

| **Reverse Transcription master mix** | | |
|---|---|---|
| Stock solution | Supplier | Final concentration |
| 10 mM dNTPs | Thermo Fisher (R0191) | 0.12 mM |
| 5X SuperScript IV Reverse Transcriptase buffer | Thermo Fisher (18090010) | 0.3X |
| 100 mM DTT | Thermo Fisher (18090010) | 1 mM |
| RNaseOUT inhibitor (40 U/μL) | Thermo Fisher (10777019) | 40 U |
| SuperScript IV Reverse Transcriptase (200 U/μL) | Thermo Fisher (18090010) | 160 U |
| Stabilyser Reagent | Sigma (PNS1010) | 1 μL |
| **qPCR master mix** | | |
| SpeedStar HS DNA polymerase (5 U/μL) | TaKaRa (RR070B) | 4 U |
| Fast Buffer I (10X) | TaKaRa (RR070B) | 1X |
| N1 forward primer/reverse primer/probe | IDT | 400 nM/400 nM/200 nM |
| N2 forward primer/reverse primer/probe | IDT | |
| RP forward primer/reverse primer/probe | IDT | |
| Nuclease Free $H_2O$ | | Add up to 100 μL |

***Table 3.*** Cycle threshold (Ct) value of SARS CoV-2 positive samples of ultrafast one-step qRT-PCR in comparison to an FDA approved test.

| | *ultrafast one-step qRT-PCR* test | | FDA approved test | |
|---|---|---|---|---|
| Positive samples | Ct of N1 | Ct of N2 | Ct of N1 | Ct of N2 |
| 1 | 12.3 | 12.8 | 13.5 | 15.2 |
| 2 | 12.7 | 13.4 | 15.4 | 16.1 |
| 3 | 14.6 | 15.5 | 19.8 | 21.3 |
| 4 | 8 | 11.7 | 11.7 | 12.1 |
| 5 | 10.6 | 11.1 | 14.1 | 14.5 |
| 6 | 5.3 | 7.9 | 9.9 | 10 |
| 7 | 12.2 | 12.2 | 14.4 | 14.7 |
| 8 | 15.4 | 16.4 | 14 | 14.5 |
| 9 | 13.5 | 26 | 12 | 12 |
| 10 | 15.2 | 15 | 16.1 | 17.1 |
| 11 | 20.1 | 20.3 | 19.7 | 20.6 |
| 12 | 17.1 | 17 | 17 | 17.8 |
| 13 | 17.1 | 16.7 | 19 | 19 |
| 14 | 14.7 | 14.8 | 13 | 14 |
| 15 | 7.7 | 12.5 | 15 | 16.4 |
| 16 | 10.9 | 10.7 | 11.4 | 12.4 |
| 17 | 15.9 | 16.4 | 13 | 14 |
| 18 | 22.3 | 22.1 | 21 | 22 |
| 19 | 22.4 | 24.7 | 23 | 24 |
| 20 | 18.9 | 18.8 | 17 | 18 |
| 21 | 19.1 | 19.4 | 18 | 19 |
| 22 | 12.2 | 12 | 14 | 15 |
| 23 | 14.2 | 13.6 | 13 | 13 |
| 24 | 13.5 | 13.6 | 13 | 13 |
| 25 | 19 | 18.9 | 19 | 19 |
| 26 | 13 | 12.2 | 15.6 | 18.4 |
| 27 | 15.9 | 15.8 | 13 | 14 |
| 28 | 16.8 | 17 | 17 | 18 |
| 29 | 12.2 | 12.1 | 13 | 13 |
| 30 | 13.1 | 13 | 17 | 19 |

**Table 4. Cycle threshold (Ct) value of SARS CoV-2 negative samples of the ultrafast one-step qRT-PCR.**

| | Ultrafast one-step qRT-PCR test | | |
|---|---|---|---|
| **Negative samples** | **Ct of N1** | **Ct of N2** | **Ct of RP** |
| 1 | NA | NA | 27 |
| 2 | NA | NA | 24 |
| 3 | NA | NA | 27 |
| 4 | NA | NA | 23 |
| 5 | NA | NA | 26 |
| 6 | NA | NA | 25 |
| 7 | NA | NA | 26 |
| 8 | NA | NA | 26 |
| 9 | NA | NA | 23 |
| 10 | NA | NA | 28 |
| 11 | NA | NA | 25 |
| 12 | NA | NA | 18 |
| 13 | NA | NA | 23 |
| 14 | NA | NA | 29 |
| 15 | NA | NA | 27 |
| 16 | NA | NA | 28 |
| 17 | NA | NA | 23 |
| 18 | NA | NA | 29 |
| 19 | NA | NA | 27 |
| 20 | NA | NA | 29 |
| 21 | NA | 39 | 38 |
| 22 | NA | 37 | 29 |
| 23 | NA | NA | 28 |
| 24 | NA | NA | 33 |
| 25 | NA | NA | 28 |
| 26 | NA | NA | 27 |
| 27 | NA | NA | 29 |
| 28 | NA | 38 | 26 |
| 29 | NA | NA | 26 |
| 30 | NA | NA | 28 |

**Table 5**: Positive and Negative Predictive Values of ultrafast one-step qRT-PCR for SARS-CoV-2 detection in nasopharyngeal samples.

| | | **Comparator Assay (FDA approved assay)** | |
|---|---|---|---|
| | | **Positive** | **Negative** |
| **Ultrafast One Step qRT-PCR** | **Positive** | 30 | 0 |
| | **Negative** | 0 | 30 |
| | **Total** | 30 | 30 |
| **Percent Positive Agreement (PPA)** | | 30/30=100% (95% CI: 88.7%-100.0%) | |
| **Percent Negative Agreement (PNA)** | | 30/30=100% (95% CI: 88.7%-100.0%) | |
| **Percent Overall Agreement (POA)** | | 60/60=100% (95% CI: 94.0%-100.0%) | |