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1 # Pooled RNA: extraction free testing of saliva for SARS-CoV-2 detection

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9

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11

12 ## Abstract

13 The key to limiting SARS-CoV-2 spread is to identify virus-infected individuals (both
14 symptomatic and asymptomatic) and isolate them from the general population. Hence,
15 routine weekly testing for SARS-CoV-2 in all asymptomatic (capturing both infected and
16 non-infected) individuals is considered critical in situations where a large number of
17 individuals congregate such as schools, prisons, aged care facilities and industrial
18 workplaces. Such testing is hampered by operational issues such as cost, test availability,
19 access to healthcare workers and throughput. We developed the SalivaDirect RT-qPCR
20 assay to increase access to SARS-CoV-2 testing via a low-cost, streamlined protocol
21 using self-collected saliva. To expand the single sample testing protocol, we explored
22 multiple extraction-free pooled saliva testing workflows prior to testing with the

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SalivaDirect assay. A pool size of five, with or without heat inactivation at 65°C for 15 minutes prior to testing resulted in a positive agreement of 98% and 89%, respectively, and an increased Ct value shift of 1.37 and 1.99 as compared to individual testing of the positive clinical saliva specimens. Applying this shift in Ct value to 316 individual, sequentially collected, SARS-CoV-2 positive saliva specimen results reported from six clinical laboratories using the original SalivaDirect assay, 100% of the samples would have been detected (Ct value >45) had they been tested in the 1:5 pool strategy. The availability of multiple pooled testing workflows for laboratories can increase test turnaround time, permitting results in a more actionable time frame while minimizing testing costs and changes to laboratory operational flow.

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33 **Introduction**

34 During the emergence and spread of the SARS-CoV-2 virus in 2020, the majority of
35 testing for the virus was aimed at diagnosing COVID-19 (the disease that it causes) in
36 patients presenting with symptoms characteristic of COVID-19. An infected person may
37 develop COVID-19 disease symptoms 3 to 8 days post infection or may never develop
38 symptoms (asymptomatic) (1-3). However, asymptomatic individuals can be infectious,
39 carrying viral loads high enough to spread the virus to uninfected individuals. Soon
40 thereafter it became clear that to control the spread of SARS-CoV-2 infection, a two-
41 pronged approach must be used in the general population to prevent viral spread from
42 infected asymptomatic individuals to the non-infected population.

43 The first prong involves utilisation of physical barriers (e.g. face masks) to minimize virus
44 spread via aerosols (4, 5). The second prong involves routine weekly testing for SARS-
45 CoV-2 in all asymptomatic (capturing both infected and non-infected) individuals at high-
46 risk for infection (6, 7). Such testing is considered critical in situations where many
47 individuals congregate such as schools, prisons, aged care facilities and industrial
48 workplaces. Testing strategies rely on obtaining a respiratory tract specimen and an assay
49 for the presence of SARS-CoV-2 antigen or genome.

50 It is generally considered that molecular tests for viral genome are more sensitive than the
51 antigen tests; however, they can be costly, can take days to return results and can be hard
52 to scale for large population testing. Furthermore, for tests requiring a swab-based
53 respiratory tract specimen, these can be uncomfortable and difficult to obtain, especially
54 under weekly self-collected specimen protocols, deterring individuals from participating in
55 testing (8). Early in the pandemic response, saliva emerged as an alternative specimen for
56 SARS-CoV-2 testing and by 2021 it became apparent that a self-collected specimen could
57 obviate the disadvantages of respiratory tract swab specimens. Importantly, the clinical

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58 sensitivities for SARS-CoV-2 detection were similar between respiratory tract swab and
59 saliva specimens(9).

60 To address some of the limitations of testing, SalivaDirect™ was developed as an open-
61 source protocol wherein clinical laboratories could adopt a streamlined, easy-to-use,
62 inexpensive, scalable and flexible genomic (RT-qPCR) assay method for SARS-CoV-2
63 detection (10). Importantly the assay is based upon a simple self or observed saliva
64 collection protocol. The SalivaDirect™ assay was developed to simplify testing individual
65 saliva specimens; however, with the momentum around testing large-scale asymptomatic
66 populations (e.g. school students, faculty and staff) where SARS-CoV-2 prevalence is low,
67 in a cost-effective manner, a more scalable protocol is required for sustainable testing
68 programs. We therefore investigated higher throughput protocols, wherein saliva
69 specimens are pooled prior to testing with RT-qPCR. These pooled testing approaches
70 were evaluated for the clinical sensitivity of SARS-CoV-2 detection.

71 Methods

72 *Ethics statement*

73 The use of de-identified saliva specimens from healthy or asymptomatic individuals was
74 approved by the Institutional Review Board of the Yale Human Research Protection
75 Program (Protocol ID. 2000028394) (11). Study participants were informed in writing about
76 the purpose and procedure of the study, and consented to study participation through the
77 act of providing the saliva sample; the requirement for written informed consent was
78 waived by the Institutional Review Board. Additionally, the Institutional Review Board of
79 the Yale Human Research Protection Program determined that the use of de-identified,
80 remnant COVID-19-positive clinical samples obtained from laboratory partners for the RT-
81 qPCR testing conducted in this study is not research involving human subjects (Protocol
82 ID: 2000028599).

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83

84 ***Sample Collection***

85 All de-identified saliva samples used in the current study were collected unsupplemented
86 into simple laboratory plastic tubes per the SalivaDirect protocol (12). All samples were
87 tested with the SalivaDirect assay(13) in our research laboratory to confirm SARS-CoV-2
88 status. Samples were stored at -80°C until further analysis.

89 ***Pooled Sample Testing***

90 To understand the effect of sample dilution by pooling, on clinical sensitivity, a total of 20
91 saliva specimens which previously tested positive with the modified CDC assay RT-qPCR
92 assay, with resulting cycle threshold (Ct) values between 22.98 and 39.43, were diluted
93 1:5, 1:10, and 1:20 with negative saliva specimens from healthcare workers(14). Undiluted
94 specimens and pools were tested with the standard SalivaDirect RT-qPCR assay.

95 After identifying the optimal pool size, we performed an initial workflow evaluation using 5
96 different pooled samples, each composed of a single SARS-CoV-2-positive saliva sample
97 pooled with 4 SARS-CoV-2-negative saliva samples, in a 1:5 dilution. To confirm our initial
98 workflow findings and assess the sensitivity of viral detection when pooling, 20 additional
99 pools (1 positive with 4 negative saliva specimens) were prepared and tested using the
100 five different workflows.

101 The five different saliva pooling workflows investigated in both the initial and confirmation
102 studies are depicted in **Figure 1**. All saliva samples were thawed on ice prior to testing
103 and all samples were tested in duplicate. For workflows A and C (**Figure 1a**), 50 µl of
104 each sample, (including the SARS-CoV-2-positive saliva) were pooled to 250 µL total
105 volume, followed by vigorous vortexing. For workflow A, 50 µl of the pooled sample was
106 tested following the standard SalivaDirect protocol(10). For workflow C the remaining

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107 sample was treated with 10 µl of proteinase K then heat inactivated before testing directly
108 without further treatment in the SalivaDirect RT-qPCR assay. Workflows B, D and E
109 (**Figure 1b**) involved incubating individual non-pooled samples at 65ºC for 15 minutes
110 before combining 50 µl of each sample into pools of 5 pre-treated samples. For workflow
111 B, 50 µl of the pool of pre-treated samples was tested through the standard SalivaDirect
112 protocol. For workflow E, 10 µl of each of these pre-treated pools was removed and tested
113 with the SalivaDirect RT-qPCR assay without proteinase K treatment. Finally, for workflow
114 D, 10 µl of proteinase K was added to the remaining volume of the pre-treated pool then
115 heat inactivated before testing in the SalivaDirect RT-qPCR assay.

116

117 ***Assessment of clinical Ct values with pooling***

118 To evaluate the real-world potential loss of sensitivity on clinical samples with pooling, we
119 estimated the average change in Ct value for each pooled testing workflow using the
120 results from the 25-sample workflow confirmation study. We then estimated how the
121 change in Ct value would potentially impact assay sensitivity by applying the ΔCt value
122 (with pooling) to real world SalivaDirect RT-qPCR SARS-CoV-2 testing results. Six SARS-
123 CoV-2 testing sites around the U.S., all testing with the standard SalivaDirect™ protocol,
124 provided sequential testing results (Ct values for positive specimens) during August 2021
125 and to these values we applied the ΔCt value that we estimated.

126 ***Statistical analyses***

127 The correlation of Ct values between each workflow and the individual positive samples
128 was assessed using the Pearson correlation coefficient and represented graphically with
129 linear regression. The negative RT-PCR of the target gene was set at the Ct value of 45
130 for the statistical analysis. All statistical analyses were performed using GraphPad Prism

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131 version 9 (GraphPad Software, San Diego, CA). For the calculation of percent positive
132 agreement, samples are considered positive at Ct < 45.

133 **Results**

134 ***Pooling sizes and workflow selection***

135 We initially performed a limit of detection range-finding study to determine the impact of
136 sample dilution via pooling with the SalivaDirect RT-qPCR assay on detection sensitivities.
137 As pool size increased the resulting assay Ct values increased as well, generally in a
138 linear manner. The smallest change in Ct values (i.e. loss of assay sensitivity) of pooled
139 versus neat saliva was obtained with 1:5 pooling (1 positive and 4 negative saliva
140 samples). Thus a 1:5 pooling strategy was employed for workflow analysis. Our
141 preliminary results indicated that the SalivaDirect assay was able to detect SARS-CoV-2
142 in pooled saliva specimens with high virus loads, but additional testing was required to
143 optimize saliva pooling and processing workflows.

144 **Table 1. Distribution of the Ct values of clinical saliva samples used for pooling.**

Ct range*	No. samples Workflow A (n=22)	No. samples Workflow B - E (n=25)
20.0-29.9	7 (32%)	7 (28%)
30.0-34.9	5 (23%)	8 (32%)
35.0-40.0**	10 (45%)	10 (40%)

145 *samples <40 Ct are considered positive for SARS-CoV-2

146 **mean Ct for the CFX96 Touch RT-qPCR instrument when determining LOD for analytical sensitivity using
147 this set of reagents was 36.7

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148 Extrapolating from previous work (Watkins et al. 2021), we selected 5 workflows
149 representing different pooling strategies. Initial analyses using 5 pools showed that 4
150 workflows (A-D) provided similar results for most of the pools (see **Table S1**). Workflow E
151 provided a much larger shift in Ct values for all five pools (5.26) and hence loss of assay
152 sensitivity. As expected, a shift in Ct values (to higher) was noted for all five pooled
153 workflows compared the standard SalivaDirect RT-qPCR assay performed on the
154 undiluted positive sample. For workflows A-E, initial analysis of the differences in Ct
155 values between the pools and individual positive samples resulted in a Ct shift of 2.17 to
156 3.50. Workflow E was omitted from further evaluations.

157 ***Workflow analysis***

158 Twenty-five SARS-CoV-2 saliva specimens were used for pooling, with each pool
159 including one unique positive specimen and 4 negative specimens, to make 25 contrived
160 pools. The Ct values (obtained at the site of saliva collection) for SARS-CoV-2-positive
161 samples ranged from 22.98 to 39.43. The majority (40 to 45%) of the specimens had Ct
162 values >35 indicating a relatively low concentration of virus, 28 to 32% of the specimens
163 had Ct values <30 indicating a higher concentration of virus (**Table 1, Table S2**).
164 We assessed the sensitivity of each workflow by comparing to see which workflow had
165 the smallest shift in Ct values between the undiluted sample processed with SalivaDirect
166 RT-qPCR assay and the workflow in question, and by comparing which workflow had
167 the smallest number of pools dropping below the sensitivity threshold (between Ct 40 to
168 45). When compared to undiluted samples processed with the standard SalivaDirect
169 assay, Workflows A and B provided the highest sensitivity (**Figure 2**). Workflows A and
170 B resulted in 3 and 1 pool(s) with Ct increases to 40 and 45, respectively. In contrast,
171 workflows C and D demonstrated the lowest in clinical sensitivity, with loss in detection
172 in 8/25 and 10/25 pools processed by these workflows respectively.

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173 Workflow A resulted in a positive agreement of 88.6% (86.4% and 91.0% for the
174 individual replicates), compared to the individual testing results using the standard
175 SalivaDirect protocol. Workflow B resulted in a 98% positive agreement (100% and 96%
176 for the replicates), compared to the individual testing results using the standard
177 SalivaDirect protocol (**Figure 2a**). The positive agreement for workflows C and D were
178 less, with averages of 76% and 62%, respectively.

179 A theoretical Ct shift of $Log_2(n)$ can be estimated for most RT-qPCR tests due to the
180 dilution of positive samples when pooled with negative samples. This means that for
181 pools of 5, a Ct value shift of 2.3 would be expected. The Ct shift observed for
182 Workflows A and B were below this expected value, with Ct value shift of 1.99 and 1.37
183 respectively, confirming a slight loss of assay sensitivity. Workflows C demonstrated the
184 worst Ct value shift of 2.81.

185 ***Impact of pooling on clinical sensitivity***

186 To determine the pragmatic loss of clinical sensitivity with pooling before performing the
187 SalivaDirect RT-qPCR assay with workflows A-D, we queried six SalivaDirect CLIA
188 laboratories across the United States for the Ct values obtained from sequential testing of
189 saliva samples for SARS-CoV-2. These values and the breakdown of samples per site are
190 presented in **Figure 3** (raw data available in **Table S3**). The average Ct value for the six
191 labs was 28.0. There was no statistical difference in Ct values across the labs. Out of a
192 total of 613 determinations across the labs, only 16 samples (2.6%) had Ct values
193 between 38-40. Considering the calculated (from the confirmation study) worst case Ct
194 shift in pooling workflows A and B of 1.99, and if all of the 613 determinations had been
195 made using workflow A or B, these 16 samples would have shifted Ct to between 40 and
196 42. While considered negative using the individual testing workflow, these 2.6% of
197 samples would fall into a grey zone of 40-45 Ct values.

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198 **Discussion**

199 Widespread surveillance of asymptomatic individuals is one of the main methods of
200 controlling the spread of SARS-CoV-2. The pooling of samples before testing is a
201 resource-saving approach to increase testing capacity, especially for surveillance in a
202 population with a low infection rate (14), such as travellers, school populations and
203 employees of large organisations. Additionally, testing these members of the community
204 serve as a proxy to the broader community, perhaps identifying larger outbreaks through
205 family members and their associated activities. As saliva is easy to collect from a large
206 number of people, pooling strategies are thus a natural extension to surveillance
207 programs. While pooling saliva does impact assay sensitivity and potentially decrease
208 virus detection, the actual impact appears to be minimal.

209 In the current study, we demonstrated that weakly positive samples (Ct values of 38 to 40)
210 may be missed when testing pools of larger sizes (pools > 5) when compared to testing
211 samples individually. However, on the basis of the calculated relative sensitivity loss
212 resulting from pooling, we looked at six datasets comprising 613 SARS-CoV-2 positive
213 samples from across the U.S. Using such real world data we found that pooling saliva in
214 groups of 5 samples prior to testing is expected to have minimal impact on clinical
215 sensitivity. Based upon the lab reported Ct values only 2.6% of these samples would have
216 shifted into a Ct 40-45 grey zone using the proposed SalivaDirect pooling workflows A or
217 B. Importantly, if these samples were pooled, none would have become undetectable. It is
218 advised that any sample pool resulting in Ct values between 40-45 should be retested
219 individually using the standard SalivaDirect protocol.

220 Surveillance programs for SARS-CoV-2 genomic testing in low prevalence populations
221 must be operationally pragmatic. First, they need to be cost-effective. Pooled testing
222 significantly reduces reagent costs, lab personnel cost, and lab resource needs. Second,

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223 these programs need to be easy to implement. Self-collection of a simple saliva specimen
224 obviates the need for healthcare workers to collect specimens and the associated
225 personal protective equipment. Third, programs should utilize existing resources for
226 sample collection. Self-collection of saliva can be performed anywhere and the resulting
227 specimen can be deposited at a drop site location (e.g. school or workplace entrance)
228 such that specimens from thousands of participants are collected in a parallel manner.
229 Pooling of specimens once received in the laboratory for testing should fit into established
230 laboratory accessioning and pre-analytic workflows. Finally, the end test results must
231 provide acceptable clinical sensitivities and specificities. We have shown that a saliva-
232 based RNA-extraction-free pooled (1:5) testing strategy results in detection of 97.4-100%
233 SARS-CoV-2-positive samples, as compared to individual testing. Large pooled testing
234 programs have already demonstrated the efficacy of pooled saliva testing for helping to
235 keep schools safely open (15, 16), with pooled samples having a similar sensitivity to the
236 molecular testing of individual samples, in terms of both qualitative and quantitative
237 (comparable Ct values between pooled and individual samples) measures.

238

239 Throughout the pandemic, clinical laboratories have been hesitant to implement pooled
240 sample testing (17) due to: 1) stringent workflows which do not fit within existing laboratory
241 operations, 2) a lack of clear guidance on how to implement such methods and 3) the
242 perception that clinical sensitivity of the assay will be lost with pooling. The methods we
243 propose in the current study demonstrate minimal impact on assay sensitivity with 5
244 samples per pool, and are straightforward extensions of a simple SARS-CoV-2 testing
245 method which can be easily conducted manually, without requiring additional investment.
246 SalivaDirect is a flexible extraction-free platform for RT-qPCR testing. For ease of
247 implementation and safety of lab personnel, multiple workflows (18) were developed for

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248 the testing of individual samples. We sought to extend this level of flexibility for labs
249 seeking to offer pooled testing. We demonstrated that workflows A and B provide the best
250 assay sensitivity, with B providing a heat pre-treatment step for labs who require it by local
251 Environmental Health and Safety guidelines. Consequently, workflows A and B were
252 selected as the proposed approaches for pooling of the SalivaDirect test.

253 Overall, surveillance testing is not generally easy, requiring a pivot by traditionally clinical
254 diagnostic labs, especially when scalable protocols do not exist. Thus, when a decrease in
255 positive cases are observed, there is a psychological and practical desire to decrease
256 testing. However, these dips in COVID-19 cases can lead to a decrease in pandemic
257 preventative measures, which inevitably leads to disease resurgence. Additionally, with
258 the introduction of different variants of concern, the need for affordable and sustainable
259 mass testing strategies only becomes more urgent. Our findings suggest that combining
260 saliva with a practical pooling protocol will enable easier SARS-CoV-2 surveillance testing,
261 especially in resource-limited settings. Such pooled testing has the potential to
262 significantly reduce the overall number of tests and associated costs. This would in turn
263 operationally permit an increased frequency of testing, meaning an increased likelihood of
264 detecting individuals earlier in their infection. This approach should allow broader
265 screening in schools and workplaces for SARS-CoV-2 testing and importantly lay the
266 foundation for managing future upper respiratory infection mediated pandemics.

267

268 **Data Availability**

269 Data from this study is available in the supplemental information.

270

271 **Author contributions**

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272 A.L.W. conceived the study and developed the study protocol. D.A.Y-C. executed the
273 study. A.L.W. co-ordinated external laboratory data. O.M.A. and A.L.W. analyzed the data.
274 O.M.A. assisted with the design of the statistical analysis. J.A.T., D.A.Y-C, O.M.A., and
275 A.L.W. wrote and edited the manuscript.

276

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281 Ventures at the Mercatus Center at George Mason University (A.L.W).

282

283 **Figures**

284 **Figure 1. The SalivaDirect™ pooled testing workflows evaluated in the study.** (A) For
285 workflows A (purple) and C (gold), 50 µl of SARS-CoV-2-positive saliva and 200 µL total
286 volume of four SARS-CoV-2-negative saliva samples (50 µl each) were pooled and
287 vigorously vortexed to mix. For workflow A, 50 µl of the pooled sample was tested
288 following the standard SalivaDirect protocol. For workflow C the remaining sample was
289 treated with 10 µl of proteinase K then heat inactivated before testing directly without
290 further treatment in the SalivaDirect™ RT-qPCR assay. (B) For workflows B, D and E,
291 individual, non-pooled samples were incubated at 65ºC for 15 minutes before combining
292 50 µl of each sample into pools of 5 pre-treated samples. For workflow B (pink), 50 µl of
293 the pool of pretreated samples was tested through the standard SalivaDirect protocol. For
294 workflow E (orange), 10 µl of each of these pre-treated pools was removed and tested

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295 with the SalivaDirect™ RT-qPCR assay without proteinase K treatment. Finally, for
296 workflow D (blue), 10 µl of proteinase K was added to the remaining volume of the pre-
297 treated pool then heat inactivated before testing in the SalivaDirect™ RT-qPCR assay.

298

299 **Figure 2. SARS-CoV-2 N1 gene detection with individual saliva-based RNA-**
300 **extraction-free RT-qPCR testing versus pooled sample testing using Workflows A-**
301 **D.** A) N1 detection of one positive sample pooled and tested with equal volumes of 4
302 negative samples correlated to the Ct value obtained when samples were tested
303 individually. B). Ct values obtained from each sample tested individually and when
304 combined with 4 negative samples and tested with each of the workflows. The N1 Ct cutoff
305 for classifying individual samples as positive is 40 (as indicated by the grey area under the
306 horizontal dashed line. No cutoff was set for the pooled samples. B) line - median values

307

308 **Figure 3 Detection of SARS-CoV-2 N1 gene persists when Ct value shift from**
309 **workflows A is applied to data from six clinical laboratories across the US in July**
310 **2021.** Each dot represents the clinical samples. The black line indicates the median value
311 of the samples, and the number of samples processed at each site in square brackets
312 above the location of the laboratory.

313

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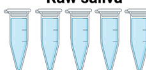
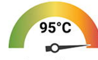




A
Raw saliva
Add 50 µL of each sample to a single tube
250 µL final volume
Workflow A
Workflow C
Add 10 µL of proteinase k to the pool
Take 50 µL for testing by standard SalivaDirect Protocol
Mix to combine
Add 2.5 µL of proteinase K
Mix to combine
95°C
Inactivate proteinase k
5 µL
Duplex RT-qPCR Targets N1+ RP
B
Raw saliva
Heat inactivate at 65°C for 15 minutes
Add 50 µL of each sample to a single tube
250 µL final volume
Workflow B
Workflow D
Add 10 µL of proteinase k to the pool
Take 50 µL for testing by standard SalivaDirect protocol
Mix to combine
Add 2.5 µL of proteinase k
Workflow E
Mix to combine
Mix to combine
95°C
Inactivate proteinase k
5 µL
Duplex RT-qPCR Targets N1+ RP
5 µL
95°C
Inactivate proteinase k

A

Workflow A

Pooled samples C_T value (N1)

Individually tested C_T value (N1)

Workflow B

Pooled samples C_T value (N1)

Individually tested C_T value (N1)

Workflow C

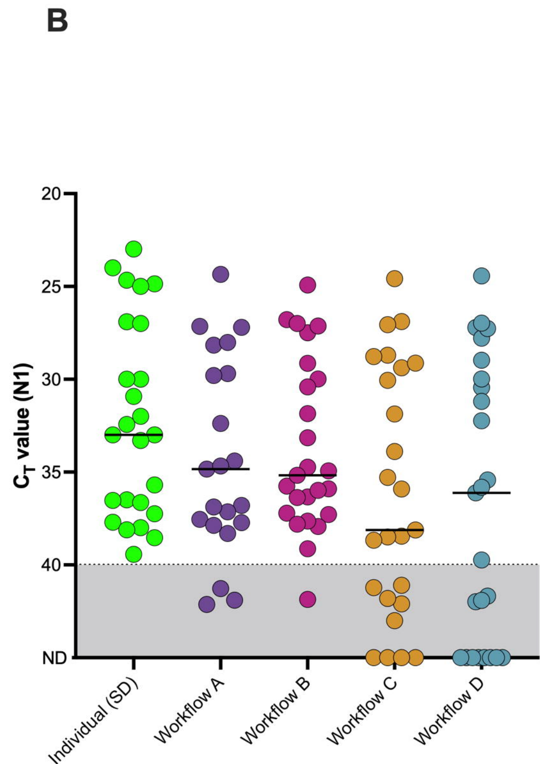
Pooled samples C_T value (N1)

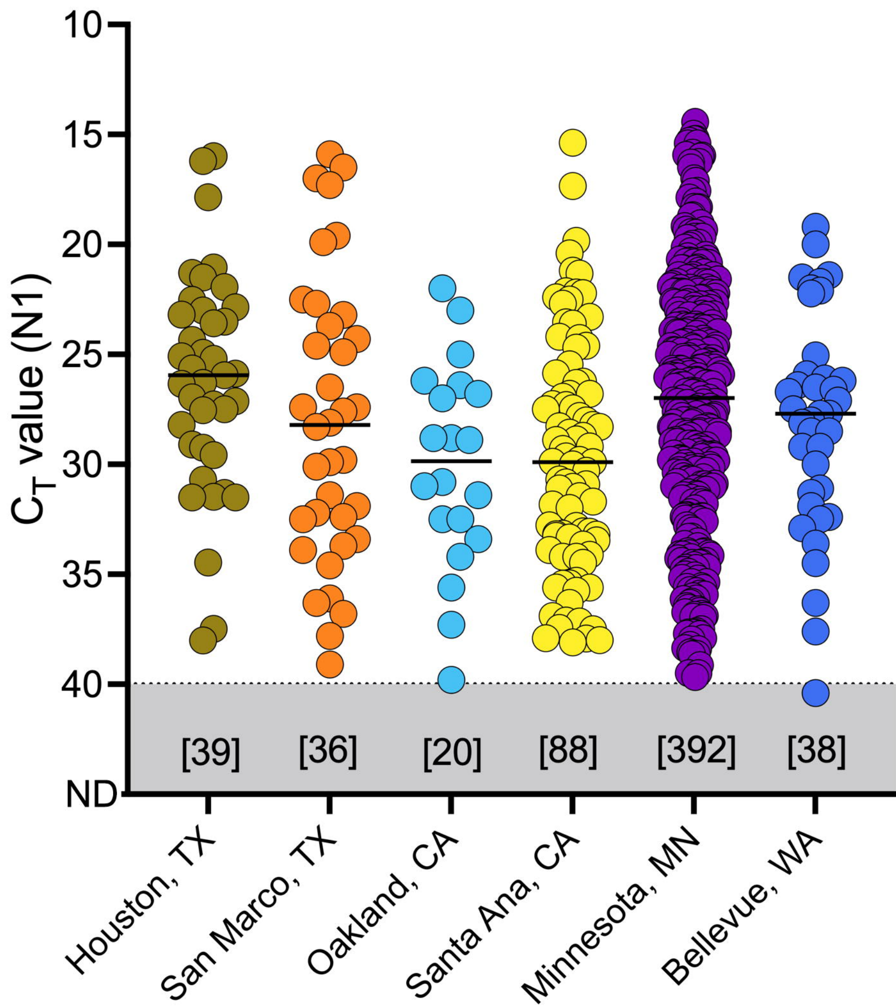
Individually tested C_T value (N1)

Workflow D

Pooled samples C_T value (N1)

Individually tested C_T value (N1)





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ND
C_T value (N1)
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Houston, TX
San Marco, TX
Oakland, CA
Santa Ana, CA
Minnesota, MN
Bellevue, WA