CoPrimer Based rRT-PCR Detection of SARS-CoV-2 Directly from Saliva

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Abstract – Saliva provides an easy to collect sample type for SARS-CoV-2 diagnostic testing and screening, and it is utilized by numerous tests with FDA Emergency Use Authorization. Numerous saliva tests include a collection process that uses stabilizers and preservatives with a sample extraction step prior to molecular testing, but in recent months several tests have been developed to utilize minimally processed raw saliva followed by direct molecular detection. Co-Diagnostics, Inc has conducted an investigation into the compatibility of its patented CoPrimer™ technology with detection of SARS-CoV-2 directly from raw saliva. We used various CoPrimer assay configurations to consistently detect SARS-CoV-2 in minimally processed saliva with confirmed sensitivity limits as low as 347 copy/mL, and determined CoPrimers to be a robust platform for the development of such tests in the future.

Introduction – Since the inception of the SARS-CoV-2 pandemic, numerous diagnostic testing strategies have been developed. Some applications involve disseminated sample collection, addition of samples to chemical cocktails designed to stabilize and preserve the specimen, followed by transport to centralized high-throughput testing laboratories. Other applications are designated for use with individual specimens near the point of care. Many tests have been developed for use with swabs, washes, or sputum collected from the upper or lower respiratory tracts. A smaller group of tests has been developed for use with alternative sample types like saliva. Saliva is a reliable, convenient sample type for detection of SARS-CoV-2, which can be self-collected. In the absence of any current FDA approved test, molecular tests with Emergency Use Authorization based on Real-time Reverse Transcription Polymerase Chain Reaction (rRT-PCR) remain the gold standard against which all other tests are compared, due to the high levels of sensitivity and specificity that can be achieved.

Processing specimens with an extraction step typically allows the testing of samples collected with harsh additives designed to disrupt degradative enzymes and thereby stabilize nucleic acids that may be present. However, extraction typically requires procurement of additional instruments and consumable reagents which can add substantial cost to testing. Extraction also requires extra processing time, which can introduce a bottleneck to testing throughput. With several groups publishing direct sample testing protocols which can save time and money [1,2], we evaluated the performance of CoPrimers in a direct saliva testing protocol.

Materials and Methods – Heat-inactivated SARS-CoV-2 was obtained from the American Type Culture Collection (ATCC VR-1986HK).

Positive and negative saliva specimens were collected from donors with consent. Due to the inhomogeneity of different specimens, initial vortexing of saliva for at least one minute prior to additional processing or pooling was determined to be an essential first step. Negative saliva was pooled and vortexed to create a uniform negative sample matrix and diluent.

For testing, saliva samples were vortexed at room temperature for 1 minute. 50 µL saliva was pipetted into a new tube. 2.5 µL of Proteinase K stock was added to each tube for a final concentration of 0.8 to 1.0 mg/mL and vortexed for 1 minute at room temperature, followed by inactivation for 5 minutes at 95°C. 2× and 4× Master Mixes were prepared from 5× PCR Buffer, MgCl2, GoTaq, and GoScript (Promega), and custom designed CoPrimers (Biosearch Technologies).

RT-PCR reactions were performed in 96-well plates on a CFX96 instrument. (Bio-Rad) with the following cycling conditions: 45°C for 15 min (reverse transcription), 95°C for 2 min (activation), 45 cycles of 95°C for 3 sec (denaturation) and 55°C for 32 sec (anneal/extension).

For experiments using a 2× Master Mix, saliva was added at a 1:1 ratio into the final reaction volume. For experiments using a 4× Master Mix, saliva was added at a 3:1 ratio into the final reaction volume.

Replicates (4 or 20) for each experiment were prepared, processed, and tested as individual samples.

Results – Without harsh transport media additives, the natural enzymes present in raw saliva immediately begin to degrade any bare nucleic acids introduced into the sample. Although intact virus particles are protected from these natural defenses, when we spiked a mixture of synthetic RNA and DNA into raw saliva, the RNA signal was quickly degraded. To establish a minimal processing protocol, raw saliva specimens were treated in several different stepwise combinations with Proteinase K (ProK) plus vortexing, 95°C incubation, and a synthetic RNA/DNA Positive Control (PC) spike, as depicted in Table 1.

Table 1 – ProK treatment removes RNAse activity but requires Inactivation

Condition	*Step 1*	*Step 2*	*Step 3*	*RNA Detected*	*DNA Detected*
1	+ PC	ProK + Vortex	95°C 5 min	-	+
2	ProK + Vortex	+ PC	95°C 5 min	+	+
3	ProK + Vortex	95°C 5 min	+ PC	+	+
4	+PC	95°C 5 min	ProK + Vortex	-	-

When synthetic PC material was spiked into the raw saliva prior to ProK treatment (Condition 1), RNA was not detectable. Treating saliva with ProK prior to PC addition restored the RNA signal (Conditions 2 and 3), regardless of whether the 95°C heat inactivation step preceded or followed that PC addition. Addition of ProK after 95°C heat inactivation led to loss of both RNA and DNA signals (Condition 4), presumably due to degradation of essential RT-PCR enzymes.

To assess the analytical sensitivity of CoPrimer detection of SARS-CoV-2, we used heat-inactivated SARS-Cov-2 reference material. This material has been rendered non-infectious by heat inactivation and is not therefore expected to contain uniformly intact viral particles, so it was spiked after ProK treatment and inactivation of pooled negative raw saliva. The lower limit of detection (LOD) was estimated with tenfold and twofold dilutions in quadruplicate reactions (data not shown). An initial LOD_{95} confirmation level of 15.6 copies per reaction was selected for further confirmation testing. CoPrimers were prepared with either a 2× or 4× Master Mix (MM), combined directly with contrived specimen at the appropriate ratio, and tested with final reaction volumes of 10, 20, or 30 µL. In all cases, 20/20 replicates were detected, as shown in Table 2.

Table 2 – CoPrimer Limit of Detection Confirmation at 15.6 copy/reaction

MM Type	*Reaction Volume (µL)*	*Detected*	*Avg. Cq ± StDev*	*LOD (copy/mL)*
2×	10	20/20	37.88 ± 0.83	3120
2×	20	20/20	37.27 ± 0.60	1560
2×	30	20/20	37.25 ± 0.69	1040
4×	10	20/20	37.83 ± 0.76	2080
4×	20	20/20	38.19 ± 1.27	1040
4×	30	20/20	37.83 ± 1.99	693

Since all replicates were detected, LOD_{95} confirmation was attempted after one additional two-fold dilution step. As shown in Table 3, 7.8 copies per reaction produced 18/20 positive replicates in two of six conditions, while the other four conditions detected 19/20 replicates. Negative replicates did not produce a Cq value and were not included in the Average Cq calculations.

Table 3 – CoPrimer Limit of Detection Confirmation at 7.8 copy/reaction

MM Type	*Reaction Volume (µL)*	*Detected*	*Avg. Cq ± StDev (Detected Only)*	*LOD (copy/mL)*
2×	10	18/20	38.97 ± 0.80	1560
2×	20	19/20	38.57 ± 0.84	780
2×	30	19/20	38.68 ± 0.95	520
4×	10	19/20	38.57 ± 1.03	1040
4×	20	18/20	38.49 ± 0.61	520
4×	30	19/20	39.14 ± 1.07	347

This LOD performance compares favorably with that reported for other direct saliva tests (5,000 copy/mL [1], 6,000 to 12,000 copy/mL [2]). Given the accurate and sensitive performance of CoPrimers with viral reference material in minimally processed saliva, a positive donor specimen was then evaluated.

A raw saliva specimen obtained from a donor (#65) went through minimal processing and was tested in quadruplicate with 2× CoPrimer Master Mix in a 10 µL final reaction volume. All four replicates were detected, with an Average Cq value of 34.89. Since our contrived LOD testing gave us confidence that CoPrimers could accurately detect positive samples at later Cq values, this low-positive #65 sample was diluted further for additional testing using pooled negative raw saliva as the diluent. Seven two-fold dilutions were prepared, minimally processed, and tested in quadruplicate reactions under the same conditions. As shown in Table 4, we observed that the original processed specimen and the next five dilutions were detected in 4/4 replicates.

Table 4 – 2-fold dilutions of Low-Positive Specimen #65

Sample Dilution	*Detected*	*Avg. Cq ± StDev (Detected Only)*
Neat	4/4	34.89 ± 0.40
2×	4/4	36.30 ± 0.75
4×	4/4	37.30 ± 0.80
8×	4/4	37.53 ± 0.85
16×	4/4	38.12 ± 1.47
32×	4/4	38.14 ± 1.18
64×	1/4	39.49
128×	0/4	-

To determine how robust the detection would be nearer the Limit of Detection, 20 additional replicates were tested at the 8×, 16×, and 32× dilution levels. As shown in Table 5, diluted saliva was consistently detected in approximately the same Cq range observed for samples contrived from reference materials.

Table 5 – Additional testing of Diluted Low-Positive Specimen #65

Sample Dilution	*Detected*	*Avg. Cq ± StDev (Detected only)*
8×	20/20	37.74 ± 0.59
16×	18/20	38.67 ± 0.81
32×	13/20	39.91 ± 0.43

Conclusion – CoPrimer technology provides an accurate and robust platform for molecular assay development. The cooperative binding kinetics of CoPrimers allow the selection of larger binding domains to improve the specificity of assays, and the lack of incorporation of CoPrimer capture sequences into the ensuing amplicon serves as a powerful impediment to prevent bypass amplification of mismatched targets [3,4]. This investigation shows that CoPrimers are also compatible with direct testing of minimally processed saliva samples without the need for extraction, which opens additional possibilities for flexible implementation of CoPrimers in various diagnostic applications.

References –

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