

Charles S. LiMandri (SBN: 110841)  
 Paul M. Jonna (SBN: 265389)  
 Jeffrey M. Trissell (SBN: 292480)  
 LIMANDRI & JONNA LLP  
 P.O. Box 9120  
 Rancho Santa Fe, CA 92067  
 Telephone: (858) 759-9930  
 Facsimile: (858) 759-9938  
 cslimandri@limandri.com  
 pjonna@limandri.com  
 jtrissell@limandri.com

Harmeet K. Dhillon (SBN:207873)  
 Mark P. Meuser (SBN: 231335)  
 Gregory R. Michael (SBN: 306814)  
 DHILLON LAW GROUP INC.  
 177 Post Street, Suite 700  
 San Francisco, CA 94108  
 Telephone: 415-433-1700  
 Facsimile: 415-520-6593  
 harmeet@dhillonlaw.com  
 mmeuser@dhillonlaw.com  
 gmichael@dhillonlaw.com

Thomas Brejcha, *pro hac vice*\*  
 Peter Breen, *pro hac vice*\*  
 THOMAS MORE SOCIETY  
 309 W. Washington St., Ste. 1250  
 Chicago, IL 60606  
 Telephone: (312) 782-1680  
 tbrejcha@thomasmoresociety.org  
 pbreen@thomasmoresociety.org  
 \*Application forthcoming

Attorneys for Plaintiffs

Attorneys for Plaintiffs

UNITED STATES DISTRICT COURT  
 SOUTHERN DISTRICT OF CALIFORNIA

SOUTH BAY UNITED PENTECOSTAL  
 CHURCH, a California non-profit  
 corporation; and BISHOP ARTHUR  
 HODGES III, an individual,

Plaintiffs,

v.

GAVIN NEWSOM, in his official capacity  
 as the Governor of California, *et al.*,

Defendants.

Case No. 3:20-cv-865-BAS

**Declaration of James Lyons-  
 Weiler in Support of Plaintiffs'  
 Renewed Motion for a  
 Temporary Restraining Order /  
 Preliminary Injunction**

Judge: Hon. Cynthia Bashant

1 I, James Lyons-Weiler, declare as follows:

2 1. My name is James Lyons-Weiler. I am a resident of Allison Park,  
3 Pennsylvania; I am over 18 years-old; and I am otherwise competent to make this  
4 declaration.

#### 5 **PROFESSIONAL BACKGROUND**

6 2. I am currently CEO and Director of The Institute for Pure and Applied  
7 Knowledge in Pittsburgh, PA, a registered not-for-profit organization that conducts  
8 biomedical research in the public interest. I was formerly Senior Research Scientist at  
9 the University of Pittsburgh, where I served as the Scientific Director of the University  
10 of Pittsburgh's Bioinformatics Analysis Core. Prior to that, I had been faculty in the  
11 Departments of Pathology and Biomedical Informatics, where I conducted grant-  
12 funded research, taught courses and advised graduate students and medical and post-  
13 doctoral fellows. I have over 54 peer-reviewed publications and have served as  
14 Associate Editor and Editor-in-Chief on two journals. I currently serve as the  
15 Founding Editor-in-Chief of the journal *Science, Public Health Policy & the Law*. I  
16 append a biosketch (a form a curriculum vitae) should it be helpful to the Court. I  
17 have no stake in the current case, as I am not involved in and do not profit from  
18 vaccine manufacture as a non-worshipping Scientist I hope to bring objectivity to the  
19 considerations being deliberated.

20 3. Plaintiffs contacted me and asked if I would be willing to offer my expert  
21 opinion about the risks associated with in-person worship services with social  
22 distancing. Children are at very low risk of serious or critical illness and death from  
23 SARS-CoV-2 infection, and they do not pose a severe risk of transmission to adults.

24 4. The CDC estimate of the population-wide death rate of COVID-19 is  
25 0.26%.<sup>1</sup> However, the vast majority of this risk is in the elderly and in people whose  
26

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27 <sup>1</sup> USA Today, <https://www.usatoday.com/story/news/factcheck/2020/06/05/fact-check-cdc-estimates-covid-19-death-rate-0-26/5269331002/>  
28

1 overall health has been significantly deteriorated prior to infection (e.g., individuals  
2 with pre-existing chronic pulmonary illness, whose death rate is 6.3%, cardiovascular  
3 illness (10.5%), high blood pressure (6%), adults with diabetes (7.3%), and cancer  
4 (5.6%).<sup>2</sup>

5       5.       However, these frightening rates—which are from the earliest reports in  
6 the outbreak, from Wuhan City in Hubei Province, China—are massively inflated  
7 because they are only symptomatic case fatality rates, not infection case fatality rates.  
8 After the peak infection wave subsided in China, scientists there estimated that as  
9 many as 85% of COVID-19 cases were undetected.<sup>3</sup>

10       6.       Even the attribution of death to COVID-19 is still in question, and it is  
11 unlikely that we will be able to determine an accurate count of those who “died  
12 from” the infection, as opposed to “died with” the infection. In Italy, 99% of deaths  
13 have had a significant comorbid condition.<sup>4</sup> Also, late-stage treatment of patients  
14 critically ill with COVID-19 initially involved ventilator use, which may have  
15 exacerbated the mortality rate. It has been observed that high-pressure “per  
16 protocol” use of ventilators in COVID-19 patients with severe infections, showing  
17 inflammation to the periphery of the alveoli, may cause further tissue damage to the  
18 patients’ already inflamed and thinned-out lung epithelial layers.<sup>5,6</sup>

19       7.       The original overall death rate estimate from COVID-19 – 3% - and the  
20 percentage of overall deaths attributed to COVID-19 – would appear to be a cause of  
21 concern worthy of drastic measures designed to reduce community transmission. We

22 <sup>2</sup> [https://markets.businessinsider.com/news/stocks/coronavirus-age-differences-millennials-](https://markets.businessinsider.com/news/stocks/coronavirus-age-differences-millennials-parents-health-social-distancing-2020-3-1029000036)  
23 [parents-health-social-distancing-2020-3-1029000036](https://markets.businessinsider.com/news/stocks/coronavirus-age-differences-millennials-parents-health-social-distancing-2020-3-1029000036)

24 <sup>3</sup> <https://www.medpagetoday.com/infectiousdisease/covid19/85448>

25 <sup>4</sup> [https://www.bloomberg.com/news/articles/2020-03-18/99-of-those-who-died-from-virus-had-](https://www.bloomberg.com/news/articles/2020-03-18/99-of-those-who-died-from-virus-had-other-illness-italy-says)  
26 [other-illness-italy-says](https://www.bloomberg.com/news/articles/2020-03-18/99-of-those-who-died-from-virus-had-other-illness-italy-says)

27 <sup>5</sup> <https://time.com/5820556/ventilators-covid-19/>

28 <sup>6</sup> [https://www.wsj.com/articles/some-doctors-pull-back-on-using-ventilators-to-treat-covid-19-](https://www.wsj.com/articles/some-doctors-pull-back-on-using-ventilators-to-treat-covid-19-11589103001)  
[11589103001](https://www.wsj.com/articles/some-doctors-pull-back-on-using-ventilators-to-treat-covid-19-11589103001)

1 now know the Infection Case Fatality Rate is about 0.26% (compared to CDC's  
2 "influenza" death rate of 0.1%). There is, however, new information revealing  
3 serious problems with the rates of diagnosis and the rates of attribution of deaths  
4 attributed to COVID-19 – all of which likely caused a per capita diagnosis and death  
5 rate overestimation.

#### 6 **PROBLEMS WITH RATES OF DIAGNOSIS**

7 8. The use of mass screening of populations using the Reverse  
8 Transcriptase Polymerase Chain Reaction (RT-PCR), such as the test used by the  
9 Centers for Disease Control, is problematic. This test serves as the gold-standard  
10 against which other RT-PCR tests are validated. Unfortunately, US CDC's test  
11 appears to be inaccurate. Dr. Sin Hang Lee (Milford, CT) determined the nucleotide  
12 sequence of the PCR product of a test designed to detect RT-PCR. To evaluate the  
13 test, he requested and received clinical samples from patients whose diagnosis had  
14 been confirmed by CDC's PCR test. These samples included "CDC-test-positive"  
15 and "CDC-test-negative" samples. His experiments showed that the SARS-CoV-2  
16 virus PCR products from the two sample sets did not match their labels; some of the  
17 "CDC-test-negative" samples could yield amplified PCR product that matched the  
18 target sequence of the SARS-CoV-2 genome. He also found that some of the "CDC-  
19 test-positive" samples did NOT contain SARS-CoV-2 nucleotide sequence. His  
20 experiments included positive and negative controls, and the sequence from the  
21 "CDC-test-negative" samples actually matched a new mutation strain of SARS-  
22 CoV-2 isolated from NYC. (See *Businesswire*: "CDC Coronavirus Test Kits Generate  
23 False Positive and 20% False Negative Results - Connecticut Pathologist's Newly Published  
24 Findings Confirm.")<sup>7</sup> I append Dr. Lee's peer-reviewed publication "Testing for

25  
26  
27 <sup>7</sup> <https://www.businesswire.com/news/home/20200717005397/en/CDC-Coronavirus-Test-Kits-Generate-30-False>  
28

1 *SARS-CoV-2 in cellular components by routine nested RT-PCR followed by DNA*  
 2 *sequencing*” published July 17, 2020.<sup>8</sup>

3 9. This means that both the rate of diagnosis and the rate of deaths  
 4 attributed to COVID-19 are both now in serious question.

5 **PROBLEMS WITH ATTRIBUTION OF CAUSE OF DEATH**  
 6 **(DIED WITH VS. DIED FROM)**

7 10. Different states have been counting and reporting COVID-19 deaths  
 8 differently. On April 16, 2020, *The Washington Post* reported that in Alabama,  
 9 officials had “ruled that one of every 10 people who died with Covid-19 did not die of  
 10 Covid-19,” contrasting the practice in Colorado, which was counting all deaths  
 11 involving respiratory complications without any type of molecular diagnostic test.<sup>9</sup>

12 11. On April 8, 2020, Dr. Deborah Birx announced that the CDC was  
 13 classifying all deaths of patients who die with coronavirus as “COVID-19 deaths”  
 14 regardless of cause.<sup>10</sup>

15 12. On May 27, 2020, *National Geographic* reported that “Regardless of  
 16 whether a death is due to a car crash or COVID-19, the process remains largely the same”  
 17 when reporting COVID-19 deaths, citing the CDC’s policy that also includes using  
 18 symptom-only diagnosis of COVID-19.<sup>11</sup>

22 <sup>8</sup> [http://www.int-soc-clin-geriat.com/info/wp-content/uploads/2020/03/Dr.-Lees-paper-on-](http://www.int-soc-clin-geriat.com/info/wp-content/uploads/2020/03/Dr.-Lees-paper-on-testing-for-SARS-CoV-2.pdf)  
 23 [testing-for-SARS-CoV-2.pdf](http://www.int-soc-clin-geriat.com/info/wp-content/uploads/2020/03/Dr.-Lees-paper-on-testing-for-SARS-CoV-2.pdf)

24 <sup>9</sup> [https://www.washingtonpost.com/investigations/which-deaths-count-toward-the-covid-19-](https://www.washingtonpost.com/investigations/which-deaths-count-toward-the-covid-19-death-toll-it-depends-on-the-state/2020/04/16/bca84ae0-7991-11ea-a130-df573469f094_story.html)  
 25 [death-toll-it-depends-on-the-state/2020/04/16/bca84ae0-7991-11ea-a130-](https://www.washingtonpost.com/investigations/which-deaths-count-toward-the-covid-19-death-toll-it-depends-on-the-state/2020/04/16/bca84ae0-7991-11ea-a130-df573469f094_story.html)  
 26 [df573469f094\\_story.html](https://www.washingtonpost.com/investigations/which-deaths-count-toward-the-covid-19-death-toll-it-depends-on-the-state/2020/04/16/bca84ae0-7991-11ea-a130-df573469f094_story.html)

26 <sup>10</sup> [https://www.foxnews.com/politics/birx-says-government-is-classifying-all-deaths-of-patients-](https://www.foxnews.com/politics/birx-says-government-is-classifying-all-deaths-of-patients-with-coronavirus-as-covid-19-deaths-regardless-of-cause)  
 27 [with-coronavirus-as-covid-19-deaths-regardless-of-cause](https://www.foxnews.com/politics/birx-says-government-is-classifying-all-deaths-of-patients-with-coronavirus-as-covid-19-deaths-regardless-of-cause)

27 <sup>11</sup> [https://www.nationalgeographic.com/science/2020/05/what-we-need-to-find-true-coronavirus-](https://www.nationalgeographic.com/science/2020/05/what-we-need-to-find-true-coronavirus-death-toll/#close)  
 28 [death-toll/#close](https://www.nationalgeographic.com/science/2020/05/what-we-need-to-find-true-coronavirus-death-toll/#close)

1           13.     On May 15, 2020, *The Washington Examiner* reported that a San Diego  
2 County supervisor reported that only six of 194 “confirmed” COVID-19 deaths to  
3 date at that time were “pure, solely coronavirus deaths.”<sup>12</sup>

4           14.     On July 16, 2020, FOX 35 Orlando reported that the Florida  
5 Department of Health had confirmed that it had counted a death of a victim of a  
6 motorcycle accident as a COVID-19 death.<sup>13</sup>

7           15.     On March 18, 2020, *Bloomberg* reported that 99% of people who died  
8 from the SARS-CoV-2 virus in Italy had other illnesses.<sup>14</sup>

9           16.     Verified reports of individuals being sent positive test responses but  
10 having never been tested exist;<sup>15</sup> most these are individuals in queue for testing who  
11 leave the queue, leading to incorrectly paired test results for themselves and everyone  
12 in queue following them.

13           17.     The complications of testing accuracy on top of the lack of consistent  
14 diagnosis and attribution of death makes assessment of the actual risk of death due to  
15 coronavirus difficult if not impossible.

16           18.     In May, 2020, a *Medpage* article proposed that excess deaths would  
17 provide an accurate estimate,<sup>16</sup> but we now know that deaths due to non-specific  
18 effects of society’s response to COVID-19 outnumber the deaths that might be due to  
19 COVID-19: deaths from suicides due to job loss and social isolation and deaths from  
20 lack of access to healthcare.

21  
22 <sup>12</sup> [https://www.washingtonexaminer.com/news/san-diego-county-supervisor-says-six-of-194-](https://www.washingtonexaminer.com/news/san-diego-county-supervisor-says-six-of-194-confirmed-deaths-are-pure-solely-coronavirus-deaths)  
23 [confirmed-deaths-are-pure-solely-coronavirus-deaths](https://www.washingtonexaminer.com/news/san-diego-county-supervisor-says-six-of-194-confirmed-deaths-are-pure-solely-coronavirus-deaths)

24 <sup>13</sup> [https://www.fox35orlando.com/news/fox-35-investigates-questions-raised-after-fatal-](https://www.fox35orlando.com/news/fox-35-investigates-questions-raised-after-fatal-motorcycle-crash-listed-as-covid-19-death)  
25 [motorcycle-crash-listed-as-covid-19-death](https://www.fox35orlando.com/news/fox-35-investigates-questions-raised-after-fatal-motorcycle-crash-listed-as-covid-19-death)

26 <sup>14</sup> [https://www.bloomberg.com/news/articles/2020-03-18/99-of-those-who-died-from-virus-had-](https://www.bloomberg.com/news/articles/2020-03-18/99-of-those-who-died-from-virus-had-other-illness-italy-says)  
27 [other-illness-italy-says](https://www.bloomberg.com/news/articles/2020-03-18/99-of-those-who-died-from-virus-had-other-illness-italy-says)

28 <sup>15</sup> [https://www.mysuncoast.com/2020/07/19/concerns-arise-some-receive-positive-covid-results-](https://www.mysuncoast.com/2020/07/19/concerns-arise-some-receive-positive-covid-results-never-got-tested/)  
[never-got-tested/](https://www.mysuncoast.com/2020/07/19/concerns-arise-some-receive-positive-covid-results-never-got-tested/)

<sup>16</sup> <https://www.medpagetoday.com/blogs/working-stiff/85925>

19. It is now understood that deaths in the US and elsewhere from COVID-19 are restricted to individuals that have potential indicators of expected severity. The medical literature of factors that place individuals at risk of serious illness and mortality from COVID-19 include an altered immune system (specifically Th2-skewed immunity vs. Th1/Th2 balanced immunity).<sup>17</sup> Patients who progress to serious COVID-19 in need of critical care undergo a type of inflammatory response known as a cytokine storm, involving cytokines IL-1 $\beta$ , TNF- $\alpha$  and IL-6.

20. Clinical care does not yet make use of an assay to determine patients' Th2/Th1 balance, but a combined risk model that includes comorbidity, age and Th2/Th1 cell counts could be useful in predicting the clinical course of SARS-CoV-2 infections, reducing mortality and streamlining resource allocation. This means we can expect dramatically improved clinical care of COVID-19 patients and lower serious illness and death as effective and tailored treatments come online.

#### CURRENT CASE FATALITY RATES AND INFECTION CASE FATALITY RATES ARE DROPPING

21. Although immunity to SARS-CoV-2 following infection was originally in doubt due to waning presence of antibodies in people previously infected,<sup>18</sup> this concern is likely overblown; long-term immunity of COVID-19 is now suspected to involve memory B-cell and T-cell immunity, like other viruses. The very best resource available on this complex issue of antibody vs. T- and B-cell immunity for the layperson is the July 20th, 2020 article in *The Atlantic* by Mr. Derek Thompson.<sup>19</sup> In short, the fear over re-infection is false, anecdote-based and rejected by the absences of millions of repeat diagnoses of COVID-19 among those previously

<sup>17</sup> <https://www.biocentury.com/article/304604/how-covid-19-vaccine-developers-can-lower-the-risk-of-being-derailed-by-antibody-dependent-enhancement>

<sup>18</sup> <https://www.reuters.com/article/us-health-coronavirus-antibodies-vaccine-idUSKCN24F261>

<sup>19</sup> <https://www.theatlantic.com/ideas/archive/2020/07/could-covid-19-immunity-really-disappear-months/614377/>



1 infected.<sup>20</sup> Due to a surge in testing, more asymptomatic COVID-19 “cases” are  
 2 being detected. Cross-protection from prior exposure to coronaviruses, including the  
 3 common cold, is now highly suspected to already have conferred immunity from  
 4 COVID-19 symptomatic disease. The cross-protection may be so effective that in the  
 5 UK, herd immunity may already have been achieved.<sup>21</sup> Herd immunity is general  
 6 immunity in the population achieved when a sufficient number of individuals are  
 7 immune, either through natural infection or via vaccination to cause viral  
 8 transmission to end. One estimate from the UK is that only 10% infection in a  
 9 population might provide herd immunity due to such high prior cross-protection due  
 10 to exposure to other coronaviruses.<sup>18</sup> Eventually, with or without a vaccine, the  
 11 population will achieve herd immunity, and during that process, the vulnerable –  
 12 those with comorbid conditions, those with Th2-skewed immunity, and the elderly,  
 13 should be cautious about exposure and become educated and ask their doctors for  
 14 recently reported, safer, potentially highly effective treatment options.<sup>22,23,24</sup>

15       22. The only elevated risks to healthy adults appear to be a hypothetical  
 16 possibility of increased risk of miscarriage if a woman is pregnant and becomes  
 17 infected with SARS-CoV-2, and a potential increased risk of hospitalization relative  
 18 to the full population if pregnant.<sup>25</sup> However, CDC has supported (without FDA  
 19 approval) the use of both the influenza vaccine and the Tdap vaccine during  
 20 pregnancy. A study has found that military personnel who received the flu vaccine  
 21

22 \_\_\_\_\_  
 23 <sup>20</sup> <https://www.sciencedirect.com/science/article/pii/S1521661620305106>

24 <sup>21</sup> <https://www.dailymail.co.uk/news/article-8532935/Britain-herd-immunity-against-coronavirus.html>

25 <sup>22</sup> <https://academic.oup.com/jid/article/191/12/2033/839720>

26 <sup>23</sup> <https://www.mediterranean-infection.com/wp-content/uploads/2020/03/COVID-IHU-2-1.pdf>

27 <sup>24</sup> <https://www.publichealthpolicyjournal.com/clinical-and-translational-research>

28 <sup>25</sup> <https://www.cnn.com/2020/06/25/health/coronavirus-pregnant-risks-cdc-study/index.html>



1 had 36 percent increased risk for coronavirus.<sup>26</sup> Other studies support the conclusion  
 2 of increased risk of viral infections, especially respiratory viral infections – following  
 3 the influenza shot.<sup>27,28,29</sup> According to the chief medical officer for England, people in  
 4 the UK who have received the influenza vaccine for this flu season were cautioned to  
 5 self-isolate for 12 weeks because they fall into the government’s “high risk” category  
 6 for acquiring a SARS-CoV-2 infection.<sup>30</sup> This “disease enhancement” from a  
 7 vaccine is known as “pathogenic priming”<sup>31</sup> which is expected to occur with  
 8 vaccination against SARS-CoV-2 coronavirus due to its occurrence in animal trials of  
 9 vaccines against SARS-CoV-1 and MERS (Middle Eastern Respiratory Syndrome  
 10 Virus). This may occur in part due to antibody dependent enhancement. It has  
 11 inaccurately been referenced as “immune enhancement.”

12 23. Contrary to the representation of risk in the mainstream media, there is  
 13 minimal risk to adults and children attending worship services due to Coronavirus.  
 14 Women receiving vaccinations during pregnancy may be at higher risk to  
 15 complications if they are vaccinated and become infected with SARS-CoV-2, but this  
 16 is only a hypothetical risk unsupported by data.

### 17 THE SWEDEN MODEL

18 24. For some time, the world has had their eye on Sweden, which never  
 19 used “lock-down” as a tool to fight SARS-CoV-2. Instead, they created special  
 20 protections for those at highest risk of death and serious illness – the elderly, people  
 21 with diabetes, and people with cardiovascular disease. Schools and churches

22 \_\_\_\_\_  
 23 <sup>26</sup> <https://www.sciencedirect.com/science/article/pii/S0264410X19313647?via%3Dihub>

24 <sup>27</sup> <https://www.sciencedirect.com/science/article/pii/S0264410X18303153>

25 <sup>28</sup> <https://insights.ovid.com/pediatric-infectious-disease/pidj/2011/02/000/vaccine-effectiveness-against-laboratory-confirmed/4/00006454>

26 <sup>29</sup> <https://academic.oup.com/cid/article/54/12/1778/455098>

27 <sup>30</sup> <https://www.mirror.co.uk/news/uk-news/coronavirus-top-medic-warns-anyone-21708701>

28 <sup>31</sup> <https://www.sciencedirect.com/science/article/pii/S2589909020300186?via%3Dihub>

1 remained open, allowing the virus to spread throughout the population, conferring  
 2 immunity to those who acquired the infection. Countries (like ours) that did employ  
 3 lock-down are now experiencing a “second surge.” Part of the surge in cases is due to  
 4 more aggressive testing, and it is known that Sweden’s increase in cases in June  
 5 coincided with more aggressive testing. While cases in Sweden are not zero, they are  
 6 not experiencing any increase in COVID-19 deaths.

7 25. *US News and World Report* outlined on July 7, 2020 that how, after a  
 8 false “surge” in cases due to increased testing in June 2020, during which the  
 9 number of deaths due to COVID-19 continued to fall, the Swedes reported the lowest  
 10 daily new case report since May.<sup>32</sup>

11 26. *Euromomo* reports that this, the 32<sup>nd</sup> week of 2020 (first week of  
 12 August), Sweden has no excess deaths – this achieved without a lock-down.<sup>33</sup>

13 27. Initially, Sweden did limit gatherings in churches to 20 people at a time,  
 14 but *America Magazine* cites “voluntary social distancing, appropriate hygiene and  
 15 self-isolation by members of high-risk groups” as the secret to their success.<sup>34</sup>  
 16 Sweden’s  $R_0$ , the basic reproduction number, has been  $<1$  for over a month, meaning  
 17 they are benefiting from herd immunity without a vaccine- and without draconian  
 18 physical isolation measures.

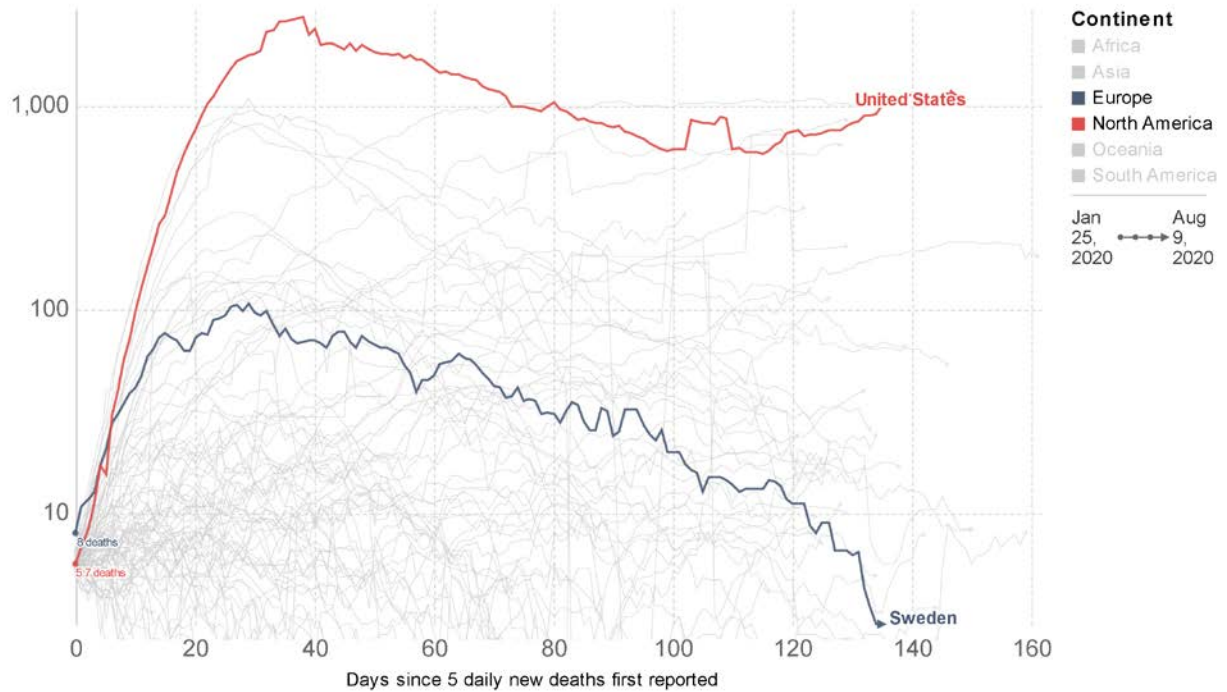
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 25 <sup>32</sup> <https://www.usnews.com/news/world/articles/2020-07-07/swedens-daily-tally-of-new-covid-19-cases-falls-to-lowest-since-may>

26 <sup>33</sup> <https://www.euromomo.eu/graphs-and-maps>

27 <sup>34</sup> <https://www.americamagazine.org/politics-society/2020/05/25/how-parish-sweden-responds-coronavirus-it-will-all-work-out>  
 28

## Daily new confirmed COVID-19 deaths

Shown is the rolling 7-day average. Limited testing and challenges in the attribution of the cause of death means that the number of confirmed deaths may not be an accurate count of the true number of deaths from COVID-19.



Source: European CDC – Situation Update Worldwide – Last updated 9 August, 10:33 (London time), Our World In Data

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**Figure 1. Comparison of numbers of deaths with United States and Sweden highlighted. Note the log scale and absolute numbers. Data Source: Our World in Data.**

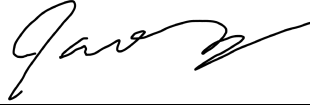
28. In the US, numerous nodes of transmission have been attributed to in-person church services, however, not all have been confirmed via contact tracing. The transmission of the virus in a church service can be minimized by appropriate social distancing, as is done in restaurants, and modifications to church services like mass via preparation of individual servings of ingested sacrament. More importantly, those at high risk of serious illness or death from COVID-19 should be protected via appropriate actions taken at the level of the individual citizen, who should know whether they have likely been exposed to SARS-CoV-2 as the population moves toward herd immunity via community transmission. It is worth keeping in mind that medical procedures, not COVID-19, were likely the cause of the majority of deaths

29. The increase in cases in the US are not as large of a concern as they were in the beginning of the pandemic. With an infection case fatality rate estimated by independent means and by CDC at 0.26% that is falling fast, COVID-19 is not the monster we initially thought it was. The overall case fatality rate was overestimated by an order of magnitude due to insufficient testing. We now know that many people have immunity to COVID-19 due to prior exposure to other coronaviruses; this immunity is so valuable that the US government is spending billions of dollars in a bid to create a vaccine for COVID-19, a venture fraught with its own slate of serious problems, not the least of which is the high likelihood of pathogenic priming (disease enhancement) caused by secondary exposure to viral proteins following vaccination.

30. Given that herd immunity seems the most likely outcome ending the transmission of the virus, and that other segments of society are allowed to assemble (protestors, shoppers, diners, etc.) the special, undue and ineffectual burden placed upon religious members of society who would otherwise be free to worship as they see fit, the harm visited upon the co-worshipping citizens of California exceeds those required of other identifiable categories of people. This arbitrary and limited physical isolation would seem to merely delay the biological inevitability.

1 spite of the cost of the investment in COVID-19 vaccines that any will be both  
2 sufficiently effective and sufficiently safe to act as a proxy for the otherwise inevitable  
3 herd immunity that need not come at the significant cost of human life given  
4 treatment protocols that are proving far more effective than earlier deadly medical  
5 protocols. A rational response to COVID-19 will not dismantle the foundations of  
6 freedom we have inherited intact from prior generations, which includes the rights to  
7 peaceable assemble, freedoms of expression, and the freedom of religion.

8 I declare until penalty of perjury under the laws of the United States and the  
9 State of California that the foregoing is true and correct. Executed on August 10,  
10 2020.



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12 James Lyons-Weiler  
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**EXHIBIT 1**

Program Director/Principal Investigator (Last, First, Middle):Lyons-Weiler, James

**BIOGRAPHICAL SKETCH**

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.  
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME James Lyons-Weiler	POSITION TITLE CEO/President/Director/Scientist Institute for Pure and Applied Knowledge		
eRA COMMONS USER NAME (credential, e.g., agency login) jameslyonsweiler			
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	MM/YY	FIELD OF STUDY
State University of New York, Oswego, NY	B.A.	8, 1991	Biology
Ohio State University	M. Sc.	6, 1993	Zoology
The University of Nevada, Reno	Ph.D.	6, 1998	Ecology, Evol & Conservation Biology
Pennsylvania State University	Postdoctoral	2000	Computational Molecular Biology

**A. Personal Statement**

My research program is focused on improving healthcare via the development of treatment efficacy and risk biomarkers, optimizing clinical interventions after adverse events, and understanding the neurological and immunological basis of vaccine injuries. I have been performing collaborative research with DNA sequence data since 1996, microarray gene expression data since 1998, proteomic data since 2003, and next generation sequencing since 2009 in cancer, immunology and neurobiology. I have participated in program-level consortia including the NCI's Early Detection Research Network, caBIG, and managed a large multi-institutional consortium in Proteomics and Bioinformatics (funded via TATRC). With a deep understanding of the effects of data preprocessing algorithms on the validity of downstream statistical inferences, and fundamental comprehension of principles of ecology and evolutionary theory, I have developed novel paradigms for high-dimensional -omic analyses, including advances in statistical methods for variant calling, transcriptional profiling, machine-learning based prediction modeling, integrative translational research via decision modeling, covariate matching for case/control selection, and for the statistical evaluation of survivorship prediction models. These advances are applied via intelligent methods optimization, in which empirically optimized data analysis protocols are arrived at routinely via systematic comparative evaluative methodological research & development to insure reproducibility. I have directed the successful analysis of at least twenty-five Next Generation Sequence data sets with various applications from whole microbial and mouse genome sequencing to from various platforms for transcriptomics, variant detection, ChipSeq, methylomics, and whole genome/exome sequencing. The algorithms I have developed are current in use by many labs as they provide information measures reflective of both data quality and algorithm performance using objective evaluation measures. I have written books on Ebola, Translational Research and Autism. I founded the Institute for Pure and Applied Knowledge to conduct biomedical and translational research in the public interest.

**Positions and Honors**

1998-2000 AP Sloan/US Department of Energy Postdoctoral Fellow in Computational Molecular Biology  
 1998-2001 (Awarded but declined) NIH National Research Service Award in Bioinformatics  
 2000-2002 Assistant Professor, University of Massachusetts, Lowell, Department of Biological Sciences  
 2000-2002 Co-Director, Center for Bioinformatics and Computational Biology, University of Massachusetts, Lowell  
 2002-2007 Assistant Professor, University of Pittsburgh School of Medicine, Department of Biomedical Informatics; Core Faculty Member, Biomedical Informatics Training Program; Department of Pathology; Fully Associated Faculty Member, University of Pittsburgh Cancer Institute  
 2007-2014 Scientific Director, Bioinformatics Analysis Core Service, University of Pittsburgh; Adjunct Assistant Professor, Department of Biomedical Informatics, University of Pittsburgh  
 2014-present CEO/Director/Scientist, Institute for Pure and Applied Knowledge



Program Director/Principal Investigator (Last, First, Middle):Lyons-Weiler, James

### Other Experience and Professional Membership

2019-present Editor in Chief, *Science, Public Health Policy & the Law*

2016-2019 Editorial Board, *Cancer Research*

2004-present Member, *Society for Experimental Biology*

2004-2006 Associate Editor, *Applied Bioinformatics*

2006-2007 Founding Editor-in-Chief, *Cancer Informatics*

2002-2012 NIH Grant reviewer

2008-2010. Grant Review Committee, New Jersey Commission on Cancer Research

2009 Member, The Society for Clinical and Translational Science

### B. Selected Peer-Reviewed Publications

#### Most relevant to the current application

**Lyons-Weiler J.** 2020. Pathogenic Priming Likely Contributes to Serious and Critical Illness and Mortality in COVID-19 via Autoimmunity. *J Transl Autoimmun.* 2020 Apr 9:100051. doi: 10.1016/j.jtauto.2020.100051.

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**EXHIBIT 2**

# Testing for SARS-CoV-2 in cellular components by routine nested RT-PCR followed by DNA sequencing

Sin Hang Lee <sup>1\*</sup>

<sup>1</sup> Affiliation

Milford Molecular Diagnostics Laboratory, 2044 Bridgeport Avenue, Milford, CT 06460, U.S.A.

\* Correspondence: [shlee01@snet.net](mailto:shlee01@snet.net)

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**Abstract:** Currently, molecular tests for SARS-CoV-2 infection are primarily based on reverse transcription-quantitative polymerase chain reaction (RT-qPCR) on cell-free fluid samples of respiratory tract specimens. These tests measure the rate of fluorescent signal accumulation as a surrogate for direct DNA sequence determination and are known to generate false-negative and false-positive results. The author has developed a routine protocol to test the cellular components of respiratory tract specimens instead of cell-free fluids only and to use conventional nested RT-PCR to amplify the target nucleic acid for high detection sensitivity. A 398-bp heminested PCR amplicon is used as the template for direct DNA sequencing to ensure no false-positive test results. Using this protocol to re-test 20 reference samples prepared by the Connecticut State Department of Public Health, the author found 2 positives among 10 samples classified as negative by RT-qPCR assays. One of these two positive samples contained a mutant with a novel single nucleotide insertion in the N gene and a wild-type parental SARS-CoV-2. Of the 10 samples classified as positive by RT-qPCR assays, only 7 (7/10) were confirmed to contain SARS-CoV-2 by heminested PCR and DNA sequencing of a 398-bp amplicon of the N gene. One of the latter 7 positive SARS-CoV-2 isolates belongs to a newly discovered mutant first isolated from a specimen collected in the State of New York on March 17, 2020, according to information retrieved from the GenBank database. Routine sequencing of a 398-bp PCR amplicon can categorize any isolate into one of 6 clades of SARS-CoV-2 strains known to circulate in the United States. The author proposes that extremely accurate routine laboratory tests for SARS-CoV-2 be implemented as businesses attempt to return to normal operation in order to avoid raising false alarms of a re-emerging outbreak. False-positive laboratory test reports can easily create unnecessary panic resulting in negative impacts on local economies.

**Keywords:** Nested RT-PCR; DNA sequencing; cellular components; SARS-CoV-2; false-negative RT-qPCR; false-positive RT-qPCR; single nucleotide insertion; 398-base; mutant; parental virus

## 1. Introduction

Accurate diagnosis and isolation of infectious patients without delay are the key steps in reducing the spread of emerging highly contagious diseases, like COVID-19. False-negative laboratory test results allow infected patients with mild clinical symptoms to spread SARS-CoV-2 among susceptible persons. False-positive test results may lead to placement of non-infected persons in the same isolation rooms with COVID-19 patients; eventually the non-infected individuals may become true-positive patients.

The lack of timely appropriate response from the Centers for Disease Control and Prevention (CDC) to the need of an accurate laboratory test for SARS-CoV-2 has been reported to be a factor contributing to the pandemic of COVID-19 in the United States in 2020 [1]. On February 2, 2020 the CDC was the only

place in the country that could perform SARS-CoV-2 tests. But on February 12, the CDC announced that the test was providing inconclusive results. By then, the United States had reported 11 COVID-19 cases.

The CDC designed its own test in March 2020. The Food and Drug Administration (FDA) picked a conservative testing strategy, allowing laboratories to use only the CDC test kits distributed under the Emergency Use Authorization (EUA) authority. When the CDC test kits failed by generating many false-positive results, neither a new strategy nor a new test was available for more than two weeks [1]. Eventually, the pandemic COVID-19 spread widely and rapidly throughout the country [2].

After an investigation, the lawyers from Department of Health and Human Services of the U. S. government concluded that the faulty CDC test kits were likely contaminated due to “time pressure” [3].

However, independent scientists have reported that at least two sets of the N primers used in the CDC test kits were found to give false positive signals even in the absence of cDNA (no template control condition) [4], indicating possible faulty designs. Yet from February 4 to July 7, 2020, the FDA issued 104 individual EUAs for Molecular Diagnostic Tests for SARS-CoV-2 to device manufacturers [5]. These commercial devices are almost all based on RT-qPCR using the CDC primers and probes or using undisclosed primers and probes which cannot be independently verified.

One group of scientists in Australia tested a commercial RT-qPCR test kit and found its positive predictive value to be only 55.56%. The authors suggested that any positive results derived from one commercial test kit should be confirmed using another nucleic acid test or nucleotide sequencing [6].

Another attempt to mitigate false-positive results generated by commercial RT-qPCR kits was to develop more conventional PCR-based protocols, using the primers of the RT-qPCR kits or newly developed primers, and apply a multiplex PCR-based protocol that allowed the simultaneous testing of primer sets for RdRP, N, E, and S genes all in one reaction [7]. However, the authors offered no means to verify the various PCR products. In diagnostic virology, the optimization of multiplex PCRs can pose several difficulties, including poor sensitivity or specificity and/or preferential amplification of certain specific targets. The presence of more than one primer pair in the multiplex PCR increases the chance of obtaining spurious amplification products, primarily because of the formation of primer dimers. These nonspecific products may be amplified more efficiently than the desired target, consuming reaction components and producing impaired rates of annealing and extension [8].

False-positive RT-qPCR for SARS-CoV-2 in preoperative screening has caused unnecessary delay of urgent otolaryngology surgeries in a university hospital [9].

When a false-negative RT-qPCR for SARS-CoV-2 is generated, the tendency is to lay blame on faulty specimen collection and handling [10]. But the cause may be more complex.

The RT-qPCR test kits distributed by the CDC, also known as the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel [11], depend on using commercial viral RNA purification devices for sample preparation. Nasopharyngeal or oropharyngeal samples should be obtained by using a Nylon flocked swab, if available, to enhance the collection and release of sufficient human cellular material to be tested.

However, these commercial viral RNA preparation kits, for example, the QIAamp® Viral RNA Mini Kit (Qiagen), are for purification of viral RNA from 180 µL of plasma, serum, cell-free body fluids and culture supernatants. Samples collected in viral transport media or saline need to be centrifuged or filtered



to remove the cellular components for proper viral RNA sample preparation [12]. The Roche DNA and Viral NA Small Volume Kit is for purifying nucleic acids from up to 200  $\mu$ L sample volumes using the MagNA Pure 96 Instrument.

SARS-CoV-2 must grow and replicate in a living cell. The viral particles are primarily located in intra-cytosolic vacuoles of the infected cell [13]. The number of SARS-CoV-2 particles per infected cell has not been published. But according to the studies on other viruses, such as the human immunodeficiency virus (HIV) [14] and the human papillomavirus (HPV) [15], one infected cell in a patient's specimen may contribute several thousands of copy numbers of viral genome equivalents to be tested. Therefore, testing the cellular components of a nasopharyngeal swab rinse or bronchoalveolar lavage rather than the cell-free supernatant may raise the detection sensitivity of nucleic acid-based tests for molecular diagnosis of SARS-CoV-2 infection. An additional advantage of testing the host cells for virus is to assure that a positive result is an indication of having detected viral particles capable of causing infection, not merely non-infective free viral RNA residues floating in the extracellular fluid.

Nucleic acid-based diagnostics for infectious diseases are designed to determine the nucleotide sequence in a unique segment of the genome of the pathogen. Methodologies available to diagnostic laboratories for sequence determination include restriction fragment length polymorphism (RFLP), probe hybridization, and direct DNA sequencing.

The CDC RT-qPCR assay panel uses TaqMan® probes to bind 2 or 3 target DNA sequences if such sequences are present in the sample. The probes are labeled at the 5'-end with a fluorescent dye reporter molecule 6-carboxyfluorescein (FAM) and with the quencher, Black Hole Quencher 1 (BHQ-1) at the 3'-end. The principle of this test relies on the 5'–3' exonuclease activity of *Taq* polymerase to cleave and degrade the dual-labeled probes which have annealed to the complementary target sequences during hybridization. Degradation of the probe releases the fluorophore from it and breaks its proximity to the quencher, thus relieving the quenching effect and allowing fluorescence of the fluorophore. It is assumed that the intensity of fluorescence detected in the qPCR thermal cycle is directly proportional to the fluorophore released and the amount of DNA template present in the PCR. However, even when a probe anneals to a partially matching sequence, enzymatic primer extension may take place with release of the fluorophore and the quencher thereafter as the probe degrades. Therefore, the fluorescent signals accumulated in RT-qPCR are merely a surrogate of the probe sequence, not the true image of the target nucleotide sequence of the template which may not exist in the sample being tested.

In contrast, automated DNA sequencing is considered the gold standard technology for nucleotide sequence determination because each fluorescent signal is emitted from a specific fluorescent dye coupled to a specific nucleotide molecule, reporting the actual position of the labeled nucleotide in a DNA sequence during capillary electrophoresis. The computer-generated sequence electropherogram represents a true image of the alignment of the 4 nucleic acid bases in the template.

According to the guidance of the U.S. Food and Drug Administration (FDA) on molecular diagnosis of viral infection caused by human papillomavirus (HPV), if the performance of a newly introduced molecular diagnostic device deviates from an FDA-approved device on the market, a conventional PCR detection followed by Sanger sequencing on both strands of the PCR amplicon (bi-directional sequencing) which contains a minimum of 100 contiguous bases is acceptable as valid diagnostics for HPV infection provided the sequence matches the reference or consensus sequence, *e.g.* with an Expected Value (E-Value)  $<10^{-30}$  for the specific HPV DNA target based on a BLAST search of the GenBank database [16]. It

implies that nucleotide sequencing of a PCR amplicon of certain size with supportive GenBank BLAST search results is a *de facto* gold standard.

The DNA probes used in the CDC RT-qPCR test kits for SARS-CoV-2 assay are about 25 bases long. This design of RT-qPCR with multiple short probes does not meet the FDA requirement for nucleic acid-based molecular diagnostics for viral disease infections. In molecular diagnostics for infectious diseases when complex human specimens are tested, the significance of detecting 4 short sequences each of 25 bases long by hybridization does not equate to that of a DNA sequence composed of 100 contiguous bases that matches a signature sequence of the genome of the pathogen. Unconnected short DNA fragments may come from different sources in a complex human specimen.

The crucial step in most nucleic acid-based assays is to amplify the target DNA or cDNA in a complex clinical sample. PCR amplification always faces problems caused by inhibitors carried over from the clinical sample. Real-time PCR or qPCR is no exception. No commercial viral RNA extraction kits can remove all PCR inhibitors from the human respiratory tract specimens. For example, the QIAamp Viral RNA Mini Kit is not designed to separate viral RNA from cellular DNA [12]. The CDC test panel [11] also requires demonstration of human DNA in the test samples. Non-target nucleic acid molecules and other PCR inhibitors are usually co-extracted and co-purified along with SARS-CoV-2 RNA [17]. Inhibition of PCR by host DNA is a well-recognized problem in molecular diagnosis of bacterial infections [18]. These PCR-inhibitory molecules may come from the host cells, and from other viruses, bacteria and fungi normally residing in the human respiratory tract. The nature and quantity of these nucleic acids and inhibitors in the respiratory tract vary from one person to another, and are totally unpredictable. Non-template nucleic acids can affect PCR amplification efficiency, the fluorescent signal growth curve and the cycle threshold (Ct) value in qPCR assays [19].

This paper introduces a protocol developed for conventional nested PCR amplification followed by sequencing of a 398-base cDNA amplicon of the nucleocapsid (N) protein gene to be used as a method for the detection of SARS-CoV-2 RNA, using cellular components of the specimens as the test material to raise the sensitivity of detection. The inhibitors carried over from the test material are greatly diluted in a nested PCR setting. Routine sequencing of the unique 398-bp nested PCR amplicon guarantees no-false positive test results. DNA sequencing of the PCR amplicon of the genomic nucleic acid of the pathogen is a well-established molecular test for detection of infectious agents which are difficult to culture [20].

## 2. Results

### 2.1. Single target sequencing for molecular diagnosis of SARS-CoV-2

An in-depth study of the region of the SARS-CoV-2 nucleocapsid (N) gene which the CDC selected to design its N1, N2 and N3 probes revealed that there is a highly conserved ~400-base segment of sequence with 5 single nucleotide polymorphisms between the N2 and N3 probes. Selection of a 398-base segment from this region for PCR detection and to prepare the template for DNA sequencing would be able to satisfy the FDA requirement for molecular diagnosis of viral infection [16]. The positional relationship between this 398-base sequence and those of the N1, N2 and N3 probes is illustrated in Figure 1.



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Figure 1 A segment of the N gene sequence of SARS-CoV-2 retrieved from the GenBank database, sequence ID# LC528233, highlighted to show the sequences and positions of the 3 probes N1, N2 and N3 (in red) with their respective flanking primers (highlighted in yellow) and a 398-base segment (in bold and underlined; position: 28728-29125) with 5 single nucleotide polymorphisms (SNPs) highlighted in green. The sequence of the N gene targeted for SARS-CoV-2 RT-qPCR detection in China [21] is shaded in gray

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28261 catctaaacg aacaaactaa aatgtctgat aatggacccc aaaatcagcg aaatgcaccc N1
28321 cgcattacgt ttgtgtggacc ctcagattca actggcagta accagaatgg agaacgcagt
28381 ggggcgcgat caaaacaacg tcggcccca ggtttacca ataatactgc gtcttggttc
28441 accgctctca ctcaacatgg caaggaagac cttaaattcc ctgaggaca aggcgttcca
28501 attaacacca atagcagtc agatgaccaa attggtact accgaagagc taccagacga
28561 attcgtggtg gtgacggtaa aatgaaagat ctgagtcgaa gatggtattt ctactaccta
28621 tgaactgggc cagaagctgg acttccttat ggtgctaaca aagacggcat cataatgggtt
28681 gcaactgagg gagccttgaa tacaccaaaa gatcacattg gcacccgcaa tcctgctaac N3
28741 aatgctgcaa tcgtgctaca acttcctcaa ggaacaacat tgccaaaagg cttctacgc
28801 gaagggagca gaggcggcag tcaagcctct tctcgttctc catcacgtag tcgcaacagt
28861 taagaaatt caactccagg cagcagtagg ggaacttctc ctgctagaat ggctggcaat
28921 ggcggtagtg ctgctcttgc tttgcgtgct cttgacagat tgaaccagct tgagagcaaa
28981 atgtctggtg aaggccaaca acaacaagcg caaactgtca ctaagaaatc tgctgctgag
29041 gcttctaaga agcctcggca aaaacgtact gccactaaag catacaatgt aacacaagct
29101 ttgggcagac gtggtccaga acaaacccaa ggaaattttg gggaccagga actaatcaga
29161 caaggaactg attacaaaca ttggcgcgcaa attgcaaat ttgccccag cgcttcagcg N2
29221 ttcttcggaa tgtcgcgcgat tggcatggaa gtcacacctt cggaacgtg gttgacctac

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The 398-base prototype sequence underlined is identical to the corresponding sequence in the N gene of the strain first isolated in Wuhan in December 2019 — GenBank Sequence ID: NC\_045512.2. This version of sequence is present in most strains of SARS-CoV-2 subsequently isolated worldwide. However, strains with the 5 single nucleotide polymorphisms (highlighted in green) were first isolated in the United States. The dates and locations of the first sample collection for these 5 strains are summarized as follows, based on information retrieved from the GenBank database.

28800 A-to-T. Nasopharyngeal swab, CA, USA, 01-23-2020. Sequence ID: MN994467  
 28829 C-to-A. Nasal swab, NY, USA, 03-17-2020. Sequence ID: MT370913  
 28862 C-to-T. Oropharyngeal swab, MA, USA, 01-29-2020. Sequence ID: MT039888  
 28886 G-to-A. Nasopharyngeal swab, CA, USA, 02-06-2020. Sequence ID: MT106052  
 29103 C-to-T. Sputum of patient, TX, USA, 02-11-2020. Sequence ID: MT106054

Based on information retrieved from the GenBank database, this 398-base N gene nucleic acid sequence is specific for SARS-CoV-2. Except in the receptor-binding domain (RBD) where there is a 97.4% amino acid similarity between Guangdong pangolin coronaviruses and SARS-CoV-2, bat coronavirus RaTG13 is most closely related to SARS-CoV-2 in the remainder of the viral genome [22]. As illustrated below, alignment of the 398-base nucleic acid segment of the N gene of bat coronavirus RaTG13 (GenBank: MN996532, in red) against that of SARS-CoV-2 (in black) shows a 96.0% (1-16/398) similarity (the 16 nucleotide dissimilarities are highlighted green).

```

CAATCCTGCTAACAATGCTGCAATCGTGCTACAACCTCCTCAAGGAACAACATTGCCAAAAGGCTTCTACGCAGAAGGGAGCAGAG
CAATCCTGCTAACAATGCTGCAATCGTGCTACAACCTCCTCAAGGAACAACATTGCCAAAAGGCTTCTACGCAGAAGGGAGCAGAG
GGGCAGTCAAGCTCTTCTCGTCTCATCACGTAGTCGCAACAGTTCAAGAAATCAACTCCAGGCAGCAGTAGGGGAACTTCT
GCGGCAGTCAAGCTCTTCTCGTTCTCATCACGTAGTCGCAACAGTTCAAGAAATCAACTCCAGGCAGCAGTAGGGGAACTTCT
CCTGCTAGATGGCTGGCAATGGCGTGATGCTGCTCTTGCTTTGCTGCTGCTTGACAGATTGAACCAAGCTTGAGAGCAAAATGTC
CCTGCTAGATGGCTGGCAATGGCGTGATGCTGCTCTTGCTTTGCTGCTGCTTGACAGATTGAACCAAGCTTGAGAGCAAAATGTC
TGGTAAAGGCCAACAACACAAGCGCAAACTGTCTACTAAGAAATCTGCTGCGAGAGGCTTCTAAGAAACCTCGGCCAAAACGTACTG
TGGTAAAGGCCAACAACACAAGCGCAAACTGTCTACTAAGAAATCTGCTGCGAGAGGCTTCTAAGAAACCTCGGCCAAAACGTACTG
CCACAAACATACAATGTAACACAAGCTTTGGCAGACGTGGTCCAGAACAA
CCACAAAGCATACAATGTAACACAAGCTTTGGCAGACGTGGTCCAGAACAA

```

The pair of PCR primers (underlined) defining this 398-base segment of amplicon are highly conserved among all known strains of SARS-CoV-2 and have not undergone mutations, based on search of the GenBank database. A single base mutation within a 21-base primer usually does not affect the result of heminested PCR unless the mutation occurs at the position binding the 3' terminus of the primer.

SARS-CoV-1 gene (GenBank: AY274119.3) is not amplified by the primary PCR primer pair used in this protocol and there is < 90% similarity between the 398-base N gene sequence of the SARS-CoV-1 genome and that of the SARS-CoV-2 genome. Molecular misdiagnosis of SARS-CoV-2 based on nucleotide sequencing of this segment of the N gene is extremely unlikely.

## *2.2. Nested RT-PCR of cell lysates is needed to raise sensitivity for SARS-CoV-2 detection*

In PCR research, a theoretically 100% efficient exponential amplification of the target DNA during repeated thermal cycling cannot be achieved even under strictly controlled experimental conditions. In the hands of most researchers, a properly designed PCR in the absence of any identifiable interfering substances in the sample matrix may amplify target DNA with a 90% efficiency [23, 24]. In molecular diagnosis of infectious diseases, there is always a possibility of false-negative results even when target DNA molecules are plentiful in the patient samples. After DNA purification steps are used before PCR, a 14% false-negative rate has been observed for hepatitis B virus detection, most probably due to incomplete removal of PCR inhibitors [25].

A high template/non-template nucleic acid ratio is one of the determinant factors in successful real-time PCR assays [19]. For example, persistent human papillomavirus (HPV) infection is the key factor in cervical carcinogenesis. During the long course of cancerous development, the viral load decreases from several thousand viral copies to about double digit per infected cell [15]. Virologic DNA real-time PCR assay is reported to be effective in identifying cervicovaginal cytology samples containing low-grade lesions with koilocytes which can have thousands of copies of human papillomavirus (HPV) per cell with a sensitivity rate of 95.5%. But the same real-time PCR test kit was only 58.26% effective in detecting high-grade CIN 3 lesions in which the viral load can be as low as 10 copies per cell [15, 26]. Non-template nucleic acids are potent PCR inhibitors which can greatly raise the Ct values of a qPCR assay when a non-template/template ratio is high in the reaction mixture.

False-negative test results for SARS-CoV-2 are expected in any PCR assays, including RT-qPCR when the viral load is low in the sample being tested. In testing low viral load samples, the laboratories must use highly sensitive assays to increase the detection sensitivity without causing false positives.

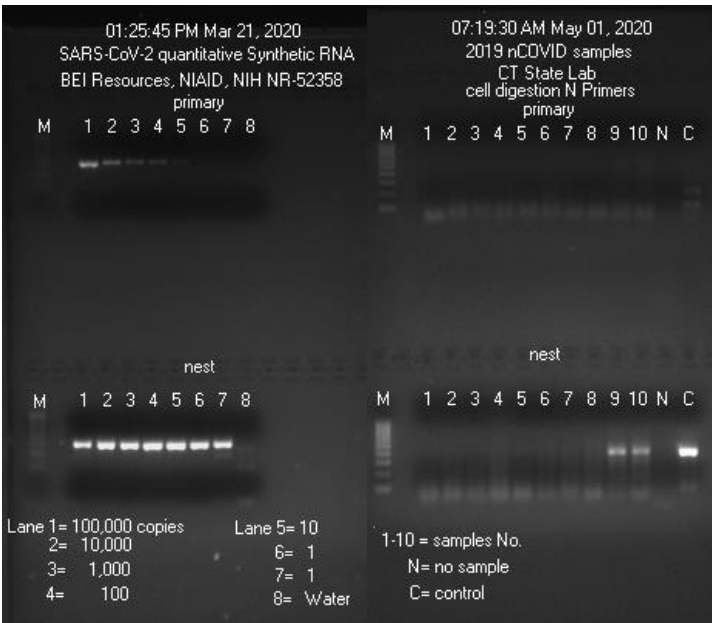
All diagnostic PCR assays depend on fluorescence intensity for evaluation. In the CDC RT-qPCR Assay Panel, the intensity of fluorescence emitted by a dye is measured by fluorometer, an instrument. In conventional PCR, the intensity of fluorescence emitted by a DNA/ethidium bromide complex is observed and evaluated with human eyes. A fluorometer used in qPCR is more sensitive than human eyes in measuring fluorescent signals, but it does not measure fluorescence emitted from a DNA complex. There are no products generated by the CDC RT-qPCR assays for further analysis whereas an amplicon of conventional PCR is composed of a mass of DNA molecules. The heminested PCR amplicon can be used as the template for DNA sequencing validation. Therefore, conventional PCR followed by DNA sequencing is the more appropriate testing platform for SARS-CoV-2 if a reliable test result is desired for patient management.

Nested, or two-round PCR, is a proven technique to mitigate the low sensitivity of conventional PCR in molecular diagnosis of infectious diseases, such as HPV infections [27] and borrelial spirochetemia [28]. This technique can be used for SARS-CoV-2 tests to increase detection sensitivity, as shown in Figure 2.

The left panel of Figure 2 is an image of agarose gel electrophoresis of the products of primary RT-PCR (upper half) and heminested PCR (lower half) showing that heminested PCR increased the sensitivity of one-round RT-PCR by ~1,000 fold in detecting a 398-base N gene segment of synthetic SARS-CoV-2 RNA. The number of copies of synthetic viral RNA added to each 25 µL primary RT-PCR mixture was calculated based on the analysis data supplied by BEI Resources, NIAD, NIH, Cat. No. NR-52358. As demonstrated, a single copy of viral RNA was detected with a robust amplicon band observed in lane 6 and lane 7 (lower half). In contrast, it needed 100-1,000 copies of viral RNA to generate a weak band in primary PCR, barely visible in lane 4 and lane 3 (upper half). The primary and heminested PCR primers and the PCR conditions are detailed in the Methods section.

The right panel of Figure 2 is an image of agarose gel electrophoresis showing two (2) positive heminested PCR products in lanes 9 and 10. There are no visible primary PCR products in any lanes. These 10 “negative” samples, each consisting of 0.5-1 mL of fluid (See Materials and Methods), were prepared and used as reference material by the Connecticut State Department of Public Health, Microbiology Laboratory Division on April 30, 2020 to assist local laboratories for developing their nucleic acid-based tests for SARS-CoV-2 under the CLIA program. Although these 10 samples were classified as negative by RT-qPCR, no Ct values were given. The cell lysates were extracted by phenol/chloroform and the RNAs were precipitated in ethanol as the test material (See Methods)

Figure 2 Agarose gel electrophoresis: Heminested PCR raises the sensitivity for detection of a 398-bp N gene segment in synthetic SARS-CoV-2 RNA (Left) and of viral RNA extracted from infected human cells in respiratory tract specimens (Right: Amplicons of cDNA of viral RNA detected in lanes 9 and 10 from reference samples #1-10 classified as negative by RT-qPCR assays )



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The laboratory data presented in Figure 2 show that two-round PCR is needed for the detection of amplicons when this technology is used for molecular identification of SARS-CoV-2 in respiratory tract samples. The material to be tested should include cellular components of the specimen for maximizing the sensitivity of detection.

### *2.3. False positives of RT-qPCR assay for SARS-CoV-2*

PCR, including qPCR, is known to generate both false-negative and false-positive results when this DNA replication tool is used for the diagnosis of infectious diseases.

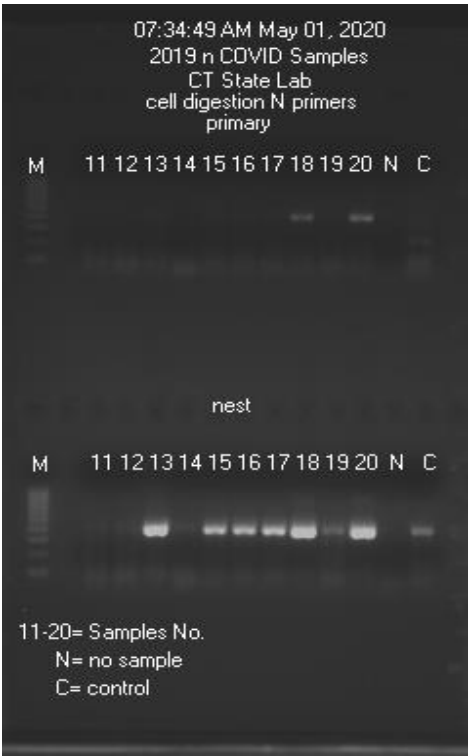
In an international external quality assessment proficiency testing program for MERS-CoV, a Coronavirus, laboratories using RT-qPCR assay on simulated samples with viral load equivalent to throat swab MERS-CoV RNA concentrations generated an 84.6% detection sensitivity and 8.1% false-positive results [29]. False-negative and false-positive rates are usually much higher in routine testing than those observed in regulatory proficiency test programs.

It is not unexpected to find false-positive results generated by RT-qPCR when this tool is used to test for SARS-CoV-2.

On April 30, 2020, in addition to the 10 negative samples referenced above the Connecticut State Department of Public Health, Microbiology Laboratory Division also prepared 10 reference samples, which were diagnosed as positive for SARS-CoV-2 by the CDC RT-qPCR assays, in support of the local laboratories to develop their tests for the virus.

According to the documents accompanying these 10 positive samples, they all generated an N1 Ct value between 16 and 29.78 and an N2 Ct value between 15.05 and 29.4 except one with an N2 Ct 67.86. The R. P. (RNase P gene) Ct values for these 10 samples ranged from 3.31 to 25.13. Based on these Ct values, all samples were interpreted as confirmed positive and used as standard reference material for developing SARS-CoV-2 assays. However, only 7 of these 10 samples were found to be positive by nested PCR amplification (Figure 3).

Figure 3 Agarose gel electrophoresis showing that only 7 of the 10 samples classified as positive for SARS-CoV-2 by RT-qPCR could be confirmed by conventional nested PCR followed by DNA sequencing of a 398-bp cDNA amplicon of the N gene



As demonstrated in Figure 3, primary PCR products after amplification of 30 thermal cycles were only visualized on 2 of 7 validated positive samples (lanes 18 and 20). Five of the 7 positives (5/7) needed nested PCR for detection. All seven positive nested PCR products were proven to consist of a 398-base segment of SARS-CoV-2 N gene by DNA sequencing. Six (6/7) were of the prototype (See Section 2.4. Figures 4 and 5). Sample #13 belongs to a newly discovered mutant first isolated from a human specimen collected in the State of New York on March 17, 2020 (See Section 2.4. Figures 6 and 7).

*2.4. Routine sequencing for PCR product validation and single nucleotide polymorphisms*

For emerging serious virus infectious diseases, such as Ebola, the European CDC’s laboratory criteria of case definition are 1) Detection of virus nucleic acid in a clinical specimen and confirmation by sequencing or a second assay on different genomic targets; or 2) Isolation of Ebola virus from a clinical specimen [30].

A recent “Report from the American Society for Microbiology COVID-19 International Summit” on the Value of Diagnostic Testing for SARS-CoV-2/COVID-19 pointed out that one of the new challenges in the current molecular diagnostics is to optimize the current multiple targets to a single target [31].

By following these recommendations and the FDA’s guidance for molecular diagnosis of another viral infection [16], routine bi-directional sequencing of a >100-bp PCR amplicon of the viral genome was designed as the proper molecular testing platform for SARS-CoV-2. Examples are presented by analysis of the computer-generated 398-base N gene sequences on the reference samples supplied by the Connecticut State Department of Public Health Microbiology Laboratory Division as follows.

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Figure 4 Electropherogram showing DNA sequence of the nested PCR amplicon visualized in Lane 20 of Figure 3. Forward Co4 PCR primer was the sequencing primer. Note: the base C in position 14 is underlined

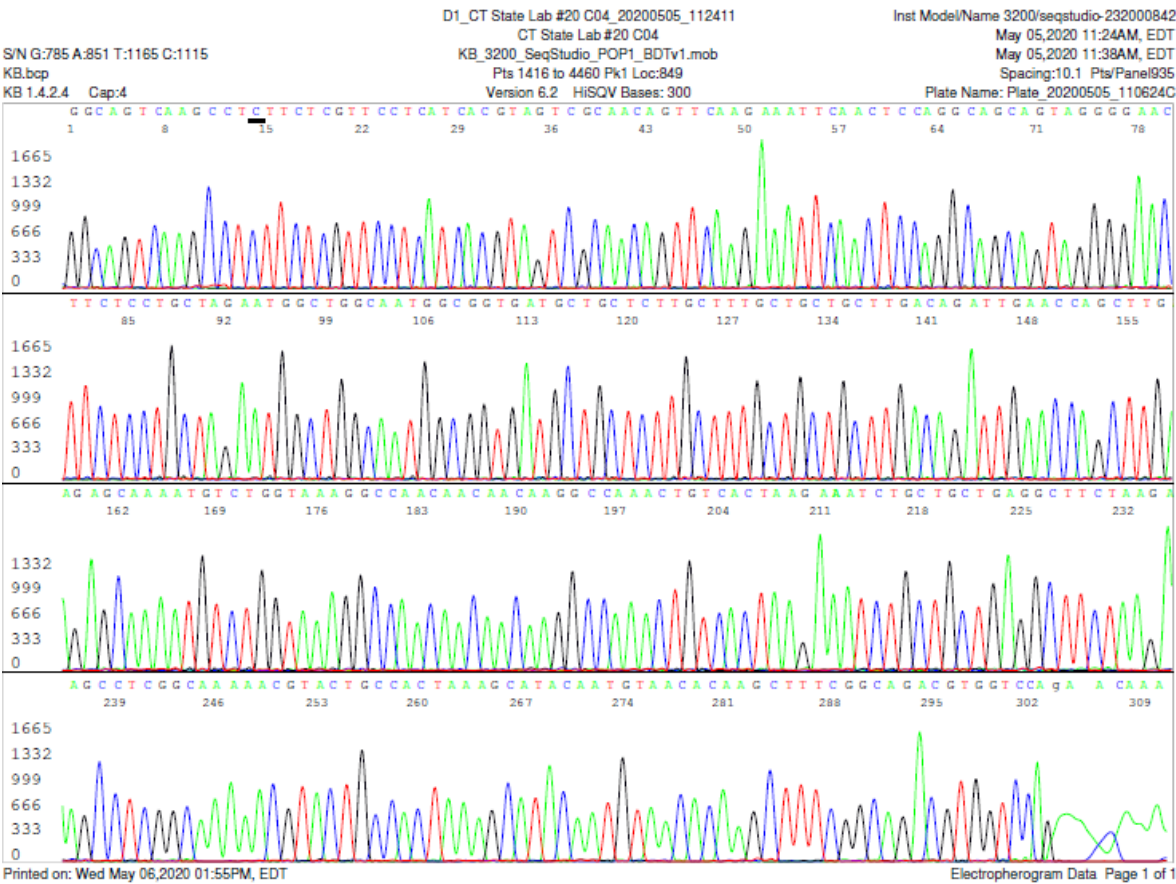
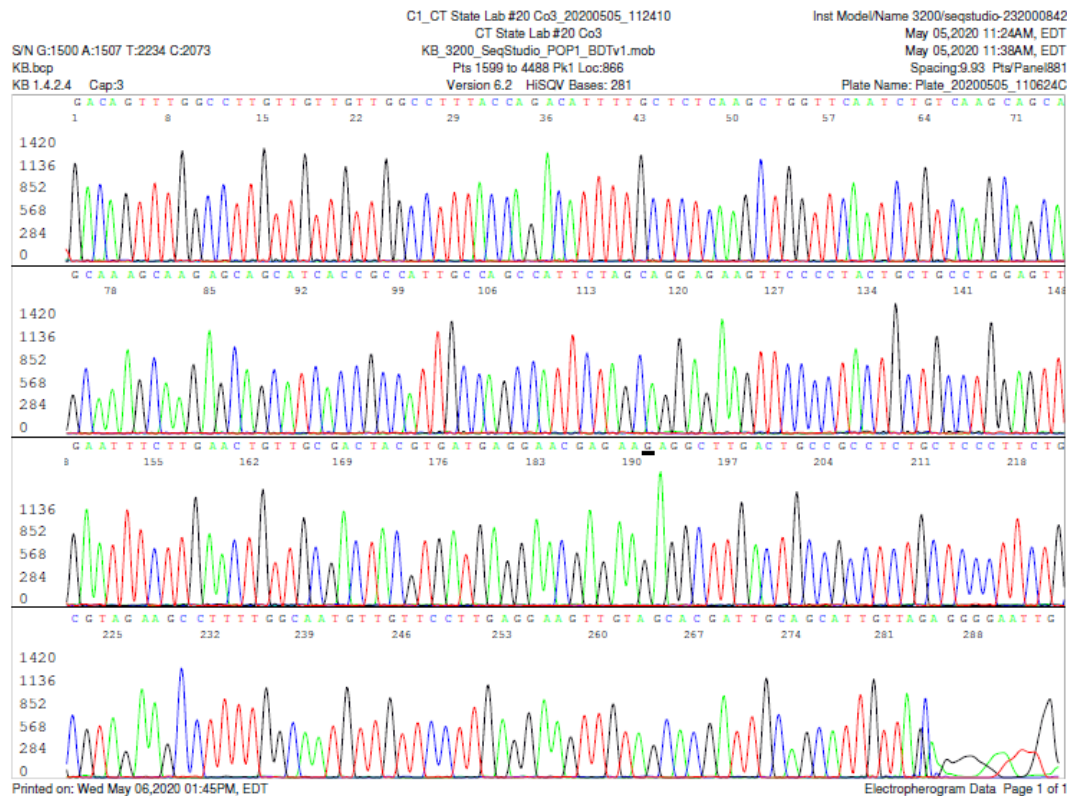




Figure 5 Electropherogram showing DNA sequence of the nested PCR amplicon visualized in Lane 20 of Figure 3. Reverse Co3 PCR primer was the sequencing primer. Note: the base G in position 191 is underlined

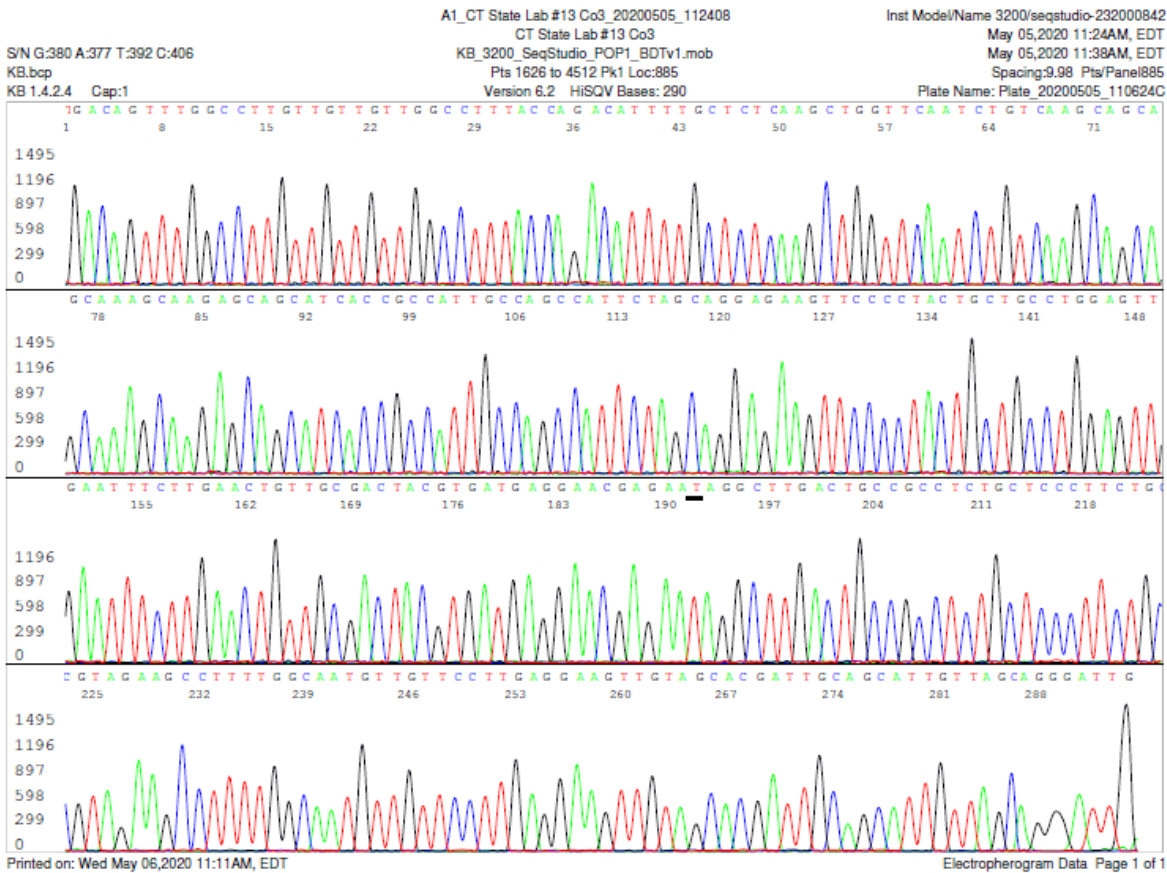


Connecting the two sequences illustrated in Figures 4 and 5 after all the complementary bases were converted to those for a 5'–3' reading resulted in a composite sequence fully matching a 398-base sequence illustrated in Figure 1, position 28728-29125 which is generally accepted as the prototype sequence for SARS-CoV-2.



Figure 6 Electropherogram showing DNA sequence of the nested PCR amplicon visualized in Lane 13 of Figure 3. Forward Co4 PCR primer was the sequencing primer. Note: the base A in position 14 is underlined

Figure 7 Electropherogram showing DNA sequence of the nested PCR amplicon visualized in Lane 13 of Figure 3. Reverse Co3 PCR primer was the sequencing primer. Note: the base T in position 191 is underlined



Connecting the two sequences illustrated in Figures 6 and 7 after all the complementary bases were converted to those for a 5'–3' reading resulted in a composite sequence fully matching a 398-base sequence illustrated in Figure 1, position 28728-29125 except for a single base mutation of C-to-A in position 28829, as annotated in GenBank Sequence ID: MT370913.

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Figure 8 Electropherogram showing two superimposed SARS-CoV-2 N gene sequences in the nested PCR amplicon visualized in Lane 10 of Figure 2, right panel. Forward Co4 PCR primer was the sequencing primer. One sequence was from a novel mutant with a single nucleotide A insertion at position 250, and the other was from a wildtype parental virus



The computer-generated electropherogram illustrated in Figure 8 shows one prototype N gene sequence of SARS-CoV-2 from position 1 to position 249. But there was a single nucleotide A insertion at position 250 in the second N gene, creating a mutant while the wildtype parental virus was infecting the human host. The chance for one patient being infected by two SARS-CoV-2 strains from the very beginning is extremely low. A virus mutant with a nucleotide A insertion in this segment of the N gene has not been reported in the literature nor deposited into the GenBank. In Figure 8, the extra nucleotide in the mutant gene caused a frameshift in DNA sequencing after the nucleotide A insertion. The two components of each double peak in different colors are aligned against each other in sequence and presented immediately below the electropherogram. The inserted base A is underlined.

This sequencing pattern showing an “A” insertion in one of the two templates at position 250 was reproduced for 3 times to rule out possible sequencing artifacts. Sequencing from the opposing direction on this amplicon composed of two homeologous DNA templates failed to generate a readable sequence due to insertion of the “A” nucleotide into the mutant gene too close to the site of the reverse Co3 sequencing primer.

In summary, the results of re-testing the cellular components of 20 reference samples of nasopharyngeal and oropharyngeal swab rinses by heminested RT-PCR amplification followed by

nucleotide sequencing showed that SARS-CoV-2 was not found in 3 of the 10 (3/10) reference samples classified as positive by RT-qPCR, and that 2 of the 10 (2/10) reference samples classified as negative by RT-qPCR in fact contained SARS-CoV-2.

Among the 9 positive samples, one isolate showed a C-to-A single nucleotide mutation in the 398-base segment of the N gene targeted for amplification. According to information retrieved from the GenBank database, this mutation was discovered first in a specimen collected in New York State on March 17, 2020. And only 5 cases with this mutation were reported to the GenBank as of May 13, 2020.

In addition, one of the two positive isolates from the 10 RT-qPCR-negative samples in fact contained two viral genomes, one from a mutant with a single nucleotide insertion in the N gene which was not found in the GenBank database, and one from a wildtype parental virus. Co-infection of one patient by two strains of SARS-CoV-2, a mutant and the wildtype parental virus, has not been reported and may pose a challenging problem in molecular diagnosis of SARS-CoV-2

### 2.5. Unintended primer extension in RT-qPCR

The CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel [11] advises to use cell-free samples prepared by the QIAamp Viral RNA Mini Kit as the material to be tested. But it also requires proof of human genomic nucleic acids in the same samples for a valid negative SARS-Cov-2 RNA result. As proof of the presence of sufficient human cell nucleic acids in the material being tested, the CDC protocol sets a cycle threshold (Ct) value within 40.00 cycles (< 40.00 Ct) in the human RNase P gene RT-qPCR channel for positive cut-off.

The sequences of the primers and probe for the RNase P gene RT-qPCR as an Internal Process Control (IPC) are given as follows [32] (color-highlighted for discussion convenience).

RNase P Forward Primer 5'-AGA TTT GGA CCT GCG AGC G-3'

RNase P Reverse Primer 5'-GAG CGG CTG TCT CCA CAA GT-3'

RNase P Probe 5'-FAM - TTC TGA CCT GAA GGC TCT GCG CG - BHQ-1-3'

These primers and probe were designed to amplify a 65-base segment of *Homo sapiens* ribonuclease P/MRP subunit p30 (RPP30). Sequence ID: NM\_001104546. The relevant sequence in this segment of the gene is retrieved from the GenBank database and pasted below with primer-matching colors to indicate the positions of the CDC-recommended primers and the probe which is typed in red in reverse complement (Figure 9).

Figure 9 Segment of DNA sequence retrieved from the GenBank database (NM\_001104546) showing the color-highlighted positions of primers and probe used in the CDC RT-qPCR assay kit and the sequence of the 160-bp (underlined) amplicon used as the template to generate the sequence illustrated in Figure 10

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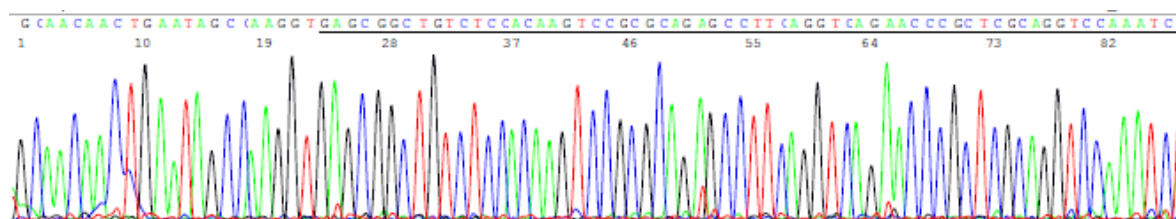
240 CTAGTTAAATTTTAATTGGTCTTGATTTTCCCTGTACAATTGGCAAAGTTGTGAAGAGT 181
180 TCAGAAACAGCTACTGGTTTTTCAATTTCTGTTTCTTTTCTTAAAGTCAACGATATGA 121
120 TTGATAGCAACAACCTGAATAGCCAAGGTGAGCGGCTGTCTCCACAAGTCCGCGCAGAGCC 61
60 TTCAGGTCAGAAACCGCTCGCAGGTCCAAATCTGCAAACACCGCCATGCTGAAGTCCCAT 1

```

It was assumed that a 65-base segment of the RPP30 gene defined by the two primers would be amplified as shown in Figure 9 in the RT-qPCR testing platform. However, it has never been

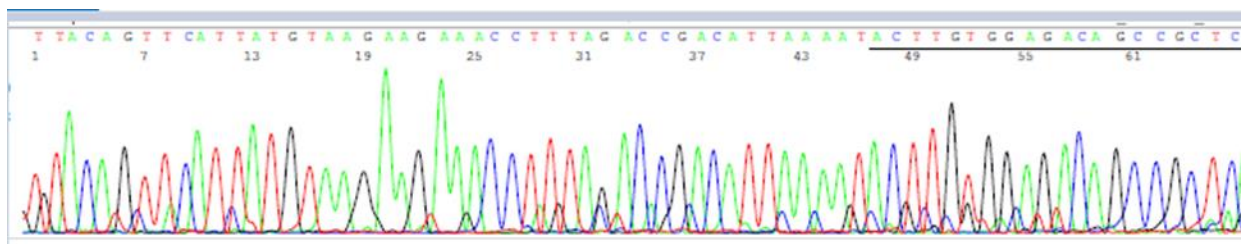
demonstrated that this sequence has actually been amplified in real practice because the inter-primer sequence is too short for the currently used sequencing technologies. To prove that this 65-base sequence is actually present in the clinical sample, a longer amplicon (160-base, underlined in Figure 9) is needed to be used as the sequencing template to generate a sequence illustrated in Figure 10.

Figure 10 Electropherogram of a 65-base sequence (underlined) presumed to be generated during RT-qPCR using the CDC SARS-CoV-2 test kit, but it has never been shown in real practice



There is a single copy of RPP30 DNA sequence located at 10q23.31 locus on chromosome 10 in each haploid human genome and there are two copies in a diploid human cell [33]. RPP30 has been used as a reference for the autosomal chromosomes in research when there is abundant human chromosomal material in the sample [33, 34]. However, in cell-free fluids derived from respiratory tract specimens there are no intact human cells in the sample being tested. RPP30 gene is detected in the viral RNA extracts only when some broken human cell nuclear parts have not been completely removed from the cell-free samples. In the absence of a preferred template, the PCR primers may anneal to a non-target DNA with partially matching sequence and initiate an unintended primer extension or PCR. Such an example is illustrated in Figure 11.

Figure 11. Sequencing electropherogram showing PCR amplification of an unintended DNA segment by the CDC RNase P Reverse Primer (underlined)



The sequence illustrated in Figure 11 was generated during an attempt of using the CDC RNase P primers to induce a 65-bp PCR amplicon for further analysis as an internal control which can be validated by DNA sequencing. In this electropherogram, the binding site for the CDC RNase P reverse primer is underlined. Submission of this sequence to GenBank for BLAST algorithmic analysis led to a return illustrated in Figure 12 (yellow and green added by author).



Figure 12 In the absence of a fully matching template, the primers designed for the CDC RT-qPCR assay kit may anneal to partially matching DNA and initiate an unintended PCR

**Homo sapiens BAC clone RP11-154F14 from 4, complete sequence**

**Sequence ID: AC092608.2 Length: 196952 Number of Matches: 1**

Range 1: 102113 to 102177 [GenBankGraphics](#) [Next Match](#) [Previous Match](#)

Score	Expect	Identities	Gaps
121 bits (65)	3e-24	65/65 (100%)	0/65 (0%)
Query 1	TTACAGTTCATTATGTAAGAAGAAACCTTTAGACCGACATTAAAAATCTTGAAGAGACCC	60	
Sbict 102113	TTACAGTTCATTATGTAAGAAGAAACCTTTAGACCGACATTAAAAATCTTGAAGAGAGACCA	102172	
Query 61	GGGCGC	65	
Sbict 102173	GGGCGC	102177	

As shown in Figure 11, PCR amplification as often applied in clinical diagnostics is not absolutely specific. There are always some irrelevant products, visible or invisible, generated as a result of enzymatic DNA replications in addition to those represented in the main sequence. The main sequence in this electropherogram shows that the CDC RNase P reverse primer found and annealed to a segment of DNA which shares 16 (highlighted in green, Figure 12) of its 20 bases in sequence to initiate an unintended PCR (compare the green-highlighted sequence in Figure 12 with the primer sequence underlined in Figure 11). The sequence highlighted in yellow is totally different from that of the probe (Figure 9).

The findings described in this section suggest that non-specific hybridization or annealing between non-target DNA and the primer or probe used in the CDC RT-qPCR test kits for SARS-CoV-2 may have contributed to the causes for false-positive results especially when the Ct cut-off value has been set as high as 40.0 [11].

Other investigators also reported that the CDC N2 and N3 primer sets can amplify irrelevant nucleic acids in RT-qPCR and in conventional PCR [4].

In an FDA publication titled "Real-Time Fluorescent RT-PCR Kit for Detecting SARS-CoV-2", the Instructions for Use repeatedly emphasize that the Ct values for positive result on all controls and samples should be no higher than 37 [35]. Setting a 40.00 Ct value as the cut-off point for the presence of RNase P gene or for the presence of SARS-CoV-2 N gene in the sample being tested deviates from common practice and from the FDA guidance. Accepting a questionable Ct value as evidence for the presence of RNase P gene in the sample being tested may have allowed many invalid test results to be reported as negatives.

## 2.6. Human BRCA1 gene as internal extraction control

Since the segment of RNase P gene selected for the CDC RT-qPCR test panel was not always PCR-amplifiable for validation by conventional PCR followed by sequencing, a segment of human BRCA1 gene was selected as the internal cellular extraction control.

A pair of AG1 and R2 PCR primers (see Materials and Methods section) was designed to initiate a 409-bp *BRCA1* gene primary PCR. The primary PCR product was re-amplified by a pair of AG2/R2 heminested PCR primers to generate a 323-bp amplicon which can be used as the template for sequencing validation. Demonstration of a segment of *BRCA1* gene amplicon was accepted as reliable physical evidence to confirm that sufficient human nucleic acids had been extracted into the sample being tested in the SARS-CoV-2 negative cases. While the RNase P gene in the 20 reference samples supplied by the Connecticut State Department of Public Health Microbiology Laboratory Division could not be detected, the *BRCA1* gene was demonstrated in all these samples as shown in Figures 13 and 14.

Figure 13 Image of agarose gel electrophoresis showing a 323-bp heminested PCR amplicon of human *BRCA1* gene in all 20 reference samples tested. Lane 11=water negative control. Lane 12= known human cell extract positive control

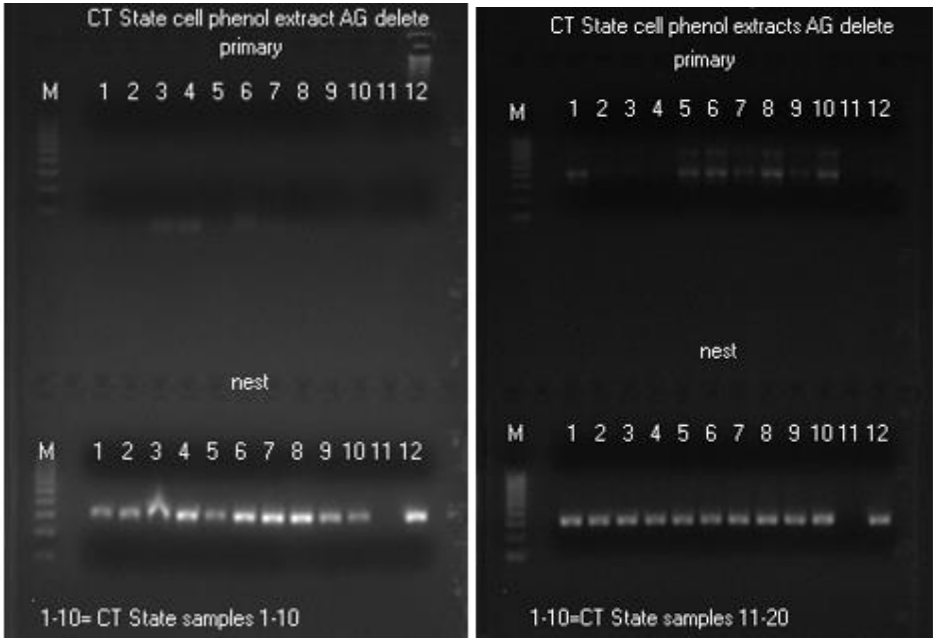
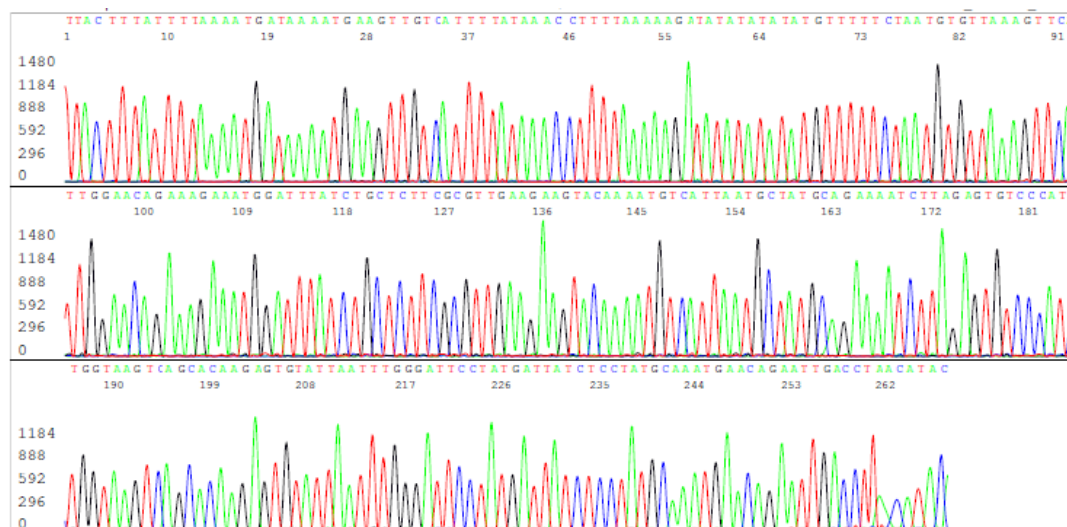




Figure 14 Sample of electropherograms confirming a segment of human *BRCA1* gene sequence of the nested PCR amplicons shown in lower half of Figure 13. The 21 bases in the end are the binding site for R2 primer (5'-GTATGTAAGGTCAATTCTGTTC-3')



### 3. Discussion

This article has demonstrated that for molecular diagnosis, testing the cellular components for SARS-CoV-2 rather than cell-free fluids only on respiratory tract specimens by conventional nested PCR and using the traditional phenol/chloroform method for RNA sample preparation can reduce the number of false-negatives. Using nucleotide sequencing to validate every positive PCR amplicon practically eliminates all false-positive results. For timely accurate molecular diagnosis and characterization of infectious agents, concerned scientists have been urging to bring microbial sequencing to the hospital laboratories to improve individual and population health [36, 37].

Nested PCR is a simple method to increase the detection sensitivity of the conventional PCR technology. Cross contamination which is often used as a reason to object using nested PCR in diagnostic laboratories can be mitigated by elimination of transferring PCR products by micropipettes and proper training of the testing staff. Cross contamination is a function of the laboratory performing PCR, not an inherent part of the nested PCR technology [28].

RT-qPCR tests are known to generate both false-negative and false-positive results [4, 6, 7, 9]. Highly sensitive and accurate laboratory tests for SARS-CoV-2 are needed for certain populations, especially for the people of advanced age living in long-term care facilities and their care takers. Patients with false-negative test results may transmit the virus to family, friends, or care-givers. Uninfected residents in long-term care facilities with false-positive results may be isolated in rooms with Covid-19 patients, which puts them at risk of becoming true positives. Convalescent hospitalized patients must be tested by an extremely sensitive, no false-positive nucleic acid test prior to being discharged into the communities.

Long-term care facilities with exceptionally high COVID-19 death tolls among their residents may consider re-testing the residues of the respiratory tract samples of the deceased collected and tested prior to their death. If false-negative and false-positive test results were found, the false test results might have contributed to spreading of SARS-CoV-2 in the institution. Then corrective measures can be made in the infection control practices to lower the rate of cross infection among the residents.

Highly sensitive, no-false positive SARS-CoV-2 tests are needed in hospitals with head-and-neck surgical department to screen patients before admission for protection of the medical staff involved [9].

Extremely accurate SARS-CoV-2 laboratory tests are especially important as businesses attempt to return to normal operation in order to avoid raising false alarms of a re-emerging outbreak. False-positive test reports can easily create unnecessary panic resulting in negative impacts on local economies.

The current SARS-CoV-2 nucleic acid test platforms overly depend on the supply of commercial RNA extraction devices, such as the QIAamp viral RNA preparation kits. However, better options are readily available. For example, the abilities of QIAamp blood kit (Qiagen, Inc., Hilden, Germany) and another commercial kit to extract the hepatitis B virus (HBV) DNA template from serum for amplification by PCR were evaluated and compared with that of the standard phenol-chloroform method. Differences in the sensitivities of the three methods were revealed by nested PCR of HBV DNA extracted from serially diluted hepatitis B e antigen (HBeAg)-positive (high-titer) serum. Phenol-chloroform was found to be a  $10^6$  times more sensitive extraction method than the QIAamp blood kit; and nested PCR was found to be  $10^6$  times more sensitive than one-round PCR in detection of HBV DNA in serum samples [25]. In nested PCR, the PCR inhibitors carried over from the sample into the primary PCR are further diluted by about 100-fold. The value of nested PCR followed by DNA sequencing for detection of SARS-CoV-2 was previously reported by Nao and colleagues [38].

As demonstrated in the Results section of this article, using the standard phenol-chloroform method to prepare viral RNA from cell lysates and nested RT-PCR amplification followed by DNA sequencing for SARS-CoV-2 detection, 2 false negatives and 3 false positives were found in 20 reference samples which had been tested by the CDC RT-qPCR kits.

In addition, one of the seven (1/7) RT-qPCR positive samples was found unexpectedly to contain an isolate which belongs to a strain of SARS-CoV-2 with single C-to-A mutation in the 398-bp N gene segment targeted for amplification. This newly discovered strain was first recognized in a specimen collected in the State of New York on March 17, 2020, and then found in specimens collected in the District of Columbia and Connecticut in the next two weeks, according to information retrieved from the GenBank database. It is not known how many of the Covid-19 patients were caused by this locally developed “mutant” in the tri-state area of New York, Connecticut and New Jersey.

Another surprise in testing the 20 reference samples prepared by the Connecticut State Department of Public Health Microbiology Laboratory Division was the finding of a SARS-CoV-2 mutant with a novel single nucleotide A insertion within the 398-base DNA segment sequenced. This novel mutant and the wildtype parental virus apparently co-infected the host cells collected for testing and their N genes were co-amplified in the nested PCR process. This novel viral mutant was present in a person with a false-negative test result and was capable of infecting the host cells as the parental virus. It is not known if such a mutant can be transmitted to another individual to cause a new infection as the wildtype parental virus can.

As of early May 2020, there were at least 198 recurrent mutations identified among global isolates of SARS-CoV-2 [39]. For patient management, it is neither practical nor necessary to routinely check all these known mutations in the genome of every isolate for diagnostic purpose.

However, it is feasible to routinely perform a bi-directional DNA sequencing of a 398-bp PCR amplicon of the N gene on every positive sample to categorize each isolate into one of the 6 SARS-CoV-2 clades which are known to circulate in the United States to track real-time movement of these viral strains in order to facilitate studies of their possible relevancy to transmissibility and pathogenicity.

The major limitation of this method is that the nested PCR and nucleotide sequencing technologies cannot be readily automated. The test takes 24 to 48 hours to generate a result because the procedures of cell digestion, two PCR amplifications and DNA sequencing take more than 10 hours of instrument time to complete. As for all PCR-based nucleic acid detection methods, unexpected mutation of the N gene in the SARS-CoV-2 genome affecting the binding site for the 3' terminus of any of the PCR primers may cause false-negative results. If a patient does not have SARS-CoV-2 infected cells in the upper respiratory tract, or the specimen does not contain any virus-infected cells, this method may generate false-negative test results.

#### **4. Materials and Methods**

##### *4.1. Test materials*

The specimens used for this study were 10 negative and 10 positive reference samples which were specially prepared by the Connecticut State Department of Public Health, Microbiology Laboratory Division, on April 30, 2020 and packaged in a Styrofoam box containing dry ice to be used in support of the local diagnostic laboratories in the State of Connecticut to develop their laboratory tests for detecting SARS-CoV-2 virus in respiratory tract specimens. All samples were in 0.5-1 mL of saline as the media in plastic vials with screw caps and processed on the same day in Milford Molecular Diagnostics Laboratory, in Milford, Connecticut.

According to the Connecticut State Department of Public Health, Microbiology Laboratory Division, these samples were all clinical specimens, namely nasopharyngeal or oropharyngeal swabs placed in saline, that were received at the Connecticut State Department of Public Health, Microbiology Laboratory Division for the purpose of testing for SARS-CoV-2. No culture, spiking/or diluents were added or performed on these specimens. These 20 samples were previously tested by the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel as negative for SARS-CoV-2 or positive for SARS-CoV-2, and were used as reference samples for evaluation of other laboratory-developed assays for SARS-CoV-2 in nasopharyngeal and oropharyngeal swab specimens.

##### *4.2. RNA extraction*

The entire content of each sample was transferred to a graduated 1.5 mL microcentrifuge tube and centrifuged at  $\sim 16,000\times g$  for 5 min to pellet all cells and cellular debris. The supernatant was discarded except the last 0.2 mL which was left in the test tube with the pellet.

To each test tube containing the pellet, 200  $\mu\text{L}$  of digestion buffer containing 1% sodium dodecyl sulfate, 20mM Tris-HCl (pH 7.6), 0.2M NaCl and 700  $\mu\text{g/mL}$  proteinase K, modified from a protocol for releasing cellular RNase P genes from HeLa cells [40], was added. The mixture was digested at  $47^\circ\text{C}$  for 1 hr in a shaker.

An equal volume (400  $\mu\text{L}$ ) of acidified 125:24:1 phenol:chloroform:isoamyl alcohol mixture (Thermo Fisher Scientific Inc.) was added to each tube. After vortexing for extraction and centrifugation at  $\sim 16,000\times$

g for 5 min to separate the phases, 200  $\mu$ L of the aqueous supernatant without any material at the interface was transferred to a new 1.5 mL microcentrifuge tube.

To the 200  $\mu$ L of phenol/chloroform-extracted aqueous solution, 20  $\mu$ L of 3M sodium acetate (pH 5.2) and 570  $\mu$ L of 95% ethanol were added. The mixture was placed into a cold metal block in a -15 to -20°C freezer for 20 min. The precipitated nucleic acids were centrifuged at  $\sim 16,000\times$  g for 5 min and washed with 700  $\mu$ L of cold 70% ethanol.

After a final centrifugation at  $\sim 16,000\times$  g for 5 min, the 70% ethanol was completely removed by a fine-tip pipette, and the microcentrifuge tubes with opened caps were put into a vacuum chamber for 10 minutes to evaporate the residual ethanol. The nucleic acid in each tube was dissolved in 50  $\mu$ L of M.B. grade water. All samples were tested immediately.

As positive control, a 0.2 mL aliquot of suspension of nasopharyngeal and oropharyngeal cells previously collected from normal individuals, tested negative for SARS-CoV-2, and stored in normal saline at -20°C was spiked with 400 copies of Quantitative Synthetic RNA from SARS-Related Coronavirus 2, Cat. No. NR-52358 supplied by BEI Resources, NIAD, NIH. This positive control tube was processed along with the reference samples for digestion and extraction.

The negative control consisted of 0.2 mL aliquot of suspension of nasopharyngeal and oropharyngeal cells previously collected from normal individuals, tested negative for SARS-CoV-2, and stored in normal saline at -20°C.

#### 4.3. PCR primers

Based on the sequence retrieved from the GenBank database and presented in Figure 1, 3 primers were designed for primary and heminested PCR amplification of a 398-bp cDNA fragment to be used as the template for nucleotide sequencing confirmation of nucleic acid from the SARS-CoV-2. Their sequences and positions are listed below (see Figure 1).

##### Primary PCR primers

Forward primer Co1	5'-ACATTGGCACCCGCAATCCTG-3'	28715-28735
Reverse primer Co3	5'-TTTGTTCTGGACCACGTCTGC-3'	29105-29125

##### Heminested PCR primers

Forward primer Co4	5'-CAATCCTGCTAACAATGCTGC-3'	28728-28748
Reverse primer Co3	5'-TTTGTTCTGGACCACGTCTGC-3'	29105-29125

The Sequences of the primary and heminested PCR primers for the *BRCA1* gene are listed as follows.

##### Primary PCR primers for a 409-bp *BRCA1* gene amplicon are:

Forward AG1 primer	5'-AAGGGGTTGGCAGCAATATGTG-3'
Reverse R2 primer	5'-GTATGTAAGGTCAATTCTGTTC-3'

##### Heminested PCR primers for a 323-bp *BRCA1* gene amplicon are:

Forward AG2 primer	5'-GACGTTGTCATTAGTTCTTTGG-3' (also for sequencing)
Reverse R2 primer	5'-GTATGTAAGGTCAATTCTGTTC-3'

*4.4. Primary RT-PCR conditions for SARS-CoV-2 gene amplification*

To initiate the primary RT-PCR, a total volume of 25  $\mu$ L mixture was made in a PCR tube containing 20  $\mu$ L of ready-to-use LoTemp® PCR mix with denaturing chemicals (HiFi DNA Tech, LLC, Trumbull, CT, USA), 1  $\mu$ L (200 units) of Invitrogen SuperScript III Reverse Transcriptase, 1  $\mu$ L (40 units) of Ambion™ RNase Inhibitor, 0.1  $\mu$ L of Invitrogen 1 M DTT (dithiothreitol), 1  $\mu$ L of 10  $\mu$ M Co1 forward primer in TE buffer, 1  $\mu$ L of 10  $\mu$ M Co3 reverse primer in TE buffer and 1  $\mu$ L of sample RNA extract, synthetic N gene RNA extract as positive control or water as negative control.

The ramp rate of the thermal cycler was set to 0.9 °C/s. The program for the temperature steps was set as: 47°C for 30 min to generate the cDNA, 85°C 1 cycle for 10 min, followed by 30 cycles of 85°C 30 sec for denaturing, 50°C 30 sec for annealing, 65°C 1 min for primer extension, and final extension 65°C for 10 minutes.

*4.5. Heminested PCR conditions for SARS-CoV-2 gene amplification*

The heminested PCR mixture was a 25  $\mu$ L volume of complete PCR mixture containing 20  $\mu$ L of ready-to-use LoTemp® mix, 1  $\mu$ L of 10  $\mu$ M Co4 forward primer, 1  $\mu$ L of 10  $\mu$ M Co3 reverse primer and 3  $\mu$ L of water.

To initiate the heminested PCR, a trace (about 0.2  $\mu$ L) of primary PCR products was transferred by a micro-glass rod to the complete heminested PCR mixture. The thermocycling steps were programmed to 85°C 1 cycle for 10 min, followed by 30 cycles of 85°C 30 sec for denaturing, 50°C 30 sec for annealing, 65°C 1 min for primer extension, and final extension 65°C for 10 minutes.

The crude heminested PCR products showing an amplicon of 398 bp in size at agarose gel electrophoresis were subject to Sanger reaction without further purification.

*4.6. Primary and heminested PCR conditions for BRCA1 gene amplification*

The primary PCR mixture contained 20  $\mu$ L of ready-to-use LoTemp® PCR mix with denaturing chemicals (HiFi DNA Tech, LLC, Trumbull, CT, USA), 2  $\mu$ L of water, 1  $\mu$ L of 10  $\mu$ M forward AG1 primer, 1  $\mu$ L of 10  $\mu$ M reverse R2 primer and 1  $\mu$ L of sample RNA extract. For thermocycling, the temperature steps were programmed for an initial heating at 85 °C for 10 min, followed by 30 cycles at 85 °C for 30 s, 50 °C for 30 s and 65 °C for 1 min. The final extension was 65 °C for 10 min.

The heminested PCR mixture contained 20  $\mu$ L of ready-to-use LoTemp® PCR mix, 1  $\mu$ L of forward AG2 primer, 0.5  $\mu$ L of reverse R2 primer and 3.5  $\mu$ L of water in a total 25  $\mu$ L volume. About 0.2  $\mu$ L of the primary PCR products was transferred into the corresponding heminested PCR mixture with a micro-glass rod. The thermocycling steps were identical to those used for the primary PCR.

*4.7. Interpretation of PCR results*

As described above, 1  $\mu$ L of RNA extract of patient sample, 1  $\mu$ L of SARS-CoV-2 synthetic N gene RNA extract as positive control (P) and 1  $\mu$ L of negative cell extract control (N) were used to initiate a set of primary PCR for viral nucleic acid detection.

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In addition, 1  $\mu$ L of each sample was used to initiate a *BRCA1* gene primary PCR; and 1  $\mu$ L of negative cell extract and 1  $\mu$ L of water were used in the N and P control (see gel electrophoresis image below), respectively, for the *BRCA1* primary PCR.

In routine testing, only the heminested PCR products were subjected to agarose gel electrophoresis because most primary PCR amplicons are not visible to the naked eyes.

An aliquot of 5  $\mu$ L of each heminested PCR product was pipetted for agarose gel electrophoresis to detect the bands of the target DNA amplicons. As illustrated in the agarose gel image pasted below, for example, the negative control in Lane N of the SARS-CoV-2 series (upper half) and the water control in the P Lane of the *BRCA1* (lower half) must show no amplicons. A 398-bp amplicon must be visualized in the P Lane of the SARS-CoV-2 series (upper half) and a 323-bp amplicon must be visualized in the N Lane of the *BRCA1* series (lower half). Otherwise, the assay results are not valid.



As shown in the above image of gel electrophoresis, when the control amplicons on the P and N lanes are correctly visualized, there will be 4 possible combinations in the sample lanes. Sample 1 and 4 patterns are considered presumptive-positive for SARS-CoV-2 RNA. Sample 2 pattern is negative for SARS-CoV-2 as human *BRCA1* gene is demonstrated in the sample. Sample 3 pattern indicates that no human cell genetic material was present in the sample being tested; the test result is invalid. Sample 4 pattern indicates that there is SARS-CoV-2 RNA in the test sample, but the human DNA has been extracted by the acidified phenol chloroform mixture. In some samples, there are more copies of viral genome equivalents than human genome copies. Since human genomic DNA is a potent PCR inhibitor, acidified phenol chloroform is used to remove most of the DNA from the sample to be tested.

The presumptive-positive crude heminested PCR products showing an amplicon of 398 bp in size in the SARS-CoV-2 series and the negative samples showing an amplicon of 323 bp in size in the *BRCA1* series at agarose gel electrophoresis are subjected to Sanger reaction and automated sequencing for validation without further purification.



#### 4.8. DNA Sequencing

The 398-bp SARS-CoV-2 heminested PCR product (about 0.2  $\mu$ L), if detected at gel electrophoresis, was transferred by a micro-glass rod into a Sanger reaction tube containing 1  $\mu$ L of 10  $\mu$ M sequencing primer (Co3 or Co4), 1  $\mu$ L of BigDye® Terminator (v 1.1/Sequencing Standard Kit), 3.5  $\mu$ L 5 $\times$  buffer, and 14.5  $\mu$ L water in a total volume of 20  $\mu$ L for 20 enzymatic primer extension/termination reaction cycles according to the protocol supplied by the manufacturer (Applied Biosystems, Foster City, CA, USA). For the SARS-CoV-2 negative samples, the 323-bp heminested PCR product was transferred by a micro-glass rod into a Sanger reaction tube containing 1  $\mu$ L of 10  $\mu$ M forward AG2 primer, 1  $\mu$ L of BigDye® Terminator (v 1.1/Sequencing Standard Kit), 3.5  $\mu$ L 5 $\times$  buffer, and 14.5  $\mu$ L water in a total volume of 20  $\mu$ L for 20 enzymatic primer extension/termination reaction cycles according to the protocol supplied by the manufacturer (Applied Biosystems, Foster City, CA, USA). After a dye-terminator cleanup with a Centri-Sep column (Princeton Separations, Adelphia, NJ, USA), the reaction mixture was loaded in an Applied Biosystems SeqStudio Genetic Analyzer for sequence analysis. Sequence alignments were performed against the standard sequences stored in the GenBank database by on-line BLAST alignment analysis.

#### 5. Conclusions

Testing the cellular components of respiratory tract specimens instead of cell-free fluids only and using conventional nested RT-PCR to amplify the target nucleic acid can reduce the number of false negatives in molecular testing for SARS-CoV-2. Routine sequencing of the nested PCR products not only can eliminate false positives, but also can categorize the positive isolate into one of the six clades of SARS-CoV-2 known to exist in the United States, based on single nucleotide polymorphisms in a 398-base segment of the N gene. Extremely accurate routine laboratory tests for SARS-CoV-2 are needed in long-term care facilities and as businesses attempt to return to normal operation. Molecular diagnosis of SARS-CoV-2 infection will become a serious matter and a positive test result must be substantiated by a DNA sequencing electropherogram showing the genomic fingerprints of the virus. False-positive test reports can easily create unnecessary panic resulting in negative impacts on local economies.

Based on an official document dated March 15, 2020, the FDA issued a letter on February 4, 2020 authorizing emergency use of the CDC 2019-Novel Coronavirus (2019-nCoV, *renamed as SARS-CoV-2*) Real-Time Reverse Transcriptase (RT)-PCR Diagnostic Panel for the presumptive qualitative detection of nucleic acid from the 2019-nCoV in upper and lower respiratory specimens [41]. The methodology presented in this paper is for definitive qualitative detection of nucleic acid from the SARS-CoV-2 infecting the cells in upper and lower respiratory specimens. The FDA also advises that in performing Clinical Evaluation of newly developed RT-qPCR assays for the SARS-CoV-2, using Recommended Comparator Method for percent agreement performance calculations, false results can be investigated using an additional EUA RT-PCR assay, and/or Sanger sequencing [42]. Routine nucleotide sequencing of all detected 398-bp N gene amplicons guarantees no-false positive results in SARS-CoV-2 assays.

#### Author Contributions

S.H.L.: Conceptualization, study design, methodology, data analysis, manuscript preparation and final review

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**Conflicts of Interest:** Sin Hang Lee is Director of Milford Molecular Diagnostics Laboratory specialized in developing DNA sequencing-based diagnostic tests implementable in community hospital laboratories. The reagents and equipment used in this work can be substituted with similar products from various suppliers. The technology presented is not patentable.

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