

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

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3 Running Title: LoD Matters

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24

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

## 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-](https://www.fda.gov/medical-devices/emergency-situations-medical-devices/emergency-use-authorizations)  
46 [devices/emergency-use-authorizations](https://www.fda.gov/medical-devices/emergency-situations-medical-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  $\geq 95\%$  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<sub>50</sub>, 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.

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

87 includes an internal control. Results are reported as positive if the Ct value is  $\leq 31.5$ , 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  $26.06 \pm 1.03$  (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

111 of interpretation); binned histogram by 0.5 log<sub>10</sub> units, and linear regression on log<sub>10</sub>-  
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

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<sub>50</sub> units, the BEI Resources  
144 control material referenced lists both TCID<sub>50</sub> 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

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.)



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

208 in manners that are often difficult to comprehend, for example, TCID<sub>50</sub> values that may related  
209 to viral copy numbers in different ways depending on the viral preparation, or units of copies/ $\mu$ L  
210 (1 copy/ $\mu$ L = 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.

## 214 **Acknowledgements**

215 K.P.S. was supported by the National Institute of Allergy and Infectious Diseases of the National  
216 Institutes of Health under award number F32 AI124590. The content is solely the responsibility  
217 of the authors and does not necessarily represent the official views of the National Institutes of  
218 Health. We would like to thank the clinical laboratory scientists and volunteers in the Beth Israel  
219 Deaconess Medical Center microbiology laboratory for generating the data used in this  
220 manuscript.

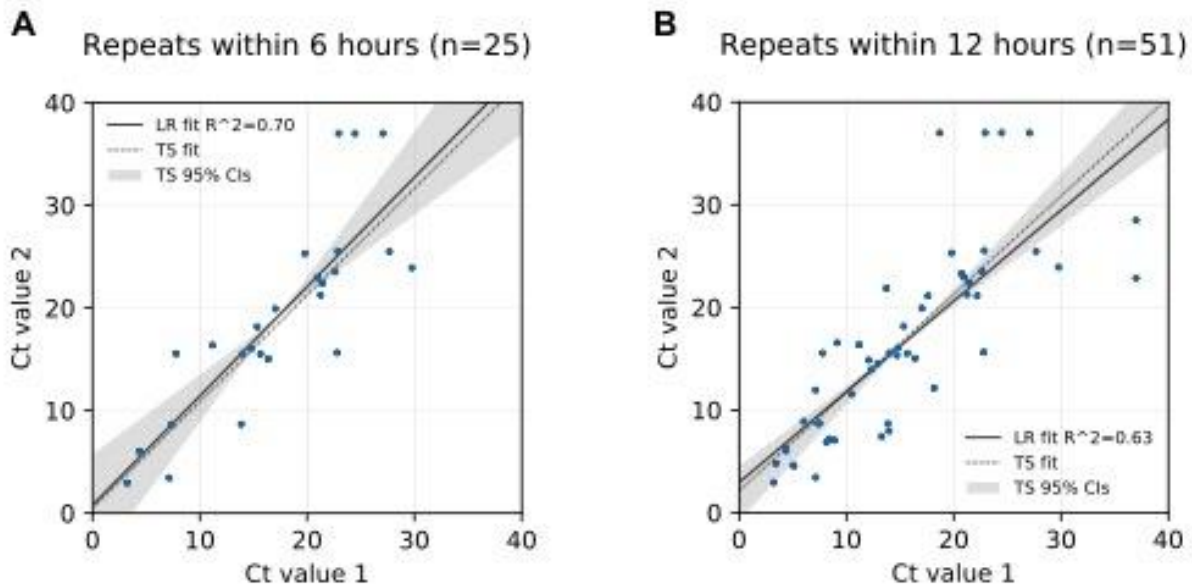
221

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268 **Figure Legends**

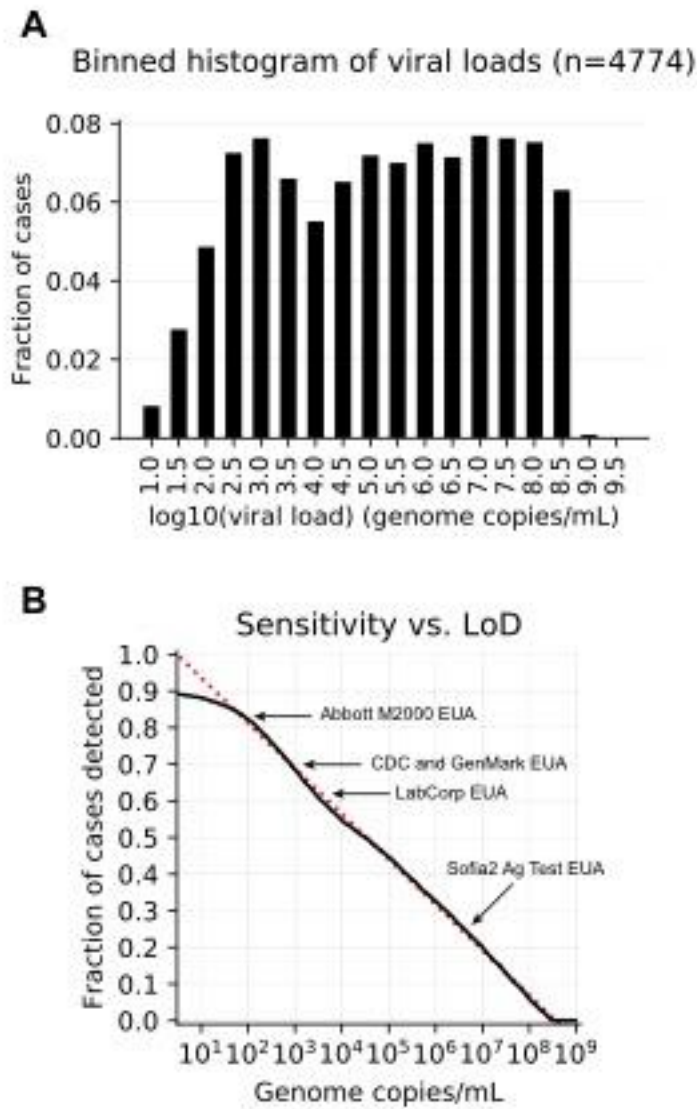


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270 **Figure 1: Ct values are highly repeatable.** Data points shown are Ct values for SARS-CoV-2  
271 testing of pairs of nasopharyngeal samples obtained within either 6 hours (**A**) or 12 hours (**B**) or  
272 each other from the same patient, 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.

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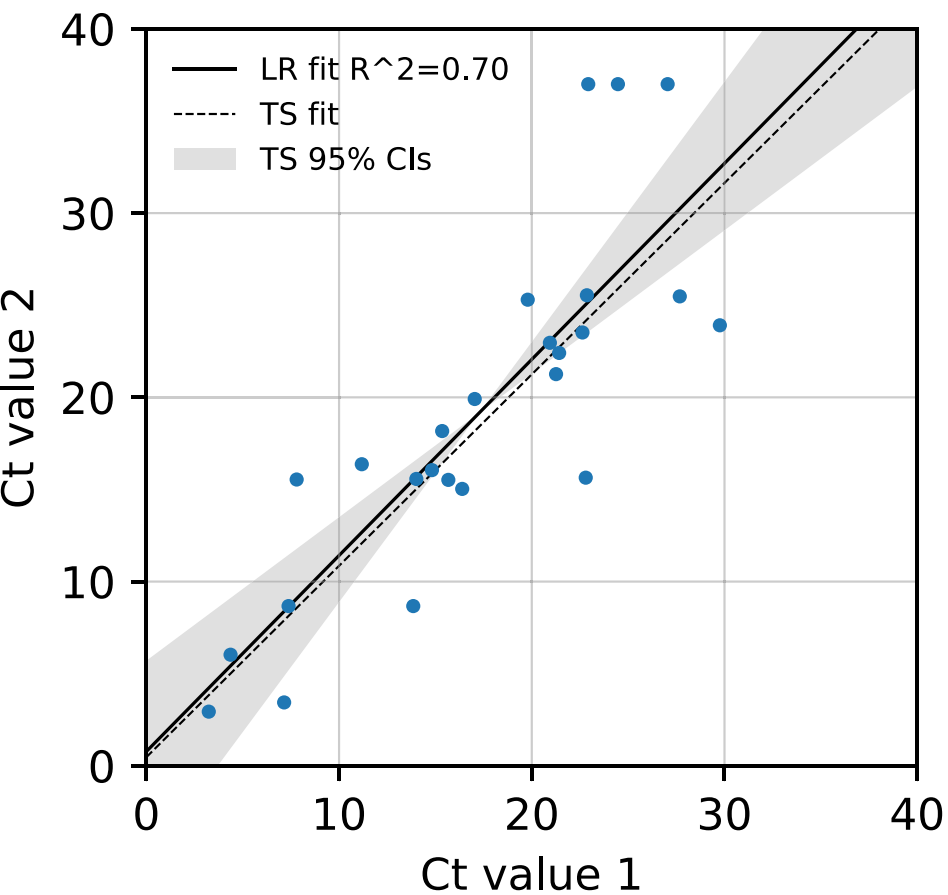


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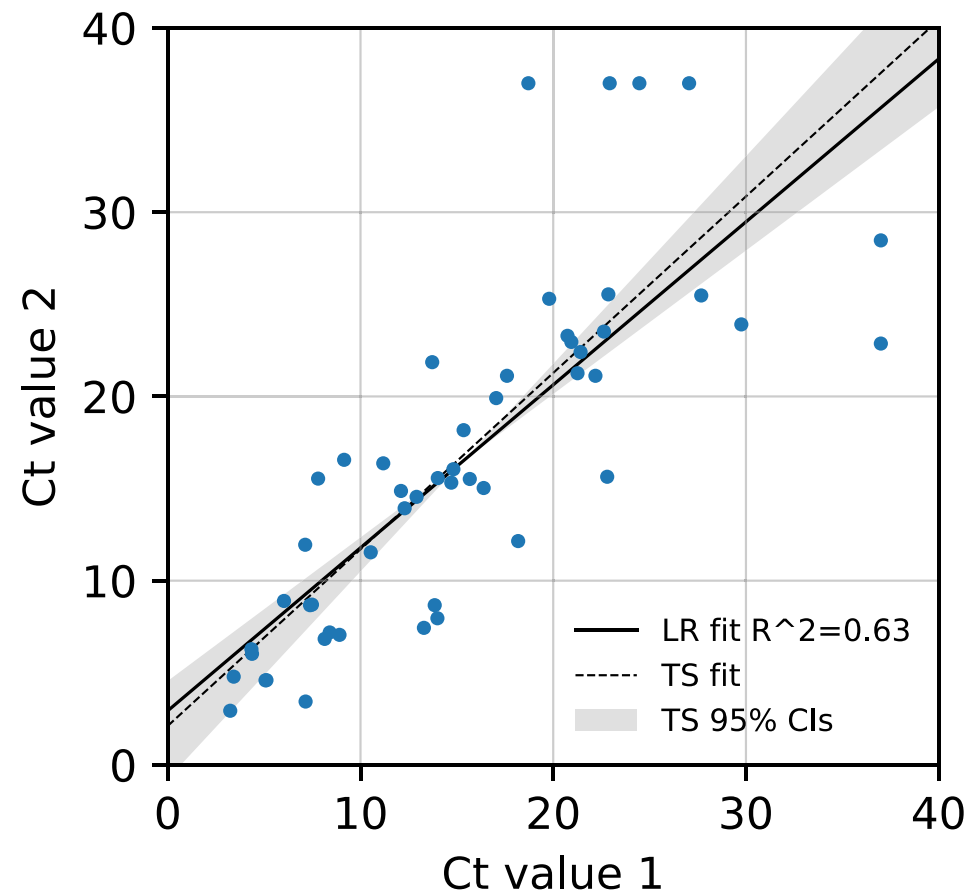
278 **Figure 2: Viral load distribution and LoD. (A)** Fraction of positive tests binned by 0.5 log<sub>10</sub>  
279 bins of viral load. **(B)** Cumulative histogram distribution of viral loads showing percent detected  
280 as a function of limit of detection - actual, solid line, and trend-line, dotted line.

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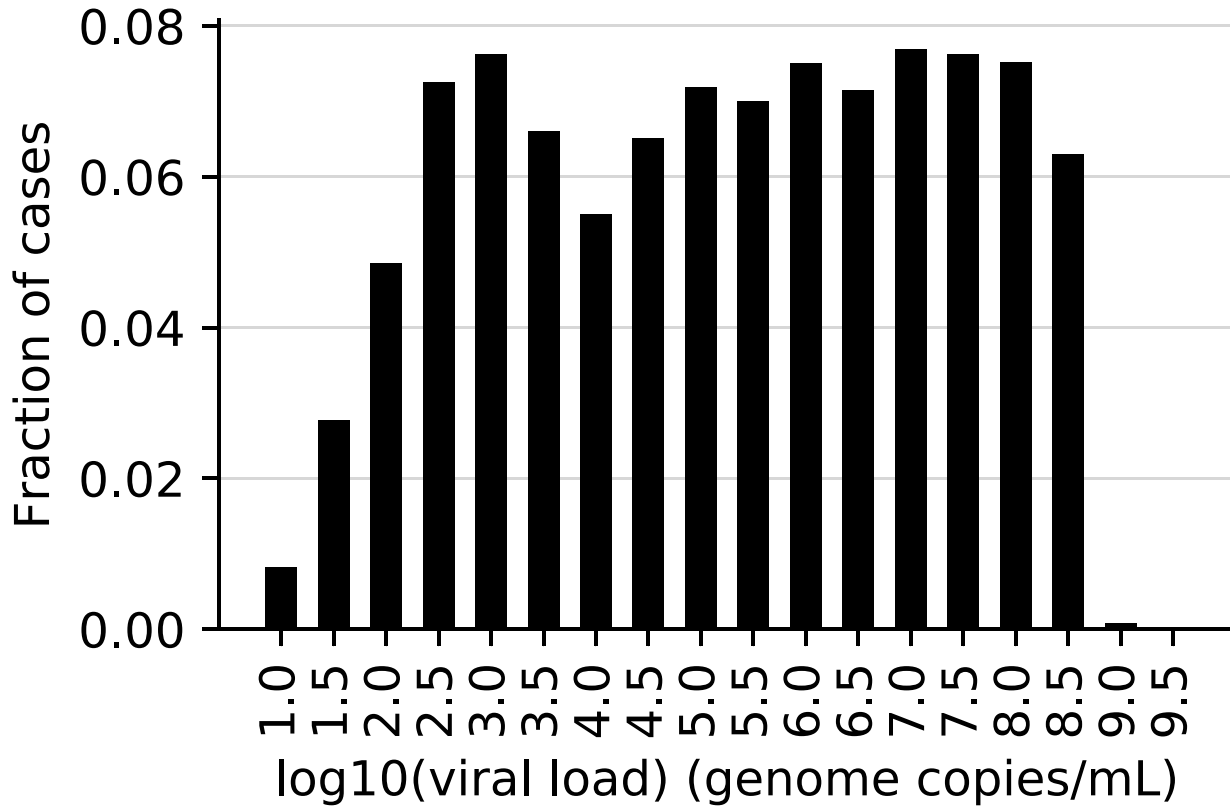
**A** Repeats within 6 hours (n=25)



**B** Repeats within 12 hours (n=51)



## Binned histogram of viral loads (n=4774)



**B**

## Sensitivity vs. LoD

