Med, Volume *2*

Supplemental information

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

Chantal B.F. Vogels, Anne E. Watkins, Christina A. Harden, Doug E. Brackney, Jared Shafer, Jianhui Wang, César Caraballo, Chaney C. Kalinich, Isabel M. Ott, Joseph R. Fauver, Eriko Kudo, Peiwen Lu, Arvind Venkataraman, Maria Tokuyama, Adam J. Moore, M. Catherine Muenker, Arnau Casanovas-Massana, John Fournier, Santos Bermejo, Melissa Campbell, Rupak Datta, Allison Nelson, Yale IMPACT Research Team, Charles S. Dela Cruz, Albert I. Ko, Akiko Iwasaki, Harlan M. Krumholz, J.D. Matheus, Pei Hui, Chen Liu, Shelli F. Farhadian, Robby Sikka, Anne L. Wyllie, and Nathan D. Grubaugh

Figure S1: Human RNA in saliva specimens degrades when stored unfrozen for 7 days, related to Figure 1. 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 **Data S1**.

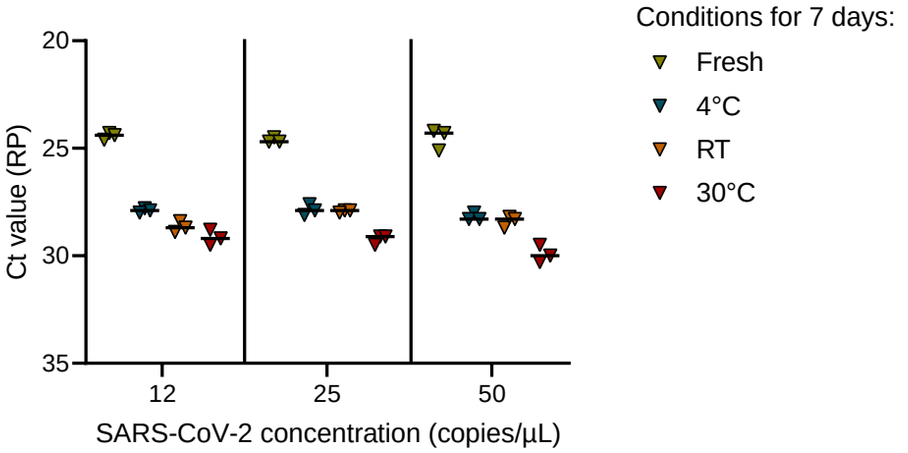

Figure S2: SARS-CoV-2 RNA is stable in saliva under different warm and cold temperature profiles, related to Figure 1. Negative saliva specimens were spiked with a SARS-CoV-2-positive saliva sample to achieve spike-in samples with concentrations of 12, 25, and 50 SARS-CoV-2 copies/µL and stored at 40°C for 72 hours, or under summer or winter profiles. 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. 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. No significant differences were found in N1 Ct values when comparing fresh samples to the 3 temperature regimes (Kruskal-Wallis; $P > 0.05$). Ct values for RP of samples kept at 40°C for 72 hours were significantly higher than fresh samples (Kruskal-Wallis; $P = 0.01$). The horizontal bars indicate the median. Data used to make this figure can be found in **Data S1**.

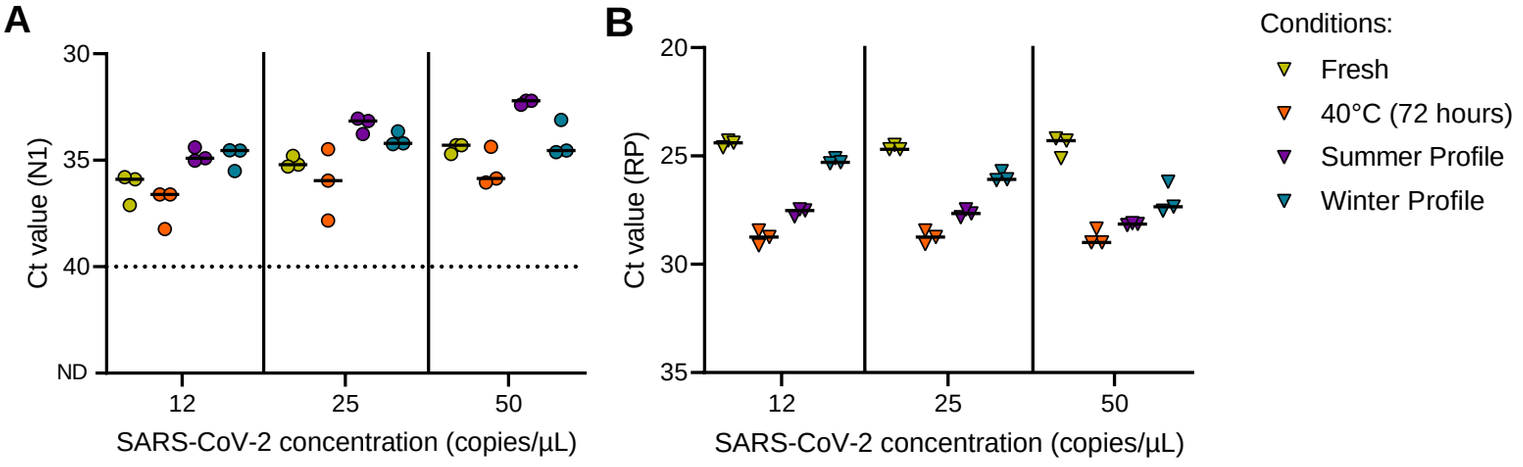

Figure S3: SalivaDirect can be run with RT-qPCR reagents from multiple vendors with universal thermocycler conditions, related to Figure 2. We selected six positive saliva specimens and tested each sample with four different RT-qPCR kits under recommended and unified thermocycler conditions. The tested 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 **Data S1.**

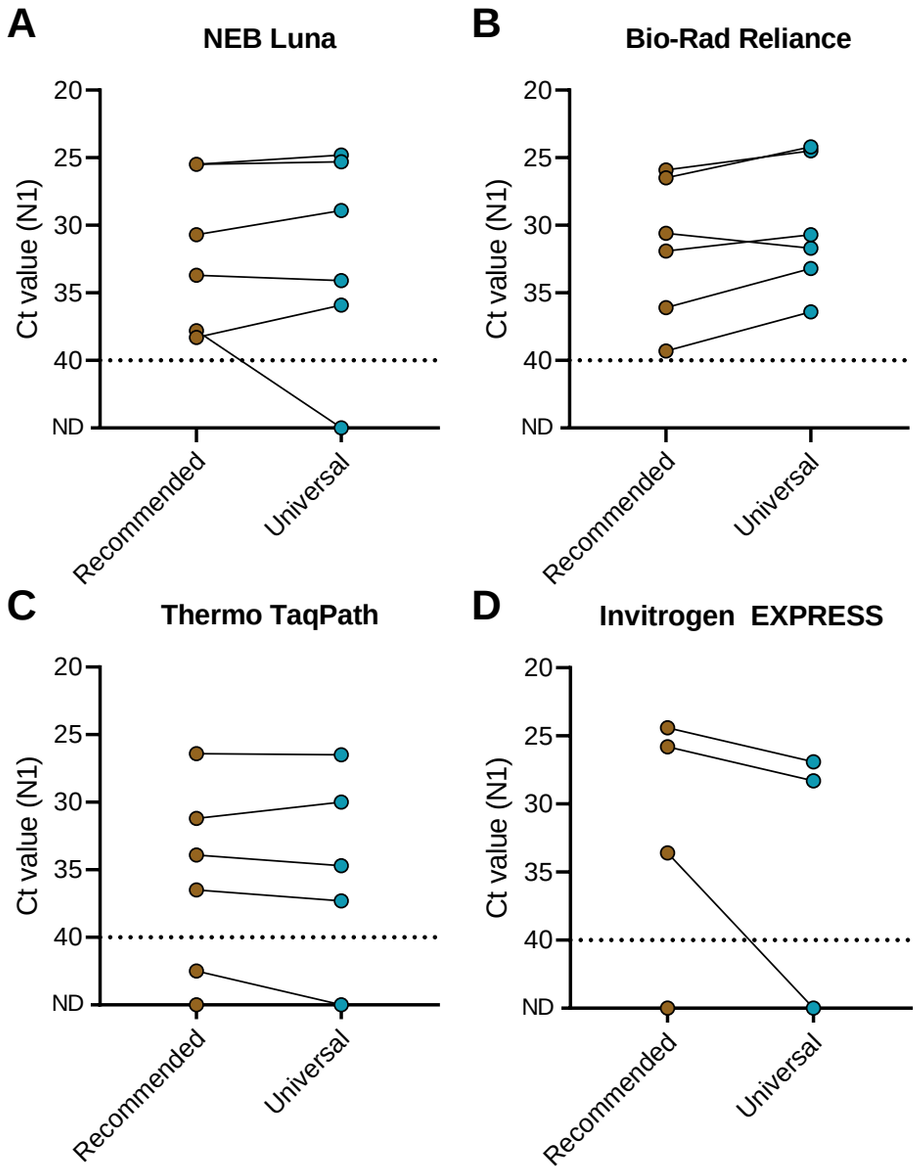

Figure S4: No background amplification when testing pre-COVID-19 saliva specimens with SalivaDirect, related to STAR methods. Saliva specimens were collected from adults during the 2018-2019 and 2019-2020 autumn/winter influenza seasons and tested with SalivaDirect. Shown are Ct values for N1 and the 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 **Data S1**.

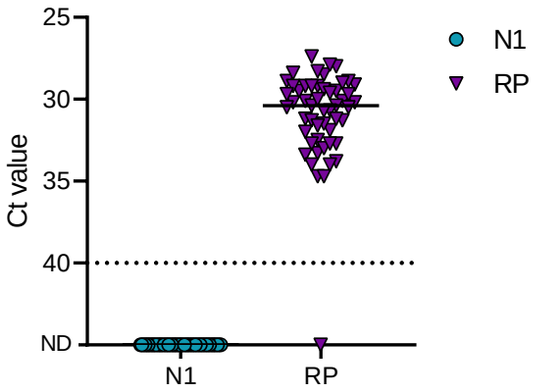

Figure S5: SalivaDirect yields mostly valid results (99.7%) and is comparable for SARS-CoV-2 detection in asymptomatic individuals, related to Table 2. A total of 3,779 saliva specimens were collected from asymptomatic NBA players, staff, and contractors and tested with SalivaDirect. (A) After initial testing 38 specimens tested invalid (RP > 35), and after retesting 12 specimens (0.3%) remained invalid. Shown are final human RNase P values. The horizontal bar indicates the median and the dotted line indicates the limit of detection. (B) We received ten paired AN/OP swabs which were identified as positive when tested by Quest/Bioreference. All ten swabs were retested with the modified multiplex CDC assay and Ct values were compared to saliva tested with SalivaDirect. No significant differences were found between Ct values of AN/OP swabs and saliva (Wilcoxon, P = 0.91). Upon retesting, two AN/OP swabs tested negative, of which the paired saliva of one also tested negative by SalivaDirect. 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**.

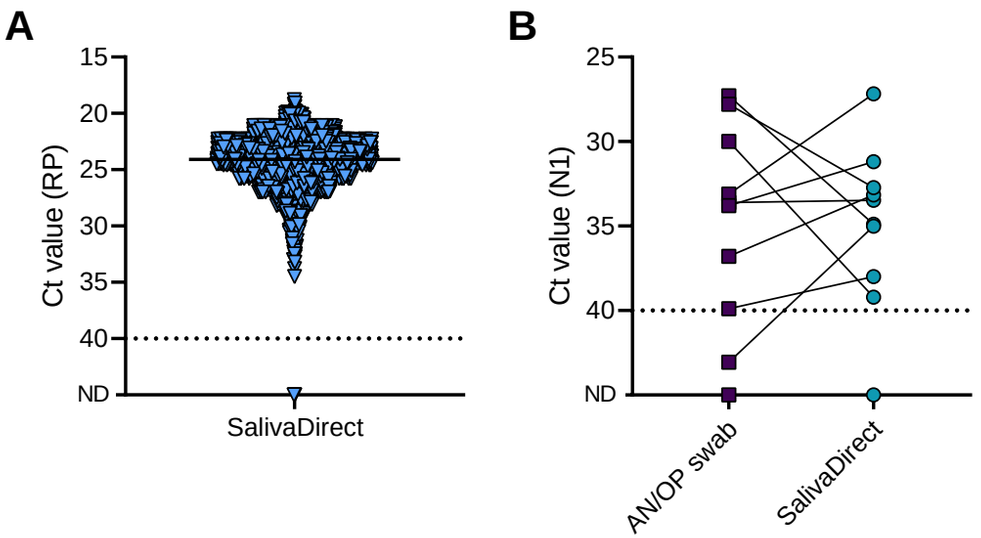

Table S1: No consistent SARS-CoV-2 detection when testing saliva with a multiplex RT-qPCR assay using a HEX-fluorophore, related to STAR methods. We compared Ct values between the modified CDC assay with 3 versions of a multiplexed assay with N2 (Fwd: TTACAAACATTGGCCGCAAA, Rev: GCGCGACATTCCGAAGAA, Probe: HEX-ACAATTTGCCCCCAGCGCTTCAG-IBFQ),[30] E (Fwd: ACAGGTACGTTAATAGTTAATAGCGT, Rev: ATATTGCAGCAGTACGCACACA, Probe: HEX-ACACTAGCCATCCTTACTGCGCTTCG-IBFQ),[65] or ORF1 (Fwd: TGGGGYTTTACRGGTAACCT, Rev: AACRCGCTTAACAAAGCACTC, Probe: HEX-TAGTTGTGATGCWATCATGACTAG-IBFQ)[66] 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