# Primer Isothermal Amplification for SARS-CoV-2 Detection

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Running Head: Molecular assays for SARS-CoV-2 detection

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**ABSTRACT**

SARS-CoV-2 is a newly emerged coronavirus that was isolated from human infections in recent months. Since drugs and vaccines of Covid-19 are still being developed, accurate pathogen detection plays a crucial role in the current public health crisis. Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay has been reliably used for the detection and confirmation of SARS-CoV-2 since the beginning of outbreak, whereas isothermal nucleic acid amplification based point of care automated assays has also been considered as a simpler and rapid alternative. However, since these assays have only been developed and applied for clinical use within a short timeframe, their analytical performance has not been adequately compared to-date. We describe a comparative study between a newly developed cross primer isothermal amplification (CPA) assay (Kit A) and five RT-PCR assays (Kits B to F), using clinical diagnosis as the reference standard to evaluate their sensitivity, specificity, predictive values and accuracy analysis. Clinical samples used (n=52) included throat swabs (n=30), nasal swabs (n=7), nasopharyngeal swabs (n=7) and sputum specimens (n=8), comprised of positive (n=26) and cleared cases (n=26) by clinical diagnosis. For the CPA assay (Kit A), the sensitivity, specificity, positive and negative predictive values and accuracy were 100%. Among the five RT-PCR kits, Kits B, C and F had good agreement with clinical diagnosis (Kappa≥0.75), Kits D and E were less congruent (0.4≤Kappa<0.75). Differences between all assays were statistically significant (P<0.001). The reproducibility of RT-PCR assays was determined using a positive sample that was verified by all assays, with standard deviations (SD) between 0.35 and 0.87, and coefficients of variation (CV) between 0.95% and 2.57%, indicating good reproducibility. To further evaluate the CPA assay (Kit A) compared to Kits B and F, throat swabs from close contacts of cases (n=200) were analyzed. All three kits identified the same positive samples and showed total agreement. This is the first comparative study to evaluate a CPA assay and RT-PCR assays for SARS-CoV-2 detection, which could serve as a reference for clinical laboratories and inform testing protocols amid the rapidly evolving COVID-19 pandemic.

Keywords: SARS-CoV-2; COVID-19; nucleic acid detection; real-time reverse transcriptase PCR (RT-PCR); cross primer isothermal amplification (CPA);

## INTRODUCTION

With currently over 1.8 million confirmed cases and over 100,000 deaths reported globally as of April 13th 2020, the ongoing COVID-19 pandemic has been rapidly escalating and straining public health systems worldwide [1]. The genome sequence of SARS-CoV-2 (Wuhan-Hu-1, GenBank accession number MN908947) (http://virological.org/t/novel-2019-coronavirus-genome/319) released for immediate public health support by the Global Initiative on Sharing All Influenza Data (GISAID) has shown that SARS-CoV-2 has 84% nucleotide homology with bat SARS-like

coronavirus, 78% homology with the human SARS virus, and approximately 50% homology with MERS virus [1-4], whereas the branch 1 of the receptor binding domain of coronavirus S protein in the B lineage of SARS-CoV-2 could bind to angiotensin-converting enzyme 2. The most commonly reported symptoms of SARS-CoV-2 infection included fever, dyspnea, leukopenia, lymphocytopenia, and ground-glass opacity commonly observed on chest computed tomography[5]. From an autopsy on the first patient who died from COVID-19, pulmonary manifestations were diffuse alveolar injury and clear membrane formation [6], which were consistent with the manifestations of acute respiratory distress syndrome and similar to the pathological features of SARS and MERS coronavirus infection [7]. From the public health standpoint, it has become clear that the most effective way to prevent spread of infections was by breaking the chains of transmission through social distancing.

Since the outbreak began, various commercial molecular assays for have been developed to provide a simple and reliable means for the laboratory detection of SARS-CoV-2. Among assay methodologies, real time reverse transcriptase PCR (RT-PCR) has been the standard method for the laboratory diagnosis of SARS-CoV-2 infection, which has already been routinely used for detecting other respiratory viruses. According to China's latest clinical guideline, the Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia (Trial Version 7)[8, 9], diagnosis of COVID-19 must be confirmed by RT-PCR or gene sequencing from respiratory or blood samples, which is a key criterion for hospitalization the Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia (Trial Version 7) [10, 11]. Conversely, cross primer isothermal amplification (CPA) assays that uses a closed-tube test has become a simple and fast SARS-CoV-2 test that can be performed by minimally trained personnel at point of care (POC) without the need for complex equipment [12]. Through specific amplification primers, fluorescent probes and DNA polymerases with high activity of reverse transcriptase and chain displacement characteristic, CPA assays could complete the specific amplification process of SARS-CoV-2 fragments at a constant temperature, and the fluorescence signal is detected by adaptive instruments and real-time fluorescence curve was automatically generated.

Due to the high global demand, the development and production of molecular assays by various manufacturers in China have rapidly scaled up, but have only been applied for clinical use in a short period of time. Hence, such assays may not have been fully validated in performance and evaluated in clinical application, where both false-negative and false-positive results have been observed [2], and there has been no comparative study between their analytical performance. In this study, our objective was to compare and evaluate six different commercially available molecular assays using clinical diagnosis as a reference standard to provide the necessary comparative evaluation.

## MATERIALS AND METHODS

**Clinical samples.** Respiratory samples (n=52) were collected from suspected cases at the Shenzhen Third People's Hospital and a compulsory quarantine facility in Shenzhen. Specimen types included throat swabs (n=30), nasal swabs (n=7), nasopharyngeal swabs (n=7) and sputum specimens (n=8), comprised of positive (n=26) and cleared (n=26) cases by clinical diagnosis.

**Case definitions**. In accordance to the latest guideline of Diagnosis and Treatment of Novel Coronavirus Pneumonia (Trial Version 7) from the National Health Commission & State Administration of Traditional Chinese Medicine of China (available at http://en.nhc.gov.cn/2020-03/29/c_78469.htm), a suspect case was defined as a person who has any of the following epidemiological history plus any two of the following clinical manifestations, or all three clinical manifestations if there were no clear epidemiological history. 1) Epidemiological history: Within 14 days prior to the onset of illness, a) traveled to or took residence, or b) having contact with those who had fever or respiratory symptoms, in Wuhan and geographical proximities or communities where cases have been reported; or c) having contact with any laboratory confirmed cases; or d) be part of a clustered of two or more cases with fever and/or respiratory symptoms. 2) Clinical manifestations: a) fever and/or respiratory symptoms; b) defined imaging characteristics of novel coronavirus pneumonia; c) Normal or decreased WBC count and/or lymphocyte count in the early stage of illness onset.

Confirmed cases were defined as suspect cases with one of the following etiological or serological evidences: (1) a positive RT-PCR test for SARS-CoV-2; (2) viral gene sequence highly homologous to SARS-CoV-2. (3) SARS-CoV-2 specific IgM and IgG antibodies detectable in serum; IgG detectable or reaches a titration of at least 4-fold increase during convalescence compared with the acute phase. Cleared cases were defined as suspect cases with two negative two nucleic acid tests taken at least 24-hour apart were and that SARS-CoV-2 specific IgM and IgG antibodies were negative after 7 days from illness onset.

**Molecular assays.** All six assays used the ORF1ab gene and the N gene as targets (only ORF1ab for Kit D) and were performed in accordance to manufacturer's instructions (Table 1). Briefly, in the automated CPA assay (Kit A), swab samples were directly applied to cartridges preloaded with reagents for nucleic acid purification, elution buffer and CPA reaction master mix, in which RNA were automatically purified, extracted and amplified. Fluorescently labeled probes binds specifically to the amplified RNA targets to produce fluorescence signal detectable by the instrument in real time for the determination of test results. In the RT-PCR assays (Kits B to F), RNA was manually extracted with the High Pure Viral Nucleic Acid (Roche Diagnostics, Mannheim, Germany). For kit B, the PCR was carried out in 25 μl reaction system containing 5 μl isolated DNA as a template, 4 μl primers and probes mix (ORF1ab/N) and 4 μl Premix ExTaq, Amplification began with 1cycle reverse transcription at 50℃ for 10min, 1cycle pre-denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C for 10s, 55°C for 40s. For kit C, the PCR was carried out in 25 μl reaction system containing 5 μl viral

cDNA as a template, 19 μl RT-PCR Buffer and 1μl Enzyme Mix. Amplification began with 1cycle reverse transcription at 42℃ for 15min, 1cycle pre-denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 5s, 60°C for 45s. For kit D, the 30 μl RT-PCR reaction mixtures contained 10μl viral cDNA as a template, 18.5 μl RT-PCR Buffer and 1.5μl Enzyme Mix. Amplification began with 1cycle reverse transcription at 50℃ for 20min, 1cycle pre-denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15s, 60°C for 30s.For kit E, the 20 μl RT-PCR reaction mixtures contained 4μl viral cDNA template, 18.5 μl RT-PCR Buffer and 1.5μl Enzyme Mix. Amplification began with one cycle reverse transcription at 55℃ for 15min, one cycle pre-denaturation step at 95°C for 30 min, followed by 45 cycles of 95°C for 10s, 60°C for 30s. For kit F, the PCR was carried out in 25 μl reaction system containing 5μl viral cDNA template, 17 μl RT-PCR Buffer A and 3μl RT-PCR Buffer B. Amplification began with one cycle reverse transcription at 50℃ for 15min, 1cycle pre-denaturation step at 95°C for 15 min, followed by 45 cycles of 94°C for 15s, 55°C for 45s. The assay was performed on the ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, CA), Fluorescence signal was detected at the end of each cycle of the extension step. The results were interpreted by CT values generated using specific cut-offs for each kit (Table 1).

**Analytical performance of assays.** Analytical sensitivity and specificity, the positive and negative predictive values (PPV and NPV) and the accuracy for each assay was assessed using clinical diagnosis as reference standard.

**Reproducibility of RT-PCR assays.** Reproducibility for the RT-PCR assays were evaluated using a positive sample by verified all assays, measured in triplicates by three different operators, by calculating the mean, standard deviations (SD) and the coefficients of variation (CV).

**Statistical analysis.** Statistical analyses were performed using the SPSS software version 21.0 (SPSS Inc. Chicago, IL). Confidence intervals (95%) were computed using the Wilson Score method. The agreement between assay results and clinical diagnosis were compared using the McNemar-Bowker test.

**Clinical application.** Additional throat swabs (n=200) from close contacts of confirmed cases for further evaluation to compare Kit A with Kits B and F (approved and authorized by the National Medical Products Administration for clinical use) was also collected from the compulsory quarantine facility. Agreement between assays was assessed.

## RESULTS

**Performance characteristics of assays.** From the clinical samples tested (n=52), which comprised both positive (n=26) and cleared (n=26) cases by clinical diagnosis, the analytical performances from the six assays were evaluated using clinical diagnosis as the reference standard (Table 2). The analytical sensitivity and negative predictive value (NPV) of all six assays were 100%. The analytical specificity and

positive predictive value (PPV) of the CPA assay (Kit A) were both 100% (95%CI, 87.1-100.0), and the kappa value was 1.0 ($P< 0.001$). For RT-PCR assays (Kits B to F), Kits B, C and F have shown high agreement with clinical diagnosis (Kappa$\geqslant$0.75, $P<0.001$); while Kits D and E were generally consistent with clinical diagnosis (0.4$\leqslant$ Kappa<0.75, $P<0.001$). Differences observe between assays were statistically significant ($P<0.001$).

**Reproducibility of RT-PCR assays.** The standard deviations (SD) and coefficients of variation (CV) for the five RT-PCR assays were between 0.35 and 0.87; and 0.95% and 2.57%, respectively, indicating highly reproducible assays.

**Clinical application.** Among throat swab samples from close contacts (n=200), one sample from a positive case by clinical diagnosis was tested positive for SARS-CoV-2 by all three (Kits A, B and F) assays and hence total agreement between the three assays.

## DISCUSSION

COVID-19 caused by SARS-CoV-2 has now become a global pandemic and caused substantial mortality and morbidity internationally due to rapid disease propagation. The rapid and accurate identification of pathogenic virus is of great significance to the selection of appropriate treatment, life saving and prevention of infectious diseases. At present, China has rapidly developed and approved more than 10 SARS-CoV-2 detection methods[10]. In this study, the analytical performance of six molecular assays, including a CPA assay and five RT-PCR assays were compared and evaluated using clinical diagnosis as the reference standard. Real-time reverse transcriptase PCR tests are often used in the detection of respiratory secretions of pathogenic viruses and COVID - 19 final diagnosis of the disease[13]. It is robust and reliable, and has been widely used in laboratorial tests and scientific experiments. However, RT-PCR also has some drawbacks. For example, it could take up to 4 hours or longer in total including nucleic acid extraction, amplification reactions and analysis to reporting results. Therefore, shortening the process of nucleic acid extraction and amplification time would be a useful improvement for RT-PCRs. Our evaluation of RT-PCR assays showed that Kits B, C and F have good agreement with clinical diagnosis, while kit D and E have lower consistencies, all of which gave false negative results. According to an estimate, the false negative rate (FNR) of RT-PCR may be as high as 30% to 50% in real COVID-19 cases. Several factors have been proposed to explain the inconsistency or high FNR.[13].For example, this could be caused by patients who were asymptomatic or only display mild symptoms after infection. The viral load sampled from patients may also vary, depending on disease progression or sample qualities, which could be one of the main reasons why clinical patients have repeatedly tested negative but tested positive later. Therefore, nucleic acid testing should be carried out by continuous collection of specimens for suspected cases. Furthermore, primers ORF1ab gene and N as the target, can be affected by

virus genetic variation[13]. And we have also found that sampling method, depth, residence time and rotation number are related to the sample quality, which will affect the results of RT-PCR. In essence, a negative result for the first time should not be considered a confirmed laboratory diagnosis, especially in areas with high disease incidence. In contrast to RT-PCRs, Cross Primer isothermal Amplification (CPA) is a super sensitive nucleic acid amplification method, that can usually detect small amounts of RNA templates with minimal sample preparation[10]. It is also often fully automated and could be used by personnel on the field or at point of care with minimal training，so the biosecurity risks of it are lower. From our evaluation, the CPA assay (Kit A) has shown excellent analytical performance, with 100% sensitivity, specificity and accuracy. One main drawback of the CPA assay is a low-throughput device, which could only process two samples at once; another minor drawback in its methodology that requires specific high temperatures.

Our current comparative evaluation also has certain limitations. Due to the small number of positive cases confirmed in our laboratory, there was a lack of sample diversity in our tests, coupled with a limited number of reagent batches used in our assays. Therefore, the limited sample size may not accurately reflect the true analytical performance. During this outbreak, samples may also have been repeatedly frozen and thawed, which may have impact on the assay results. If possible, the sample size of clinical specimens should be increased for more robust evaluation of the molecular assays for SARS-CoV-2 detection .

## CONCLUSION

Each analysis method has its unique advantages and inevitable disadvantages. CPA method has the advantages of fast amplification, simple operation and convenient detection.is an ideal candidate for the development of portable molecular diagnostics, which is suitable for field screening detection and point of care test (POCT). RT-PCR assays on the other hand, are highly sensitive and specific with high-throughput capabilities, making it a more common choice for a laboratory setting. For COVID-19, we still need to develop more effective and practical methods to overcome the shortcomings of existing methods. At the same time, only according to the specific purpose of weighing the advantages and disadvantages of various detection methods, in order to get the most economical and optimal choice.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

All samples used in this study were de-identified and anonymous to protect patient privacy and confidentiality. This study was approved by the Ethics Committee of

Shenzhen Center for Disease Control and Prevention (Permit No. 2020-003A).

## AUTHOR CONTRIBUTIONS

Conceived and designed the experiment: SW, QH; Performed the experiments: SW, QC, MJ, YJ, LZ, LW, YL, SF, BP, WW; Contributed analysis: SW, XS, RZ, HL; Wrote the paper: SW, QH, PK

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## CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Table 1. Assay parameters for each of the six molecular assays evaluated

| Kit | Target | Volume (uL) | | Results interpretation | | LOD | Ref |
|---|---|---|---|---|---|---|---|
| | | sample | RNA | Positive | Negative | (copies/mL) | |
| A | ORF 1ab, N | 500 | / | Tt≤40 | No Tt value | 1000 | Y |
| B | ORF 1ab, N | / | 5 | Ct≤38 | No Ct value or Ct>38 | 1000 | N |
| C | ORF 1ab, N | / | 5 | Ct≤35 | Ct>38 | 500 | N |
| D | ORF 1ab | / | 10 | Ct≤32 | FAM：No Ct value<br>VIC/HEX：Ct≤32 | 100 | Y |
| E | ORF 1ab, N | / | 4 | Ct<38 | No Ct value or Ct=45 | 300 | N |
| F | ORF 1ab, N | / | 5 | Ct≤40 | No Ct value or Ct>40 | 500 | Y |

[a] Tt value is unique to the CPA assay (Kit A) and represents the time when fluorescence value exceeds the threshold line.

375 Table 2. Performance characteristics of six molecular assays for the detection of SARS-CoV-2 using clinical diagnosis as the reference standard.

| | No. of RT-PCR result | No. of clinical diagnosis | | Sensitivity （95% CI) | Specificity （95% CI) | PPV （95% CI) | NPV （95% CI) | Diagnostic Accuracy （95% CI) | kappa |
|---|---|---|---|---|---|---|---|---|---|
| | | Positive | Negative | | | | | | |
| A | Positive | 26 | 0 | 100 | 100 | 100 | 100 | 100.0 | 1.000 |
| | Negative | 0 | 26 | (87.1-100) | (87.1-100) | (87.1-100) | (87.1-100) | (93.1-100) | （p<0.001) |
| B | Positive | 23 | 0 | 100 | 89.7 | 88.5 | 100 | 94.2 | 0.885 |
| | Negative | 3 | 26 | (85.7-100) | (73.6-96.4) | (71.0-96.0) | (87.1-100) | (84.4-98.0) | （p<0.001) |
| C | Positive | 25 | 0 | 100 | 96.3 | 96.2 | 100 | 98.1 | 0.962 |
| | Negative | 1 | 26 | (86.7-100) | (81.7-99.3) | (81.1-99.3) | (87.1-100) | (89.9-99.7) | （p<0.001) |
| D | Positive | 18 | 0 | 100 | 76.5 | 69.2 | 100 | 84.6 | 0.692 |
| | Negative | 8 | 26 | (82.4-100) | (60.0-87.6) | (50.0-83.5) | (87.1-100) | (72.5-92.0) | （p<0.001) |
| E | Positive | 20 | 0 | 100 | 81.3 | 76.9 | 100 | 88.5 | 0.769 |
| | Negative | 6 | 26 | (83.9-100) | (64.7-91.1) | (57.6-89.0) | (87.1-100) | (77.0-94.6) | （p<0.001) |
| F | Positive | 24 | 0 | 100 | 92.9 | 92.3 | 100 | 96.15 | 0.923 |
| | Negative | 2 | 26 | (86.2-100) | (77.4-98.0) | (75.9-97.9) | (87.1-100) | (87.0-98.9) | （p<0.001) |

376 Table 3. Reproducibility of five RT-PCR assays (Kits B to F)

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| Kit | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | Mean（℃） | SD | CV（%） |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B | 34.56 | 33.80 | 34.79 | 35.49 | 34.37 | 33.99 | 35.32 | 33.43 | 33.72 | 34.39 | 0.72 | 2.09 |
| C | 31.72 | 30.38 | 31.90 | 31.61 | 32.41 | 31.85 | 30.35 | 32.31 | 31.61 | 31.57 | 0.74 | 2.34 |
| D | 34.54 | 34.34 | 34.36 | 35.77 | 33.19 | 33.45 | 33.88 | 33.26 | 33.06 | 33.98 | 0.87 | 2.57 |
| E | 32.74 | 31.78 | 33.03 | 32.55 | 31.99 | 32.66 | 32.61 | 32.99 | 33.91 | 32.70 | 0.62 | 1.89 |
| F | 36.15 | 36.54 | 36.34 | 36.87 | 37.22 | 36.81 | 36.29 | 36.61 | 36.95 | 36.64 | 0.35 | 0.95 |

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