In silico analysis of RT-qPCR designs recommended by WHO for detection of SARS-CoV-2 and a commercial kit validated following UNE/EN ISO 17025:2005 and two reference laboratories

Antonio Martínez-Murcia[1,2], Gema Bru[2], Aaron Navarro[2], Patricia Ros-Tárraga[2], Adrián García-Sirera[2], Laura Pérez[2]

[1]Department of Microbiology, University Miguel Hernández, 03300-Orihuela, Alicante, Spain

[2]Genetic PCR Solutions™, 03206-Elche, Alicante, Spain

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***Corresponding author:**

Dr Antonio J. Martínez-Murcia

Department of Microbiology,

University Miguel Hernández,

03300-Orihuela, Alicante, Spain

Phone: +34-965429901

Fax: +34-966661040

Electronic address: ammurcia@umh.es

ABSTRACT

The Corona Virus Disease 2019 (COVID-19), caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), has become a serious infectious disease affecting human health worldwide and rapidly declared a pandemic by WHO. Early, because the urgent need of reliable and rapid SARS-CoV-2 detection strategies and once the first SARS-CoV-2 genome sequence was available, several reference laboratories developed RT-qPCR methods which have been deposited in the WHO website. Only weeks later, after additional SARS-CoV-2 genomes were sequenced by several unrelated laboratories, the kit GPS™ CoVID-19 dtec-RT-qPCR Test developed by scientific team from Genetic PCR Solutions™ (GPS™, Alicante, Spain) was one of the first commercially available worldwide. The parameters specificity (inclusivity/exclusivity), quantitative phase analysis (10-10^6 copies), reliability (repeatability/reproducibility) and sensitivity (detection/quantification limits) of this qPCR kit were validated with the strict acceptance criteria recommended by the UNE/EN ISO 17025:2005 and ISO/IEC 15189:2012. Diagnostic validation was achieved by two independent reference laboratories, the Instituto de Salud Carlos III (ISCIII), (Madrid, Spain) and the Public Health England (PHE; Colindale, London, UK). The present study approached the *in silico* specificity of the protocols currently recommended by WHO (16, 18, 23-27), a recently published RT-qPCR method, and the GPS™ CoVID-19 dtec-RT-qPCR Test. The analysis suggested the later RT-qPCR design as the more exclusive by far.

1. INTRODUCTION

Last 30th January, the Emergency Committee of the World Health Organization (WHO) under the International Health Regulations (IHR) declared an outbreak of pneumonia, lately named Corona Virus Disease 2019 (COVID-19), as a "Public Health Emergency of International Concern" (PHEIC). The disease was caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), linked to the Huanan Seafood Wholesale Market in Wuhan, which was previously notified by the Chinese Health Authorities on 31st December 2019. Chinese scientists were able to isolate this novel coronavirus and the first genome was rapidly provided (http://virological.org/t/novel-2019-coronavirus-genome/319). A few weeks later, the disease spread worldwide and become a serious infectious disease affecting human health worldwide which forced the WHO to declare a Pandemic on March 11 when more than 118,000 positives were already registered in 114 countries and 4,291 deaths. Today, 26th April, the number of positive cases globally amounts to more than 2.9 million people with more than 203,000 deaths. Faced with the aggressiveness of this global alarm, the massive, reliable, and rapid diagnosis is undoubtedly vital and foremost priority for decision-making at each stage to facilitate public health interventions, and the needs have overwhelmed any forecast.

SARS-CoV-2 is a *Betacoronavirus* subgenus *Sarbecovirus* of group 2B and, in many ways, it resembles SARS-CoV, Bat-SARS-CoV and other Bat SARS-like-CoV (1-6). Similar to SARS-CoV, a recent study confirmed that Angiotensin Converting Enzyme 2 (ACE2), a membrane exopeptidase, is the receptor used by SARS-CoV-2 for entry into the human cells (7-9). There are some theories about the SARS-CoV-2 origins. One of them is the natural selection in an animal host before zoonotic transfer. SARS-CoV-2 have a high similarity to Bat SARS-like-CoV, such as Bat coronavirus isolate RaTG13 (GenBank No.: MN996532.1), which showed a 96% of nucleotide similarity with SARS-CoV-2. This indicates bats can act as reservoir hosts for its progenitor (10).

Current molecular diagnostic tools for viral detection are typically based upon the amplification of target-specific genetic sequences using the Polymerase Chain Reaction (PCR). In acute respiratory infection, real time PCR (so-called quantitative PCR; qPCR) is the gold-standard and routinely used to detect causative viruses as, by far, is the most sensitive and reliable method (11-15). On the 17th January, the WHO published the very first primers and probes for Reverse Transcriptase qPCR (RT-qPCR) developed by the Charité – Universitätsmedizin Berlin Institute of Virology, Berlin, Germany and German Centre for Infection Research (DZIF), Berlin, Germany and collaborators. They used known genomic data from SARS-CoV and SARS-CoV related (Bat viruses) to generate a non-redundant alignment. The candidate diagnostic RT-PCR assay was

designed upon the first SARS-CoV-2 sequence release, based on their matching to SARS-CoV of the sequence alignment. Because only a single SARS-CoV-2 genome was available, the two monoplex PCR protocols (ORF1ab and N genes) designed to detect SARS-CoV-2 are also reactive to SARS-CoV and Bat SARS-like-CoV. A few days later, 23rd January, the same laboratory together with reference laboratories from the Netherlands, Hong Kong, France, United Kingdom, and Belgium, added a third monoplex-RT-qPCR (16). Many laboratories worldwide are currently using this RT-qPCR protocol (17) and also it has been the basis to develop many commercial kits. Almost simultaneously, other primers and probes were designed and available by Institut Pasteur, París; Centers for Disease Control and Prevention (CDC), Division of Viral Diseases, Atlanta, USA; National Institute for Viral Disease Control and Prevention (CDC), China; The University of Hong Kong – LKS Faculty of Medicine, Hong Kong; Department of Medical Sciences, Ministry of Public Health, Thailand; the National Institute of Infectious Diseases, Japan (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/laboratory-guidance), and some laboratories are publishing new primers and probes, as Hong Kong University – State Key Laboratory of Emerging Infectious Disease (17). The Respiratory Viruses Branch, Division of Viral Diseases, CDC, Atlanta, recently (4th February) updated a manual of Real-Time RT-PCR Panel for detection of this 2019-Novel Coronavirus (SARS-CoV-2), which was modified 15th March. The SARS-CoV-2 primer and probe sets were designed for the universal detection of SARS-like coronaviruses (N3 assay) and for specific detection of SARS-CoV-2 (N1 and N2 assays). Lately, the N3 assay has been removed (https://www.who.int/docs/default-source/coronaviruse/whoinhouseassays.pdf?sfvrsn=de3a76aa_2). Finally, Institut Pasteur, Paris, based on the first sequences of SARS-CoV-2 available on the GISAID database (Global Initiative on Sharing All Influenza Data) on 11th January, updated a protocol for the detection of SARS-CoV-2 for two RdRp targets (IP2 and IP4) (18).

Some biotechnology-based companies have recently developed kits for detection of SARS-CoV-2, based on RT-qPCR and provided easy transfer of technology to laboratories worldwide. A fully SARS-CoV-2-specific RT-qPCR thermostable kit was early launched on 27th January by Genetic PCR Solutions™ (GPS™), a brand of Genetic Analysis Strategies SL. (Alicante, Spain). The alignments used at that time included 13 SARS-CoV-2 genome sequences released by 6 different laboratories, deposited in GISAID and available since 19th January 2020. With the purpose to discriminate this new SARS-CoV-2 of present outbreak from previous related SARS, a second independent monoplex RT-qPCR test to detect any other non-SARS-CoV-2 was also produced and provided (not shown). On this study, we have performed a deep analytical and diagnostic

validations of the GPS™ COVID-19 dtec-RT-qPCR Test, following the UNE/EN ISO 17025:2005 and ISO/IEC 15189:2012, respectively. A comparative analysis of the specificity (inclusivity and exclusivity) of the designed primers and probes with most previously published RT-qPCR methods is also here reported.

2. MATERIALS AND METHODS

2.1. GENOME SEQUENCES ALIGNMENT AND PHYLOGENETIC ANALYSIS

Full alignments of ten SARS-CoV-2 genomic sequences (EPI_ISL_406595, EPI_ISL_403931, EPI_ISL_403934, EPI_ISL_406594, EPI_ISL_406596, EPI_ISL_406973, EPI_ISL_406844, EPI_ISL_407084, EPI_ISL_406034, EPI_ISL_407193) were selected to represent intra-species SARS-CoV-2 variability. With the aim to analyse the phylogenetic relationships of SARS-CoV-2 to the most closely related coronaviruses, the genomic sequences of different Betacoronavirus strains, available on NCBI and/or GISAID websites, were selected: Bat-CoV (MK211377.1, MK211378.1, MK211375.1, KY770859.1, JX993988.1, JX993987.1, KJ473813.1, KY770860.1, MN996532.1, KF569996.1), Bat SARS-like-CoV (MG772933.1, MG772934.1, KY417146.1, KY417152.1, KC881005.1), SARS-CoV (AY395003.1, AY394996.1, AY304488.1, AY278554.2, AY394985.1, AY394983.1) and Pangolin-CoV (EPI_ISL_410721, EPI_ISL_410538, EPI_ISL_410539, EPI_ISL_410541). The complete alignment (35 genomic sequences, ca. 18,141 bp) was performed by Clustal W (19). The evolutionary distances were computed using the Maximum Composite Likelihood method (20) and the corresponding phylogenetic tree (Figure 1) was obtained by Neighbour joining method (21), with bootstrap values for 1000 replicates, using the MEGA 5.2.2 software (22).

2.2. *IN SILICO* COMPARATIVE ANALYSIS OF PRIMERS/PROBES SPECIFICITY

The genomic sequences of 63 SARS-CoV-2 strains retrieved from GISAID, were preliminarily selected to analyse the *in silico* inclusivity of the primers and probes of GPS™ COVID-19 dtec-RT-qPCR Test and the RT-qPCR designs recently published, some of them recommended by the WHO (16-18, 23-27). The target sequences of all RT-qPCR designs were aligned to the corresponding homologous regions of closely related Betacoronavirus (Bat-CoV, Bat SARS-like-CoV, SARS-CoV, Pangolin-CoV) using the Basic Local Alignment Search Tool (BLAST) software available on the National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi) website databases (Bethesda, MD, USA). The specificity

in silico of the primers and probes provided in the GPS™ COVID-19 dtec-RT-qPCR kit was updated at time of writing the present article against the 705 entries currently available at the NCBI. The three sequences representing the groups of Bat SARS-like-CoV, SARS-CoV, Bat-CoV, and Pangolin-CoV, which showed the highest sequence similarities to these of SARS-CoV-2, were selected to perform a comparative *in silico* specificity analysis by counting the number of mismatches of the primers and probes of the GPS™ COVID-19 dtec-RT-qPCR Test and the other RT-qPCR designs recently published (Table 1). An illustration of the mismatching of primers/probe sequences of the GPS™ CoVID-19 dtec-RT-qPCR Test, respect of the SARS-CoV-2 (MN975262.1), Bat SARS-like-CoV (MG772934.1), SARS-CoV (AY304489.1), Bat-CoV (KY770859.1) and Pangolin-CoV (EPI_ISL_410539) groups is shown in Figure 2.

2.3. GPS™ COVID-19 dtec-RT-qPCR Test

Assays for qPCR were prepared as described in the manual provided in the GPS™ COVID-19 dtec-RT-qPCR kit (Alicante, Spain). Internal, positive, and negative PCR controls were included. The reaction mixtures were subjected to qPCR in a StepOnePlus™ or a QuantStudio3 (ABI), programmed with a standard regime composed by first retrotranscription step at 50 ºC for 10 min, followed by a denaturation step at 95 °C for 1 min, and 40 cycles of amplification as follows: denaturation at 95 ºC for 10 second, annealing/extension at 60 ºC for 60 seconds. Standard curve calibration of the qPCR was performed by preparing ten-fold dilution series containing 10 to 10^6 copies of standard template provide in the kit, but also using $5{\cdot}10^6$ to $5{\cdot}10$ copies of two complete synthetic RNA genomes from SARS-CoV-2 isolate Australia/VIC01/2020 (GenBank No.: MT007544.1) and isolate Wuhan-Hu-1, (GenBank No.: MN908947.3), synthesized by Twist Bioscience (South San Francisco, United States of America).

2.4. ANALYTICAL AND DIAGNOSTIC VALIDATION OF THE GPS™ CoVID-19 dtec-RT-qPCR Test

The method for SARS-CoV-2 detection using the GPS™ CoVID-19 dtec-RT-qPCR Test was subjected to strict validation according to guidelines of the UNE/EN ISO/IEC 17025:2005 and ISO/IEC 15189 (28, 29). Validation terms included *in vitro* specificity (inclusivity/exclusivity), a quantitative phase analysis, reliability (repeatability/reproducibility), and sensitivity (detection and quantification limits) with the acceptance criteria shown in Table 2. The linearity analysis of the calibration curve was carried out with a ten-fold decimal dilution series (ranging 10-10^6 copies) of the standard template provided in the kit. The full calibration curve was repeated 10 times. The validity of the standard curve was assessed using a linear regression in a semi-logarithmic graph

and the following equation: $Y = a * X + b$, where Y is the obtained Ct for each decimal dilution of the range; X, the copy number logarithm; a, slope of the linear regression and b, the cut-off respect of Y axis. Average data from the fitted regression lines showed the slope (a) and regression coefficient (R^2) values. Criteria for acceptance involve a slope value between $-3.587 < a < -3.103$, and R^2 higher than 0.98. The Fisher test was performed in order to validate the linear model. The model was accepted when F obtained from assay (F_{assay}) was lower than F given by a Fisher distribution table (F_{fisher}) for [$v_1 = k-1$; $v_2 = k * (n - 1)$], with a 95% Confidence Interval. Efficiency (E) was calculated according to equations where *slope* was obtained from the linear regression (a): $E = 10^{-1/slope}$; e = *% Efficiency* = (E – 1) * 100. Efficiency was accepted if 90% < e < 110%. Reliability of the qPCR was estimated through the assessment of repeatability and reproducibility. Both parameters should show variation coefficient values (CV) of < 10 % to be considered acceptable. To evaluate the repeatability of the method, 10 replicates of ten-fold standard dilution series (from 10^6 to 10 standard template copies) were prepared. The test for each replicated dilution serie was performed independently. The coefficient of variation (CV) was estimated as follows, where S is the standard deviation and $\bar{x}$, the average of Ct values: $CV = S / \bar{x} * 100$. For reproducibility, values obtained in the previous test (repeatability evaluation) were compared with values obtained in a new experimental set composed by five standard calibration curves (n = 5) made by a different technician on a different date and a different PCR equipment. The coefficient of variation (CV) was estimated to assess the reproducibility as follows, where S_{ab} is standard deviation between technician a and b, and $\bar{x}_{ab}$ is the Ct value averaged from data sets obtained from technician a and b: $CV = S_{ab} / \bar{x}_{ab}$. The limit of detection (LOD) of the qPCR at a 90% confidence level according to the laboratory's procedure test was estimated by performing 15 tests with 10 copies of standard template. Estimation of the limit of quantification (LOQ) of the method generating a repeatability result was achieved by analysing the results with a t-Student test with a 95% Confidence Interval. Assays were performed for two sets of 15 tests for both, 10 and 5 copies of standard templates. Results of quantification were used to calculate the experimental t value as follows, where $\bar{x}$ is the average of the sample, μ is the reference value of copy number; s is the typical deviation and n is the number of samples: $t = (\bar{x} - \mu) / (s / \sqrt{n})$. The accuracy of the LOQ was acceptable if the t value obtained from the assays was lower than the theoretical value from a Student table (t value < $t_{Student}$; freedom degree n - 1).

Diagnostic validation of GPS™ CoVID-19 dtec-RT-qPCR Test was a service performed by the Instituto de Salud Carlos III (ISCIII), reference laboratory for biomedical investigation and Public Health (Madrid, Spain), by testing 80 breath specimens retrieved from the anonymous biobank of

Centro Nacional de Microbiología (CNM, Madrid, Spain). These specimens included 41 positive and 39 negative, previously characterized by a reference protocol (16). The GPS™ CoVID-19 dtec-RT-qPCR Test was also evaluated by the Public Health England (PHE; Colindale, London, UK) with a sample-panel of 195 specimens, including respiratory clinical specimens negative for SARS-CoV-2 as determined by the validated in-house PHE PCR assay (targeting the RNA-dependent RNA polymerase; RdRP) and three dilutions of material positive for SARS-CoV-2.

3. RESULTS

A preliminary SARS-CoV-2 genome sequence alignment by BLAST showed over 99.9% of overall sequence similarity between all SARS-CoV-2 sequences available. The closest relatives to SARS-CoV-2 sequences were these belonging to Betacoronavirus strains of SARS-CoV, Bat SARS-like-CoV, Bat-CoV, and Pangolin-CoV. Therefore, 10 SARS-CoV-2 sequences and 25 of the most closely related strains representing these Betacoronaviruses, were selected to build the phylogenetic tree shown in Figure 1. The analysis indicated that Bat-CoV RaTG13 (MN996532.1) and Pangolin-CoV (EPI_ISL_410721) showed the highest sequence similarity to SARS-CoV-2 (96.70% and 90.74%, respectively). Similarities with three other Pangolin-CoV sequences available (EPI_ISL_410538, EPI_ISL_410539 and EPI_ISL_410541), however, showed a lower homology (85.21%). The rest of related Betacoronaviruses were related as follows: 88.79% and 88.50% for two Bat SARS-like-CoV sequences (MG772933.1 and MG772934.1, respectively), a range of 81.16%-81.13% for human SARS-CoV (AY395003.1, AY394996.1, AY304488.1, AY278554.2, AY394985.1, AY394983.1), and 81.01-80.70% for Bat-CoV (MK211377.1, MK211378.1, MK211375.1, KY770859.1, JX993988.1, JX993987.1, KJ473813.1, KY770860.1, KF569996.1).

The primers and probes sequences of the GPS™ COVID-19 dtec-RT-qPCR Test and the other RT-qPCR designs recently published, including those recommended by WHO, were located in the alignments containing 63 SARS-CoV-2 and other selected sequences of Betacoronaviruses (SARS-CoV, Bat-CoV, Bat SARS-like-CoV, and Pangolin-CoV) for *in silico* specificity analysis. Number of mismatches found with sequences of SARS-CoV-2 (inclusivity) and these for the rest of Betacoronaviruses were annotated in Table 1. All RT-qPCR designs subjected to study showed they are inclusive for SARS-CoV-2. Nevertheless, it is worth of note that the probe for N gene designed by Hong Kong University (25), showed 4 mismatches with all SARS-CoV-2 sequences analysed. In terms of exclusivity however, the highest number of mismatches was found in the GPS™ RT-qPCR design, ranging from 19 to 48 (Pangolin-CoV and Bat-CoV, respectively), often

with a considerable number of indels. The second more exclusive RT-qPCR design was this recently published by Chan JF, et al., from Hong Kong University – State Key Laboratory of Emerging Infectious Diseases (17), whose target gene is the S gene of SARS-CoV-2 showing a range of values between 11 and 25 mismatches (Pangolin-CoV and Bat SARS-like-CoV, respectively). The Institut Pasteur, Paris (18) has proposed two RT-qPCR designs for the RdRp gene, so-called IP2 and IP4. Although in overall IP2 and IP4 showed a total of 6-12 and 12-17 mismatches respectively, both exhibited only 4 mismatches with some Pangolin-CoV sequences. The CDC, Atlanta (23), published 3 primers/probe sets for SARS-CoV-2 N gene (N1, N2 and N3). The N1 set showed 8-10 mismatches with Bat-CoV and Bat SARS-like-CoV, 11 with SARS-CoV, and 2-11 with Pangolin-CoV. N2 primers/probe showed 6-7 mismatches with Bat-CoV, Bat SARS-like-CoV, and SARS-CoV, but only 4 with Pangolin-CoV. Finally, for the N3-design, 2-5 mismatches were found for the other coronavirus, but a single mismatch with Pangolin-CoV. The RT-qPCR designs of Charité – Universitätsmedizin Berlin Institute of Virology, Berlin, Germany and German Centre for Infection Research (DZIF), Berlin, Germany, and other collaborators (16), used the RdRp gene (with two probes, P1 and P2) and the E gene of SARS-CoV-2. The set for RdRp with P2, showed 4 mismatches with Bat-CoV, 3-5 with Bat SARS-like-CoV, 2-3 with SARS-CoV and 2-5 with Pangolin-CoV. When using P1 probe showed 2-3 mismatches (Bat-CoV, Bat SARS-like-CoV and Pangolin-CoV) but only 1 with SARS-CoV. Moreover, the E gene set showed 0 mismatches with many sequences from all other coronaviruses. The designs from the National Institute for Viral Disease Control and Prevention, China (24), were based on ORF1ab and N genes. While the first showed 7-9 mismatches with SARS related CoV (2-4 with Pangolin-CoV), the second exhibited a range of 3-10 with all coronaviruses analysed. Scientists from the Faculty of Medicine of Hong Kong University (25), suggested RT-qPCR based on the ORF1b and N genes. While ORF1b showed full matching with some sequences of other coronaviruses, the N gene-target differed in 4-5 positions of the sequences. Other designs targeting the N gene were those by the Ministry of Public Health of Thailand (26) (6-7 mismatches in overall; 2-6 with Pangolin-CoV) and the National Institute of Infectious Diseases, Japan (27) (9-11 mismatches; 3-7 with Pangolin-CoV).

In order to illustrate the extent of mismatching, an alignment of primers/probe sequences of the GPS™ CoVID-19 dtec-RT-qPCR Test, respect to the SARS-CoV-2 and selected sequences of Bat SARS-like-CoV (MG772934.1), SARS-CoV (AY304489.1), Bat-CoV (KY770859.1) and Pangolin-CoV (EPI_ISL_410539) has been shown in Figure 2. The total number of mismatches were, 31 for Bat SARS-like-CoV (Figure 2b), 37 for SARS-CoV (Figure 2c), 40 for Bat-CoV (Figure

2d) and 20 for Pangolin-CoV (Figure 2e), including considerable indels in probe and reverse primer sequences.

A complete analytical and diagnostic validation of the GPS™ CoVID-19 dtec-RT-qPCR Test for the detection of SARS-CoV-2 was undertaken and the results were summarized in Table 2. Empirical validation terms were evaluated for a minimum of 10 assays (15 in the case of LOD and LOQ) and results were subjected to stablished criteria for acceptance (Table 2), according to the guidelines of the UNE/EN ISO/IEC 17025:2005 and ISO/IEC 15189 (28, 29). The standard curve calibration of the qPCR was performed from ten-fold dilution series containing 10 to 10^6 copies of standard template (Figure 3a, b). The inclusivity *in vitro* was assessed by testing two complete synthetic RNA genomes from isolate Australia/VIC01/2020 (GenBank No.: MT007544.1) and isolate Wuhan-Hu-1, (GenBank No.: MN908947.3) SARS-CoV2 and results obtained from $5 \cdot 10$ to $5 \cdot 10^6$ copies are shown in Figure 3c, d. The slope and coefficient (R^2) values obtained were -3.534 and 0.9986, respectively, the F_{assay} (0.014) was lower than the F_{fisher} (5.318), and efficiency obtained was 93.1%, considering these values acceptable. The CV values, to assess the reliability of the method, ranged from 0.53% to 1.31% for repeatability, and 0.59% to 1.83% for reproducibility. LOD for 10 copies was 100% reproducible and accuracy of LOQ of 10 copies was acceptable as the *t* value (0.582) was lower than the theoretical value from a Student table ($t_{Student}$ = 2.145). Finally, the results obtained in the diagnostic validation of the GPS™ CoVID-19 dtec-RT-qPCR Test by the Instituto de Salud Carlos III (ISCIII) are shown in Table 3. Full agreement was obtained by comparing data resulting from this commercial kit and these from the reference qPCR used by ISCIII. Consequently, 100% of diagnostic sensitivity and 100% of diagnostic specificity was assigned. The GPS™ kit was also evaluated by the Public Health England (PHE; Colindale, London, UK) and results completely correlated (100%) with these determined by the PHE in house assay targeting the RNA-dependent RNA polymerase (RdRP) of SARS-CoV-2 (not shown).

4. DISCUSSION

Only three months ago, an outbreak of severe pneumonia caused by the novel coronavirus SARS-CoV-2 started in Wuhan (China) and rapidly expanded to almost all areas worldwide. Early, Chinese scientists were able to isolate a novel coronavirus and a first genome was provided on the 7th January. Due to the need of urgent detection tools, several laboratories developed RT-qPCR methods by designing primers and probes from the alignment of a single SARS-CoV-2 genome sequence with known SARS-CoV, and the protocols were published at the WHO website

(16-18, 23-27). As of 19th January 2020, 13 other genome sequences from 6 different laboratories were released on to the GISAID, scientists from Genetic PCR Solutions™ (GPS™, Alicante, Spain) analysed the set data and found a more specific target for SARS-CoV-2 qPCR-detection. Consequently, at the end of January, the kit GPS™ CoVID-19 dtec-RT-qPCR Test was available worldwide, being this company one of the pioneers able to commercialize a PCR-kit for the CoVID-19. The sensibility of the test is also crucial as positive patients should be found at early stages of CoVID-19 and in asymptomatic patients.

A purpose on this study was the comparison of designed primers and probes for the GPS™ CoVID-19 dtec-RT-qPCR Test, the seven RT-qPCR protocols currently published by WHO (16, 18, 23-27), and an improved RT-qPCR method recently published (17) in order to ascertain their degree of *in silico* specificity. The phylogenetic analysis performed in the present study indicated that, while SARS-CoV-2 shows a high sequence homology (over 99.91-99.97%), the closest relatives were strains of several Betacoronavirus, i.e., SARS-CoV, SARS-like-CoV, Bat-CoV, and Pangolin-CoV, which confirmed previous results (1, 6, 30-32). The sequence identity with coronaviruses isolated from Pangolin was considerably high (85-90%) which, according to other authors (32-34), could be taken as a step in the course of evolutive zoonosis of SARS-CoV-2 from bats. We have found that a single genome sequence (MN996532.1) of the Bat coronavirus RaTG13 isolated from *Rhinolophus affinis* in Wuhan, showed the highest homology level (96.70%) to SARS-CoV-2, as previously described (1, 2, 4, 6, 34, 35). However, because only a single Bat-CoV sequence showing this high identity is available, and it was deposited after the outbreak started (27th January), the possibility of RNA contamination during genome sequencing should be ruled-out before take further conclusions. The sequence alignments performed from representative sequences have served to analyse in detail the *in silico* specificity of these RT-qPCR designs and the number of mismatches retrieved were calculated (Table 1). In overall, all qPCR designs were inclusive for SARS-CoV-2 as primers and probes showed a good matching. Only the probe for N gene designed by Chu et al., from Hong Kong University - LKS Faculty of Medicine (25) showed 4 mismatches which may affect to its binding, particularly considering the low melting temperature according to the primary structure of this probe. In some cases, single nucleotide mismatching was observed in some primers, but none of them were located close to primer 3'-end. Considering all updated alignments, the SARS-CoV-2 isolate Australia/VIC01/2020 (GenBank No.: MT007544.1) was the only sequence which showed a unique mismatch to the probe designed for the GPS™ CoVID-19 dtec-RT-qPCR Test. Therefore, the test was run by using ten-fold dilution series containing $5{\cdot}10$ to $5{\cdot}10^6$ of the synthetic RNA genomes from SARS-CoV-2

isolate Australia/VIC01/2020 and the resulting Ct values of calibration curve correlated with this obtained with synthetic RNA genome of isolate Wuhan-Hu-1 (Figure 3), indicating that this mismatch in the probe did not affect to the method.

The *in silico* analysis for exclusivity was more complex, showing a wide range of discriminative power for the methods subjected to analysis (Table 1). For instance, the two RT-qPCR designs IP2 and IP4 developed by Institut Pasteur seems to discriminate well between SARS-CoV-2 and other respiratory virus as confirmed for a panel of specimens (18). The present *in silico* analysis showed a considerably mismatching respect to other SARS, Bat SARS and related coronavirus. The CDC from Atlanta (USA) designed 3 different primer/probes sets named N1, N2 and N3 (23). We found a low exclusivity in the N3 primer/probe, but a few weeks ago, this set was removed from the panel (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/laboratory-guidance). Both N1 and N2 showed a good level of mismatching with most coronaviruses except for some Pangolin-CoV sequences which showed very few nucleotide differences. In overall, there is no prediction of potential false positive RT-qPCR results when these primers and probes are combined. In fact, no cross-reactivity was observed in several human CoV, MERS-CoV and SARS-CoV specimens tested (23). The RT-qPCR proposed by authors from Charité-Berlín and other collaborators, designed to detect SARS-CoV-2, SARS-CoV and Bat SARS-like-CoV (16), is probably the most used worldwide. They recommend the use of E gene assay as the first-line screening tool, followed by confirmatory testing with the two probes P1 and P2 in the RdRp gene assay. While P1 probe reacts with both SARS-CoV-2 and SARS-CoV, P2 probe is specific only for SARS-CoV-2. Although our *in silico* results confirmed that purpose for P1 (Table 1), the RdRp_P2 assay may also react with some other coronavirus. The National Institute for viral Disease Control and Prevention in China developed two RT-qPCR assays for ORF1ab and N genes (24). Both showed a good overall mismatching to consider they are exclusive, except for some Pangolin-CoV sequences. A similar conclusion may be taken for the N-gene RT-qPCR at the Ministry of Public Health of Thailand (26). The data of Table 1 indicated that primer/probe sets of Faculty of Medicine, Hong Kong University (25), may be reactive with SARS-CoV-2, SARS-CoV and Bat SARS-like-CoV. The exclusivity of the RT-qPCR design developed at the National Institute of Infectious Diseases of Japan (27) clearly resided in the reverse primer as showed 7 mismatches with all SARS related coronavirus. Finally, Chan et al., 2020 (17) developed three RT-qPCR assays targeting RdRp/Hel, S and N genes of SARS-CoV-2. They select the RdRp/Hel assay as considered to give the best amplification performance and was tested in parallel with the RdRp-P2 from Charité-Berlin (16). All positive patients with the

RdRp-P2 assay were positive with the RdRp/Hel design. However, 42 patients negative for the RdRp-P2 assay were positive with RdRp/Hel and they found that only RdRp-P2 assay, but not RdRp/Hel, cross-reacted with SARS-CoV culture lysates (17). Above findings agreed with expected exclusivity derived from the present study. Two additional comparative *in vitro* analysis of some RT-qPCR designs have recently been published (36, 37). These primer/probes of ORF1ab from The National Institute for Viral Disease Control and Prevention from China (24), seems to be the most sensitive but the assays N2, N3 from the Centers for Disease Control and Prevention, Atlanta, and the N-targeted assay from the National Institute of Infectious Disease, Japan, were the most recommended (36). This partially disagrees our findings as the N3 design may react with other coronaviruses than SARS-CoV-2 (recently removed for the CDC panel). In the study by Arun et al., 2020 (37), the specificity of methods from Charité (Berlin) and CDC (Atlanta) was tested finding no false positive results but differences in the sensitivity was observed. The most sensitive were N2 (CDC) and E (Charité-Berlin). However, the present study indicates the RT-qPCR for E target may react with different SARS coronavirus. Finally, the commercially available kit GPS™ COVID-19 dtec-RT-qPCR Test have shown the highest number of mismatches (i.e., 19-48) for all CoV sequences described so far, including these of Pangolin-CoV which showed a range of 19-31 mismatches. In addition, considerable indels were discerned which enlarge even more the exclusivity of this design.

The kit GPS™ CoVID-19 dtec-RT-qPCR Test was subjected to analytical and diagnostic validation and criteria for acceptance were those recommended by the guidelines of the UNE/EN ISO/IEC 17025:2005 and ISO/IEC 15189. The results are summarized in Table 2. The study of the quantitative PCR phase within the standard curve is essential to afford quantification experiments. The analysis was repeated a minimum of ten times and average value for *a* (slope) was the optimum according to the limits of standards, as well as the regression coefficient (R^2=1). The linear model was evaluated by the Fisher test, resulting in an obtained value for F_{assay} notably lower than this of F_{fisher} (given by a Fisher table), therefore, the model was accepted. The efficiency or yield of the PCR amplification was found to be e = 93.1%, fitting well in the range of 90-110%. Reliability, considered as the ability of a method to produce results free of random errors, was estimated through the assessment of repeatability and reproducibility. While the first was evaluated by testing independently the prepared 10 replicates of standard ten-fold dilution series (from 10^6 to 10 standard template copies), the reproducibility was assayed comparing the results obtained in the ten previous tests (repeatability evaluation) with the results obtained from five standard calibration curves (n = 5) made by a different technician on a different date and a different

PCR equipment. The coefficient of variation (CV) obtained in all cases for both, repeatability and reproducibility, was always much lower than 10% (Table 2), therefore the test was considered repeatable and reproducible. To accept a PCR limit of detection (LOD) we should ascertain the smallest number of target units generating a positive amplification at a 90% confidence level. The LOD was tested with the usual protocol for 10 copies repeated 15 times with a positive result in all cases (100%). The estimation of the PCR limit of quantification (LOQ) involved determination of the lowest number of target units able to generate a repeatability result of quantification. Assays were performed for two sets of 15 tests for both 10 copies of standard templates. The LOQ measurement in both cases was validated with a t-Student test with a confidence interval of 95%, considering the acceptable accuracy limit of quantification. Consequently, the calculated parameters to evaluate repeatability/reproducibility (reliability) and sensitivity (detection and quantification limits) were all accepted and the kit GPS™ CoVID-19 dtec-RT-qPCR Test passed this validation. The kit received diagnostic validation at two different reference laboratories (ISCIII, Madrid; and PHE, London). The results shown in Table 3 indicated 100% of diagnostic sensitivity and 100% of diagnostic specificity was assigned, demonstrating the reliability of the GPS™ CoVID-19 dtec-RT-qPCR Test for the detection of SARS-CoV-2.

Obviously, a lack of SARS-CoV-2 genomes available, or positive controls, at the time of designing the RT-qPCR primers and probes published since CoVID-19 appeared (12, 13, 16, 17, 19-22), may explain the scarce exclusivity found for some of them. Despite the greater or lesser *in silico* specificity of the different RT-qPCR designs published in the WHO website, due to host specificity of Bat-CoV, Bat SARS-like, Pangolin-CoV, together with the fact of that no human-SARS have been reported since 2004, all positive results obtained from these designs would be considered as SARS-CoV-2 infections (25, 38). However, RNA viruses in particular show substantial genetic variability. Although efforts were made to design RT-qPCR assays in conserved regions of the viral genomes, variability resulting in mismatches between the primers and probes and the target sequences can result in diminished assay performance and possible false negative results. Primers and probes should be reviewed and updated according to new data, which will increase exponentially during the next few weeks/months.

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FIGURE LEGENDS

Figure 1. Phylogenetic neighbour-joining tree showing relationships of SARS-CoV-2 and the most related strains of some Betacoronavirus, including SARS-CoV, Bat-CoV, Bat SARS-like-CoV and Pangolin-CoV. The analysis was derived from the alignment of 18,141 nucleotides. Numbers at nodes indicate bootstrap values (percentage of 1000 replicates).

Figure 2. Illustrative alignment representation of the primers/probes sequences of GPS™ CoVID-19 dtec-RT-qPCR Test with a) SARS-CoV-2 (MN975262.1); b) Bat SARS-like-CoV (MG772934.1); c) SARS-CoV (AY304489.1); d) Bat-CoV (KY770859.1); and e) Pangolin-CoV (EPI_ISL_410539).

Figure 3. Quality Control of the GPS™ CoVID-19 dtec-RT-qPCR Test with data of six ranges of decimal dilution from 10^6 copies to 10 copies, and negative control. a) Amplification plot and b) a representative calibration curve with stats. Inclusivity of the GPS™ CoVID-19 dtec-RT-qPCR Test using six ranges of decimal dilution from $5{\cdot}10^6$ copies to $5{\cdot}10$ copies, and negative control. Amplification plot of synthetic RNA of c) Australian strain of SARS-CoV-2 (MT007544.1); and d) Wuhan-Hu-1 strain of SARS-CoV-2 (MN908947.3).

Table 1. Number of mismatches found in the primers/probes sets of the GPS™ COVID-19 dtec-RT-qPCR Test and recently published in WHO and scientific journals, from the comparative *in silico* analysis with Bat-CoV, Bat SARS-like-CoV, SARS-CoV and Pangolin-CoV. Numbers in bold show the sum of the mismatches found in the primers/probes of the RT-qPCR designs. Numbers in brackets show the mismatches found in forward primer (FP), probe (P) and reverse primer (RP) following this format: [FP / P / RP].

			TOTAL MISMATCHES									
Reference	**INSTITUTION**	**TARGET**	**SARS-CoV-2**		**Bat coronavirus**		**Bat SARS-like coronavirus**		**SARS coronavirus**		**Pangolin coronavirus**	
	Genetic PCR solutions™ (Spain)	**-**	**0-1**	[0 / 0-1 / 0]	**37-48**	[8-11 / 17-23 / 12-14]	**26-37**	[6-9 / 12-17 / 8-11]	**36-38**	[7-8 / 19 / 9]	**19-31**	[5-9 / 4-11 / 10-11]
(18)	**Institut Pasteur (París)**	**RdRp (IP2)**	**0**	[0 / 0 / 0]	**6-9**	[1-2 / 2-3 / 3-4]	**10-11**	[4 / 2-3 / 4]	**7-12**	[1-4 / 3 / 3-5]	**4-8**	[0-4 / 2 / 2]
		RdRp (IP4)	**0**	[0 / 0 / 0]	**12-13**	[1 / 6 / 5-6]	**12-17**	[1-2 / 5-7 / 6-8]	**14**	[2 / 6 / 6]	**4-11**	[0-1 / 2-3 / 2-7]
(23)	**Centers for Disease Control and Prevention (Atlanta)**	**N (1)**	**0-1**	[0-1 / 0 / 0]	**8-10**	[3-4 / 2 / 3-4]	**8-10**	[3-4 / 2 / 3-4]	**11**	[7 / 2 / 2]	**2-11**	[1-7 / 0-1 / 1-3]
		N (2)	**0**	[0 / 0 / 0]	**6**	[0 / 5 / 1]	**6**	[0 / 5 / 1]	**7**	[0 / 5 / 2]	**4**	[1 / 3 / 0]
		N (3)	**0-1**	[0 / 0-1 / 0]	**3-5**	[1-2 / 1 / 1-2]	**2-5**	[1-3 / 1 / 0-1]	**4**	[1 / 1 / 2]	**1-3**	[0-1 / 1-2 / 0]
(16)	**Charité (Berlin)**	**RdRp_P1**	**3**	[0 / 2 / 1]	**2**	[0 / 2 / 0]	**2-3**	[0-1 / 1 / 1]	**1**	[0 / 1 / 0]	**2-3**	[0-1 / 1 / 1]
		RdRp_P2	**1**	[0 / 0 / 1]	**4**	[0 / 4 / 0]	**3-5**	[0-1 / 2-3 / 1]	**2-3**	[0 / 2-3 / 0]	**2-5**	[0-1 / 1-3 / 1]
		E	**0**	[0 / 0 / 0]	**0-3**	[0-1 / 0-1 / 0-1]	**0**	[0 / 0 / 0]	**0-5**	[0-3 / 0-1 / 0-1]	**0**	[0 / 0 / 0]
(24)	**National Institute for Viral Disease Control and Prevention (China)**	**ORF1ab**	**0**	[0 / 0 / 0]	**7-8**	[2 / 1 / 4-5]	**7-9**	[1-2 / 1-2 / 5]	**7-8**	[2 / 1 / 4-5]	**2-4**	[0-1 / 0-1 / 2]
		N	**0**	[0 / 0 / 0]	**8-10**	[2 / 4-5 / 2-3]	**5-10**	[0-3 / 3-4 / 2-3]	**8**	[2 / 4 / 2]	**3-8**	[1 / 1-4 / 1-3]
(25)	**Hong Kong University Faculty of Medicine (Hong Kong)**	**ORF1b**	**0**	[0 / 0 / 0]	**0-1**	[0 / 0-1 / 0]	**0-1**	[0 / 0-1 / 0]	**0-6**	[0 / 0-2 / 0-4]	**2**	[1 / 0 / 1]
		N	**4**	[0 / 4 / 0]	**4**	[0 / 4 / 0]	**4**	[0 / 4 / 0]	**5**	[1 / 4 / 0]	**4-5**	[0-1 / 4 / 0]
(26)	**Ministry of Public Health (Thailand)**	**N**	**0**	[0 / 0 / 0]	**6**	[1 / 2 / 3]	**6-7**	[1-2 / 2 / 3]	**6**	[2 / 2 / 2]	**2-6**	[0-1 / 1-3 / 1-2]
(27)	**National Institute of Infectious Diseases (Japan)**	**N**	**1**	[0 / 0 / 1]	**9-12**	[2-4 / 1 / 6-7]	**10**	[2 / 1 / 7]	**11**	[3 / 1 / 7]	**3-7**	[2-4 / 0 / 1-3]
(17)	**Hong Kong University State Key Laboratory of Emerging Infectious Diseases (Hong Kong)**	**RdRp/Hel**	**1**	[0 / 0 / 1]	**12-18**	[1-2 / 8-12 / 3-4]	**11-15**	[2 / 8-9 / 1-4]	**11**	[1 / 10 / 0]	**4-6**	[1 / 2-3 / 1-2]
		S	**0**	[0 / 0 / 0]	**14-22**	[6-8 / 6-9 / 2-5]	**24-25**	[8-9 / 9 / 7]	**23**	[8 / 7 / 8]	**11-19**	[3-7 / 4-8 / 4]
		N	**0-1**	[0 / 0 / 0-1]	**10-13**	[2-3 / 7 / 1-3]	**10-11**	[1-2 / 7/ 2]	**11-12**	[2-3 / 7 / 2]	**2-7**	[1 / 0-3 / 1-3]

Table 2. Summarized results of CoVID-19 dtec-RT-qPCR Test validation according with the guidelines of the UNE/EN ISO/IEC 17025:2005 and ISO/IEC 15189:2012, and acceptance criteria adopted.

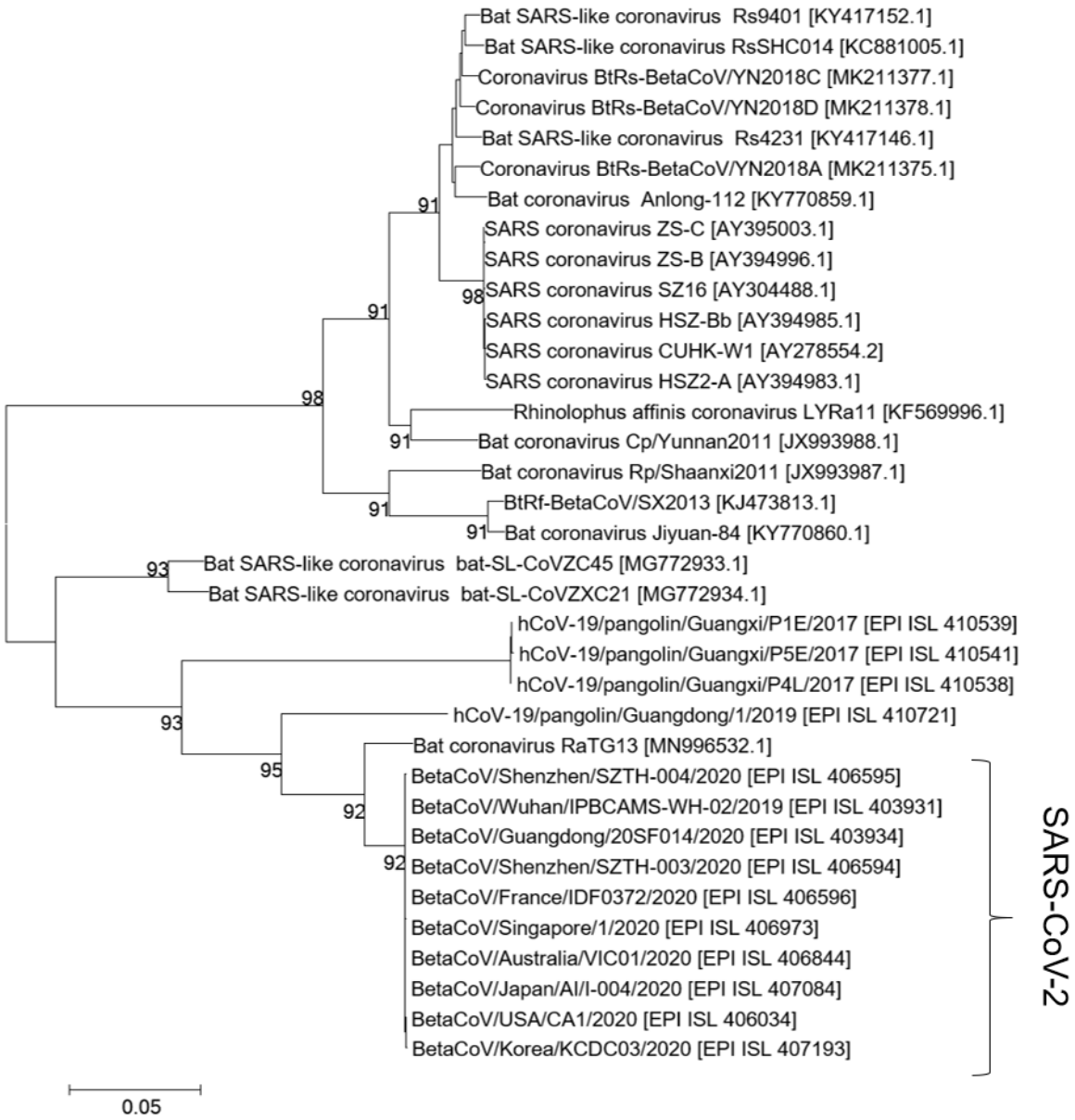
Term of validation	Obtained values	Acceptance criteria	Result
Specificity	**Positive**: SARS-CoV-2 isolate Australia/VIC01/2020 (GenBank No.: MT007544.1) and isolate Wuhan-Hu-1 (GenBank No.: MN908947.3)	**Inclusiveness:** Positive for both SARS-CoV-2 strains	**ACCEPTED**
	Negative: 39 negative specimens from ISCIII, previously characterized by reference protocol (16)	**Exclusiveness:** Negative for all negative specimens,	**ACCEPTED**
Standard curve	Y = -3.534 · m + 37.534 a = -3.534 R^2 = 0.9986	-3.587 < a < -3.103	**ACCEPTED**
	F_{assay} = 0.014 F_{fisher} = 5.318	$F_{assay} < F_{fisher}$	**ACCEPTED**
	Efficiency (*e*) = 93.1 %	90 % < *e* < 110%	**VALIDATED**
Reliability	**Repeatability** Conc. — CV (%) 10^6 copies — 1.18 10^5 copies — 1.08 10^4 copies — 0.68 10^3 copies — 0.53 10^2 copies — 0.54 10 copies — 1.31	CV < 10%	**REPEATABLE**
	Reproducibility Conc. — CV (%) 10^6 copies — 1.13 10^5 copies — 0.91 10^4 copies — 0.93 10^3 copies — 0.59 10^2 copies — 0.66 10 copies — 1.83	CV < 10%	**REPRODUCIBLE**
Limit of Detection (LOD)*	10 copies — Positive = 15/15 (100%)	Positives ≥ 90 %	**ACCEPTED**
Limit of Quantification (LOQ)*	10 copies — t_{value} = 0.582 $t_{student}$ = 2.145	$t_{value} < t_{student}$	**ACCEPTED**
Diagnostic Validation	Diagnostic Specificity: 100% Diagnostic Sensitivity: 100% Diagnostic Efficiency: 100%	≥ 90 %	**ACCEPTED**

Table 3. Results obtained with GPS™ CoVID-19 dtec-RT-qPCR Test in 80 breath specimens compared with the Ct values determined by using a reference protocol (16), at the Instituto de Salud Carlos III (Madrid)

CNM Code	CNM Result	CNM Ct GEN1	CNM Ct GEN2	CoVID-19 dtec-RT-qPCRT Test	Ct CoVID-19 dtec-RT-qPCR Test
#01	NEG	0	0	NEG	0
#02	NEG	0	0	NEG	0
#03	POS	24	28	POS	29.43
#04	POS	24	28	POS	23.06
#05	POS	23.19	26.10	POS	27.56
#06	NEG	0	0	NEG	0
#07	NEG	0	0	NEG	0
#08	POS	27.16	30.41	POS	32.18
#09	NEG	0	0	NEG	0
#10	POS	20.19	25.17	POS	21.42
#11	POS	28	32	POS	30.63
#12	NEG	0	0	NEG	0
#13	POS	28	31	POS	30.06
#14	NEG	0	0	NEG	0
#15	POS	23.19	26.1	POS	24.72
#16	NEG	0	0	NEG	0
#17	POS	27.48	31.16	POS	19.15
#18	NEG	0	0	NEG	0
#19	NEG	0	0	NEG	0
#20	POS	23	25	POS	16.07
#21	POS	23	25	POS	19.32
#22	NEG	0	0	NEG	0
#23	NEG	0	0	NEG	0
#24	POS	25	29	POS	24.37
#25	POS	20	22	POS	26.47
#26	NEG	0	0	NEG	0
#27	POS	23	25	POS	24.86
#28	NEG	0	0	NEG	0
#29	NEG	0	0	NEG	0
#30	POS	24	27	POS	23.45
#31	NEG	0	0	NEG	0
#32	POS	24.29	27.08	POS	16.2
#33	NEG	0	0	NEG	0
#34	NEG	0	0	NEG	0
#35	POS	27.16	30.41	POS	29.22
#36	NEG	0	0	NEG	0
#37	NEG	0	0	NEG	0
#38	POS	31	34	POS	33.41
#39	NEG	0	0	NEG	0
#40	POS	23.13	26.49	POS	25
#41	POS	16.61	19.06	POS	16.58
#42	NEG	0	0	NEG	0
#43	POS	22.14	25.35	POS	24.01
#44	POS	26.47	29.47	POS	27.17
#45	NEG	0	0	NEG	0
#46	POS	25.59	28.03	POS	27.23
#47	POS	24.16	26.44	POS	25.96
#48	NEG	0	0	NEG	0

#49	POS	24.27	26.48	POS	25.99
#50	NEG	0	0	NEG	0
#51	NEG	0	0	NEG	0
#52	NEG	0	0	NEG	0
#53	POS	24.40	26.61	POS	26.37
#54	NEG	0	0	NEG	0
#55	POS	25.33	26.75	POS	25.66
#56	NEG	0	0	NEG	0
#57	NEG	0	0	NEG	0
#58	POS	25.69	28.39	POS	27.08
#59	NEG	0	0	NEG	0
#60	NEG	0	0	NEG	0
#61	POS	25.73	28.61	POS	27.24
#62	POS	25.91	28.43	POS	27.46
#63	NEG	0	0	NEG	0
#64	POS	26.11	28.2	POS	27.98
#65	POS	25.29	28.17	POS	27.84
#66	NEG	0	0	NEG	0
#67	POS	24.24	27.33	POS	26.19
#68	NEG	0	0	NEG	0
#69	NEG	0	0	NEG	0
#70	POS	24.25	26.87	POS	26.81
#71	POS	26.5	29.08	POS	26.35
#72	POS	25.29	28.17	POS	26.91
#73	POS	26.11	28.20	POS	26.45
#74	NEG	0	0	NEG	0
#75	POS	24.24	27.33	POS	26.41
#76	POS	24.19	26.67	POS	26.65
#77	NEG	0	0	NEG	0
#78	POS	25.23	30.18	POS	31.72
#79	NEG	0	0	NEG	0
#80	POS	24.03	26.88	POS	26.43

Figure 1.


Bat SARS-like coronavirus Rs9401 [KY417152.1]
Bat SARS-like coronavirus RsSHC014 [KC881005.1]
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Bat SARS-like coronavirus Rs4231 [KY417146.1]
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Bat coronavirus Anlong-112 [KY770859.1]
SARS coronavirus ZS-C [AY395003.1]
SARS coronavirus ZS-B [AY394996.1]
SARS coronavirus SZ16 [AY304488.1]
SARS coronavirus HSZ-Bb [AY394985.1]
SARS coronavirus CUHK-W1 [AY278554.2]
SARS coronavirus HSZ2-A [AY394983.1]
Rhinolophus affinis coronavirus LYRa11 [KF569996.1]
Bat coronavirus Cp/Yunnan2011 [JX993988.1]
Bat coronavirus Rp/Shaanxi2011 [JX993987.1]
BtRf-BetaCoV/SX2013 [KJ473813.1]
Bat coronavirus Jiyuan-84 [KY770860.1]
Bat SARS-like coronavirus bat-SL-CoVZC45 [MG772933.1]
Bat SARS-like coronavirus bat-SL-CoVZXC21 [MG772934.1]
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hCoV-19/pangolin/Guangxi/P5E/2017 [EPI ISL 410541]
hCoV-19/pangolin/Guangxi/P4L/2017 [EPI ISL 410538]
hCoV-19/pangolin/Guangdong/1/2019 [EPI ISL 410721]
Bat coronavirus RaTG13 [MN996532.1]
BetaCoV/Shenzhen/SZTH-004/2020 [EPI ISL 406595]
BetaCoV/Wuhan/IPBCAMS-WH-02/2019 [EPI ISL 403931]
BetaCoV/Guangdong/20SF014/2020 [EPI ISL 403934]
BetaCoV/Shenzhen/SZTH-003/2020 [EPI ISL 406594]
BetaCoV/France/IDF0372/2020 [EPI ISL 406596]
BetaCoV/Singapore/1/2020 [EPI ISL 406973]
BetaCoV/Australia/VIC01/2020 [EPI ISL 406844]
BetaCoV/Japan/AI/I-004/2020 [EPI ISL 407084]
BetaCoV/USA/CA1/2020 [EPI ISL 406034]
BetaCoV/Korea/KCDC03/2020 [EPI ISL 407193]
SARS-CoV-2
91
98
91
98
91
91
91
93
93
95
92
92
0.05

Figure 2.

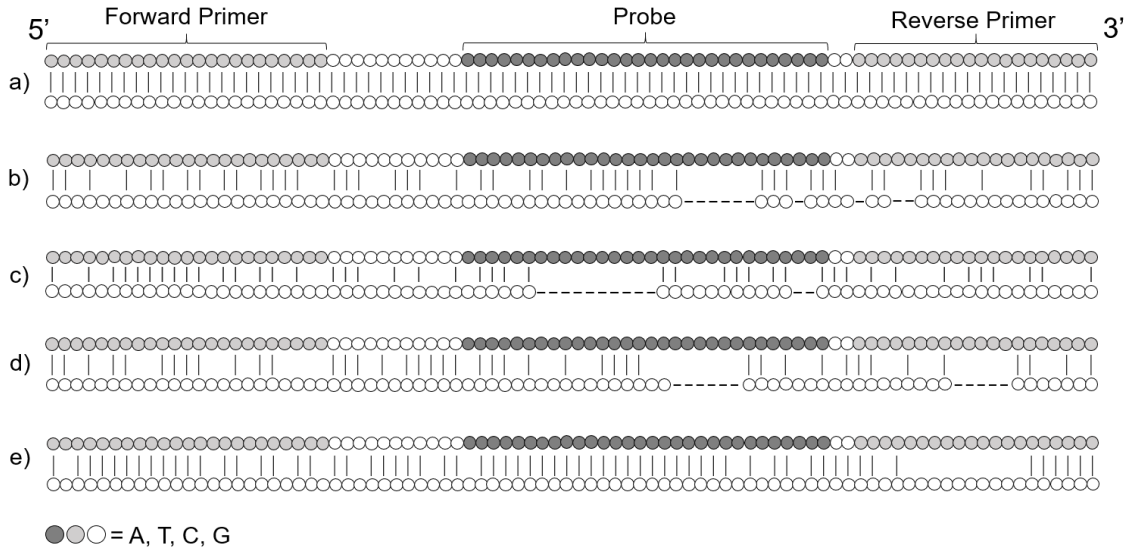
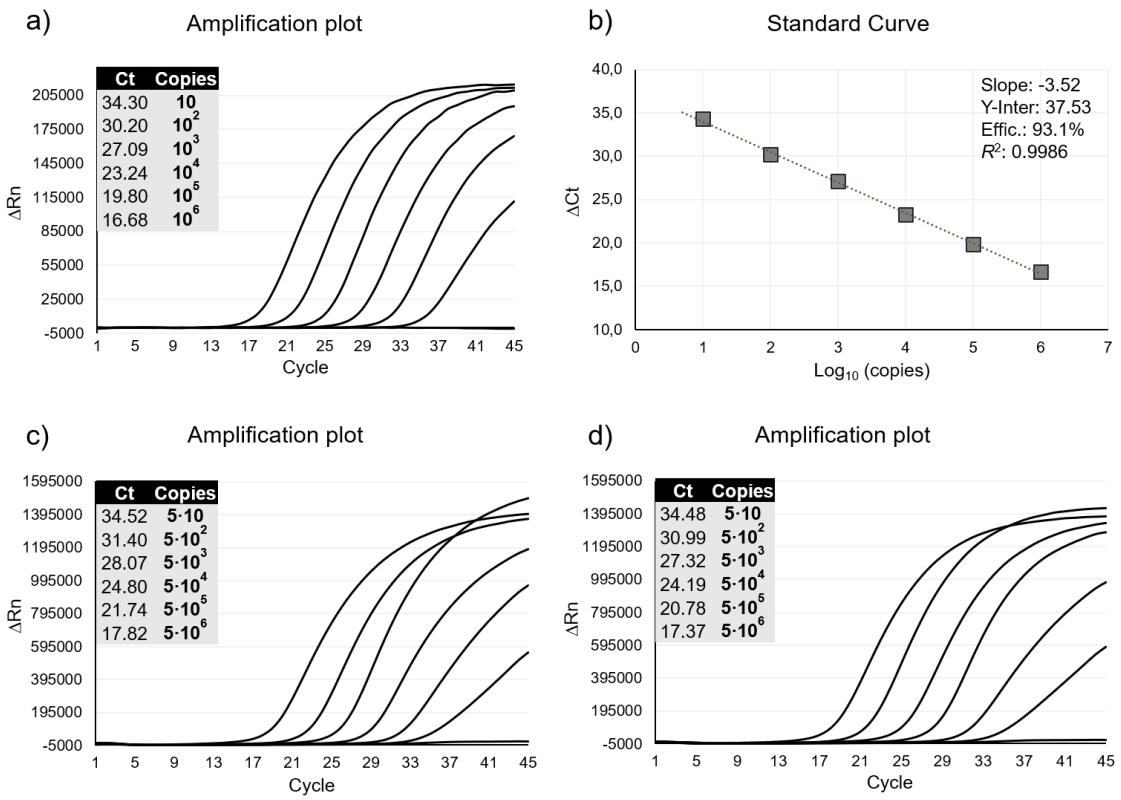


Figure 3.


a)
Amplification plot
Ct Copies
34.30 10
30.20 10^2
27.09 10^3
23.24 10^4
19.80 10^5
16.68 10^6
ΔRn
Cycle
b)
Standard Curve
Slope: -3.52
Y-Inter: 37.53
Effic.: 93.1%
R²: 0.9986
ΔCt
Log10 (copies)
c)
Amplification plot
Ct Copies
34.52 5·10
31.40 5·10^2
28.07 5·10^3
24.80 5·10^4
21.74 5·10^5
17.82 5·10^6
ΔRn
Cycle
d)
Amplification plot
Ct Copies
34.48 5·10
30.99 5·10^2
27.32 5·10^3
24.19 5·10^4
20.78 5·10^5
17.37 5·10^6
ΔRn
Cycle