

RT-PCR Test Targeting the Conserved 5'-UTR of SARS-CoV-2 Overcomes Shortcomings of the First WHO-Recommended RT-PCR Test

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Abstract

For the first time in medical history, a laboratory assay (RT-PCR) was used as the sole criterion to diagnose a disease (COVID-19) and to define infectivity of a virus (SARS-CoV-2) without rating clinical symptoms and proof of replication-competent virus to justify implementing population-wide, untested interventions. The aims here are (1) to evaluate a robust quantitative RT-PCR (RT-qPCR) protocol that overcomes major concerns raised within the scientific community on the first WHO-recommended RT-qPCR protocol for SARS-CoV-2 sequences, (2) to characterize individual SARS-CoV-2 strains circulating in the Czech Republic from autumn 2020 to spring 2021 applying next generation sequencing and (3) to re-initiate scientific dialogue and return to reason and evidence-based medicine. We present a RT-qPCR test designed for the detection of all SARS-CoV-2 variants known so far without producing false-positives. Based on the genomic mutation profile, we demonstrate that the three individual waves (autumn 2020 to spring 2021) in the Czech Republic were successive, but lacked direct genomic relationship between each other. This became obvious with the omicron variant, which did not reveal direct evolutionary connection to any of the previous SARS-CoV-2 variants. In addition, we provide evidence that neglected principles of good scientific practice resulted not only in the publication of the WHO-recommended Charité RT-qPCR protocol, but also in health-related problems. Unnecessary quarantine of healthy individuals, as well as lockdowns and atrocious collateral damage on societies and economies worldwide due to a high number of false-positive “PCR-cases.” Otherwise, infectious symptomatic individuals were given a false sense of security by false-negative test results, which could lead to COVID-19 clusters. Both our results and literature data confirm that validation of any PCR-based diagnostic test by sequencing is mandatory on a regular basis. To prevent future misconduct, science needs a reality check and must re-initiate the scientific dialogue and liberate itself from political influence and dogma.

Keywords: *Charité protocol, COVID-19, next generation sequencing (NGS), RT-PCR, SARS-CoV-2, scientific misconduct.*

INTRODUCTION

Since March 2020, COVID-19 (Coronavirus disease-2019) has been keeping the world on edge mostly due to collateral damage with a catastrophic impact on health, society, and economies. From the beginning of the putative pandemic, and for the first time in medical history, there was a global political consensus (Hedges & Lasco, 2021) that disease status, infection and infectivity could be diagnosed solely by a Reverse Transcription – Polymerase Chain Reaction (RT-PCR) laboratory assay without any independent clinical evaluation of symptoms of individuals diagnosed with COVID-19 disease (China CDC, 2020; Paul-Ehrlich-Institut, 2020). Due to the critical importance of the RT-PCR results, one could readily anticipate that the highest quality standards for accuracy and reliability would be adopted. However, in this paper, we question the first WHO (World Health Organization) recommended and, therefore, most frequently applied RT-PCR test protocol used at the beginning of the pandemic, hereafter referred to as the Charité protocol (Corman et al., 2020; WHO, 24 Jan 2021). In addition, we present an alternative and robust RT-PCR assay targeting the 5'-UTR (UnTranslated Region) of SARS-CoV-2 (Severe Acute Respiratory Syndrome — CoronaVirus-2), which overcomes shortcomings of the Charité protocol. To keep the focus, this study does not consider tests other than RT-PCR. Readers interested in rapid antigen tests other than RT-PCR assays are referred to a recent review (Puhach et al., 2022). In the following, we outline the early phase of COVID-19 and the WHO test strategy to combat the pandemic.

TIMELINE OF THE SO-CALLED COVID-19 PANDEMIC

On December 30, 2019, a hospital in the Chinese city of Wuhan reported that seven of their patients suffered from a severe pneumonia of unknown origin (Reuters, 2019). The local health authorities immediately informed the WHO and had already identified the causative agent as a coronavirus applying whole genome sequencing and RT-PCR (Ren et al., 2020; Zhu et al., 2020; Lu et al., 2020). On January 7, 2020, the identified virus was named 2019-nCoV (2019-novel CoronaVirus) and, on February 1, 2020, was renamed to SARS-CoV-2 (Coronaviridae Study Group, 2020), despite the protest of the Chinese scientists, who preferred the name HCoV-19 (Jiang et al., 2020). Subsequently, the Chinese Center of Disease Control (CCDC) reported that they had established an RT-PCR assay to detect the novel virus in patient samples (China CDC, 2020). Sequencing results placed the identified virus in the beta-Coronaviridae of the subgenus Sarbecoviruses (Ren et al., 2020). On January 9, 2020, the Chinese scientists shared their findings with the WHO (Tan et al., 2020) and uploaded the full-length virus sequence into the Global Initiative on Sharing Avian Influenza Data (GISAID) database headquartered in the US (NC_045512.1).

The WHO published the first diagnostic RT-PCR test protocol for a RT-qPCR online on January 13, 2020 (WHO, 13 Jan 2020). Notably, the published RT-PCR test guidelines were *not* based on the protocols established and shared by the Chinese scientists (China CDC, 2020), but were an artefactual product of several cooperating laboratories in Europe (Reusken et al., 2020). Authors used the Wuhan sequences deposited in the GISAID database by the CCDC for primer and probe design *without any positive patient samples*, and *without the virus itself being available to validate the test* (Corman et al., 2020). The European protocol, which we are referring to as the “Charité protocol”, recommended three targets (located in the nucleocapsid (N) gene, the envelope (E) gene and corresponding to the RNA-dependent RNA-polymerase (RdRp) from the first and second Open Reading Frames (ORF1a and ORF1b) gene. The latter were intended to detect the whole SARS beta-coronavirus subgroup (Sarbeco) with one RdRp probe as discriminating oligonucleotide only. This enabled the use of the SARS-CoV strain Frankfurt-1 as a positive control, however, proof of accuracy of the amplified targets by proper controls and sequencing were not shown.

On January 17, 2020, an update was published on the WHO webpage correcting the former protocol by omitting the most specific primer pair for the N-gene target due to “lack of sensitivity” (WHO, 24 Jan 2021). Six days later, on January 23, 2020, this RT-qPCR protocol — which quickly became the standard for the detection of SARS-CoV-2 RNA in Europe, and many other regions of the world — was published as a research manuscript by Eurosurveillance after an alleged full peer-review process that took only one day (Corman et al., 2020). Primers and probes from the Charité protocol were made commercially available almost immediately, as a laboratory in Slovenia reported (Poljak et al., 2020):

“After extensive evaluation, our laboratory implemented LightMix-based SARS-CoV-2 testing on 17 January 2020. Routine SARS-CoV-2 testing started on 27 January 2020, and the first positive sample was detected on 4 March 2020 after testing 353 routine samples. As of 8 April 2020, a total of 30,669 SARS-CoV-2 tests have been performed in Slovenia (15,330 tests per million inhabitants), 1,103 laboratory-confirmed cases of COVID-19 have been detected, and 40 deaths have been reported.”

At that time, there was not a single COVID-19 case in Europe and the “decision for a diagnostic approach switch” was made nearly a week prior to the publication of the Charité protocol by Eurosurveillance. However, just three days after the “diagnostic switch”, on January 30, 2020, the WHO Emergency Commission (WHO, 30 Jan 2020) declared a Public Health Emergency of International Concern (PHEIC), despite as few as 1,651 test-positive cases in China including 38 deaths, and 98 cases in 18 countries outside China without any reported deaths (Our World in Data). On March 11, 2020, the number of positive SARS-CoV-2 test-cases had risen to 4,670 worldwide, including 280 deaths attributed to this new virus-induced disease COVID-19 (Our World in Data) and the WHO Director-General, Dr. Tedros Adhanom Ghebreyesus, declared COVID-19 a “pandemic” (WHO, 11 Mar 2020) — a worldwide crisis affecting fewer than one person per million (approximately 8 billions divided by 4,670). This was possible because the WHO definition of a “pandemic”, was rephrased in 2009, so that it no longer required “enormous numbers of deaths and illness” (Doshi, 2010).

WHO STRATEGY TO COMBAT THE DECLARED COVID-19 PANDEMIC

On March 16, 2020, Ghebreyesus announced the global strategy for handling COVID-19:

“We have a simple message to all countries: test, test, test. Test every suspected case. If they test positive, isolate them and find out with whom they have been in close contact, up to two days before they developed symptoms, and test those people too (WHO, 16 Mar 2020).”

In parallel, the WHO launched the COVID-19 dashboard feature allowing real-time tracking of RT-PCR positive test results in absolute numbers independent of the number of tests performed and without further specifying the test system applied. This was intended to support planning, implementation and resourcing of country preparedness and response activities — on the basis of worldwide RT-PCR testing, 13,982 positive cases including 871 COVID-19 attributed deaths were supposedly identified (Our World in Data). The strategy recommended by the WHO — to test as many people as possible independent of clinical symptoms by this molecular test — seems puzzling, as the RT-PCR technology by itself has insufficient sensitivity and specificity to verify an intact infectious pathogen like a virus and thus to identify a contagious person, nor can it be used validly to diagnose a disease. What it does is to amplify any genetic material of interest independent of the “viability” of the source (**Box 1**). Further, page 9 of a consensus document on the Epidemiology of Severe Acute Respiratory Syndrome (SARS) from the WHO (2003), a highly related virus, reads as follows: “Data linkage is required to determine whether there is a direct relationship between clinical severity and viral load and excretion.” This implies that an RT-PCR positive tested individual cannot be automatically considered to be or become symptomatic or infectious without corroborating clinical symptoms. Therefore, it is incomprehensible that the WHO committed to the RT-PCR test strategy even

after publication of a systematic review demonstrating that patient characteristics (i.e., severity of symptoms) and test parameters (i.e., cycle threshold) radically restricts the reliability of the RT-PCR test results (Jefferson et al., 2020). Furthermore, the WHO researchers should have been aware of the pitfalls connected to PCR testing, as in 2007, false-positive results caused a pseudo-pandemic of the whooping cough in the Dartmouth-Hitchcock Medical Center (New York Times, 2007). Nevertheless, mass testing by RT-PCR became the strategy of choice to monitor COVID-19 and, from this time on, the number of positive tests was used to justify massive restrictions of human rights and nation-wide lockdowns.

Box 1: No diagnostic value of RT-PCR for the proof of an infectious virus

Of utmost importance and irrespective of any protocol design, RT-PCR solely detects the reverse-transcribed and amplified RNA target(s) selected by applied primers and, therefore, can by no means prove that a replication-competent, infectious virus is actually present in a given sample. Of note, due to the high sensitivity of RT-PCR, residual, non-infectious viral RNA remains detectable even in the absence of infectious viruses. When applying external standards with defined viral RNA copy numbers, RNA viral loads can be correlated with Ct-values obtained by RT-qPCR. However, neither a certain RNA copy number, nor a specific Ct-value used as a threshold can enable any secure conclusion even concerning whether the viral load is increasing or decreasing.

As early as May 23, 2020, the foregoing basic information on RT-PCR was published in a position statement by the National Centre for Infectious Diseases (2020). Subsequently, it was confirmed in a podcast from November 26, 2020 by Marion Koopmans (2020), co-author of the Charité protocol (Corman et al., 2020), in a video statement from December 30, 2021 by Anthony Fauci (2021), the chief medical advisor to President Biden in the USA, and very recently in a comprehensive review with corresponding author Isabella Eckerle as pointed out by Puhach et al., (2022). The latter contains a detailed outline showing why infectiousness has to be determined by the assessment of viral replication in cell culture representing the gold standard for replication-competent, infectious virus. The latter paper concludes that “to date, no diagnostic tests exist that reliably determine the presence of infectious virus”.

In the final analysis, for determining the amount of RNA, RT-PCR can only be used as a proxy because cell culture with SARS-CoV-2 requires level 3 biosafety laboratory conditions (Risi et al., 2010). Also, any diagnosis must be confirmed by one or more clinicians who must show agreement of any laboratory test with clinical symptoms of the RT-PCR tested individual, as is the case for any other laboratory assay.

Of note, any laboratory assay, even when exhibiting both high specificity and high sensitivity, will generate false-positives that may even outnumber true-positives when the prevalence is low, i.e., when performing mass testing of asymptomatic individuals (Skitttrall et al., 2020; Lyons-Weiler, 2021). Positive-tested, asymptomatic individuals represent low initial target numbers associated with high Ct-values. Even in the case that the test readout is correct, these individuals will not be infectious, but represent clinical false-positives comprising either recovered individuals, who still show viral remnants, or immune individuals, who will not be contagious due to a low viral load (Cevik et al., 2020; Lyons-Weiler, 2021). Basile et al. (2020) reported a false-positive rate of RT-PCR testing of 11% (13/122), at a time when COVID-19 prevalence was 2%. Only two of the 13 false-positives had SARS-CoV-2 serology available, both were negative for SARS-CoV-2, while one was positive for a rhinovirus. The problem of contamination has been addressed very early by Wernicke et al. (2020), who reported Ct-values as low as 17 for negative controls indicating high levels of contamination in reagents obtained from oligonucleotide suppliers. Therefore, each batch of PCR reagents must be pre-tested before using it in routine diagnostics.

The only approach that would drive false-positives to zero requires performance of Sanger sequencing (Lee, 2021). Using nested RT-PCR followed by Sanger sequencing to retest 50 samples sold as RT-qPCR positive reference confirmed 21 (42%) false-positives (Lee, 2022).

As a plethora of scientists, physicians and medical advisors have reported their complaints on the shortcomings of the first WHO-recommended RT-PCR, we further challenged the seminal protocol for the detection of SARS-CoV-2. To improve RT-PCR testing of SARS-CoV-2 RNA as a proxy for viral load, to escape the problems of the Charité protocol and to implement a reliable and verifiable PCR approach, an alternative test regime was established. Hereafter, we refer to it as the “5'-UTR RT-qPCR protocol”. Based on the inter-individual genomic heterogeneity of the SARS-CoV-2 strains alpha, beta, gamma, and delta (Ong et al., 2022), we identified a unique consensus region located in the 5'-UTR as both a specific and sensitive target for real-time quantitative polymerase chain reaction (RT-qPCR) detection of SARS-CoV-2 related RNA. Subsequently, we characterized the genome sequences of individual SARS-CoV-2 strains that were circulating in the Czech Republic from autumn 2020 to spring 2021. With this experience, we address critical comments raised within the scientific community following our request for retraction of the Charité protocol (Borger et al., 2020) and we discuss comprehensively our major concerns with the Charité protocol and how the politicization of science obliterates the principles of good scientific practice with damaging and deadly societal effect worldwide.

Methods

PRIMER DESIGN TARGETING THE 5'-UTR REGION OF SARS-COV-2 FOR RT-QPCR

Using alignment of genomic sequences available in February 2020 relative to Bat/SARS/nCoV-19 coronaviruses, we selected a unique region located within the conserved and specific 5'-UTR of SARS-CoV-2 to serve as a specific and sensitive target for real-time RT-qPCR detection of the viral RNA resulting in a 207 base pairs (bp) amplicon. We validated the assay in-house, according to the European ISO 13485 guidelines for the production of in-vitro diagnostics. As detailed below, 50 positive cases identified in authentic symptomatic patient samples (nasopharyngeal swabs), were confirmed by direct Sanger sequencing of the obtained PCR products. The assay fulfilled all requirements for specificity (zero false-specific products observed) and also for sensitivity (seven copies of the targets in a PCR reaction). In addition, our assay underwent external validation by the UK NEQAS official authority for quality assessment and, subsequently, was deployed in routine diagnostics of 31,028 authentic samples tested so far. Characteristics of primers and probe are summarized in **Table 1**.

The PCR profile comprised an initial denaturation step at 94 °C for 5 minutes, followed by 45 cycles at 94 °C for 30 seconds, at 58 °C for 30 seconds (acquiring at FAM) and at 72 °C for 30 seconds. The identity of the generated amplicons was confirmed by Sanger sequencing (**Supplementary Figure 1**). In each sample analyzed, the Sanger chromatograph revealed authentic SARS-CoV-2 sequence, thus corroborating the required specificity of the 5'-UTR RT-qPCR assay. The copy number of the SARS-CoV-2 genome in 1 ml of the initial sample eluate was calculated using a calibration curve constructed from 4-log-dilutions of a synthetic genomic sequence covering the 5'-UTR sequence and its immediate flanking region (custom-synthesized by Eurofins Genomics, Germany) using the following calibration curve equation: $10^{(-0.279 * Ct + 11.244)}$. **Supplementary Figure 2** shows the log-dilution of synthetic SARS-CoV-2 template starting from 10e10 copies per PCR reaction down to 10e1 copies per PCR reaction. Negative control is negative, i.e., no non-specific amplicons produced by unwanted inter- and intra-molecular interactions were present. Subsequently, Ct-values measured for the authentic patient samples were used to calculate the number of copies of the SARS-CoV-2 genome in 1 ml of initial sample eluate. Given the fact that the quantitation method used was the so-called ABSOLUTE (which is a typical set-up in molecular microbiology), the copy number of SARS-CoV-2 in each patient sample was determined using the calibration curve equation, constructed from serially diluted synthetic SARS-CoV-2 genome, which is, as per the state of the art, the most exact method to construct a calibration curve in molecular microbiology.

Table 1

Diagnostic primer-set and TaqMan hybridization probe for routine SARS-CoV-2 real-time qPCR (5'-UTR), as well as primers for Sanger genotyping, located in the S-gene SARS-CoV-2, as performed at Tilia Laboratories from March 2020 to April 2022. Given the intrinsic genomic stability of the 5'-UTR region of coronaviruses, the assay did not require any redesign despite the various SARS-CoV-2 strains emerging since March 2020. T_m and GC-content values of oligonucleotides are as calculated by the manufacturer (Eurofins Genomics, Germany). T_m: Melting temperature of primer; GC: Proportion of guanines and cytosines of primer.

Oligo name	Sequence 5'-3'	T _m [°C]	GC [%]
5'-UTRforward	CGATCTCTTGTAGATCTGTTCTC	58.9	43
5'-UTRreverse	CACCCGGACGAAACCTAGATGTGC	66.1	58
5'-UTR TaqMan probe	FAM– TACTGTCGTTGACAGGACACGAGTAACTCGTCT- BHQ1	70.6	48
SARS-CoV-2 forward	CACACGTGGTGTATTATTACCCTGAC	58.0	36
SARS-CoV-2 delta-reverse	FAM-TCAAAAGTGCAATTATTCGCACTAG	58.1	36
SARS-CoV-2 UK-forward	GTAATTAGAGGTGATGAAGTCAGAC	59.7	40
SARS-CoV-2 UK-reverse	CCACAAACAGTTGCTGGTGCATGTAG	64.8	50

MOLECULAR TRACING OF THE SARS-COV-2 CIRCULATING STRAINS IN THE CZECH REPUBLIC

We genomically characterized 260 authentic SARS-CoV-2 samples from symptomatic individuals (no clinical data available) collected between September 2020 and April 2021 using Sanger sequencing and fragment analysis of the SARS-CoV-2 S-protein gene, which allowed us to distinguish three distinct genomic clusters of SARS-CoV-2 that directly correspond to the individual “waves.” Primers used for the genotyping of the individual SARS-CoV-2 strains, applicable for all circulating strains, are summarized in **Table 1**.

From each of the three clusters (September 2020, November 2020, December 2020), we selected four random representatives and subjected them to NGS whole genome sequencing (SRA Bioproject, accession no. PRJNA742374).

WHOLE GENOME SEQUENCING OF SARS-COV-2 ISOLATES USING NGS

Whole RNA was isolated from authentic patient samples, using QIAamp Viral RNA Mini Kit (Qiagen, Germany), according to the manufacturer’s recommendations. Total RNA was reversely transcribed using Verso cDNA kit (Thermo Fisher Scientific, USA) and tailing products covering the whole SARS-CoV-2 genome were PCR amplified using the ARTIC v3 primers (IDT, USA). Obtained PCR pools (fragments of approximately 400 bp) were gel-purified using QIAquick Gel Extraction Kit (Qiagen, Germany) and subjected to NGS library building using NEBNext® Fast DNA Library Prep Set for Ion Torrent kit (NEB, USA), according to the manufacturer’s instructions. Libraries were quantified applying Ion Plus Fragment Library kit (Thermo Fisher Scientific, USA) and 10 pM library pool was used as a template for emulsion

PCR (emPCR) using Ion PGM™ Hi-Q™ View OT2 Kit (Thermo Fisher Scientific, USA). After bead enrichment (OT2 instrument, Thermo Fisher Scientific, USA), a v316 NGS sequencing chip was loaded in order to obtain sufficient base coverage. NGS sequencing was performed applying the Ion Torrent PGM platform (Thermo Fisher Scientific, USA) using the Ion PGM™ Hi-Q™ View Sequencing Kit chemistry (Thermo Fisher Scientific, USA). Raw data obtained were end- and quality-trimmed and used for direct alignment with the SARS-CoV-2 reference genome (MT192773) to identify genomic mutations characteristic for the September, November and December 2020 waves.

Controls

All experiments performed included internal standard (control), specifically the human albumin transcript. Given the fact that the quantitation was ABSOLUTE, the internal standard was used only to evaluate the quality and integrity of the clinical samples tested. Thus, for the absolute quantitation of SARS-CoV-2 in each clinical sample, calibration curve equation as given above was used, not the $\Delta\Delta C_t$ method, which is typically used for RELATIVE quantitation.

Ethics statement

Samples for routine molecular diagnostics were taken upon informed consent and following governmental legal directives in regards to the COVID-19 pandemic testing strategy which were issued and archived by the referring Clinics. Anonymized samples were processed in accordance with the Declaration of Helsinki.

Literature review

On November 27, 2020, an international group of 22 scientists (including UK, PB, RJK and KS of this paper) submitted an external review report (Borger et al., 2020) together with a retraction request for the Charité protocol (Corman et al., 2020) to the Eurosurveillance Editorial Board. As documents were, in parallel, placed online on both a scientific preprint server and at ResearchGate.net, the scientific community provided both additional references and reasonable criticism concerning the lack of wet-lab experimental data supporting the concerns that we raised. This information was summarized in an addendum and submitted to Eurosurveillance on January 11, 2021. All documents are available online (Borger et al., 2020). In our comprehensive discussion, we include publications from a PubMed search using the terms “PCR” or “RT-PCR” or “RT-qPCR” and “SARS-CoV-2” or “COVID-19.” We also include numerous WHO recommendations with regard to the test strategy that are available at the WHO webpage.

Results

In contrast to the WHO-recommended Charité protocol - recommending 45 cycles without defining a C_t for positive versus negative discrimination, but resulting in a high rate of false-positives at $C_t > 35$, the proposed 5'-UTR assay clearly outperformed the Charité protocol in terms of specificity (no non-specific PCR products, even when applying 45 cycles), while retaining the necessary sensitivity. In contrast to downstream genes, which are prone to mutations, the 5'-UTR genomic sequence is crucial for regulating viral protein synthesis by the human host and inherently remains stable in coronaviruses (Yang & Leibowitz, 2015; Fields, 2021), as it also does in SARS-CoV-2 (Baldassarre et al., 2020; Miao et al., 2021; Mohammadi-Dehcheshmeh et al., 2021). Therefore, we were able to use the 5'-UTR assay successfully throughout the putative COVID-19 pandemic irrespective of the downstream genomic heterogeneity of the individual circulating strains observed over time (i.e., alpha, beta, gamma, delta, and, also, omicron). The 5'-UTR assay

is distinguished by no false background (i.e., a negative sample really is PCR negative). In total, 31,028 samples have been tested with 2,737 of them being positive (8.8%).

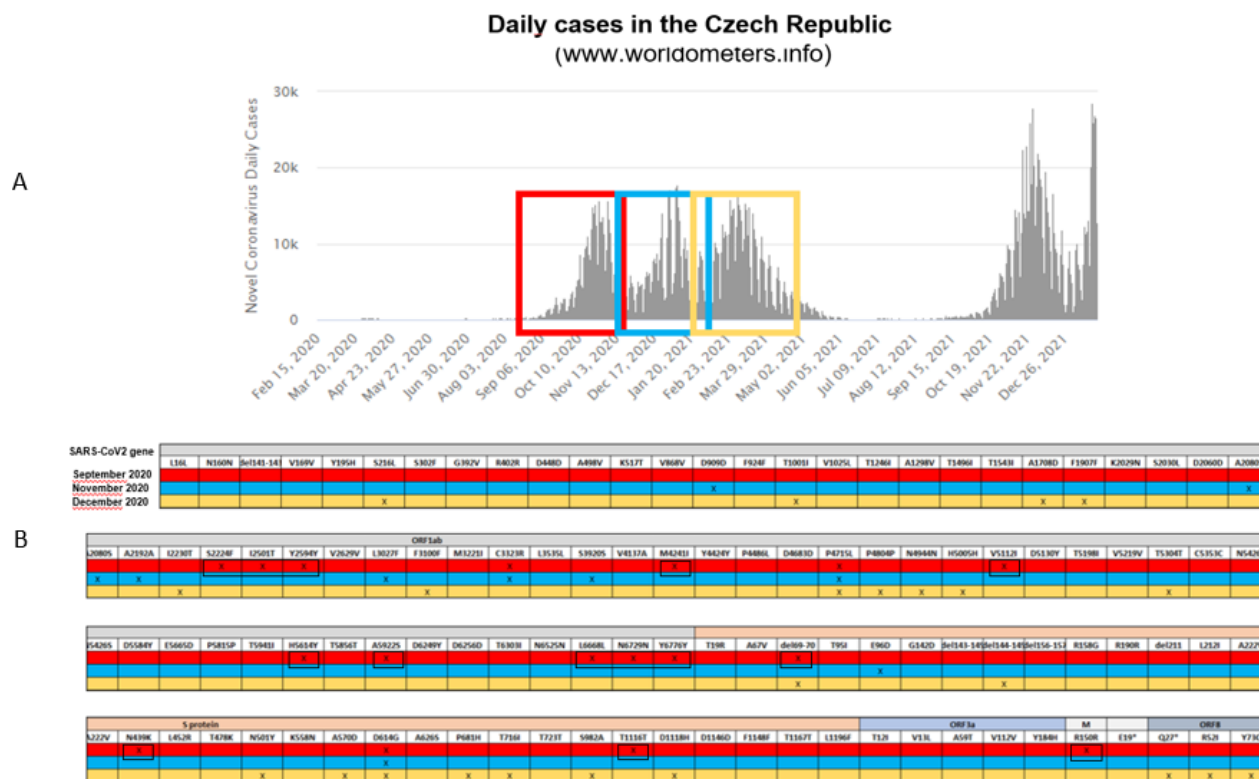


Figure 1. SARS-CoV-2 NGS whole genome sequencing data of three randomly selected representative genomes collected between September 2020 and April 2021 in the Czech Republic. A. Individual SARS-CoV-2 autumn 2020 waves are highlighted in red (September 2020), blue (November 2020) and yellow (December 2020). Graph has been adopted from www.worldometers.info open source. B. Table shows (from 5' to 3') the wave-specific SARS-CoV-2 mutations found throughout the respective genomes (Orf1ab, S, Orf3a, M, Orf8). Mutations are translated into amino acids and highlighted by a black “x”. Black boxes highlight those mutations that were present in the September 2020 SARS-CoV-2 strain, but were absent in the directly following November 2020 SARS-CoV-2 strain. A similar pattern is discernible with the December 2020 SARS-CoV-2 strain (already dubbed as “British”, or later on as “Alpha”). Given the fact that at that time the Czech Republic territory, from the genetic point of view, corresponded to a genetically confined area, the observation that the individual strains were not directly genetically interrelated, is rather astounding.

Based on our years of experience in the field of quantitative molecular microbiology, we adopted the following routine criterion to evaluate the clinical significance of the measured SARS-CoV-2 viral load: Ct <25, highly positive (>10e6 copies/ml); Ct 25-30, positive; Ct 30-35, positive traces, but unlikely to be infectious; Ct >35, negative, not infectious (<10e2 copies/ml). Note that “copy numbers” might differ between laboratories, as there is a difference between copy numbers present in a PCR reaction tube and in an authentic patient sample. In the latter, various dilution steps have to be considered: RNA contained in 1 ml swab is transferred into 50 µl elution buffer, from which 4 µl is used for the cDNA synthesis, from which 2 µl is used for the PCR reaction. We re-calibrated by recalculating the initial sample volume in order to make all our PCR results comparable to standard classical cell culture measurements. We rated Ct 25 to equate our standard at Ct 20 (see **Supplementary Figure 2**) representing the patient’s sample cut-off for a plausible correlation with the infectious viral load.

Using genomic follow-up and NGS whole genome sequencing of random representatives from each of the three autumn 2020 waves, we were able to demonstrate that each wave was represented by a different SARS-CoV-2 strain. Indeed, mutations found in the September 2020 wave were not present in the directly following November 2020, and December 2020, waves (aka “British”, “B.1.1.7.”, or later on “alpha”). The disparity is most prominent between the September 2020 and the November 2020 SARS-CoV-2 strain. Notably, the November wave “lost” 14 mutations that had been present in the preceding September wave (Figure 1).

Discussion

In January 2020, a group of scientists from Europe and Hong Kong (China) developed an RT-PCR protocol that was uploaded on the WHO webpage (WHO, 13 Jan 2020) before publication in Eurosurveillance (Corman et al., 2020). This so-called Charité protocol served as a blueprint for most of the subsequent protocols at least in Europe and was aimed “to develop and deploy a robust diagnostic methodology enabling its use in a public health laboratory setting without having virus material available”. Already at this time, it was apparent that the proposed objective was misleading due to the following: Specific biological material (here, the virus of interest) is essential to assure specificity and sensitivity of the test design, but was not available despite its necessity having already been recognized in the published work of the Chinese scientists largely responsible for the creation of the Charité protocol (Zhu et al., 2019; Ren et al., 2020). Further, a proper test design, which respects international standards and includes extremely specific test materials (primers and probes), was not fulfilled by the Charité protocol. Instead of being designed and checked for maximum specificity without cross-reactivity, the primer design of the Charité protocol allowed a group-specific detection of different coronaviruses of the subgenus Sarbeco. This is questionable in view of the fact that Chinese scientists had already shared the whole genome sequence of the Wuhan virus with the WHO on January 5, 2020 (WHO, 28 Feb 2020), and had submitted the sequence of the full-length genome to the National Center for Biotechnology Information (NCBI) Nucleotide Database (NC_045512.1), thus allowing the establishment of highly specific primers and probes. However, the Charité protocol and primer design was based on a set of synthetic sequences and the SARS-CoV strain Frankfurt-1 as a positive control without even verifying the PCR products by sequencing. Worse still, no cut-off window for the Ct-value was determined in relation to a well-defined, specific viral load control for any of the different target genes. As Puhach et al., (2022) noted, a viral load of 1,00E+06 RNA copies is now generally accepted to correlate with the minimum viral load of infectious individuals. This was already reported by the CCDC protocol (2020) and corresponded with a mean Ct-value of 25. In regard with the Charité Sarbeco E gene, the aforementioned RNA copy number corresponds with a Ct-value of approximately 28.19, but was not communicated (Corman et al., 2020) opening the door for Ct-values up to 45 reported as positive by commercial labs and thus fueling “waves” of implausible (probably false) “positive” test results.

Summarized, none of the expected requirements, usually mandatory, for a “robust diagnostic test” in any Standard Operating Procedure (SOP) was met in the Charité protocol. Indeed, the need for that protocol was questionable to begin with because the CCDC (2020; also see Chan et al., 2020) had already established an efficient RT-PCR protocol on January 3, 2020 which included specific control samples.

Incomprehensibly, this protocol was uploaded on the WHO webpage after the Charité protocol and, in parts, only in Chinese (WHO, 24 Jan 2021). Although the addition of that Chinese part in English would have been appropriate, and desirable, it still would not have been sufficient to solve the key problem: RT-PCR can only detect RNA even in fragments containing the intact target region of a positive amplicon. Thus, by its very nature, RT-PCR is powerless to distinguish between replication-competent, infectious virus

particles, and non-infectious residuals of virus genome fragments that are basically biological noise in the system (**Box 1**).

Given the fact that the Charité protocol suffered from a high non-specific background noise producing false-positive results above a Ct-value of 35 for all target regions and because such a suboptimal design cannot be reasonably be relied on in routine diagnostics, we rejected the Charité design and instead established a new assay. While the Charité protocol recommends amplification of three target sequences located within the E-gene, the RdRp-gene (which by nomenclature is the ORF1ab-gene coding for the RdRp protein) and the N-gene, our 5'-UTR assay targets only one region to amplify, but it consists of the well-conserved sequence within the 5'-UTR of SARS-CoV-2. Thus, our test design has proved to be highly specific without any sensitivity to the noisy background of relatively meaningless and non-infectious fragments, as corroborated by Sanger sequencing of the obtained PCR amplicons.

ASSESSMENT OF THE INFECTIOUS VIRAL LOAD BY CELL CULTURE, NOT BY RT-PCR

Previous contact with a specific virus can be checked by immunological tests, which search an immune response in the host triggered by the virus of interest, and manifested in specific antibodies (i.e., IgM, IgG, IgA) or T-cells reacting against the antigen. Despite the fact that such specific tests were available very early during the pandemic (Amanat et al., 2020; Braun et al., 2020; Okba et al., 2020), politicians worldwide did not adopt them in their testing policies, but instead promoted molecular tests claiming to be able to detect viral RNA with RT-PCR or, later from viral protein using rapid antigen tests. It is noteworthy that PCR is a very sensitive technique for DNA amplification, which according to its inventor, Kary Mullis (1990) serves to multiply specific sequences (i.e., genes) from extremely small sample amounts within a short time (Mullis, 1990). However, RT-PCR for the detection of RNA targets requires the reverse transcription of RNA to convert it into the DNA form, which PCR was designed to detect. In the case of quantitative PCR (qPCR), as applied in testing for SARS-CoV-2, the gene amplification in mass testing involves, a third primer, the so-called probe, that is labelled with a fluorescent dye, and that specifically binds within the amplified target sequence. Upon destruction of the probe by polymerase during the elongation step, a light signal is produced, which is used as a surrogate marker for amplification rounds (Bustin & Nolan, 2017; Lee, 2021).

Importantly, sample preparation prior to all types of PCR requires complete break-up of any biological structures in order to separate nucleic acids, proteins, lipids and cell debris. Extraction protocols and commercial kits for RNA extraction prior to RT-PCR are mainly based on the so-called “Chomscynski” isolation (Chomscynski & Sacchi, 1987), which treats samples with a mixture of acid guanidinium thiocyanate, phenol, and chloroform that completely destroys any complex organisms. Consequently, any PCR, even if performed properly, can solely test for the presence of the genetic target in question, but not for the “viability” of the underlying pathogen organism. Therefore, PCR can by no means serve as any kind of measure to assess the infectiousness of an individual.

The gold standard for determining infectiousness and infectious viral load is represented by the reproducibility of the virus of interest in a proper cell culture (Berczuk et al., 2020; Case et al., 2020; Puhach et al., 2022; EVAg Portal; NIH BEI Resources Repository). Possible replication activity of a virus within a tested individual may be provided by an RT-PCR assay that is based on the detection of subgenomic RNA (sgRNA) transcripts, which will only be generated during virus replication in infected cells (Bruce et al., 2022; Puhach et al., 2022). Of note, because sgRNA has been verified up to 17 days after detection of infection, absence of sgRNA indicates absence of viral replication, but presence of sgRNA does not necessarily indicate infectiousness (Bruce et al., 2022). Remarkably, a sgRNA-specific RT-PCR for SARS-CoV-2 was described in a manuscript submitted on March 1, 2020 by Wölfel et al., (2020), which was co-

authored by the first and the senior authors of the Charité protocol. From that point in time forward, all WHO-recommended protocols should have been substantially changed. They were not.

In general, PCR may nonetheless contribute to improving differential diagnostics, i.e., when applying multiplex testing for a broad range of pathogens in order to discriminate between distinct pulmonary infections, which are often manifested in similar clinical symptoms. As is the case for any other laboratory assay, the outcome of the PCR test must be interpreted within the context of the patient's symptoms. Because PCR represents a highly sensitive technique, it was actually possible to detect an underrepresented pathogen within a pathogen mixture found in a bodily fluid or tissue in a patient sample, as was in fact reported for two of the first five COVID-19 patients in Wuhan (Ren et al., 2020). Moreover, remnants in the form of remaining nucleic acid fragments of SARS-CoV-2, or whatever other coronavirus or prior pathogen may have been formerly present, may be detected by PCR after the patient's natural immune systems have already cleared any infectious agents, and thus can result in a clear positive PCR signal which is actually false (Puhach et al., 2022).

RT-PCR CAN ONLY SUPPORT, BUT NOT REPLACE MEDICAL DIFFERENTIAL DIAGNOSES

To sum up, it is important to keep in mind the key problems associated with PCR-based laboratory assays:

- First, there is a clear difference in the key question between clinical and epidemiological testing. While clinical testing aims at a high sensitivity in order to confirm or decline a suspected infection in a symptomatic individual, epidemiological testing aims at the specific detection of truly infectious individuals, which are able to spread the viral infection. As PCR is not able to detect or predict whether a positively tested individual will be infectious or not (Puhach et al., 2022; **Box 1**), it does not represent a “robust diagnostic tool.” Accordingly, PCR-based tests should never be used for surveilling an asymptomatic healthy population with the aim to “detect” nucleic acid sequences of SARS-CoV-2 or any other virus.
- Second, the PCR test result can by no means replace a thorough medical diagnosis considering patient characteristics, i.e., symptoms, contact history, co-morbidities, drug history, age and Ct-value. COVID-19 shares common symptoms of respiratory tract infections and, therefore, could not accurately diagnosed by a single distinct symptom or sign, as recently published by a Cochrane report (Struyf et al. 2020).
- Third, even in the case in which a PCR test, like ours, exhibits both excellent specificity and sensitivity, there remains the risk of false-positive and false-negative results that can arise from technical and clinical errors. As has been reported in a systematic review and meta-analysis (Cevik et al., 2021), the infectious period of SARS-CoV-2 seems to begin about two days after exposure and continues up to 12 days after onset of symptoms. By contrast, PCR may remain positive for up to 90 days according to the CDC (2019a). Consequently, PCR-positive individuals are not necessarily individuals that can transmit the virus. This discrepancy becomes obvious in the group of positive tested, but asymptomatic individuals representing low initial target numbers and high Ct-values in the test readout. Here, the vast majority will not be infectious, but represent clinical false-positives comprising both recovered individuals, who still show viral remnants, and immune individuals, who will not be contagious due to an insufficient viral load (Cevik et al., 2020; Lyons-Weiler, 2021). For a comprehensive review on the problem of clinical false-positives and false-negatives, refer to Figure 2 in Verna et al. (Verna et al., 2021). Rather, as recommended by both WHO and CDC, the PCR test will help the physician in confirming or declining an initial suspicion whether a symptomatic patient is suffering from a possible SARS-CoV-2 infection (WHO, 20 Jan 2021; CDCb; CDCc).

CT-VALUE CORRELATES WITH TARGET GENE AMOUNT

The cycle threshold (Ct), also called cycle quantification (Cq), is the cycle number when the fluorescence of the amplified PCR product can be specifically detected above the background signal. It represents a measure of the amount of a specific nucleic acid sequence that was present in the original sample. The lower the Ct-value, the more target material was initially present.

As reverse transcription, priming conditions, and secondary structures at the primer binding sites represent stochastic processes, the Ct-value may vary between different RT-PCR runs and different laboratories. Therefore, reference genes of defined amounts must be included to measure relative quantification between various study groups. This represents a minimum requirement for a “robust diagnostic tool”, but inexplicably was not performed by the authors of the Charité protocol. In addition, absolute quantification of a defined viral load present in a specific sample requires qPCR methodology with a dilution series of known amounts of inactivated virus. Subsequently, the Ct-value of an unknown sample can be correlated with the Ct-values of the dilution series and the virus number can be estimated to determine the viral copies per ml — the so-called “viral load”.

For SARS-CoV-2, it was reported as early as April 2020 (Wölfel et al., 2020; co-authored by first and senior authors of the Charité protocol) that a minimum of 10^6 RNA copies/ml are comparable with a viral load that can be used to infect a proper cell culture and thus is defined as “potentially infectious”. Performing 45 PCR cycles, the Charité protocol was described to detect as little as four RNA copies per sample (approximately 10^3 RNA copies/ml) according to Corman et al., (2020). Some commercial test kits report a detection threshold of 10 RNA copies per sample (Tib Molbiol). This is roughly between 10^3 and 10^5 -fold more sensitive than the infectious viral load required to cultivate SARS-CoV-2 in a proper cell culture setting. Incomprehensibly, the Charité protocol counts any sample with a PCR signal up to 45 cycles explicitly positive without defining any Ct cut-off value that discriminates between a positive/negative decision, and without any correlation to control samples comprising defined RNA copy numbers. By contrast, our 5'-UTR assay correlates quantified RNA copy numbers with defined Ct values based on a calibration curve constructed from serial log dilutions of synthetic SARS-CoV-2 genome with precisely given concentrations of the targeted sequence. In addition, sequencing is performed to confirm amplicons and to determine the type of virus variant that shows up in the testing. Importantly, a positive PCR signal per se does not allow us to make inferences about a possible infectious viral load, if no Ct-value is provided, and if the result is not specifically related to a defined standard curve (Kohmer et al., 2021). Both these requirements must be met. Ct-values less than 25 reflect more than 10^6 RNA copies per ml sample, which can be correlated with a potentially infectious viral load.

In a systematic review published in December 2020 by Jefferson et al., it was suggested that samples with $Ct > 25$ will not contain enough genetic material to correlate with an infectious potential. In June 2021, Jaafar et al., correlated 3,790 positive RT-PCR samples with subsequent successful cell cultures. Those authors demonstrated that the virus can be cultivated in 70%, 20% and 3% of the samples when applying Ct-values of 25, 30 and 35, respectively. In August 2021, Stang et al., reported that Ct-values between symptomatic and asymptomatic subjects typically differ by more than four cycles (25.5 versus 29.6, respectively). They concluded that the Ct-value for defining potentially infectious individuals should be lowered from 30 to 25.

TARGET GENE NUMBER AND SPECIFICITY

To reliably detect a specific virus genome by RT-PCR, a well-defined, highly specific and ideally conserved target region is mandatory (such as we have included in our 5'-UTR assay). In case such a region cannot be identified, alternatively, more than one gene or specific sequence of that virus must be targeted by distinct sets of specific primers. The Charité protocol (Corman et al., 2020) properly specified three target genes to detect SARS-CoV-2, namely the E-gene, the RdRp-gene (which is the ORF-1ab gene coding for the RdRp protein) and the N-gene. However, the primers selected for the Charité E-gene target were defined as

specific for the whole Sarbeco group of coronaviruses including SARS and Bat-derived Sarbecoviruses. This target clearly fails to fulfil the requirements for a specific SARS-CoV-2 detection by design and, consequently, was excluded from analysis in a manuscript testing all WHO-recommended RT-PCR protocols by Tao et al., (2022). Furthermore, the probe from the RdRp target was defined as “Pan-Sarbeco” making it even less specific as a test for SARS-CoV-2.

Table 2

Specificity of the SARS-CoV-2 target genes E, RdRp and N, as evaluated in a round robin test by the German Institute Instand (Zeichhardt & Kammel, 2020) demonstrating a high risk of false-positive results, especially via cross-reactivity with common cold coronaviruses like HCoV 229E. *Of note, HCoV229E belongs to the genus alpha-Coronaviridae, while SARS-CoV-2 and other Sarbecoviruses belong to the genus beta-Coronaviridae. The “common cold” viruses HCoV-HKU1 and OC43 are classified in the latter genus with their genome organization being much closer to Sarbecoviruses (Liu et al., 2021), therefore, one of these two viruses would have been a better control.

Target gene	No. of tests performed with different test kits	Specificity-Test 1 Cell culture (virus-free) Correctly identified as negative Cases [%]	Specificity-Test 2 Cell culture (with HCoV229E*) Correctly identified as SARS-CoV-2 negative Cases [%]	Mean specificity from samples 1 and 2 [%]	Mean error rate (false-positives) (100 – mean specificity) [%]
SARS-CoV-2					
E-gene	373	371 [99.46]	355 [95.17]	97.31	2.69
RdRp-gene	182	178 [97.80]	165 [90.66]	94.23	5.77
N-gene	166	164 [98.20]	146 [87.95]	93.08	6.92

Despite the fact that their design was ostensibly aiming not to be exclusively specific to the new SARS-CoV-2, the targets they chose to amplify all have their own absolute specificity and error rate (**Table 2**). The number of false-positive results can be calculated for each individual gene and also for any combination of them. Putting it briefly, the risk of false-positives for non-specific primers such as the ones used in the Charité protocol depends on the number of target genes tested. The fewer target genes are tested, the greater the number of false-positive results that will be generated. It is therefore completely incomprehensible why the WHO, during the course of the pandemic, recommended reducing the number of target genes without improving the specificity of the primer design. In the original Charité protocol (published January 13, 2020), testing for three gene targets (E-gene, RdRp-gene, N-gene) was recommended by the WHO, (24 Jan 2021). Doing so, could have limited the number of false-positives. However, in the first modification (published January 17, 2020), PCR-detection of the N-gene (the most specific and in dilution series the least sensitive according to Muenchhoff et al., 2020), and therefore the least frequently occurring false-positive target, was omitted (WHO, 13 Jan 2020). Worse still, with the second modification (published March 2, 2020), the WHO recommended that “in areas, where COVID-19 virus is widely spread, a simpler algorithm might be adopted, in which for example screening by RT-PCR of a single discriminatory target (i.e., the E-gene) is considered sufficient” (2 Mar 2020). These modifications — especially the final recommendation to test for the highly unspecific E-gene as the sole target — was certain to increase the risk of false-positives thus driving up the case numbers, and, it also was likely to increase the number of false-negatives (Finn & Lucey, 2021; Kanji et al., 2021; Pecoraro et al., 2021).

DISPUTABLE PRIMER DESIGN

Any reliable RT-PCR assay critically depends on the quality of primers designed to amplify the region selected as specific target within a sequence. For diagnostic purposes, it is essential that primers are 100% specific for the sequence of interest in order to avoid cross-reactivity with nearly homologous sequences of closely related genes which may be virus variants (Bustin & Nolan, 2017). When two or more primer-sets are applied, amplification efficiencies per primer-set may nevertheless be distinct. This may result in different assay sensitivities due to differences in priming efficiency associated with secondary structure or stability (Chan et al., 2020). The Charité protocol describes primers containing up to six unspecified positions. The unspecified positions result in the design of several different alternative primer sequences (two distinct RdRp_SARSr_F primers + 8 distinct RdRp_SARS_P1 probes + 4 distinct RdRp_SARSr_R). In addition, one of the N-primers (sequence GCAGACGTGGTCCAGAACAAA) shares 10 bases with a sequence of human chromosome-1 (sequence GCAGACTCTGAGGGGATGCCA), of which six bases are located at the 3'-end and therefore pose a high risk of unspecific priming (Borger et al., 2020). However, the RdRp reverse primer of the Charité protocol is even more problematic, since it is 100% identical to a sequence present on human chromosome-18 (Borger et al., 2020). This may generate unintended PCR amplification even in the complete absence of any SARS-CoV-2 sequences. In addition, the RdRp-gene has been reported to be problematic because it has the lowest rate of positive detection and the highest Ct-value (Anantharajah et al., 2021; Zimmermann et al., 2022). Another important aspect of PCR primer design is the annealing temperature (T_m), which critically depends on the GC-content of the primer sequence. The lower the T_m , the higher the risk of false-positive results. Further, the T_m of a primer pair should be very close, preferably not further apart than 2° C according to Bustin et al., (2009). It must be noted that the aforementioned in-silico T_m prediction serves only as a first indication. It cannot take account of all the possible secondary structures in the broader genomic context of the template, or in the exact composition of PCR master mixes, which contain chemicals influencing the T_m of PCR hybrids, thus allowing for a more flexible use of primers and probes, while supposedly retaining the necessary stringency of target detection. Despite this considerable degree of flexibility thanks to modern PCR compositions, each PCR design requires a design in the end that is absolutely specific and adequately sensitive — specified in the ISO13485 guidelines and rigorously tested via external quality assessment (UK NEQAS in our case). Several of the Charité protocol primers have a high degree of T_m flexibility according to Borger et al., (2020) and Corman et al., (2020). In this specific case, however, the T_m flexibility of the primers has been accompanied by a notably suboptimal performance of the PCR assay in terms of specificity. For a “robust diagnostic test,” this is unacceptable. Therefore, the Charité assay should have been re-designed prior to global implementation.

VALIDATION OF GENERATED AMPLICONS BY SEQUENCING IS MANDATORY

Assessment of the absolute virus amount in a patient sample, which is necessary to correlate the Ct-value with the infectious viral load estimated in cell culture, requires quantitative PCR, which is different from qualitative PCR. Qualitative PCR generates a defined amplicon, which normally is detected by size-dependent analysis in an agarose gel and can then be used as the template for sequencing analysis. In quantitative PCR, a Ct-value is generated, which represents the kinetics of an accumulated fluorescing signal that correlates with probe degradation. Consequently, no confirmation of the correct amplification of the target gene is possible in this process. Especially for newly designed protocols, amplification is usually followed by agarose gel and sequencing analysis in order to confirm the amplified sequence. Although whole or at least partial genome sequencing is recommended by the WHO (19 Mar 2020; 8 Jan 2021; 20 Jan 2021), the authors of the Charité protocol did not include this important confirmation step, i.e., Sanger sequencing.

Hence, the specificity of the primers and probes used in their protocol that would be necessary to produce a reliable target-specific amplification has never been produced (Corman et al., 2020).

Remarkably, as early as in April 2020, it was known within the scientific community that the Charité protocol suffered from specificity problems of “unknown origin” (Konrad et al., 2020). Even under standardized laboratory conditions, authors of the Charité protocol (Corman et al., 2020) themselves reported that four positive samples were negative after re-testing representing the classic example of false-positives or random laboratory contaminations with PCR products due to handling issues. The fact that the authors indeed explained their false-positives as “handling issues,” which even occurred in the very beginning of the Charité protocol in a laboratory setting with experienced personnel, raises serious questions about contamination rates in large government laboratories as well as in newly established commercial laboratories that were put under pressure to do many PCR tests since the onset of the so-called “pandemic”.

Consequently, to assess the reliability of different laboratories supposedly using the same PCR protocol, an experiment was performed by Muenchhoff et al., (2020). They submitted a dilution series of a SARS-CoV-2 PCR-positive sample to seven participating laboratories. All seven of them reported that all samples were negative at a $C_t \geq 32$. The authors reported:

“Based on computation using Primer Express v3.0 (Applied Biosystems, Dreieich, Germany) annealing temperatures were predicted to be 64 °C for the RdRp forward and 51 °C for the RdRp reverse primer of the Charité protocol. This temperature difference may result in reduced PCR efficiency.”

Despite these facts, the authors concluded the following:

“The majority of RT-PCR assays for SARS-CoV-2 examined detected five RNA copies per reaction reflecting a high sensitivity and their suitability for screening purposes worldwide.”

This conclusion is valid, when PCR sensitivity is considered by itself. However, the remarkable sensitivity reported points to the risk of amplifying even small residues of a past-infection. Doing that is not suitable for a diagnostic tool aiming to test for positive and negative individuals. Repercussions of the consequent errors can be lethal. Hence, with RT-PCR testing, the specificity is far more important (Klement & Bandyopadhyay, 2021). Excellent specificity for the targeted genes in combination with sensitive C_t -values (≤ 25) are the features necessary for a robust and reliable PCR test.

Another well-known diagnostic challenge is the occurrence of mutations and new virus variants. Their existence requires regular validation and possibly primer re-design in order to avoid potential primer-sample mismatches. This has been demonstrated by Osorio and coworkers, who aligned 1825 SARS-CoV-2 genome sequences deposited in the GISAID database (March 2020) against the Wuhan-Hu-1 reference sequence (NC_045512). Subsequently, they annotated, in the alignments, the binding sites of 33 oligonucleotides shared by the WHO for the use in RT-PCR and found that approximately 79% of the analyzed primer-binding sequences exhibited mutations in at least one targeted gene (Osorio et al., 2020).

In addition, our own NGS data generated in the Czech Republic from October 2020 to January 2021 confirmed that validation of a PCR-based diagnostic test by sequencing is mandatory. It is essential, not only during the initial phase of establishment, but also on a regular basis subsequently. The necessity for such an approach was shown plainly by our NGS data, which revealed that each of the three individual waves culminating in the Czech Republic during October 2020, November 2020 and December 2020/January 2021 was genomically different from the preceding wave. Although SARS-CoV-2 reveals a high mutation rate, with our 5'-UTR assay, we were able to reliably identify all circulating strains, i.e., alpha, beta, gamma, delta, and omicron.

Interestingly, mutations found in the September 2020 wave were not present in the directly following November 2020 and December 2020 waves. This discrepancy has become fully obvious with the omicron variant, which reportedly lacks direct evolutionary connection to any of the previous SARS-CoV-2 variants (Sun et al., 2022; also, Perez et al., 2023 in this journal). This is more than strange, given the fact that the Czech Republic had implemented all restriction measures, namely lockdown, massively restricted traveling, social distancing, and facial masks. In this way, the Czech Republic territory behaved as a genetically confined, isolated population that would not support such a SARS-CoV-2 diversity. Namely, subsequent waves originating in a confined area should carry all the successive mutations of its predecessors. It could acquire more mutations during its evolution, but logically it cannot magically erase mutations that occurred in prior waves of the same viral succession. Odder still was the fact that the observed disparity was most prominent between the September 2020 and the November 2020 SARS-CoV-2 strains. The peculiar development consisted in the fact that the November wave “lost” 14 mutations that were present in the immediately preceding September wave. We can only speculate about these unusual genomic characteristics of the successive SARS-CoV-2 “waves,” however, due to available literature on reverse genetics of RNA viruses (Perez, 2017) and especially on how easy it is to genetically manipulate infectious coronaviruses (Cockrell et al., 2017; Muth et al., 2018), artificial or man-made intervention cannot be ruled out.

GOOD LABORATORY PRACTICE REQUIRES A STANDARD OPERATING PROCEDURE (SOP)

The high sensitivity of PCR-based technology goes along with a severe bottleneck in the performance. Even in case of a 100% test specificity, tantamount to a false-positive rate of 0%, the outcome solely refers to a lack of reaction with sequences other than the selected target(s). However, this analytical specificity calculated in certified laboratories cannot be equated to specificity in real-world testing, where contaminants, which are also amplified exponentially, and handling errors by untrained personnel will unavoidably result in the generation of false-positives. For example, Layfield and colleagues (2021) reported false-positive specimens in a plate map that were located adjacent to high viral load specimens ($Ct < 20$). Moreover, when the positivity rate approaches the false-positive rate, the reliability of a positive test result falls toward zero. This is especially important when the prevalence is low, as there are more uninfected than infected individuals. In this scenario, small changes in specificity will have a much larger impact on the probability that an individual testing positive has the infection than variations in sensitivity (Cohen et al., 2020).

To avoid errors inherent to any laboratory analysis, detection procedures always must be brought together, thoughtfully designed by competent researchers, with a reliable SOP. The RT-PCR test is only suitable as a diagnostic tool for virus detection, if it is standardized and controlled on all levels. A SOP is essential if erroneous results are to be excluded to a reasonable extent. For the detection of SARS-CoV-2, the SOP should include an anonymized panel of test samples containing inactivated virus material made available by an external provider (i.e., a reference laboratory), a negative sample and samples with closely related virus in order to check for specificity (these samples must remain negative). Ideally, the SOP should also contain a dilution series of inactivated virus to determine the sensitivity of the test (with Ct -value corresponding with the infectious viral load). In the context of a worldwide pandemic, the requisite demands can only be achieved by experimentally testing such a procedure for its worldwide validity. The latter validity requirement can only be met in a so-called round robin test (**Box 2**).

Box 2: Internal controls for each RT-PCR run should include:

- a blank swab to exclude contamination during sample collection;
- an RNA extraction control to ensure correct RNA isolation;
- negative control with the kit components only to guard against production or clinical kit contamination;
- a “waterproof” as internal negative control;
- a reference gene (e.g., human RNaseP) as internal positive control;
- positive controls of inactivated SARS-CoV-2 isolated from cell culture supernatants to correlate the Ct-value with the copy number of the replication-competent infectious viral load, e.g., by plaque assays (Mendoza et al., 2020). This includes (1) a concentration that corresponds with the infectious viral load (10^7) with a Ct < 30 in all amplified target genes and (2) a probe that corresponds with a non-infectious concentration (e.g., 5×10^5) in order to define the Ct-value above which RT-PCR gain negative results. These positive controls must be quality-control checked, as the virus will involve in the cell line and may not reflect emerging viruses.
- cross-reactivity control (must remain negative), e.g., “normal seasonal flu” coronavirus lines, such as OC43 and 229E, which like SARS-CoV-2 positive controls have been inactivated at a viral load of 10^7 . Ideally, these should be Sanger-sequence proven target negative controls of human samples.

The need for a SOP was made obvious by studies in Germany coordinated by Instand eV, an organization, which grants certificates for good laboratory practice. Their first round robin test to validate the Charité RT-PCR, involving 488 laboratories, revealed considerable problems (Zeichhardt & Kammel, 2020). Of note, during an ongoing round robin test, three of seven samples were excluded from the blind panel due to “urgent requests from Germany and abroad to reveal the properties of the samples to be tested before the end of the extended submission period, i.e., before April 28, 2020, so that laboratories can improve their test method in the short term in case of possible incorrect measurements”. Intervention in a round robin procedure is very unusual and the report cannot be regarded as representing an independent external validation procedure of the participating laboratories. An additional concern, even in this controlled round robin test procedure, with the samples already prepared and a reduced test scope, is sample mix-ups that occurred in 24 laboratories, which interestingly enough always affected the same SARS-CoV-2 positive probe with the control probe containing the common cold coronavirus HCoV229E (Zeichhardt & Kammel, 2020). Finally, it was noticed that detection of the target genes showed enormous variation between laboratories with respect to Ct-values. For example, the Ct-values for the same diluted sample of SARS-CoV-2 (sample number 340061) ranged between 15-40 for the E-gene, 20-40.7 for the N-gene and 19.5-42.8 for the RdRp-gene. Obviously, the largely different Ct-values for the different gene targets based on the same target demonstrate the different sensitivity of the targets and are largely influenced by the initial amplification success. Since PCR is a 2-logarithmic process, any small initial problem will increase logarithmically as well. This highlights the need for a control standard curve to be included in every PCR to rate the results. Together, these data impressively demonstrate an extreme lack of test standardization within the participating, certified laboratories. As there is no worldwide (or at least EU-wide or USA-wide) SOP, we can only imagine the enormous variation generated in laboratories detecting “SARS-CoV-2 RNA” in real patient samples.

Conclusion

THE VIOLATION OF PRINCIPLES OF GOOD SCIENTIFIC PRACTICE ENABLED POLITICIANS TO APPLY NON-EVIDENCE-BASED MEDICINE ON ENTIRE POPULATIONS

The Charité protocol represents an infringement of all internationally recognized principles of good scientific practice (**Box 3**). First, the primers and probes used for RT-PCR were forwarded to testing companies, i.e., Labor Berlin and Tib Molbiol, and made commercially available as Light Mix Diagnostic Test Kits, i.e., LightMix® Modular SARS-CoV-2/COVID-19, RdRp; LightMix® Modular SARS-CoV-2/COVID-19, E-gene; Tib Molbiol, Roche Diagnostics before scientific publication and without mentioning this fact and the related conflict of interest in the scientific publication. Second, the test protocol was published online as a WHO guideline (WHO, 24 Jan 2021) prior to peer-review and publication in *Eurosurveillance* (Corman et al., 2020). It was only then that it underwent a rapid 24-hour peer-review, retrospectively justified by the imminent pandemic. Nevertheless, there were only six deaths worldwide on January 21, 2020, the day the manuscript was submitted (Our World in Data). Furthermore, the Tib Molbiol LightMix Kit was already available in Slovenia a week before publication of the Charité protocol (Poljak et al., 2020). At that time, not a single case of SARS-CoV-2 was documented in Europe (Our World in Data). Third, two of the authors are members of the *Eurosurveillance* Editorial Board, another is the managing director of Tib Molbiol, while yet another is a senior researcher at GenExpress and a scientific advisor for Tib Molbiol — none of these potential conflicts of interest were disclosed when submitting the manuscript (Borger et al., 2020).

The neglect of internationally recognized principles of good scientific practice prompted the publication of a severely flawed laboratory assay. Subsequently, RT-PCR positive outcomes were equated with “COVID-19 cases” even in the absence of any disease symptoms. However, a “case” implies symptoms and diagnosis of an illness, here COVID-19, not the presence of (parts of) SARS-CoV-2. Also from a scientific perspective, the daily reports of so-called “new cases” or “new infections” did not make any sense, since it was neither established whether they were “new”, nor that they were “infectious.” The high sensitivity of PCR enables the detection of viral fragments of any origin, but PCR cannot diagnose “cases” or “infections.” Indeed, more than half of the positive test outcomes are likely not to be infectious (Jaafar et al., 2021). Nevertheless, governments implemented quarantines of healthy people and installed lockdowns with significant collateral damage to populations and economies worldwide based on a deeply flawed RT-PCR laboratory assay.

As a consequence of the lack of proofreading activity of polymerase, the nucleotide mutation rate of SARS-CoV-2 is estimated to be 8×10^{-4} substitutions per site per year (The Open Science Prize, 2020). There is a high probability that new genetic variations of SARS-CoV-2 could compromise both sensitivity and specificity of established RT-PCR assays. This is all the more likely, given that 8.5% of all mutations (new nucleotide differences) in SARS-CoV-2 variants around the world have been demonstrated to map to known PCR primer locations (Penarrubia et al., 2020). Thus, we recommend continuous monitoring of genomic variations in order to provide a rapid response in case assay re-design is needed.

Therefore, from a national public health perspective, agencies should require constant Sanger sequencing surveillance of RT-PCR positive and negative, symptomatic individuals to detect decays in Sanger-positive and Sanger-negative over time in order to prevent evolution-based PCR escape. Here, official numbers of case estimates should be adjusted downward based on loss of Sanger-positive and upward based on loss of Sanger-negative (both reported with confidence intervals).

In summary, positive test results comprise a mixture of true COVID-19 cases (i.e., contact with SARS-CoV-2 at least 9 days prior to the test, probably infectious with symptoms of a respiratory disease), untrue cases (i.e., contact with virus or virus fragments less than 9 days ago, probably not infectious, sometimes with symptoms), and false cases (healthy, for sure not infectious). The distribution of these three categories depends on laboratory distinctions, kits used, skills of the technicians, etc. We thus strongly argue against using the RT-PCR technology to measure “cases” or “infections” without appropriate and independent “old-fashioned” physician-made diagnosis. We conclude that the application of this technology as a population-wide mass testing instrument unnecessarily exaggerated and prolonged the COVID-19 pandemic and should be omitted in similar future scenarios.

Box 3: Internationally recognized principles of good scientific practice include:

- formal aspects, e.g., thorough review of research results by independent referees before publication and expression of any kind of existing conflict of interest by all co-authors, such as project funding by the pharmaceutical industry,
- research aspects, e.g., the implementation of a valid protocol, including positive and negative controls, as well as the confirmation of results and deployment of appropriate and sound techniques,
- quality assurance and the establishment of standards. The latter of which are of particular importance upon the development of new methods or diagnostic tests, especially when a test result may decide upon patient treatment.

LACK OF TRANSPARENCY WAS AND STILL IS AN OMNIPRESENT COMPANION OF THE CRISIS ENDING UP BY CAUSING MORE HARM THAN GOOD

Two BMJ Editorials argued that during COVID-19 politicians and governments were suppressing science to accelerate the commercial availability of diagnostics and treatments (Abbasi, 2020; Jureidini & McHenry, 2022). While in light of an imminent “killer-virus pandemic” the publication and marketing of a suboptimal RT-PCR test for SARS-CoV-2 detection may be defended, any errors and inappropriate scientific standards discovered after publication must be reported and corrected immediately. A request to retract the Charité protocol (Corman et al., 2020) based on ten formal and technical concerns was submitted to the Eurosurveillance Editorial Board in November 2020, but seems never to have received serious consideration (Borger et al., 2020). The request was declined in an online statement based on five undisclosed expert reviews, although none of the concerns were addressed appropriately (Editorial Note Eurosurveillance). Moreover, an addendum containing 20 peer-reviewed, published papers supporting these concerns was not even mentioned. The subsequent appeal to disclose the peer-review reports of the five peer reviewers was declined by the Eurosurveillance Editorial Board and thus violated key scientific standards that guarantee a transparent peer-review process to enable an honest scientific dialogue. Why they did not choose to inform the scientific community about conceivable shortcomings and pitfalls of the Charité protocol remains elusive.

Finally, the decision-making processes of the WHO lacks transparency, being applied 12 years after the swine flu, it is still unclear why the WHO modified the definition of a pandemic. According to the old definition, it would not have been possible to declare COVID-19 a pandemic at all. It is also incomprehensible why the WHO did not immediately publish an English version of the RT-PCR test developed by the CCDC, but rather published a different European test. Another unanswered question is

why the WHO did not immediately report on the importance of the Ct-value and interpretation of the RT-PCR test results when the PCR technology was adopted as the “gold standard” to detect SARS-CoV-2? Why did they only do so a full year after the onset of the pandemic (WHO, 20 January 2021)? Why did influential scientists, such as Marion Koopmanns and Anthony Fauci, not call attention to the misuse of the PCR as the so-called “gold standard” for the detection of “infectious” individuals, although they must have known better, as they demonstrated in interviews and podcasts (see **Box 1**)? If the lack of transparency is not due to scientific ignorance, which we find a reasonable conclusion, it seems to involve an undesirable intrusion of politics into science and medical practice. If so, this would be disturbing. At all costs, science itself at least must stay free from political ideologies, from dogmas and from financial interests.

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Author Contributions

UK, SP, PB, KS: Study design. SP: NGS analysis. RJK, RL, KS: Review design. All authors were involved in reviewing the literature and writing the manuscript.

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Competing Interest Declaration

SP is owner and head of Tilia Laboratories, which is focused on diagnostics and research in molecular microbiology and genetics. RL, Erasmus MC and BioCoS, have signed a joint-ownership agreement related to SARS-CoV-2 detection methods. All other authors declare that they have received no support from any organization for the submitted work; that they have had no financial relationships with any organizations that might have had an interest in the submitted work during the previous three years; and they have no other relationships, nor have they engaged in any activities that could even appear to have influenced the submitted work.

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https://en.chinacdc.cn/special/COVID19_Response/discoveries_guidelines/202205/t20220516_259215.html but the link to the PowerPoint does not seem to go anywhere; later changed to <http://www.chinacdc.cn/en/COVID19/202003/P020200323390321297894.pdf>, but neither of those links works at the time of this writing. Therefore, we have made the pdf file available as a separate document at <https://doi.org/10.56098/ijvtp.v3i1.74>].

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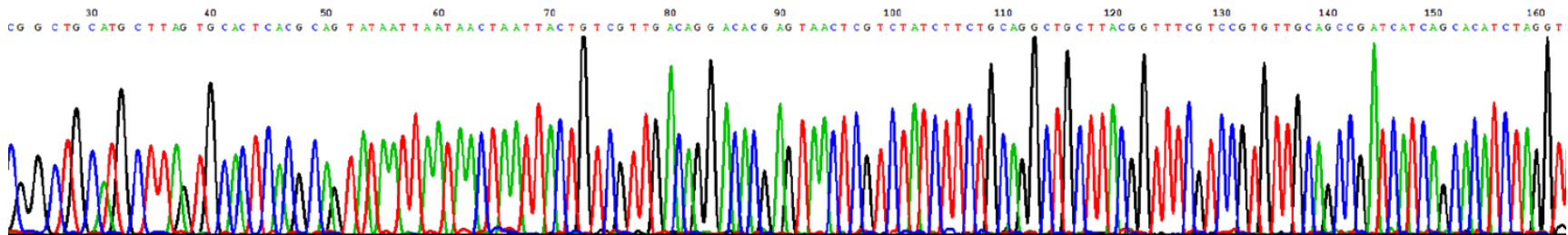
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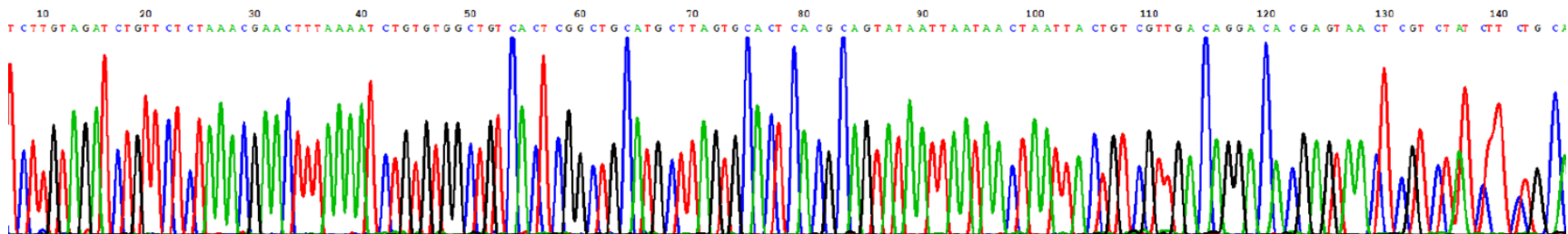
Supplementary Figure 1A. The chromatograms from Sanger forward and reverse sequencing of the 5'-UTR sequence of the index patient BD103, diagnosed with a load of 5×10^7 copies of the virus/ml of sample eluate (nasopharyngeal swab).

A

BD103 5' UTR forward:



BD103 5' UTR reverse:



Supplementary Figure 1B. BLAST alignment of the BD103 5'-UTR sequence to the 5'-UTR sequences of the closest neighbours. Note the 100% homology with the SARS-CoV-2 5'-UTR and the striking heterology when compared with Bat-coronavirus and Bat-SARS-coronavirus, respectively. Query: The authentic sequence obtained from the BD103 index patient; Subject: Reference sequence of the particular coronavirus deposited in NCBI ref seq library (<https://www.ncbi.nlm.nih.gov/>).

Download GenBank Graphics	
Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/nCoV-19-02S/human/2020/VNM	
Sequence ID: MT192773.1 Length: 29890 Number of Matches: 1	
Range 1: 43 to 249 GenBank Graphics Next Match Previous Match	
Score	Expect
383 bits(207)	2e-102
Identities	Gaps
207/207(100%)	0/207(0%)
Strand	Plus/Plus
Query 1	CGATCTCTTGTAGATCTGTTCTCTAAACGAACCTTAAATCTGTGGCTGTCACTCGGC 60
Sbjct 43	CGATCTCTTGTAGATCTGTTCTCTAAACGAACCTTAAATCTGTGGCTGTCACTCGGC 102
Query 61	TGCATGCTTAGTGCACTCACGAGTATAATTAATACTAATTAAGTCTGTTGACAGGACA 120
Sbjct 103	TGCATGCTTAGTGCACTCACGAGTATAATTAATACTAATTAAGTCTGTTGACAGGACA 162
Query 121	CGAGTAACTCGTCTATCTTCTGCAGGCTGCTACGGTTTCGTCGGTGTGACGCCGATCA 180
Sbjct 163	CGAGTAACTCGTCTATCTTCTGCAGGCTGCTACGGTTTCGTCGGTGTGACGCCGATCA 222
Query 181	TCAGCACATCTAGGTTTCGTCGGGTG 207
Sbjct 223	TCAGCACATCTAGGTTTCGTCGGGTG 249

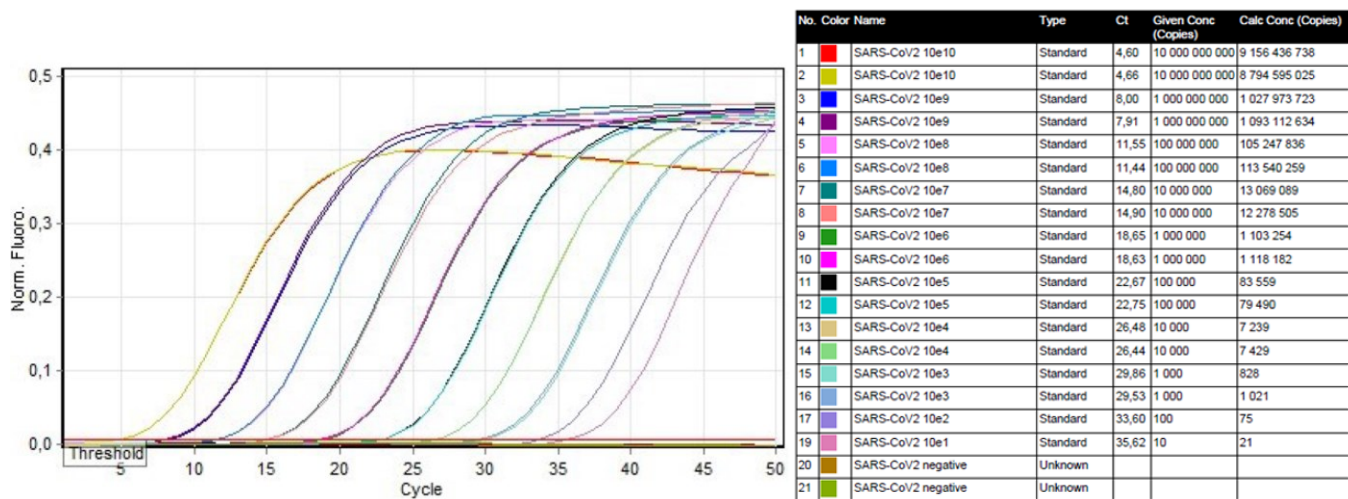
Download GenBank Graphics	
Bat coronavirus (BtCoV/279/2005), complete genome	
Sequence ID: DQ648857.1 Length: 29741 Number of Matches: 1	
Range 1: 41 to 248 GenBank Graphics Next Match Previous Match	
Score	Expect
283 bits(153)	2e-72
Identities	Gaps
191/209(91%)	3/209(1%)
Strand	Plus/Plus
Query 1	CGATCTCTTGTAGATCTGTTCTCTAAACGAACCTTAAATCTGTGGCTGTCACTCGGC 60
Sbjct 41	CGATCTCTTGTAGATCTGTTCTCTAAACGAACCTTAAATCTGTGGCTGTCACTCGGC 100
Query 61	TGCATGCTTAGTGCACTCACGAGTATAATTAATACTAA--TTACTGTCGTTGACAGGA 118
Sbjct 101	TGCATGCTTAGTGCACTCACGAGTATAATTAATACTAA--TTACTGTCGTTGACAGGA 159
Query 119	CACGAGTAACTCGTCTATCTTCTGCAGGCTGCTACGGTTTCGTCGGTGTGACGCCGAT 178
Sbjct 160	TACGAGTAACTCGTCTATCTTCTGCAGGCTGCTACGGTTTCGTCGGTGTGACGCCGAT 219
Query 179	CATCAGCACATCTAGGTTTCGTCGGGTG 207
Sbjct 220	CATCAGCACATCTAGGTTTCGTCGGGTG 248

Download GenBank Graphics	
Bat SARS coronavirus HKU3-12, complete genome	
Sequence ID: GQ153547.1 Length: 29704 Number of Matches: 1	
Range 1: 40 to 246 GenBank Graphics Next Match Previous Match	
Score	Expect
281 bits(152)	7e-72
Identities	Gaps
190/208(91%)	3/208(1%)
Strand	Plus/Plus
Query 2	GATCTCTTGTAGATCTGTTCTCTAAACGAACCTTAAATCTGTGGCTGTCACTCGGC 61
Sbjct 40	GATCTCTTGTAGATCTGTTCTCTAAACGAACCTTAAATCTGTGGCTGTCACTCGGC 99
Query 62	GCATGCTTAGTGCACTCACGAGTATAATTAATACTAA--TTACTGTCGTTGACAGGAC 119
Sbjct 100	GCATGCTTAGTGCACTCACGAGTATAATTAATACTAA--TTACTGTCGTTGACAGGAC 158
Query 120	ACGAGTAACTCGTCTATCTTCTGCAGGCTGCTACGGTTTCGTCGGTGTGACGCCGATC 179
Sbjct 159	ACGAGTAACTCGTCTATCTTCTGCAGGCTGCTACGGTTTCGTCGGTGTGACGCCGATC 218
Query 180	ATCAGCACATCTAGGTTTCGTCGGGTG 207
Sbjct 219	ATCAGCACATCTAGGTTTCGTCGGGTG 246

Supplementary Figure 2A. SARS-CoV-2 Quantitation Report.

Standard Curve (1)	$\text{conc} = 10^{(-0,279 \cdot \text{CT} + 11,244)}$
Standard Curve (2)	$\text{CT} = -3,587 \cdot \log(\text{conc}) + 40,327$
Reaction efficiency (*)	$(* = 10^{(-1/m)} - 1) 0,90024$
M	-3,58668
B	40,3274
R Value	0,99919
R^2 Value	0,99839

Supplementary Figure 2B. Standard curve and log dilution of synthetic SARS-CoV-2 template from 10e10 copies/PCR to 10e1 copies/PCR is shown. Quantitation Cycling. A.Green.



Supplementary Figure 2C. Standard Curve.

