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1 **SARS-CoV2 Testing: The Limit of Detection Matters**
2

3 Running Title: LoD Matters

4

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25 **Abstract**

26 Resolving the COVID-19 pandemic requires diagnostic testing to determine which individuals
27 are infected and which are not. The current gold standard is to perform RT-PCR on
28 nasopharyngeal samples. Best-in-class assays demonstrate a limit of detection (LoD) of ~100
29 copies of viral RNA per milliliter of transport media. However, LoDs of currently approved
30 assays vary over 10,000-fold. Assays with higher LoDs will miss more infected patients,
31 resulting in more false negatives. However, the false-negative rate for a given LoD remains
32 unknown. Here we address this question using over 27,500 test results for patients from across
33 our healthcare network tested using the Abbott RealTime SARS-CoV-2 EUA. These results
34 suggest that each 10-fold increase in LoD is expected to increase the false negative rate by
35 13%, missing an additional one in eight infected patients. The highest LoDs on the market will
36 miss a majority of infected patients, with false negative rates as high as 70%. These results
37 suggest that choice of assay has meaningful clinical and epidemiological consequences. The
38 limit of detection matters.

39

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40 **Introduction**

41 In response to the SARS-CoV-2 pandemic being declared a public health emergency, clinical
42 and commercial laboratories as well as test kit manufacturers have been submitting diagnostic
43 devices and assays for expedited Emergency Use Authorization by the Food and Drug
44 Administration (FDA EUA). As of June 2020, there were over 85 such EUA issuances for
45 COVID-19 diagnostics (https://www.fda.gov/medical-devices/emergency-situations-medical-
46 devices/emergency-use-authorizations, accessed June 1, 2020). However, optimal use of these
47 assays requires consideration of several issues.

48 First, NP swabs are generally considered to provide optimal detection early in disease.
49 However, even for this sample type, there is currently no ideal reference standard to establish
50 clinical sensitivities of the available EUA SARS-CoV-2 diagnostic assays (1). Second, details
51 about assay limit of detection (LoD) are often not provided with sufficient detail and
52 transparency to allow facile comparisons. For molecular diagnostic assays, the LoD is generally
53 considered the lowest concentration of target that can be detected in ≥95% of repeat
54 measurements. The LoD is a measure of analytic sensitivity, as opposed to clinical sensitivity,
55 which measures the fraction of infected people detected by a given test. LoDs are sometimes
56 reported in units other than copies of viral genomic RNA per milliliter of transport media
57 (copies/mL), such as $TCID_{50}$, copies/microliter, copies per reaction volume, or molarity of assay
58 target, making comparisons difficult. Third, the LoDs of currently approved EUA nucleic acid
59 amplification and antigen detection tests for SARS-CoV-2 vary up to 10,000 fold (see below)
60 and likely are associated with meaningful differences in clinical sensitivity for these tests.
61 Fourth, although LoDs are quantitative, and RT-PCR tests are inherently quantitative, in practice
62 results for SARS-CoV-2 testing are generally reported qualitatively, as positive or negative,
63 even though viral load may provide both clinically and epidemiologically important information.

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64 Two barriers to quantitative reporting are demonstration that qPCR cycle threshold (Ct) values
65 are repeatable with acceptably low variance and a reliable means of converting from Ct value to
66 viral load. The latter is complicated by a traditional requirement for a standard curve that must
67 span a range of viral loads at least as large as what is observed in the patient population, which
68 can be expensive and time-consuming, especially in a pandemic where the limits of this range
69 are unknown; however, there have been reports demonstrating how appropriate measurements,
70 based on the principles of RT-PCR, can be used as an alternative for reliable conversion of Ct
71 values to viral loads (2, 3).

72 Here we report on the reliability of Cts for the Abbott SARS-CoV-2 EUA (LoD 100 copies viral
73 RNA/mL transport medium, among the best in class) (4) and a conversion from Ct to viral load,
74 which together support the use of reporting viral loads clinically, and also on an observation
75 based on over 4,700 first-time positive results that makes it possible to estimate the clinical
76 sensitivity and false-negative rate of both this assay and other assays that have received EUA
77 for detecting SARS-CoV-2 infection. These findings have clear implications for patient care,
78 epidemiology, and the social and economic management of the ongoing pandemic.

79 **Methods**

80 **Setting and time period.** All SARS-CoV-2 testing data from The Beth Israel Lahey Health
81 Network from March 26th to May 2nd, 2020 was included in our analysis. The study was
82 deemed exempt by our hospital institutional review board.

83 **Testing.** Tests were performed using the Abbott RealTime SARS-CoV-2 assay, a real-time
84 reverse transcriptase (RT) polymerase chain reaction (PCR) test for qualitative detection of
85 SARS-CoV-2 in NP and oropharyngeal swabs (5). The dual target assay detects both the
86 SARS-CoV-2 RdRp and N genes with a reported LoD of 100 copies/mL. The assay also

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87 includes an internal control. Results are reported as positive if the Ct value is ≤31.5, based upon
88 the signal threshold determined by the manufacturer. Ct values for all first-time positive test
89 results were analyzed. Repeat tests were excluded in order to more accurately estimate the
90 range of Ct values of the infected population upon presentation at our medical center. In our
91 internal validation we determined that the LoD with 100% detection for the Abbott m2000
92 platform was 100 copies/mL (n=80), with Ct mean and standard deviation at this LoD,
93 26.06±1.03 (4). Note, the Ct determination on Abbott M2000rt platform is alternatively called the
94 fractional cycle number (FCN) and is specifically one way of determining the cycle number at
95 the maximum amplification efficiency inflection point, i.e, the maxRatio, of each amplification
96 curve (6). The FCN has been reported to be a more robust measure for Ct determination than a
97 fixed fluorescence threshold.

98 **Statistics**. Variance was estimated by R^2 of Ct values for repeat tests obtained within 6 hours
99 (n=25 patients, excluding one obvious outlier that by itself accounted for half the total variance:
100 initial Ct 4.4, but repeat negative and attributed to pre-analytic or analytic technical error) and 12
101 hours (n=51 patients, excluding the same outlier). The conversion from Ct value to viral load
102 was performed using the definition of exponential growth with variable efficiency (2, 3).
103 Efficiency was measured from plots of fluorescence intensity vs. cycle number for 50 positive
104 samples chosen at random, yielding an expression for viral load in copies/mL as a function of Ct
105 (Eq. 6, Supplementary Methods). Per this expression, the expected negative cutoff corresponds
106 to 9.2 copies per mL or ~2 virions per RT-PCR reaction volume (0.5mL), supporting the validity
107 of our parameter estimation.

108 We used Python (v3.6) and its NumPy, SciPy, Matplotlib, and Pandas libraries to plot linear
109 regression and Theil-Sen slopes with 95% confidence intervals on repeat positives; a
110 normalized cumulative distribution (histogram) of positive results (with reversed x-axis for ease

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111 of interpretation); binned histogram by 0.5 log10 units, and linear regression on log10-
112 transformed data.

113 **Results**

114 Of the 27,098 tests performed on 20,076 patients over the testing period, 6,037 tests were
115 positive (22%), representing 4,774 unique patients. Analysis of repeats within 6 or 12 hours of
116 each other (7) demonstrated high repeatability of Ct values over these short time windows (R^2
117 0.70 and 0.63, n=25 and 51, respectively), supporting the validity of this quantitative measure as
118 a basis for assessment of viral load in patients (Fig. 1). We used basic principles of PCR and
119 detailed measurements of PCR efficiency on 50 randomly chosen positive samples to convert
120 from Ct values to viral load, in units of copies of viral RNA per mL of viral transport medium. In
121 order to study the patient population upon presentation without confounding by repeat
122 measurements on the same patients, the remainder of the analysis was on the first positive
123 value for the above 4,774 unique patients.

124 Viral loads spanned nearly nine orders of magnitude, from 9 copies/mL to 2.5 billion copies/mL
125 (Fig. 2). Notably, patients were almost equally likely to exhibit low, medium, or high viral loads
126 upon initial testing, with remarkable uniformity down to the LoD of 100 copies/mL (R^2=0.99). The
127 reason for this uniformity is unknown. Fewer patients had viral loads below the LoD, as reflected
128 by the curve's departure from the trend in this range. Because the LoD is a 95% confidence
129 limit, the difference between the curve and the trend likely reflects false negatives: the lower the
130 viral load, the greater the likelihood that infection will be missed. By definition, only 5% of
131 patients with viral load at the LoD are expected to be missed (1 in 20 patients); this percentage
132 grows for patients with viral loads below this threshold. Thus, extending the observed trend
133 leftward to the assay's positive cutoff, which corresponds to approximately two virions per

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134 reaction, yields an estimate of the total false negative rate for this assay of 10%, and thus a
135 clinical sensitivity of 90%, or 9 in 10 infected individuals.

136 This method can be used to estimate the clinical sensitivity of assays with other LoDs. For
137 example, an assay with LoD of 1,000 copies/mL, such as that of the CDC assay (8) or Genmark
138 ePlex EUA (9), is expected to detect 77%, or 3 in 4, of infected individuals, for a false-negative
139 rate of 22%. With an LoD of 6,250 copies/mL, the LabCorp COVID-19 RT-PCR EUA test has an
140 estimated clinical sensitivity of 67% and a false-negative rate of 33%, missing approximately 1
141 in 3 infected individuals. The first EUA antigen detection assay, the Quidel Sofia2 SARS Antigen
142 FIA, has an LoD of approximately 6 million in a contrived universal transport medium sample
143 collection. Although the package insert indicates the LoD using $TCID_{50}$ units, the BEI Resources
144 control material referenced lists both $TCID_{50}$ and genome copies/mL, allowing the calculation of
145 the latter and an associated estimated clinical sensitivity of 31%, i.e., it would miss 7 in 10
146 infected patients.

147 **Discussion**

148 The diagnostic priorities in the COVID-19 pandemic are to robustly identify three populations:
149 the infected, the infectious, and the susceptible. Our study addresses the first of these.
150 Specifically, it illustrates the clinical and epidemiologic impact of assay LoD on SAR-CoV-2
151 diagnosis and the challenges of interpreting and comparing molecular assay results across
152 various platforms. First, viral loads vary widely among infected individuals, from individuals with
153 extremely high viral loads, potential “super-spreaders” who presumably would be picked up by
154 even the least sensitive assays, to those whose viral loads are near, at, or even below the LoD
155 of many assays. Therefore, a substantial fraction of infected patients will be missed by less
156 sensitive assays. Concerningly, some of these missed patients are, have been, or will become
157 infectious, and such misses will undermine public health efforts and put patients and their

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158 contacts at risk. This must give pause in the rush to approve additional testing options and
159 increase testing capacity, and emphasizes the importance of defining infectivity as a function of
160 viral load and other factors (e.g. time of exposure), which remains a critical unknown in this
161 pandemic.

162 Antigen detection assays promise rapid turnaround time, point-of-care implementation, and low
163 cost. For influenza detection, such tests have exhibited substantially lower analytical and clinical
164 sensitivity compared with NAAT tests (10). The poor historical performance for influenza
165 detection led to reclassification of influenza rapid antigen detection tests as Class II devices with
166 a new minimal performance standard of at least 80% sensitivity compared with NAAT (11).
167 Previously, clinical sensitivity of 50-88% for the Quidel Sofia influenza test was noted in several
168 studies in different influenza seasons compared to RT-PCR comparators (12-14). The same
169 trend was observed in our analysis of the single SARS-CoV-2 antigen test introduced thus far
170 with EUA status. Tests with such performance characteristics will identify individuals with the
171 highest viral burden. However, such a high detection threshold will be unlikely to fully meet
172 public or individual health goals in the COVID-19 pandemic.

173 Our findings also suggest that Ct values and imputed viral loads have clinical utility. Real-time
174 PCR methods in particular are inherently quantitative, and we demonstrate here that they are
175 quite reproducible during repeated clinical sampling over a short time period, with R^2 of 0.70 for
176 repeats within six hours (as a proxy for immediate repeats). We note that because PCR
177 efficiency can fall substantially with PCR cycle number, as we observed here, viral load is
178 ideally calculated not simply as a powers-of-2 transformation of Ct value but based on the
179 observed trend between efficiency and Ct number. This trend may differ by assay: for example,
180 the assay used here includes an internal control whose product may contribute to polymerase
181 inhibition. (This method can be extended to provide confidence limits that incorporate the
182 variance in, e.g., the Ct of the LoD, but this extension is beyond the scope of the current work.)

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183 As yet it is unclear whether or how viral loads affect prognosis, but they at least suggest a
184 measure of infectivity, as well as possibly severity of illness, and, therefore may have value for
185 public health efforts, as we learn which cutoffs may imply minimal or inconsequential infectivity,
186 especially during clearance of infection. We make explicit our assumption that ~2 virions per
187 reaction, translating to a viral load of 9 copies/mL, reflects a 100% detection rate. With stricter
188 cutoffs, clinical sensitivity falls slightly (e.g., from 90% to 86% for an assay with an LoD of 100
189 copies/mL, if using a cutoff of 4 copies/mL, or a single virion per reaction, and to 79% if using a
190 cutoff of 0.7 copies/mL, or a single virion per 3mL transport tube). Regardless, these different
191 assumptions have essentially no effect on the relative clinical sensitivities of different assays.
192 While it is theoretically possible that even lower levels of infection are possible, making our
193 estimates of clinical sensitivity upper limits, we believe potential for contagion at these levels is
194 highly unlikely, as that would assume that breathing, a cough, or a sneeze would transmit more
195 particles than can be obtained by dedicated and vigorous physical swabbing of the actual
196 nasopharynx.

197 To control the pandemic, ultimately we will need diagnostics for all three populations of interest,
198 infected, infectious, and susceptible, and for that we will need to understand whether and how
199 viral load relates to infectiousness. As we have shown, assays with higher LoD are likely to miss
200 non-negligible fractions of infected individuals. However, individuals with viral burdens low
201 enough to be missed by some assays may prove to be less infectious. *In vitro,* approximately
202 only 1 of 10,000 genome copies in viral cultures may be associated with a tissue culture
203 infectious viral particle based on standard preparation such as BEI Resources NR-52866(15).
204 However, it is unclear how or whether this fraction might change with viral load for patients *in*
205 *vivo*.

206 The ultimate lesson from these studies bears repetition: LoD matters and directly impacts efforts
207 to identify, control, and contain outbreaks during this pandemic. Various assays report out LoDs

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208 in manners that are often difficult to comprehend, for example, $TCID_{50}$ values that may related
209 to viral copy numbers in different ways depending on the viral preparation, or units of copies/µL
210 (1 copy/µL = 1,000 copies/mL) or attomolar quantities (1 attomolar = 602 copies/mL). We
211 therefore suggest that viral copies/mL be used as a universal standard metric, so that cross
212 comparison between assays can readily be made. It is clear that viral load matters, and
213 therefore LoD values should be readily evaluable and in the public domain.

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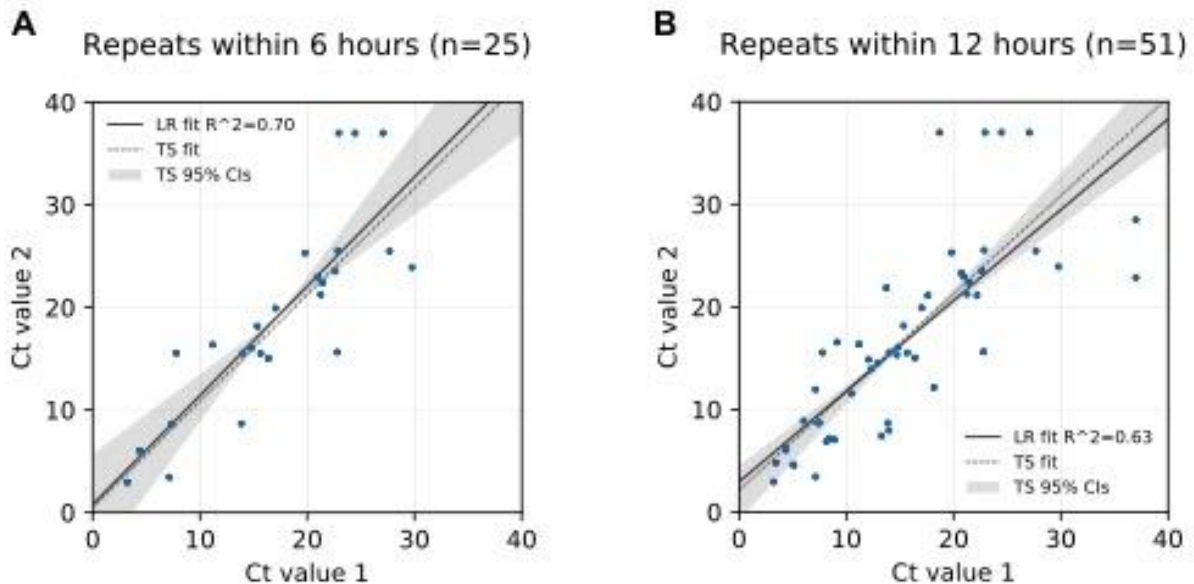
268 **Figure Legends**



269

270 **Figure 1: Ct values are highly repeatable.** Data points shown are Ct values for SARS-CoV-2
271 testing of pairs of nasopharyngeal samples obtained within either 6 hours **(A)** or 12 hours **(B)** or
272 each other from the same patient, represented by the X and Y coordinates of each data point.
273 LR = Linear Regression Fit. TS = Theil-Sen Linear Regression Fit. Shade areas indicate 95%
274 confidence interval for TS fit.

275

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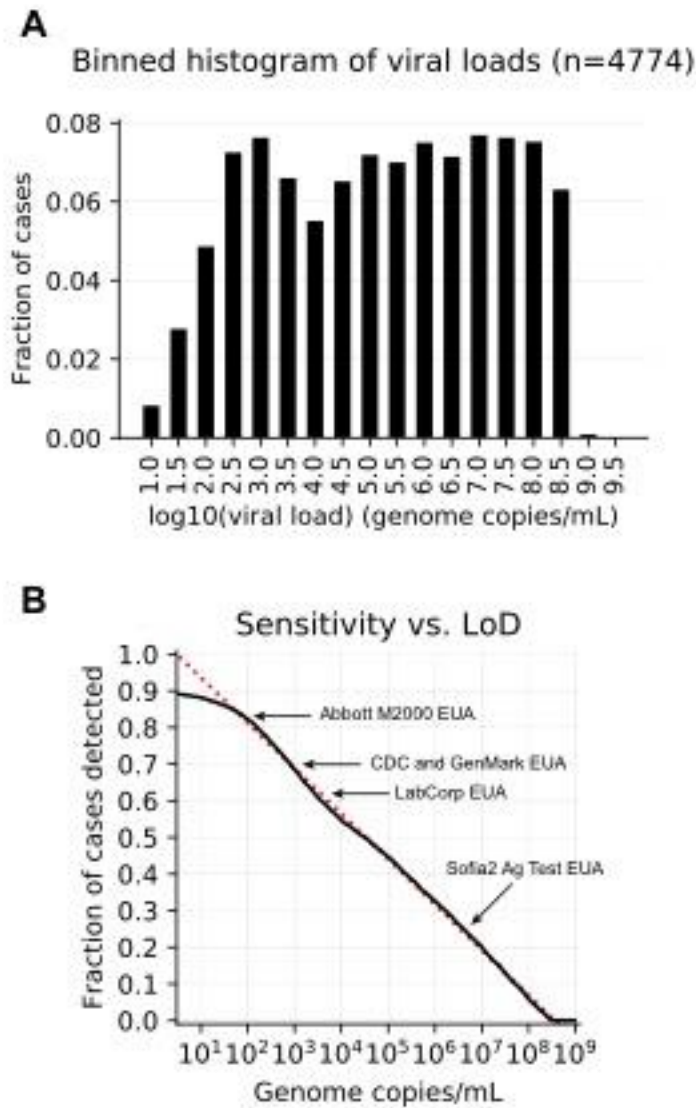
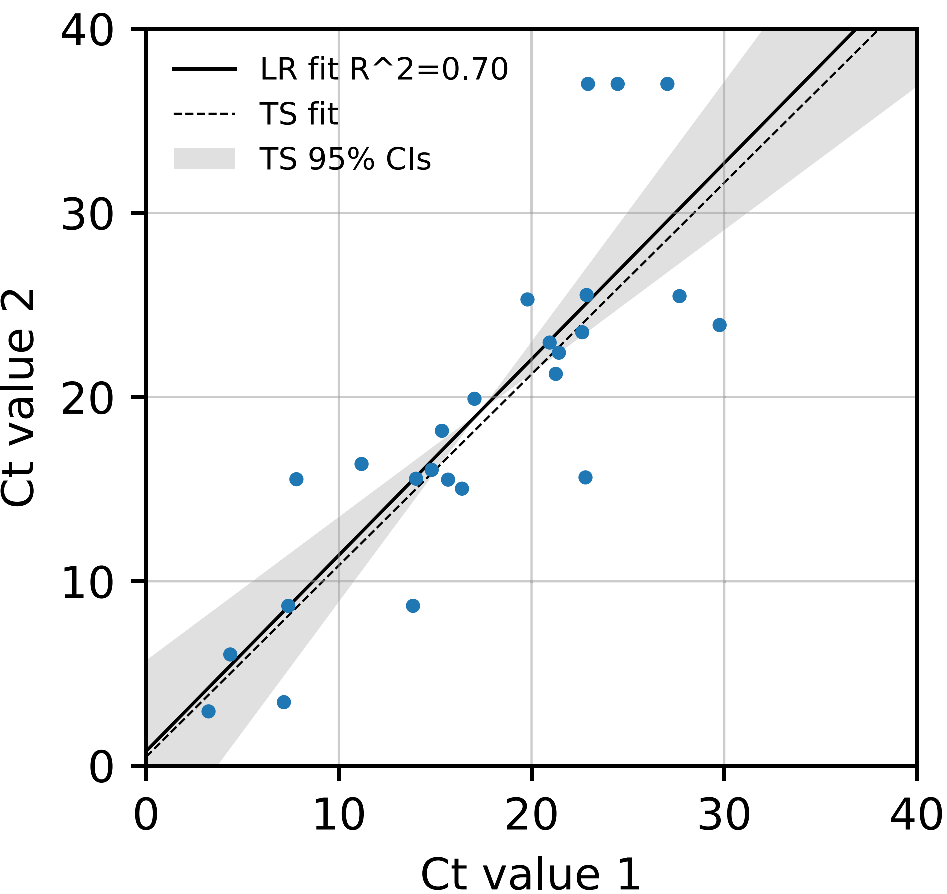
276

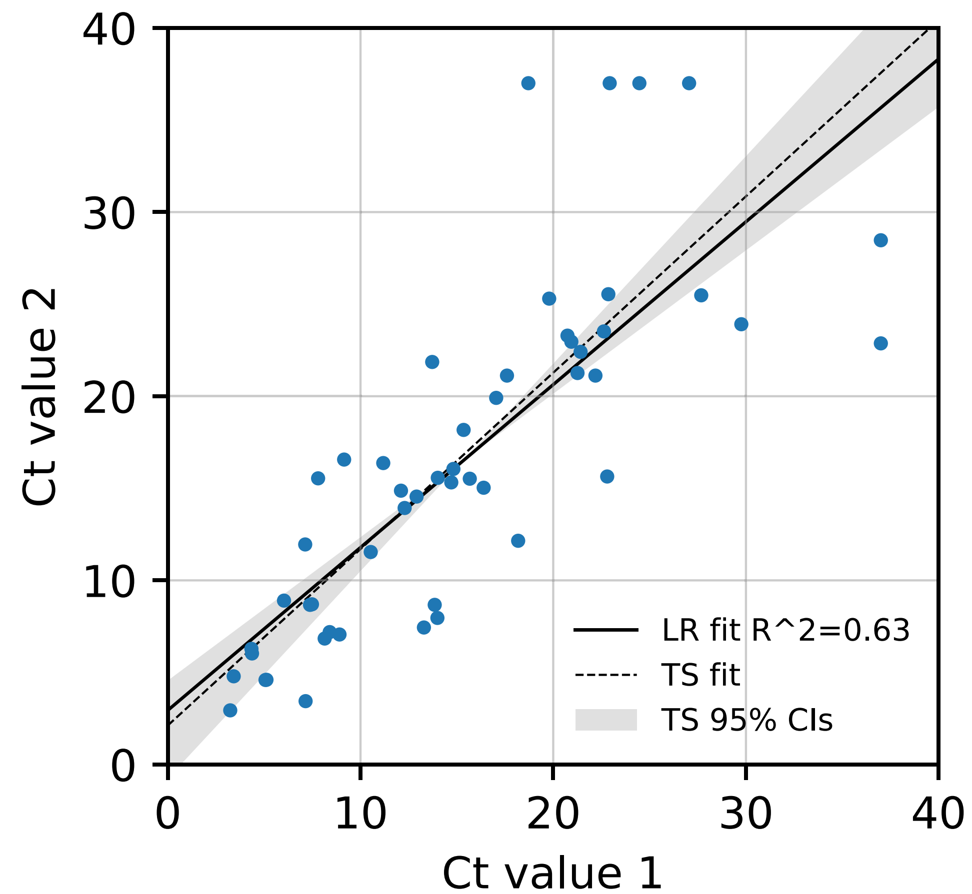


277

278 **Figure 2**: **Viral load distribution and LoD. (A)** Fraction of positive tests binned by 0.5 log10
279 bins of viral load. **(B)** Cumulative histogram distribution of viral loads showing percent detected
280 as a function of limit of detection - actual, solid line, and trend-line, dotted line.

281


A
Repeats within 6 hours (n=25)
LR fit R^2=0.70
TS fit
TS 95% CIs
Ct value 2
Ct value 1
0
10
20
30
40


B
Repeats within 12 hours (n=51)
LR fit R^2=0.63
TS fit
TS 95% CIs
Ct value 2
Ct value 1
0
10
20
30
40

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