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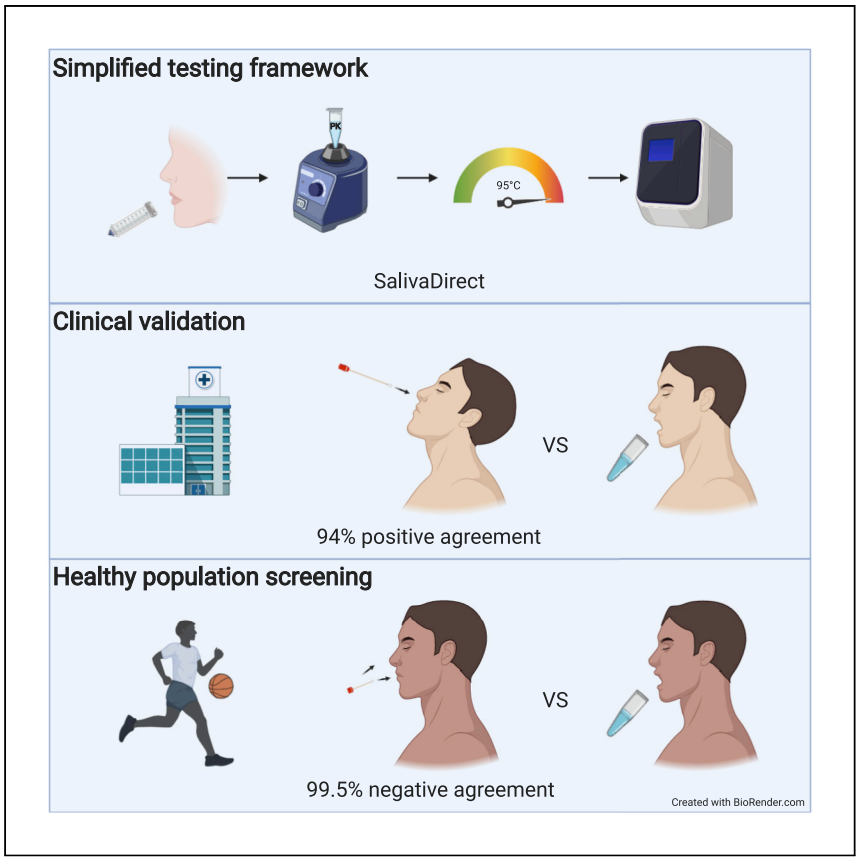
SalivaDirect: A simplified and flexible platform to enhance SARS-CoV-2 testing capacity



SalivaDirect is a sensitive saliva-based COVID-19 diagnostic test, which received Emergency Use Authorization from the U.S. FDA. With the ability to designate other laboratories, the flexible and simplified framework helps to increase the capacity of existing laboratory infrastructure for SARS-CoV-2 testing.

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HIGHLIGHTS

SalivaDirect is a simplified saliva-based test for detection of SARS-CoV-2

The testing framework is flexible to minimize the risk of supply chain issues

SalivaDirect is sensitive, with low rates of invalid and false-positive results

Laboratories can be designated to use SalivaDirect to increase testing capacity

Translation to Patients

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Clinical and Translational Article

SalivaDirect: A simplified and flexible platform to enhance SARS-CoV-2 testing capacity

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SUMMARY

Background: Scaling SARS-CoV-2 testing to meet demands of safe re-openings continues to be plagued by assay costs and supply chain shortages. In response, we developed SalivaDirect, which received Emergency Use Authorization (EUA) from the U.S. Food and Drug Administration (FDA).

Methods: We simplified our saliva-based diagnostic test by (1) not requiring collection tubes with preservatives, (2) replacing nucleic acid extraction with a simple enzymatic and heating step, and (3) testing specimens with a dualplex qRT-PCR assay. Moreover, we validated SalivaDirect with reagents and instruments from multiple vendors to minimize supply chain issues.

Findings: From our hospital cohort, we show a high positive agreement (94%) between saliva tested with SalivaDirect and nasopharyngeal swabs tested with a commercial qRT-PCR kit. In partnership with the National Basketball Association (NBA) and National Basketball Players Association (NBPA), we tested 3,779 saliva specimens from healthy individuals and detected low rates of invalid (0.3%) and false-positive (<0.05%) results.

Conclusions: We demonstrate that saliva is a valid alternative to swabs for SARS-CoV-2 screening and that SalivaDirect can make large-scale testing more accessible and affordable. Uniquely, we can designate other laboratories to use our sensitive, flexible, and simplified platform under our EUA (https://publichealth.yale.edu/salivadirect/).

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Context and significance

Frequent testing is critical to limit SARS-CoV-2 transmission. In response to this need, we developed SalivaDirect, a sensitive, simplified, and flexible testing framework, which received Emergency Use Authorization (EUA) from the U.S. Food and Drug Administration (FDA). We tested saliva collected from a hospital cohort and showed a high positive agreement (94%) as compared to paired nasopharyngeal swabs tested with a commercial diagnostic kit. Then, we partnered with the National Basketball Association (NBA) to test a large cohort of mostly healthy individuals, and we detected low rates of invalid (0.3%) and false-positive (0.03%–0.05%) results. Our study shows that SalivaDirect can help to increase testing capacity by providing access to an affordable framework that is less prone to supply chain shortages.

INTRODUCTION

SARS-CoV-2, a novel beta-coronavirus, emerged in late 2019 in Wuhan, China, and the subsequent coronavirus disease 2019 (COVID-19) pandemic rapidly followed.[1,2] In many parts of the world, including the United States, COVID-19 cases continue to rise.[3,4] The implementation of mass testing efforts followed by contact tracing will be

necessary to quell the pandemic. Routine state-level screening and surveillance of healthy individuals is particularly important for safe reopening of the economy and schools and can minimize the risk of relapsing local outbreaks. However, the scalability and availability of currently authorized assays for SARS-CoV-2 diagnostic testing are still limited, and large-scale application is hampered by worldwide supply chain issues.[5] To overcome these challenges, mass testing efforts must be (1) safe, both at the point of specimen collection and specimen processing; (2) affordable; (3) flexible, without the need for specific reagents or instrumentation from specific vendors; (4) adaptable to high-throughput workflows; and (5) amenable to quick turnaround times. While several different types of diagnostic assays have been recently authorized for emergency use by the U.S. Food and Drug Administration (FDA) such as qRT-PCR, LAMP, CRISPR, and sequencing-based assays, alternatives are still needed for large-scale testing efforts.[6]

Based on established diagnostic practices for other respiratory infections, the nasopharyngeal swab was initially adopted as the preferred sampling technique for SARS-CoV-2. However, we and others have shown that saliva can serve as an alternative upper respiratory tract specimen type for SARS-CoV-2 detection.[7–14] This is significant as saliva offers a number of advantages over nasopharyngeal swabs when considering the aforementioned criteria for mass testing efforts. Specifically, saliva does not require a certified swab and collection receptacle and does not necessarily have to be obtained by a skilled healthcare provider, both of which increase diagnostic-associated costs. Nasopharyngeal sampling requires a swab being inserted into the back of the nares, which can cause irritation that could promote sneezing and coughing. Thus, the noninvasive collection of saliva is safer, as it protects healthcare workers from being inadvertently exposed to potentially infectious droplets. In addition to being more affordable and safer, collection of nasopharyngeal swabs has been associated with variable, inconsistent, and false-negative test results due to the technical difficulties of taking a proper swab.[10,15–19]

To increase testing capacity for large-scale screening efforts, we developed SalivaDirect, a saliva-based, nucleic-acid-extraction-free, dualplex qRT-PCR method for SARS-CoV-2 detection. Our approach can be broadly implemented, as it does not require expensive saliva collection tubes containing preservatives[20] and does not require specialized reagents or equipment for nucleic acid extraction. We validated SalivaDirect for use with products from multiple vendors. Thus, the simplicity and flexibility of SalivaDirect mean that it will not be as affected by supply chain bottlenecks as some other assays that rely on swabs and/or nucleic acid extraction. We show that SalivaDirect has a low limit of detection (6–12 copies/μL) and yields highly concordant results as compared to currently validated qRT-PCR assays. The unique features of SalivaDirect is that it is noninvasive, less expensive ($1.21–$4.39/sample in reagents), and validated for use with reagents and instruments from multiple vendors. Through our partnerships with the National Basketball Association (NBA) and the National Basketball Players Association (NBPA), we conducted a large usability study of SalivaDirect and comparison to standard qRT-PCR testing of paired anterior nares/oropharyngeal (AN/OP) swabs for asymptomatic/presymptomatic detection of SARS-CoV-2. Our results demonstrate how our specialized protocols for saliva collection produce mostly valid specimens for testing (99.7%), and the specificity of SalivaDirect leads to very few false-positive results (0.03%–0.05%), showcasing that SalivaDirect, and saliva testing in general, can be used to enhance large-scale testing of symptomatic and asymptomatic individuals. The data presented here were used to support our Emergency Use Authorization (EUA) for SalivaDirect granted by the FDA on August 15th, 2020.[21]

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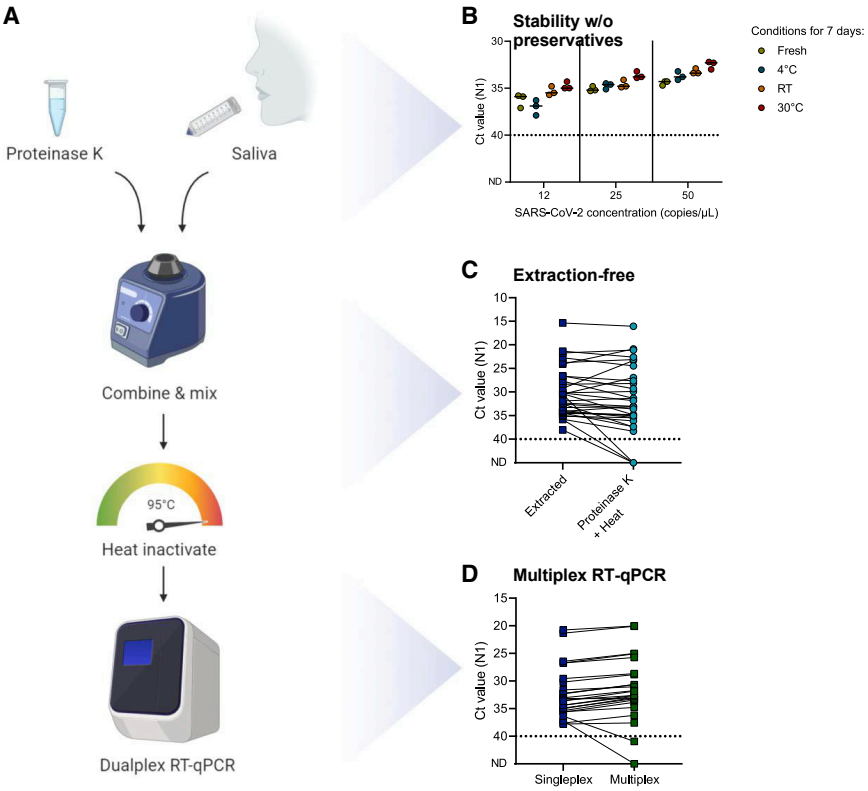

Figure 1. SalivaDirect is a simplified method for SARS-CoV-2 detection

(A) Schematic overview of SalivaDirect workflow depicting the main steps of mixing saliva with proteinase K, heat inactivation, and dualplex qRT-PCR testing. Figure created with Biorender.com.

(B) SARS-CoV-2 is stable in saliva for at least 7 days at 4°C, room temperature (RT; ~19°C), and 30°C without addition of stabilizing buffers. Spiked-in saliva samples of low virus concentrations (12, 25, and 50 SARS-CoV-2 copies/μL) were kept at the indicated temperature for 7 days and then tested with SalivaDirect. N1 cycle threshold (Ct) values were lower when kept for 7 days at 30°C as compared to fresh specimens (Kruskal-Wallis; $p = 0.03$). Horizontal bars indicate the median.

(C) Comparing Ct values for saliva treated with proteinase K and heat as compared to nucleic extraction yields higher N1 Ct values without extraction (Wilcoxon; $p < 0.01$).

(D) Testing extracted nucleic acid from saliva with the N1 primer-probe set (singleplex) as compared to a multiplex assay showed stronger N1 detection in multiplex (Wilcoxon; $p < 0.01$). The dotted line in (B)–(D) indicates the limit of detection.

Data used to make this figure can be found in Data S1.

RESULTS

Development of a simplified SARS-CoV-2 molecular diagnostic framework

To reduce the cost, time, and effort for SARS-CoV-2 detection, we developed SalivaDirect (https://publichealth.yale.edu/salivadirect/), a simplified and flexible saliva-based platform. SalivaDirect consists of three steps: (1) collecting saliva without preservative buffers, (2) proteinase K treatment and heat inactivation in place of nucleic acid extraction, and (3) dualplex qRT-PCR SARS-CoV-2 detection (Figure 1A).

Stability of SARS-CoV-2 detection in saliva without preservatives

Several protocols imply that stabilizing buffers (e.g., Tris-borate-EDTA [TBE], Tris-EDTA [TE], or PBS) and additives (e.g., Triton X-100, Tween 20, or NP-40) are required to preserve the detection of SARS-CoV-2 RNA in saliva specimens, while other studies suggest that these buffers are not required and may even inhibit

qRT-PCR.[20,22,23] To determine the stability of SARS-CoV-2 RNA detection using SalivaDirect, we stored saliva specimens for 7 days at 4°C, room temperature, or 30°C without the addition of preservatives. We quantified the virus copies from a positive saliva specimen and spiked-in different concentrations of the positive sample to achieve concentrations of 12, 25, and 50 SARS-CoV-2 copies/μL into negative saliva collected from healthcare workers.[10] After 7 days, we tested the spiked-in saliva specimens with SalivaDirect and compared results to "fresh" samples. We found that SARS-CoV-2 detection was stable in saliva for at least 7 days at each of the three thermal conditions (Figure 1B). Surprisingly, we even detected significantly lower N1 cycle threshold (Ct) values (e.g., better detection) when saliva was kept for 7 days at 30°C as compared to fresh specimens (median difference across concentrations of 1.4 Ct, $p = 0.03$; Figure 1B). In contrast, we found that Ct values for human *RNase P* (RP) were significantly higher after 7 days at room temperature (median difference of 3.8 Ct, $p < 0.01$) or 30°C (median difference of 5.0 Ct, $p < 0.001$) as compared to fresh specimens, which suggests that the human RNA degraded over time (Figure S1). We observed similar patterns when testing additional temperature profiles that the samples may encounter during transport, with no significant differences between N1 Ct values for fresh samples as compared to samples kept at 40°C for 72 h ($p > 0.99$) or samples kept under summer (alternating 28°C–40°C; $p = 0.54$) or winter profiles (alternating −20°C to room temperature; $p > 0.99$; Figure S2). Moreover, in a parallel clinical study, we showed that SARS-CoV-2 RNA from saliva collected from COVID-19 patients without preservatives is stable at 30°C for at least 3 days ($n = 20$) and at room temperature for up to 25 days ($n = 20$), though the samples were tested by a standard PCR test and not SalivaDirect.[20] Thus, our data suggest that SARS-CoV-2 RNA, or at least the targeted nucleocapsid RNA, is stable in saliva without preservatives for at least 3 days, and likely longer, when stored at temperatures of up to 40°C.

Nucleic-acid-extraction-free PCR detection of SARS-CoV-2

Nucleic acid extraction is included in most authorized PCR diagnostic assays to detect SARS-CoV-2 RNA by qRT-PCR. However, nucleic acid extraction is relatively expensive, time-consuming, and subject to supply chain bottlenecks, which limit the scalability of testing that is critical for safe reopenings. Previous studies have shown that the nucleic acid extraction step can be omitted with a relatively small impact on analytical sensitivity.[23–28] Therefore, we explored the potential of proteinase K and heat as an affordable, fast, and easy alternative to nucleic acid extraction. We used the modified Centers for Disease Control and Prevention (CDC) assay[29] to compare qRT-PCR detection of SARS-CoV-2 in saliva specimens processed with nucleic acid extraction or by simply mixing the specimen with proteinase K followed by heat inactivation (Figure 1C). As compared to nucleic acid extraction, our data show that our extraction-free approach minimally decreases detection (median N1 Ct increase = 1.8 Ct; $p < 0.01$). The reduction in detection that we observed is equivalent to what we would expect from omitting the ~4-fold concentration step that occurs during nucleic acid extraction. Our findings demonstrate that proteinase K and heat can be used as an alternative to nucleic acid extraction with only a minor loss in sensitivity.

Dualplex PCR detection of SARS-CoV-2 and a human control gene

Our final modification to improve the scalability of SARS-CoV-2 diagnostic assays was to increase the high-throughput testing potential of the qRT-PCR step. We previously found that the U.S. CDC primer-probe sets are among the most sensitive and reliable for SARS-CoV-2 detection.[29] The CDC assay consists of three separate reactions targeting two regions of the SARS-CoV-2 nucleocapsid (N1 and N2) and a

human RP control.[30] We previously modified the CDC assay by multiplexing the three primer-probe sets, thereby reducing the number of tests from three to one, without a significant impact on its sensitivity.[31] When testing the multiplex qRT-PCR assay on saliva treated with proteinase K and heat, however, we were not able to detect consistent results for the N2 primer-probe set or the Sarbeco-E (E) or HKU-ORF1 (ORF1) primer-probe sets with HEX fluorophores (Table S1). We previously showed that SARS-CoV-2 detection by the N1 primer-probe set is more consistent and stronger as compared to N2.[29] Therefore, to further simplify the qRT-PCR assay, we developed a dualplex qRT-PCR assay based on N1 and RP and modified the fluorophore (Cy5, ATTO647, or Quasar670 instead of FAM) on the RP probe. When comparing the modified singleplex CDC assay with the dualplex assay on extracted nucleic acid, median N1 Ct values were 0.9 Ct lower when tested in multiplex ($p < 0.01$; Figure 1D). Thus, SalivaDirect allows for a reduction in the number of qRT-PCR reactions to one reaction per sample.

Lower limit of detection using reagents and equipment from multiple vendors

Tests that depend on specific reagents from single vendors leave them vulnerable to supply chain shortages, as happened throughout the COVID-19 pandemic. Thus our approach was to validate SalivaDirect using reagents and instruments from multiple vendors to avoid dependence on a single vendor for each step (Table 3). In addition to what is shown here, we will continue to amend our SalivaDirect EUA through the use of bridging studies to ensure that it remains free of supply chain issues and to provide cheaper alternatives. A current list of validated products can be found in our updated EUA summary.[21]

To validate reagents and instruments, we spiked a known concentration of SARS-CoV-2-positive saliva into negative saliva from healthcare workers to prepare a 2-fold dilution series of 400, 200, 100, 50, 25, 12, and 6 virus copies/μL. By testing each concentration in triplicate, we determined the preliminary limit of detection, which was then confirmed by testing another 20 replicates (Figure 2). Treating saliva with proteinase K from three different vendors resulted in a limit of detection of 6 SARS-CoV-2 copies/μL, suggesting that SalivaDirect is not dependent on proteinase K from a specific vendor (Figures 2A–2C).

Next, we determined the limit of detection by comparing three different qRT-PCR kits obtained from New England Biolabs, Bio-Rad, and ThermoFisher Scientific (Table 3). As each kit specifies the use of slightly different PCR cycle times and temperatures, we first sought to standardize these into a "universal" thermocycler program to make it easier to switch between products when needed. Comparing the results from each kit using the manufacturer's protocol and the universal qRT-PCR program, we found no significant differences in Ct values (Luna: $p = 0.69$, Reliance: $p = 0.06$, TaqPath: $p = 0.44$; Figure S3). One additional qRT-PCR kit, Invitrogen EXPRESS One-Step SuperScript qRT-PCR kit, which we tested under their recommended protocol as well as our universal program, did not seem compatible with SalivaDirect and was therefore excluded from our limit-of-detection experiment. Using the universal thermocycler program with the Bio-Rad CFX96 instrument, New England Biolabs (NEB) Luna Universal Probe One-Step kit and Bio-Rad Reliance One-Step Multiplex RT-qPCR Supermix had a lower limit of detection of 6 SARS-CoV-2 copies/μL, whereas the ThermoFisher Scientific TaqPath 1-Step RT-qPCR Master Mix resulted in a slightly higher limit of detection of 12 SARS-CoV-2 copies/μL (Figures 2D–2F). Importantly, this indicates that the specific qRT-PCR kit can influence the lower limit of virus detection and that not all kits may be suitable for use with SalivaDirect.

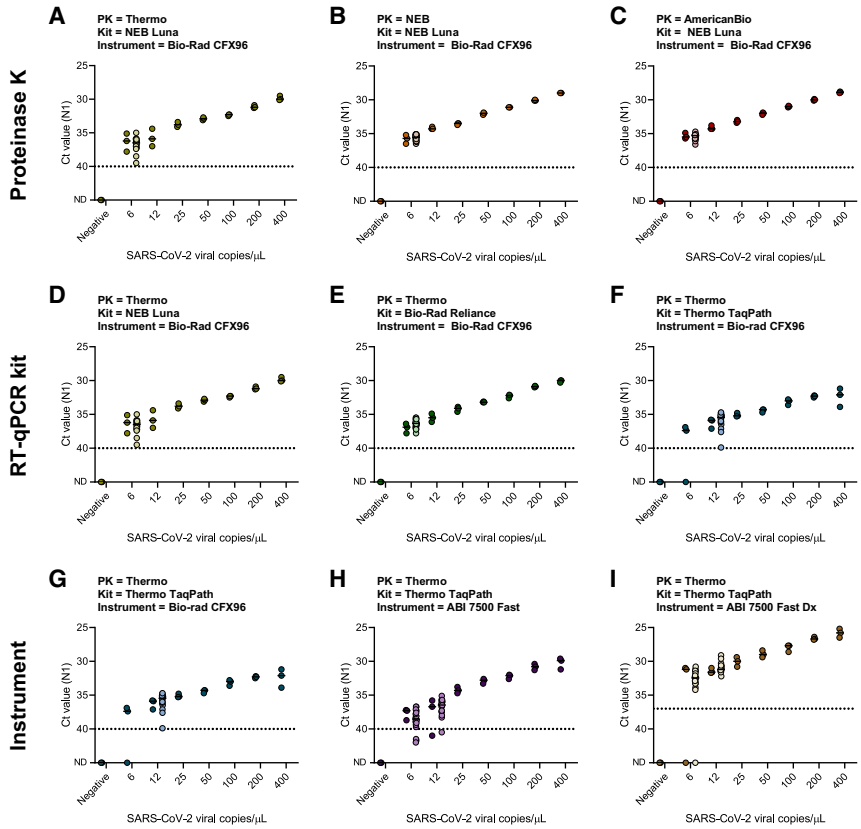

Figure 2. SalivaDirect is validated for use with reagents and instruments from multiple vendors

We determined the lower limit of detection of SalivaDirect with a 2-fold dilution series (400, 200, 100, 50, 25, 12, and 6 copies/µL) of positive saliva spiked-in negative saliva. Initially, each concentration and negative saliva were tested in triplicate to determine the preliminary limit of detection (dark-colored dots). The limit of detection was confirmed with 20 additional replicates (light-colored dots) for which 19 out of 20 needed to be detected. Limit of detection when tested with (A–C) proteinase K, (D–F) RT-qPCR kits, and (G–I) qRT-PCR instruments from different vendors, while keeping the other conditions constant. (A) and (D), as well as (F) and (G), are duplicates to enable comparisons between the different combinations of reagents or instruments within a single row. Shown are the Ct values for the N1 primer-probe set. The horizontal bars indicate the median and the dotted line indicates the limit of detection. Data used to make this figure can be found in Data S1.

Using the qRT-PCR kit with the highest limit of detection, TaqPath 1-Step RT-qPCR Master Mix, we compared the detection across three commonly used qRT-PCR thermocycler instruments: Bio-Rad CFX96, Applied Biosystems (ABI) 7500 Fast, and ABI 7500 Fast Dx. We found that the Bio-Rad CFX96 and ABI 7500 Fast had similar lower limits of detection at 12 SARS-CoV-2 copies/µL, whereas the ABI 7500 Fast Dx had a slightly lower limit of detection of 6 SARS-CoV-2 copies/µL (Figures 2G–2I). Interestingly, when determining the preliminary limit of detection for the ABI 7500 Fast Dx, we found that Ct values were on average 4.7 lower than Ct values generated on the ABI 7500 Fast. This suggests a difference in the auto-threshold that the machine sets, and therefore, we have increased the positive threshold to 37 Ct for the ABI 7500 Fast Dx to correspond to the positive threshold for the ThermoFisher Scientific TaqPath COVID-19 combo kit using the ABI 7500 Fast Dx. Changing the threshold did not affect the confirmed lower limit of detection of 6 copies/µL for the ABI 7500 Fast Dx. Overall, we found that SalivaDirect has a low limit of detection (6–12 SARS-CoV-2 copies/µL) using reagents and instruments from multiple vendors.

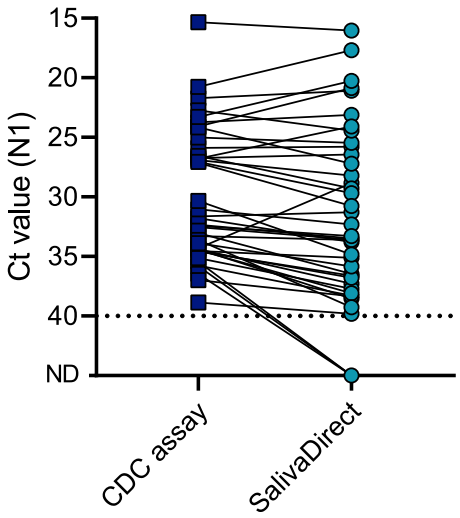

Figure 3. Sensitivity of SalivaDirect is comparable to a standard approach for SARS-CoV-2 detection in saliva

We compared Ct values for N1 between the modified CDC assay (nucleic acid extraction and singleplex qRT-PCR) and SalivaDirect for 41 saliva specimens tested with both methods. Overall, detection of SARS-CoV-2 with SalivaDirect is weaker (median 1.2 Ct, Wilcoxon; $p < 0.001$) than the modified CDC assay, but with a high agreement in outcomes of both tests of (93%). Shown are the Ct values for the N1 primer-probe set and the dotted line indicates the limit of detection. Data used to make this figure can be found in Data S1.

Sensitivity of SalivaDirect compared to saliva tested using a standard qRT-PCR assay

After determining the lower limit of detection of SalivaDirect, we compared the sensitivity of SARS-CoV-2 detection from saliva using a standard approach, a modified CDC assay with nucleic acid extraction and singleplex qRT-PCR.[29] We found that the median N1 Ct values were 1.2 higher (e.g., weaker detection) for SalivaDirect as compared to the modified CDC assay ($p < 0.001$; Figure 3). Overall, the reduction in analytical sensitivity contributed to a 7.3% (3/41) false-negative rate for SalivaDirect, but only for weakly positive samples (all three false-negative specimens had N1 Ct values of 35–40 when using the modified CDC assay; Figure 3). Our findings show ~93% positive agreement of SalivaDirect compared to a standard testing approach.

Clinical validation with paired nasopharyngeal swabs and saliva

For consideration of an EUA, the FDA requires clinical validation of any new laboratory developed test by comparing to currently authorized tests. For our validation study, we compared both across tests (SalivaDirect to the authorized ThermoFisher Scientific TaqPath COVID-19 combo kit)[32] and across sample types (saliva to nasopharyngeal swabs) (Figure 4; Tables 2 and 3). We collected 37 paired positive and 30 paired negative nasopharyngeal swabs and saliva specimens from inpatients and healthcare workers at the Yale-New Haven Hospital. The ThermoFisher Scientific TaqPath COVID-19 combo kit combines nucleic acid extraction using the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit with a multiplex qRT-PCR diagnostic assay targeting three regions of the SARS-CoV-2 genome on the ABI 7500 Fast Dx instrument. For SalivaDirect, we used the ThermoFisher Scientific proteinase K, ThermoFisher Scientific TaqPath RT-PCR kit, and Bio-Rad CFX96 instrument. We selected the positive and negative pairs based on preliminary results of our modified CDC assay.

First, when we compared nasopharyngeal swabs and saliva specimens when tested with the TaqPath COVID-19 combo kit, we found a positive agreement of 91.2% (Figure 4A). For both sample types, there were three specimens that tested negative, invalid, or inconclusive, while the other sample type tested positive. However, we did not find significant differences in Ct values for the three virus targets between both sample types ($p = 0.39–0.72$), with the median difference for each of the virus

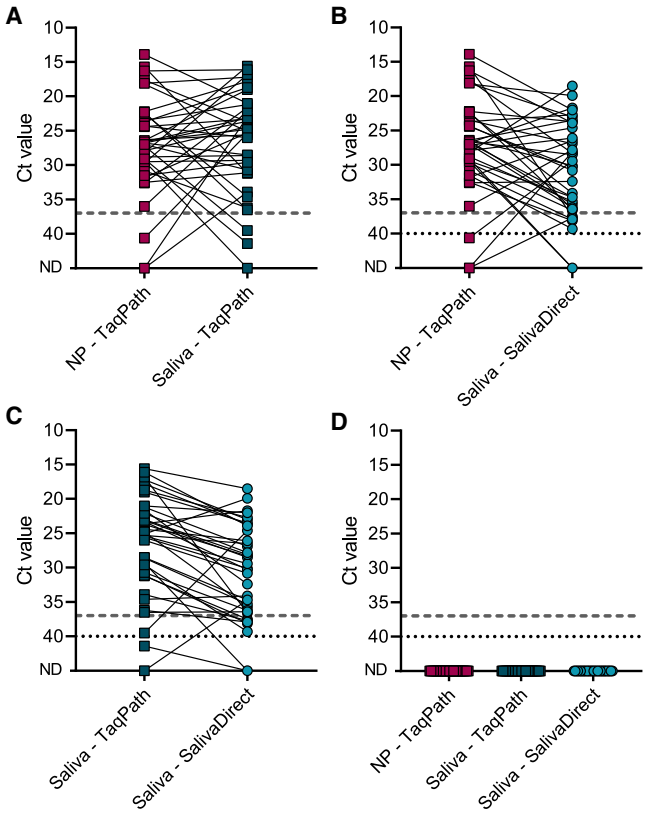

Figure 4. SalivaDirect is highly comparable to standard qRT-PCR tests with nucleic acid extraction from nasopharyngeal swabs and saliva

We selected 37 paired positive and 30 paired negative nasopharyngeal swabs and saliva specimens. Paired samples were collected a maximum 4 days apart. Nasopharyngeal swabs and saliva specimens were tested with the ThermoFisher Scientific TaqPath COVID-19 combo kit, and average Ct values for N, S, and ORF1ab were compared to N1 Ct values for saliva specimens tested with SalivaDirect.

(A) Comparison of 37 paired nasopharyngeal swabs and saliva tested with the TaqPath COVID-19 combo kit showed 84% positive agreement and no significant differences in each of the three virus targets (Wilcoxon; N: $p = 0.51$, S: $p = 0.72$, ORF1ab: $p = 0.39$).

(B) Comparison of nasopharyngeal swabs tested with the TaqPatch COVID-19 combo kit and saliva tested with SalivaDirect showed 94% positive agreement. Median N1 Ct values were 3.3 Ct higher for SalivaDirect (Wilcoxon; $p < 0.01$).

(C) Comparison of saliva tested with TaqPath COVID-19 combo kit and SalivaDirect again shows that SalivaDirect showed 97% positive agreement. Median N1 Ct values were 5.0 Ct higher for SalivaDirect (Wilcoxon; $p < 0.001$).

(D) 30 paired nasopharyngeal swabs and saliva specimens tested negative with both the TaqPath COVID-19 combo kit and SalivaDirect. Shown are average Ct values for N, S, and ORF1ab for the TaqPath combo kit and N1 Ct values for SalivaDirect. The dashed line indicates the limit of detection for the TaqPath combo kit (37 Ct) and the dotted line indicates the limit of detection for SalivaDirect (40 Ct).

Data used to make this figure can be found in Data S1.

targets <2 Ct. This again confirms that some variation exists between sample types but that saliva is a valuable alternative.[7–10]

Next, we found a 94% positive agreement with SalivaDirect compared to nasopharyngeal swabs tested with the TaqPath COVID-19 combo kit (Table 1). The N1 Ct values were higher using SalivaDirect (median difference of 3.3 Ct; $p < 0.01$; Figure 4B), and the increased Ct values are likely due to a combination of removing

Table 1. Parallel testing of nasopharyngeal swabs and saliva from inpatients and healthcare workers with SalivaDirect and a commercial qRT-PCR kit

		TaqPath COVID-19					
		Nasopharyngeal Swab		Saliva			
		Positive	Negative	Positive	Inconclusive	Invalid	Negative
SalivaDirect	positive	32	3[a]	33	0	2	0
Saliva	negative	2	30	1	1	0	30
Total		34	33	34	1	2	30

NP-Saliva: positive agreement = 94.1% (32/34) and negative agreement = 90.9% (30/33). Saliva-Saliva: positive agreement = 97.1% (33/34) and negative agreement = 100% (30/30).

[a]Three nasopharyngeal swabs tested negative, while previous outcomes of the modified CDC assay indicated that they were weakly positive.

the nucleic acid step (Figures 1C and 3) and using different thermocycler instruments (Figure 2). Out of the 37 nasopharyngeal swabs that were tested with the TaqPath COVID-19 combo kit, three specimens tested negative (Table 1 and Figure 4B). However, earlier results with the modified CDC assay indicated a (weakly) positive signal, and the paired saliva specimen tested positive with both SalivaDirect and the TaqPath COVID-19 kit. While this is not captured in the percentage of positive agreement, SalivaDirect was able to detect SARS-CoV-2 in saliva of three individuals for which the nasopharyngeal swab tested negative.

When we directly compared the results of SARS-CoV-2 detection from saliva using SalivaDirect and the TaqPath COVID-19 combo kit, we found a high positive (97.1%) as well as negative agreement (100%; Table 1). Ct values for N1 were higher when comparing SalivaDirect with the TaqPath COVID-19 combo kit (median difference of 5.0 Ct, $p < 0.001$; Figure 4C), likely for the reasons described above. We intentionally included this comparison to enable a direct comparison of test results based on the same input specimen.

Finally, we compared results of negative paired nasopharyngeal swabs and saliva specimens tested with both the TaqPath COVID-19 combo kit and SalivaDirect (Figure 4D). No SARS-CoV-2 was detected in any of the specimens, while we did detect the internal controls. Thus, we did not detect any false-positive results with any of the assays.

Evaluation of off-target amplification

Background amplification or cross-reactivity of primer-probe sets with related human respiratory pathogens can cause false-positive results. Previous *in vitro* evaluations by the CDC showed no cross-reactivity with other human coronaviruses (229E, OC43, NL63, and HKU1), MERS-coronavirus, SARS-coronavirus, and 14 additional human respiratory viruses.[30] These findings are in accordance with our previous investigation of nine primer-probe sets, including the N1 set, which did not detect any background amplification.[29] To test for possible cross-reactivity of the dualplex qRT-PCR assay, we tested 52 saliva specimens collected from adults in the 2018/2019 and 2019/2020 fall and winter (pre-COVID-19; Figure S4). We did not detect off-target amplification or false positives, which is in agreement with previous findings from the CDC.[30]

Asymptomatic validation with paired AN/OP swabs and saliva

To conduct effective SARS-CoV-2 screening programs to allow populations to return to school and work, the test must be able to detect asymptomatic and/or

Table 2. Parallel testing of anterior nares/oropharyngeal swabs and saliva from NBA players, staff, and contractors

		Quest/BioReference	
		AN/OP Swab	
		Positive	Negative
SalivaDirect Saliva	positive	17	2
	negative	2	3,746
	invalid	0	12
Total		19	3,760

Invalid samples = 0.3% (12/3,779). Positive agreement = 89.5% (17/19). Negative agreement = 99.9% (3,746/3,748 valid samples). Overall agreement = 99.9% (3,763/3,767 valid samples).

presymptomatic cases and have a low false-positive rate. To evaluate SalivaDirect for these uses, we compared 3,779 saliva samples to paired combined AN/OP swabs from healthy NBA players, staff, and contractors (Table 2). The saliva samples were sent to the Yale School of Public Health for testing by SalivaDirect, and the AN/OP swabs were tested by commercial clinical diagnostic laboratories (Quest Diagnostics or BioReference Laboratories) using standard qRT-PCR assays.

There are concerns with using saliva as a testing specimen, as accidental collection of sputum and/or remnants from food or drinks can interfere with sample processing or inhibit PCR. For example, in a study with 124 symptomatic outpatients without optimized saliva collection protocols, approximately one-third of the samples were difficult to pipet.[33] In our NBA cohort study, we developed explicit instructions on how to collect true saliva,[34,35] used a saliva collection aid (a straw that fits into the collection tube) to promote providing true saliva and minimize potential aerosolization, and added proteinase K as the first laboratory step to help degrade any mucus. As a result, all 3,779 saliva samples collected from the cohort could be tested by SalivaDirect. Furthermore, the use of the RP human control gene in the dualplex PCR helps to determine if there are inhibitors in the specimens. We found that 12 of the 3,779 saliva samples had RP values above a Ct of 35, the threshold for an invalid sample (Table 2; Figure S5A). Thus 0.3% of the saliva samples collected from our cohort were invalid by PCR. Overall, we had a high rate of success for testing saliva by SalivaDirect.

During the study, 19 AN/OP swabs were positive for SARS-CoV-2, and 17 of those were also positive from saliva tested by SalivaDirect (89.5% positive agreement; Table 2). Out of the 19 AN/OP swabs that tested positive by commercial clinical diagnostic laboratories, we received 10 for comparative testing in our lab with the modified multiplex CDC assay (Figure S5B). When comparing the Ct values for the paired AN/OP swabs and saliva, we found no significant differences between both sample types ($p = 0.91$; Figure S5B). Upon retesting with the modified multiplex CDC assay, we found that two AN/OP swabs tested below our positive threshold, indicating that these samples may have been weakly positive or false positive from the commercial labs. Paired saliva of one of these two swab specimens also tested negative with SalivaDirect. Thus, the true sensitivity of SalivaDirect to AN/OP swabs by standard qRT-PCR for asymptomatic/presymptomatic detection may be higher than 90%; however, a larger positive sample size is needed to further evaluate.

Out of the 3,748 valid samples that tested negative by AN/OP swabs, 3,746 were also identified as negative by SalivaDirect, resulting in a negative agreement of 99.9%. For one of the samples that was negative by AN/OP swabs but positive by

SalivaDirect, the subsequent saliva and AN/OP swabs from the same individual tested positive, suggesting that the previous SalivaDirect result was a true positive. For the other incongruent result (AN/OP negative, SalivaDirect positive), however, subsequent saliva and AN/OP swabs tested negative, suggesting that the previous SalivaDirect result was a false positive. Thus, our data indicate that the false-positive rate for SalivaDirect is between 1 and 2 per 3,748 (0.03%–0.05%) samples tested.

Supply costs for SalivaDirect testing

We aimed to develop a simplified testing method that is not dependent on commercialized kits, which may be subject to supply chain issues. Therefore, we reduced the number of steps and initially validated SalivaDirect with reagents and instruments from three different vendors. By doing so, we have reduced the cost per sample to a minimum of $1.21, if saliva is collected without a saliva collection aid, and a maximum of $4.39 when using a saliva collection aid (Table 3). These cost estimates are based on list prices; therefore, the actual costs may be lower. Additional reagents and instruments can be validated by performing a bridging study to show an equal limit of detection and can be submitted to the FDA as an amendment to the authorized EUA. Thus, the supply costs for SalivaDirect are relatively inexpensive, though these prices do not include labor or other laboratory expenses.

DISCUSSION

SalivaDirect is a simplified and flexible platform

We developed SalivaDirect to adapt to the needs and budgets of heterogeneous SARS-CoV-2 surveillance systems. Testing saliva as an alternative to invasive swabs allows for safe and easy specimen collection. Furthermore, high-throughput testing can be maximized without the need for expensive saliva collection tubes with stabilizing reagents and nucleic acid extraction kits and a reduction in qRT-PCR reagents needed per specimen. We validated SalivaDirect with multiple reagents and instruments from different vendors to provide alternative options to minimize bottlenecks associated with supply chain issues. Furthermore, we demonstrated its effectiveness using multiple cohorts. (1) From our hospital cohort consisting of inpatients and healthcare workers with known SARS-CoV-2 infections, we found a positive agreement (94%) between SalivaDirect and NP swabs tested using a commercial qRT-PCR kit. (2) From our large NBA cohort consisting of mostly healthy individuals, our results showed that SalivaDirect had low rates of invalid (0.3%) and false-positive (0.03%–0.05%) samples when compared to AN/OP swabs tested by commercial clinical diagnostic laboratories. Together, our clinical studies showcase how our saliva collection and testing protocols are conducive for large-scale and repeated SARS-CoV-2 screening in mostly healthy individuals.

Uniquely, the U.S. FDA authorized us to designate other high-complexity Clinical Laboratory Improvement Amendments (CLIA) certified laboratories to use SalivaDirect, allowing our flexible and simplified framework to help increase the capacity of existing laboratory infrastructure for SARS-CoV-2 testing. As of December 7, 2020, 52 high-complexity CLIA certified laboratories from 25 U.S. states have been designated to use SalivaDirect, and another 265 laboratories have initiated the designation process. An outline of the designation process and a list of the designated labs can be found on our website (https://publichealth.yale.edu/salivadirect/).

To adapt to interested laboratories, avoid supply chain interruptions, or expand product availability, additional reagents and instruments can be added to our SalivaDirect FDA EUA. This can be done by performing bridging studies to establish

Table 3. SalivaDirect is a relatively inexpensive method for SARS-CoV-2 diagnostic testing

Vendor	Item	Catalog Number	Price/Sample
Sample Collection (Pick one of the Listed Options)			
Thomas Scientific	screw-cap tube, 5 mL, sterile	1188R46	$0.22
VWR	5 mL screw-cap centrifuge tubes, sterile	10002-738	$0.25
Eppendorf	Eppendorf tubes 5.0 mL with screw cap, sterile	0030122321	$0.41
Salimetrics	saliva collection aid	5016.02	$1.40
Sample Processing (Pick one of the Listed Options)			
AmericanBio	proteinase K	AB00925-00100	$0.13
ThermoFisher Scientific	MagMAX viral/pathogen proteinase K	A42363	$0.16
New England Biolabs	proteinase K, molecular biology grade	P8107S	$0.26
qRT-PCR Primers and Probes (Pick one of the Listed Sets)			
Eurofins	SalivaDirect primer probe set, 50–100 nmol	12YS-010YST	$0.12
Integrated DNA technologies	nCOV_N1 forward primer aliquot, 50 nmol	10006821	$0.02
	nCOV_N1 forward primer aliquot, 100 nmol	10006830	$0.02
	nCOV_N1 reverse primer aliquot, 50 nmol	10006822	$0.02
	nCOV_N1 reverse primer aliquot, 100 nmol	10006831	$0.02
	nCOV_N1 probe aliquot, 25 nmol	10006823	$0.04
	nCOV_N1 probe aliquot, 50 nmol	10006832	$0.03
	RNase P forward primer aliquot, 50 nmol	10006827	$0.01
	RNase P forward primer aliquot, 100 nmol	10006836	$0.01
	RNase P reverse primer aliquot, 50 nmol	10006828	$0.01
	RNase P reverse primer aliquot, 100 nmol	10006837	$0.01
	RP probe (Cy5-IBRQ)	custom	$0.10
	RP probe (ATTO657-IBRQ), 25 nmol	10007061	$0.09
	RP probe (ATTO657-IBRQ), 50 nmol	10007062	$0.06
LGC Biosearch Technologies	nCOV_N1 forward primer, 100 nmol	nCoV-N1-F-100	$0.01
	nCOV_N1 forward primer, 1000 nmol	nCoV-N1-F-1000	$0.01
	nCOV_N1 reverse primer, 100 nmol	nCoV-N1-R-100	$0.01
	nCOV_N1 reverse primer, 1000 nmol	nCoV-N1-R-1000	$0.01
	nCOV_N1 probe, 25 nmol	nCoV-N1-P-25	$0.04
	nCOV_N1 probe, 250 nmol	nCoV-N1-P-250	$0.03
	RNase P forward primer, 20 nmol	RNP-F-20	<$0.01
	RNase P forward primer, 100 nmol	RNP-F-100	<$0.01
	RNase P forward primer, 1000 nmol	RNP-F-1000	<$0.01
	RNase P reverse primer, 20 nmol	RNP-R-20	<$0.01
	RNase P reverse primer, 100 nmol	RNP-R-100	<$0.01
	RNase P reverse primer, 1000 nmol	RNP-R-1000	<$0.01
	RP probe, 25 nmol	RNP-PQ670-25	$0.06
	RP probe, 250 nmol	RNP-PQ670-250	$0.03
RT-qPCR Kits (Pick One of the Listed Options)			
New England Biolabs	Luna Universal Probe One-Step RT-qPCR Kit	E3006S	$0.75–$1.08
		E3006L	
		E3006X	
		E3006E	
Bio-Rad	Reliance One-Step Multiplex RT-qPCR Supermix	12010176	$1.84–$2.11
		12010220	
		12010221	
ThermoFisher Scientific	TaqPath 1-Step RT-qPCR Master Mix, GC	A15299	$1.94–$2.06
		A15300	
Controls			
Twist Bioscience	synthetic SARS-CoV-2 RNA control 2	102024	<$0.01

The price per sample is calculated based on prices listed on the vendor websites and does not include additional costs for general laboratory consumables (e.g., pipette tips) or required equipment and instruments (e.g., pipette and qRT-PCR instruments). Total minimum reagent cost per sample is $1.21–$4.39

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equivalent performance between parallel testing of saliva specimens with new and previously validated components.[36] The FDA recommends testing 2- to 3-fold serial dilutions of SARS-CoV-2-spiked saliva specimens in a pooled negative saliva matrix in triplicate until a hit rate of <100% is reached. Both tests can be considered to have equivalent performance if the resultant limit of detection is the same (e.g., ≤2–3 times the limit of detection) as the unmodified authorized test. Thus, our SalivaDirect EUA can continue to be modified to fill future needs.

Sensitivity and cost comparison to other authorized tests

In the rush to develop and authorize SARS-CoV-2 diagnostic tests,[37] sparse and unstandardized data made it difficult to compare the performance. This led to several independent evaluations of SARS-CoV-2 diagnostic tests or test components,[29,38–43] though these were not always comprehensive enough to help clinicians or public health officials to determine which to use in the sea of growing options. To better establish true performance and directly compare among assays, the FDA developed reference material to establish an absolute limit of detection for each assay.[37] We participated in this post-authorization validation using our least sensitive combination of reagents and equipment: ThermoFisher proteinase K, ThermoFisher TaqPath RT-qPCR Master Mix, and the Bio-Rad CFX96 thermocycler (detailed in our EUA summary[21]). Our measured limit of detection with the FDA reference material was 18 detectable units/μL (18,000 units/mL), similar to what we measured in this study (12 copies/μL). Compared to other FDA EUA authorized SARS-CoV-2 PCR tests, SalivaDirect has a limit of detection similar to or better than most manufactured PCR assays for swabs with RNA extraction (range = 540–540,000 units/mL), including the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel (18,000 units/mL).[44] Furthermore, SalivaDirect is one of only seven PCR tests that completed the reference panel for saliva, and based on the detection limit ranges (600–180,000 units/mL), SalivaDirect has intermediate overall performance and is approximately three times more sensitive than the Fluidigm Advanta Dx SARS-CoV-2 RT-PCR Assay (54,000 units/mL).[44] Finally, as expected for a PCR assay, the limit of detection for SalivaDirect is substantially better than antigen based tests, including the Abbott ID NOW COVID-19 test (300,000 units/mL) and the Quidel Lyra Direct SARS-CoV-2 Assay (540,000 units/mL).[44] Thus the analytical sensitivity of SARS-CoV-2 detection for SalivaDirect is similar to many other PCR tests with RNA extraction using swabs or saliva, and it is more sensitive than the rapid antigen tests.

One of our motivating factors behind developing SalivaDirect was to help reduce the costs of PCR testing. Most manufacturers and laboratories do not make their SARS-CoV-2 testing prices easily available to the public. However, those that do show that the cost to the individual being tested is typically between $100 and $250, sometimes with additional expenses for sample collection and/or shipping.[45–49] While these prices may be justifiable for diagnostic testing covered by health insurance, they are not conducive for large-scale screening. By removing the need for swabs, expensive saliva collection devices,[20] RNA extraction reagents, and kits manufactured specifically for SARS-CoV-2, we reduced the raw supply costs to $1.21–4.39 per sample. Even with that, some have argued that the labor of processing saliva samples ends up making SalivaDirect more expensive than swab-based tests.[50] In reality, most of our more than 50 designated laboratories are offering SalivaDirect to the public for $25 or less, citing the inexpensive reagents and simplified workflow as the driving forces for the reduced costs. While these prices are similar to isothermal methods (e.g., LAMP assays),[51] SalivaDirect will be more expensive than some of the less sensitive rapid antigen tests, such as the Abbott BinaxNOW being offered at a subsidized cost of $5 per test.[52] Our hope is that other

government subsidies and/or pooling approaches will further reduce the costs associated with testing saliva for SARS-CoV-2 by PCR to aid large-scale screening programs.[53,54]

Target populations and testing approaches

There is an increasing debate over the appropriate uses of high-sensitivity diagnostic tests (primarily PCR) and rapid lower sensitivity screening tests (primarily antigen).[55] This revolves around the need to shift from clinical diagnostics, in which the demand has mostly been met, to population-level routine screening for safe reopenings.[56] There is a reason why most schools, for example, do not have testing programs in place, despite the obvious need for them: most available local testing options are from low-capacity clinical labs offering primarily expensive swab-based tests, sometimes with long (>48 hour) turnaround times. Until rapid and inexpensive tests become widely available, there is a critical need to increase the capacities of existing labs and expand access to saliva-based tests.

To date, there are only a few SARS-CoV-2 laboratory diagnostic tests authorized by the FDA for asymptomatic screening.[57–60] While we validated SalivaDirect using our hospital cohort and our EUA is in the category of a high-sensitivity diagnostic test for suspected COVID-19 cases, the simplification, reduced costs, and flexibility of the platform was designed to facilitate routine screening of mostly healthy populations. Through our partnerships with the NBA and NBPA, we conducted the largest evaluation to date of saliva to swabs (AN/OP combined) for screening of healthy individuals. Importantly, the saliva collection for this study was observed by individuals without healthcare training, and still 99.7% of the samples were valid for PCR testing (i.e., samples could be pipetted and human control values were acceptable), showcasing how saliva could be used in non-healthcare settings. We will use this study to apply for an EUA for asymptomatic screening, and we are already working with organizations to use SalivaDirect in school settings.

Safety precautions for saliva collection and testing

Like with all clinical samples, we strongly recommend that anyone handling saliva for SARS-CoV-2 testing to follow "universal precautions": treat all samples as infectious, wear proper personal protective equipment, and decontaminate any spills or exposed areas. Additional precautions should be taken during saliva collection where the individual may contaminate the sample collection tube through their breath or by directly spilling saliva on the outside surfaces. Because of this, we outline in our protocol (which was reviewed by the FDA) to disinfect the outside of the tube with 70% ethanol and for the clinical and laboratory staff to always wear gloves when handling the tubes. We do not believe that our method of collecting saliva without preservatives, as some other methods use,[32,61–63] makes the samples any more hazardous to handle. First, anything added to the inside of the tubes would not help with saliva spilled on the outside. Second, preservatives were included in some tests to stabilize the genetic material, and to our knowledge, none of the commercial preservatives have been evaluated for inactivating SARS-CoV-2. Finally, SARS-CoV-2 RNA is still considered as a biosafety level 2 risk, and thus, even inactivated samples should be handled with caution. Thus, as similar to nasal swabs, collecting saliva contains risks to the collector and laboratory staff that can be minimized through following proper safety protocols.

Limitations of study

Our intended use of SalivaDirect is for the clear and liquid saliva that naturally pools in the mouth. The protocol as currently written is not intended for use with

individuals who are unable to produce "true" saliva, whether this is due to illness or other reasons. While our previous analysis indicates that saliva is more sensitive for SARS-CoV-2 detection than nasopharyngeal swabs in COVID-19 inpatients,[10] saliva can contain blood, mucus, or foreign substances (e.g., food, drink, or tobacco), which can interfere with PCR or make it difficult to pipet.[33] We can overcome many of these issues by having specific collection procedures for saliva collection and using proteinase K to make samples easier to pipet, but some samples can still be invalid if they are not collected properly. While we show that invalid samples are rare from our NBA cohort, our study was biased toward adult men. Thus we were not able to test our approach in two key demographics: elderly individuals (>65 years of age) who are the most at risk for disease and school-aged children (<18 years of age) who critically need testing to help support in-person learning, though others have recently demonstrated that saliva is a suitable specimen for pediatric testing.[64] Moreover, including professional athletes in our trial of repeat SARS-CoV-2 screening may not be representative of the general population, and we will therefore continue to monitor any laboratories using SalivaDirect for asymptomatic screening.

Conclusions

We designed SalivaDirect to be a simplified approach for sample collection and PCR testing for SARS-CoV-2. By using many different vendors, not seeking commercialization, and making the protocol completely open, our goal is to make SalivaDirect as accessible as possible. We encourage other groups to work with us or make their own adjustments to fit their specific needs. In particular, development and validation of automated liquid handling platforms could significantly enhance testing throughput, or replacing PCR with isothermal amplification techniques could make SalivaDirect more accessible for lower complexity laboratory settings. Furthermore, our ability to designate laboratories to use SalivaDirect under our EUA application provides a direct pathway for organizations looking to use noninvasive sampling coupled with a simplified molecular testing scheme without having to submit their own application to the FDA. Thus, SalivaDirect is not only a unique assay but also a unique way to approach testing during a pandemic.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Ethics
  - Sample size and replication
- METHOD DETAILS
  - Clinical specimens
  - SalivaDirect protocol
  - Limit of detection
  - Stability
  - Cross-reactivity
  - Clinical validation
  - Asymptomatic testing
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

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CONSORTIA

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AUTHOR CONTRIBUTIONS

C.B.F.V., D.E.B., A.L.W., and N.D.G. designed the laboratory experiments. C.B.F.V., A.E.W., C.A.H., D.E.B., J.W., C.C.K., J.R.F., and I.M.O. performed the experiments. J.S., E.K., P.L., A.V., M.T., A.J.M., M.C.M., A.C.-M., J.F., S.B., M.C., R.D., and A.N. provided clinical samples. C.S.D.C., A.I.K., A.I., J.M., S.F.F., and R.S. are members of the Yale IMPACT Research Team. J.S., C.C., H.M.K., J.M., S.F.F., R.S., A.L.W., and N.D.G. designed the NBA study. C.B.F.V., A.E.W., C.A.H., J.W., and J.R.F. analyzed the data. C.S.D.C., A.I.K., A.I., J.M., P.H., C.L., S.F.F., R.S., A.L.W., and N.D.G. supervised the project. C.B.F.V., A.E.W., C.A.H., D.E.B., A.L.W., and N.D.G. wrote and edited the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
Clinical samples	Yale New Haven Health	N/A
Clinical samples	National Basketball Association	N/A
Critical Commercial Assays		
Proteinase K	American Bio	AB00925-00100
MagMAX Viral/Pathogen Proteinase K	ThermoFisher Scientific	A42363
Proteinase K, Molecular Biology Grade	New England Biolabs	P8107S
Luna Universal Probe One-Step RT-qPCR Kit	New England Biolabs	E3006S; E2006L; E3006X; E3006E
Reliance One-Step Multiplex RT-qPCR Supermix	Bio-Rad	12010176; 12010220; 12010221
TaqPath 1-Step RT-qPCR Master Mix, GC	ThermoFisher Scientific	A15299; A15300
Synthetic SARS-CoV-2 RNA Control 2	Twist Bioscience	102024
Deposited Data		
Raw and analyzed data	This paper; and GitHub	Table S1; Data S1; https://github.com/grubaughlab/paper_2021_salivadirect
Oligonucleotides		
Forward primer SARS-CoV-2 Nucleocapsid 1: N1-F: GACCCCAAAATCAGCGAAAT	Eurofins; IDT; LGC Biosearch Technologies	See Table 3
Reverse primer SARS-CoV-2 Nucleocapsid 1: N1-R: TCTGGTTACTGCCAGTTGAATCTG	Eurofins; IDT; LGC Biosearch Technologies	See Table 3
Probe SARS-CoV-2 Nucleocapsid 1: N1-P: FAM-ACCCCGCATTACGTTTGGTGGACC-IBFQ	Eurofins; IDT; LGC Biosearch Technologies	See Table 3
Forward primer human RNase P: RP-F: AGATTTGGACCTGCGAGCG	Eurofins; IDT; LGC Biosearch Technologies	See Table 3
Reverse primer human RNase P: RP-R: GAGCGGCTGTCTCCACAAGT	Eurofins; IDT; LGC Biosearch Technologies	See Table 3
Probe human RNase P: Cy5-TTCTGACCTGAAGGCTCTGCGCG-IBRQ	Eurofins; IDT; LGC Biosearch Technologies	See Table 3
Forward primer SARS-CoV-2 Nucleocapsid 2: N2-F: TTACAAACATTGGCCGCAAA	IDT	https://www.idtdna.com/pages
Reverse primer SARS-CoV-2 Nucleocapsid 2: N2-R: GCGCGACATTCCGAAGAA	IDT	https://www.idtdna.com/pages
Probe SARS-CoV-2 Nucleocapsid 2: N2-P: HEX-ACAATTTGCCCCCAGCGCTTCAG-IBFQ	IDT	https://www.idtdna.com/pages
Forward primer SARS-CoV-2 Envelope: E-F: ACAGGTACGTTAATAGTTAATAGCGT	IDT	https://www.idtdna.com/pages
Reverse primer SARS-CoV-2 Envelope: E-R: ATATTGCAGCAGTACGCACACA	IDT	https://www.idtdna.com/pages
Probe SARS-CoV-2 Envelope: E-P: HEX-ACACTAGCCATCCTTACTGCGCTTCG-IBFQ	IDT	https://www.idtdna.com/pages
Forward primer SARS-CoV-2 ORF1: ORF1-F: TGGGGYTTTACRGGTAACCT	IDT	https://www.idtdna.com/pages
Reverse primer SARS-CoV-2 ORF1: ORF1-R: AACRCGCTTAACAAAGCACTC	IDT	https://www.idtdna.com/pages
Probe SARS-CoV-2 ORF1: ORF1-P: HEX-TAGTTGTGATGCWATCATGACTAG-IBFQ	IDT	https://www.idtdna.com/pages
Software and Algorithms		
GraphPad Prism 8.3.0	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
Bio-Rad CFX Maestro 1.1 V4.1.2435.1219	Bio-Rad	https://www.bio-rad.com/en-us/category/qpcr-analysis-software?ID=42a6560b-3ad7-43e9-bb8d-6027371de67a

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
ABI 7500 Software v2.3	ThermoFisher Scientific	https://www.thermofisher.com/us/en/home/technical-resources/software-downloads/applied-biosystems-7500-real-time-pcr-system.html
ABI 7500 Fast System SDS software v1.4.1	ThermoFisher Scientific	https://www.thermofisher.com/us/en/home/technical-resources/software-downloads/applied-biosystems-7500-real-time-pcr-system.html
Other		
SalivaDirect: RNA extraction-free SARS-CoV-2 diagnostics V.5	Protocols.io	https://www.protocols.io/view/salivadirect-rna-extraction-free-sars-cov-2-diagno-bkjgkujw
CFX96 Touch Real-Time PCR Detection System	Bio-Rad	https://www.bio-rad.com/en-us/product/cfx96-touch-deep-well-real-time-pcr-detection-system?ID=LZJTUJ15
Applied Biosystems 7500 Fast Real-Time PCR System	ThermoFisher Scientific	https://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr-instruments/7500-fast-real-time-pcr-system.html
Applied Biosystems 7500 Fast Dx Real-Time PCR System	ThermoFisher Scientific	https://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr-instruments/7500-fast-real-time-pcr-system.html

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Nathan Grubaugh (nathan.grubaugh@yale.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Additional Supplementary Items are available from GitHub at https://github.com/grubaughlab/paper_2021_salivadirect.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethics

The collection of clinical samples from COVID-19 inpatients and healthcare workers at the Yale-New Haven Hospital was approved by the Institutional Review Board of the Yale Human Research Protection Program (Protocol ID. 2000027690). Informed consent was obtained from all patients and healthcare workers prior to sample collection. We used deidentified saliva specimens collected pre-COVID-19 to test for possible cross-reactivity of SalivaDirect. The collection of these saliva specimens was approved by the Institutional Review Board of the Yale Human Research Protection Program (Protocol ID. 0409027018). The collection of deidentified specimens from healthy or asymptomatic individuals from the NBA was approved by the Institutional Review Board of the Yale Human Research Protection Program (Protocol ID. 2000028394). Study participants were informed in writing about the purpose and procedure of the study, and consented to study participation through the act of providing the saliva sample; the requirement for written informed consent was waived by the Institutional Review Board.

Sample size and replication

We followed guidelines from the U.S. FDA to determine sample size and replication for each of the components of the performance evaluation. To determine the lower limit of detection, we initially tested each concentration in triplicate, and confirmed the lowest concentration for which all 3 replicates were detected with an additional 20 replicates for confirmation. For the clinical evaluation, the FDA recommends to test a minimum of 30 paired nasopharyngeal and saliva specimens to compare SalivaDirect to a test that previously received Emergency Use Authorization. For the asymptomatic validation we tested all samples we received from the NBA, and we will continue testing to meet the requirements of the FDA for a minimum of 20 positive specimens.

METHOD DETAILS

Clinical specimens

Clinical samples were collected from COVID-19 diagnosed patients and healthcare workers at the Yale-New Haven Hospital as described earlier.[10,29] Briefly, nasopharyngeal swabs were collected in viral transport medium, and saliva was collected in containers without the addition of stabilizing reagents. All specimens were aliquoted upon arrival in the laboratory, with nucleic acid extracted from one aliquot,[35] tested using a modified CDC RT-qPCR assay,[29] and the remainder stored at −80°C. We modified the CDC assay by using the 2019-nCoV_N1 (N1), 2019-nCoV-N2 (N2), and human RNase P (RP) primer-probe sets (500 nM of forward and reverse primer and 250 nM of probe per reaction; Integrated DNA Technologies, Coralville, IA, US) with the Luna Universal Probe One-Step RT-qPCR Kit (New England Biolabs, Ipswich, MA, US). Thermocycler conditions were reverse transcription for 10 minutes at 55°C, initial denaturation for 1 min at 95°C, followed by 45 cycles of 10 s at 95°C and 30 s at 55°C on the CFX96 qPCR machine (Bio-Rad, Hercules, CA, US).

SalivaDirect protocol

A detailed step-by-step SalivaDirect protocol has been published.[34] SalivaDirect has been validated with proteinase K (American Bio, ThermoFisher Scientific, New England Biolabs) and RT-qPCR kits (New England Biolabs, Bio-Rad, and ThermoFisher Scientific) from three vendors, as well as three RT-qPCR instruments (Table 3; Key Resources Table). At least 500 μL of saliva that naturally pools in the mouth was collected in tubes without preservatives. The sample provider was observed by a trained healthcare professional, and was instructed not to eat, drink, smoke, or conduct dental hygiene for 30 minutes prior to the saliva collection. Saliva collection tubes were decontaminated with 70% ethanol or a disinfecting wipe. Saliva specimens were then transferred to the laboratory for testing. Processing of saliva specimens which could potentially be positive for SARS-CoV-2 should be conducted in BSL2+ settings. A total of 2.5 μL (50 mg/mL) or 6.5 μL (20 mg/mL) of Proteinase K was added to 50 μL of saliva in 8-strip tubes. The tubes were placed in a rack and vortexed for 1 minute at 3200 RPM. Samples were heated for 5 minutes at 95°C on a thermocycler, and then 5 μL of processed saliva was used as input for the dualplex RT-qPCR assay. The dualplex RT-qPCR assay includes the 2019-nCoV_N1 (N1) primer-probe set that targets the nucleocapsid (N1-F: GACCCCAAAATCAGCGAAAT, N1-R: TCTGGTTACTGCCAGTTGAATCTG, N1-P: FAM-ACCCCGCATTACGTTTGGTGGACC-IBFQ) and the human RNase P control (RP) primer-probe set (RP-F: AGATTTGGACCTGCGAGCG, RP-R: GAGCGGCTGTCTCCACAAGT, RP-P: Cy5-TTCTGACCTGAAGGCTCTGCGCG-IBRQ) developed by the CDC (Integrated DNA Technologies, Coralville, IA, US), which are highly specific for SARS-CoV-2 detection (Figure S4). The fluorophore on the human RNase P probe was modified to combine both primer-probe sets in a dualplex assay, reducing the

number of tests to a single assay. Additional fluorophores on the human RNase P probe have been validated and include ATTO647 and Quasar670. Each of these fluorophores are detected by the Cy5 channel and have equal performance (Table 3). We will continue to monitor the performance of our primer-probe sets and make adjustments if mismatches are identified with newly emerging SARS-CoV genotypes.

In the initial development, we included N2 (Fwd: TTACAAACATTGGCCGCAAA, Rev: GCGCGACATTCCGAAGAA, Probe: HEX-ACAATTTGCCCCCAGCGCTT CAG-IBFQ),[30] E (Fwd: ACAGGTACGTTAATAGTTAATAGCGT, Rev: ATATTGCAG CAGTACGCACACA, Probe: HEX-ACACTAGCCATCCTTACTGCGCTTCG-IBFQ),[65] or ORF1 (Fwd: TGGGGYTTTACRGGTAACCT, Rev: AACRCGCTTAACAAAG CACTC, Probe: HEX-TAGTTGTGATGCWATCATGACTAG-IBFQ)[66] as a second virus target with HEX-fluorophore. However, this second virus target was removed from the final assay, because unlike the promising results with extracted nucleic acid,[31] we were not able to consistently detect SARS-CoV-2 in saliva treated with proteinase K and heat (Table S1). Thus, the final SalivaDirect dualplex RT-qPCR assay consisted of the N1 and RP primer-probe sets.

The RT-qPCR master mix was prepared following the vendor's recommended instructions, with 400 nM of N1 forward and reverse primer, 200 nM of N1 probe, 150 nM of RP forward and reverse primer, and 200 nM of RP probe per reaction. Thermocycler conditions were unified for all three RT-qPCR kits (universal protocol) with 10 minutes at 52°C, 2 minutes at 95°C, and 45 cycles of 10 s at 95°C and 30 s at 55°C. Specimens were considered positive if N1 Ct $<$ 40 (or $<$ 37 on the ABI 7500 Fast Dx) and any value for RP, negative if N1 Ct $\geq$ 40 (or $\geq$ 37 on the ABI 7500 Fast Dx) and RP $<$ 35, and invalid if N1 Ct $\geq$ 40 and RP $\geq$ 35. Thus, RP is considered as a sample quality control and invalid samples should be retested on a new aliquot of saliva re-run through the entire SalivaDirect protocol.

Limit of detection

We spiked a positive saliva specimen from a confirmed COVID-19 patient with a known virus concentration (3.7×10^4 copies/μL) into saliva collected from 25 healthcare workers who tested negative for SARS-CoV-2 using the modified CDC assay.[29] We tested a 2-fold dilution series of 400, 200, 100, 50, 25, 12, and 6 SARS-CoV-2 copies/μL in triplicate to determine the preliminary limit of detections, and confirmed the final limit of detection with 20 additional replicates. We used this approach to determine the lower limit of detection of different proteinases K, RT-qPCR kits, and RT-qPCR instruments from multiple vendors (Key Resources Table), by using the same input volumes, matrices and RT-qPCR programs for each combination of reagents and instruments. We found no differences in the limit of detection between proteinase K from three vendors and therefore selected one (ThermoFisher Scientific MagMAX proteinase K) to validate the three RT-qPCR kits. The RT-qPCR kit (ThermoFisher TaqPath) with the weakest limit of detection was then used to validate additional RT-qPCR instruments.

Stability

We determined the stability of SARS-CoV-2 RNA detection in spiked-in saliva samples (positive saliva spiked into a pool of negative saliva from 25 individuals to achieve concentrations of 12, 25, and 50 copies/μL; as prepared for the limit of detection experiment) by placing them for 7 days at 4°C, room temperature (RT, ~19°C), or 30°C. In addition, we tested stability at 40°C for 72 hours, and under summer and winter profiles - conditions which could be encountered during sample

transport. The summer profile consisted of 40°C for 8 hours, room temperature for 4 hours, 40°C for 2 hours, 28°C for 36 hours, and 40°C for 6 hours. The winter profile consisted of −20°C for 8 hours, room temperature for 4 hours, −20°C for 2 hours, 4°C for 36 hours, and −20°C for 6 hours. Results were compared to results obtained in the limit of detection experiment (fresh). Saliva specimens were tested in triplicate and were treated with ThermoFisher Scientific proteinase K and tested with the ThermoFisher TaqPath RT-qPCR kit on the Bio-Rad CFX96.

Cross-reactivity

We tested 52 saliva specimens, collected from adults during the 2018/2019 and 2019/2020 (pre-COVID19) autumn/winter influenza seasons in New Haven, CT to test for possible cross-reactivity of SalivaDirect with other human respiratory pathogens. Saliva specimens were treated with ThermoFisher Scientific proteinase K and tested with the NEB Luna Universal Probe One-Step RT-qPCR kit on the Bio-Rad CFX96.

Clinical validation

Paired nasopharyngeal swabs and saliva specimens (collected maximum 4 days apart) were selected from the Yale IMPACT biorepository. In total 67 paired nasopharyngeal swabs and saliva specimens were tested with the US FDA EUA ThermoFisher Scientific TaqPath COVID-19 combo kit following the vendor's protocol. Briefly, nucleic acid was extracted using the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit on the KingFisher Flex Magnetic Particle Processor. In total 200 μL of specimen was used as input and eluted in 50 μL. For each reaction, 5 μL of extracted nucleic acid was used as input and tested with the ThermoFisher Scientific TaqPath RT-qPCR reaction on the ABI 7500 Fast Dx. Ct values were exported through the 7500 Fast System SDS software v1.4.1. For saliva specimens that were too thick to pipette, 100 μL sample was mixed with 100 μL PBS, and 10 μL was used in the RT-qPCR reaction. For the clinical validation of SalivaDirect, saliva samples were treated with ThermoFisher Scientific proteinase K and tested with the ThermoFisher Scientific TaqPath RT-qPCR kit on the Bio-Rad CFX96.

Asymptomatic testing

Paired AN/OP swabs and saliva specimens (collected maximum 4 days apart) were collected from NBA players, staff, and other vendors. Combined AN/OP swabs were collected by Quest and followed their EUA specimen collection guidelines.[67,68] Saliva was collected, under observation of Drug Free Sport International collectors, who are not trained healthcare workers, using a Salimetrics Saliva Collection Aid (Salimetrics, State College, PA, US) into a 2 mL screw-top tube with O-ring cap (Millipore Sigma, Burlingston, MA, US). Saliva was shipped overnight in NanoCool cooling system boxes to our laboratory at the Yale School of Public Health for testing.

A total of 3,779 paired samples were tested by SalivaDirect for saliva specimens at Yale and with Quest's protocol for swabs, in their labs during the in-market phase of the study while swab testing was completed by BioReference while the teams were in Orlando, FL. Additional swab testing was performed in our lab using the modified CDC multiplex assay protocol for positive swabs sent on for sequencing.[31] In total 10 matched samples, reported positive by the NBA, were tested in-house.

QUANTIFICATION AND STATISTICAL ANALYSIS

We used the Bio-Rad CFX Maestro 1.1 V4.1.2435.1219, ABI 7500 Software v2.3, and ABI 7500 Fast System SDS Software v1.4.1 to analyze and export Ct values.

GraphPad Prism 8.3.0 was used to make the figures and perform all statistical analyses. Kruskal-Wallis tests were used to test for statistical differences in SARS-CoV-2 RNA stability kept at different temperatures and multiple comparisons were corrected with Dunn's test. The Wilcoxon matched pairs test was used to test for statistical differences between paired samples. If a virus target was not detected, the Ct value was set to 45 Ct. In all statistical tests, $p \leq 0.05$ was considered significant.