Reliable detection of SARS-CoV-2 RNA using RT-(q)PCR critically depends on primer design and PCR test parameters: an evaluation study of novel primers

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ABSTRACT

Objectives To assess the performance of newly developed polymerase chain reaction (PCR) primers to detect severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA, using gel electrophoresis and sequencing. Our results were compared against those obtained with the primers developed by Charité Berlin and ones commercially available in the Applex^{TM} SARS-CoV-2 assay.

Design Evaluation study

Setting This evaluation study was conducted at the Erasmus MC an academic hospital in the southwest of the Netherlands. Samples were obtained from a Medical Diagnostic Center also stationed in the South-West of the Netherlands that offers routine microbiology diagnostics (e.g., serology, molecular testing, bacterial cultures) for approximately 1,500 primary health care facilities. The primer sequences were designed by BioCoS, a biotechnology company providing bioinformatics services for biomarker discovery and primer design.

Participants 150 symptomatic patients suspicious for a SARS-CoV-2 infection who presented themselves at a general practitioner or at a geriatric specialist were included.

Main outcome measures Presence or absence of SARS-CoV-2 RNA in oro-nasopharyngeal swabs as detected by RT-(q)PCR, gel electrophoresis and sequencing of the PCR amplicons after which the positive predicted value (PPV), negative predicted value (NPV), positive percentage agreement (PPA) and negative percentage agreement (NPA) of each primerset was determined.

Results Gel electrophoresis of RT-(q)PCR amplicons and sequencing methods demonstrated that the newly discovered and designed triplet STAMINA primersets by BioCoS in the ORF1ab (PPV,100%; NPV, 80%), E- (PPV 100%; NPV 73.85%) and N-gene (PPV 100%; NPV 60%) harbored an increased PPA compared to the triplet Charité Berlin primersets designed in the RdRp- (PPV 100%; NPV 67.61%), E- (PPV 100%; NPV 71.64%) and N-gene (PPV 96.97%; NPV 39.17%), by using the Allplex^{TM} SARS-CoV-2 assay as a criterion standard. Moreover, calculating the PPA by using our own constructed composite reference as a standard confirmed that the STAMINA primersets outperformed the Charité Berlin primersets, which came with a trade-off in NPA. Sequencing of the RT-(q)PCR amplicons revealed the presence of aspecific products e.g., Homo sapiens, bacteria and viruses other than SARS-CoV-2, but excluded the presence of related coronaviruses in the amplicons generated with the STAMINA primersets.

Conclusion This evaluation study reveals that reliable detection of SARS-CoV-2 RNA using RT-(q)PCR critically depends on primer design and PCR test parameters. Moreover, our work shows that the newly developed primers, despite outperforming the ones designed by Charité Berlin in PPA, are still suboptimal to detect SARS-CoV-2 RNA.

INTRODUCTION

The emergence of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) and the associated pandemic has dramatically affected human health, society and economics worldwide [1–4]. SARS-CoV-2 is a single-stranded, positive-sense RNA virus, which is closely related to the beta-coronavirus-2B lineage of the *Coronavirinae* subfamily [5]. In early 2020, it was shown that the SARS-CoV-2 genome encodes for the coronavirus-typical essential nucleocapsid (N), membrane (M), spike (S), envelope (E) proteins and expresses 16 additional non-structural proteins, including a RNA-dependent RNA-polymerase (RdRp) gene [5–8]. During the early stages of the pandemic, the reverse transcription-(quantitative) PCR (RT-(q)PCR) method, designed by Charité Berlin [9], quickly provided support to monitor the pandemic and was advised to be used as a reference test for the detection of SARS-CoV-2 RNA [10]. The RT-(q)PCR assay was selected as a result of achieved performances during previous coronavirus outbreaks, because other techniques like antibody-based detection still required optimization for SARS-CoV-2 identification [11]. The RT-(q)PCR assay is based on the detection of the RdRp-, E- and N-genes as present in SARS-CoV-2 [9], which was introduced into the market in a relative short-time window after whole-genome sequencing data became available on Jan 5th 2020 [12]. A challenge to the development of this detection test was the lack of patient samples at that time. So the designed primersets were validated on a set of synthetic sequences only, which subsequently turned into a limitation [9,11,13]. Despite this, the nucleic acid detection test offered valuable support in monitoring the spread of SARS-CoV-2 during the early stages of the pandemic. Logically, as time progressed, data and new knowledge accumulated inevitably, revealing that the protocol by the World Health Organization had space for improvements [10,14]. The main concerns related to lower sensitivity and specificity levels as seen with other developed methods [11,13,15,16] was in part driven by the genomic nature of SARS-CoV-2, in terms of sequence variations and mutations that affected the test results [16-19]. The observation that a specific mutation reduced the performance of the WHO recommended assay underlines also the necessity to further validate the SARS-CoV-2 positive test results using sequencing methods on the generated PCR amplicons [18]. This type of validation is fundamental to keep improving the nucleic acid detection methods, since among other factors that affect pandemic management, also the test accuracy has its important role to prevent misjudgment of an outbreak situation [14]. Indeed, a high number of false positives may force decision makers to apply unnecessarily measures and regulations [19,20]. For obvious reasons, high number of false negative results (undetected infected subjects) also interfere with an appropriate response of decision makers [20,21], which led to important remarks that need to be considered to improve such nucleic acid detection tests [14,19,22–28]. Moreover, the more reliable a detection test is, the better the development of treatment options can be validated to tackle later stages of a pandemic [29–37].

The development of nucleic acid detection tests was also part of STAMINA (ID: 883441), an EU funded project focused on management and intelligent decision support to tackle a pandemic crisis within and across European borders. In this paper, we present data on the first of the two nucleic acid detection tests on SARS-CoV-2 developed in STAMINA. The herein test involves the validation of three novel primersets discovered and designed in the ORF1ab-, E- and N-gene.

The obtained results were evaluated against primersets designed in the RdRp-, E- and N-gene to detect SARS-CoV-2 RNA by Charité Berlin or as available in the Allplex™ SARS-CoV-2 assay [9,38,39]. Moreover, gel electrophoresis and sequencing methods were applied to increase the resolution of detection of the generated PCR amplicons.

METHODS

Study population

A Medical Diagnostic Center that provides laboratory services in the South-West of the Netherlands was involved, which performs for approximately 1,500 primary health care facilities diagnostic services (e.g., serology, molecular testing, bacterial cultures). During the pandemic, patients presenting at a general practitioner or geriatric medicine specialist with signs and symptoms suspicious for a SARS-CoV-2 infection, were sampled from both the oral and nasal cavity, subsequently using a single oro-nasopharyngeal swab (Aptima® Multitest Swab Transport Media, Hologic Inc., Marlborough, MA, USA). The Allplex™ SARS-CoV-2 assay (Seegene Inc., Seoul, Republic of Korea) was used, since it was thoroughly validated [38,39]. Oro-nasopharyngeal samples were stored at –20°C until assayed.

Sample collection

Oro-nasopharyngeal samples (*n* = 150), in Aptima® Multitest Swab Transport Media, were collected based on results obtained from the three genes (RdRp-, E, and N-gene) targeted in the Allplex™ SARS-CoV-2 assay and several patients' characteristics (e.g., gender, age and the day of sample collection). SARS-CoV-2 was detected in 102 and remained undetected in 48 samples, respectively. In addition, data on cycle threshold (Ct)-values for each of the three genes were collected (**Table 1**) and on average, a Ct-value ≥ 35 was considered as negative. A SARS-CoV-2 reference sample (inactivated) with known viral load was kindly provided by the Virology department of Erasmus University Medical Center Rotterdam, Netherlands.

Nucleic acid extraction

First, nucleic acids were extracted on the MagNA Pure 96 Instrument (Roche, Almere, Netherlands) using the "Viral NA Plasma ext Lys SV 4.0 protocol'' from the ''MagNA Pure 96 DNA and Viral NA Small Volume kit'' (Roche). 450 µl of each sample was processed to obtain an elution volume of 50 µl, whereafter the nucleic acid samples were stored at -20 ^{0}C.

SARS-CoV-2 RNA detection using reverse transcription polymerase chain reaction (RT-PCR)

The SensiFast Probe No-ROX One-step kit (Meridian Bioscience®, Boxtel, Netherlands) was executed using six different sets of primer pairs (**Table 2**). The RT-PCR forward and reverse primersets designed by the STAMINA partner BioCoS in the ORF1ab, N- and E-gene from now on referred to as the STAMINA primers and the Charité Berlin SARS-CoV-2 forward and reverse primersets designed by Corman *et al*., [9] in the RdRp-, N- and E-gene were used (**Table 2**). Briefly, the reaction mixture of the STAMINA or the Charité Berlin primersets contained 1x SensiFAST™ Probe No-ROX One-Step mix

(Meridian Bioscience®), 0.4 µM forward and reverse primer, 0.4 µl Ribosafe RNase inhibitor (Meridian Bioscience®), 0.2 µl reverse transcriptase (Meridian Bioscience®) and 5 µl extracted nucleic acids in a final volume of 20 µl. The RT-PCR program used included ten minutes of reverse transcription at 45 ^{0}C, two minutes of polymerase activation at 95 ^{0}C, 45 cycles of five seconds of denaturation at 95 ^{0}C together with 30 seconds of annealing/extension at 60 ^{0}C and a final step of 30 seconds of cooling at 40 ^{0}C. For the Charité Berlin N-gene primerset, the same reaction mixture was used with a concentration of 0.6 µM forward primer and 0.8 µM reverse primer as stated in their protocol [9]. The RT-PCR program used included ten minutes of reverse transcription at 45 ^{0}C, three minutes of polymerase activation at 95 ^{0}C, 45 cycles of 15 seconds of denaturation at 95 ^{0}C together with 30 seconds of annealing/extension at 55 ^{0}C and a final step of 30 seconds of cooling at 40 ^{0}C. The human RNase P gene used as an internal control was detected by PCR using 1x Dreamtaq Green buffer (ThermoFisher Scientific (TFS), Breda, Netherlands)), 1.0 µM forward and reverse primer, 0.2 mM dNTP (TFS), 1.25 U DreamTaq DNA polymerase (TFS) and 2 µl extracted nucleic acids with a final volume of 50 µl. The PCR program used included five minutes of initial denaturation, 35 cycles of 40 seconds of denaturation at 95 ^{0}C together with 40 seconds of annealing/extension at 57 ^{0}C and one minute of extension at 72 ^{0}C and a final step of 30 seconds of cooling at 40 ^{0}C. All PCR reactions were executed using the Veriti 96 Well Thermal Cycler (Applied Biosystems, Nieuwerkerk aan den IJssel, Netherlands) and all amplified products were analysed by gel electrophoresis and sequencing.

Limit of detection

To investigate the limit of detection (LOD) of the STAMINA and the Charité Berlin primersets, the SensiFast Probe No-ROX One-step kit was executed according to the 45 cycles RT-PCR SARS-CoV-2 RNA detection protocol, testing serial reference sample dilutions.

Agarose gel analysis

The 2.5% agarose gels were prepared using agarose (SphaeroQ, Gorinchem, Netherlands), 1x TBE Electrophoresis buffer (TFS) and SYBR safe (Invitrogen, Carlsbad, USA). Agarose gels were run using a Bio-Rad SUB-CELL® GT tank and Bio-Rad Power Pac 300 in 1x TBE Electrophoresis buffer. The gels were analysed using an Isogen Life sciences Proxima 16 Phi+ gel reader. GeneRuler 100 bp plus DNA ladders (TFS) and samples were prepared using a 6x Orange DNA loading dye (Fermentas, Vilnius, Lithuania). The agarose gels were run at 60 mA.

Sequence analysis

All in-house generated RT-(q)PCR products were sequenced by BaseClear (Leiden, Netherlands). The identity of the sequences was analysed via the Basic Local Alignment Search Tool for Nucleotides (BLASTN) from the National Center of Biotechnological Information (NCBI) [40]. The produced results from BLASTN were reported as: ‘Confirmed’, ‘No significant result’ and ‘To repeat’. Based on the outcomes of the sequencing analyses, a final overall conclusion considering the identity of each individual primerset and all primersets combined was formulated. In total, a set of two sequencing runs were performed. During the first sequencing analysis all 102 positive samples and a selection of

negative samples that generated positive results were sequenced. A second sequencing run was executed to validate the positive and negative reported samples that produced a weak signal during the first run using a low primer concentration.

Ethical approval

This study involving participants' residual materials was conducted in accordance with the 1964 Helsinki declaration. Anonymous data corresponding to Allplex™ SARS-CoV-2 assay run 1 and 2 were courtesy received from a medical diagnostic center that provides laboratory services in the South-West of the Netherlands, in support to the EU project STAMINA. Separate approval by an ethics review committee was therefore not required.

RESULTS

Limit of detection

To assess the limit of detection (LOD) of the STAMINA and Charité Berlin primersets, we generated serial dilutions of a reference sample known to contain 8.56E06 infectious units of SARS-CoV-2 per microliter. Both the RdRp- and the ORF1ab-gene had a LOD of 85 infectious units per microliter (**Supplementary Figure 1A**). The LOD for the E-gene using STAMINA primerset was 8,560, while for the Charité Berlin primerset this number was 856 infectious units of SARS-CoV-2 per microliter, respectively (**Supplementary Figure 1B**). For the N-gene, both primersets revealed a LOD of 8,560 infectious units of SARS-CoV-2 per microliter (**Supplementary Figure 1C**).

The Allplex™ SARS-CoV-2 assay and agarose banding pattern analyses

Oro-pharyngeal samples (*n* = 150) were analysed using the Allplex™ SARS-CoV-2 assay and agarose gel electrophoresis (**Table 1**). In the first Allplex™ SARS-CoV-2 assay run, hundred patient samples were found to be positive and 50 were found to be negative for the genetic material of SARS-CoV-2, as measured by the presence of the RdRp/S-, E- and N-gene in a RT-(q)PCR setting (**Table 1**). In the second Allplex™ SARS-CoV-2 assay run, a discrepancy was detected for three negative samples (sample 101, 107 and 127). In addition, multiple negative samples (*n* = 13) identified in the first Allplex™ SARS-CoV-2 assay run were found to give a signal for one or more SARS-CoV-2 genes in the second Allplex™ SARS-CoV-2 assay run, which were not detected in the first run (**Table 1**). We used gel electrophoresis to visualize the RT-(q)PCR products banding patterning of all and these dubious negative samples (**Fig 1A**), one of them (sample 103) revealed amplicons resembling a RT-(q)PCR product generated from the RdRp/S-, E- and N- and an internal control gene. For two other samples (sample 101 and 127) we first obtained negative data on their Ct-values in the first Allplex™ SARS-CoV-2 assay run, which was later on corrected (**Table 1**). Moreover, sample 103 had Ct-values around 37 and was actually counted as negative earlier (**Table 1**). All the positive samples identified in the Allplex™ SARS-CoV-2 assay, including the two samples (101 and 127) (*n* = 102) were run on an agarose gel revealing positive banding patterns of RT-(q)PCR products obtained from the RdRp/S-, N-, E- and internal control gene (**Fig 1B**).

RT-PCR and agarose banding pattern analyses

We then analyzed the performance of the STAMINA primers designed in the ORF1ab, E- and N-gene by agarose banding pattern analysis using gel electrophoresis (**Fig 2A-C**). The ORF1ab primerset (167 bp amplicon) resulted in 12 negative PCR samples, whereas the E-gene primerset (181 bp amplicon) revealed 17 and the N-gene primerset (193 bp amplicon) revealed 32 negative PCR samples out of the 102 that were found to be positive in the Allplex™ SARS-CoV-2 assay, which we used as our criterion standard (**Table 1**), see American Medical Association manual of style for additional info on this standard [41]. Hereafter, we analyzed the primer performance of the primers (RdRp-, E- and N-gene) as mentioned in the Charité Berlin protocol by agarose banding pattern analysis (**Fig 3A-C**). The RdRp-gene primerset (100 bp amplicon) resulted in 23 negative PCR samples, whereas the E-gene (113 bp amplicon) and the N-gene (128 bp amplicon) primersets revealed 19 and 73 negative PCR samples, respectively, out of the 102 that were found to be positive in the Allplex™ SARS-CoV-2 assay (**Table 1**). In contrast, the N-gene primerset revealed one positive PCR sample out of the 48 that were found to be negative in the Allplex™ SARS-CoV-2 assay (**Table 1**).

The Ct-value cut-off is important to eliminate false positives and negatives from true positives and negatives with respect to the ability to identify infectious persons and therefore we made advantage of available literature in which such cut-off values were established [42,43]. We therefore reanalysed our STAMINA and Charité Berlin primer results against the Allplex™ SARS-CoV-2 assay data with Ct-values at different cut-offs at 25 and 20 cycles (**Table 1**). At a Ct-value ≤ 25 the number of false positives for the STAMINA primers were for the ORF1ab-, E- and N-gene 23, 18 and 5, respectively, whereas the number of false negatives was 0, 0 and 2. For the Charité Berlin primers the number of false positives were for the RdRp-, E- and N-gene 14, 16 and 4 respectively, whereas the number of false negatives was 2, 0 and 41, respectively. At a Ct-value ≤ 20 the number of false positives for the STAMINA primers rose for the ORF1ab-, E- and N-gene to 49, 44 and 29, respectively, whereas the number of false negatives was zero for all three genes. The number of false positives for the Charité Berlin primers rose for the RdRp-, E- and N-gene to 38, 42 and 8, respectively, whereas the number of false negatives was 0, 0 and 19. Our data thus reveals that by lowering the Ct-value cut-off and using the Allplex™ SARS-CoV-2 assay as a criterion standard, there is a trade-off for the six primer pairs (STAMINA and Charité Berlin) in the number of false negatives and false positives.

RT-PCR amplicon sequencing

We then analyzed the RT-PCR generated amplicons for each of the used primerset (ORF1ab-, RdRp-, E- and N-gene) by sequencing. After two sequencing runs out of the 102 samples that were found to be positive in the Allplex™ SARS-CoV-2 assay, the STAMINA primersets resulted in three SARS-CoV-2 negative RT-PCR samples for the ORF1ab gene obtained amplicons, whereas for the E-gene and N-gene obtained RT-PCR amplicons, the number of samples negative for genetic material of SARS-CoV-2 was 8 and 22, respectively (**Supplementary data 1-3**). For the Charité Berlin related RdRp-gene primerset, out of the 102 samples that were found to be positive in the Allplex™ SARS-CoV-2 assay, the number of negative amplicons for SARS-CoV-2 obtained after RT-PCR and sequencing was 29, whereas the number of SARS-CoV-2 negative RT-PCR amplicons for the E- and N-gene amplicons was

23 and 52, respectively (**Supplementary data 4-6**). From our sequencing results it thus becomes clear, that by taking care in primer design, the accuracy in detection of the genetic material of SARS-CoV-2 can be improved, e.g., by reducing potential false positive hits initiated by primer cross-reactivity. By not doing so, as can be seen in the results obtained after sequencing of the Charité Berlin amplicons, there will be an increase in the detection of genetic material of species other than SARS-CoV-2, ranging from Hepatitis and Rotaviruses, to *Solobacterium* spp., *Rothia mucilaginosa* to Homo sapiens, amongst others (**Supplementary data 4-6**). Furthermore, the STAMINA primersets for ORF1ab-, E- and N-gene generated amplicons in two samples that were corrected (sample 101 and 127). These amplicons (*n* = 6) were sequenced and found all to be positive for SARS-CoV-2 (**Supplementary data 1-3**), confirming that the correction of the error found in the Allplex™ SARS-CoV-2 assay run 1 dataset was valid. The Charité Berlin primersets (RdRp-, E- and N-gene) also generated for each SARS-CoV-2 gene RT-PCR amplicons in three samples, the two corrected samples (101 and 127) and a negative sample, number 102. The RT-PCR related amplicons (*n* = 9) were all sequenced and five of these amplicons (101 and 127) revealed to be positive for a SARS-CoV-2 gene (**Supplementary data 4-6**). Number six, the N-gene amplicon of sample number 127 provided unexpectedly a signal for Homo sapiens genomic DNA (**Supplementary data 6**).

We then constructed a composite reference standard [44] by combining the results of the STAMINA and Charité Berlin tests (both with their limitations) and found when compared to the test results obtained earlier that eight of the 102 samples (36, 45, 52, 82, 83, 101, 127 and 143) shown to be positive and one of the 48 samples (number 103) found to be negative in the Allplex™ SARS-CoV-2 assay criterion standard were dubious (**Table 1 & 4**). Subsequently, these results made us reanalyse these samples again by sequencing. Noteworthy, the Allplex™ SARS-CoV-2 assay harbors primersets that enabled the detection of the RdRp/S-, E- and N-gene with primer sequences that were unknown to us, complicating the sequencing process at the start of this analyses. To overcome this problem we tried using the Charité Berlin primersets for the RdRp-, E- and N-gene on all nine RT-(q)PCR amplicons obtained in the Allplex™ SARS-CoV-2 assay and to our surprise successfully discovered that eight out of nine generated a positive agarose banding result for the RdRp- and E-gene, respectively, whereas the N-gene generated only three aspecific PCR products (**Supplementary Figure 2A-C**). After tackling this problem all nine samples were found to be positive for the E-gene, surprisingly also the PCR negative sample 143 (**Table 4**)**.** This indicates that sequencing of the PCR amplicons can increase the sensitivity of detection, but also revealed that for these nine dubious samples only the SARS-CoV-2 E-gene was detected in all the PCR amplified samples (**Supplementary Figure 2A-C & Table 4**).

Positive and negative percentage agreement analysis

We then calculated the positive (PPA) and negative (NPA) percentage agreement for each primerset tested and used the Allplex™ SARS-CoV-2 assay as a criterion standard or our own constructed composite standard as a reference (**Table 1**). PPA and NPA nomenclature were preferred in use instead of sensitivity and specificity, since the SARS-CoV-2 reference test was brought to the market with an emergency use authorization [9,45,46]. The composite standard was used to control whether the Allplex™ SARS-CoV-2 assay was truly positive for SARS-CoV-2 RNA or not. Firstly, by using the

Allplex™ SARS-CoV-2 assay with a Ct-value 35 cut-off, we calculated the positive (PPV) and negative (NPV) predicted value and the PPA and NPA for the STAMINA and Charité Berlin primersets (**Table 3A**). We found that the performance of the STAMINA primersets in eliminating false negatives was increased compared to the Charité Berlin primersets (**Table 3A**). We then calculated the PPV, NPV and PPA and NPA of the RT-PCR tests (STAMINA and Charité Berlin) for each primerset validated, using our own constructed composite reference as a standard (**Table 1**). This calculation confirmed that the STAMINA primersets outperformed the Charité Berlin primersets in PPA (**Table 3B**). By using the Allplex™ SARS-CoV-2 assay as the criterion standard with a Ct-value 25 cut-off, we found that the STAMINA primersets still outperformed the Charité Berlin primers in PPA, which came with a trade-off in NPA. The number of false positives obtained with the STAMINA primersets was increased compared to the Charité Berlin primers (**Table 3A**), which was further established by using the Allplex™ SARS-CoV-2 assay as the criterion standard with a Ct-value 20 cut-off (**Table 3A**), respectively.

DISCUSSION

In the present work we assessed the performance of new PCR primers discovered and designed in the STAMINA project against primers developed by Charité Berlin and ones commercially available (Applex™ assay) to detect the genetic material of SARS-CoV-2. Gel electrophoresis and sequencing methods were applied to increase the resolution of detection of the generated PCR amplicons. When the commercial Applex™ assay was used as a criterion standard, we found that the STAMINA primersets harbored an increased PPA to detect the RNA of SARS-CoV-2 in symptomatic patients. Results that we could confirm by establishing our own constructed composite reference standard. Indeed, specifically the N-gene primerset was improved in performance by increasing the PPA from 28% as observed for the Charité Berlin primerset to a 100% for the STAMINA primerset, depending on the condition validated. There against, the increase in PPA was accompanied with a trade-off in NPA in which the STAMINA primers were less well performing compared to the Charité Berlin primers. On the other hand, our sequencing data did reveal that the STAMINA primers were more specific in detecting SARS-CoV-2 RNA, whereas the Charité Berlin PCR amplicons were more often associated with a-specific products. Indeed, we identified hits with species other than SARS-CoV-2, ranging from Hepatitis and Rotaviruses, to *Solobacterium* spp., *Rothia mucilaginosa* to Homo sapiens, amongst others, but we also excluded the presence of coronaviruses other than SARS-CoV-2 in the amplicons generated with the STAMINA primersets. Increasing the PPA of the SARS-CoV-2 RT-PCR test is important, because in a situation where a virus spreads in the community, it is mandatory, specifically from a track and trace situation or clinic point of view, not to miss real potential positive (infectious) cases. Our evaluation study thus demonstrated that in symptomatic patients suspicious for a SARS-CoV-2 infection, the STAMINA RT-PCR test protocol harbors an increased PPA, indicating that genetic material of this pathogen will be less often missed compared to the Charité Berlin protocol. Unfortunately, the NPA of both tests still exhibits problems, which became obvious when we reduced the Ct-value cut-off, to correct for infectious persons only [42,43]. Overall, our data shows that the RT-(q)PCR tests used in this work are still suboptimal in detecting SARS-CoV-2 RNA. However, we do demonstrate that by optimizing primer design and increasing the resolution of detection, the performance of the RT-(q)PCR test can be

substantially improved to trace back the genetic material of SARS-CoV-2, particularly by substantially reducing false negatives.

After the WHO recommended the usage of the Charité Berlin RT-(q)PCR protocol at the beginning of the pandemic, many colleagues in the field started to consider this protocol as a 'gold-standard' to detect SARS-CoV-2 RNA [47], whereas Corman *et al*., nor the WHO explicitly mentioned to treat this protocol as a gold standard in their documents [9,10]. Based on findings by us and others [14,19,22–28], it is clear that the Charité Berlin protocol required improvement despite having a crucial role at the start of the pandemic. On the other hand this protocol is acceptable as a criterion standard, a test for a particular disease or condition that can be used as a basis of comparison for new tests to further optimize the technology, as described in the American Medical Association manual of style [41].

In the EU funded STAMINA project, we aimed to develop tools that facilitate intelligent and evidence-based decision support to assist end-users and optimize pandemic management by decision makers. The current pandemic crisis revealed that while this project was executed many problems and gaps were identified in tackling a viral outbreak in a coordinated manner. Indeed, care is required in all processes involved in tackling a pandemic crisis from which lessons needs to be learned [48], because in the end they will influence healthcare, policy and decision making accordingly [49].

Strengths and limitations of this study

A clear strength of our study is that we cross-validated the performance of the new primersets designed by BioCoS in the ORF1ab-, E- and N-gene, on their ability to detect the genetic material of SARS-CoV-2 in symptomatic patients with the Charité Berlin primers designed in the RdRp-, E- and N-gene, and the commercially available Allplex™ assay primersets. The oro-nasopharynx swab samples obtained from symptomatic patients were put in Aptima transport media that enabled the inactivation, but also the preservation of the genetic material of SARS-CoV-2, to guarantee the quality of the samples for longer periods of storage. Furthermore, after RT-(q)PCR amplification we increased the resolution of detection by performing combined gel electrophoresis and sequencing of the PCR amplicons, our main parameters tested in this study. The short fragments of some of the PCR amplicons might have affected the reliability of the sequencing results [50], although a second run was added, next to forward and reverse sequencing of the PCR amplicons to validate the findings. Another limitation that might have influenced our study outcome is that, although the PCR amplicons were all small sized < 200 bp, those obtained with the Charité Berlin protocol were at least 50 bp smaller when compared to the PCR amplicons obtained with the primers generated by BioCoS, which might have affected our sequencing results as well. Finally, different standards were used, one based on the commercially available Allplex™ SARS-CoV-2 assay and one based on or our own constructed composite reference standard. In this way, we obtained insight in the performance of our newly developed primersets during the STAMINA project, the WHO recommended RT-(q)PCR protocol as developed by Charité Berlin [10] and the ones commercially available as provided with Allplex™ SARS-CoV-2 assay. Moreover, the adaptation of the Ct-value cut-off helped us to study the effect on viral infectiousness [42,43], revealing that there is a trade-off in PPA and NPA. There against the main limitation of this study is the lack of an *in vitro* assay, to control for the presence of infectious virus particles in the patient samples, and its correspondence to

the Ct-value cut-off. In this respect, existing literature to this topic was of support [42,43], with a recent study even showing prolonged time of positive RT-PCR results in comparison to a negative viral culture already at Ct-values < 36 [51]. Moreover, the performance and interpretation of the assays also depends on disease status of the patients, which was not available to us, except that they were suspicious for a SARS-CoV-2 infection. Finally, our results and findings are limited to the 150 samples tested, therefore follow up work with more samples and a broader variety of commercial test kits will be of importance to establish our findings by gel electrophoresis and sequencing.

Comparisons with other studies

Our findings are in agreement with a multitude of studies questioning the reliability of the WHO recommended protocol overtime [11,13–16], and with a series of studies showing that the Charité Berlin primers were not optimal [19–28,52]. Of note, it is important to clarify that our work does not aim to criticize the work performed by Charité Berlin, where the primers design and the test were developed in an emergency state and without any prior genomic knowledge of SARS-CoV-2 as well as the lack of patient samples. Our findings were solely compared to the above test, due to the availability of data and results from other studies. Indeed, in our work we noticed that by adapting the Ct-values the number of false positives and false negatives became altered, a finding reported and discussed before in relation to infectivity and the Charité Berlin protocol [42,43,51,53–55]. This narrative indicates there is still space for an optimized and validated diagnostic nucleic acid detection test [14]. However, we cannot completely exclude that among the several diagnostic tests developed during the pandemic, such issues have already been taken into consideration. This is why more research studies evaluating (non-) commercial tests are fundamental to keep improving the scientific knowledge that will serve to empower the monitoring of SARS-CoV-2 or any other emerging pathogen.

Health care and policy implications

During the STAMINA project (ID: 883441), although submitted and funded before the SARS-CoV-2 pandemic, our team from Erasmus MC and BioCoS became by coincidence involved in a real-time unfolding pandemic. Part of this project was focused to develop, validate and apply point of care tests to anticipate on potential pandemic threats and to plan daily efforts to enhance health security of the European citizens. However, the unfolding pandemic also provided a unique opportunity to critically analyze the suitability of molecular tests implemented under an emergency state. Summarized, our results point towards the need of a thorough cross-validation of different tests, but also a continuous improvement of diagnostic laboratory assays as the virus continuously evolves. Indeed, the more accurate a test applied on a global scale the better the healthcare response, and the management of a pandemic will become, even if the latter is a multidimensional process. A suboptimal diagnostic test can both over- and under-interpret the severity of a pandemic [21,56], and moreover, can affect (mislead) the validation process of medical treatment options [23–30], that will be desperately sought to control or even eliminate a pandemic causing pathogen. In that respect, our work adds additional insights and knowledge to enhance the accuracy in pandemic monitoring, an important factor in supporting health-care system and decision-making processes in which communication based on solid data is mandatory.

Conclusion

This evaluation study reveals that reliable detection of SARS-CoV-2 RNA using RT-(q)PCR critically depends on primer design and PCR test parameters. Moreover, we found that the STAMINA primers outperform the ones as designed by Charité Berlin in PPA, but are still suboptimal to detect SARS-CoV-2 RNA.

What is already known on this topic

A substantial number of publications reported on the shortcomings of the RT-(q)PCR laboratory assay implemented at the start of the pandemic to detect SARS-CoV-2 RNA, revealing certain risks in using nucleic acid detection test in interpreting the severity of an outbreak. Moreover, the RT-(q)PCR test implemented at the start of the SARS-CoV-2 pandemic played a crucial role in healthcare, economics, policy and decision making and in the clinical validation of different treatment options targeting SARS-CoV-2.

What this study adds

This work reveals the importance of wet lab data on how to increase the resolution of detection by gel electrophoresis and sequencing analysis of the generated RT-PCR amplicons obtained of tested suspects suspicious for a SARS-CoV-2 infection. Furthermore, our new primersets show that the detection of SARS-CoV-2 RNA can be improved, but also reveals that all the RT-PCR tests analyzed in this work remain suboptimal. The more the SARS-CoV-2 RT-(q)PCR tests are optimized the more sophisticated the accuracy of monitoring a pandemic will become. Indeed, solid laboratory assays will not only help us to understand how pathogens are spreading, but will also minimize collateral effects that may appear in the short and long run, affecting healthcare, economies, and most importantly societies [49]. As a final note we would like to suggest that future studies should specifically focus on technological developments that act faster and better and search for infectious viral particles only, so that future pandemics or outbreaks can be monitored more precise.

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Competing interest

Erasmus MC and BioCoS, have signed a joint ownership agreement related to SARS-CoV-2 detection methods. All other authors declare no competing interest.

Author contributions

RL Study management, Experimental design, result interpretation, manuscript writing, editing and reviewing; **SV** Experimental design, primer validation, execution, result interpretation, manuscript reviewing and editing; **IL** bioinformatic analysis, biomarker discovery, primer design, manuscript reviewing; **PB** Experimental design, result interpretation, manuscript writing, editing and reviewing; **AMD** primer design, manuscript editing and reviewing and **SA** Bioinformatic analysis, primer design, manuscript editing and reviewing

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Figures

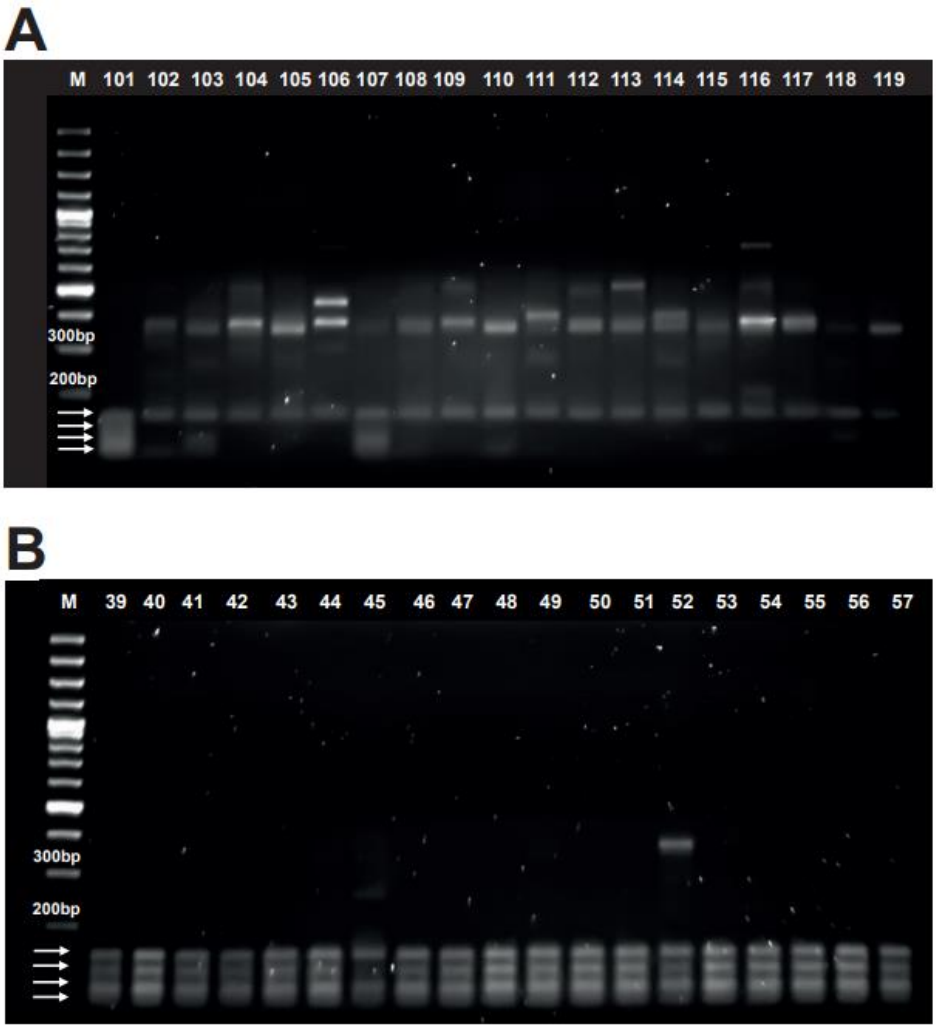

Fig 1 SARS-CoV-2 Allplex™ assay and agarose banding pattern analyses.

Examples of (**A**) negative and **(B)** positive Allplex™ assay samples are shown**.** The presence or absence of the RdRp/S-, E-, N- and control gene is visualised (white arrow) using gel electrophoresis. (M) 100 base pairs Plus DNA size marker; (pos) positive control sample; (neg) negative control sample; (NTC) no template control sample; (number) patient sample numbers.

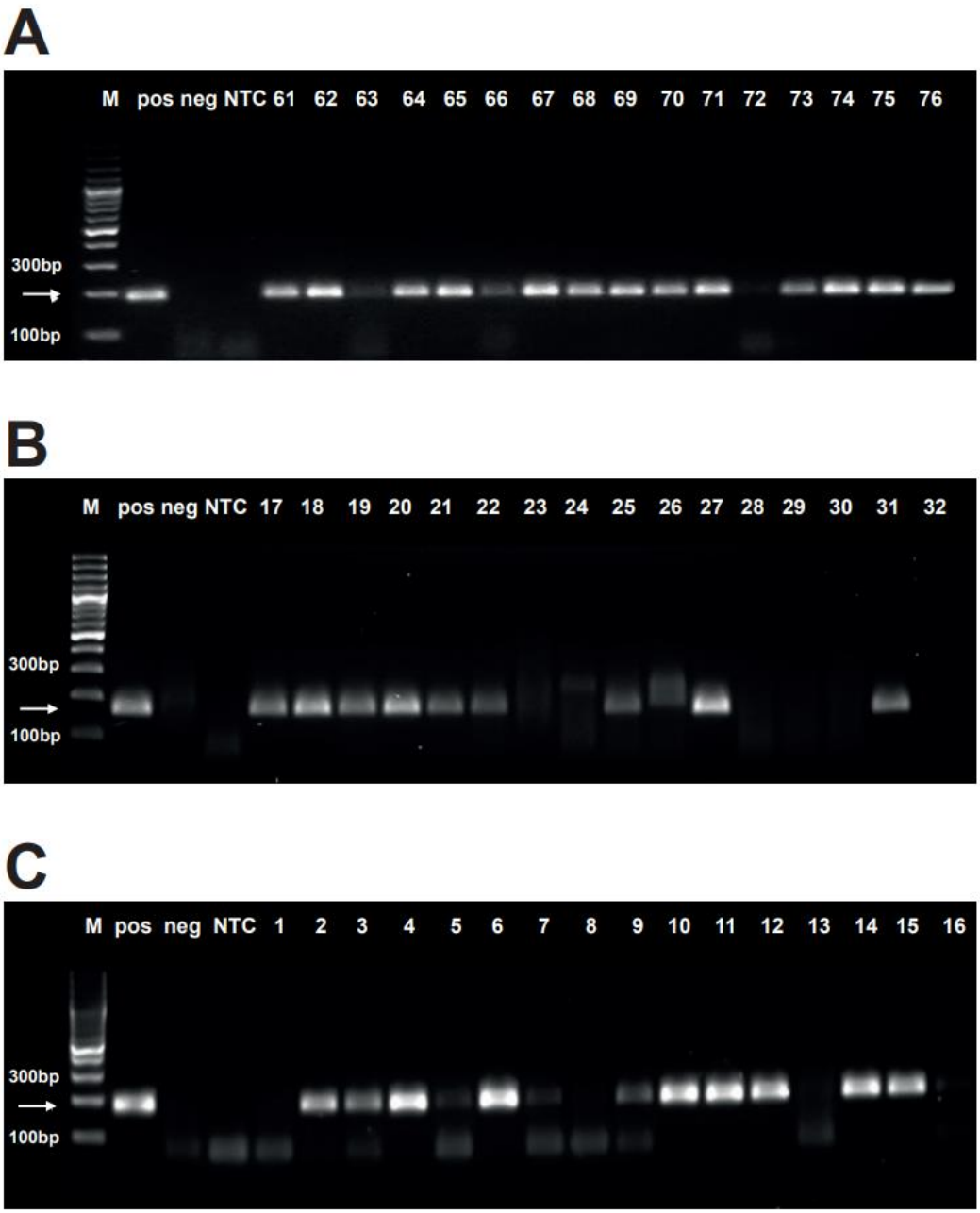

Fig 2 SARS-CoV-2 STAMINA RT-PCR agarose banding pattern analyses.

Examples of (**A**) ORF1ab, (**B**) E-gene and (**C**) N-gene RT-PCR agarose banding pattern results are shown**.** The presence or absence of the ORF1ab-, E-, and N-gene is visualised (white arrow) using gel electrophoresis. (M) 100 base pairs Plus DNA size marker; (pos) positive control sample; (neg) negative control sample; (NTC) no template control sample; (number) patient sample numbers.

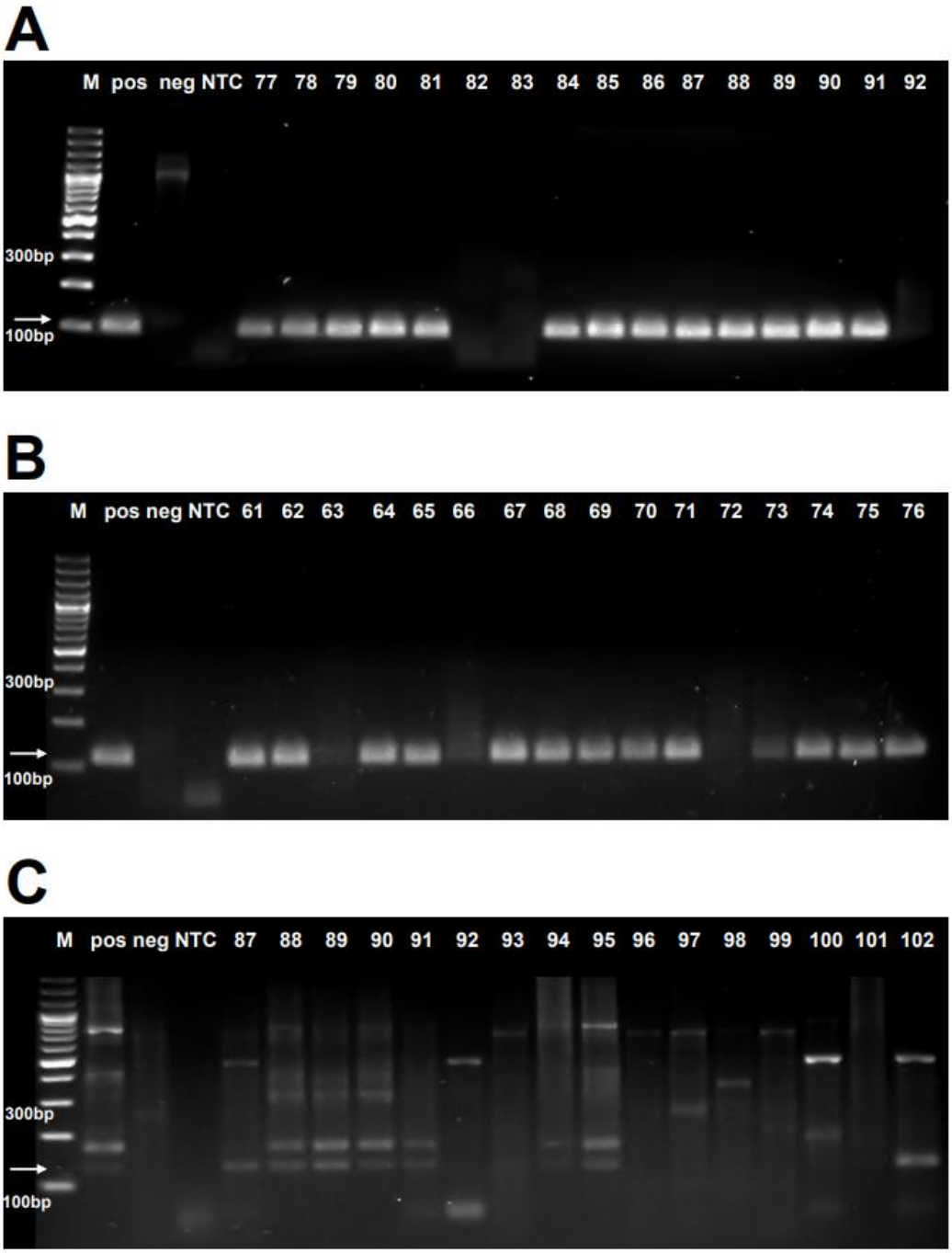

Fig 3 SARS-CoV-2 Charité Berlin RT-PCR agarose banding pattern analyses.

Examples of (**A**) RdRp-, (**B**) E-gene and (**C**) N-gene RT-PCR agarose banding pattern results are shown**.** The presence or absence of the RdRp-, E-, and N-gene is visualised (white arrow) using gel electrophoresis. (M) 100 base pairs Plus DNA size marker; (pos) positive control sample; (neg) negative control sample; (NTC) no template control sample; (number) patient sample numbers.

679 **Supplemental Figures**

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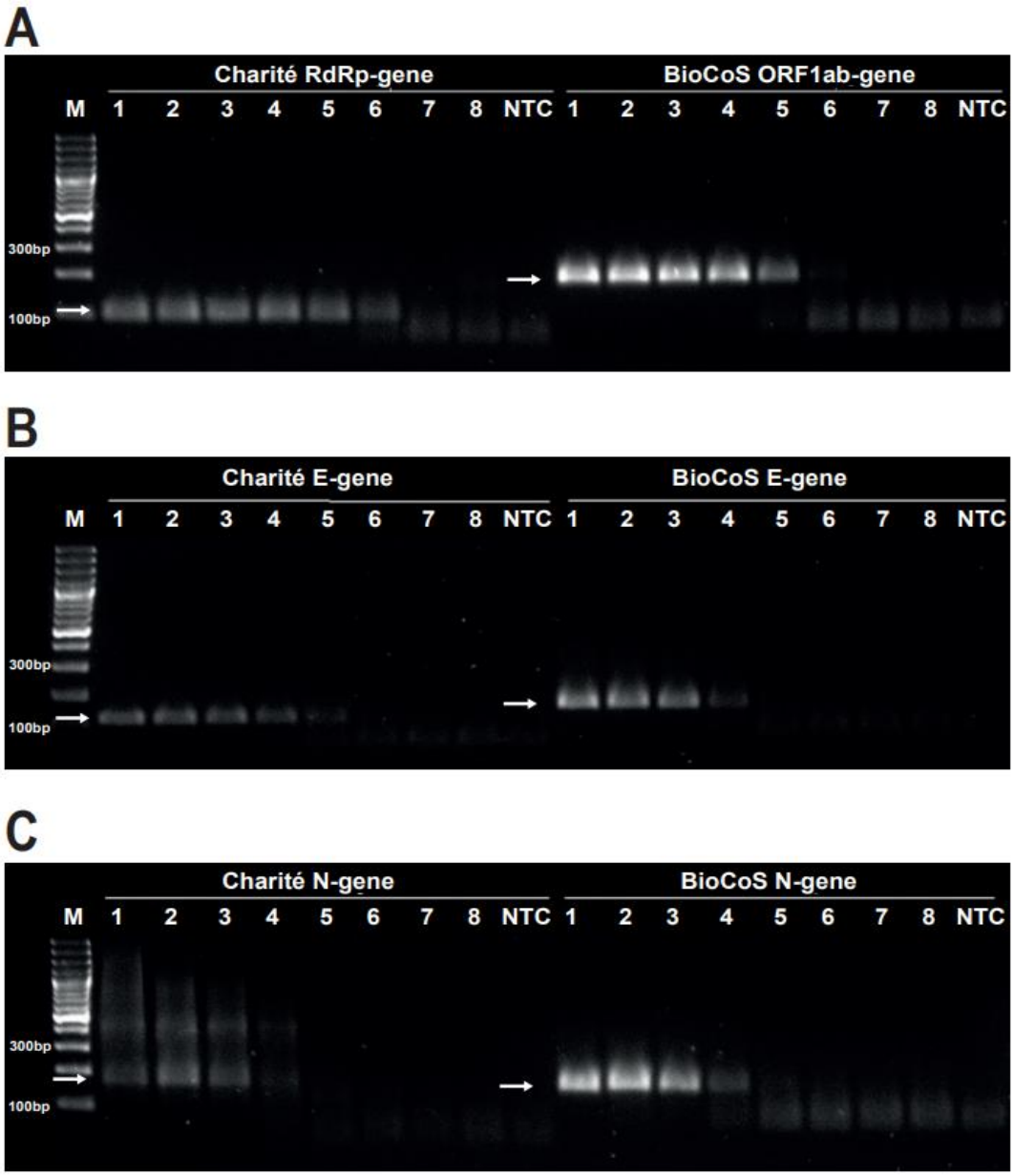

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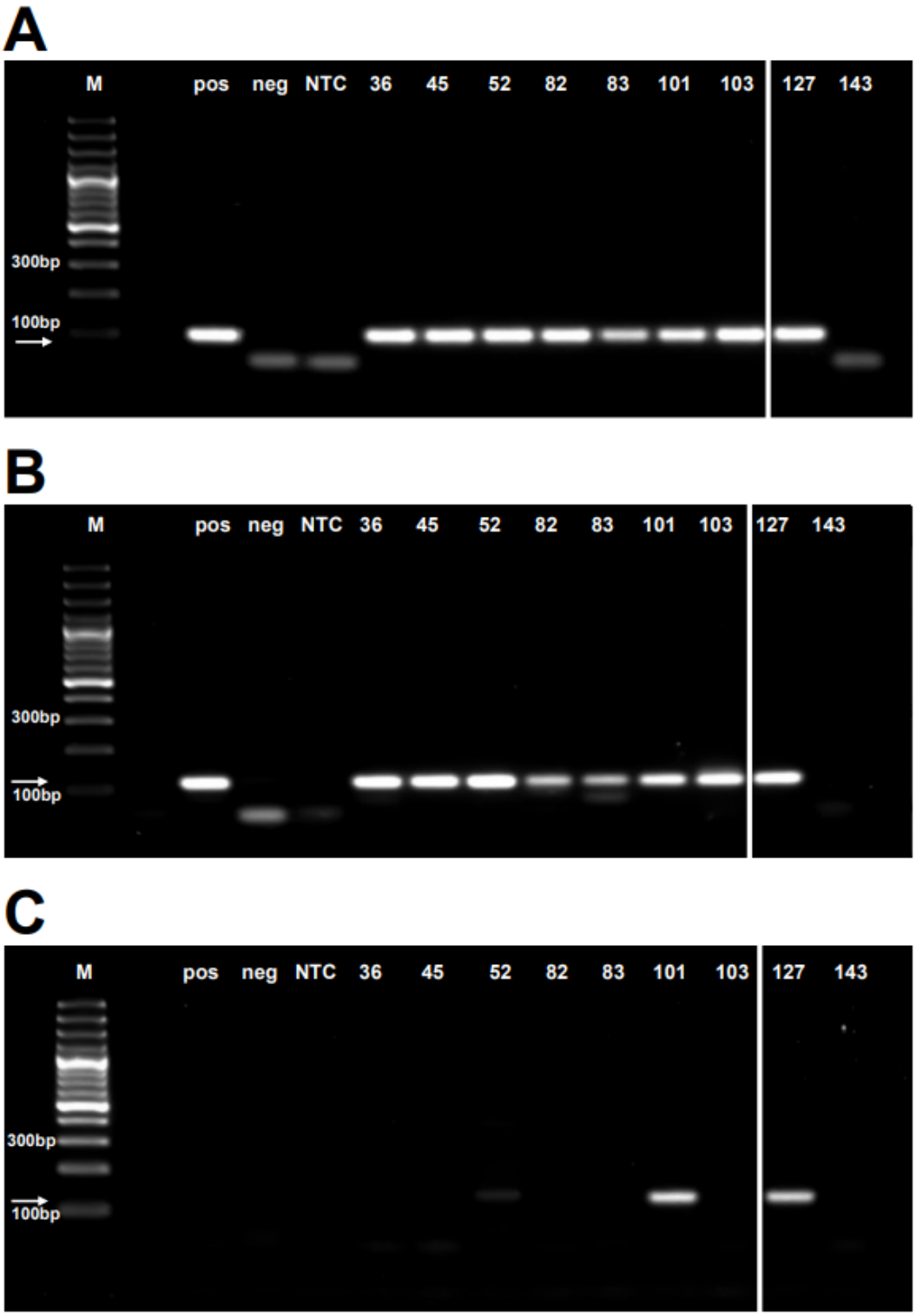
683 **Supplementary Figure 1 SARS-CoV-2 RT-PCR Limit of detection agarose banding pattern**
684 **analyses.** (**A**) Limit of detection of the Charité Berlin and STAMINA SARS-CoV-2 RdRp- (100 bp) and
685 ORF1ab gene (167 bp), (**B**) E-gene (113 bp and 181 bp) and (**C**) N-gene (128 bp and 193 bp) primers
686 are shown (white arrow). (M) 100 base pairs Plus DNA size marker; (1) reference sample SARS-CoV-
687 2 Delta 8.56E6 IU/uL; (2) reference sample SARS-CoV-2 Delta 8.56E5 IU/uL; (3) reference sample
688 SARS-CoV-2 Delta 8.56E4 IU/uL; (4) reference sample SARS-CoV-2 Delta 8.56E3 IU/uL; (5) reference
689 sample SARS-CoV-2 Delta 8.56E2 IU/uL; (6) reference sample SARS-CoV-2 Delta 8.56E1 IU/uL; (7)
690 reference sample SARS-CoV-2 Delta 8.56 IU/uL; (8) reference sample SARS-CoV-2 Delta 0.856 IU/uL;
691 (NTC) no template control sample.

692



Supplementary Figure 2 SARS-CoV-2 PCR on nine dubious Allplex™ assay samples. PCR result of the (**A**) RdRp-, (**B**) E-gene and (**C**) N-gene on the Allplex™ assay obtained RT-qPCR amplicons are shown**.** The presence or absence of the RdRp-, E-, and N-gene is visualised (white arrow) using gel electrophoresis. (M) 100 base pairs Plus DNA size marker; (pos) positive control sample; (neg) negative control sample; (NTC) no template control sample; (number) patient sample numbers that were found to be dubious.

Tables

Table 1: Analyses of 150 symptomatic patients suspicious for a SARS-CoV-2 infection using RT-(q)PCR and sequencing

Sample number	*RT-(q)PCR Allplex™ assay run 1*	*Ct-value E-gene*	*Ct-value RdRp/S-gene*	*Ct-value N-gene*	*RT-(q)PCR Allplex™ assay run 2*	*Ct-value E-gene*	*Ct-value RdRp/S-gene*	*Ct-value N-gene*	*Agarose gel*	*RT-PCR STAMINA ORF1ab-gene*	*RT-PCR STAMINA E-gene*	*RT-PCR STAMINA N-gene*	*RT-PCR Charité Berlin RdRp-gene*	*RT-PCR Charité Berlin E-gene*	*RT-PCR Charité Berlin N-gene*	*Composite reference standard*
1	pos	31.81	31.06	31.00	pos	29,84	30,92	29,87	pos	neg	neg	neg	neg	neg	neg	pos
2	pos	21.33	21.20	20.57	pos	20,60	21,26	20,03	pos	pos	pos	pos	pos	pos	neg	pos
3	pos	24.47	25.75	23.88	pos	23,30	24,90	23,24	pos	pos	pos	pos	neg	pos	neg	pos
4	pos	16.70	16.40	13.43	pos	15,99	16,58	14,12	pos	pos	pos	pos	pos	pos	neg	pos
5	pos	27.18	30.91	26.16	pos	27,57	28,51	26,60	pos	pos	pos	neg	pos	pos	neg	pos
6	pos	16.64	16.83	15.14	pos	15,45	16,24	13,97	pos	pos	pos	pos	pos	pos	pos	pos
7	pos	27.74	30.91	27.97	pos	26,48	27,47	26,88	pos	pos	pos	neg	pos	pos	neg	pos
8	pos	31.06	30.74	29.70	pos	29,26	30,38	28,19	pos	pos	neg	neg	neg	pos	neg	pos
9	pos	26.22	28.70	26.05	pos	24,89	25,76	24,33	pos	pos	pos	neg	neg	pos	neg	pos
10	pos	16.41	16.42	13.27	pos	14,23	14,96	12,39	pos	pos	pos	pos	pos	pos	neg	pos
11	pos	17.16	17.13	15.50	pos	15,63	16,36	14,54	pos	pos	pos	pos	pos	pos	neg	pos
12	pos	16.85	17.09	15.73	pos	15,54	17,18	14,50	pos	pos	pos	pos	pos	pos	neg	pos
13	pos	30.18	29.77	28.87	pos	27,71	29,01	26,74	pos	pos	neg	neg	pos	pos	neg	pos
14	pos	18.18	17.58	17.15	pos	16,68	17,11	16,05	pos	pos	pos	pos	pos	pos	neg	pos
15	pos	19.78	19.17	19.08	pos	19,02	19,13	18,23	pos	pos	pos	pos	pos	pos	neg	pos
16	pos	22.46	22.57	23.10	pos	22,06	23,07	22,55	pos	pos	pos	pos	pos	pos	neg	pos
17	pos	21.19	20.76	20.62	pos	20,25	20,67	19,73	pos	pos	pos	pos	pos	pos	neg	pos
18	pos	18.26	18.27	16.09	pos	16,49	17,39	14,61	pos	pos	pos	pos	pos	pos	neg	pos
19	pos	20.95	21.47	18.75	pos	21,03	22,19	18,64	pos	pos	pos	pos	pos	pos	neg	pos
20	pos	16.89	17.03	15.66	pos	16,91	17,42	14,78	pos	pos	pos	pos	pos	pos	neg	pos
21	pos	20.40	20.64	19.65	pos	20,02	18,86	18,69	pos	pos	pos	pos	pos	pos	neg	pos
22	pos	22.62	22.46	21.02	pos	21,39	21,83	19,86	pos	pos	pos	pos	pos	pos	neg	pos
23	pos	26.82	26.65	27.43	pos	25,93	27,12	26,28	pos	pos	pos	neg	neg	neg	neg	pos
24	pos	29.72	29.83	29.19	pos	29,25	30,09	28,88	pos	pos	neg	neg	neg	neg	neg	pos
25	pos	23.22	23.17	22.31	pos	21,84	23,67	20,86	pos	pos	pos	pos	pos	pos	neg	pos
26	pos	27,91	27,78	28,28	pos	25,98	27,10	26,14	pos	pos	pos	neg	neg	neg	neg	pos
27	pos	24.76	24.90	22.94	pos	20,03	21,20	17,22	pos	pos	pos	pos	pos	pos	neg	pos
28	pos	27.15	27.32	26.43	pos	27,01	28,31	26,52	pos	pos	pos	neg	neg	pos	neg	pos
29	pos	31.27	31.58	31.85	pos	30,40	31,12	31,04	pos	neg	neg	neg	neg	neg	neg	pos
30	pos	30.95	31.38	30.13	pos	29,36	30,50	28,54	pos	neg	neg	neg	neg	neg	neg	pos
31	pos	23.05	22.61	21.83	pos	21,96	22,72	20,67	pos	pos	pos	pos	pos	pos	neg	pos
32	pos	28.34	27.69	28.41	pos	26,98	27,99	27,02	pos	pos	pos	neg	neg	pos	neg	pos
33	pos	21.13	20.40	20.85	pos	20,50	20,74	20,86	pos	pos	pos	neg	pos	pos	neg	pos
34	pos	17.25	18.06	15.59	pos	16,16	17,23	13,95	pos	pos	pos	pos	pos	pos	neg	pos
35	pos	18.06	18.92	18.75	pos	16,80	18,48	18,10	pos	pos	pos	pos	pos	pos	neg	pos

36	pos	30.25	30.03	30.16	pos	29,49	30,41	28,50	pos	neg	neg	neg	neg	neg	neg	pos
37	pos	24.50	23.99	23.58	pos	23,12	24,28	22,47	pos	pos	pos	pos	pos	pos	neg	pos
38	pos	15.35	15.22	12.12	pos	14,33	14,76	12,32	pos	pos	pos	pos	pos	pos	neg	pos
39	pos	26.06	27.16	25.03	pos	24,99	26,63	24,96	pos	pos	pos	pos	pos	pos	pos	pos
40	pos	26.43	27.39	25.32	pos	26,09	27,05	24,77	pos	pos	pos	neg	pos	pos	neg	pos
41	pos	24.39	23.69	23.93	pos	22,98	23,92	22,61	pos	pos	pos	pos	pos	pos	neg	pos
42	pos	25.61	26.07	23.26	pos	24,27	26,15	21,92	pos	pos	pos	pos	neg	pos	neg	pos
43	pos	20.36	21.09	18.31	pos	15,58	16,10	13,99	pos	pos	pos	pos	pos	pos	neg	pos
44	pos	26.96	28.30	25.06	pos	26,40	28,05	24,51	pos	pos	pos	neg	pos	pos	neg	pos
45	pos	34.35	34.65	32.58	pos	33,81	34,55	32,53	pos	neg	neg	neg	neg	neg	neg	pos
46	pos	30.54	30.48	30.55	pos	28,80	29,97	28,80	pos	neg	neg	neg	neg	neg	neg	pos
47	pos	16.98	17.75	15.45	pos	15,68	17,42	14,51	pos	pos	pos	pos	pos	pos	neg	pos
48	pos	18.99	19.56	17.20	pos	18,31	18,92	16,38	pos	pos	pos	pos	pos	pos	neg	pos
49	pos	27.22	27.84	25.58	pos	25,89	27,28	24,24	pos	pos	pos	neg	pos	pos	neg	pos
50	pos	24.43	24.32	22.56	pos	22,76	24,42	21,19	pos	pos	pos	pos	pos	pos	neg	pos
51	pos	21.35	22.23	19.31	pos	19,91	20,56	19,11	pos	pos	pos	pos	pos	pos	neg	pos
52	pos	33.55	33.54	33.16	pos	31,68	32,56	31,01	pos	neg	neg	neg	neg	neg	neg	pos
53	pos	19.47	19.16	18.83	pos	18,10	19,20	17,58	pos	pos	pos	pos	pos	pos	neg	pos
54	pos	19.70	19.12	19.93	pos	18,84	19,26	19,02	pos	pos	pos	pos	pos	pos	neg	pos
55	pos	12.79	13.98	11.24	pos	12,60	13,98	11,32	pos	pos	pos	pos	pos	pos	neg	pos
56	pos	20.34	20.36	18.81	pos	19,63	20,37	18,16	pos	pos	pos	pos	pos	pos	pos	pos
57	pos	28.02	27.84	28.10	pos	27,79	28,63	27,61	pos	neg	pos	neg	neg	neg	neg	pos
58	pos	18.30	18.98	16.39	pos	17,65	19,70	15,69	pos	pos	pos	pos	pos	pos	pos	pos
59	pos	26.17	26.28	26.49	pos	24,94	26,11	25,20	pos	pos	pos	neg	pos	pos	neg	pos
60	pos	18.49	17.80	17.95	pos	17,84	18,10	17,17	pos	pos	pos	pos	pos	pos	pos	pos
61	pos	18.41	18.21	17.36	pos	18,37	18,88	17,20	pos	pos	pos	pos	pos	pos	pos	pos
62	pos	17.49	17.43	15.47	pos	15,96	17,07	14,47	pos	pos	pos	pos	pos	pos	pos	pos
63	pos	29.89	29.46	28.38	pos	28,83	30,44	27,55	pos	pos	pos	neg	neg	neg	neg	pos
64	pos	19.35	19.84	18.36	pos	19,29	19,92	17,70	pos	pos	pos	pos	pos	pos	pos	pos
65	pos	17.70	18.09	16.26	pos	17,39	18,21	15,67	pos	pos	pos	pos	pos	pos	pos	pos
66	pos	29.51	29.48	30.04	pos	28,18	29,07	28,57	pos	pos	pos	pos	pos	neg	neg	pos
67	pos	13.12	14.61	12.16	pos	14,32	15,08	13,07	pos	pos	pos	pos	pos	pos	pos	pos
68	pos	13.01	14.31	11.39	pos	13,84	14,83	11,90	pos	pos	pos	pos	pos	pos	pos	pos
69	pos	24.14	24.53	21.50	pos	21,84	23,23	18,81	pos	pos	pos	pos	pos	pos	pos	pos
70	pos	22.54	22.07	20.98	pos	21,36	22,08	20,39	pos	pos	pos	neg	pos	pos	neg	pos
71	pos	16.24	16.29	13.29	pos	15,72	16,98	13,75	pos	pos	pos	pos	pos	pos	pos	pos
72	pos	32.26	32.24	31.34	pos	32,44	32,63	31,23	pos	neg	neg	neg	neg	neg	neg	pos
73	pos	26.16	26.17	25.79	pos	25,36	27,18	25,27	pos	pos	pos	pos	pos	pos	neg	pos
74	pos	20.18	20.18	18.23	pos	19,30	19,36	16,74	pos	pos	pos	pos	pos	pos	pos	pos
75	pos	20.54	20.04	20.34	pos	19,29	19,03	19,12	pos	pos	pos	pos	pos	pos	pos	pos
76	pos	17.41	17.97	15.40	pos	15,50	16,07	13,67	pos	pos	pos	pos	pos	pos	neg	pos
77	pos	24.53	24.25	23.80	pos	23,46	23,31	22,54	pos	pos	pos	pos	pos	pos	neg	pos
78	pos	24.52	24.44	24.45	pos	23,10	23,49	23,2	pos	pos	pos	pos	pos	pos	neg	pos
79	pos	27.91	27.78	28.28	pos	19,99	20,07	18,4	pos	pos	pos	pos	pos	pos	pos	pos
80	pos	27.91	27.78	28.28	pos	18,39	18,57	17,09	pos	pos	pos	pos	pos	pos	pos	pos
81	pos	19.68	19.44	17.95	pos	20,46	20,41	20,22	pos	pos	pos	pos	pos	pos	pos	pos
82	pos	34.59	34.23	34.94	pos	33,86	33,54	34,44	pos	neg	neg	neg	neg	neg	neg	pos
83	pos	30.66	31.04	30.30	pos	30,14	31,20	29,84	pos	neg	neg	neg	neg	neg	neg	pos

84	pos	19.46	19.23	18.81	pos	18,51	18,11	18,07	pos	pos	pos	pos	pos	pos	pos	pos
85	pos	21.30	21.27	20.20	pos	20,37	20,17	19,24	pos	pos	pos	pos	pos	pos	pos	pos
86	pos	16.28	17.26	16.32	pos	15,47	15,51	14,87	pos	pos	pos	pos	pos	pos	pos	pos
87	pos	22.50	24.50	25.06	pos	21,64	20,9	24,21	pos	pos	pos	pos	pos	pos	pos	pos
88	pos	18.10	20.82	18.43	pos	17,08	17,84	17,08	pos	pos	pos	pos	pos	pos	pos	pos
89	pos	17.41	17.65	15.91	pos	16,66	16,87	15,33	pos	pos	pos	pos	pos	pos	pos	pos
90	pos	14.09	15.36	12.61	pos	14,73	15,05	12,65	pos	pos	pos	pos	pos	pos	pos	pos
91	pos	20.29	20.30	18.79	pos	20,03	20,43	17,63	pos	pos	pos	pos	pos	pos	pos	pos
92	pos	31.02	30.61	30.42	pos	30,54	30,58	30,55	pos	pos	neg	neg	neg	neg	neg	pos
93	pos	24.30	24.50	24.27	pos	23,46	22,84	24,47	pos	pos	pos	pos	pos	pos	neg	pos
94	pos	16.60	16.37	15.63	pos	15,19	14,61	24,22	pos	pos	pos	pos	pos	pos	pos	pos
95	pos	16.10	15.97	13.50	pos	15,32	14,91	14,44	pos	pos	pos	pos	pos	pos	pos	pos
96	neg				neg				neg	neg	neg	neg	neg	neg	neg	
97	neg				neg				neg	neg	neg	neg	neg	neg	neg	
98	neg				neg	37,49	38,47	35,3	neg	neg	neg	neg	neg	neg	neg	
99	neg				neg				neg	neg	neg	neg	neg	neg	neg	
100	neg				neg				neg	neg	neg	neg	neg	neg	neg	neg
101	pos	17,52	18,2	16,43	pos	16,30	17,50	15,24	pos	pos	pos	pos	pos	pos	neg	pos
102	neg				neg			37,71	neg	neg	neg	neg	neg	neg	pos	neg
103	neg				neg	37,60	38,51	36,8	pos	neg	neg	neg	neg	neg	neg	pos
104	neg				neg				neg	neg	neg	neg	neg	neg	neg	
105	neg				neg				neg	neg	neg	neg	neg	neg	neg	
106	neg				neg				neg	neg	neg	neg	neg	neg	neg	
107	neg		36,14		pos	34,50	35,80	33,56	pos	neg	neg	neg	neg	neg	neg	
108	neg				neg			37,53	neg	neg	neg	neg	neg	neg	neg	
109	neg				neg				neg	neg	neg	neg	neg	neg	neg	
110	neg				neg			36,60	neg	neg	neg	neg	neg	neg	neg	
111	neg				neg				neg	neg	neg	neg	neg	neg	neg	
112	neg				neg				neg	neg	neg	neg	neg	neg	neg	neg
113	neg				neg				neg	neg	neg	neg	neg	neg	neg	
114	neg				neg				neg	neg	neg	neg	neg	neg	neg	
115	neg				neg				neg	neg	neg	neg	neg	neg	neg	
116	neg				neg	37,58			neg	neg	neg	neg	neg	neg	neg	
117	neg				neg				neg	neg	neg	neg	neg	neg	neg	
118	neg				neg		38,60		neg	neg	neg	neg	neg	neg	neg	pos
119	neg				neg			37,61	neg	neg	neg	neg	neg	neg	neg	
120	neg				neg				neg	neg	neg	neg	neg	neg	neg	
121	neg				neg			38,10	neg	neg	neg	neg	neg	neg	neg	
122	neg				neg				neg	neg	neg	neg	neg	neg	neg	
123	neg				neg	36,95		36,56	neg	neg	neg	neg	neg	neg	neg	
124	neg				neg				neg	neg	neg	neg	neg	neg	neg	
125	neg				neg				neg	neg	neg	neg	neg	neg	neg	
126	neg				neg			37,80	neg	neg	neg	neg	neg	neg	neg	
127	pos	20,86	21,53	19,04	pos	19,83	21,24	18,06	pos	pos	pos	pos	pos	pos	pos	pos
128	neg				neg				neg	neg	neg	neg	neg	neg	neg	
129	neg				neg				neg	neg	neg	neg	neg	neg	neg	
130	neg				neg				neg	neg	neg	neg	neg	neg	neg	
131	neg				neg				neg	neg	neg	neg	neg	neg	neg	

132	neg				neg				neg	neg	neg	neg	neg	neg	neg	
133	neg				neg				neg	neg	neg	neg	neg	neg	neg	
134	neg				neg			37,49	neg	neg	neg	neg	neg	neg	neg	
135	neg				neg				neg	neg	neg	neg	neg	neg	neg	
136	neg				neg				neg	neg	neg	neg	neg	neg	neg	
137	neg				neg			37,45	neg	neg	neg	neg	neg	neg	neg	
138	neg				neg				neg	neg	neg	neg	neg	neg	neg	
139	neg				neg				neg	neg	neg	neg	neg	neg	neg	neg
140	neg				neg				neg	neg	neg	neg	neg	neg	neg	
141	neg				neg				neg	neg	neg	neg	neg	neg	neg	
142	pos	21.51	21.2	20.24	pos	21,40	21,75	19,78	pos	pos	pos	pos	pos	pos	neg	pos
143	pos	32.36	32.7	31.24	pos	31,57	32,63	30,77	pos	neg	neg	neg	neg	neg	neg	pos
144	pos	20.41	19.5	20.65	pos	17,59	17,54	18,72	pos	pos	pos	pos	pos	pos	neg	pos
145	neg				neg				neg	neg	neg	neg	neg	neg	neg	
146	neg				neg				neg	neg	neg	neg	neg	neg	neg	neg
147	neg				neg				neg	neg	neg	neg	neg	neg	neg	
148	neg				neg				neg	neg	neg	neg	neg	neg	neg	
149	pos	29.83	30.33	29.95	pos	28,66	30,23	29,10	pos	pos	neg	neg	pos	neg	neg	pos
150	pos	28.59	29.48	27.87	pos	27,42	29,24	26,49	pos	pos	neg	neg	pos	pos	neg	pos

Table 2: SARS-CoV-2 RT-PCR primers

A. Charité Berlin

Target	Oligonucleotide	Sequence (5' – 3')
SARS-CoV-2 RdRp-gene	FW, Charité Berlin	GTGAAATGGTCATGTGTGGCGG
	RV, Charité Berlin	CAAATGTTAAAAACACTATTAGCATA
SARS-CoV-2 E-gene	FW, Charité Berlin	ACAGGTACGTTAATAGTTAATAGCGT
	RV, Charité Berlin	ATATTGCAGCAGTACGCACACA
SARS-CoV-2 N-gene	FW, Charité Berlin	CACATTGGCACCCGCAATC
	RV, Charité Berlin	GAGGAACGAGAAGAGGCTTG

B. STAMINA

Target	Oligonucleotide	Sequence (5' – 3')
SARS-CoV-2 ORF1ab-gene	FW, STAMINA	Confidential
	RV, STAMINA	Confidential
SARS-CoV-2 E-gene	FW, STAMINA	Confidential
	RV, STAMINA	Confidential
SARS-CoV-2 N-gene	FW, STAMINA	Confidential
	RV, STAMINA	Confidential

Table 3: Comparison of the STAMINA and Charité Berlin primersets using the Allplex™ assay as a criterion standard

A. Results based on the Allplex™ assay

Ct value	Assay	Target gene	PPA[a]	NPA[b]	PPV[c]	NPV[d]
35	STAMINA	ORF1ab-gene	88.24% (90/102)	100.00% (48/48)	100.00% (90/90)	80.00% (48/60)
		E-gene	83.33% (85/102)	100.00% (48/48)	100.00% (85/85)	73.85% (48/65)
		N-gene	68.63% (70/102)	100.00% (48/48)	100.00% (70/70)	60.00% (48/80)
	Charité Berlin	RdRp-gene	77.45% (79/102)	100.00% (48/48)	100.00% (79/79)	67.61% (48/71)
		E-gene	81.37% (82/102)	100.00% (48/48)	100.00% (83/83)	71.64% (48/76)
		N-gene	28.43% (29/102)	97.92% (47/48)	100.00% (29/30)	39.17% (47/120)
25	**Assay**	**Target gene**	**PPA[a]**	**NPA[b]**	**PPV[c]**	**NPV[d]**
	STAMINA	ORF1ab-gene	100.00% (67/67)	72.29% (60/83)	74.44% (67/90)	100.00% (60/60)
		E-gene	100.00% (67/67)	78.31% (65/83)	78.82% (67/85)	100.00% (65/65)
		N-gene	97.01% (65/67)	93.98% (78/83)	92.86% (65/70)	97.50% (78/80)
	Charité Berlin	RdRp-gene	97.01% (65/67)	83.13% (69/83)	82.28% (65/79)	97.18% (69/71)
		E-gene	100.00% (67/67)	80.72% (67/83)	80.72% (67/83)	100.00% (67/67)
		N-gene	38.81% (26/67)	95.18% (79/83)	86.67% (26/30)	65.83% (79/120)
20	**Assay**	**Target gene**	**PPA[a]**	**NPA[b]**	**PPV[c]**	**NPV[d]**
	STAMINA	ORF1ab-gene	100.00% (41/41)	55.05% (60/109)	45.56% (41/90)	100.00% (60/60)
		E-gene	100.00% (41/41)	59.53% (65/109)	48.24% (41/85)	100.00% (65/65)
		N-gene	100.00% (41/41)	73.39% (80/109)	58.57% (41/70)	100.00% (80/80)
	Charité Berlin	RdRp-gene	100.00% (41/41)	65.14% (71/109)	51.90% (41/79)	100.00% (71/71)
		E-gene	100.00% (41/41)	61.47% (67/109)	49.40% (41/83)	100.00% (67/67)
		N-gene	53.66% (22/41)	92.66% (101/109)	73.33% (22/30)	84.17% (101/120)

B. Results based on our composite reference standard

Assay	Target gene	PPA[a]	NPA[b]	PPV[c]	NPV[d]
STAMINA	ORF1ab-gene	86.54% (90/104)	100.00% (46/46)	100.00% (90/90)	76.67% (46/60)
	E-gene	81.73% (85/104)	100.00% (46/46)	100.00% (85/85)	70.77% (46/65)
	N-gene	67.31% (70/104)	100.00% (46/46)	100.00% (70/70)	57.50% (46/80)
Charité Berlin	RdRp-gene	75.96% (79/104)	100.00% (46/46)	100.00% (79/79)	64.79% (46/71)
	E-gene	79.81% (83/104)	100.00% (46/46)	100.00% (83/83)	68.66% (46/67)
	N-gene	27.88% (29/104)	97.83% (45/46)	96.97% (29/30)	37.50% (45/120)

[a] PPA: positive percentage agreement; [b] NPA: negative percentage agreement
[c] PPV: positive predictive value; [d] NPV: negative predictive value

Table 4: RT-qPCR, RT-PCR, banding patterning and sequencing analysis results of nine dubious Allplex™ assay samples

	Sample 36	*Sample 45*	*Sample 52*	*Sample 82*	*Sample 83*	*Sample 101*	*Sample 103*	*Sample 127*	*Sample 143*
STAMINA PCR ORF1ab-gene	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Pos	Neg
STAMINA PCR E-gene	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Pos	Neg
STAMINA PCR N-gene	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Pos	Neg
Charité Berlin PCR RdRp-gene	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Pos	Neg
Charité Berlin PCR E-gene	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Pos	Neg
Charité Berlin PCR N-gene	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg
RT-(q)PCR Allplex™ assay run 1	Pos	Pos	Pos	Pos	Pos	Pos	Neg	Pos	Pos
RT-(q)PCR Allplex™ assay run 2	Pos	Pos	Pos	Pos	Pos	Pos	Neg	Pos	Pos
Gel electrophoresis Allplex™ assay run 2	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
Sequencing Allplex™ RdRp-gene	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Sequencing Allplex™ E-gene	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
Sequencing Allplex™ N-gene	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Pos	Neg
Composite reference standard	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Supplementary Data 1: Sequencing analyses of 150 STAMINA SARS-CoV-2 ORF1ab-gene RT-PCR samples

Sample number	First run Highly similar	First run Somewhat similar	Second run Highly similar	Second run Somewhat similar
1	To repeat		Confirmed	
2	Confirmed			
3	Confirmed		Confirmed	
4	Confirmed			
5	Confirmed		Confirmed	
6	Confirmed			
7	Confirmed		Confirmed	
8	Confirmed			
9	Confirmed		Confirmed	
10	Confirmed			
11	Confirmed			
12	Confirmed			
13	Confirmed			
14	Confirmed			
15	Confirmed			
16	Confirmed			
17	Confirmed			
18	Confirmed			
19	Confirmed			
20	Confirmed			
21	Confirmed			
22	Confirmed			
23	Confirmed			
24	Confirmed			
25	Confirmed			
26	Confirmed			
27	Confirmed			

28	Confirmed			
29	Confirmed (very low query cover)	Confirmed (very low query cover)	Confirmed (very low query cover)	Confirmed (very low query cover)
30	Confirmed	Confirmed	Rotavirus A	
31	Confirmed			
32	Confirmed			
33	Confirmed			
34	Confirmed			
35	Confirmed			
36	No significant similarity found	No significant similarity found	Confirmed	
37	Confirmed			
38	Confirmed			
39	Confirmed			
40	Confirmed			
41	Confirmed			
42	Confirmed			
43	Confirmed			
44	Confirmed			
45	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
46	Confirmed		Confirmed	
47	Confirmed			
48	Confirmed			
49	Confirmed		Confirmed	
50	Confirmed			
51	Confirmed			
52	To repeat		No significant similarity found	No significant similarity found
53	Confirmed			
54	Confirmed			
55	Confirmed			
56	Confirmed			
57	Confirmed		Confirmed	
58	Confirmed			

59	Confirmed			
60	Confirmed			
61	Confirmed			
62	Confirmed			
63	Confirmed			
64	Confirmed			
65	Confirmed			
66	Confirmed			
67	Confirmed			
68	Confirmed			
69	Confirmed		Confirmed	
70	Confirmed		Confirmed	
71	Confirmed		Confirmed	
72	Confirmed		Confirmed	
73	Confirmed		Confirmed	
74	Confirmed		Confirmed	
75	Confirmed		Confirmed	
76	Confirmed			
77	Confirmed			
78	Confirmed			
79	Confirmed			
80	Confirmed			
81	Confirmed			
82	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
83	No significant similarity found	No significant similarity found	Confirmed	
84	Confirmed			
85	Confirmed			
86	Confirmed			
87	Confirmed			
88	Confirmed			
89	Confirmed			

90	Confirmed			
91	Confirmed			
92	Confirmed			
93	Confirmed			
94	Confirmed		Confirmed	
95	Confirmed		Confirmed	
96				
97				
98				
99				
100	No significant similarity found	No significant similarity found		
101	Confirmed			
102	No significant similarity found	No significant similarity found		
103	No significant similarity found	No significant similarity found	Confirmed	Confirmed
104				
105				
106				
107				
108				
109				
110				
111				
112	No significant similarity found	No significant similarity found		
113				
114				
115				
116				
117				
118	Confirmed			
119				
120				

121				
122				
123				
124				
125				
126				
127	Confirmed			
128				
129				
130				
131				
132				
133				
134				
135				
136				
137				
138				
139	Homo sapiens		No significant similarity found	No significant similarity found
140				
141				
142	Confirmed			
143	No significant similarity found	Confirmed	Homo sapiens	Confirmed
144	Confirmed			
145				
146	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
147				
148				
149	Confirmed			
150	Confirmed			

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Supplementary Data 2: Sequencing analyses of 150 STAMINA SARS-CoV-2 E-gene RT-PCR samples

Sample number	First run Highly similar	First run Somewhat similar	Second run Highly similar	Second run Somewhat similar
1	Confirmed	Confirmed	No significant similarity found	Confirmed
2	Confirmed			
3	Confirmed			
4	Confirmed		No significant similarity found	No significant similarity found
5	Confirmed			
6	Confirmed		Confirmed	
7	Confirmed			
8	Confirmed		Confirmed	
9	Confirmed			
10	Confirmed		Confirmed	
11	No significant similarity found	No significant similarity found	Confirmed	
12	Confirmed	Confirmed	Confirmed	
13	Confirmed		Confirmed	
14	Confirmed		Confirmed	
15	Confirmed		Confirmed	
16	Confirmed			
17	No significant similarity found	Confirmed	Confirmed	
18	Confirmed		No significant similarity found	No significant similarity found
19	Confirmed		Confirmed	
20	Confirmed		Confirmed	
21	Confirmed		Confirmed	
22	Confirmed		Confirmed	
23	Confirmed		Confirmed	
24	No significant similarity found	No significant similarity found		
25	Confirmed		Confirmed	
26	Confirmed		Confirmed	
27	Confirmed		Confirmed	

28	Confirmed		Confirmed	
29	Confirmed		Confirmed	
30	No significant similarity found	No significant similarity found		
31	Confirmed	Confirmed	Confirmed	
32	Confirmed			
33	Confirmed		Confirmed	
34	No significant similarity found	No significant similarity found	Confirmed	
35	Confirmed		Confirmed	
36	No significant similarity found	No significant similarity found	No significant similarity found	Confirmed
37	Confirmed		Confirmed	
38	No significant similarity found	No significant similarity found	Confirmed	
39	No significant similarity found	Confirmed	Confirmed	
40	Confirmed		Confirmed	
41	Confirmed		Confirmed	
42	Confirmed		Confirmed	
43	Confirmed	Confirmed	Confirmed	
44	Confirmed		Confirmed	
45	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
46	Confirmed		Confirmed	
47	Confirmed		Confirmed	
48	Confirmed	Confirmed	Confirmed	
49	Confirmed			
50	Confirmed		Confirmed	
51	Confirmed			
52	No significant similarity found	Confirmed	No significant similarity found	No significant similarity found
53	Confirmed			
54	Confirmed			
55	Confirmed		Confirmed	
56	Confirmed		Confirmed	
57	Confirmed			
58	Confirmed			

59	Confirmed			
60	Confirmed		Confirmed	
61	Confirmed		Confirmed	
62	Confirmed		Confirmed	
63	Confirmed		Confirmed	
64	Confirmed		Confirmed	
65	Confirmed		Confirmed	
66	Confirmed		Confirmed	
67	Confirmed		Confirmed	
68	Confirmed		Confirmed	
69	Confirmed		Confirmed	
70	Confirmed		Confirmed	
71	Confirmed		No significant similarity found	No significant similarity found
72	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
73	Confirmed		Confirmed	
74	Confirmed		Confirmed	
75	Confirmed		Confirmed	
76	Confirmed		Confirmed	
77	Confirmed		Confirmed	
78	Confirmed		Confirmed	
79	No significant similarity found	No significant similarity found	Confirmed	Confirmed
80	Confirmed		Confirmed	
81	Confirmed		Confirmed	
82	No significant similarity found	Confirmed	No significant similarity found	No significant similarity found
83	No significant similarity found	No significant similarity found		
84	Confirmed		Confirmed	
85	Confirmed	Confirmed	Confirmed	
86	Confirmed		Confirmed	
87	Confirmed		Confirmed	
88	Confirmed	Confirmed	Confirmed	
89	Confirmed		Confirmed	

Confirmed		Confirmed		
Confirmed		Confirmed		
Confirmed		No significant similarity found	No significant similarity found	
Confirmed		Confirmed		
Confirmed				
Confirmed		Confirmed		
No significant similarity found	Confirmed			
Confirmed		Confirmed		
No significant similarity found	No significant similarity found			
No significant similarity found	Confirmed	No significant similarity found	No significant similarity found	
Confirmed		No significant similarity found	No significant similarity found	
No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found	

121				
122				
123				
124				
125				
126				
127	Confirmed	Confirmed	No significant similarity found	No significant similarity found
128				
129				
130				
131				
132				
133				
134				
135				
136				
137				
138				
139	No significant similarity found	Confirmed	Confirmed	No significant similarity found
140				
141				
142	Confirmed			
143	Confirmed		Confirmed	
144	No significant similarity found	No significant similarity found	Confirmed	
145				
146	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
147				
148				
149	Confirmed			
150	Confirmed			

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749

Supplementary Data 3: Sequencing analyses of 150 STAMINA SARS-CoV-2 N-gene RT-PCR samples

Sample number	First run Highly similar	First run Somewhat similar	Second run Highly similar	Second run Somewhat similar
1	No significant similarity found	Confirmed	Cloning vectors	Cloning vectors
2	Confirmed		Confirmed	
3	Confirmed		Confirmed	
4	Confirmed		Confirmed	
5	Legionella	Confirmed	Legionella	
6	Confirmed		Confirmed	
7	Cloning vectors		Neisseria	
8	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
9	Confirmed		Confirmed	
10	Confirmed		Confirmed	
11	Confirmed		Confirmed	
12	Confirmed		Confirmed	
13	Rothia		Rothia	
14	Confirmed		Confirmed	
15	Confirmed		Confirmed	
16	Confirmed		Confirmed	
17	Confirmed		Confirmed	
18	Confirmed		Confirmed	
19	Confirmed		Confirmed	
20	Confirmed		Confirmed	
21	Confirmed		Confirmed	
22	Confirmed		Confirmed	
23	Confirmed	Confirmed	No significant similarity found	Confirmed
24	Neisseria		Neisseria	
25	Confirmed		Confirmed	
26	Confirmed	Species		
27	Confirmed		Confirmed	

28	Confirmed	Confirmed	No significant similarity found	No significant similarity found
29	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
30	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
31	Confirmed		Confirmed	
32	No significant similarity found	Rothia	No significant similarity found	No significant similarity found
33	No significant similarity found	Confirmed	Confirmed	
34	Confirmed		Confirmed	
35	Confirmed		Confirmed	
36	Cloning vectors	No significant similarity found	Cloning vectors	No significant similarity found
37	Confirmed		Confirmed	
38	Confirmed		Confirmed	
39	Confirmed		Confirmed	
40	No significant similarity found	Confirmed	Confirmed	
41	Confirmed		Confirmed	
42	Confirmed		Confirmed	
43	Confirmed		Confirmed	
44	Confirmed		Confirmed	
45	Confirmed (primer sequence)		Rothia	Rothia
46	Confirmed	Confirmed	Cloning vectors	
47	Confirmed		Confirmed	
48	Confirmed		Confirmed	
49	Confirmed		Confirmed	
50	Confirmed		Confirmed	
51	Confirmed		Confirmed	
52	Confirmed	Confirmed	Confirmed	
53	Confirmed		Confirmed	
54	Confirmed		Confirmed	
55	Confirmed		Confirmed	
56	Confirmed		Confirmed	
57	No significant similarity found	No significant similarity found	No significant similarity found	Confirmed
58	Confirmed		Confirmed	

59	Confirmed		Confirmed	
60	Confirmed		Confirmed	
61	Confirmed		Confirmed	
62	Confirmed		Confirmed	
63	No significant similarity found	Confirmed	No significant similarity found	Confirmed
64	Confirmed		Confirmed	
65	Confirmed		Confirmed	
66	Confirmed		Confirmed	
67	Confirmed		Confirmed	
68	Confirmed		Confirmed	
69	Confirmed		Confirmed	
70	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
71	Confirmed		Confirmed	
72	No significant similarity found	Rothia	Rothia	Rothia
73	Confirmed		Confirmed	
74	Confirmed		Confirmed	
75	Confirmed		Confirmed	
76	Confirmed		Confirmed	
77	Confirmed		Confirmed	
78	Confirmed		Confirmed	
79	Confirmed		Confirmed	
80	Confirmed		Confirmed	
81	Confirmed		Confirmed	
82	No significant similarity found	No significant similarity found	No significant similarity found	Species
83	Neisseria		Neisseria	
84	Confirmed		Confirmed	
85	Confirmed		Confirmed	
86	Confirmed		Confirmed	
87	Confirmed		Confirmed	
88	Confirmed		Confirmed	
89	Confirmed		Confirmed	

Confirmed		Confirmed		
Confirmed		Confirmed		
Veillonella		Veillonella		
Confirmed		Confirmed		
Confirmed		Confirmed		
Confirmed		Confirmed		
Veillonella		Veillonella		
Confirmed		Confirmed		
No significant similarity found	Rothia	No significant similarity found	Veillonella	
Cloning vectors		No significant similarity found	Cloning vectors / species	
No significant similarity found	No significant similarity found	No significant similarity found	Confirmed	
No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found	

	Confirmed		Confirmed	
	No significant similarity found	Homo sapiens	No significant similarity found	No significant similarity found
	Confirmed		Confirmed	
	No significant similarity found	Species	No significant similarity found	Veillonella
	Confirmed		Confirmed	
	Rothia		Rothia	
	No significant similarity found	Homo sapiens	No significant similarity found	Homo sapiens / species
	No significant similarity found	No significant similarity found	Veillonella	Confirmed

Supplementary Data 4: Sequencing analyses of 150 Charité Berlin SARS-CoV-2 RdRp-gene RT-PCR samples

Sample number	First run Highly similar	First run Somewhat similar	Second run Highly similar	Second run Somewhat similar
1	No significant similarity found		Hepatitis A virus / Hepatovirus A	Hepatitis A virus / Hepatovirus A
2	To repeat		Hepatitis A virus / Hepatovirus A	
3	Confirmed			
4	Confirmed			
5	To repeat			
6	Confirmed			
7	Confirmed			
8	To repeat		No significant similarity found	Rotavirus A
9	Error / Cannot be determined			
10	Confirmed			
11	Confirmed			
12	Confirmed			
13	To repeat		No significant similarity found	Rotavirus A and SARS-CoV-2 (query cover 10%)
14	To repeat		Confirmed	
15	Confirmed			
16	No significant similarity found	Confirmed	No significant similarity found	Rotavirus A and SARS-CoV-2 (query cover 15%)
17	Confirmed	Confirmed	Confirmed	Confirmed
18	Confirmed	Confirmed	Confirmed	Confirmed
19	No significant similarity found	Confirmed	No significant similarity found	Confirmed (very low query cover)
20	Confirmed			
21	Confirmed			
22	Confirmed			
23	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
24	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
25	No significant similarity found	Solobacterium	Solobacterium	
26	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
27	To repeat		Confirmed	Confirmed

28	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
29	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
30	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
31	No significant similarity found			
32	16S ribosomal RNA genes			
33	Confirmed			
34	Confirmed			
35	Confirmed			
36	No significant similarity found	No significant similarity found		
37	No significant similarity found	Confirmed		
38	Confirmed			
39	No significant similarity found	Confirmed		
40	Confirmed	Confirmed		
41	Confirmed			
42	No significant similarity found	Confirmed		
43	Confirmed			
44	Confirmed	Confirmed		
45	No significant similarity found	No significant similarity found		
46	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
47	Confirmed			
48	Confirmed			
49	No significant similarity found	No significant similarity found		
50	Confirmed			
51	Confirmed			
52	To repeat		No significant similarity found	No significant similarity found
53	Confirmed			
54	Confirmed			
55	Confirmed			
56	Confirmed			
57	To repeat		No significant similarity found	No significant similarity found
58	Confirmed			

59	Confirmed	Confirmed	Confirmed	
60	Confirmed			
61	Confirmed		Confirmed	
62	Confirmed		Confirmed	
63	Confirmed		Confirmed	
64	Confirmed			
65	No significant similarity found	Confirmed	Confirmed	
66	No significant similarity found	Confirmed	Confirmed	
67	Confirmed		Confirmed	
68	Confirmed		Confirmed	
69	Confirmed			
70	No significant similarity found	Confirmed	Confirmed	
71	No significant similarity found	Confirmed	Confirmed	
72	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
73	Confirmed			
74	No significant similarity found	Confirmed	Confirmed	
75	No significant similarity found	Confirmed	Confirmed	
76	Confirmed			
77	Confirmed			
78	Confirmed			
79	Confirmed			
80	Confirmed			
81	Confirmed			
82	No significant similarity found	No significant similarity found	Homo sapiens	No significant similarity found
83	Solobacterium	No significant similarity found	No significant similarity found	Solobacterium
84	Solobacterium			
85	Confirmed			
86	No significant similarity found	No significant similarity found	Confirmed	
87	Confirmed			
88	Confirmed			
89	Confirmed			

90	Confirmed	Confirmed		
91	Confirmed	No significant similarity found		
92	Confirmed / Staphylococcus	Confirmed		
93	Confirmed		Confirmed	Confirmed
94	No significant similarity found	Confirmed	Confirmed	
95	Confirmed		Confirmed	
96				
97				
98				
99				
100	Solobacterium		Solobacterium	
101	Confirmed		Confirmed	
102	Solobacterium			
103	No significant similarity found	No significant similarity found		
104				
105				
106				
107				
108				
109				
110				
111				
112	No significant similarity found	No significant similarity found		
113				
114				
115				
116				
117				
118	No significant similarity found	No significant similarity found		
119				
120				

121				
122				
123				
124				
125				
126				
127	Confirmed			
128				
129				
130				
131				
132				
133				
134				
135				
136				
137				
138				
139	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
140				
141				
142	Confirmed			
143	To repeat	No significant similarity found	No significant similarity found	No significant similarity found
144	Confirmed			
145				
146	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
147				
148				
149	Confirmed			
150	No significant similarity found	Uncultured bacterium clones		

Supplementary Data 5: Sequencing analyses of 150 Charité Berlin SARS-CoV-2 E-gene RT-PCR samples

Sample number	First run Highly similar	First run Somewhat similar	Second run Highly similar	Second run Somewhat similar
1	To repeat		Rotavirus A	
2	Confirmed			
3	Confirmed			
4	Confirmed			
5	Confirmed / also Bat SARS-like corona-virus			
6	Confirmed			
7	Confirmed			
8	No significant similarity found	Confirmed	Rotavirus A	
9	Confirmed			
10	Confirmed			
11	Confirmed			
12	Confirmed			
13	Confirmed			
14	Confirmed			
15	Confirmed / also Bat SARS-like corona-virus			
16	Confirmed			
17	Confirmed			
18	Confirmed			
19	Confirmed			
20	Confirmed			
21	Confirmed			
22	Confirmed			
23	Confirmed			
24	To repeat	Confirmed (very low query cover)	Confirmed	
25	Confirmed			
26	Confirmed			
27	Confirmed			

28	Confirmed	Confirmed		
29	No significant similarity found	No significant similarity found	No significant similarity found	Corynebacterium
30	No significant similarity found	Confirmed	No significant similarity found	Confirmed
31	No significant similarity found	Confirmed (very low query cover)		
32	No significant similarity found	No significant similarity found	Error / Cannot be determined	
33	To repeat		Error / Cannot be determined	
34	To repeat		Error / Cannot be determined	
35	No significant similarity found	No significant similarity found	Error / Cannot be determined	
36	No significant similarity found	No significant similarity found	Error / Cannot be determined	
37	To repeat		Error / Cannot be determined	
38	No significant similarity found	No significant similarity found	Error / Cannot be determined	
39	To repeat		Error / Cannot be determined	
40	No significant similarity found	No significant similarity found	Error / Cannot be determined	
41	To repeat		Error / Cannot be determined	
42	To repeat		Error / Cannot be determined	
43	To repeat		Error / Cannot be determined	
44	To repeat		No significant similarity found	No significant similarity found
45	To repeat		Error / Cannot be determined	
46	No significant similarity found	Confirmed	Confirmed	
47	Confirmed		Confirmed	
48	Confirmed			
49	Confirmed			
50	Confirmed			
51	Confirmed			
52	To repeat		No significant similarity found	No significant similarity found
53	Confirmed			
54	Confirmed			
55	Confirmed			
56	Confirmed			
57	To repeat		Confirmed	
58	Confirmed			

59	Confirmed			
60	Confirmed			
61	Confirmed			
62	Confirmed		Confirmed	
63	Confirmed (low query cover%)			
64	Confirmed		Confirmed	
65	Confirmed			
66	Confirmed			
67	Confirmed		Confirmed	
68	Confirmed			
69	Confirmed			
70	Confirmed		No significant similarity found	Confirmed
71	Confirmed		Confirmed	
72	No significant similarity found	Confirmed		
73	Confirmed		Confirmed	
74	Confirmed	Confirmed	Confirmed	
75	Confirmed		Confirmed	
76	Confirmed		Confirmed	
77	Confirmed			
78	Confirmed			
79	Confirmed			
80	Confirmed			
81	Confirmed			
82	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
83	No significant similarity found	No significant similarity found		
84	Confirmed		Confirmed	
85	Confirmed	No significant similarity found	Confirmed	
86	Confirmed	No significant similarity found	Confirmed	
87	Confirmed	No significant similarity found	Confirmed	
88	Confirmed	No significant similarity found	Confirmed	
89	Confirmed	Confirmed	Uncultured archaeon clone	

90	Confirmed		Confirmed	
91	Confirmed		Confirmed	
92	Confirmed / Sarbecovirus	Confirmed		
93	Confirmed			
94	Confirmed			
95	Confirmed			
96				
97				
98				
99				
100	No significant similarity found			
101	Confirmed			
102	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
103	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
104				
105				
106				
107				
108				
109				
110				
111				
112	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
113				
114				
115				
116				
117				
118	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
119				
120				

121				
122				
123				
124				
125				
126				
127	Confirmed			
128				
129				
130				
131				
132				
133				
134				
135				
136				
137				
138				
139	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
140				
141				
142	Confirmed			
143	No significant similarity found	Confirmed	No significant similarity found	Confirmed
144	Confirmed			
145				
146	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
147				
148				
149	Confirmed		Confirmed	
150	Confirmed			

762 **Supplementary Data 6: Sequencing analyses of 150 Charité Berlin SARS-CoV-2 N-gene RT-PCR samples**
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Sample number	First run Highly similar	First run Somewhat similar	Second run Highly similar	Second run Somewhat similar
1	Homo sapiens	Homo sapiens		
2	Confirmed			
3	No significant similarity found	Confirmed		
4	Confirmed			
5	No significant similarity found			
6	Confirmed			
7	No significant similarity found			
8	No significant similarity found	Homo sapiens		
9	Homo sapiens			
10	Confirmed		Confirmed	
11	Confirmed			
12	Rothia mucilaginosa	Confirmed	Rothia mucilaginosa	
13	No significant similarity found			
14	Confirmed			
15	Confirmed			
16	Rothia mucilaginosa	Rothia mucilaginosa		
17	Confirmed			
18	Confirmed		Confirmed	
19	Confirmed			
20	Confirmed			
21	Confirmed			
22	Confirmed			
23	No significant similarity found	No significant similarity found		
24	Homo sapiens	No significant similarity found		
25	No significant similarity found			
26	No significant similarity found			
27	Confirmed			

28	Homo sapiens		
29	Homo sapiens		
30	No significant similarity found		
31	Confirmed		
32	No significant similarity found		
33	No significant similarity found		
34	Confirmed		
35	Confirmed		
36	No significant similarity found		
37	Homo sapiens		
38	Confirmed		
39	Homo sapiens		
40	Homo sapiens		
41	Confirmed		
42	Homo sapiens		
43	Confirmed		
44	Rothia mucilaginosa		
45	Homo sapiens		
46	No significant similarity found		
47	Confirmed		
48	Confirmed		
49	No significant similarity found		
50	Confirmed		
51	Confirmed		
52	No significant similarity found	No significant similarity found	
53	Confirmed		
54	Confirmed		
55	No significant similarity found	No significant similarity found	No significant similarity found
56	Confirmed		
57	Homo sapiens	Homo sapiens	
58	Confirmed		

59	Streptococcus	Homo sapiens	Homo sapiens
60	Confirmed		
61	Confirmed		Confirmed
62	Confirmed		
63	No significant similarity found		
64	Confirmed		
65	Confirmed		
66	Rothia mucilaginosa		
67	Confirmed		
68	Confirmed		
69	Homo sapiens		
70	No significant similarity found		Homo sapiens
71	Confirmed		
72	Rothia mucilaginosa		
73	Homo sapiens	Confirmed	
74	Confirmed		
75	Confirmed		Confirmed
76	Confirmed		
77	Homo sapiens	Confirmed	
78	Homo sapiens		
79	Confirmed		
80	Confirmed		
81	Confirmed		
82	Homo sapiens		
83	Homo sapiens		
84	Confirmed		
85	Confirmed		
86	Confirmed		Confirmed
87	Confirmed		Rothia mucilaginosa
88	Confirmed		
89	Confirmed		Confirmed

90	Confirmed	Confirmed	
91	Confirmed	Confirmed	
92	Rothia mucilaginosa		
93	Confirmed		
94	Confirmed		
95	Confirmed		
96			
97			
98			
99			
100	Homo sapiens		
101	Homo sapiens		
102	Homo sapiens		
103	Confirmed		
104			
105			
106			
107			
108			
109			
110			
111			
112	Homo sapiens	No significant similarity found	No significant similarity found
113			
114			
115			
116			
117			
118	Homo sapiens	No significant similarity found	
119			
120			

121	
122	
123	
124	
125	
126	
127	Confirmed
128	
129	
130	
131	
132	
133	
134	
135	
136	
137	
138	
139	Homo sapiens
140	
141	
142	Homo sapiens
143	Homo sapiens
144	Confirmed
145	
146	Homo sapiens
147	
148	
149	Homo sapiens
150	Homo sapiens

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Supplemental data 7

Raw sequencing data of positive RT-PCR amplicons obtained from symptomatic patients suspicious on SARS-CoV-2 (Confidential)