SalivaDirect: Simple and sensitive molecular diagnostic test for SARS-CoV-2 surveillance

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One sentence summary:

SalivaDirect is an extraction-free, dualplex RT-qPCR laboratory developed test validated with reagents and instruments from multiple vendors and submitted for FDA Emergency Use Authorization.

Abstract

Current bottlenecks for improving accessibility and scalability of SARS-CoV-2 testing include diagnostic assay costs, complexity, and supply chain shortages. To resolve these issues, we developed SalivaDirect. The critical component of our approach is to use saliva instead of respiratory swabs, which enables non-invasive frequent sampling and reduces the need for trained healthcare professionals during collection. Furthermore, we simplified our diagnostic test by (***1***) not requiring nucleic acid preservatives at sample collection, (***2***) replacing nucleic acid extraction with a simple proteinase K and heat treatment step, and (***3***) testing specimens with a dualplex quantitative reverse transcription PCR (RT-qPCR) assay. We validated SalivaDirect with reagents and instruments from multiple vendors to minimize the risk for supply chain issues. Regardless of our tested combination of reagents and instruments from different vendors, we found that SalivaDirect is highly sensitive with a limit of detection of 6-12 SARS-CoV-2 copies/µL. When comparing paired nasopharyngeal swabs and saliva specimens using the authorized ThermoFisher Scientific TaqPath COVID-19 combo kit and our SalivaDirect protocol, we found high agreement in testing outcomes (>94%). Being flexible and inexpensive ($1.29-$4.37/sample), SalivaDirect is a viable and accessible option to help alleviate SARS-CoV-2 testing demands. We submitted SalivaDirect as a laboratory developed test to the US Food and Drug Administration for Emergency Use Authorization on July 14th, 2020, and current details can be found on our website (covidtrackerct.com/about-salivadirect/).

Introduction

SARS-CoV-2, a novel beta-coronavirus, emerged in late 2019 in Wuhan, China, and the subsequent 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 re-opening 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 and achieve the stated goals, 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 turn-around 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 RT-qPCR, LAMP, CRISPR, and sequencing-based assays, simple and inexpensive options are still needed for mass 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 *(7–10)*. 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. Furthermore, because nasopharyngeal sampling requires a swab being inserted into the back of the nares, it can cause irritation that could promote sneezing and coughing. Thus, the non-invasive 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)*.

To address the need for a mass testing workflow, we developed SalivaDirect, a nucleic acid extraction-free, dualplex RT-qPCR method for SARS-CoV-2 detection to reduce the cost, time, and effort of most currently approved RT-qPCR assays. Our approach can be broadly implemented as it does not require saliva collection tubes containing preservatives, and does not require specialized reagents or equipment for nucleic acid extraction. Furthermore, we validated SalivaDirect for use with products from multiple vendors. Thus, the simplicity and flexibility of SalivaDirect means 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 RT-qPCR assays. The unique feature of

SalivaDirect is that it is non-invasive, inexpensive ($1.29-$4.37/sample), simple, and is validated for use with reagents and instruments from multiple vendors. We submitted SalivaDirect as a laboratory developed test (LDT) to the US FDA for Emergency Use Authorization (EUA) on July 14th, 2020. SalivaDirect can help to realize large-scale testing of the general public to facilitate isolation and contact tracing of cases with the ultimate goal of preventing the spread of SARS-CoV-2.

Results

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

To reduce cost, time, and effort for SARS-CoV-2 detection, we developed SalivaDirect (covidtrackerct.com/about-salivadirect/), a saliva-based, nucleic acid extraction-free, dualplex RT-qPCR method. SalivaDirect consists of three steps: (***1***) collecting saliva without preservative buffers, (***2***) proteinase K treatment and heat inactivation, and (***3***) dualplex RT-qPCR virus detection (**Fig. 1a**).

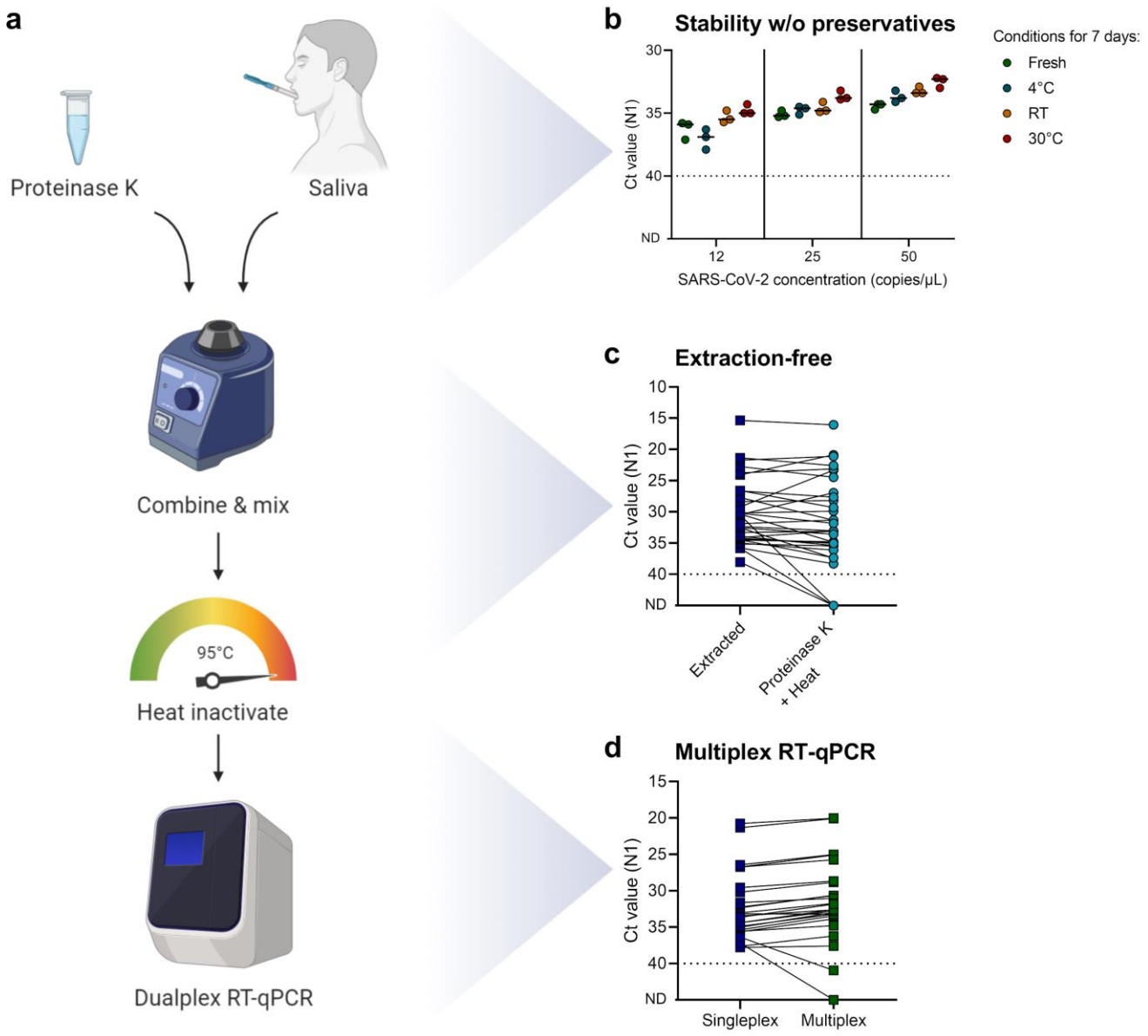

Fig. 1: SalivaDirect is a simplified and sensitive method for SARS-CoV-2 detection. (**a**) Schematic overview of SalivaDirect workflow depicting the main steps of mixing saliva with proteinase K, heat inactivation, and dualplex RT-qPCR testing. Figure created with Biorender.com. (**b**) SARS-CoV-2 is 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 panels b, c, d indicates the limit of detection. Data used to make this figure can be found in **Source Data Fig. 1**.

Several protocols imply that stabilizing buffers (*e.g.* TBE, 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 RT-qPCR *(16, 17)*. 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 addition of preservatives. We quantified the virus copies from a positive saliva specimen and spiked-in different 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 (**Fig. 1b**). Surprisingly, we even detected significantly lower N1 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, $P = 0.03$; **Fig 1b**). In contrast, we found that Ct values for human *RNase P* (RP) were significantly higher after 7 days at RT (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 (**Supplementary Fig. 1**). Thus, our data suggest that SARS-CoV-2 RNA is stable in saliva without preservatives for at least 7 days when stored at temperatures of up to 30°C.

Nucleic acid extraction is included in US FDA EUA-approved PCR diagnostic assays to detect SARS-CoV-2 RNA by RT-qPCR. However, nucleic acid extraction is relatively expensive, time-consuming, and subject to supply chain bottlenecks which limits the scalability of testing which 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 *(17–20)*. 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 CDC assay *(21)* to compare RT-qPCR 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 (**Fig. 1c**). As compared to nucleic acid extraction, our data show that direct testing does decrease detection (median N1 Ct increase = 1.8 Ct; $P < 0.01$). By leaving the nucleic acid extraction step, an increase in Ct values is expected as specimens are typically ~4-fold concentrated 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.

Our final modification to improve the scalability of SARS-CoV-2 diagnostic assays was to increase the high-throughput testing potential of the RT-qPCR step. We previously found that the US CDC primer-probe sets are among the most sensitive and reliable for SARS-CoV-2 detection *(21)*. The CDC assay consists of three separate reactions targeting two regions of the SARS-CoV-2 nucleocapsid (N1 and N2) and a human *RNase P* (RP) control *(22)*. 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 *(23)*. When testing the multiplex RT-qPCR assay on saliva treated with proteinase K and heat, however, we were not able to detect consistent results for the N2 primer-

probe set, nor for the Sarbeco-E (E) or HKU-ORF1 (ORF1) primer-probe sets with HEX fluorophores (**Supplementary Table 1**). By comparing the N1 and N2 primer-probe sets for 613 clinical samples COVID-19 patients and infected healthcare workers, we found that the median N1 Ct values were 1.2 Ct lower as compared to N2 (**Supplementary Fig. 2**), indicating more consistent and significantly stronger detection ($P < 0.001$). Therefore, to further simplify the RT-qPCR assay we developed a dualplex RT-qPCR assay based on N1 and RP, and modified the fluorophore (Cy5 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 ($P < 0.01$; **Fig. 1d**). Thus, SalivaDirect allows for a reduction in the number of RT-qPCR reactions to one reaction per sample.

Lower limit of detection using reagents and equipment from multiple vendors

We determined the lower limit of detection of SalivaDirect using reagents and instruments from multiple vendors to avoid dependence on a single vendor for each step (**Table 1**). A current list of validated products can be found on our website (covidtrackerct.com/salivadirect-results/). 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 (**Fig. 2**). Treating saliva with proteinase K from three different vendors resulted in a limit of detection of 6 SARS-CoV-2 copies/µL, and suggests that SalivaDirect is not dependent on proteinase K from a specific vendor (**Fig. 2a-c**).

Next, we determined the limit of detection by comparing three different RT-qPCR kits obtained from New England Biolabs, Bio-Rad, and ThermoFisher Scientific **(Table 1**). 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 RT-qPCR program, we found no significant differences in Ct values (Luna: $P = 0.69$, Reliance: $P = 0.06$, TaqPath: $P = 0.44$; **Supplementary Fig. 3**). One additional RT-qPCR 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 (**Fig. 2d-f**). Importantly, this indicates that the specific RT-qPCR kit can influence the lower limit of virus detection and not all kits may be suitable for use with SalivaDirect.

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 RT-qPCR thermocycler instruments: Bio-rad CFX96, Applied Biosystems (ABI) 7500 Fast, 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 (**Fig. 2g-i**). 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 US FDA EUA-approved 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.

Table 1: Validated reagents and instruments for use with SalivaDirect.

Item	Vendor	Product Name	Catalog number
Proteinase K	ThermoFisher Scientific	MagMAX Viral/Pathogen Proteinase K	A42363
	New England Biolabs	Proteinase K, Molecular Biology Grade	P8107S
	AmericanBio	Proteinase K	AB00925-00100
RT-qPCR kit	New England Biolabs	Luna Universal Probe One-Step RT-qPCR Kit	E3006E
	Bio-Rad	Reliance One-Step Multiplex RT-qPCR Supermix	12010176
	ThermoFisher Scientific	TaqPath 1-Step RT-qPCR Master Mix, GC	A15299
RT-qPCR instrument	Bio-Rad	CFX96 Touch Real-Time PCR Detection System	
	ThermoFisher Scientific	Applied Biosystems 7500 Fast Real-Time PCR System	
	ThermoFisher Scientific	Applied Biosystems 7500 Fast Dx Real-Time PCR System	

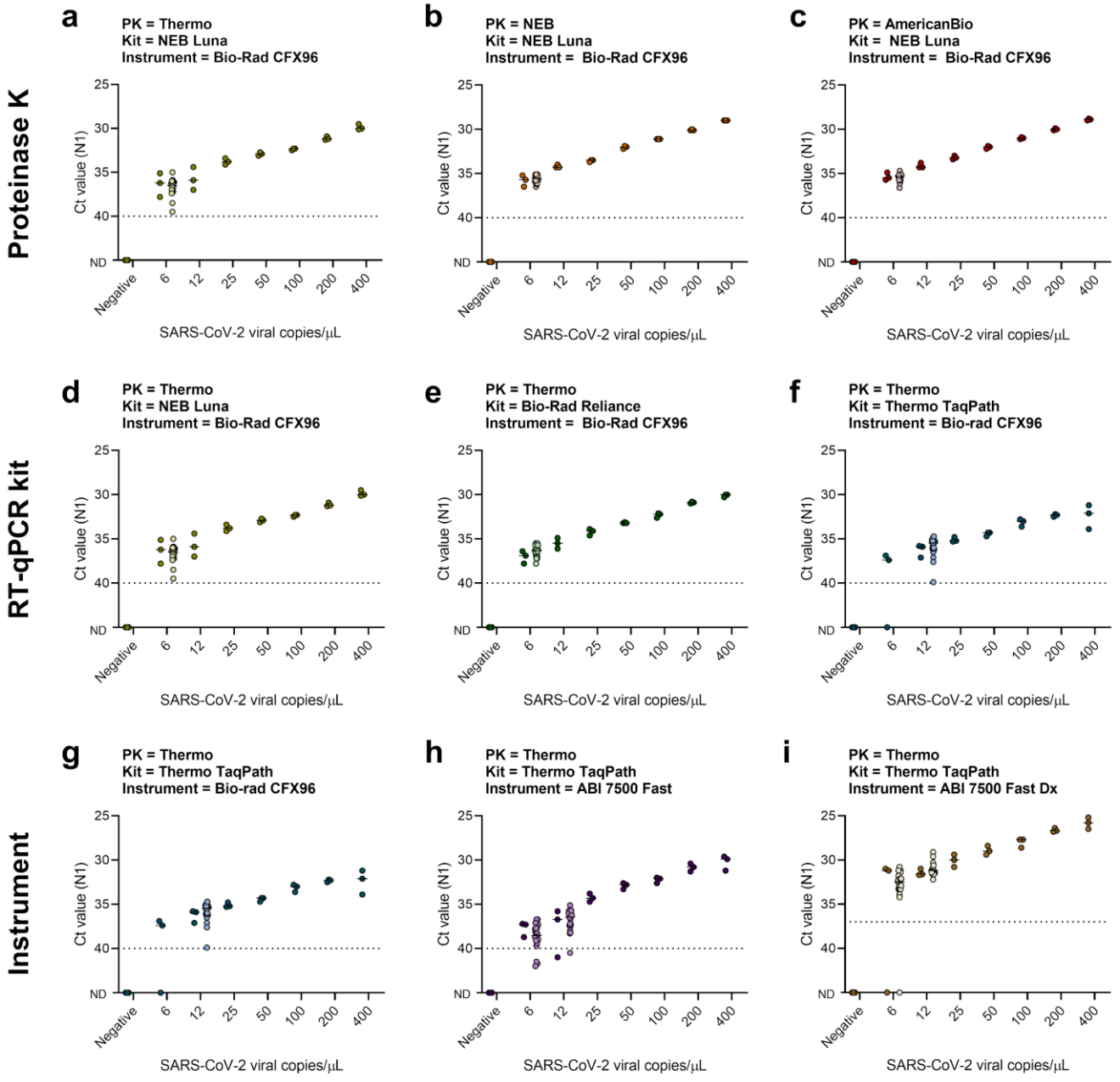

Fig. 2: SalivaDirect has a lower limit of detection of 6-12 SARS-CoV-2 copies/µL when tested with reagents and instruments from multiple vendors. We determined the lower limit of detection of SalivaDirect with a two-fold dilution series (400, 200, 100, 50, 25, 12, and 6 copies/µL) of positive saliva 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**) RT-qPCR instruments from different vendors, while keeping the other conditions constant. Panels 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 **Source Data Fig. 2**.

Sensitivity of SalivaDirect compared to saliva tested using the modified CDC RT-qPCR assay

After determining the lower limit of detection of SalivaDirect, we compared Ct values for N1 of saliva specimens tested with the modified CDC assay (*e.g.* nucleic acid extraction and singleplex RT-qPCR) *(21)* with SalivaDirect. Median N1 Ct values were 1.2 higher (*e.g.* weaker detection) for SalivaDirect as compared to the modified CDC assay ($P < 0.001$; **Fig. 3**). Overall, the reduction in analytical sensitivity led to three out of 41 (7.3% false negative) tested saliva specimens which were not detected by SalivaDirect. All three specimens that tested negative had N1 Ct values of 35-40 when using the modified CDC assay. Our findings show ~93% positive agreement of SalivaDirect compared to the modified CDC assay.

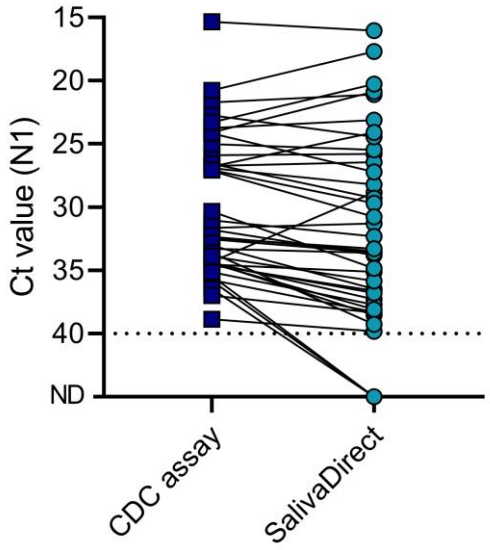

Fig. 3: SalivaDirect is comparable to the modified CDC RT-qPCR assay for SARS-CoV-2 detection. We compared Ct values for N1 between the modified CDC assay (nucleic acid extraction and singleplex RT-qPCR) and SalivaDirect for 41 saliva specimens tested with both methods. Overall, detection of SARS-CoV-2 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 **Source Data Fig. 3**.

Clinical validation with paired nasopharyngeal swabs and saliva

We compared the performance of SalivaDirect to the US FDA EUA-approved ThermoFisher Scientific TaqPath COVID-19 combo kit by testing 37 paired positive and 30 paired negative nasopharyngeal swabs and saliva specimens (**Fig. 4, Tables 2-3**). Nasopharyngeal swabs and saliva were collected

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 RT-PCR diagnostic assay targeting 3 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 83.8% (**Fig. 4a**). For both sample types there were 3 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 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 2**). The N1 Ct values were higher using SalivaDirect (median difference of 3.3 Ct; $P < 0.01$; **Fig. 4b**), and the increased Ct values are likely due to a combination of removing the nucleic acid step (**Fig. 1c, Fig. 3**) and using different thermocycler instruments (**Fig. 2**). Out of the 37 nasopharyngeal swabs that were tested with the TaqPath COVID-19 combo kit, three specimens tested negative (**Table 2** and **Fig. 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 3**). Ct values for N1 were higher when comparing SalivaDirect with the TaqPath COVID-19 combo kit (median difference of 5.0 Ct, $P < 0.001$; **Fig. 4c**) for likely reasons as 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 (**Fig. 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.

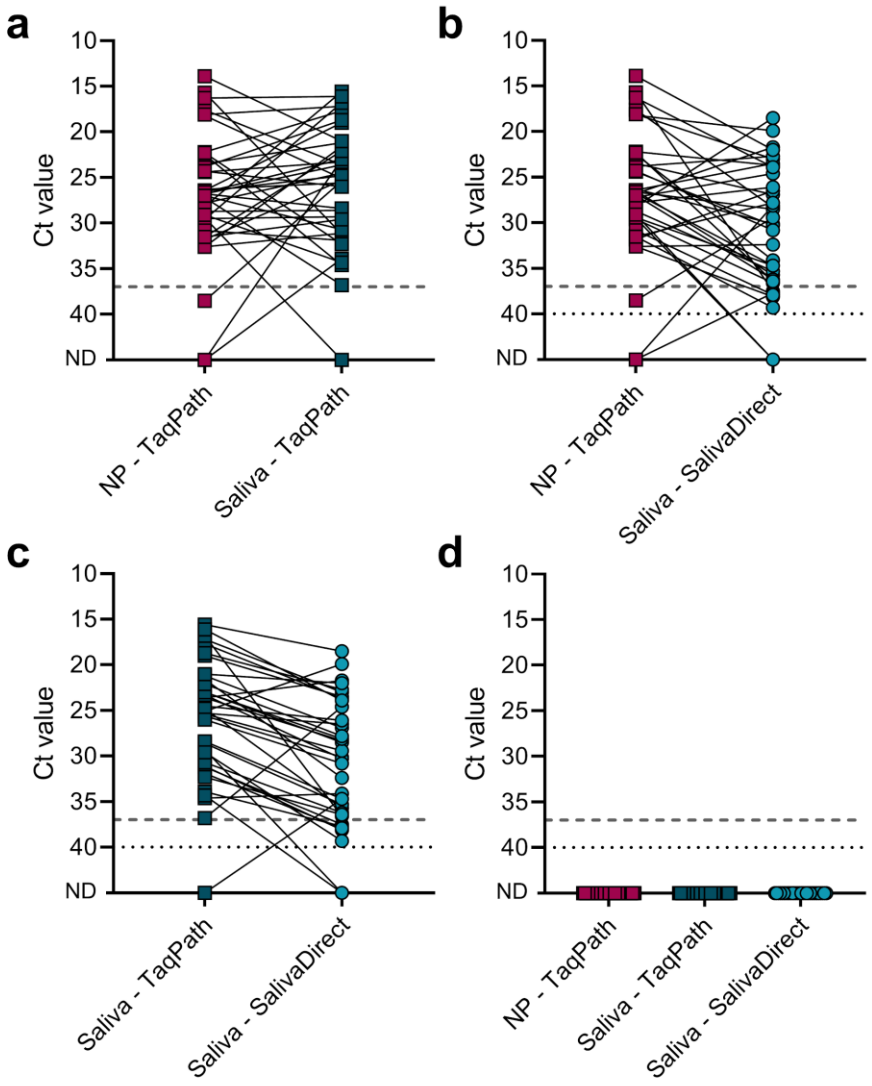

Fig. 4: Clinical evaluation of SalivaDirect in comparison to the US FDA EUA-approved ThermoFisher Scientific TaqPath COVID-19 combo kit with paired nasopharyngeal and saliva specimens indicates high agreement between both methods. We selected 37 paired positive and 30 paired negative nasopharyngeal swabs 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 **Source Data Fig. 4**.

Table 2: Qualitative outcome of parallel testing of paired nasopharyngeal swabs and saliva with SalivaDirect and the ThermoFisher Scientific TaqPath COVID-19 combo kit. Three nasopharyngeal swabs tested negative while previous outcomes of the modified CDC assay indicated that they were weakly positive.

		TaqPath COVID-19 Nasopharyngeal swab	
		Positive	Negative
SalivaDirect Saliva	Positive	32	**3***
	Negative	2	30
Total		34	33
Positive agreement = 94.1% (32/34) Negative agreement = 90.9% (30/33)			

Table 3: Qualitative outcome of parallel testing of saliva with SalivaDirect and the ThermoFisher Scientific TaqPath COVID-19 combo kit.

		TaqPath COVID-19 Saliva			
		Positive	Inconclusive	Invalid	Negative
SalivaDirect Saliva	Positive	33		2	
	Negative	1	1		30
Total		34	1	2	30
Positive agreement = 97.1% (33/34) Negative agreement = 100% (30/30)					

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 *(22)*. 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 *(21)*. To test for possible cross-reactivity of the dualplex RT-qPCR assay, we tested 52 saliva specimens collected from adults in the 2018/2019 and 2019/2020 fall and winter (pre-COVID-19; **Supplemental Fig. 4**). We did not detect off-target amplification or false positives, which is in agreement with previous findings from the CDC.

Supply costs for SalivaDirect testing

We aimed to develop a simple and quick diagnostic 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 $1.29, if saliva is collected without a saliva collection aid, and a maximum of $4.37 when using a saliva collection aid (**Table 4**). 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 approved EUA. Thus, SalivaDirect provides a relatively inexpensive alternative to current RT-qPCR-based assays.

Table 4: SalivaDirect is a flexible and relatively inexpensive method for SARS-CoV-2 diagnostic testing. The price per sample is calculated based on prices listed on the vendor websites and does not include additional costs for general laboratory consumables such as pipet tips or required equipment and instruments such as pipette and RT-qPCR instruments.

Vendor	Item	Catalog number	Price/sample
Sample collection			
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			
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
RT-qPCR			
Integrated DNA technologies	nCOV_N1 Forward Primer Aliquot, 100 nmol	10006830	$0.02
	nCOV_N1 Reverse Primer Aliquot, 100 nmol	10006831	$0.02
	nCOV_N1 Probe Aliquot, 50 nmol	10006832	$0.03
	RNase P Forward Primer Aliquot, 100 nmol	10006836	$0.01
	RNase P Reverse Primer Aliquot, 100 nmol	10006837	$0.01
	RP probe (Cy5-IBRQ)		$0.10
New England Biolabs	Luna Universal Probe One-Step RT-qPCR Kit	E3006S E3006L E3006X E3006E	$0.75-$1.08
Bio-Rad	Reliance One-Step Multiplex RT-qPCR Supermix	12010176 12010220	$1.84-$2.11

ThermoFisher Scientific	TaqPath 1-Step RT-qPCR Master Mix, GC	12010221 A15299 A15300	$1.94-$2.06
Total minimum reagent cost per sample			$1.29-$4.37

Discussion

SalivaDirect is a low cost, 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 RT-qPCR 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. Overall, SalivaDirect has a low limit of detection of 6-12 SARS-CoV-2 copies/μL and has a 94% positive agreement with paired nasopharyngeal swabs tested with the FDA EUA-approved ThermoFisher TaqPath COVID-19 combo kit.

Additional reagents and instruments can be added to SalivaDirect by performing FDA EUA bridging studies to establish equivalent performance between parallel testing of saliva specimens with new and previously validated components (https://www.fda.gov/media/135659/download). The FDA recommends testing 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.* ≤3x limit of detection) as the unmodified authorized test. Thus, SalivaDirect provides a simplified testing workflow without the trade-off of decreased sensitivity and with the flexibility of adding additional reagents and equipment.

Target populations and limitations of use

The 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 of hospitalized COVID-19 patients who are unable to produce “true” saliva. While our previous analysis indicates that saliva is more sensitive for SARS-CoV-2 detection than nasopharyngeal swabs in COVID-19 patients *(10)*, saliva from patients can contain blood or mucus, which can interfere with PCR or make it difficult to pipet. These issues can be overcome by diluting with PBS or treating the complete specimen with proteinase K in its collection tube, but these steps are not included in the SalivaDirect EUA application to the FDA. Despite the simplified protocol, which has advantages for testing in low- and middle-income countries, our

method still requires electricity and specialized RT-qPCR instruments which can be a limiting factor when such equipment is not available.

Future improvements and validation

Large-scale testing of a mostly uninfected population will likely require validation with asymptomatic and pre-symptomatic cases to evaluate whether automation and/or pooling is cost-effective. Through our partnerships with the National Basketball Association and the National Basketball Players Association, called SWISH (covidtrackerct.com/swish/), we are conducting a large trial to compare the negative and positive agreements of SalivaDirect to a combined anterior nares/oropharyngeal swab tested by an approved RT-qPCR assay following nucleic acid extraction. In addition, we will use these specimens to investigate pooled testing strategies. To date, there is only one SARS-CoV-2 laboratory diagnostic test FDA EUA-approved for both asymptomatic testing and pooled testing *(24)*, and the data from our ongoing study could be used to approve SalivaDirect for both tests. Furthermore, we are currently working with testing facilities to evaluate liquid handling robots to decrease sample processing time, and will seek FDA bridging studies to validate these processes if successful. Finally, we are initiating preliminary studies to evaluate saliva collection and SARS-CoV-2 detection from infants and children in daycare centers and schools in Connecticut, US. These key improvements and validation steps will help expand access to testing.

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 make their own adjustments to fit their specific needs or to improve capacity. Thus, our broad FDA EUA application provides a basis for organizations looking to use non-invasive sampling coupled with a simplified molecular testing scheme for SARS-CoV-2 surveillance.

Methods

Ethics

Collection of clinical samples from COVID-19 patients and healthcare workers at the Yale-New Haven Hospital was approved by the Institutional Review Board of the Yale Human Research Protection Program (FWA00002571, Protocol ID. 2000027690). Consent was obtained from all patients and healthcare workers. We used deidentified saliva specimens collected pre-COVID-19 to test for possible cross-reactivity of SalivaDirect. Collection of these saliva specimens was approved by the Institutional Review Board of the Yale Human Research Protection Program (Protocol ID. 0409027018).

Clinical specimens

Clinical samples were collected from COVID-19 diagnosed patients and healthcare workers at the Yale-New Haven Hospital as described earlier *(10, 21)*. Briefly, nasopharyngeal swabs were collected

in viral transport medium, and saliva was collected in containers without addition of stabilizing reagents. All specimens were aliquoted upon arrival in the laboratory, with nucleic acid extracted from one aliquot *(25)*, tested using a modified CDC RT-qPCR assay *(21)*, 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 seconds at 95°C and 30 seconds at 55°C on the CFX96 qPCR machine (Bio-Rad, Hercules, CA, US).

SalivaDirect protocol

A detailed SalivaDirect protocol has been published *(26)*. SalivaDirect has been validated with proteinase K and RT-qPCR kits from three vendors, as well as three RT-qPCR instruments (**Table 4**). At least 500 µL of saliva that naturally pools in the mouth was collected in tubes without preservatives. 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. The fluorophore on the human RNase P probe was modified (Cy5 instead of FAM) to combine both primer-probe sets in a dualplex assay, reducing the number of tests to a single assay. In the initial development, we included N2 (Fwd: TTACAAACATTGGCCGCAAA, Rev: GCGCGACATTCCGAAGAA, Probe: HEX-ACAATTTGCCCCCAGCGCTTCAG-IBFQ) *(22)*, E (Fwd: ACAGGTACGTTAATAGTTAATAGCGT, Rev: ATATTGCAGCAGTACGCACACA, Probe: HEX-ACACTAGCCATCCTTACTGCGCTTCG-IBFQ) *(27)*, or ORF1 (Fwd: TGGGGYTTTACRGGTAACCT, Rev: AACRCGCTTAACAAAGCACTC, Probe: HEX-TAGTTGTGATGCWATCATGACTAG-IBFQ) *(28)* 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 *(23)*, we were not able to consistently detect SARS-CoV-2 in saliva treated with proteinase K and heat. 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 seconds at 95°C and 30 seconds 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 ≥40 and RP <35, and invalid if N1 Ct ≥40 and RP ≥35.

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 *(21)*. 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 (**Table 1**), 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 (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. 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-approved 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.

Statistical analysis

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.

Data availability

All data are included in this article, the supplementary files, and the Source Data.

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Competing interests

ALW has received research funding through grants from Pfizer to Yale and has received consulting fees for participation in advisory boards for Pfizer. The other authors declare no competing interests.

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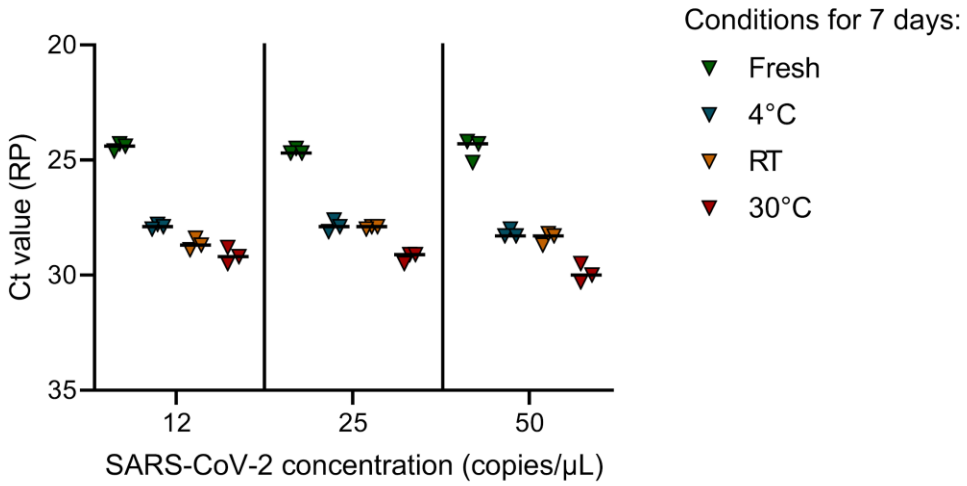
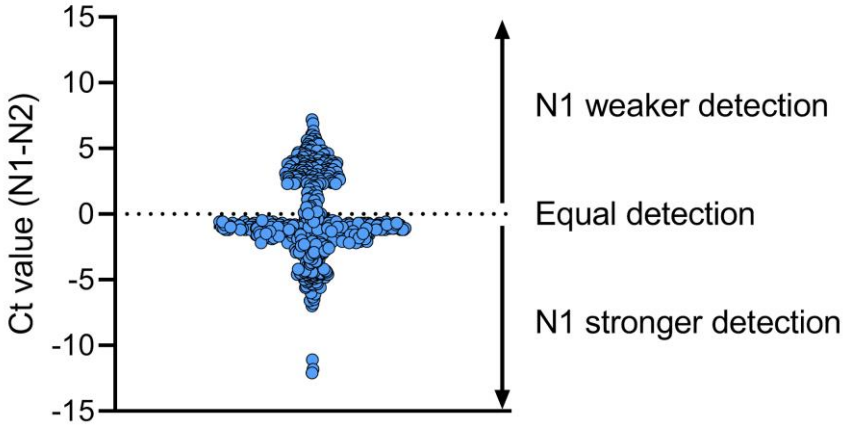
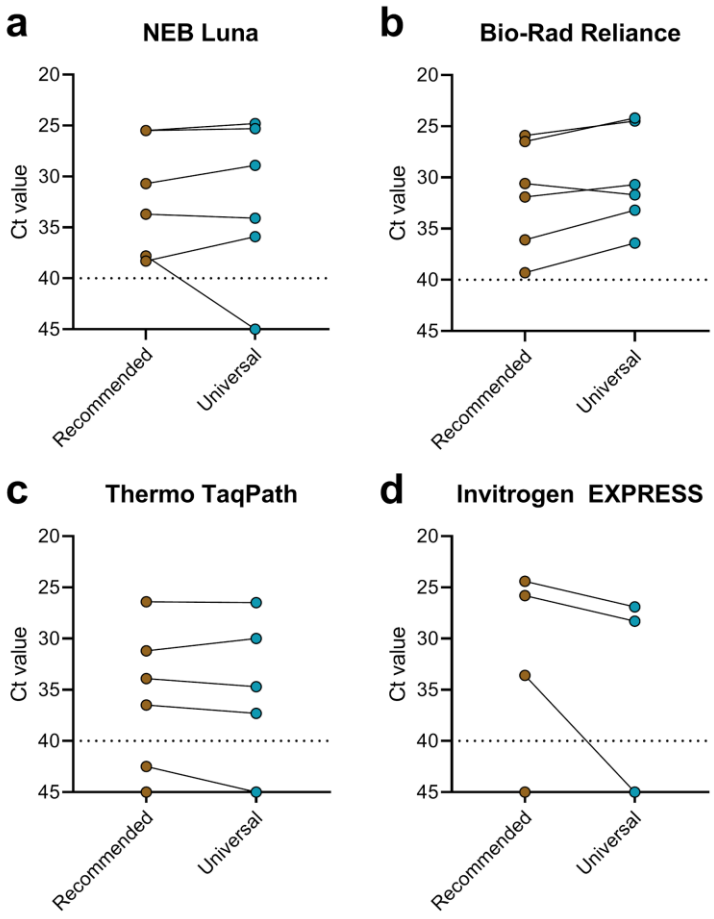
Supplementary figures



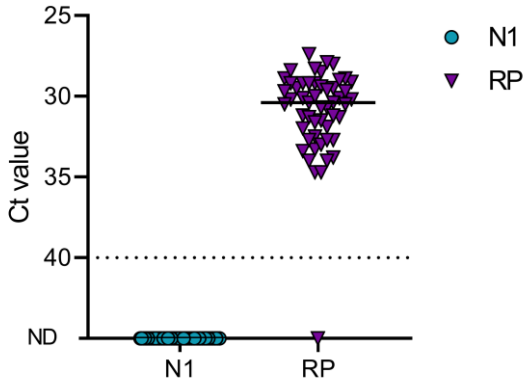
Supplementary Fig. 1: Decreased detection of human RNA in saliva specimens after storage at 4°C, room temperature (~19°C), or 30°C for 7 days. When saliva specimens with 12, 25, and 50 SARS-CoV-2 copies/µL were stored under different temperatures for 7 days, we found a significant increase in Ct values for human RNAse P (RP) at RT (Kruskal-Wallis; $P < 0.01$) and 30°C (Kruskal-Wallis; $P < 0.001$), while SARS-CoV-2 N1 Ct values were significantly decreased after 7 days at 30°C (Kruskal-Wallis, $P = 0.03$). This suggests that SARS-CoV-2 is stable in saliva, whereas human RNA seems to degrade over time. The horizontal bars indicate the median. Data used to make this figure can be found in **Source Data Supplementary Fig. 1**.



Supplementary Fig. 2: The N1 primer-probe set is more reliable than N2 for SARS-CoV-2 detection. We compared Ct values for N1 and N2 primer-probe sets for 613 clinical specimens, and found that overall the N1 primer-probe set detects a stronger signal as compared to N2. Shown is the difference in Ct value between N1 and N2 and the dotted line indicates equal Ct values for N1 and N2. Data used to make this figure can be found in **Source Data Supplementary Fig. 2**.



Supplementary Fig. 3: Comparisons between vendor recommended and SalivaDirect universal thermocycler conditions for four RT-qPCR kits. We selected six positive saliva specimens and tested each sample with four different RT-qPCR kits under recommended and unified thermocycler conditions. The tested kits included the (**a**) NEB Luna Universal Probe One-Step RT-qPCR Kit, (**b**) Bio-Rad Reliance One-Step Multiplex RT-qPCR Supermix, (**c**) TaqPath 1-Step RT-qPCR Master Mix, GC, and (**d**) Invitrogen EXPRESS One-Step SuperScript qRT-PCR kit. Overall, modifying the thermocycler conditions did not affect the Ct values generated with the N1 primer-probe set (Wilcoxon; Luna: P=0.69, Reliance: P=0.06, TaqPath: P=0.44, EXPRESS: P=0.25). One out of the four evaluated RT-qPCR kits (*e.g.* Invitrogen EXPRESS) was not suitable for SARS-CoV-2 detection with SalivaDirect and was therefore not included in further validation. 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 **Source Data Supplementary Fig. 3**.



Supplemental Fig. 4: No background amplification of the SalivaDirect dualplex RT-qPCR assay when testing pre-COVID-19 saliva specimens. Saliva specimens were collected from adults during the 2018-2019 and 2019-2020 autumn/winter influenza seasons and tested with SalivaDirect. Shown are Ct values for N1 and the human RNase P (RP) primer-probe sets. All samples tested negative for N1, indicating no cross-reactivity, while detection of RP indicated proper sample processing. One specimen tested invalid with no detection for both N1 and RP. Shown are the Ct values for the N1 and RP (specimen quality control) primer-probe sets. The horizontal bar indicates the median and the dotted line indicates the limit of detection. Data used to make this figure can be found in **Source Data Supplementary Fig. 4**.

Supplementary tables

Supplementary Table 1: No consistent SARS-CoV-2 detection when testing saliva with a multiplex RT-qPCR assay using a HEX-fluorophore. We compared Ct values between the modified CDC assay with 3 versions of a multiplexed assay with N2 (Fwd: TTACAAACATTGGCCGCAAA, Rev: GCGCGACATTCCGAAGAA, Probe: HEX-ACAATTTGCCCCCAGCGCTTCAG-IBFQ) *(22)*, E (Fwd: ACAGGTACGTTAATAGTTAATAGCGT, Rev: ATATTGCAGCAGTACGCACACA, Probe: HEX-ACACTAGCCATCCTTACTGCGCTTCG-IBFQ) *(27)*, or ORF1 (Fwd: TGGGGYTTTACRGGTAACCT, Rev: AACRCGCTTAACAAAGCACTC, Probe: HEX-TAGTTGTGATGCWATCATGACTAG-IBFQ) *(28)* as a second virus target with HEX-fluorophore. Eight samples were tested in duplicate with the modified CDC assay (singleplex) as well as each multiplex assay, and average Ct values are shown. No consistent detection of SARS-CoV-2 was achieved for N2, E, or ORF1 with the HEX-fluorophore.

	Singleplex			**Multiplex**								
	N1-FAM	N2-FAM	RP-FAM	N1-FAM	N2-HEX	RP-Cy5	N1-FAM	E-HEX	RP-Cy5	N1-FAM	ORF1-HEX	RP-Cy5
1	33.3	34.6	25.3	31.8	ND	21.4	31.9	32.5	21.2	32.0	ND	21.9
2	33.2	34.6	21.5	33.5	ND	19.6	ND	ND	20.3	ND	ND	21.6
3	37.5	37.9	25.9	ND	ND	20.3	ND	ND	20.1	ND	ND	20.1
4	30.2	33.0	18.5	30.4	ND	19.9	29.9	ND	19.2	29.8	ND	19.1
5	30.2	32.1	22.1	33.3	ND	21.6	31.0	ND	19.7	31.8	ND	20.6
6	29.2	30.2	26.9	23.6	26.2	22.2	23.3	23.8	20.8	23.2	ND	20.7
7	35.0	35.1	23.7	40.4	ND	22.9	ND	ND	22.7	35.9	ND	22.9
8	40.0	43.9	19.4	38.1	ND	19.7	ND	ND	19.1	ND	ND	19.1