Rapid detection of SARS-CoV-2 by low volume real-time single tube reverse transcription recombinase polymerase amplification using an exo probe with an internally linked quencher (exo-IQ)

Running head: Rapid detection of SARS-CoV-2 by RPA

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List of abbreviations:

SARS-CoV-2 – Severe Acute Respiratory Syndrome Coronavirus 2

RT-RPA – Reverse Transcription Recombinase Polymerase Amplification

Exo-IQ – Exonuclease probe, Internally Quenched

RT-qPCR – Reverse transcription quantitative Polymerase Chain Reaction

CI – Confidence Interval

WHO – World Health Organization

LAMP – Loop Mediated Isothermal Amplification

MERS-CoV – Middle East Respiratory Syndrome Coronavirus

Abstract

Background: The current outbreak of SARS-CoV-2 has spread to almost every country with more than three million confirmed cases and over two hundred thousand deaths as of April 28, 2020. Rapid first-line testing protocols are needed for outbreak control and surveillance. **Methods:** We used computational and manual design to generate a suitable set of RT-RPA primer and exo-IQ probe sequences targeting the SARS-CoV-2 N gene. RT-RPA sensitivity was determined by amplification of in vitro transcribed RNA standards. Assay selectivity was demonstrated by means of a selectivity panel of 32 nucleic acid samples derived from common respiratory viruses. To validate the assay against full-length SARS-CoV-2 RNA, total viral RNA derived from cell culture supernatant and 19 nasopharyngeal swab samples (8 positive and 11 negative for SARS-CoV-2) were screened. All results were compared to established RT-qPCR assays. **Results:** The 95 % detection probability of the RT-RPA assay was determined to be 7.74 (95% CI: 2.87 - 27.39) RNA copies per reaction. The assay showed no cross-reactivity to any other screened coronaviruses as well as respiratory viruses of clinical significance. The developed RT-RPA assay produced 100% diagnostic sensitivity and specificity when compared to RT-qPCR (n=20). **Conclusions:** With a run time of 15 to 20 minutes and first results being available in under 7 minutes for high RNA concentrations, the reported assay constitutes one of the fastest nucleic acid based detection methods for SARS-CoV-2 to date and may provide a simple to use alternative to RT-qPCR for first-line screening at the point of need.

1 Introduction

In December 2019, several cases of a new form of respiratory disease were first described in the central Chinese city of Wuhan (Hubei Province, People's Republic of China) (1). The initial outbreak soon spread to other regions and has now led to a global pandemic. The causative agent was identified as a novel coronavirus of possible bat origin closely related to SARS-CoV (2) and designated as SARS-CoV-2 by the WHO.

Current protocols for the diagnosis of SARS-CoV-2 infection rely on reverse transcription quantitative PCR (RT-qPCR) for the detection of viral RNA (3,4). However, due to the need for energy intensive thermocycling, PCR-based methods are cumbersome to implement for rapid and decentralized screening at the point of need. Recently, isothermal nucleic acid amplification methods such as loop-mediated isothermal amplification (LAMP) (5) or recombinase polymerase amplification (RPA) (6) have become available. These methods do not require sophisticated thermocycling instrumentation and rely on enzymatic processes for all stages of DNA amplification. Due to its comparatively simple design approach and incubation requirements (39 - 42 °C), speed (15 to 20 minutes), and reagent availability in long shelf life lyophilized form, RPA is considered one of the most promising isothermal DNA detection methods.

RPA in its basic form uses two opposing DNA primers, like PCR, that are designed to be complementary to the target sequence of interest. By complexation of the primers with recombinase proteins, a D-loop is formed in the double stranded target sequence and the primers anneal to their respective complementary sequences. The

primers are then extended by a mesophilic DNA polymerase resulting in two copies of the target DNA region. The process is then initiated again on the copies leading to exponential amplification. With the addition of a fluorescently labeled probe, RPA can be monitored in real-time (7).

RT-RPA assays for the detection of other coronaviruses such as MERS-CoV (8) and bovine coronavirus (BCoV) (9) have been reported. With a proven track record for first line screening using a suitcase lab for the detection of H7N9 avian influenza virus (10) and Ebola virus (11), RT-RPA technology has been demonstrated to be of great potential for early diagnostics in rapidly evolving and resource limited outbreak situations.

In this study we demonstrate the design of a simple to use RT-RPA assay for the specific detection of SARS-CoV-2 and introduce a novel internal structure for RPA exo probe construction (exo-IQ) that removes a major assay design constraint. Inspired by the results reported in (12) that RPA performs very well in low reaction volumes, we demonstrate successful amplification of both *in vitro* transcribed RNA as well as total SARS-CoV-2 RNA derived from nasopharyngeal swab samples and cell culture supernatant in volumes of only 6.2 µl. Our results show that the proposed assay may be a viable alternative to RT-qPCR that is both faster and requires less complex and energy-intensive instrumentation.

2 Methods

2.1 RNA standards

The sequences of the SARS-CoV-2 N gene was downloaded from GenBank (RefSeq: NC_045512.2). A T7 RNA polymerase promoter sequence was then added *in silico* to the 5' end and the construct was ordered as double stranded DNA from a commercial supplier (GeneArt, Germany). We then produced RNA transcripts by incubation with T7 polymerase (HiScribe T7 High Yield RNA Synthesis Kit, NEB, USA) followed by DNAse I (NEB, USA) digestion and spin-column based purification (RNA Clean-Up and Concentration Kit, Norgen Biotek, Canada). Purified RNA was then quantified by RiboGreen (Invitrogen, USA) assay and standards ranging from 10^7 to 10^0 RNA copies per µl were prepared in TE buffer. The standards were confirmed to be free of residual DNA from the transcription reaction by qPCR.

A coronavirus RNA specificity panel containing full virus RNA (from cell culture) of HCoV-Nl63, HCoV-229E, HCoV-OC43, MERS-CoV and SARS-CoV was obtained through the European Virus Archive global (EVAg). RT-qPCR threshold cycle (C_t) values for each RNA isolate were provided to the authors by personal communication (A. van der Linden, University Medical Center Rotterdam, February 2020). Due to its difficult availability, HCoV-HKU1 was not included in this study. However, BLAST alignment of SARS-CoV-2 (RefSeq: NC_045512.2) and HCoV-HKU1 (RefSeq: NC_006577.2) N

genes showed almost no sequence homology making off-target amplification highly unlikely.

Twenty-seven nucleic acid samples for further specificity testing covering a broad range of other respiratory viruses that may cause a similar clinical picture as SARS-CoV-2 were screened (Supplemental Table 1). The samples were provided by Quality Control for Molecular Diagnostics, Glasgow, Scotland, UK; Landesgesundheitsamt Niedersachsen, Hannover, Germany; Robert Koch Institute, Berlin, Germany, and the Friedrich-Loeffler-Institute, Greifswald-Insel Riems, Germany.

SARS-CoV-2 viral RNA was provided as inactivated Vero E6 cell culture supernatant of the patient isolate MUC-IMB-1 by the Bundeswehr Institute of Microbiology (IMB), Munich, Germany. The complete sequence of this isolate is available through GISAID under the accession ID_EPI_ISL_406862 and name “hCoV-19/Germany/BavPat1/2020”.

Eight total RNA extracts (QIAamp Viral RNA Mini Kit, Qiagen, Germany) from nasopharyngeal swabs were provided by Labor Staber, Kassel, Germany as anonymized leftover material from routine clinical diagnostics. All samples were confirmed to be positive for SARS-CoV-2 by the providing laboratory using RT-qPCR based on the LightMix E and RdRp assays (TIB Molbiol, Germany). Upon arrival in our lab, all samples were analyzed with the N1 RT-qPCR assay.

Eleven further nasopharyngeal swab samples were collected from healthy laboratory personnel and total RNA was extracted with the QIAamp Viral RNA Mini Kit

(Qiagen, Germany) according to the manufacturer's instructions. All samples were confirmed to be free of SARS-CoV-2 RNA by the N1 and/or N3 RT-qPCR assays.

2.2 Reverse transcription quantitative PCR

The primers and probes targeting SARS-CoV-2 used in this work are listed in Supplemental Table 2. Primers were synthesized exactly as presented in the table, whereas an internal quenching group located between the 8th and 9th nucleotides from the 5´-end was integrated in the fluorescent probes. This modification, together with the second quencher located at the 3' end of the probe, strongly decreases the background fluorescence of unbound probes leading to improved signal quality. All oligonucleotides were synthesized by biomers.net (Ulm, Germany).

For the N1/N2/N3 assays (CDC, Atlanta, USA) (4), a 40X primer/probe mix (PPM) was prepared at concentrations of 20 µM for each primer and 10 µM for the probe. Reactions of 20 µl total volume were then assembled by combining 7.5 µl DEPC treated water, 10 µl 10X Luna OneStep probe PCR Mix (NEB, USA), 1µl 20X WarmStart RT enzyme mix (NEB, USA), 0.5 µl 40X PPM and 1 µl RNA template. Thermal cycling was then performed on a LightCycler 480 II instrument (Roche Diagnostics, Germany). Cycling conditions were 10 minutes at 50 °C for reverse transcription followed by one minute at 95 °C for initial denaturation and RT enzyme inactivation and PCR for 45 cycles of 95 °C for 3 seconds and 55 °C for 30 seconds. Fluorescence was read at the end of the 55 °C step. All qPCR data was analyzed by the Cy0 method (13) implementation included in the R language qpcR package (14).

2.3 Low volume single tube RT-RPA

2.3.1 Reaction setup and conditions

All RT-RPA reactions were performed using the TwistAmp exo-RT kit (TwistDx, United Kingdom). Optically clear 8-tube strips with individually attached lids were placed on ice and 10 µl of PCR-grade mineral oil were pipetted into each lid. For each series of 8 reactions with identical targets, a rehydration mix consisting of 31 µl rehydration buffer, 2.21 µl (10 µM stocks) of each primer and 0.63 µl exo-IQ probe (10 µM stock), 2.63 µl RNAse inhibitor (20 U/µl, Bioron, Germany) and 8.4 µl RNA template were combined and 45 µl of this were used to rehydrate an exo-RT reagent pellet. The reaction mix was then placed on ice and 5 µl of a freshly prepared 140mM MgOAC solution were added. Finally, 6.2 µl of the activated reaction mix were distributed to each tube of the previously prepared 8-tube strip.

In case each tube was to contain a different RNA template, the rehydration mix was prepared as described previously while omitting the RNA template. In this case, 37 µl rehydration mix were then used to rehydrate an exo-RT pellet, and 4.6 µl of the reaction mix was distributed to each reaction tube and 1 µl of individual RNA template was pipetted into each tube. A total of 0.63 µl of 140 mM MgOAC solution were then carefully pipetted in a single drop onto the inside wall of each reaction tube so that the drop was not in contact with the reaction mix.

After closing the lids, the 8-tube strips were centrifuged to overlay the reaction mix with the oil and, in the case of individual RNA targets, mix the MgOAC solution with the reaction mix. The tubes were then quickly placed into an ESEQuant isothermal

fluorescence reader (Qiagen Lake Constance GmbH, Stockach, Germany) preheated to 42 °C and let to equilibrate for one minute. Fluorescence (Ex: 470 nm / Em: 550 nm) was then recorded every 20 seconds for a total of 20 minutes. To enhance reaction efficiency, tubes were removed from the instrument after 320 sec. (240 sec. for reactions containing 10^4 template copies and 160s for 10^5 template copies), mixed by vortexing, centrifuged and immediately placed back into the instrument.

For unknown samples we suggest keeping the mixing time fixed at 320 sec., as this will guarantee highest assay sensitivity although some of the initial fluorescence signal may be lost.

2.3.2 Fluorescence data processing

RPA fluorescence data was first background corrected by subtracting the first measurement value taken after the mixing step from all other values. All measurement points taken before and during the mixing step were then set to zero.

To establish a fluorescence threshold, twelve no template control (NTC) reactions were run and averaged. In addition, the standard deviation was calculated. Three standard deviations were then added to the average NTC and the threshold was then defined by the highest value rounded to the nearest integer. Thresholding of the RT-RPA fluorescence data was performed with the R language qpcR package (14).

Probit analysis for assay sensitivity determination was performed according to (15) using the R language.

3 Results

3.1 Design of the exo-IQ RPA probe

RPA exo probes are oligonucleotides of a length of at least 46 nt with an internal structure consisting of both a fluorophore and a quencher that are separated by an apurinic/apyrimidinic (AP) site. Once the probe hybridizes with its target sequence, the AP endonuclease activity of Exonuclease III (ExoIII) (16) cleaves the AP site leading to spatial separation of the fluorophore and quenching groups. This mechanism causes an increase in fluorescence that is directly related to the amount of RPA product allowing for monitoring of the RPA reaction in real time.

The commercial availability of internal fluorophore and quenching groups for DNA synthesis is largely limited to modified thymine phosphoramidites, which leads to the constraint that exo probes can only be designed for sequences that contain two thymines within 2 to 5 nt from each other. This hampers design of RPA exo assays, as suitable regions that also satisfy all further assay design rules (17) are often difficult to find. Furthermore, it has been shown that exo probe performance is strongly dependent on the distance between the fluorophore and quenching groups with the optimal distance being only 1-2 nt (18). To overcome this limitation, we introduce the concept of the exo-IQ (**I**nternally **Q**uenched) probe (Figure 1) where the quenching group is attached between two nucleotides by an internal linker (see Supplemental Figure 1). This strategy greatly simplifies exo probe design as the quencher can always be placed

at a constant distance downstream of the thymine linked fluorophore without the need for a second proximal thymine. We propose that an exo-IQ probe can thus be designed from any single conveniently located thymine as a starting point with the next 3' nucleotide being replaced by an AP site followed by the internal quencher between the following two nucleotides. The active site is then simply extended for 30 nt in the 5' direction and 14 nt in the 3' direction. To prevent unwanted amplification, the free 3'-end is blocked by a C3 spacer. Since AP sites are susceptible to acid and heat mediated cleavage, exo-IQ probes should be stored cold and dark in a buffering solution such as Tris-EDTA (TE).

3.2 SARS-CoV-2 RPA assay design

Upon release of the first SARS-CoV-2 genome (GenBank MN908947), we designed an exo-IQ RT-RPA assay targeting the nucleocapsid (N) gene both by computational and manual methods. PrimedRPA (19) was used to generate a list of possible primer/probe combinations. Of the 18 generated combinations, two contained probe sequences suitable for exo-IQ construction with a thymine at position 30 and were investigated further for SARS-CoV-2 specificity by BLAST alignment. The first probe showed almost complete sequence homology with SARS-CoV and was discarded. Alignment of the second probe with SARS-CoV (Figure 2) shows numerous mismatches resulting in a design that is highly specific for SARS-CoV-2. Two primer pairs that are selective for SARS-CoV-2 and compatible with the selected probe sequence were chosen for evaluation. All oligonucleotides were synthesized by biomers.net (Ulm, Germany) and

primer and probe combinations were screened for amplification efficiency (data not shown). The best performing primers are listed in Table 1 and are shown in their relation to SARS-CoV in Figure 2. While the reverse primer has complete sequence homology with SARS-CoV and could be used as part of a possible assay that detects both SARS-CoV and SARS-CoV-2, the forward primer was designed to be highly selective for SARS-CoV-2 as the first three 3' nucleotides are mismatched to SARS-CoV rendering extension by DNA polymerase highly inefficient (20).

3.3 RT-RPA assay sensitivity and specificity

Assay sensitivity was determined by amplification of *in vitro* transcribed (IVT) SARS-CoV-2 N gene templates. Eight low-volume RT-RPA reactions were run in parallel for target copy numbers of 10^5 down to 10^0 molecules/reaction (Figure 3). Strong amplification of each replicate reaction was observed for target copy numbers from 10^5 down to 10^2 (Figure 3 top and bottom left) while the results for 10^1 and 10^0 (Figure 3, bottom center and right) were, not surprisingly, more variable. Final fluorescence values were found to be different for each replicate with the highest values being observed for a target copy number of 10^3 copies (Figure 3, top right).

Except for 10^0 copies with a detection rate of 25%, all other tested template copy numbers yielded positive results for each replicate. Figure 4C shows the probit regression of these results which was used to compute the 95% detection probability of the assay to be 7.74 (95% CI: 2.87 - 27.39) RNA copies/reaction.

The threshold times (TT) for each experiment presented in Figure 3 were plotted against the initial RNA target copy number to evaluate assay linearity. For copy numbers from 10^5 to 10^2, the assay was found to be linear and a simple linear model for the calculation of initial target copy numbers from measured TT values of unknown samples was constructed by linear regression.

Assay selectivity was determined using the European Virus Archive global (EVAg) coronavirus selectivity panel. No cross-reactivity was observed for any of the tested RNAs while the positive control of IVT SARS-CoV-2 RNA showed strong amplification (Figure 4A). In addition, assay selectivity was further confirmed by testing of 27 nucleic acid samples (listed in Supplemental Table 1) derived from common respiratory viruses. No amplification was observed (data not shown).

3.4 RT-RPA detection of SARS-CoV-2

Total RNA extracted from nasopharyngeal swabs (Pat. 1-19) and cell culture supernatant (BavPat1) was subjected to both RT-RPA and RT-qPCR (Figure 5) in an open-label study. Nine out of 20 screened samples showed strong amplification in the RT-RPA and RT-qPCR assays, whereas 11 samples did not amplify in either assay. The linear model for the N1 RT-qPCR assay (Supplemental Figure 2) was used to estimate the initial SARS-CoV-2 RNA copy numbers which are presented in the inset table in Figure 5. The mixing time for RT-RPA was kept at 320 sec. demonstrating that this value is suitable for the detection of a sample with an unknown concentration of target RNA.

4 Discussion

In the present study we describe the design and characterization of a low volume real-time RT-RPA assay for the detection of SARS-CoV-2 RNA and a simplified approach for RPA exo probe design we termed exo-IQ. This approach allows a much more flexible RPA exo probe design since it removes the constraint that exo probes can only be designed for sequences that contain two thymines within 2 to 5 nt from each other.

The newly developed SARS-CoV-2 assay using an exo-IQ probe provided performance comparable to that reported for the detection of MERS-CoV by RT-RPA (8). In agreement with the findings presented in (12), we found that RPA reactions of low volume (6.2 µl) worked well, most likely due to the higher template to reagent ratio. It was beneficial to cover the reaction mix with mineral oil to prevent reagent evaporation in order to achieve consistent results.

By demonstrating the detection of genuine full-length viral RNA derived from real patient samples, we confirmed that the assay can successfully detect SARS-CoV-2 RNA from complex clinical specimens.

In comparison to the (non-RT)RAMP assay published by El-Tholoth et al. (21) (non peer-reviewed preprint) who report a lower detection limit of 7 copies of a synthetic SARS-CoV-2 DNA analog within about 50 minutes, our assay is both faster, of a much simpler design and does not require incubation at the elevated temperatures needed for LAMP while having the ability to directly detect viral RNA without a separate reverse transcription step. When compared to the second detection scheme for SARS-CoV-2

using RPA chemistry published by Zhang et al. (22) (non peer-reviewed online protocol) who report the detection of 120 RNA copies in one hour by a simple colorimetric, non-quantitative endpoint readout, our assay is more sensitive, faster and has much more simple handling requirements.

With a total run time between 15 and 20 minutes, our newly developed assay is one of the fastest nucleic acid based method that has been reported for the detection of SARS-CoV-2. Assuming an assay setup time of 5 minutes, the total assay time is only 20 to 25 minutes and thus much shorter than RT-qPCR. Due to the small reaction volume (6.2 µl) and the fixed reaction temperature (42 °C), our assay is also an ideal candidate for use in microfluidic devices which will further simplify and improve point of need diagnostics.

5 Acknowledgements

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6 Ethics statement

All samples were collected from suspected COVID-19 patients during medical examination. The samples were screened with licensed RT-qPCR in an accredited laboratory. Leftover material was screened with the developed RT-RPA assay. Results of this study did not influence the clinical discussion between patient and physician. All samples were handled anonymously.

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8 Author contributions

Conceptualization: O.B., F.H., G.Da., M.Sp., A.A.

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Project administration: F.H., G.Da.

Supervision: O.B., F.H., G.Da., A.A., G.Do.

Writing – original draft: O.B.

All authors have critically reviewed the manuscript and have given their approval for publication.

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Table 1 Sequences of the RPA primers and exo-IQ probe used in this work. Due to the proprietary nature of the internally linked quencher, exo-IQ probes can be supplied commercially by biomers.net. However, exo-IQ construction with internally linked quenching groups offered by other manufacturers may also be possible.

Name	**Sequence 5'-3'**
RPA-SARS-2-FW	CCTCTTCTCGTTCCTCATCACGTAGTCGCAAC
RPA-SARS-2-RV	AGTGACAGTTTGGCCTTGTTGTTGTTGGCCTT
RPA-SARS-2-P	CCTGCTAGAATGGCTGGCAATGGCGGTGA(dT-FAM)(AP site)C(BMN-Q535)TGCTCTTGCTTTGC-C3

Figure 1: Design principle of the exo-IQ probe. The need for two closely spaced thymine bases is removed using an internal quenching group.

Figure 2: Alignment of the SARS-CoV-2 RPA amplicon with SARS-CoV. Gray highlight: Matching bases; White background: Mismatching bases. The primers and exo-IQ probe are denoted by arrows above the aligned sequences.

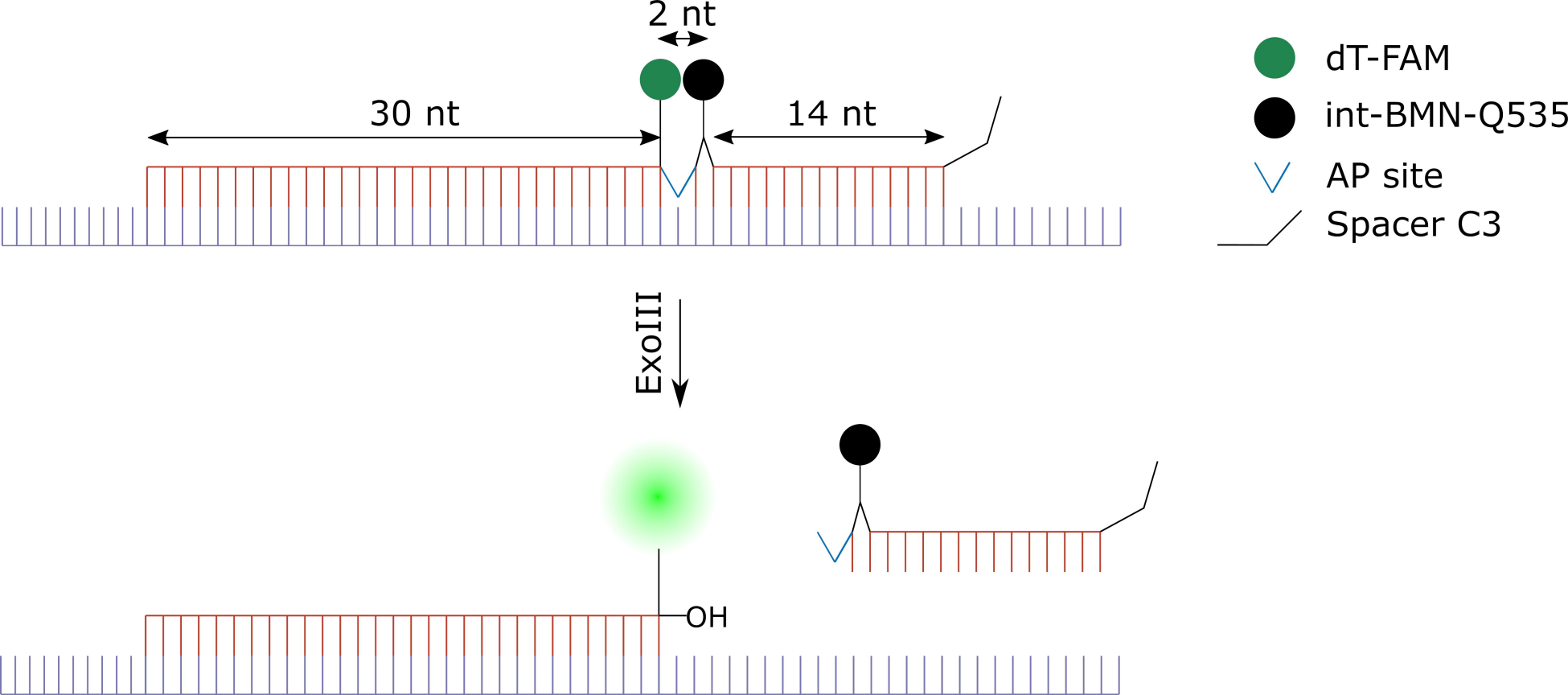
Figure 3: RT-RPA amplification of IVT SARS-CoV-2 N gene RNA. The number of RNA target copies in each reaction is indicated in the top left corner of each panel. Red: Reactions containing target RNA; Blue: NTC; Black: Threshold. Only reactions yielding fluorescence values above the threshold are considered to be positive.

Figure 4A: Specificity analysis using the EVA coronavirus selectivity panel. Indicated C_t values were determined by specific RT-qPCR assays. Black:Threshold.

Figure 4B: Linear model (calibration curve) of the RT-RPA assay.

Figure 4C: Black, solid: Probit regression. Black, dashed: 95 % confidence interval of the probit regression. Red, dashed: 95% detection probability of the RT-RPA assay.

Figure 5: RT-RPA amplification of total RNA extracted from nasopharyngeal swabs (Patients 1-19) and cell culture supernatant (BavPat1). Red: SARS-CoV-2 positive samples; Blue: SARS-CoV-2 negative samples; Black: Threshold. The inset table shows the RT-RPA threshold time (TT) as well as the Cy0-values and RNA copies/µl derived from the N1 RT-qPCR assay.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int-BMN-Q535
AP site
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ExoIII
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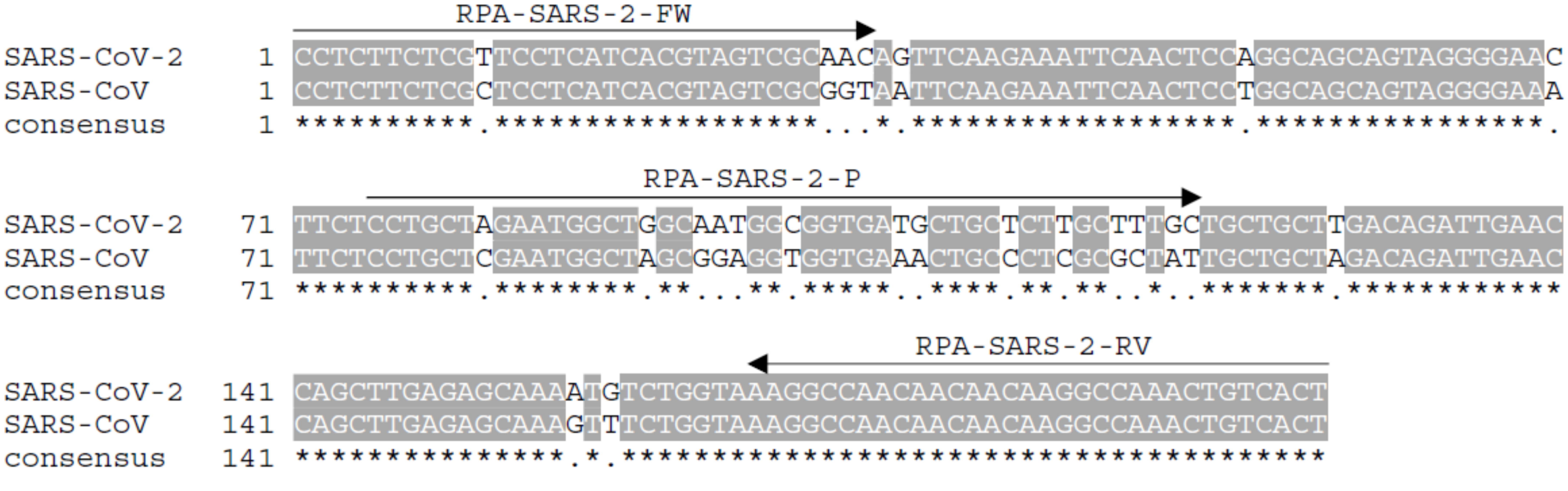
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SARS-CoV-2 1 CCTCTTCTCGTTCCTCATCACGTAGTCGCAACAGTTCAAGAAATTCAACTCCAGGCAGCAGTAGGGGAAC
SARS-CoV 1 CCTCTTCTCGCTCCTCATCACGTAGTCGCGGTAATTCAAGAAATTCAACTCCTGGCAGCAGTAGGGGAAA
consensus 1 **********.******************...*.******************.****************.
RPA-SARS-2-P
SARS-CoV-2 71 TTCTCCTGCTAGAATGGCTGGCAATGGCGGTGATGCTGCTCTTGCTTTGCTGCTGCTTGACAGATTGAAC
SARS-CoV 71 TTCTCCTGCTCGAATGGCTAGCGGAGGTGGTGAACTGCCTCGCGCTATTGCTGCTAGACAGATTGAAC
consensus 71 **********.********.**...**.*****..****.**.**..*..*******.************
RPA-SARS-2-RV
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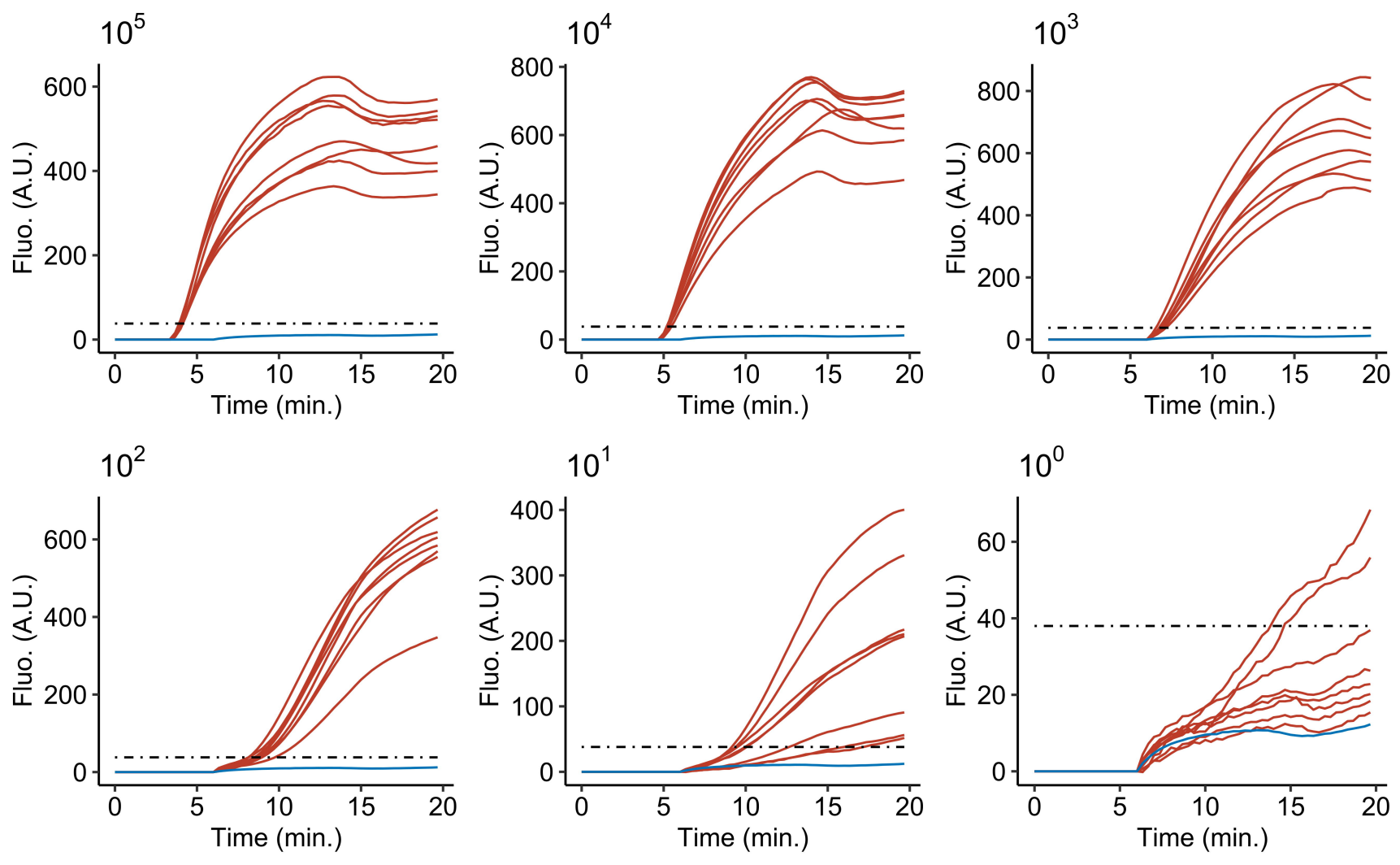
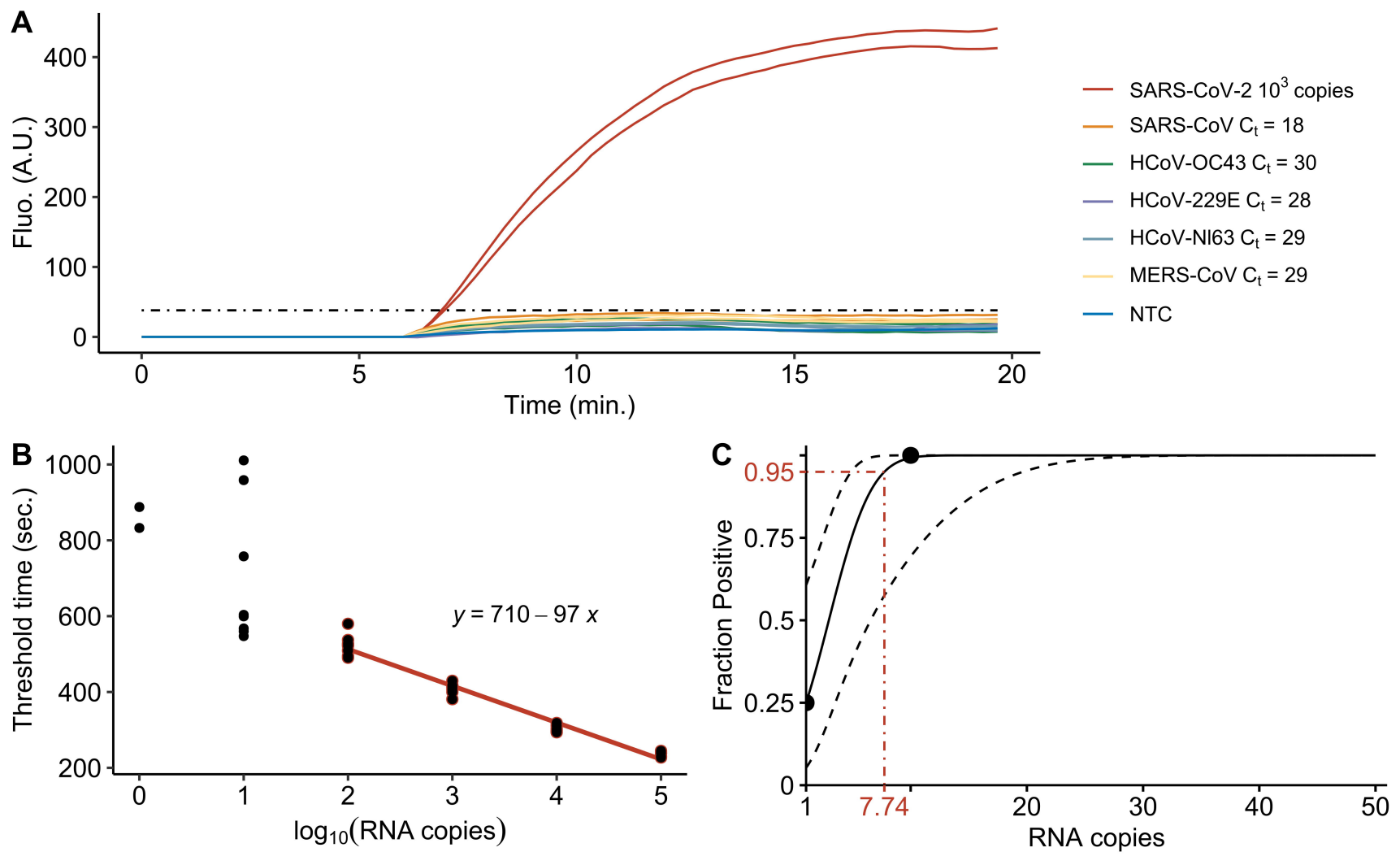
SARS-CoV 141 CAGCTTGAGAGCAAAGTTCTGGTAAAGGCCAACAACAACAAGGCCAAACTGTCACT
consensus 141 ****************.*.**********************************


10^5
10^4
10^3
10^2
10^1
10^0
Fluo. (A.U.)
Time (min.)


A
Fluo. (A.U.)
400
300
200
100
0
0
5
10
15
20
Time (min.)
SARS-CoV-2 10^3 copies
SARS-CoV Ct = 18
HCoV-OC43 Ct = 30
HCoV-229E Ct = 28
HCoV-NI63 Ct = 29
MERS-CoV Ct = 29
NTC
B
Threshold time (sec.)
1000
800
600
400
200
0
1
2
3
4
5
log10(RNA copies)
y = 710 – 97 x
C
0.95
Fraction Positive
0.75
0.5
0.25
0
1
7.74
20
30
40
50
RNA copies

Patient	Cy0	RNA copies/µl	TT (min.)
BavPat1	29.4	3.27E+03	6.28
1	35.13	6.01E+01	10.18
2	28.48	6.22E+03	6.57
3	33.14	2.41E+02	8.65
4	33.87	1.45E+02	10.25
5	27.91	9.26E+03	6.97
6	24.36	1.10E+05	6.48
7	31.34	8.46E+02	8.10
8	27.82	9.86E+03	6.51
9-19	-	-	-